Alteration of Macrophage Signalling and Functions by the Protozoan Parasite *Leishmania*

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To my supportive father **Ayoub Issa Abu-Dayyeh**, my one-of-a-kind mother **Yasmin Farah Sweiss**, and to all the beloved people in Jordan, Lebanon, and Canada who made a difference in my life one way or the other!

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List of Abbreviations

A: alanine

Ag: antigen

AMP: anti-microbial peptide

AP-1: activating protein 1

APC: antigen presenting cell

Asc: apoptosis-associated speck-like protein

ATF-2: activating transcription factor 2

ATP: adenosine triphosphate

BAFFR: B-cell-activating factor receptor

BALB/c: Bagg albino c

BCR: B-cell receptor

BLNK: B-cell linker

BMDM: bone marrow-derived macrophage

C: cysteine

cAMP: cyclic adenosine monophosphate

CCR: chemokine CC motif receptor

CD: cluster of differentiation

c-fos: cellular finkel, osteogenic, and sarcoma

CHO: Chinese hamster ovary

CIS: cytokine-inducible SH2-containing protein

c-jun: cellular JU-Nana

COX: cyclooxygenase

CP: cysteine proteinase

CpG: cytosine-phosphate-guanine

CR: complement receptor

CRE: c-AMP-regulated enhancer

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

CRP: C-reactive protein

CRPR: C-reactive protein receptor

c-SH2: C-terminal SH2 domain

DAG: diacylglycerol

DC: dendritic cell

DD: death domain

DFADD: *Drosophila* Fas-associated protein with death domain

Dif: dorsal-related immunity factor

dMyD88: *Drosophila* myeloid differentiation factor 88

DREDD: death-related ced-3 / NEDD2-like protein

dTAK1: *Drosophila* TGF-β activated kinase 1

dTRAF6: *Drosophila* TNF receptor-associated factor 6

E: glutamic acid

EF-1α: elongation factor 1 alpha

Elk-1: Ets-like transcription factor 1

EMSA: electromobility shift assay

Erk: extracellular signal-regulated kinase

Ets: E twenty-six specific family of transcription factors

F: phenylalanine

FBS: fetal bovine serum

FcγR: fragment crystallization gamma receptor

fos: finkel, osteogenic, and sarcoma

FR: fibronectin receptor

Fra: fos-related antigen

G: glycine

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GIPL: glycosylinositol phospholipid

Glu: glutamine

gp63: glycoprotein 63

GPI : glycosylphosphatidylinositol

GST: glutathione S-transferase

H: histidine

HRP: horseradish peroxidase

I: isoleucine

IFN-γ: interferon gamma

IFN-γR: interferon gamma receptor

Ig: immunoglobulin

IκB: inhibitory kappa B

IKK: inhibitory kappa B kinase

IL: interleukin

IMD: immunodeficiency

iNOS: inducible nitric oxide synthase

IP: immunoprecipitate

IP-10: interferon-inducible protein 10

IRAK: interleukin 1 receptor-associated kinase

IRAK-M: interleukin 1 receptor-associated kinase restricted to cells of monomyeloic origin

ird5: immune response deficient gene 5

IRF-3: interferon regulatory transcription factor 3

ITAM: immunoreceptor tyrosine-based activation motif

ITIM: immunoreceptor tyrosine-based inhibitory motif

JAK: Janus kinase

JNK: jun N-terminal kinase

jun: JU-Nana

Kenny: *Drosophila* homologue of mammalian IKK-γ

KIR: killer cell IgG-like receptor

KTIM: kinase tyrosine-based inhibitory motif

L: leucine

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

LBP: LPS-binding protein

LM-1: littermate 1

lmcp: *Leishmania mexicana* cysteine proteinase

L-NMMA: L-N-monomethyl arginine

LPG: lipophosphoglycan

LPS: lipopolysaccharide

LRR: leucine-rich repeat

LTBR: lymphotoxin beta receptor

LYN: v-yes-1 Yamaguchi sarcoma viral-related oncogene homolog

M: methionine

MAA: medium for axenically-grown amastigotes

Mac-1: macrophage antigen 1 (alpha)

Mal: MyD88 adaptor-like protein

MALP-2 : macrophage-activating lipopeptide 2

MAPK : mitogen-activated protein kinase

MAPKAP-K2: MAPK-activated protein kinase 2

MAPKK: mitogen-activated protein kinase kinase

MAPKKK: mitogen-activated protein kinase kinase kinase

MCP-1: monocyte chemotactic protein 1

MD: myeloid differentiation protein

me-3: motheaten 3

MØ: macrophage

MFR: mannose-fucose receptor

MHC: major histocompatibility complex

MIP-1α: macrophage inflammatory protein 1 alpha

MKK: MAPKK

MyD88: myeloid differentiation factor 88

MyD88s: myeloid differentiation factor 88 short

NAP1: NF-κB-activating kinase associated protein 1

NF-κB: nuclear factor kappa B

NIK: NF-κB-inducing kinase

NK: natural killer

Nlrp2: Nod-like receptor family pyrin domain-containing 2

NO: nitric oxide

N-SH2: N-terminal SH2 domain

O/N: overnight

p130CAS: Crk-associated substrate

PAMP: pathogen-associated molecular pattern

PBS: phosphate-buffered saline

PD-1: programmed death receptor 1

PfEMP1: *Plasmodium falciparum* erythrocyte membrane protein 1

PFTG: profilin-like protein from *T. gondii*

PG: phosphoglycan

PGE2: prostaglandin E2

PGN: peptidoglycan

PGRP: peptidoglycan recognition protein

phox: phagocyte NADPH oxidase

PIP2: phosphatidylinositol 4, 5 bisphosphate

PIP3: phosphatidylinositol 3,4,5-triphosphate

PIR-B: B cell inhibitory receptor

PKC: protein kinase C

PLC: phospholipase C

PMA: phorbol 12-myristate 13-acetate

pNPP: para-nitrophenyl phosphate

poly I:C: poly inosine:cytosine

PP2A: protein phosphatase 2A

PRR: pattern-recognition receptor

PS: phosphatidylserine

PSR: phosphatidylserine receptor

PTP: protein tyrosine phosphatase

PTP-1B: protein tyrosine phosphatase 1B

PTPN6: protein tyrosine phosphatase, non-receptor type 6

p-Y: phospho-tyrosine

qRT-PCR: quantitative real-time polymerase chain reaction

R: arginine

RANTES: regulated on activation normal T cell expressed and secreted

rel: relish

RIP: receptor-interacting protein

ROI: reactive oxygen intermediate

RP105: radioprotective 105

RPA: RNAase protection assay

RT: room temperature

RT-PCR: reverse-transcriptase polymerase chain reaction

S: serine

SAcP: secretory acid phosphatase

SAM: sterile α-motif

SAP-1: SRF accessory protein 1

SARM: sterile α- and armadillo-motif-containing protein

SD: standard deviation

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM: standard error of the means

SHIP: Src homology 2 domain-containing inositol-5-phosphatase

SHP-1: Src homology 2 domain-containing protein tyrosine phosphatase 1

SHP-2: Src homology 2 domain-containing protein tyrosine phosphatase 2

SIGIRR: single immunoglobulin IL-1-related protein

SIGLEC: sialic acid-binding immunoglobulin-like lectin

SLE: systemic lupus erythematosus

SOCS: suppressor of cytokine signalling

Sp1: signal protein 1

Spz: spaetzle

Src: Schmidt-Ruppin A-2 oncogene

SRF: serum response factor

ST2: suppressor of tumorigenicity 2

STAT: signal transducer and activator of transcription

sTLR: soluble TLR

Syk: spleen tyrosine kinase

T: threonine

TAB: TAK1-binding protein

TAK1: TGF-β activated kinase 1

TBK1: TNFR-associated factor (TRAF)-family-member-associated NF-κB-activator-binding kinase 1

Tc-PTP: T-cell protein tyrosine phosphatase

TCR: T-cell receptor

TF: transcription factor

TGF-β: transforming growth factor beta

Th: T- helper

Thr: threonine

TIR: Toll / IL-1 receptor

TIRAP: TIR domain-containing adaptor protein

TLR: Toll-like receptor

TNF: tumor necrosis factor

TOLLIP: Toll-interacting protein

TRAF6: TNF receptor-associated factor 6

TRAILR: TNF-related apoptosis-inducing ligand receptor

TRAM: TRIF-related adaptor molecule

TRIF: TIR-domain-containing adaptor-inducing interferon-β

TYK2: tyrosine kinase 2

Tyr: tyrosine

Ubc13: ubiquitin-conjugating enzyme E2 13

Uev1A: ubiquitin-conjugating enzyme E2 variant 1A

V: valine

Vpu: viral protein U

VV: Vaccinia virus

WT: wildtype

x: any amino acid

X-CGD: X-linked chronic granulomatous disease

Y: tyrosine

Yop: *Yersinia* outer protein

ZAP-70: Zeta-chain-associated protein kinase 70

Abstract

Parasites of the genus *Leishmania* are able to secure their survival and propagation within their host by altering key signalling pathways involved in the ability of macrophages (MØs) to directly kill pathogens or to activate cells of the adaptive immune system. One important step in this immune evasion process is the *Leishmania*-induced activation of host protein tyrosine phosphatase SHP-1. SHP-1 has been shown to directly inactivate JAK2 and Erk1/2, and to play a role in the negative regulation of several transcription factors involved in MØ activation such as: NF-κB, STAT-1α, and AP-1. These signalling alterations contribute to the inactivation of critical MØ functions such as the production of IFN-γ-induced nitric oxide (NO), a free radical associated with parasite killing and clearance. In addition to interfering with IFN-γ receptor signalling, *Leishmania* is able to alter several LPS-mediated responses (e.g. IL-12, TNF-α, NO production) through mechanisms not yet fully understood. A main goal of this study was to better understand the mechanisms used by the parasite to block Toll-like receptor (TLR)-mediated functions. Experiments performed revealed a pivotal role for SHP-1 in the inhibition of TLR-induced MØ activation through binding to and inactivating IL-1 receptor-associated kinase 1 (IRAK-1). We identified the binding site as an evolutionarily conserved ITIM-like motif, which we named kinase tyrosine-based inhibitory motif (KTIM). Further experiments and sequence analysis revealed that several cytosolic kinases other than IRAK-1 possess potential KTIMs, suggesting it could represent a regulatory mechanism widely used by kinases. The final experimental section aimed to explore the differential ability of the two different stages of *Leishmania*, promastigotes and amastigotes, to alter MØ signalling and function. In conclusion, this work uncovers a new mechanism

whereby *Leishmania* is able to interfere with TLR-mediated activation of MØs by inducing host SHP-1 activity. The SHP-1 binding site on IRAK-1 was named KTIM, a motif we believe might play a major role in regulating a wide range of kinases other than IRAK-1. In addition, we describe important similarities and differences in the ability of promastigotes and amastigotes to alter several MØ signalling molecules in order to inhibit IFN-γ-mediated NO production in MØs. Taken together, the experiments performed in this work are aimed to improve our understanding of evasion mechanisms employed by promastigotes and/or amastigotes of *Leishmania*, hoping such findings will help in the development of more efficient anti-leishmanial therapies in the near future.

Résumé

Les parasites du genre *Leishmania* assurent leur survie et leur propagation par l'altération de voies de signalisation impliquées dans la capacité des macrophages (MØs) à détruire directement les pathogènes ou à activer les cellules du système immunitaire acquis. Une étape critique de ce mécanisme d'inactivation est l'activation par *Leishmania* de la protéine phosphatase SHP-1 de la cellule hôte. Il a été démontré que la protéine SHP-1 peut inactiver directement JAK2 ainsi que Erk1/2 et joue un rôle dans la régulation négative de plusieurs facteurs de transcription, tels que NF-κB, STAT-1α et AP-1, impliqués dans l'activation des MØs. L'altération de ces voies de signalisation contribue à l'inactivation de fonctions critiques des MØs telle que la production d'oxyde nitrique (NO) induite par l'IFN-γ, un radical-libre impliqué dans l'anéantissement du parasite. En plus d'inhiber les fonctions engendrées par l'IFN-γ, *Leishmania* est capable d'inhiber de nombreuses fonctions induites par le LPS, incluant la production d'IL-12, de TNF-α et de NO, et cela par des mécanismes encore peu compris. Le but principal de cette étude était de mieux comprendre les stratégies employées par le parasite afin d'inhiber les fonctions induites par les Toll-like receptors (TLRs). Nos résultats révèlent le rôle critique de SHP-1 dans l'inhibition de l'activation des MØs induite par les TLRs, par l'interaction et l'inactivation de la kinase 1 associée au récepteur IL-1 (IRAK-1). Nous avons également identifié le site de liaison qui semble être un motif conservé lors de l'évolution ressemblant à un ITIM, que nous avons nommé motif de kinase à base de tyrosine inhibiteur (KTIM). Des expériences supplémentaires et l'analyse de séquences ont révélées que plusieurs autres kinases cytosoliques autres qu'IRAK-1 possèdent un motif potentiel KTIMs, suggérant que le KTIM pourrait représenter un mécanisme de

régulation utilisé abondamment par les kinases. La dernière section d'expériences avait pour but d'explorer les différences entre les deux stages de *Leishmania,* promastigote et amastigote, au niveau de leur efficacité à altérer la signalisation intracellulaire et les fonctions des MØs. En conclusion, cette étude met à jour un nouveau mécanisme par lequel *Leishmania* est capable d'interférer avec l'activation des MØs via les TLRs par l'activation de la protéine hôte SHP-1. Le site de liaison de SHP-1 contenu dans la protéine IRAK-1 a été nommé KTIM, un motif qui joue possiblement un rôle primordial dans la régulation d'un vaste nombre de kinases autres qu'IRAK-1. De plus, nous soulignons d'importantes différences dans l'efficacité des promastigotes et amastigotes à altérer plusieurs molécules signalétiques des MØs afin d'inhiber la production de NO induite par l'IFN-γ dans les MØs. En somme, cette étude avait pour but d'améliorer notre compréhension des mécanismes d'évasion utilisés par les promastigotes et/ou amastigotes de *Leishmania* et ces découvertes vont possiblement contribuer au développement de thérapies anti-*leishmania* plus efficaces.

Preface and Acknowledgements

As described in the *Thesis Preparation Guidelines* of the Graduate and Postdoctoral Studies Office of McGill University, the author is presenting a manuscript-based thesis. Issa Abu Dayyeh is the principal author of all the manuscripts included in this thesis, indicating that he performed the majority of the work presented.

The manuscript presented in Chapter 2 has been published in the *Public Library of Science (PLoS) Neglected Tropical Diseases*, a peer-reviewed open-access journal (PLoS Negl Trop Dis, 2008, 2(12): e305). The author performed the majority of the experiments in this study and all the sequence analysis. Special thanks go to Dr. Marina Tiemi Shio for the help she provided in the immunoprecipitation experiments in figures 10 and 14 of the manuscript, and to Marceline Côté from Dr. Liu's laboratory (McGill University, Canada) for her help in designing primers and trouble-shooting for the site-directed mutagenesis experiments. I would also like to thank Dr. Shintaro Sato and Dr. Shizuo Akira (Osaka University, Japan) for providing generous amounts of the IRAK-1 antibody used for the kinase assay, Dr. Benoit Cousineau (McGill University) for his extremely helpful input regarding the evolutionary aspects of the manuscript and for his help in the manuscript preparation, and Dr. Martin Olivier (McGill University) for his vital role in directing the project, providing ideas, discussing results, and preparing the manuscript.

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General Introduction

Activation of host SHP-1 is an important event leading to successful *Leishmania* infections. Compared to wildtype mice, SHP-$1^{-/-}$ mice have been shown to have reduced footpad lesions and significantly lower parasite loads when infected with *L. major*, and this was associated with increased neutrophil recruitment and augmented iNOS expression in the site of infection. These findings go hand in hand with the ability of SHP-$1^{-/-}$ mice to produce higher amounts of several pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α, and chemokines in response to *Leishmania* infection or LPS stimulation, compared to wildtype mice. Taken together, these observations confirm the role of SHP-1 in the negative regulation of several leishmanicidal molecules produced by macrophages (MØs), therefore providing evidence that help explain why the activation of SHP-1 by *Leishmania* is crucial to the parasite.

Interestingly, accumulating evidence from our laboratory suggest that SHP-1 might have an important role to play in the regulation of TLR signalling. This conclusion is based on two main observations: Firstly, the previously mentioned finding that SHP-$1^{-/-}$ mice, and MØs derived from them, produce higher amounts of LPS-induced NO and pro-inflammatory cytokines, which are functions mediated by TLR signalling. Secondly, the previously reported roles for SHP-1 in the direct or indirect regulation of MAPKs and the transcription factors (TFs) NF-κB and AP-1, all of which are critical proteins involved in TLR signalling.

In Chapter 2, we explored the possibility that SHP-1 is a negative regulator of TLR signalling and found out that this was the case. We demonstrated that SHP-1 was able to

directly bind to IL-1-receptor associated kinase 1 (IRAK-1) causing the inhibition of its kinase activity. Given the key role of IRAK-1 in the activation of signalling downstream most TLRs, we were able to show that one mechanism by which *Leishmania* is known to block LPS-mediated functions in MØs is indeed the inactivation of IRAK-1. We further identified the binding site of SHP-1 on IRAK-1 to be an immunoreceptor tyrosine-based inhibitory motif (ITIM)-like motif, which we termed kinase tyrosine-based inhibitory motif (KTIM). Sequence analysis that we performed revealed that IRAK-1's KTIM emerged in early vertebrates and was evolutionarily conserved up to humans.

In Chapter 3, we pursued the new regulatory concept of KTIM. The high conservation of this motif in IRAK-1 suggested to us that it was subjected to a remarkable evolutionary pressure due to its useful function, leading to the stability of its amino acid components. It was therefore very plausible that this motif could represent a novel regulatory mechanism used by a wide range of kinases. Towards that end, we analyzed the sequences of key cellular kinases and indeed found that many of them contain one or more potential KTIMs, the majority of which were strikingly found in the kinase domain, as in IRAK-1. We then followed the relative time in which those KTIMs appeared and found that, consistent with what we observed in IRAK-1, most KTIMs appeared at the early vertebrate level (fish or amphibian). Interestingly, the KTIMs in JNK and p38 appeared at the invertebrate level providing insights about different potential roles of this motif when present in invertebrates (as invertebrates do not have SHP-1).

Having explored the modulation of IRAK-1 signalling by *Leishmania*, we wanted to build a more comprehensive view of how other signalling molecules are altered by the parasite. A considerable amount of work on how *Leishmania* can interfere with many signalling

molecules of the host such as protein tyrosine phosphatases (PTPs) and TFs has been done with the promastigote stage of the parasite. This is important in understanding the events occurring at the time of disease onset, but does not help in understanding the evasion mechanisms utilized by the *Leishmania* form found in established infections, the amastigote stage. Although quite a number of studies have been done using amastigotes, comparative studies between the two forms is lacking.

In Chapter 4, we performed a comparative study between the ability of promastigotes and amastigotes to alter MØ signalling and function. The study helped to confirm some previous findings that dealt with either promastigote or amastigote infections only, but in a comparative context. In addition, the study revealed new stage-specific evasion mechanisms and some that are shared by both. Importantly, we reported, for the first time, that amastigotes of *Leishmania* were able to activate host PTPs including SHP-1 as early as promastigotes and that they did not seem to activate PTP-1B in early infection time. We also uncovered interesting similarities and differences in the way the two stages modulate the iNOS-binding TFs: NF-κB, STAT-1α, and AP-1. Finally, the role of the parasite's cysteine proteinase (CP) lmcpb was explored, and data implicating this CP in the inhibition of the three previously-mentioned TFs was obtained, further increasing our knowledge about this proteinase as a *Leishmania* virulence factor.

Taken together, we believe that the data presented in this thesis provide new insights into molecular and cellular mechanisms that can help explain the long-observed ability of *Leishmania* to inhibit LPS-mediated MØ functions. This study also challenges the notion that ITIMs are negative regulatory motifs strictly associated with transmembrane proteins by providing strong evidence that ITIM-like motifs (KTIMs) can be found in cytosolic

kinases, and are able to regulate their activity. Such a finding broadens our knowledge about immunoregulatory motifs and opens the door to the discovery of novel substrates of SH2-domain-containing proteins like SHP-1. Finally, our comparative study between promastigotes and amastigotes helps to expand our knowledge about similar mechanisms used by *Leishmania* to alter MØ signalling, but more importantly identify stage-specific differences that could contribute to effective drug design, especially since amastigotes are the diagnostic stage of the parasite in infected organisms.

Chapter 1

Literature Review

1- Abstract

Apart from the impact of *Leishmania* on world health, leishmaniasis represents an elegant infection model that can teach us a lot about host-parasite interactions and immune evasion. This parasite has the ability to enter host macrophages (MØs) safely and replicate inside the very same phagocytes that were recruited to destroy it. The inability of MØs to kill the parasite and activate cells of the adaptive immune system is a product of the parasite's long-reported capacity to alter several key signalling pathways in the host. Many signalling alterations are seen early in the course of infection suggesting they start upon the initial contact between the parasite and the MØ. These rapid alterations of signalling pathways serve at least two main functions: Firstly, inhibition of MØ killing mechanisms that are triggered upon phagocytosis of foreign particles (e.g. production of reactive oxygen species) and secondly, inhibition of leishmanicidal functions that can be triggered in response to MØ activation in infected tissues in response to stimuli such as lipopolysaccharides (LPS) or interferon-γ (e.g. nitric oxide production). In this literature review, we will discuss the roles of promastigotes and amastigotes of *Leishmania* in disease establishment, focusing on the signalling pathways that they interfere with and the MØ functions that are affected by the alteration of these pathways. We then pay special attention to Toll-like receptor signalling and its role in the triggering of innate immunity and in the activation of the adaptive immune system. Finally, we devote the last section of this review to discuss the various known mechanisms by which pathogens are able to interfere with Toll-like receptor signalling to prevent the activation of immune cells via these evolutionarily-conserved receptors whose main function is to detect pathogens and contribute to their clearance.

2- Leishmaniasis

2.1 Epidemiology

Leishmaniasis is a protozoan disease widespread in more than 80 countries in tropical and subtropical regions of the world. It is estimated that over 12 million people are infected world-wide with two million new clinical cases emerging every year [1]. This disease continues to be a major health concern due to the fact that the majority of affected areas are poor developing countries that often lack the funds and/or awareness to fight it. This results in the lack of serious efforts and effective methods to treat infected individuals and to control the spread of the insect vector and animal reservoirs. This situation is further complicated by the lack of an effective vaccine against human leishmaniasis.

2.2 Life Cycle of *Leishmania*

The *Leishmania* parasite alternates between two main forms: the extracellular promastigote and obligate intracellular amastigote stages. Promastigotes are elongated flagellated forms transmitted by bites of female sandflies (*Phlebotomus* or *Lutzomyia* species). While having a blood meal, the infected sandfly injects a small number of infectious-stage, metacyclic promastigotes into the skin. Upon their entry, promastigotes find their way into mammalian MØs where they enter and transform to smaller, round, aflagellated amastigotes. Amastigotes start dividing within the phagolysosome and ultimately lyse the MØ and proceed to infect other MØs or be picked up by another sandfly during blood feeding where they transform again in the midgut of the fly to rapidly dividing non-infectious procyclic promastigotes (Figure 1) [2].

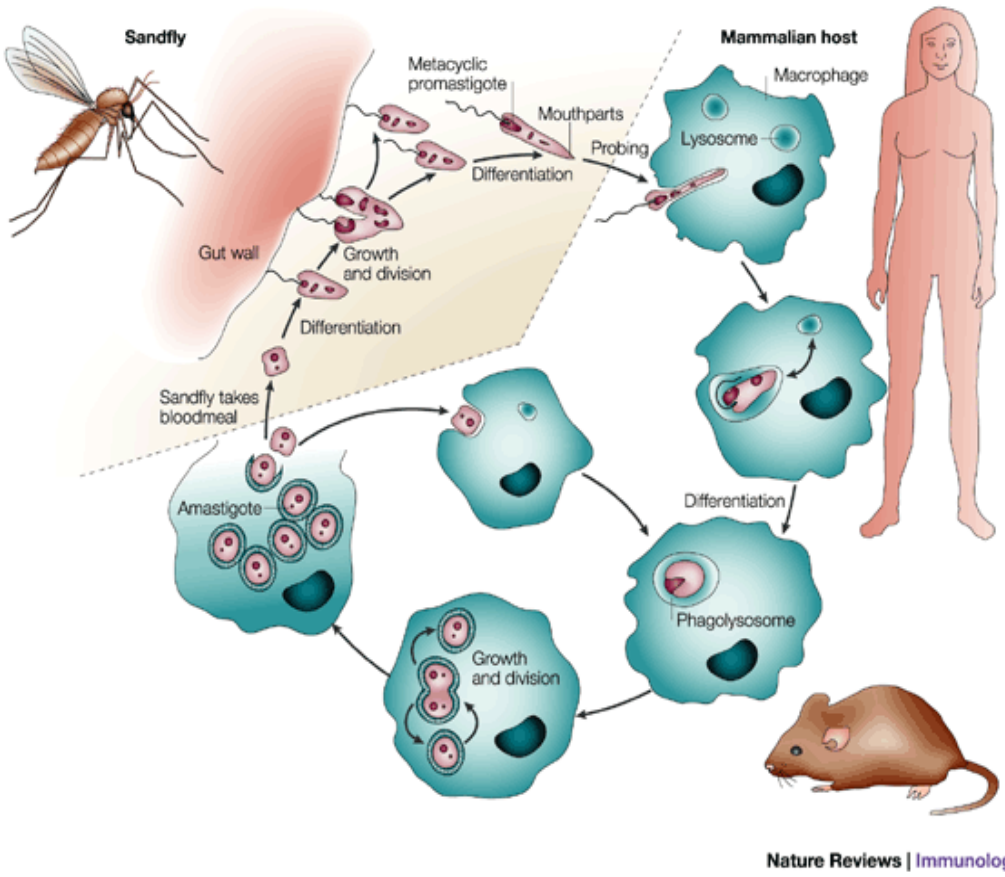


Figure 1: Life cycle of *Leishmania*. A schematic explaining the life cycle of *Leishmania* alternating between the promastigote stage in the sandfly and the amastigote stage in host macrophages. (Adapted from Sacks D *et al.* (2002) Nat. Rev. Immunol. 2: 845-858)

In addition to being morphologically different, the two life stages of the parasite have different surface molecule compositions. While infectious metacyclic promastigotes have a thick glycocalyx, this cover is almost completely absent in amastigotes [3]. The glycocalyx is made of glycoproteins and other glycosylated species anchored to the surface membrane by a glycosylphosphatidylinositol (GPI) linkage [4]. The promastigote

surface is predominantly covered by lipophosphoglycan (LPG), a GPI-anchored molecule made of repeating units of a disaccharide and a phosphate. Buried in a sea of LPG, promastigotes have another important GPI-anchored molecule, the surface protease glycoprotein 63 (gp63). Interestingly, amastigotes have been shown to produce very little LPG compared to promastigotes [5], and have reduced gp63 production [6]. Although LPG and gp63 are down-regulated by amastigotes, other proteins such as cysteine proteinases (CPs) are upregulated in the amastigote stage of several *Leishmania* species [7] indicating that some of these peptidases might play a crucial role in the intracellular survival of the parasite. Despite several differences in surface composition, promastigotes and amastigotes both express glycosylinositol phospholipids (GIPLs), a GPI-linked glycolipid. GIPLs have a small size compared to LPG, are located close to the parasite membrane, have a long half-life, and are believed to play a protective role on the parasite surface [8].

2.3 Disease manifestations

Depending on the species of *Leishmania* involved and the immune response of the host, the parasite can cause disease that can manifest itself in three main forms:

1- Cutaneous leishmaniasis: caused by parasites such as *L. tropica, L. major, and L. aethiopica* in the old world, and *L. mexicana* in the new world. Two to eight weeks following a bite from an infected sandfly, a red papule appears at the site of the bite. The lesion becomes irritated, with intense itching, and begins to enlarge and ulcerate. Ultimately, the patient can either mount a response where the ulcer can self-heal

leaving a disfiguring scar or can have an anergic response where the nodule grows and spreads over large areas of the skin [9].

2- Mucocutaneous leishmaniasis: caused by parasites of the *L. brazilensis* complex. Initially, mucocutaneous leishmaniasis manifests itself in a way similar to that of cutaneous leishmaniasis except that weeks to years following the healing of the skin ulceration, lesions start appearing in mucocutaneous tissues mainly in the mouth and nose causing disfiguring facial mutilation [9].

3- Visceral leishmaniasis: caused by parasites such as *L. donovani donovani* and *L. donovani infantum* in the old world, and *L. donovani chagasi* in the new world. The incubation period may be several weeks to a year, with a gradual onset of fever, diarrhea, and anemia. Chills and sweating that may resemble malaria symptoms are common early symptoms. As organisms proliferate and invade cells of the liver and spleen, hepatomegaly, splenomegaly, and weight loss occur. Kidney damage may also happen as cells of the glomeruli become infected. When the disease persists, patients develop granulomatous areas in their skin known as post-kala-azar dermal leishmaniasis. If untreated, visceral leishmaniasis develops into a debilitating lethal disease that can kill the patient within one to two years [9].

2.4 *Leishmania* virulence factors

In order to survive in the sandfly midgut and inside the mammalian host, *Leishmania* requires a set of virulence factors that aid its survival. We will hereby discuss the major ones.

1- **LPG**: It is the major surface glycoconjugate of promastigotes. It consists of a polymer of repeating Galβ1, 4Man-PO4 units attached to a glycan core that is inserted into the membrane via a phosphatidylinositol anchor. The molecule has a terminal branch of a small oligosaccharide structure which varies among species [10]. Although the backbone of the repeating units, the glycan core, and the lipid anchor are conserved among *Leishmania* species, they differ in the additional oligosaccharide chains branching off the backbone sugars [10]. One important feature that draws the attention to LPG as a virulence factor is its ability to undergo several important modifications during the life cycle of the parasite. As the parasite changes from a non-infective procyclic to an infective metacyclic promastigote in the sandfly midgut, LPG doubles its length by increasing the number of its repeating units [11]. The structural changes of LPG are believed to protect the parasites from the gut's digestive enzymes and help the infectious ones detach from the sandfly's midgut and migrate to the salivary glands so they can be transported into the mammalian host during the fly's blood meal [12]. As promastigotes enter the mammalian host, LPG has been shown to play a key role in protecting the parasite against complement-mediated lysis [13] while maintaining the ability to bind members of the complement system [13,14] and C-reactive protein [15] which helps in the opsonization of the parasite and its uptake by MØs through complement receptors (CRs) and C-reactive protein receptors (CRPRs), respectively. Interestingly, it has also been shown that LPG protects parasites from the oxidative burst by blocking protein kinase C (PKC) activity [16] and scavenging hydroxyl radicals and superoxide anions [11]. Furthermore, this surface glycoconjugate was shown to inhibit phagosome maturation and phagolysosome formation in MØs [17,18]. Other reported functions of LPG include its ability to inhibit IL-12 synthesis

and release at the transcriptional level [19] and its ability to reduce inducible nitric oxide synthase (iNOS) mRNA expression and nitric oxide (NO) production when incubated with MØs prior to IFN-γ stimulation [20]. Although the requirement of LPG for virulence varies from one species of *Leishmania* to another, it was demonstrated that LPG-defective mutants of *L. donovani* and *L. major* are destroyed following phagocytosis and that they are able to survive following the restoration of LPG expression using genetic complementation of the LPG gene [21,22]. Despite the many functions performed by LPG to help in the survival of promastigotes, this molecule is strongly down-regulated in amastigotes, with the exception of *L. major* which has been shown to express LPG that is structurally different from its promastigote counterpart [23]. Nevertheless, the down-regulation of LPG in the amastigote stage of most *Leishmania* species suggests that it is dispensable for amastigote survival, most probably due to the presence of other mechanisms utilized by this form of the parasite to survive the harsh environment of MØs.

2- **GP63**: It is a zinc-dependent metalloprotease, found on the surface of the *Leishmania* parasite, that has a wide range of substrates including casein, gelatin, albumin, haemoglobin, and fibrinogen [24]. This protease belongs to the metzincin class [25] whose members include a sequence motif HExxHxxGxxH, and an N-terminal pro-peptide that renders the proenzyme inactive during translation, and is removed during maturation and activation[26]. Gp63 is abundant in promastigotes but has been shown to be down-regulated in amastigotes [6]. Nevertheless, it is speculated that the reduced expression of gp63 might be compensated for by the absence of LPG on the amastigote surface [3,27] where gp63 is no longer buried in a sea of LPG and can

therefore play an important role in the ability of amastigotes to modulate the host response despite its lower numbers compared to promastigotes.

Given its presence on both forms of the parasite, gp63 is likely to play different roles depending on the parasite stage. For instance, promastigotes of *L. amazonensis and L. major* have been shown to utilize their gp63 to cleave C3b into iC3b and therefore help the parasite avoid complement-mediated lysis [28]. Generation of iC3b can also act as an opsonin aiding the parasite to interact with MØs through complement receptors 1 and 3 (CR1 / CR3) [29,30]. Gp63 also interacts with the fibronectin receptor (FR) and can thus help the parasite adhere to MØs that way [31]. Interestingly, incorporating gp63 into liposomes was able to protect intraliposomal serum bovine albumin from degradation. This observation can suggest a similar mechanism whereby gp63 can help amastigotes survive within the harsh environment of MØ phagolysosomes [32,33]. Work in our laboratory has shown that gp63 plays an important role in the cleavage and activation of several MØ protein tyrosine phosphatases (PTPs) including the Src homology 2 domain-containing protein tyrosine phosphatase 1 (SHP-1) (M.A. Gomez and M. Olivier, manuscript under review). This novel role for gp63 can help explain the rapid ability of *Leishmania* to activate SHP-1 and negatively regulate several key signalling pathways in MØs [34].

3- **Cysteine proteinases (CPs)**: They are mostly studied in *L. mexicana*, which was shown to exhibit high activity of CPs. This family includes: *L. mexicana* cysteine proteinase a (lmcpa), *L. mexicana* cysteine proteinase b (lmcpb), and *L. mexicana* cysteine proteinase c (lmcpc). lmcpb is a cathepsin L-like CP whose genes are multicopy and occur in a tandem array of 19 genes [7]. *lmcpa* (cathepsin L-like) and

lmcpc (cathepsin C-like), on the other hand, are single copy genes [35,36]. Although lmcpb is expressed in metacyclic promastigotes, which might indicate a role for these CPs in the virulence of this life-cycle stage [37], the expression of this cysteine proteinase is significantly increased in amastigotes [7].

The roles of lmcpa, lmcpb, and lmcpc in *Leishmania* virulence was studied through the generation of mutants deficient for the *cpa*, *cpb*, and *cpc* genes. Although lmcpc did not act as a virulence factor, lmcpa and lmcpb did. *L. mexicana* deficient in the multicopy *cpb* gene array had reduced virulence with poor lesion growth in BALB/c mice [38,39]. The lmcpa was also implicated as a virulence factor based on the observation that *lmcpa / lmcpb* double null mutant parasites were less infective to BALB/c mice than lmcpb mutants only. This was concluded based on the observation that the inoculation of double mutants did not result in the formation of skin lesions in infected mice while *lmcpb* mutants caused a delayed appearance (week 37) of very small lesions [39].

Several studies have suggested methods by which lmcpb can act as a virulence factor. In fact, a role for lmcpb was proposed in the degradation of inhibitory kappa B- alpha (IκB-α), inhibitory kappa B- beta (IκB-β), and nuclear factor kappa B (NF-κB) in MØs. This degradation was not seen with lmcpb mutants and was reversed when CP inhibitors were used [40]. The authors concluded that this could represent one mechanism by which *L. mexicana* can inhibit Lipopolysaccharide (LPS)-mediated IL-12 production in MØs. Another interesting report provided evidence that *L. amazonensis* amastigotes were able to internalize major histocompatibility complex (MHC) class II molecules found in the parasitophorous vacuoles of their host cells and

degrade them within their megasomes in order to block antigen (Ag) presentation [41]. The role of CPs in the degradation of these molecules was confirmed when more MHC class II accumulation was observed inside amastigotes pre-treated with an irreversible CP inhibitor. In addition to the role of lmcpa and lmcpb as virulence factors, these proteinases can act as immunomodulators favouring a non-healing Th2 response during the course of infection. It has been shown that lmcpb inoculated into footpads of BALB/c mice increases IL-4 in the draining lymph nodes and polarizes splenocytes towards a Th2 response measured by their increased IL-5 production compared to controls [42]. This increase in IL-4 production was paralleled by an lmcpb-dependent reduction in the expression of both the IL-2R on activated T cells and the low-affinity IgER on mature resting B cells, and an increase in plasma IgE levels in mice injected with active recombinant lmcpb for two weeks [42]. It was also shown that wildtype (WT) but not *lmcpa / lmcpb* double mutants can induce IL-4 production in splenocytes cultured from BALB/c mice. The double mutants but not WT parasites, on the other hand, induced IL-2 production [39].

4- **Acid phosphatases**: These phosphatases are membrane-bound or secreted by pathogenic species of *Leishmania* and are also found to be constitutively released into the culture medium during *in vitro* growth [43]. One of the well-studied acid phosphatases is the histidine secretory acid phosphatase (SAcP) produced by *L. donovani* [44]. This acid phosphatase has been shown to dephosphorylate a wide range of substrates including glycerol phosphates, mono- and di- phosphorylated sugars, inositol phosphates and phosphorylated proteins [45-49]. Hydrolysis of such substrates by SAcP could generate essential nutrients and/or modify the host environment to the

advantage of the parasite [50]. These modifications of host environment include the ability of acid phosphatases to inhibit toxic oxidative metabolite production by neutrophils [51].

2.5 *Leishmania* modes of entry to macrophages

Phagocytosis by host MØs is a prerequisite to successful infections by promastigotes and amastigotes. Promastigotes have to do so upon entry to the mammalian host and amastigotes when they rupture infected MØs and go on to infect other ones. It is generally accepted that entry of *Leishmania* to host MØs is a receptor-mediated event, and several receptors have been shown to play a role in this process. We will hereby discuss the main ones whose involvement in parasite attachment and entry has been well-established and supported by a respectable amount of previous work.

1- **Complement receptor (CR)**: Serum complement has been shown to improve parasite adhesion to MØs [29]. Importantly, the binding of the third component of complement to the parasite helps in its adhesion to host complement receptors [52]. Human MØs have two classes of complement receptors: CR1 (CD35) and CR3 (Mac-1, CD18/11b), which bind to C3b and iC3b, respectively [53]. The adhesion of parasites to CR1 is transient because of the factor I cofactor activity of CR1 and the parasite's gp63, which are both able to cleave C3b to iC3b [28,54]. Based on this cleavage, the main complement receptor involved in the phagocytosis of *Leishmania* appears to be CR3 and not CR1. In addition to helping the parasite get phagocytosed, C3 fixation on parasites has been shown to aid in the intracellular survival of the parasite through enabling it to block the MØ respiratory burst [55].

2- **Mannose-fucose receptor (MFR)**: Sugar receptors play an important role in the recognition of pathogens due to their ability to detect carbohydrate moieties expressed on the surface of those organisms. One Important receptor implicated in the phagocytosis of *Leishmania* is the MFR. This receptor mediates the uptake of mannose- and fucose- terminated glycoproteins [56]. Further evidence regarding the ligand specificity of this receptor was obtained when rat fibroblasts transfected with mannose receptor c-DNA were rendered able to endocytose and degrade mannose-BSA in a specific manner [57]. The MFR was first identified in MØs [58], but was afterwards found to be expressed on other cell types such as dendritic cells (DCs) [59]. Upon binding of the ligand to the MFR, the receptor is rapidly internalized, the ligands are released into early endosomes and are quickly seen to co-localize with MHC class II-enriched compartments and lysosomes while the receptors recycle to the surface [60]. As LPG of *Leishmania* contains terminal mannose sugar moieties, it is not surprising to find out that the MFR can mediate the binding of the parasite to MØs [61]. This has been shown by reversing parasite binding (attachment and ingestion) through the use of soluble inhibitors of MFR activity, namely mannan and ribonuclease B [61]. It is noteworthy that this receptor plays a more important role in promastigote binding to MØs since it is this parasite stage that primarily expresses LPG on its surface.

3- **C-reactive protein receptor (CRPR)**: C-Reactive Proteins (CRPs) are acute phase proteins mainly secreted by the liver. They are named so because they were initially discovered to be able to bind to and precipitate the C-polysaccharide of the *Streptococcus pneumonia* cell walls due to their ability to bind phosphorylcholine, a

component of the C-polysaccharide, which they bind to in a calcium-dependent manner [62]. Indications that CRP can bind to *Leishmania* were supported by observations whereby CRP was found to bind some promastigotes in a calcium-dependent manner [63]. In fact, it was later found that the repeating phosphorylated disaccharides of LPG in metacyclic *L. donovani* promastigotes but not *L. major* promastigotes were responsible for the binding to CRP [15]. It has been suggested that the modifications of the LPG sugar backbone in the metacyclic stage might explain why CRP binds to metacyclic but not procyclic promastigotes. As for why CRPs bind to *L. donovani* but not *L. major* promastigotes, the substitutions that take place in the 3-position of the galactose of LPG of *L. major* that are known not to take place in *L. donovani* have been proposed to mediate the decreased binding affinity of CRP to LPG of *L. major* [15]. The discovery of LPG as a ligand of CRP directly implicated the CRP receptor in the binding and uptake of the *Leishmania* parasite. It is very interesting to note that although *Leishmania* is able to exploit CRP as an opsonin, the entry of the parasite via the CRPR does not trigger MØ activation as would normally happen in CRP-mediated uptake of other particles. It has been demonstrated that the CRP-mediated entry of *Leishmania* does not affect parasite survival in MØs and does not lead to the production of pro-inflammatory cytokines such as TNF-α and IL-12 [64].

4- **Fibronectin receptor (FR)**: FRs belong to the β1 integrin family, with two abundant members VLA-4 and VLA-5 [65,66]. It has been shown that gp63 can exhibit fibronectin-like properties and interact with the FR [31]. This interaction suggests a role for the FR in the adhesion of *Leishmania* to MØs and in their uptake as seen by

the slower and decreased parasite internalization upon the use of antibodies directed against β1 integrins [31]. The fact that parasites still manage to ultimately get internalized in the absence of functional fibronectin receptors suggests that these receptors might not be the primary mediators of parasite adhesion but can facilitate the process by stabilizing complement-mediated adhesion of parasites to MØs through the ability of the FR to bind to gp63 of *Leishmania* [31]. The binding of gp63 to integrins represents yet another mechanism by which gp63 can help in parasite adhesion and entry, the first being its ability to cleave complement components and use the cleaved forms as opsonins to adhere to MØs through CRs.

5- **Fc receptor (FcR):** These are protein receptors found on several immune cells including MØs and have binding affinity to the Fc portion of immunoglobulins. Initial experiments showed that opsonising *Leishmania* with parasite-specific IgG significantly enhanced the ability of promastigotes to enter MØs but did not have any enhancing effect on the ability of amastigotes derived from BALB/c mice to do so [67]. The initial conclusion was that the FcR might not play a role in amastigote infection. Further experiments revealed that the reason IgG opsonisation did not augment the entry of amastigotes is the fact that amastigotes derived from mice lesions are already highly coated with immunoglobulins. Interestingly, amastigotes harvested from bone-marrow derived MØs (BMDMs) cultured *in vitro* or those collected from SCID mice, have enhanced entry to MØs in the presence of specific IgG due to the absence of immunoglobulins on the surface of these amastigotes prior to the opsonisation step [67]. It is important to mention that amastigotes are still able to enter MØs in the absence of immunoglobulins as opsonins suggesting a role for other

receptors including CRs. Based on receptor blocking experiments, it appears that amastigotes have the ability to switch binding receptors based on availability. Blocking the FcR or CR3 one at a time is not sufficient to observe a strong decrease in amastigote entry, and only when both receptors are blocked simultaneously that we observe a drastic decrease in amastigote entry to MØs [67]. It remains to be mentioned that entry by FcR seems to be an important mechanism by which the already established amastigotes can enter and infect new cells, but most likely is not a mechanism utilized by promastigotes as when they first enter the host, no specific antibody response against the parasite is present.

6- **Phosphatidylserine receptor (PSR)**: This is a receptor that detects phosphatidylserine (PS) on apoptotic cells, therefore enabling phagocytes to clear them in a non-inflammatory fashion. PS has been reported to be expressed on the surface of amastigotes in what is referred to as "apoptotic mimicry". By expressing PS, amastigotes of *L. amazonensis* have been shown to be able to bind to MØs through the PSR, mimicking apoptotic cells and therefore triggering an anti-inflammatory response by inhibiting NO production and increasing IL-10 and transforming growth factor-beta (TGF-β) secretion [68,69]. By doing so, amastigotes are able to more readily attach to and infect MØs while avoiding any harmful inflammatory responses. It is worth mentioning that PS has been regarded as a contributor to amastigote virulence as it has been shown that amastigotes derived from susceptible BALB/c mice display more PS than those derived from resistant C57BL/6 mice [68].

3. Alteration of macrophage signalling and functions by *Leishmania*

3.1 Signalling molecules altered by *Leishmania*

Cells can alter their physiology and function by activating and inhibiting their intracellular signalling pathways. A typical pathway is usually triggered by a ligand such as a cytokine or growth factor or pathogen-associated molecule. The binding of ligands to their receptors causes activation of the receptor via its phosphorylation and/or conformational change which in turn leads to the activation of second messengers in the cytosol. These second messengers ultimately lead to the activation of several transcription factors (TFs) that can translocate to the nucleus and bind to the promoters of their target genes generating a change in the cell's response. On the other hand, other signalling molecules like phosphatases are able to dephosphorylate molecules and can therefore counteract effects caused by protein phosphorylation. Using this negative feedback loop, the cell can establish a balance between activation and inhibition, and is able to return to the resting state following activation. Interestingly, many pathogens are able to alter the signalling of their target cells to their own advantage and *Leishmania* is no exception. *Leishmania* achieves this by either employing strategies to inhibit proteins that play a positive role in immune cell activation or by activating molecules known to play key roles in the negative regulation of immune cell signalling and function (Figure 2) [34]. We will discuss below the main signalling molecules altered by *Leishmania* in an effort of the parasite to survive inside host MØs.

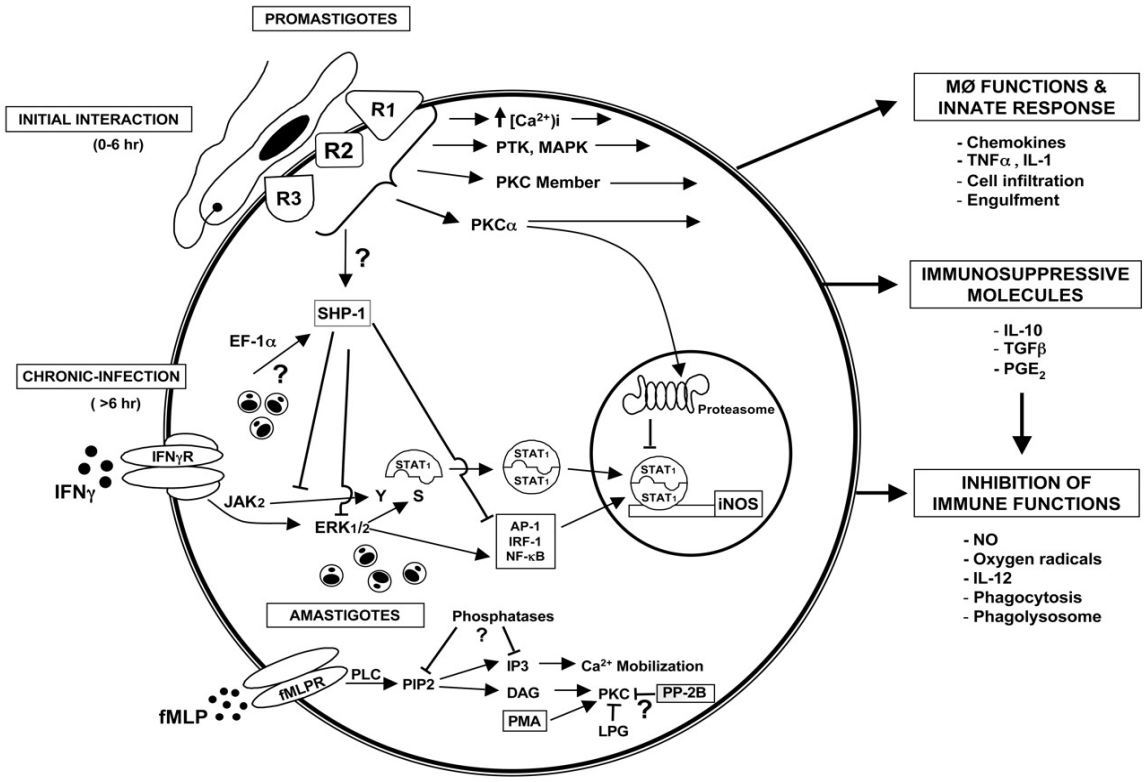


Figure 2: Macrophage signalling and functions altered by *Leishmania*. (Adapted from Olivier M *et al.* (2005) Clin. Microbiol. Rev. 18(2): 293-305)

1- **Protein kinase C (PKC)**: It is a family that comprises 10 serine / threonine kinases, first characterized as Ca^{+2} and phospholipid-dependent [70,71]. The PKC family consists of three sub-families: conventional, novel, and atypical isoforms. Conventional isoforms include: PKC-alpha, -beta I, -beta II, and –gamma and these isoforms require Ca^{+2} and diacylglycerol (DAG) to function. Novel isoforms include: PKC-delta, -epsilon, -eta, and –theta and these isoforms require DAG but not Ca^{+2}. Atypical isoforms include: PKC-zeta and –lambda and these require neither DAG nor Ca^{+2} [72]. PKC signalling plays a key role in the response of MØs to activating cytokines such as IFN-γ and TNF-α [73,74], cytokines which have important roles in driving several MØ functions including NO production [73] and oxidative burst [75].

Given the leishmanicidal effect of these functions, it is remarkable that *Leishmania* is able to block PKC activity in infected MØs. Promastigote LPG has been described to be able to block PKC activity [16,76,77]. This inhibition is achieved through the binding of LPG to the regulatory domain of PKC which contains the DAG, Ca^{+2}, and phospholipid binding sites [11]. It is interesting to observe that amastigotes, which lack LPG, are also able to inhibit PKC activity in monocytes [78], suggesting that factors other than LPG can also mediate this inhibitory effect. Indeed, *Leishmania*-induced ceramide generation [79] and GIPLs [77] have been shown to be able to inhibit PKC, providing a possible mechanism by which amastigotes can inhibit the activity of this kinase.

2- **Janus kinase 2 (JAK2)**: It is one of four members of the Janus family of tyrosine kinases (JAK1, JAK2, JAK3, TYK2). As with the other members of the JAK family, JAK2 has two kinase-homologous domains at its C-terminus, the first is non-catalytic and has a regulatory function and the second exhibits kinase activity. JAK activation plays an important role in cell proliferation, differentiation, migration, apoptosis, as well as immune activation [80]. The JAK signalling pathway is initiated when a ligand (cytokine or growth factor) binds to its receptor inducing receptor multimerization. This process brings the cytoplasmic domains of the receptor subunits -which are associated with JAKs- into close proximity to each other, allowing the JAKs to transphosphorylate and therefore activate each other. Activated JAKs are then able to phosphorylate the receptors themselves on a conserved tyrosine residue (Tyr-440 in the case of IFNγRI) providing a docking site for the TF signal transducer and activator of transcription (STAT). JAKs then phosphorylate the docked STATs on a conserved

tyrosine near their C-terminus. Phosphorylated STATs are then able to dimerize with the help of their conserved SH2 domains and proceed to translocate to the nucleus to bind their target regulatory sequences to activate or repress transcription [80,81].

The promoter of the iNOS gene responsible for NO production has binding sites for several TFs including STAT-1 [82,83]. Given the leishmanicidal effect of NO as a free radical, it is not surprising that *Leishmania* has the ability to block the JAK/STAT signalling pathway in response to IFN-γ stimulation. Indeed, it has been reported that infection with *L. donovani* amastigotes was able to block IFN-γ-induced JAK1, JAK2, and STAT-1 phosphorylation in PMA-differentiated U-937 promoncytic cells and human monocytes [84]. Our laboratory has gone further in studying the effect of *Leishmania* on JAK2 phosphorylation by reporting that *L. donovani* promastigotes were able to rapidly activate host SHP-1 and that this activation was associated with increased binding of SHP-1 to JAK2 and the subsequent inhibition of the phosphorylation of this kinase in response to IFN-γ stimulation [85]. Another study suggested that the *Leishmania*-induced unresponsiveness to IFN-γ stimulation can be due to the inhibition of the IFN-γ receptor (IFN-γR) complex formation. The authors observed decreased phosphorylation of IFN-γR-α and decreased association of the receptor with JAK2 caused by a downregulation of the receptor expression itself, but provided no clues on how the parasite could do so [86]. Another complication with this report is that the authors infected cells for 24 hours to see an appreciable effect on receptor expression and phosphorylation, which cannot explain the rapid dephosphorylation of JAK2 seen when BMDMs are infected with *Leishmania* promastigotes [85]. This supports the notion that early JAK/STAT inhibition must

depend on parasite-induced alterations of existing signalling molecules of the host and not on alterations at the transcriptional level.

3- **Mitogen-activated protein kinases (MAPKs)**: These are serine / threonine kinases activated by phosphorylation on two residues (threonine and tyrosine) in their kinase activation loop. These two residues are separated by a single residue unique for each group of MAPKs: glutamate for the extracellular signal-regulated kinase1/2 (Erk1/2), proline for Jun N-terminal kinase (JNK), and glycine for p38 [87]. The signalling cascade usually starts by an external stimulus causing the activation of a MAPK kinase kinase (MAPKKK) which is a serine / threonine kinase. The MAPKKK in turn phosphorylates a MAPK kinase (MAPKK) which is a dual specificity kinase that can phosphorylate both the threonine and tyrosine found in the T-X-Y motif found in the activation loop of MAPKs. Phosphorylation of the threonine and tyrosine causes a conformational change in the activation loop allowing it to clear the ATP binding site of MAPKs which it obstructs in the inactive state thus activating the kinase [88]. Upon their activation, MAPKs can phosphorylate cytosolic targets or translocate to the nucleus to phosphorylate TFs and thus can directly affect gene expression. We will hereby discuss the three main MAPKs in terms of their signalling roles and then review some of the mechanisms by which *Leishmania* can interfere with their activity.

Erk1/2: Upon its activation by a MAPKK such as MEK1/2, Erk1/2 is able to phosphorylate more than 70 different substrates including TFs [89]. Erk1/2 has the ability to translocate to the nucleus where it is able to phosphorylate several members of the Ets family of proteins in the transactivation domain [90]. Activated Ets1/2, for example, interact with activating protein-1 (AP-1) and NF-κB in binding to gene

promoters therefore enhancing transcription [87]. Other substrates of Erk1/2 from the Ets family include Elk-1 and the SRF accessory protein-1 (SAP-1) which play an important role in the regulation of c-fos (a subunit of AP-1) transcription [91]. Erk1/2 can also activate Fra1/2 [92], therefore having yet another way to activate AP-1.

JNK: JNK was first cloned as a kinase that phosphorylates and activates the Jun AP-1 subunit [93], but was soon found to have a wider range of substrates. There are three JNK genes that can give rise to several splice variants [94]: JNK1 and JNK2 which are widely expressed, and JNK3 which is found only in brain tissue. JNK is activated by MAPKKs such as MKK4 and MKK7 [87] and activated JNK can phosphorylate c-Jun in addition to its ability to phosphorylate activating transcription factor-2 (ATF-2) on threonines 69 and 71 preventing its ubiquitination and degradation [95]. The phosphorylation of c-Jun has a direct effect on AP-1 activation while phosphorylation of ATF-2 enhances its ability to dimerize with c-Jun to drive c-Jun expression [96], or dimerize with ATF-6 to mediate signal transduction of stress signals associated with protein misfolding [87].

p38: This MAPK contains at least four members: p38α and p38β which are ubiquitously expressed, p38γ found in skeletal muscles, and p38δ found in the prostate, testes, pancreas, and salivary, pituitary, and adrenal glands [97]. One of the main targets of p38 is the MAPK-activated protein kinase 2 (MAPKAP-K2) [98] which is a kinase able to phosphorylate several TFs such as: ATF-2 and ATF-1/cAMP response element binding (CREB) [87] which is a TF that binds to c-AMP-regulated enhancer (CRE) regions in cAMP-inducible genes like c-fos [99]. Notably, MAPKAP-

K2 is also able to phosphorylate the SRE-binding TF serum response factor (SRF) that forms a complex with Elk-1 and regulates c-fos gene expression [87,100].

In addition to playing important roles in the proliferation and differentiation of cells, MAPKs play a crucial role in the activation of immune cells including MØs through their ability to activate several TFs that control pro-inflammatory mediators [101]. As was the case with the JAK family, it is remarkable, though not unexpected, that the *Leishmania* parasite developed tactics to render several MAPK members inactive in response to parasite entry to MØs or to activating stimuli that follow infection.

Indeed, it was reported that the phagocytosis of *L. donovani* promastigotes by naive MØs does not lead to the activation of any of the three MAPKs (Erk1/2, JNK, p38) [102]. Additionally, several MAPKs have been shown to be inhibited in infected cells in response to activating stimuli such as LPS. For example, *L. amazonensis* amastigotes are able to block LPS-mediated Erk1 phosphorylation in infected MØs [103] and *L. donovani* amastigotes can block PMA-induced Erk1/2 phosphorylation in RAW264 MØs leading to the inhibition of Elk-1 and c-fos expression [104]. The authors of the latter study suggested a role for host PTPs in Erk1/2 inactivation, a hypothesis supported and more deeply explored by our laboratory where we provided evidence that PTP-SHP-1 is able to dephosphorylate and inactivate Erk1/2 through demonstrating that this MAPK was still able to be activated in *Leishmania*-infected SHP-1-deficient MØs in response to IFN-γ stimulation [105]. Furthermore, it has been suggested that the increased ceramide production in *L. donovani*-infected MØs can lead to reduced Erk1/2 phosphorylation ultimately leading to enhanced parasite survival [106]. Interestingly, amastigotes of *L. mexicana* were also reported to inhibit

Erk1/2 signalling not by inhibiting their phosphorylation but rather by degrading them using the parasite's cysteine proteinases. Similar cysteine proteinase-dependent degradation was observed for JNK [40].

Concerning p38, it has been shown that this MAPK is non-responsive when MØs infected with *L. major* are stimulated with a CD40 antibody to mimic the MØ-T cell interaction. p38 inactivation correlated with impaired iNOS2 expression and NO production and therefore impaired leishmanicidal functions [107]. In fact, this inactivation makes sense in the light of experiments showing the importance of p38 activation in the control of *Leishmania* infection. The use of anisomycin, a p38 activator, enhanced parasite killing in MØs by triggering p38-dependent anti-leishmanial effects [107,108].

4- **Transcription factors (TFs)**: In order to inhibit gene expression of pro-inflammatory cytokines and microbicidal molecules, *Leishmania* developed several strategies to interfere with TFs that bind to the promoters of those genes. Several TFs are involved in this process including: NF-κB, STAT-1α, and AP-1, all of which have been shown to be modulated by the parasite. We will hereby discuss the role of each of these TFs in signalling and describe the known mechanisms by which the parasite is able to interfere with their functions.

NF-κB: This TF is composed of five subunits: p65 (RelA), RelB, c-Rel, p50 (NF-κB1), and p52 (NF-κB2). These subunits associate together as homo- or heterodimers forming NF-κB [109]. Each subunit has three distinct structures: a Rel homology

domain used for DNA binding, a dimerization domain, and a nuclear localization signal [110]. In addition to these structures p65, Rel B, and c-Rel have a transactivation domain suggesting their role in transcription activation [109]. NF-κB is held in an inactive state by binding to inhibitory kappa B (IκB) [111]. NF-κB is released from its inhibitor when IκB gets phosphorylated by IκB kinases (IKK), which are a family of three proteins: IKK-α and IKK-β, which phosphorylate IκB, and IKK-γ, which serves as a regulatory subunit of the IKK complex [109].

