Leishmania-Macrophage Interactions: Regulation of Protein Tyrosine Phosphatases and its Implication in the Outcome of Infection.

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ABSTRACT

The outcome of *Leishmania* infection depends both on host and pathogen factors. Macrophages, the specialized host cell for uptake and intracellular development of *Leishmania* parasites, play a central role in the control of infection. Underlying their effector and accessory functions is the activation of signalling pathways, which in turn are largely controlled by events of protein phosphorylation. Consequently, the regulation of protein kinase and phosphatase activities results critical for the sequential progression of the signalling cascade, and therefore for the control of antimicrobial and inflammatory phagocyte functions.

This doctoral thesis discusses novel mechanisms of protein tyrosine phosphatase (PTP) regulation in the context of *Leishmania*-macrophage interactions. Herein are presented two events in which, independently, host and pathogen factors orchestrate the differential regulation of macrophage PTP activity. Chapter 2 describes the role of NRAMP-1 on macrophage PTP activity modulation. These investigations led to discover that iron, a metal substrate of NRAMP-1, inhibits PTP activity, resulting in the upregulation of leishmanicidal macrophage functions, through the positive regulation of JAK/STAT and MAPK signalling. Furthering these observations, an in depth study of the mechanisms underlying iron-dependent PTP inhibition (presented as Chapter 3), identified mononuclear dicitrate iron citrate complexes as specific PTP inhibitors.

Despite the role of macrophages as efficient accessory and effector immune cells, *Leishmania* has evolved strategies to downregulate host cell functions. This is largely mediated by the parasite-induced activation of macrophage PTPs. In Chapter 4 we identified PTP1B and TCPTP as two novel PTPs engaged upon *Leishmania* infection. More importantly, we unravel an intimate interaction between the *Leishmania* surface protease GP63 and host PTPs, revealing a novel mechanism of PTP cleavage-dependent activation.

Collectively, our investigations have provided evidence for novel mechanisms of PTP regulation by host and parasite factors. Early activation of macrophage PTPs via GP63-dependent cleavage promotes an environment for the proper establishment of *Leishmania* parasites. Conversely, long-term iron-dependent regulation of PTP activity, ultimately contributes to the successful control of the intracellular pathogen. These results illustrate the importance of balanced signal transduction in the outcome of *Leishmania* infection, and provide insights for the development of novel control strategies.

RÉSUMÉ

L'issue d'une infection avec *Leishmania* dépend de la réponse de l'hôte ainsi que des facteurs pathogéniques. Le macrophage, la cellule hôte, est spécialisée dans l'internalisation et le développement intracellulaire du parasite *Leishmania* et joue un rôle clé dans le contrôle de l'infection. Les fonctions effectrices et accessoires du macrophage proviennent de l'activation des voies signalétiques, qui à leur tour sont largement contrôlées par des événements de phosphorylation de protéines. Donc, la régulation de l'activité des protéines kinases et phosphatases devient essentielle dans la progression séquentielle d'une cascade signalétique et, par conséquent, dans le contrôle des fonctions inflammatoires et antimicrobiales du phagocyte.

Cette thèse doctorale propose de nouveaux mécanismes de régulation des protéines tyrosine phosphatases (PTPs) dans le cadre des interactions entre le macrophage et *Leishmania*. Dans cette étude, nous présentons deux événements dans lesquels des facteurs de l'hôte ainsi que du parasite influencent, de façon indépendante, la régulation différentielle de l'activité PTP du macrophage. Le Chapitre 2 décrit le rôle du NRAMP-1 dans la modulation de l'activité PTP du macrophage. Ces recherches nous ont conduits à découvrir que le fer, un métal substrat du NRAMP-1, inhibe l'activité PTP, ayant comme effet l'augmentation des fonctions leishmanicides du macrophage en régulant de façon positive les voies signalétiques JAK/STAT et MAPK. En plus de ces observations, dans une étude plus approfondi des mécanismes responsables de l'inhibition des PTPs dépendents du fer (présentées dans le Chapitre 3), nous avons identifié les complexes mononucléaires dicitrate fer citrate comme des inhibiteurs spécifiques des PTPs.

Malgré le rôle central du macrophage comme cellule accessoire et effectrice du système immunitaire, *Leishmania* a développé des stratégies afin de réguler de façon négative les fonctions de sa cellule hôte. Celles-ci sont en grande partie mediées par l'activation des PTPs du macrophage causée par le parasite.

Dans le Chapitre 4, nous décrivons l'identification de PTP1B et TCPTP comme deux nouvelles PTPs activées au cours de l'infection avec *Leishmania*. Notamment, nous avons découvert une interaction intime entre la protéase de surface GP63 de *Leishmania* et des PTPs de la cellule hôte, ce qui révèle un nouveau mécanisme d'activation de PTPs dépendent de leur clivage.

Dans l'ensemble, nos recherches fournissent des évidences qui appuient l'existence d'un nouveau mécanisme de régulation de PTPs medié par des facteurs du parasite ainsi que de l'hôte. L'activation rapide des PTPs du macrophage *via* leur clivage dépendent du GP63 favorise un environnement pour l'établissement du parasite *Leishmania*. Par contre, la régulation à long terme de l'activité PTP mediée par le fer contribue au contrôle efficace du pathogène intracellulaire. Ces résultats illustrent l'importance d'une équilibre dans transduction des signaux dans l'issue de l'infection avec *Leishmania*, et fournissent un aperçu qui pourrait contribuer au développent de nouvelles strategies du contrôle.

CONTRIBUTIONS OF AUTHORS

In compliance with the guidelines of the Faculty of Graduate and Postdoctoral Studies of McGill University, concerning thesis preparation, I am presenting the experimental part of this doctoral thesis in a manuscript-based form. Chapters 2 through 4 correspond to manuscripts which have been published (Chapter 2), submitted (Chapter 4), or to-be-submitted (Chapter 3).

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

- **Gomez M.A**., Li, S., Tremblay, M.L., Olivier, M. *NRAMP 1 Expression Modulates Protein Tyrosine Phosphatase Activity in Macrophages: Impact on Host Cell Signalling and Functions J.Biol.Chem.* 2007 Dec 14; 282(50):36190-8. (Presented as Chapter 2)

    - Gomez, M.A.: All the experimental work and manuscript writing.
    - Li, S.: Contributed with STAT and MAPK western blots, STAT and NFκB Electromobility Shift Assays.
    - Tremblay, M.: Assisted with discussion regarding protein tyrosine phosphatases and manuscript editing.
    - Olivier, M.: Manuscript editing and constant experimental supervision.

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    - Alisaraie, L.: Computational modelling of iron citrate complexes and SHP-1 interactions. Manuscript editing.
    - Berghuis, A.: Manuscript editing and discussion regarding structural and computational approaches.
    - Lebrun, C.: Electrospray Ionization Mass Spectrometry.
    - Gautier-Luneau, I.: Electrospray Ionization Mass Spectrometry and discussion on chemistry of iron citrate complexes. Manuscript editing.
    - Olivier, M.: Manuscript editing and constant experimental supervision.

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    - **Gomez, M.A.:** All the experimental work and manuscript writing.
    - Contreras, I.: Performed nitric oxide determinations and JAK-2 western blot.

- Halle, M.: Assisted with manuscript editing.
- Tremblay, M.: Provided GST-PTP1B, GST-TCPTP and GST-PTP PEST constructs.
- McMaster, R.W.: Provided *L. major, L. major* GP63$^{-/-}$ and *L. major* GP63^{R} strains, and recombinant GP63.
- Olivier, M.: Manuscript editing and constant experimental supervision.

CONTRIBUTIONS TO ORIGINAL SCIENTIFIC KNOWLEDGE.

1. Unravelled a novel mechanism of NRAMP-1-mediated regulation of macrophage functions and control of *Leishmania* infection, revealing a direct linking between its biochemical metal transport function and the modulation of phagocyte signalling pathways.

2. Showed both *in vitro* and *in vivo,* that Fe^{3+}-containing solutions modulate macrophage PTP activity and MAPK signalling, strongly suggesting an important biological role for an iron-dependent regulation of cellular protein tyrosine phosphatase activity.

3. Identified, by biochemical and computational approaches, mononuclear iron citrate complexes as potent protein tyrosine phosphatase inhibitors.

4. Identified PTP1B and TCPTP as two macrophage protein tyrosine phosphatases engaged upon *Leishmania* infection, and showed the important contribution of PTP1B during the early development of murine cutaneous leishmaniasis.

5. Demonstrated a direct interaction between the macrophage protein tyrosine phosphatases PTP1B, TCPTP and SHP-1, and the *Leishmania* protease GP63, resulting in the cleavage and modulation of protein tyrosine phosphatase activity, responsible for the downstream downregulation of host signalling pathways.

6. Showed that *Leishmania* GP63 is internalized into host macrophages in an event mediated in part via lipid raft domains, revealing for the first time the internalization of a *Leishmania* surface protein and its interaction with intracellular host protein substrates. This observation opens interesting new perspectives into the host-pathogen interactions governing *Leishmania* infection.

CHAPTER 1

INTRODUCTION

“Approximately 1 billion people – one sixth of the world's population – suffer from one or more neglected tropical diseases […] Nonetheless, under extremely challenging conditions, dramatic achievements have been made in recent decades: 14.5 million people have been cured of leprosy; the number of people infected with guinea-worm disease has dropped from 3.5 million to just 10 000; more than 25 million hectares of land previously infested with black flies leading to river blindness are available for resettlement and cultivation; millions of people are now protected from lymphatic filariasis. Schistosomiasis has been effectively controlled in Brazil, China and Egypt and eliminated from the Islamic Republic of Iran, Mauritius and Morrocco. Intestinal helminths have been eliminated in the Republic of Korea and are under control in many endemic countries. **These successes demonstrate that interventions against neglected tropical diseases are technically feasible, immediate, visibly powerful and highly cost effective** […] We now need to urgently work together with endemic countries and the international community to improve neglected communities’ access to the rapid-impact interventions and quality care to protect them from neglected tropical diseases and to ensure their human and social development. The need to do so is incontestable from all perspectives: moral, human rights, economic and global public good. The task is feasible and must be done.”

Extracted from the WHO Brochure

“*Neglected tropical diseases, hidden successes, emerging opportunities”*

Dr Lorenzo Savioli

Director

Department of Control of Neglected Tropical Diseases

World Health Organization, Geneva [1]

Infectious and parasitic diseases represent the second leading cause of deaths in the world after cardiovascular diseases [2]. Among these is leishmaniasis, a neglected tropical disease which currently affects more than 12 million people globally, with over 2 million new infections occurring per year [3]. In the absence of an available vaccine, the increased emergence of drug resistance, and the toxicity, elevated costs and inaccessibility of current chemotherapy, research on transmission, drug targets, vaccine development and host-pathogen interactions are international priorities of high relevance to public health in the developing world. During my doctoral thesis research, I have investigated the mechanisms by which *Leishmania* parasites modulate the host immune response, in an effort to better understand the interactions between this parasite and its host and to provide novel insights for future strategies of disease control.

1. LEISHMANIASIS

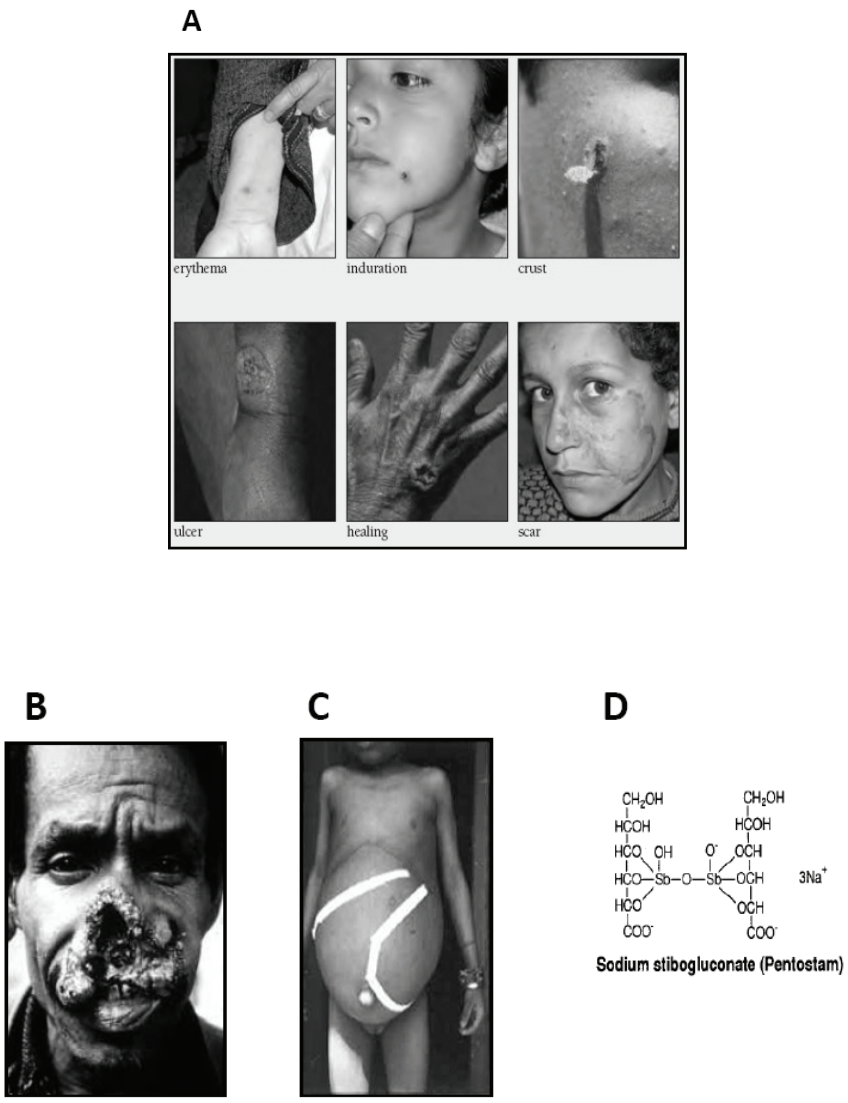
Leishmaniasis is a sandfly-borne infectious disease caused by eukaryotic protozoan parasites of the genus *Leishmania* [4]. It comprises a complex of diseases which, depending on the infecting *Leishmania* species, could range from self healing cutaneous ulcers, disfiguring mucocutaneous lesions, to the often fatal visceral form. Although more than 15 *Leishmania* species are pathogenic to humans [5, 6], *L. major, L. donovani* and *L. braziliensis* are three prototypes of the main clinical outcomes (cutaneous, visceral and mucocutaneous leishmaniasis, respectively) described below.

1.1 Clinical Manifestations

Cutaneous leishmaniasis (CL).

CL is manifested by papules, nodules or ulcerative skin lesions (Figure 1A), usually localized to the site of sandfly bite, and is the most prevalent form of the

FIGURE 1. Clinical Outcomes of Leishmaniasis.



A) Manifestations of cutaneous leishmaniasis ranging from small papules, to enlarged ulcerating lesions. Adapted from adapted from [7] **B)** Mucocutaneous leishmaniasis compromising the nasopharyngeal region and manifested by facial disfiguration. Adapted from http://www.wehi.edu.au/facweb/faculty/emanuela_handman/images/visceralMucoLesion.jpg **C)** Visceral leishmaniasis. Adapted from Adapted from [8] **D)** Pentavalent antimony-based drugs are still the first line treatment against leishmaniasis in most afflicted countries. Adapted from [9]

disease. It is widely distributed, present in Central and South America; Mediterranean and Caspian Sea regions; North, Central Africa and the Middle East. CL is caused by a broad range of *Leishmania* species including *L. major, L. tropica*, *L. mexicana*, *L. amazonenesis*, *L. panamensis*, and *L. guyanensis* [4]. Although most of the cases of CL self-heal within 2-15 months post-infection, serious disability and permanent scarring represent a major socio-cultural and psychological stigma [10].

Mucocutaneous leishmaniasis (MCL)

MCL is the result of the parasite dissemination from the site of sandfly inoculation into mucosal areas of the nasopharyngeal region, ultimately resulting in facial disfiguration and potential life threatening inflammatory disease [4] (Figure 1B). Cases of MCL are only associated with infections with members of the *Leishmania Viannia* subgenus such as *L.(V) braziliensis, L.(V) panamensis* and *L.(V) guyanensis,* restricted to South American countries. Mucosal dissemination occurs in 1-10% of infections, developing 1-5 years after the initial cutaneous lesion has healed.

Visceral leishmaniasis (VL)

90% of VL cases, caused by *L. donovani* and *L. chagasi,* occur in poor rural and suburban areas of 5 countries: Bangladesh, India, Nepal, Sudan and Brazil [10]. Infection outcomes range from subclinical asymptomatic cases, to the fatal viscerotropic disease (Kala Azar). Hepatosplenomegaly and severe immunodeficiency are hallmarks of VL, often leading to death from cachexia and secondary infection (Figure 1C). Although antimonial-based chemotherapy (Figure 1D) -first line of treatment against leishmaniasis- successfully controls VL, drug resistance has emerged as a major issue for disease control. Recent epidemiological studies have shown that in extreme cases such as the hyperendemic district of Bihar (India), refractory VL to antimonial drug treatment can reach up to 50% of reported cases [9]. Moreover, even in situations where primary

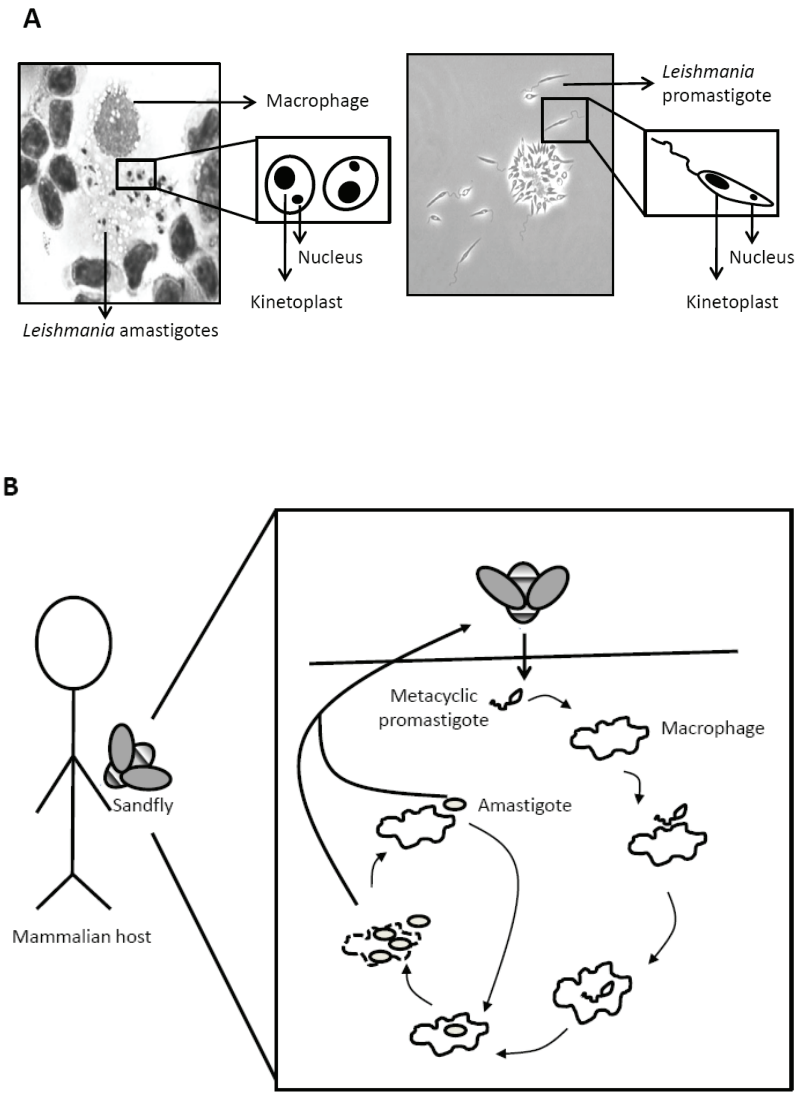
treatment has been successful in controlling the infection, a considerable percentage of VL patients present symptoms of disease relapse [4].

1.2. Life Cycle

Leishmania parasites alternate between two main life stages: one within its insect vector as an extracellular flagellated promastigote (Figure 2A *left panel*) and the second inside the vertebrate host as an obligate intracellular aflagellated parasite, the amastigote (Figure 2A *right panel*). *Leishmania* infection is established in the mammalian host (including humans, dogs and rodents) following the inoculation of a small number (100-1000) of infective metacyclic promastigotes upon a female sandfly blood meal (Figure 2B). Transmission between mammalian hosts is dependent on more than 50 different sandfly species (*Phlebotomus spp.* and *Lutzomia spp.*), each of which is specific to one or multiple *Leishmania* species [11].

Metacyclic promastigotes are phagocytized primarily by cells of the monocyte/macrophage lineage, and reside inside the phagolysosome or parasitophorous vacuole, within which differentiation into the proliferating amastigote form takes place. Although macrophages are regarded as the canonical host cell, neutrophils [12, 13], dendritic cells [14] and fibroblast [15] have also been described as *Leishmania* harbouring cells. Inside its host cell, *Leishmania* amastigotes replicate by binary fission, and are ultimately liberated after macrophages rupture, continuing then to infect neighbouring cells. Although still unclear the mechanisms governing parasite tropism from the local area of sandfly feeding to distal body sites (spleen, liver, bone marrow or mucosal areas), it is suggested that infected host cells may act as vehicles for parasite dissemination within the vertebrate host. It has been shown that Langerhans cells (epithelial dendritic cells -DCs-) engulf and transport the parasite to the draining lymph node for antigen presentation [16].

FIGURE 2. *Leishmania* Life Cycle.



A) The small (2-6 µm) round-shaped amastigotes (left panel) are the aflagellated obligate intracellular stage found in the vertebrate host. Amastigotes replicate within the parasitophorous vacuole or phagosome of infected macrophages, and thus are highly adapted to acidic pH, high temperature (37°C), and nutrient limitations. Promastigotes (right panel) are flagellated slender forms 15-30 µm in length, who survive and replicate within the midgut of the sandfly vector. **B)** The life cycle of *Leishmania* parasites alternates between its development within the sandfly vector and the mammalian host. Transmitted to humans by *Lutzomia sp.* -New World- or *Phlebotomus sp.* -Old World-, promastigotes are injected via the bite of the sandfly, and are rapidly internalized into host cells where they differentiate into the amastigote form. Adapted from [17] [18]

Closing the *Leishmania* life cycle, a sandfly takes a blood meal from an infected vertebrate. The insect vector acquires host cells harbouring intracellular amastigotes. Once inside the sandfly gut, amastigotes will differentiate into procyclic promastigotes 12-18 hours following the blood meal. After extensive replication and consecutive differentiation for about 7 days, promastigotes will develop into the infective metacyclic stage (metacyclogenesis). Metacyclogenesis involves the upregulation of parasite factors involved in host-pathogen interactions, including lipophosphoglycan (LPG) and the major surface protease GP63 [19]. Following another blood meal, metacyclic parasites are injected to a new vertebrate host starting a new round of infection.

1.3 Disease outcome: host and pathogen determinants.

Underlying the disease outcome is the infecting *Leishmania* species, parasite and species-specific factors, and importantly, the host immune response [4]. The observation of asymptomatic clinical cases of CL and VL [20, 21], in addition to the differential disease outcome of inbred mice strains [22, 23], suggest that the immune status/immune response of the host plays an essential role in the development and progression of leishmaniasis.

The immunology and host genetics governing the pathogenesis of leishmaniasis are complex. Comparative studies of resistant and susceptible mouse strains, predominantly to *L. major* or *L. donovani* infections, have clearly shown the association of one or multiple genes/genetic loci to the susceptible phenotype [22]. Of similar importance is the innate and acquired immunity, where the type of T cell response elicited results critical for the subsequent disease progression; IFN-γ / IL-12 driven T-cell activation results in the polarization of a T Helper 1 (Th1) -response and control of the disease [24], whereas a predominantly IL-4-driven polarization and IL-10-dependent maintenance of a T Helper 2 (Th2) response [25] will inevitably result in disease progression and a non-curing phenotype [23].

1.3.1 Determinants of genetic resistance and susceptibility to *Leishmania* infection.

As varied as the pathologies associated to different *Leishmania* species, are the host genetic determinants of susceptibility. For example, association studies in humans have shown that polymorphisms in genes that encode HLA class I and class II molecules influence susceptibility to CL or MCL by *L. braziliensis* and *L. guyanensis* [26], but not to VL caused by *L. donovani* or *L. chagasi.* Conversely, polymorphisms in the human *Slc11A1* gene, formerly known as NRAMP-1 (Natural Resistance Associated Macrophage Protein-1), have been associated with the development of VL caused by *L. donovani* infection [27], but not with the CL or MCL disease caused by *L. major* or *L. braziliensis* infection (reviewed in [22]).

Adding to the complexity of host genetics to leishmaniasis is the multigenic nature of disease control [28]. Human and mouse VL caused by *L. donovani* together with *L. major* infection in the highly susceptible BALB/c mice, show characteristics of a metastatic disease with parasite migration to the draining lymph nodes, spleen, liver and in extreme cases to the bone marrow and lungs. Genetic control of parasite burden in the mouse model of *L. major* infection is organ-specific (Table 1). As an example, the *Lmr3* locus controls serum levels of IFN-γ and IgE, splenomegaly and hepatomegaly. Alternatively, *Lmr5* controls serum levels of IFN-γ, IL-12 and IgE, splenomegaly but not hepatomegaly [29].

The contribution of genetic control to VL caused by *L. donovani* is somewhat clearer. Genes contributing to disease resistance and susceptibility in the early and late stages of the infection have been identified, and although the response seems to be multigenic in nature, single gene effects markedly determine disease outcome.

In the acute phase of *L. donovani* infection (early stage from 2-4 weeks post-inoculation), parasite migration to the liver and spleen takes place. During this initial stage, parasite burden and disease control is largely attributed to the effects of the *Nramp-1/Slc11a1* gene, where resistant mice show more than 100 fold decrease in

TABLE 1.

Genetic determinants of *L. major* infection in mice

Gene / Genetic loci	Trait controlled	Reference
Lmr1	Skin lesion	[30]
Lmr2	Skin lesion	[30]
Lmr5	IFN-γ, IgE, IL-12, splenomegaly but not hepatomegaly.	[29]
Scl-1 (candidate gene Irf-1)	IL-12 responsiveness	[31]
Lmr12	IgE, TNF-α, IL-4, proliferation	[29]
Lmr3	IFN-γ, IgE, splenomegaly, hepatomegaly.	[29]
Lmr30	Skin lesion	[32]

This does not represent an exhaustive list of mapped gene associated to resistance/susceptibility to L. major *infection in mice. Refer to [22].*

parasite numbers in the spleen and liver and no subsequent development of disease, compared to susceptible strains [33]. Identification of a glycine-to-aspartic-acid substitution at position 169 in the NRAMP-1 protein was associated with disease susceptibility in BALB/c mice [34].

NRAMP-1 is a pH dependent divalent cation transporter [35] localized to the late endosome/lysosomal compartment of macrophages from the reticuloendothelial system [36] and present in gelatinase positive tertiary granules of neutrophils [37]. Upon phagocytosis, NRAMP-1 is rapidly recruited to the phagolysosomal membrane where it mediates the transport of Mn^{2+}, Fe^{2+}, Co^{2+}, and potentially other metals including Zn^{2+} [38-41]**.** The direction of metal flux is still controversial [35, 42, 43]; however, transport studies, sequence and structural similarities with NRAMP-2, together with topology and thermodynamic considerations, suggest that metal transport occurs from the vesicular lumen to the cytoplasm [35, 42, 43]. NRAMP-1 expression has been shown to promote phagosome maturation [44-46]. In addition, efflux of essential metals from the phagosome may restrain the pathogen's development by interfering with essential microbial enzymes such as superoxide

dismutase (SOD), and/or by promoting the upregulation of host pro-inflammatory molecules [38].

During the late stages of *L. donovani* infection, parasite numbers in the liver are influenced, although not exclusively, by the haplotype at the *H2* region (the major histocompatibility complex (MHC)) [47] and the immune response locus-2 (*Ir2*). Conversely, parasite burden in the spleen is controlled by the lysosomal trafficking regulator (*Lyst*) [48]. However, the precise mechanisms by which these genes/gene loci influence disease susceptibility are still unknown.

All of the above point to a critical role for genetic control in the development of leishmaniasis both in mice and humans. However, not mutually exclusive, a clear involvement of the innate and acquired immune response in the outcome of infection is established. Certainly, genetic determinants controlling levels of IL-12, IFN-γ, TNF-α, IL-10 and IL-4 production among others influence the re-direction of a healing vs. non-healing response (discussed below).

1.3.2 Th1/Th2 response in *L. major* infection.

Studies of the *in vivo* model of resistance and susceptibility to *L. major* infection, largely helped to understand the important contribution of the polarization of a Th1 or Th2 T-cell response towards the fate of intracellular pathogens [49]. Whereas a predominant Th1 response results in a healing phenotype in *L. major* infection and long-term immunity to re-infection, skewing towards Th2 will ultimately lead to disease progression and a non-healing phenotype [23, 50]. Although this Th1 vs. Th2 dichotomy is clear for infections with *L. major,* a predominant IL-12-driven Th1 response or the maintenance of a Th2 response via IL-10 production, respectively contribute, however not exclusively, to resistance and susceptibility to *L. donovani.* [25].

Following *L. major* infection, an initial type-2 response takes place in both resistant and susceptible mice. Rapidly upon parasite inoculation and interaction with

TABLE 2.

Parasite and host factors involved in the establishment and progression of leishmaniasis.

Molecules	Gene disrupted and Infection Model	Disease Outcome	Reference
	Host		
IFN-γ	IFN-$\gamma^{-/-}$C57BL/6 - *L. major*	Susceptibility	[51]
	IFN-γ receptor $^{-/-}$129/Sv - *L. major*	Susceptibility	[52]
IL-4	IL-$4^{-/-}$ BALB/c - *L. major*	Resistance	[53]
IL-13	IL-$13^{-/-}$BALB/c - *L.major*	Resistance	[54]
	IL-13-overexpressing C57BL/6-*L.major*	Susceptibility	
IL-12	IL-12 $p35^{-/-}$ and IL-12 $p40^{-/-}$ 129/Sv - *L.major*	Susceptible	[24]
IL-6	IL-$6^{-/-}$C57BL/6 - *L.major*	Resistance	[55]
IL-10	IL-$10^{-/-}$BALB/c - *L.major*	Resistance	[56]
iNOS	$iNOS^{-/-}$ C57BL6 x 129/Sv -*L. donovani*	Susceptibility	[57]
TNF-α	TNF-$\alpha^{-/-}$C57BL/6 - *L.major*	Susceptibility	[58]
MyD88	MyD$88^{-/-}$C57BL/6-*L .major*	Susceptibility	[59]
Nramp-1	Nramp-$1^{-/Asp169}$129/Sv -*L.donovani*	Susceptibility	[27]
	Leishmania		
LPG	*Lpg-$1^{-/-}$L.major* -BALB/c	Virulence attenuation	[60]
LACK	*Lack$1^{-/-}$ or Lack$2^{-/-}$L. major*-BALB/c	Virulence attenuation	[61]
GP63	*gp$63^{-/-}$L. major* -BALB/c	Delayed lesion formation	[62]
HASPB	*haspb null L. major* -BALB/c	Virulence enhancement	[63]
CPA/CPB	Δ*cpb L.mexicana*- BALB/c	Virulence attenuation	[64, 65]
	Δ*cpa*Δ*cpb L.mexicana*- BALB/c	No lesion formation	
SIR	*Sir$^{-/-}$L. infantum* BALB/c	Reduced infectivity	[66]
LMPK	Δ*sap1-sap2 L.mexicana*- BALB/c	No lesion formation	[67]
A2	Antisense RNA A2 *L. donovani* - BALB/c	Reduced survival	[68]
LIT	Δ*lit1 L. amazonensis* - BALB/c	Reduced virulence	[69]

host macrophages, host cell functions are inhibited (discussed below) including macrophage IL-12 production [70, 71]. Inhibition of the development of a Th1 response by *Leishmania* is largely mediated by a subset of $CD4^+$ T-cells which recognize the *Leishmania* LACK (*Leishmania* Homologue of Receptors for Activated Kinase C) antigen [72]. This $CD4^+$ T-cell population seems to be responsible for the high levels of IL-4 and to some extent IL-10 production detected in infected mice, preventing T-cell-dependent IFN-γ secretion and subsequent macrophage activation and parasite killing. Although this initial Th2 polarization is not necessarily the decisive event for the healing vs. non-healing phenotype, the persistence of a Th2 response undoubtedly is responsible for susceptibility.

During the late events of *L. major* infection in a susceptible mouse background, IL-4, IL-13 and IL-10 may act in an additive fashion to promote maintenance of a Th2 response [23], as deficiency of any of these three anti-inflammatory cytokines results in enhanced resistance to infection [53, 54, 56] (Table 2). In addition, skewing towards a Th2 response may be enhanced by i) neutrophil recruitment and maintenance, providing a source of anti-inflammatory IL-10 and TGF-β; ii) visceralization, where an environment for sustained Th2 could be more suitable given the presence of lineage specific DCs that can prime a Th2 response; iii) unstable expression of the IL-12 receptor in $CD4^+$ T-cells [23].

Given that an initial Th2 response is established in both resistance and susceptible mouse strains, how is a protective type-I response acquired? Recent investigations provide evidence for a critical role of dendritic cells in the re-direction towards a Th1 response. Rapid deactivation of macrophage functions is a hallmark of *Leishmania* infection [73]. However, DCs are still capable of producing IL-12, necessary to activate T-cells for IFN-γ production and macrophage activation [74]. Additionally, highly susceptible BALB/c mice have a deficiency in IL-1α production by skin DCs compared to resistant C57BL/6 mice [75]. This suggests that IL-1, in conjunction with IL-12, act to polarize a Th1 response. More recently, reports have shown the

downregulation of DC functions by *L. amazonensis* [76], suggesting that species-specific factors may influence the DC response [77]. Although not critical, the IFN-γ production activity of Natural Killer cells (NK) may contribute to Th1 T-cell activation as well, by enhancing DC IL-12 production and expression of T-cell IL-12 receptor [78].

1.3.3 *Leishmania* virulence factors in the development of leishmaniasis.

Genetic and immunological studies in mice have almost exclusively been performed with *L. major* and *L. donovani* as infecting species. The difficulty of disease onset in mouse models challenged with New World species of the *Leishmania Viannia* complex (which include *L. (V) braziliensis, L. (V) guyanenesis, L.(V) panamensis* and *L.(V) peruviana* among others), the chronic nature of *L. mexicana* infection in mouse strains resistant to *L. major* (e.g. C57BL/6 or C3H) [79], and the absence of pathology following inoculation of heat-killed *Leishmania* parasites or parasite homogenates, suggest that intact species-specific factors play an essential role in the establishment of disease and its progression. Not surprisingly the *Leishmania* complexes (*L. Viannia, L. Leishmania (L) major, L.(L) donovani and L.(L) mexicana*) diverged 40-80 million years ago [50], thus suggesting that species-specific determinants control vector competence and disease pathology in the mammalian host (Table 2).

With the recent completion of the genome sequences of three representative etiological agents of cutaneous, mucocutaneous and visceral leishmaniasis (*L. major, L. braziliensis,* and *L. infantum,* respectively) [80, 81], and a total content of ~8,300 genes in the *Leishmania* genome, surprisingly only 2.5% represent species-specific genes. These include elements of an RNAi machinery that are found only in *L.braziliensis,* suggesting that few species-specific virulence factors control disease tropism and pathogenicity. Among the molecules recognized as important virulence factors are the *L. donovani* A2 proteins [82], the *L. mexicana* cysteine peptidases [83], the major surface protease (leishmanolysin or GP63) [84] and LPG [85].

Three different types of *Leishmania* molecular determinants can be subcategorized based on their role during pathogenesis: 1) Infection related determinants, which allow *Leishmania* to establish disease upon initial interaction with the host. These include surface and secreted parasite molecules such as LPG, GP63, Glycosylinositol phospholipid (GIPL) and Cysteine Proteases. 2) Pathogenic determinants, which represent unique parasite epitopes that are involved in eliciting the immunopathology associated to leishmaniasis, often inducing strong B and T cell responses, and include Heat Shock Protein (HSP) 60 and 70, histones H2A/B, H3 and H4 and the LACK antigen among others [86], and 3) Determinants for parasite clearance: this last group of *Leishmania* molecules have been implicated in directing the host immune system towards clearance and resolution of the disease and include the *Leishmania* homologue of the eukaryotic elongation factor 4A (LeIF) and HSP70 [86].

Although many molecules have been described to be involved in *Leishmania* pathogenicity, I will discuss some of the major findings concerning host-pathogen interactions, where clear functions of specific *Leishmania* molecules have been identified, and present recent discoveries of proteins involved in parasite virulence.

Lipophosphoglycan (LPG)

LPG is the most predominant glycoconjugate of infective metacyclic promastigotes, at approximately 5 million copies per cell [87], and distributed along the entire cell surface and the flagellum. Its main structure consists of a polymer of Galβ1-4Man-PO_4 units, attached to the cell surface via a glycophosphatidylinositol (GPI) anchor. Although present throughout the *Leishmania* genus, its structure varies among species suggesting a role in virulence [88, 89]. Its importance as a virulence factor was later confirmed by targeted gene deletion strategies, where Beverley and colleagues showed attenuation of virulence following *Lpg-1* (limiting enzyme in LPG

biosynthesis) gene knockout in *L. major* [60]. However, surprisingly LPG-deficient *L. mexicana* promastigotes are as virulent as wild type parasites, suggesting that LPG plays a selective role in a species-specific manner [90].

In the interface of host-pathogen interactions, LPG has been shown to play an essential role. As a first line of defence, the compact LPG glycocalix contributes to the evasion of complement mediated lysis [91] and, although not essential, binding to the C3b component of the complement cascade, and in conjunction with GP63-mediated conversion to C3bi, promotes parasite internalization via the macrophage C3 receptor (C3R) [92]. LPG has also been shown to downregulate macrophage signal transduction, including, but not restricted to, inhibition of LPS-dependent c-fos gene expression, ERK 1/2 phosphorylation and PKC inhibition [93-96]. This downregulation of macrophage signalling in part contributes to the inhibition of macrophage functions including the nitric oxide (NO) [97], and IL-12 production [98]. Inhibition of macrophage apoptosis [99] and phagolysosomal maturation [87] are also functions attributed to LPG. The mechanism by which LPG prevents macrophage apoptosis remains unclear. Conversely, inhibition of phagolysosomal maturation has been shown to occur via inhibition of PKC signalling and impaired recruitment of endocytic markers, together with the rapid and non-transient accumulation of F-actin around the phagosomal membrane, preventing subsequent vesicle-vesicle interactions [87]. More recently, LPG has been shown to inhibit the proper assembly of the NADPH oxidase complex in the phagosomal membrane, impairing the intraphagosomal generation of superoxide [100]. This finding has important implications, as inhibition of reactive oxygen species accumulation in the site of *Leishmania* development, will contribute to parasite survival.

Recently, two novel roles for LPG have been described; the disruption of host cell lipid raft domains [101] and the induction of mast cell degranulation [102]. It is suggested that disruption of host lipid rafts will impair proper phagosome maturation, given the presence of these microdomains in the phagosomal membrane [103]. On the other hand, mast cell degranulation induced by LPG is thought to differentially favour disease susceptibility in BALB/c and C57BL/6 mice, in each case by an

exacerbated inflammatory response, and activation and maintenance of a protective immune response, respectively.

GP63 (Major surface protease or Leishmanolysin)

Leishmania GP63 (standing for glycoprotein of 63 KDa), otherwise known as leishmanolysisn or major surface protease (MSP), is the most abundant surface protein of *Leishmania* promastigotes, contributing in some species to approximately 1% of the total protein content [104], and to a much lesser extent expressed in the amastigote stage [84, 105]. GP63 is a GPI-anchored zinc metalloproteinase, where a histidine-mediated coordination of a Zn^{2+} atom in the active site allows for catalytic activity [106]. It is expressed as a pro-enzyme, and activated by removal of the N-terminal pro-peptide [107]. Although present in all *Leishmania* species studied to date, varying degrees of GP63 expression, and in some instances, subtle variations in substrate cleavage sites [84, 108], have been described for different species. As an example, one of the most heterogeneous GP63 gene cluster is found in *L. mexicana,* comprising a tandem array of 10 GP63 genes, subdivided in 3 different gene groups [109]. Additionally, 22 GP63 genes are found *in L. guyanensis,* 7 in *L. major,* at least 10 in *L. donovani* [84], and more than 18 in *L. chagasi* [110]. Interestingly, the lizard pathogen *L. tarentolae* has GP63 coding genes, however, no protease activity was detected in this parasite, correlating with its lack of virulence in mammalian systems [111].

The subcellular localization of GP63 is divided in three different pools: the major component being a C-terminal GPI anchored surface GP63, followed by an internal and a released pool [112-115]. Although the mechanism by which GP63 is released to the extracellular milieu is still not fully understood, it has been shown that cleavage by phopholipase C (PLC) seems not to be necessary for release, and a mechanism involving autoproteolysis of surface GP63 is favoured [114]. Nevertheless, alternative mechanisms of release/secretion may be involved including a membrane

or small vesicle-bound GP63 release [113], and a pool of internal GP63 available for rapid secretion through the flagellar pocket [115].

GP63 has been, together with LPG, one of the most well studied molecules of *Leishmania* parasites, identified as a virulence factor by targeted gene deletion of the *L. major* GP63 genes [62]. This protease recognizes a consensus site in its target substrates ($P_1\downarrow P'_1\text{-}P'_2\text{-}P'_3$) where P'_1 corresponds to a hydrophobic amino acid residue, P'_2 and P'_3 to basic residues and P_1 preferentially a polar amino acid [116]. Many substrates have been described including the intracellular macrophage proteins Myristoilated Alanine-Rich C Kinase Substrate (MARCKS), MARCKS-related protein (MRP) [117], and recently NFκB $p65^{RelA}$ subunit [118], as well as extracellular proteins such as fibrinogen, haemoglobin, C3b and albumin among others [116]. It has been therefore implicated in parasite binding to macrophages via a C3bi-C3 receptor mechanisms thus enhancing phagocytosis, evasion of complement mediated lysis, migration through the extracellular matrix [119], induction of a Th2 type immune response demonstrated by the activity of a subset of $CD4^+$ which activated IL-10, IL-4 and TGF-β production [84, 120] and induction of a protective type 1 immune response in vaccine trials [62, 121].

Cysteine Peptidases (CPs)

In addition to GP63, the *Leishmania* Cysteine Peptidases (CPs), whose name makes reference to their catalytic activity dependent on a cysteine residue [122], represent another group of important proteases for parasite virulence. Based on the genome sequence of *L. major [80],* about 65 different genes encode CPs, subdivided into clan CA, CD, CF and PC peptidases [83]. Most of the studies have focused on the role of three CP *Leishmania* family members: CPA, CPB -both Cathepsin L-like CPs- and CPC -Cathepsin B-like-. More recently, investigations have highlighted the importance of metacaspases (distant orthologues of caspases) in organelle (nucleus and kinetoplast)

duplication and segregation during the parasite´s life cycle, rather than cell death. Absence of metacaspases from mammalian cells allows potential for a role as putative drug target [123].

Of the three main CPs, CPB has been described as an important virulence factor [64, 70, 124]. Although present in *L. major* and *L. donovani*, the main role of CPB in virulence has been demonstrated in *L. mexicana,* suggested to be species-specific due to the 19 CPB coding genes, as opposed to 8 of *L. major* [83] and 5 of *L. donovani* [125]. Isoforms of *L. mexicana* CPB are differentially expressed throughout the parasite life cycle, predominantly present in the amastigote stage confined to large lysosomal compartments, and to a much lesser extent in metacyclic promastigotes [126].

Targeted gene deletion of the complete array of CPB genes in *L. mexicana* evidenced its role in virulence, where Δ*cpb* mutants exhibited poor lesion formation in the highly susceptible BALB/c mice [64, 65]. Interestingly, non-redundant but rather complementary functions of the different *cpb* genes was hypothesized, as reconstitution with a single gene did not rescue the virulent phenotype, as opposed to a multiple-gene complementation strategy [127]. Its role as an immunomodulatory molecule roots from the capacity of CPB to favour a Th2 type response by promoting IL-4 production [127], cleavage of the IL-12 receptor and MHC II molecules [128], and inhibiting a Th1 response through the cleavage-dependent abrogation of NFκB activity and consequent inhibition of IL-12 production [70].

The role of CPC in parasite virulence is not clear as Δ*cpc L. mexicana* induces a similar pathology to the wild type strain in BALB/c mice [65]. Nevertheless, a recent role for *L. infantum* CPC as a potential vaccine candidate was elucidated, suggesting that CPCs rather than enhancing parasite virulence, may act to boost a protective immune response in the host [129].

L .donovani A2

The *L. donovani* A2 locus encodes a family of at least 7 cytoplasmic amastigote-specific proteins, interestingly characterized by a predominant 10 amino acid sequence which is repeated 40-90 times in individual genes [130]. Although A2 genes are also present in *L. major,* they lack the amino acid repeats found in *L.donovani* and appear to be non-expressing pseudogenes [131]. Its role as a virulence factor was demonstrated by inhibition of protein and mRNA expression using an antisense RNA approach [68], and showed that A2 deficient parasites were unable to survive neither in *in vitro* macrophage culture or *in vivo.* More interestingly is the discovery of *L. donovani* A2 involvement in parasite tropism and visceralization [131]. Not only *L. major* expressing the *L. donovani* A2 proteins have an impaired capacity to develop the cutaneous infection in susceptible BALB/c mice [131], but exhibited increased visceralization and survival in the spleen of BALB/c mice [82].

Lastly, the A2 proteins have been extensively studied for their strong antigenic capacity, as observed by seroreactivity with serum from patients with visceral leishmaniasis [132], and in several occasions has been studied as a vaccine candidate. Vaccination studies have shown the capacity of A2 to direct an IFN-γ-driven Th1 response [133, 134] and an effective anti-A2 antibody response [135].

Leishmania Homologue of Receptors for Activated C Kinase (LACK)

The importance of the *Leishmania* LACK antigen (the *Leishmania* homologue of mammalian RACKs -receptor for activated C kinase-), is based on its ability to direct an early Th2 response (2-3 days after infection), by specifically activating a subset of IL-4 secreting $CD4^+$ T-cells [72]. LACK localizes close to the kinetoplast (the unique mitochondrial organelle) and is suggested to interact with proteins involved in DNA replication and RNA synthesis [136]. A vital function for this protein in parasite survival is suggested, as targeted gene deletion of both *L. major lack* genes did not

generate viable parasites. Nevertheless, independent deletion of *lack-1 or lack*-2 genes generated highly attenuated *L. major* parasites, as evaluated by infection in BALB/c mice [61].

An important characteristic of the LACK protein is its high antigenicity, a property that has been exploited for vaccine development [137, 138], and most recently shown to enhance protection if provided in combination with CPA, TSA (homologue to eukaryotic thiol-specific-antioxidant protein) and LmSTI (*L. major* homologue to eukaryotic stress-inducible protein) as a DNA vaccine [139].

Hydrophilic Acylated Surface Protein (HASP)

The HASP genes (*HASPA1, HASPA2* and *HASPB*) are one of the few chromosomal regions absent in *L.braziliensis,* but present in *L. major* and *L. infantum* [81] and exclusively regulated for stage-specific expression[140], localizing to the plasma membrane of amastigotes and metacyclic promastigotes [140-144]. Functional analysis of the HASP proteins, using a transgenic parasite approach, has revealed putative roles in parasite virulence and maintenance within the host; in an *in vitro* model of infection, HASP null parasites infect and survive within macrophages as efficiently as wild type parasites, regardless of their higher sensitivity to complement-mediated lysis. Interestingly however, null parasites lead to an early onset of footpad lesion formation in BALB/c [63], suggesting that rather than inducing pathogenicity, HASPB may play a role in attenuating the immune response, possibly promoting parasite persistence.

The central domain of HASPB contains a series of amino acid repeats [145], shown to be immunomodulatory in the mouse model, by leading to the activation of $CD8^+$ T-cells through induction of IL-4 production [146]. Vaccination studies with *L. major* and *L. donovani* recombinant HASPB have shown protection in mice and dogs, a major reservoir for visceralizing *Leishmania* species [147, 148].

Kinases and others

In *Leishmania,* signal transduction plays an important role in the control of multiple intracellular events, predominantly controlling parasite differentiation, cell cycle, and stress responses [149]. Additionally, a function of *Leishmania* protein kinases in parasite virulence has been described, adding to the importance of signal transduction in this group of parasites [67]. Deletion of the *L. mexicana* soluble acid phosphatase (SAP) locus, comprising the two SAP genes separated by an intergenic region of approximately 11.5 kb, impaired the parasite´s ability to cause a progressive disease in BALB/c mice. Interestingly, re-complementation with the intergenic region, later identified as a *Leishmania* Mitogen-Activated Protein Kinase (MAPK) homologue, rescued the virulent phenotype, identifying an essential function of this protein in amastigote proliferation[67].

In addition to protein kinases, “metabolic kinases” have also been shown to be important for parasite virulence. Recently, the identification and targeted gene deletion of the *Leishmania* thymidine kinase (deoxyribonucleotide kinases), rate-limiting enzyme in deoxyribonucleotides synthesis, showed its partial requirement for parasite survival within BALB/c mice peritoneal exudate cells [150].

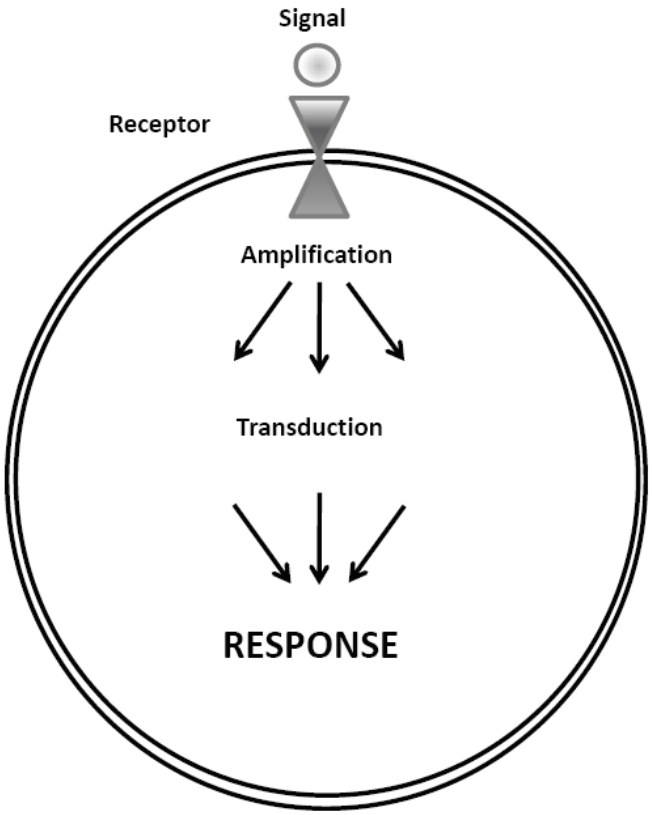
Lastly, the activities of trypanothione reductase (essential for the red-ox defence system of *Leishmania*) [151], cytosolic Silent Information Regulator 2 deacetylase (SIR2, proposed to act as a metabolic sensor in *Leishmania* parasites) [66], β1-2 mannan and the glucose transporter (important for the uptake of glucose and other hexoses) [152], were found necessary for infection and survival within the host.

1.4 Modulation of host cell signalling by *Leishmania* parasites.

The engulfment and internalization process of *Leishmania* parasites by host macrophages occurs within minutes of the initial cell-cell interaction [153]. Therefore, in order to survive, this protozoan parasite has developed ingenious strategies to overcome the innate immune response, rapidly triggering the downregulation of key host cellular functions. Although as a general rule *Leishmania* parasites inactivate the pro-inflammatory components of the innate immune response, in very specific instances it also leads to the activation of some functions such as macrophage chemokine gene expression and anti-inflammatory cytokine production. The balance of positive and negative signals, will ultimately define the course of infection.

In higher eukaryotes, rapid control of cellular functions in response to extracellular stimuli (microbe, hormone, protein, lipid, carbohydrate, crystal, etc.) is determined by signalling events, largely regulated by protein-protein interactions, protein phosphorylation and second messengers such as Ca^{2+}, cAMP and phosphoinositides. The triggering of a signal transduction cascade usually results from a receptor mediated event, where detection of the external stimulus by a transmembrane receptor in the cell, initializes the amplification and integration of signals, resulting in a specific cellular response such as changes in enzyme activity, gene expression, or ion-channel activity and an active communication of the cell with its immediate environment (Figure 3) [154].

FIGURE 3. Signal Transduction.



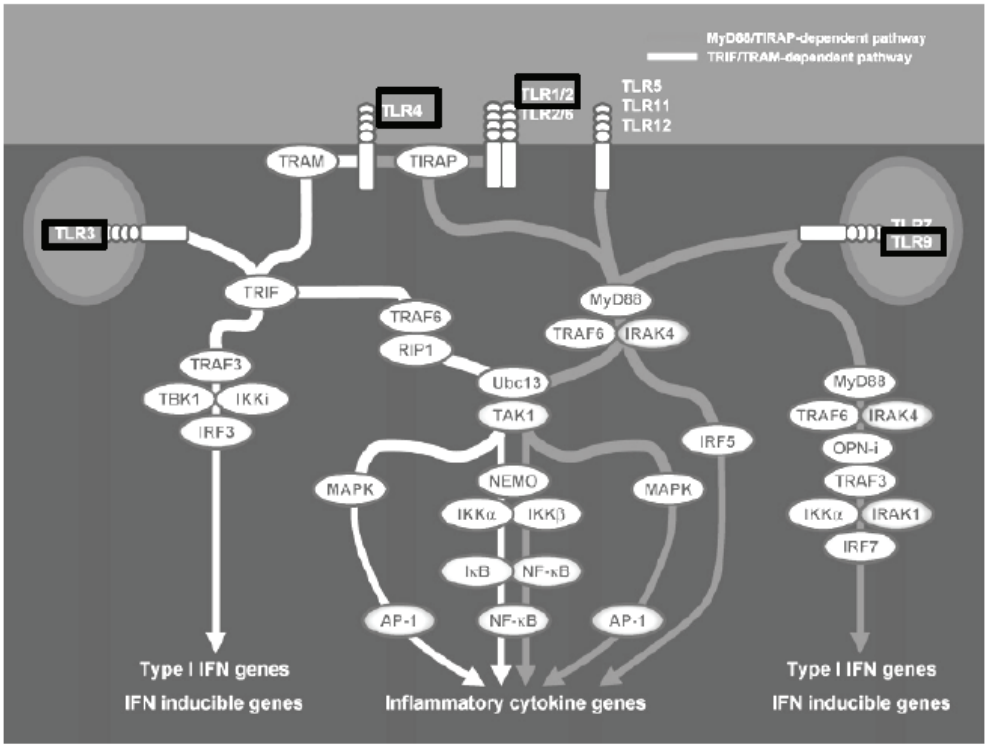
Cellular responses to the extracellular environment are coordinated by signalling events which begin from conformational changes, activation, or protein modifications of transmembrane receptors in response an external stimulus. These modifications are the trigger of a series of protein-protein interactions, protein-lipid interactions, second messenger signalling, post-translational modifications, etc. which result in the amplification and transduction of the extracellular signal, finally leading to specific gene transcription and protein translation which coordinate individual cellular responses.

1.4.1 Host cell receptors that interact with *Leishmania* parasites.

The initial interaction of *Leishmania* promastigotes with multiple macrophage receptors has been described [155]. It has been shown that the *Leishmania* surface protease GP63 interacts with the receptors for fibronectin (β1-integrins) [156] and complement receptors [92, 157], potentially cooperating to promote parasite adhesion and internalization into the host cell. Additionally, the Fcγ receptor has been also implicated in the initial host-pathogen interaction [158]. Although previous studies have identified a role for the carbohydrate mannose-fucose receptor in the initial interaction and internalization of *Leishmania* [159, 160], recent investigations have demonstrated that the mannose receptor is not necessary for triggering the downregulatory signals hallmark of this parasitic infection [161]. All together, these "promiscuous" interactions lead to an array of signalling events which orchestrate an end result of general downregulation of normal macrophage functions such as the inhibition of nitric oxide (NO) and reactive oxygen species (ROS) production; inhibition of pro-inflammatory cytokines including IL-12 and TNF-α; inhibition of phagolysosome maturation and MHC II-mediated antigen presentation [73].

More recently, an important role for Toll-Like Receptor (TLR) mediated signalling events in *Leishmania* infection has emerged. Cell membrane (TLR 1, 2, 4, 5, 6, 1 and 12) or intracellular (TLR 3, 7 and 9) TLRs recognize pathogen associated molecular patterns (PAMPs), triggering signal transduction mediated in all instances, with the exception of TLR3, by the recruitment of MyD88, IRAKs and TRAF6. This protein recruitment ultimately activates NFκB, AP-1, IRF-3 and IRF-7 transcription factors, resulting in the expression of inflammatory cytokine genes, type I interferons (IFN-α and IFN-β) and IFN-inducible genes (Figure 4) [162]. In *Leishmania* infection, deficiency in the adaptor protein MyD88 in C57BL/c mice renders this genetically resistant mouse strain completely susceptible to *L. major.* This observation prompted to the identification of LPG as a TLR2 ligand [59] and activator of the signalling cascade. Parallel observations in support of this finding showed an LPG-TLR2

FIGURE 4. Toll-Like Receptor signalling in *Leishmania* Infection.



Shown to be involved in *Leishmania* infection

Signaling through TLRs 2, 3, 4 and 9 has been independently shown to occur following *Leishmania* infection. Specific parasite molecules have been implicated in such events including LPG (see text for details). Adapted from [162]

mediated activation of NK cells, resulting in higher NFκB nuclear translocation and enhanced IFN-γ and TNF-α production [163]. More recently, TLR9-dependent activation of NK cells was also reported in a model of *L. major* infection, where a TLR9-dependent IL-12 production by DCs activated IFN-γ production and NK cytotoxicity. This event was elicited both by *L. major* DNA or intact *L. major* promastigotes. However, although TLR9 is necessary for *L. major*-mediated NK cell activation, it is not essential for ultimate parasite control [164, 165]. TLR4 was shown to play an important role in the successful control of *L. major* infection largely through NO production. However, the specific *Leishmania* ligand has not yet been identified [166]. TLR2 and TLR3 have also been involved in the activation of IFN-γ primed *L. donovani* infected macrophages, resulting in enhanced secretion of NO and TNF-α [167].

As opposed to the activation observed in IFN-γ primed *Leishmania* infected macrophages, *Leishmania* downregulates TLR-signalling in naïve macrophages. Recently, our group has reported an important breakthrough in the negative regulation of TLR-signalling upon *Leishmania* infection [168]. Abu-Dayyeh and colleagues have shown that *Leishmania* infection mediates the inactivation of the central TLR signalling molecule (with the exception of TLR 3) IRAK-1. IRAK-1 inactivation results from SHP-1 activation and direct binding to IRAK-1, inhibiting its intrinsic kinase activity and preventing its dissociation from the MyD88 complex, therefore dowregulating downstream LPS-induced functions such as NO and TNF-α production.

1.4.2 Macrophage signalling pathways altered by *Leishmania* infection.

Rapidly following *Leishmania*-macrophage interactions, inhibition of host cell functions provides a suitable environment for the establishment of the intracellular pathogen. Among these is the inhibition of ROS and NO production; inhibition of phagolysosome maturation; inhibition of TNF-α, IL-12 and IL-1β production [73].

Common to these pro-inflammatory macrophage responses is their control by upstream signalling pathways including the JAK/STAT, MAPK and PI3K pathways. Adequate signal transduction, in all instances, is in its most part achieved by the fine tuning of phosphorylation events, controlled by the balanced action of protein phosphatases and protein kinases. Here I will discuss the current knowledge on the impact of *Leishmania* infection on these signalling pathways and their respective enzymatic regulators, providing a comprehensive view of a global mechanism of signalling-dependent inhibition of macrophage functions.

MAPK (Mitogen-activated protein kinases)

The MAP Kinases - extracellular signal-regulated kinases 1 and 2 (ERK1/2), p38 MAPK and c-Jun N-terminal kinase (JNK) - are the effector kinases of signalling cascades responsible for the regulation of cellular functions in response to various extracellular stimuli [169], and in macrophages, for the regulation of functions involved in inflammation and host defence. Inhibition of MAPK signalling (ERK, JNK and p38 pathways) by *Leishmania* promastigotes has been reported in several instances. In naïve macrophages, *L. donovani* infection fails to induce the phosphorylation/activation of p38, JNK and ERK1/2, for the later in an LPG-dependent manner, terminating in the inactivation of NFκB and AP-1-dependent gene transcription [95]. Following *L. donovani* infection, downregulation of ERK 1/2 signalling has been attributed to an increased macrophage ceramide synthesis following infection [170], and more recently inhibition of ERK 1/2 phosphorylation was reported to be dependent on the rapid activation of the host protein tyrosine phosphatase (PTP) SHP-1 [171]. Interestingly, a cleavage-dependent inactivation mechanism of ERK 1/2 and JNK, but not p38 was reported following infection with *L. mexicana* amastigotes, and this was dependent on CPB expression [70]. This observation suggests that Old Word and New World *Leishmania* species may utilize additional or different mechanisms to inactivate MAPK signalling,

but will ultimately converge in one same end result: the downregulation of host cell functions.

JAK/STAT (Janus Kinases / Signal Transducers and Activators of Transcription)

IFN-γ inducible functions including NO and IL-12 production, together with MHC II expression, are controlled upstream by the JAK-2/STAT-1 signalling pathway. Alterations in this cascade have been extensively reported as a result of the *Leishmania*-induced activation of SHP-1 and subsequent dephosphorylation of JAK2 [172], and to a PKC-α-dependent proteasome-mediated degradation of STAT-1 [173]. Activation of SHP-1 results in the tyrosine dephosphorylation of JAK2 and abrogates the recruitment of STAT-1, its subsequent dephosphorylation, translocation to the nucleus and regulation of gene transcription. Added to these results, de Veer and colleagues have shown that LPG mediates induction of the Suppressor of Cytokine Signalling (SOCS) [174] SOCS-1 and SOCS-3 mRNA expression [59], possibly contributing to the negative regulation of IFN-γ signalling. This, together with the proteolytic degradation of STAT-1 and the dephosphorylation/inactivation of JAK2 ensures an efficient downregulation of IFN-γ signalling in *Leishmania*-infected macrophages.

PKC (Protein Kinase C)

Classic PKCs are phospholipid (phosphotydilserine -PS- and diacylglycerol -DAG-) and calcium-dependent serine/threonine kinases, ubiquitously expressed and involved in the regulation of multiple signalling cascades including the MEK-ERK and Akt-PI3K pathways [175]. Relevant to the infection with intracellular pathogens, PKC has been shown to positively regulate receptor mediated phagocytosis and the process of phagosome maturation [176, 177].

PKC inactivation following *L. donovani* infection has been reported [178], and shown to be dependent on the expression of LPG [96]. Using a macrophage cell line overexpressing a dominant-negative mutant of PKCα, the intracellular survival of *L.donovani* was enhanced 10-20 fold 48 hours after infection [179]. PKC inactivation by *Leishmania* contributes to the inhibition of phagosomal maturation [177] and generation of ROS [180].

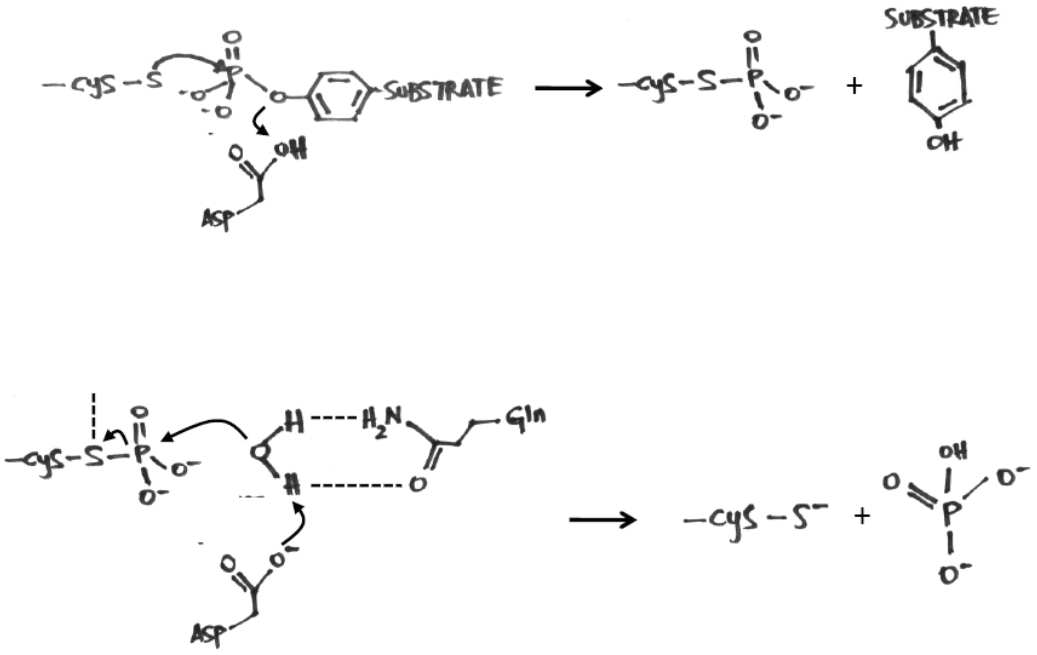
Leishmania infection rapidly triggers the augmentation of intracellular Ca^{2+} concentration [181], modulating signalling dependent on this secondary messenger. Ca^{2+} acts as a cofactor for a number of enzymes including the serine/threonine phosphatase calcineurin (PP2B) and PKC. Correlating with this increase in intracellular [Ca^{2+}], a rapid PKCα phosphorylation/activation following *L. donovani* infection (10 minutes up to 1 hour following parasite-macrophage contact) was recently reported, shown to contribute to the proteasome degradation of STAT-1 [173]. These results support an important role of PKC in the regulation of macrophage functions and the control of infection, and suggest that *Leishmania* can rapidly and transiently activate PKC resulting in STAT degradation, but more stably and at later points will inactivate PKC for inhibition of phagosome maturation and ROS production.

2. PROTEIN TYROSINE PHOSPHATASES (PTPs)

With the completion of genomic sequences of higher eukaryotes including plants and mammals, an estimated 30% of all proteins are targeted for post-translational modifications by phosphorylation on serine, threonine or tyrosine residues. The balance of protein phosphorylation, maintained by the concerted action of protein kinases and protein phosphatases, is fundamental in determining the outcome of multiple cellular functions ranging from cell proliferation to cell death, by influencing enzymatic activation, substrate specificity, protein localization, and resulting in the modulation of signalling pathways. In this line, tight regulation of protein phosphatase (PP) and protein kinase (PK) activities is crucial for the proper unfold of signal transduction. Alterations in this balance however, lead to aberrant responses resulting in the pathogenesis of multiple diseases including cancer, diabetes and autoimmune diseases [182]. As a generalized observation, protein phosphorylation and dephosphorylation results in positive and negative signal transduction respectively. However, both PKs and PPs have been shown to have roles as both positive and negative regulators of signalling.

Compared to protein phosphorylation in general, protein tyrosine phosphorylation plays an essential role in coordinating and controlling the duration of cellular functions including cell-cell communication, adhesion and migration, proliferation and immune-related functions [183]. The regulation of tyrosine phosphorylation was initially thought to be largely controlled by the activity of Protein Tyrosine Kinases (PTKs). However, after the cloning and purification of the first Protein Tyrosine Phosphatase (PTP) (PTP1B) 18 years ago [184], and ten years after cloning of the first PTK (src) [185], their equally important role as regulators of tyrosine phosphorylation and signal transduction was revealed.

FIGURE 5. Molecular Mechanism of PTP Catalytic Activity.



Schematic representation of the mechanisms of action of PTPs. PTP catalysis takes place in a two step mechanism. First there is a nucleophilic attack to the incoming phosphate group by the sulfur atom of the active cysteine residue in the catalytic pocket, and release of the now dephosphorylated substrate; this forms a PTP phospho-cysteine intermediate. The second step follows the coordination of a water molecule, allowing the hydrolysis of the phospho-intermediate and release of the phosphate group. Adapted from [186]

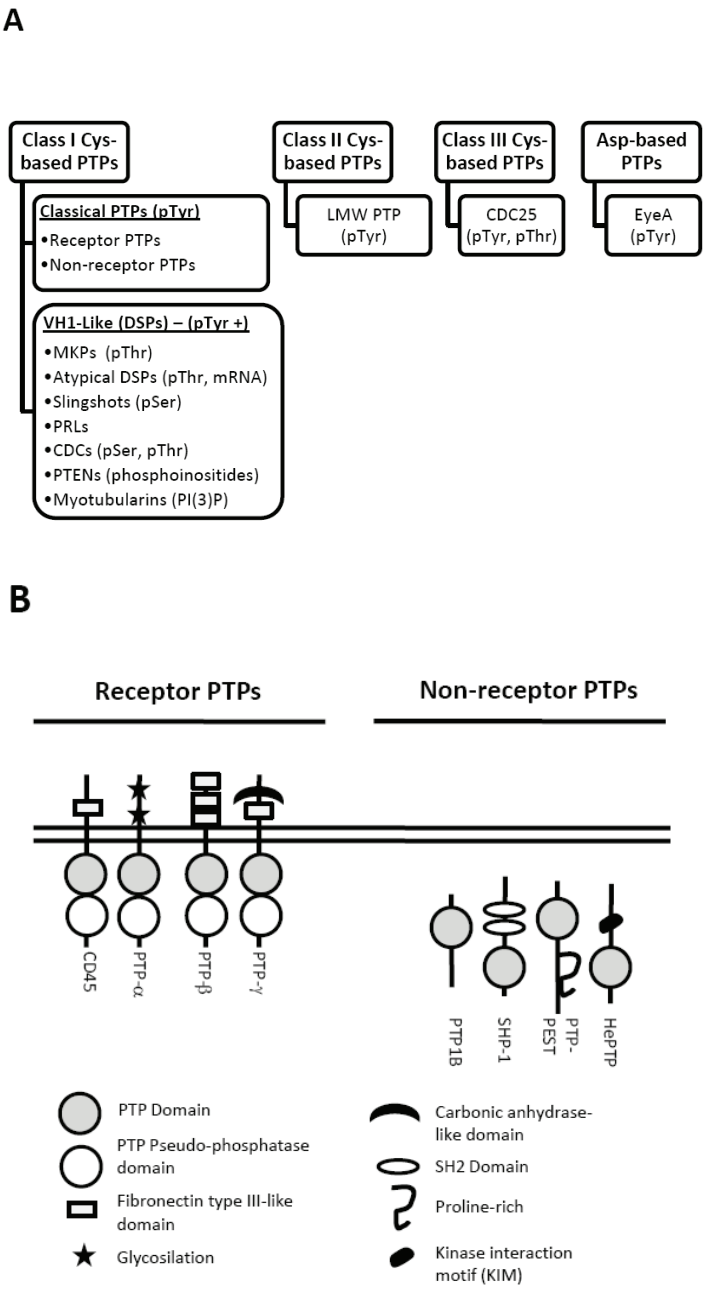
2.1 PTP classification

PTPs constitute a large family of enzymes ubiquitously expressed from viruses to mammalian cells. The PTP signature motif [I/V]H**C**xxGxxR[S/T], which defines this group of proteins, is localized in the catalytic domain of the enzymes, and is characterized by the presence of a conserved cysteine residue acting in the nucleophilic attack of the incoming phosphate group (Figure 5) [186]. The completion of the human genome [187] revealed that PTPs represent the largest phosphatase gene family, consisting of a broad group of enzymes encoded by 107 genes (Figure 6A), with 105 homologues in the mouse genome. Of the 107 genes, 11 encode catalytically inactive proteins, 2 dephosphorylate mRNA, and 13 dephosphorylate phospholipids, reducing the total number of active PTPs capable of dephosphorylating tyrosine residues to 81. PTPs are distributed ubiquitously or in a tissue/cell-specific manner, with an average cellular expression of 30-60% of the total PTP repertoire. Subcellular distribution of PTPs is as broad as its expression, and can be found as transmembrane proteins, in the cytoplasmic membrane periphery, associated with intracellular vesicles, in the mitochondria, cytosol and nucleus [188].

PTPs are important regulators of the immune system. They play an essential role in controlling the outcome of immune-related functions including IFN-inducible functions (TCPTP, PTP1B and SHP-1), cytokine production (PAC1, MKPs and HEPTP), T and B cell activation (positive regulators: CD45 and SHP-2, and negative regulators: SHP-1, LYP and PTP-PEST), among others. Importantly, all cells of the immune system have high levels of tyrosine phosphorylation and express more genes encoding PTKs and PTPs than any other cell type; remarkably, lymphocytes express at least 45 different PTPs. [188]. Adding to the importance of PTPs in the control of immune-related functions is the restricted expression of important PTPs such as SHP-1, HEPTP and CD45 in hematopoietic cells.

PTPs are subdivided into four different families (Figure 6A) based on the amino acid sequence of their catalytic domain [183]: Class I cysteine-based PTPs (discussed

FIGURE 6. PTP classification



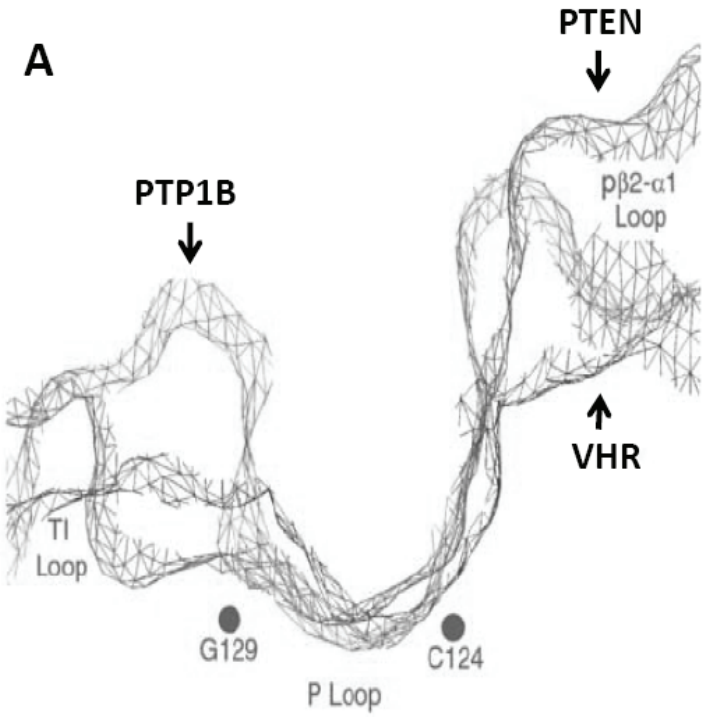
A) Classification of the mammalian PTP family. In parenthesis are detailed the individual substrate specificities. **B)** Sub-classification Class I Cysteine-based classic PTPs. This is not an exhaustive classification of all classic PTPs. Examples are shown to portrait the diversity among the family members. Adapted from [183] [182].

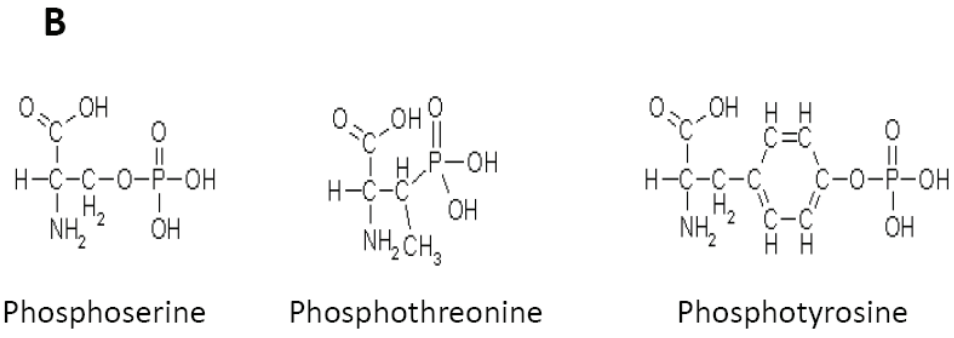
below), class II cysteine-based PTPs, represented in the human genome by a single gene encoding a low molecular weight PTP (LMWPTP); class III cysteine-based PTPs, comprising 3 cell cycle regulators (CDCs) involved in the dephosphorylation and activation of cyclin-dependent kinases (Cdks) allowing cell cycle progression, and the Asp-based PTPs: a recently discovered group of PTPs harbouring a catalytic aspartic acid residue instead of a conserved cysteine [183].

Class I cysteine-based PTPs.

This group is by far the largest PTP family (99 of the 107 human PTPs). It is subdivided into two groups based on their ability to strictly dephosphorylate tyrosine residues (classic PTPs), serine/threonine or phosphoinositides (PIs) in addition to tyrosine (dual specificity PTPs -DSPs-). The capacity of these highly divergent enzymes to dephosphorylate additional residues results from the structure of the catalytic domain. It has been suggested that the depth of the active site pocket, harbouring the catalytic cysteine residue, is an important determinant of phospho-amino acid specificity. The shallow and wide pocket of DSPs, compared to the narrow and deeper cleft of classic tyrosine-specific PTPs, allows for the accommodation of smaller phospho-residues such as serine and threonine, and with an even wider pocket PI-phosphatases can accommodate phosphoinositides [189] (Figure 7). Interestingly, 11 of the 61 DSP genes code for PTPs with specific activities for MAPK, referred to as MAPK phosphatases (MKPs). MKPs catalyze the dephosphorylation of both phoshotyrosine and phoshothreonine residues in the activation loop of protein kinases [182]. Knowledge on the activity of this large group of PTPs strengthens the importance for the need of a tight regulation of MAPK activities, who represent the converging point of numerous signalling pathways involved in the regulation of gene transcription in the immune response. Other interesting subgroups of DSPs are the PTENs and myotubularins, who catalyze the dephosphorylation of inositol phospholipids. PTEN has been extensively studied for its implication in the negative regulation of the Akt-PI3K signalling pathway

FIGURE 7. Comparative structures of PTP active site pockets.





A) Structural representation of the catalytic pocket of classic PTPs (PTP1B) and dual specificity PTPs (VHR and PTEN). The depth and width of each pocket determines the ability to dephosphorylate **B)** tyrosine, serine or threonine residues. Adapted from [189]

suppressing cell survival and proliferation, and hence its important role as a tumour suppressor [190].

Classic PTPs represent the most common group of PTPs, and contribute to about 50% of all PTPs expressed in cells of the immune system [188]. They are divided in transmembrane receptor-like (RPTPs) and non-transmembrane classic PTPs (Figure 6B). RPTPs have the potential to regulate signalling through ligand-dependent protein dephosphorylation, mediating cell-cell interaction and cell-matrix contact. CD45, a highly abundant surface receptor of hematopoietic cells and a prototypical member of the RPTPs, is implicated in the control of immune-related functions, together with the regulation of T and B cell receptor signal transduction. CD45 is one of the few PTPs with a clear positive regulatory function; deficiency in CD45 completely abrogates T and B cell development [191]. The main molecular substrates of CD45 are Src-family kinases (first line PTKs activated through the stimulation of immunoreceptors) such as Lck and Fyn, whereby dephosphorylation of a C-terminal inhibitory phosphotyrosine residue promotes enzymatic activation and positive signal transduction. Although most of the attention has been centered on the role of CD45 as a positive regulator of T and B cell-receptor signalling, lesser characterized negative regulatory actions have also been described [192]. In macrophages, CD45 in part regulates integrin mediated adhesion, shown by the hyper-adhesion observed in macrophages from $CD45^{-/-}$ mice [193], and has been also shown to dephosphorylate JAK family members in response to IL-3, IL-4 and IFN-α, downregulating JAK/STAT pathways[194]. Non-receptor classic PTPs (which include prototypical PTPs such as SHP-1 SHP-2, TCPTP and PTP1B) are represented by 17 members in the human genome. Their functions, substrates and subcellular localization vary widely and will be discussed throughout the text.

Interestingly the genetic distribution of classic PTPs is not random; no genes are found in the X or Y chromosome, or in chromosomes 16, 17, 21 or 22. Moreover, most of the classic PTP genes are clustered on chromosomes 1 and 12, summing 12 out of the 38 human coding genes. Based on sequence similarity, closely related PTPs (e.g. SHP-1/SHP-2, PTP1B/TCPTP, PTPα/PTPε) seem to have appeared by gene duplication of a

common ancestral gene [195]. Structural and sequence analysis has shown that the catalytic domain of classic PTPs comprises ~280 amino acid residues and is defined by 10 conserved motifs all playing a specific role in the catalytic mechanism [196]. Additional to the catalytic site, non-catalytic domains such as SH2, KIM and PDZ domains (no identification of SH3-domain containing PTPs), play a key role in substrate specificity, subcellular distribution and, importantly, in activity regulation [182].

2.2 Regulation of PTP activity

Post-translational modifications are important for activity regulation, substrate specificity and subcellular localization of signalling proteins. Multiple mammalian PTPs are post-translationally modified by glycosylation (e.g. CD45), myristoylation (e.g. DUSP15), oxidation, and more commonly by phosphorylation (at least 15 PTPs including SHP-1 and SHP-2) [183]. Among these, oxidation and phosphorylation (however not demonstrated in all instances), together with protein cleavage and dimerization have shown to play a role in PTP activity regulation.

Although progress has been recently achieved in understanding the mechanisms of PTP regulation, particularly trying to identify ligands directing ligand-dependent RPTP activation/inhibition, and the discovery of a mechanism for *in vivo* PTP inhibition by ROS, many questions remain unanswered: In a ROS-depleted environment (resting physiological state), how are PTPs regulated? How are specific PTPs regulated by ROS? The complexity of PTP activity modulation argues that more than a unified mechanism of PTP regulation, a plethora of molecular systems may be involved in controlling PTP activity. This suggests that regulatory mechanisms may not even be family specific, but may need to be considered in an individual protein manner.

RPTP dimerization

Ten years ago, the crystal structure of RPTPα revealed an interesting mechanism of RPTP regulation by protein dimerization [197]. In contrast to the activation of RPTKs via ligand-dependent dimerization, the homophilic dimerization of RPTPs has been shown to impair enzymatic activity due to a physical occlusion of the active site [182].

Many RPTPs contain two catalytic domains, D1 and D2, arranged in tandem in the intracellular tail of the protein. D1 is situated most proximal to the membrane periphery and is enzymatically active, whereas D2 has been shown, in most instances, to be catalytically inactive given the lack of fundamental residues in the active site [182] (Figure 6B). Dimerization of RPTPs leads to a reciprocal inhibition of the enzymatically active D1 domains by a physical interaction between the "wedge motif" (a helix-turn-helix motif) of one molecule and the catalytic site of the adjacent one [197, 198]. Two ligand-dependent mechanisms of RPTP regulation are proposed: a ligand-induced protein dimerization and inhibition, and the constitutive dimerization and ligand-dependent activation via a zipper type mechanism; ligand binding will promote the disruption of the protein-protein interaction, liberating the intracellular domains promoting enzymatic activation [199, 200]. Such is the case of RPTPσ which requires PTP dimerization for interaction with extracellular ligands, and in such a way potentially regulates PTP activity [201]

PTP oxidation

Professional phagocytes, such as macrophages and neutrophils produce ROS as a mechanism of defence against invading pathogens. Moreover, both phagocytic and non-phagocytic cells produce ROS in response to extracellular stimuli such as growth factors, hormones or cytokines, contributing to signal propagation and downstream responses [202]. It has been recently found that signal transduction initiated by growth

factors, cytokines, or receptor mediates events can be controlled by ROS-dependent regulation of PTP activity [203].

The oxidation state of the conserved catalytic cysteine residue ([I/V]H**C**xxGxxR[S/T]) localized at the base of the active site of PTPs, is critical for enzymatic activity. At neutral pH, its reduced state (Cys-S^-) is necessary for the nucleophilic attack on the incoming phosphate group, where the sulfur atom of the cysteine residue binds to the incoming phosphorous atom of the phospho-amino acid forming a transient phospho-cys intermediate. Completing the enzymatic activity is the action of a conserved aspartic acid residue present in the conserved motif 8 (WPD loop) of the catalytic domain and coordinates the hydrolysis of the phosphate group (Figure 5) [186]. Oxidation of the catalytic cysteine impairs enzymatic activity by preventing the nucleophilic attack and the formation of the phospho-cys intermediate. The oxidation state of the active Cys ranges from a reversibly oxidized form (Cys-SOH) to higher order oxidation states (Cys-SO_2H and Cys-SO_3H) that result in the irreversible inhibition of PTPs. Reversible oxidation is accompanied by a profound conformational change in the active site impairing interaction with the incoming substrate, and can be reversed by biological or chemical antioxidants including glutathione, thioredoxin, or DTT [203].

Reversible PTP oxidation is induced by ligation of PDGF [204], TNF-α [205] and insulin [206] stimulation [182], or by treatment of cells with H_2O_2 [207]. Cytokine and growth factor stimulation lead to the induction of a localized oxidative stress and the generation of ROS, rapidly targeting neighbouring PTPs, reversibly inhibiting their enzymatic activity. This event, which has been documented for a number of PTPs including classic PTPs such as PTP1B, TCPTP and SHP-2; RPTPs such as LAR and CD45, and in DSP such as MKPs and PTEN [182], has been proposed to contribute to the biological regulation PTPs and the physiological modulation of signalling pathways. Positive regulation of signal transduction will occur upon local and temporary inhibition of PTP activity, promoting the expression of cellular functions.

One of the few complete mechanisms of ROS-dependent PTP inactivation that has been elucidated is the insulin/ROS-mediated inhibition of PTP1B. Insulin stimulation

leads to a transient increase in ROS. This was found to be mediated via the activity of Nox4, a homologue in the family of NADPH oxidase catalytic subunit, which catalyzes the generation of ROS, transiently inactivating PTP1B. Nox4 and PTP1B co-localize in intracellular membrane compartments, suggesting a spatial control over PTP regulation [208].

Another interesting mechanism is the oxidation-dependent inhibition of some RPTPs, which results in the stabilization of PTP dimmers by forming an intramolecular disulfide bond between the two adjacent D2 domains [209, 210].

Although the oxidation model represents a very interesting mechanism of biologically relevant PTP activity regulation, it is still unclear whether inhibition will occur in a PTP-specific manner, or if in a ROS enriched environment all neighbouring PTPs will be similarly affected. An interesting hypothesis resulted from observations by Groen and collaborators, suggesting that PTPs can be differentially oxidized, that is differentially regulated by ROS-dependent oxidation, showing that the membrane-proximal D1 domain of RPTPα was catalytically active but not readily oxidized, whereas the membrane-distal RPTPα-D2, was readily oxidized [211]. The authors also showed that the potential for oxidation was dependent on pH, suggesting that different cellular micro-environments may influence ROS-dependent regulation.

PTP phosphorylation

As is the case of multiple signalling molecules, phosphorylation of PTPs plays an important role in activity regulation. PTP phosphorylation in serine, threonine or tyrosine residues is the most common post-translational modification, and has been documented in at least 15 different phosphatases; however, the biological significance remains unclear in most of the cases [183].

Serine and tyrosine phosphorylation of the Src-homology 2 (SH2) domain containing PTPs SHP-1 and SHP-2 have been shown to regulate enzymatic activity and

substrate specificity by promoting the interaction with adaptor proteins such as Grb2. Although structurally very similar, SHP-1 and SHP-2 have different roles in the regulation of signal transduction: SHP-1 has been shown to largely play a negative regulatory role principally in hematopoietic cells by downregulating JAK/STAT, MAPK and immuno-receptor mediated signalling, leading to the inactivation of cell functions. SHP-2, on the other hand, is commonly regarded as a positive regulator of cells signalling [212], activating Src-family kinases and promoting signal transduction downstream of immunoreceptors.

The important regulatory role of SHP-1 is evidenced by the severe immunopathology phenotype of the *motheaten* mice (Table 3), which results from a mutation in the *Shp1* alleles leading to the aberrant splicing of the *Shp-1* gene. Augmented numbers of circulating blood leukocytes are detected. Their massive infiltration into organs such as liver, spleen and lungs, leads to an exacerbated inflammatory response, tissue damage and eventually death [213]. Conversely, SHP-2 function has been implicated in the positive regulation of cell differentiation, proliferation, haematopoiesis [214], cell migration, adhesion and cytoskeletal architecture [215]. Disruption of the SHP-2 gene in mice results in early embriogenic lethality [214, 216, 217] evidencing its essential function in development. SHP-2 regulates signalling downstream the insulin receptor (IR), platelet derived growth factor receptor (PDGFR) and fibroblast growth factor receptor (FGFR), activating Src-family kinases and Ras/ERK signalling [212].

Both SHP-1 and SHP-2 have C-terminal tyrosine and serine residues that can be phosphorylated, and have been implicated in activity regulation. The role of C-terminal tyrosine phosphorylation of both SHP-1 and SHP-2 is twofold: it has been proposed to modulate enzymatic activity, or serve as post-translational modification for the recruitment of SH2-containing adaptor proteins such as Grb2. Tyr^{536} and Tyr^{564} phosphorylation of SHP-1 in T cells has been proposed to be mediated by the PTK Lck

TABLE 3.