There exist two distinct pathways for NF-κB signalling: the classical pathway and the alternative pathway [110]. The classical pathway involves activation of receptors such as p55TNFR, IL-1R, or TLR. This results in the activation of IKK-β which in turn phosphorylates IκBα and leads to its polyubiquitination by an E3-ubiquitin ligase causing its degradation and the release of p65/p50 dimers that are now free to translocate to the nucleus to drive gene expression [112]. The alternative pathway, on the other hand, involves the activation of receptors such as B cell-activating factor receptor (BAFFR), CD40, and lymphotoxin beta receptor (LTBR) [113]. This activates NF-κB-inducing kinase (NIK) which phosphorylates IKK-α which in turn phosphorylates p100 that gets processed to a p52 subunit. This pathway leads to the accumulation of RelB/p52 dimers in the nucleus that drive gene expression [109].

NF-κB has more than 150 target genes, many of them code for inflammatory cytokines, chemokines, immunoreceptors, and cell adhesion molecules. Examples of these genes include: IFN-γ, IL-1, IL-6, IL-12, MIP-1α, MIP-1β, MIP-2, MCP-1, RANTES, and MHC class I [114]. It is therefore no surprise that this transcription factor is referred to as a "central mediator of the human immune response" [114].

Several groups have reported different strategies employed by *Leishmania* to alter NF-κB. *Leishmania*-induced ceramide generation by MØs was shown to play a role in NF-κB inhibition [106]. One study provided evidence that *L. major* amastigotes blocked the nuclear translocation of the p65/p50 complex selectively favouring the c-Rel/p50 complex that they proposed plays a role in the gene expression of immunosuppressive cytokines in MØs such as IL-10 [115]. Another study showed that cysteine proteinases of *L. mexicana* mediated NF-κB degradation and caused its inability to bind its DNA consensus sequence, thus partially explaining how the parasite can inhibit LPS-mediated IL-12 production [40]. Work from our laboratory showed that promastigotes of several pathogenic *Leishmania* species were able to cleave the p65 RelA subunit to generate a p35 RelA fragment that is able to translocate to the nucleus and bind DNA. This p35 fragment was suggested to be involved in the parasite's ability to drive NF-κB-mediated chemokine gene expression in infected MØs [116].

STAT: the STAT family consists of STAT-1, STAT-2, STAT-3, STAT-4, STAT-5α, STAT-5β, and STAT-6 [117]. These STATs are activated by distinct cytokines, for example, STAT-1 mediates responses to IFN-γ, STAT-3 to IL-10, STAT-4 to IL-12, and STAT-6 to IL-13 [118]. The signalling cascade involving STATs has been discussed previously in the JAK section. Briefly, upon the binding of the appropriate ligand to its receptor, JAKs associated to the cytoplasmic portion of the receptor will get activated and phosphorylate the receptor on a conserved tyrosine residue. This phosphorylation provides a docking site for STATs, which bind via their SH2 domains. STATs are phosphorylated by JAKs and are able to dimerize and translocate to the nucleus where they can activate gene expression [117].

We have previously described the ability of *Leishmania* to inhibit the JAK/STAT pathway through SHP-1-mediated JAK2 dephosphorylation [85]. Interestingly, our laboratory has also reported that the parasite is able to repress IFN-γ-mediated signalling in MØs through its ability to interfere with STATs. *L. donovani* promastigotes were shown to be able to cause proteasome-dependent STAT-1 degradation in infected MØs. However, whereas STAT-1 degradation was reversed using proteasome inhibitors [119], its capacity to respond to IFN-γ was still altered due to JAK2 inactivation (unpublished data).

AP-1: AP-1 is a structurally complex TF whose dimers are principally made of proteins belonging to the Jun (c-Jun, JunB, JunD) and fos (c-fos, fosB, Fra-1, Fra-2) subfamilies, although proteins of the Maf and ATF families can also participate in the formation of the AP-1 complex [120]. While members of the Jun subfamily can homodimerize, members of the fos subfamily have to dimerize with members of other subfamilies, mainly the Jun subfamily [120].

AP-1 can be activated by many kinds of stimuli such as growth factors, cytokines, hormones, and pathogens, which do so using several signalling molecules. PKC has been shown to play a role in c-fos expression [121]. Furthermore, MAPKs play a key role in AP-1 activation. Erk1/2 for example, can activate AP-1 either by activating elk which binds to and activate the c-fos gene [91] or by directly phosphorylating Fra1 and Fra2 enhancing their binding to c-Jun [122]. JNK can activate AP-1 by either phosphorylating c-Jun or ATF2 which dimerizes with c-Jun [123]. In addition, p38 is also able to phosphorylate c-Jun [124].

Keeping these AP-1 activators in mind, we can see that the previously mentioned tactics employed by *Leishmania* to interfere with PKC, Erk1/2, JNK, and p38 activities have a direct impact on the ability of the parasite to block AP-1 signalling in MØs. In addition, work from our group demonstrated a role for SHP-1 in AP-1 inhibition [105,125] and a key role for the parasite's surface protease gp63 in the cleavage and degradation of key AP-1 subunits (I. Contreras and M. Olivier, manuscript in preparation). The latter finding provides the first demonstration that a parasite-derived molecule can directly interfere with AP-1 in host MØs in order to block its downstream functions.

5- **Protein tyrosine phosphatases (PTPs)**: PTPs are proteins that have the ability to dephosphorylate substrates and are divided into receptor-like and non-receptor PTPs. Non-receptor PTPs can either dephosphorylate tyrosines only or can possess dual specificity dephosphorylating tyrosines as well as serines / threonines (Figure 3) [126]. One common feature of PTPs is the presence of a PTP catalytic domain in which a critical cysteine is found within a conserved signature motif (I/V)HCxxGxxR(S/T) and mediates the hydrolysis via the formation of a thio-phosphate intermediate [127]. Receptor-like PTPs include: RPTP-α, CD45, and CD148 and the functions of some like CD45 in immune cell signalling is well-known [128]. We will however focus on a selected group of soluble PTPs who have been shown to play a role in *Leishmania* host evasion mechanisms namely: PTP-1B, TC-PTP, PTP-PEST, and most importantly SHP-1.

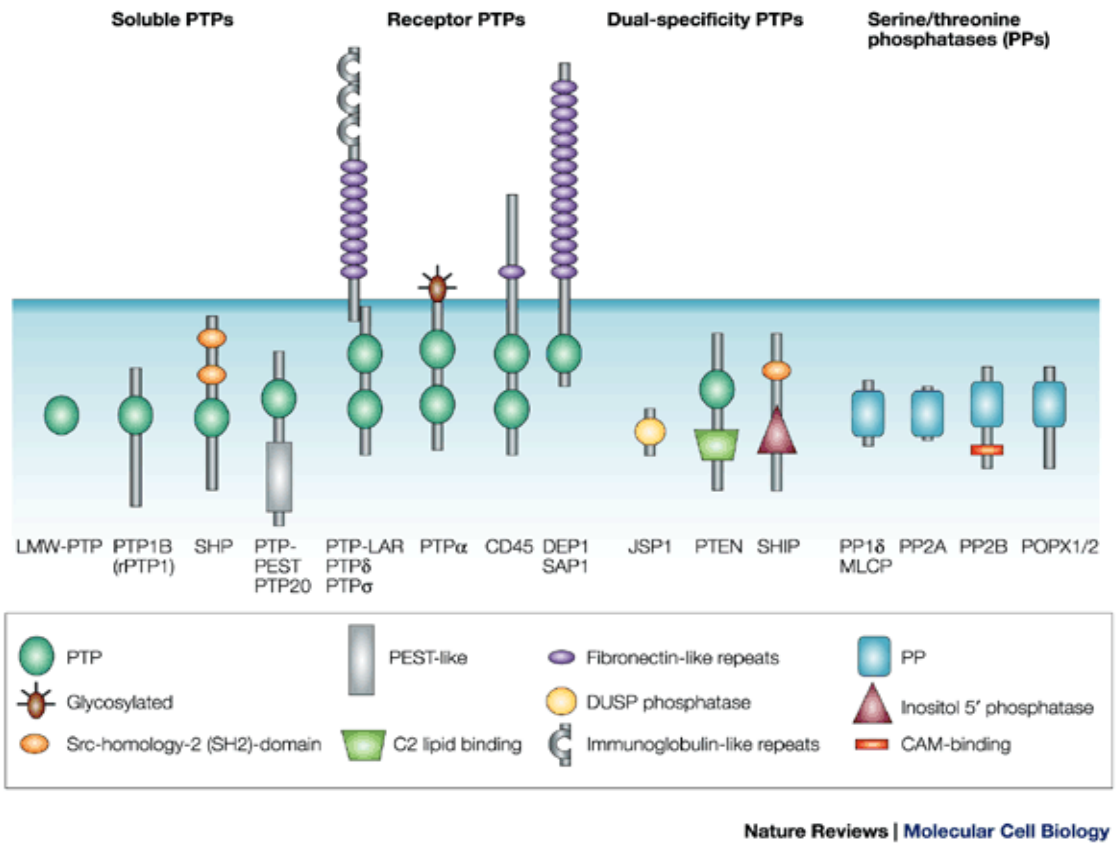


Figure 3: Different phosphatase structures. (Adapted from Larsen M *et al.* (2003) Nat. Rev. Mol. Cell. Biol. 4: 700-711)

PTP-1B and TC (T cell)-PTP: They are two ubiquitously expressed PTPs that have more than 73% identity in their catalytic domain [129]. PTP-1B is known to play important regulatory functions in metabolism, as demonstrated by the insulin hypersensitivity of PTP-1B$^{-/-}$ mice and their resistance to high fat diet-induced obesity [130,131]. This insulin hypersensitivity was shown to be due to the ability of PTP-1B to dephosphorylate the insulin receptor [132]. PTP-1B also seems to play a role in the regulation of cytokine signalling through its ability to interact with and dephosphorylate members of the JAK family namely: JAK2 and TYK2 [133]. In addition to PTP-1B's role in the regulation of JAK/STAT signalling, a role for this

phosphatase in the regulation of TLR4 signalling was proposed. PTP1B$^{-/-}$ MØs had increased LPS-induced iNOS expression and NO production compared to WT MØs and were more susceptible to endotoxic shock following low-dose LPS injection [128]. Given the ability of PTP-1B to regulate several cellular processes including signalling pathways of high importance in MØ function, it is rather surprising that no work has been published on the role that PTP-1B plays during *Leishmania* infection. In fact, very recent work from our laboratory showed that *Leishmania* gp63 was able to enhance PTP-1B activation by cleaving it. PTP-1B activity seems to inhibit MØ activation and help in parasite survival as seen in the delayed onset of footpad swelling and reduced parasite burden in PTP-1B$^{-/-}$ mice infected with *L. major* (M.A. Gomez and M. Olivier, manuscript under revision).

TC-PTP$^{-/-}$ mice suffer from multiple immunological defects and die within five weeks of birth [134]. This observation and the findings that TC-PTP plays important roles in the negative regulation of JAK1, JAK3 [135], and nuclear STAT-1 [136] suggest the important role of this PTP in the regulation of immune responses. As is the case with PTP-1B, it is equally surprising that data is lacking regarding the role of TC-PTP during *Leishmania* infection. Our laboratory has shown that gp63 of *Leishmania* was able to enhance TC-PTP activation by cleaving it in host MØs (M.A. Gomez and M. Olivier, manuscript under revision). This gp63-mediated TC-PTP cleavage along with the cleavage of PTP-PEST were very recently reported, by our group and M.L. Tremblay's group, to occur in fibroblasts infected with *Leishmania* and were suggested to enhance the catalytic activity of the PTPs in question and/or allow them to access additional substrates that might help the parasite establish itself [137].

SHP-1: It is a PTP that contains two N-terminal SH2 domains (N-SH2, C-SH2), followed by a PTP domain responsible for dephosphorylating substrates, and a C-terminal tail [128]. This phosphatase is mostly expressed in hematopoietic cells [138,139], but is also expressed at lower levels in epithelial [139], endothelial [140,141], and central nervous system cells [142]. The SH2 domains have two main functions: Firstly, the N-SH2 domain plays an important auto-inhibitory role by interacting intramolecularly with the PTP domain keeping the PTP in the inactive state. Secondly, both SH2 domains have the ability to bind to phospho-tyrosine (p-Y) residues usually found within immunoreceptor tyrosine-based inhibitory motifs (ITIMs) whose consensus sequence is (I/V/L/S)xYxx(L/V) [143]. This second feature of SH2-domains is thought to play a role in the detachment of the N-SH2 from the PTP domain once the C-SH2 domain binds to a target p-Y, therefore opening up and activating the PTP (Figure 4) [128].

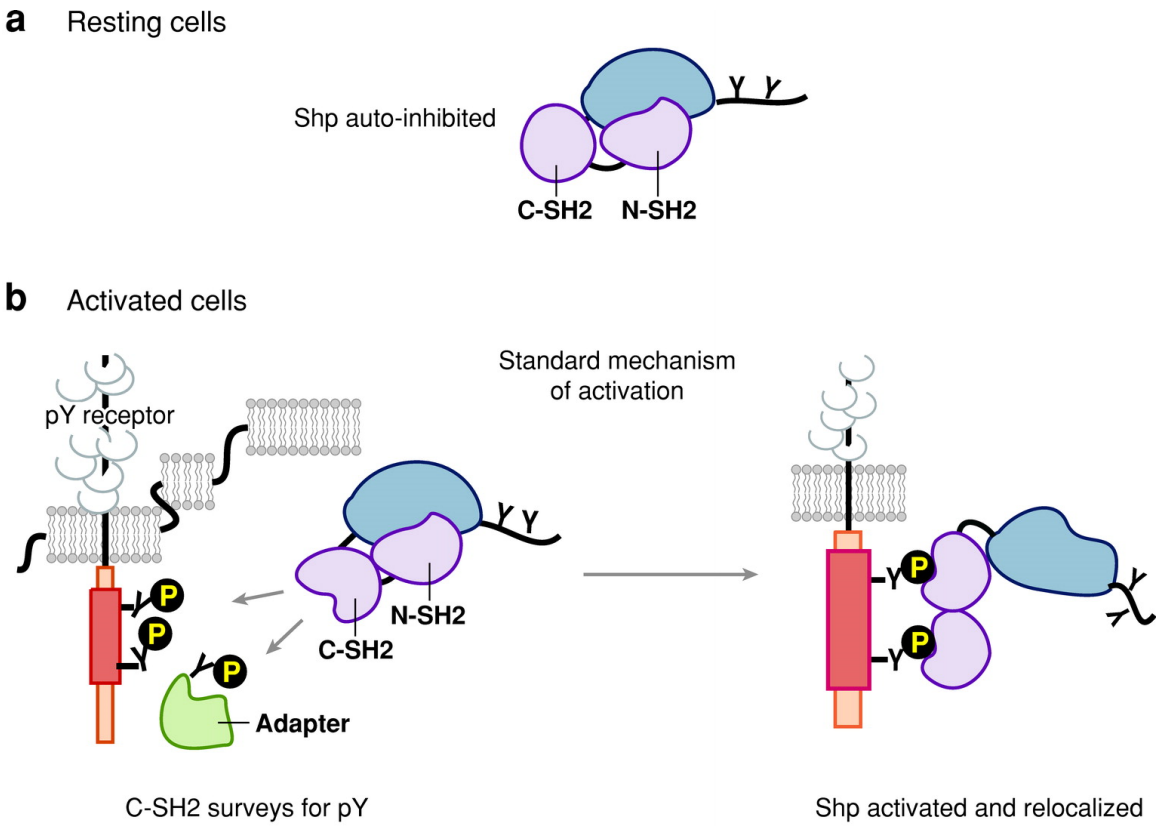


Figure 4: Regulation of SHP-1 by its N-SH2 domain. **(Reprinted, with permission, from the *Annual Review of Immunology*, Volume 25 © 2007 by Annual Reviews www.annualreviews.org)**

SHP-1 plays a key role in immune cell signalling, this is supported by the autoimmune and immunodeficiency syndrome exhibited by SHP-$1^{-/-}$ mice (motheaten mice). These mice are named so because of the patchy hair loss that they suffer from due to displacement of hair follicles by MØs and neutrophils that infiltrate the subepidermal tissue. These myeloid cells also migrate to several other sites causing severe inflammation and tissue damage [128,144]. In addition to its role in BCR [145] and

TCR [146-148] signalling, SHP-1 has been shown to be able to interact with non-receptor targets such as JAK2, JAK3 [149], several MAPKs [150,151], and transcription factors such as STAT [152]. It is therefore an elegant tactic that *Leishmania* activates SHP-1 in infected MØs in order to inhibit several signalling pathways that can otherwise be deadly to the parasite (see figure 2).

At the signalling level, our laboratory has clearly demonstrated that *Leishmania* was able to rapidly activate host SHP-1 causing SHP-1-mediated JAK2 inactivation in MØs [85]. Additionally, we and others have implicated SHP-1 in the negative regulation of Erk1/2 activity [104,105] and in the regulation of the downstream TFs NF-κB and AP-1 [105] during *Leishmania* infection. At the functional level, our laboratory showed that the injection of PTP inhibitors (bis-peroxovanadium compounds) to mice infected with *L. major* or *L. donovani* helped control the infection [153] in a manner dependent on iNOS expression and NO production [154]. Furthermore, we demonstrated that SHP-1-deficient viable motheaten mice, infected with *L. major*, did not develop footpad swelling and had significantly reduced parasitic loads [155]. This decreased pathology was associated with more neutrophil recruitment to the footpad and more iNOS mRNA expression [155].

As to how *Leishmania* is able to activate SHP-1, it has been proposed that *Leishmania*'s Elongation Factor-1α (EF-1α) is responsible for the activation of host SHP-1 seen 16 hours post-infection [156]. This report cannot explain; however, how SHP-1 is activated in earlier infection times nor does it explain how EF-1α of the parasite can shuttle from the phagolysosome where the parasite is to the cytosol where SHP-1 is found. A more plausible mechanism has been recently suggested by our

group, where SHP-1 was shown to be activated via cleavage by the parasite's protease gp63, which gains access to the cytosol by going through the lipid raft of host MØs (M.A. Gomez and M. Olivier, manuscript under review). In conclusion, it appears that the rapid activation of SHP-1 by *Leishmania* is a key host evasion step whereby the parasite is able to utilize this phosphatase to negatively regulate several key MØ pathways and render it unresponsive to activating stimuli such as: IFN-γ and LPS. By doing so, the parasite is able to block several MØ functions such as NO production and the synthesis of many pro-inflammatory cytokines that can be deadly to the parasite if allowed to be produced.

6- **Suppressors of cytokine signalling (SOCS)**: This family is made of eight members: cytokine-inducible SH2-containing protein (CIS) and suppressors of cytokine signalling 1 to 7 (SOCS1-7). These proteins have a central SH2 domain and a SOCS box motif in their C-terminal and have been shown to play an important role in the regulation of over 30 cytokines including IL-6 and IFN-γ [157]. Both SOCS1 and SOCS3 are involved in the negative regulation of JAK/STAT signalling through the ability of both proteins to interact with Y1007 in the catalytic loop of JAK2 [158,159]. SOCS1 and SOCS3 not only bind JAK2 but also have strong binding affinities to phosphotyrsoines located within several receptor subunits such as the IFNγR1 [160] and the IL-12 Rβ2 [161]. SOCS gene expression is driven by JAK/STAT signalling where SOCS can inhibit this pathway in a negative feedback loop. This negative regulation can occur either by direct binding and inhibition of JAK (e.g. SOCS1), SH2-mediated binding of SOCS to the cytoplasmic domain of the receptor, followed by JAK inactivation (e.g. SOCS3), or by competing with STAT SH2 domains in the

binding to critical phosphotyrsoines in the cytoplasmic domains of receptors (e.g. SOCS2). An additional regulation mechanism is the ability of the E3 ubiquitin-ligase complex to bind to the SOCS box motif targeting receptors or receptor-associated proteins to proteasome-mediated degradation [157].

The involvement of SOCS in *Leishmania* infection remains unclear due to the paucity of published research in that subject. One study reported the ability of live and heat-killed *L. donovani*, but not purified LPG, to transiently induce SOCS3 expression in MØs. This expression was independent of phagocytosis and cytokine release by the infected MØs [162]. This report does not explain which parasite component is able to induce SOCS3 expression nor does it demonstrate any downstream functional consequences of this induction. Further work is certainly required to explore the role of SOCS proteins in the inhibition of cytokine signalling in *Leishmania*-infected MØs.

3.2 Macrophage functions altered by *Leishmania*

Modulation of signalling pathways by *Leishmania* are intended to alter critical MØ functions to the advantage of the parasite. Upon the initial contact of *Leishmania* with the MØ, certain functions such as the production of chemokines and chemokine receptors are induced whereas others are inhibited. Among the functions inhibited by the parasite are those related to MØ activation and to their ability to present Ag and communicate with cells of the adaptive immune system. Hereby, we will discuss the main functions that *Leishmania* can interfere with upon initial interaction (0-6 h) or chronic infection (> 6 h) of host MØs.

1- **Induction of chemokine expression**: One of the important early challenges confronted by *Leishmania* is the ability to preferentially recruit cells of the immune system to the site of inoculation in order to infect them and establish disease in the host without getting killed. One key mechanism by which the parasite is able to do so is the induction of chemokine expression and production by host immune cells. One study showed that infection of mice with *L. major* upregulated the gene expression of several chemokines in cells collected from the footpad and their draining lymph nodes. These chemokines were measured by quantitative PCR and RNAase Protection Assay (RPA) and include: RANTES / CCL5, MIP-1α / CCL3, IP-10 / CXCL10, and MCP-1 / CCL2 [163]. In addition, our laboratory has clearly shown that *L. major* infection caused an upregulation in the expression of several chemokines in cells recruited to the air pouch of infected mice. These chemokines include: RANTES, MIP-1α, MIP-1β / CCL4, IP-10, MCP-1, and MIP-2 / CXCL1) [164]. It is interesting to see that most of these chemokines are monocyte chemoattractants, recruiting MØs to infected tissues and helping the parasite get installed. It is equally interesting to see that none of these chemokines, with the exception of MIP-2, attract neutrophils. This is in accordance with our previous finding that neutrophil recruitment to infection sites is associated with parasite killing in SHP-1 deficient viable motheaten mice [155]. In addition, our laboratory has shown that cells recruited to the air pouch in *L. major*-infected mice not only upregulate chemokine expression but also the expression of chemokine receptors including: CCR1, CCR2, CCR3, and CCR5 [164].

So far, we have considered chemokine upregulation as beneficial to the parasite, yet it is important to bear in mind that secreted chemokines during leishmaniasis can act as a

double-edged sword. While early selective activation of monocyte-chemotactic factors can help the parasite recruit MØs that they can infect, treatment of susceptible BALB/c mice infected with *L. major* with recombinant IP-10 in the early course of infection, for example, has been shown to increase NK cell cytotoxic activity in the draining lymph nodes and can drive a healing IFN-γ-mediated Th1 response [165]. In chronic infections, chemokine types, amounts, and duration of chemotactic effect have been implicated in parasite clearance or persistence. For instance, in visceral leishmaniasis, clearance of parasites from the liver is strongly associated with increased late phase IP-10 production and the Th1 effects associated with its presence [165]. Parasite persistence in the spleen, on the other hand, has been correlated with sustained MCP-1 but not IP-10 levels [166].

2- **Inhibition of microbicidal free radical production**: One of the dangers that *Leishmania* encounters recruiting and entering MØs is the ability of these cells to produce free radicals that are deadly to the parasite. Two main free radical molecules have been shown to have leishmanicidal effects: NO [167] and reactive oxygen intermediates (ROIs) [168]. NO is produced by NOS which converts one of the terminal nitrogens of the guanidino group of L-arginine to NO producing L-citrulline [169,170]. The importance of this free radical in leishmaniasis was demonstrated by several groups. An early study showed the ability of activated MØs to kill *L. major* amastigotes by an L-arginine-dependent mechanism [171]. Another study confirmed this observation by showing that L-N-monomethyl arginine (L-NMMA), an L-arginine analogue and inhibitor of the NO pathway, was able to inhibit the leishmanicidal effect of MØs activated *in vitro* with IFN-γ or LPS. The authors also showed the ability of

NO in cell-free suspensions to kill the parasite. Importantly, the same group demonstrated the importance of NO *in vivo* by rendering resistant CBA mice susceptible to *L. major* infection upon local administration of L-NMMA [167].

The question that comes to mind next is: how can NO contribute to parasite killing? Modes of action seem to include the ability of NO to cause modifications of proteins, injury to mitochondria, oxidation of membranes, DNA damage (e.g. depurination), modulation of cytokine production, and interference with maturation of immune cells [172]. Examples of *Leishmania* proteins inactivated by NO include the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [173] and the Krebs cycle enzyme, aconitase, which NO can inhibit through triggering iron loss from its iron-sulfur prosthetic group [174].

Having discussed the detrimental effects of NO on *Leishmania*, it is comprehensible that the parasite is able to block its synthesis in response to stimuli such as IFN-γ [20], but how can *Leishmania* achieve this inhibition? A critical role for host SHP-1 has been proposed. As previously stated, *Leishmania* has the ability to rapidly activate SHP-1 in infected MØs and by doing so can interfere with several molecules involved in NO production including: JAK2, Erk1/2, and the TFs NF-κB and AP-1. Indeed, SHP-1 deficient MØs infected with *L. donovani* are still able to produce NO in response to IFN-γ stimulation unlike infected WT MØs which are refractory to a similar stimulation [105]. Expectedly, the IFN-γ-mediated NO production in infected SHP-1 deficient MØs correlated with successful phosphorylation of JAK2 and Erk1/2, and the activation of NF-κB and AP-1. These findings further elucidate the role of

SHP-1 activation in parasite survival and propagation through its ability to contribute to NO inhibition [105].

We have previously mentioned that the iNOS promoter contains binding sites for NF-κB, STAT-1α, and AP-1. It therefore follows that all *Leishmania*-mediated signalling alterations of these TFs or of signalling molecules that activate them may contribute to NO inhibition in response to activating stimuli. We have discussed many of these alterations in the previous section, but will reiterate the ones of direct relevance to NO inhibition. These include: the ability of the parasite to inhibit PKC activity, inhibit MAPK activation, inhibit LPS-mediated NF-κB activation, induce ceramide formation, and cause proteasome-mediated STAT-1α degradation. Additionally, the *Leishmania*-induced production of the immunosuppressive molecules IL-10 and TGF-β by MØs may contribute to the ability of the parasite to inhibit NO [175,176].

ROIs represent another source of danger to *Leishmania*. These intermediates include the superoxide radical and hydrogen peroxide produced by cells of the immune system such as: neutrophils and MØs in response to phagocytosis of foreign particles. Although important in parasite killing, the activity of the respiratory burst in mice was shown to have an early and transient effect only. This conclusion is based on the delayed granuloma formation and resolution of infection seen in respiratory burst-deficient X-CGD mice infected with *L. donovani* compared to WT [177]. Despite the critical role that NO seems to play in *Leishmania* killing [177], ROIs do contribute to parasite clearance and are therefore a target to be inhibited by the parasite. Indeed, *L. donovani* has been shown to inhibit the oxidative burst in infected MØs [78,178,179], and this inhibition was mediated by the parasite surface molecules LPG and gp63

[11,180] and involve PKC inactivation [78]. Interestingly, it was later shown that LPG of *L. donovani* promastigotes is able to block NADPH oxidase assembly at the phagosome membrane without interfering with p47(phox) phosphorylation and its ability to form complexes with p67(phox) [181]. *L. donovani* amastigotes, on the other hand, were shown to effectively block superoxide release through inhibiting the phosphorylation of the NADPH oxidase component p47(phox), leading to defective recruitment of p47(phox) and p67(phox) to the phagosome [182]. The inhibition of p47(phox) phosphorylation could be a result of the previously reported ability of *Leishmania* amastigotes to inhibit PKC activity [78], which is reported to be required for p47(phox) phosphorylation [183].

3- **Inhibition of pro-inflammatory cytokine production:** We will focus in this section on three main cytokines: IL-1, TNF-α, and IL-12. IL-1 and TNF-α have been correlated with antimicrobial activities against bacteria and parasites *in vitro* and *in vivo* [184-187], and IL-12 is well-known for its ability to promote Th1 differentiation and to activate NK cells [188]. Concerning IL-1 and TNF-α, it has been shown that these molecules are not produced upon a 12 h *in vitro* infection of human monocytes with *L. donovani* amastigotes [189]. Interestingly, preinfection of those cells diminished LPS-mediated IL-1 production but not IL-1 m-RNA, suggesting inhibition at the translational level [189]. Another study showed that pre-incubation of human monocytes with purified LPG was able to cause inhibition of LPS-mediated IL-1β secretion [190]. The role of LPG in IL-1β inhibition was later shown to involve the ability of LPG to inhibit the transcription of the IL-1β gene in a manner dependent on the nucleotide region -310 to -57 of the promoter region [191]. This inhibitory effect of

LPG on IL-1β gene transcription was suggested to involve an inhibition of the binding of an activation factor or an induction of an unknown transcription repressor [191]. Interestingly, an *in vivo* study using the mouse air pouch system showed that *L. major* was able to cause production of IL-1, IL-6, and TNF-α in the early stage of infection, an event that was less notable with *L. donovani* [192]. These findings could help explain the different pathologies caused by both species including the ability of *L. donovani* to visceralize and the restriction of *L. major* to the inoculation site. A later study by our laboratory clearly demonstrated that SHP-1 deficient mice stimulated with LPS or infected with *L. major* produced significantly higher amounts of IL-1, IL-6, and TNF-α compared to their littermates [164]. This suggests that SHP-1, activated by *Leishmania*, plays a pivotal role in the attenuation of the inflammatory response via repressing the production of these pro-inflammatory cytokines possibly contributing to parasite survival and pathogenesis.

IL-12 is another key molecule inhibited by *Leishmania*. This inhibitory effect is necessary for parasite survival given the established role of this molecule in driving Th1 differentiation and production of IFN-γ by T cells and NK cells, which in turn can activate MØs to kill the parasite. It has been reported that infection of BMDMs with promastigotes of *L. major* or *L. donovani* fails to induce IL-12 production both following infection alone and upon subsequent LPS or heat-killed bacterial stimulation of MØs [193]. Similar observations were seen when murine MØs were infected with amastigotes of *L. major* and *L. mexicana* [194]. Furthermore, incubation of activated murine MØs with LPG led to the inhibition of IL-12 production by these cells, with the inhibition occurring at the transcriptional level [19]. The mechanism by which IL-

12 is inhibited by *Leishmania* remains unclear. Roles for the MØ CR3 [195] and FcγR [196] have been proposed. Additionally, we have described in the previous section the role of *L. mexicana*'s cysteine proteinases in the cleavage of NF-κB and the possible involvement of this process in the inhibition of LPS-induced IL-12 production by infected MØs [40].

4- **Production of immunosuppressive molecules:** In addition to being able to suppress pro-inflammatory molecules like IL-1, IL-6, IL-12, and TNF-α, *Leishmania* is also able to induce the production of immunosuppressive molecules that can help further the survival and propagation of the parasite. Three immunosuppressive molecules have been reported to play important roles in disease establishment: prostaglandin E2 (PGE2), TGF-β, and IL-10.

PGE2 is produced from arachidonic acid with the help of enzymes known as cyclooxygenases (COXs). COXs have two isoforms: the constitutive COX-1 and the inducible COX-2 [197]. PGE2 has been demonstrated to inhibit MØ activation through interfering with several functions including generation of oxygen radicals, expression and release of LPS-induced TNF-α, and response to several pro-inflammatory cytokines [198]. It is therefore understandable why *Leishmania* has been reported to induce PGE2 production in infected MØs [199-201]. Following this line of thought, our laboratory showed that *Leishmania* was able to increase PGE2 production in infected MØs through its ability to induce COX-2 expression in a PKC-dependent manner [202]. This finding raises the interesting notion that PKC seems to be rapidly and transiently activated upon *L. donovani* infection resulting in increased PGE2 synthesis, and then gets inactivated afterwards to inhibit some of the PKC-dependent

leishmanicidal functions described earlier in the literature review under the PKC section. Another interesting report correlated PGE2 production with increased visceralization of *L. donovani* in malnourished mice [203].

TGF-β is a potent suppressor of the immune system, with effects on a wide range of immune cells including: MØs, DCs, NK, and T cells [204]. Given the important role of these cells in the progression of *Leishmania* infections, it is not surprising to find out that several species of the parasite are able to induce TGF-β production and promote an anti-inflammatory environment [205]. This *Leishmania*-induced increase in TGF-β production has been correlated with reduced iNOS expression in immune cell infiltrates found in the skin lesion and draining lymph nodes of infected mice [176] and with impaired IL-12-driven IFN-γ production and cytotoxic abilities of NK cells derived from mice infected with *L. major* [206]. In support of the previous roles of TGF-β in suppressing various innate and adaptive immune functions during leishmaniasis, one study reported high levels of TGF-β in the local environment surrounding *L. chagasi*-infected MØs [207]. Furthermore, the same group showed that *in vitro* infection of human MØs with *L. chagasi* was associated with an increase in the biologically active form of TGF-β rather than an increase in m-RNA or total protein levels [207]. *L. chagasi*'s cysteine proteinase cathepsin B was implicated in the production of active TGF-β from the latent form [207], a mechanism reported to be shared by the *L. donovani* complex [208]. Additionally, as previously described under the phosphatidylserine receptor section, interaction of PS-expressing amastigotes with the PSR has been shown to trigger TGF-β production in infected MØs [69].

IL-10 is yet another anti-inflammatory cytokine induced by *Leishmania*. This cytokine has established roles in the inhibition of effector functions of key immune cells, importantly: T cells and monocytes / MØs. The principal function of this cytokine is to control and ultimately terminate inflammatory responses [209], making the induction of this cytokine of clear benefit to *Leishmania*. Ligation of the FcγR seems to play a role in the induction of IL-10 in MØs [210]. The binding of amastigote PS to its receptor can also trigger IL-10 production [69] as previously mentioned in the PSR section. The production of IL-10 leads to some similar consequences as those encountered by TGF-β production, importantly the ability to inhibit MØ activation and NO production [175]. In addition, IL-10 suppresses the production of pro-inflammatory cytokines such as: IL-1, IL-12, and TNF, and the expression of costimulatory molecules such as B7-1 and B7-2 [175]. The role of IL-10 in the progression of leishmaniasis *in vivo* is established based on reports where resistant mice were rendered more susceptible to *L. major* infection when they were designed to express an IL-10 transgene [211], and other reports where susceptible BALB/c mice were rendered more resistant to infection when their IL-10 gene was knocked-out [212]. All this accumulating data suggest IL-10 induction to be one effective mechanism used by *Leishmania* to evade MØ activation and killing.

5- **Interference with antigen presentation:** MØs are antigen presenting cells (APCs) that can link innate and adaptive immunity by phagocytosing foreign objects, digesting them in lysosomes, and coupling pathogen-associated Ags to MHC II molecules and presenting them on their surface to CD4+ T cells (T helper cells). The activation of T helper cells causes activation of other immune cells including B cells, cytotoxic CD8+

T cells, and MØs. Therefore, and in order to persist inside MØs and cause chronic infections, *Leishmania* had to develop ways to interfere with the ability of these cells to present foreign Ags.

One remarkable tactic the parasite utilizes is its ability to inhibit IFN-γ-induced MHC class II expression in infected MØs. Indeed, *L. chagasi* and *L. donovani* were both shown to inhibit MHC II expression in response to IFN-γ stimulation [213-215]. Surprisingly, MØs infected with *L. major* or *L. amazonensis* showed normal phagocytosis, Ag processing, and MHC II production, yet these cells failed to present parasitic Ags to T cell hybridomas [216,217]. Authors of both studies concluded that the failure to present Ags to T cells is due to the parasite's ability to interfere with the loading of Ags onto MHC II molecules. Another interesting mechanism to control Ag presentation was presented previously in the section related to cysteine proteinases of *Leishmania*. Briefly, amastigotes of *L. amazonensis* have been shown to be able to internalize MHC II molecules and to degrade them using their cysteine proteinases [41].

Activation of CD4+ T cells involves a "two-signal model" whereby two signals are required to activate the T helper cell. The first signal is triggered by the binding of the T-cell receptor (TCR) to the MHC II-Ag complex on the APC, and the second is provided by the binding of CD28 or CD40L on T cells to costimulatory molecules of APCs such as those of the B7 family or CD40. Interestingly, apart from interfering with the first signal by inhibiting MHC II presentation, *Leishmania* has been demonstrated to interfere with MØ costimulatory signals. *L. donovani* infection was reported to block LPS-mediated B7-1 expression in infected MØs [218], a mechanism

that seems to be mediated by prostaglandins [219]. Furthermore, *L. major* was reported to interfere with CD40 signalling in infected MØs in a p38-dependent manner [107]. This result is very interesting, especially that previous studies have established a protective role for CD40 in *Leishmania major* infections [220,221], while others have reported that the disruption of CD40 / CD40L ligation results in increased susceptibility to *L. amazonensis* infection [222]. The increased susceptibility caused by the disruption of CD40 / CD40L ligation was in part due to the inhibition of iNOS expression [220,222] and IL-12 production [223] by infected MØs.

So far in the previous sections, we have discussed several mechanisms by which *Leishmania* can interfere with key signalling pathways involved in MØ activation such as the JAK/STAT pathway. We also discussed alterations that occur to signalling molecules involved in TLR signalling such as MAPKs and the TFs NF-κB and AP-1. However, this does not give justice to TLR signalling, given its extremely important role in the activation of APCs to kill invading pathogens and/or activate cells of the adaptive immune system. Equally important are the strategies developed by pathogens to block TLR signalling pathways that can lead to undesirable activation of immune functions. We will therefore dedicate the rest of this literature review to discuss TLR signalling and what is known about its modulation by pathogens, with special emphasis on parasites including *Leishmania*, the subject of this thesis.

4. Toll-like receptor (TLR) signalling

4.1 Toll signalling and functions in *Drosophila*

Unlike vertebrates which have innate and adaptive immune systems, invertebrates, including the fruit fly *Drosophila melanogaster*, have no adaptive immune system and rely only on innate immunity to fight off pathogens. The innate immune system of the fruit fly has cellular and non-cellular components. The cellular component being MØ-like blood cells and the non-cellular part being antimicrobial peptides (AMPs) secreted by the fat body (insect equivalent of mammalian liver) into the hemolymph [224]. *Drosophila* has seven different AMPs and their expression is driven by two distinct pathways: the Toll pathway, triggered by Gram-positive bacteria and fungi, and the immune deficiency (IMD) pathway triggered by Gram-negative bacteria [225]. Both pathways lead to the activation of NF-κB, yet of different homologues. While Toll signalling causes the degradation of cactus (insect homologue of mammalian IκB) and the translocation of Dif to the nucleus, the IMD pathway causes the proteolytic cleavage and activation of Relish (insect homologue of mammalian p105 precursor of NF-κB p50), whose N-terminal part can translocate to the nucleus and drive gene expression [226]. The existence of different components in those two pathways suggests a good level of signalling independence and specificity in the response to invading pathogens [227]. While Toll was originally found to be involved in the dorsal-ventral patterning of fruit flies during early embryogenesis, a striking finding was the ability of insect Toll receptors and mammalian TLRs to mediate host defence. The ability of TLR4 to recognize bacterial LPS raised the possibility that *Drosophila*'s Toll was also a pattern-recognition receptor (PRR), but further studies revealed that this was not the case. Unlike TLR4 which can directly detect LPS with the

help of MD-2 and CD14, *Drosophila*'s Toll can detect pathogens through the proteolytic cleavage of a cytokine-like molecule known as spaetzle (Spz). Spz is found in its inactive form in the blood of the fly and is cleaved upon immune challenge by a serine protease cascade. The cleaved form corresponds to the 106 carboxyl-terminal residues and will be referred to as C-106 [228]. The binding of C-106 to Toll causes receptor dimerization and activation of downstream signalling. The cytoplasmic portions of Tolls then interact with the *Drosophila* homologues of the adaptor protein myeloid differentiation factor 88 (dMyD88) through their Toll / IL-1 receptor (TIR) domain [229]. The adaptor protein Tube is then recruited to dMyD88 via a death domain / death domain (DD/DD) interaction allowing it to activate Pelle, the IRAK homologue of *Drosophila.* Pelle activation leads to the activation of *Drosophila* TNF receptor-associated factor 6 (dTRAF6) which, in turn, ultimately leads to the phosphorylation of cactus and the translocation of NF-κB homologues to the nucleus to drive gene expression (Figure 5) [224].

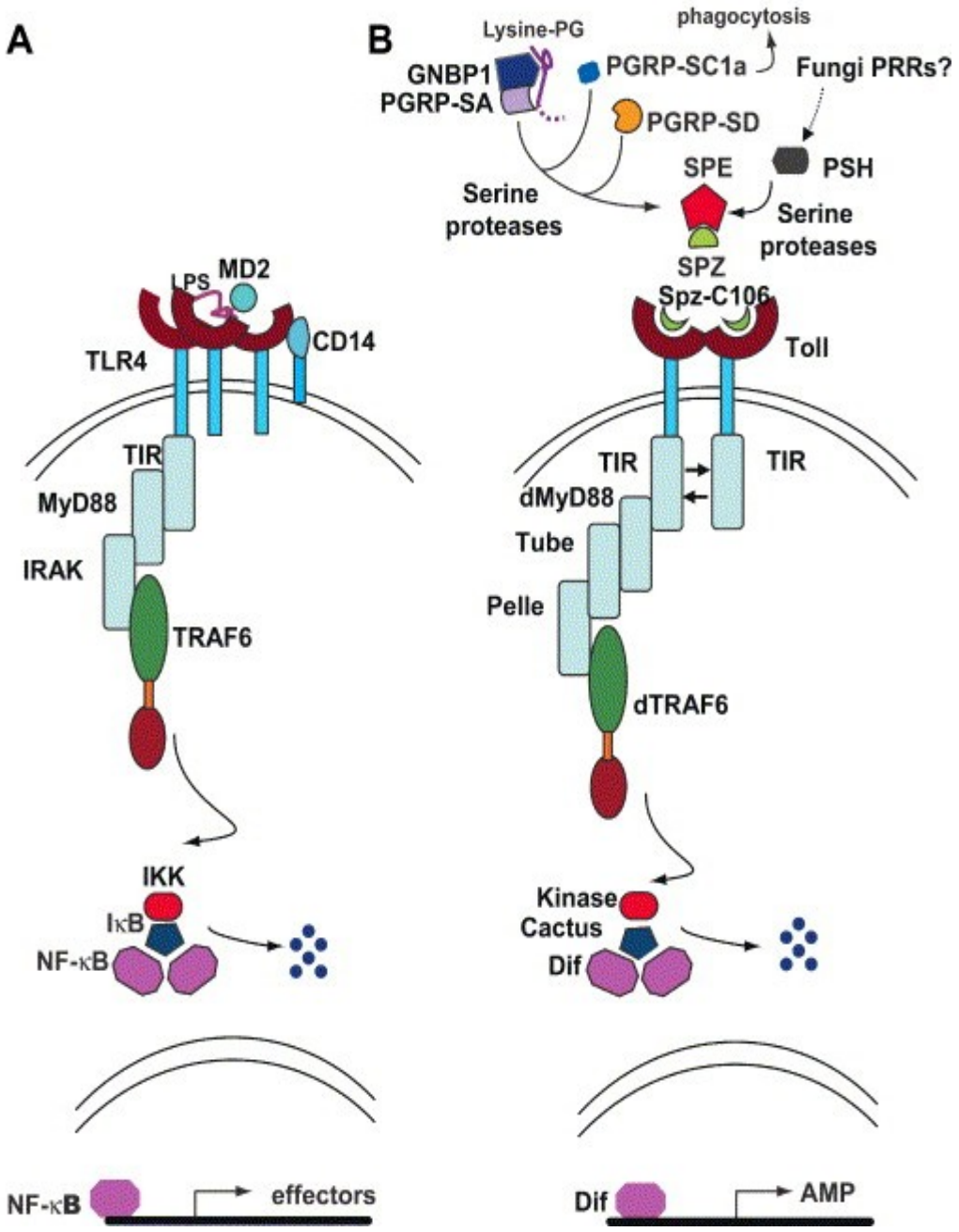


Figure 5: Insect and mammalian Toll and TLR pathways. A: Schematic of mammalian TLR4 signalling. B: Schematic of *Drosophila* Toll signalling. (Adapted from Wang L *et al.* (2006) Immunobiology 211: 251-261)

The IMD pathway, on the other hand, resembles the mammalian TNFR1 pathway. Following the binding of Gram negative peptidoglycans (PGNs) to peptidoglycan recognition proteins (PGRPs), PGRP-LC isoforms dimerize and relay the signal to IMD (insect homologue of mammalian RIP). IMD recruits *Drosophila*'s Fas-associated protein with death domain (DFADD) which interacts with death-related ced-3 / NEDD2-like protein (DREDD), a protein that shares homology with mammalian caspase 8. DREDD is also able to activate *Drosophila* TGF-β activated kinase 1 (dTAK1), a kinase that has the ability to activate the *Drosophila* JNK pathway and the IKK complex (ird5 and Kenny). Activation of IKK ultimately leads to the freedom of Relish to translocate to the nucleus and regulate transcription (Figure 6) [224].

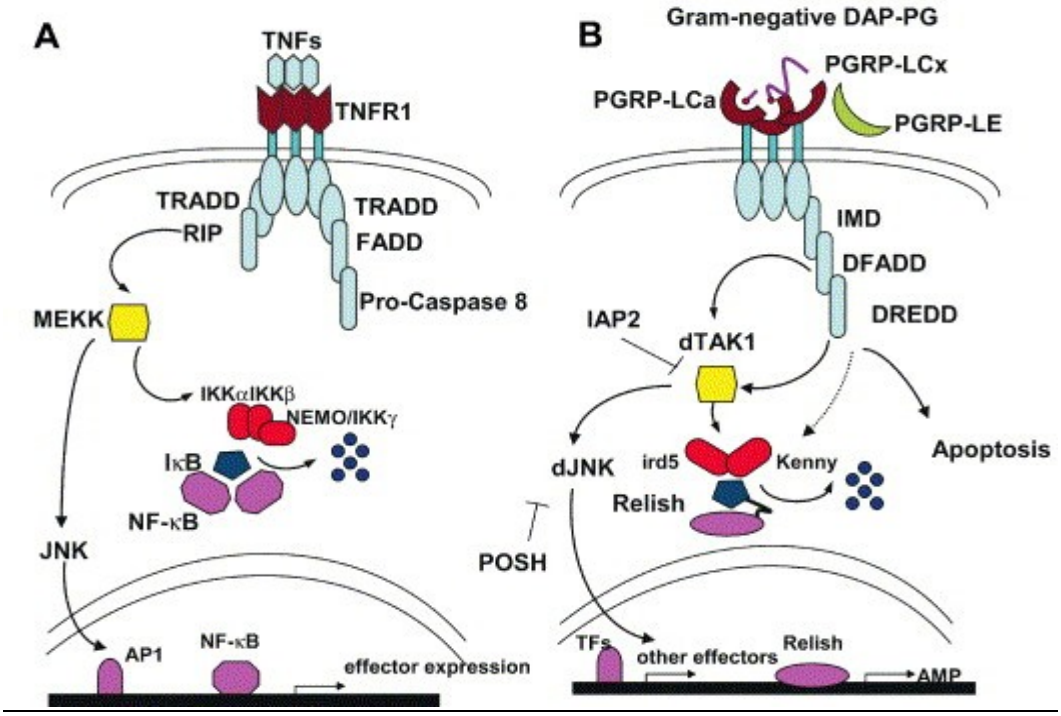


Figure 6: *Drosophila* IMD and mammalian TNFR-1 pathways. A: Schematic of mammalian TNFR-1 pathway. B: Schematic of *Drosophila* IMD pathway. (Adapted from Wang L *et al.* (2006) Immunobiology 211: 251-261)

4.2 TLR signalling and role in innate and adaptive immunity

Similar to Toll receptors in *Drosophila*, organisms from *Caenorhabditis elegans* to *Homo sapiens* have TLRs that are able to sense diverse pathogen-associated molecular patterns (PAMPs) including: proteins, lipids, lipoproteins, and nucleic acids (Table 1) [230].

Microbial Components	**Species**	**TLR Usage**
Bacteria		
LPS	Gram-negative bacteria	TLR4
Diacyl lipopeptides	*Mycoplasma*	TLR6/TLR2
Triacyl lipopeptides	Bacteria and mycobacteria	TLR1/TLR2
LTA	Group B *Streptococcus*	TLR6/TLR2
PG	Gram-positive bacteria	TLR2
Porins	*Neisseria*	TLR2
Lipoarabinomannan	Mycobacteria	TLR2
Flagellin	Flagellated bacteria	TLR5
CpG-DNA	Bacteria and mycobacteria	TLR9
ND	Uropathogenic bacteria	TLR11
Fungus		
Zymosan	*Saccharomyces cerevisiae*	TLR6/TLR2
Phospholipomannan	*Candida albicans*	TLR2
Mannan	*Candida albicans*	TLR4
Glucuronoxylomannan	*Cryptococcus neoformans*	TLR2 and TLR4
Parasites		
tGPI-mutin	*Trypanosoma*	TLR2
Glycoinositolphospholipids	*Trypanosoma*	TLR4
Hemozoin	*Plasmodium*	TLR9

Microbial Components	Species	TLR Usage
Profilin-like molecule	*Toxoplasma gondii*	TLR11
Viruses		
DNA	Viruses	TLR9
dsRNA	Viruses	TLR3
ssRNA	RNA viruses	TLR7 and TLR8
Envelope proteins	RSV, MMTV	TLR4
Hemagglutinin protein	Measles virus	TLR2
ND	HCMV, HSV1	TLR2
Host		
Heat-shock protein 60, 70		TLR4
Fibrinogen		TLR4

Table 1: TLR ligands and their corresponding receptors. (Adapted from Akira S *et al.* (2006) Cell 124: 783-801)

In mammals, TLRs are expressed on several types of immune cells including: MØs, DCs, B cells, and certain types of T cells [231] and therefore play an important role in the triggering of the innate and adaptive immune response. Upon binding of PAMPs to their receptor, signalling pathways that lead to the induction of several pro-inflammatory cytokines (e.g. IL-1β, IL-6, TNF-α, IL12), chemokines, interferons, and co-stimulatory molecules can be activated [230]. The activation of APCs such as MØs not only increases their ability to phagocytose and kill foreign objects, but also enhances their ability to

present antigens and activate cells of the adaptive immune system. It therefore follows that TLRs play a very important role in bridging innate immunity to adaptive immunity.

TLRs are type I membrane proteins that have leucine rich repeats (LRRs) in their ectodomain responsible for the recognition of PAMPs, and a cytoplasmic region similar to that of the IL-1 receptor known as the TIR domain responsible for the binding to adaptor proteins and the triggering of downstream signalling events [232]. To date, 11 human and 13 mouse TLRs have been identified. TLR 1, 2, 4, and 6 recognize lipids, TLR 5 and 11 recognize proteins, and TLR 3, 7, 8, and 9 are intracellular receptors which recognize nucleic acids (see Table 1). TLRs homo- or hetero-dimerize and the binding of their specific ligands causes a conformational change that brings the two TIR domains in the cytosol into close proximity generating a platform on which signalling complexes can form. The molecules that are first recruited to TIR domains are adaptor proteins and the next section will discuss in details the different types of the TIR domain-containing adaptor proteins involved in TLR signalling.

4.3 Adaptor proteins in TLR signalling

1- **Myeloid differentiation factor 88 (MyD88)**: This adaptor protein was named so because it was coded by the 88^{th} gene found to be induced in myeloid precursor cells in response to IL-6 [233]. It plays a crucial role in IL-1R signalling [234], and was later found to play an equally important role in TLR signalling as seen by the unresponsiveness of $MyD88^{-/-}$ mice to several TLR ligands including TLR2 and TLR4 ligands, and the resistance of those mice to LPS-induced shock [235,236]. In fact, MyD88 signals downstream all TLRs except TLR3, and the absence of MyD88 in

mice has been associated with a decreased ability to fight off different pathogens including *Leishmania* (Table 2) [237].

Adaptor	Infection	Phenotype
MyD88		
	Staphylococcus aureus	↓ Survival after intravenous infection ↑ Bacterial load in blood and organs ↑ Cytokines produced by MØs
	Plasmodium berghei	Normal parasitaemia and survival ↓ Serum IL-12 and liver injury
	Toxoplasma gondii	↓ Survival after intraperitoneal infection ↓ Serum IL-12 and IFN-γ
	Listeria monocytogenes	↓ Survival after intraperitoneal infection ↑ Bacteraemia in spleen and liver ↓ Serum TNF, IFN-γ, and NO
	Leishmania major	↓ Resistance to skin inoculums ↓ IL-12-mediated Th1 cell response

Table 2: Effects of different pathogens on MyD88$^{-/-}$ mice. (Adapted from O'Neill LA *et al.* (2007) Nat. Rev. Immunol. 7: 353-364)

Once MyD88 binds to the TIR domain of TLRs, it is able to recruit members of the IL-1 receptor-associated kinase (IRAK) family. Interestingly, IRAK-4 –the closest human homologue to Pelle- was discovered to be the first IRAK member to be required to the complex [238]. The kinase activity of IRAK-4 is required for its function and necessary for the phosphorylation of IRAK-1 and the induction of NF-

κB, as shown by the blockage of IL-1-induced activation of IRAK-1 and NF-κB when 293 cells are transfected with a dominant negative form of IRAK-4 [238]. The authors of the previous work rightfully proposed that IRAK-4 signals upstream of IRAK-1 and has a signalling role that is distinct from that of IRAK-1. Soon after, another study demonstrated by peptide phosphorylation assays that IRAK-4 is able to phosphorylate IRAK-1 on Threonine^{209} (Thr^{209}) and Thr^{387} [239]. The first phosphorylation event on Thr^{209} was shown to cause a conformational change in the kinase domain of IRAK-1 allowing a second phosphorylation event on Thr^{387}, leading to its full enzymatic activity [239]. Once IRAK-1 is fully active, it is able to autophosphorylate itself in the proline- serine-, and threonine-rich ProST region causing an increase in the total negative charge of the protein responsible for its detachment from MyD88 and attachment to TRAF6 to activate signalling (Figure 7) [239]. It is important to note that whereas IRAK-4 is thought to perform those critical threonine phosphorylations *in vivo*, IRAK-1 seems to be able to perform those two critical phosphorylations on its own in *in vitro* and overexpression systems, by autophosphorylation. The result is also a fully active IRAK-1 able to autophosphorylate in the ProST region causing a dramatic increase in its molecular weight and a shift in its electromobility, a hallmark of IRAK-1 activation [239].

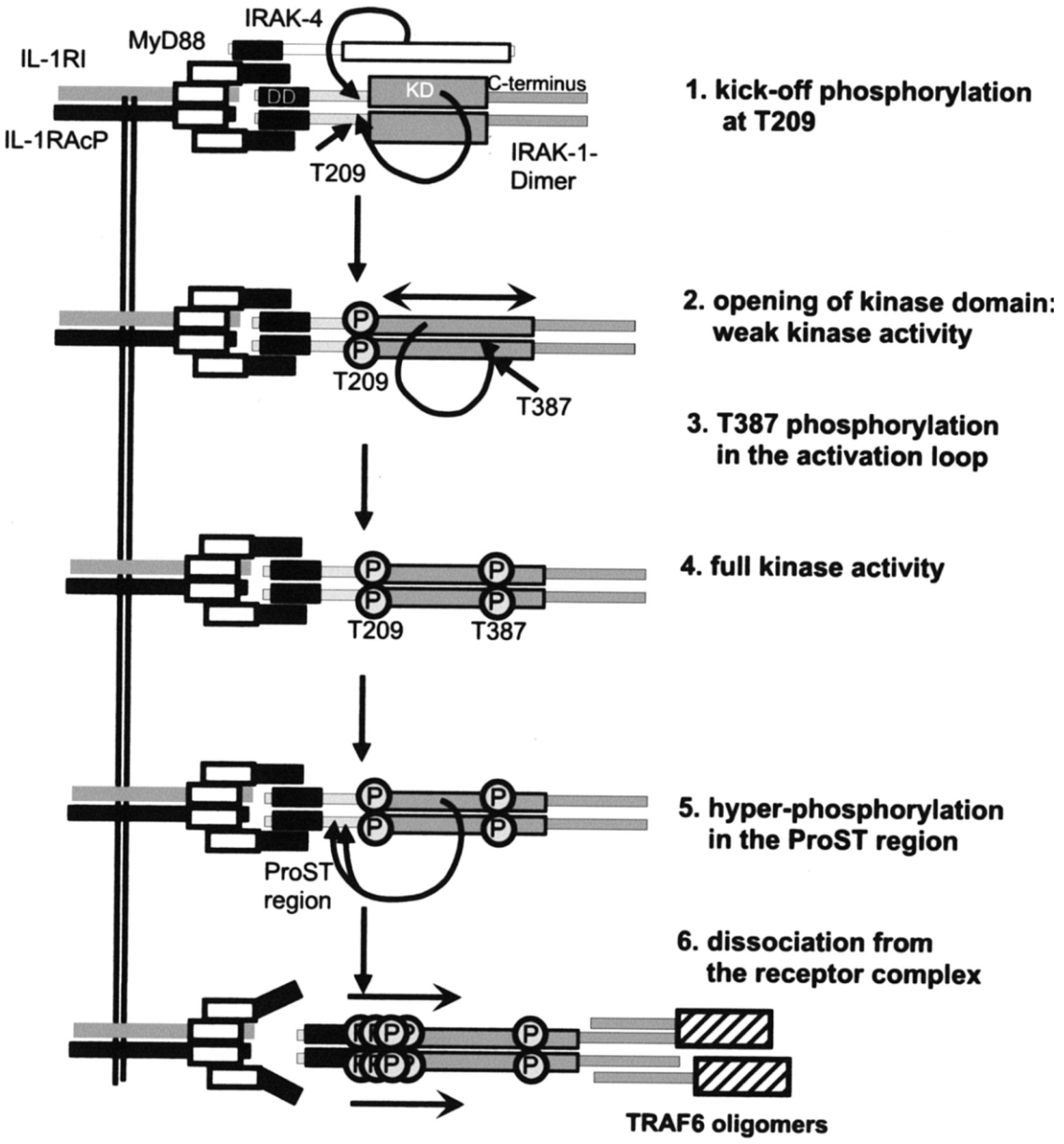


Figure 7: Simplified model explaining the role of IRAK-4 in IRAK-1 autoactivation.

The two vertical lines on the left represent the cell membrane. IRAK-1 is made of an N-terminal (contains the death domain (DD) which binds to MyD88), the ProST region, the kinase domain, and the C-terminal. (Adapted from Kollewe C *et al.* (2004) J. Biol. Chem. 279: 5227-5236)

The detachment of IRAK-1 from the MyD88 complex and its attachment to TRAF6 represents a key step in the transmission of the signal transduction pathway from the IL-1R and TLRs to the nucleus. TRAF6 is an E3 ubiquitin ligase which forms a complex with Ubc13 and Uev1A to synthesize lysine-63-linked polyubiquitin chains responsible for the activation of a pivotal MAPKKK known as TAK1 [240]. TAK1 associates with TAK1-binding proteins 1, 2, and 3 (TAB1, TAB2, TAB3) and activates two downstream pathways: the NF-κB and the MAPK pathways [232]. TAK1 activates NF-κB through its ability to activate the IKK complex, which in turn phosphorylates IκB proteins leading to their degradation and the consequent translocation of the now free NF-κB to the nucleus. It also activates several MAPKs, importantly JNK and p38, through the activation of the MAPKKs that phosphorylate them [232,237]. The activation of the NF-κB and MAPK pathways via MyD88 therefore contributes to the ability of immune cells such as MØs to produce pro-inflammatory cytokines and free radicals such NO and ROIs in response to LPS stimulation.