Mouse models of PTP deficiencies: discovering the role as key regulators of cell functions

PTP	Enzyme Family	Substrate[188, 218]*	Outcome	Reference
SHP-1$^{-/-}$ (*me^{v}*)	Classic non-receptor	JAK2, Actin, β-catenin, ITIM-receptors, ZAP70, Fyn	Severe immunopathology. Hyper-activated macrophages and hyper-responsive T and B cells	[219]
PTP1B$^{-/-}$	Classic non-receptor	Insulin receptor, JAK2, TYK2, STAT5, p130Cas	Resistance to diet-induced obesity	[220]
PTP-PEST$^{-/-}$	Classic non-receptor	p130cas, WASP, PYK2, VAV2, FAK, CSK.	Embriogenic lethality. Defect in proliferation and migration. Impaired integrin signalling	[221]
TCPTP$^{-/-}$	Classic non-receptor	STAT1, 5 and 3, JAK 1 and 3, PDGFR	Exacerbated inflammation due to increased production o pro-inflammatory cytokines. Abnormal B lymphopoiesis.	[222]
SHP-2$^{-/-}$	Classic non-receptor	Src-family Kinases, PDGF, EGF.	Early embriogenic lethality. Defective lymphopoiesis	[217]
RPTPα$^{-/-}$	RPTP	Src, Fyn, p130cas	Regulatory function in learning and neuroplasticity	[223]
RPTPε$^{-/-}$	RPTP	Kv 2.1 and 1.5, Src, Fyn, Yes	Hypomyelination, increased activity of Kv channels.	[224]
CD45$^{-/-}$	RPTP	Src-family Kinases (Lck, lyn and Fyn)	Immunodeficiency, defect in T and B cell development and activation	[225]
MKP1$^{-/-**}$	DSP	p38, JNK, ERK	Increased innate immune response.	[226]
PAC 1$^{-/-**}$	DSP	ERK, p38	Partial immunodeficiency. Protection from rheumatoid arthritis	[227]
PTEN$^{-/-}$	DSP	PI(3,4,5)P3	Early embriogenic lethality. High incidence of cancer in heterozygous.	[189]

* This does not represent an exhaustive list of PTP substrates. **[228]

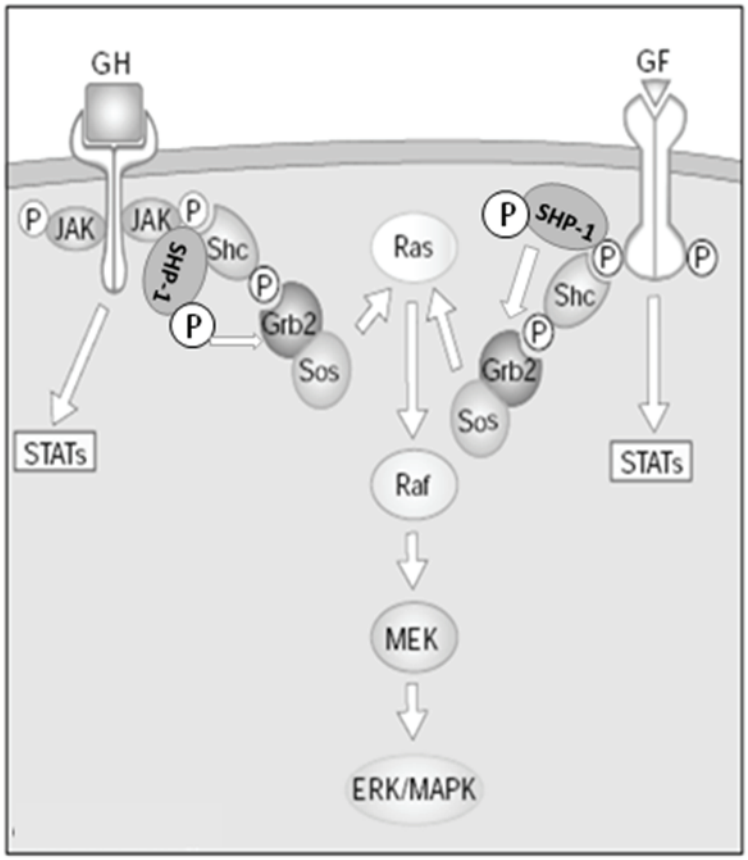
[229]. However, phosphorylation at these sites undergoes a rapid autodephosphorylation [230], questioning their relevance. More recently, site directed mutagenesis of Tyr^{536} and Tyr^{564} has revealed role in enzymatic activation possibly through an enhanced recognition of the "appropriate substrates" [231], or through its interaction with the N-terminal SH2 domain, which at resting state sits in the catalytic pocket inhibiting PTPase activity [232]. Interaction of the phospho-tyrosine residue and the SH2 domain will impair its interaction with the catalytic site, allowing enzymatic activity.

The adaptor function of SHP-1 tyrosine phosphorylation has been shown to occur via the recruitment of Grb2, which may lead to the connection of receptor PTK signalling and the Ras/ERK pathway. Additionally, these adaptor proteins will allow SHP-1 recognition of alternate substrates including ERK family members, leading to their negative regulation by SHP-1 (Figure 8). Similar to SHP-1, tyrosine phosphorylation of SHP-2 (Tyr^{542}) has been shown to promote the recruitment of adaptor proteins, enabling recruitment and coupling to the MAPK pathway [233].

Serine phosphorylation has also been documented for SHP-1 (Ser^{591}) [234] and SHP-2 ($Ser^{576,591}$). In T cells, T cell receptor ligation induces a fast and transient serine phosphorylation of SHP-1, which could be mediated by PKC, and leads to inhibition of phosphatase activity [234]. PKCα-mediated C-terminal serine phosphorylation of SHP-1 led to decreased PTP activity in platelets [235]. Similarly, SHP-2 serine phosphorylation seems to be mediated via PKC [236]. However, whether this modification affects enzymatic activity is still unclear.

Another case of PTP activity regulation by phosphorylation is PTP1B. PTP1B is the prototypical classic non-receptor PTP. Being the first PTP to be cloned, the great majority of structural and mechanistic evidences of PTP function, enzymatic activity and regulation have been based on findings in PTP1B. PTP1B is ubiquitously expressed and found associated to the endoplasmic reticulum (ER) with the catalytic domain facing the cytosolic space [237], or as a free cytosolic phosphatase [238]. The importance of PTP1B in the regulation of metabolic functions came from $PTP1B^{-/-}$ mice, which showed

FIGURE 8. JAK/STAT and MEK/ERK signaling pathways crosstalk



SHP-1 tyrosine phosphorylation may serve as a link between receptor signalling and the ERK pathway. SHP-1 binds to phosphorylated Immunoreceptor Tyrosine-based Inhibitory Motifs (ITIM), by interaction with its SH2 domains (right panel). Alternatively, SHP-1 interacts with substrates such as JAK-2 (left panel). Tyrosine phosphorylation of SHP-1 will recruit Grb-2 adaptor protein, initializing a signalling cascade that will lead to ERK activation. Adapted from **[239]**

resistance to diet-induced obesity and hypersensitivity to insulin, defining a critical role in the negative regulation of insulin signalling, by dephosphorylation of the insulin receptor [240]. PTP1B has also been shown to downregulate cytokine signalling, dephosphorylating JAK2 and TYK2 kinases, and growth factor receptor signalling, dephosphorylating the EGFR and PDGFR among others [240]. Interestingly, PTP1B has been shown to regulate macrophage activation *in vivo*, where higher levels of iNOS and NO production, serum IL-12 and IFN-γ were detected in $PTP1B^{-/-}$ mice [241].

Regulation of PTP1B activity is tightly controlled, and among the regulatory mechanisms is protein phosphorylation. PTP1B phosphorylation has been shown to occur both in serine and tyrosine residues following insulin stimulation [242, 243]. Interestingly, while tyrosine phosphorylation has been associated with enhanced enzymatic activity, phosphorylation at serine residues seems to play an inhibitory role. This suggests that upon insulin stimulation phosphorylation at both sites may occur resulting in a balanced level of phosphatase activity, in order to drive the appropriate signal transduction. Alternatively, phosphorylation at one or the other residue will drive a positive or negative feedback loop, resulting in the continuation or inhibition of insulin signalling.

PTP cleavage

Cleavage-mediated regulation of PTP activity has been described in several occasions. The best studied model is the calpain-mediated cleavage regulation of PTP1B. Although conflicting results regarding whether PTP1B cleavage enhances or inhibits enzymatic activity, it is clearly demonstrated that PTP1B is indeed a calpain substrate. Under conditions of platelet aggregation, PTP1B is cleaved between its C-terminal and PTP domain by calpain [244]; a calcium-regulated cysteine protease, which removes 60 to 70 amino acids from the C-terminal region causing PTP1B relocalization from the ER to the cytoplasmic space and enhancement of enzymatic activity [244]. A

more recent study identified PTP1B as a specific substrate of calpain-1. In contrast to previous reports, the authors show that using calpain-1 deficient platelets there is an enhanced accumulation of PTP1B, correlating with induced PTPase activity and substrate dephosphorylation, thus suggesting that calpain-1-dependent PTP1B cleavage impairs its phosphatase activity [245]. Lastly, an interesting study evaluating the enzymatic activity of the full length or a C-terminal truncated version of PTP1B showed that when using the small synthetic phosphate analogue p-Nitrophenyl Phosphate (pNPP) as a PTP substrate, no difference in the enzymatic activity was observed in the absence or presence of the C-terminal region. However, when a synthetic tyrosine phospho-peptide was used as a substrate, the PTPase activity was markedly enhanced in the C-terminal deficient PTP1B [246]. This suggests that calpain-mediated cleavage may alter PTPase activity, but may be substrate-dependent.

Other PTPs have also been shown to be regulated by a calpain-mediated proteolytic cleavage. Among these are the non-receptor PTP SHP-1, and the receptor PTPs RPTPα, RPTPε and STEP. Calpain-mediates cleavage of platelet SHP-1 in its C-terminal domain, and is associated with platelet activation and microvesicle release [247]. However, whether calpain-mediated SHP-1 cleavage enhances its enzymatic activity still remains elusive. Cleavage of RPTPα occurs within the intracellular juxtamembrane domain, releasing the phosphatase catalytic domains from the membrane anchor and translocating them to the cytoplasm. Releasing the catalytic domains from the membrane region prevents their interaction with classic substrates including voltage-gated potassium channels and Src-family kinases [248]. However, this opens an interesting possibility of interaction with alternate cytosolic substrates differentially regulating alternative signalling pathways. Another calpain-substrate is the RPTP STEP, which is highly expressed in the central nervous system. In response to a rapid calcium influx, STEP is cleaved in a calpain-dependent manner, releasing a smaller isoform to the cytoplasm. Whether this cleavage induces enzymatic activation awaits verification [249].

PTP-MEG is a two member family of classic non-receptor PTPs whose functions (at least of PTP-MEG2) seems to be the positive regulation of secretory vesicle trafficking and fusion [240]. *In vitro* cleavage of the C-terminal domain by trypsin or calpain has been shown to enhance enzymatic activity [250].

Two PTPs, PTP-PEST and PTEN have been shown to be proteolytically cleaved by caspases. In the case PTEN, it was found to be a substrate of caspase-3, with cleavage sites located to the C-terminal region of the phosphatase reducing protein stability. PTEN cleavage was enhanced by TNF-α treatment, but inhibited by phosphorylation in the C-terminal tail [251]. PTP-PEST is involved in the regulation of the actin cytoskeleton. During apoptosis, PTP-PEST was also recognized as a proteolytic substrate of caspase-3, regulating protein-protein interactions and enhancing enzymatic activity [252].

Together, these results indicate that PTP cleavage is an important post-translational modification that acts to positively or negatively regulate PTP activity in a PTP-specific manner. It is interesting to consider that PTP cleavage may play an important role in potentiating the recognition of alternate phospho-substrates and/or adaptor molecules, regulating signalling pathways which may not necessarily be the canonical pathways regulated by the full length enzyme.

Metal and metal-derived compounds PTP inhibition.

The importance of metals such as zinc (Zn), manganese (Mn) and iron (Fe) in the control of immune-related functions and protein phosphorylation [253-258] suggest that they could have a potential role as coordinators of signalling pathways by interacting with PTKs and/or PTPs. Interestingly, the mechanism of action of protein serine/threonine phosphatases such as PP2A and PP2B, important regulators of cell signalling, requires the presence of two metal ions in the catalytic site, facilitating the direct hydrolysis of the phospho-substrate [259]. Furthermore, the importance of these

biologically active metals in regulating protein activities is evidenced by their action as enzymatic cofactors of superoxide dismutase (SOD), ribonucleotide reductase, cytochrome oxidase, transcription factors, among many others [260]. The role of metals in the control of signalling pathways through PTP activity regulation will be further discussed.

Zinc **----** Although a few metal ions or metal-derived compounds have been shown to modulate PTP activity, only zinc (Zn^{2+}) has been recently recognized as a physiological regulator of PTPase activity. The first evidence of a Zn^{2+}-mediated PTP inhibition appeared in 1981, where Brautigan et.al., showed *in vitro* that low concentrations of Zn^{2+} (10 μM) inhibited PTP activity in membrane vesicles of the epidermiod carcinoma cell line A-431 [261]. In this report, PTP inhibition was shown to be specific to Zn^{2+} and to a lesser extent Co^{2+} cations, as no other divalent metal (Mn^{2+}, Mg^{2+} or Ca^{2+}) prevented protein dephosphorylation. These observations were more recently revisited by Haase and Maret [262-264], who have unequivocally shown that *in vivo* Zn-dependent inhibition of PTP activity results in the modulation of signalling pathways and cellular functions. Addition of extracellular zinc to C6 rat glioma cells has an insulinomimmetic effect, promoting general protein tyrosine hyperphosphorylation, but specifically that of the Insulin Growth Factor-1 (IGF-1) receptor via PTP1B inactivation [262]. In addition the authors reported the specific inhibition of SHP-1, and showed that the inhibitory effect was limited to the catalytic domain, possibly by a direct interaction of Zn ions with putative Zn binding motifs in the PTP domain [262]. Interestingly, Zn^{2+} has been shown to have no effect, or even a stimulatory effect over CD45 phosphatase activity [265], thus suggesting that zinc-mediated PTP inhibition is specific for particular PTP subsets.

In a model of pulmonary inflammation induced by inhaled toxicants (arsenic, zinc and vanadium), inhibition of PTP activity by Zn and vanadium may help explain some of the effects of metal exposure in the activation of signalling pathways and the induced inflammatory response. It is suggested that a mechanism of PTP inhibition by a

direct metal-dependent oxidation of the sulfhydryl groups, or via an indirect metal-induced generation of ROS must take place [266].

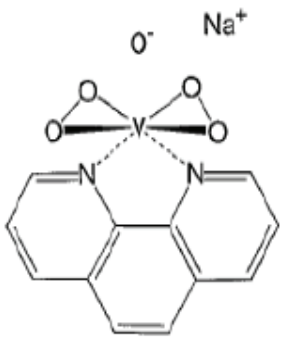
All together, these results suggest that a tight control of cellular zinc homeostasis is required for the maintenance of balanced cellular activities, in part exerted through its inhibitory action over PTPs, and provide further evidence for the importance of these cations in the regulation of cell functions through the control of signalling pathways.

Vanadium and vanadium-derived compounds ---- The PTP inhibitory effect of vanadate (VO_4^{3-}) was described more than 15 years ago [267]. Vanadium (V) is a transition metal that has been mostly studied for its toxic and carcinogenic effects after prolonged and high dose exposure [268]. It can be found in some foods including mushrooms, shellfish, parsley and black pepper. Recently, attention has shifted, and vanadium may be regarded as an important trace element with anti-diabetic and anti-carcinogenic characteristics [269].

Vandate, as well as Zn, has insulinomimmetic effects *in vitro* [270]. I*n vivo,* intraperitoneal injections reduce hyperglycemia in rats and stimulation of glucose uptake and fatty acid synthesis in adipocytes [271]. It was also demonstrated that vanadate synergized with peroxide to promote insulin signalling, resulting from the formation of peroxovanadium (pV) complexes, which exert a specific and strong inhibition of PTP activity [270]. These studies led to the identification of the very specific PTP inhibitor bpV(phen) (Figure 9), latter shown to irreversibly inhibit PTP activity by the oxidation of the critical cysteine residue (Cys-SO_3H) present in the catalytic pocket of PTPs [272].

PTP inhibition by pV-derived compounds has been shown to promote signalling through Syk-family kinases, NFκB [273] and MAPK [274] pathways. This upregulation of signal transduction and downstream cellular functions such as NO production [274], chemokine and cytokine gene expression [275, 276], has led to the identification of pV-derived compounds as potent inhibitors for the control of experimental visceral and

FIGURE 9. Structure of bpV(Phen)



The specific PTP inhibitor bpV(phen) irreversibly inhibits PTP activity by oxidizing the critical catalytic cysteine residue (Cys-SO_3H) in the active site. Adapted from **[273].**

cutaneous leishmaniasis [275, 277], and the inhibition of *in vivo* tumour progression and development [278].

Antimony (Sb) **----** One last metal-compound that has been reported to inhibit PTP activity is Sodium Antimony Gluconate, also known as Sodium Stibogluconate (SSG). SSG has been used for more than 50 years as the first line of treatment against human leishmaniasis, and although its precise mechanism of action is not fully understood, following its intracellular reduction from Sb^{V} to Sb^{III}, it directly affects *Leishmania* parasites possibly by inhibiting macromolecular biosynthesis, inhibition of glycolysis, fatty acid β-oxidation and perturbation of antioxidant metabolism [9].

In vitro, it has been shown that SSG has a strong inhibitory effect over SHP-1, SHP-2 and PTP1B, but not MKP-1 [279]. Of particular interest, SSG was able to almost fully inhibit SHP-1 activity at doses used therapeutically for the treatment of leishmaniasis, however, SHP-2 and PTP1B inhibition was only reached using 10 fold higher doses. Inhibition of SHP-1 was suggested to occur via the direct interaction of

SSG and the catalytic pocket, as incubation with a mutant SHP-1 construct harbouring only the catalytic domain, showed a similar degree of inhibition as with the full enzyme. In the murine hematopoietic Baf3 cell line, SSG treatment promoted general protein hyperphosphorylation, and specifically JAK2 and STAT5 hyperphosphorylation in the context IL-3 stimulation [279]. These observations are particularly interesting in the context that SSG may exert its antileishmanial action both by directly affecting *Leishmania* parasites, as well as promoting the induction of host cell cytokine-induced signalling and downstream immune-related functions.

Most recently, SSG in combination with IL-2 has been shown to inhibit tumour growth in a murine renal cancer model, correlated with an increase in tumour infiltrating macrophages. [280]. In addition, a role in the recuperation of refractory sensitivity of cancer cells to interferon treatment was suggested, by a targeted inhibition of SHP-1 and SHP-2, negative regulators of JAK/STAT signalling [281].

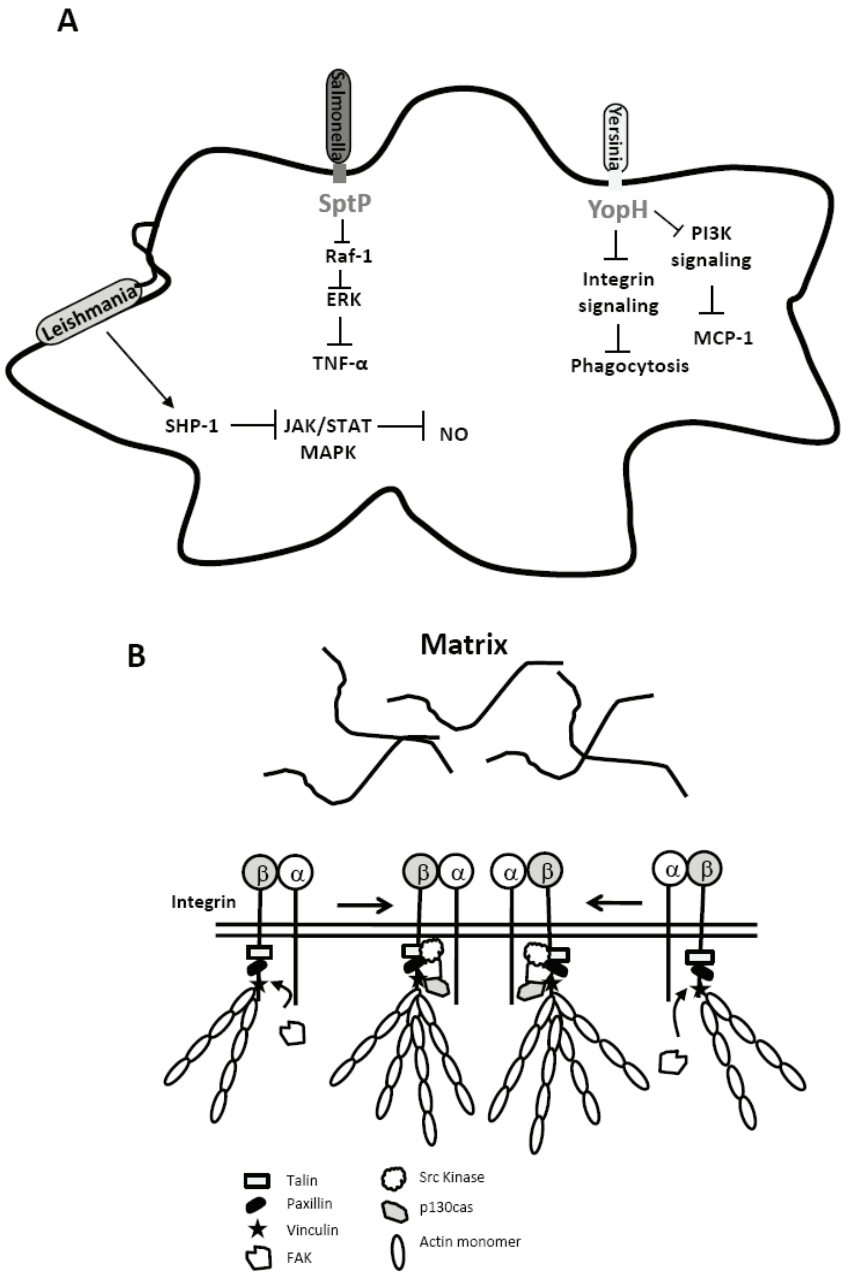
2.3 PTPs and infectious diseases

In order to survive within its host, pathogens require a variety of ingenious tactics to evade the innate and acquired immune response. Among some of these strategies, one of the most sophisticated mechanisms discovered to date is the antigenic variation of *Trypanosoma brucei* [282], causative agent of African trypanosomiasis or sleeping sickness. This extracellular protozoan parasite expresses a highly abundant glycoprotein -variant surface glycoprotein, VSG- coating its entire cell surface. VSG has been involved in the evasion of an antibody response and the modulation of innate immune functions in part by targeting NFκB activation [283]. *T. brucei* has more than 1000 *VSG* genes and pseudogenes, of which only one is transcribed at a time from one of multiple telomeric *VSG* expression sites. Switching the active *VSG* gene occurs spontaneously with a frequency of 1 switch every 10^2 to 10^6 cells, and can involve DNA rearrangements or, alternatively, transcriptional control. This

provides the parasite with a “variant antigenic coat”, allowing the evasion of antibody-mediated recognition. In addition to its tremendous variability, the VSG coat is so densely packed at the cell surface that it avoids antibody recognition of any other plasma membrane determinants [282, 284].

On the other hand, intracellular pathogens have been well recognized by their ability to escape the harsh intracellular environment of host cells like macrophages. Evasion strategies used by these pathogens include inhibition of phagolysosomal maturation, physical adaptations for survival within acidic subcellular compartments, escaping to the cytosolic space, and modulation of innate immune functions by alteration of host signalling pathways [284, 285]. This last mechanism is most avidly utilized by pathogens such as *Leishmania, Trypanosoma cruzi* and *Salmonella*. Here I will discuss a very specific case of pathogen-induced modulation of signal transduction by differential regulation of PTP activities, and examine particular situations where this modulation requires the activity of a host cell or pathogen PTPs (Figure 10A).

FIGURE 10. PTP-dependent modulation of host cell signaling induced by pathogens.



A) Various pathogens (bacteria and protozoan parasites) have been shown to modulate host cell signalling in PTP-dependent manner. Whether it is a host PTP (*Leishmania*) or a pathogen-derived PTP (*Yersinia* and *Salmonella*) the coordinator of downregulating host cell functions, these strategies have served pathogens to favour their survival within the host. **B)** Integrin signalling is downregulated by *Yersinia* in order to avoid phagocytosis (see text for full details). Adapted from [286]

Pathogen PTPs and the modulation of host cell signalling.

Salmonella SptP ---- Several pathogenic gram-negative bacteria share homology in regions called pathogenicity islands (PI). Encoded in PIs are proteins that can be injected into host cells via a type III secretion system which work as a "molecular syringe" [287]. Human pathogenic *Salmonella* species are intracellular pathogens causative agents of a variety of diseases ranging from gastro-intestinal to systemic febrile illnesses. Type III secretions systems have been very well studied in *Salmonella* and have provided clues into the effector functions of the injected bacterial molecules. Among these, is the bacterial protein tyrosine phosphatase SptP [288]. SptP has been shown to interfere with host cell signalling. This results in the alteration of actin cytoskeleton [289, 290] and inhibition of macrophage TNF-α secretion [288], by directing the targeted inhibition of MAPK signalling through the inhibition of Raf-1 and subsequent inactivation of ERK [288, 290]. This provides a safe intracellular environment for bacterial replication and delays the induction of a pro-inflammatory response.

Yersinia YopH **----** *Y. enterocolitica, Y. pseudotuberculosis*, and *Y. pestis* are the three *Yersinia* species that are pathogenic to humans, the first two causing usually self-limiting gastro-intestinal infections, whereas *Y. pestis* causes the acute and often fatal plague [291]. Rapidly upon infection, monocytes, macrophages, and specially neutrophils are recruited and mediate the innate immune response. *Yersinia* has evolved a mechanism to downregulate effector host cell functions, delaying an adaptative Th1 response, via the Yop proteins. Perhaps one of the most studied bacterial virulence factors is the *Yersinia* PTP YopH. YopH is encoded in a virulence plasmid forming part of a type III secretion system, and plays an essential role in inhibiting the phagocytosis of bacterial cells by macrophages [292]. This process is accomplished by the negative regulation of β1-integrin signalling (Figure 10B), resulting in the altered rearrangement of the actin cytoskeleton [286, 293]. Host proteins

identified as substrates of YopH include Fak and the adaptor proteins Cas and paxillin. In macrophages, YopH dephosphorylates Cas, Fyb, and the Fak-homolog Pyk, and in doing so, interrupt integrin-mediated phagocytosis at an early stage [291]. Additionally, YopH activity has been involved in the inhibition of MCP-1 chemokine (involved in the recruitment of monocytes and macrophages) gene expression and T-cell activation, by inhibiting the PI3K signalling pathway [294] and potentially Src-family kinase signalling [295].

Trypanosoma cruzi ---- In 1998, Furuya and colleagues reported PTP activity of membrane-anchored proteins facing the extracellular milieu, in infective stages (trypomastigotes and amastigotes) of *T. cruzi* [296]. Later on in the same year, the group reported protein dephosphorylation in the non-phagocytic myoblast cell line L_6E_9 following infection with *T.cruzi* [297]. Correlating with this observation, they continued to show that parasite invasion was markedly reduced in the presence of the PTP inhibitors sodium ortho-vanadate and Zn, and suggested that either the *T. cruzi* ecto-PTP or an activated host PTP may coordinate this dephosphorylation event. More recently, it was shown that macrophage infection by *T. cruzi* induced the generation of ROS, resulting in the inhibition of host PTP activity and downstream induction of SAPK/JNK signalling [298]. Together these results suggest that *T. cruzi* may modulate host cell tyrosine phosphorylation and signalling in a cell-type specific manner, in order to avoid innate immune defence mechanisms of phagocytic cells, or alternatively to promote parasite uptake and “safe” survival in more inert non-phagocytic cells.

Vaccinia virus VH1 ---- Type I (α and β) and type II (γ) IFNs plays a key role in host defence against viruses [299]. It is therefore likely that viruses have developed mechanisms to avoid IFN signalling to survive and replicate within mammalian host cells. An interesting mechanism is exemplified by the vaccinia virus (VV), a linear double-stranded DNA (dsDNA) poxvirus, used in its attenuated form as the smallpox vaccine. The VV genome encodes the first ever identified dual specificity PTP; VH1 [300]. VH1 is expressed late in the virus life cycle and is packaged within the virion. In

2001, Najarro and colleagues showed that VH1 interferes with IFN-γ signal transduction early during the infection process. They suggest that following entry and uncoating of the viral core, VH1 is released into the host cell cytoplasm where it interacts with and dephosphorylate STAT-1, preventing its nuclear translocation and DNA-binding activity, thus inhibiting downstream pro-inflammatory functions [301].

Pathogen-induced modulation of host PTP activity.

***Leishmania* ----** Perhaps the most striking ability of *Leishmania* parasites is their capacity to modulate host cell signalling in order to subvert normal macrophage functions and establish intracellularly. In the early stages of *Leishmania*-macrophage interactions (within the first 24 hours post parasite-macrophage contact), alterations in the JAK/STAT and MAPK signalling pathways occur as a result of the *Leishmania*-induced activation of the host PTP SHP-1. SHP-1 activation targets the dephosphorylation of JAK 2 and ERK 1/2, leading to the downregulation of nitric oxide production which is fundamental for controlling the intracellular parasite [171, 276, 302]. Despite the critical role of SHP-1 in the *Leishmania*-induced negative regulation of macrophage signalling, additional PTP enzymatic activation is still evident in infected SHP-1 deficient macrophages [171]. This observation, in addition to the differential regulation of macrophage gene expression [303, 304] [305], and the negative regulation of multiple pro-inflammatory functions including IL-12 and TNF-α production (reviewed in [73]), suggests that other macrophage PTPs in addition to SHP-1 could play a role in negatively regulating key host cell functions during the early stages of infection. In this line of thought, Suzuki and colleagues recently reported that the tyrosine phosphatase PTEN, a negative regulator of PI3K signalling, plays a role in the macrophage mediated control of *Leishmania* infection, correlated with a downregulation in pro-inflammatory cytokine production [306].

To date, the exact mechanism whereby *Leishmania* parasites induce PTP activation is not completely understood. Recent reports propose the *Leishmania*

elongation factor 1 alpha (EF1α) and fructose-1,6-bisphosphate aldolase as SHP-1 activators [307, 308]. However, the mechanism by which these proteins could access to, interact with, and activate host PTPs remains elusive.

A *Leishmania* PTP (LPTP1) has been identified and implicated in parasite virulence. However, whether this PTP plays a role in virulence by maintaining parasite fitness or in the direct interactions with its host remains to be established [309].

Mycobacterium tuberculosis **----** *M. tuberculosis* is an obligate intracellular pathogen, which resides and multiplies exclusively within mammalian mononuclear cells, and similarly to *Leishmania* parasites, evades natural macrophage functions in order to survive within its host [310]. *M. tuberculosis* lipoarabinomannam (LAM) is a complex cell wall anchored molecule which, in large part, composes the outer coat of these bacterial cells, and was identified as a virulence factor for its capacity to modulate host cell functions [311]. In the human monocytic cell line THP-1, LAM stimulation induced the rapid tyrosine dephosphorylation of host cell proteins, and was correlated with the induction of PTP activity in membrane-fractions. Induction of membrane PTP activity was associated with the *in vivo* LAM-dependent translocation of the host PTP SHP-1 to the cell membrane, and direct enhancement of SHP-1 enzymatic activity *in vitro.* Induction of SHP-1 activity was correlated with the downstream inhibition of LPS-induced TNF-α and IL-12 production. Additional evidence was provided by our group, whereby using SHP-1 deficient cells was shown that LAM inhibits *M. tuberculosis*-induced macrophage apoptosis in part by SHP-1 activation [312]. All together, these data suggest that LAM plays an essential and direct role in the negative regulation of multiple host cell functions by modulating signal transduction [313, 314].

Entamoeba histolytica **----** The protozoan parasite *E. histolytica* is the causative agent of amebiasis. Among the strategies used by *E. histolytica* to evade the host immune response is the secretion of active cysteine proteases, involved in complement and immunoglobulin degradation. In addition, *E. histolytica* is responsible, in a contact-

dependent manner, for inducing the cell death of immune competent cells such as neutrophils and T cells. Host cell death is largely mediated by secretion of amoeba pore-forming proteins and induction of intracellular calcium concentrations, and dependent on the direct contact of host and amoebic cells mediated by an *Entamoeba* surface lectin. In 2002, Teixeira and colleagues have shown that during *E. histolytica* attachment to Jurkat T-cells, a rapid host protein dephosphorylation was induced preceding host cell death. This was shown to occur via calpain activation following calcium influx, resulting in the cleavage-dependent activation of host PTP1B. In addition to a role of PTP induction in the parasite-mediated host cell apoptosis, the authors suggest that activation of PTP1B may also be involved in the inhibition of phagocytosis, similarly to what is observed in *Yersini*a infection [315].

Viruses **----** In addition to protozoan parasites, viruses, as strict intracellular pathogens, have adapted to their host in part manipulating signalling pathways in order to control their replication, survival and propagation. Although not extensively studied, modulation of host PTPs seems to play an important role in some of these events. The human cytomegalovirus (HCMV), a member of the β-herpesvirus subfamily, can persist in the host thanks to its capacity to enter into latency and the inhibition of host cell functions including MHCI and MHC II expression [316]. Recently it was reported that HCMV inhibits IFN-γ signalling via induction of SHP-2 activity and STAT-1 dephosphorylation in a mechanism dependent on viral replication [317]. However, the identity of viral proteins/molecules involved in this activation process remains unknown.

Another interesting case is the induction of host cell cycle arrest by the HIV-1 virus, which precedes cellular apoptosis. In this process, the viral protein R (Vrp) plays an important role by promoting the hyper-phosphorylation (in this case rendering the protein inactive) of cell-cycle dependent kinase (CDK-1). This is in part achieved through the inhibition of the central PTP coordinator of the cell cycle CDC25 [318], a positive regulator of cell cycle progression [183].

All together, this section provides insight into the clever ways in which pathogens evade host immune responses by modulating host cell signalling via activation of host protein tyrosine phosphatases or delivery of pathogen´s PTPs. Although to date this list is limited to a few pathogens, it is likely that others such as *Toxoplasma, Listeria* or *Plasmodium*, all of which survive successfully within mammalian host cells, may use similar strategies to manipulate host cell signalling via regulation of protein phosphorylation and PTP activity.

3. IRON METABOLISM: IMPLICATIONS IN CELL SIGNALLING AND INFECTIOUS DISEASES

As so far discussed, the outcome of infectious diseases is influenced by host genetic factors, determinants of innate and acquired immune responses, as well as pathogen fitness and virulence factors. However, underlying disease onset and progression are also environmental conditions, socio-cultural practices and nutritional status.

Iron is one of the most important metal nutrients in biological systems, acting as a central constituent of haemoproteins, iron-sulfur (Fe-S) and other iron-containing proteins, playing a fundamental role in the regulation of a wide array of cellular processes including ATP and *de novo* DNA synthesis, oxygen transport, control of cell division cycles, and importantly in the control of immune functions [319]. Imbalances in iron homeostasis are associated with the pathogenesis of neurodegenerative and hematopoietic disorders [320], and altered host responses to microbial infections [321], among others. In the following sections I will discuss the implications of body/cellular iron homeostasis in the onset and development of infectious diseases, examining its impact from a signalling point of view.

3.1 Body and cellular iron homeostasis

The importance of iron as a biologically active transition metal roots from its ability to engage in one-electron oxidation-reduction reactions, fluctuating between its oxidized and reduced state. However, this same property is responsible for the toxic effects associated with iron overload, where free reactive Fe^{2+} ions can catalyze the formation of ROS via the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$), leading to lipid peroxidation, DNA and protein damage, and ultimately cell death [322]. An average adult human requires an estimated 25 mg of iron for daily usage, of which 80% is destined for haemoglobin synthesis and haematopoiesis. Of these 25 mg, only 1-2 mg

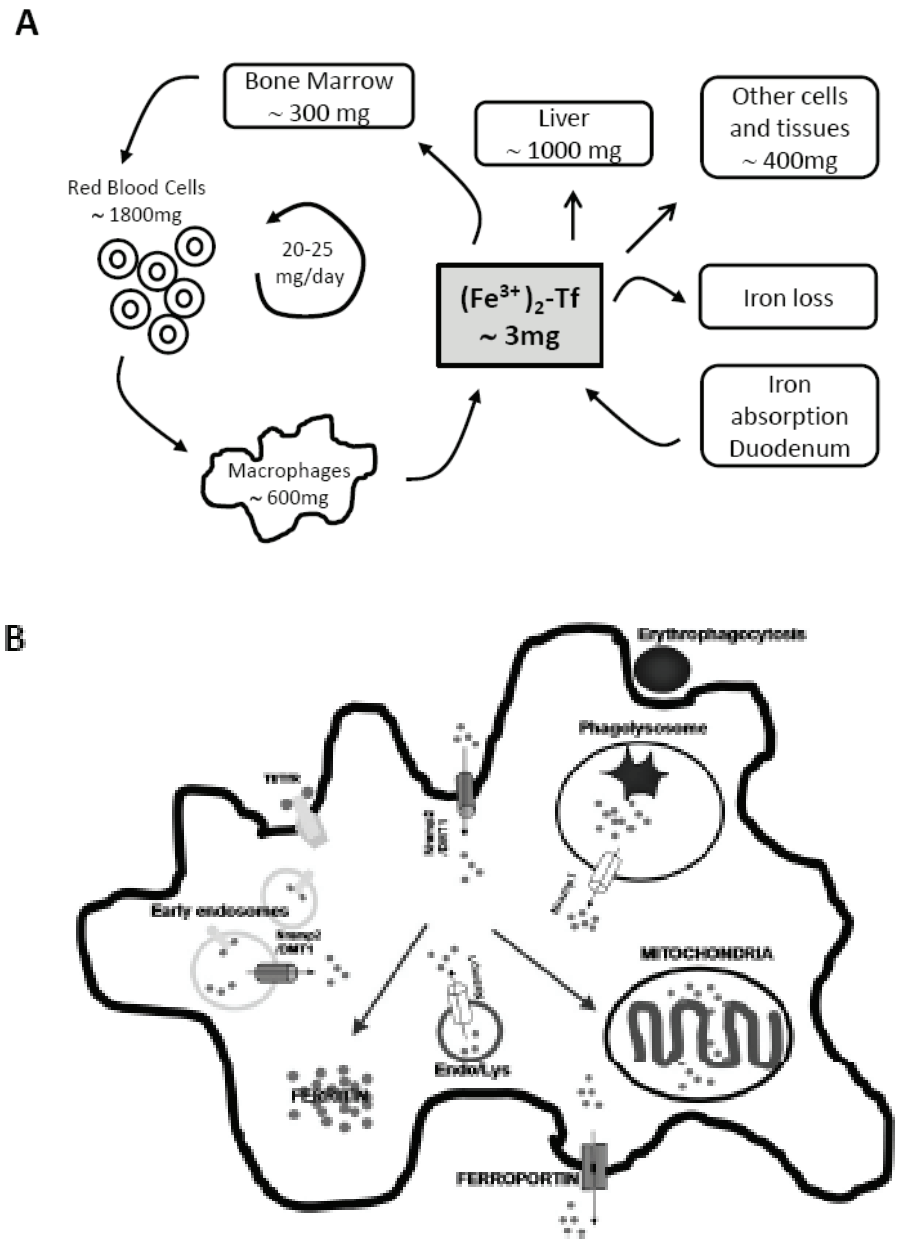
are absorbed from dietary sources [319]. All of the above requires that iron absorption, recycling, storage and distribution be efficiently controlled at the cellular and molecular levels, providing the sufficient amount of iron required for fulfilling cellular functions, avoiding a toxic excess.

Dietary iron absorption takes place at the proximal portion of the duodenum, and it is carried out by epithelial duodenal enterocytes. It is believed that the great majority of iron is absorbed as free -non-heme bound- ferric iron (Fe^{3+}), and it is still controversial the possibility of direct intestinal absorption of heme as an iron source [323, 324]. For transmembrane transport, Fe^{3+} is enzymatically reduced by the ferrireductase Dcytb (duodenal cytochrome-b) to ferrous iron (Fe^{2+}), allowing transport into the enterocyte via DMT-1 (formerly called NRAMP-2), a divalent metal/H^+ symporter homologue to NRAMP-1 [325-327] (Figure 11). Once inside the cytoplasmic space of enterocytes, iron has three fates: it is utilized for immediate cellular functions, it is stored, or it is exported to be distributed systemically by transferrin (Tf) and stored in tissue macrophages and hepatocytes. Iron export from enterocytes is mediated by ferroportin. Fe^{2+} is rapidly oxidized back to Fe^{3+} by the ferroxidase hephestin, allowing loading of two Fe^{3+} atoms onto one Tf molecule initializing its voyage for systemic iron distribution [319].

A central regulator of intestinal iron absorption is hepcidin; a small cysteine-rich peptide hormone produced in the liver. Hepcidin inhibits iron uptake in isolated duodenal segments [328], and reduces serum iron levels by binding to the iron exporter ferroportin, targeting its lysosomal degradation, and resulting in inhibition of cellular iron export [329, 330]. In addition, hepcidin levels are themselves controlled by serum iron levels, where decreased serum iron concentrations leads to lower hepcidin synthesis and increased plasma membrane ferroportin, resulting in iron export and higher serum iron concentrations [330] [331] (Figure 11).

Once in circulation, iron homeostasis is also tightly controlled by regulated mechanisms of iron internalization, storage and export. Cellular uptake of serum iron

FIGURE 11. Iron metabolism.



A) Body iron metabolism. Normal iron content and distribution between human tissues and cells is depicted. Adapted from [319]. **B)** Cellular (macrophage) iron homeostasis. Shown are the mechanisms of cellular iron uptake, storage and export.

proceeds by transferrin-dependent and independent mechanisms. Iron-loaded Tf binds to the ubiquitously expressed transferrin receptor-1 (TfR-1) or TfR-2 (exclusively expressed in hepatocytes, duodenal crypt cells and erythroid cells). This Tf-TfR complex is then internalized by endocytosis, followed by endosome acidification and liberation of the iron atoms from Tf [332]. Next, Fe^{3+} is enzymatically reduced to Fe^{2+} for subsequent transport by the endosomal DMT-1 into the cytoplasmic space [326], and Tf together with TfR are recycled back to the cell surface for subsequent rounds or iron transport.

Additional to Tf-dependent iron internalization, Tf-independent mechanisms also take place, including haemoglobin internalization by macrophages [333], cytoplasmic membrane DMT-1-mediated iron import, and a recently discovered mechanism of iron import mediated by the neutrophil-gelatinase-associated lipocalin (NGAL) [334]. NGAL protein is secreted by neutrophils and endothelial cells and is believed to complex iron through its binding to small molecules including bacterial siderophores. NGAL-iron complex is then internalized by endocytosis and localized to late endosomes [334], suggesting that further transport to the cytoplasm could occur through NRAMP-1 (at least in macrophages and neutrophils).

Once inside the cytoplasm, iron can be found in two major pools, one directed for rapid storage within ferritin, which can retain up to 4500 Fe^{3+} atoms/multimeric protein [335], and a second transient “labile iron pool” (LIP) comprised of redox-active iron complexes which are readily available for extracellular export, association with iron-containing proteins, sensing and regulation of iron-dependent functions, or storage within ferritin [336]. This LIP increases once the saturation storage level is reached and at high pathologic concentrations the homeostatic level will be surpassed, inevitably leading to the generation of ROS and cell death [337]. In order to avoid high toxicity of the LIP, Fe^{2+} and Fe^{3+} atoms are complexed to small molecular weight compounds including ascorbic acid, citrate [338] and phosphates [339], as well as small polypeptides or lipid moieties [336]. Importantly, the high blood plasma citrate levels (~0.1 mM)

allow the bioavailability of dietary non-heme iron [340, 341] and it is believed to represent the major non-transferrin Fe^{3+} plasma pool [338].

Of the total average intracellular iron concentration (10 µM - 100 µM), about 0.4 µM - 16 µM (10 - 20%) represent the LIP [337, 339, 342], differently distributed into intracellular compartments. The main subcellular localization of LIP is found within the cytosol, although a small portion is also found in mitochondria, nucleus [343, 344], endosomes and lysosomes (following ferritin degradation) [339]. Within these compartments, iron is utilized for Fe-S cluster and heme biogenesis, electron transport and ATP biosynthesis, and *de novo* DNA synthesis among others. Finally, in order to allow for proper iron distribution of dietary or recycled iron, cellular export mechanism also play an essential role in the homeostatic regulation of this metal. In this respect ferroportin -a cytoplasmic membrane-associated Fe^{2+} exporter- coordinates the export of Fe^{2+} after which it is rapidly oxidized to Fe^{3+} by ceruloplasmin (Cp), allowing availability for transferrin loading [319].

Parallel to the regulation of iron absorption, storage and export, is the expression and functional regulation of proteins involved in the coordination of cellular iron homeostasis. The regulation of ferritin, ferroportin, TfR-1 and DMT-1 synthesis occurs post-transcriptionally and it is orchestrated by the IRP/IRE system. Iron Regulatory Elements (IREs) are secondary hairpin structures with a canonical 5´-CAGUGN - 3' apical loop present in the 5' or 3' UTR mRNA region of iron-regulated genes. Iron Regulatory Proteins (IRPs) interact with IREs repressing or promoting translation; 5´UTR IRP/IRE complex inhibits mRNA translation, whereas 3´UTR IRP/IRE complexes induce mRNA stability promoting translation [345, 346]. IRPs (IRP-1 and IRP-2) themselves are regulated by the LIP concentrations, where at high intracellular [iron], an Fe-S cluster assembles in IRP-1 inhibiting its binding ability to IREs. Conversely, in low [iron], IRP-1 binds IREs, repressing the translation of storage molecules (ferritin) and promoting iron import via induction of TfR-1 and potentially DMT-1 translation. Although IRP-2 activity is independent of the assembly of an Fe-S cluster, its protein

stability is compromised at high iron concentrations [319]. This iron-dependent regulation of IRPs further emphasizes the importance of a tight modulation of iron metabolism, where clear molecular control check points respond to intracellular iron concentrations and determine the need for iron storage, absorption or export.