2- **MyD88 adaptor-like protein (Mal)**: This adaptor molecule is also known as TIR domain-containing adaptor protein (TIRAP) and has been shown to play an important role in MyD88-dependent signalling [241,242]. Interestingly, Mal does not seem to participate in all MyD88-dependent signalling pathways, which adds specificity to the role it plays in TLR signalling. Mal-deficient mice have defective TLR4-induced cytokine production and impaired TLR2 but not TLR9 signalling [243,244]. These studies suggest a specific role for Mal in TLR2 and TLR4 signalling only.

The mechanism of action of Mal was largely unknown for a few years after its discovery. The mystery surrounding Mal's function started to be solved when it was found that Mal contained a phosphatidylinositol 4, 5 bisphosphate (PIP2) binding domain that mediates its recruitment to the plasma membrane. Upon the recruitment of Mal, this adaptor protein serves as a bridge to facilitate MyD88 delivery to activated TLR4 to initiate signal transduction [245].

3- **TIR-domain-containing adaptor-inducing interferon-β (TRIF)**: This is a key adaptor protein in TLR signalling as it mediates MyD88-independent signalling leading to the production of type I interferons and is the exclusive adaptor protein used in TLR3 signalling [237]. The role of TRIF in MyD88-independent signalling was confirmed using TRIF-deficient mice, which were shown to have impaired TLR3- and TLR4- induced IFN-β production and activation of interferon regulatory transcription factor 3 (IRF3) [246]. In addition to the induction of type I interferons, the MyD88-independet pathway also drives NF-κB activation as was shown by the complete abolition of LPS- induced NF-κB activation in cells deficient for both TRIF and MyD88 [247].

How does TRIF perform its functions at the signalling level? TRIF has different interaction motifs that allow it to recruit several proteins including: TNFR-associated factor (TRAF)-family-member-associated NF-κB-activator-binding kinase 1 (TBK1), TRAF6, and receptor-interacting protein 1 (RIP1) [237]. TRIF seems to associate with TBK1 through NF-κB-activating kinase associated protein 1 (NAP1) [248] and therefore activate the TF IRF-3 that way. To activate NF-κB, TRIF has a RIP interaction motif in its C-terminal that allows it to bind to RIP1 and therefore activate

NF-κB [249]. Although TRAF6 was shown to have a binding site in the N-terminal of TRIF and to play a role in TRIF-mediated NF-κB activation [250,251], the role of TRAF6 in TRIF signalling remains controversial [252]. The latter study demonstrated that poly inosine:cytosine (poly I:C) was able to activate NF-κB in MØs derived from $TRAF6^{-/-}$ mice as efficiently as in MØs derived from $TRAF6^{+/-}$ mice [252].

4- **TRIF-related adaptor molecule (TRAM)**: This adaptor protein is involved in TLR4 signalling only, as shown by the defect of TRAM-deficient mice in the production of cytokines in response to TLR4 but not other TLR ligands [253]. Just like Mal has a PIP2 binding domain to associate with cellular membranes, TRAM was shown to become membrane-associated through its ability to undergo constitutive myristoylation [254]. Another similarity between Mal and TRAM lies in their functions, similar to Mal serving as a bridge to MyD88, TRAM bridges TRIF, recruiting it to the activated TLR4 complex [255].

5- **Sterile α- and armadillo-motif-containing protein (SARM):** Unlike the other four TIR-containing adaptor proteins which drive TLR signalling, SARM plays a negative role in the regulation of NF-κB and IRF signalling. This negative regulatory role targets MyD88-independent but not MyD88-dependent pathways through the direct interaction between SARM and TRIF [256]. It is not exactly understood how SARM can inhibit TRIF signalling. One possibility is that by binding to TRIF, SARM blocks the ability of the protein to bind to downstream signalling molecules such as: TBK1, RIP1, or TRAF6. The other possibility is that SARM can recruit an as-yet-unknown TRIF inhibitor using its sterile α-motif (SAM) domains [237]. It is important to mention that SARM protein levels were found to be increased following LPS

stimulation, suggesting that it functions as part of a negative feedback loop to control the TRIF pathway [237].

To conclude this part, we have shown that TLR signalling is mediated by several TIR domain-containing adaptor proteins that ultimately signal in one of two main sub-pathways: the MyD88-dependet pathway, utilized by all TLRs except TLR3, and the MyD88-independent pathway utilized by TLR3 and TLR4 (Figure 8) [232]. The MyD88-dependent pathway leads to the induction of pro-inflammatory cytokines and the production of free radicals such as NO and ROIs through its ability to activate NF-κB and MAPKs, and the MyD88-independent pathway leads to NF-κB activation and most notably the activation of the IRF family of TFs that induces the production type I interferons. The following section will discuss the known mechanisms by which those two pathways are negatively regulated following immune cell activation.

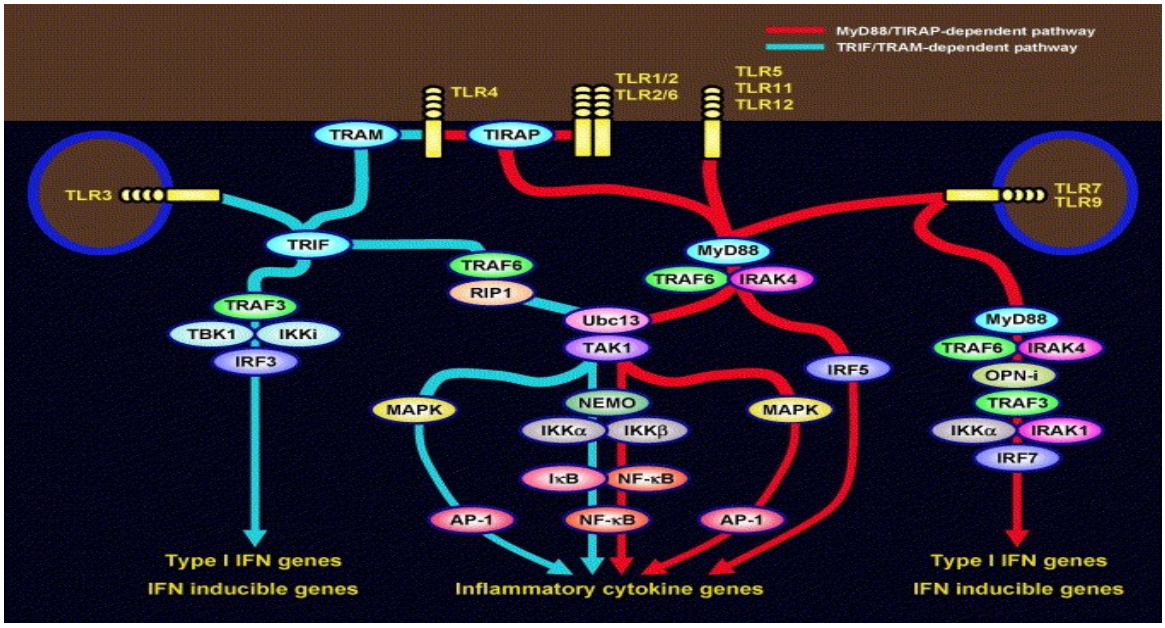


Figure 8: MyD88-dependent and –independent pathways. (Adapted from Kawai T *et al.* (2007) Semin. Immunol. 19: 24-32)

4.4 Negative regulation of TLR signalling

The activation of TLR signalling is clearly a double-edged sword. On one hand, it is important in the early detection of many pathogens by the innate immune response, and is crucial for the activation of the adaptive immune response and immunological memory. On the other hand, if left uncontrolled, immune activation by TLRs can have detrimental outcomes. One of the most serious diseases is sepsis which is mainly caused by LPS of Gram negative bacteria, a TLR4 ligand [257]. The strong inflammation produced by LPS-induced cytokines such as TNF-α, IL-1, and IL-6 leads to massive vasodilation and tissue hypoxia, which can result in septic shock and death [258]. TLR signalling may also contribute to heart failure in young patients by activating DCs [259] and to atherosclerosis in a MyD88-dependent fashion [260,261]. In addition, a role for TLR signalling has been proposed in the development of diabetes [262,263], experimental autoimmune encephalomyelitis [264], systemic lupus erythematosus (SLE) [265], asthma [266,267], and rheumatoid arthritis [268,269].

In order to keep TLR signalling under tight control, cells have developed several strategies to negatively regulate it at different levels. Negative regulators can be assigned to the following cellular compartments: extracellular, transmembrane, and intracellular [270]. We will hereby discuss these three classes and how they work.

1- **Extracellular negative regulators**: The only extracellular regulators known to-date are soluble decoy receptors (sTLRs). Two of these have been identified: sTLR2 and sTLR4 [271,272]. sTLR2 was demonstrated to inhibit lipopeptide-induced IL-8 and TNF-α production in monocytes by binding to the PGN of *Staphylococcus aureus* and

to sCD14 [273], and sTLR4 has been proposed to block the interaction between TLR4 and its co-receptor MD-2 by binding to the latter [274].

2- **Transmembrane negative regulators**: These regulators are defined by their ability to inhibit TLR signalling by either interfering with the binding of TLRs to their ligands or by sequestering TLR adaptors. The ones identified so far are: suppressor of tumorigenicity 2 (ST2), single immunoglobulin IL-1-related protein (SIGIRR), radioprotective 105 (RP105), and TNF-related apoptosis-inducing ligand receptor (TRAILR).

ST2: It is found in two main forms: ST2L and sST2. Both forms are type I transmembrane receptors with a cytoplasmic TIR domain and an extracellular domain containing immunoglobulin (Ig)-like domains [275-277]. The only difference between both forms is that sST2 contains an additional nine amino acid stretch in the C-terminal [275-277]. The negative regulatory role of ST2 was based on the finding that mice deficient in both ST2L and sST2 produced extensive amounts of pro-inflammatory cytokines in response to IL-1, LPS, bacterial polypeptides, and cytosine-phosphate-guanine (CpG), but not in response to poly I:C (TLR3 ligand), clearly demonstrating that ST2 is a negative regulator of MyD88-dependent pathways only [278]. This conclusion was further confirmed by demonstrating the ability of ST2 to directly bind MyD88 and Mal but not TRIF [278]. By doing so, ST2 sequesters MyD88 and Mal and prevents them from otherwise initiating TLR signalling.

SIGIRR: This regulator is highly expressed in epithelial and dendritic cells but not MØs and is a member of the TIR superfamily with one Ig domain in its extracellular

domain [279,280]. It is LPS-inducible [281], and its role in negative regulation is derived from the findings that its overexpression in DCs leads to the inhibition of IL-1 and IL-18-mediated NF-κB activation [282]. Mice deficient for SIGIRR displayed increased LPS and CpG-mediated (but not poly I:C-mediated) NF-κB activation and cytokine production, further confirming the role of SIGIRR in the negative regulation of TLR signalling and confining its role to MyD88-dependent pathways [281]. In fact, SIGIRR has been shown to form complexes with TRAF6 and members of the IRAK family in response to IL-1 stimulation *in vitro* [279].

RP105: It is a TLR4-homologue induced by LPS and able to directly interact with the TLR4 / MD-2 complex; therefore occupying sites that otherwise could bind LPS and trigger TLR4 signalling [283]. Interestingly, RP105 was shown to require binding to its own co-receptor MD-1 prior to binding to the TLR4 / MD-2 complex [283].

TRAILR: This regulator belongs to the TNF superfamily and has no TIR domains [284]. Mice deficient for TRAILR showed increased cytokine production when MØs were stimulated with ligands of TLR2, 3, and 4 [285], suggesting that TRAILR could regulate both MyD88-dependent and –independent pathways. This was confirmed by the finding that TRAILR works at the level of IκB, stabilizing IκB-α and causing a decrease in the nuclear translocation of NF-κB [285].

3- **Intracellular negative regulators**: These proteins include: MyD88 short (MyD88s), IRAK-M, SOCS1, NOD2, PI3K, Toll-interacting protein (TOLLIP), and A20 [286]. We will hereby discuss them one at a time, explaining their modes of action.

MyD88s: We have previously discussed the pivotal role of MyD88 in TLR signalling, but we have not discussed its structure in enough detail. MyD88 has a DD in its N-terminal required for interaction with the IRAK family (IRAK-4 and IRAK-1), a central interdomain also required for the binding to IRAK-4, and a C-terminal TIR domain used to interact with the cytoplasmic portion of TLRs. MyD88s is a MyD88 protein that lacks the interdomain and cannot therefore bind IRAK-4 [286]. Unlike MyD88 which is ubiquitously expressed, MyD88s was found only in the spleen and brain [286], and was reported to be induced in THP-1 human monocytes following LPS stimulation [287]. The regulatory effect was deduced from the inhibition of IL-1 and LPS-induced NF-κB activation in HEK293T cells overexpressing MyD88s [287]. As for how MyD88s works, this alternatively-spliced adaptor protein was found to form heterodimers with MyD88 which compete in binding to TLRs with MyD88 homodimers. IRAK-1 is still recruited to the complex but is no longer activated by IRAK-4 as the latter is unable to associate with MyD88s due to the absence of the interdomain necessary for IRAK-4's ability to bind MyD88 [288].

IRAK-M: It is an LPS-induced protein [289] that is predominantly expressed in peripheral blood leukocytes and only weakly in other tissues [286]. Its negative regulatory function has been suggested based on the findings that IRAK-M-deficient mice have increased inflammatory responses to bacterial infection and reduced LPS tolerance and that MØs derived from these mice produce more pro-inflammatory cytokines in response to LPS and CpG DNA [289]. IRAK-M's mode of action involves the inhibition of IRAK-1 / TRAF6 complex formation without interfering with the ability of IRAK-1 to be recruited to MyD88 [286]. This implies that IRAK-M

can either interfere with the phosphorylation of IRAK-1 and/or stabilize the TLR-MyD88-IRAK-4 complex [286].

Interestingly, it has also been reported that two splice variants of IRAK-2 (IRAK-2c and 2d) have a negative regulatory function in TLR signalling. Although IRAK-2a and IRAK-2b seem to induce LPS-mediated NF-κB activity when overexpressed in fibroblasts, overexpression of IRAK-2c and IRAK-2d inhibit this activation [286,290]. The latter two isoforms are LPS-inducible in MØs and lack death domains, as opposed to full length IRAK-2 and all other isoforms, suggesting a possible negative-feedback effect on TLR signalling. To sum up, IRAK-2 and its isoforms -2a and -2b signal downstream of IRAK-4 and are involved in positive signalling in parallel with IRAK-1 [290-292], while IRAK-2c and -2d are involved in the negative regulation of TLR signalling [290]. Despite this knowledge, IRAK-2 is among the least explored and least understood IRAK members, and future studies are needed to better understand its role in TLR siganlling.

SOCS1: We have previously discussed SOCS1 when we listed mechanisms by which *Leishmania* can interfere with JAK/STAT signalling. Interestingly, SOCS1's expression in MØs is also induced in response to LPS and CpG stimulation suggesting a role in the negative regulation of TLR signalling [286]. In fact, MØs from SOCS1-deficient mice produce increased amounts of NO and pro-inflammatory cytokines in response to LPS and CpG, and the mice are more susceptible to sepsis compared to WT mice [293,294]. However, it remains a matter of debate whether SOCS1 plays a direct role in the regulation of TLR signalling or whether this regulatory effect is an

indirect effect arising when SOCS1 is activated to inhibit type I IFN signalling triggered by TLR ligands. Few studies support the latter suggestion [295,296].

NOD2: It is a member of the nucleotide-binding oligomerization domain family (Caterpiller family) and contains, like other members of this family, a C-terminal leucine-rich repeat domain [297]. Compared to WT mice, spleen cells from NOD2-deficient mice have an elevated TLR2 ligand-induced Th1 response marked by elevated IL-12 and IFN-γ production. This elevated Th1 response was seen in response to bacterial peptidoglycan but not LPS, suggesting a specific role for NOD2 in the regulation of TLR2 signalling [298]. The mechanism by which NOD2 works is not well understood, and future research in this area is required.

PI3K: It is a protein expressed in most cells and is a heterodimer that consists of a p85 regulatory subunit and a p110 catalytic chain [299]. Similar to what was observed with NOD2-deficient mice, PI3K-deficient mice exhibit an elevated Th1 response marked by IL-12 production. Unlike NOD2 deficiency however, this elevated Th1 was observed in response to several TLR ligands including peptidoglycan, LPS, and CpG [300]. The mechanism of action of PI3K in the inhibition of TLR signalling is not well-understood, but is thought to involve inactivation of all three MAPKs and the TF NF-κB [300].

TOLLIP: TOLLIP represents the only known negative regulator of IRAK-1 at resting state. Its regulatory role was proposed based on experiments where overexpression of TOLLIP led to the inhibition of TLR2- and TLR4-mediated NF-κB activation [301]. In addition to its ability to bind TLR2 and TLR4, TOLLIP was reported to be able to bind

the DD and kinase domain of IRAK-1 suppressing its ability to autophosphorylate [301]. Upon TLR stimulation, IRAK-1 phosphorylates the TOLLIP attached to it causing its release so the kinase can get fully activated and bind TRAF6 to activate downstream signalling pathways [301].

A20: Although A20 was initially identified as a TNF-induced zinc-finger protein involved in the negative regulation of TNF-α-induced NF-κB, this protein was later shown to be induced by LPS stimulation as well [286]. Experiments on MØs derived from A20-deficient mice showed increased production of pro-inflammatory cytokines in response to many TLR ligands including peptidoglycan and poly I:C [302]. In addition, transfection of cells with A20 suppressed TLR-induced NF-κB activation [302]. The mechanism of action of A20 makes it unique among all others in that it can regulate both MyD88-dependent and -independent pathways through regulating TRAF6. A20 was shown to be a cysteine proteinase capable of blocking TLR signalling by cleaving the ubiquitin chain of TRAF6 [302]. Ubiquitination of TRAF6 is crucial for its ability to interact with TAB1 and TAB2 and therefore its role in the activation of TAK1 and ultimately NF-κB. For a summary of all the intracellular negative regulators of TLR signalling, see figure 9 [286].

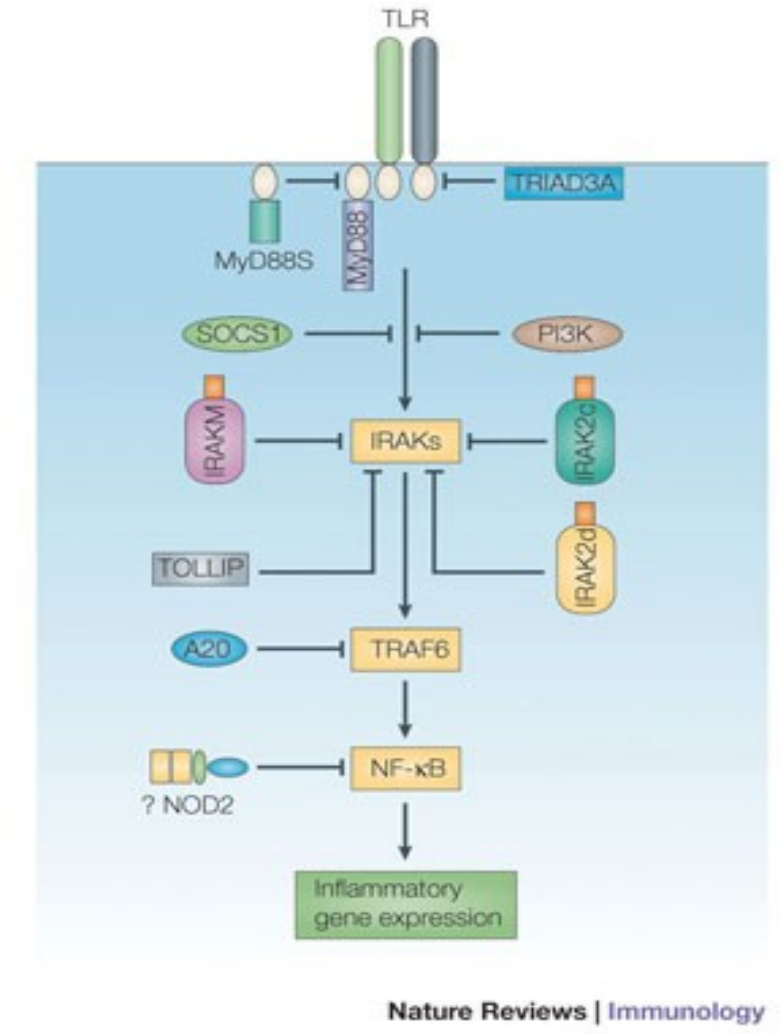


Figure 9: Intracellular negative regulators of TLR signalling. A schematic summarizing all the previously discussed negative regulators of TLR signalling, indicating the molecules they act upon. (Adapted from Liew FY *et al.* (2005) Nat. Rev. Immunol. 5:446-458)

5. Modulation of TLR signalling by pathogens

We have previously explained how TLR signalling is invaluable for the activation of innate immunity and the initiation of an adaptive immune response towards invading pathogens. However, pathogens also have their say! In fact, several pathogens have been shown to have the ability to interfere with TLR-mediated immunity by avoiding detection, down-regulating TLR expression, or by blocking signalling pathways triggered by the ligation of PAMPs to TLRs [303]. This ability of pathogens to circumvent TLR-mediated immunity was acquired during the evolutionary arms race between pathogens and their hosts (represented here by the host's immune system) in a continuous effort of both parties not to fall behind, become less competitive, and eventually lose the race. In this section, we will discuss strategies employed by bacteria, viruses, and parasites to alter TLR-signalling to their advantage.

5.1 Modulation of TLR signalling by bacteria

One of the main reasons behind the success of TLRs is their detection of PAMPs, but why is that so? The answer to this question lies in the fact that PAMPs are highly conserved molecules of big importance to the virulence of pathogens. In other words, pathogens that have major mutations affecting the antigenicity of their PAMPs are most likely penalized by natural selection due to their decreased virulence. This results in the conservation of most PAMPs which can explain how TLRs remain successful in detecting them. This argument applies to all currently known bacterial PAMPs with one possible exception: flagellin. Although flagellin has been shown to represent an important virulence factor involved in the attachment and entry of several bacteria [304-307], some

such as *Salmonella enterica*, for example, was shown not to require flagellin for its pathogenicity [308], opening the room for modifications to occur to flagellin and giving it a potential to escape detection.

Based on the fact that the vast majority of bacterial PAMPs can be detected by TLRs, bacterial evasion mechanisms are expected to exist, and this will be the topic of this section. Certain bacterial components have been shown to down-regulate the expression of TLRs and/or their co-receptors. Indeed, it has been shown that LPS of the oral pathogen *Porphyromonas gingivalis* is able to downregulate TLR4 expression in human gingival fibroblasts [309] and that *P. gingivalis'* adhesion molecule fimbrillin A is able to bind TLR4 and induce the downregulation of its co-receptor CD14 [310]. *Ehrlichia chaffeensis*, an intracellular bacterium, was shown to cause LPS tolerance by downregulating TLR2, TLR4, and CD14 expression. It is not to be understood that LPS tolerance (the ability of LPS to cause tolerance to subsequent LPS stimulation) is always advantageous to the pathogen. On the contrary, it is more plausible that LPS tolerance is a mechanism used by immune cells to decrease the heightened level of inflammation to avoid its detrimental effects on the host while maintaining the ability to resist pathogens [311].

Mechanisms that seem to clearly work in favour of the pathogen include those directed towards preventing PAMP detection or inhibiting the signalling cascade it generates. For instance, a glycolipid preparation of *Treponema medium* was shown to inhibit the binding of LPS to immobilized CD14 and LPS-binding protein (LBP) and to inhibit LPS-mediated NO production in murine MØs [312]. *Bacillus anthracis*, the causative agent of anthrax, contains a metalloproteinase called the lethal factor that can cleave the N-

terminal of MKKs, therefore inhibiting MAPK signalling [313]. *Francisella tularensis*, on the other hand, upregulates a 23 kDa protein during its intracellular infection that is able to block the degradation of IκB, therefore inhibiting the NF-κB pathway. This 23 kDa protein also plays a role in the inhibition of LPS- or bacterial lipopeptide-mediated p38 and c-jun phosphorylation and in the inhibition of TNF-α and IL-1 production by J774 MØs [314]. However, the authors of the previous study did not identify this protein or define its mode of action.

Mycobacterium tuberculosis expresses a 19 kDa lipoprotein that inhibits MHC II expression and antigen processing in a TLR2-dependent manner [315]. Further studies showed that the activation of TLR2 by this 19 kDa lipoprotein leads to the inactivation of IFN-γR signalling in human MØs which can help explain the effect it has on MHC II expression and antigen presentation [316]. Interestingly, this lipoprotein has been also implicated in the inhibition of phagosome maturation in a MyD88-dependent manner [317].

All virulent *Yersinia* species express the virulence antigen known as LcrV. This antigen was shown to cause immunosuppression by inducing IL-10 expression [318] in a TLR2- and CD14-dependent manner [319]. This was further confirmed when IL-10 knock-out mice were found to mount a strong pro-inflammatory response and to be resistant to *Yersinia* infection [320]. Other famous virulence factors that *Yersinia* possesses are known as *Yersinia* outer proteins (Yops). These proteins are made of several components: a type III secretion system called Ysc, a system to deliver bacterial proteins to eukaryotic target cells such as YopB and YopD, a control element called YopN, and a set of intracellularly-delivered proteins that interfere with host cell signalling such as YopE and

YopH [321]. Interestingly, YopH is a PTP capable of dephosphorylating target proteins in the host such as Crk-associated substrate (p130Cas) [322], therefore disrupting focal adhesion points involved in phagocytosis [323]. Another reported function of Yops is the inactivation of host MAPK signalling pathways [323]. Whether or not this represents direct interference with TLR signalling is a matter of debate, but the inactivation of MAPK signalling and the existence of possible TLR-related substrates of YopH certainly mean that YopH could play an important role in the inhibition of TLR-related functions.

5.2 Modulation of TLR signalling by viruses

Few TLRs have been implicated with viral PAMP detection, and the information regarding the ability of viruses to alter TLR signalling is scant. one study using *Drosophila* fat body cells showed that HIV-1's viral protein U (Vpu) is able to interfere with the NF-κB pathway through blocking the degradation of cactus (insect homologue of IκB) [324]. The authors of the previous study relied on the fact that many aspects of NF-κB signalling is conserved between the fly and mammals to propose that Vpu could potentially play a role in TLR signalling evasion in mammals in a similar manner. This awaits further investigation.

Vaccinia virus (VV) is reported to encode proteins known as A46R and A52R that share amino acid sequence similarity to TIR and can therefore compete with TLR signalling by associating with TIR domains of TLRs (Similar to the mechanism of action that we previously described for MyD88s). These proteins have been shown to inhibit IL-1 and TLR4-mediated NF-κB activation in mammalian cells [325].

Viral PAMPs are now known to be recognized by several TLRs including: TLR2, TLR3, TLR4, TLR7, TLR8, and TLR9 (see Table 1). Nevertheless, not much data is available on how viruses can evade signalling through these TLRs. It is therefore very likely that many viral evasion mechanisms related to signalling through those TLRs will be reported in the future.

5.3 Modulation of TLR signalling by parasites

As shown previously in Table 1, several parasite PAMPs are ligands of TLRs. In the light of the host-pathogen arms race that we previously touched upon, the detection of parasite PAMPs by TLRs has two main implications: First, the ability of cells of the immune system to detect parasites and eliminate them when favourable conditions are present. Second, the ability of parasites to counteract TLR detection by interfering with TLR signalling keeping immune cells in an inactive state and rendering them refractory to subsequent TLR stimulation.

One of the main parasite-derived molecules involved in TLR binding and activation are GPI-anchored proteins. *Trypanosoma cruzi*-derived GPI-anchors were shown to be detected by TLR2 / TLR6 and CD14 and to activate NF-κB [326,327], while GIPLs of *T. cruzi* activated Chinese hamster ovary (CHO) cells in a TLR4 / CD14-dependent manner [328]. It has been also shown that GPI-mucin of *T. cruzi* is able activate TLR signalling on first exposure and induce tolerance to secondary TLR stimulation [329]. This was later shown to be mediated by the ability of GPI-mucin to induce the expression and activation of the serine / threonine phosphatase PP2A that acts on cellular IRAK-1, MAPKs, and IκB causing their inhibition and leading to tolerance [330]. The induction of PP2A was

shown to require p38 and NF-κB, the very same molecules PP2A is induced to inhibit therefore giving rise to an autoregulatory loop [330]. LPG of *Leishmania* is another GPI-anchored protein detected by TLRs. It has been shown that LPG of *L. major* directly binds to TLR2 of MØs and NK cells [331,332] and that LPG of *L. donovani* is also detected by TLR2 of activated MØs [333]. Interestingly, GPI-anchors derived from *Plasmodium falciparum* merozoites can induce TNF production in human monocytes and mouse MØs through interacting with TLR1 / TLR2 and to a lesser extent TLR4 [334,335]. Moreover, GPI- anchors of *Toxoplasma gondii* are detected by TLR2 and TLR4, which can thus play an important role in host defense against *T. gondii* infections [336].

Although less numerous than GPI-anchored ligands, non-GPI-related ligands represent an important group of parasite-related molecules detected by TLRs. An example is the *T. cruzi*-derived protein Tc52, which is able to induce pro-inflammatory cytokine production in DCs in a TLR2-dependent manner [337]. Other important non-GPI ligands include the DNA of *T. cruzi*, *T. brucei*, and *Babesia bovis*, which are able to activate MØs and DCs [338,339], possibly through unmethylated CpG motifs [340] detected by TLR9 [341,342]. TLR3 was recently shown to be upregulated in IFN-γ-primed MØs and to play a role in their leishmanicidal activity. The silencing of TLR3 led to impaired NO and TNF-α production in IFN-γ-primed MØs in response to *L. donovani* infection and increased parasite survival [333]. Given that the only known ligand of TLR3 is dsRNA, the parasite component that activates TLR3 remains unclear. The authors ruled out the presence of dsRNA *leishmania*virus infection in their parasite strain and also failed to detect natural *Leishmania*-derived double-stranded RNA structures such as rRNA or tRNA [333]. As

far as apicomplexans are concerned, *Plasmodium*-derived hemozoin crystals were shown to induce pro-inflammatory cytokines in MØs [343,344]. Initially, TLR9 was proposed as the binding receptor of hemozoin [345], this remains controversial as it has been later shown that TLR9 activation by hemozoin is mediated by malaria DNA attached to the crystal and that the activation of TLR9 by hemozoin was abolished upon treatment with nucleases [346]. In fact, recent data from our laboratory show that the pro-inflammatory cytokine IL-1β is induced by hemozoin through the Nod-like receptor family, pyrin domain containing 2 (Nlrp2) and the adaptor protein Asc, which lead to caspase 1 activation (M.T. Shio and M. Olivier, manuscript under review). Concerning *Toxoplasma*, a profilin-like protein from *T. gondii* (PFTG) activates TLR11 in mouse cells [347], and heat shock proteins and partially purified preparations isolated from tachyzoites activate TLR4 and TLR2, respectively [348,349].

The many parasite-related molecules that are detected by TLRs suggest an important role for TLR-related signalling molecules in the resistance to parasitic infections [350]. Given the fact that Th1-driving pro-inflammatory responses are beneficial to the host in several types of parasitic infections, it is not surprising that the activation of the MyD88-dependent pathway is crucial in the resistance to many protozoan diseases. Indeed, MyD88-deficient mice are highly susceptible to *T. cruzi* [351], *T. brucei* [342], *L. major* [352], and *T. gondii* [353] infections due to the decreased inflammatory response and the impaired production of Th1-associated cytokines such as IL-12 and IFN-γ in these mice. It is important to mention that MyD88-driven pro-inflammatory events are not always favourable to the host in the fight against protozoans. The decreased inflammatory and Th1 responses in MyD88-deficient mice were seen to improve pathology and outcome of

P. berghei infection in mice. This suggests that *Plasmodium*, in this case, utilizes the MyD88-dependent pathway to cause tissue injury and worsen disease symptoms [354].

It is quite remarkable that the amount of susceptibility to several protozoan infections conferred by the absence of MyD88 is significantly higher than that observed when mice lacking a single TLR are used. This strongly suggests that several TLRs are simultaneously involved in the recognition of parasites, thus explaining why the loss of MyD88 can have a bigger impact on susceptibility compared to the loss of a single TLR [350]. Nevertheless, deficiency of relevant TLRs increases susceptibility to certain infections. For example, TLR9-deficient mice have higher parasitemia and mortality when infected with *T. cruzi* [341] or *T. brucei* [342]. TLR4-deficient mice are more susceptible to *L. major* infection with bigger lesion size and parasite loads compared to WT mice [355,356], and TLR11-deficient mice are more susceptible to *T. gondii* infection manifesting increased cyst formation in the central nervous system and decreased IL-12 and IFN-γ production compared to WT mice [347].

The ability of TLRs to detect parasite PAMPs put together with the fact that many successful infections are associated with silent entry to target cells suggests that parasites must have evasion tactics to block TLR signalling and functions. Some of these mechanisms have been already described while others are still to be discovered. We will hereby discuss some evasion strategies employed by *Leishmania*, *Plasmodium,* and *Toxoplasma.*

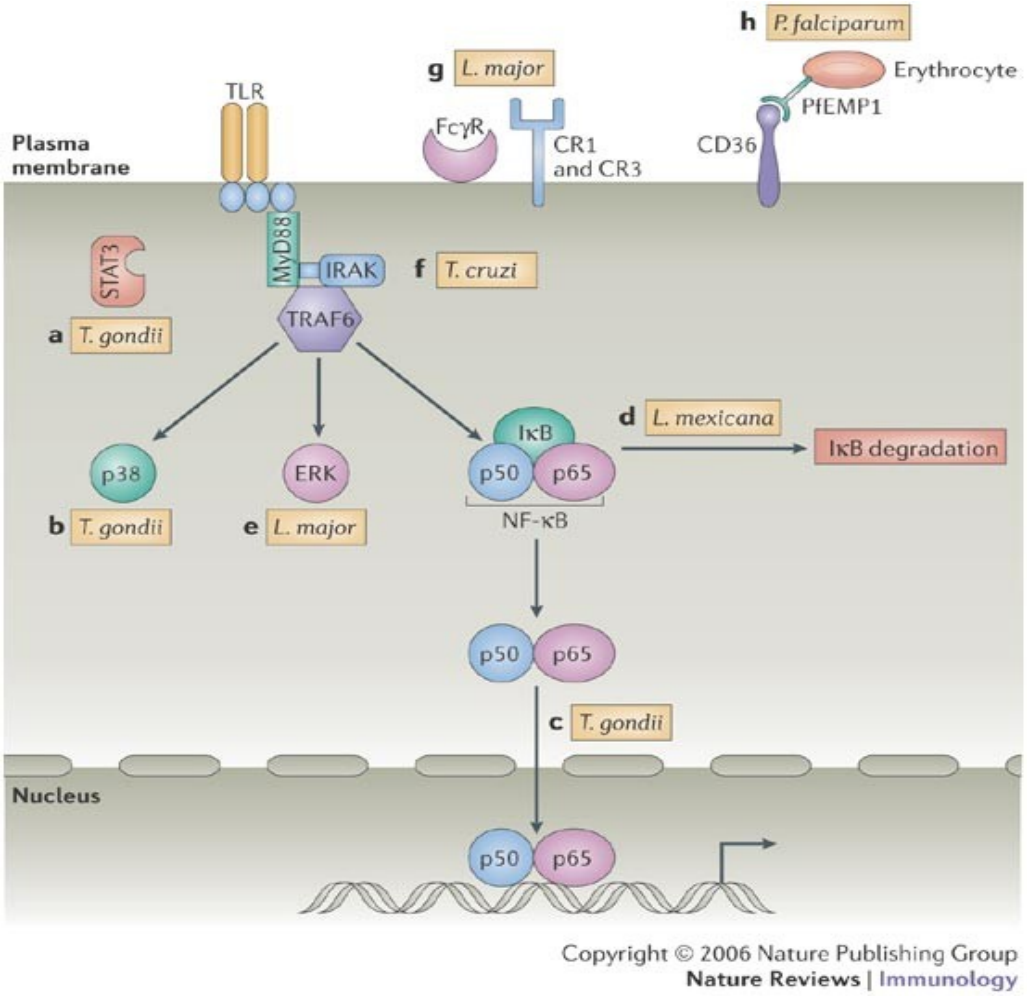
The ability of *Leishmania* to interfere with TLR signalling components has been already discussed in this thesis under the "signalling pathways altered by *Leishmania*" section.

These evasion mechanisms include the previously discussed ability of the parasite to interfere with the activation of all three MAPKs (Erk1/2, JNK, p38) (see MAPK section), and its ability to interfere with IκB, NF-κB, and AP-1 (see TF section). There is also evidence that signalling through CR1 and CR3, which *Leishmania* is known to bind to, can inhibit LPS- and IFN-γ-induced IL-12 production through impaired STAT-1 phosphorylation [195]. A similar role for FcγR ligation has been proposed [196,357].

P. falciparum causes infected erythrocytes to express *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) which was shown to interact with the scavenger receptor CD36 on the surface of DCs [358] making the cells that phagocytose these infected erythrocytes become unresponsive to LPS stimulation, ultimately leading to defects in T cell activation [359-361] .

T. gondii is yet another parasite able to block LPS-mediated IL-12 and TNF-α production, the upregulation of co-stimulatory molecules, and the activation of T cells [362-365]. One way the parasite is able to do so is by activating STAT3 in IL-10-dependent and –independent manners [366,367]. Although this *T. gondii*-induced inhibition of subsequent LPS stimulation might somehow resemble LPS tolerance in that it inhibits MAPKs like p38 [368], important differences between infection and LPS tolerance exist. Unlike LPS tolerance, *T. gondii* infection followed by LPS stimulation resulted in the activation of MKK3 and MKK6 (upstream activators of p38) and in the degradation of IκB [368]. This suggests that the inactivation of p38 observed when LPS stimulation is preceded by *Toxoplasma* infection is either due to the inhibition of another p38-activating kinase such as MKK4, or is mediated by a *T. gondii*-induced MAPK phosphatase that prevents the phosphorylation-dependent activation of p38 [350]. It is interesting to note that although

T. gondii infection followed by LPS stimulation causes IκB activation, the liberated NF-κB fails to translocate to the nucleus [369,370]. Later studies suggested that the lack of NF-κB translocation might actually be due to increased nuclear export of this TF rather than inhibition of nuclear import [371]. For a summary of the previously-mentioned TLR signalling manipulation tactics by parasites, refer to figure 10 [350].



<u>Figure 10: Modulation of TLR signalling by protozoan parasites.</u> Note that other species of *Leishmania* such as *L. donovani* can also interfere with Erk1/2 and NF-κB activation and have not been included for simplicity. (Adapted from Gazzinelli RT *et al.* (2006) Nat. Rev. Immunol. 6: 895-906)

Based on the previous sections describing the importance of TLR signalling in fighting disease and the many tactics developed by pathogens to circumvent this, it is rather tempting to hope that by using TLR ligands in vaccination or treatment regiments, we can activate the immune system to fight pathogens and clear them more efficiently. For example, monophosphoryl lipid A (TLR4 ligand) is in an advanced stage of development for use in vaccines in combination with antigens of *P. falciparum* [372] and *Leishmania* species [373]. Other TLR ligands that demonstrated promising results in vaccination experiments include imiquimod and R848 (resiquimod) (TLR7 / TLR8 ligands) which mounted a protective Th1 response against *L. major* antigens [374], and CpG-containing oligodeoxynucleotides (TLR9 ligand), which induced protective immunity against *T. cruzi*, *Leishmania*, *Plasmodium,* and *T. gondii* [350,375-379]. Additionally, flagellin (TLR5 ligand) was successfully used to stimulate mucosal immunity [380], and might turn out to be important in developing vaccines that target pathogens of mucosal tissue such as *T. gondii* which infects the intestinal tract.

As far as treatment is concerned, the use of imiquimod and CpG-containing oligodeoxynucleotides as immunomodulators, for example, has been proven effective in the treatment of leishmaniasis in experimental models [381-383].

In conclusion, TLRs play a crucial role in mounting innate and adaptive immunity against invading pathogens. Alteration of TLR signalling by pathogens or by clinical drugs can play a key role in the outcome of infections. We have discussed in good detail strategies used by pathogens or by the clinic to alter TLR signalling. The activation of MyD88-dependent signalling and Th1 responses can turn out very useful in the elimination of many pathogens including *Leishmania*. However, these efforts must always be perceived

with caution as exaggerated activation of inflammation can cause edema, pain, tissue injury, and in severe conditions could be deadly. In addition, certain infectious models like malaria seem to benefit from MyD88-dependent signalling and inflammation in their pathology and thus a completely different approach should be used when trying to fight *Plasmodium*. As opposed to using TLR ligands which can worsen the disease, TLR agonists could prove clinically effective in treating malaria. Nevertheless, the effects of blocking TLR-TLR-L interactions on the ability of the immune system to fight off other pathogens that can be / become present have to be taken into serious consideration.

Chapter 2

Leishmania-Induced IRAK-1 Inactivation is Mediated by SHP-1 Interacting with an Evolutionarily Conserved KTIM Motif

Although there is a good deal of knowledge about how *Leishmania* can utilize host SHP-1 to alter signalling triggered by IFN-γ stimulation, mechanisms underlying the ability of *Leishmania* to block TLR signalling, especially in response to LPS, and the role of SHP-1 in the process remain largely unexplored. It has been reported that *Leishmania* can interfere with some signalling molecules involved in downstream TLR signalling such as the MAPKs Erk1/2 and JNK and the transcription factors NF-κB and AP-1, nevertheless, nothing is known about how the parasite can interfere with critical upstream proteins unique to IL-1 / TLR signalling such as members of the IL-1-receptor-associated kinase (IRAK) family. In this chapter, we were interested to explore mechanisms utilized by *Leishmania* to block TLR signalling in MØs, and to evaluate the role of host SHP-1 in this process. The study permitted us to identify SHP-1 as a novel regulator of TLR signalling utilized by *Leishmania* to inhibit IRAK-1 kinase activity leading to the inability of MØs to respond to a wide range of TLR stimulation. We also identified the binding site for SHP-1 on IRAK-1 as an evolutionarily conserved immunoreceptor tyrosine-based inhibitory motif (ITIM)-like motif that we named kinase tyrosine-based inhibitory motif (KTIM). Finally, we proposed that such a motif (KTIM) could play a pivotal role in the regulation of many kinases other than IRAK-1.

Leishmania-Induced IRAK-1 Inactivation is Mediated by SHP-1 Interacting with an Evolutionarily Conserved KTIM Motif

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Running title: *Leishmania-Induced IRAK-1 Inactivation*

Abstract

Parasites of the *Leishmania* genus can rapidly alter several macrophage (MØ) signalling pathways in order to tame-down the innate immune response and inflammation, therefore favouring their survival and propagation within their mammalian host. Having recently reported that *Leishmania* and bacterial LPS generate a significantly stronger inflammatory response in animals and phagocytes functionally deficient for the Src homology 2 domain-containing protein tyrosine phosphatase (SHP-1), we hypothesized that *Leishmania* could exploit SHP-1 to inactivate key kinases involved in Toll-like receptor (TLR) signalling and innate immunity such as IL-1 receptor-associated kinase 1 (IRAK-1). Here we show that upon infection, SHP-1 rapidly binds to IRAK-1 completely inactivating its intrinsic kinase activity and any further LPS-mediated activation as well as MØ functions. We also demonstrate that the SHP-1 / IRAK-1 interaction occurs via an evolutionarily conserved ITIM-like motif found in the kinase domain of IRAK-1, which we named KTIM (Kinase Tyrosine-based Inhibitory Motif). This regulatory motif appeared in early vertebrates and is not found in any other IRAK family member. Our study additionally reveals that several other kinases (e.g. Erk1/2, IKK-α/β) involved in downstream TLR signalling also bear KTIMs in their kinase domains and interact with SHP-1. We thus provide the first demonstration that a pathogen can exploit a host protein tyrosine phosphatase, namely SHP-1, to directly inactivate IRAK-1 through a generally conserved KTIM motif.

Author Summary

Leishmania developed several methods to seize control of macrophage signalling pathways in an effort to inactivate their killing abilities. One effective method utilized by the parasite is the activation of host protein tyrosine phosphatases, specifically SHP-1. This increased phosphatase activity contributes to the inactivation of signalling molecules involved in critical macrophage functions such as NO and cytokines production. Interestingly, the absence of SHP-1 results in stronger macrophage inflammatory responses to a bacterial cell wall component known as LPS, a molecule detected by macrophages through Toll-like receptors (TLRs). This observation suggested a role for SHP-1 in the regulation of TLR signalling. Our study reveals that upon *Leishmania* infection, SHP-1 is able to rapidly bind to and inactivate a critical kinase (IRAK-1) in this pathway. This regulatory binding was shown to be mediated by an evolutionarily conserved motif identified in the kinase. This motif was also present in other kinases involved in TLR signalling and therefore could represent a regulatory mechanism of relevance to many kinases. This work not only reports a unique mechanism by which *Leishmania* can avoid harmful TLR signalling, but also provides a platform on which extensive investigation on host evasion mechanisms and regulation of cellular kinases can be gained.

Introduction

Innate inflammatory responses play a critical role in controlling pathogens [1]. However, protozoan parasites such as *Leishmania* evolved strategies to avoid phagocyte activation by seizing control of key signalling pathways, therefore favouring their invasion and survival within the host cell [2]. We recently reported that the protein tyrosine phosphatase (PTP) SHP-1 plays a pivotal role in taming down phagocyte-mediated inflammatory responses [3]. For instance, we showed that in the absence of SHP-1, several pro-inflammatory cytokines (e.g. IL-1β, IL-6, TNF-α) and chemokines, as well as inflammatory neutrophil recruitment were all exacerbated by *Leishmania* infection [3]. Of interest, we also found that LPS mediates an excessive inflammatory response in the absence of SHP-1, therefore suggesting that SHP-1 could exert its negative regulatory action via Toll like receptor (TLR) signalling.

As SHP-1 can interact with various members of the JAK and MAP kinase families in physiological, immune response, and infection contexts [2,3], we explored the possibility that the capacity of *Leishmania* to block the macrophage (MØ) inflammatory response could result from rapid IRAK-1 kinase inactivation through SHP-1 action. This hypothesis is further reinforced by the fact that several LPS-mediated MØ functions (e.g. TNF-α, NO, IL-12), critical for the containment of pathogens and adaptive immune response development, are inhibited upon *Leishmania* infection [2,4,5].

Whereas invertebrates depend mainly on the evolutionarily conserved innate immune system to fight off pathogens, vertebrates have developed a sophisticated adaptive immune system, hence the need to regulate the innate immune response. The TLR family

has been shown to play a key role in triggering innate immunity as well as the subsequent induction of adaptive immune responses in vertebrates [6]. Our previous findings reporting augmented *Leishmania*- and LPS-induced innate inflammatory response in the absence of SHP-1 (PTPN6) [3], and the several reports that key transcription factors (NF-κB and AP-1) related to TLR signalling were strongly activated in the absence of SHP-1 [7-9], suggested the importance of SHP-1 in the negative regulation of TLR signalling and its subsequent inflammatory response in vertebrates. Of interest, a mutation in the *PTPN6* gene coding for SHP-1 in humans has been recently linked to Sezary syndrome [10], a T-cell cutaneous lymphoma arising from chronic inflammatory state.

From these observations, and given the fact that IRAK-1 serves as a crucial kinase in all MyD88-dependent pathways leading to the activation of innate inflammatory responses, we hypothesised that SHP-1 is a critical player in the negative regulation of this kinase that can be exploited by *Leishmania*. For instance, until recently there was no indication that SHP-1 could interact with IRAK-1. However, a recent study by Cao's laboratory [11] provided strong evidence that SHP-1 can interact with IRAK-1.

Here, we provide evidence that SHP-1 negatively regulates IRAK-1 intrinsic kinase activity in its resting state and upon *Leishmania* infection through binding to an evolutionarily conserved ITIM-like motif located within IRAK-1's kinase domain. In addition, it is important to stress that this is the first mention of this motif to be found within a kinase, as to date it has only been found within the intracytoplasmic portion of immunoglobin (Ig)-like receptors. Of interest, we also discovered that this ITIM-like motif was present in several other kinases. Finally, our study also provides evidence from *in silico* sequence analyses that both IRAK-1 and SHP-1 evolutionarily emerged in

vertebrates concomitantly with the development of a better-controlled innate immune response. Therefore the appearance of this key interaction in early vertebrates may have also contributed to the development of the more complex adaptive immune response.

Materials and Methods

Cell Culture and Reagents. The immortalized me-3 (SHP-$1^{-/-}$) and LM-1 (WT) bone marrow-derived MØs (BMDMs) were generated from motheaten mice (*Ptpn6*$^{me/me}$; C3HeBFeJ *me*/*me*) and their respective wild-type littermates (C3HeBFeJ *me*/+) as described [7]. The immortalized B10R BMDMs were derived from B10A.Bcg^r mice [12]. L929 cells used for the TNF bioassays were grown in RPMI-1640 medium (5% FBS). MØ-activating lipopeptide-2 (MALP-2) and lipopolysaccharide (LPS) from *E.coli* were purchased from Alexis Biochemicals, San Diego, CA. Flagellin and CpG DNA were purchased from Invivogen, San Diego, CA. IRAK1/4 inhibitor (N-(2-Morpholinylethyl)-2-(3-nitrobenzoylamido)-benzimidazole) was purchased from Calbiochem, La Jolla, CA.

***In vitro* infection.** *L. donovani infantum, L. mexicana* (MNYC/BZ/62/M379)*, L. major* Friedlin strain (MHOM/JL/80/Friedlin)*,* and *L. tarentolae* strain TAR II promastigotes were kept in SDM medium (10% FBS), and stationary phase parasites were used to infect cells in a parasite to MØ ratio of 20:1. Non-internalized parasites were removed by washing the plates with phosphate-buffered saline (PBS), after which MØs were collected for subsequent experiments.

Western Blot Analysis. Western blotting was performed as previously described [13]. Proteins were detected using antibodies directed against IRAK-1 (generated in the

laboratory of Dr. Akira), SHP-1 and phospho-tyrosine (clone 4G10) (Upstate, Charlottesville, VA), and actin (Sigma-Aldrich, ON, Canada). Proteins were detected using an anti-rabbit or anti-mouse horseradish peroxidase (HRP)-conjugated antibody (Amersham, QC, Canada) and visualized using ECL western blotting detection system (Amersham).

IRAK-1/IRAK-4 kinase assay. $6x10^6$ MØs were lysed in cold lysis buffer (20mM Tris (pH 7.5), 1mM EDTA, 150mM NaCl, 1% Igepal, 10mM β-glycerophosphate, 1mM sodium orthovanadate, 25μg/ml aprotinin and 25μg/ml leupeptin). Lysates were precleared with protein A/G agarose beads (Santa Cruz, CA). Samples were then centrifuged (13,000 x g, 10 min) and supernatants kept. IRAK-1 or IRAK-4 antibody and protein A/G agarose beads were added to the supernatant and samples were incubated O/N at 4°C. Beads were spun down and washed with the lysis buffer described above, followed by washes with the kinase assay buffer (20mM HEPES pH 7.5, 20mM $MgCl_2$, 3mM $MnCl_2$, and 10mM β-glycerophosphate). Kinase assay buffer (20μl) containing 10μCi of γ-^{32}P (Amersham) was then added to the beads and samples incubated (30 min, 30°C). The reaction was stopped by the addition of 4x sample loading buffer (12.5% Tris-HCl (pH 6.8), 10% glycerol, 10% SDS, 5% β-mercaptoethanol, 0.05% bromophenol blue). Samples were boiled and run on SDS-PAGE. Bands were detected using X-ray Kodak films (Amersham) or by image analyzer (BioRad, Canada).

In gel PTP assay. For immunoprecipitation samples, $6x10^6$ MØs were lysed as described previously for the IRAK-1 kinase assay without the addition of sodium orthovanadate to the lysis buffer. Cell lysate controls (25µg) were obtained using a PTP lysis buffer (50mM Tris (pH 7.0), 0.1mM EDTA, 0.1mM EGTA, 0.1% β-mercaptoethanol, 1% Igepal, 25µg/ml aprotinin and 25µg/ml leupeptin). Samples were loaded on a gel containing a γ-^{32}P-labelled poly(Glu4Tyr) peptide (Sigma-Aldrich) and the SHP-1 band was observed by in gel PTP assay as previously described [14].

Co-immunoprecipitation. Samples were lysed in the western blot lysis buffer (no sodium orthovanadate was added when immunoprecipitating SHP-1) and immunoprecipitated using protein A/G agarose beads (Santa Cruz) and 4µg of the IRAK-1, SHP-1 antibody, or anti-rat antibody (Sigma-Aldrich) for non-specific binding. Beads were spun down and washed three times with lysis buffer. Beads were resuspended in the 4x western sample loading buffer previously described and boiled supernatants were loaded on SDS-PAGE and western blot analysis was performed as described above.

GST pull-down assay. Wildtype mouse IRAK-1 gene and the IRAK-1 genes of the different KTIM mutants (all in PCDNA3 vectors) were *in vitro* translated using the Promega TNT Quick Coupled Transcription/Translation kit (Fisher Scientific, ON, Canada) using 20µCi ^{35}S (Amersham). The active or the trapping mutant of GST SHP-1 was produced in BL21 bacteria. Bacterial lysates were extracted using the BugBuster Protein Extraction Reagent (VWR CANLAB, ON, Canada), and the GST protein (5µg)

was pulled down from bacterial lysates using glutathione sepharose beads (30µl) (Amersham). The active/trapping mutant of GST-SHP-1 bound to glutathione beads was left to interact with immunoprecipitates (IPs) or *in vitro* translated IRAK-1 protein in a PTP reaction buffer (50mM Hepes (pH 7.5), 0.1% β-mercaptoethanol) for 1 h at RT. When in vitro translation of IRAK-1 was performed, GST-SHP-1 was allowed to interact with IRAK-1 in a 5:1 ratio. Beads were then spun down, washed 3x with the PTP lysis buffer, then resuspended in 4x sample loading buffer (20µl), boiled, and loaded on SDS-PAGE. IRAK-1 bands were revealed by exposing to X-ray film (Amersham).

Alkali-resistance phosphoprotein assay. Kinase assays were run on SDS-PAGE as described above, pre-treatment image is taken by exposing the gel to a phospho-imager screen. Next, gels were fixed overnight at RT in a 10% methanol / 7% acetic acid solution. Gels were then soaked in a 10% glutaraldehyde solution (30 min, RT) with gentle shaking and rinsed in water prior to incubation with KOH. The alkali treatment of ^{32}P-labelled IRAK-1 was performed as previously described [15].

Generation of IRAK-1 mutants. The mouse IRAK-1 gene cloned into a PCDNA3 plasmid was mutated at different sites within the KTIM using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) as instructed by the manufacturer. The primers (all synthesized by Genome Québec, Montréal, QC, Canada) designed to create the mutants were:

For the tyrosine to phenylalanine mutation;

sense: 5'GGCTTATACTGCCTTGTTTTTGGCTTCTTGCCCAATGG3';

anti-sense: 5'CCATTGGGCAAGAAGCCAAAAACAAGGCAGTATAAGCC3'.

For the leucine to methionine mutation;

sense: 5'GGCTTATACTGCCTTGTTTATGGCTTCATGCCCAATGG3';

anti-sense: 5'CCATTGGGCATGAAGCCATAAACAAGGCAGTATAAGCC3'.

For the glycine to alanine, phenylalanine to tyrosine, leucine to methionine triple mutation, sequential mutagenesis was performed where the above-mentioned leucine mutation was used as the template to generate an additional glycine to alanine mutation using the primers:

sense: 5'GGCTTATACTGCCTTGTTTATGCCTTCATGCCCAATGG3';

anti-sense: 5'CCATTGGGCATGAAGGCATAAACAAGGCAGTATAAGCC3'.

Finally, a phenylalanine to tyrosine mutation was generated using the previously described double mutation as a template using the primers:

sense: 5'GGCTTATACTGCCTTGTTTATGCCTACATGCCCAATGG3';

anti-sense: 5'CCATTGGGCATGTAGGCATAAACAAGGCAGTATAAGCC3'.

All mutations were verified by sequencing the entire plasmid using the T7 and SP6 primers (provided by Genome Quebec, Montreal, QC, Canada) and the internal primers 5'TTCCTCCACCAAGTCAAG3' and 5'CCTGAGGAGTACATCAAGAC3'.

IL-12 mRNA expression analysis. RNA was extracted from MØs using TRIzol reagent (Invitrogen Canada, ON, Canada). Reverse transcription was performed using oligodT. Quantitative Real-Time PCR (qRT-PCR) was performed with a Corbett Research Rotorgene (Corbett Life Science, Sydney, Australia), using Invitrogen Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and 0.4 µM primer in 25 µl. qPCR program is: 50^{o}C 2 min; 95°C 3 min; (95°C 20 sec, 60°C 30 sec, 72°C 20 sec) for 40 cycles followed by a melting curve. All primers annealing temperature was 60°C. Oligo sequences are: 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 AGG GAG AAG TAG GAA TGG-5'.

TNF bioassay. TNF bioassay was performed as previously described [16]. Briefly, TNF-sensitive L929 fibroblasts were seeded in 96-well plates in a concentration of 3.5 x 10^4 cells/100 µl/well in RPMI-1640 (5% FBS) medium and incubated for 24 h until obtaining a monolayer. Supernatants from designated experiments were added to L929 cells and serially diluted in the presence of actinomycin D (2 µg/ml). After incubation (18 - 24 h, 37°C), the L929 monolayers were stained with crystal violet, washed with distilled water, and left to dry. Then, methanol was added to dissolve the stain and cytotoxicity was determined by measuring absorbance at 595 nm. One unit of TNF was referred to as the reciprocal of the dilution that induced 50% of L929 cell lysis.

NO assay. NO production was evaluated by measuring the accumulation of nitrite in the culture medium by the Griess reaction, as previously described [3].

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared by a standard protocol, and EMSAs were performed as previously described [17]. Briefly, nuclear extracts were incubated with binding buffer containing 1.0 ng of [γ-^{32}P] dATP radiolabeled double-stranded DNA oligonucleotide for 20 min at room temperature. The DNA binding consensus sequence used for NF-κB was (5'-AGTTGAGGGGACTTTCCCAGGC-3'). Sp1 consensus oligonucleotide was used as non-specific control (5'-ATTCGATCGGGGCGGGGCGAGC-3') (Santa Cruz). DNA-protein complexes were resolved by electrophoresis in native 4% (w/v) polyacrylamide gels. The gels were then dried and autoradiographed.

pNPP phosphatase assay. MØs were collected, lysed in the PTP lysis buffer described previously and kept on ice for 45 min. Lysates were cleared by centrifugation, and protein content was determined by Bradford reagent followed by IP. Equal amounts of IPs were incubated in a phosphatase reaction mix (50mM Hepes (pH 7.5), 0.1% β-mercaptoethanol, 10mM pNPP) overnight at 37°C. OD was then read at 405 nm.

Sequence alignments. Sequences were obtained from the NCBI protein database. Sequence alignments used to calculate identity and similarity percentages were generated

by EMBOSS local pair-wise alignment algorithms program (http://www.ebi.ac.uk/Tools/emboss/index.html). The accession numbers of the protein sequences included in the study are: human (*Homo sapiens*) IRAK-1 (P51617), chimpanzee (*Pan troglodytes*) IRAK-1 (XP_521332), dog (*Canis familiaris*) IRAK-1 (XP_549367), bull (*Bos Taurus*) IRAK-1 (Q2LGB3), mouse (*Mus musculus*) IRAK-1 (Q62406), rat (*Rattus norvegicus*) IRAK-1 (XP_001057078), tropical frog (*Xenopus tropicalis*) IRAK-1 (NP_001006713), zebrafish (*Danio rerio*) IRAK-1 (XP_697688), human IRAK-4 (Q9NWZ3), chimpanzee IRAK-4 (XP_001166114), rhesus monkey (*Macaca mulatta*) IRAK-4 (XP_001091707), dog IRAK-4 (XP_543727), bull IRAK-4 (Q1RMT8), mouse IRAK-4 (Q8R4K2), rat IRAK-4 (XP_217026), chicken (*Gallus gallus*) IRAK-4 (NP_001025909), zebrafish IRAK-4 (AAT37635), squid (*Euprymna scolopes*) IRAK-4 (AAY27972), sea urchin (*Strongylocentrotus purpuratus*) IRAK-4 (XP_784716), *Caenorhabditis elegans* IRAK-4 (NP_502587), honeybee (*Apis mellifera*) pelle-like protein (XP_624002), *Drosophila melanogaster* pelle (NP_476971), chicken IRAK-2 (NP_001025776), mouse JAK2 (Q62120), mouse JAK3 (Q62137), mouse TAK1 (Q62073), mouse Erk1 (Q63844), mouse Erk2 (P63085), mouse JNK (CAC88132), mouse p38 (P47811), mouse IKK-α (Q60680), mouse IKK-β (O88351), mouse LYN (AAH31547).

Band quantification. All densitometric analyses were performed using the Quantity One software, Biorad Laboratories Inc. Values and standard deviations observed represent scans of three independent experiments.

Ethical Oversight: The bone marrow-derived macrophages described in this study have been previously derived from WT and SHP-1-deficient mice (see reference 7), and immortalized as cell lines. However, experiments done on the animals used in that study (reference 7) adhered to McGill University's guidelines for animal husbandry and was approved by the institutional research ethics committee.

Results

SHP-1 regulates IRAK-1 kinase activity by direct interaction

To investigate the effect of SHP-1 on IRAK-1 kinase activity, we immunoprecipitated IRAK-1 from the lysates of SHP-$1^{-/-}$ MØs and their wildtype (WT) counterparts and subjected the IP to an IRAK-1 kinase assay. Results indicated that IRAK-1 kinase activity in SHP-$1^{-/-}$ cells was significantly higher compared to WT (Figure 1, top panel). The increase in IRAK-1 basal kinase activity observed in SHP-$1^{-/-}$ cells is not due to a differential expression of IRAK-1 as supported by loading controls provided (Figure 1, lower panels).

Then, to evaluate whether the SHP-1 regulatory effect on IRAK-1's kinase activity involved their interaction, we performed immunoprecipitation assays and observed that IRAK-1 and SHP-1 co-IP (Figure 2A). Their association was further confirmed as we have detected PTP activity corresponding to SHP-1 in the IRAK-1 IP (Figure 2B, top panel), and IRAK-1 kinase activity in the IP of SHP-1 (Figure 2B**,** bottom panel). A secondary rat antibody was used as a negative control (Figures 2A and B). These experiments suggested the presence of IRAK-1 and SHP-1 in the same multi-protein complex. To test whether they directly interact, we *in vitro* translated IRAK-1 using radiolabelled methionine, and put the radiolabelled IRAK-1 in contact with GST-SHP-1. IRAK-1 was pulled down specifically by GST-SHP-1 and not by GST alone, showing that this interaction is direct (Figure 2C).

Next, we examined whether the binding of SHP-1 is sufficient to regulate IRAK-1 kinase activity. To do so, IRAK-1 was immunoprecipitated and put in contact with increasing concentrations of active GST-SHP-1. IRAK-1 kinase activity was inhibited in a dose-dependent manner by GST-SHP-1 and not by GST alone (Figure 2D, left panel). Interestingly, the highest dose of GST-SHP-1 used to inhibit IRAK-1 activity did not alter IRAK-4's kinase activity (Figure 2D, right panel).

The fact that the PTP-SHP-1 dephosphorylates tyrosine residues raised the possibility that IRAK-1 is tyrosine phosphorylated. To investigate this hypothesis, alkali-resistance phosphoprotein assays were performed. Treatment of IRAK-1 kinase assay gels with KOH permits the in-gel dephosphorylation of pSer and pThr, but not pTyr allowing us to evaluate the contribution of tyrosine phosphorylation to the overall phosphorylation signal. Although IRAK-1 is known to be phosphorylated on Ser/Thr residues [18], our results represent the first demonstration that IRAK-1 is also tyrosine phosphorylated at resting state, and that LPS increases IRAK-1 tyrosyl phosphorylation by 46 ±15% SD (Figure 2E, upper panels). This finding was further confirmed by western blot using the 4G10 pTyr-specific antibody (Figure 2E, lower two panels).

SHP-1 binds to the kinase domain of IRAK-1 via an ITIM-like motif

At the view of our observations, we screened the mouse IRAK-1 sequence for possible SHP-1 binding sites. We discovered that IRAK-1 contains an ITIM-like motif ($_{286}$**L**V**Y**GF**L**$_{291}$) located in its kinase domain (Figure 3). This motif was found to be absent in all the other IRAK family members since the last residue is a methionine instead

of a leucine (Figure 4). To determine the involvement of this ITIM-like motif in the SHP-1 / IRAK-1 binding, we used the full-length IRAK-1 sequence to introduce site-specific mutations within the motif followed by *in vitro* binding assays (Figure 5). Firstly, a Y288F mutation slightly decreased SHP-1 binding suggesting that possible phosphorylation of the motif's central tyrosine may increase binding affinity but is not absolutely necessary for the binding to occur. Secondly, an L291M mutation, which renders the site no more ITIM-like, significantly decreased SHP-1 binding. Thirdly, the G289A/F290Y/L291M triple mutation, which also disrupts the ITIM-like motif, completely abrogated the binding of SHP-1. Interestingly, this triple mutant of IRAK-1 is identical to the corresponding site within IRAK-4. Collectively, these site-specific mutations confirm the role of the ITIM-like motif in the binding of SHP-1 to IRAK-1. This represents the first description of such a motif in a kinase that we now call KTIM (**K**inase **T**yrosine-based **I**nhibitory **M**otif). Importantly, these experiments also suggest that the SHP-1-mediated regulation of IRAK-1 is a mechanism not shared with IRAK-4.

Leishmania inhibits LPS-mediated MØ functions by rapidly inactivating IRAK-1

The biological relevance of this regulatory interaction between IRAK-1 and SHP-1 was investigated using the ability of N-(2-Morpholinylethyl)-2-(3-nitrobenzoylamido)-benzimidazole, a potent IRAK-1 inhibitor [19], to reduce NO production in WT and SHP-$1^{-/-}$ MØs. As mentioned earlier, SHP-1 deficiency in MØs results in an increase in NF-κB and AP-1 activity [7-9] leading to NO production at basal level and in response to LPS when compared to WT [8]. Addition of the IRAK-1 inhibitor abrogated IRAK-1 activity

in a dose-dependent manner (Figure 6A), and was paralleled by a reduction of basal NO production in SHP-1$^{-/-}$ cells and in LPS-mediated NO production in both cell lines (Figure 6B). In addition to demonstrating the essential role of IRAK-1 signalling in NO generation, our data also show that SHP-1-mediated IRAK-1 regulation is critical for the control of MØ activation.

Using *Leishmania* as an infectious model, we studied its ability to inhibit key MØ LPS-mediated functions namely: IL-12 expression, TNF production, and NO generation. Our results confirmed that infection with *Leishmania* caused a significant inhibition of LPS-mediated expression of IL-12 (Figure 7A), TNF production (Figure 7B), and NO generation (Figure 7C) in MØs.

As *Leishmania* activates host SHP-1 and blocks many LPS-mediated functions known to be detrimental to the parasite, we investigated the possibility that *Leishmania* inactivates IRAK-1. Kinase assays comparing IRAK-1 activity in MØs infected with *L. donovani* to uninfected cells revealed that the parasite caused a rapid time-dependent inactivation of IRAK-1 seen by reduced basal IRAK-1 activity in infected MØs (Figure 8A). To investigate whether IRAK-1 inactivation is a common mechanism utilized by other infectious *Leishmania* species, MØs were infected for 1 h with various *Leishmania* species promastigotes and IRAK-1 kinase activity was measured. *L. donovani* decreased IRAK-1 activity by 65±11% SD, and consistent with our expectation, *L. mexicana* and *L. major* were also able to inactivate IRAK-1 as they decreased IRAK-1 kinase activity by 65±7% SD and 52±4% SD, respectively (Figure 8B). Interestingly, *L. tarentolae*, a lizard non-pathogenic *Leishmania* did not inhibit IRAK-1 and seemed to even slightly activate it (increase of 20±11% SD).

In light of these observations, we were interested to evaluate whether the *Leishmania*-mediated IRAK-1 kinase inactivation could alter LPS-mediated functions in infected MØs. Our results indicated that unlike LPS stimulation *per se* that activates IRAK-1, infection with *Leishmania* rendered IRAK-1 activation refractory to this TLR4 agonist (Figure 8C). Since IRAK-1 signals downstream of all TLRs with the exception of TLR3, we investigated whether this *Leishmania*-induced IRAK-1 inactivation is persistent upon stimulation with other TLR ligands. As expected, *Leishmania* was able to render IRAK-1 unresponsive to MALP (TLR2), flagellin (TLR5), and CpG (TLR9) (Figure 8D). These results suggest that alteration of IRAK-1-dependent signalling by *Leishmania* causes a general unresponsiveness to a broad range of TLR ligands. All TLR ligands used were shown to be functional using an NF-κB nuclear translocation assay (Figure 9).

Leishmania infection enhances the IRAK-1 / SHP-1 interaction leading to IRAK-1 inactivation

Having previously reported that *Leishmania* can rapidly induce host PTP SHP-1 to inactivate JAK and MAP kinase pathways [8,20], we hypothesized that the *Leishmania*-induced IRAK-1 inactivation observed was associated with an increased SHP-1 / IRAK-1 interaction. We indeed noticed by Western blot that a significantly greater amount of SHP-1 was co-immunoprecipitated with IRAK-1 upon *Leishmania* infection (Figure 10A). Similarly, using in gel PTP assay, we were able to detect more SHP-1 activity in IRAK-1 IP from lysates of *Leishmania*-infected MØs (Figure 10B). Higher SHP-1

activity in *Leishmania* infected MØs was further supported when equal IP fractions were subjected to a pNPP phosphatase assay (Figure 10C).

To demonstrate that the increased SHP-1 / IRAK-1 binding upon *Leishmania* infection is responsible for IRAK-1 inactivation, IRAK-1 kinase activity was monitored in infected WT and SHP-$1^{-/-}$ MØs. In accordance with our finding in B10R MØs (Figure 8), *Leishmania* was able to inactivate IRAK-1 in WT MØs (67± 9% SD decrease in IRAK-1 activity). Interestingly, this *Leishmania*-induced inactivation was not detected in the absence of SHP-1 (2± 6% SD decrease in IRAK-1 activity) (Figure 10D**)**. This rescue of IRAK-1 activity was correlated with an inability of the parasite to block LPS-induced NO production in SHP-$1^{-/-}$ MØs (Figure 10E). Collectively, this set of data shows that the *Leishmania*-activated SHP-1 is responsible for IRAK-1 inactivation leading to the unresponsiveness of infected MØs to LPS stimulation.

To further understand the impact of IRAK-1 inactivation on LPS-mediated activation in infected MØs, we monitored the association and dissociation events of IRAK-1 with known key signalling molecules (MyD88, TRAF6) in response to LPS in naïve and *Leishmania*-infected cells. The result showed that IRAK-1 inactivation by *Leishmania*-induced SHP-1 is associated with the inability of IRAK-1 to detach from MyD88 and attach to TRAF6 in response to LPS stimulation (Figures 10F and G).

IRAK-1 and SHP-1 emerged in early vertebrates while IRAK-1 KTIM appeared only in amphibians

Given the important regulatory function of the KTIM present within IRAK-1, we speculated that it would be evolutionarily conserved. *In silico* sequence comparisons of available IRAK-1 sequences revealed that KTIM (**L**V**Y**GF**L**) was fully conserved from rodents to human (Figure 11). However, while the KTIM in *Xenopus tropicalis* showed some variations compared to the other vertebrate sequences (**L**I**Y**LY**L**), it was absent in zebrafish due to the presence of a methionine at the last position (**V**I**Y**VYM). Next, we addressed the origin of IRAK-1 and SHP-1 as they are only present in vertebrates. Sequence similarity analyses, including available IRAK-4 sequences from vertebrates and invertebrates (Figure 12), indicate that IRAK-1 evolved from IRAK-4 by gene duplication (Figure 13A). Similar sequence similarity comparisons suggest that SHP-1 evolved from SHP-2 and its orthologues found in invertebrates and that the ancestral SHP-1 gene also appeared through gene duplication in lower vertebrates (zebrafish) (Figure 13B).

From these observations, we raised the question whether other kinases may also have a KTIM within their kinase domain. Although several proteins involved in MyD88-dependent signalling (e.g. MyD88, TIRAP, TRAF6) did not contain KTIMs in their amino acid sequence (data not shown), we were intrigued to discover that several kinases from the JAK, MAP, Src, and IKK kinase families (JAK2, JAK3, Erk1/2, JNK, p38, LYN, IKK-α/β) contained one or more potential KTIMs, the majority located within their kinase domains (Figure 14A). This finding raises the possibility that KTIMs play important regulatory functions for many kinases by favouring their interaction with SHP-

1, as we herein report for IRAK-1. SHP-1 binding may control the activity of these kinases at resting state or regulate their activity upon activation. In gel phosphatase assays that we performed support this possibility as they demonstrate that IPs of IKK-β, Erk, JNK, and p38 indeed exhibit SHP-1 activity (Figure 14B), indicating that these kinases interact with SHP-1. Interestingly, Syk – a kinase that has no KTIM in its amino acid sequence – did not show interaction with SHP-1 at resting state.

Discussion

Leishmania has been reported to inhibit critical LPS-mediated MØ functions such as NO and pro-inflammatory cytokines (e.g. IL-12 and TNF) production [2,4,5]. Although mechanisms whereby NO is inhibited by *Leishmania* in response to IFN-γ have been well explored [2], our knowledge concerning the negative regulatory mechanisms leading to the down-regulation of LPS-mediated MØ functions in *Leishmania*-infected cells is limited. Herein, we provide the first demonstration that the *Leishmania* parasite can rapidly inactivate IRAK-1 kinase activity with the participation of SHP-1, therefore inhibiting MØ LPS-mediated functions. We further reveal that the mechanism by which this inactivation occurs is through the binding of SHP-1 to an evolutionarily-conserved ITIM-like motif located in the kinase domain of IRAK-1. This is the first demonstration that a pathogen can use a host PTP to inactivate IRAK-1 and therefore block signalling pathways ultimately leading to free radicals and pro-inflammatory cytokines production known to be detrimental to its survival.

Given that TNF is a potent MØ activator, NO is leishmanicidal, and IL-12 is a critical cytokine that drives Th1 responses essential for the development of immunity against *Leishmania*, it is not surprising that the parasite has evolved means to block the production of these molecules [2]. A role for *Leishmania* phosphoglycans (PG) has been proposed in the inhibition of NO [21]. In addition, roles for promastigote PG [22,23] and amastigote cysteine peptidases [24] in the inhibition of LPS-mediated IL-12 production have been reported. Nevertheless, apart from very few reports about *Leishmania*-induced alterations in the Erk MAPK [22] and the downstream transcription factor NF-κB [24], very little is known about how LPS-mediated functions are inhibited by *Leishmania*. In

this study, we confirmed that all three LPS-mediated MØ functions were inhibited by *Leishmania*. Importantly, looking at NO production as a key function involved in the killing of *Leishmania* parasites, we were able to show that IRAK-1 signalling is key for its production. In fact, our finding that *Leishmania* inactivates IRAK-1 kinase activity and that this inactivation is persistent upon subsequent LPS-stimulation supports the fact that the parasite is able to successfully block LPS-mediated NO production in MØs. Interestingly, consistent with the fact that IRAK-1 signals downstream of many TLRs, we showed that IRAK-1 was also unresponsive in *Leishmania*–infected cells subjected to stimulation with TLR2, TLR5, and TLR9 ligands. This result suggests that the parasite causes wide range unresponsiveness to TLR signalling upon infection possibly allowing *Leishmania* to avoid any harmful MØ activation involving TLR engagement. Interestingly, *L. donovani* has been shown to activate IRAK-1 in IFN-γ-primed MØs [25] suggesting that the activation state of the MØ can play an important role in the ability of the parasite to inactivate IRAK-1. In an effort to understand how *Leishmania* inactivates IRAK-1, we were able to identify SHP-1 as a key player in this process as there was almost a complete rescue of IRAK-1 activity in SHP-$1^{-/-}$ MØs infected with *Leishmania*. This rescue was corroborated by the parasite's inability to block LPS-mediated NO production in SHP-$1^{-/-}$ MØs. These results suggest a new evasion mechanism whereby *Leishmania* can avoid detrimental MØ functions driven by MyD88-dependent pathways by blocking IRAK-1, a key kinase in this pathway.

Our observation that the *Leishmania*–mediated IRAK-1 inactivation was associated with enhanced SHP-1 binding to IRAK-1 fits with our finding that SHP-1 binds to and regulates IRAK-1 at resting state. We clearly showed that IRAK-1's intrinsic kinase

activity was higher in SHP-1$^{-/-}$ compared to WT MØs identifying SHP-1 as a novel regulator of IRAK-1 activity, a finding supported by recent work of Cao and colleagues [11]. The fact that SHP-1 interacts with and also dephosphorylates tyrosine residues raised the possibility that IRAK-1 is tyrosine phosphorylated. Here, we show that IRAK-1 is indeed tyrosine phosphorylated at resting state, and further so in response to LPS stimulation. Given that IRAK-1 was previously shown to be phosphorylated on Ser / Thr residues only [18], our findings represent the first demonstration that IRAK-1 is also tyrosine phosphorylated.

Having identified an ITIM-like motif in the kinase domain of IRAK-1 as the binding site of SHP-1, its functionality was demonstrated by generating mutations within the motif providing valuable information about the role of its amino acid components in the binding affinity of SHP-1. Firstly, the tyrosine to phenylalanine (Y288F) mutation suggested that the phosphorylation of the motif's central tyrosine is not necessary for the binding of SHP-1 to occur. Indeed it has been previously reported that tyrosyl phosphorylation within ITIMs is not always required for the binding of SH2 domain-containing proteins [26]. Secondly, the observation that the G289A-F290Y-L291M mutation caused a total abrogation of SHP-1 binding, and that the L291M mutation partially reduced binding suggested that the amino acids between the central tyrosine and the terminal leucine in the motif play an important role in the binding affinity of SHP-1. Lastly, as the triple mutant was designed to render the ITIM-like site in IRAK-1 identical to its corresponding site in IRAK-4, the loss of SHP-1 binding in this mutant suggested that the SHP-1-mediated regulation of IRAK-1 is a regulatory mechanism not shared with IRAK-4. It is noteworthy to emphasize that ITIMs have been named so due to their presence in

intracytoplasmic portions of transmembrane receptors [27]. Given that here we describe this motif to be found in a cytosolic kinase and show that it mediates SHP-1 binding and IRAK-1's negative regulation, we propose to rename it KTIM (**K**inase **T**yrosine-based **I**nhibitory **M**otif).

In MyD88-dependent signalling pathways, binding of a TLR ligand to its corresponding receptor causes a rearrangement of the receptor complex and triggers the recruitment of the adaptor protein MyD88, which in turn recruits the kinases IRAK-4 and IRAK-1 to the receptor complex [1]. Upon critical phosphorylations of IRAK-1 by IRAK-4 [18], IRAK-1 is partially activated and is able to get fully activated by autophosphorylation. This autophosphorylation causes IRAK-1 to detach from the MyD88 complex and attach to TRAF6, activating downstream signalling pathways. Therefore, the IRAK-1 inactivation by *Leishmania*-induced SHP-1 had to interfere somehow with the integrity of the previous signalling events. Of utmost interest, we have been able to show that although IRAK-1 was still able to bind MyD88 in *Leishmania*-infected MØs in response to LPS stimulation, the kinase was unable to detach from the MyD88 complex and bind to TRAF6 as the stimulation persisted. This is the first demonstration that a pathogen can interfere with TLR signalling by altering IRAK-1's capacity to dissociate from the MyD88 complex. This inability of IRAK-1 to detach from MyD88 is supported by our observation that the binding of *Leishmania*-induced SHP-1 to the kinase domain of IRAK-1 causes a strong inactivation of this kinase seen by its inability to autophosphorylate, a process required for IRAK-1 to detach from the receptor complex and activate downstream signalling cascades.

Finally, it was remarkable to find out that the KTIM in IRAK-1 was evolutionarily conserved from human to rodents. The absence of KTIM in fish and its appearance in amphibians suggests that this motif emerged rapidly after the appearance of the ancestral IRAK-1 gene in early branching vertebrates (amphibians) and was highly conserved thereafter (Figure 15). Our findings thus raise the possibility that during the course of evolution, the emergence of a mechanism to regulate the innate immune response by targeting IRAK-1 activity (e.g. SHP-1) may have favoured the development of a more sophisticated adaptive immune system in higher vertebrates (Figure 15). In addition, it is important to note that Toll-interacting protein (TOLLIP) [28], the only other negative regulator of IRAK-1 at resting state has emerged very early in invertebrates as opposed to SHP-1 [29], IRAK-1 [30] and the KTIM motif which all appeared only in early vertebrates. Noteworthy, we found that several other kinases from the JAK, MAP and IKK kinase families contained one or more potential KTIMs raising the possibility that KTIMs play important regulatory functions in many kinases (other than IRAK-1) by favouring their interaction with SHP-1. In fact, it has been previously reported that some of these kinases (e.g. JAK2, JAK3, JNK, Erk1/2) are negatively regulated by SHP-1 [31-33]. However, none of these studies paid great attention to the mechanism whereby SHP-1 either interacts or regulates these kinases. It remains to be mentioned that whereas some of these kinases are also present in invertebrates, IRAK-1 and its KTIM only appeared in vertebrates. This observation supports the idea that the appearance of this motif in IRAK-1 has favoured the development of a mechanism to control the innate immune response. In this context, regulation of IRAK-1 kinase activity would have prevented abnormal and exacerbated microbicidal and inflammatory immune responses that could have been detrimental to vertebrates and to the development of the adaptive immune response. It is

also tempting to speculate that the appearance of an improved control over kinases by SHP-1 may have influenced the global development of vertebrates, as several of these kinases play pivotal roles in the regulation of cellular, molecular, developmental, and metabolic processes.

In conclusion, we have identified a new evasion mechanism whereby *Leishmania*-activated SHP-1 binds to an evolutionarily conserved KTIM located in IRAK-1's kinase domain leading to its inactivation. This abrogation was associated with the inability of IRAK-1 to detach from the MyD88 complex to bind TRAF6, consequently resulting in the unresponsiveness of *Leishmania*-infected macrophages to several TLR ligand stimulation including LPS. By doing so, the parasite is not only able to block LPS-mediated MØ production of NO and pro-inflammatory cytokines known to be involved in *Leishmania* killing, but also terminate the extremely important roles played by these molecules in the development of an effective adaptive immune response. At the evolutionary level, we propose that the appearance of SHP-1 as a key regulator of IRAK-1 kinase activity represented a pivotal evolutionary step that could have favoured the development of the adaptive immune response in vertebrates.

Figure 1: Regulation of IRAK-1 kinase activity by SHP-1. Upper Panel represents an *in vitro* kinase assay comparing the basal kinase activity of IRAK-1 in WT littermates versus *Ptpn6*$^{me/me}$ MØ (SHP-1$^{-/-}$). A fraction of the IP was kept and subjected to a western blot as a control for equal IRAK-1 IP (2nd panel from top). Cell lysates of WT and SHP-1$^{-/-}$ MØs were blotted for SHP-1 to demonstrate the presence / absence of the SHP-1 protein (3rd panel from top). The membrane was stripped and reblotted for IRAK-1 to monitor its expression level in both cell lines (4th panel from top). Actin levels are shown as loading controls (bottom panel). All results are representative of at least three independent experiments.

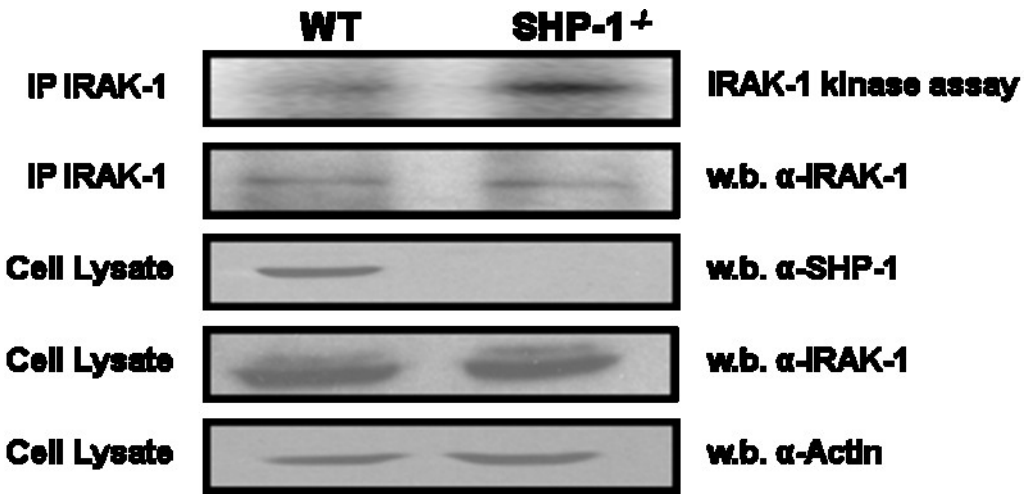

WT
SHP-1 -/-
IP IRAK-1
IRAK-1 kinase assay
IP IRAK-1
w.b. α-IRAK-1
Cell Lysate
w.b. α-SHP-1
Cell Lysate
w.b. α-IRAK-1
Cell Lysate
w.b. α-Actin

Figure 2: Demonstration of the IRAK-1 / SHP-1 interaction. (A) Western blot analysis demonstrating the co-IP of IRAK-1 and SHP-1. IRAK-1 and SHP-1 were immunoprecipitated then blotted against SHP-1 and IRAK-1 antibodies. Rabbit IgG anti-rat was used as a control. (B) In gel PTP activity assay of SHP-1 and IRAK-1 IPs (top blot). Rabbit IgG anti-rat was used as an IP control. Cell lysates of WT and SHP-$1^{-/-}$ were added in the last two lanes to confirm that the signal was SHP-1. Lower blot represents IRAK-1 kinase activity in reciprocal IPs. (C) *In vitro* transcription/translation of the IRAK-1 gene was performed using radiolabelled methionine. First lane shows the IRAK-1 input. The last two lanes show methionine-labelled IRAK-1 pulled down after 1 h incubation with either a GST-SHP-1 or GST respectively. (D) Kinase assay measuring IRAK-1 activity upon its interaction with either GST alone or increasing concentrations of an active GST-SHP-1 construct (left panel). Kinase assay measuring IRAK-4 activity (right panel) upon its interaction with either GST alone or GST-SHP-1 (5µg). (E) Kinase assay showing IRAK-1 activity at basal level and upon LPS treatment subjected to alkali treatment to evaluate tyrosine phosphorylation in IRAK-1. Tyrosyl phosphorylation was confirmed by western blot using an anti-phosphotyrosine antibody. An IP fraction was kept and blotted for IRAK-1 as a loading control.

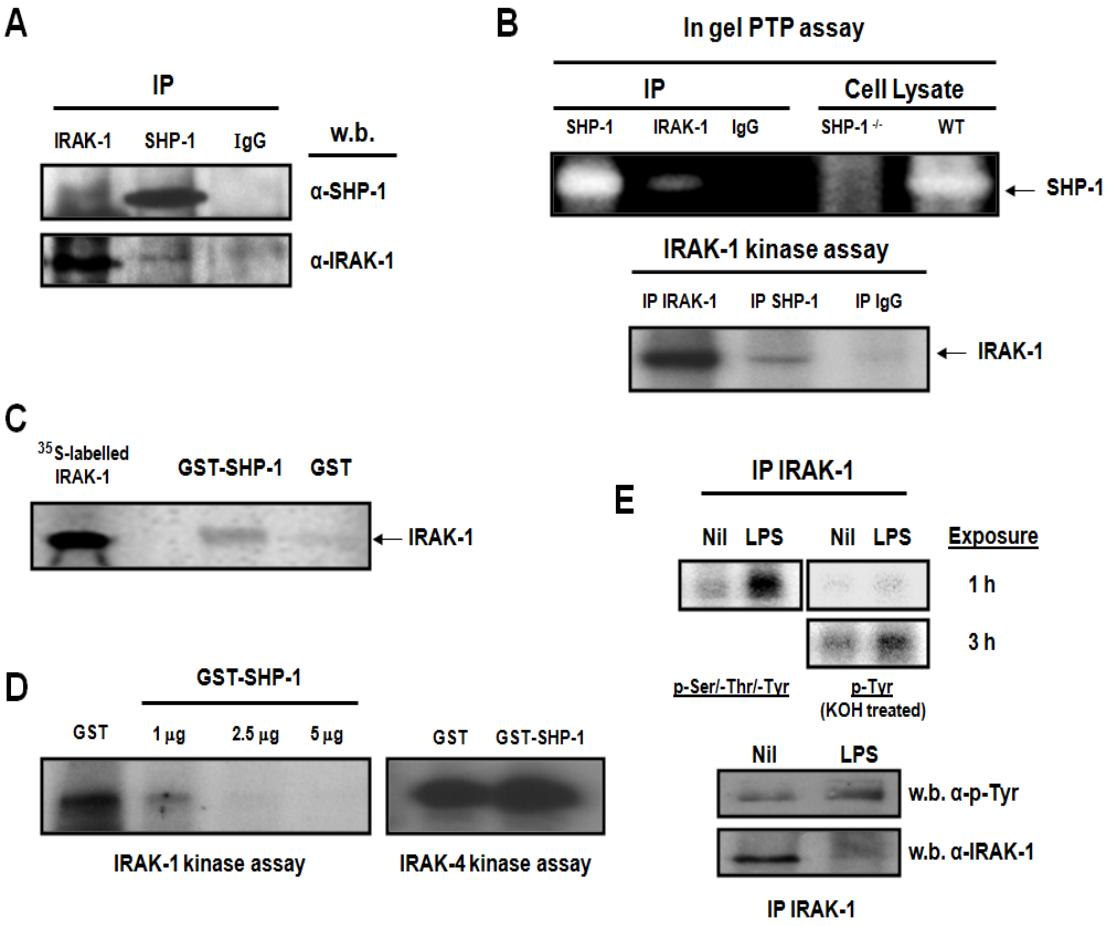

A
IP
IRAK-1
SHP-1
IgG
w.b.
α-SHP-1
α-IRAK-1
B
In gel PTP assay
IP
Cell Lysate
SHP-1
IRAK-1
IgG
SHP-1 -/-
WT
SHP-1
IRAK-1 kinase assay
IP IRAK-1
IP SHP-1
IP IgG
IRAK-1
C
35S-labelled IRAK-1
GST-SHP-1
GST
IRAK-1
D
GST-SHP-1
GST
1 µg
2.5 µg
5 µg
GST
GST-SHP-1
IRAK-1 kinase assay
IRAK-4 kinase assay
E
IP IRAK-1
Nil
LPS
Nil
LPS
Exposure
1 h
3 h
p-Ser/-Thr/-Tyr
p-Tyr
(KOH treated)
Nil
LPS
w.b. α-p-Tyr
w.b. α-IRAK-1
IP IRAK-1

Figure 3: IRAK-1 contains a KTIM motif in its kinase domain. The full amino acid sequence of mouse IRAK-1 has been obtained from the NCBI protein database (Ref. no. Q62406). The newly identified KTIM is in violet. Bottom drawing is a schematic representation of the IRAK-1 protein showing the locations of the different domains and critical residues. KTIM motif is shown as a violet rectangle. ProST, Proline/Serine/Threonine –rich.

IRAK-1 mouse sequence

(IL-1 receptor-associated kinase 1)

MAGGPGPGEP VVPGAQHFLY EVPPWVMCRF YKVMDALEPA DWCQFAALIV RDQTELRLCE
RSGQRTASVL WPWINRNARV ADLVHILTHL QLLRARDIIT AWHPPAPVVP PSTAAPRPSS
ISAGSEAGDW SPRKLQSSAS TFLSPAFPGS QTHSESELLQ VPLPVSLGPP LPSSAPSSTK
SSPESPVSGL QRAHPSPFCW PFCEISQGTC NFSEELRIGE GGFGCVYRAV MRNTTYAVKR
LKEEADLEWT MVKQSFLTEV EQLSRFRHPN IVDFAGYCAE SGLYCLVYGFL PNGSLEDQL
HLQTQACSPL SWPQRLDILL GTARAIQFLH QDSPSLIHGD IKSSNVLLDE RLMPKLGDFG
LARFSRFAGA KASQSSTVAR TSTVRGTLAY LPEEYIKTGR LAVDTDTFSF GVVILETLAG
QRAVRTQGAK TKYLKDLIED EAEEAGVTLK STQPTLWVGV ATDAWAAPIA AQIYKKHLDS
RPGPCPPQLG LALAQLACCC MHRRAKKRPP MTQVYKRLEG LQAGPPWELE VAGHGSPSPQ
ENSYMSTTGS AQSGDEPWQP LVVTTRAPAQ AAQQLQRSPN QPVESDESVP GLSATLHSWH
LTPGSHPSPA SFREASCTQG GTTRESSVRS SPGFQPTTME GSPTGSSSLL SSEPPQIIIN
PARQKMVQKL ALYEEGVLDS LQLLSSGFFP GLDLEPEKQG PEESDEFRQS

Death domain (28-103)

ProST region (110-211)

Pkinase (212-521)

K: ATP-binding lysine[239]

D: Conserved Aspartate[340]

T: Thr[209] and Thr[387]

ITIM motif Usual consensus: (I/V/L/S) X Y X X (L/V), motif recognized by SH2-domain containing proteins. Renamed KTIM motif (Kinase Tyrosyl-based Inhibitory Motif)

Figure 4: Among the IRAK family, KTIM is unique to IRAK-1. All other IRAK family members (IRAK-2, IRAK-M, and IRAK-4) whose sequences are available for various invertebrate and vertebrate organisms lack a KTIM. A sequence comparison in the KTIM region among the different IRAK family members is shown. Mouse was chosen as a representative organism.

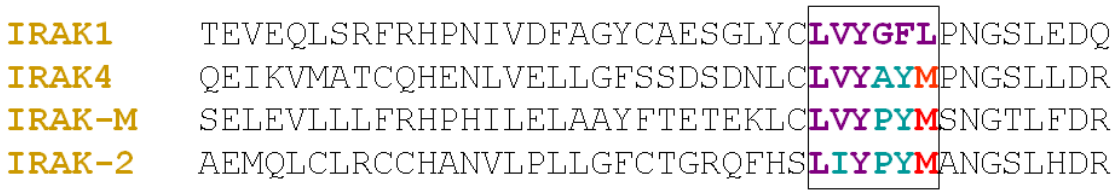

IRAK1 TEVEQLSRFRHPNIVDFAGYCAESGLYCLVYGFLPNGSLEDQ
IRAK4 QEIKVMATCQHENLVELLGFSSDSDNLCLVYAYMPNGSLLDR
IRAK-M SELEVLLLFRHPHILELAAYFTETEKLCLVYPYMSNGTLFDR
IRAK-2 AEMQLCLRCCHANVLPLLGFCTGRQFHSLIYPYMANGSLHDR

Figure 5: Mutation of IRAK-1's KTIM abrogates its ability to bind SHP-1. Top panel represents methionine-labelled WT-IRAK-1 as well as the different IRAK-1 mutants *in vitro* translated and put in contact with 5µg of either GST alone or a trapping GST-SHP-1 construct. Bottom panel represents equal fractions of the *in vitro* translated products ran on an SDS gel to show equal input. All results are representative of at least three independent experiments.

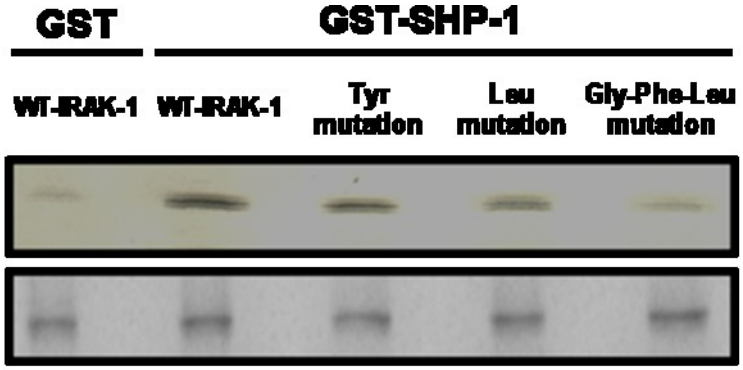

GST
GST-SHP-1
WT-IRAK-1
WT-IRAK-1
Tyr mutation
Leu mutation
Gly-Phe-Leu mutation
In vitro translation (Input, 0.2 μg)

Figure 6: Effect of pharmacological inhibition of IRAK-1 on macrophage nitric oxide production. (A) IRAK-1 was immunoprecipitated from SHP-$1^{-/-}$ MØ lysates and incubated (1 h, RT) with increasing concentrations of the IRAK-1 inhibitor. A kinase assay was then performed to show functionality of the inhibitor. Data are representative of three experiments. (B) NO assay showing that the IRAK-1 inhibitor blocks, in a dose-dependent manner, basal production of NO by SHP-$1^{-/-}$ cells as well as LPS-mediated (O/N stimulation) NO production in both WT and SHP-$1^{-/-}$ MØs.

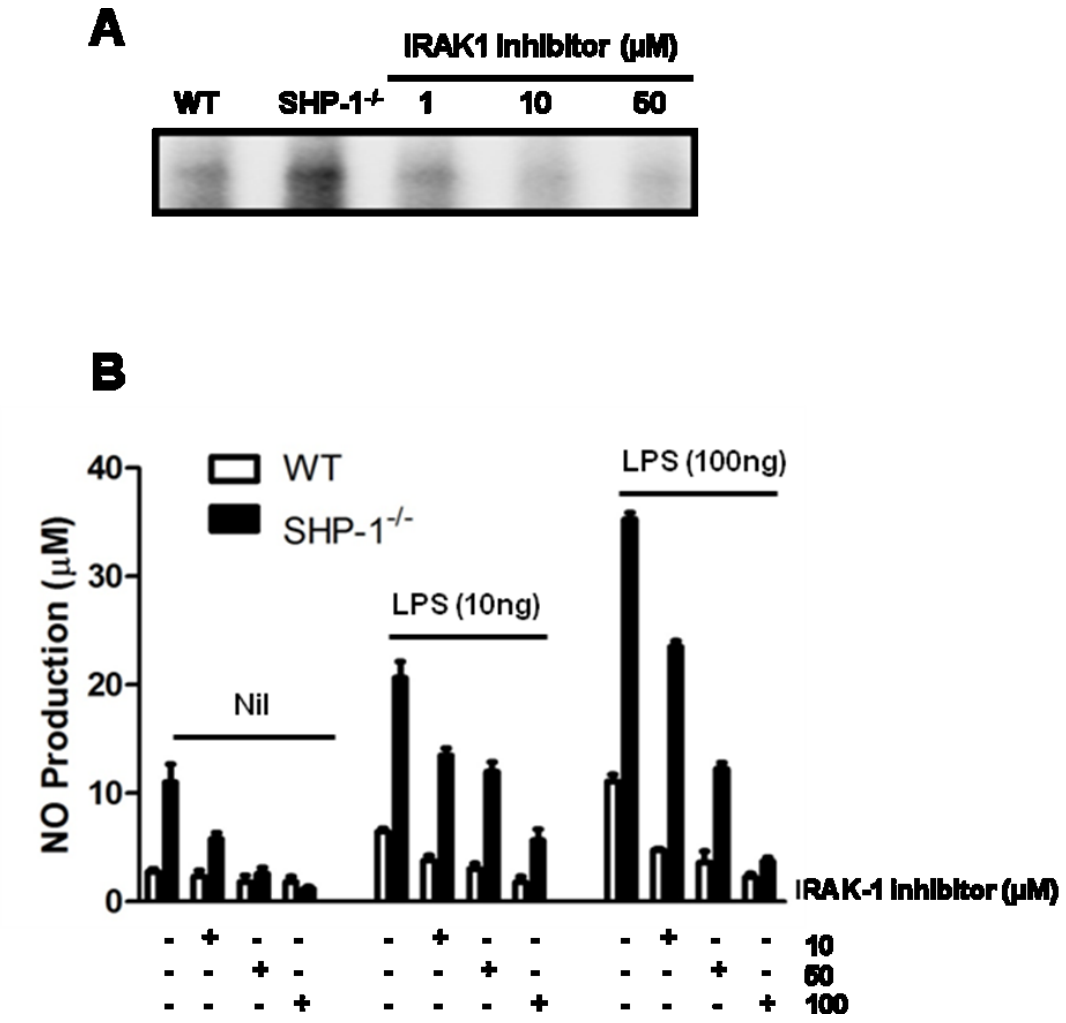

A
IRAK1 Inhibitor (μM)
WT
SHP-1-/-
1
10
50
B
WT
SHP-1-/-
LPS (100ng)
LPS (10ng)
Nil
NO Production (μM)
40
30
20
10
0
IRAK-1 Inhibitor (μM)
10
50
100

Figure 7: Inhibition of LPS-mediated functions by *Leishmania*. (A) LPS-mediated MØ IL-12 mRNA expression was analyzed by RT-PCR in uninfected and *Leishmania*-infected MØs. Cells were infected with *L. donovani* O/N followed by LPS stimulation (10 and 100 ng/ml, 12 h). (B) LPS-mediated TNF production by MØs infected with *Leishmania*. Cells have been infected as above and stimulated with LPS for 3 h. (C) NO production by *Leishmania*-infected MØs in response to LPS. Cells have been infected as above and stimulated with LPS for 24 h. (A - C) *, significant at $P < 0.05$, Anova test, error bar SEM. Mean of three independent experiments.

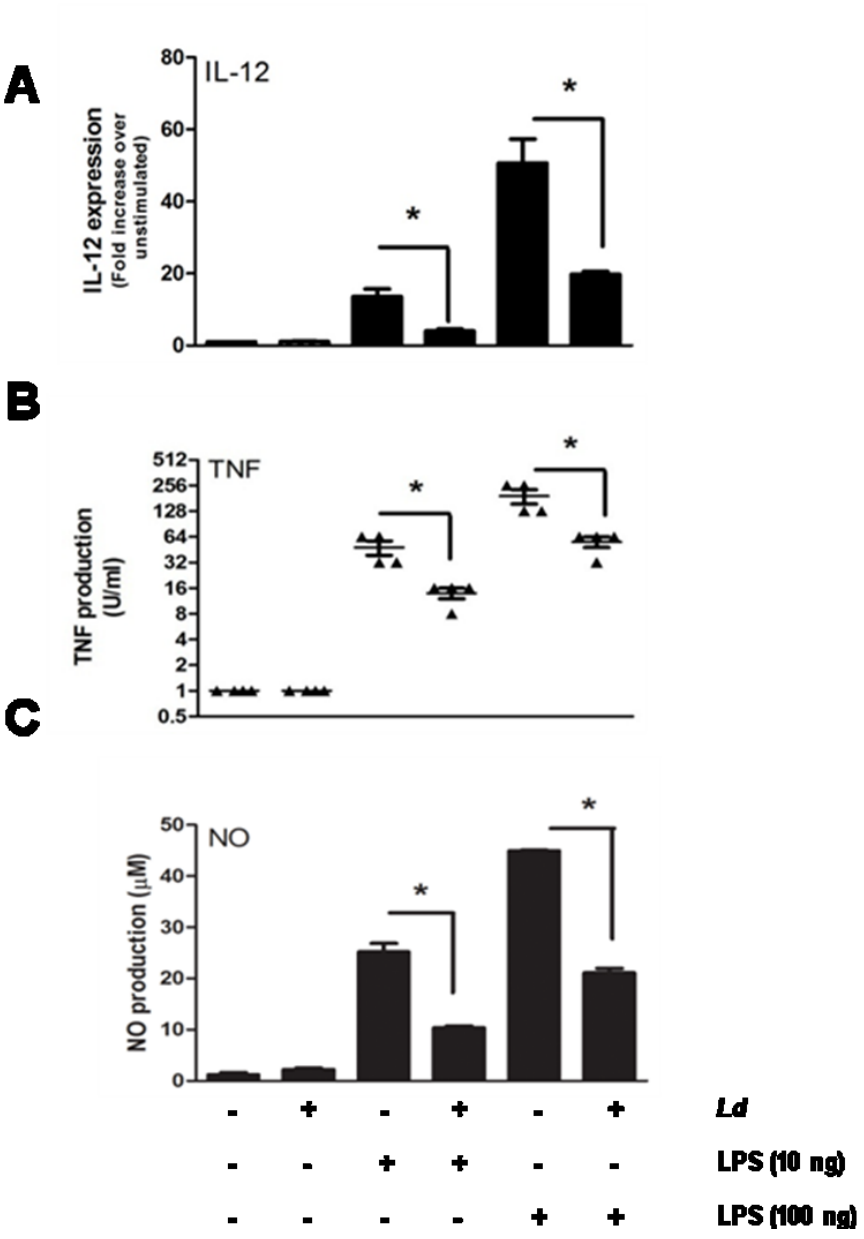

A
IL-12
IL-12 expression
(Fold increase over unstimulated)
80
60
40
20
0
*
*
B
TNF
TNF production
(U/ml)
512
256
128
64
32
16
8
4
2
1
0.5
*
*
C
NO
NO production (μM)
50
40
30
20
10
0
*
*
- + - + - + Ld
- - + + - - LPS (10 ng)
- - - - + + LPS (100 ng)

Figure 8: Inhibition of LPS-induced IRAK-1 kinase activity by *Leishmania*. (A) Kinase assay performed on IRAK-1 IPs from lysates of MØs uninfected and infected with *L. donovani* over a 6 h time-period. LPS stimulation (100 ng/ml, 30 min), positive control. (B) IRAK-1 kinase activity detected in IPs from lysates of MØs infected or not with pathogenic *Leishmania* species (*L. donovani, L. mexicana, L. major*) (20:1 parasite to cell ratio, 1 h). Non-pathogenic lizard *L. tarentolae* was used as negative control. (C) Kinase assay of IRAK-1 IPs from lysates of naïve and *L. donovani*-infected MØs (O/N infection) subjected or not to LPS stimulation (100 ng/ml, 30 min). (D) IRAK-1 kinase activity in IPs from naïve and *L. donovani*-infected MØs (O/N infection) stimulated or not with various TLR ligands (MALP (100 ng/ml), LPS (100 ng/ml), flagellin (100 ng/ml), CpG (5µg/ml); 30 min). All results are representative of at least three independent experiments.

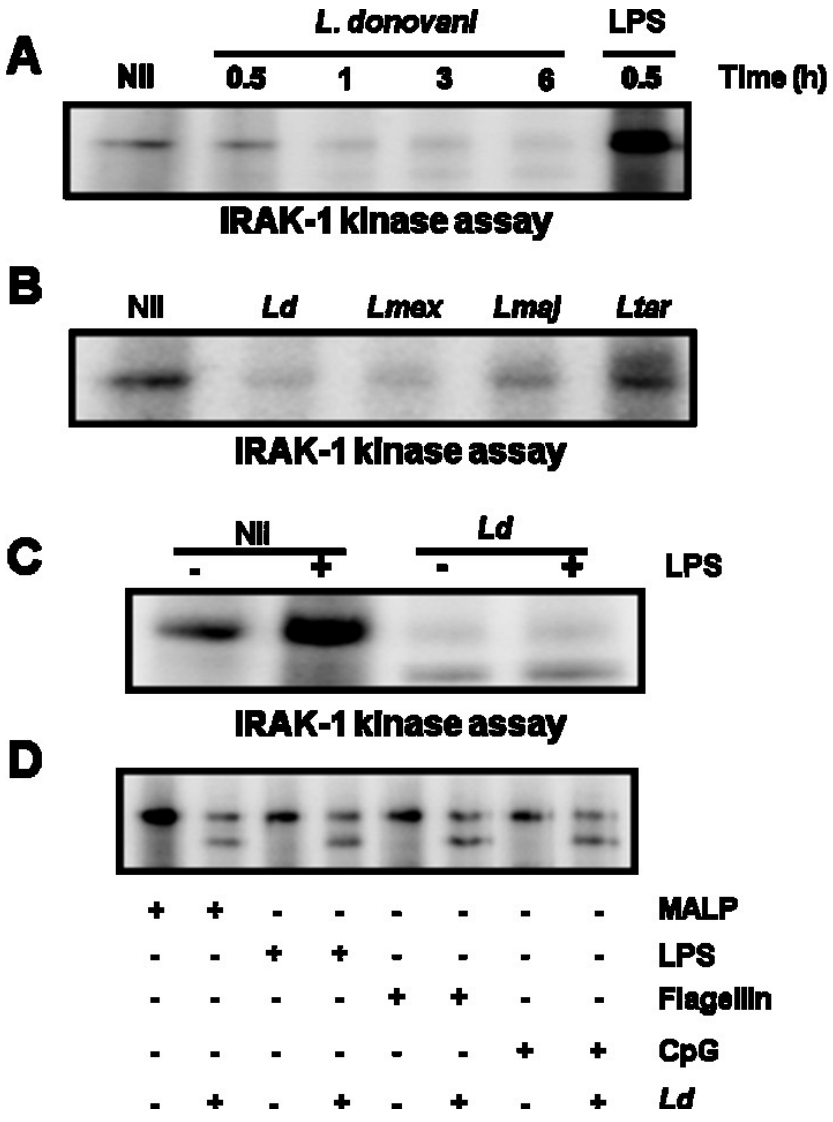

A
L. donovani
LPS
Nil
0.5
1
3
6
0.5
Time (h)
IRAK-1 kinase assay
B
Nil
Ld
Lmex
Lmaj
Ltar
IRAK-1 kinase assay
C
Nil
Ld
-
+
-
+
LPS
IRAK-1 kinase assay
D
+ + - - - - - - MALP
- - + + - - - - LPS
- - - - + + - - Flagellin
- - - - - - + + CpG
- + - + - + - + Ld

Figure 9: TLR ligands activate NF-κB in stimulated macrophages. Gel represents an electromobility shift assay (EMSA) showing NF-κB nuclear translocation in response to a 2 h stimulation with the different TLR ligands used in Figure 8D. The EMSA confirms that the ligands are functional and activating at the concentrations used. MALP, macrophage-activating lipopeptide-2. Flag, flagellin. S, specific competition (100X cold oligo). NS, non-specific competition (SP1 oligo).

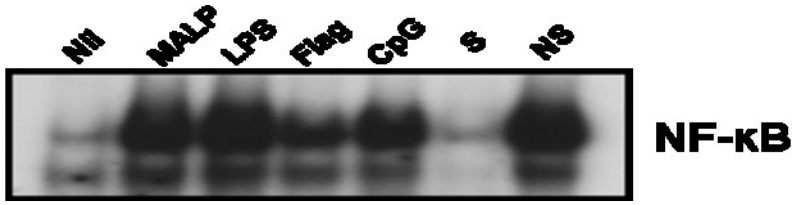

Nil
MALP
LPS
Flag
CpG
S
NS
NF-κB

Figure 10: IRAK-1 / SHP-1 interaction is enhanced by *Leishmania* infection and leads to IRAK-1 signalling alteration and macrophage functional inhibition. (A) Western blot analysis demonstrating the enhanced co-IP of IRAK-1 and SHP-1 in response to *Leishmania* infection. IRAK-1 was immunoprecipitated from uninfected and *Leishmania*-infected MØ lysates (30 min post-infection). The IPs were run on SDS-PAGE and blotted against SHP-1 (upper panel). Membrane was then stripped and blotted against IRAK-1 as a control for equal IP (lower panel). (B) A fraction of the IPs (a, upper panel) was subjected to in gel PTP assay showing higher SHP-1 activity associated with the IP of *Leishmania*-infected MØs over uninfected. (C) Fraction of the IP (a, upper panel) was also subjected to a phosphatase assay based on pNPP hydrolysis demonstrating a significantly higher total phosphatase activity in the IP of *Leishmania*-infected cells compared to uninfected controls. *, $P < 0.05$; error bar SD. Data are the mean of four independent experiments. (D) IRAK-1 kinase assay of WT and SHP-$1^{-/-}$ MØs infected or not with *L. donovani* for 1 h (upper panel). IP fraction was kept and subjected to western blot as a loading control of IRAK-1 immunoprecipitation (lower panel). (E) NO production by *Leishmania*-infected WT and SHP-$1^{-/-}$ MØs in response to LPS. Cells have been infected with *L. donovani* (O/N) and stimulated with LPS for 24 h. Significant difference $P < 0.05$, Anova test, error bar SEM. Mean of three independent experiments. (F and G) IRAK-1 inactivation by *Leishmania* causes its inability to bind TRAF6. Western blot analysis showing that *Leishmania* causes an abrogation of the ability of IRAK-1 to bind TRAF6, but not MyD88, upon LPS stimulation. IRAK-1 was immunoprecipitated from lysates of naïve and *L. donovani*-infected MØ (1 h infection) stimulated or not with LPS (100 ng/ml, 1 h). The IPs were run on SDS-PAGE and blotted

against MyD88 (F) and TRAF6 (G). Membranes were stripped and blotted against IRAK-1 to demonstrate equal IP.

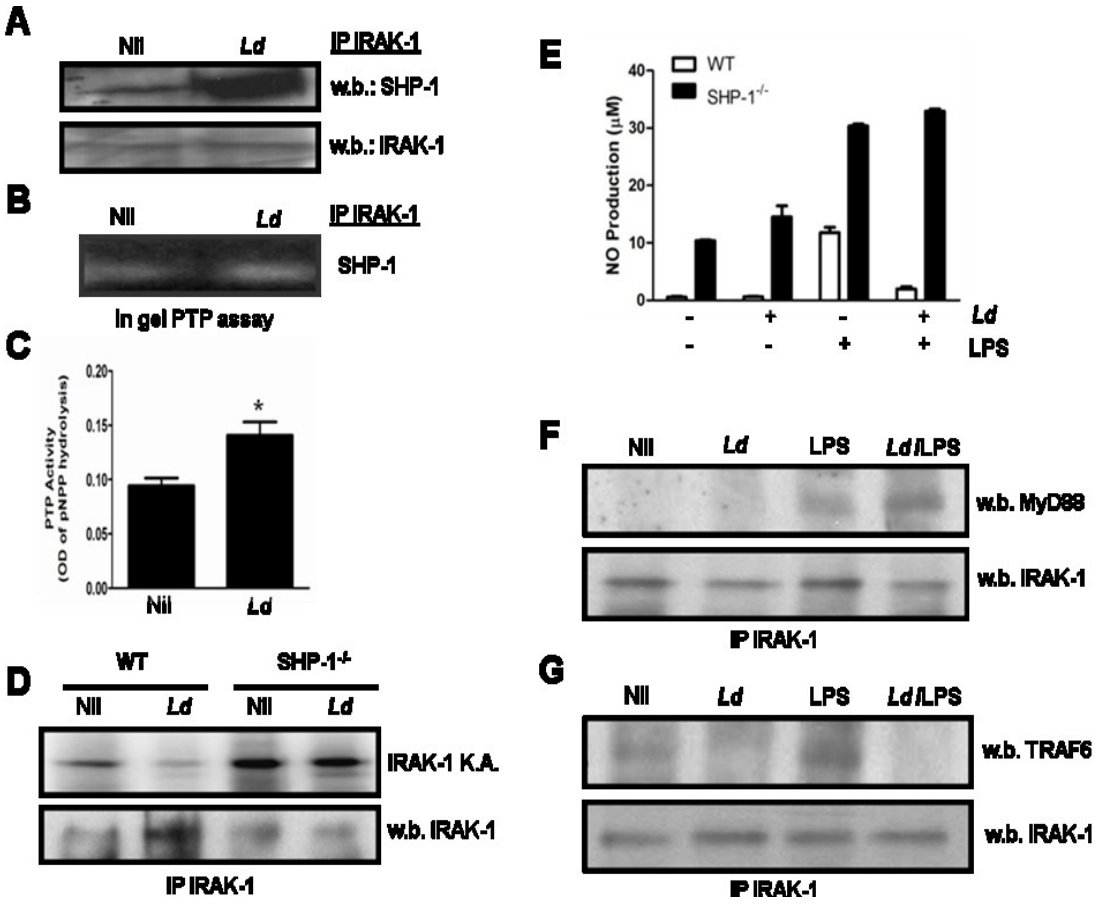
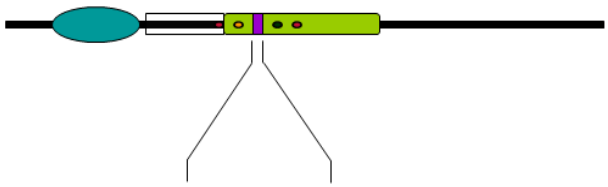

A
Nil
Ld
IP IRAK-1
w.b.: SHP-1
w.b.: IRAK-1
B
Nil
Ld
IP IRAK-1
SHP-1
In gel PTP assay
C
PTP Activity
(OD of pNPP hydrolysis)
0.20
0.15
0.10
0.05
0.00
*
Nil
Ld
D
WT
SHP-1-/-
Nil
Ld
Nil
Ld
IRAK-1 K.A.
w.b. IRAK-1
IP IRAK-1
E
WT
SHP-1-/-
NO Production (μM)
40
30
20
10
0
-
+
-
+
Ld
-
-
+
+
LPS
F
Nil
Ld
LPS
Ld/LPS
w.b. MyD88
w.b. IRAK-1
IP IRAK-1
G
Nil
Ld
LPS
Ld/LPS
w.b. TRAF6
w.b. IRAK-1
IP IRAK-1

Figure 11: IRAK-1's KTIM is evolutionarily conserved in vertebrates. Sequence comparison of IRAK-1 in various vertebrates reveal that the KTIM is conserved in all vertebrates down to *Xenopus tropicalis* (amphibian) but is absent in *Danio rerio* (zebrafish). All homology percentages were calculated using the human IRAK-1 sequence as a reference. Human: *Homo sapiens*; Chimpanzee: *Pan troglodytes*; Dog: *Canis familiaris*; Bull: *Bos Taurus*; Mouse: *Mus musculus*; Rat: *Rattus norvegicus*.



		% IRAK-1 Overall Sequence Identity/Similarity	% IRAK-1 Kinase Domain Identity/Similarity
Human	NGFYCLVYGFLPNGSLEDRL	100%, 100%	100%, 100%
Chimpanzee	NGFYCLVYGFLPNGSLEDRL	95%, 96%	94%, 96%
Dog	SGFYCLVYGFLPNGSLEDRL	81%, 86%	91%, 95%
Bull	SGFYCLVYGFLPNGSLEDRL	80%, 86%	87%, 93%
Mouse	SGLYCLVYGFLPNGSLEDQL	81%, 85%	88%, 93%
Rat	SGFYCLVYGFLPNGSLEDQL	80%, 85%	88%, 93%
Xenopus	GEEYCLIYLYLPNGSLEDRL	39%, 52%	54%, 70%
Zebrafish	GQTYCVIYVYMPNGSLDDRL	39%, 52%	53%, 70%

Figure 12: IRAK-4 shows homology to IRAK-1 but does not bear a KTIM due to a single amino acid substitution. IRAK-4 sequence comparison of various vertebrates and invertebrates reveal that IRAK-4 has no KTIM due to a single leucine to methionine / isoleucine substitution. All IRAK-4 homology percentages were calculated using the human IRAK-4 sequence as a reference. IRAK-1/IRAK-4 homology percentages were calculated within the same species. Rhesus monkey: *Macaca mulatta*; Chicken: *Gallus gallus*; Squid: *Euprymna scolopes*; Sea urchin: *Strongylocentrotus purpuratus*; Worm: *Caenorhabditis elegans*; Honeybee: *Apis mellifera*; Fly: *Drosophila melanogaster*.