3.2 Iron and immunity

Several lines of evidence point to an important interplay between the immune system and iron metabolism; the regulation of different aspects of iron metabolism by inflammatory cytokines [319], the altered response to infectious diseases in the context of iron deficiency and iron overload [321], and the central role of immune cells in the control of iron homeostasis [347], represent key elements linking iron and immunity.

Perhaps one of the most compelling facts is the central function of macrophages in the storage and recycling of body iron via erythrophagocytosis. Senescent or damaged RBCs are removed from the blood by macrophages from the reticuloendothelial system (bone marrow, spleen and liver). Following phagocytosis RBCs are lysed releasing haemoglobin. Haemoglobin is then degraded allowing iron liberation from heme, via heme oxygenase-1 (HO-1), after which iron can be further utilized and transported. Free iron is exported from the phagosomal compartment by NRAMP-1 joining afterwards the LIP [348]. Extracellular transport via the iron transporter ferroportin, returns the iron into circulation [347] (Figure 11B).

The involvement of macrophages as host, accessory and effector cells in the development of infectious diseases, together with their role in iron homeostasis, place these immune cells as central players in the interface between iron and infection. Similar to mammalian cells, iron is an important growth factor for pathogenic microorganisms by acting as a cofactor or prosthetic group in a number of enzymes required for cellular replication, glycolysis and defence against the host toxic oxygen radicals. In this sense, extracellular and intracellular pathogens have to compete with

their host for iron acquisition. In doing so, microorganisms have developed efficient iron uptake mechanisms such as siderophore-mediated iron chelation [349] or interference with host iron uptake systems [350]. Interestingly, the latter is most avidly exploited by intracellular pathogens which depend on their host cell for iron acquisition, clearly exemplified by *Mycobacterium tuberculosis* and *Leishmania* parasites.

M. tuberculosis successfully resides within host macrophages in part by inhibiting phagolysosomal maturation [351]. Therefore, in the state of early phagosome, the pathogen can exploit the host´s Tf-TfR-iron uptake system, incorporating iron from Tf presumably through affinity competition of mycobacterial siderophores (mycobactin) and Tf [352].

Both the amastigote and promastigote stages of the protozoan parasite *Leishmania* have developed mechanisms to obtain iron from its host. Extracellular promastigotes have been shown to uptake iron via haemoglobin binding and internalization through the flagellar pocket via an endocytic mechanism [353]. Conversely, the intracellular amastigote survives and replicates within the phagosome of infected macrophages. Here *Leishmania* parasites subvert macrophage Tf trafficking, by delivering iron-loaded Tf to the parasitophorous vacuole. Although it is not clear which mechanism is utilized by the parasite to divert Tf recycling, it was shown that phagosomal Tf interacts with and binds amastigotes allowing iron-loaded Tf (holo-Tf) internalization via an endocytic pathways mediated through the parasite´s flagellar pocket [354]. In addition, it has been previously reported that promastigotes have a Tf receptor which can interact with Tf for iron acquisition [355, 356]. Moreover the interesting finding that holo-Tf rather than apo-Tf (Tf lacking iron atoms) promotes parasite survival, suggest that *Leishmania* clearly utilizes host Tf as an iron source for its intracellular development. More recently, a novel amastigote-specific *Leishmania* iron transported (LIT) was identified, localizing to the plasma membrane, and involved in the transport Fe^{2+} from the phagosomal milieu into the amastigote cell [69]. Interestingly, LIT expression was induced in iron-poor environments, correlating with an important

role in iron acquisition, and further identified this transporter as an important virulence factor; LIT-deficient *Leishmania* parasites had poor ability to induce lesion formation in BALB/c mice footpads [69].

During inflammatory responses, both Th1 and Th2 cytokines influence iron metabolism [357] (Table 4). Pro-inflammatory cytokines such as IFN-γ, IL-1β, TNF-α and IL-6 promote iron retention and storage by inducing ferritin gene transcription and downregulating TfR-1 expression [335]. In the case of TNF-α, induction of ferritin expression is dependent on NFκB binding and activation of gene transcription [358]. Cytokines also regulate ferritin levels post-transcriptionally, however, in the case of IL-1β independently of IRE/IRP-mediated regulation [335]. Moreover inflammatory stimuli such as bacterial LPS and IFN-γ induce DMT-1 expression [359] and repress ferroportin [360], promoting additional intracellular iron retention. Interestingly, the anti-inflammatory cytokine IL-10 had a reverse effect on iron metabolism compared to pro-inflammatory cytokines, as it induced TfR-1 and ferroportin expression [360]. Another interesting regulatory mechanism is the IL-6 and LPS-induced upregulation of hepcidin expression. As mentioned before, hepcidin binds ferroportin (iron exporter) promoting its internalization and degradation, resulting in a net intracellular iron accumulation [329]. Collectively, these data further emphasize the role of pro-inflammatory cytokines in the uptake and cytosolic retention of intracellular iron aiding, for example, in the defence strategies against pathogens by retaining essential nutrients limiting in this way their development and survival.

The complex interactions between iron and the immune system are also reflected in the modulation of immune functions by iron overload or iron deficiency. Iron overload prior to IFN-γ stimulation has been shown to inhibit macrophage TNF-α production and MHC II expression [357]. The same group has continued to show that inhibition of type I cytokines by iron overload results in a balance towards a Th2 response with induction of IL-4 production [357]. However, many other reports have

TABLE 4.

Iron and the modulation of immune functions.

Treatment	Outcome	Putative mechanism	Reference
IFN-γ + FeTf IFN-γ + FeNTA	Control of intracellular *Brucella*	IFN-γ synergism with ROS	[361, 362]
TNF-α + FeNTA FeNTA →TNF-α	Enhanced TNF-α cytotoxicity in L929 cells	---	[363]
$FeSO_4$	Enhanced secretion of IL-1β	Interaction of oxidized lipids with the cell	[364]
Iron-dextran	Inhibition of IL-4 and IL-10 production. Control of *Leishmania* infection.	Increased oxidative burst, enhanced NFκB signaling.	[365-367]
$FeSO_4$	Enhanced secretion of TNF-α.	Induced NFκB signaling	[368]
FeTf → IFN-γ	Inhibition of TNF-α production and MHC II expression.	---	[357] [369]
IFN-γ, IL-1β, TNF-α or IL-6	Iron retention and storage.	Induction of ferritin transcription and downregulation of TfR-1.	[335]
LPS and IFN-γ	Iron import.	Induction of DMT-1 expression	[370]
IL-6 and LPS	Intracellular iron accumulation	Upregulation of hepcidin expression.	[329]
IL-10	Increased iron export.	Induced TfR-1 and ferroportin	[360]

shown that iron promotes macrophage functions. The microbicidal capacity to control the intracellular bacteria *Brucella abortus* was shown to be enhanced in iron-loaded macrophages, where IFN-γ synergized with iron-catalyzed ROS to eliminate the intracellular organism [361, 362]. As well, TNF-α-mediated cytotoxicity was shown to be enhanced by co-administration with iron [363]; IL-1β release was induced in macrophages treated with ferrous sulfate [364], and iron-dextran loaded mice showed inhibition of IL-4 and IL-10 production [366]. Moreover, iron chelation leads to inhibition of TNF-α and IL-6 [258] and inhibited T-cell IFN-γ production along with IL-12 and IL-4-mediated T-cell proliferation [371]. Albeit contradictory arguments of whether iron promotes or inhibits the pro-inflammatory immune response, it is clear that imbalance in its metabolism leads to pathological conditions. Differences observed

between groups may be a result of the experimental iron concentrations, iron donor species, cellular models and kinetics of the reactions.

As a consequence of the complex regulation of immune functions by systemic or cellular iron levels, the outcome of infectious diseases is similarly complex in response to iron overload or iron deficiency. In addition to therapeutic treatments where iron supplementation or iron chelation are imperative, conditions such as chronic alcoholism, hemochromatosis or β-thalassemias have a great impact on iron absorption and accumulation, and will therefore impact the onset, progression and ultimate outcome of infections [372]. Although iron overload has been shown to exacerbate infectious diseases including tuberculosis, malaria and yersiniosis [321], it has also been associated with resistance to *Leishmania* infection mediated by induction of oxidative burst and enhanced IFN-γ production by T-cells [365-367]. In parallel, iron chelation has been shown to exacerbate intracellular *Listeria* growth [373] and *Salmonella* infection in mice [374], the latter by abrogating NADPH-oxidase-dependent ROS generation; whereas iron chelation therapy successfully promotes cure for malaria [375] and TB [376, 377]. All these reports exemplify the complex interaction between iron, the immune system and the response to infection, and call to a very careful study of the impact of iron treatment in the course of specific infectious diseases.

3.3 Iron and cell signalling

As discussed in previous sections, the cellular response to inflammatory stimulus is controlled by signalling cascades; therefore, the differential modulation of cytokine production, T and B-cell activities, as well as macrophage functions in response to iron treatment or iron chelation should be upstream regulated by differential modulation of signal transduction. In its role as an enzyme co-factor, iron modulates the activity of protein kinases and protein phosphatases. In this way, it regulates the phosphorylation

and activation of signalling molecules and transcription factors, ultimately leading to the differential regulation of gene transcription and expression.

Serine/Threonine Phosphatases

Protein phosphatases comprise two large groups of enzymes: Serine/Threonine phosphatases of the PPP and PPM family, and PTPs. Although not as critical as PTPs in the regulation of immune responses, Serine/Threonine phosphatases have been shown to play an essential role in glycogen metabolism, muscle contraction [378, 379] and in neuronal systems controlling learning and memory [380]. Serine/Threonine phosphatases of the PPP family comprise three important regulators of cell signalling in eukaryotic cells: PP1, PP2A and PP2B, all of which share a common catalytic domain and mechanism of catalysis, however maintaining great diversity in the N and C-terminal domains [378].

Crystallographic studies provided evidence that the catalytic activity of serine/threonine phosphatases was dependent on the coordination of two metal ions in the enzyme´s catalytic site. Although the exact composition and oxidation state of the metal core is still controversial (Zn^{2+}-$Fe^{2+ \text{ or } 3+}$, or Mn^{2+}-$Fe^{2+ \text{ or } 3+}$) the presence of an iron atom in the enzymatic core is undoubted. Serine/threonine phosphatases catalyze the dephosphorylation of serine and threonine residues in a single step mechanism, where the metal core plays a central role [378, 381]. Consistent with this observation, site directed mutagenesis of amino acid residues involved in the coordination of the metal ions greatly reduced phosphatase activity [382]. Interestingly, it is proposed that oxidation of the binuclear metal center represents a mechanism of activity regulation [383, 384].

Surprisingly, to the best of our knowledge, no investigations so far have addressed the impact of differential iron metabolism on the regulation of

serine/threonine phosphatase activity. This line of research may shed important insights into the molecular mechanisms by which iron homeostasis regulates cellular functions.

Protein kinases

For more than a decade, it has been known that ROS can induce the activation of protein tyrosine kinases [385]. Stimulation of COS-7 cells with H_2O_2 induces the tyrosine phosphorylation and activation of PKC family members [386], suggesting that upstream kinases need to be activated in response to peroxide treatment. Indeed it has been shown that the MAPKs p38, ERK and JNK [385, 387], in addition to tyrosine kinases such as Syk and Lck [388, 389] are activated upon oxidative stress. Although the exact molecular mechanism remains largely unknown, this activation may be a result of ROS-dependent PTP inhibition or direct activation of protein kinases as ROS targets. In such a way, iron may indirectly promote signal transduction by activating protein kinases catalyzing the generation of ROS; however to our knowledge, this hypothesis has not been extensively researched.

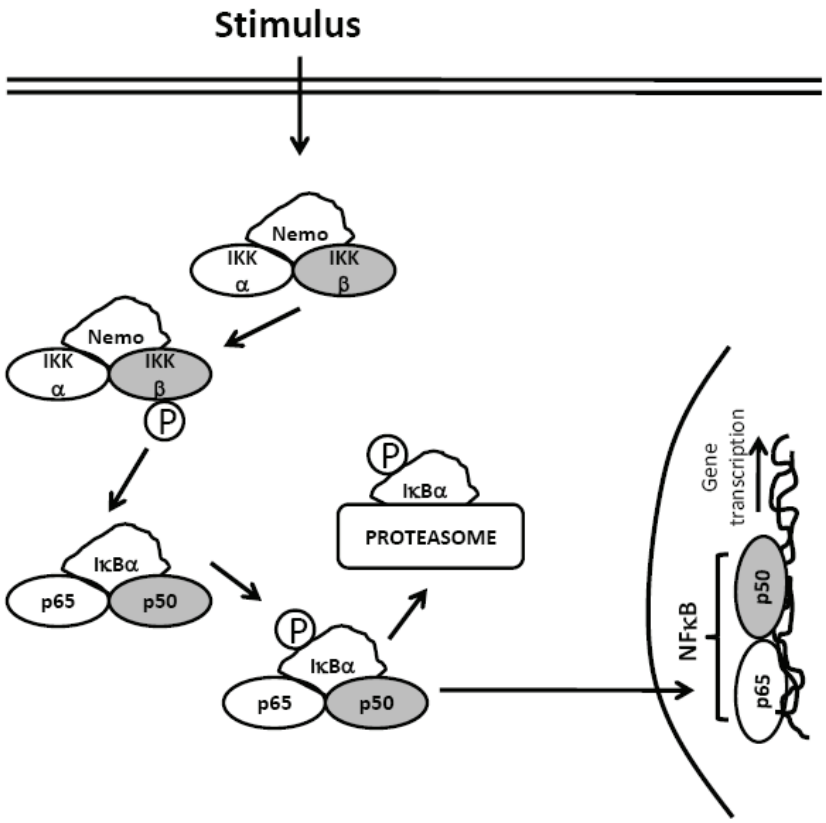
NFκB signalling pathway

Various reports have identified the transcription factor NFκB as an upstream target regulating some of the effects associated to iron and inflammation. Treatment of human intestinal epithelial cells (HT-29) or THP-1 monocytes with deferroxamine (DFO) - a bacterial siderophore commonly used as a potent chelator in iron-chelation therapy - was shown to promote pro-inflammatory cell functions by inducing IL-8 secretion; an effect that was inhibited by the addition of ferric citrate. IL-8 induction was further correlated with upstream hyperphosphorylation of the MAPKs ERK 1/2 and p38. Although downstream of these MAPK signalling pathways is the induction of gene transcription by NFκB, no NFκB activation was observed and IL-8 secretion was rather suggested to occur by induction of mRNA stability [390]. Paradoxically, Zhang and

colleagues showed that iron chelation with DFO in microglia (phagocytes of the central nervous system) inhibits induction of pro-inflammatory cytokine production induced by LPS, and was mediated by the inhibition of NFκB activation [391]. In line with these observations, Chen and colleagues have recently demonstrated that low molecular weight iron complexes (LMW·Fe) induce NFκB translocation and DNA binding activity, by an upstream activation of IKK (Figure 12). IKK activation leads to the phosphorylation of IκB targeting its ubiquitination and proteasomal degradation, releasing NFκB for nuclear translocation and activity [368]. Interestingly, iron-dependent IKK activation and signalling was shown to be upstream regulated by p21ras, a G-protein family member which functions as an adaptor molecule central for signal transduction through Ras-Raf-MAPK and Ras-PI3K-AKT pathways. The authors suggest that p21ras activation is mediated by iron-dependent generation of oxygen and nitrogen radicals conducting to p21ras S-nitrosylation, previously shown to activate this G-protein [392]. Moreover, p21ras activation was correlated with an iron-induced activation of the PI3K and ERK-MEK pathways, detected by the hyperphosphorylation of ERK 1/2 and AKT [368].

Of particular interest is the finding that ligand-receptor interaction in the context of LPS and TNF-α stimulation induces a rapid and transient increase in intracellular LMW·Fe complexes in macrophages. Increased $[LMW{\cdot}Fe]_I$ led to upregulation of NFκB nuclear translocation and DNA binding, correlating with higher TNF-α release [257]. The authors suggest that increase $[LMW{\cdot}Fe]_I$ in response to ligand-receptor interactions may represent an event similar to the modulation of intracellular calcium concentrations in response to various exogenous stimuli, and its suggested to act as signalling trigger event in macrophages.

FIGURE 12. NFκB signaling.



The five members of the mammalian NFκB family, p65 (RelA), RelB, c-Rel, p50/p105 and p52/p100 exist in unstimulated cells as homo- or heterodimers bound to IκB family proteins, preventing the translocation of NFκB to the nucleus, thus maintaining it inactive. Upon stimulation, IκB kinase (IKK) gets phosphorylated which in turn phosphorylates IκB, targeting it for ubiquitination and subsequent proteasomal degradation. This event releases NFκB allowing its translocation to the nucleus where it binds a consensus sequence in promoter regions of DNA, stimulating gene transcription. Adapted from [393]

RATIONALE AND OBJECTIVES OF THE RESEARCH

Although major advancements have been achieved in understanding the immunomodulatory events regulating the outcome of leishmaniasis, little is known on the basic molecular events controlling signal transduction, which in turn delineate the altered host cell functions associated with *Leishmania* infection. This thesis aims to address this point from the perspective of host protein tyrosine phosphatases and the regulation of signalling pathways; how does *Leishmania* induce the activation of host PTPs? In addition to SHP-1, do other macrophage PTPs intervene in the negative regulation of host cell functions exerted by *Leishmania* parasites? How are macrophage PTPs regulated in the context of innate resistance to infection? Data presented herein seek to put in evidence novel mechanisms of PTP regulation by host and parasite factors, in an effort to better understand the host-pathogen interaction governing this infectious disease.

Macrophages, the specialized host cell for uptake and intracellular development of *Leishmania* parasites, play a central role in the control of infection. This is largely delineated by their capacity to produce nitric oxide and reactive oxygen intermediates, together with their ability to mediate leukocyte chemotaxis and T-cell activation. Despite the role of macrophages as efficient accessory and effector immune cells, *Leishmania* has evolved strategies to downregulate host cell functions including nitric oxide and IL-12 production, together with MHC II expression, promoting its development as an intracellular pathogen. From a signalling perspective, *Leishmania* alters Ca^{2+}, MAPK, JAK/STAT and PKC signalling, and importantly, activates host protein tyrosine phosphatases (PTPs) [73]. One of the early events regulating the *Leishmania*-dependent inhibition of macrophage effector and accessory functions is the rapid activation of the host PTP SHP-1, which results in the downregulation of JAK/STAT and MAPK signalling. Although PTP activation is a hallmark of the *Leishmania*-induced

negative regulation of phagocyte functions, its role in the context of innate resistance to *Leishmania* infection remains unknown.

NRAMP-1 has been associated with innate resistance to *Leishmania*, and with the upregulation of pro-inflammatory macrophage functions such as MHC II expression and NO production [394]. As a divalent cation transporter, it is suggested that efflux of metals from the phagosome arrests parasite development by interfering with essential microbial functions [38]. However, it is not clear how NRAMP-1 metal transport function regulates the multiple pleiotropic effects associated with its functional expression. Given that modulation of PTP activity greatly influences signalling and phagocyte functions**,** we sought to determine whether NRAMP-1 expression impacts on macrophage PTP activity and as a result, differentially regulates signalling pathways and downstream macrophage functions. Chapter 2 details the mechanism of NRAMP-1 mediated PTP modulation as an upstream event controlling host cell functions and possibly the outcome disease. We have demonstrated that macrophages functionally expressing NRAMP-1 negatively regulate PTP activity resulting in the hyperphosphorylation and activation of STAT1, ERK1/2 and MEK1/2 following IFN-γ and LPS stimulation, and leading to the induction of nitric oxide production and control of *Leishmania* infection.

Decreased PTP activity observed in NRAMP-1 expressing macrophages does not result from changes in PTP protein expression, but instead involves the direct inhibition of PTP activity by iron, a substrate of this metal transporter. Chapter 3 describes the molecular mechanism underlying the iron-mediated inhibition of macrophage PTP activity. Herein, we have identified mononuclear iron-citrate complexes as the active species which specifically interact with the macrophage PTPs SHP-1, PTP1B, TCPTP and PTP-PEST. By using biochemical and computational approaches, we have identified the PTP catalytic cleft as the putative site for iron-PTP interaction. Moreover, we extend our *in vitro* observations to *in vivo* models of iron delivery, and show that iron treatment results in lower PTP activity and increased MAPK signalling both in macrophage cell

culture and in BALB/c mice. A model of *in vivo* iron-dependent regulation of PTP activity and its implications in the outcome of signalling pathways is discussed.

Despite the critical role of SHP-1 in the *Leishmania*-mediated negative regulation of macrophage signalling [73], additional PTP enzymatic activation is observed in SHP-1 deficient macrophages infected with *Leishmania* parasites, suggesting that other host PTPs could play a role in this downregulation process. Although identification of SHP-1 as a host target of *Leishmania* infection was reported 10 years ago [172, 395], the exact mechanism whereby *Leishmania* induces PTP activation is not completely understood. Chapter 4 describes the identification of TCPTP and PTP1B as two additional macrophage PTPs engaged upon *Leishmania* infection. Moreover, we unravel a novel mechanism of *Leishmania*-dependent macrophage PTP regulation which involves active cleavage of SHP-1, PTP1B and TCPTP by the *Leishmania* protease and virulence factor GP63. The molecular mechanisms underlying GP63 internalization into host cells and PTP-GP63 interactions are further discussed. Collectively, the results presented throughout this thesis illustrate the importance of balanced signal transduction in the outcome of *Leishmania* infection, and provide evidence of novel mechanisms of host-pathogen interactions. In an ideal situation, the new knowledge generated with the present investigations would shed lights into understanding the underlying mechanisms by which other intracellular pathogens interact with their hosts, opening new avenues for the rational design of pathogen control strategies.

PREFACE TO CHAPTER 2

NRAMP-1 has been associated with innate resistance to *Leishmania* infection and with the upregulation of pro-inflammatory macrophage functions. However, it remained unclear how this divalent metal transporter regulates the pleiotropic effects associated with its functional expression. Controlling cellular functions is the activation of signaling pathways, where events of protein phosphorylation play a decisive role in the enzymatic activation, localization and protein-protein interactions regulating signal transduction. Understanding the central role of protein tyrosine phosphatases in the regulation of protein phosphorylation, we examined the impact of NRAMP-1 expression on macrophage PTP activity as a putative mechanism contributing to the control of the innate immune response. Experiments presented in this chapter show that macrophage NRAMP-1 expression negatively regulates PTP activity, resulting in the phosphorylation/activation of signaling molecules and the induction of downstream phagocyte functions, which contribute to the killing of intracellular *Leishmania* parasites. Importantly, we show that NRAMP-1 mediated PTP activity regulation results from an iron-dependent inhibition of PTP activity, providing a direct link for NRAMP-1 metal transport and the differential expression of pro-inflammatory phagocyte functions.

CHAPTER 2

NRAMP-1 EXPRESSION MODULATES PROTEIN TYROSINE PHOSPHATASE ACTIVITY IN MACROPHAGES: IMPACT ON HOST CELL SIGNALING AND FUNCTIONS.

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ABSTRACT

Natural Resistance Associated Macrophage Protein-1 (NRAMP-1) has been associated with innate resistance to unrelated intracellular pathogen infections, upregulation of proinflammatory phagocyte functions, and susceptibility to autoimmune diseases. It is still unclear how the divalent cation transport function of NRAMP-1 accounts for the associated pleiotropic effects. In this study, we evaluated the impact of murine macrophage NRAMP-1 expression on the activity of protein tyrosine phosphatases (PTPs), as an upstream event contributing to the NRAMP-1 regulation of signal transduction and control of effector macrophage functions. Functional expression of NRAMP-1 results in lower macrophage PTP activity and increased protein phosphorylation. Decreased PTP activity is not a result of changes in protein expression, but rather, a reversible regulatory mechanism involving the interaction with NRAMP-1 metal substrates. In the context of intracellular infections, NRAMP-1 expression prevents full macrophage PTP induction by *Leishmania* infection, correlating with higher nitric oxide production and lower parasite survival. We suggest that NRAMP-1 divalent cation transport leads to transient inhibition of PTPs, via direct PTP-metal interaction, and/or by reactive oxygen species dependent PTP oxidation, consequently promoting positive signal transduction, as a backbone for the induction of pro-inflammatory phagocyte functions.

INTRODUCTION

Natural Resistance Associated Macrophage Protein-1 (NRAMP-1, previously known as *Bcg/Ity/Lsh*, and recently re-named Slc11a1) has been associated with host resistance to unrelated intracellular pathogens [27, 396] including *Leishmania* [397, 398]*, Mycobacterium* [399] and *Salmonella* [400]**.** It has also been associated with the upregulation of pro-inflammatory macrophage (Mϕ) functions such as MHC II expression [401, 402], KC chemokine [403, 404]**,** IL-1β [403]**,** TNF-α [405, 406], nitric

oxide (NO) production [407, 408] and increased respiratory burst [409, 410]. NRAMP-1 is a pH dependent divalent cation transporter [28] localized to the late endosome/lysosomal compartment of Mϕs from the reticuloendothelial system [29] and present in gelatinase positive tertiary granules of neutrophils [30]. Upon phagocytosis, NRAMP-1 is rapidly recruited to the phagolysosomal membrane where it mediates the transport of Mn^{2+}, Fe^{2+}, Co^{2+}, and potentially other metals including Zn^{2+} [31-34]. It is still controversial the direction of metal flux [28, 35, 36], however, transport studies, sequence and structural similarities with NRAMP-2, together with topology and thermodynamic considerations, suggest that metal transport occurs from the vesicular lumen to the cytoplasm [28]. NRAMP-1 expression has been shown to promote phagosome maturation [37-39]. In addition, efflux of essential metals from the phagosome may restrain the pathogen's development by interfering with essential microbial enzymes such as superoxide dismutase (SOD), and/or by promoting the upregulation of host pro-inflammatory molecules. Although more than 10 years have passed since the positional cloning and characterization of NRAMP-1 [396, 411], it still remains unclear how the divalent cation transport function of this protein contributes to, and mediates the multiple pleiotropic events associated with its functional expression.

Protein phosphorylation plays a fundamental role in signaling pathways. The fine balance of protein kinase and protein phosphatase activities promotes the homeostasis of protein phosphorylation. Alterations in this balance, however, permit the unfolding of signal transduction. It has been previously shown that NRAMP-1 expressing Mϕs have a higher protein kinase C (PKC) activity compared to NRAMP-1 deficient cells. This was associated with the induction of NO production, respiratory burst and NRAMP-1 mRNA stability [412, 413]. Having formerly observed that modulation of protein tyrosine phosphatases (PTP) greatly influences signaling and phagocyte functions [158, 251, 252, 277], we sought to determine whether NRAMP-1 expression impacts PTP activity, as a mechanism for regulating signaling pathways and downstream cellular events. Results from the present study reveal that NRAMP-1 expression has an effect on Mϕ PTP

activity, in turn modulating the phosphorylation/activation of signaling proteins associated with LPS and IFN-γ dependent NO production and the Mϕ response to *Leishmania* infection. Our data and ongoing research suggests that the mechanism underlying this regulation involves a metal-dependent reversible inhibition of PTP activity.

EXPERIMENTAL PROCEDURES

Materials. 4-Nitrophenylphosphate disodium salt hexahydrate (pNPP), LPS (*Escherichia coli*, serotype 0111:B4), Poly(Glu,Tyr), phosphotyrosine peptide substrate (TRDIpYETDYYRK), N,N,N′,N′-Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), and deferoxamine (DFO) were purchased from Sigma-Aldrich. [γ-^{32}P]dATP (3000 Ci/mmol) was obtained from GE Healthcare. Guanidine ydrochloride was purchased from Laboratoire MAT (Beauport, Qc, Canada). Recombinant interferon gamma (IFN-γ) was obtained from Cedarlane Laboratories.

Cell culture. RAW 264.7 murine Mϕ cell line was kept in DMEM (Gibco-BRL) supplemented with 10% heat inactivated fetal bovine serum (FBS), 100μg/ml streptomycin, 100U/ml penicillin, and 2mM L-glutamine at 37°C and 5% CO_2. *Nramp-1* transfected RAW 264.7 (RAW *Nramp-1)* were maintained in selective DMEM containing 1mg/ml G418 [414]. *Leishmania donovani* promastigotes strain 2211 (Ld) and a *L. donovani* strain stably transfected with the luciferase reporter gene (Ld-Luc) were kept in SDM-79 medium at 25°C supplemented with 10% heat inactivated FBS and 5 mg/ml of hemin[415, 416].

In vitro infections. Stationary phase Ld promastigotes were used to infect Mϕs for 1h at 37°C in a 20:1 Ld-Mϕ ratio (unless noted). Non-internalized parasites were removed by washing the plates with Phosphate Buffer Saline (PBS), after which Mϕs were collected for subsequent procedures. For parasite survival determinations, Mϕs

were infected with a 10:1 Ld-Mϕ ratio for 6 hours, washed with PBS and followed by a chase period of 24h. Parasite survival was estimated by determining luciferase activity [416].

Phosphatase assays. As previously described [159]**,** infected and non-infected Mϕs were collected, lysed in PTP lysis buffer (50mM Tris pH 7, 0.1mM EDTA, 0.1mM EGTA, 0.1% 2-ME, 1% Igepal, 25 μg/ml aprotinin, and 25 μg/ml leupeptin) and kept on ice for 45 minutes. Lysates were cleared by centrifugation, and protein content was determined by Bradford's method [417]. Ten μg of protein extract were incubated in phosphatase reaction buffer (50mM Hepes pH 7.5, 0.1% 2-ME, 10mM pNPP) for 30 minutes. OD was read at 405nm. Specific PTP activity was determined by the capacity of protein lysates to dephosphorylate a monophosphorylated phosphotyrosine peptide substrate (TRDIpYETDYYRK) for 10 minutes at 37°C. Free inorganic phosphate was detected with malachite green (Sigma-Aldrich) and OD was taken at 620nm.

In-gel PTP assay. In-gel PTP assay was performed as previously described [418, 419]. Briefly, Poly(Glu,Tyr) substrate was tyrosine phosphorylated by overnight (ON) incubation with 10 μg of purified GST-FER protein kinase and 150 μCi [γ-^{32}P]dATP. The radiolabeled-phosphorylated substrate was incorporated in a 10-12% SDS-polyacrylamide gel mixture at a concentration of 2 x 10^5 CPM/ml of gel solution. Mϕ protein extracts were prepared as described above, denatured by standard SDS-PAGE procedures and loaded onto the gel. After electrophoresis, the gel was incubated ON in Buffer A (50mM Tris-HCl pH 8.0, 20% isopropanol). Gels were washed twice with Buffer B (50mM Tris-HCl pH 8.0, 0.3% β-mercaptoethanol), followed by full protein denaturation in Buffer B containing 6M guanidine hydrochloride and 1mM EDTA. The gels were washed twice in Buffer C (50mM Tris-HCl pH 8.0, 1mM EDTA, 0.3% β-mercaptoethanol and 0.04% Tween 20). Final renaturation was obtained after a ON incubation in Buffer C. Gels were dried and exposed to X-ray film.

Western Blotting. Western blots were performed as previously described [420]. Primary antibodies used were α-phosphotyrosine clone 4G10, α-SHP-1 and α-PTP1B (Upstate), α-SHP-2 and α-STAT1 (Santa Cruz Biotechnology). α-phosphotyrosine STAT1 (Tyr 701) (Cell Signaling Technologies), α-phospho-ERK1/2 (Thr202/Tyr204), α-ERK1/2, α-phospho-MEK1/2 (Ser217/221), α-MEK1/2 (New England Biolabs). α-TCPTP 3E2 [421] and α-PTP-PEST 2530 [422] were obtained as described.

Electrophoretic Mobility Shift Assay (EMSA). EMSA was performed as described before [420]. Briefly, nuclear extracts were incubated with binding buffer containing 1.0ng of [γ-^{32}P]dATP radiolabeled dsDNA oligonucleotide for 20 min at RT. The oligonucleotides used were the DNA binding consensus sequences for NF-κB (5'-AGTTGAGGGGACTTTCCCAGGC-3') and GAS/ISRE (5'-AAGTACTTTCAGTTTCATATT ACTCTA-3'). Sp1 consensus oligonucleotide was used as unspecific control (5'-ATTCGATCGGGGCGGGGCGAGC-3') (Santa Cruz Biotechnology). DNA-protein complexes were resolved by electrophoresis in native 4% (w/v) polyacrylamide gels. The gels were subsequently dried and autoradiographed.

Confocal microscopy. Cells were plated ON in glass cover slips, washed with cold PBS, fixed with 4% formaldehyde at 4°C and permeabilized for 5 minutes in PBS containing 1%BSA and 0.05% NP-40. After blocking in 5% non-fat evaporated milk in PBS the cover slips were incubation with α-phosphotyrosine antibody. Slides were washed with PBS incubated with AlexaFluor 488 α-mouse antibody (Molecular Probes). Nuclear stain was performed with propidium iodide. After mounting, cells were visualized by confocal microscopy (X63), using a Zeiss LSM 510 system.

Nitric oxide (NO) production. Mϕs were seeded in 12-well plates and cultured in the presence or absence of purified *E.coli* LPS for 24h or IFN-γ for 48 hours. NO production was evaluated by measuring the accumulation of nitrite in the culture medium by the Griess reaction, as previously described [250].

Statistical analysis. Data were analyzed by one-way ANOVA. Statistically significant difference between groups was considered when $P < 0.05$ or $P < 0.01$. All data are presented as the mean ± standard error of the mean (SEM).

RESULTS

NRAMP-1 regulation of NO production is associated with modulation of signaling pathways. One of the major Mϕ functions associated with NRAMP-1 expression and the successful control of intracellular pathogens is NO production [407, 408]. By using the Mϕ cell line RAW 264.7 (naturally deficient in a functional NRAMP-1) and NRAMP-1-transfected RAW cells (RAW *Nramp-1*) [414]**,** we have studied the signaling pathways underlying IFN-γ and LPS dependent NO production as a means to functionally validate the resistance phenotype of our Mϕ cell line system.

LPS stimulation induced a higher and dose dependent (2-10 fold) nitrite accumulation in RAW *Nramp-1* cells, compared to its NRAMP-1 deficient counterpart (Figure 1A). As with LPS, NO production following IFN-γ stimulation was significantly higher in RAW *Nramp-1* cells, (Figure1D). Previous reports have shown that IFN-γ and LPS dependent NO production in NRAMP-1 expressing Mϕs results from increased iNOS mRNA stability and transcription [408]**.** Evaluation of MEK/ERK and JAK/STAT signaling pathways, responsible for the induction of iNOS expression [423-425], showed that LPS and IFN-γ strongly induced the respective phosphorylation and activation of MEK1/2, ERK1/2 (Figure 1B) and STAT1 (Figure 1E) in RAW *Nramp-1* cells at all time points during a one-hour time course stimulation, and was reflected downstream in an increased and sustained nuclear translocation and DNA binding activity of NF-κB (Figure 1C) and STAT1 (Figure 1F). These results are in support of previous observations [360, 426] validating our *in-vitro* system.

FIGURE 1

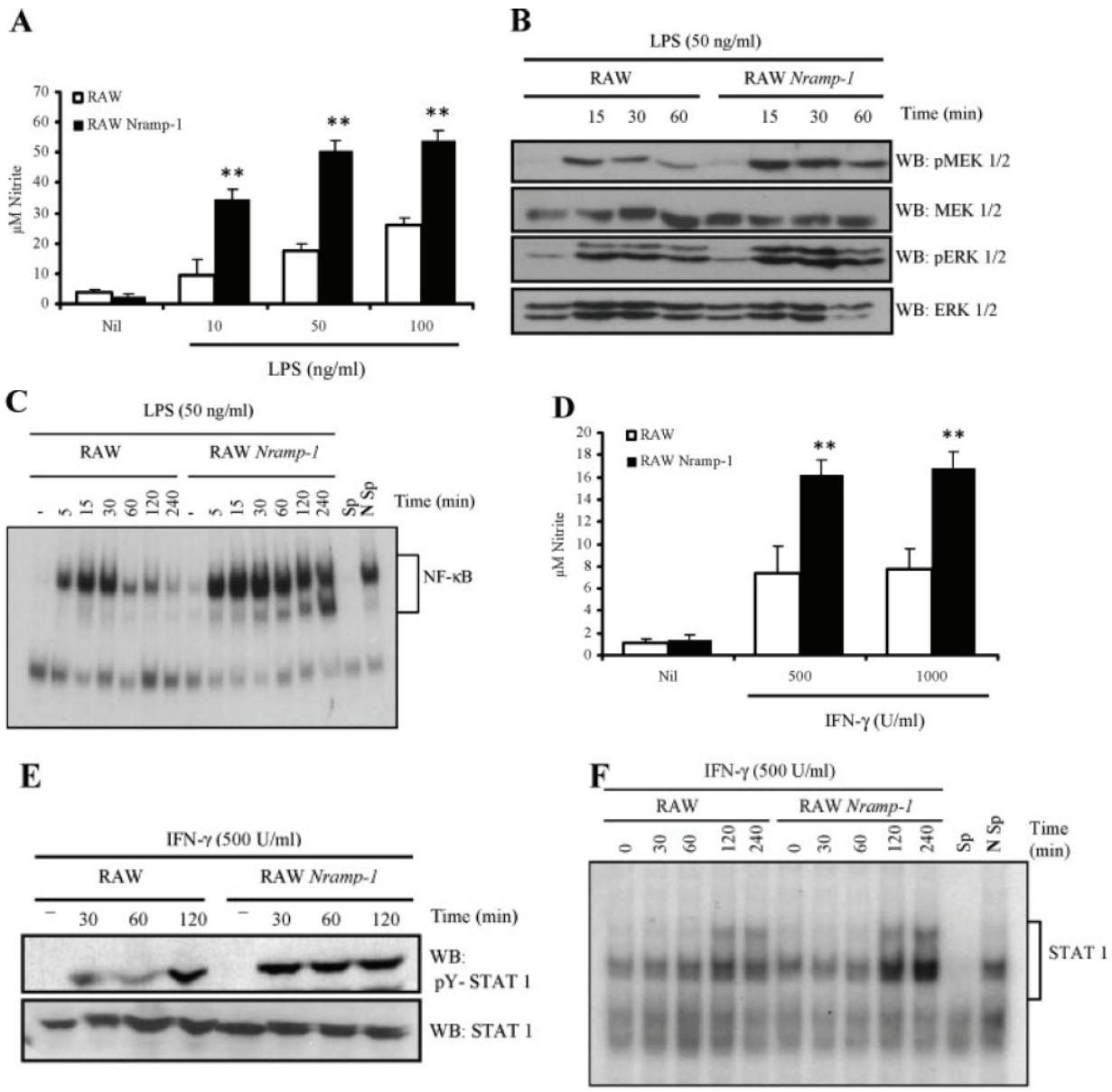

Figure 1. ***NRAMP-1 expression modulates NO production by regulating the phosphorylation of signaling proteins.*** Mϕs were stimulated with 10-100 ng/ml LPS **(A)** or 500-1000 U/ml IFN-γ **(D)** for 24h. NO production evaluated by quantification of nitrite accumulation in the supernatant of cultured Mϕs using Griess reaction [250]. MEK1/2, ERK1/2 **(B)** and STAT1 **(E)** phosphorylation was evaluated by western blot of total Mϕ protein lysates following 0-60 min stimulation with LPS (50 ng/ml)or IFN-γ (500 U/ml). Shown are the loading controls using anti-MEK1/2, anti-ERK1/2 and anti-STAT1 antibodies. Kinetic studies (0-2 h) of the translocation and DNA binding activity of the transcription factors NF-κB **(C)** and STAT1 **(F)** following LPS or IFN-γ stimulation were performed by EMSA analysis of nuclear extracts. Data are representative of three individual experiments (B,C,E,F) or mean values of three independent experiments performed in duplicate ± SEM (A,D). Statistically significant differences (**) were considered when *P<0.05.* **Sp**: specific competitor. **N Sp**: non-specific competitor.

Macrophage PTP activity is affected by the functional expression of NRAMP-1. In order to understand the mechanism by which NRAMP-1 mediates the regulation of protein phosphorylation and activation of signaling pathways, we studied its impact on the direct coordinators of tyrosine phosphorylation. Protein tyrosine phosphorylation is tightly controlled by the balanced activity of protein tyrosine kinases (PTK) and protein tyrosine phosphatases (PTP). PTPs, comprising the largest gene family of phosphatases in the human genome [169, 170]**,** are key regulators of signal transduction in multiple cellular processes, including differentiation, activation, and response to infection. Basal Mϕ PTP activity was determined by the capacity of total Mϕ protein lysates to dephosphorylate the phosphate analogue pNPP, and a synthetic tyrosine monophosphorylated peptide substrate. Dephosphorylation of the two PTP substrates showed a significant 35% reduction of PTP activity in response to NRAMP-1 expression (Figure 2A), contributing to the higher phosphorylation levels of some phospho-proteins including STAT1, ERK1/2, MEK1/2 and increased kinase activities [360, 413]. To evaluate the effect of the differential PTP activity, tyrosine phosphorylation of Mϕ proteins from both cell lines was monitored by immunofluorescence and confocal microscopy. As shown in Figure 2B, RAW *Nramp-1* Mϕs had higher levels of total tyrosine phosphorylation at basal level and upon latex beads phagocytosis (data not shown). Furthermore, anti-phosphotyrosine western blot showed increased phosphorylation in RAW *Nramp-1*protein bands ranging from 30 KDa to 150 KDa (Figure 2C), suggesting that inhibition of more than one PTP is involved in the maintenance of higher protein phosphorylation.

Modulation of PTP activity in NRAMP-1 deficient Mϕs reverts their functional phenotype. Peroxovanadium derived compounds have been shown by our laboratory [252] and others [245] to be specific and potent PTP inhibitors which modulate phagocyte functions *in-vitro* and *in-vivo*. To demonstrate the contribution of differential PTP activity in the signaling and functional events associated with NRAMP-1 expression, RAW and RAW *Nramp-1* cells were incubated with the peroxovanadium derived compound bpV(phen) to reach equivalent PTP activity levels in both cell lines. As seen in

FIGURE 2

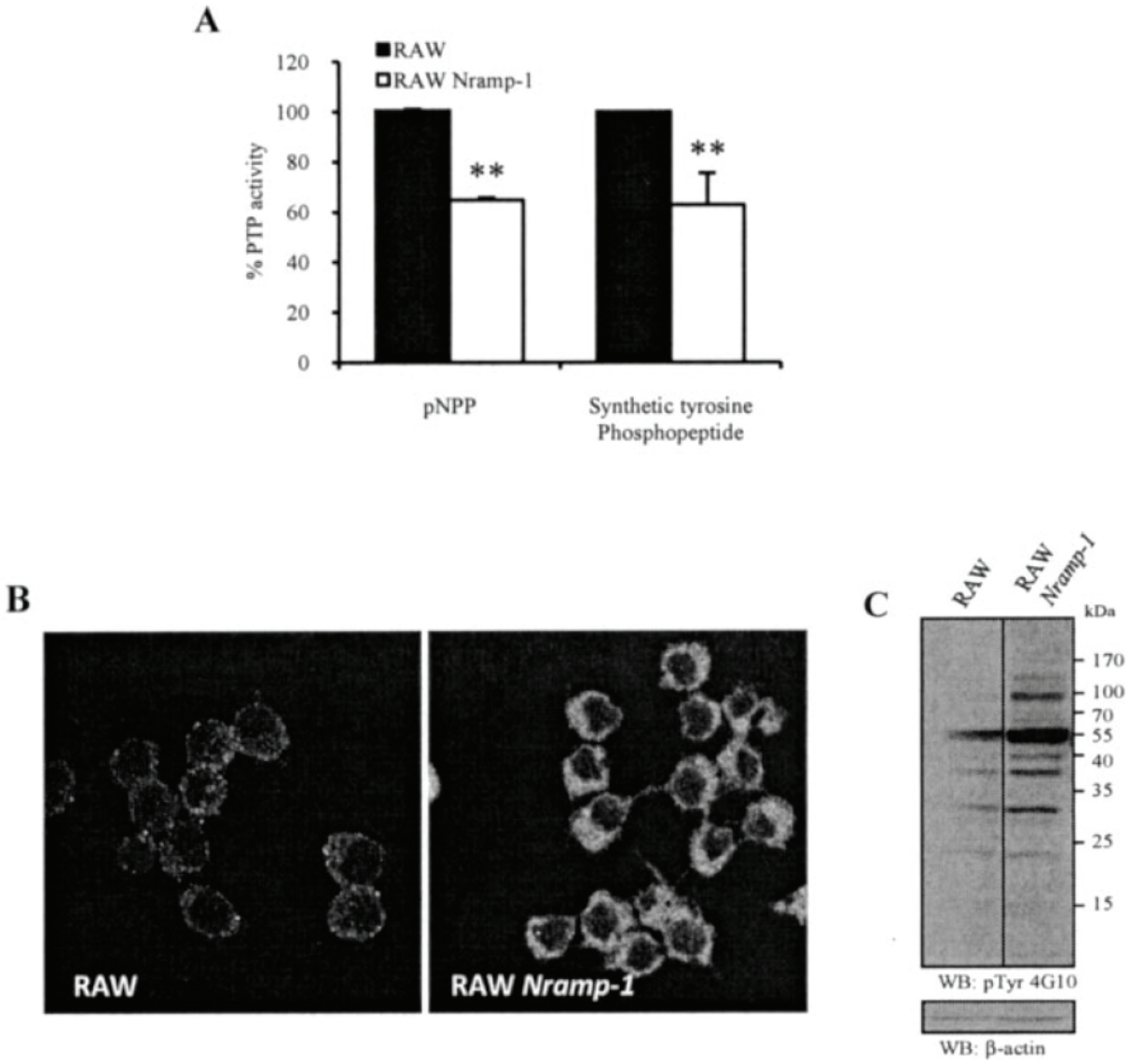

Figure 2. ***Mϕ PTP activity is affected by the functional expression of NRAMP-1.*** Total phosphatase and specific PTP activity of RAW and RAW *Nramp-1* was evaluated by the capacity of Mϕ cell lysates to hydrolyze pNPP or a synthetic tyrosine phosphopeptide. OD readings were taken at 405nm and 620nm respectively. Data are presented as percentage values ± SEM of three independent experiments done in duplicate. Statistically significant differences (**) were considered when *P<0.05* **(A).** 4G10 anti-phosphotyrosine Ab was used to evaluate the phosphotyrosine content of Mϕs by confocal microscopy X63 (image presented in gray scale format of the color image) **(B)** and western blot of total Mϕ protein lysates**(C)**. Loading control was performed using anti-β-actin Ab.