Vertebrate
Invertebrate

		% IRAK-4 Overall Sequence Identity/Similarity	% IRAK-4 Kinase Domain Identity/Similarity	% IRAK1/IRAK4 Within Species Identity/Similarity	% IRAK1/IRAK4 Kinase Domain Identity/Similarity
Human	GDDLCLVYVYMPNGSLLD	100%, 100%	100%, 100%	29%, 46%	35%, 52%
Chimpanzee	GDDLCLVYVYMPNGSLLD	99%, 99%	99%, 100%	29%, 46%	34%, 57%
Rhesus monkey	GDDLCLVYVYMPNGSLLD	99%, 99%	99%, 99%	N/A	N/A
Dog	GDDLCLVYVYMPNGSLLD	93%, 96%	96%, 98%	29%, 46%	35%, 53%
Bull	GDDLCLVYVYMPNGSLLD	90%, 94%	96%, 99%	29%, 44%	33%, 51%
Mouse	SDNLCLVYAYMPNGSLLD	83%, 91%	89%, 93%	29%, 46%	34%, 53%
Rat	SDNLCLVYAYMPNGSLLD	84%, 91%	90%, 95%	29%, 46%	35%, 54%
Chicken	GAQPCLVYEYMPNGSLLD	66%, 81%	78%, 91%	N/A	N/A
Zebrafish	GPPLCVVFELMVNGSLLE	48%, 68%	55%, 75%	28%, 43%	33%, 50%
Squid	GPKFCLVYTYMVNGSLED	34%, 51%	45%, 63%	--	--
Sea Urchin	PPQLCLVYELITGGALRD	37%, 51%	43%, 59%	--	--
C. elegans	GSEPCLVYQFMSNGSLED	29%, 49%	38%, 58%	--	--
Honeybee	KIP-CLIYQLMKNGSLED	33%, 49%	43%, 59%	--	--
Drosophila	GGKPCLVYQLMKGGSLEA	29%, 45%	36%, 52%	--	--

Figure 13: Evolution of vertebrate IRAK-1 and SHP-1 from IRAK-4 and SHP-2 ancestor genes. Schematic representation of the appearance of IRAK-1 and SHP-1 from gene duplication events of the IRAK-4 (A) and SHP-2 (B) ancestor genes, respectively. The emergence of the KTIM in IRAK-1 occurred after this gene duplication event took place as the motif only appeared in amphibians. Similarity percentages were calculated using the mouse IRAK-1 and IRAK-4 sequences.

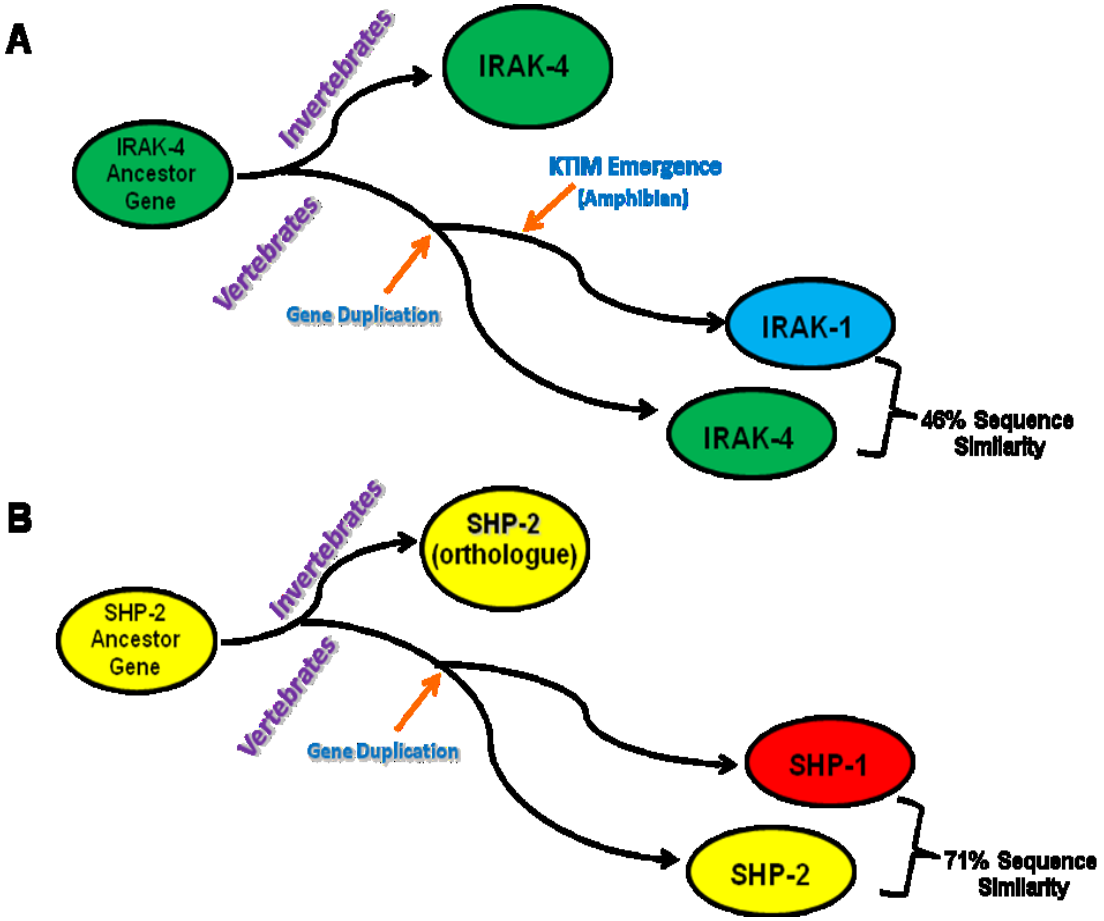

A
IRAK-4
Ancestor
Gene
Invertebrates
IRAK-4
KTIM Emergence
(Amphibian)
Vertebrates
Gene Duplication
IRAK-1
IRAK-4
46% Sequence
Similarity
B
SHP-2
Ancestor
Gene
Invertebrates
SHP-2
(orthologue)
Vertebrates
Gene Duplication
SHP-1
SHP-2
71% Sequence
Similarity

Figure 14: Several kinases possess KTIMs. (A) Table showing that several kinases from the JAK, MAP, Src, and IKK kinase families possess potential KTIMs in their amino acid sequences. Screening was done using published mouse protein sequences found in the NCBI protein database. (B) An in gel phosphatase assay (upper panel) demonstrating that IPs of IKK-β, Erk1/2, JNK, and p38 all exhibit SHP-1 activity. Syk IP was added as a control for a kinase that has no KTIM in its sequence and rabbit IgG was used as a negative control. Fractions of all IPs were kept and run on SDS-PAGE and blotted against their corresponding antibody to demonstrate the success of the IP procedure (lower panel).

A

Kinase	KTIM Sequence & Location	KTIM Within Kinase Domain
JAK2	911VKYKGV916	Yes
	932LPYGSL937	
JAK3	575VSYPHL580	
	779SDYELL784	Yes
	818LKYISL823	
	880VKYRGV885	
TAK1	111SLYNVL116	Yes
Erk1	59SAYDHV64	Yes
Erk2	39SAYDNL44	Yes
JNK	131LLYQML136	Yes
	379LIYKEV384	No
P38	130LIYQIL135	Yes
	340LTYDEV345	No
IKK-α	519IHYSEV524	No
IKK-β	607VIYTQL612	No
Lyn	432LLYEIV437	Yes

B

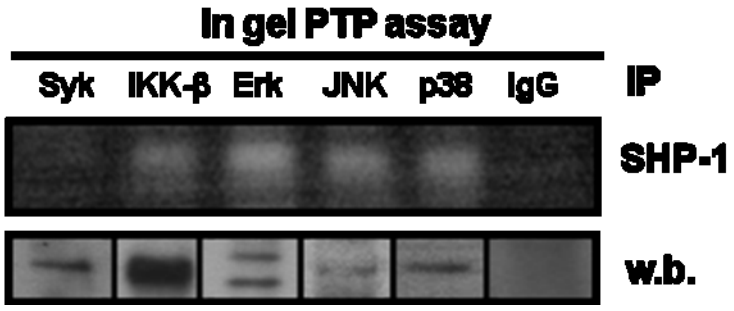
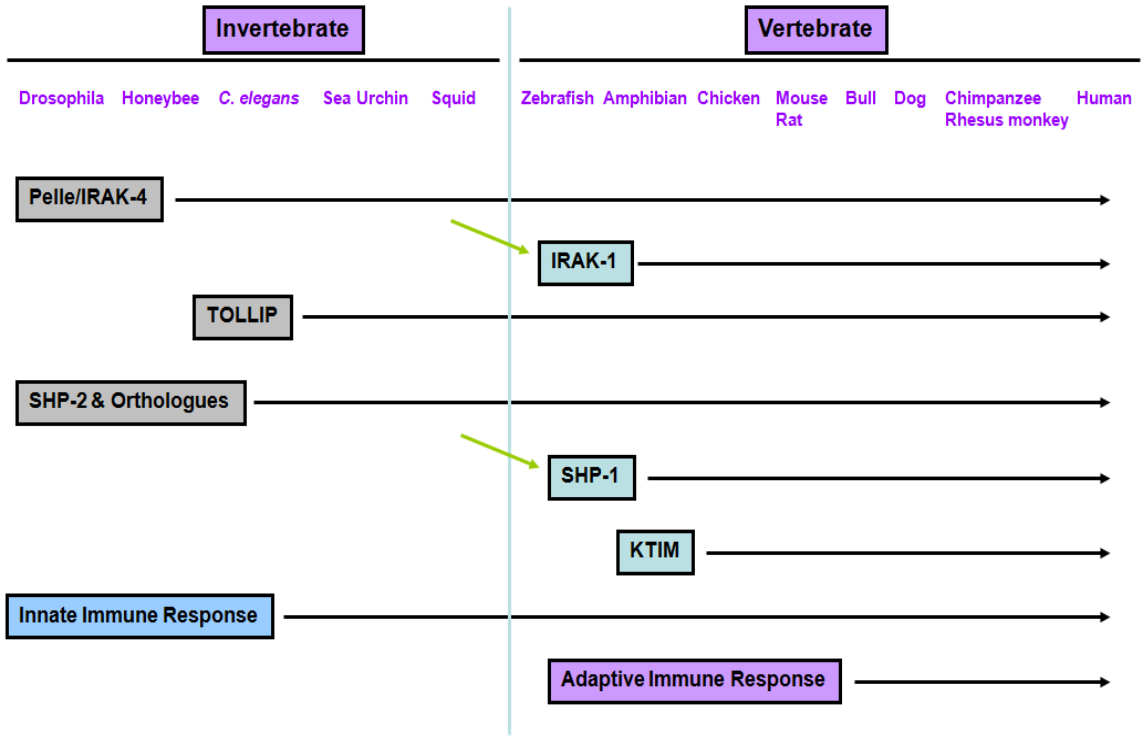


Figure 15: Regulation of IRAK-1 by SHP-1 through its binding to KTIM is unique to vertebrates and may have favoured the development of their adaptive immune response. Schematic representation of the emergence of IRAK-1 and SHP-1 from IRAK-4 and SHP-2, respectively. Unlike TOLLIP and SHP-2 which are found in invertebrates, SHP-1 arose in vertebrates just like IRAK-1 and KTIM, coinciding with the emergence of the adaptive immune response.


Invertebrate
Vertebrate
Drosophila
Honeybee
C. elegans
Sea Urchin
Squid
Zebrafish
Amphibian
Chicken
Mouse
Rat
Bull
Dog
Chimpanzee
Rhesus monkey
Human
Pelle/IRAK-4
IRAK-1
TOLLIP
SHP-2 & Orthologues
SHP-1
KTIM
Innate Immune Response
Adaptive Immune Response

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References

1. Takeda K, Akira S (2001). Roles of Toll-like receptors in innate immune responses. *Genes Cells* **6**: 733-742.
2. Olivier M, Gregory DJ, Forget G (2005). Subversion mechanisms by which *Leishmania* parasites can escape the host immune response: a signaling point of view. *Clin Microbiol Rev* **18**: 293-305.
3. Forget G, Matte C, Siminovitch KA, Rivest S, Pouliot P, et al. (2005). Regulation of the *Leishmania*-induced innate inflammatory response by the protein tyrosine phosphatase SHP-1. *Eur J Immunol* **35**: 1906-1917.
4. Cameron P, McGachy A, Anderson M, Paul A, Coombs GH, et al. (2004). Inhibition of lipopolysaccharide-induced macrophage IL-12 production by *Leishmania* mexicana amastigotes: the role of cysteine peptidases and the NF-κB signaling pathway. *J Immunol* **173**: 3297-3304.
5. Carrera L, Gazzinelli RT, Badolato R, Hieny S, Muller W, et al. (1996). *Leishmania* promastigotes selectively inhibit interleukin 12 induction in bone marrow-derived macrophages from susceptible and resistant mice. *J Exp Med* **183**: 515-526.
6. Lang T, Mansell A (2007). The negative regulation of Toll-like receptor and associated pathways. *Immunol Cell Biol* **85**: 425-434.
7. Forget G, Siminovitch KA, Brochu S, Rivest S, Radzioch D, et al. (2001). Role of host phosphotyrosine phosphatase SHP-1 in the development of murine leishmaniasis. *Eur J Immunol* **31**: 3185-3196.
8. Forget G, Gregory DJ, Whitcombe LA, Olivier M (2006). Role of host protein tyrosine phosphatase SHP-1 in *Leishmania* donovani-induced inhibition of nitric oxide production. *Infect Immun* **74**: 6272-6279.
9. Massa PT, Wu C (1998). Increased inducible activation of NF-κB and responsive genes in astrocytes deficient in the protein tyrosine phosphatase SHP-1. *J Interferon Cytokine Res* **18**: 499-507.
10. Alonso A, Sasin J, Bottini N, Friedberg I, Osterman A, et al. (2004). Protein tyrosine phosphatases in the human genome. *Cell* **117**: 699-711.
11. An H, Hou J, Zhou J, Zhao W, Xu H, et al. (2008). Phosphatase SHP-1 promotes TLR- and RIG-I-activated production of type I interferon by inhibiting the kinase IRAK1. *Nat Immunol* **9**: 542-550.
12. Radzioch D, Hudson T, Boule M, Barrera L, Urbance JW, et al. (1991). Genetic resistance/susceptibility to mycobacteria: phenotypic expression in bone marrow derived macrophage lines. *J Leukoc Biol* **50**: 263-272.
13. Olivier M, Romero-Gallo BJ, Matte C, Blanchette J, Posner BI, et al. (1998). Modulation of interferon-gamma-induced macrophage activation by phosphotyrosine

phosphatases inhibition. Effect on murine Leishmaniasis progression. *J Biol Chem* **273**: 13944-13949.

14. Markova B, Gulati P, Herrlich PA, Bohmer FD (2005). Investigation of protein-tyrosine phosphatases by in-gel assays. *Methods* **35**: 22-27.
15. Bourassa C, Chapdelaine A, Roberts KD, Chevalier S (1988). Enhancement of the detection of alkali-resistant phosphoproteins in polyacrylamide gels. *Anal Biochem* **169**: 356-362.
16. Ribeiro-Dias F, Russo M, Marzagao Barbuto JA, Fernandes do Nascimento FR, Timenetsky J, et al. (1999). Mycoplasma arginini enhances cytotoxicity of thioglycollate-elicited murine macrophages toward YAC-1 tumor cells through production of NO. *J Leukoc Biol* **65**: 808-814.
17. Jaramillo M, Olivier M (2002). Hydrogen peroxide induces murine macrophage chemokine gene transcription via extracellular signal-regulated kinase- and cyclic adenosine 5'-monophosphate (cAMP)-dependent pathways: involvement of NF-κB, activator protein 1, and cAMP response element binding protein. *J Immunol* **169**: 7026-7038.
18. Kollewe C, Mackensen AC, Neumann D, Knop J, Cao P, et al. (2004). Sequential autophosphorylation steps in the interleukin-1 receptor-associated kinase-1 regulate its availability as an adapter in interleukin-1 signaling. *J Biol Chem* **279**: 5227-5236.
19. Powers JP, Li S, Jaen JC, Liu J, Walker NP, et al. (2006). Discovery and initial SAR of inhibitors of interleukin-1 receptor-associated kinase-4. *Bioorg Med Chem Lett* **16**: 2842-2845.
20. Blanchette J, Racette N, Faure R, Siminovitch KA, Olivier M (1999). *Leishmania*-induced increases in activation of macrophage SHP-1 tyrosine phosphatase are associated with impaired IFN-gamma-triggered JAK2 activation. *Eur J Immunol* **29**: 3737-3744.
21. Proudfoot L, Nikolaev AV, Feng GJ, Wei WQ, Ferguson MA, et al. (1996). Regulation of the expression of nitric oxide synthase and leishmanicidal activity by glycoconjugates of *Leishmania* lipophosphoglycan in murine macrophages. *Proc Natl Acad Sci USA* **93**: 10984-10989.
22. Feng GJ, Goodridge HS, Harnett MM, Wei XQ, Nikolaev AV, et al. (1999). Extracellular signal-related kinase (ERK) and p38 mitogen-activated protein (MAP) kinases differentially regulate the lipopolysaccharide-mediated induction of inducible nitric oxide synthase and IL-12 in macrophages: *Leishmania* phosphoglycans subvert macrophage IL-12 production by targeting ERK MAP kinase. *J Immunol* **163**: 6403-6412.
23. Piedrafita D, Proudfoot L, Nikolaev AV, Xu D, Sands W, et al. (1999). Regulation of macrophage IL-12 synthesis by *Leishmania* phosphoglycans. *Eur J Immunol* **29**: 235-244.

24. Weinheber N, Wolfram M, Harbecke D, Aebischer T (1998). Phagocytosis of *Leishmania* mexicana amastigotes by macrophages leads to a sustained suppression of IL-12 production. *Eur J Immunol* **28**: 2467-2477.
25. Flandin, JF, Chano, F, and Descoteaux, A. (2006). RNA interference reveals a role for TLR2 and TLR3 in the recognition of *Leishmania donovani* promastigotes by interferon-gamma-primed macrophages. *Eur J Immunol* **36**(2): 411-20.
26. Avril T, Freeman SD, Attrill H, Clarke RG, Crocker PR (2005). Siglec-5 (CD170) can mediate inhibitory signaling in the absence of immunoreceptor tyrosine-based inhibitory motif phosphorylation. *J Biol Chem* **280**: 19843-19851.
27. Staub E, Rosenthal A, Hinzmann B (2004). Systematic identification of immunoreceptor tyrosine-based inhibitory motifs in the human proteome. *Cell Signal* **16**: 435-456.
28. Mushegian A, Medzhitov R (2001). Evolutionary perspective on innate immune recognition. *J Cell Biol* **155**: 705-710.
29. Neel BG, Gu H, Pao L (2003). The 'Shp'ing news: SH2 domain-containing tyrosine phosphatases in cell signaling. *Trends Biochem Sci* **28**: 284-293.
30. Li L (2004). Regulation of innate immunity signaling and its connection with human diseases. *Curr Drug Targets Inflamm Allergy* **3**: 81-86.
31. Bedecs K, Elbaz N, Sutren M, Masson M, Susini C, et al. (1997). Angiotensin II type 2 receptors mediate inhibition of mitogen-activated protein kinase cascade and functional activation of SHP-1 tyrosine phosphatase. *Biochem J* **325** (Pt 2): 449-454.
32. Jiao H, Berrada K, Yang W, Tabrizi M, Platanias LC, et al. (1996). Direct association with and dephosphorylation of Jak2 kinase by the SH2-domain-containing protein tyrosine phosphatase SHP-1. *Mol Cell Biol* **16**: 6985-6992.
33. Matsubara H, Shibasaki Y, Okigaki M, Mori Y, Masaki H, et al. (2001). Effect of angiotensin II type 2 receptor on tyrosine kinase Pyk2 and c-Jun NH2-terminal kinase via SHP-1 tyrosine phosphatase activity: evidence from vascular-targeted transgenic mice of AT2 receptor. *Biochem Biophys Res Commun* **282**: 1085-1091.

Chapter 3

Identification of Key Cytosolic Kinases Containing Potential Evolutionarily Conserved Kinase Tyrosine-based Inhibitory Motifs (KTIMs)

In the previous chapter, we showed for the first time that tyrosine-based inhibitory motifs are not restricted to immunoreceptors. Indeed, we were able to demonstrate that the evolutionarily conserved ITIM-like motif located in the kinase domain of IRAK-1 is able to mediate binding to SHP-1 and is responsible for the regulation of the kinase activity of the protein. Based on this finding, we proposed to name this motif a kinase tyrosine-based inhibitory motif (KTIM). We ended the first chapter by revealing that several key cellular kinases such as: JAK2, JAK3, and all three MAPKs (Erk1/2, JNK, p38) possess one or more potential KTIMs, and that they are able to interact with SHP-1. In this chapter, we decided to explore in more depth the potential KTIMs that we identified in these kinases. Towards that end, we studied the number and location of these motifs in the different kinases, their relative time of emergence in evolution, and their conservation from the species they arise in up to human. Results indicated that in the majority of the cases, KTIMs appeared in the amino acid sequence of kinases at the early vertebrate level (fish or amphibian) and was highly conserved afterwards. These results are in accordance to what we observed with IRAK-1 and support the evolutionary role that we proposed it could have played, and that is the regulation of the innate immune response in early vertebrates. Furthermore, we performed experiments that suggested that the few KTIMs identified in invertebrates may have constituted readily available sites that performed new regulatory functions as soon as their binding partners (e.g. SHP-1) arose in vertebrate cells.

Identification of Key Cytosolic Kinases Containing Potential Evolutionarily Conserved Kinase Tyrosine-based Inhibitory Motifs (KTIMs)

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Running title: *Evolutionarily-conserved kinase KTIMs*

Abstract

We have previously reported that the protein tyrosine phosphatase SHP-1 regulates the kinase activity of IL-1 receptor-associated kinase 1 (IRAK-1) through binding to an ITIM-like motif found in its kinase domain. As IRAK-1 is a cytosolic kinase and not a membrane-associated immunoreceptor, we proposed to call this motif a kinase tyrosine-based inhibitory motif (KTIM). Herein, we further investigated the presence, number, location, and evolutionary time of emergence of potential KTIMs in a number of cytosolic kinases including members of the JAK, MAPK, IKK, and Src families, all known to play important roles in the signalling and function of various cells of the immune system. Here, we unveil that many cytosolic kinases contained one or more potential KTIMs, mostly located within their kinase domain. Additionally, we found that most of KTIMs appeared at the level of early vertebrates (fish, amphibian) and were highly conserved thereafter possibly due to the crucial role they played in controlling complex cellular and physiological immune functions. Regarding KTIMs that were found conserved in invertebrates, we provide experimental data suggesting that such motifs may have constituted readily available sites that performed new regulatory functions as soon as their binding partners (e.g. SHP-1) appeared in vertebrate cells. We thus propose KTIMs as novel regulatory motifs found in a wide range of cytosolic kinases regulating their activity through binding to SH2 domain-containing proteins such as: SHP-1 and SHIP.

Introduction

It is as important to inhibit an immune response as to activate it. It is therefore not surprising to find a complex system of activating and inhibitory receptors associated with different cells of the immune system. One important manner by which these receptors can function is through the presence of immunoreceptor tyrosine-based activation motifs (ITAMs) [1] and immunoreceptor tyrosine-based inhibitory motifs (ITIMs) [1,2] in their cytoplasmic domains. ITIMs have the consensus sequence (I/V/L/S)-x-Y-x-x-(L/V) [3], where x denotes any amino acid. They play a crucial role in the regulation of immune responses as supported by the autoimmunity seen when inhibitory receptors containing ITIMs are lacking [4,5].

The important role of ITIMs in the negative regulation of immune responses is further reinforced by the fact that they are evolutionarily conserved and found in many types of immune cells such as: B, T, and NK cells, as well as cells of myeloid origins. The B cell inhibitory receptor (PIR-B) for example, contains ITIMs that are able to recruit Src-homology 2 domain-containing protein tyrosine phosphatase 1 (SHP-1) leading to the attenuation of B cell antigen receptor (BCR)-triggered activation responses through the dephosphorylation of BCR, Syk, B-cell linker (BLNK), and phospholipase C (PLC) [6-8]. The binding of SHP-1 to ITIMs on immunoreceptors is an early event that can effectively shut down many subsequent events such as: calcium mobilization, cytokine release, transcriptional activation, and cellular proliferation [3]. Another SH2 domain-containing phosphatase known to be recruited to ITIMs is the SH2 domain-containing inositol 5-phosphatase (SHIP). SHIP has been shown to be recruited to the inhibitor IgG Fc receptor (FcγRIIB) and to mediate the hydrolysis of phosphatidylinositol 3,4,5-

triphosphate (PIP3), which can ultimately lead to the negative regulation of signalling events associated with this receptor [9].

In addition to B cells, NK and T cells have inhibitory receptors that have ITIMs in their cytoplasmic domains. Many of the NK Ly49 receptors that belong to the C lectin-like family contain ITIMs and their presence is crucial for the inhibitory function of this receptor [10]. Half of the killer cell IgG-like receptor (KIRs) found on NK and memory T cells have two ITIMs in their cytoplasmic domain. These ITIMs have been shown to bind SHP-1 thus inhibiting cell-mediated cytotoxicity and cytokine secretion [11-13]. Additionally, CD94/NKG2A receptors, found in half of NK cells and a subset of memory CD8+ cells, contain two ITIMs and are able to recruit SHP-1 or SHP-2 [14,15].

Importantly, monocytes, macrophages (MØs), and dendritic cells have also been reported to possess ITIM-containing receptors such as the leukocyte inhibitory receptor (LIR, also known as ILT, MIR, and CD85), which is able to associate to SHP-1 and SHP-2 [3]. Other receptors containing ITIMs on myeloid cells include: CD33, SIGLEC5 [16], SIGLEC6 [17], SIGLEC7 [18,19], and PD-1 [20].

It is striking however, that all reports of ITIMs have been restricted to transmembrane receptors, and no attention was paid to the possibility that such a motif could perform regulatory functions in non-receptor proteins. We have recently reported that SHP-1 was able to regulate IRAK-1 kinase activity in MØs through its ability to bind an evolutionarily conserved ITIM-like motif located in the kinase domain [21]. Given that this motif was present in a cytosolic kinase and not an immunoreceptor, we decided to name it kinase tyrosine-based inhibitory motif (KTIM). Anticipating KTIM to be a

regulatory mechanism of relevance to several kinases other than IRAK-1, we searched the sequences of other key cytosolic kinases and reported that several of them such as members of the Janus kinase (JAK) and MAP kinase families contained one or more potential KTIMs mainly located in their kinase domain [21].

In this study, we investigated in more depth the KTIMs found in members of the JAK family (JAK2 / JAK3), TGF-β-activated kinase (TAK1), MAPK family (Erk1/2, JNK, p38), inhibitory kappa B kinases (IKK-α / IKK-β), and the Src family member LYN. Furthermore, we monitored the relative evolutionary time in which each of these KTIMs emerged in the amino acid sequence of its corresponding kinase. Of utmost interest, the kinase JNK, known to be conserved from the fruit fly *Drosophila melanogaster* to *Homo sapiens*, bears a KTIM within its kinase domain and was found to bind SHP-1, a negative regulator only observed in vertebrates. This suggests that although this type of motif could have been present long time ago in some invertebrate kinases, the emergence of SHP-1 in early vertebrates may have brought about a better control over kinases involved in the innate immune response, possibly favouring the development of a fine-tuned adaptive immune response in higher vertebrates.

Materials and Methods

Cell Culture and Reagents. The immortalized B10R BMDMs were derived from B10A.Bcg^r mice as previously described [22]. The S2 macrophage-like cell-line derived from a primary culture of late stage (20-24 h old) *Drosophila melanogaster* embryos was kindly provided by Dr. Paul Lasko, Department of Biology, McGill University, Canada.

Western Blot Analysis. Western blotting was performed as previously described [23]. Proteins were detected using antibodies directed against SHP-1 (Chemicon, CA, USA) and JNK (Santa Cruz, CA, USA). Proteins were detected using an anti-mouse or anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (Amersham, QC, Canada) and visualized using ECL western blotting detection system (Amersham).

In gel PTP Assay. MØs were lysed with a PTP lysis buffer (50mM Tris (pH 7.0), 0.1mM EDTA, 0.1mM EGTA, 0.1% β-mercaptoethanol, 1% Igepal, 25μg/ml aprotinin and 25μg/ml leupeptin) and samples were loaded on a gel containing a γ-^{32}P-labelled poly(Glu4Tyr) peptide (Sigma-Aldrich, ON, Canada). SHP-1 activity was assessed by in gel PTP assay as previously described [24].

GST Pull-down Assay. GST and the trapping mutant of GST-SHP-1 were produced in the BL21 strain of *Escherichia coli*. Bacterial lysates were obtained using the BugBuster protein extraction reagent (VWR CANLAB, ON, Canada). For pull-down experiments, GST or GST-SHP-1 (5μg) was purified from bacterial lysates using glutathione sepharose

beads (Amersham). GST or GST-SHP-1 bound to glutathione beads were left shaking (O/N, 4°C) with 500µl of B10R or S2 lysates (2 mg) obtained using the previously mentioned PTP lysis buffer. The beads were then spun down, washed 3x with the PTP lysis buffer, resuspended in 4x sample loading buffer (20µl), boiled (95°C, 5 min.), and subjected to SDS-PAGE.

Sequence Alignments. Sequences were obtained from the NCBI protein database. Sequence alignments were generated by EMBOSS local pair-wise alignment algorithms program (http://www.ebi.ac.uk/Tools/emboss/index.html). The accession numbers of the protein sequences included in the study are: human (*Homo sapiens*) JAK2 (AAC23653), chimpanzee (*Pan troglodytes*) JAK2 (XP_001139368), rhesus monkey (*Macaca mulatta*) JAK2 (XP_001082883), pig (*Sus scrofa*) JAK2 (NP_999278), bull (*Bos taurus*) JAK2 (XP_870226), dog (*Canis familiaris*) JAK2 (XP_541301), mouse (*Mus musculus*) JAK2 (Q62120), rat (*Rattus norvegicus*) JAK2 (NP_113702), chicken (*Gallus gallus*) JAK2 (Q75R65), tropical frog (*Xenopus tropicalis*) JAK2 (AAI25683), zebrafish (*Danio rerio*) JAK2 (NP_571168), honey bee (*Apis mellifera*) JAK2 (hopscotch-like) (XP_001121783), fruit fly (*Drosophila melanogaster*) JAK2 (hopscotch) (Q24592), mosquito (*Culex tritaeniorhynchus*) JAK (AAQ18517), human JAK3 (NP_000206), chimpanzee JAK3 (XP_512502), rhesus monkey JAK3 (XP_001115037), dog JAK3 (XP_852473), mouse JAK3 (Q62137), rat JAK3 (NP_036987), chicken JAK3 (NP_990327), human TAK1 (O43318), chimpanzee TAK1 (predicted) (XP_001160579), rhesus monkey TAK1 (XP_001099849), pig TAK1 (NP_001107752), bull TAK1 (A2VDU3), mouse TAK1 (Q62073), chicken TAK1 (XP_001233491), tropical frog TAK1 (AAI36217), zebrafish TAK1 (AAH95335), fruit fly TAK1 (Q9V3Q6), *Caenorhabditis elegans* TAK1 (MOM-

4) (AAD39816), human Erk1 (P27361), chimpanzee Erk1 (XP_510921), bull Erk1 (NP_001103488), dog Erk1 (XP_854045), mouse Erk1 (Q63844), rat Erk1 (P21708), tropical frog Erk1 (NP_001017127), zebrafish Erk1 (BAB11812), fruit fly Erk-A (Protein rolled) (P40417), *Caenorhabditis elegans* Erk homologue (mpk-1) (NP_001022583), human Erk2 (P28482), chimpanzee Erk2 (XP_515005), bull Erk2 (P46196), dog Erk2 (NP_001104270), mouse Erk2 (EDK97436), rat Erk2 (P63086), tropical frog Erk2 (CAJ81851), zebrafish Erk2 (CAM16297), human JNK (NP_620637), chimpanzee JNK (XP_001136927), rhesus monkey JNK (XP_001108815), bull JNK (XP_869760), dog JNK (XP_855700), mouse JNK (BAA85875), rat JNK (P49185), chicken JNK (XP_421650), tropical frog JNK (AAI67282), zebrafish JNK (BAB11810), fruit fly JNK (Basket) (NP_723541), *Caenorhabditis elegans* JNK (NP_741434), human p38 (CAG38743), chimpanzee p38 (NP_001009065), rhesus monkey p38 (XP_001112423), bull p38 (NP_001095644), dog p38 (O02812), mouse p38 (p47811), rat p38 (NP_112282), chicken p38 (XP_001232616), tropical frog p38 (AAH75368), zebrafish p38 (AAQ91248), fruit fly p38 (BAA35141), *Caenorhabditis elegans* p38 (AAB00664), Yeast (*Kluyveromyces marxianus*) (Hog1p-like protein) p38 (ACD02022), human IKK-α (O15111), bull IKK-α (Q95KV1), dog IKK-α (XP_861524), mouse IKK-α (Q60680), chicken IKK-α (Q5ZJB4), tropical frog IKK-α (Q28DZ1), zebrafish IKK-α (Q4G3H4), human IKK-β (O14920), bull IKK-β (Q95KV0), dog IKK-β (XP_539954), mouse IKK-β (O88351), rat IKK-β (NP_445807), tropical frog IKK-β (NP_001005651), zebrafish IKK-β (XP_692018), fruit fly IKK-β (ird5) (NP_524751), human LYN (AAH59394), chimpanzee LYN (XP_528143), rhesus monkey LYN (XP_001087049), bull LYN (XP_614963), dog LYN (XP_535078), mouse LYN (AAA39470), rat LYN

(AAA41549), chicken LYN (NP_001006390), tropical frog LYN (CAJ83326), zebrafish LYN (NP_001013288).

Results

Identification of potential KTIMs in members of the JAK family

To find out whether members of the JAK family of kinases contain potential KTIMs, we screened all available JAK1, JAK2, JAK3, and TYK2 amino acid sequences for the presence of this motif. While no KTIMs were found in any of the JAK1 or TYK2 sequences, JAK2 contains two potential KTIMs both located within its kinase domain, one emerging in fish and the other in amphibians (Figure 1). Additionally, JAK3 contains three potential KTIMs, one located in its kinase domain and appearing in early mammals (mouse / rat) and the other two present as early as chicken and found within or close to the kinase domain (Figure 2). KTIMs in JAK2 and JAK3 are conserved upon emergence all the way up to human.

Identification of potential KTIMs in the TAK1 and IKK family of kinases

Given the crucial role of TAK1 and IKK in the ultimate activation of NF-κB, it was important to explore the possibility that those two kinases could contain potential KTIMs that can play a role in the regulation of their kinase activity. Upon analyzing the available TAK1 amino acid sequences from *C. elegans* to human, we found that TAK1 contains one potential KTIM located within its kinase domain. The motif appeared in fish and is conserved up to human (Figure 3). Furthermore, IKK-α contains one potential KTIM that emerged in mouse and is conserved up to human while the KTIM present in IKK-β appeared in amphibians and is conserved thereafter. Interestingly, the KTIMs in IKK-α and IKK-β did not lie within their respective kinase domain (Figure 4).

MAPK and Src family of kinases also contain potential KTIMs

MAP kinases and those of the Src family play pivotal roles in signalling pathways that affect several cellular processes, therefore, identifying potential KTIMs in their sequences would be of high significance. Sequence analysis revealed that all three MAP kinases Erk, JNK, and p38, as well as LYN have potential KTIMs. Erk1 and Erk2 contain one KTIM each located within their kinase domain. This motif appeared in amphibians and is highly conserved thereafter (Figure 5). Two KTIMs are present in JNK, one in the kinase domain, highly conserved from as early as *C. elegans*, and a second one located outside the kinase domain that emerged in *Drosophila* (Figure 6). p38 bears two KTIMs, one found in the kinase domain and highly conserved from as early as *C. elegans*. The second KTIM is located outside the kinase domain and emerged in the fish sequence, becoming highly conserved thereafter (Figure 7). As far as the Src family is concerned, LYN was found to contain one potential KTIM within its kinase domain. The motif is absent in lower vertebrates (fish and amphibians), but appears in chicken and is conserved thereafter (Figure 8).

SHP-1 is able to bind *Drosophila*'s JNK *in vitro*

Our sequence analysis revealed the emergence of KTIMs in two key kinases in invertebrates (JNK, p38). This finding is interesting since SHP-1 emerged after the divergence between invertebrates and vertebrates and is only found in the latter [21]. This suggests that the KTIMs found in JNK and p38 may have emerged before their regulators and that they could have been readily available upon appearance of SHP-1. We therefore hypothesized that SHP-1 may be able to recognize and bind JNK from *Drosophila*, which

contains two KTIMs. To address that question, we first confirmed by western blot and in gel PTP assay that unlike mouse MØs, no SHP-1 protein or SHP-1 phosphatase activity could be detected in *Drosophila* S2 MØs (Figure 9A). Next, we incubated purified GST-SHP-1 with mouse and *Drosophila* MØ lysates, then GST-SHP-1 was pulled down and the recuperated proteins were run on SDS-PAGE. Western blotting was then performed using an antibody that detects both mouse and *Drosophila* JNK. Results clearly showed that while mouse JNK interacted with SHP-1 as expected, *Drosophila*'s JNK was also able to interact with SHP-1 as efficiently as vertebrate JNK even though the phosphatase was artificially introduced into the S2 MØ lysates (Figure 9B).

Discussion

We have recently reported that IRAK-1 possesses a KTIM motif in its kinase domain and that this motif emerged in the amino acid sequence of IRAK-1 at the amphibian level and was conserved thereafter until human [21]. In addition, we have shown that other kinases known to play pivotal roles in signalling of immune cells have potential KTIMs [21]. In this study, we analyzed the number, location, and time of emergence of potential KTIMs in several important kinases. We included members of the JAK and MAPK families due to their established role in signalling pathways of immune cells. On the other hand, TAK1 and members of the IKK family are known to activate NF-κB and therefore modulate the transcription of several important genes involved in immune functions. Additionally, the Src family of proteins also plays important signalling functions including their ability to phosphorylate ITIMs creating docking sites for SH2 domain-containing proteins [6,25,26].

It was remarkable to find that all the kinases studied, with the exception of IKK-α and IKK-β, had some or all of their KTIMs located in the kinase domain. This is in accordance to what we observed previously with IRAK-1 [21] and may represent one way by which their regulation can take place. The steric hindrance caused by binding of SH2 domain-containing proteins such as SHP-1 to the kinase domain can itself represent a powerful way to interfere with the kinase activity of these proteins. The presence of potential KTIMs outside the kinase domain in IKK-α and IKK-β suggests that this is not the only way KTIMs work, but that the phosphatase activity of recruited proteins like SHP-1 or SHIP must play an important role in the inactivation process, either by dephosphorylating tyrosine residues on the KTIM-containing kinase or by docking itself

on this kinase and dephosphorylating other proteins that come in close proximity to the protein complex.

In accordance to what we observed previously with IRAK-1 [21], the majority of the identified KTIMs emerged in the early vertebrate level (fish and amphibian) (Figure 10). These findings support our previous suggestion that the emergence of KTIMs in early vertebrates might have played an important role in the development of adaptive immunity in vertebrates by tightly controlling innate immune responses through their ability to modulate the activity of kinases involved in innate immune functions [21]. It was interesting to note that KTIMs appeared later in evolution in kinases that were found exclusively in vertebrates (JAK3, LYN, and IKK-α). This is possibly due to the shorter period of time available for the favourable mutations to occur compared to kinases present in invertebrates. Even more interesting was the finding that some kinases namely JNK and p38 contained potential KTIMs present in invertebrates and conserved all the way to human. The fact that these motifs emerged in invertebrates and were highly conserved suggests evolutionary pressure that maintained them due to important functions they may have played in invertebrates. These functions might have included binding to SH2 domain-containing proteins like SHP-2 whose orthologues are found in invertebrates [27] but not SHP-1 which is found exclusively in vertebrates [21]. This raises the interesting possibility that such motifs were theoretically able to bind SHP-1, except that SHP-1 was not found in invertebrates. This implies that once SHP-1 emerged in early vertebrates, these conserved KTIMs constituted novel and readily available regulatory sites that could bind to this phosphatase.

To test the possibility that an invertebrate KTIM-containing kinase could theoretically bind SHP-1 in a similar manner to a vertebrate KTIM-containing kinase, we performed pull-down assays to measure the ability of the mouse GST-SHP-1 to pull down *Drosophila* or mouse JNK. JNK was chosen for this experiment for two main reasons: Firstly, JNK is the only kinase we identified whose KTIMs all appeared early in invertebrates and were highly conserved throughout species and secondly because JNK has been previously shown to be regulated by SHP-1 in mouse cells [28]. Indeed, our pull-down assays showed that *Drosophila* JNK was able to bind SHP-1 as efficiently as mouse JNK. This result is a good indication that one or both KTIMs found in the JNK sequence since invertebrates could mediate the binding to SHP-1.

It is important to stress that several kinases that we screened did not contain any KTIM, these kinases include JAK1 and TYK2 from the JAK family, IKK-γ from the IKK family, Syk from the ZAP-70 family, and Src, Yes, Fyn, Fgr, Lck, Hck from the Src family (Data not shown). This finding supports the fact that the consensus sequence of KTIM is not very flexible and is unlikely to appear in kinases by chance alone.

Although mutagenesis assays are further required to confirm the ability of the reported KTIMs to mediate binding to SH2 domain-containing proteins such as SHP-1, SHP-2, and SHIP, this work sheds light on the potential importance of these motifs as regulatory sites in many kinases and opens the door to the discovery of new SHP-1 substrates. KTIMs can help explain some of the results obtained in previous studies where SHP-1 was implicated in the regulation of kinases such as JAK2, JAK3, Erk, and JNK but where no clear regulatory mechanism was proposed [28-30]. Importantly, this work introduces for the first time the notion that KTIMs can be a regulatory mechanism widely used by

kinases and that the KTIMs found conserved as early as invertebrates might have constituted readily available motifs that were able to bind regulatory proteins as they appeared in vertebrates (e.g. SHP-1). Nevertheless, despite the few KTIMs we found conserved in invertebrates, it is not surprising to find that most of KTIMs appeared in early vertebrates where they must have performed critical functions that guaranteed their evolutionary conservation. In performing their negative regulatory functions, KTIMs could have played an important role in the development of adaptive immune responses in vertebrates by fine-tuning innate immune responses and helping to avoid excessive activation of the immune system that could have been detrimental to the host.

Figure 1. JAK2 contains two KTIMs within its kinase domain. (A) Amino acid sequence comparison of JAK2 in several invertebrates and vertebrates reveals that JAK2 possesses two KTIMs located in its kinase domain, appearing in early vertebrates. Conserved amino acid residues appear in red, non-conserved residues in critical positions in the motif appear green, and non-conserved residues in variable positions in the motif appear blue. A red box is drawn around the motif in the amino acid sequence of the organism in which it first emerges. (B) Schematic diagram of human JAK2 indicating the important domains and the location of the KTIMs. N, N-terminal. SH2, Src-homology 2 domain. KD, kinase domain. C, C-terminal.

A

Janus kinase 2 (JAK2)

Vertebrate
Invertebrate

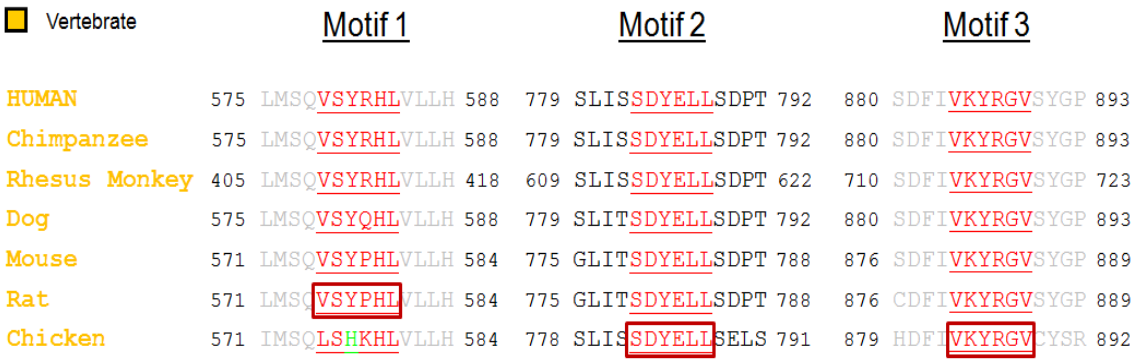
Human	903	KSLQHDNIVKYKGVCYSAGRRNLKLIMEYLPYGSLRDYLQKHK	945
Chimpanzee	903	KSLQHDNIVKYKGVCYSAGRRNLKLIMEYLPYGSLRDYLQKHK	945
Rhesus monkey	903	KSLQHDNIVKYKGVCYSAGRRNLKLIMEYLPYGSLRDYLQKHK	945
Pig	903	KSLQHDNIVKYKGVCYSAGRRNLRLIMEYLPYGSLRDYLQKHK	945
Bull	827	KSLQHDNIVKYKGVCYSAGRHNLRLIMEYLPYGSLRDYLQKHK	869
Dog	903	KSLQHDNIVKYKGVCYSAGRRNLRLIMEYLPYGSLRDYLQKHK	945
Mouse	903	KSLQHDNIVKYKGVCYSAGRRNLRLIMEYLPYGSLRDYLQKHK	945
Rat	903	KSLQHDNIVKYKGVCYSAGRRNLRLIMEYLPYGSLRDYLQKHK	945
Chicken	900	KSLQHDNIVKYKGVCYSAGRRNLRLIMEYLPYGSLRDYLQKHK	942
Xenopus	900	KSLQHDNIVRYKGVCYSAGRRNLRLIMEYLPYGSLRDYLQKHK	942
Zebrafish	866	RSLQHENIVRYKGVCYSAGRNNLRLVMEFLPFGSLRDYLSKNR	908
Honey bee	910	KTLNHPNVVKILGVIS---EPEVCLVMEYVKHGSLQSYLAIHK	949
Drosophila	945	RTLSHPNIVKFK---YWAEKSH-CIIMEYLQSGSFDIYLRFTA	983
Mosquito	956	KKLNHRNIVRLLEFVDE--PDRMVVVMEYIEHGSLERYLQYKR	996

B

Figure 2. JAK3 contains three KTIMs close or within its kinase domain. (A) Amino acid sequence comparison of JAK3 in several vertebrates reveals that JAK3 possesses three KTIMs located close or within its kinase domain. Conserved amino acid residues appear in red and non-conserved residues in critical positions in the motif appear green. A red box is drawn around the motif in the amino acid sequence of the organism in which it first emerges. (B) Schematic diagram of human JAK3 indicating the important domains and the location of the KTIMs. N, N-terminal. SH2, Src-homology 2 domain. KD, kinase domain. C, C-terminal.

Janus kinase 3 (JAK3)

A



B

Figure 3. TAK1 contains one KTIM within its kinase domain. (A) Amino acid sequence comparison of TAK1 in several invertebrates and vertebrates reveals that TAK1 possesses one KTIM located in its kinase domain, appearing in early vertebrates. Conserved amino acid residues appear in red, non-conserved residues in critical positions in the motif appear green, and non-conserved residues in variable positions in the motif appear blue. A red box is drawn around the motif in the amino acid sequence of the organism in which it first emerges. (B) Schematic diagram of human TAK1 indicating the important domains and the location of the KTIM. N, N-terminal. KD, kinase domain. C, C-terminal.

TGF-β activated kinase 1 (TAK1)

A

Vertebrate
Invertebrate

Human	103	VMEYAEGGSLYNVLHGAEPLPY	124
Chimpanzee	103	VMEYAEGGSLYNVLHGAEPLPY	124
Rhesus Monkey	103	VMEYAEGGSLYNVLHGAEPLPY	124
Pig	103	VMEYAEGGSLYNVLHGAEPLPY	124
Bull	103	VMEYAEGGSLYNVLHGAEPLPY	124
Mouse	103	VMEYAEGGSLYNVLHGAEPLPY	124
Chicken	93	VMEYAEGGSLYNVLHGAEPLPH	114
Xenopus	92	VMEYAEGGSLYNVLHGAEPLPY	113
Zebrafish	85	VMEYAEGGSLYNVLHGAEPLPH	106
Drosophila	88	IMEFAEGGSLHNFLHGKVKPAY	109
C. elegans	127	VMEYMDCGSMADLLYDRTHINY	148

B

Figure 4. IKK-α and IKK-β each contain one KTIM outside their kinase domain (A) Amino acid sequence comparison of IKK-α and IKK-β in several invertebrates and vertebrates reveals that IKK-α and IKK-β each possess one KTIM located outside the kinase domain. Conserved amino acid residues appear in red, non-conserved residues in critical positions in the motif appear green, and non-conserved residues in variable positions in the motif appear blue. A red box is drawn around the motif in the amino acid sequence of the organism in which it first emerges. (B) Schematic diagram of human IKK-α and IKK-β indicating the important domains and the location of the KTIM. N, N-terminal. KD, kinase domain. C, C-terminal.

Inhibitor of kappa B kinase (IKK-α/β)

A

Vertebrate
Invertebrate

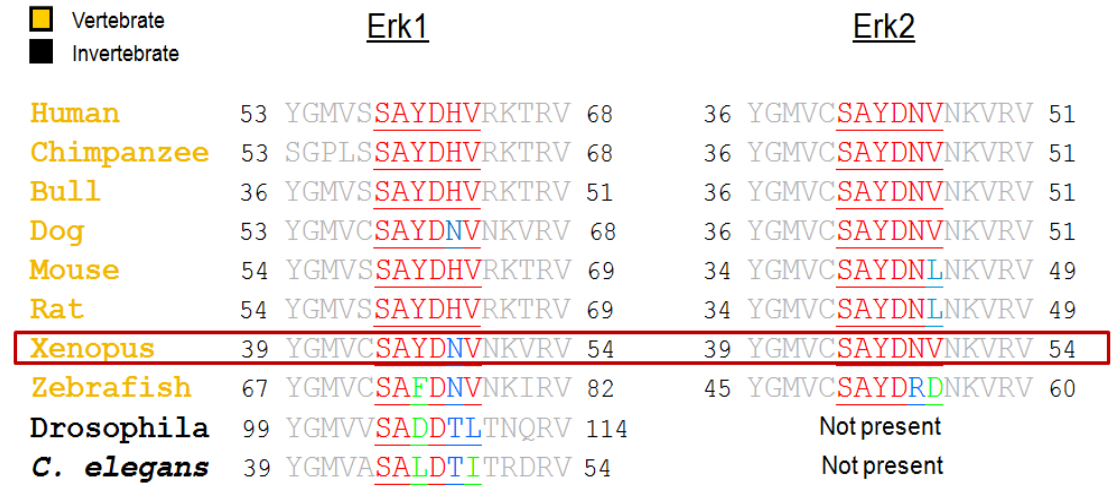
	IKK-α	IKK-β
Human	514 MEEKAIHYAEVGVIGY 529	602 EKKVRVIYTQLSKTVV 617
Bull	514 MEEKAIHYAEVGVIGY 529	602 EKKVRVIYTQLSKTVV 617
Dog	514 MEEKAIHYAEVGVIGY 529	602 EKKVRVIYTQLSKTVV 617
Mouse	514 MEEKAIHYSEVGVIGY 529	602 EKKVRVIYTQLSKTVV 617
Chicken	528 MEEKASHCAQAEDIGY 543	602 EKKVRVIYSQLSKTVV 617
Xenopus	512 MEDKAVCFGKAGKIHF 527	613 EKRVILIYDQLSKTVA 628
Zebrafish	515 NEERAAAFAQVAEIGH 530	604 ERKLRDFYTHLSKTMV 619
Drosophila	Not present	636 AKSRFKLYNEGEARHL 651

B

Figure 5. Erk1 and Erk2 each contain one KTIM within their kinase domain. (A) Amino acid sequence comparison of Erk1/2 in several invertebrates and vertebrates reveals that Erk1 and Erk2 each possess one KTIM located in the kinase domain, appearing in early vertebrates. Conserved amino acid residues appear in red, non-conserved residues in critical positions in the motif appear green, and non-conserved residues in variable positions in the motif appear blue. A red box is drawn around the motif in the amino acid sequence of the organism in which it first emerges. (B) Schematic diagram of human Erk1 and Erk2 indicating the important domains and the location of the KTIM. N, N-terminal. KD, kinase domain. C, C-terminal.

Extracellular signal-regulated kinase 1/2 (Erk1/2)

A



B

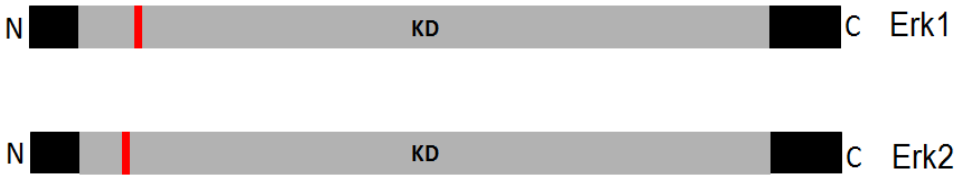
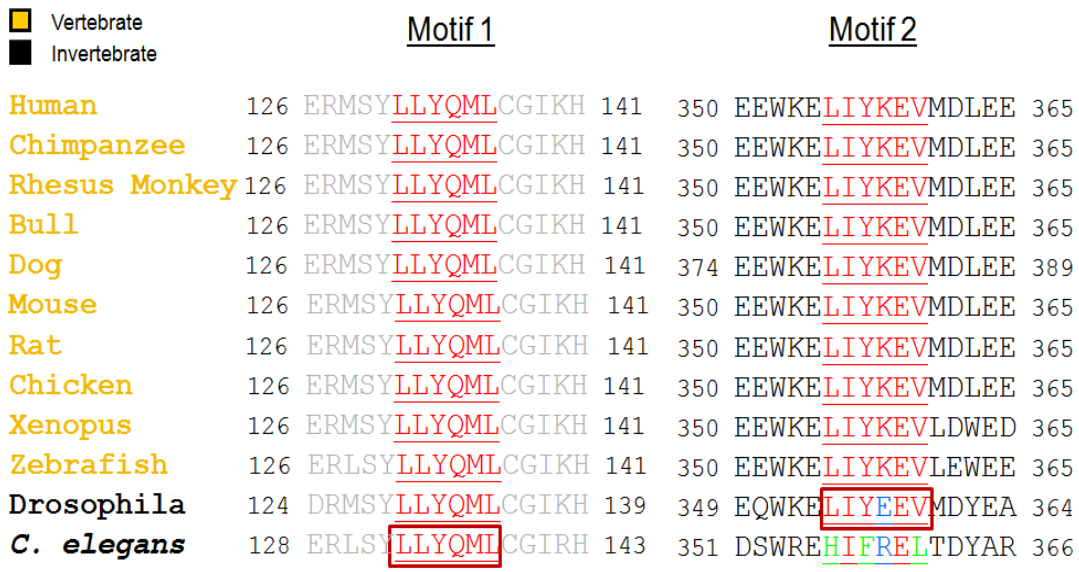


Figure 6. JNK contains two KTIMs one of which within the kinase domain. (A) Amino acid sequence comparison of JNK in several invertebrates and vertebrates reveals that JNK possesses two KTIMs in *Drosophila* and that they are conserved in vertebrates. Conserved amino acid residues appear in red, non-conserved residues in critical positions in the motif appear green, and non-conserved residues in variable positions in the motif appear blue. A red box is drawn around the motif in the amino acid sequence of the organism in which it first emerges. (B) Schematic diagram of human JNK indicating the important domains and the location of the KTIMs. N, N-terminal. KD, kinase domain. C, C-terminal.

Jun N-terminal kinase (JNK)

A

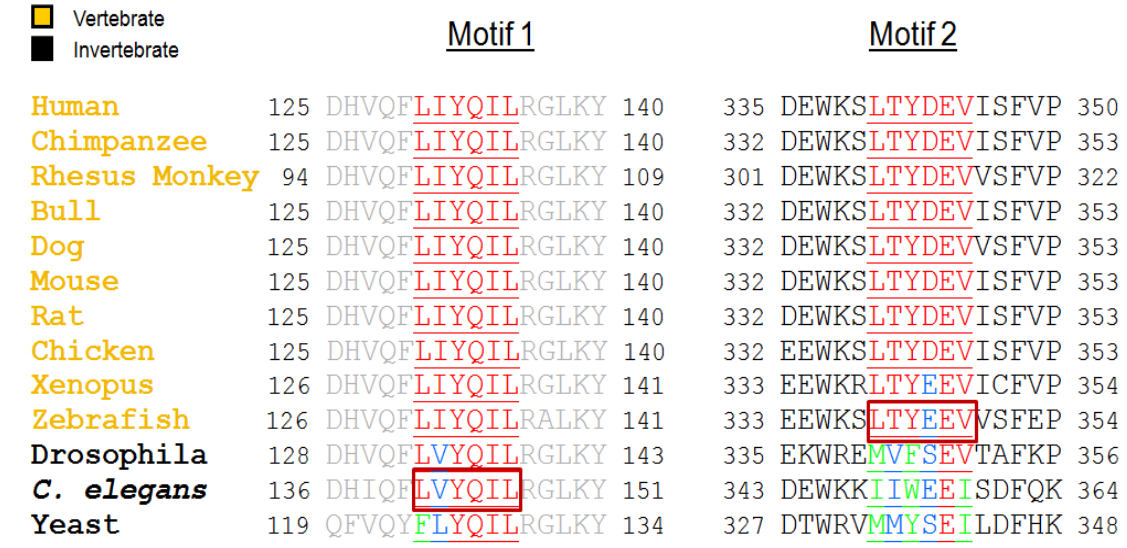


B

Figure 7. p38 contains two KTIMs one of which within the kinase domain. (A) Amino acid sequence comparison of p38 in several invertebrates and vertebrates reveals that p38 possesses two KTIMs one present in *Drosophila* and conserved in vertebrates and the other appearing in early vertebrates. Conserved amino acid residues appear in red, non-conserved residues in critical positions in the motif appear green, and non-conserved residues in variable positions in the motif appear blue. A red box is drawn around the motif in the amino acid sequence of the organism in which it first emerges. (B) Schematic diagram of human p38 indicating the important domains and the location of the KTIMs. N, N-terminal. KD, kinase domain. C, C-terminal.

p38

A


Vertebrate
Invertebrate
Motif 1
Motif 2
Human 125 DHVQFLIYQILRGLKY 140 335 DEWKSLTYDEVISFVP 350
Chimpanzee 125 DHVQFLIYQILRGLKY 140 332 DEWKSLTYDEVISFVP 353
Rhesus Monkey 94 DHVQFLIYQILRGLKY 109 301 DEWKSLTYDEVVSFVP 322
Bull 125 DHVQFLIYQILRGLKY 140 332 DEWKSLTYDEVISFVP 353
Dog 125 DHVQFLIYQILRGLKY 140 332 DEWKSLTYDEVVSFVP 353
Mouse 125 DHVQFLIYQILRGLKY 140 332 DEWKSLTYDEVISFVP 353
Rat 125 DHVQFLIYQILRGLKY 140 332 DEWKSLTYDEVISFVP 353
Chicken 125 DHVQFLIYQILRGLKY 140 332 EEWKSLTYDEVISFVP 353
Xenopus 126 DHVQFLIYQILRGLKY 141 333 EEWKRLTYEEVICFVP 354
Zebrafish 126 DHVQFLIYQILRALKY 141 333 EEWKSLTYEEVVSFEP 354
Drosophila 128 DHVQFLVYQILRGLKY 143 335 EKWREMVFSEVTAFKP 356
C. elegans 136 DHIQFLVYQILRGLKY 151 343 DEWKKIIWEEISDFQK 364
Yeast 119 QFVQYFLYQILRGLKY 134 327 DTWRVMMYSEILDFHK 348

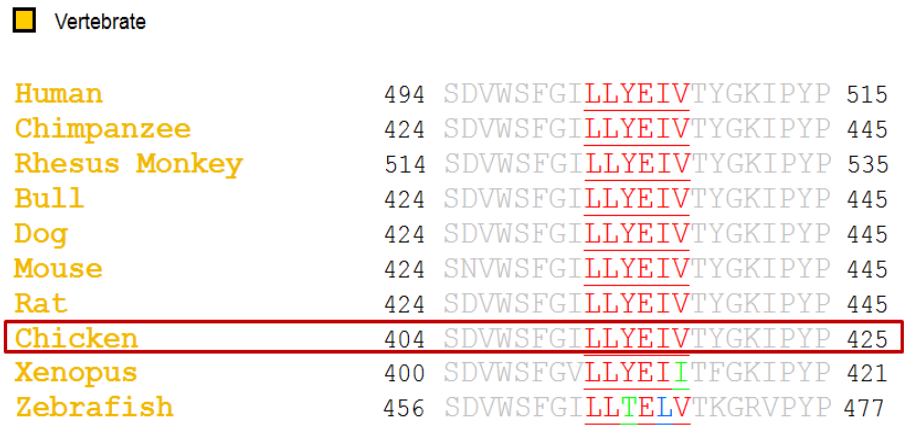
B


N
KD
C
p38

Figure 8. LYN contains one KTIM within its kinase domain. (A) Amino acid sequence comparison of LYN in several vertebrates reveals that LYN possesses one KTIM located in its kinase domain. Conserved amino acid residues appear in red, non-conserved residues in critical positions in the motif appear green, and non-conserved residues in variable positions in the motif appear blue. A red box is drawn around the motif in the amino acid sequence of the organism in which it first emerges. (B) Schematic diagram of human LYN indicating the important domains and the location of the KTIM. N, N-terminal. SH2, Src-homology 2 domain. KD, kinase domain. C, C-terminal.

LYN

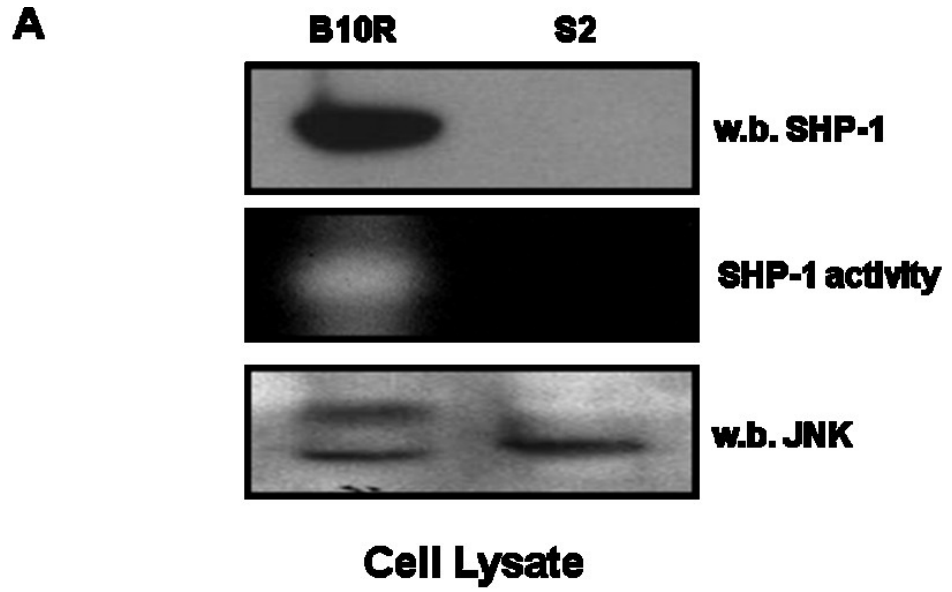
A


Vertebrate
Human 494 SDVWSFGILLYEIVTYGKIPYP 515
Chimpanzee 424 SDVWSFGILLYEIVTYGKIPYP 445
Rhesus Monkey 514 SDVWSFGILLYEIVTYGKIPYP 535
Bull 424 SDVWSFGILLYEIVTYGKIPYP 445
Dog 424 SDVWSFGILLYEIVTYGKIPYP 445
Mouse 424 SNVWSFGILLYEIVTYGKIPYP 445
Rat 424 SDVWSFGILLYEIVTYGKIPYP 445
Chicken 404 SDVWSFGILLYEIVTYGKIPYP 425
Xenopus 400 SDVWSFGVLLYEIITFGKIPYP 421
Zebrafish 456 SDVWSFGILLTELVTKGRVPYP 477

B


N
SH2
KD
C
LYN

Figure 9: SHP-1 is able to bind *Drosophila*'s JNK *in vitro*. (A) Upper panel represents a western blot demonstrating the presence of SHP-1 in murine B10R MØ lysates and its absence in *Drosophila* S2 MØ lysates (30 µg of protein per lane). Middle panel is an in gel PTP assay detecting SHP-1 activity in murine but not *Drosophila* MØ lysates (30 µg of protein per lane). Bottom panel is a western blot where the membrane in upper panel was stripped and re-blotted for JNK to show its presence in both murine and *Drosophila* MØ lysates. (B) Western blot using JNK antibody of a pull-down of GST or GST-SHP-1 put in contact with 2 mg of murine B10R or *Drosophila* S2 MØ lysates. All results are representative of at least three independent experiments.


A
B10R
S2
w.b. SHP-1
SHP-1 activity
w.b. JNK
Cell Lysate

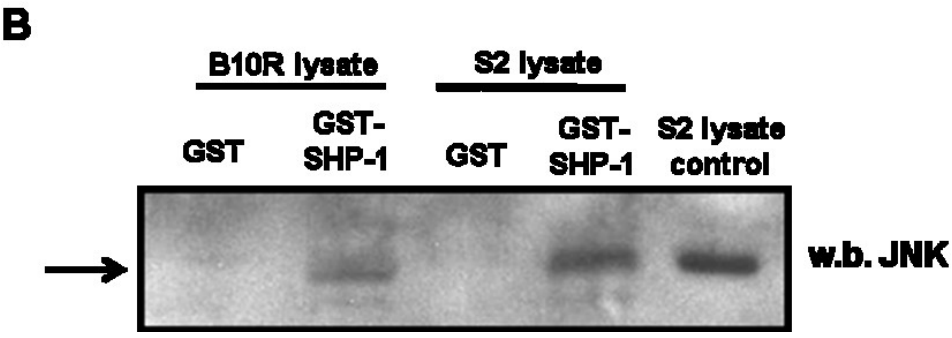
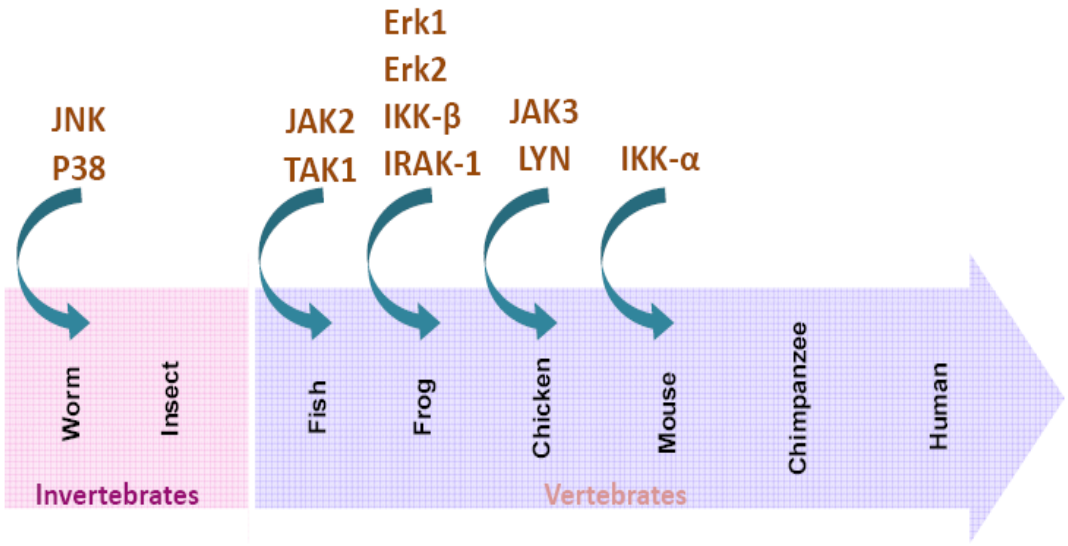

B
B10R lysate
S2 lysate
GST
GST-SHP-1
GST
GST-SHP-1
S2 lysate control
w.b. JNK

Figure 10: KTIM appearance in evolution. A schematic showing the relative evolutionary time in which KTIM got introduced into the amino acid sequences of the kinases investigated in this study.


JNK
P38
JAK2
TAK1
Erk1
Erk2
IKK-β
IRAK-1
JAK3
LYN
IKK-α
Worm
Insect
Fish
Frog
Chicken
Mouse
Chimpanzee
Human
Invertebrates
Vertebrates

Acknowledgements

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References

1. Vely F, Vivier E (1997). Conservation of structural features reveals the existence of a large family of inhibitory cell surface receptors and noninhibitory/activatory counterparts. *J Immunol* **159**: 2075-2077.
2. Bolland S, Ravetch JV (1999). Inhibitory pathways triggered by ITIM-containing receptors. *Adv Immunol* **72**: 149-177.
3. Ravetch JV, Lanier LL (2000). Immune inhibitory receptors. *Science* **290**: 84-89.
4. O'Keefe TL, Williams GT, Batista FD, Neuberger MS (1999). Deficiency in CD22, a B cell-specific inhibitory receptor, is sufficient to predispose to development of high affinity autoantibodies. *J Exp Med* **189**: 1307-1313.
5. Bolland S, Ravetch JV (2000). Spontaneous autoimmune disease in Fc(gamma)RIIB-deficient mice results from strain-specific epistasis. *Immunity* **13**: 277-285.
6. Maeda A, Scharenberg AM, Tsukada S, Bolen JB, Kinet JP, et al. (1999). Paired immunoglobulin-like receptor B (PIR-B) inhibits BCR-induced activation of Syk and Btk by SHP-1. *Oncogene* **18**: 2291-2297.
7. Maeda A, Kurosaki M, Ono M, Takai T, Kurosaki T (1998). Requirement of SH2-containing protein tyrosine phosphatases SHP-1 and SHP-2 for paired immunoglobulin-like receptor B (PIR-B)-mediated inhibitory signal. *J Exp Med* **187**: 1355-1360.
8. Blery M, Kubagawa H, Chen CC, Vely F, Cooper MD, et al. (1998). The paired Ig-like receptor PIR-B is an inhibitory receptor that recruits the protein-tyrosine phosphatase SHP-1. *Proc Natl Acad Sci USA* **95**: 2446-2451.
9. Ono M, Bolland S, Tempst P, Ravetch JV (1996). Role of the inositol phosphatase SHIP in negative regulation of the immune system by the receptor Fc(gamma)RIIB. *Nature* **383**: 263-266.
10. Long EO (1999). Regulation of immune responses through inhibitory receptors. *Annu Rev Immunol* **17**: 875-904.
11. Olcese L, Lang P, Vely F, Cambiaggi A, Marguet D, et al. (1996). Human and mouse killer-cell inhibitory receptors recruit PTP1C and PTP1D protein tyrosine phosphatases. *J Immunol* **156**: 4531-4534.
12. Burshtyn DN, Scharenberg AM, Wagtmann N, Rajagopalan S, Berrada K, et al. (1996). Recruitment of tyrosine phosphatase HCP by the killer cell inhibitor receptor. *Immunity* **4**: 77-85.
13. Fry AM, Lanier LL, Weiss A (1996). Phosphotyrosines in the killer cell inhibitory receptor motif of NKB1 are required for negative signaling and for association with protein tyrosine phosphatase 1C. *J Exp Med* **184**: 295-300.

14. Carretero M, Palmieri G, Llano M, Tullio V, Santoni A, et al. (1998). Specific engagement of the CD94/NKG2-A killer inhibitory receptor by the HLA-E class Ib molecule induces SHP-1 phosphatase recruitment to tyrosine-phosphorylated NKG2-A: evidence for receptor function in heterologous transfectants. *Eur J Immunol* **28**: 1280-1291.
15. Le Drean E, Vely F, Olcese L, Cambiaggi A, Guia S, et al. (1998). Inhibition of antigen-induced T cell response and antibody-induced NK cell cytotoxicity by NKG2A: association of NKG2A with SHP-1 and SHP-2 protein-tyrosine phosphatases. *Eur J Immunol* **28**: 264-276.
16. Cornish AL, Freeman S, Forbes G, Ni J, Zhang M, et al. (1998). Characterization of siglec-5, a novel glycoprotein expressed on myeloid cells related to CD33. *Blood* **92**: 2123-2132.
17. Patel N, Brinkman-Van der Linden EC, Altmann SW, Gish K, Balasubramanian S, et al. (1999). OB-BP1/Siglec-6. a leptin- and sialic acid-binding protein of the immunoglobulin superfamily. *J Biol Chem* **274**: 22729-22738.
18. Falco M, Biassoni R, Bottino C, Vitale M, Sivori S, et al. (1999). Identification and molecular cloning of p75/AIRM1, a novel member of the sialoadhesin family that functions as an inhibitory receptor in human natural killer cells. *J Exp Med* **190**: 793-802.
19. Vitale C, Romagnani C, Falco M, Ponte M, Vitale M, et al. (1999). Engagement of p75/AIRM1 or CD33 inhibits the proliferation of normal or leukemic myeloid cells. *Proc Natl Acad Sci USA* **96**: 15091-15096.
20. Ishida Y, Agata Y, Shibahara K, Honjo T (1992). Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *EMBO J* **11**: 3887-3895.
21. Abu-Dayyeh I, Shio MT, Sato S, Akira S, Cousineau B, et al. (2008). *Leishmania*-Induced IRAK-1 Inactivation Is Mediated by SHP-1 Interacting with an Evolutionarily Conserved KTIM Motif. *PLoS Negl Trop Dis* **2**: e305.
22. Radzioch D, Hudson T, Boule M, Barrera L, Urbance JW, et al. (1991). Genetic-Resistance Susceptibility to Mycobacteria - Phenotypic-Expression in Bone-Marrow Derived Macrophage Lines. *J Leukoc Biol* **50**: 263-272.
23. Blanchette J, Abu-Dayyeh I, Hassani K, Whitcombe L, and Olivier M (2008). Regulation of macrophage nitric oxide production by the protein tyrosine phosphatase Src homology 2 domain phosphotyrosine phosphatase 1 (SHP-1). *Immunology* **127**: 123-133.
24. Markova B, Gulati P, Herlich PA, Bohmer FD (2005). Investigation of protein-tyrosine phosphatases by in-gel assays. *Methods* **35**: 22-27.
25. Malbec O, Fong DC, Turner M, Tybulewicz VL, Cambier JC, et al. (1998). Fc epsilon receptor I-associated lyn-dependent phosphorylation of Fc gamma receptor IIB during negative regulation of mast cell activation. *J Immunol* **160**: 1647-1658.

26. Smith KG, Tarlinton DM, Doody GM, Hibbs ML, Fearon DT (1998). Inhibition of the B cell by CD22: a requirement for Lyn. *J Exp Med* **187**: 807-811.
27. Neel BG, Gu H, Pao L (2003). The 'Shp'ing news: SH2 domain-containing tyrosine phosphatases in cell signaling. *Trends Biochem Sci* **28**: 284-293.
28. Matsubara H, Shibasaki Y, Okigaki M, Mori Y, Masaki H, et al. (2001). Effect of angiotensin II type 2 receptor on tyrosine kinase Pyk2 and c-Jun NH2-terminal kinase via SHP-1 tyrosine phosphatase activity: evidence from vascular-targeted transgenic mice of AT2 receptor. *Biochem Biophys Res Commun* **282**: 1085-1091.
29. Bedecs K, Elbaz N, Sutren M, Masson M, Susini C, et al. (1997). Angiotensin II type 2 receptors mediate inhibition of mitogen-activated protein kinase cascade and functional activation of SHP-1 tyrosine phosphatase. *Biochem J* **325** (Pt 2): 449-454.
30. Jiao H, Berrada K, Yang W, Tabrizi M, Platanias LC, et al. (1996). Direct association with and dephosphorylation of Jak2 kinase by the SH2-domain-containing protein tyrosine phosphatase SHP-1. *Mol Cell Biol* **16**: 6985-6992.

Chapter 4

Comparative Study of the Ability of *Leishmania mexicana* Promastigotes and Amastigotes to Alter Macrophage Signalling and Functions

Having explored the modulation of IRAK-1-dependent signalling by *Leishmania*, we wanted to build a more comprehensive view of how the parasite can alter other MØ signalling molecules involved in various pathways to establish itself in the host. The proteins we decided to study were: protein tyrosine phosphatases (PTPs) and selected transcription factors (TFs): NF-κB, STAT-1α, and AP-1. This choice is based on the fact that PTPs are established negative regulators of MØs and the previously-mentioned TFs are involved in the production of leishmanicidal molecules such as NO and pro-inflammatory cytokines. The data available in the literature regarding the modulation of these signalling molecules deal with one or the other form of the parasite, and comparative studies are lacking. Therefore, a major goal of this chapter is to compare the ability of promastigotes and amastigotes to alter cell signalling and function. Results yielded several interesting similarities between the two forms including the ability of both to rapidly activate SHP-1 and to inhibit STAT-1α and AP-1. Of utmost interest, certain differences were unveiled including the differential ability of promastigotes to activate PTP-1B and to strongly induce the generation of a p35 NF-κB subunit in early infection time. Finally, this work uncovers novel roles for *L. mexicana*'s cysteine proteinase lmcpb in the inhibition of STAT-1α and AP-1, therefore expanding our knowledge about this proteinase as a *Leishmania* virulence factor.

Comparative Study of the Ability of *Leishmania mexicana* Promastigotes and Amastigotes to Alter Macrophage Signalling and Functions

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Running title: *Alteration of Macrophage signalling by Leishmania*

Abstract

Leishmania alternates between two morphologically different stages: promastigotes and amastigotes. While the majority of reports focused on how the promastigote form can alter macrophage (MØ) signalling and function, fewer reports investigated signalling alterations mediated by amastigotes, and comparative studies are lacking. In this study, we performed a comparison between the ability of both forms of the parasite to alter MØ signalling and functions. Here, we show that promastigotes and amastigotes were both able to rapidly activate host protein tyrosine phosphatases (PTPs), importantly the Src-homology 2 domain-containing PTP (SHP-1). However, we found that PTP-1B is specifically activated by promastigote but not amastigote infection. We also show a similarity in the way promastigotes and amastigotes inactivate the transcription factors (TFs) STAT-1α and AP-1, but differences in the modulation of NF-κB with promastigotes cleaving the p65 subunit generating a smaller p35 subunit while amastigotes fully degrading the p65 subunit with no p35 production. Importantly, we show that the cysteine proteinase lmcpb plays a key role in the alteration of NF-κB, STAT-1α, and AP-1 by promastigote and amastigote infections, ultimately leading to the inability of these TFs to translocate to the nucleus in response to IFN-γ stimulation and thus contributing to the ability of both parasite forms to effectively block IFN-γ-mediated NO production in MØs.

Introduction

Leishmania parasites are endemic in more than 80 countries in the world. It is estimated that there are 12 million cases of leishmaniasis worldwide, with 2 million new cases emerging every year [1]. The most common manifestations of the disease are: visceral leishmaniasis caused by *L. donovani* and *L. chagasi* and responsible for the majority of mortality cases, cutaneous leishmaniasis caused principally by *L. major* and *L. mexicana* and is the most common manifestation of the disease, and the disfiguring mucocutaneous leishmaniasis caused by *L. braziliensis* [2].

Leishmania is a dimorphic protozoan alternating between the promastigote and amastigote stages. Promastigotes have an elongated shape with long flagella and live inside the sandfly vector. Once promastigotes are deposited into mammalian skin while the sandfly is having a blood meal, they are phagocytosed by cells of the monocyte / MØ lineage and rapidly transform into round amastigotes which are smaller in size and lack flagella [2]. In addition to being morphologically different, the two life stages of the parasite have different surface molecule compositions. While infectious metacyclic promastigotes have a thick glycocalyx, this cover is almost completely absent in amastigotes [3]. The glycocalyx is made of glycoproteins and other glycosylated species anchored to the surface membrane by a glycosylphosphatidylinositol (GPI) linkage [4]. The promastigote surface is predominantly covered by lipophosphoglycan (LPG), a GPI-anchored molecule made of repeating units of a disaccharide and a phosphate. Buried in a sea of LPG, promastigotes have another important GPI-anchored molecule, the surface protease gp63. Interestingly, amastigotes have been shown to produce very little LPG compared to promastigotes [5], and have reduced gp63 [6] that, in the case of *L.*

mexicana, has been shown to lack a GPI anchor and to be confined to the flagellar pocket [7].

Although LPG, and to a lesser extent gp63, are the most studied virulence factors in *Leishmania*, other important virulence factors found in several *Leishmania* species include cysteine proteinases (CPs) [8]**.** *L. mexicana* has been shown to have cathepsin L-like CP genes (lmcpb) that are a multicopy and occur in a tandem array [9] and two single copy CP genes, one is cathepsin L-like (lmcpa) and the other cathepsin B-like (lmcpc). Unlike LPG and gp63 which are plentiful in promastigotes and are downregulated in amastigotes, lmcpb is expressed at low levels in metacyclic promastigotes and is strongly upregulated in amastigotes [9] indicating that the protein may play a crucial role in the intracellular survival of the parasite.

Given that promastigotes have to avoid MØ microbicidal action in order to establish themselves in the host and that amastigotes have to suppress MØ killing abilities when they try to invade new MØs in the course of a persistent *Leishmania* infection, it is not surprising that both forms of the parasite can alter key MØ signalling pathways [10]. Indeed several studies have previously shown that promastigotes [11-14] and amastigotes [12,15,16], or molecules derived from them, are able to block Nitric Oxide (NO) production by host MØs in response to activating stimuli such as IFN-γ or bacterial lipopolysaccharide (LPS). The promastigote surface contains several glycoconjugates allowing interaction with MØs and internalization via several types of receptors such as: complement receptors 1, 3, and 4 [17,18], the mannose fucose receptor [19], the C-reactive protein receptor [20], and the fibronectin receptor [21]. On the other hand, amastigotes lack many of those glycoconjugates and seem to interact with MØs mainly

through glycosylinositol phospholipids (GIPLs) and to be phagocytosed via the Fc receptor following opsonisation by antibodies or via complement receptors. Although redundancies might exist in the way promastigotes and amastigotes interact with MØs and modulate their signalling in order to block their killing functions, the differences between both forms, whether at the gene expression, metabolic, or surface molecule levels suggest to us that some differences ought to exist in the way those two forms can modulate MØ signalling to their own favour. This work is an effort to compare the similarities and differences between promastigotes and amastigotes of *L. mexicana* in terms of their ability to alter key signalling molecules namely: protein tyrosine phospahatases (PTPs), and the transcription factors (TFs): nuclear factor kappa B (NF-κB), signal transducer and activator of transcription-1 alpha (STAT-1α), and activating protein 1 (AP-1), known to play a pivotal role in NO production [22,23] as well as other MØ functions detrimental to the survival of the *Leishmania* parasite [10]. In addition, given the established role of lmcpb as a virulence factor and immunomodulator [24,25], this work explores the role of this proteinase in the *Leishmania*-induced alterations of signalling molecules that we observed.

Materials and Methods

Cell Culture and Reagents. The immortalized B10R BMDMs were derived from B10A.Bcg^{r} mice [26]. The immortalized me-3 (SHP-1$^{-/-}$) and LM-1 (WT) bone marrow-derived MØs (BMDMs) were generated from motheaten mice (*Ptpn6*$^{me/me}$; C3HeBFeJ *me*/*me*) and their respective wild-type littermates (C3HeBFeJ *me*/+) as previously described [27]. Recombinant murine IFN-γ was purchased from Cedarlane, NC, USA. Antibodies used to immunoprecipitate SHP-1 and PTP-1B were purchased from Upstate, NY, USA.

***In vitro* infection.** Promastigotes of *L. mexicana* (MNYC/BZ/62/M379)*, and L. mexicana* deficient for lmcpb generated by targeted gene deletion as previously described [24] were kept in SDM medium (10% FBS), and stationary phase parasites were used to infect cells in a parasite to MØ ratio ranging from 5:1 to 20:1. Axenic amastigotes of WT and lmcpb$^{-/-}$ *L. mexicana* were transformed from promastigote cultures, kept in MAA (medium for axenically grown amastigotes, pH 5.6, 20% FBS), and incubated at a temperature of 32°C. When parasites were used for infections, non-internalized ones were removed by washing the plates with phosphate-buffered saline (PBS), after which MØs were collected for subsequent experiments.

Western Blot Analysis. Western blotting was performed as previously described [28]. Proteins were detected using antibodies directed against gp63 (provided by Dr. Robert McMaster, University of British Columbia, Canada)**,** LACK (provided by Dr. Eric Prina, Institut Pasteur, France), A2 (provided by Dr. Greg Matlashewski, McGill University, Canada), SHP-1 (Chemicon, CA, USA), and PTP-1B (Upstate). Proteins were detected using an anti-rabbit or anti-mouse horseradish peroxidase (HRP)-conjugated antibody (Amersham, QC, Canada) and visualized using ECL western blotting detection system (Amersham).

NO assay. NO production was evaluated by measuring the accumulation of nitrite in the culture medium by the Griess reaction, as previously described [29].

pNPP phosphatase assay. MØs were collected, lysed in PTP lysis buffer (50mM Tris (pH 7.0), 0.1mM EDTA, 0.1mM EGTA, 0.1% β-mercaptoethanol, 1% Igepal, 25µg/ml aprotinin and 25µg/ml leupeptin), and kept on ice for 45 min. Lysates were cleared by centrifugation, and protein content was determined by Bradford reagent. For experiments measuring phosphatase activity in total cell lysates, 30µg of lysates were incubated in a phosphatase reaction mix (50mM Hepes (pH 7.5), 0.1% β-mercaptoethanol, 10mM pNPP) for 30 min at 37°C and OD was read at 405 nm. For experiments measuring phosphatase activity in immunoprecipitates (IPs), cell lysates were subjected to immunoprecipitation using protein A/G agarose beads (Santa Cruz, CA, USA) and 3µg of the SHP-1, PTP-1B, or the anti-rat (Sigma-Aldrich, ON, Canada) antibody for non-specific binding. Beads

were spun down and washed 3x with the PTP lysis buffer and then incubated with the phosphatase reaction mix for 4-6 h at 37°C and OD was read at 405 nm.

In gel PTP assay. Cell lysates (30μg) were obtained using the PTP lysis buffer previously described. Samples were loaded on a gel containing a γ-^{32}P-labelled poly(Glu4Tyr) peptide (Sigma-Aldrich) and PTP bands were observed by in gel PTP assay as previously described [30].

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared by a standard protocol and EMSAs were performed as previously described [31]. Briefly, nuclear extracts were incubated with binding buffer containing 1.0 ng of [γ-^{32}P] dATP radiolabeled double-stranded DNA oligonucleotide for 20 min at room temperature. The DNA binding consensus sequence used for NF-κB was (5'-AGTTGAGGGGACTTTCCCAGGC-3'), for STAT-1 (GAS/ISRE consensus) was (5'-AAGTACTTTCAGTTTCATATTACTCTA-3'), and for AP-1 was (5'-AGCTCGCGTGACTCAGCTG-3'). Sp1 consensus oligonucleotide was used as non-specific control (5'-ATTCGATCGGGGCGGGGCGAGC-3') (All DNA oligonucleotides were purchased from Santa Cruz). DNA-protein complexes were resolved by electrophoresis in native 4% (w/v) polyacrylamide gels. The gels were then dried and autoradiographed.

Results

Promastigotes and amastigotes of *L. mexicana* efficiently block IFN-γ-mediated NO production in MØs

To investigate whether both promastigotes and amastigotes were able to inhibit IFN-γ-mediated NO production in our experimental system, NO levels were measured in B10R MØs infected with one form or the other, followed by IFN-γ stimulation. Results showed that both promastigotes and amastigotes of *L. mexicana* were able to significantly block IFN-γ-mediated NO production in MØs (Fig. 1A). Successful differentiation of *L. mexicana* promastigotes to amastigotes was confirmed by the remarkably decreased production of gp63 in amastigotes compared to promastigotes (Fig. 1B, upper panel) and also by the detection of the amastigote-specific A2 protein in amastigote but not promastigote lysates (Fig. 1B, middle panel). The LACK D protein was used as a loading control (Fig. 1B, lower panel). Morphological differences between the two forms were also confirmed using phase-contrast light microscopy which shows that promastigotes are greater in size, elongated, and flagellated (Fig. 1C, upper picture) while amastigotes are smaller in size, round, and aflagellated (Fig. 1C, lower picture).

Promastigotes and amastigotes of *L. mexicana* activate PTPs in MØs

We have previously reported that *Leishmania donovani* promastigotes can rapidly increase total PTP activity in MØs to alter their signalling pathways [32]. Here, were performed pNPP phosphatase assays to compare the ability of promastigotes and amastigotes of *L. mexicana* to rapidly activate PTPs in MØs. Results indicated that both

promastigotes and amastigotes were able to rapidly increase total PTP activity in infected MØs reaching a peak activation value at 6 h post-infection and decreasing thereafter (Fig. 2A). To have a better understanding of the specific PTPs involved in this activation, we performed in gel PTP assays screening for different MØ PTPs and monitoring their modifications with promastigote or amastigote infections over a 24 h infection period. In gel PTP assays showed that both promastigotes and amastigotes were able to rapidly activate the Src-homology 2 domain-containing protein tyrosine phosphatase (SHP-1) seen by the appearance of a cleavage product associated with SHP-1's activation (Fig. 2B, two upper arrows). Interestingly, only promastigote infection activated PTP-1B resulting in a cleavage fragment (Fig. 2B, two lower arrows).

L. mexicana's cysteine proteinase is not involved in SHP-1 or PTP-1B activation in MØs.

One important virulence factor expressed in metacyclic promastigotes and in amastigotes of *L. mexicana* is the cysteine proteinase lmcpb. To evaluate a possible role for lmcpb in the activation of SHP-1 and PTP-1B, we performed in gel PTP assays, western blots, and pNPP phosphatase assays on lysates and IPs of MØs infected with WT or lmcpb$^{-/-}$ promastigotes and amastigotes and evaluated the effect of these parasites on SHP-1 and PTP-1B cleavage and activation. Although there was some reduced cleavage of SHP-1's upper band in MØs infected with lmcpb$^{-/-}$ promastigotes and amastigotes (Fig. 3A, first arrow from top and Fig 3B, upper panel), its lower cleavage band was still prominent (Fig. 3A, second arrow from top and Fig 3B, upper panel), and SHP-1's immunoprecipitate (IP) from lmcpb$^{-/-}$ infected MØs exhibited an elevated phosphatase

activity similar to that caused by WT parasites (Fig. 3C, upper graph), suggesting that lmcpb is not required in the *Leishmania*-induced SHP-1 activation.

Infection with lmcpb$^{-/-}$ parasites did not lead to the cleavage of the upper PTP-1B band seen with WT promastigotes (Fig. 3A, third arrow from top and Fig. 3B, lower panel), resulting in a strong reduction in the cleavage band that we observe in WT promastigote infection (Fig. 3A, bottom arrow and Fig. 3B, lower panel). This decrease in PTP-1B cleavage was associated with the inability of lmcpb$^{-/-}$ parasites to cause PTP-1B activation, measured by pNPP assays, when compared to WT promastigote infection (Fig. 3C, lower graph). Surprisingly, no PTP-1B cleavage was observed when MØs were infected with WT amastigotes which are known to express *lmcpb* in higher levels than promastigotes. This controversy can be explained by our finding that *lmcpb*$^{-/-}$ parasites express lower gp63 levels compared to WT parasites (data not shown), thus explaining why PTP-1B cleavage was reversed when MØs were infected with lmcpb$^{-/-}$ promastigotes.

Ability of *L. mexicana* promastigotes and amastigotes to alter MØ TFs

Being strong modulators of MØ signalling, we decided to study the ability of promastigotes and amastigotes of *L. mexicana* to alter key MØ TFs namely: NF-κB, STAT-1α, and AP-1. We also evaluated the lowest parasite to cell ratio required to cause these alterations. EMSAs revealed that both promastigotes and amastigotes were able to cause the disappearance of the p65-containing subunit of NF-κB, however with different end results. Promastigotes cleaved the p65 into a p35-containing subunit, while amastigotes caused a total degradation of this subunit with no observable production of

the p35 subunit (Fig. 4A). Interestingly, both promastigotes and amastigotes inhibited STAT-1α and AP-1 DNA-binding, but with a generally stronger effect seen with amastigote infection (Figs. 4B and 4C). Additionally, a parasite to cell ratio of 5:1 was sufficient to observe the previously mentioned alterations (Figs 4A, 4B, and 4C) and was thus selected for the rest of the EMSA experiments conducted.

Lmcpb plays a crucial role in the ability of promastigotes and amastigotes of *L. mexicana* to modulate NF-κB, STAT-1α, and AP-1

To study the role of lmcpb in the modulation of the different TFs, we performed EMSAs to evaluate the ability of lmcpb$^{-/-}$ parasites to alter NF-κB, STAT-1α, and AP-1. Results revealed that both promastigotes and amastigotes of lmcpb$^{-/-}$ parasites were not able to alter the p65 subunit of NF-κB (Fig. 5A) or to inhibit STAT-1α (Fig. 5B) and AP-1 (Fig. 5C) DNA-binding. The previously mentioned TFs of MØs infected with lmcpb$^{-/-}$ parasites remained intact and similar to that of uninfected MØs suggesting a crucial role for lmcpb in the cleavage / degradation of NF-κB, and in the blockage of STAT-1α and AP-1 DNA-binding in host MØs.

Unlike infection by WT parasites, TFs of MØs infected with lmcpb$^{-/-}$ parasites retain their ability to be activated by IFN-γ

To evaluate whether the alteration of NF-κB, STAT-1α, and AP-1 by WT promastigotes and amastigotes interferes with the ability of those TFs to translocate to the nucleus, and to evaluate the role of lmcpb in this context we performed EMSAs. Results indicated that infection with WT promastigotes and amastigotes of *L. mexicana* rendered p65-containing subunits of NF-κB, STAT-1α, and AP-1 unresponsive to IFN-γ stimulation

(Figs. 6A, 6B, and 6C, respectively). Interestingly, these TFs were able to translocate to the nucleus and bind their consensus sequences in a manner similar to the IFN-γ only positive control when MØs were infected with lmcpb$^{-/-}$ parasites (Figs. 6A, 6B, and 6C).

Promastigotes and amastigotes of WT *L. mexicana* but not lmcpb$^{-/-}$ parasites are able to inhibit MØ NO production in response to IFN-γ stimulation

To evaluate the impact of lmcpb on MØ function, we performed NO assays to test whether lmcpb$^{-/-}$ parasites are able to block IFN-γ-mediated NO production by MØs in a fashion similar to that observed by WT parasites. Results showed that while WT promastigotes and amastigotes successfully inhibited IFN-γ-mediated NO production as illustrated in Figure 1A, lmcpb$^{-/-}$ parasites were not able to do so. Levels of NO produced in response to lmcpb$^{-/-}$ parasites were comparable to those produced by uninfected cells stimulated with IFN-γ (Fig. 7).

Discussion

NO production by MØs plays a key role in the resolution of *Leishmania* infections. Indeed, there are several publications reporting the ability of promastigotes, amastigotes, and various parasite molecules such as LPG [11] and GIPLs [12] to inhibit MØ NO production in response to activating stimuli. Of utmost interest, none of those previous studies compared the ability of both forms of the parasite to inhibit MØ NO production and the negative regulatory mechanisms they employ to cause this inhibition.

We have previously reported the ability of *Leishmania* promastigotes to rapidly activate host PTPs [32]. Furthermore, we and others have shown that host SHP-1 is one of the key PTPs activated by *Leishmania* [13,27,29,32,33]. However, very little work has been done concerning the ability of amastigotes to activate host PTPs and SHP-1 in particular. The key role that SHP-1 plays in amastigote infection can be deduced from in vivo studies where amastigotes are exclusively found and where the absence of SHP-1 was associated with increased resistance to *Leishmania* infection [27]. Additionally, it has been previously reported that *Leishmania donovani* amastigotes are able to activate host SHP-1, yet the effect was claimed to be observable after 17 hours of infection [34]. In this work, we confirm previous observations regarding the ability of promastigotes to activate host PTPs (e.g. SHP-1). Importantly, we demonstrate that amastigotes can rapidly activate host PTP activity (as early as 0.5 h post-infection) (Fig. 2A), and that SHP-1 is a key phosphatase rapidly activated by amastigote infection (Fig. 2B and Figs. 3A, 3B, 3C). This suggests that SHP-1 is a critical phosphatase utilized by both forms of the parasite to inactivate key MØ signalling pathways.

Interestingly, PTP-1B was activated by promastigote but not amastigote infection (Fig. 2B and Figs. 3A, 3B, 3C). This phosphatase has been reported to bind to and negatively regulate JAK2 [35] while knock-down and overexpression studies suggested its role in the regulation of MyD88-dependent pro-inflammatory cytokines such as TNF-α and the inhibition of NF-κB and STAT-1 activation in TLR-triggered MØs [36]. Given that SHP-1 has also been associated with its ability to negatively regulate JAK2 [32], block LPS-mediated TNF production [14], and control NF-κB and AP-1 DNA-binding activity [37], this finding suggests that PTP-1B activated by promastigotes might have an additive effect to SHP-1 action to help establish the parasite in host cells. Indeed, data from our laboratory demonstrated that *Leishmania* promastigote gp63 activated PTP-1B in a cleavage-dependent manner and that PTP1B$^{-/-}$ mice infected with *L. major* promastigotes showed a significant delay in the onset and progression of footpad inflammation as well as lower parasite burden (M.A. Gomez and M. Olivier, manuscript under review). Therefore, the additional necessity of PTP-1B action by promastigotes is plausible given that they are less adapted to the host environment compared to amastigotes that might find SHP-1 action sufficient to ensure their safe entry to new uninfected phagocytes especially that they seem to more drastically affect some signalling molecules upon initial contact with MØs such as the strong inhibition they cause to NF-κB, STAT-1, and AP-1 compared to promastigotes (Fig. 4). It is worth mentioning; however, that the utilization of host PTPs other than SHP-1 by amastigotes is a possibility that cannot be disregarded and deserves further investigation.

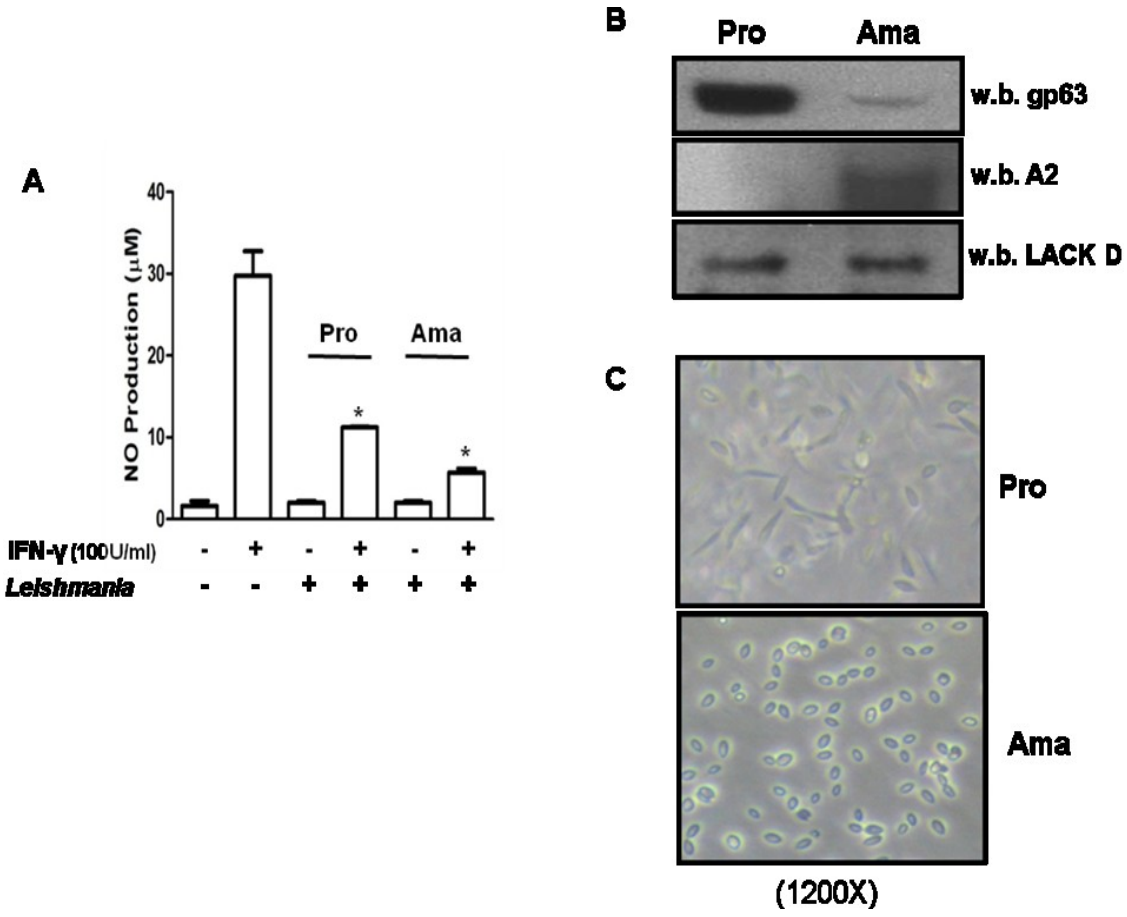
Consistent with a previous finding from our group**,** we have shown that promastigotes cause a cleavage of NF-κB's p65 subunit generating a smaller p35 subunit [38]. On the

other hand, amastigotes caused complete NF-κB degradation (Fig. 4A) as previously shown [39]. Also, consistent with previous findings, promastigotes were able to cause degradation of STAT-1α [40] and inhibition of AP-1 [13] in MØs. Importantly, we were able to demonstrate for the first time that amastigotes of *L. mexicana* can also cause rapid STAT-1α and AP-1 inhibition (Fig. 4). Very interestingly, all the alterations caused by promastigotes and amastigotes to NF-κB, STAT-1α, and AP-1 were reversed when infecting MØs with lmcpb$^{-/-}$ parasites. Lmcpb has been previously reported to cause the degradation of: IκB and NF-κB [39], IL-2R, and the IgER [41]. Our data confirm the role of lmcpb in NF-κB degradation (Fig. 5A), and demonstrate for the first time that lmcpb leads to the inhibition of STAT-1α and AP-1 DNA-binding activity possibly by causing their degradation (Fig. 5B and 5C). The protection of these transcription factors seen when infecting MØs with lmcpb$^{-/-}$ parasites is reflected by the ability of those transcriptions factors to translocate to the nucleus and bind their target sequences in response to IFN-γ stimulation (Fig. 6), which correlated with the ability of MØs to produce NO when stimulated with IFN-γ following infection with lmcpb$^{-/-}$ parasites (Fig. 7). This finding could help partially explain the previously observed low infectivity of cpb mutants [24], as well as further confirm the role of this cysteine proteinase as a *Leishmania* virulence factor.

In conclusion, our comparison of promastigotes and amastigotes of *L. mexicana* in terms of their ability to alter MØ signalling yielded several interesting similarities as well as differences. While both forms were able to activate total PTP activity and to rapidly activate SHP-1, PTP-1B was only activated by promastigotes. Additionally, while both forms caused full inhibition of STAT-1α and AP-1, NF-κB seemed to be altered

differently. Promastigotes cleaved NF-κB's p65 subunit to p35 while amastigotes caused complete p65 inhibition with no significant production of the smaller subunit. Importantly, the study also revealed new roles for lmcpb as a virulence factor by demonstrating its ability to interfere with STAT-1α and AP-1 DNA-binding activity, therefore contributing to the parasite's ability to block IFN-γ-induced NO production in MØs, securing its survival and propagation within its mammalian host.

Figure 1: Inhibition of IFN-γ-mediated nitric oxide production by *L. mexicana* promastigotes and amastigotes. (A) NO assay of B10R MØs left untreated, stimulated with IFN-γ (100U/ml) for 24 h, infected with *Leishmania* (O/N), or infected with *Leishmania* (O/N) then stimulated with IFN-γ (100U/ml) for 24 h. *, significant at $P < 0.05$, Anova test, error bar SEM. (B) Western blot analysis of *L. mexicana* promastigote and amastigote cell lysates (40μg). Membrane was cut and blotted for gp63 (upper panel) and A2 (middle panel). Membrane was then stripped and reblotted for LACK D (lower panel) to demonstrate equal loading. (C) Phase contrast light microscopy pictures of stationary phase *L. mexicana* promastigotes (upper picture) and *L. mexicana* axenic amastigotes (lower picture). Both pictures were taken under a magnification of 1200X. Pro, promastigotes; Ama, amastigotes. All results are representative of at least three independent experiments.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Ama
(1200X)

Figure 2: Activation of host PTPs by *L. mexicana* promastigotes and amastigotes. (A) B10R MØs were infected with *L. mexicana* promastigotes or amastigotes for the specified time course (0-24 h) and total PTP activity of MØ lysates (20µg) was measured using the pNPP phosphatase assay. (B) Cell lysates (30µg) of MØs infected with promastigotes or amastigotes of *L. mexicana* for the specified time course (0-24 h) subjected to an in gel PTP assay. Last two lanes are lysates (30 µg) from WT and SHP-$1^{-/-}$ MØs to confirm the band corresponding to SHP-1. All results are representative of at least three independent experiments.

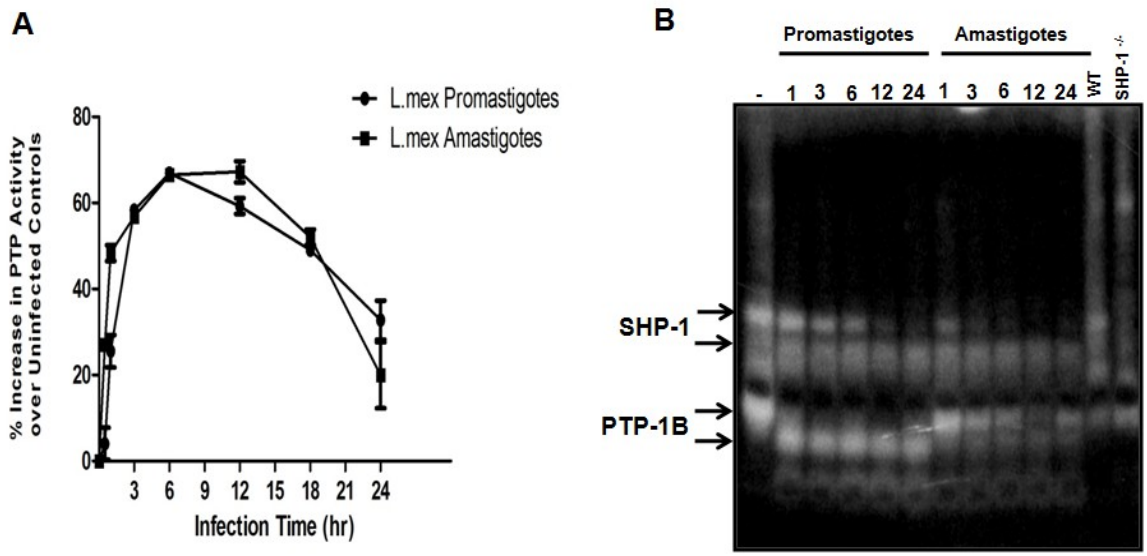
A
L.mex Promastigotes
L.mex Amastigotes
% Increase in PTP Activity over Uninfected Controls
80
60
40
20
0
3 6 9 12 15 18 21 24
Infection Time (hr)
B
Promastigotes
Amastigotes
- 1 3 6 12 24 1 3 6 12 24 WT SHP-1 -/-
SHP-1
PTP-1B

Figure 3: lmcpb is not involved in SHP-1 and PTP-1B activation. (A) B10R MØs were infected with *L. mexicana* promastigotes and amastigotes of WT and lmcpb$^{-/-}$ parasites for the specified time course (0-6 h) and cell lysates (30µg) were subjected to an in gel PTP assay. Last two lanes are lysates (30 µg) from WT and SHP-1$^{-/-}$ MØs to confirm the band corresponding to SHP-1. (B) Samples in A ran on SDS-PAGE and blotted for SHP-1 (upper panel) and PTP-1B (lower panel). (C) B10R MØs were infected with *L. mexicana* promastigotes and amastigotes of WT and lmcpb$^{-/-}$ parasites (3 h) followed by lysis and immunoprecipitation of SHP-1 or PTP-1B. The SHP-1 and PTP-1B IPs were subjected to a pNPP phosphatase assay (upper and lower graphs, respectively). *, significant at $P < 0.05$, Anova test, error bar SEM. Rabbit IgG anti-rat was used as a negative control for the IPs. P, promastigotes; A, amastigotes. All results are representative of at least three independent experiments.

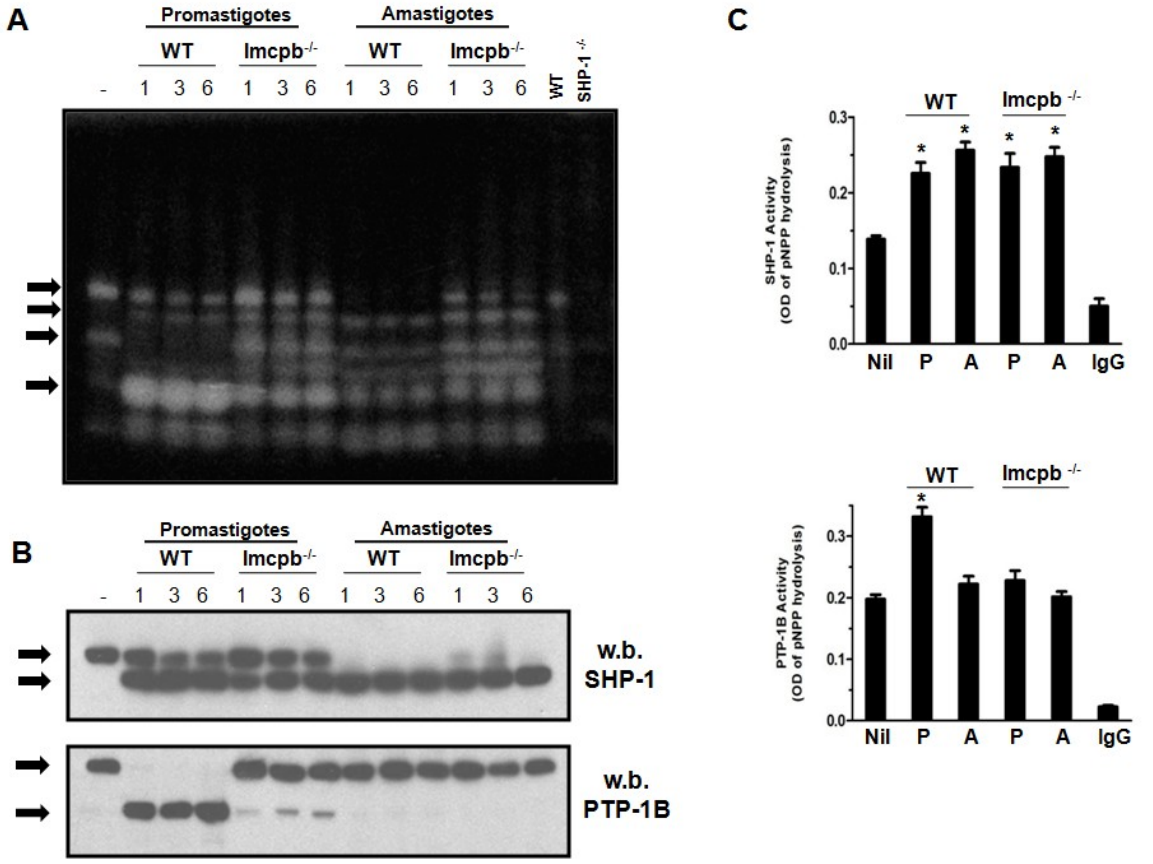
A
Promastigotes
Amastigotes
WT
lmcpb-/-
WT
lmcpb-/-
- 1 3 6 1 3 6 1 3 6 1 3 6
WT
SHP-1-/-
B
Promastigotes
Amastigotes
WT
lmcpb-/-
WT
lmcpb-/-
- 1 3 6 1 3 6 1 3 6 1 3 6
w.b.
SHP-1
w.b.
PTP-1B
C
WT
lmcpb -/-
SHP-1 Activity
(OD of pNPP hydrolysis)
0.3
0.2
0.1
0.0
Nil P A P A IgG
WT
lmcpb -/-
PTP-1B Activity
(OD of pNPP hydrolysis)
0.3
0.2
0.1
0.0
Nil P A P A IgG

Figure 4: Modulation of macrophage transcription factors by *L. mexicana* promastigotes and amastigotes. B10R MØs infected in the specified parasite to cell ratios (3 h infection) with *L. mexicana* promastigotes and amastigotes. Nuclear proteins were extracted and subjected to EMSA to evaluate DNA-binding activity of NF-κB (A), STAT-1α (B), and AP-1 (C). S, specific competition (100X of specific non-radioactive oligo); NS, non-specific competition (100X of non-specific, non-radioactive Sp-1 oligo).

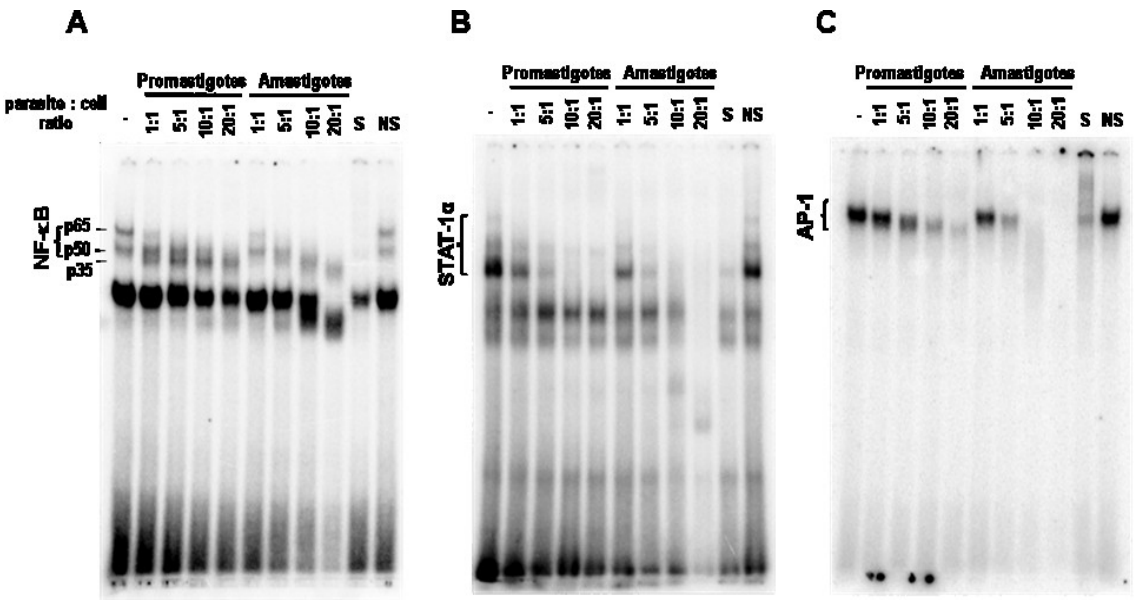
A
B
C
Promastigotes
Amastigotes
parasite : cell ratio
-
1:1
5:1
10:1
20:1
S
NS
NF-κB
p65
p50
p35
STAT-1α
AP-1

Figure 5: Role of lmcpb in the modulation of macrophage transcription factors. B10R MØs infected for the specified time course (0.5 - 3 h) with WT or lmcpb$^{-/-}$ promastigotes and amastigotes of *L. mexicana* in a 5:1 parasite to cell ratio. Nuclear proteins were extracted and subjected to EMSA to evaluate DNA-binding activity of NF-κB (A), STAT-1α (B), and AP-1 (C). S, specific competition (100X of specific non-radioactive oligo); NS, non-specific competition (100X of non-specific, non-radioactive Sp-1 oligo).

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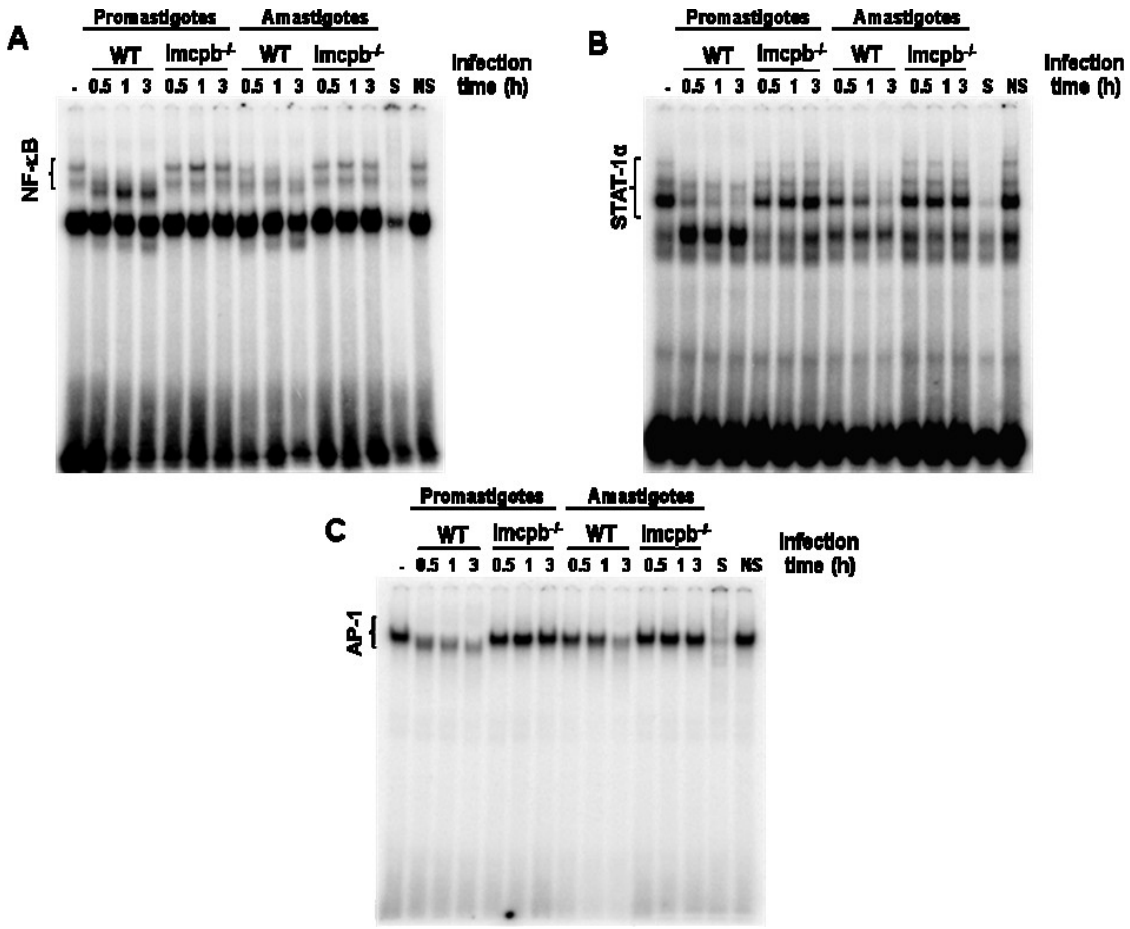

A
Promastigotes
Amastigotes
WT
lmcpb-/-
WT
lmcpb-/-
- 0.5 1 3 0.5 1 3 0.5 1 3 0.5 1 3 S NS
Infection time (h)
NF-κB
B
Promastigotes
Amastigotes
WT
lmcpb-/-
WT
lmcpb-/-
- 0.5 1 3 0.5 1 3 0.5 1 3 0.5 1 3 S NS
Infection time (h)
STAT-1α
C
Promastigotes
Amastigotes
WT
lmcpb-/-
WT
lmcpb-/-
- 0.5 1 3 0.5 1 3 0.5 1 3 0.5 1 3 S NS
Infection time (h)
AP-1

Figure 6: Nuclear translocation of transcription factors in response to IFN-γ in macrophages infected with wildtype or lmcpb$^{-/-}$ *L. mexicana* promastigotes and amastigotes. B10R MØs left uninfected, stimulated with IFN-γ (100U/ml) for 6 h, infected (O/N) with WT or lmcpb$^{-/-}$ promastigotes and amastigotes of *L. mexicana* in a 5:1 parasite to cell ratio, or infected (O/N) then stimulated with IFN-γ (100U/ml) for 6 h. Nuclear proteins were extracted and subjected to EMSA to evaluate DNA-binding activity of NF-κB (A), STAT-1α (B), and AP-1 (C). P, promastigotes; A, amastigotes; S, specific competition (100X of specific non-radioactive oligo); NS, non-specific competition (100X of non-specific, non-radioactive Sp-1 oligo).