Figure 3A, treatment with doses ranging from 5 to 25 μM bpV(phen) result in equivalent PTP activity for RAW and RAW *Nramp-1* Mϕs. Interestingly, one hour bpV(phen) treatment prior to LPS stimulation, promotes similar and enhanced capacity of both cell lines to produce NO (Figure 3B). Moreover, inhibition of PTP activity in RAW Mϕs even with the lower dose of bpV(phen) (5μM), rescues the functional phenotype and induces NO production to the same level of bpV(phen) untreated RAW *Nramp-1* Mϕs. This data suggest that the basal difference in PTP activity, is crucial for the differential regulation of NO given that equivalent levels of PTP activity in both cell lines, achieved by partial inhibition with bpV(phen), rescues the capacity of NRAMP-1 deficient Mϕs to produce NO.

NRAMP-1 expression prevents full induction of Mϕ PTP activity upon Leishmania infection. As previously shown by our group, *Leishmania* infection strongly induces Mϕ PTP activity particularly that of the SH2 domain containing PTP SHP-1, as a mechanisms to downregulate Mϕ functions and promote successful intracellular survival [158, 159, 250-252, 277]. In light of the observation that NRAMP-1 expression plays a role in the regulation of Mϕ PTP activity, we sought to determine if the difference in PTP activity among RAW and RAW *Nramp-1* cells was maintained in response to a strong PTP inducing stimulus, and whether this difference plays an active role in the control of *L. donovani* (Ld) infection.

Infection of RAW Mϕs promotes a 35% induction of PTP activity above basal level. However, functional NRAMP-1 expression limits the capacity of Mϕs to reach full PTP activation in response to Ld (Figure 4A), suggesting that the classical downregulation of host cell signaling may be partially blocked in NRAMP-1 expressing cells. Knowing that parasite-induced Mϕ PTP activation is fundamental for its intracellular development, we studied the capacity of Ld to survive in both cell lines by infecting Mϕs with luciferase transfected Ld promastigotes (Ld-Luc) [416]. Following 6h of initial Mϕ-parasite contact and a chase period of 24h, no difference in intracellular survival was observed between RAW and RAW *Nramp*-1 Mϕs. However, when Mϕs were activated with LPS following

FIGURE 3

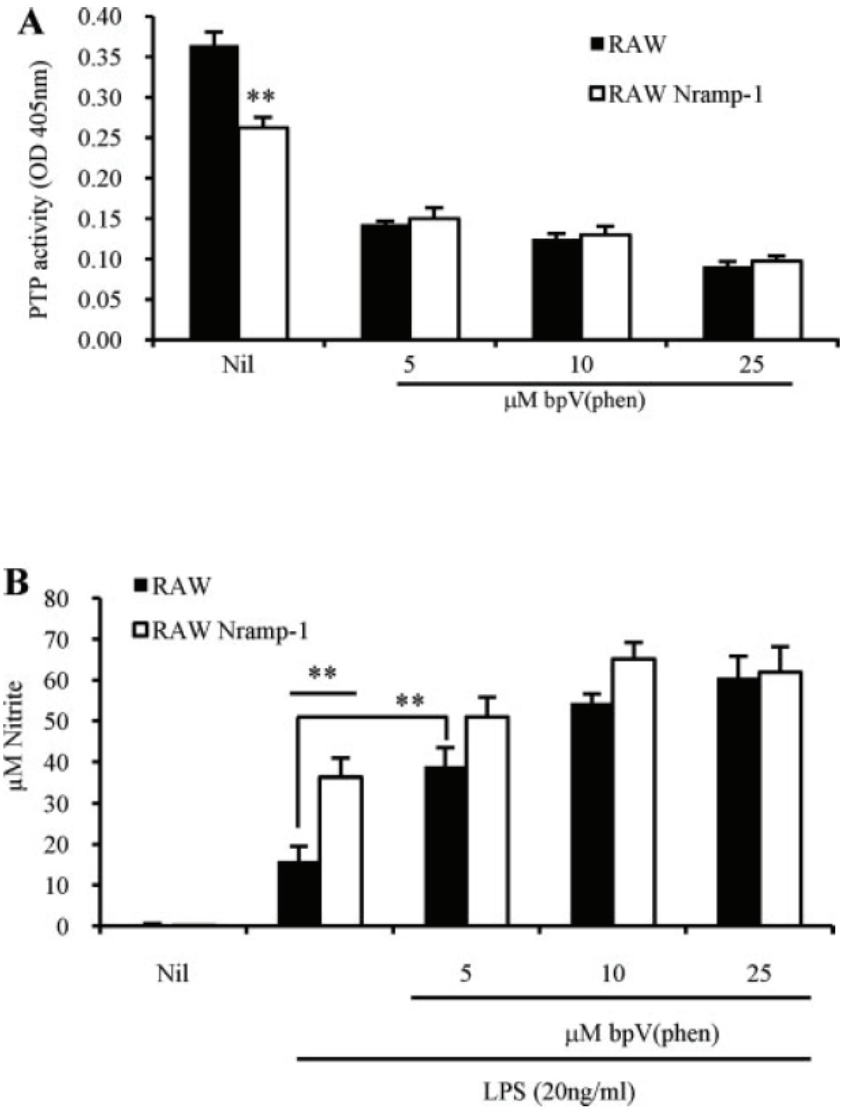

Figure 3. ***Modulation of PTP activity in NRAMP-1 deficient cells induces NO production to the levels of NRAMP-1 expressing Mϕs.*** RAW and RAW *Nramp-1* Mϕs were treated for 1h with increasing doses of bpV(phen). PTP activity was evaluated by pNPP hydrolysis and OD readings at 405 nm. **(A)**. Mϕs were left untreated or pre-treated with 5-25 μg bpV(phen) for 1h. Cells were washed 3 times with PBS and stimulated with 20 ng/ml LPS over night. NO production was evaluated by Griess reaction and OD was measured at 540nm **(B)**. Data represent mean values of three independent experiments performed in duplicate ± SEM. Statistically significant differences (**) were considered when *P<0.01.*

the initial 6h Mϕ-parasite contact, the intracellular parasite killing capacity of RAW *Nramp-1* Mϕs was greatly increased as a result of higher NO production. In contrast RAW cells showed a limited capacity to fully control the infection, in line with low NO levels (Figure 4B and 4C). When RAW and RAW *Nramp-1* Mϕs were pre-treated with bpV(phen) prior to infection and LPS stimulation, the intracellular Ld survival rate was greatly reduced in both cell lines as a result of general inhibition of PTP activity and downstream induction of NO (Figure 4B and 4C). However, a significant difference in the killing capacity of both cell lines was still evident, suggesting that although differential PTP activation plays an important role in the intracellular parasite killing, other PTP-independent events act together to fully control the infection.

Insights into the mechanism of NRAMP-1-dependent PTP activity regulation. In an effort to understand the mechanism underlying the differential PTP activity associated with NRAMP-1, we sought to determine whether PTP protein expression was altered. Western blot analysis of a broad panel of PTPs, roughly accounting for an estimated 20% of leukocyte PTPs [175], showed no difference among RAW and RAW *Nramp-1* cells in the expression levels of SHP-1, SHP-2, PTEN, TCPTP, PTP-PEST, PTP1B (Figure 5A), MKP2, CD45 and KAP (data not shown). However, the specific activity of individual immunoprecipitated PTPs, shown here PTP1B and SHP-1, was 50% and 25% respectively lower in RAW *Nramp-1* Mϕs (Figure 5B).

Similar PTP expression profiles in RAW and RAW *Nramp-1* Mϕs suggest that a post-translational regulatory mechanism may be responsible for the differential PTP activity. To address this, an in-gel PTP activity assay was performed. In this assay, PTPs are fully denatured and subsequently renatured, thus recovering their enzymatic activity. A post-translational modification affecting PTP activity such as reversible inhibition by catalytic cysteine oxidation, protein-protein interactions or protein phosphorylation, will be abrogated during the steps of in-gel protein denaturation and renaturation, and in-gel PTP activity will be restored [418, 419]. As seen in Figure 5C, a reversible mechanism of PTP inhibition in RAW *Nramp-1* is favored, as similar intensity

FIGURE 4

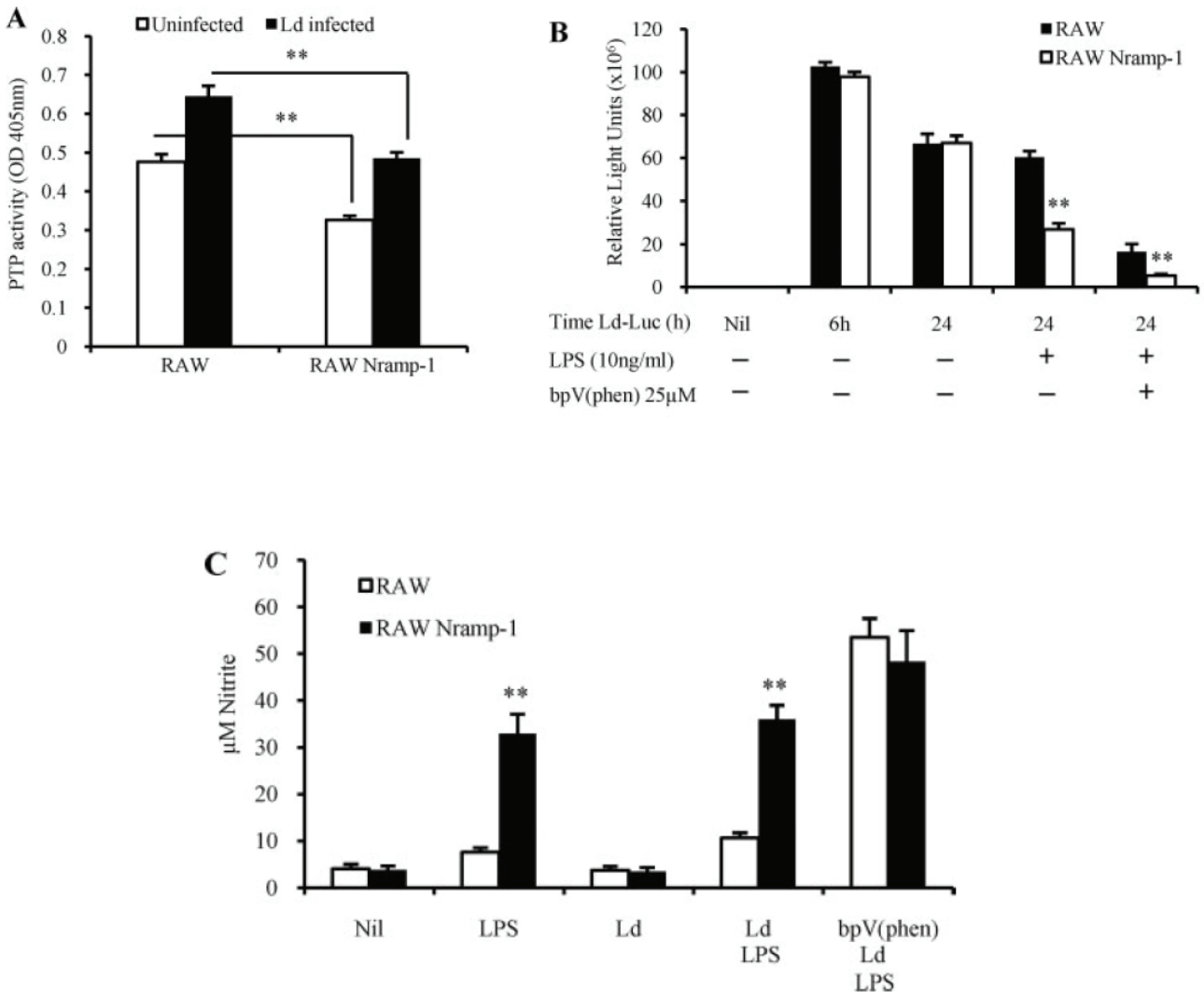

Figure 4. ***NRAMP-1 expression affects Ld-dependent induction of Mϕ PTPs.*** RAW and RAW *Nramp-1* Mϕs were infected for 1h with stationary Ld promastigotes. Mϕs were washed three times with PBS, lysed and PTP activity was evaluated by pNPP hydrolysis **(A)**. Mϕs were pre-treated or not with 25μM bpV(phen) for 1h followed by 6h infection with stationary Ld-Luc promastigotes (10:1 Ld:Mϕ ratio). Cells were washed to remove non-internalized parasites. LPS (10 ng/ml) stimulation followed, after which infection was allowed to progress for 24h. Intracellular parasite survival was determined by luciferase activity after 6h and 24h of infection **(B)**. Intracellular Ld killing capacity was correlated with Mϕ NO production **(C).** Data represent mean values of three independent experiments performed in duplicate ± SEM. Statistically significant differences (**) were considered when *P<0.01.*

FIGURE 5

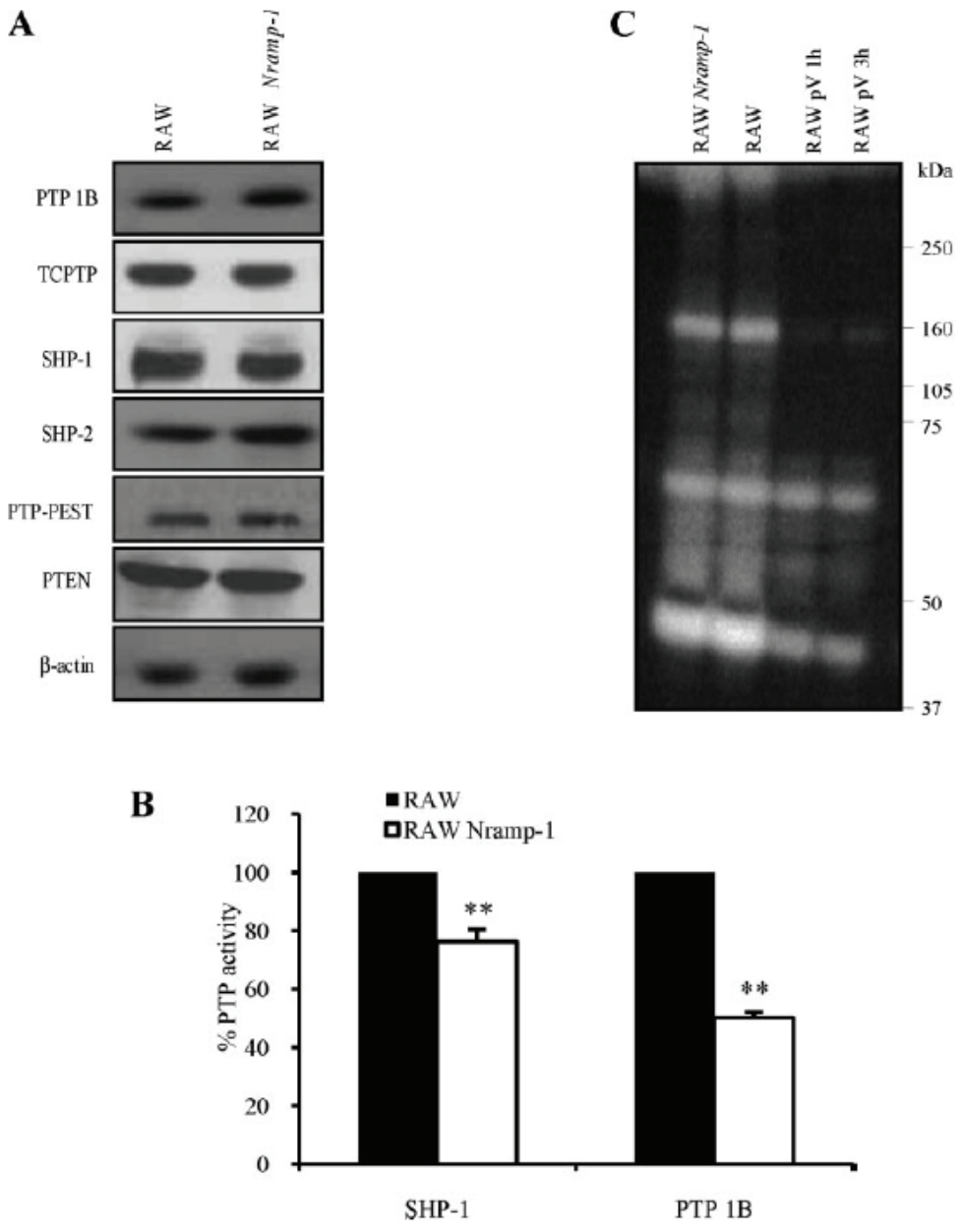

Figure 5. ***Insights into the mechanism of NRAMP-1 regulated Mϕ PTP activity.*** PTP expression was evaluated by western blot of total Mϕ lysates. Anti-β-actin Ab was used as loading control **(A).** PTP 1B and SHP-1 specific activity was measured after immunoprecipitation from 1 mg of total protein lysates. Activity of immunoprecipitated PTPs was determined by pNPP hydrolysis and OD reading at 405nm. **(B)**. 50μg of total protein lysates from RAW *Nramp-1*(lane 1) and bpV(phen) treated (0-3 h) RAW cells (lanes 2-4) were subjected to in-gel PTP assay. Bands of dephosphorylation represent active PTPs. Lanes 3 and 4 show the irreversible PTP inhibition following bpV(phen) treatment**(C).**

of PTP bands ranging from 37 to 160 KDa is seen when comparing RAW and RAW *Nramp-1* protein extracts. In contrast, a positive control of irreversible PTP inhibition is shown in lanes 3 and 4, were incubation of Mϕs with the irreversible PTP inhibitor bpV(phen) prior to gel running, results in the reduction of intensity and almost disappearance of PTP bands (75-160 KDa). The importance of divalent cations such as calcium, zinc and manganese in the control of enzymatic reactions and intracellular signaling is well known. We therefore sought to determine whether metal substrates of NRAMP-1 may act as regulators of PTP activity. Total RAW *Nramp*-1 Mϕ protein lysates were extracted and incubated with ferric citrate, $ZnCl_2$ and $MnCl_2$, or with chelators for iron (deferoxamine-DFO) or zinc (TPEN), after which PTP activity was determined by pNPP hydrolysis. Interestingly, iron treatment inhibited PTP activity to the level of the PTP inhibitor bpV(phen), while zinc showed a modest 20% inhibition and manganese no significant effect (Figure 6A). Of interest, the specific activity of SHP-1 and PTP1B, two PTPs intimately involved in IFN-γ and LPS signaling, was significantly inhibited by iron treatment (Figure 6B). Supporting the hypothesis of a reversible mechanism of PTP inhibition by metal substrates of NRAMP-1, TPEN treatment augments PTP activity by 14% over basal level, and most interestingly, and reinforcing the regulatory effect of iron, iron chelation by addition of DFO induced PTP activity more than 50% over basal level (Figure 6A).

Following a dose response analysis of the iron-dependent PTP inhibition in RAW and RAW *Nramp-1* Mϕ cell lysates, a stronger inhibition of PTP activity was observed in RAW *Nramp-1* Mϕs using lower doses of ferric citrate when compared to NRAMP-1 deficient macrophages, where even a high dose of 250μM, partial inhibition of PTP activity only reached the level of resting RAW *Nramp-1* cells. Maximal PTP inhibition in both cell lines was reached at a dose of 500μM, resulting from iron excess (Figure 6C).

Cellular iron homeostasis is a tightly control processes which involves the coordinated activity of extracellular iron import, intracellular storage, mobilization, utilization and export [296]**.** To mimic the effect of NRAMP-1 expression on PTP activity,

FIGURE 6

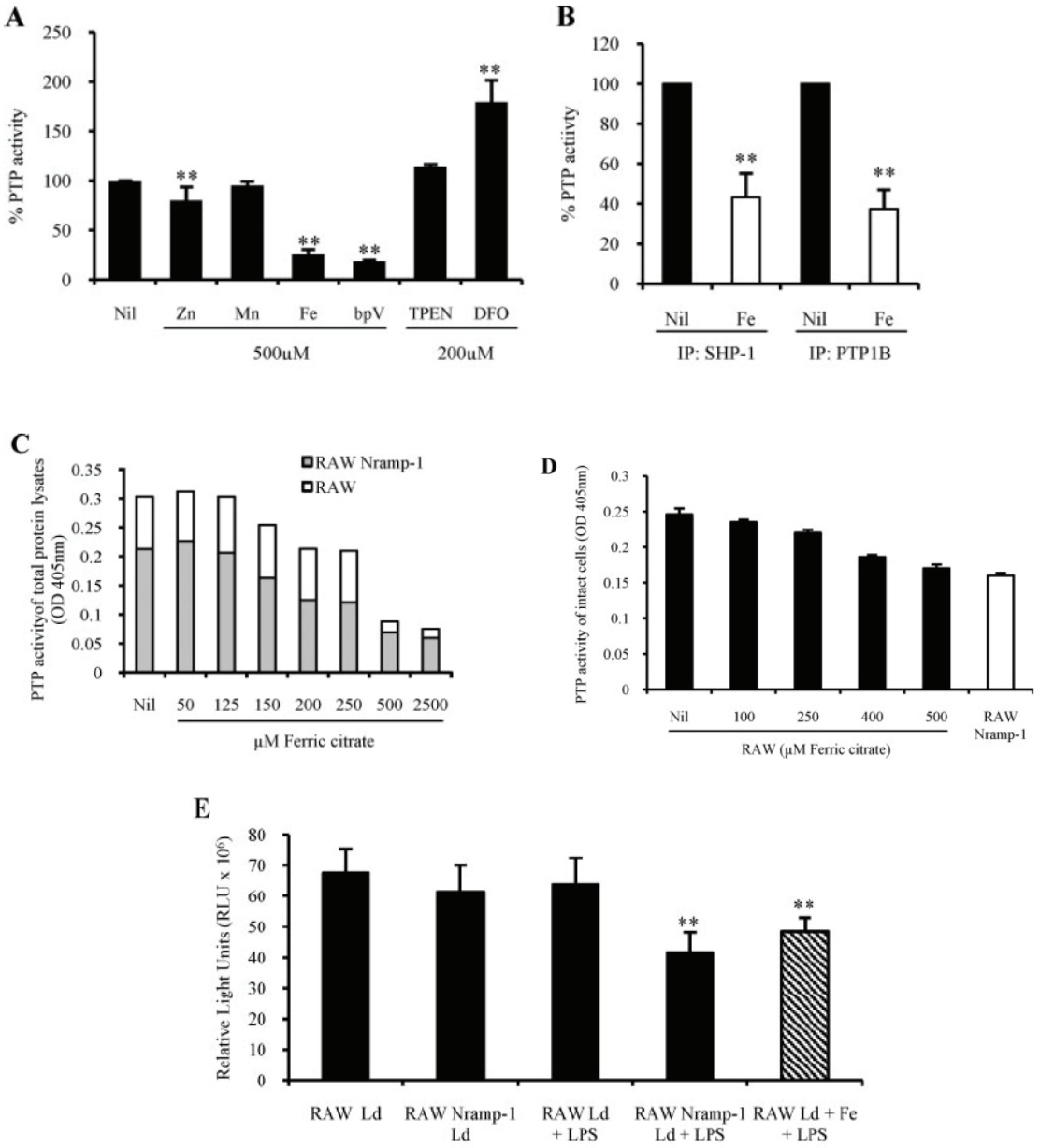

Figure 6. ***NRAMP-1 metal substrates regulate PTP activity.*** Mϕ PTP activity was determined following incubation of 10 µg of RAW *Nramp-1* total protein lysates **(A)** or specific immunoprecipitated PTPs (**B**) for 10 min at RT with ferric citrate, $ZnCl_2$ $MnCl_2$, TPEN or DFO**.** A dose response curve of RAW and RAW *Nramp-1* protein extracts with 50-2500µM ferric citrate was performed **(C)** RAW Mϕs were incubated ON with increasing concentrations of iron solution **(D).** PTP activity was evaluated in all instances by pNPP hydrolysis and OD reading at 405nm. *Leishmania* killing capacity was determined by luciferase activity of Mϕs incubated ON with 500µM ferric citrate, infected and LPS stimulated **(E)**. Statistically significant differences (**) were considered when *P<0.01* where applicable.

we incubated RAW Mϕs ON with doses of ferric citrate ranging from 100-1000μM. Despite the use of high doses of extracellular iron, only partial PTP inhibition was obtained at a subcytotoxic dose of 500μM, where PTP activity of RAW cells was inhibited to the level of basal activity in RAW *Nramp-1* Mϕs (Figure 6D). Incubation of RAW cells with ferric citrate prior to Ld-Luc infection and LPS stimulation, promoted intracellular parasite killing similar to LPS stimulated RAW *Nramp-1* cells as determined by luciferase activity (Figure 6E). These data clearly support an effect of iron, a metal substrate of NRAMP-1, in the regulation of PTP activity and downstream in the control of *Leishmania* infection.

DISCUSSION

NRAMP-1 has been associated with host resistance to intracellular pathogens, upregulation of pro-inflammatory functions in Mϕs, and more recently with susceptibility to autoimmune diseases [427, 428]. Although major understanding has accumulated regarding the biochemistry and effector functions linked to NRAMP-1 [429], it is still unclear how as a divalent cation transporter can mediate the pleiotropic effects associated with its functional expression. Lafuse and colleagues have proposed that NRAMP-1 expression influences the mRNA stability of several NRAMP-1 regulated genes including MHC II, TNF-α, iNOS and NRAMP-1 itself [412, 430], via a mechanism involving a ROS-dependent signaling pathway requiring PKC and MAPKs [360]. However it remained elusive how NRAMP-1 metal transport influences protein kinases activities and ROS generation in order to promote the stabilization of certain mRNA species. In the present study we propose a mechanism whereby NRAMP-1 mediated iron transport contributes to the regulation of Mϕ functions through the modulation of PTP activity, linking divalent cation transport, signaling and cellular functions.

Protein phosphorylation as a result of the balanced action of protein kinases and protein phosphatases, is fundamental for the activation, localization, and substrate

specificity of signaling molecules. In extension of previous observations [408, 426], we show that the higher nuclear translocation and DNA binding activity of NF-κB and STAT1 in response to LPS and IFN-γ in NRAMP-1 expressing cells, correlates with higher phosphorylation of protein members of the MAPK and JAK/STAT signaling pathways. Several PTPs have been shown to regulate LPS and IFN-γ signaling, including SHP-1 [158, 159, 251, 277, 431], PTP1B [216, 432], TCPTP [433, 434], MKPs [435] and SHP-2 [436]. In this line of thought, we sought to evaluate the status of PTP activity in response to NRAMP-1 expression. RAW *Nramp*-1 Mϕs had 35% lower PTP activity compared to NRAMP-1 deficient RAW cells. Of interest, the specific activity of two PTPs involved in the JAK/STAT and MEK/ERK signaling pathways, SHP-1 and PTP1B, was significantly lower in RAW *Nramp-1* cells, which may contribute to the higher phosphorylation of MEK1/2, ERK1/2 and STAT1, and therefore to the enhanced NO production. Together with SHP-1 and PTP1B, TCPTP is involved in the regulation of JAK/STAT signaling [214, 433, 434, 437]. Although protein expression of TCPTP was observed in both RAW and RAW *Nramp-1* (Figure 5A), the enzymatic activity of the immunoprecipitated protein was undetectable by biochemical substrate dephosphorylation assays (data not shown). This suggests that in our Mϕ system SHP-1 and PTP1B may exert a stronger regulatory function in the NRAMP-1 dependent modulation of IFN-γ signaling.

Natural resistance to *Leishmania* infection, as with other intracellular parasites, is associated with the functional expression of NRAMP-1. In addition, disease progression is exacerbated by a strong parasite-induced Mϕ PTP activation, which leads to the negative regulation host cell functions [50]. Several pathogens are capable of interfering with host cell signaling by modifying tyrosine phosphorylation, as is the case of *Yersinia* [438, 439], *Salmonella* [440] and *Trypanosoma cruzi* [271]. However, only two cases have been documented where the pathogen itself [159, 290] induces host PTP activity as a mechanism to downregulate host cell functions. We have used *L. donovani* infection to evaluate the contribution of NRAMP-1 in the regulation of Mϕ PTP activation. As shown here, NRAMP-1 expression prevents full induction of PTP activity in infected Mϕs, promoting positive signal transduction which leads to NO production and

greater leishmanicidal capacity upon LPS stimulation. The importance of NRAMP-1-dependent regulation of PTP activity in the outcome of Ld infection is moreover supported given that NRAMP-1 deficient cells rescued the capacity to produce NO and control the intracellular parasite after their PTP activity was partially inhibited following treatment with the PTP inhibitor bpV(phen). This suggests that NRAMP-1 regulation of PTP activity is maintained even in a scenario where PTPs are strongly induced as is the case of Ld infection, limiting the negative regulation of host cell functions induced by the parasite and promoting the control of infection.

Changes in cellular PTP activity may result from reversible or irreversible events including differential protein expression, phosphorylation, oxidation and dimerization [190, 441]. Western blot analysis of a broad panel of Mϕ PTPs suggest that NRAMP-1 regulation of PTP activity does not result from differential PTP expression, however, we do not discard the possibility that other PTPs not evaluated in this study may be differentially expressed. The dynamic nature of signaling cascades requires a tight regulation of PTP activity. An irreversible inhibition of PTPs will be detrimental for cellular stability as no negative control of signal transduction will occur. Therefore a reversible mechanism of PTP regulation by NRAMP-1 expression results fundamental for the proper display of Mϕ functions. Our data suggest that the reduced PTP activity of RAW *Nramp-1* Mϕs indeed results from a reversible inhibition. Following PTP denaturation and renaturation in an in-gel PTP activity assay, active PTP bands in RAW *Nramp-1* showed similar intensities to NRAMP-1 deficient cells, this explained by the fact that any reversible PTP inhibition including oxidation or phosphorylation will be abrogated after protein denaturation.

The PTP signature motif [I/V]HCxxGxxR[S/T] localized to the catalytic domain of the enzymes, is characterized by the presence of a conserved cysteine residue acting in the nucleophilic attack of the incoming phosphate group [173]. The reduced state of this residue is fundamental for PTP activity and when oxidized impairs enzymatic activity [190]. It has been recently demonstrated that ROS play a pivotal role in the reversible inhibition and regulation of PTPs [169, 190, 193, 194]. Given that NRAMP-1

expression has been associated with increased intracellular ROS [410, 442], this event may contribute to the decreased PTP activity seen in our experimental system. In addition to ROS, recent reports show that zinc inhibits PTP activity i*n- vitro* and *in-vivo* [237-239]. Following this line of thought, we have evaluated the impact of NRAMP-1 metal substrates on Mϕ PTP activity. As shown in Figure 6A, Zn inhibits PTP activity by 20%, however, iron a well studied metal substrate of NRAMP-1, inhibited total PTP activity up to the levels of the specific PTP inhibitor bpV(phen). This interesting finding led us to further investigate the role of iron in PTP activity regulation. In support of a mechanism where NRAMP-1 regulation of PTP activity is mediated by its iron transport function, stronger PTP inhibition was observed in RAW *Nramp-1* Mϕs at lower doses of ferric citrate compared to NRAMP-1 deficient cells. (Figure 6C). In intact cells iron pre-treatment of RAW Mϕs mimicked the effect of NRAMP-1 expression by partially inhibiting PTP activity and promoting *Leishmania* killing, in accordance with previous reports where iron overload in BALB/c mice prevented the onset and development of lesion formation following *L.major* infection [341, 345]. Moreover, the finding that iron treatment by itself inhibits the activity of both PTP1B and SHP-1, suggests that differential iron transport may play a role in the regulation of LPS and IFN-γ signaling and consequently in NO production, by directly affecting PTP activity. These data give clear evidence for the involvement of iron in the NRAMP-1 regulation of signaling pathways and control of *Leishmania* infection.

In conclusion, our findings suggest a model which directly links NRAMP-1 metal transport and the regulation of Mϕ functions. NRAMP-1 mediated transport of zinc and iron from the late endosome/lysosome and phagolysosomal compartment of Mϕs, directly or indirectly (by catalyzing the formation of ROS) inhibit PTP activity in a reversible fashion, promoting protein phosphorylation and positive signal transduction, leading to the upregulation of effector Mϕ functions, in our particular case of study, nitric oxide production. In addition to NRAMP-1 mediated regulation of protein kinase activities and mRNA stability, our current study on the regulation of PTP activity broadens our understanding of the complex mechanism responsible for the NRAMP-1

mediated modulation of cellular functions, bringing together basic metal transport and signal transduction.

FOOTNOTES

We thank Dr. Philippe Gros (Centre for Host Resistance and Department of Biochemistry, McGill University) who kindly provided us with the macrophage cell lines RAW 264.7 and RAW *Nramp-1.* We also thank Dr. Keith Burridge (University of North Carolina, Chapel Hill) for kindly providing us with the GST-FER construct.

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PREFACE TO CHAPTER 3

The involvement of macrophages as host, accessory and effector cells in the development of infectious diseases, together with their central role in iron homeostasis, place these immune cells as central players in the interphase between iron and infection. Having shown that iron, a metal substrate of NRAMP-1, plays a role in the differential regulation of macrophage PTP activity, we sought to study the mechanism(s) underlying this specific event. In this chapter we identify the mononuclear dicitrate iron complex $[Fecit_2H_{4-x}]^{(1+x)-}$ as the active species responsible for specifically inhibiting PTP activity, in a mechanism that involves its interaction within the PTP active site. We show that upon iron citrate treatment, ERK and JNK signaling pathways are modulated in part by an iron-dependent inhibition of PTP activity. Additionally, we propose that this novel mechanism may represent a biologically relevant regulatory event important for the control of signal transduction both, at resting sate and in the scenario of an inflammatory response.

CHAPTER 3

IRON CITRATE COMPLEXES MODULATE MACROPHAGE SIGNAL TRANSDUCTION BY DIFFERENTIALLY REGULATING PROTEIN TYROSINE PHOSPHATASE ACTIVITY

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ABSTRACT

The involvement of macrophages as host, accessory, and effector cells in the development of infectious diseases, together with their central role in iron homeostasis, place these immune cells as central players in the interphase between iron and infection. Having previously shown that the functional expression of NRAMP-1 results in increased protein phosphorylation mediated in part by an iron-dependent inhibition of macrophage protein tyrosine phosphatase (PTP) activity, we sought to study the mechanism(s) underlying this specific event. Herein we have identified the mononuclear dicitrate iron complex $[Fecit_2H_{4-x}]^{(1+x)-}$ as the species responsible for the specific inhibition of macrophage PTP activity. By using biochemical and computational approaches, we show that $[Fe(Cit)_2]^{5-}$ targets the catalytic pocket of the PTP SHP-1, competitively inhibiting its interaction with an incoming phospho-substrate. *In vitro* and *in vivo* inhibition of PTP activity by iron-citrate and iron-dextran respectively, results in protein hyperphosphorylation and enhanced MAPK signaling in response to LPS stimulation. We propose that iron-citrate-mediated PTP inhibition represents a novel and biologically relevant regulatory mechanism of signal transduction.

INTRODUCTION

The balance of protein phosphorylation, maintained by the concerted action of protein kinases and protein phosphatases, is fundamental in determining the outcome of multiple cellular functions ranging from cell proliferation to cell death [443]. In recent years the importance of protein tyrosine phosphatases (PTPs) as coordinators of signaling pathways and the immune response has become evident [175, 333]. Progress has been achieved in understanding the mechanisms of PTP regulation, including receptor PTP dimerization [184], oxidation [190] and PTP phosphorylation; however, with more than 100 family members, a broad structural diversity, varying subcellular localization and substrate specificities [170], the complexity of PTP activity regulation is far from being completely unraveled.

For more than 15 years, our laboratory has studied the involvement of macrophage PTPs in the outcome of *Leishmania* infection. In the early stages of *Leishmania*-macrophage interaction, alterations in the JAK/STAT and MAPK signalling pathways occur as a result of parasite-induced activation of the host PTP SHP-1. This event leads to the attenuation of innate inflammatory responses and the downregulation of macrophage nitric oxide (NO) production, favouring intracellular parasite survival and disease progression. However, susceptibility to infection is not always the case; resistance/susceptibility of the host to *Leishmania major* infection seems to be multigenic by nature [24], and innate resistance to *Leishmania donovani* has been associated with the functional expression of NRAMP-1 (Natural Resistance Associated Macrophage Protein-1).

NRAMP-1 is a divalent metal transporter localized to the late endosome/lysosomal compartment of macrophages [29] and present in gelatinase positive tertiary granules of neutrophils [30]. Upon phagocytosis, NRAMP-1 is recruited to the phagolysosomal membrane where it mediates the transport of Mn^{2+}, Fe^{2+}, Co^{2+}, and potentially other metals including Zn^{2+} [31-34]**.** In addition of mediating innate resistance to *Leishmania* and other unrelated intracellular pathogens [27, 396], NRAMP-1 expression has also been associated with the upregulation of pro-inflammatory macrophage functions such as MHC II expression [401, 402], IL-1β [403]**,** TNF-α [405] and NO production [407, 408]. We have recently reported that functional expression of NRAMP-1 results in lower macrophage PTP activity and increased protein phosphorylation, in part explaining the associated pleiotropic effects [444]. Our findings suggest a model which directly links metal transport and the regulation of macrophage functions; NRAMP-1 mediated transport of iron from the late endosome/lysosome and phagolysosomal compartment of macrophages, directly or indirectly (by catalyzing the formation of reactive oxygen species -ROS-) inhibit PTP activity in a reversible fashion, promoting protein phosphorylation and positive signal transduction, leading to the upregulation of effector macrophage functions such as NO production [444].

In the current research article we have further our observations, and studied the mechanisms underlying the iron-dependent PTP regulation in macrophages. The involvement of macrophages as host, accessory and effector cells in the development of infectious diseases, together with their central role in iron homeostasis, place these immune cells as central players in the interphase between iron and infection [333]. We here show that macrophages regulate MAPK signaling pathways in part by modulating PTP activity in an iron-dependent manner. Moreover, we propose that this novel mechanism may represent a biologically relevant PTP regulatory event which could be exploited as a therapeutic approach, given the essential function of iron in cell viability, replication and immune-related functions [294].

METHODS

Materials. 4-Nitrophenylphosphate disodium salt hexahydrate (pNPP), LPS (*Escherichia coli*, serotype 0111:B4), catalase, superoxide dismutase, Poly(Glu,Tyr), iron (III) chloride ($FeCl_3$), iron dextran and iron (II) sulfate ($FeSO_4$) were purchased from Sigma-Aldrich. [γ-^{32}P]dATP (3000 Ci/mmol) was obtained from GE Healthcare. Guanidine hydrochloride and citric acid were purchased from Laboratoire MAT (Beauport, Qc, Canada).

Cell culture and bone marrow derived macrophage (BMM) differentiation. B10R murine macrophage cell line was kept in DMEM (Gibco-BRL) supplemented with 10% heat inactivated fetal bovine serum (FBS), 100μg/ml streptomycin, 100U/ml penicillin, and 2mM L-glutamine at 37°C and 5% CO_2. 6-8 week old male BALB/c mice were purchased from Charles River (Pointe-Claire, Qc.). Mice were kept in pathogen-free housing. All animal work was carried out according with the regulations of the Canadian Council of Animal Care and approved by the McGill Animal Care Committee. Mice were injected intraperitoneally with 8 mg/animal of iron-dextran daily for a total of 3 days. For BMM differentiation, bone marrows of treated and untreated animals were

cultured for 5 days in DMEM containing 30% L929-cell conditioning medium (LCCM) and 10% FBS, and an additional 2 days with refreshed LCCM.

Phosphatase assays. As previously described [159]**,** B10R or BMM were collected, lysed in PTP lysis buffer (50mM Tris pH 7.0, 0.1mM EDTA, 0.1mM EGTA, 0.1% 2-ME, 1% Igepal, 25 μg/ml aprotinin, and 25 μg/ml leupeptin) and kept on ice 45 min. Lysates were cleared by centrifugation, and protein content determined by Bradford's method. Ten μg of protein extract were incubated in phosphatase reaction buffer (50mM Hepes pH 7.5, 0.1% β-ME, 10mM 4-Nitrophenylphosphate disodium salt hexahydrate -pNPP-) for 30 minutes. OD was read at 405nm.

Preparation of Iron-Citrate solutions. Three different stock solutions of iron-citrate (Fe-Cit) were prepared with variations in the iron-to-citrate (Fe:Cit) molar ratios -1:1, 1:4 and 1:10 -. A stock solution of 0.5 M $FeCl_3$ was prepared and added drop wise to citric acid (H_4cit) solutions to obtain a final concentration of 100 mM Fe^{3+} in all solutions. 100 mM, 400 mM and 1 M citric acid solutions were used to generate 1:1, 1:4 and 1:10 Fe-Cit preparations respectively. Solutions were slowly stirred for 1h at RT and protected from light to avoid photoreduction of Fe^{3+}. Fe-Cit preparations were kept at RT and used for a maximum of one month after preparation.

Electrospray Ionization Mass Spectrometry (ESI-MS). The ESI-MS experiments were performed on a LCQ-ion trap (Finnigan-Thermoquest, San Jose, CA, USA) equipped with an electrospray source. Electrospray full scan spectra, in the range of *m/z* 50-1200, were obtained by infusion through fused silica tubing at 2-10 μL min^{-1}. The LCQ calibration (*m/z* 50-2000) was achieved according to the standard calibration procedure from the manufacturer (mixture of caffeine, MRFA and Ultramark 1621). The temperature of the heated capillary of the LCQ was set to 100 °C, the ion spray voltage was in the range of 1-6 kV with an injection time of 5-200 ms. The solutions were analyzed in the negative mode. Experimental peak values throughout this study are identified by the *m/z* ratio of the most abundant peak in the parent group. Calculated

m/z values tabulated are those based on the most abundant isotopes. Peak intensities are cited as percentages of the base major peak intensity. When citric acid was in excess in the solution (1:4 and 1:10), the peaks ($[H_3cit]^-$ *m/z* 191; $([H_4cit][H_3cit]^-$ *m/z* 383) become the major, so spectra were represented in the range of 200 (or 400) to 900 for clarity. As protonation occurs during the ionization process in the spectrometer, a single species can be detected with different degrees of protonation. The nuclearity of complexes is in accordance with the result of isotopic pattern calculation.

For ESI-MS analyses, 1:1, 1:4 and 1:10 Fe-Cit solutions (1mM Fe^{3+}) were prepared 20h prior to be analyzed, and kept in the dark. The pH of the 1:1 and 1:4 solutions was 3.5, and 2.4 for Fe-Cit 1:10. Results do not vary with time, showing that the mixture has reached equilibrium at the time when the experiments were performed, consistent with data obtained from solutions prepared with the iron(III) salt ($Fe(ClO_4)_3 \cdot 9H_2O$) used in previous analysis [445].

Molecular docking. The three-dimensional structures of the catalytic domain of the PTP SHP-1 (1GWZ.pdb) [446] and its complex with the phosphotyrosyl decapeptide PY469 derived from SHPS-1 (1FPR.pdb) [447] were retrieved from the Protein Data Bank (http://www.pdb.org). Molecular docking simulations of the Fe-Cit complexes with SHP-1 were carried out using the program FlexX [448] embedded in the SYBYL7.3 (Tripos, Inc). The water molecules were removed from the proteins and the essential hydrogen atoms were added prior to docking. Protein conformations were kept rigid during all experiments. H_2O, NH_4 and Him residues were removed from the Fe-Cit complexes (**C7**, $(NH_4)_5[Fe(cit)_2]. 2H_2O$; **C2**, $(Him)_2[Fe_2(cit)_2]. 2H_2O$) [445]. Ligand charges were calculated using Gasteiger-Huckel method and their structures were minimized using Tripos Force Field. For each individual ligand the first thirty docking solutions were ranked and the results of top ranked conformation were analyzed.