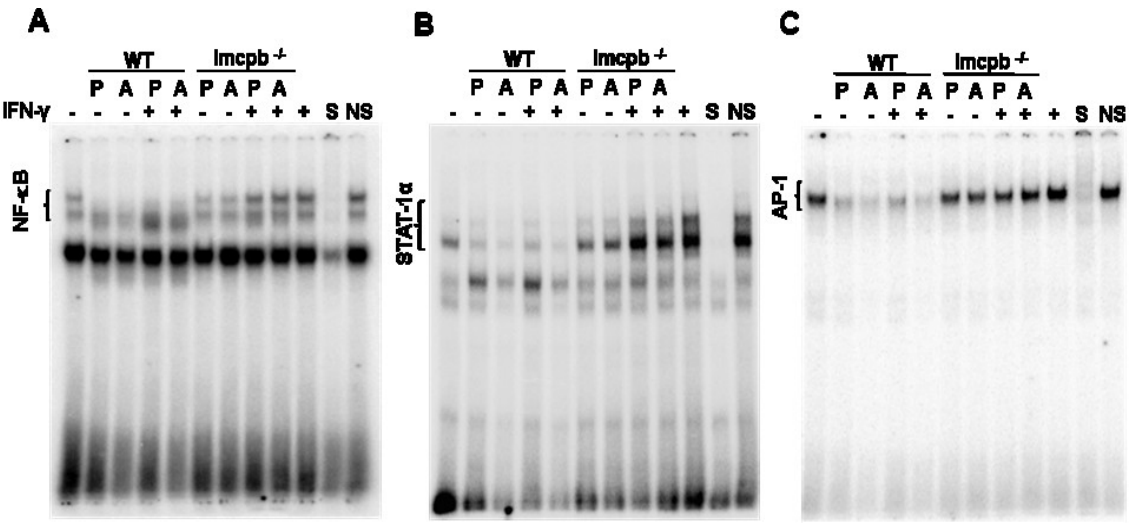
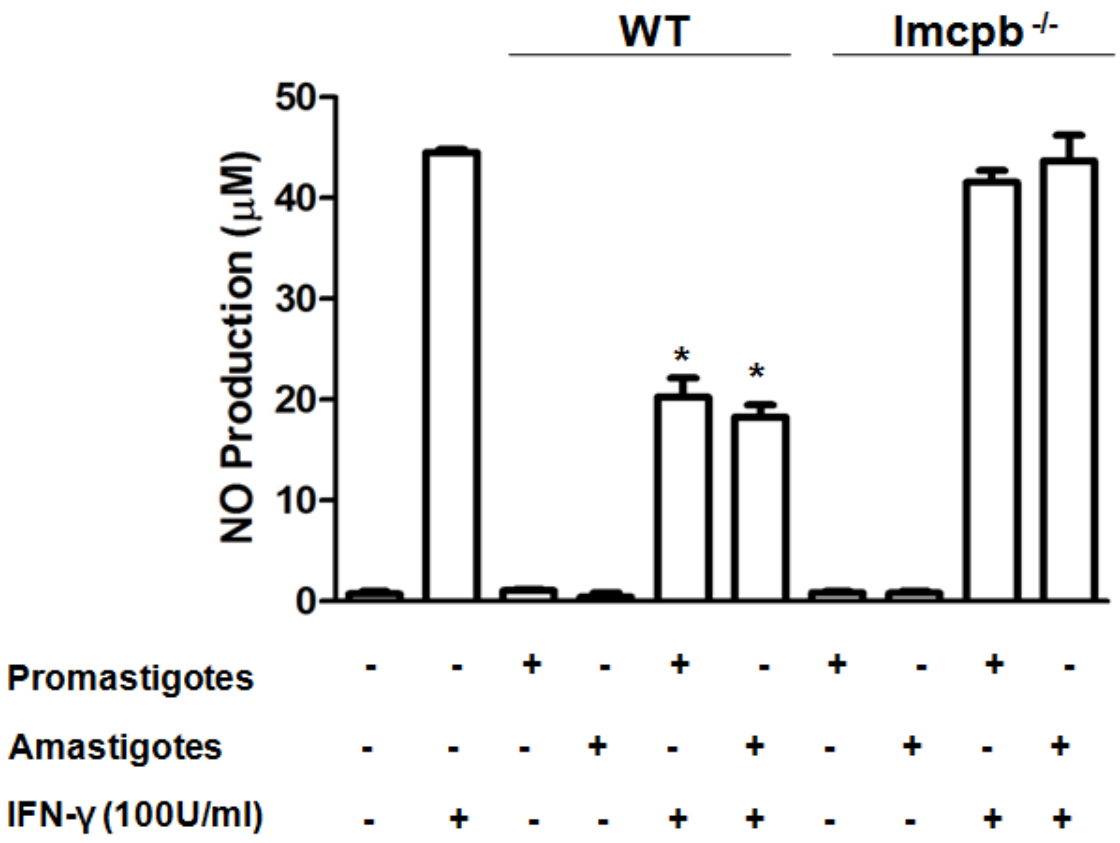

A
WT
Imcpb -/-
P A P A P A P A
IFN-γ - - - + + - - + + + S NS
NF-κB
B
WT
Imcpb -/-
P A P A P A P A
- - - + + - - + + + S NS
STAT-1α
C
WT
Imcpb -/-
P A P A P A P A
- - - + + - - + + + S NS
AP-1

Figure 7: Inability of lmcpb$^{-/-}$ parasites to inhibit IFNγ-mediated macrophage nitric oxide production. NO assay of B10R MØs left untreated, stimulated with IFN-γ (100U/ml) for 24 h, infected with WT or lmcpb$^{-/-}$ *L. mexicana* (O/N), or infected (O/N) then stimulated with IFN-γ (100U/ml) for 24 h. *, significant at $P < 0.05$, Anova test, error bar SEM. Result is representative of at least three independent experiments.


WT
lmcpb -/-
NO Production (μM)
50
40
30
20
10
0
*
*
Promastigotes - - + - + - + - + -
Amastigotes - - - + - + - + - +
IFN-γ (100U/ml) - + - - + + - - + +

Acknowledgements

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References

1. Choi CM, Lerner EA (2001). Leishmaniasis as an emerging infection. *J Investig Dermatol Symp Proc* **6**: 175-182.
2. Melby PC (2002). Recent developments in leishmaniasis. *Curr Opin Infect Dis* **15**: 485-490.
3. Pimenta PFP, Saraiva EMB, Sacks DL (1991). The Comparative Fine-Structure and Surface Glycoconjugate Expression of 3 Life Stages of *Leishmania major*. *Exp Parasitol* **72**: 191-204.
4. Ferguson MA (1997). The surface glycoconjugates of trypanosomatid parasites. *Philos Trans R Soc Lond B Biol Sci* **352**: 1295-1302.
5. McConville MJ, Blackwell JM (1991). Developmental-Changes in the Glycosylated Phosphatidylinositols of *Leishmania donovani* - Characterization of the Promastigote and Amastigote Glycolipids. *J Biol Chem* **266**: 15170-15179.
6. Schneider P, Rosat JP, Bouvier J, Louis J, Bordier C (1992). *Leishmania major* - Differential Regulation of the Surface Metalloprotease in Amastigote and Promastigote Stages. *Exp Parasitol* **75**: 196-206.
7. Medinaacosta E, Karess RE, Schwartz H, Russell DG (1989). The Promastigote Surface Protease (Gp63) of *Leishmania* Is Expressed but Differentially Processed and Localized in the Amastigote Stage. *Mol Biochem Parasitol* **37**: 263-273.
8. North MJ, Mottram JC, Coombs GH (1990). Cysteine Proteinases of Parasitic Protozoa. *Parasitol Today* **6**: 270-275.
9. Souza AE, Waugh S, Coombs GH, Mottram JC (1992). Characterization of a Multicopy Gene for a Major Stage-Specific Cysteine Proteinase of *Leishmania mexicana*. *FEBS Lett* **311**: 124-127.
10. Olivier M, Gregory DJ, Forget G (2005). Subversion Mechanisms by Which *Leishmania* Parasites Can Escape the Host Immune Response: a Signaling Point of View. *Clin Microbiol Rev* **18**: 293-305.
11. Proudfoot L, Nikolaev AV, Feng GJ, Wei XQ, Ferguson MAJ, et al. (1996). Regulation of the expression of nitric oxide synthase and leishmanicidal activity by glycoconjugates of *Leishmania* lipophosphoglycan in murine macrophages. *Proc Natl Acad Sci USA* **93**: 10984-10989.
12. Proudfoot L, Odonnell CA, Liew FY (1995). Glycoinositolphospholipids of *Leishmania major* Inhibit Nitric-Oxide Synthesis and Reduce Leishmanicidal Activity in Murine Macrophages. *Eur J Immunol* **25**: 745-750.
13. Forget G, Gregory DJ, Whitcombe LA, Olivier M (2006). Role of host protein tyrosine phosphatase SHP-1 in *Leishmania donovani*-induced inhibition of nitric oxide production. *Infect Immun* **74**: 6272-6279.

14. Abu-Dayyeh I, Shio MT, Sato S, Akira S, Cousineau B, et al. (2008). *Leishmania*-Induced IRAK-1 Inactivation Is Mediated by SHP-1 Interacting with an Evolutionarily Conserved KTIM Motif. *PLoS Negl Trop Dis* **2**: e305.
15. Wanderley JLM, Moreira MEC, Benjamin A, Bonomo AC, Barcinski MA (2006). Mimicry of Apoptotic Cells by Exposing Phosphatidylserine Participates in the Establishment of Amastigotes of *Leishmania (L) amazonensis* in Mammalian Hosts. *J Immunol* **176**: 1834-1839.
16. Melby PC, Chandrasekar B, Zhao WG, Coe JE (2001). The hamster as a model of human visceral leishmaniasis: Progressive disease and impaired generation of nitric oxide in the face of a prominent Th1-like cytokine response. *J Immunol* **166**: 1912-1920.
17. Kane MM, Mosser DM (2000). *Leishmania* parasites and their ploys to disrupt macrophage activation. *Curr Opin Hematol* **7**: 26-31.
18. Talamas-Rohana P, Wright SD, Lennartz MR, Russell DG (1990). Lipophosphoglycan from *Leishmania mexicana* promastigotes binds to members of the CR3, p150,95 and LFA-1 family of leukocyte integrins. *J Immunol* **144**: 4817-4824.
19. Blackwell JM, Ezekowitz RAB, Roberts MB, Channon JY, Sim RB, et al. (1985). Macrophage Complement and Lectin-Like Receptors Bind *Leishmania* in the Absence of Serum. *J Exp Med* **162**: 324-331.
20. Culley FJ, Harris RA, Kaye PM, McAdam K, Raynes JG (1996). C-reactive protein binds to a novel ligand on *Leishmania donovani* and increases uptake into human macrophages. *J Immunol* **156**: 4691-4696.
21. Brittingham A, Chen G, McGwire BS, Chang KP, Mosser DM (1999). Interaction of *Leishmania* gp63 with cellular receptors for fibronectin. *Infect Immun* **67**: 4477-4484.
22. Xie QW, Whisnant R, Nathan C (1993). Promoter of the mouse gene encoding calcium-independent nitric-oxide synthase confers inducibility by interferon-gamma and bacterial lipopolysaccharide. *J Exp Med* **177**: 1779-1784.
23. Lowenstein CJ, Alley EW, Raval P, Snowman AM, Snyder SH, et al. (1993). Macrophage Nitric-Oxide Synthase Gene - 2 Upstream Regions Mediate Induction by Interferon-Gamma and Lipopolysaccharide. *Proc Natl Acad Sci USA* **90**: 9730-9734.
24. Mottram JC, Souza AE, Hutchison JE, Carter R, Frame MJ, et al. (1996). Evidence from disruption of the lmcpb gene array of *Leishmania mexicana* that cysteine proteinases are virulence factors. *Proc Natl Acad Sci USA* **93**: 6008-6013.
25. Alexander J, Coombs GH, Mottram JC (1998). *Leishmania mexicana* cysteine proteinase-deficient mutants have attenuated virulence for mice and potentiate a Th1 response. *J Immunol* **161**: 6794-6801.
26. Radzioch D, Hudson T, Boule M, Barrera L, Urbance JW, et al. (1991). Genetic-Resistance Susceptibility to Mycobacteria - Phenotypic-Expression in Bone-Marrow Derived Macrophage Lines. *J Leukoc Biol* **50**: 263-272.

27. Forget G, Siminovitch KA, Brochu S, Rivest S, Radzioch D, et al. (2001). Role of host phosphotyrosine phosphatase SHP-1 in the development of murine leishmaniasis. *Eur J Immunol* **31**: 3185-3196.
28. Olivier M, Romero-Gallo BJ, Matte C, Blanchette J, Posner BI, et al. (1998). Modulation of interferon-gamma-induced macrophage activation by phosphotyrosine phosphatases inhibition - Effect on murine Leishmaniasis progression. *J Biol Chem* **273**: 13944-13949.
29. Forget G, Matte C, Siminovitch KA, Rivest S, Pouliot P, et al. (2005). Regulation of the *Leishmania*-induced innate inflammatory response by the protein tyrosine phosphatase SHP-1. *Eur J Immunol* **35**: 1906-1917.
30. Markova B, Gulati P, Herlich PA, Bohmer FD (2005). Investigation of protein-tyrosine phosphatases by in-gel assays. *Methods* **35**: 22-27.
31. Jaramillo M, Olivier M (2002). Hydrogen peroxide induces murine macrophage chemokine gene transcription via extracellular signal-regulated kinaseand cyclic adenosine 5 '-monophosphate (cAMP)-dependent pathways: Involvement of NF-kappa B, activator protein 1, and cAMP response element binding protein. *J Immunol* **169**: 7026-7038.
32. Blanchette J, Racette N, Faure R, Siminovitch KA, Olivier M (1999). *Leishmania*-induced increases in activation of macrophage SHP-1 tyrosine phosphatase are associated with impaired IFN-gamma-triggered JAK2 activation. *Eur J Immunol* **29**: 3737-3744.
33. Nandan D, Yi TL, Lopez M, Lai C, Reiner NE (2002). *Leishmania* EF-1 alpha activates the Src homology 2 domain containing tyrosine phosphatase SHP-1 leading to macrophage deactivation. *J Biol Chem* **277**: 50190-50197.
34. Nandan D, Lo R, Reiner NE (1999). Activation of phosphotyrosine phosphatase activity attenuates mitogen-activated protein kinase signaling and inhibits c-FOS and nitric oxide synthase expression in macrophages infected with *Leishmania donovani*. *Infect Immun* **67**: 4055-4063.
35. Myers MP, Andersen JN, Cheng A, Tremblay ML, Horvath CM, et al. (2001). TYK2 and JAK2 Are Substrates of Protein-tyrosine Phosphatase 1B. *J Biol Chem* **276**: 47771-47774.
36. Xu HM, An HZ, Hou J, Han CF, Wang P, et al. (2008). Phosphatase PTP1B negatively regulates MyD88- and TRIF-dependent proinflammatory cytokine and type I interferon production in TLR-triggered macrophages. *Mol Immunol* **45**: 3545-3552.
37. Blanchette J, Abu-Dayyeh I, Hassani K, Whitcombe L, and Olivier M. (2008). Regulation of macrophage nitric oxide production by the protein tyrosine phosphatase Src homology 2 domain phosphotyrosine phosphatase 1 (SHP-1). *Immunology*, **127**: 123-133.

38. Gregory DJ, Godbout M, Contreras I, Forget G, Olivier M (2008). A novel form of NF-kappa B is induced by *Leishmania* infection: Involvement in macrophage gene expression. *Eur J Immunol* **38**: 1071-1081.
39. Cameron P, McGachy A, Anderson M, Paul A, Coombs GH, et al. (2004). Inhibition of Lipopolysaccharide-Induced Macrophage IL-12 Production by *Leishmania mexicana* Amastigotes: The Role of Cysteine Peptidases and the NF-κB Signaling Pathway. *J Immunol* **173**: 3297-3304.
40. Forget G, Gregory DJ, Olivier M (2005). Proteasome-mediated degradation of STAT1 alpha following infection of macrophages with *Leishmania donovani*. *J Biol Chem* **280**: 30542-30549.
41. Pollock KGJ, McNeil KS, Mottram JC, Lyons RE, Brewer JM, et al. (2003). The *Leishmania mexicana* cysteine protease, CPB2.8, induces potent Th2 responses. *J Immunol* **170**: 1746-1753.

Chapter 5

Summary and General Discussion

Among the major findings in this thesis is our discovery that SHP-1 negatively regulates IRAK-1's kinase activity by directly interacting with an ITIM-like motif in its kinase domain, which we called kinase tyrosine-based inhibitory motif (KTIM). IRAK-1's KTIM appears in amphibians and is evolutionarily conserved, suggesting that this motif might have played a key role in the control of innate immune responses of early vertebrates and in the development of a fine-tuned adaptive immune response in higher vertebrates. Furthermore, KTIM represnts the first report of an ITIM-like motif not present in a transmembrane protein. This finding led us to discover that several key kinases other than IRAK-1 (e.g. JAK2, JAK3, TAK1, IKK-α, IKK-β, LYN, and the MAPKs Erk1/2, JNK, and p38) possess potential KTIMs and that this motif might be a regulatory mechanism used by a wide range of cellular kinases.

Very interestingly, this newly-identified role of SHP-1 as a negative regulator of TLR signalling was found to be utilized by *Leishmania* in order to inhibit MØ functions that are harmful to the parasite. In this thesis, we uncovered that the parasite was able to utilize its ability to activate host SHP-1 to increase the binding of this phosphatase to IRAK-1 causing the inhibition of its intrinsic kinase activity and downstream functions (e.g. NO production) in response to various TLR ligands including LPS. This observed inactivation of IRAK-1 by *Leishmania* was associated with the inability of the kinase to detach from MyD88 to bind TRAF6. Taken together, these experiments propose a novel evasion mechanism used by this protozoan parasite that can help explain its long-reported ability to inhibit LPS-mediated MØ functions.

In an effort to explore the ability of *Leishmania* to alter other signalling molecules in MØs and to better understand which life stage of the parasite can cause these alterations,

we performed experiments to compare promastigotes and amastigotes in terms of their ability to activate PTPs and alter TFs in host MØs. Data presented in this thesis indicate that unlike PTP-1B, which is activated by promastigotes alone in early infection, the rapid induction of SHP-1 activity is a common strategy employed by both forms. In addition, we demonstrated that although promastigotes and amastigotes inhibit the transcription factors STAT-1α and AP-1 similarly, NF-κB is modulated differently. While amastigotes of *L. mexicana* mainly caused the degradation of the p65 subunit of NF-κB, promastigotes cleaved it to produce a p35 DNA-binding subunit. Finally, we explored the role of *L. mexicana*'s CP, lmcpb, in these signalling alterations and uncovered that it plays a pivotal role in the early modulation events of the transcription factors NF-κB, STAT-1α, and AP-1, which correlated with the parasite's ability to block IFN-γ-mediated NO production in MØs.

Over the past years, our laboratory has accumulated evidence that support the involvement of SHP-1 in the regulation of TLR signalling. SHP-$1^{-/-}$ mice, and MØs derived from them, exhibit higher LPS-mediated NO and pro-inflammatory cytokine production. In addition, key components of TLR signalling such as MAPKs and the TFs NF-κB and AP-1 are activated in the absence of SHP-1. Furthermore, SHP-$1^{-/-}$ mice have lower parasite survival when infected with *Leishmania* compared to their littermates, and this was associated with increased iNOS expression and higher production of several pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α. Based on all these observations, one of the main goals of this study was to understand the exact role of SHP-1 in the negative regulation of TLR siganlling and how this can be exploited by *Leishmania* to secure its survival in the host.

One of the major findings of Chapter 2 is the identification of SHP-1 as a novel negative regulator of TLR signalling. This result was corroborated by Cao and colleagues who showed that in the absence of SHP-1, DCs and MØs produce a higher amount of TNF-α and a lower amount of IFN-β in response to LPS, and that this was due to the absence of the negative regulatory effect of SHP-1 on IRAK-1's kinase activity [384]. Although this study provided good support to our findings, important differences exist in the experimental approach used to demonstrate the mechanism underlying the IRAK-1 / SHP-1 interaction. While we showed the involvement of the ITIM-like motif in the binding of SHP-1 to IRAK-1 by performing point mutations to full-length IRAK-1 rendering the motif similar to the corresponding site within IRAK-4 (which we showed is not regulated by SHP-1), the latter study study showed the interaction by deleting the motif completely from a heavily truncated form of IRAK-1 (a construct that includes amino acids 149-386 of IRAK-1, whose murine WT protein has 710 amino acids). Our approach remains more physiologically and biochemically relevant as we used the full-length protein for the interaction assays, verified the functionality of the mutant IRAK-1 proteins produced (data not shown), and performed carefully chosen mutations that gave a deep insight into the contribution of individual amino acids within the motif to the binding affinity of SHP-1. Importantly, we were the first to realize and report that the ITIM-like motif in IRAK-1 should not be called ITIM at all! We clearly emphasized that this functional ITIM-like motif is found in a cytosolic kinase, and so we proposed to name it kinase tyrosine-based inhibitory motif (KTIM). In fact, we went further to identify several important kinases that contained potential KTIMs and interacted with SHP-1. This finding paved the road to the research conducted in Chapter 3.

Our finding that SHP-1 interacts with and regulates IRAK-1 activity at resting state led us to propose that an important function of SHP-1 is to keep IRAK-1 in-check and prevent its activation in the absence of appropriate stimuli. In support of this hypothesis, preliminary data that we generated showed that SHP-1 seems to detach from IRAK-1 following LPS stimulation (data not shown). A better understanding of the kinetics of this process and the specificity of TLR ligands that can induce it is a future direction our laboratory would like to pursue.

Results derived from this study carry another major finding and a first demonstration that a pathogen can rapidly interfere with TLR signalling through inactivating IRAK-1 using a host PTP. We have convincingly showed that IRAK-1 inhibition is an evasion mechanism specific to pathogenic *Leishmania* species and is not observed in non-pathogenic strains like *L. tarentolae*. We then provided evidence that SHP-1 activity is essential for the parasite's ability to block IRAK-1 and ultimately the LPS-mediated functions downstream of it. Therefore, this work provides a novel mechanism by which *Leishmania* can inhibit several LPS-mediated functions detrimental for parasite survival such as IL-12, TNF-α, and NO production. In fact, we were able to show that IRAK-1 in infected cells was not only unresponsive to LPS stimulation but also to several other TLR ligands including ligands of TLR2, TLR5, and TLR 9. This result is interesting given that TLR2 has been shown to be a ligand of *Leishmania*'s LPG [331,332], TLR5 detects flagellin [385] and could be activated along with TLR4 when bacteria are present, and TLR9 has been previously shown to play a favourable role in the resolution of leishmaniasis [386]. Keeping this in mind, we propose a dual function for IRAK-1 inactivation by *Leishmania*: First, as some parasite components can be detected by TLRs that signal through IRAK-1,

inactivation of this kinase might have a key role in the ability of the parasite to enter MØs silently without triggering their activation. Second, this mechanism provides protection to the internalized parasites from subsequent MØ activation that can be mediated by LPS or flagellin of bacteria accidentally injected by the sandfly or introduced to the infection site afterwards one way or the other.

Another interesting finding is the way IRAK-1 signalling is inhibited in response to *Leishmania*. We have uncovered in Chapter 2 that the inability of IRAK-1 to autophosphorylate upon SHP-1 binding abrogates its ability to detach from the MyD88 complex to bind TRAF6. This is very similar to the suggested mode of action of a naturally-occurring TLR negative regulator: TOLLIP (see TOLLIP section in the literature review for more details). Consequently, the manuscript in Chapter 2 represents, to the best of our knowledge, the first report that a pathogen can utilize this cellular regulatory concept as an evasion mechanism.

The evolutionary consequences of KTIMs are truly exciting. We have shown that IRAK-1's KTIM is not found in fish and emerges in amphibians remaining highly conserved thereafter up to human. Very interestingly, although we only displayed the zebrafish IRAK-1 sequence in our publication, we also know that IRAK-1 of the aquarium pet the green puffer fish (*Tetraodon nigroviridis*) lacks KTIMs too (data not shown), further supporting our report that the motif's appearance in IRAK-1 seems to have occurred in amphibians. In fact, we have obtained fish MØs from collaborators and intend to evaluate the ability of their IRAK-1 to interact with SHP-1. If no interaction is found, a very exciting additional support to the evolutionary role of amphibian-emerging KTIM in binding SHP-1 will be generated. In addition and to explore the effect of KTIM *in vivo*,

plans are on the way to generate transgenic mice that express IRAK-1 mutated in its KTIM and compare them to mice expressing WT IRAK-1 in terms of their inflammatory status, response to LPS, and resistance / susceptibility to *Leishmania* infection.

We discussed in Chapter 2 the suggested significance of KTIM appearance in the regulation of innate immune responses and possibly the appearance of adaptive immunity in lower vertebrates, and I will not repeat the arguments here. What is worth mentioning, however, is that our search for potential KTIMs in other kinases in Chapter 3 uncovered that the majority of KTIMs appear in the early vertebrate stage and are found in or near the kinase domain (similar to IRAK-1). Taken together, these findings re-enforce our suggestion that KTIMs could represent a regulatory mechanism widely used by cellular kinases. The interesting exceptions, where KTIM was found conserved in the sequence of invertebrate kinases, suggest that invertebrate KTIMs could have performed different functions other than binding SHP-1, but nevertheless transformed into readily available regulatory sites as soon as their regulators emerged in vertebrates. Our experiments that show the ability of *Drosophila*'s JNK to bind mammalian SHP-1 support this hypothesis. Finally, it is important to stress that the involvement of all the reported potential KTIMs explored in Chapter 3 in SHP-1 binding needs to be confirmed by site-directed mutagenesis, in an experimental manner similar to that used with IRAK-1 in Chapter 2. These experiments will be of utmost importance in unraveling which of these KTIMs are truly involved in SHP-1 binding and in the regulation of the kinase they lie within.

Going back to the main focus of this thesis and that is to explore immune evasion mechanisms developed by *Leishmania*, one important question that arises is: how many of these tactics are shared by both forms of the parasite (promastigotes and amastigotes)

and how many are unique to one form or the other? This knowledge is essential given the differences in biochemical and cellular properties of the two stages of the parasite and the different roles they play in the course of infection (amastigotes being the diagnostic stage of the disease in infected hosts).

Having discovered that *Leishmania* promastigotes inactivate IRAK-1, we performed additional experiments and found that amastigotes are also capable of doing so (data not shown). This is not surprising in the light of our finding in Chapter 4 that amastigotes are equally able to activate host SHP-1 in infected MØs. This finding establishes SHP-1 as a key molecule triggered in early infection by the two *Leishmania* forms to alter phagocyte signalling pathways. Chapter 4 goes deeper in exploring further similarities and differences between evasion tactics used by promastigotes and amastigotes. The end result of this work is the characterization of mechanisms that they both share such as STAT-1α and AP-1 inhibition, and others that are unique to one form only such as the early induction of PTP-1B and the generation of a p35 subunit of NF-κB by promastigotes. We will not go through the possible implications of these similarities and differences as they have been sufficiently discussed in Chapter 4, but should definitely stress the potential importance of this work in the success of future efforts to design amastigote-specific anti-leishmanial therapies that interfere with signalling pathways reported to be exploited by the parasite. It is important to mention that the study in Chapter 4 additionally provided valuable data about novel roles for the cysteine protease lmcpb as a virulence factor. To date, roles for lmcpb in the degradation of the IgER and NF-κB have been reported [37]. We have provided evidence that lmcpb is also involved in STAT-1α and AP-1 DNA-binding inhibition. Whether this effect is directly mediated

by lmcpb's protease activity or not remains to be determined. Given the previously reported ability of lmcpb to access the cytoplasm from the parasitophorous vacuole and to mediate degradation of the TF NF-κB [40], we suspect a similar mechanism to cause STAT-1α and AP-1 degradation by *L. mexicana*, but this is to be confirmed. Finally, future directions for this project include comparing the effect of promastigotes and amastigotes on signalling molecules upstream of the studied TFs such as JAK2 and MAPKs as well as exploring whether the proteins targeted by the activated PTPs are of the same nature in oder to further understand the differential manners whereby promastigotes and amastigotes interfere with host MØ physiology.

In conclusion, we believe this thesis brings about valuable additions to the fields of parasite immunology and cell signalling. We provide a novel mechanism whereby *Leishmania* can inhibit TLR-mediated MØ activation through its ability to cause SHP-1-mediated IRAK-1 inhibition (see figure 11 for a cartoon summary). This implies that pharmacological targeting of SHP-1 or IRAK-1 in the future could prove useful in the fight against leishmaniasis. Additionally, the identification of SHP-1 as a novel regulator of TLR signalling widens our knowledge about how signalling pathways associated with these PAMP-detecting receptors can be controlled. This finding therefore implicates potential applications for SHP-1 in controlling exacerbated TLR-mediated inflammation and serious conditions related to it such as septic shock. Importantly, this study breaks the dogma that has prevailed until the publication of the manuscript in Chapter 2 that ITIMs are only associated with transmembrane receptors. Indeed, a study published in August 2008 screening for potential ITIMs in the genomes of various organisms started their quest by excluding all proteins that did not have a transmembrane domain [387]. This

strongly demonstrates the new avenues that KTIM will bring to many fields as an ITIM-like motif shown for the first time to be present in non-receptor proteins involved in various cellular, molecular, developmental, and metabolic processes.

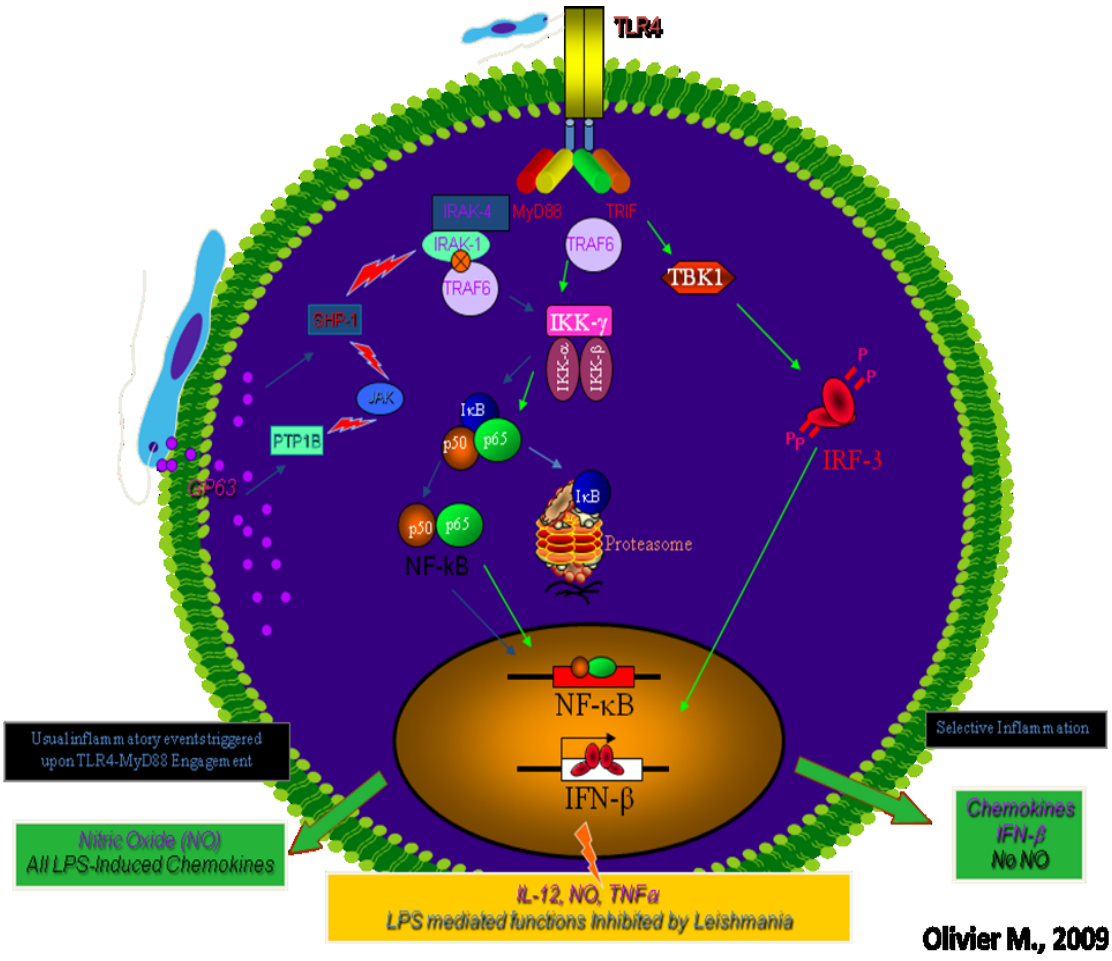

Figure 11: Proposed model by which *Leishmania* inhibits MyD88-dependent signalling in macrophages. (Adapted from Olivier M (2009), unpublished)

Claims to Originality

The work presented here increases the scientific knowledge in the fields of cell signalling and host-pathogen interactions. Although the discoveries were made while studying the *Leishmania* infection model, many of the findings reported in this thesis can have an important impact on our understanding of signalling pathways and the way they are regulated in various cell types. In addition, our identification of novel host evasion mechanisms utilized by *Leishmania* raises the possibility that similar tactics could be employed by other pathogens. To the best of our knowledge, the findings that bring originality to this work can be summarized as follows:

- The discovery that SHP-1 binds to and regulates IRAK-1's kinase activity. This finding classified SHP-1 as a novel negative regulator of TLR signalling.

- The first emphasis that an ITIM-like motif is found in a cytosolic kinase, and the introduction of the concept of kinase tyrosine-based inhibitory motif (KTIM).

- The first report that IRAK-1 is tyrosine phosphorylated.

- The first report that a pathogen can rapidly interfere with TLR signalling by using a host PTP to inhibit IRAK-1 activity. The exact step at which IRAK-1 signalling is inhibited was also identified.

- The first report to implicate the *Leishmania*-induced IRAK-1 inactivation in the ability of the parasite to inhibit LPS-mediated MØ functions.

- The first work to explore the conservation of IRAK-1's KTIM in evolution, and to investigate the presence and conservation of KTIMs in several other kinases.

- The first report of the ability of *Leishmania* amastigotes to rapidly activate MØ PTPs, importantly SHP-1.

- The first report that amastigotes do not activate PTP-1B in early infection time.

- The first work to suggest a role for *Leishmania*'s cysteine proteinase, lmcpb, in the inhibition of STAT-1α and AP-1 activity in infected MØs.

Bibliography

1. Choi CM, Lerner EA (2001) Leishmaniasis as an emerging infection. J Investig Dermatol Symp Proc 6: 175-182.
2. Sacks D, Noben-Trauth N (2002) The immunology of susceptibility and resistance to *Leishmania major* in mice. Nat Rev Immunol 2: 845-858.
3. Pimenta PFP, Saraiva EMB, Sacks DL (1991) The Comparative Fine-Structure and Surface Glycoconjugate Expression of 3 Life Stages of *Leishmania major*. Exp Parasitol 72: 191-204.
4. Ferguson MA (1997) The surface glycoconjugates of trypanosomatid parasites. Philos Trans R Soc Lond B Biol Sci 352: 1295-1302.
5. McConville MJ, Blackwell JM (1991) Developmental-Changes in the Glycosylated Phosphatidylinositols of *Leishmania donovani* - Characterization of the Promastigote and Amastigote Glycolipids. J Biol Chem 266: 15170-15179.
6. Schneider P, Rosat JP, Bouvier J, Louis J, Bordier C (1992) *Leishmania major* - Differential Regulation of the Surface Metalloprotease in Amastigote and Promastigote Stages. Exp Parasitol 75: 196-206.
7. Souza AE, Waugh S, Coombs GH, Mottram JC (1992) Characterization of a Multicopy Gene for a Major Stage-Specific Cysteine Proteinase of *Leishmania mexicana*. FEBS Lett 311: 124-127.
8. Proudfoot L, Odonnell CA, Liew FY (1995) Glycoinositolphospholipids of *Leishmania major* Inhibit Nitric-Oxide Synthesis and Reduce Leishmanicidal Activity in Murine Macrophages. Eur J Immunol 25: 745-750.
9. Murray PR, Rosenthal KS, Kobayashi GS, Pfaller MA (2002) Medical Microbiology. Fourth ed: Mosby Inc.
10. Lodge R, Descoteaux A (2005) Modulation of phagolysosome biogenesis by the lipophosphoglycan of *Leishmania*. Clin Immunol 114: 256-265.
11. Descoteaux A, Turco SJ (1999) Glycoconjugates in *Leishmania* infectivity. Biochim Biophys Acta 1455: 341-352.
12. Sacks DL (2001) *Leishmania*-sand fly interactions controlling species-specific vector competence. Cell Microbiol 3: 189-196.
13. Puentes SM, Sacks DL, da Silva RP, Joiner KA (1988) Complement binding by two developmental stages of *Leishmania major* promastigotes varying in expression of a surface lipophosphoglycan. J Exp Med 167: 887-902.
14. Green PJ, Feizi T, Stoll MS, Thiel S, Prescott A, et al. (1994) Recognition of the major cell surface glycoconjugates of *Leishmania* parasites by the human serum mannan-binding protein. Mol Biochem Parasitol 66: 319-328.
15. Culley FJ, Harris RA, Kaye PM, McAdam K, Raynes JG (1996) C-reactive protein binds to a novel ligand on *Leishmania donovani* and increases uptake into human macrophages. J Immunol 156: 4691-4696.
16. Descoteaux A, Matlashewski G, Turco SJ (1992) Inhibition of macrophage protein kinase C-mediated protein phosphorylation by *Leishmania donovani* lipophosphoglycan. J Immunol 149: 3008-3015.

17. Desjardins M, Descoteaux A (1997) Inhibition of phagolysosomal biogenesis by the *Leishmania* lipophosphoglycan. J Exp Med 185: 2061-2068.
18. Scianimanico S, Desrosiers M, Dermine JF, Meresse S, Descoteaux A, et al. (1999) Impaired recruitment of the small GTPase rab7 correlates with the inhibition of phagosome maturation by *Leishmania donovani* promastigotes. Cell Microbiol 1: 19-32.
19. Piedrafita D, Proudfoot L, Nikolaev AV, Xu DM, Sands W, et al. (1999) Regulation of macrophage IL-12 synthesis by *Leishmania* phosphoglycans. European Journal of Immunology 29: 235-244.
20. Proudfoot L, Nikolaev AV, Feng GJ, Wei XQ, Ferguson MAJ, et al. (1996) Regulation of the expression of nitric oxide synthase and leishmanicidal activity by glycoconjugates of *Leishmania* lipophosphoglycan in murine macrophages. Proc Natl Acad Sci USA 93: 10984-10989.
21. Spath GF, Epstein L, Leader B, Singer SM, Avila HA, et al. (2000) Lipophosphoglycan is a virulence factor distinct from related glycoconjugates in the protozoan parasite *Leishmania major*. Proc Natl Acad Sci U S A 97: 9258-9263.
22. McNeely TB, Turco SJ (1990) Requirement of lipophosphoglycan for intracellular survival of *Leishmania donovani* within human monocytes. J Immunol 144: 2745-2750.
23. Turco SJ, Sacks DL (1991) Expression of a stage-specific lipophosphoglycan in *Leishmania major* amastigotes. Mol Biochem Parasitol 45: 91-99.
24. McMaster WR, Morrison CJ, MacDonald MH, Joshi PB (1994) Mutational and functional analysis of the *Leishmania* surface metalloproteinase GP63: similarities to matrix metalloproteinases. Parasitology 108 Suppl: S29-36.
25. Schlagenhauf E, Etges R, Metcalf P (1998) The crystal structure of the *Leishmania major* surface proteinase leishmanolysin (gp63). Structure 6: 1035-1046.
26. Button LL, McMaster WR (1988) Molecular cloning of the major surface antigen of *leishmania*. J Exp Med 167: 724-729.
27. McConville MJ, Schnur LF, Jaffe C, Schneider P (1995) Structure of *Leishmania* lipophosphoglycan: inter- and intra-specific polymorphism in Old World species. Biochem J 310 (Pt 3): 807-818.
28. Brittingham A, Morrison CJ, McMaster WR, McGwire BS, Chang KP, et al. (1995) Role of the *Leishmania* surface protease gp63 in complement fixation, cell adhesion, and resistance to complement-mediated lysis. J Immunol 155: 3102-3111.
29. Mosser DM, Edelson PJ (1985) The mouse macrophage receptor for C3bi (CR3) is a major mechanism in the phagocytosis of *Leishmania* promastigotes. J Immunol 135: 2785-2789.
30. Wozencraft AO, Blackwell JM (1987) Increased infectivity of stationary-phase promastigotes of *Leishmania donovani*: correlation with enhanced C3 binding

capacity and CR3-mediated attachment to host macrophages. Immunology 60: 559-563.

31. Brittingham A, Chen G, McGwire BS, Chang KP, Mosser DM (1999) Interaction of *Leishmania* gp63 with cellular receptors for fibronectin. Infect Immun 67: 4477-4484.
32. Chaudhuri G, Chaudhuri M, Pan A, Chang KP (1989) Surface acid proteinase (gp63) of *Leishmania mexicana*. A metalloenzyme capable of protecting liposome-encapsulated proteins from phagolysosomal degradation by macrophages. J Biol Chem 264: 7483-7489.
33. Seay MB, Heard PL, Chaudhuri G (1996) Surface Zn-proteinase as a molecule for defense of *Leishmania mexicana* amazonensis promastigotes against cytolysis inside macrophage phagolysosomes. Infect Immun 64: 5129-5137.
34. Olivier M, Gregory DJ, Forget G (2005) Subversion Mechanisms by Which *Leishmania* Parasites Can Escape the Host Immune Response: a Signaling Point of View. Clin Microbiol Rev 18: 293-305.
35. Mottram JC, Robertson CD, Coombs GH, Barry JD (1992) A developmentally regulated cysteine proteinase gene of *Leishmania mexicana*. Mol Microbiol 6: 1925-1932.
36. Bart G, Coombs GH, Mottram JC (1995) Isolation of lmcpc, a gene encoding a *Leishmania mexicana* cathepsin-B-like cysteine proteinase. Mol Biochem Parasitol 73: 271-274.
37. Mottram JC, Coombs GH, Alexander J (2004) Cysteine peptidases as virulence factors of *Leishmania*. Curr Opin Microbiol 7: 375-381.
38. Mottram JC, Souza AE, Hutchison JE, Carter R, Frame MJ, et al. (1996) Evidence from disruption of the lmcpb gene array of *Leishmania mexicana* that cysteine proteinases are virulence factors. Proc Natl Acad Sci USA 93: 6008-6013.
39. Alexander J, Coombs GH, Mottram JC (1998) *Leishmania mexicana* cysteine proteinase-deficient mutants have attenuated virulence for mice and potentiate a Th1 response. J Immunol 161: 6794-6801.
40. Cameron P, McGachy A, Anderson M, Paul A, Coombs GH, et al. (2004) Inhibition of Lipopolysaccharide-Induced Macrophage IL-12 Production by *Leishmania mexicana* Amastigotes: The Role of Cysteine Peptidases and the NF-κB Signaling Pathway. J Immunol 173: 3297-3304.
41. De Souza Leao S, Lang T, Prina E, Hellio R, Antoine JC (1995) Intracellular *Leishmania amazonensis* amastigotes internalize and degrade MHC class II molecules of their host cells. J Cell Sci 108: 3219-3231.
42. Pollock KGJ, McNeil KS, Mottram JC, Lyons RE, Brewer JM, et al. (2003) The *Leishmania mexicana* cysteine protease, CPB2.8, induces potent Th2 responses. J Immunol 170: 1746-1753.

43. Shakarian AM, Dwyer DM (2000) Structurally conserved soluble acid phosphatases are synthesized and released by *Leishmania major* promastigotes. Exp Parasitol 95: 79-84.
44. Shakarian AM, Ellis SL, Mallinson DJ, Olafson RW, Dwyer DM (1997) Two tandemly arrayed genes encode the (histidine) secretory acid phosphatases of *Leishmania donovani*. Gene 196: 127-137.
45. Das S, Saha AK, Remaley AT, Glew RH, Dowling JN, et al. (1986) Hydrolysis of phosphoproteins and inositol phosphates by cell surface phosphatase of *Leishmania donovani*. Mol Biochem Parasitol 20: 143-153.
46. Gottlieb M, Dwyer DM (1981) *Leishmania donovani*: surface membrane acid phosphatase activity of promastigotes. Exp Parasitol 52: 117-128.
47. Gottlieb M, Dwyer DM (1982) Identification and partial characterization of an extracellular acid phosphatase activity of *Leishmania donovani* promastigotes. Mol Cell Biol 2: 76-81.
48. Lovelace JK, Dwyer DM, Gottlieb M (1986) Purification and characterization of the extracellular acid phosphatase of *Leishmania donovani*. Mol Biochem Parasitol 20: 243-251.
49. Remaley AT, Das S, Campbell PI, LaRocca GM, Pope MT, et al. (1985) Characterization of *Leishmania donovani* acid phosphatases. J Biol Chem 260: 880-886.
50. Joshi MB, Mallinson DJ, Dwyer DM (2004) The human pathogen *Leishmania donovani* secretes a histidine acid phosphatase activity that is resistant to proteolytic degradation. J Eukaryot Microbiol 51: 108-112.
51. Remaley AT, Glew RH, Kuhns DB, Basford RE, Waggoner AS, et al. (1985) *Leishmania donovani*: surface membrane acid phosphatase blocks neutrophil oxidative metabolite production. Exp Parasitol 60: 331-341.
52. Mosser DM, Springer TA, Diamond MS (1992) *Leishmania* promastigotes require opsonic complement to bind to the human leukocyte integrin Mac-1 (CD11b/CD18). J Cell Biol 116: 511-520.
53. Kane MM, Mosser DM (2000) *Leishmania* parasites and their ploys to disrupt macrophage activation. Curr Opin Hematol 7: 26-31.
54. Sutterwala FS, Rosenthal LA, Mosser DM (1996) Cooperation between CR1 (CD35) and CR3 (CD 11b/CD18) in the binding of complement-opsonized particles. J Leukoc Biol 59: 883-890.
55. Mosser DM, Edelson PJ (1987) The third component of complement (C3) is responsible for the intracellular survival of *Leishmania major*. Nature 327: 329-331.
56. Shepherd VL, Campbell EJ, Senior RM, Stahl PD (1982) Characterization of the mannose/fucose receptor on human mononuclear phagocytes. J Reticuloendothel Soc 32: 423-431.

57. Taylor ME, Conary JT, Lennartz MR, Stahl PD, Drickamer K (1990) Primary structure of the mannose receptor contains multiple motifs resembling carbohydrate-recognition domains. J Biol Chem 265: 12156-12162.
58. Stahl P, Schlesinger PH, Sigardson E, Rodman JS, Lee YC (1980) Receptor-mediated pinocytosis of mannose glycoconjugates by macrophages: characterization and evidence for receptor recycling. Cell 19: 207-215.
59. Sallusto F, Lanzavecchia A (1994) Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. J Exp Med 179: 1109-1118.
60. Tan MC, Mommaas AM, Drijfhout JW, Jordens R, Onderwater JJ, et al. (1997) Mannose receptor-mediated uptake of antigens strongly enhances HLA class II-restricted antigen presentation by cultured dendritic cells. Eur J Immunol 27: 2426-2435.
61. Blackwell JM, Ezekowitz RAB, Roberts MB, Channon JY, Sim RB, et al. (1985) Macrophage Complement and Lectin-Like Receptors Bind *Leishmania* in the Absence of Serum. J Exp Med 162: 324-331.
62. Volanakis JE, Kaplan MH (1971) Specificity of C-reactive protein for choline phosphate residues of pneumococcal C-polysaccharide. Proc Soc Exp Biol Med 136: 612-614.
63. Raynes JG, Curry A, Harris RA (1994) Binding of C-reactive protein to *Leishmania*. Biochem Soc Trans 22: 3S.
64. Bodman-Smith KB, Mbuchi M, Culley FJ, Bates PA, Raynes JG (2002) C-reactive protein-mediated phagocytosis of *Leishmania donovani* promastigotes does not alter parasite survival or macrophage responses. Parasite Immunol 24: 447-454.
65. Ruoslahti E (1991) Integrins. J Clin Invest 87: 1-5.
66. Wayner EA, Garcia-Pardo A, Humphries MJ, McDonald JA, Carter WG (1989) Identification and characterization of the T lymphocyte adhesion receptor for an alternative cell attachment domain (CS-1) in plasma fibronectin. J Cell Biol 109: 1321-1330.
67. Guy RA, Belosevic M (1993) Comparison of receptors required for entry of *Leishmania major* amastigotes into macrophages. Infect Immun 61: 1553-1558.
68. Wanderley JLM, Moreira MEC, Benjamin A, Bonomo AC, Barcinski MA (2006) Mimicry of Apoptotic Cells by Exposing Phosphatidylserine Participates in the Establishment of Amastigotes of *Leishmania (L) amazonensis* in Mammalian Hosts. J Immunol 176: 1834-1839.
69. de Freitas Balanco JM, Moreira ME, Bonomo A, Bozza PT, Amarante-Mendes G, et al. (2001) Apoptotic mimicry by an obligate intracellular parasite downregulates macrophage microbicidal activity. Curr Biol 11: 1870-1873.
70. Takai Y, Kishimoto A, Inoue M, Nishizuka Y (1977) Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. I.

Purification and characterization of an active enzyme from bovine cerebellum. J Biol Chem 252: 7603-7609.
71. Inoue M, Kishimoto A, Takai Y, Nishizuka Y (1977) Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. II. Proenzyme and its activation by calcium-dependent protease from rat brain. J Biol Chem 252: 7610-7616.
72. Reyland ME (2009) Protein kinase C isoforms: Multi-functional regulators of cell life and death. Front Biosci 14: 2386-2399.
73. Severn A, Wakelam MJ, Liew FY (1992) The role of protein kinase C in the induction of nitric oxide synthesis by murine macrophages. Biochem Biophys Res Commun 188: 997-1002.
74. Warzocha K, Bienvenu J, Coiffier B, Salles G (1995) Mechanisms of action of the tumor necrosis factor and lymphotoxin ligand-receptor system. Eur Cytokine Netw 6: 83-96.
75. Turco SJ, Descoteaux A (1992) The lipophosphoglycan of *Leishmania* parasites. Annu Rev Microbiol 46: 65-94.
76. McNeely TB, Turco SJ (1987) Inhibition of protein kinase C activity by the *Leishmania donovani* lipophosphoglycan. Biochem Biophys Res Commun 148: 653-657.
77. McNeely TB, Rosen G, Londner MV, Turco SJ (1989) Inhibitory effects on protein kinase C activity by lipophosphoglycan fragments and glycosylphosphatidylinositol antigens of the protozoan parasite *Leishmania*. Biochem J 259: 601-604.
78. Olivier M, Brownsey RW, Reiner NE (1992) Defective stimulus-response coupling in human monocytes infected with *Leishmania donovani* is associated with altered activation and translocation of protein kinase C. Proc Natl Acad Sci U S A 89: 7481-7485.
79. Ghosh S, Bhattacharyya S, Das S, Raha S, Maulik N, et al. (2001) Generation of ceramide in murine macrophages infected with *Leishmania donovani* alters macrophage signaling events and aids intracellular parasitic survival. Mol Cell Biochem 223: 47-60.
80. Rawlings JS, Rosler KM, Harrison DA (2004) The JAK/STAT signaling pathway. J Cell Sci 117: 1281-1283.
81. Woldman I, Varinou L, Ramsauer K, Rapp B, Decker T (2001) The Stat1 binding motif of the interferon-gamma receptor is sufficient to mediate Stat5 activation and its repression by SOCS3. J Biol Chem 276: 45722-45728.
82. Lowenstein CJ, Alley EW, Raval P, Snowman AM, Snyder SH, et al. (1993) Macrophage Nitric-Oxide Synthase Gene - 2 Upstream Regions Mediate Induction by Interferon-Gamma and Lipopolysaccharide. Proc Natl Acad Sci USA 90: 9730-9734.

83. Xie QW, Whisnant R, Nathan C (1993) Promoter of the mouse gene encoding calcium-independent nitric-oxide synthase confers inducibility by interferon-gamma and bacterial lipopolysaccharide. J Exp Med 177: 1779-1784.
84. Nandan D, Reiner NE (1995) Attenuation of gamma-interferon-induced tyrosine phosphorylation in mononuclear phagocytes infected with *Leishmania-donovani* - selective-inhibition of signaling through Janus kinases and STAT1. Infection and Immunity 63: 4495-4500.
85. Blanchette J, Racette N, Faure R, Siminovitch KA, Olivier M (1999) *Leishmania*-induced increases in activation of macrophage SHP-1 tyrosine phosphatase are associated with impaired IFN-gamma-triggered JAK2 activation. Eur J Immunol 29: 3737-3744.
86. Ray M, Gam AA, Boykins RA, Kenney RT (2000) Inhibition of interferon-gamma signaling by *Leishmania donovani*. J Infect Dis 181: 1121-1128.
87. Boldt S, Kolch W (2004) Targeting MAPK signalling: Prometheus' fire or Pandora's box? Curr Pharm Des 10: 1885-1905.
88. Chen Z, Gibson TB, Robinson F, Silvestro L, Pearson G, et al. (2001) MAP kinases. Chem Rev 101: 2449-2476.
89. Lewis TS, Shapiro PS, Ahn NG (1998) Signal transduction through MAP kinase cascades. Adv Cancer Res 74: 49-139.
90. Chang F, Steelman LS, Lee JT, Shelton JG, Navolanic PM, et al. (2003) Signal transduction mediated by the Ras/Raf/MEK/ERK pathway from cytokine receptors to transcription factors: potential targeting for therapeutic intervention. Leukemia 17: 1263-1293.
91. Gille H, Sharrocks AD, Shaw PE (1992) Phosphorylation of transcription factor p62TCF by MAP kinase stimulates ternary complex formation at c-fos promoter. Nature 358: 414-417.
92. Treinies I, Paterson HF, Hooper S, Wilson R, Marshall CJ (1999) Activated MEK stimulates expression of AP-1 components independently of phosphatidylinositol 3-kinase (PI3-kinase) but requires a PI3-kinase signal To stimulate DNA synthesis. Mol Cell Biol 19: 321-329.
93. Hibi M, Lin A, Smeal T, Minden A, Karin M (1993) Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. Genes Dev 7: 2135-2148.
94. Gupta S, Barrett T, Whitmarsh AJ, Cavanagh J, Sluss HK, et al. (1996) Selective interaction of JNK protein kinase isoforms with transcription factors. Embo J 15: 2760-2770.
95. Fuchs SY, Tappin I, Ronai Z (2000) Stability of the ATF2 transcription factor is regulated by phosphorylation and dephosphorylation. J Biol Chem 275: 12560-12564.

96. van Dam H, Duyndam M, Rottier R, Bosch A, de Vries-Smits L, et al. (1993) Heterodimer formation of cJun and ATF-2 is responsible for induction of c-jun by the 243 amino acid adenovirus E1A protein. Embo J 12: 479-487.
97. Lee JC, Kumar S, Griswold DE, Underwood DC, Votta BJ, et al. (2000) Inhibition of p38 MAP kinase as a therapeutic strategy. Immunopharmacology 47: 185-201.
98. Rousseau S, Morrice N, Peggie M, Campbell DG, Gaestel M, et al. (2002) Inhibition of SAPK2a/p38 prevents hnRNP A0 phosphorylation by MAPKAP-K2 and its interaction with cytokine mRNAs. Embo J 21: 6505-6514.
99. Hunter T (1995) Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. Cell 80: 225-236.
100. Ling Y, West AG, Roberts EC, Lakey JH, Sharrocks AD (1998) Interaction of transcription factors with serum response factor. Identification of the Elk-1 binding surface. J Biol Chem 273: 10506-10514.
101. Rao KM (2001) MAP kinase activation in macrophages. J Leukoc Biol 69: 3-10.
102. Prive C, Descoteaux A (2000) *Leishmania donovani* promastigotes evade the activation of mitogen-activated protein kinases p38, c-Jun N-terminal kinase, and extracellular signal-regulated kinase-1/2 during infection of naive macrophages. Eur J Immunol 30: 2235-2244.
103. Martiny A, Meyer-Fernandes JR, de Souza W, Vannier-Santos MA (1999) Altered tyrosine phosphorylation of ERK1 MAP kinase and other macrophage molecules caused by *Leishmania* amastigotes. Mol Biochem Parasitol 102: 1-12.
104. Nandan D, Lo R, Reiner NE (1999) Activation of phosphotyrosine phosphatase activity attenuates mitogen-activated protein kinase signaling and inhibits c-FOS and nitric oxide synthase expression in macrophages infected with *Leishmania donovani*. Infect Immun 67: 4055-4063.
105. Forget G, Gregory DJ, Whitcombe LA, Olivier M (2006) Role of host protein tyrosine phosphatase SHP-1 in *Leishmania donovani*-induced inhibition of nitric oxide production. Infect Immun 74: 6272-6279.
106. Ghosh S, Bhattacharyya S, Sirkar M, Sa GS, Das T, et al. (2002) *Leishmania donovani* suppresses activated protein 1 and NF-κB activation in host macrophages via ceramide generation: involvement of extracellular signal-regulated kinase. Infect Immun 70: 6828-6838.
107. Awasthi A, Mathur R, Khan A, Joshi BN, Jain N, et al. (2003) CD40 signaling is impaired in L. major-infected macrophages and is rescued by a p38MAPK activator establishing a host-protective memory T cell response. J Exp Med 197: 1037-1043.
108. Junghae M, Raynes JG (2002) Activation of p38 mitogen-activated protein kinase attenuates *Leishmania donovani* infection in macrophages. Infect Immun 70: 5026-5035.
109. Goetz CA, Baldwin AS (2008) NF-kappaB pathways in the immune system: control of the germinal center reaction. Immunol Res 41: 233-247.