In vitro and in vivo iron treatment. 10 µg of total B10R macrophages cell lysates were incubated for 10 min at RT with $FeCl_3$, Fe:Cit 1:1, Fe:Cit 1:4, Fe:Cit 1:10 or $FeSO_4$ to a final iron concentration of 500 µM, and PTP activity assay performed as described

above. Alternatively, B10R macrophages were incubated for different time periods with Fe:Cit 1:1 or Fe:Cit 1:10 in serum-free DMEM. Cells were then lysed in PTP lysis buffer and PTP activity evaluated by pNPP hydrolysis.

In-gel PTP assay. In-gel PTP assay was performed as we and other previously described [418, 444]. Briefly, Poly(Glu,Tyr) substrate was tyrosine phosphorylated by overnight (O/N) incubation with GST-FER protein kinase (10 μg) and 150 μCi [γ-^{32}P]dATP. The substrate was then incorporated in a 10-12% SDS-polyacrylamide gel mixture at a concentration of 2 x 10^5 CPM/ml. Macrophage protein extracts, prepared as described above, were denatured for SDS-PAGE and loaded onto the gel. After electrophoresis, the gel was incubated O/N in Buffer A (50mM Tris-HCl pH 8.0, 20% isopropanol), washed twice with Buffer B (50mM Tris-HCl pH 8.0, 0.3% β-ME), and followed by full protein denaturation in Buffer B containing 6M guanidine hydrochloride and 1mM EDTA. Gels were washed twice in Buffer C (50mM Tris-HCl pH 8.0, 1mM EDTA, 0.3% β-ME and 0.04% Tween 20) and final renaturation O/N in Buffer C with or without 500 μM Fe:Cit 1:10. Gels were dried and exposed to X-ray film. Active PTPs were detected as clear bands on the film.

Western Blotting and GST-pulldown. Western blots were performed as previously described [420]. Primary antibodies used were α-phosphotyrosine clone 4G10 (Millipore), α-phospho-ERK1/2 (Thr202/Tyr204), α-ERK1/2, α-phospho-JNK (Thr 183/Tyr 185) and α-JNK (Cell Signaling). GST-tagged PTP1B 1-321 a.a., PTP-PEST 1-453 a.a. and full length TCPTP and SHP-1 constructs were expressed in *E.coli* BL21 (PTP1B, TCPTP and PTP-PEST constructs were kindly provided by Dr. Michel L. Tremblay at the department of Biochemistry at McGill University). Fusion proteins were isolated by 1h incubation of bacterial lysates and Glutathione-Sepharose beads (GE Healthcare) at 4°C. Isolated fusion proteins were incubated with Fe-Cit for 10 min at RT and PTP activity evaluated by pNPP hydrolysis.

Statistical analysis. Data were analyzed by one-way ANOVA. Statistically significant difference between groups was considered when $P < 0.05$ or $P < 0.01$. All data are presented as the mean ± standard error of the mean (SEM).

RESULTS

Iron-dependent PTP inhibition is specific to iron-citrate chelates.

Cellular iron homeostasis is maintained by a fine balance of iron uptake, storage and export [294]. Dietary iron, found in its great majority as heme or free -non-heme bound- ferric iron (Fe^{3+}), is absorbed by epithelial duodenal enterocytes [298, 299]. For transmembrane transport, Fe^{3+} is enzymatically reduced to Fe^{2+} allowing transport via DMT-1 (formerly named NRAMP-2) [300-302]. Following intracellular iron usage and storage, excess iron is exported to be systemically distributed by plasma transferrin (Tf) [294] and, in conditions of iron excess, non-Tf-bound low molecular weight iron complexes (LMW·Fe), in its most as ferric citrate (Fe-Cit) [449].

Having previously observed that Fe-Cit strongly inhibits PTP activity in vitro [444], we investigated whether different iron donors exerted a similar effect. Interestingly, only iron in the form of Fe-Cit, but not $FeSO_4$, $FeCl_3$ or heme (data not shown) was able to inhibit total macrophage PTP activity as evaluated by the capacity of total cell lysates to dephosphorylate the phosphate analogue pNPP (Figure 1A). Moreover, Fe-Cit specifically inhibited, by more than 50% of their basal level, the enzymatic activity of the classic PTPs SHP-1, TCPTP, PTP1B and PTP-PEST (Figure 1B), central regulators of immune-related functions by acting on JAK/STAT, MAPK and cytoskeleton signalling [433, 450, 451].

The complexity of Fe-Cit solutions is evidenced by the formation of numerous Fe-Cit species found in equilibrium in any given solution, which may vary in the number

FIGURE 1

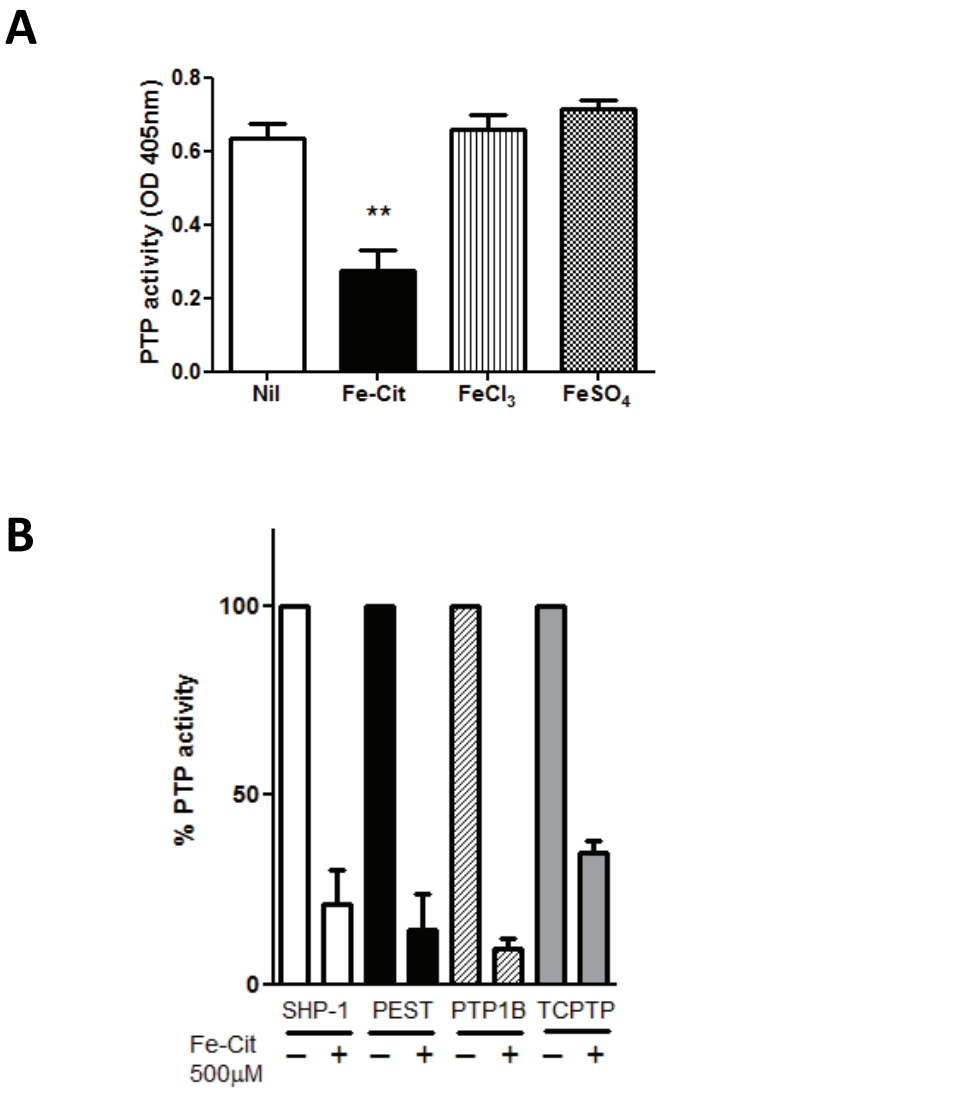


Figure 1. ***Iron-dependent PTP inhibition is specific to Fe-Cit.*** Macrophage PTP activity was determined following incubation of 10 µg of B10R total protein lysates **(A)** or GST-purified recombinant PTPs (**B**) for 10 min at RT with Fe-Cit, Fe_2SO_4 or $FeCl_3$ to a final concentration of 500µM**.** PTP activity was evaluated by pNPP hydrolysis and OD reading at 405nm. Statistically significant differences (**) were considered when *P<0.01.*

of coordinated iron nuclei, citrate molecules and protonation states [452]. The formation of specific Fe-Cit complexes is dependent on the pH, temperature, and particularly on the molar ratio of iron-to-citrate (Fe:Cit), among others [445]. These complexes can range from simple mononuclear complexes such as $[Fe(cit)_2]^{5-}$ or $[Fe(Hcit)(cit)]^{4-}$ [445], binuclear complexes such as $[Fe_2(cit)_2(H_2O)_2]^{2-}$ or $[Fe_2(Hcit)_3]^{3-}$, or trinuclear species characterized in solid state as the $[Fe_9O(cit)_8(H_2O)_3]^{7-}$nonanuclear complex [445, 453]. In this line of thought, we evaluated the impact of Fe:Cit ratios on the PTP inhibitory capacity of different Fe-Cit solutions. As shown in Figure 2A, the Fe:Cit ratio is critical for the capacity of Fe-Cit solutions to inhibit macrophage PTP activity; whereas Fe-Cit solutions prepared with 1:1 and 1:2 ratios (data not shown) did not inhibit PTP activity even at 500 μM, strong enzymatic inhibition was observed with 1:4, 1:10, 1:50 and 1:80 Fe:Cit preparations (Figure 2A and data not shown). In order to evaluate whether the Fe-Cit-mediated inhibition of PTPs was specific to the Fe-Cit complexes or to an unspecific effect of citric acid or the iron salt used in the preparation, we incubated total macrophage cell lysates with citric acid (Figure 2B) or $FeCl_3$ (Figure 1A) alone. As shown, neither citric acid alone nor $FeCl_3$ inhibit PTP activity, clearly implying that a specific Fe-Cit complex is responsible for the inhibitory effect.

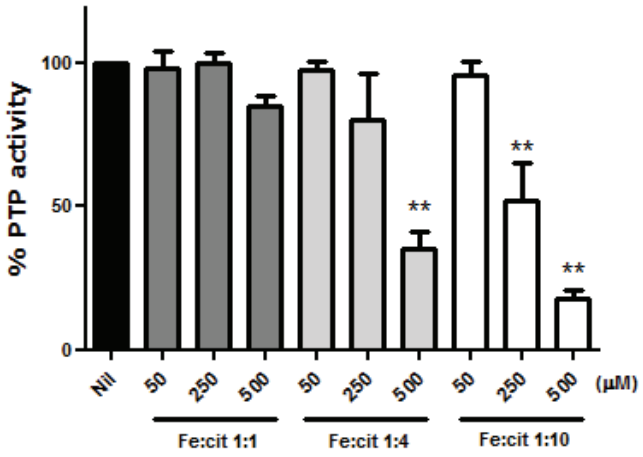
Identification of a mononuclear dicitrate iron complex in PTP inhibitory preparations.

Having observed specific PTP inhibition with Fe-Cit solutions prepared with Fe:Cit ratios ≥ 1:4, we studied the composition of each individual preparation in order to unravel putative Fe-Cit complexes responsible for the enzymatic inhibition. Electrospray Ionization Mass Spectrometry (ESI-MS) spectra of 1:1, 1:4 and 1:10 Fe:Cit solutions (Figure 3) revealed the multi-complex nature of these preparations (Table 1).

The composition of 1:1 Fe:Cit solution consisted mainly of binuclear and trinuclear iron complexes. The peaks at *m/z* 243.9 and 488.9 are assigned to $[Fe_2cit_2]^{2-}$ and $[Fe_2cit_2H]^-$ anions respectively, corresponding to the $[Fe_2cit_2(H_2O)_2]^{2-}$ binuclear complex characterized in solid state. The peaks at *m/z* 366.5 and 375.5 are assigned to

FIGURE 2

A



B

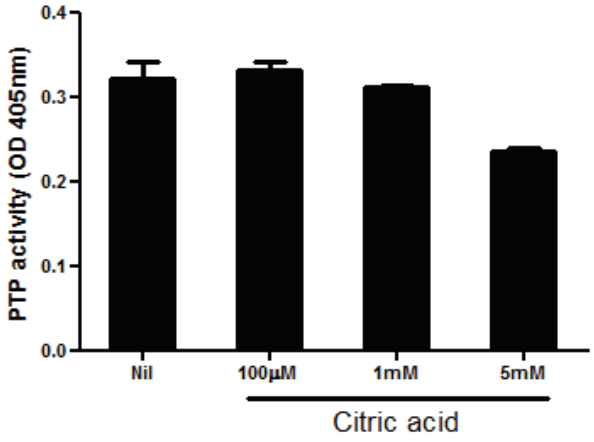


Figure 2. ***Effect of iron-to-citrate ratio in PTP inhibition.*** Three Fe-Cit solutions were prepared modifying the iron-to-citrate ratios to obtain 1:1, 1:4 and 1:10 Fe:Cit solutions. A dose response curve of B10R macrophage protein extracts incubated with 50-500µM 1:1, 1:4 and 1:10 Fe:Cit **(A)** or 0.1 - 5 mM citric acid **(B)** was performed. PTP activity was evaluated by pNPP hydrolysis and OD reading at 405nm. Statistically significant differences (**) were considered when *P<0.01.*

FIGURE 3

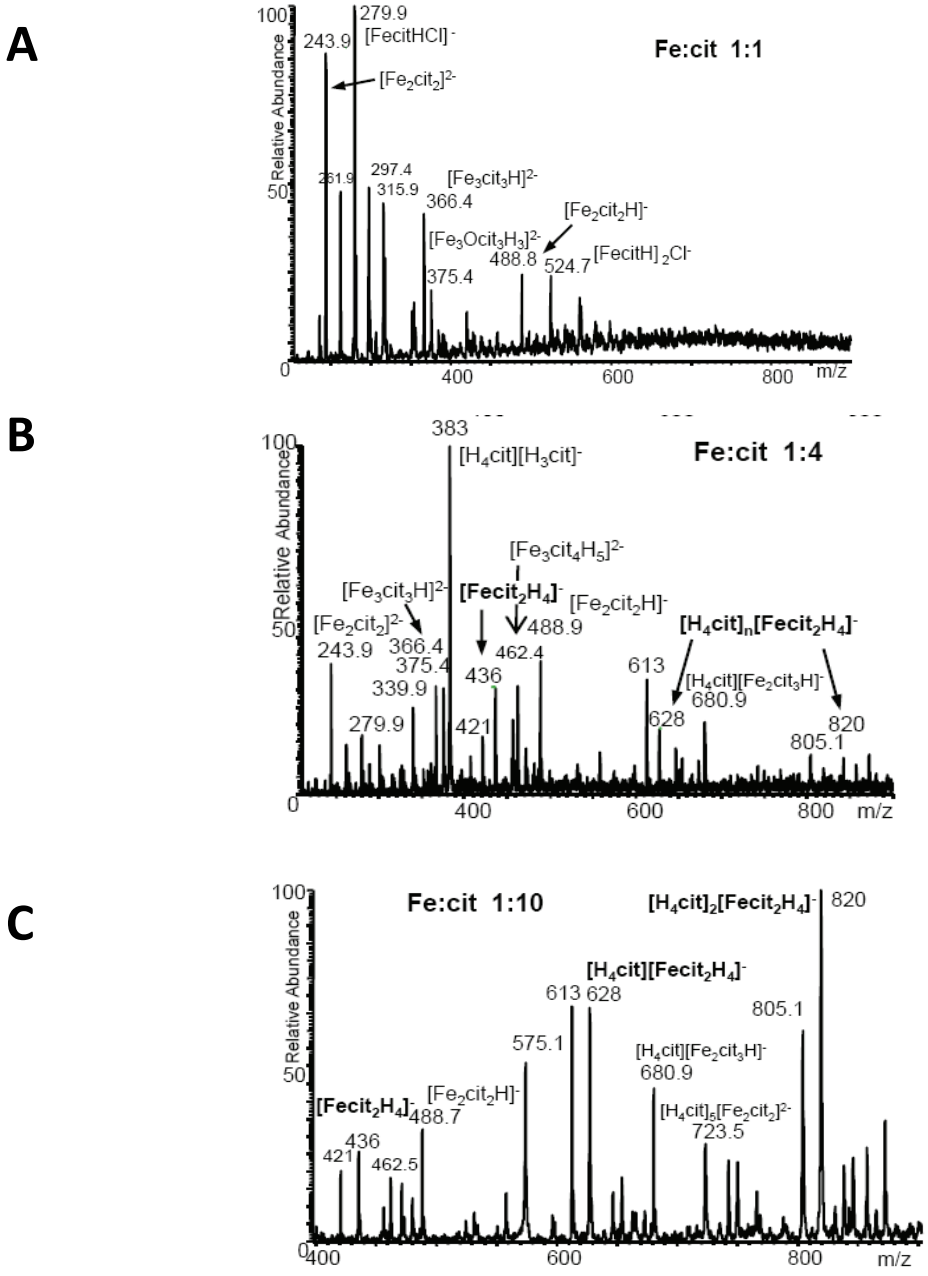


Figure 3. ***Specific inhibition of PTPs by mononuclear iron dicitrate.*** Electrospray mass spectra of 1:1 **(A)** 1:4 **(B)** and 1:10 **(C)** Fe:Cit solutions**.**

Table 1.

Fe-Cit species detected by ESI-MS.

Fe: cit ratio	m/z calculated	m/z measured	Species*
1:1	**279.9**	**279.9**	**$[FecitHCl]^-$**
	315.9	31 5.9	$[FecitH_2Cl_2]^{2-}$
	524.8	524.7	$([FecitH]_2Cl)^-$
	243.9	**243.9**	**$[Fe_2(cit)_2]^{2-}$**
	261.9	261.9	$[Fe_2(cit)_2H\ Cl]^{2-}$
	488.9	488.8	$[Fe_2(cit)_2H]^-$
	297.4	297.4	$[Fe_3O_3(cit)_2H_3]^{2-}$
	366.4	366.4	$[Fe_3(cit)_3H]^{2-}$
	375.4	375.3	$[Fe_3O(cit)_3H_3]^{2-}$
1:4	279.9	279.9	$[Fe(cit)H\ Cl]^-$
	436.0	435.9	$[Fe(cit)_2H_4]^-$
	628.0	627.7	$[H_4cit]\ [Fe(cit)_2H_4]^-$
	820.0	819.3	$[H_4cit]_2\ [Fe(cit)_2H_4]^-$
	243.9	**243.9**	**$[Fe_2(cit)_2]^{2-}$**
	339.9	339.9	$[H_4cit]\ [Fe_2(cit)_2]^{2-}$
	488.9	**488.8**	**$[Fe_2(cit)_2H]^-$**
	462.4	462.3	$[Fe_3(cit)_4H_5]^{2-}$
	366.4	366.4	$[Fe_3(cit)_3H]^{2-}$
	375.4	375.3	$[Fe_3O(cit)_3H_3]^{2-}$
	680.9	680.8	$[H_4cit]\ [Fe_2(cit)_2H]^-$
1:10	436.0	435.9	$[Fe(cit)_2H_4]^-$
	628.0	627.7	$[H_4cit]\ [Fe(cit)_2H_4]^-$
	820.0	**819.3**	***$[H_4cit]_2\ [Fe(cit)_2H_4]^-$***
	488.9	488.8	$[Fe_2(cit)_2H]^-$
	680.9	680.8	$[H_4cit]\ [Fe_2(cit)_2H]^-$
	724.0	723.5	$[H_4cit]_5[Fe_2(cit)_2]^{2-}$

* Species with major peak intensity are in bold characters. In italics are represented mononuclear dicitrate species.When the concentration of citric acid increases in the solution, free citric acid is associated with Fe-Cit species or other ions present in solution : ($[H_3cit]^-$ *m/z* 191) ($[H_4cit][H_3cit]^-$ *m/z* 283) ($[H_4cit]_2[H_3cit]^-$ *m/z* 575.5) ($(K[H_2cit])^-$ *m/z* 229) ($(K[H_3cit]_2)^-$ *m/z* 421) ($(K[H_4cit][H_3cit]_2)^-$ *m/z* 613) ($(K[H_4cit]_2[H_3cit]_2)^-$ *m/z* 805.1)

$[Fe_3cit_3H]^{2-}$, $[Fe_3Ocit_3H_3]^{2-}$ trinuclear species, which are the precursor building blocks observed in the $[Fe_9O(cit)_8(H_2O)_3]^{7-}$ nonanuclear complex. However, some mononuclear species (($[FecitHCl]^-$ *m/z* 279.9); ($[FecitH_2Cl_2]^{2-}$ *m/z* 315.9); $([FecitH]_2Cl)^-$ *m/z* 524.8)) associated with chloride coming from the iron salt, are observed. Chloride anion is a strong ligand and is coordinating the iron at acidic pH. In these species, the iron is in tetrahedral geometry as suggested by the presence of the $[FeCl_4]^-$ species (*m/z* 198) in the 1:1 Fe:Cit solution, citrate ligand being bidentate or tridentate. The peak's intensity of the $[FecitHCl]^-$ species is lowered in the 1:4 Fe:Cit solution, and is not observed in 1:10 Fe:Cit.

The 1:4 Fe:Cit solution had a predominant abundance of bi and trinuclear complexes. Moreover, the peaks at *m/z* 436, 628 and 820 are respectively assigned to the mononuclear dicitrate species $[Fecit_2H_4]^-$ and associated with free citric acid $[H_4cit][Fecit_2H_4]^-$ and $[H_4cit]_2[Fecit_2H_4]^-$. Effectively, when the concentration of citrate increases, the species are associated with free citric acid. This mononuclear dicitrate complex has been characterized in solid state as $[Fe(cit)_2]^{5-}$ and $[Fe(Hcit)(cit)]^{4-}$ crystallized from solution at pH >7 and at pH 6, respectively [445]. At more acidic pH, in solution, this species should be $[Fe(Hcit)_2]^{3-}$, $[Fe(H_2cit)(Hcit)]^{2-}$ or $[Fe(H_2cit)_2]^-$, and for the remaining of the text we refer to the mononuclear iron dicitrate species with the $[Fecit_2H_{4-x}]^{(1+x)-}$ general formula. Interestingly, the 1:10 Fe:Cit preparation, which *in vitro* exerts a stronger PTP inhibitory activity, contained mononuclear dicitrate complexes in greater relative abundance compared to binuclear or trinuclear species (Figure 3). Most interestingly, is the absence of mononuclear dicitrate species in the 1:1 Fe:Cit preparation -which *in vitro* has no PTP inhibitory activity-, strongly suggesting that mononuclear dicitrate complexes could represent the active PTP inhibitory species.

The species observed in this study by ESI-MS are in accordance with previous results obtained by crystal growth, ESI-MS and kinetic studies [445]. Polynuclear complexes are predominant for low Fe:Cit ratios (1:1 to 1:4), whereas the mononuclear dicitrate becomes predominant for the 1:10 Fe:Cit ratio. Its concentration largely

increases, from 40 % to 90 %, with the increasing concentration of citrate (from 1:5 to 1:80 Fe:Cit ratio), while the reverse holds true for the generation of polynuclear species.

Mechanism of PTP inhibition.

The importance of iron as a biologically active transition metal roots from its ability to engage in one-electron oxidation-reduction reactions, fluctuating between its oxidized and reduced state. However, this same property is responsible for the toxic effects associated with iron overload, where free reactive ions can catalyze the formation of ROS via Habber Weiss and Fenton chemistry (1) $O_2^{\bullet-} + Fe^{3+} \leftrightarrow Fe^{2+} + O_2$; (2) $2\ O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$; (3) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$, leading to lipid peroxidation, DNA and protein damage, and ultimately cell death [297]. It has been recently demonstrated that ROS play an important role in the regulation of PTP activity. Oxidation of the conserved catalytic cysteine residue, localized at the base of the active site of PTPs ([I/V]H**C**xxGxxR[S/T]), impairs enzymatic activity by preventing the nucleophilic attack to the incoming phosphate group [190]. We then hypothesized that the Fe-Cit PTP inhibition was an indirect result of PTP oxidation by ROS. To test this, we incubated macrophage protein lysates with 1:10 Fe-Cit solution in the presence or absence of superoxide dismutase (SOD), catalase (CAT), SOD+CAT or DTT (data not shown) in order to scavenge any possible ROS formed in solution, and for the latter ensure a reduced state environment propitious for PTP enzymatic activity. As shown in Figure 4A, PTP activity was not rescued following treatment with SOD, CAT, SOD+CAT or 5mM DTT (data not shown), suggesting that generation of ROS is likely not the mechanism of Fe-Cit PTP inhibition.

In-gel PTP activity assays have provided a good tool for studying the regulation of cellular PTPs. In this assay, PTPs are fully denatured and subsequently renatured following a series of washes in strong reducing conditions, thus recovering PTP enzymatic activity [418, 419, 444]. We evaluated the effect of 1:10 Fe-Cit when added during the renaturation steps in an in-gel PTP assay of total macrophage PTPs.

FIGURE 4

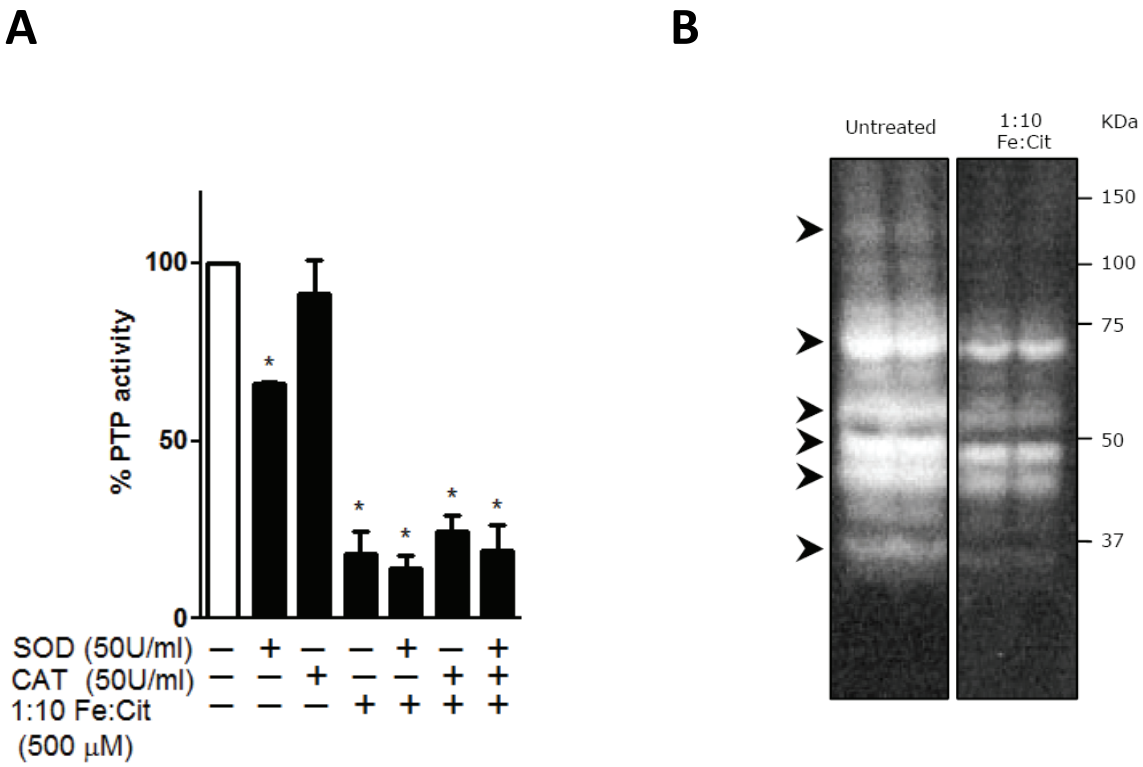


Figure 4. ***Fe-Cit-dependent PTP inhibition is independent of Fe-catalyzed ROS.*** B10R macrophage protein lysates were incubated for 10 min at RT with 500μM 1:10 Fe:Cit, in the presence or absence of superoxide dismutase (SOD) and/or catalase (CAT). PTP activity was evaluated by pNPP hydrolysis and OD reading at 405nm. Statistically significant differences (*) were considered when *P<0.05* **(A)**. 50μg of total B10R protein lysate were subjected to in-gel PTP assay. During the final renaturation step, gels were incubated with 500μM 1:10 Fe:Cit (right panel) or left untreated (left panel). Bands of dephosphorylation represent active PTPs. Arrows represent PTP targeted by Fe-Cit. The two lanes in each panel are duplicate samples **(B).**

Interestingly, full renaturation of enzymatic activity was not accomplished, suggesting that Fe-Cit could physically interact with the PTP active site impairing the dephosphorylation of the tyrosine phospho-peptide embedded in the gel (Figure 4B).

To further investigate this possibility, we performed computational molecular docking of Fe-Cit complexes on the prototypical macrophage PTP SHP-1 (one of the targets of Fe-Cit inhibition -Figure 2B-). The most energetically favorable interaction between the mononuclear dicitrate Fe-Cit complex **C8** ($[Fe(Cit)_2H]^{4-}$ (data not shown) and SHP-1 (1GWZ.pdb) and **C7** ($[Fe(cit)_2]^{5-}$) with SHP-1 (Figure 5A) occurred within the center of the catalytic pocket. Docking results reveal that $[Fe(cit)_2]^{5-}$ appears in the vicinity of Asp^{421} and His^{422} from SHP-1 WPD loop (Trp^{419}-Pro^{428}). Asp^{421} plays a key role in dephosphorylation reaction by acting as a hydrogen donor, allowing the release of the tyrosine peptide in the early stage of the reaction [183]. Within its binding pocket, the C7-ligand shares Cys^{455}, Ile^{459} and Arg^{461} with the PTP active site (Lys^{451}, His^{454}, Cys^{455}, Ile^{459} and Arg^{461}) and inhibits the access of any incoming phospho-substrate to the nucleophilic residue Cys^{455} (Figure 5B).

Docking experiments of SHP-1 co-crystallized with the *in vitro* tyrosine phospho-peptide substrate PY469 derived from SHPS-1, and $[Fe(Cit)_2]^{5-}$ (Figure 5C-D), excludes the mononuclear dicitrate complex from the active site, implying that the specific inhibition of PTP activity by $[Fe(Cit)_2]^{5-}$ follows a mechanism of direct and competitive interaction within the PTP catalytic pocket, preventing its interaction with an incoming phospho-peptide. In spite of the WPD loop being in its open conformation, the Fe-Cit complex stays away from the substrate binding pocket, allowing the correct interaction of the decapaptide with the amino acids of the active site. This experiment was repeated using a simulated annealing technique, and the protein was allowed to be relaxed within 0-0.5 Å during minimization. The flexibility of the WPD loop throughout minimization steps did not show a significant conformational change and therefore the ligand was kept away of any access to protein active site (data are not shown).

Of utmost interest, modeling experiments of SHP-1 (1GWZ.pdb) and the binuclear Fe-Cit complex **C2** ($[Fe_2(Cit)_2]^{2-}$) showed no interaction with the PTP catalytic

FIGURE 5

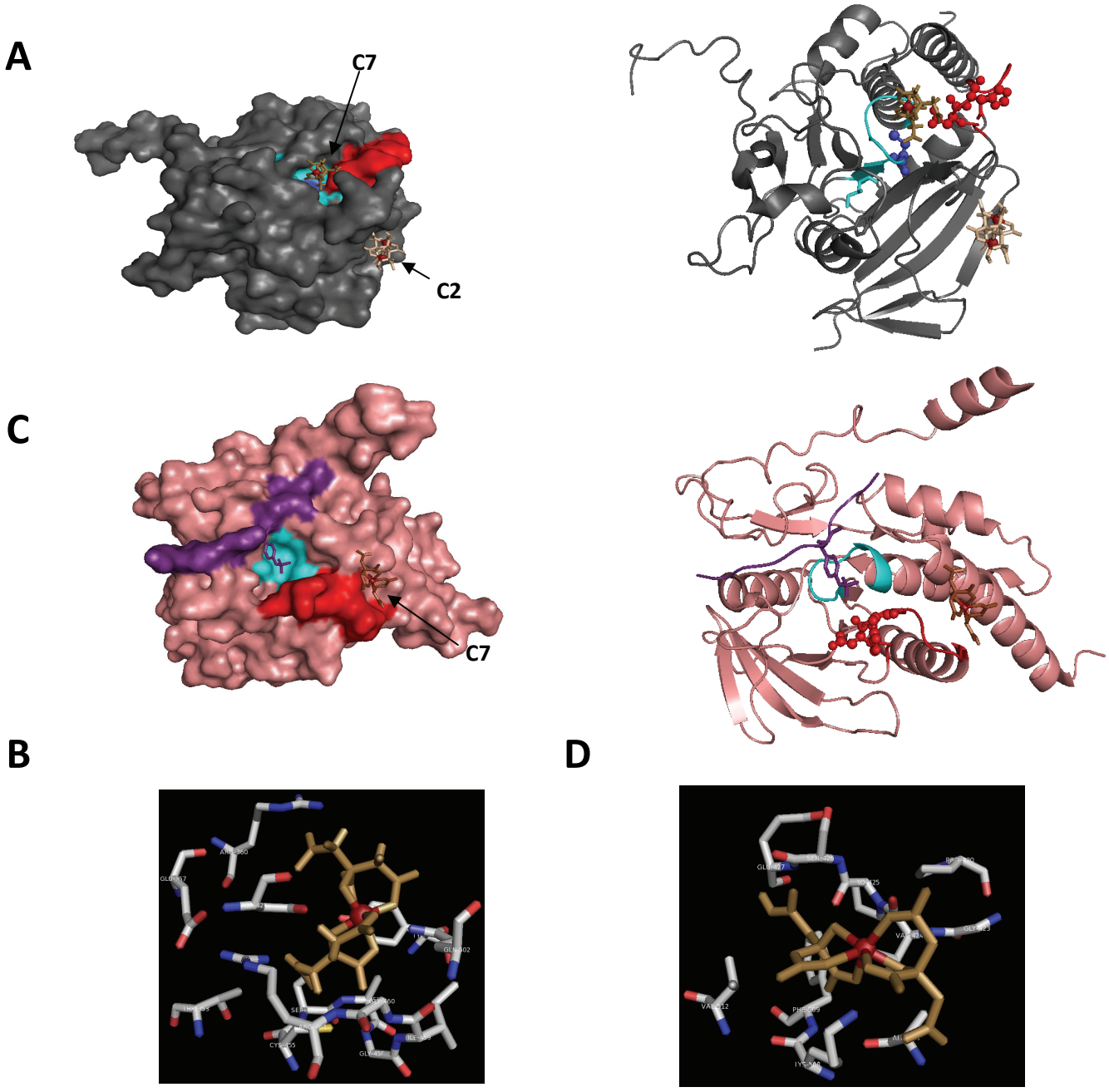


Figure 5. ***Computational docking of SHP-1 and Fe-Cit complexes.*** Docking solutions of SHP-1 (1GWZ.pdb) and the mononuclear dicitrate iron complex C7 $[Fe(Cit)_2]^{5-}$ and with the dinuclear dicitrate iron complex C2 $[Fe_2(Cit)_2]^{2-}$ **(A)** Detail of C7-SHP-1 interaction, denoting key residues of SHP-1 active site **(B)** SHP-1 co-crystallized with the *in vitro* tyrosine phospho-peptide substrate PY469 (purple) derived from SHPS-1 (1FPR.pdb) was modeled with $[Fe(Cit)_2]^{5-}$ **(C)** Detail of C7-SHP-1/PY469 interaction **(D)** Dark blue represents the catalytic Cys^{455} residue; Asp^{421} and His^{422} of WPD loop (red) are shown in sphere representation; in magenta is the PY469 phospho-peptide. Fe-Cit complexes are shown in firebrick color with the iron nuclei represented in red.

domain (Figure 5A), and binding to SHP-1 was located far away from the mononuclear binding pocket (~18.2 Å), correlating with its inability to inhibit PTP activity in a biochemical reaction. Collectively, these results corroborate our biochemical observations, and identify the mononuclear dicitrate complex $[Fe(Cit)_2H_{4-x}]^{(1+x)-}$ as the active PTP inhibitory species.

Fe-Cit transiently inhibits macrophage PTP activity resulting in protein hyperphosphorylation.

To investigate the effects of PTP inhibition in the modulation of intracellular macrophage signaling pathways, we incubated B10R murine macrophages with 1:1 or 1:10 Fe:Cit solutions and studied the kinetics of intracellular PTP inhibition. As shown in Figure 6A, PTP inhibition was detected as early as 5 minutes following 1:10 Fe:Cit delivery. This effect was transient, returning almost to basal PTP activity levels after 1h of incubation, in agreement with the activation of intracellular mechanisms of iron homeostasis (e.g. storage and export). Surprisingly, intracellular PTP inhibition also occurred with a 1:1 Fe:Cit preparation (which shows no inhibitory capacity *in vitro* -Figure 2A-) although with a slower kinetic, detecting significant levels of enzymatic inhibition after 15 minutes of incubation.

Western blot analysis of total phospho-tyrosine evidenced the hyperphosphorylation of macrophage proteins as early as 5 minutes following incubation with 1:10 Fe:Cit (Figure 6B). Moreover, the specific phosphorylation of ERK 1/2 and JNK MAP kinases, key signalling molecules during infection and inflammatory processes [454], was detected after 30 min of 1:10 Fe:Cit treatment (Figure 6C). When macrophages were treated for different time periods with 1:10 Fe:Cit prior to LPS stimulation, ERK1/2 and JNK hyperphosphorylation, in response to iron treatment, was even more evident and occurred as early as 5 minutes. These data directly correlate with iron-dependent PTP inhibition, favouring protein kinase activity and ultimately leading to a net result of protein hyperphosphorylation and positive signal transduction.

FIGURE 6

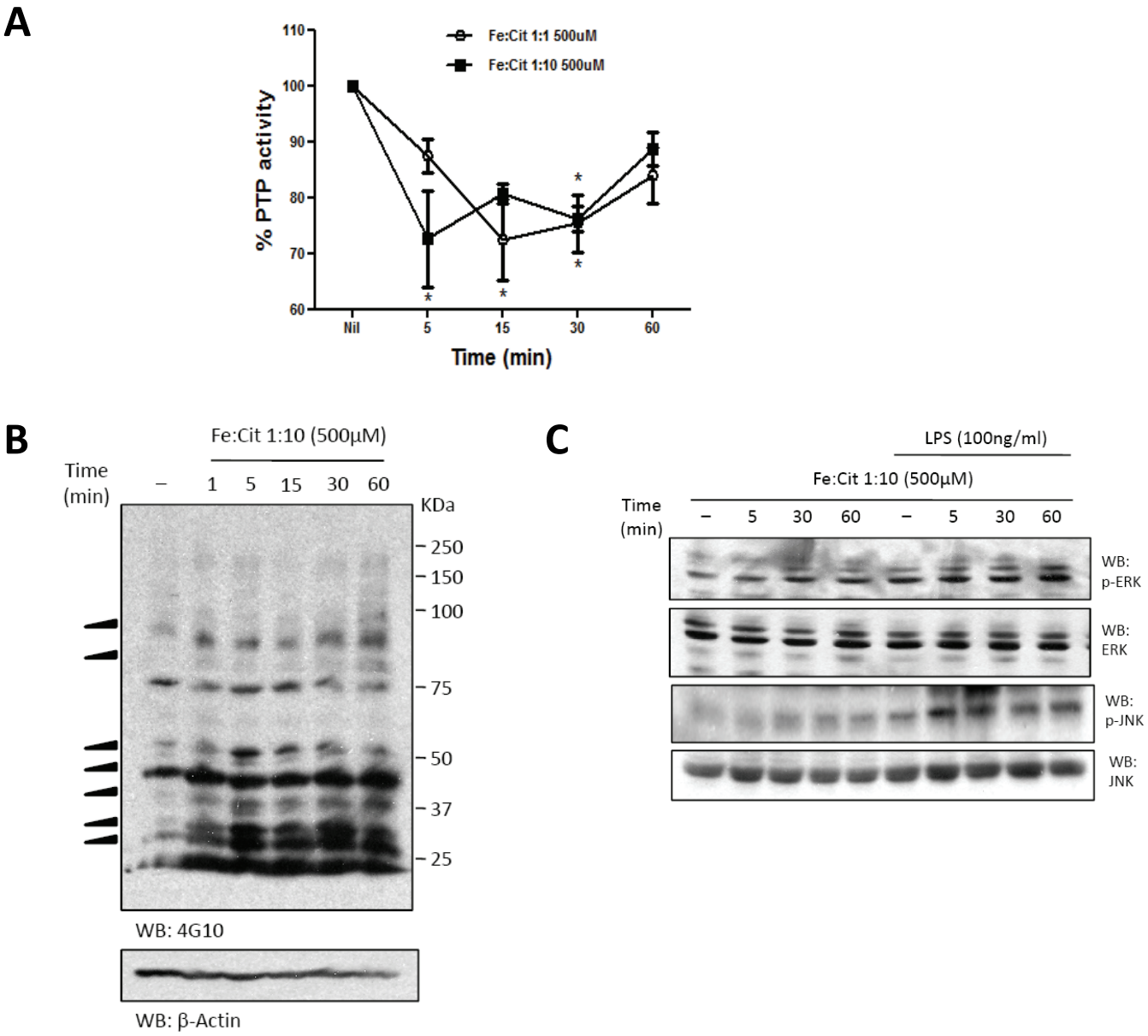


Figure 6. ***Fe-Cit inhibits PTP activity in macrophages and modulates protein phosphorylation.*** B10R macrophages were incubated with 500µM 1:10 or 1:1 Fe:Cit in serum-free DMEM, during a time course of 5 - 60 minutes**.** PTP activity was evaluated by pNPP hydrolysis and OD reading at 405nm. Statistically significant differences (**) were considered when *P<0.01* **(A)**. 4G10 anti-phosphotyrosine Ab was used to evaluate the phosphotyrosine content of 1:10 Fe:Cit treated macrophages. Loading control was performed using anti-β-actin Ab **(B)**. Macrophages were pre-treated with 500µM 1:10 in serum-free medium for 5 - 60 min or left untreated. Next, cells were stimulated with 100 ng/ml LPS and JNK and ERK1/2 phosphorylation was evaluated by western blot. Shown are the loading controls using anti-JNK and anti-ERK1/2 antibodies **(C)**.

Iron overload in mice induces macrophage PTP inhibition, ERK1/2 and JNK hyperphosphorylation

Parental iron dextran (Fe-Dex) therapy has been extensively used to treat iron deficiencies in patients where oral iron administration results unsuccessful due to poor dietary iron absorption, chronic hemodialysis, or chronic gastrointestinal loss [455]. Exogenous iron delivery as Fe-Dex has been shown to protect against *Leishmania major* infection in the highly susceptible BALB/c mice, correlated with inhibition of anti-inflammatory cytokines (IL-10 and IL-4) and upregulation of IFN-γ, iNOS expression and oxidative burst [341]. However, the precise molecular mechanism(s) by which this protection is acquired are not fully understood, and it is suggested to involve a ROS-dependent activation of the transcription factor NFκB [344, 345]. As has been previously shown by our laboratory and others, *Leishmania*-induced activation of macrophage PTPs represents a successful mechanism used by this parasite to downregulate host signaling pathways and downstream pro-inflammatory responses [50, 456]. Accordingly, ablation of individual PTPs [277, 281, 457], or pharmacological inhibition of host PTPs with the peroxovanadium compound bpV(phen) [250], results in delayed diseases onset and progression, or full protection (bpV(phen) treatment) against *Leishmania* infection in BALB/c mice [250]. Consequently, we have investigated the effect of systemic Fe-Dex administration in mice in the context of PTP regulation and protein phosphorylation, in an effort to understand the mechanism(s) underlying its capacity to protect against *L. major* infection.