110. Hayden MS, Ghosh S (2004) Signaling to NF-kappaB. Genes Dev 18: 2195-2224.
111. Yamamoto Y, Gaynor RB (2004) IkappaB kinases: key regulators of the NF-kappaB pathway. Trends Biochem Sci 29: 72-79.
112. Viatour P, Merville MP, Bours V, Chariot A (2005) Phosphorylation of NF-kappaB and IkappaB proteins: implications in cancer and inflammation. Trends Biochem Sci 30: 43-52.
113. Siebenlist U, Brown K, Claudio E (2005) Control of lymphocyte development by nuclear factor-kappaB. Nat Rev Immunol 5: 435-445.
114. Pahl HL (1999) Activators and target genes of Rel/NF-kappaB transcription factors. Oncogene 18: 6853-6866.
115. Guizani-Tabbane L, Ben-Aissa K, Belghith M, Sassi A, Dellagi K (2004) *Leishmania major* amastigotes induce p50/c-Rel NF-kappa B transcription factor in human macrophages: involvement in cytokine synthesis. Infect Immun 72: 2582-2589.
116. Gregory DJ, Godbout M, Contreras I, Forget G, Olivier M (2008) A novel form of NF-kappa B is induced by *Leishmania* infection: Involvement in macrophage gene expression. Eur J Immunol 38: 1071-1081.
117. Matsukawa A (2007) STAT proteins in innate immunity during sepsis: lessons from gene knockout mice. Acta Med Okayama 61: 239-245.
118. Reich NC, Liu L (2006) Tracking STAT nuclear traffic. Nat Rev Immunol 6: 602-612.
119. Forget G, Gregory DJ, Olivier M (2005) Proteasome-mediated degradation of STAT1 alpha following infection of macrophages with *Leishmania donovani*. J Biol Chem 280: 30542-30549.
120. Shaulian E, Karin M (2002) AP-1 as a regulator of cell life and death. Nat Cell Biol 4: E131-136.
121. Kuo NT, Agani FH, Haxhiu MA, Chang CH (1998) A possible role for protein kinase C in CO2/H+-induced c-fos mRNA expression in PC12 cells. Respir Physiol 111: 127-135.
122. Gruda MC, Kovary K, Metz R, Bravo R (1994) Regulation of Fra-1 and Fra-2 phosphorylation differs during the cell cycle of fibroblasts and phosphorylation in vitro by MAP kinase affects DNA binding activity. Oncogene 9: 2537-2547.
123. Gupta S, Campbell D, Derijard B, Davis RJ (1995) Transcription factor ATF2 regulation by the JNK signal transduction pathway. Science 267: 389-393.
124. Yamagishi S, Yamada M, Ishikawa Y, Matsumoto T, Ikeuchi T, et al. (2001) p38 mitogen-activated protein kinase regulates low potassium-induced c-Jun phosphorylation and apoptosis in cultured cerebellar granule neurons. J Biol Chem 276: 5129-5133.
125. Blanchette J, Abu-Dayyeh I, Hassani K, Whitcombe L, Olivier M (2009) Regulation of macrophage nitric oxide production by the protein tyrosine phosphatase Src

homology 2 domain phosphotyrosine phosphatase 1 (SHP-1). Immunology 127: 123-133.
126. Larsen M, Tremblay ML, Yamada KM (2003) Phosphatases in cell-matrix adhesion and migration. Nat Rev Mol Cell Biol 4: 700-711.
127. Denu JM, Dixon JE (1998) Protein tyrosine phosphatases: mechanisms of catalysis and regulation. Curr Opin Chem Biol 2: 633-641.
128. Pao LI, Badour K, Siminovitch KA, Neel BG (2007) Nonreceptor protein-tyrosine phosphatases in immune cell signaling. Annu Rev Immunol 25: 473-523.
129. Bourdeau A, Dube N, Tremblay ML (2005) Cytoplasmic protein tyrosine phosphatases, regulation and function: the roles of PTP1B and TC-PTP. Curr Opin Cell Biol 17: 203-209.
130. Elchebly M, Payette P, Michaliszyn E, Cromlish W, Collins S, et al. (1999) Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase-1B gene. Science 283: 1544-1548.
131. Klaman LD, Boss O, Peroni OD, Kim JK, Martino JL, et al. (2000) Increased energy expenditure, decreased adiposity, and tissue-specific insulin sensitivity in protein-tyrosine phosphatase 1B-deficient mice. Mol Cell Biol 20: 5479-5489.
132. Elchebly M, Cheng A, Tremblay ML (2000) Modulation of insulin signaling by protein tyrosine phosphatases. J Mol Med 78: 473-482.
133. Myers MP, Andersen JN, Cheng A, Tremblay ML, Horvath CM, et al. (2001) TYK2 and JAK2 Are Substrates of Protein-tyrosine Phosphatase 1B. J Biol Chem 276: 47771-47774.
134. You-Ten KE, Muise ES, Itie A, Michaliszyn E, Wagner J, et al. (1997) Impaired bone marrow microenvironment and immune function in T cell protein tyrosine phosphatase-deficient mice. J Exp Med 186: 683-693.
135. Simoncic PD, Lee-Loy A, Barber DL, Tremblay ML, McGlade CJ (2002) The T cell protein tyrosine phosphatase is a negative regulator of janus family kinases 1 and 3. Current Biology 12: 446-453.
136. ten Hoeve J, de Jesus Ibarra-Sanchez M, Fu Y, Zhu W, Tremblay M, et al. (2002) Identification of a nuclear Stat1 protein tyrosine phosphatase. Mol Cell Biol 22: 5662-5668.
137. Halle M, Gomez MA, Stuible M, Shimizu H, McMaster WR, et al. (2009) The *Leishmania* surface protease GP63 cleaves multiple intracellular proteins and actively participates in p38 mitogen-activated protein kinase inactivation. J Biol Chem 284: 6893-6908.
138. Matthews RJ, Bowne DB, Flores E, Thomas ML (1992) Characterization of hematopoietic intracellular protein tyrosine phosphatases: description of a phosphatase containing an SH2 domain and another enriched in proline-, glutamic acid-, serine-, and threonine-rich sequences. Mol Cell Biol 12: 2396-2405.
139. Plutzky J, Neel BG, Rosenberg RD (1992) Isolation of a src homology 2-containing tyrosine phosphatase. Proc Natl Acad Sci U S A 89: 1123-1127.

140. Kroll J, Waltenberger J (1997) The vascular endothelial growth factor receptor KDR activates multiple signal transduction pathways in porcine aortic endothelial cells. J Biol Chem 272: 32521-32527.
141. Guo DQ, Wu LW, Dunbar JD, Ozes ON, Mayo LD, et al. (2000) Tumor necrosis factor employs a protein-tyrosine phosphatase to inhibit activation of KDR and vascular endothelial cell growth factor-induced endothelial cell proliferation. J Biol Chem 275: 11216-11221.
142. Massa PT, Saha S, Wu C, Jarosinski KW (2000) Expression and function of the protein tyrosine phosphatase SHP-1 in oligodendrocytes. Glia 29: 376-385.
143. Ravetch JV, Lanier LL (2000) Immune inhibitory receptors. Science 290: 84-89.
144. Shultz LD, Rajan TV, Greiner DL (1997) Severe defects in immunity and hematopoiesis caused by SHP-1 protein-tyrosine-phosphatase deficiency. Trends Biotechnol 15: 302-307.
145. Pani G, Kozlowski M, Cambier JC, Mills GB, Siminovitch KA (1995) Identification of the tyrosine phosphatase PTP1c as a B-cell antigen receptor-associated protein involved in the regulation of B-cell signaling. Journal of Experimental Medicine 181: 2077-2084.
146. Carter JD, Neel BG, Lorenz U (1999) The tyrosine phosphatase SHP-1 influences thymocyte selection by setting TCR signaling thresholds. Int Immunol 11: 1999-2014.
147. Chiang GG, Sefton BM (2001) Specific dephosphorylation of the Lck tyrosine protein kinase at Tyr-394 by the SHP-1 protein-tyrosine phosphatase. J Biol Chem 276: 23173-23178.
148. Plas DR, Johnson R, Pingel JT, Matthews RJ, Dalton M, et al. (1996) Direct regulation of ZAP-70 by SHP-1 in T cell antigen receptor signaling. Science 272: 1173-1176.
149. Jiao H, Berrada K, Yang W, Tabrizi M, Platanias LC, et al. (1996) Direct association with and dephosphorylation of Jak2 kinase by the SH2-domain-containing protein tyrosine phosphatase SHP-1. Mol Cell Biol 16: 6985-6992.
150. Bedecs K, Elbaz N, Sutren M, Masson M, Susini C, et al. (1997) Angiotensin II type 2 receptors mediate inhibition of mitogen-activated protein kinase cascade and functional activation of SHP-1 tyrosine phosphatase. Biochem J 325 (Pt 2): 449-454.
151. Matsubara H, Shibasaki Y, Okigaki M, Mori Y, Masaki H, et al. (2001) Effect of angiotensin II type 2 receptor on tyrosine kinase Pyk2 and c-Jun NH2-terminal kinase via SHP-1 tyrosine phosphatase activity: evidence from vascular-targeted transgenic mice of AT2 receptor. Biochem Biophys Res Commun 282: 1085-1091.
152. Ram PA, Waxman DJ (1997) Interaction of growth hormone-activated STATs with SH2-containing phosphotyrosine phosphatase SHP-1 and nuclear JAK2 tyrosine kinase. J Biol Chem 272: 17694-17702.

153. Olivier M, Romero-Gallo BJ, Matte C, Blanchette J, Posner BI, et al. (1998) Modulation of interferon-gamma-induced macrophage activation by phosphotyrosine phosphatases inhibition - Effect on murine Leishmaniasis progression. J Biol Chem 273: 13944-13949.
154. Matte C, Marquis JF, Blanchette J, Gros P, Faure R, et al. (2000) Peroxovanadium-mediated protection against murine leishmaniasis: role of the modulation of nitric oxide. European Journal of Immunology 30: 2555-2564.
155. Forget G, Siminovitch KA, Brochu S, Rivest S, Radzioch D, et al. (2001) Role of host phosphotyrosine phosphatase SHP-1 in the development of murine leishmaniasis. Eur J Immunol 31: 3185-3196.
156. Nandan D, Yi TL, Lopez M, Lai C, Reiner NE (2002) *Leishmania* EF-1 alpha activates the Src homology 2 domain containing tyrosine phosphatase SHP-1 leading to macrophage deactivation. J Biol Chem 277: 50190-50197.
157. Croker BA, Kiu H, Nicholson SE (2008) SOCS regulation of the JAK/STAT signalling pathway. Semin Cell Dev Biol 19: 414-422.
158. Yasukawa H, Misawa H, Sakamoto H, Masuhara M, Sasaki A, et al. (1999) The JAK-binding protein JAB inhibits Janus tyrosine kinase activity through binding in the activation loop. Embo J 18: 1309-1320.
159. Sasaki A, Yasukawa H, Suzuki A, Kamizono S, Syoda T, et al. (1999) Cytokine-inducible SH2 protein-3 (CIS3/SOCS3) inhibits Janus tyrosine kinase by binding through the N-terminal kinase inhibitory region as well as SH2 domain. Genes Cells 4: 339-351.
160. Qing Y, Costa-Pereira AP, Watling D, Stark GR (2005) Role of tyrosine 441 of interferon-gamma receptor subunit 1 in SOCS-1-mediated attenuation of STAT1 activation. J Biol Chem 280: 1849-1853.
161. Yamamoto K, Yamaguchi M, Miyasaka N, Miura O (2003) SOCS-3 inhibits IL-12-induced STAT4 activation by binding through its SH2 domain to the STAT4 docking site in the IL-12 receptor beta2 subunit. Biochem Biophys Res Commun 310: 1188-1193.
162. Bertholet S, Dickensheets HL, Sheikh F, Gam AA, Donnelly RP, et al. (2003) *Leishmania donovani*-induced expression of suppressor of cytokine signaling 3 in human macrophages: a novel mechanism for intracellular parasite suppression of activation. Infect Immun 71: 2095-2101.
163. Antoniazi S, Price HP, Kropf P, Freudenberg MA, Galanos C, et al. (2004) Chemokine gene expression in toll-like receptor-competent and -deficient mice infected with *Leishmania major*. Infect Immun 72: 5168-5174.
164. Forget G, Matte C, Siminovitch KA, Rivest S, Pouliot P, et al. (2005) Regulation of the *Leishmania*-induced innate inflammatory response by the protein tyrosine phosphatase SHP-1. Eur J Immunol 35: 1906-1917.

165. Teixeira MJ, Teixeira CR, Andrade BB, Barral-Netto M, Barral A (2006) Chemokines in host-parasite interactions in leishmaniasis. Trends Parasitol 22: 32-40.
166. Rousseau D, Demartino S, Anjuere F, Ferrua B, Fragaki K, et al. (2001) Sustained parasite burden in the spleen of *Leishmania infantum*-infected BALB/c mice is accompanied by expression of MCP-1 transcripts and lack of protection against challenge. Eur Cytokine Netw 12: 340-347.
167. Liew FY, Millott S, Parkinson C, Palmer RM, Moncada S (1990) Macrophage killing of *Leishmania* parasite in vivo is mediated by nitric oxide from L-arginine. J Immunol 144: 4794-4797.
168. Murray HW (1982) Cell-mediated immune response in experimental visceral leishmaniasis. II. Oxygen-dependent killing of intracellular *Leishmania donovani* amastigotes. J Immunol 129: 351-357.
169. Marletta MA, Yoon PS, Iyengar R, Leaf CD, Wishnok JS (1988) Macrophage oxidation of L-arginine to nitrite and nitrate - nitric-oxide is an intermediate. Biochemistry 27: 8706-8711.
170. Hibbs JB, Taintor RR, Vavrin Z, Rachlin EM (1988) Nitric-oxide - a cyto-toxic activated macrophage effector molecule. Biochemical and Biophysical Research Communications 157: 87-94.
171. Green SJ, Meltzer MS, Hibbs JB, Jr., Nacy CA (1990) Activated macrophages destroy intracellular *Leishmania major* amastigotes by an L-arginine-dependent killing mechanism. J Immunol 144: 278-283.
172. Bogdan C, Rollinghoff M, Diefenbach A (1999). Nitric Oxide and infection. NY: Kluwer Academic/ Plenum Publishers.
173. Bourguignon SC, Alves CR, Giovanni-De-Simone S (1997) Detrimental effect of nitric oxide on Trypanosoma cruzi and *Leishmania major* like cells. Acta Trop 66: 109-118.
174. Lemesre JL, Sereno D, Daulouede S, Veyret B, Brajon N, et al. (1997) *Leishmania* spp.: nitric oxide-mediated metabolic inhibition of promastigote and axenically grown amastigote forms. Exp Parasitol 86: 58-68.
175. Cunningham AC (2002) Parasitic adaptive mechanisms in infection by *leishmania*. Exp Mol Pathol 72: 132-141.
176. Stenger S, Thuring H, Rollinghoff M, Bogdan C (1994) Tissue expression of inducible nitric oxide synthase is closely associated with resistance to *Leishmania* major. J Exp Med 180: 783-793.
177. Murray HW, Nathan CF (1999) Macrophage microbicidal mechanisms in vivo: reactive nitrogen versus oxygen intermediates in the killing of intracellular visceral *Leishmania donovani*. J Exp Med 189: 741-746.
178. Buchmuller-Rouiller Y, Mauel J (1987) Impairment of the oxidative metabolism of mouse peritoneal macrophages by intracellular *Leishmania* spp. Infect Immun 55: 587-593.

179. Olivier M, Baimbridge KG, Reiner NE (1992) Stimulus-response coupling in monocytes infected with *Leishmania*. Attenuation of calcium transients is related to defective agonist-induced accumulation of inositol phosphates. J Immunol 148: 1188-1196.
180. Sorensen AL, Hey AS, Kharazmi A (1994) *Leishmania major* surface protease Gp63 interferes with the function of human monocytes and neutrophils in vitro. Apmis 102: 265-271.
181. Lodge R, Diallo TO, Descoteaux A (2006) *Leishmania donovani* lipophosphoglycan blocks NADPH oxidase assembly at the phagosome membrane. Cell Microbiol 8: 1922-1931.
182. Lodge R, Descoteaux A (2006) Phagocytosis of *Leishmania donovani* amastigotes is Rac1 dependent and occurs in the absence of NADPH oxidase activation. Eur J Immunol 36: 2735-2744.
183. Fontayne A, Dang PM, Gougerot-Pocidalo MA, El-Benna J (2002) Phosphorylation of p47phox sites by PKC alpha, beta II, delta, and zeta: effect on binding to p22phox and on NADPH oxidase activation. Biochemistry 41: 7743-7750.
184. De Titto EH, Catterall JR, Remington JS (1986) Activity of recombinant tumor necrosis factor on Toxoplasma gondii and Trypanosoma cruzi. J Immunol 137: 1342-1345.
185. Czuprynski CJ, Brown JF, Young KM, Cooley AJ, Kurtz RS (1988) Effects of murine recombinant interleukin 1 alpha on the host response to bacterial infection. J Immunol 140: 962-968.
186. Bermudez LE, Young LS (1988) Tumor necrosis factor, alone or in combination with IL-2, but not IFN-gamma, is associated with macrophage killing of Mycobacterium avium complex. J Immunol 140: 3006-3013.
187. Ozaki Y, Ohashi T, Minami A, Nakamura S (1987) Enhanced resistance of mice to bacterial infection induced by recombinant human interleukin-1a. Infect Immun 55: 1436-1440.
188. Del Vecchio M, Bajetta E, Canova S, Lotze MT, Wesa A, et al. (2007) Interleukin-12: biological properties and clinical application. Clin Cancer Res 13: 4677-4685.
189. Reiner NE, Ng W, Wilson CB, McMaster WR, Burchett SK (1990) Modulation of in vitro monocyte cytokine responses to *Leishmania donovani*. Interferon-gamma prevents parasite-induced inhibition of interleukin 1 production and primes monocytes to respond to *Leishmania* by producing both tumor necrosis factor-alpha and interleukin 1. J Clin Invest 85: 1914-1924.
190. Frankenburg S, Leibovici V, Mansbach N, Turco SJ, Rosen G (1990) Effect of glycolipids of *Leishmania* parasites on human monocyte activity. Inhibition by lipophosphoglycan. J Immunol 145: 4284-4289.
191. Hatzigeorgiou DE, Geng J, Zhu B, Zhang Y, Liu K, et al. (1996) Lipophosphoglycan from *Leishmania* suppresses agonist-induced interleukin 1 beta gene expression in

human monocytes via a unique promoter sequence. Proc Natl Acad Sci U S A 93: 14708-14713.
192. Matte C, Olivier M (2002) *Leishmania*-induced cellular recruitment during the early inflammatory response: Modulation of proinflammatory mediators. Journal of Infectious Diseases 185: 673-681.
193. Carrera L, Gazzinelli RT, Badolato R, Hieny S, Muller W, et al. (1996) *Leishmania* promastigotes selectively inhibit interleukin 12 induction in bone marrow-derived macrophages from susceptible and resistant mice. Journal of Experimental Medicine 183: 515-526.
194. Weinheber N, Wolfram M, Harbecke D, Aebischer T (1998) Phagocytosis of *Leishmania mexicana* amastigotes by macrophages leads to a sustained suppression of IL-12 production. European Journal of Immunology 28: 2467-2477.
195. Marth T, Kelsall BL (1997) Regulation of interleukin-12 by complement receptor 3 signaling. J Exp Med 185: 1987-1995.
196. Sutterwala FS, Noel GJ, Clynes R, Mosser DM (1997) Selective suppression of interleukin-12 induction after macrophage receptor ligation. J Exp Med 185: 1977-1985.
197. Murray RK, Granner DK, Mayes PA, Rodwell VW (1999). Harper's Biochemistry. 25th ed. Norwalk: Appelton & Lange.
198. Belley A, Chadee K (1995) Eicosanoid production by parasites: from pathogenesis to immunomodulation? Parasitol Today 11: 327-334.
199. Farrell JP, Kirkpatrick CE (1987) Experimental cutaneous leishmaniasis. II. A possible role for prostaglandins in exacerbation of disease in *Leishmania major*-infected BALB/c mice. J Immunol 138: 902-907.
200. Reiner NE, Malemud CJ (1984) Arachidonic acid metabolism in murine leishmaniasis (Donovani): ex-vivo evidence for increased cyclooxygenase and 5-lipoxygenase activity in spleen cells. Cell Immunol 88: 501-510.
201. Reiner NE, Malemud CJ (1985) Arachidonic acid metabolism by murine peritoneal macrophages infected with *Leishmania donovani*: in vitro evidence for parasite-induced alterations in cyclooxygenase and lipoxygenase pathways. J Immunol 134: 556-563.
202. Matte C, Maion G, Mourad W, Olivier M (2001) *Leishmania donovani*-induced macrophages cyclooxygenase-2 and prostaglandin E-2 synthesis. Parasite Immunology 23: 177-184.
203. Anstead GM, Chandrasekar B, Zhao W, Yang J, Perez LE, et al. (2001) Malnutrition alters the innate immune response and increases early visceralization following *Leishmania donovani* infection. Infect Immun 69: 4709-4718.
204. Teicher BA (2007) Transforming growth factor-beta and the immune response to malignant disease. Clin Cancer Res 13: 6247-6251.

205. Bogdan C, Rollinghoff M (1998) The immune response to *Leishmania*: mechanisms of parasite control and evasion. Int J Parasitol 28: 121-134.
206. Scharton-Kersten T, Afonso LC, Wysocka M, Trinchieri G, Scott P (1995) IL-12 is required for natural killer cell activation and subsequent T helper 1 cell development in experimental leishmaniasis. J Immunol 154: 5320-5330.
207. Gantt KR, Schultz-Cherry S, Rodriguez N, Jeronimo SM, Nascimento ET, et al. (2003) Activation of TGF-beta by *Leishmania chagasi*: importance for parasite survival in macrophages. J Immunol 170: 2613-2620.
208. Somanna A, Mundodi V, Gedamu L (2002) Functional analysis of cathepsin B-like cysteine proteases from *Leishmania donovani* complex. Evidence for the activation of latent transforming growth factor beta. J Biol Chem 277: 25305-25312.
209. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A (2001) Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol 19: 683-765.
210. Sutterwala FS, Noel GJ, Salgame P, Mosser DM (1998) Reversal of proinflammatory responses by ligating the macrophage Fcgamma receptor type I. J Exp Med 188: 217-222.
211. Groux H, Cottrez F, Rouleau M, Mauze S, Antonenko S, et al. (1999) A transgenic model to analyze the immunoregulatory role of IL-10 secreted by antigen-presenting cells. J Immunol 162: 1723-1729.
212. Kane MM, Mosser DM (2001) The role of IL-10 in promoting disease progression in leishmaniasis. J Immunol 166: 1141-1147.
213. De Almeida MC, Cardoso SA, Barral-Netto M (2003) *Leishmania* (*Leishmania*) *chagasi* infection alters the expression of cell adhesion and costimulatory molecules on human monocyte and macrophage. Int J Parasitol 33: 153-162.
214. Reiner NE, Ng W, McMaster WR (1987) Parasite-accessory cell interactions in murine leishmaniasis. II. *Leishmania donovani* suppresses macrophage expression of class I and class II major histocompatibility complex gene products. J Immunol 138: 1926-1932.
215. Reiner NE, Ng W, Ma T, McMaster WR (1988) Kinetics of gamma interferon binding and induction of major histocompatibility complex class II mRNA in *Leishmania*-infected macrophages. Proc Natl Acad Sci U S A 85: 4330-4334.
216. Fruth U, Solioz N, Louis JA (1993) *Leishmania major* interferes with antigen presentation by infected macrophages. J Immunol 150: 1857-1864.
217. Prina E, Jouanne C, de Souza Lao S, Szabo A, Guillet JG, et al. (1993) Antigen presentation capacity of murine macrophages infected with *Leishmania amazonensis* amastigotes. J Immunol 151: 2050-2061.
218. Kaye PM, Rogers NJ, Curry AJ, Scott JC (1994) Deficient expression of co-stimulatory molecules on *Leishmania*-infected macrophages. Eur J Immunol 24: 2850-2854.

219. Saha B, Das G, Vohra H, Ganguly NK, Mishra GC (1995) Macrophage-T cell interaction in experimental visceral leishmaniasis: failure to express costimulatory molecules on *Leishmania*-infected macrophages and its implication in the suppression of cell-mediated immunity. Eur J Immunol 25: 2492-2498.
220. Kamanaka M, Yu P, Yasui T, Yoshida K, Kawabe T, et al. (1996) Protective role of CD40 in *Leishmania major* infection at two distinct phases of cell-mediated immunity. Immunity 4: 275-281.
221. Campbell KA, Ovendale PJ, Kennedy MK, Fanslow WC, Reed SG, et al. (1996) CD40 ligand is required for protective cell-mediated immunity to *Leishmania major*. Immunity 4: 283-289.
222. Soong L, Xu JC, Grewal IS, Kima P, Sun J, et al. (1996) Disruption of CD40-CD40 ligand interactions results in an enhanced susceptibility to *Leishmania amazonensis* infection. Immunity 4: 263-273.
223. Heinzel FP, Rerko RM, Hujer AM (1998) Underproduction of interleukin-12 in susceptible mice during progressive leishmaniasis is due to decreased CD40 activity. Cell Immunol 184: 129-142.
224. Wang L, Ligoxygakis P (2006) Pathogen recognition and signalling in the Drosophila innate immune response. Immunobiology 211: 251-261.
225. Leclerc V, Reichhart JM (2004) The immune response of Drosophila melanogaster. Immunol Rev 198: 59-71.
226. Silverman N, Maniatis T (2001) NF-kappaB signaling pathways in mammalian and insect innate immunity. Genes Dev 15: 2321-2342.
227. De Gregorio E, Spellman PT, Tzou P, Rubin GM, Lemaitre B (2002) The Toll and Imd pathways are the major regulators of the immune response in Drosophila. Embo J 21: 2568-2579.
228. Weber AN, Tauszig-Delamasure S, Hoffmann JA, Lelievre E, Gascan H, et al. (2003) Binding of the Drosophila cytokine Spatzle to Toll is direct and establishes signaling. Nat Immunol 4: 794-800.
229. Horng T, Medzhitov R (2001) Drosophila MyD88 is an adapter in the Toll signaling pathway. Proc Natl Acad Sci U S A 98: 12654-12658.
230. Akira S, Uematsu S, Takeuchi O (2006) Pathogen recognition and innate immunity. Cell 124: 783-801.
231. Brikos C, O'Neill LA (2008). Toll-Like Receptors (TLRs) and Innate Immunity: Springer Berlin Heidelberg. pp. 21-50.
232. Kawai T, Akira S (2007) TLR signaling. Semin Immunol 19: 24-32.
233. Lord KA, Hoffman-Liebermann B, Liebermann DA (1990) Nucleotide sequence and expression of a cDNA encoding MyD88, a novel myeloid differentiation primary response gene induced by IL6. Oncogene 5: 1095-1097.
234. Hultmark D (1994) Macrophage differentiation marker MyD88 is a member of the Toll/IL-1 receptor family. Biochem Biophys Res Commun 199: 144-146.

235. Kawai T, Adachi O, Ogawa T, Takeda K, Akira S (1999) Unresponsiveness of MyD88-deficient mice to endotoxin. Immunity 11: 115-122.
236. Takeuchi O, Takeda K, Hoshino K, Adachi O, Ogawa T, et al. (2000) Cellular responses to bacterial cell wall components are mediated through MyD88-dependent signaling cascades. Int Immunol 12: 113-117.
237. O'Neill LA, Bowie AG (2007) The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. Nat Rev Immunol 7: 353-364.
238. Li S, Strelow A, Fontana EJ, Wesche H (2002) IRAK-4: a novel member of the IRAK family with the properties of an IRAK-kinase. Proc Natl Acad Sci U S A 99: 5567-5572.
239. Kollewe C, Mackensen AC, Neumann D, Knop J, Cao P, et al. (2004) Sequential autophosphorylation steps in the interleukin-1 receptor-associated kinase-1 regulate its availability as an adapter in interleukin-1 signaling. J Biol Chem 279: 5227-5236.
240. Chen ZJ (2005) Ubiquitin signalling in the NF-kappaB pathway. Nat Cell Biol 7: 758-765.
241. Horng T, Barton GM, Medzhitov R (2001) TIRAP: an adapter molecule in the Toll signaling pathway. Nat Immunol 2: 835-841.
242. Fitzgerald KA, Palsson-McDermott EM, Bowie AG, Jefferies CA, Mansell AS, et al. (2001) Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction. Nature 413: 78-83.
243. Horng T, Barton GM, Flavell RA, Medzhitov R (2002) The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors. Nature 420: 329-333.
244. Yamamoto M, Sato S, Hemmi H, Sanjo H, Uematsu S, et al. (2002) Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4. Nature 420: 324-329.
245. Kagan JC, Medzhitov R (2006) Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling. Cell 125: 943-955.
246. Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, et al. (2003) Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science 301: 640-643.
247. Hirotani T, Yamamoto M, Kumagai Y, Uematsu S, Kawase I, et al. (2005) Regulation of lipopolysaccharide-inducible genes by MyD88 and Toll/IL-1 domain containing adaptor inducing IFN-beta. Biochem Biophys Res Commun 328: 383-392.
248. Sasai M, Oshiumi H, Matsumoto M, Inoue N, Fujita F, et al. (2005) Cutting Edge: NF-kappaB-activating kinase-associated protein 1 participates in TLR3/Toll-IL-1 homology domain-containing adapter molecule-1-mediated IFN regulatory factor 3 activation. J Immunol 174: 27-30.

249. Cusson-Hermance N, Khurana S, Lee TH, Fitzgerald KA, Kelliher MA (2005) Rip1 mediates the Trif-dependent toll-like receptor 3- and 4-induced NF-{kappa}B activation but does not contribute to interferon regulatory factor 3 activation. J Biol Chem 280: 36560-36566.
250. Sato S, Sugiyama M, Yamamoto M, Watanabe Y, Kawai T, et al. (2003) Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF) associates with TNF receptor-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF-kappa B and IFN-regulatory factor-3, in the Toll-like receptor signaling. J Immunol 171: 4304-4310.
251. Jiang Z, Mak TW, Sen G, Li X (2004) Toll-like receptor 3-mediated activation of NF-kappaB and IRF3 diverges at Toll-IL-1 receptor domain-containing adapter inducing IFN-beta. Proc Natl Acad Sci U S A 101: 3533-3538.
252. Gohda J, Matsumura T, Inoue J (2004) Cutting edge: TNFR-associated factor (TRAF) 6 is essential for MyD88-dependent pathway but not toll/IL-1 receptor domain-containing adaptor-inducing IFN-beta (TRIF)-dependent pathway in TLR signaling. J Immunol 173: 2913-2917.
253. Yamamoto M, Sato S, Hemmi H, Uematsu S, Hoshino K, et al. (2003) TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. Nat Immunol 4: 1144-1150.
254. Rowe DC, McGettrick AF, Latz E, Monks BG, Gay NJ, et al. (2006) The myristoylation of TRIF-related adaptor molecule is essential for Toll-like receptor 4 signal transduction. Proc Natl Acad Sci U S A 103: 6299-6304.
255. Oshiumi H, Sasai M, Shida K, Fujita T, Matsumoto M, et al. (2003) TIR-containing adapter molecule (TICAM)-2, a bridging adapter recruiting to toll-like receptor 4 TICAM-1 that induces interferon-beta. J Biol Chem 278: 49751-49762.
256. Carty M, Goodbody R, Schroder M, Stack J, Moynagh PN, et al. (2006) The human adaptor SARM negatively regulates adaptor protein TRIF-dependent Toll-like receptor signaling. Nat Immunol 7: 1074-1081.
257. Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, et al. (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 282: 2085-2088.
258. Latto C (2008) An overview of sepsis. Dimens Crit Care Nurs 27: 195-200; quiz 2001-2002.
259. Eriksson U, Ricci R, Hunziker L, Kurrer MO, Oudit GY, et al. (2003) Dendritic cell-induced autoimmune heart failure requires cooperation between adaptive and innate immunity. Nat Med 9: 1484-1490.
260. Michelsen KS, Wong MH, Shah PK, Zhang W, Yano J, et al. (2004) Lack of Toll-like receptor 4 or myeloid differentiation factor 88 reduces atherosclerosis and alters plaque phenotype in mice deficient in apolipoprotein E. Proc Natl Acad Sci U S A 101: 10679-10684.

261. Bjorkbacka H, Kunjathoor VV, Moore KJ, Koehn S, Ordija CM, et al. (2004) Reduced atherosclerosis in MyD88-null mice links elevated serum cholesterol levels to activation of innate immunity signaling pathways. Nat Med 10: 416-421.
262. Kolek MJ, Carlquist JF, Muhlestein JB, Whiting BM, Horne BD, et al. (2004) Toll-like receptor 4 gene Asp299Gly polymorphism is associated with reductions in vascular inflammation, angiographic coronary artery disease, and clinical diabetes. Am Heart J 148: 1034-1040.
263. Zipris D, Lien E, Xie JX, Greiner DL, Mordes JP, et al. (2005) TLR activation synergizes with Kilham rat virus infection to induce diabetes in BBDR rats. J Immunol 174: 131-142.
264. Kerfoot SM, Long EM, Hickey MJ, Andonegui G, Lapointe BM, et al. (2004) TLR4 contributes to disease-inducing mechanisms resulting in central nervous system autoimmune disease. J Immunol 173: 7070-7077.
265. Leadbetter EA, Rifkin IR, Hohlbaum AM, Beaudette BC, Shlomchik MJ, et al. (2002) Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. Nature 416: 603-607.
266. Eisenbarth SC, Piggott DA, Huleatt JW, Visintin I, Herrick CA, et al. (2002) Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. J Exp Med 196: 1645-1651.
267. Dabbagh K, Dahl ME, Stepick-Biek P, Lewis DB (2002) Toll-like receptor 4 is required for optimal development of Th2 immune responses: role of dendritic cells. J Immunol 168: 4524-4530.
268. Iwahashi M, Yamamura M, Aita T, Okamoto A, Ueno A, et al. (2004) Expression of Toll-like receptor 2 on CD16+ blood monocytes and synovial tissue macrophages in rheumatoid arthritis. Arthritis Rheum 50: 1457-1467.
269. Pierer M, Rethage J, Seibl R, Lauener R, Brentano F, et al. (2004) Chemokine secretion of rheumatoid arthritis synovial fibroblasts stimulated by Toll-like receptor 2 ligands. J Immunol 172: 1256-1265.
270. Lang T, Mansell A (2007) The negative regulation of Toll-like receptor and associated pathways. Immunol Cell Biol 85: 425-434.
271. LeBouder E, Rey-Nores JE, Rushmere NK, Grigorov M, Lawn SD, et al. (2003) Soluble forms of Toll-like receptor (TLR)2 capable of modulating TLR2 signaling are present in human plasma and breast milk. J Immunol 171: 6680-6689.
272. Colotta F, Dower SK, Sims JE, Mantovani A (1994) The type II 'decoy' receptor: a novel regulatory pathway for interleukin 1. Immunol Today 15: 562-566.
273. Iwaki D, Mitsuzawa H, Murakami S, Sano H, Konishi M, et al. (2002) The extracellular toll-like receptor 2 domain directly binds peptidoglycan derived from Staphylococcus aureus. J Biol Chem 277: 24315-24320.
274. Hyakushima N, Mitsuzawa H, Nishitani C, Sano H, Kuronuma K, et al. (2004) Interaction of soluble form of recombinant extracellular TLR4 domain with MD-2

enables lipopolysaccharide binding and attenuates TLR4-mediated signaling. J Immunol 173: 6949-6954.
275. Kropf P, Herath S, Klemenz R, Muller I (2003) Signaling through the T1/ST2 molecule is not necessary for Th2 differentiation but is important for the regulation of type 1 responses in nonhealing *Leishmania major* infection. Infect Immun 71: 1961-1971.
276. Bergers G, Reikerstorfer A, Braselmann S, Graninger P, Busslinger M (1994) Alternative promoter usage of the Fos-responsive gene Fit-1 generates mRNA isoforms coding for either secreted or membrane-bound proteins related to the IL-1 receptor. Embo J 13: 1176-1188.
277. Tominaga S (1989) A putative protein of a growth specific cDNA from BALB/c-3T3 cells is highly similar to the extracellular portion of mouse interleukin 1 receptor. FEBS Lett 258: 301-304.
278. Brint EK, Xu D, Liu H, Dunne A, McKenzie AN, et al. (2004) ST2 is an inhibitor of interleukin 1 receptor and Toll-like receptor 4 signaling and maintains endotoxin tolerance. Nat Immunol 5: 373-379.
279. Wald D, Qin J, Zhao Z, Qian Y, Naramura M, et al. (2003) SIGIRR, a negative regulator of Toll-like receptor-interleukin 1 receptor signaling. Nat Immunol 4: 920-927.
280. Thomassen E, Renshaw BR, Sims JE (1999) Identification and characterization of SIGIRR, a molecule representing a novel subtype of the IL-1R superfamily. Cytokine 11: 389-399.
281. Garlanda C, Riva F, Polentarutti N, Buracchi C, Sironi M, et al. (2004) Intestinal inflammation in mice deficient in Tir8, an inhibitory member of the IL-1 receptor family. Proc Natl Acad Sci U S A 101: 3522-3526.
282. Polentarutti N, Rol GP, Muzio M, Bosisio D, Camnasio M, et al. (2003) Unique pattern of expression and inhibition of IL-1 signaling by the IL-1 receptor family member TIR8/SIGIRR. Eur Cytokine Netw 14: 211-218.
283. Divanovic S, Trompette A, Atabani SF, Madan R, Golenbock DT, et al. (2005) Inhibition of TLR-4/MD-2 signaling by RP105/MD-1. J Endotoxin Res 11: 363-368.
284. Guo F, Li Y, Wu S (1999) Antisense IRAK-2 oligonucleotide blocks IL-1-stimulated NF-kappaB activation and ICAM-1 expression in cultured endothelial cells. Inflammation 23: 535-543.
285. Diehl GE, Yue HH, Hsieh K, Kuang AA, Ho M, et al. (2004) TRAIL-R as a negative regulator of innate immune cell responses. Immunity 21: 877-889.
286. Liew FY, Xu D, Brint EK, O'Neill LA (2005) Negative regulation of toll-like receptor-mediated immune responses. Nat Rev Immunol 5: 446-458.
287. Janssens S, Burns K, Tschopp J, Beyaert R (2002) Regulation of interleukin-1- and lipopolysaccharide-induced NF-kappaB activation by alternative splicing of MyD88. Curr Biol 12: 467-471.

288. Burns K, Janssens S, Brissoni B, Olivos N, Beyaert R, et al. (2003) Inhibition of interleukin 1 receptor/Toll-like receptor signaling through the alternatively spliced, short form of MyD88 is due to its failure to recruit IRAK-4. J Exp Med 197: 263-268.
289. Kobayashi K, Hernandez LD, Galan JE, Janeway CA, Medzhitov R, et al. (2002) IRAK-M is a negative regulator of toll-like receptor signaling. Cell 110: 191-202.
290. Hardy MP, O'Neill LA (2004) The murine IRAK2 gene encodes four alternatively spliced isoforms, two of which are inhibitory. J Biol Chem 279: 27699-27708.
291. Kawagoe T, Sato S, Matsushita K, Kato H, Matsui K, et al. (2008) Sequential control of Toll-like receptor-dependent responses by IRAK1 and IRAK2. Nat Immunol 9: 684-691.
292. Meylan E, Tschopp J (2008) IRAK2 takes its place in TLR signaling. Nat Immunol 9: 581-582.
293. Kinjyo I, Hanada T, Inagaki-Ohara K, Mori H, Aki D, et al. (2002) SOCS1/JAB is a negative regulator of LPS-induced macrophage activation. Immunity 17: 583-591.
294. Nakagawa R, Naka T, Tsutsui H, Fujimoto M, Kimura A, et al. (2002) SOCS-1 participates in negative regulation of LPS responses. Immunity 17: 677-687.
295. Baetz A, Frey M, Heeg K, Dalpke AH (2004) Suppressor of cytokine signaling (SOCS) proteins indirectly regulate toll-like receptor signaling in innate immune cells. J Biol Chem 279: 54708-54715.
296. Gingras S, Parganas E, de Pauw A, Ihle JN, Murray PJ (2004) Re-examination of the role of suppressor of cytokine signaling 1 (SOCS1) in the regulation of toll-like receptor signaling. J Biol Chem 279: 54702-54707.
297. Inohara N, Nunez G (2003) NODs: intracellular proteins involved in inflammation and apoptosis. Nat Rev Immunol 3: 371-382.
298. Watanabe T, Kitani A, Murray PJ, Strober W (2004) NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses. Nat Immunol 5: 800-808.
299. Katso R, Okkenhaug K, Ahmadi K, White S, Timms J, et al. (2001) Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. Annu Rev Cell Dev Biol 17: 615-675.
300. Fukao T, Tanabe M, Terauchi Y, Ota T, Matsuda S, et al. (2002) PI3K-mediated negative feedback regulation of IL-12 production in DCs. Nat Immunol 3: 875-881.
301. Zhang G, Ghosh S (2002) Negative regulation of toll-like receptor-mediated signaling by Tollip. J Biol Chem 277: 7059-7065.
302. Boone DL, Turer EE, Lee EG, Ahmad RC, Wheeler MT, et al. (2004) The ubiquitin-modifying enzyme A20 is required for termination of Toll-like receptor responses. Nat Immunol 5: 1052-1060.
303. Alvarez JI (2005) Inhibition of Toll Like Receptor immune responses by microbial pathogens. Front Biosci 10: 582-587.

304. Attridge SR, Rowley D (1983) The role of the flagellum in the adherence of Vibrio cholerae. J Infect Dis 147: 864-872.
305. Drake D, Montie TC (1988) Flagella, motility and invasive virulence of Pseudomonas aeruginosa. J Gen Microbiol 134: 43-52.
306. Milton DL, O'Toole R, Horstedt P, Wolf-Watz H (1996) Flagellin A is essential for the virulence of Vibrio anguillarum. J Bacteriol 178: 1310-1319.
307. Chua KL, Chan YY, Gan YH (2003) Flagella are virulence determinants of Burkholderia pseudomallei. Infect Immun 71: 1622-1629.
308. Schmitt CK, Ikeda JS, Darnell SC, Watson PR, Bispham J, et al. (2001) Absence of all components of the flagellar export and synthesis machinery differentially alters virulence of Salmonella enterica serovar Typhimurium in models of typhoid fever, survival in macrophages, tissue culture invasiveness, and calf enterocolitis. Infect Immun 69: 5619-5625.
309. Wang PL, Oido-Mori M, Fujii T, Kowashi Y, Kikuchi M, et al. (2001) Heterogeneous expression of Toll-like receptor 4 and downregulation of Toll-like receptor 4 expression on human gingival fibroblasts by Porphyromonas gingivalis lipopolysaccharide. Biochem Biophys Res Commun 288: 863-867.
310. Hajishengallis G, Martin M, Sojar HT, Sharma A, Schifferle RE, et al. (2002) Dependence of bacterial protein adhesins on toll-like receptors for proinflammatory cytokine induction. Clin Diagn Lab Immunol 9: 403-411.
311. Lehner MD, Hartung T (2002). Reviews of Physiology, Biochemistry, and Pharmacology. 1st ed: Springer Berlin Heidelberg.
312. Asai Y, Hashimoto M, Ogawa T (2003) Treponemal glycoconjugate inhibits Toll-like receptor ligand-induced cell activation by blocking LPS-binding protein and CD14 functions. Eur J Immunol 33: 3196-3204.
313. Duesbery NS, Webb CP, Leppla SH, Gordon VM, Klimpel KR, et al. (1998) Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor. Science 280: 734-737.
314. Telepnev M, Golovliov I, Grundstrom T, Tarnvik A, Sjostedt A (2003) Francisella tularensis inhibits Toll-like receptor-mediated activation of intracellular signalling and secretion of TNF-alpha and IL-1 from murine macrophages. Cell Microbiol 5: 41-51.
315. Noss EH, Pai RK, Sellati TJ, Radolf JD, Belisle J, et al. (2001) Toll-like receptor 2-dependent inhibition of macrophage class II MHC expression and antigen processing by 19-kDa lipoprotein of Mycobacterium tuberculosis. J Immunol 167: 910-918.
316. Gehring AJ, Rojas RE, Canaday DH, Lakey DL, Harding CV, et al. (2003) The Mycobacterium tuberculosis 19-kilodalton lipoprotein inhibits gamma interferon-regulated HLA-DR and Fc gamma R1 on human macrophages through Toll-like receptor 2. Infect Immun 71: 4487-4497.

317. Tobian AA, Potter NS, Ramachandra L, Pai RK, Convery M, et al. (2003) Alternate class I MHC antigen processing is inhibited by Toll-like receptor signaling pathogen-associated molecular patterns: Mycobacterium tuberculosis 19-kDa lipoprotein, CpG DNA, and lipopolysaccharide. J Immunol 171: 1413-1422.
318. Nedialkov YA, Motin VL, Brubaker RR (1997) Resistance to lipopolysaccharide mediated by the Yersinia pestis V antigen-polyhistidine fusion peptide: amplification of interleukin-10. Infect Immun 65: 1196-1203.
319. Sing A, Rost D, Tvardovskaia N, Roggenkamp A, Wiedemann A, et al. (2002) Yersinia V-antigen exploits toll-like receptor 2 and CD14 for interleukin 10-mediated immunosuppression. J Exp Med 196: 1017-1024.
320. Sing A, Roggenkamp A, Geiger AM, Heesemann J (2002) Yersinia enterocolitica evasion of the host innate immune response by V antigen-induced IL-10 production of macrophages is abrogated in IL-10-deficient mice. J Immunol 168: 1315-1321.
321. Boland A, Sory MP, Iriarte M, Kerbourch C, Wattiau P, et al. (1996) Status of YopM and YopN in the Yersinia Yop virulon: YopM of Y.enterocolitica is internalized inside the cytosol of PU5-1.8 macrophages by the YopB, D, N delivery apparatus. Embo J 15: 5191-5201.
322. Black DS, Bliska JB (1997) Identification of p130Cas as a substrate of Yersinia YopH (Yop51), a bacterial protein tyrosine phosphatase that translocates into mammalian cells and targets focal adhesions. Embo J 16: 2730-2744.
323. Brubaker RR (2003) Interleukin-10 and inhibition of innate immunity to Yersiniae: roles of Yops and LcrV (V antigen). Infect Immun 71: 3673-3681.
324. Leulier F, Marchal C, Miletich I, Limbourg-Bouchon B, Benarous R, et al. (2003) Directed expression of the HIV-1 accessory protein Vpu in Drosophila fat-body cells inhibits Toll-dependent immune responses. EMBO Rep 4: 976-981.
325. Bowie A, Kiss-Toth E, Symons JA, Smith GL, Dower SK, et al. (2000) A46R and A52R from vaccinia virus are antagonists of host IL-1 and toll-like receptor signaling. Proc Natl Acad Sci U S A 97: 10162-10167.
326. Campos MA, Almeida IC, Takeuchi O, Akira S, Valente EP, et al. (2001) Activation of Toll-like receptor-2 by glycosylphosphatidylinositol anchors from a protozoan parasite. J Immunol 167: 416-423.
327. Ropert C, Gazzinelli RT (2004) Regulatory role of Toll-like receptor 2 during infection with Trypanosoma cruzi. J Endotoxin Res 10: 425-430.
328. Oliveira AC, Peixoto JR, de Arruda LB, Campos MA, Gazzinelli RT, et al. (2004) Expression of functional TLR4 confers proinflammatory responsiveness to Trypanosoma cruzi glycoinositolphospholipids and higher resistance to infection with T. cruzi. J Immunol 173: 5688-5696.
329. Ropert C, Almeida IC, Closel M, Travassos LR, Ferguson MA, et al. (2001) Requirement of mitogen-activated protein kinases and I kappa B phosphorylation for induction of proinflammatory cytokines synthesis by macrophages indicates

functional similarity of receptors triggered by glycosylphosphatidylinositol anchors from parasitic protozoa and bacterial lipopolysaccharide. J Immunol 166: 3423-3431.

330. Ropert C, Closel M, Chaves AC, Gazzinelli RT (2003) Inhibition of a p38/stress-activated protein kinase-2-dependent phosphatase restores function of IL-1 receptor-associate kinase-1 and reverses Toll-like receptor 2- and 4-dependent tolerance of macrophages. J Immunol 171: 1456-1465.
331. de Veer MJ, Curtis JM, Baldwin TM, DiDonato JA, Sexton A, et al. (2003) MyD88 is essential for clearance of *Leishmania major*: possible role for lipophosphoglycan and Toll-like receptor 2 signaling. Eur J Immunol 33: 2822-2831.
332. Becker I, Salaiza N, Aguirre M, Delgado J, Carrillo-Carrasco N, et al. (2003) *Leishmania* lipophosphoglycan (LPG) activates NK cells through toll-like receptor-2. Mol Biochem Parasitol 130: 65-74.
333. Flandin JF, Chano F, Descoteaux A (2006) RNA interference reveals a role for TLR2 and TLR3 in the recognition of *Leishmania donovani* promastigotes by interferon-gamma-primed macrophages. Eur J Immunol 36: 411-420.
334. Krishnegowda G, Hajjar AM, Zhu J, Douglass EJ, Uematsu S, et al. (2005) Induction of proinflammatory responses in macrophages by the glycosylphosphatidylinositols of Plasmodium falciparum: cell signaling receptors, glycosylphosphatidylinositol (GPI) structural requirement, and regulation of GPI activity. J Biol Chem 280: 8606-8616.
335. Naik RS, Branch OH, Woods AS, Vijaykumar M, Perkins DJ, et al. (2000) Glycosylphosphatidylinositol anchors of Plasmodium falciparum: molecular characterization and naturally elicited antibody response that may provide immunity to malaria pathogenesis. J Exp Med 192: 1563-1576.
336. Debierre-Grockiego F, Campos MA, Azzouz N, Schmidt J, Bieker U, et al. (2007) Activation of TLR2 and TLR4 by glycosylphosphatidylinositols derived from Toxoplasma gondii. J Immunol 179: 1129-1137.
337. Ouaissi A, Guilvard E, Delneste Y, Caron G, Magistrelli G, et al. (2002) The Trypanosoma cruzi Tc52-released protein induces human dendritic cell maturation, signals via Toll-like receptor 2, and confers protection against lethal infection. J Immunol 168: 6366-6374.
338. Harris TH, Cooney NM, Mansfield JM, Paulnock DM (2006) Signal transduction, gene transcription, and cytokine production triggered in macrophages by exposure to trypanosome DNA. Infect Immun 74: 4530-4537.
339. Shoda LK, Kegerreis KA, Suarez CE, Roditi I, Corral RS, et al. (2001) DNA from protozoan parasites Babesia bovis, Trypanosoma cruzi, and T. brucei is mitogenic for B lymphocytes and stimulates macrophage expression of interleukin-12, tumor necrosis factor alpha, and nitric oxide. Infect Immun 69: 2162-2171.

340. Brown WC, Corral RS (2002) Stimulation of B lymphocytes, macrophages, and dendritic cells by protozoan DNA. Microbes Infect 4: 969-974.
341. Bafica A, Santiago HC, Goldszmid R, Ropert C, Gazzinelli RT, et al. (2006) Cutting edge: TLR9 and TLR2 signaling together account for MyD88-dependent control of parasitemia in Trypanosoma cruzi infection. J Immunol 177: 3515-3519.
342. Drennan MB, Stijlemans B, Van den Abbeele J, Quesniaux VJ, Barkhuizen M, et al. (2005) The induction of a type 1 immune response following a Trypanosoma brucei infection is MyD88 dependent. J Immunol 175: 2501-2509.
343. Jaramillo M, Gowda DC, Radzioch D, Olivier M (2003) Hemozoin increases IFN-gamma-Inducible macrophage nitric oxide generation through extracellular signal-regulated kinase- and NF-kappa B-dependent pathways. Journal of Immunology 171: 4243-4253.
344. Jaramillo M, Plante I, Ouellet N, Vandal K, Tessier PA, et al. (2004) Hemozoin-inducible proinflammatory events in vivo: Potential role in malaria infection. Journal of Immunology 172: 3101-3110.
345. Coban C, Ishii KJ, Kawai T, Hemmi H, Sato S, et al. (2005) Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin. J Exp Med 201: 19-25.
346. Parroche P, Lauw FN, Goutagny N, Latz E, Monks BG, et al. (2007) Malaria hemozoin is immunologically inert but radically enhances innate responses by presenting malaria DNA to Toll-like receptor 9. Proc Natl Acad Sci U S A 104: 1919-1924.
347. Yarovinsky F, Zhang D, Andersen JF, Bannenberg GL, Serhan CN, et al. (2005) TLR11 activation of dendritic cells by a protozoan profilin-like protein. Science 308: 1626-1629.
348. Aosai F, Chen M, Kang HK, Mun HS, Norose K, et al. (2002) Toxoplasma gondii-derived heat shock protein HSP70 functions as a B cell mitogen. Cell Stress Chaperones 7: 357-364.
349. Del Rio L, Butcher BA, Bennouna S, Hieny S, Sher A, et al. (2004) Toxoplasma gondii triggers myeloid differentiation factor 88-dependent IL-12 and chemokine ligand 2 (monocyte chemoattractant protein 1) responses using distinct parasite molecules and host receptors. J Immunol 172: 6954-6960.
350. Gazzinelli RT, Denkers EY (2006) Protozoan encounters with Toll-like receptor signalling pathways: implications for host parasitism. Nat Rev Immunol 6: 895-906.
351. Campos MA, Closel M, Valente EP, Cardoso JE, Akira S, et al. (2004) Impaired production of proinflammatory cytokines and host resistance to acute infection with Trypanosoma cruzi in mice lacking functional myeloid differentiation factor 88. J Immunol 172: 1711-1718.
352. Muraille E, De Trez C, Brait M, De Baetselier P, Leo O, et al. (2003) Genetically resistant mice lacking MyD88-adapter protein display a high susceptibility to

Leishmania major infection associated with a polarized Th2 response. J Immunol 170: 4237-4241.

353. Scanga CA, Aliberti J, Jankovic D, Tilloy F, Bennouna S, et al. (2002) Cutting edge: MyD88 is required for resistance to Toxoplasma gondii infection and regulates parasite-induced IL-12 production by dendritic cells. J Immunol 168: 5997-6001.
354. Adachi K, Tsutsui H, Kashiwamura S, Seki E, Nakano H, et al. (2001) Plasmodium berghei infection in mice induces liver injury by an IL-12- and toll-like receptor/myeloid differentiation factor 88-dependent mechanism. J Immunol 167: 5928-5934.
355. Kropf P, Freudenberg MA, Modolell M, Price HP, Herath S, et al. (2004) Toll-like receptor 4 contributes to efficient control of infection with the protozoan parasite *Leishmania major*. Infect Immun 72: 1920-1928.
356. Kropf P, Freudenberg N, Kalis C, Modolell M, Herath S, et al. (2004) Infection of C57BL/10ScCr and C57BL/10ScNCr mice with *Leishmania major* reveals a role for Toll-like receptor 4 in the control of parasite replication. J Leukoc Biol 76: 48-57.
357. Grazia Cappiello M, Sutterwala FS, Trinchieri G, Mosser DM, Ma X (2001) Suppression of Il-12 transcription in macrophages following Fc gamma receptor ligation. J Immunol 166: 4498-4506.
358. Urban BC, Willcox N, Roberts DJ (2001) A role for CD36 in the regulation of dendritic cell function. Proc Natl Acad Sci U S A 98: 8750-8755.
359. Ocana-Morgner C, Mota MM, Rodriguez A (2003) Malaria blood stage suppression of liver stage immunity by dendritic cells. J Exp Med 197: 143-151.
360. Perry JA, Olver CS, Burnett RC, Avery AC (2005) Cutting edge: the acquisition of TLR tolerance during malaria infection impacts T cell activation. J Immunol 174: 5921-5925.
361. Urban BC, Ferguson DJ, Pain A, Willcox N, Plebanski M, et al. (1999) Plasmodium falciparum-infected erythrocytes modulate the maturation of dendritic cells. Nature 400: 73-77.
362. Butcher BA, Denkers EY (2002) Mechanism of entry determines the ability of Toxoplasma gondii to inhibit macrophage proinflammatory cytokine production. Infect Immun 70: 5216-5224.
363. Denkers EY, Kim L, Butcher BA (2003) In the belly of the beast: subversion of macrophage proinflammatory signalling cascades during Toxoplasma gondii infection. Cell Microbiol 5: 75-83.
364. McKee AS, Dzierszinski F, Boes M, Roos DS, Pearce EJ (2004) Functional inactivation of immature dendritic cells by the intracellular parasite Toxoplasma gondii. J Immunol 173: 2632-2640.
365. Bennouna S, Sukhumavasi W, Denkers EY (2006) Toxoplasma gondii inhibits toll-like receptor 4 ligand-induced mobilization of intracellular tumor necrosis factor alpha to the surface of mouse peritoneal neutrophils. Infect Immun 74: 4274-4281.

366. Williams L, Bradley L, Smith A, Foxwell B (2004) Signal transducer and activator of transcription 3 is the dominant mediator of the anti-inflammatory effects of IL-10 in human macrophages. J Immunol 172: 567-576.
367. Butcher BA, Kim L, Panopoulos AD, Watowich SS, Murray PJ, et al. (2005) IL-10-independent STAT3 activation by Toxoplasma gondii mediates suppression of IL-12 and TNF-alpha in host macrophages. J Immunol 174: 3148-3152.
368. Kim L, Butcher BA, Denkers EY (2004) Toxoplasma gondii interferes with lipopolysaccharide-induced mitogen-activated protein kinase activation by mechanisms distinct from endotoxin tolerance. J Immunol 172: 3003-3010.
369. Butcher BA, Kim L, Johnson PF, Denkers EY (2001) Toxoplasma gondii tachyzoites inhibit proinflammatory cytokine induction in infected macrophages by preventing nuclear translocation of the transcription factor NF-kappa B. J Immunol 167: 2193-2201.
370. Shapira S, Speirs K, Gerstein A, Caamano J, Hunter CA (2002) Suppression of NF-kappaB activation by infection with Toxoplasma gondii. J Infect Dis 185 Suppl 1: S66-72.
371. Shapira S, Harb OS, Margarit J, Matrajt M, Han J, et al. (2005) Initiation and termination of NF-kappaB signaling by the intracellular protozoan parasite Toxoplasma gondii. J Cell Sci 118: 3501-3508.
372. Richards RL, Rao M, Wassef NM, Glenn GM, Rothwell SW, et al. (1998) Liposomes containing lipid A serve as an adjuvant for induction of antibody and cytotoxic T-cell responses against RTS,S malaria antigen. Infect Immun 66: 2859-2865.
373. Skeiky YA, Coler RN, Brannon M, Stromberg E, Greeson K, et al. (2002) Protective efficacy of a tandemly linked, multi-subunit recombinant leishmanial vaccine (Leish-111f) formulated in MPL adjuvant. Vaccine 20: 3292-3303.
374. Zhang WW, Matlashewski G (2008) Immunization with a Toll-like receptor 7 and/or 8 agonist vaccine adjuvant increases protective immunity against *Leishmania major* in BALB/c mice. Infect Immun 76: 3777-3783.
375. Araujo AF, de Alencar BC, Vasconcelos JR, Hiyane MI, Marinho CR, et al. (2005) CD8+-T-cell-dependent control of Trypanosoma cruzi infection in a highly susceptible mouse strain after immunization with recombinant proteins based on amastigote surface protein 2. Infect Immun 73: 6017-6025.
376. Stacey KJ, Blackwell JM (1999) Immunostimulatory DNA as an adjuvant in vaccination against *Leishmania major*. Infect Immun 67: 3719-3726.
377. Coban C, Ishii KJ, Stowers AW, Keister DB, Klinman DM, et al. (2004) Effect of CpG oligodeoxynucleotides on the immunogenicity of Pfs25, a Plasmodium falciparum transmission-blocking vaccine antigen. Infect Immun 72: 584-588.
378. Cunha MG, Rodrigues MM, Soares IS (2001) Comparison of the immunogenic properties of recombinant proteins representing the Plasmodium vivax vaccine candidate MSP1(19) expressed in distinct bacterial vectors. Vaccine 20: 385-396.

379. Kumar S, Jones TR, Oakley MS, Zheng H, Kuppusamy SP, et al. (2004) CpG oligodeoxynucleotide and Montanide ISA 51 adjuvant combination enhanced the protective efficacy of a subunit malaria vaccine. Infect Immun 72: 949-957.
380. McSorley SJ, Ehst BD, Yu Y, Gewirtz AT (2002) Bacterial flagellin is an effective adjuvant for CD4+ T cells in vivo. J Immunol 169: 3914-3919.
381. Arevalo I, Ward B, Miller R, Meng TC, Najar E, et al. (2001) Successful treatment of drug-resistant cutaneous leishmaniasis in humans by use of imiquimod, an immunomodulator. Clin Infect Dis 33: 1847-1851.
382. Buates S, Matlashewski G (1999) Treatment of experimental leishmaniasis with the immunomodulators imiquimod and S-28463: efficacy and mode of action. J Infect Dis 179: 1485-1494.
383. Flynn B, Wang V, Sacks DL, Seder RA, Verthelyi D (2005) Prevention and treatment of cutaneous leishmaniasis in primates by using synthetic type D/A oligodeoxynucleotides expressing CpG motifs. Infect Immun 73: 4948-4954.
384. An HZ, Hou J, Zhou J, Zhao W, Xu HM, et al. (2008) Phosphatase SHP-1 promotes TLR- and RIG-I-activated production of type I interferon by inhibiting the kinase IRAK1. Nature Immunology 9: 542-550.
385. Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, et al. (2001) The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature 410: 1099-1103.
386. Liese J, Schleicher U, Bogdan C (2007) TLR9 signaling is essential for the innate NK cell response in murine cutaneous leishmaniasis. Eur J Immunol 37: 3424-3434.
387. Daeron M, Jaeger S, Du Pasquier L, Vivier E (2008) Immunoreceptor tyrosine-based inhibition motifs: a quest in the past and future. Immunol Rev 224: 11-43.