Following a 3-day course of i.p. Fe-Dex administration to young adult BALB/c mice, bone marrows were collected and *in vitro* differentiated into macrophages (bone marrow-derived macrophages, BMM). PTP activity of BMM from Fe-Dex-treated animals, evaluated by pNPP hydrolysis, was significantly lower compared to control mice (Figure 7A). Consequently, total protein tyrosine phosphorylation, at basal level or upon *in vitro* LPS stimulation, was significantly increased in Fe-Dex BMM (Figure 7B). Moreover, in response to LPS stimulation, ERK1/2 and JNK exhibited stronger hyperphosphorylation in Fe-Dex BMM (Figure 7C), suggesting that the intracellular

FIGURE 7

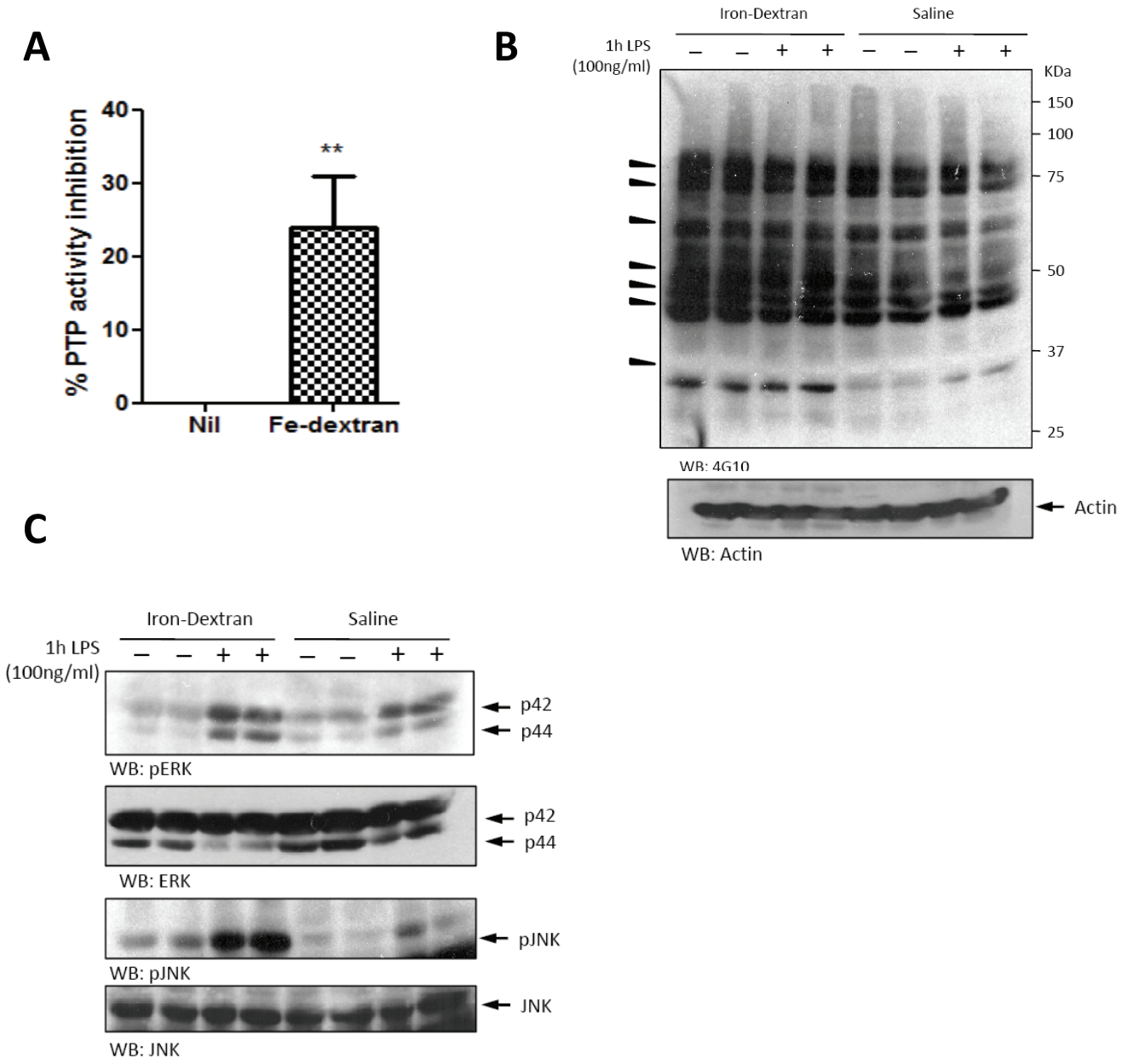


Figure 7. **In-vivo *treatment with Fe-Dex partially modulates macrophage signaling through PTP inhibition.*** 6-8 week old BALB/c mice we injected i.p. daily with iron-dextran (8mg/animal) for 3 days. BMM were differentiated and PTP activity evaluated by pNPP hydrolysis **(A)**. BMM from untreated and Fe-Dex treated mice were stimulated 1h with 100ng/ml LPS or left untreated. Phospho-tyrosine content was evaluated by western blot using 4G10 anti-phosphotyrosine Ab. Loading control was performed using anti-β-actin Ab **(B).** Specific phosphorylation of JNK and ERK1/2 was determined by western blot. Shown are the loading controls using anti-JNK and anti-ERK1/2 antibodies **(C)**. Western blots are presented with replicas from different animal subjects.

environment of these macrophages is prone for positive signal transduction, in line with lower PTP activity. Together, these observations suggest a novel mechanism of iron-dependent PTP inhibition and signaling enhancement, which could contribute to the protective phenotype of Fe-Dex treatment in the mouse model of cutaneous leishmaniasis.

DISCUSSION

Knowledge on the mechanisms underlying intracellular PTP activity regulation is pivotal for understanding the course of signal transduction in response to extracellular and intracellular stimuli, and will provide insights into how and when this group of enzymes can be considered as potential targets for therapeutic interventions. The PTP family encompasses a group of enzymes diverse in structural features, cellular distribution, subcellular localization and substrate specificities, however, maintaining a conserved catalytic domain [170]. Similarly, the mechanisms of activity regulation can be diverse, specific and broad. To date, three main processes controlling PTP activity have been described: protein phosphorylation, dimerization and oxidation. Whereas the first two have been shown to play a role in the regulation of specific PTPs or PTP sub-families (PTP dimerization occurs only within receptor PTPs) [184, 205, 212, 217], ROS-mediated PTP oxidation is thought to represent a broad mechanism of PTP regulation, as it involves oxidation of the conserved catalytic cysteine within the active site ([I/V]H**C**xxGxxR[S/T]) of all PTPs [190].

Metals such as zinc and vanadium have been known to play an important role in the regulation of PTP activity [236, 242]; however, only modulation by Zn^{2+} has been proposed to play a biologically relevant role. Recently, Haase and colleagues have shown that Zn inhibits PTP activity *in vivo,* resulting in the modulation of signaling pathways and cellular functions. Extracellular Zn delivery was shown to have insulinomimmetic effects, promoting general protein tyrosine hyperphosphorylation, but specifically that of the Insulin Growth Factor-1 (IGF-1) receptor, via PTP1B

inactivation [237-239]. The authors suggest that the mechanism of inhibition may involve the direct interaction of Zn ions with putative Zn binding motifs in the PTP domain; however, no definitive proof has thus far been provided.

In the current research article we reveal a novel mechanism of PTP regulation which involves the inhibition of PTP activity by mononuclear iron dicitrate (with the general formula $[Fe(Cit)_2H_{4-x}]^{(1+x)-}$). We have shown both, *in vitro* and *in vivo*, that 1:10 Fe:Cit solution inhibits PTP activity, in turn promoting general protein tyrosine hyperphosphorylation. Of particular interest was the finding that following LPS stimulation, the phosphorylation levels of both ERK 1/2 and JNK were significantly increased in 1:10 Fe:Cit treated cells (Figure 6), indicating that Fe-Cit-mediated PTP inhibition enhances pro-inflammatory macrophage signaling. Recently, Xiong, *et al.*, have shown that extracellular stimulation of macrophages with LPS or TNF-α induces a rapid and transient increase in intracellular LMW·Fe complexes. This results in the upregulation of NFκB nuclear translocation and DNA binding, correlating with higher TNF-α release [232]. Although the precise upstream triggering event remains unknown, the authors suggest that NFκB activation follows the p21ras-dependent activation of IKK, possibly through the generation of ROS or reactive nitrogen intermediates (RNI) [366]. Given our current results, these observations lead to a provocative hypothesis: rapid, local and transient accumulation of LMW·Fe complexes, possibly in the form of mononuclear dicitrate $[Fecit_2H_{4-x}]^{(1+x)-}$, inhibits PTPs in the periphery of receptor signaling complexes skewing the pathways to positive signal transduction, allowing and inducing downstream gene expression.

The intracellular labile iron pool (LIP) is a transient and redox-active pool of LMW·Fe complexes which are readily available for extracellular export, association with iron-containing proteins and regulation of iron-dependent functions [311]. The LIP is distributed into the cytosol, mitochondria, nucleus [318, 319], endosomes and lysosomes (following ferritin degradation) [314]. Although the exact composition of the LIP is still a matter of controversy, iron can complex intracellularly with LMW chelates

including ascorbate, citrate [313, 458], small polypeptides and lipid moieties [311]. To date, the identity of the bioactive Fe-Cit complex(es) remains unknown. However, based on intracellular citrate concentrations ranging from 0.1 mM-0.4 mM [313] and labile iron concentrations of 0.4 µM - 16 µM [312, 314, 317], the intracellular Fe:Cit ratios could range from 1:6 - 1:1000. As previously shown, high Fe:Cit ratios (≥ 1:10) allow for increased relative abundance of mononuclear Fe-Cit complexes [445]. This, together with the co-crystallization of the *E. coli* outer membrane receptor FecA complexed with the binuclear complex $[Fe_2(cit)_2]^{2-}$ [459, 460], and the importance of mononuclear Fe-Cit species as bioavailable iron sources, suggest that mononuclear and binuclear Fe-Cit complexes could represent the biologically relevant species. In line with this, using ESI-MS and computational modeling (Figures 3 and 5), we have identified the mononuclear dicitrate iron complex $[Fecit_2H_{4-x}]^{(1+x)-}$ as the active species in the inhibition of macrophage PTP activity, providing further evidence of its physiological relevance. Moreover, we show that the mechanism of PTP inhibition by mononuclear iron dicitrate is not dependent on ROS-mediated oxidation, but rather, it follows the direct interaction of the mononuclear dicitrate iron complex with the PTP catalytic domain, impairing the entrance of an incoming phospho-protein substrate by physical competition with the active site (Figure 5 A-C).

Surprisingly, in contrast to what is observed *in vitro*, the 1:1 Fe:Cit preparation (which mainly contains bi and trinulcear complexes) inhibits PTP activity in B10R macrophages, although at a slower rate than 1:10 Fe:Cit (Figure 6A). This suggests that internalized molecular iron or iron citrate complexes may be intracellularly metabolized to mononuclear Fe-Cit species, either spontaneously or as part of the process of iron mobilization. This same scenario could be responsible for the effects observed *in vivo* after parental administration of Fe-Dex. Similarly to what is observed in 1:10 Fe:Cit-treated macrophages, BMM isolated from Fe-Dex-treated mice showed lower PTP activity as early as 24 hours post-treatment (data not shown), correlating with general protein tyrosine hyperphosphorylation and enhanced sensitivity to LPS stimulation (Figure 7). Given the current unavailability of precise methodological approaches to

determine the composition of intracellular Fe-Cit species, intracellular metabolism of Fe^{3+} or Fe-Cit complexes towards mononuclear dicitrate remains thus far hypothetical.

In light of our current data, it seem interesting to revisit the role of Fe-Dex in the treatment of diseases such as leishmaniasis, where strong pathogen-induced downregulation of cell functions leads to disease establishment and progression. It is pivotal, however, to keep into consideration the toxic side effects of iron overload, the differential outcomes of other infectious diseases in the context of host iron status, and the different degrees of host iron metabolism [294, 296]. During *Leishmania* infection, pathogen-induced activation of macrophage PTPs is a major landmark of the negative regulation of host cell functions associated with the initial stages of the disease [50]. As previously shown [341, 344, 345], onset and development of *L. major* infection in mice is completely abrogated by pre-treatment with Fe-Dex, suggesting that inhibition of PTP activity may contribute to the adequate induction of host cell responses during the initial pathogen challenge.

Collectively, our data reveal a novel mechanism of PTP activity regulation by $[Fecit_2H_{4-x}]^{(1+x)-}$ mononuclear iron dicitrate. Of utmost interest, is the biological relevance of this event, given the nature of iron-citrate as a ubiquitous source of bioavailable iron. Similarly to ROS, Fe-Cit-dependent regulation of cellular PTP activity may represent an important mechanism for the control of signal transduction both, at resting sate and in the scenario of an inflammatory response, which could potentially be exploited for the manipulation of signaling pathways by iron chelation or iron delivery therapies.

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PREFACE TO CHAPTER 4

As previously mentioned, the outcome of *Leishmania* infection depends both on host and pathogen factors. Given the critical role of host signaling in the disease onset and progression, and having identified iron homeostasis as an important player in the regulation of host PTPs in the context of infection, this chapter aims to evaluate and identify putative *Leishmania* factors responsible for the modulation of host cell PTPs. Results here presented show that in addition to SHP-1, two other host PTPs intimately involved in the negative regulation of JAK/STAT and MAPK signaling pathways, PTP1B and TCPTP, are modulated upon *Leishmania* infection. Moreover, an important role for PTP1B in the onset and progression of cutaneous leishmaniasis is revealed, suggesting a concerted action of PTP1B and SHP-1 in the *Leishmania*-induced negative regulation of host functions. We have identified the molecular mechanism responsible for PTP modulation in infected macrophages, an event which depends on the functional expression of the *Leishmania* surface protease GP63. A detailed discussion on this mechanism is further provided.

CHAPTER 4

LEISHMANIA GP63 ALTERS HOST SIGNALLING THROUGH CLEAVAGE-ACTIVATED PROTEIN TYROSINE PHOSPHATASES

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ABSTRACT

Rapidly upon *Leishmania*-macrophage (MØ) interaction, activation of the protein tyrosine phosphatase (PTP) SHP-1 leads to the downregulation of JAK and MAPK signalling, playing an essential role in the attenuation of host innate inflammatory responses and various microbicidal MØ functions. Here, we report results revealing that in addition to SHP-1, PTP1B and TCPTP are modulated and post-translationally modified in infected MØs, and identify an essential role for PTP1B in the *in vivo* progression of *Leishmania* infection. The mechanism underlying PTP modulation involves the functional expression of the *Leishmania* surface protease GP63. The direct interaction of PTP1B, TCPTP and SHP-1 with GP63 is mediated by a lipid raft-dependent GP63 internalization into host MØ, resulting in PTP cleavage and activity regulation. Collectively, our data present a novel mechanism of cleavage-dependent regulation of MØ PTPs by an obligate intracellular pathogen, and show for the first time the internalization of a key *Leishmania* virulence factor into host MØs, revealing a new mechanism whereby this parasite interacts and survives within its host.

INTRODUCTION

The strategic modulation of host cell signalling pathways by *Leishmania*, in which parasite-induced activation of MØ protein tyrosine phosphatases (PTPs) plays a critical role, results in the inhibition of several phagocyte functions, providing a suitable environment for intraphagosomal parasite development and survival [50]. In the early stages of *Leishmania*-MØ interactions, alterations in the JAK/STAT and MAPK signalling pathways occur as a result of parasite-induced activation of the host PTP SHP-1[158]. This event leads to the attenuation of innate inflammatory responses and the downregulation of nitric oxide (NO) production, fundamental for controlling the intracellular parasite and its progression within the host [158, 251, 277]. Despite the critical role of SHP-1 in the *Leishmania*-mediated negative regulation of MØ signalling, additional PTP enzymatic activation is observed in SHP-1 deficient MØs infected with

the parasite [158], thus suggesting that additional MØ PTPs could play a role in this downregulation process.

PTP1B and TCPTP, in addition to SHP-1, are intimately involved in the negative regulation of JAK/STAT and MAPK signalling [433, 450]. The role of PTP1B in metabolic, oncogenic and immunological processes [216, 385, 461] highlights is importance as a key regulator of signal transduction. Of importance, PTP1B has been shown to regulate MØ activation *in vivo*, where higher levels of MØ NO production, serum IL-12 and IFN-γ were detected in PTP1B$^{-/-}$ mice [216]. Considering that downregulation of IL-12, TNFα and NO are hallmarks of *Leishmania* infections [50], PTP1B stands as a rational candidate mediating *Leishmania*-dependent inhibition of host functions.

The initial interaction between *Leishmania* and its host cell is largely mediated by abundant promastigote surface molecules including lipophosphoglycan (LPG) and the major surface protease GP63 [50]. Whereas the role of LPG as virulence factor has been extensively explored [64], *Leishmania* GP63 has been implicated in binding to MØs enhancing parasite phagocytosis, evasion of complement mediated lysis, migration through the extracellular matrix [98] and induction of a Th1 type immune response, amongst others [61, 94, 100]. In the present study we identified GP63 as the key *Leishmania* virulence factor modulating host PTPs and revealed an essential role for PTP1B in the progression of cutaneous leishmaniasis in infected mice. Of utmost interest, our findings have permitted to discover a new mechanism whereby *Leishmania* GP63 can access the MØ intracellular milieu in a lipid raft dependent manner, allowing a direct interaction with host protein substrates. Collectively, our findings provide important understanding about strategies utilized by pathogens to exploit host cell negative regulatory mechanisms resulting in the evasion of innate immune response activation.

RESULTS

Leishmania infection modulates multiple PTPs.

Activation of SHP-1, a key negative regulator of MØ signalling, is a prerequisite for the *Leishmania*-induced alterations of MØ JAK and MAPK signalling [50, 456]. Although SHP-1 activation seems to suffice for the parasite-mediated inhibition of NO production, induction of PTP activity is still observed in SHP-$1^{-/-}$ MØs [158], suggesting that other host PTPs are modulated by *Leishmania*. To address this point, an in-gel PTP activity assay was performed enabling a profiled study of MØ PTPs in infected and uninfected cells. B10R MØs were infected with *L. mexicana* (0-4-hr), and protein extracts subjected to in-gel PTP activity assay. As shown in Figure 1A, modulation of multiple PTPs could be detected as early as 1 min following *Leishmania*-MØ interaction and sustained up to 4h post-infection, evidenced by the appearance of new active PTP bands (~75, 50, 37 and 35 KDa) and disappearance of basally active PTPs (~120 and 40 KDa). Of interest, infection with *L.major, L.donovani* and *L.mexicana* showed that mammalian cutaneous and viscerotropic species similarly modulate MØ PTPs, whereas infection with the lizard pathogen *L.tarentolae* had no effect on PTP activity (Figure 1B). This suggests that the parasite-dependent PTP modulation is associated with *Leishmania* mammalian pathogenicity, but not with tropism in the host.

Modulation of host PTPs is associated with protein cleavage and is independent of Leishmania internalization.

The PTP activity profile seen by in-gel PTP assay is suggestive of PTP cleavage. To investigate the nature of PTP modulation, we performed western blot analysis of MØ PTPs implicated in the regulation of signalling pathways previously reported to be modulated by *Leishmania* infection. Upon infection, the rapid and time-dependent cleavage of SHP-1, PTP1B, TCPTP (Figure 2A) and PTP-PEST (data not shown) was observed. Full length TCPTP was completely lost 5 min following infection, whereas

FIGURE 1

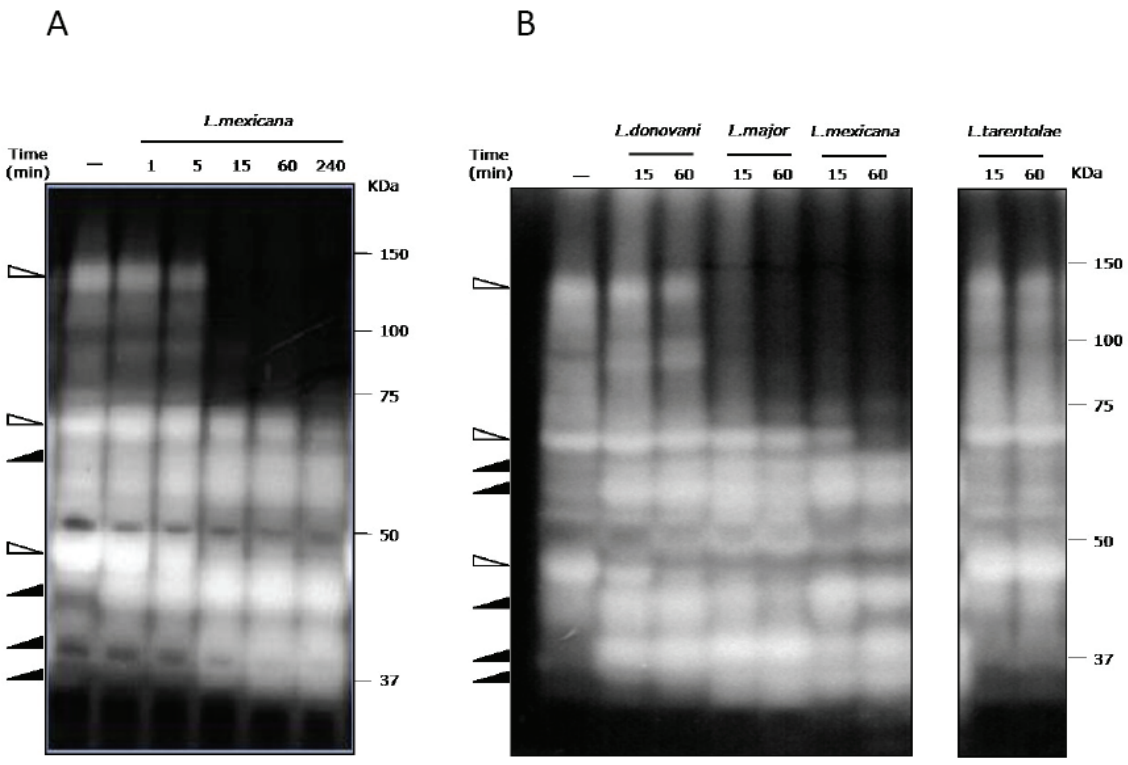

Fig 1. *Leishmania infection modulates multiple MØ PTPs.* **(A)** MØs were infected with *L.mexicana* (0-240min), **(B)** *L.donovani, L.major,* or *L.tarentolae* (15 and 60 min). 40μg of total protein lysates were loaded for in-gel PTP assay, where bands of dephosphorylation (clear bands) represent active PTPs. Modulation of MØ PTPs is represented by arrows, where black and white respectively represent active or loss of active PTP bands following *Leishmania* infection.

disappearance of the full length SHP-1 and PTP1B was visualized at 30 min post-infection. Of interest a hierarchical cleavage of PTP1B and TCPTP takes place, as the appearance of a second smaller cleavage product was detected at later time points (Figure 2A). This effect was not generalized amongst MØ phosphatases; no cleavage products or protein degradation was detected even 1h post-infection for SHP-2, PTEN and MKP-1 (data not shown), nor for the serine/threonine phosphatase PP2A (Figure 2B).

Leishmania internalization into MØs is rapidly completed within 10 min of initial MØ-parasite contact[139]. Given that PTP cleavage occurs as fast as 5 min post-infection, we asked whether parasite internalization was required for PTP cleavage. As shown in Figure 2C, parasite internalization was not required for PTP cleavage as incubation with parasite culture supernatant led to SHP-1, PTP1B and TCPTP cleavage. Although the extent of PTP cleavage was lower, the reaction followed similar kinetics as with whole parasite infection, where cleavage products appeared as early as 5 min (Figure 2A). Supporting this observation, PTP cleavage does not result from a phagocytosis-dependent mechanism, as cleavage was undetectable in B10R MØs fed with 0.8µm latex beads. However, when latex beads were coated with *L.mexicana* culture supernatant, PTP cleavage was restored (Figure 2D). These findings suggest that a soluble *Leishmania* factor is responsible and sufficient for triggering MØ PTP cleavage, independently of parasite phagocytosis.

Leishmania-induced PTP cleavage is dependent on the functional expression of GP63.

Essential *Leishmania* surface molecules, including LPG, GIPLs, and the major surface protease GP63, are widely recognized by their abundance and importance in the interphase of host-pathogen interactions. Given the importance of GP63 protease as a virulence factor and its nature as surface bound and excreted protein [92, 93], we investigated whether GP63 could be involved in MØ PTP cleavage.

FIGURE 2

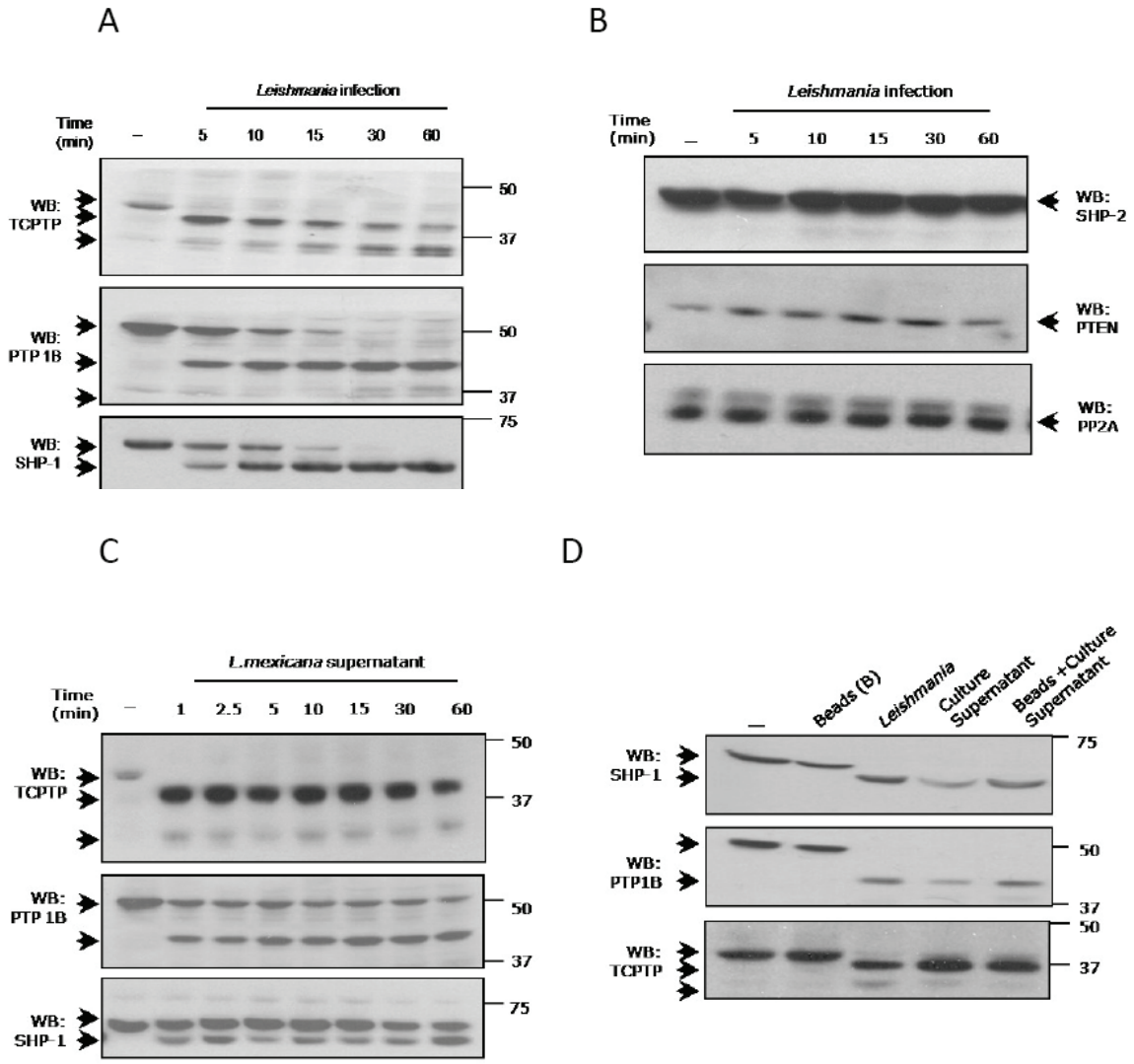

Fig 2. *Modulation of MØ PTPs is associated with protein cleavage and independent of parasite internalization.* The kinetic (5-60 min) of PTP cleavage was evaluated by western blot of *Leishmania* infected or uninfected total MØ protein lysates. **(A)** TCPTP, PTP1B and SHP-1 were cleaved as early as 5 min post-infection. **(B)** PTEN, SHP-2 and the ser/thr phosphatase PP2A were not cleaved or degraded upon infection. **(C)** MØs were incubated with stationary phase *L.mexicana* supernatant for 1-60 min, or **(D)** fed with 0.8µm latex beads or parasite culture supernatant-coated beads for 1h. Total protein lysates were evaluated by western blot. Black arrows represent full length PTPs and cleavage fragments

As seen in Figure 3A, GP63 gene knockout completely abrogates PTP cleavage. However, reinsertion of *L.major* GP63 *gene 1* into the GP63 knockout strain (*L. major* $GP63^{R}$), rescues its PTP cleavage ability (Figure 3A, lane 4). Moreover, in-gel PTP activity assay of *L. major* $GP63^{-/-}$ infected MØs shows that modulation of host PTP activity is abrogated in the absence of GP63 expression, as seen by a pattern of PTP activity identical to the uninfected control (Figure 3B). Collectively, these data show that GP63 is responsible and sufficient for the cleavage of target PTPs.

GP63 directly cleaves target PTPs.

When dissecting the mechanism of GP63-dependent PTP cleavage, we evaluated whether GP63 can directly interact and cleave target PTPs. GP63 protease recognizes a consensus site in its target substrates ($P_1\downarrow P'_1\text{-}P'_2\text{-}P'_3$) where P'_1 corresponds to a hydrophobic amino acid residue, P'_2 and P'_3 to basic residues and P_1 preferentially a polar amino acid [95]. Sequence analysis of mouse and human (data not shown) SHP-1, PTP1B and TCPTP predicted putative cleavage sites for the three target proteins (Figure 4A). The predicted molecular weights for cleavage at these sites closely correspond to cleavage fragments observed by western blot. Interestingly, localization of the target motifs in the 3D structure representation of TCPTP and PTP1B (Figure 4B) revealed that putative cleavage sites 1 and 2 found in TCPTP and 1-3 in PTP1B were present in the periphery of the globular molecules, implying structural permissiveness for protein-protein (PTP-GP63) interactions. Sequence analysis of SHP-1 localize the first putative cleavage motif at an accessible site in the C-terminal SH2 domain. However, cleavage at this site seems not to take place, as the cleavage product identified by western blot is ~60KDa (Figure 2A), likely corresponding to the molecular weight of the fragment from cleavage at sequence-predicted site 2 (Figure 4A). The unavailability of a complete SHP-1 crystal structure impeded the subsequent structural analysis of SHP-1 cleavage at site 2.

FIGURE 3

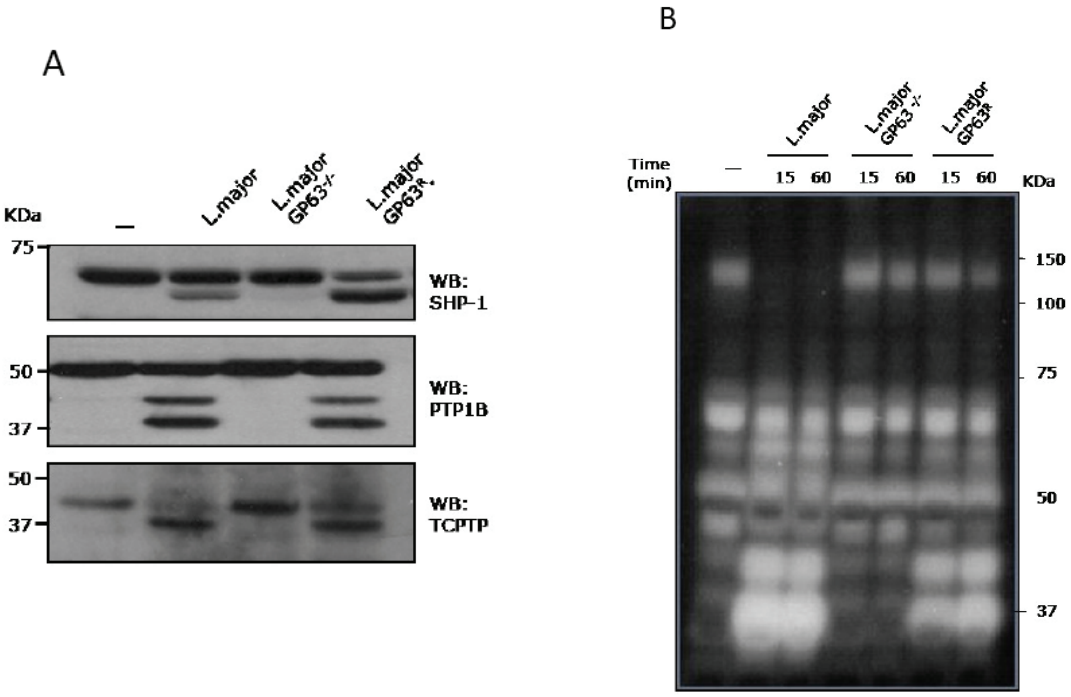

Fig 3. *Leishmania-induced PTP modulation is dependent on the functional expression of GP63.* B10R MØs were infected with *L.major, L.major* GP63$^{-/-}$ or *L.major* GP63^{R} for 1h. Total MØ protein lysates were evaluated by **(A)** western blot and **(B)** in-gel PTP activity assay.

FIGURE 4

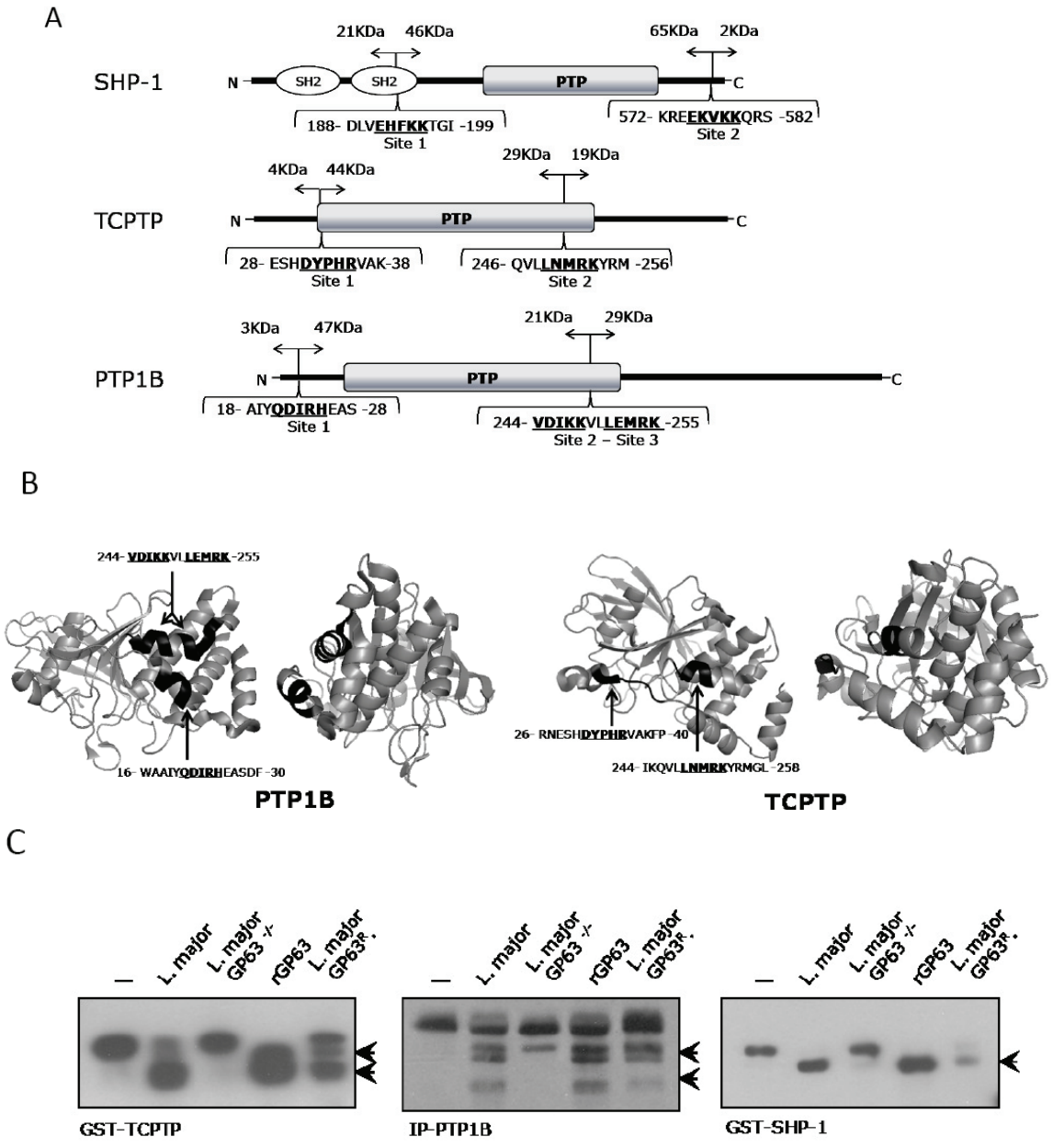

Fig 4. *Identification of putative GP63 cleavage sites and direct GP63-PTP interactions.* Sequences of mouse SHP-1 (P29350), PTP1B (P18031), and TCPTP (P17706) were retrieved from UniProtKB/Swiss-Prot database. **(A)** GP63 consensus cleavage site (P_1-P_1'-P_2'-P_3' 4 aminoacid motif: Polar-Hydrophobic-Basic-Basic) was identified in the three target PTPs. **(B)** Structural analyses of PTP1B (PDB 1OEO) and TCPTP (PDB 1L8K) predicted cleavage sites accessible for protein-protein interaction. **(C)** 2.5µg of purified GST-SHP-1, GST-TCPTP or IP PTP1B were incubated with 30 x 10^6 *L.major, L.major* GP63$^{-/-}$, *L.major* GP63^{R} or 1µg rGP63 for 1h at RT in a rocking platform. TCPTP, SHP-1 and PTP1B cleavage was evaluated by western blot. Arrows indicate cleavage fragments.

The direct PTP-GP63 interaction was confirmed by an *in-vitro* cleavage approach. Purified GST tagged full length SHP-1 [462] and TCPTP fusion proteins, together with IP PTP1B where incubated with 3 x 10^7 *L. major, L. major* GP63$^{-/-}$*L. major* GP63^{R} or with recombinant GP63 (rGP63; 1µg) [463], and cleavage evaluated by western blot. PTP cleavage was observed after incubation with WT or GP63^{R} *L.major* strains and absent when treated with *L.major* GP63$^{-/-}$ (Figure 4C). Most importantly, cleavage was detected upon incubation of target PTPs with rGP63, further confirming the specificity of GP63 in this process. These data shows that the direct interaction of PTPs and GP63 is responsible and sufficient for the *Leishmania*-induced MØ PTP cleavage.

Leishmania GP63 access the host cell and rapidly targets MØ PTPs in the cytoplasmic membrane periphery.

TCPTP, PTP1B and SHP-1 play a central role as negative regulators of the JAK/STAT signalling pathway by dephosphorylating JAK family members [159, 432, 433]. Induction of JAK/STAT signalling follows interferon (IFN) ligation to the IFN receptor (IFNR), where the JAK kinases, constitutively bound to the IFNR, are autophosphorylated and activated [274]. Negative regulation of signal transduction at this stage requires the shuttling of PTPs to the inner face of the cytoplasmic membrane where phosphorylated JAKs could act as PTP substrates. Given that upon *Leishmania*-MØ contact GP63-PTP interaction takes place as fast as 5 minutes post infection (Figure 2A), we hypothesized that this rapid interaction should occur at the stage of initial cell-cell contact in the MØ cytoplasmic membrane level, where GP63 could access shuttling or resident PTPs.

JAK2 was IP from *Leishmania* infected or uninfected MØs. In-gel PTP activity assay of IP JAK2 shows the co-IP of multiple PTPs (Figure 5A), two of which by molecular weight and comparison with PTP profiles of SHP-1$^{-/-}$ and PTP1B$^{-/-}$ MØs, were identified as PTP1B and SHP-1. Of particular interest, one more active PTP (~120 KDa) co-IPed

FIGURE 5

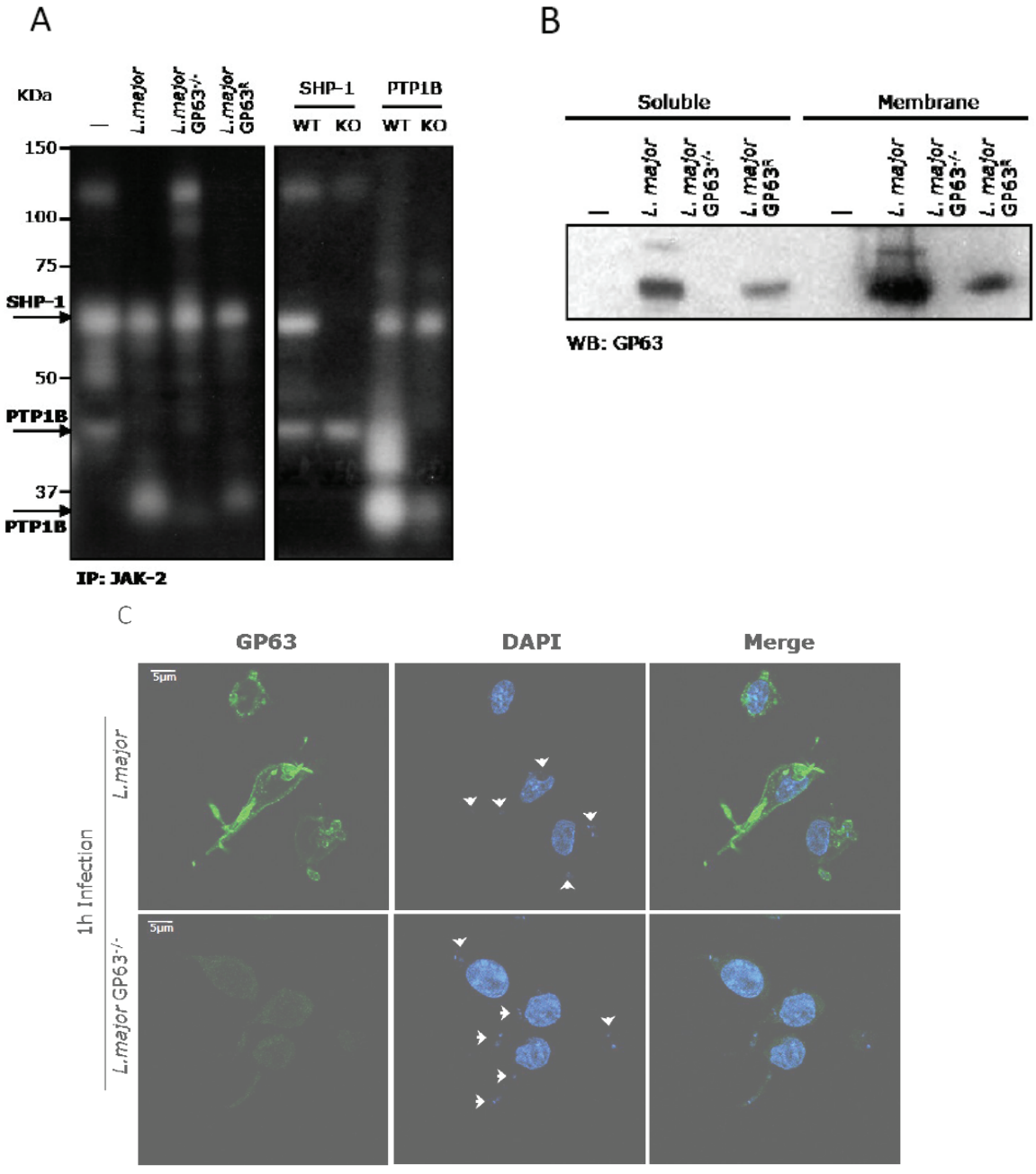

Fig 5. *Leishmania GP63 access the host cell.* MØs were infected for 1h. **(A)** JAK2 was IP and immune complexes run in an in-gel PTP activity assay. **(B)** Cells were lysed and protein extraction was performed. Crude membrane and cytosolic fractions were collected. Membrane pellets were washed 5 times with lysis buffer and solubilized in SDS-PAGE sample loading buffer. Fractions were run on gel and blotted for *Leishmania* GP63. **(C)** α-GP63 Ab clone #96 was used to evaluate GP63 localization in *Leishmania*-infected MØs by confocal microscopy. Arrows indicate *Leishmania* parasites.

with JAK2 at resting state or upon *L.major* GP63$^{-/-}$ infection, suggesting that JAK signalling may also be regulated by other phosphatases in addition to those previously described. Furthermore, following *L. major* infection the profile of PTPs that co-IP with JAK2 involved PTP cleavage. We identified cleavage fragments of PTP1B associated with JAK2 and the total disappearance of the 120 KDa PTP, an event that was abrogated in *L.major* GP63$^{-/-}$ infected cells (Figure 5A). Conversely, JAK2 associated full length SHP-1 remained unaffected, suggesting that the "free-cytosolic" SHP-1 pool is the target for cleavage, at least in the context of JAK2-SHP-1 interaction (Figure 5A).

Western blot analysis of *Leishmania* infected or uninfected MØ crude membrane and cytosolic fractions, showed that *Leishmania* GP63 can access MØs and is found both associated with membrane fractions and as a soluble GP63 pool (Figure 5B). To exclude that detection of GP63 was a contribution of lysed *Leishmania* parasites, confocal microscopy of *L.major* infected MØs was performed. Within 1h of *Leishmania* infection, GP63 gains access to the host cell localizing to the cytosolic membrane, perinuclear area (Contreras and Olivier, unpublished observations) and dispersed along the cytoplasm in a punctuated distribution (Figure 5C). GP63 internalization begins within 5 min of *Leishmania*-MØ contact and is maximal after 3h post-infection where GP63 can be detected in more than 90% of infected cells (data not shown).

Host lipid raft integrity is important for GP63 internalization.

In *Leishmania* parasites, GP63 is found in three different pools: the major component being a C-terminal GPI anchored protein followed by an internal and a released pool [91-93]. Lipid rafts, dynamic membrane domains enriched in cholesterol, sphingolipids and GPI anchored proteins, mediate the internalization of cargo proteins and lipids from the cell surface of mammalian cells [464]. To test whether the integrity of host lipid raft domains was required for GP63 entry into the host cell we pre-treated MØs with the cholesterol chelating compound Methyl-β-cyclodextrin (MβCD), and

performed a time course *Leishmania* infection. Following MβCD lipid raft disruption, GP63-dependent PTP1B and SHP-1 cleavage were markedly reduced (Figure 6A). Moreover, confocal microscopy revealed co-localization of the lipid raft marker cholera toxin B (CTxB) and GP63 in infected MØs. (Figure 6B). Consequently, MβCD pre-treatment abrogated GP63 detection in infected cells, supporting the involvement of lipid raft domains in the MØ trafficking of *Leishmania* GP63. Surprisingly, only the generation of a second cleavage product of TCPTP and a modest rescue of degradation of the first cleavage product was evident following lipid raft disruption. This suggests that another host or pathogen protease could be involved in this cleavage process.

PTP cleavage fragments are enzymatically active.

In-gel PTP activity assay of IP PTP1B, SHP-1 and TCPTP from naïve and *Leishmania* infected MØs shows that PTP cleavage fragments are enzymatically active (Figure 7A). Following *L.major* infection cleavage-induced PTP activation, measured by the capacity of protein lysates to hydrolyze the substrate p-Nitrophenyl Phosphate (pNPP), was induced by 40% above basal level. Induction of PTP activity was markedly reduced when MØs were infected with *L.major* GP63$^{-/-}$, and consistently recovered upon *L.major* GP63^{R} infection (Figure 7B). In agreement with these data, phospho-tyrosine western blot showed a strong dephosphorylation of MØ proteins when infected with *L.major* WT or GP63^{R} strain (Figure 7C). However, when infected with *L.major* GP63$^{-/-}$, PTP activation and its consequent substrate dephosphorylation were abrogated.

Using IFN-γ-primed MØs we studied the impact of GP63-dependent PTP activation on the specific JAK2 tyrosine-phosphorylation and its functional outcome. As shown in Figure 7D, JAK2 was fully dephosphorylated upon infection with GP63 expressing *Leishmania* strains. In contrast, JAK2 hyperphosphorylation was evident in *L.major* GP63$^{-/-}$ infected cells. In contrast to the silent response and downregulation of

FIGURE 6

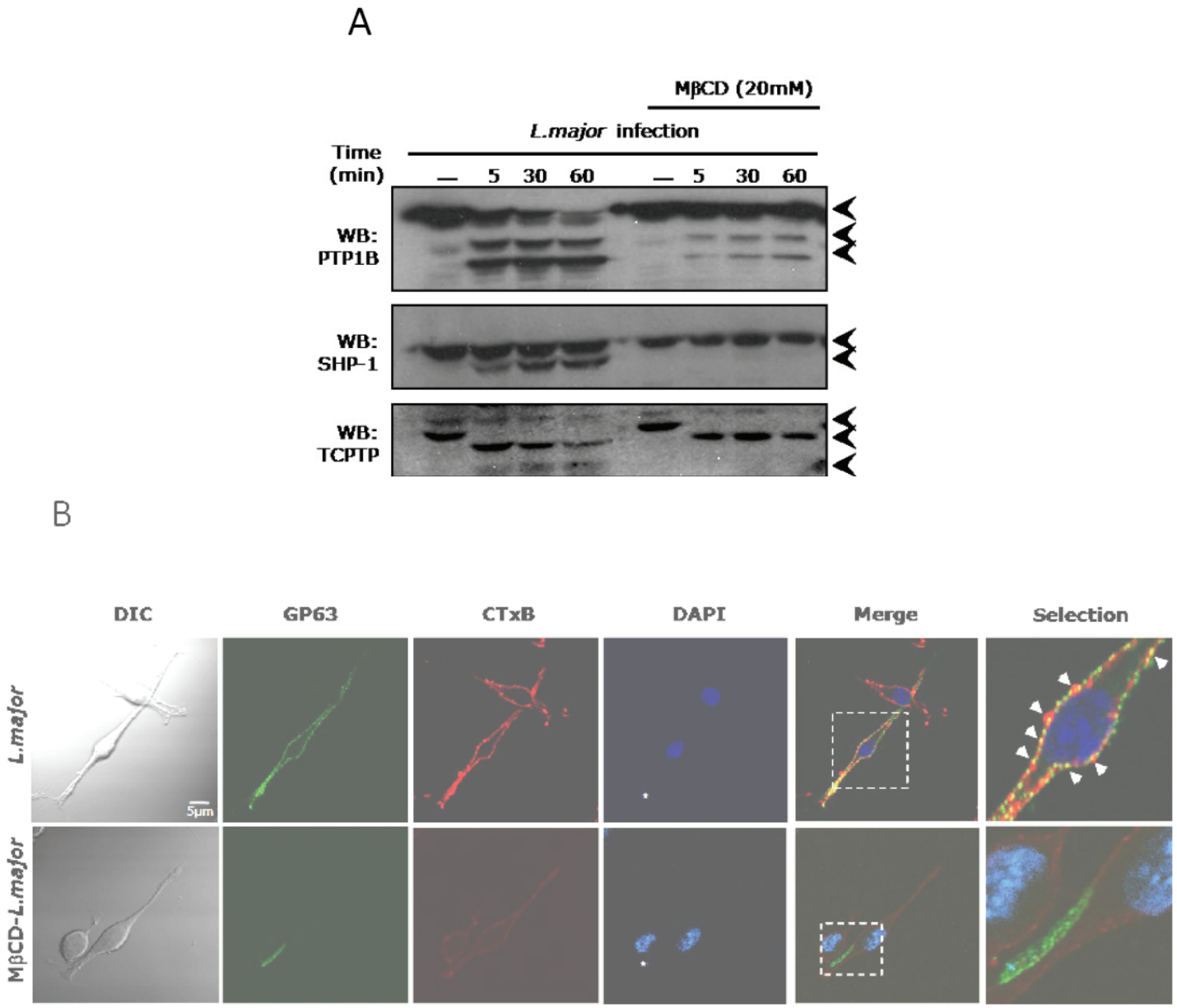

Fig 6. M*Ø lipid raft integrity is important for GP63 internalization and PTP cleavage*. **(A)** MØs were seeded in 6 well plates or **(B)** glass coverslips. Cells were pre-treated for 1h with 20mM MβCD in serum-free medium and subsequently infected. Non-internalized parasites were washed and samples were collected for **(A)** western blot or **(B)** prepared for confocal microscopy. The lipid raft marker cholera toxin B (CTxB) is shown in red, GP63 is labelled in green, and nuclei were stained with DAPI (blue). Arrows represent areas of GP63-CTxB colocalization. *indicates *Leishmania* parasites.

FIGURE 7

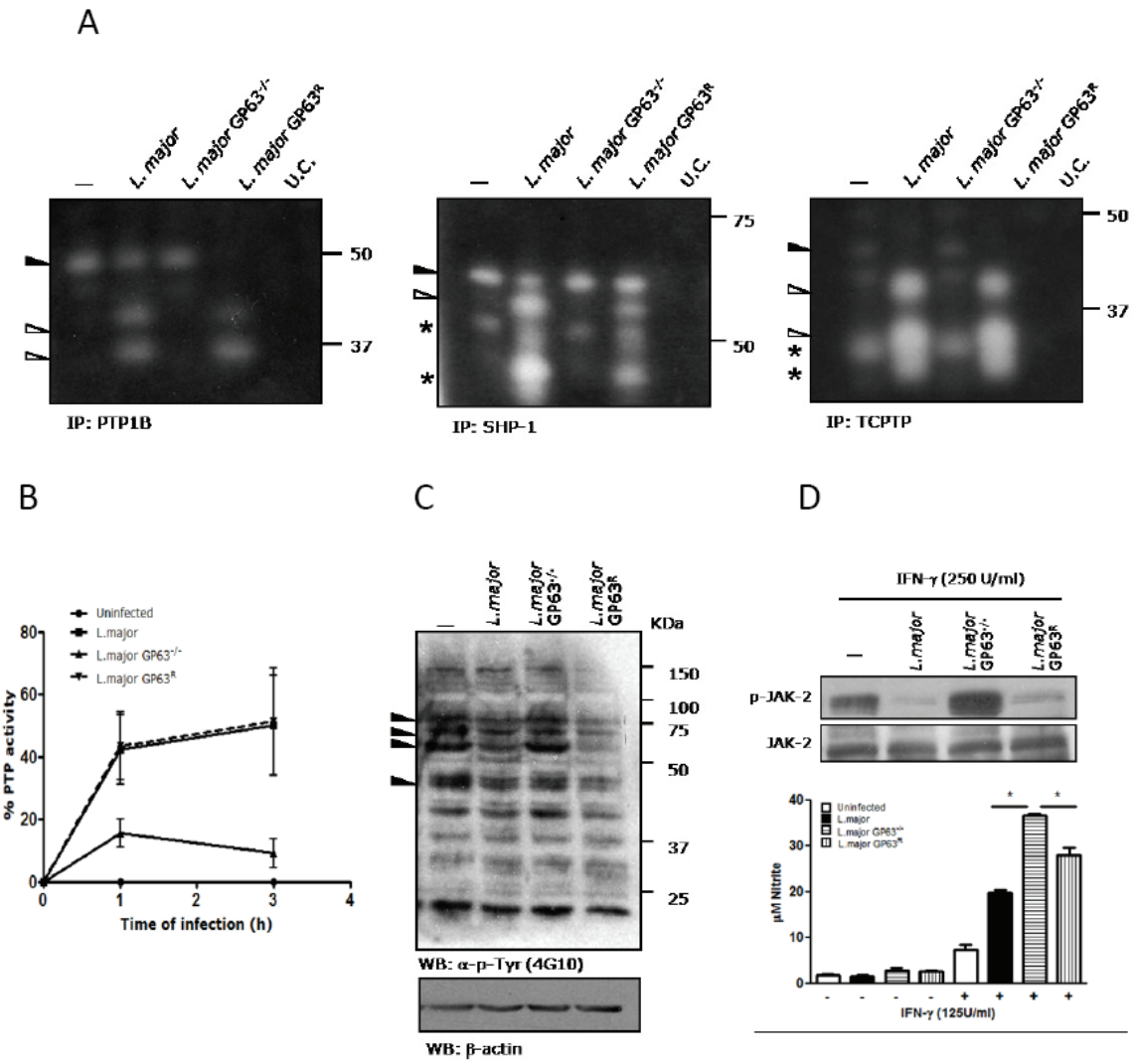

Fig 7. *PTP cleavage products are enzymatically active.* **(A)** MØs were infected for 1h. PTP1B, TCPTP and SHP-1 were IP, and immune complexes run in an in-gel PTP activity assay. White and black arrows represent full length and cleavage PTP fragments respectively. *Putative co-IP PTPs. U.C. (Unspecific Control) **(B)** Total phosphatase activity was evaluated by the capacity of cell lysates to hydrolyze pNPP. OD readings were taken at 405nm. Data are presented as percentage values ± SEM of three independent experiments done in duplicate. **(C)** Total tyrosine phosphorylation of infected or uninfected MØs was evaluated by western blot using the 4G10 anti-phosphotyrosine antibody. Loading control was performed using anti-β-actin antibody. Arrows represent modulated phospho-proteins **(D)** MØs were primed for 1h with 125 U/ml IFN-γ, followed by 1h *Leishmania* infection. JAK2 phosphorylation was evaluated. Similarly, IFN-γ primed MØs were infected for 24h and NO production quantified by nitrite accumulation in the supernatant of cultured cells using Griess reaction as previously described (bottom panel) [158]. Data are representative of three individual experiments performed in duplicate ± SEM. Statistically significant differences (*) were considered when *P<0.05.*

pro-inflammatory functions in naïve *Leishmania* infected MØs, IFN-γ primed infected cells induce NO production through TNF-α [465] and NF-κB signalling (Whitcombe and Olivier, unpublished data). In this context, *L.major* GP63$^{-/-}$ infection induces significantly higher levels of NO production compared to IFN-γ primed *L.major* or *L.major* GP63^{R} infected MØs (Figure 7D *lower panel*), as result of additional JAK/STAT signalling via JAK2 hyperphosphorylation.

PTP1B is necessary for cutaneous the development leishmaniasis.

Among the functions attributed to PTP1B are the downregulation of IFN-γ signaling [432] and MØ activation [216]. Having identified PTP1B as a novel target of *Leishmania* parasites, we evaluated its role *in vivo* in the progression of *Leishmania* infection. *L. major* infected PTP1B$^{-/-}$ showed a significant delay in the onset and progression of footpad inflammation (Figure 8A) and reduced parasite burden (Figure 8B), compared to WT mice. Significant differences were observed within the first 5 weeks post infection, suggesting that similarly to SHP-1 [277], PTP1B is required for disease progression during the initial stages of *Leishmania* infection. As we have previously reported[466], *L. major* induces inflammatory cell recruitment in a murine air pouch model. Correlating with delayed pathology progression and reduced parasite burden, basal and *L. major*-induced leukocyte recruitment in PTP1B$^{-/-}$ was, respectively, 5 and 3 fold higher than in WT mice (Figure 8C). Consistently, BMMØ from PTP1B$^{-/-}$ exhibited higher potential for activation, as seen by an increased production of NO in response to LPS (Figure 8D) and consequently, a stronger capacity to control the intracellular parasite (Figure 8E).

Collectively, these data imply a key role for PTP1B during the initial stages of *Leishmania* infection, suggesting that *Leishmania* exploits this host PTP in concert with SHP-1, promoting maximal downregulation of host cell functions for the successful establishment of infection.

FIGURE 8

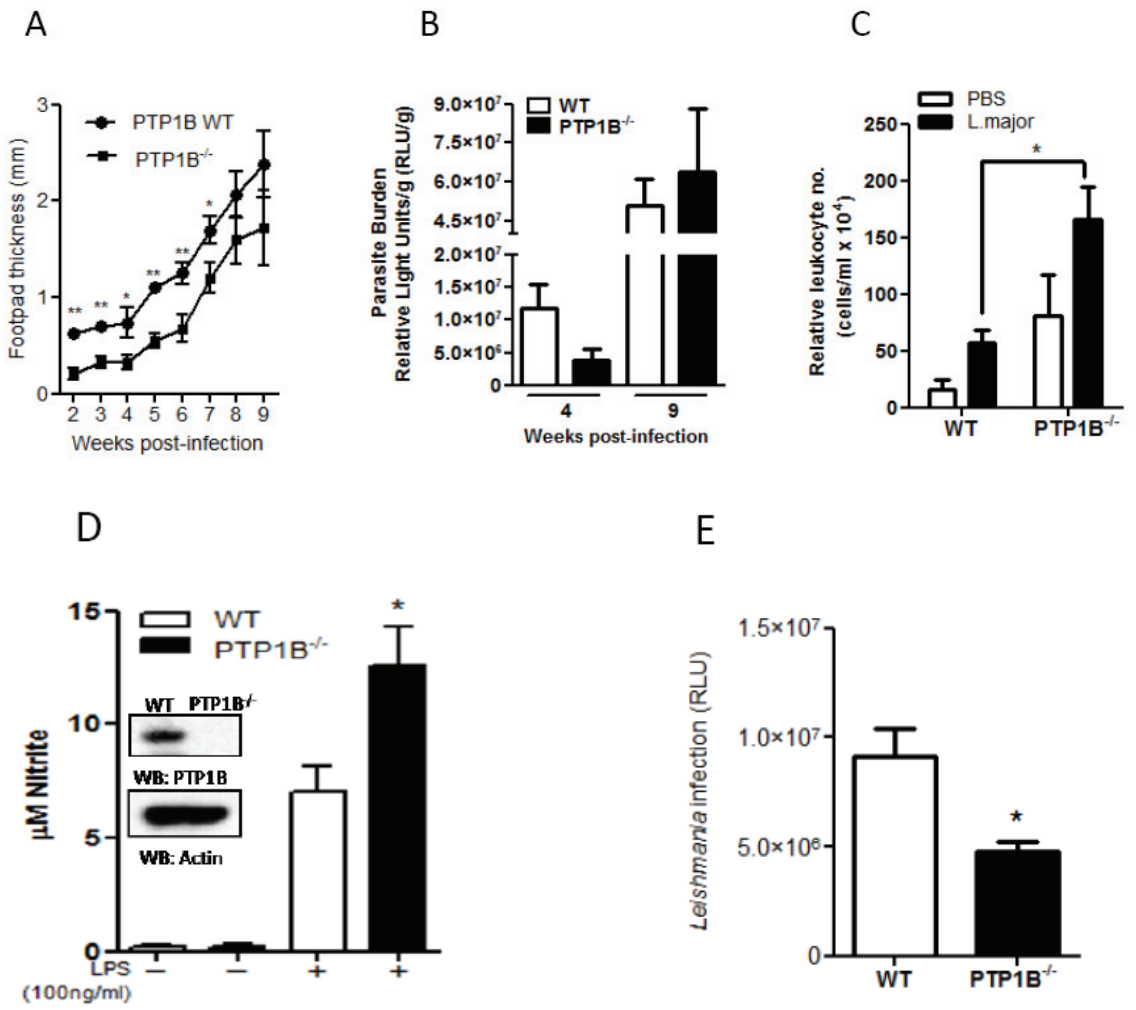

Fig 8. PTP1B is important for cutaneous lesion progression. **(A)** PTP1B$^{-/-}$ and littermate WT BALB/c control mice were infected by injection in the right hind footpad of 5 x 10^6 stationary phase L.m-LUC promastigotes. Lesion progression was evaluated by measurement of footpad swelling. Measurements are expressed as Δ between the infected footpad and the uninfected control. Results are presented as the mean $\pm$ SEM (n=7). **(B)** Parasite burden was measured by luciferase activity in the footpads after 4 or 9 weeks post-infection. Results are presented as the mean $\pm$ SEM (n=5). **(C)** *In-vivo* leukocyte recruitment was evaluated by injection of endotoxin-free PBS or *L.major* promastigotes in the air pouch of PTP1B$^{-/-}$ and WT mice. Total leukocytes were enumerated by microscope count. **(D)** BMMØs were stimulated O/N with 100ng/ml LPS or left untreated. NO production quantified by nitrite accumulation in the supernatant of cultured cells. **(E)** BMMØs were infected for 6h with stationary Ld-LUC promastigotes (10:1 Ld:MØ ratio). Cells were washed to remove non-internalized parasites. LPS (20 ng/ml) stimulation followed, after which infection was allowed to progress for 24h. Intracellular parasite survival was determined by luciferase activity after 24h of infection. Statistically significant differences (**,*) were considered when *P<0.01* and *0.05* respectively.

DISCUSSION

The balance of protein phosphorylation, maintained by the concerted action of protein tyrosine kinases (PTK) and PTPs, modulates signalling pathways fundamental to determine the outcome of multiple cellular functions. Tight regulation of PTP and PTK activities is crucial for the proper unfold of signal transduction. Results presented herein show that the *Leishmania* surface protease GP63, a key virulence factor important for lesion development [94] and initial interaction with its host cell [70], is internalized by host MØs where it interacts with, and modulates SHP-1, PTP1B and TCPTP.

Rapidly upon *Leishmania*-MØ interaction, SHP-1 activation leads to the downregulation of JAK/STAT and MAPK signalling and downstream inhibition of MØ functions including NO production [158, 159, 251, 277, 456]. To date, the exact mechanism whereby *Leishmania* parasites induce PTP activation is not completely understood. Recent reports propose the *Leishmania* elongation factor 1 alpha (EF1α) and fructose-1,6-bisphosphate aldolase as SHP-1 activators [282, 283]. However, the mechanism by which these proteins could access to, interact with and activate host PTPs remains elusive.

Previous studies, in addition to our current findings, have reported the GP63-dependent cleavage of intracellular MØ proteins including the myristoylated alanine-rich C kinase substrate (MARKS), MARKS-related protein (MRP) [96] and NF-κB p65^{RelA} subunit [97]. Herein we report the internalization of GP63 into host MØs, providing mechanistic evidence for direct GP63-host protein interactions. Co-localization of the lipid raft marker CTxB and GP63 in *Leishmania* infected MØs (Figure 7B), together with PTP1B and SHP-1 cleavage rescued by membrane cholesterol depletion with MβCD, suggest a mechanism whereby GP63 is rapidly introduced into host MØs by a lipid raft-dependent event. Given the nature of GP63 as a GPI anchored protein, we hypothesize that GP63 internalization through lipid rafts is mediated via its GPI core allowing membrane protein binding and cytoplasmic translocation. Similarly, the *Trypanosoma brucei* variant surface glycoprotein (VSG) -highly abundant GPI-anchored protein

important for parasite´s evasion of the immune response- is released and internalized by MØs. VSG internalization is mediated by a component of its GPI core, and is subsequently required for the modulation of MØ NF-κB signal transduction [258].

Surprisingly, TCPTP cleavage, although fully dependent on *Leishmania* GP63 expression, was not rescued by lipid raft disruption with MβCD. Alternative splicing of TCPTP generates a 48 KDa PTP associated to the ER through its C-terminal tail, and a 45 KDa nuclear phosphatase [467]. Given the predominance of nuclear TCPTP in our MØ system (data not shown), we suggest that ER-TCPTP will be the favoured target for direct GP63 cleavage. Conversely, nuclear TCPTP cleavage could in part result from the GP63-dependent activation of a host cell protease allowing cleavage in the absence of full GP63 internalization. However, we do not exclude the possibility that an additional lipid raft-independent mechanism of host cell GP63 import takes place, allowing for preferential TCPTP cleavage.

Caspase-dependent cleavage of PTP-PEST [227] and calpain-mediated cleavage of PTP1B and SHP-1 [219, 222] have been associated with PTP activity regulation. The rapid appearance of PTP cleavage fragments following *Leishmania*-MØ contact and the partial restoration of PTP integrity 24h post-infection (data not shown), time at which GP63 expression has dramatically decreased due to promastigote-to-amastigote differentiation, argue for an important role of PTP cleavage and activation in the initial interaction and “silent entry” of this parasite into its host cell. This is further supported by its effect on MØ phosphor-tyrosine dephosphorylation, downregulation of JAK/STAT signalling via JAK2 dephosphorylation, and downstream NO production (Figure 7B-D).

In vitro cleavage assays strongly support the identification of GP63 cleavage sites by sequence analysis in TCPTP and PTP1B. The putative interaction site of TCPTP with GP63 falls into the α1 helix of the PTP domain, important for p-Tyr substrate specificity [183]. Cleavage between residues Tyr^{32} and Pro^{33} at positions P1´and P1 respectively, avoids disruption of PTP domain motif 1 (residues 42-48), and maintains integrity of the active site and p-Tyr recognition loop, explaining the PTP enzymatic activity of the cleavage

product (Figure 7A). Previously reported, TCPTP cleavage at the extreme C-terminus greatly enhances enzymatic activity [468]. In-gel PTP activity assay (Figure 7A), shows that GP63-dependent TCPTP cleavage enhanced phosphatase activity, suggesting that the N-terminal region of the protein may also play a role in activity regulation.

Analysis of SHP-1 cleavage sites provided interesting insights into the possible roles for its GP63 cleavage-dependent regulation. The C-terminal tail of SHP-1 has been associated with the regulation of enzymatic activity. PKC-mediated phosphorylation of Ser^{591} inhibits SHP-1 activity [210], contrary to tyrosine phosphorylation at residues 536 and 564, previously shown to enhance enzymatic activation [208]. In agreement with these reports, GP63-mediated SHP-1 cleavage between residues Lys^{577} and Val^{578} would predict SHP-1 activation, as corroborated by in-gel PTP activity assay (Figure 7A), by removing the inhibitory phospho-serine site, but maintaining the activator tyrosine residues. In T cells, a portion of SHP-1 has been shown to associate with lipid rafts through its C-terminal tail where it is thought to interact with some of its substrates including Lck and ZAP70 [199]. This suggests that GP63-dependent SHP-1 cleavage, in addition to enhance its enzymatic activity, will potentially allow for interaction with alternate substrates by modifying its cellular localization. Interestingly, following an in-gel PTP activity assay of IP SHP-1 (Figure 7A), two additional PTP bands are co-IPed in *Leishmania* infected MØs, supporting an alteration in substrate specificity. In depth studies on this line are subject of our current research.

A major finding of our investigation was the *Leishmania*-dependent modulation of PTP1B and its role in the *in vivo* disease progression. Previous reports have shown that $PTP1B^{-/-}$ mice exhibit higher levels of serum IL-12, IFN-γ and MØ NO production in response to LPS [216]. Interestingly, together with NO, IL-12 and IFN-γ play a central role in the control of leishmaniasis by favouring a protective type Th1 immune response [19]. Not surprisingly, the strategic downregulation of IL-12 and NO by *Leishmania* parasites allows for its development within the host. Herein we show that the absence of PTP1B delays footpad thickening, correlating with reduced parasite burden and

higher leukocyte recruitment. Additionally, PTP1B$^{-/-}$ BMMØ presented an enhanced *Leishmania* killing capacity, paralleled by increased NO production (Figure 8). Based on our *in vivo* observations (Figure 8), the GP63-dependent modulation of PTP1B by *Leishmania* promotes a permissive environment for its initial establishment and development in the mammalian host. As observed in SHP-1 deficient mice [277], the control of disease progression takes place in the initial stages of infection. This suggests that the concerted action of both PTP1B and SHP-1 result in the *Leishmania*-induced downregulation of signalling pathways an ultimately in the development of disease. It is therefore tempting to speculate that specific targeted deletion/inhibition of both PTP1B and SHP-1 could abrogate disease development.

Collectively our data shows that in addition to SHP-1, TCPTP and PTP1B are modulated upon *Leishmania* infection in a GP63-dependent cleavage manner, and identify PTP1B as necessary for the initial stages of disease development. Interestingly, it also reports the first demonstration that a *Leishmania* surface molecule can access the MØ intracellular 'milieu' mediated in part through host lipid rafts, further supporting the importance of GP63 as a key *Leishmania* virulence factor, which favours its successful development as an obligate intracellular parasite.

METHODS

Cell culture and in vitro infections. B10R murine MØ cell line was cultured in DMEM (Gibco-BRL) supplemented with 10% heat inactivated foetal bovine serum (FBS), 100μg/ml streptomycin, 100U/ml penicillin, and 2mM L-glutamine at 37°C and 5% CO_2. *Leishmania mexicana, L. donovani* 2211*, L.major* strain stably transfected with the luciferase reporter gene (Lm-LUC) *[416], L. major, L.major* GP63 knockout -GP63$^{-/-}$- and *L. major* GP63$^{-/-}$ re-complemented (*L.major* GP63^{R}) [94] promastigotes were kept in SDM-79 medium at 25°C supplemented with 10% heat inactivated FBS and 5 mg/ml of hemin [415]. Stationary phase promastigotes were used to infect MØs in a 20:1

Leishmania-MØ ratio for various time periods. Unattached and non-internalized parasites were removed by washing the plates with phosphate buffer saline (PBS).

In vivo *infections and bone marrow derived macrophage (BMMΦ) differentiation. Ptpn1*$^{-/-}$ mice (PTP1B$^{-/-}$) [385] and WT BALB/c littermates were kept in pathogen-free housing. All animal work was carried out according with the regulations of the Canadian Council of Animal Care and approved by the McGill Animal Care Committee. 5 x 10^6 stationary phase L.m-LUC parasites were injected in the right hind footpad of 6-8 week old mice and footpad swelling was evaluated weekly for up to 10 weeks post-infection. Parasite burden was evaluated by luciferase activity in infected footpads [416]. BMMΦ were differentiated from bone marrows (BM) of 6-8 week old uninfected WT and PTP1B$^{-/-}$ mice. BMs were cultured for 5 days in DMEM containing 30% L929-cell conditioning medium (LCCM) and 10% FBS, and an additional 2 days with refreshed LCCM.

Airpouch and leukocyte migration. Air pouches were raised on the dorsum of WT and PTP1B$^{-/-}$ mice by s.c. injection of 3 ml sterile air on day 0 and 2.5 ml on day 3 as previously described [469]. At day 7, 1 ml endotoxin-free PBS containing or not 5×10^6 *L. major* stationary phase promastigotes was injected into the pouch. Six hours post-inoculation, pouches were washed with 5 ml PBS to collect leukocytes recruited into the exudate. Cells were enumerated by direct count with a hematocytometer.

Phosphatase assays. As previously described [159]**,** infected and non-infected MØs were collected, lysed in PTP lysis buffer (50mM Tris pH 7.0, 0.1mM EDTA, 0.1mM EGTA, 0.1% 2-ME, 1% Igepal, 25 µg/ml aprotinin, and 25 µg/ml leupeptin) and kept on ice 45 min. Lysates were cleared by centrifugation, and protein content determined by Bradford's method. Ten µg of protein extract were incubated in phosphatase reaction buffer (50mM Hepes pH 7.5, 0.1% β-ME, 10mM 4-Nitrophenylphosphate disodium salt hexahydrate -pNPP-) for 30 minutes. OD was read at 405nm.

In-gel PTP assay. In-gel PTP assay was performed as we and other previously

described [418, 444]. Briefly, Poly(Glu,Tyr) substrate was tyrosine phosphorylated by overnight (O/N) incubation with GST-FER protein kinase (10 μg) and 150 μCi [γ-^{32}P]dATP. The substrate was then incorporated in a 10-12% SDS-polyacrylamide gel mixture at a concentration of 2 x 10^5 CPM/ml. MØ protein extracts, prepared as described above, were denatured for SDS-PAGE and loaded onto the gel. After electrophoresis, the gel was incubated O/N in Buffer A (50mM Tris-HCl pH 8.0, 20% isopropanol), washed twice with Buffer B (50mM Tris-HCl pH 8.0, 0.3% β-ME), and followed by full protein denaturation in Buffer B containing 6M guanidine hydrochloride and 1mM EDTA. Gels were washed twice in Buffer C (50mM Tris-HCl pH 8.0, 1mM EDTA, 0.3% β-ME and 0.04% Tween 20) and final renaturation O/N in Buffer C. Gels were dried and exposed to X-ray film. Active PTPs were detected as clear bands on the film.

Immunoprecipitation (IP) and Western Blotting. Primary antibodies used were α-SHP-1 MAB1128 and α-PTP1B (Millipore), α-SHP-2, (Santa Cruz Biotechnology). α-PTEN, α-JAK-2 (Cell Signaling), α-PP2A (06-222, Upstate), α-TCPTP 3E2 was obtained as described [421] and α-GP63 monoclonal antibody clone #253. Protein IP was performed by pre-clearing 1mg of protein extract from *Leishmania* infected or uninfected MØ protein lysates with 1μg of rabbit-α-rat antibody and protein A/G beads for 1h at 4°C. Pre-cleared lysates were then incubated O/N with 3μg of α-SHP-1, α-PTP1B, α-TCPTP or α-JAK2 antibody and 2h with protein A/G beads. IPs were washed with protein lysis buffer and denatured in SDS-PAGE sample loading buffer. Western blots were performed as previously described [158].

GST-pulldown and in-vitro cleavage assay. GST-tagged full length TCPTP and SHP-1 constructs were expressed in *E.coli* BL21. Fusion proteins were isolated by 1h incubation of bacterial lysates and Glutathione-Sepharose beads (GE Healthcare) at 4°C. Fusion proteins were incubated with 3 x 10^7 *L. major, L. major* GP63$^{-/-}$*L. major* GP63^{R} or with recombinant GP63 (rGP63; 1μg) [463] for 1h at room temperature in a final volume of 200 μl. GST-tagged proteins were re-precipitated by centrifugation and denatured in SDS-sample loading buffer. PTP cleavage was evaluated by western blot.

Confocal microscopy. Cells were plated on glass cover slips and infected for 1h. MØs were washed with ice-cold PBS, fixed with 4% formaldehyde at 4°C and permeabilized for 5 minutes in PBS containing 1%BSA and 0.05% NP-40. After blocking in 5% non-fat evaporated milk in PBS, the cover slips were incubated with α-GP63 mouse monoclonal antibody clone # 96 [85]. Slides were washed with PBS and incubated with AlexaFluor 488 α-mouse antibody (Molecular Probes, Inc., Eugene, OR). 10μg/ml Cholera Toxin B (CTxB)-Alexa 594 (Molecular Probes) was used to trace lipid raft domains. DAPI (Molecular Probes) was used for nuclear stain. After mounting, cells were visualized by confocal microscopy using a Zeiss LSM 510 system.

Statistical analysis. Data were analyzed by one-way ANOVA. Statistically significant difference between groups was considered when $P < 0.05$ or $P < 0.01$. All data are presented as the mean ± standard error of the mean (SEM).

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CHAPTER 5

CONCLUSIONS AND FINAL DISCUSSION

Signalling pathways constitute the backbone of the immune response by controlling the outcome of cellular functions in response to a plethora of extracellular and intracellular stimuli. Protein phosphorylation represents the main post-translational modification influencing enzymatic activation, protein-protein interactions and protein localization, delineating in this way the course of signal transduction. To this end, the balanced action of protein kinases and protein phosphatases results essential for the sequential progression of signalling cascades and the final cellular response.

Protein phosphatases were initially considered as mere signal terminators, believed to be constitutively active proteins serving the function of reversing the activity of protein kinases. However, for the past 10 years fundamental understanding of PTPs has revealed their central function in the positive and negative regulation of signalling pathways, unravelling critical roles in the outcome of cellular functions ranging from cellular proliferation to cell death [182]. Advancements have been achieved in understanding the mechanisms that regulate PTPs. However, we are far from having a complete panorama of cellular PTP regulation, given the abundant variables influencing PTP activity which include cell type, activation state, and conditions such as infectious diseases, inflammation or cancer, to name a few. This thesis research aimed to further our understanding on the mechanisms of macrophage PTP activity regulation in the context of *Leishmania* infection. We have revealed two important mechanisms by which host and pathogen factors, independently and differently, modulate macrophage PTP activity; iron-dependent inhibition and cleavage-mediated activation of PTPs.

NRAMP-1 is a pH dependent divalent cation transporter [35] localized to the late endosome/lysosomal compartment of macrophages from the reticuloendothelial system [36]. Upon phagocytosis, NRAMP-1 is rapidly recruited to the phagolysosomal membrane where it mediates the transport of Mn^{2+}, Fe^{2+}, Co^{2+}, and potentially other metals including Zn^{2+} [38-41]. The functional expression of NRAMP-1 has been associated with host resistance to unrelated intracellular pathogens including

Leishmania, Mycobacterium and *Salmonella*, and the upregulation of pro-inflammatory macrophage functions. Although more than 10 years have passed since the positional cloning and characterization of NRAMP-1 [396, 397]**,** it still remains unclear how the divalent cation transport function of this protein contributes to, and mediates the multiple pleiotropic events associated with its functional expression. Using an *in-vitro* cell culture approach, coupled to confocal microscopy, biochemical and in-gel PTP activity assays, we have shown for the first time a mechanism linking NRAMP-1 mediated iron transport and macrophage functions.

In chapter 2, we showed that NRAMP-1 mediated transport of zinc and particularly iron inhibits macrophage PTP activity promoting protein phosphorylation and positive signal transduction, resulting in the upregulation of leishmanicidal macrophage functions [398]. Importantly, these findings open interesting questions regarding the impact of host PTP activity in the outcome of other NRAMP-1-controlled diseases such as that caused by *Mycobacterium* or *Salmonella.* Together with *Leishmania, Mycobacterium* and *Salmonella* survive as successful intracellular pathogens, in part by interfering with host cell functions and intracellular host cell environments through the modulation of signaling pathways [73, 399, 400]. Surprisingly, only two reports have addressed the impact of *Mycobacterium* infection on host PTP activity, and showed that *M. tuberculosis* lipoarabinomannan (LAM) induces SHP-1 activation, attenuating JAK/STAT signaling, TNF-α and MHC II expression [312, 314]. More intriguing, however, is the lack of investigations addressing the effect of *Salmonella* on host PTPs. Despite the known role of bacterial PTPs (SptP) on host substrates, essential for intracellular bacterial survival [288], it is unknown whether *Salmonella* infection modulates host cell signaling by altering host PTP activity. In this line of thought, it would not be surprising that the differential iron homeostasis provided by NRAMP-1 expression promotes resistance to *Salmonella* infection, as it will also conduct to the inhibition of pathogen or, alternatively, host PTPs implicated in the negative regulation of host cell functions. Studies in this direction will potentially

unravel new mechanisms underlying the early events of bacterial host-pathogen interactions, providing insights for novel therapeutic approaches.

As previously discussed, iron homeostasis is maintained by the fine balance between iron absorption, storage, utilization and export [319]. Consequently, additional models of differential iron homeostasis may provide further proof of the impact of iron on cellular PTP activity, and importantly may contribute to explain, from a signaling perspective, the various effects exerted by altered iron metabolism on the immune response [321]. Our preliminary results show that, similarly to NRAMP-1 overexpressing cells, NRAMP-2 (or DMT-1) overexpressing RAW 264.7 macrophages have lower basal PTP activity compared to wild type cells. This further corroborates our previous observations, and supports a model where augmented intracellular iron negatively regulates PTP activity. It would be interesting to validate this data in the "mk mouse"; a mouse model of severe microcytic anemia due to a spontaneous Gly185Arg loss of function mutation in the NRAMP-2 protein, impairing intestinal iron absorption and Tf-mediated cellular iron uptake [401, 402]. Conceivably, reduced intracellular iron concentration will result in increased PTP activity and downregulation of cellular functions.

Of particular relevance to our studies, is the possible correlation between iron-mediated PTP inhibition and iron-dependent control of *Leishmania* infection. It has been previously shown that parental iron dextran pre-treatment protects against *Leishmania major* infection in the highly susceptible BALB/c mice. This was correlated with inhibition of anti-inflammatory cytokines (IL-10 and IL-4) and upregulation of IFN-γ, iNOS expression and oxidative burst [366] [365, 367]. Interestingly and somehow surprisingly, this cytokine pattern and immune response balance is very similar to the effects of NRAMP-1 expression [403], suggesting that iron supplementation may functionally rescue the effect of NRAMP-1 deficiency in BALB/c mice. Our finding that iron dextran treatment modulates macrophage PTP activity is in accordance with a mechanism of iron-mediated cell function regulation by PTP-dependent modulation of

signal transduction. We have shown *in vitro* that the leishmanicidal capacity of NRAMP-1 deficient cells was recovered upon extracellular iron citrate delivery prior to infection, correlating with a partial inhibition of PTP activity [398]. Similarly, it is likely that by inhibiting macrophage PTPs and repressing the *Leishmania*-induced PTP activation, iron dextran treatment prevents the installment of infection, by abrogating the initial PTP-driven downregulation of host cell functions required for *Leishmania* survival.

The discovery that differential iron homeostasis (provided by lack of NRAMP-1, overexpression of NRAMP-1 or NRAMP-2, or parental iron-dextran administration) plays an important role in the regulation of macrophage PTP activity, led us to study the molecular mechanisms underlying the iron-mediated PTP inhibition. Using chemical and biochemical approaches, we have identified mononuclear iron-citrate complexes as the specific species responsible for PTP inhibition (Chapter 3). In support of this observation, computational modeling of PTP-iron-citrate complexes revealed a specific and competitive interaction of the mononuclear, but not binuclear complex with the catalytic pocket of SHP-1, preventing in this way the entrance of an incoming phosphate group. This observation prompted us to propose iron-citrate PTP inhibition as a novel mechanism of cellular PTP activity regulation of important biological relevance. We suggest that iron in the form of iron salt, iron-dextran or Tf-Fe^{3+}, will provide an Fe^{3+} source for the formation of intracellular mononuclear iron-citrate complexes as part of the labile iron pool (LIP), available for interaction with cellular PTPs. Although at the moment this remains speculative, the intracellular concentrations of iron -as part of the LIP- and citrate (0.4 μM - 16 μM [337, 339, 342] and 0.1 mM-0.4 mM [338], respectively), indeed suggest that mononuclear and potentially binuclear iron-citrate species are more prone to be formed than, for example, polynucleated and oligomeric complexes.

All together, these findings open interesting avenues for subsequent research in the interplay between cellular/macrophage iron homeostasis, signaling pathways and immune functions. Interesting questions arise; are NRAMP-2 deficient mice, similar to

NRAMP-1 deficient ones, more susceptible to *Leishmania* infection? Are all macrophage PTPs (classic, DUS PTPs and RPTPs) similarly regulated by mononuclear iron-citrate? Further experimentation will help us to completely understand the effects of differential iron homeostasis and the regulation of PTPs, potentially providing novel iron-based therapeutic approaches. Importantly, lessons learned from the *Leishmania* model could apply to other pathogens, helping identify novel signaling-based mechanisms involved in disease control.

As discussed throughout this thesis, *Leishmania* has evolved ingenious strategies to downregulate host cell functions which, from a signalling perspective, include alteration in Ca^{2+}, MAPK, JAK/STAT and PKC pathways, and importantly, activation of the host PTP SHP-1 [73]. Although *Leishmania*-induced SHP-1 activation has been shown to play a central role in the onset and development of leishmaniasis, the involvement of additional host PTPs and the mechanism(s) by which they are engaged following infection, remained poorly understood. In chapter 4, we have shown that in addition to SHP-1, TCPTP and particularly PTP1B play an important role in the initial stages of *Leishmania*-macrophage interaction. Moreover, we have identified a mechanism responsible for PTP activation, which involves the proteolytic cleavage of PTP1B, TCPTP and SHP-1 by the *Leishmania* surface protease GP63.

One of our most interesting observations was the lipid raft-mediated internalization of *Leishmania* GP63 into host macrophages. Contrary to the secretion systems utilized by pathogenic bacteria such as *Yersinia* or *Salmonella* (e.g. type III secretion systems) to deliver virulence factors into their host cell [404], kinetoplastids lack these so called “molecular syringes”, relying on alternate methods to directly interact with, and modulate host signalling pathways. Lipid rafts are dynamic membrane domains enriched in cholesterol, sphingolipids and GPI anchored proteins, which mediate the internalization of cargo proteins and lipids from the cell surface of mammalian cells, and act as important signalling platforms [405]. The finding that GP63 was internalized into host macrophages by lipid raft domains suggested to us the

involvement of its GPI anchor as entry mediator. Similarly, the *Trypanosoma brucei* variant surface glycoprotein (VSG) is released and internalized by macrophages. VSG internalization is mediated by a component of its GPI core, and is subsequently required for the modulation of NF-κB signal transduction [283]. Additionally, it has been recently shown that *L. major* LPG is translocated to host membranes, co-localizing with GM1 positive (lipid raft-containing) phagosomes in infected macrophages [406]. All these data suggest that other GPI-containing *Leishmania* molecules (or alternatively other pathogen molecules such as that of *Trypanosoma* or *Toxoplasma)* may be internalized into host macrophages via lipid raft domains, providing an appealing new mechanism of host-pathogen interactions.

A major finding of our investigation was the engagement of PTP1B upon *Leishmania* infection, and its role in the *in vivo* disease progression. Previous reports have shown that PTP1B$^{-/-}$ mice exhibit higher levels of serum IL-12, IFN-γ and macrophage NO production in response to LPS [241]. Similarly, TCPTP$^{-/-}$ mice display higher IL-12, IFN-γ, TNF-α and NO production, resulting however, in exacerbated inflammation and early death after birth [222]. Interestingly, together with NO, IL-12 and IFN-γ play a central role in the control of leishmaniasis by favouring a protective type Th1 immune response [23]. Not surprisingly, the strategic downregulation of IL-12 and NO by *Leishmania* parasites allows for its development within the host. In Chapter 4, we show that the absence of PTP1B delays footpad thickening, correlating with reduced parasite burden and higher leukocyte recruitment. Additionally, PTP1B$^{-/-}$ BMMØ presented an enhanced *Leishmania* killing capacity, paralleled by increased NO production. The similar cytokine profile of PTP1B$^{-/-}$ and TCPTP$^{-/-}$ suggest that TCPTP could also play a seemingly important role in the early stages of *Leishmania* infection. This suggests that the concerted action of PTP1B, SHP-1 and possibly TCPTP results in the *Leishmania*-induced downregulation of signalling pathways and ultimately in the development of disease.

Having identified TCPTP and PTP1B as additional host PTPs engaged upon *Leishmania* infection, and having provided novel insights into the mechanism of *Leishmania*-induced host PTP regulation, many new questions arise: do TCPTP, PTP1B and SHP-1 have redundant functions in downregulation of cellular functions, onset and progression of leishmaniasis? Are these PTPs similarly important for the development of the cutaneous and visceral disease? Do SHP-1, PTP1B and TCPTP physically co-exist in the same multi-protein complex upon *Leishmania* infection? Does GP63-mediated PTP cleavage alters PTP substrate specificity? Experiments in this direction will provide fruitful understanding into the delicate roles of these three critical macrophage PTPs in the context of leishmaniasis.

All together, our investigations have elucidated novel mechanisms of PTP regulation by host and parasite factors in the context of *Leishmania* infection. Early activation of PTPs via GP63-mediated cleavage promotes an environment for the successful establishment of *Leishmania* parasites inside its host cell. Conversely, long-term regulation of PTP activity mediated by an NRAMP-1/iron-dependent inhibition, ultimately contributes to the successful control of the intracellular pathogen. These results illustrate the importance of balanced signal transduction in the outcome of *Leishmania* infection, and provide insights for the development of novel control strategies.

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