The Pathological Effects of *Leishmania mexicana* Infection on Macrophage Cell Signalling and Immune Responses

A thesis presented by

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ABSTRACT

The activation of macrophages during *Leishmania* infection results in phagocytosis, activating innate cell signalling pathways and pro-inflammatory cytokines such as IL-12 and TNF-α, in addition to NO. In this study we examined the effect of *L. mexicana* amastigotes and promastigotes on macrophage intracellular signalling pathways. Infection with amastigotes abolished subsequent LPS-induced ERK and JNK phosphorylation due to CPB-mediated degradation of JNK and MEK1. In contrast, pre-incubation with promastigotes did not affect LPS-induced signalling. However, promastigotes alone stimulated MAP kinase phosphorylation including JNK, ERK and p38 MAP kinase in addition to a marked increase in NFκB activation. This effect was mimicked by using cysteine peptidase B-deficient (ΔCPB) amastigotes. Surprisingly, activation of these pathways was reduced in macrophages from TLR4- but not TLR2-deficient macrophages.

The expression of the disease regulatory proteins iNOS and COX-2 was also studied. Amastigote infection followed by LPS stimulation reduced COX-2 expression, whilst completely abolishing iNOS expression. In contrast, promastigotes enhanced LPS-induced COX-2 and iNOS expression and associated PGE_2 and NO production. Again, this effect was replicated by ΔCPB amastigotes and dependent upon TLR4. In addition, infection with promastigotes alone or in combination with LPS or IL-4 also enhanced macrophage arginase-1 expression and activity. This effect was reduced in $TLR4^{-/-}$ but not in $TLR2^{-/-}$ macrophages.

The production of the pro and/or anti-inflammatory regulatory cytokines IL-12, IL-6 and IL-10 was also studied. Both amastigotes and promastigotes inhibited LPS-induced IL-12 production in macrophages. In contrast, IL-10 production was enhanced whilst IL-6 production was not affected. This pattern was replicated using ΔCPB amastigotes. Pharmacological inhibitors to prevent either PGE_2 or NO production caused a reversal of promastigote inhibition of LPS induced IL-12 production. Interestingly, inhibition of arginase using nor-NOHA also caused a reversal of promastigote mediated inhibition of macrophage IL-12 production. Finally, the results in this thesis demonstrate key differences in the regulation of

signalling events by amastigotes and promastigotes and reveal an important role for TLR4 in promastigote responses. The results also demonstrate the potential of promastigotes to regulate the alternative macrophage pathway via the same receptor.

PUBLICATIONS

Poster presentation

Muhannad Shweash, James Alexander and Robin Plevin (2009). "Differential Effects of *Leishmania mexicana* Amastigotes and Promastigotes on Macrophage Cell Signalling Involved in Inflammation". Poster Presentation at SIPBS RESEARCH DAY, Glasgow, UK

Muhannad Shweash, H. Adrienne McGachy, Laurence C. Cadalbert, James Alexander and Robin Plevin (2009). "MAP kinase phosphatase-2 Regulates iNOS and Arginase-1 Expression in Macrophages and Protects against *Leishmania* Infection". Europhosphatases, Egmond aan Zee, The Netherlands.

Muhannad Shweash, Clare Bryant, James Alexander and Robin Plevin (2010). "Effect of Amastigotes and Promastigotes of *Leishmania mexicana* on Macrophage Signalling: Role of TLR-4", Woods Hole ImmunoParasitology (WHIP), Marine Biological Laboratory, Woods Hole, MA, USA.

Papers

Differential modulation of TLR-3 and TLR-4 mediated dendritic cell maturation and function by progesterone. Leigh A. Jones, Shrook Kreem, **Muhannad Shweash**, Andrew Paul, James Alexander and Craig W. Roberts. Journal of Immunology 2010.

Deletion of the DUSP-4 gene reveals a critical role for MAP kinase phosphatase-2 in protection against an intracellular pathogen. Mashael S Al-Mutairi, Laurence C. Cadalbert, H. Adrienne McGachy, **Muhannad Shweash**, Julianne Schroeder Magdelena K Kurnik, Callum M Sloss, Clare E. Bryant, James Alexander and Robin Plevin. PloS pathogens 2010 (in the press).

Leishmania mexicana promastigotes inhibit macrophage IL-12 production via TLR-4 dependent COX-2, iNOS and Arginase-1 expression. **Muhannad Shweash**, H. Adrienne McGachy, Clare E. Bryant, Owain Millington, Jeremy C. Mottram, James Alexander and Robin Plevin. 2010 (submitted).

DEDICATION

I would like to dedicate this thesis to my father and mother, who supported and encouraged me to achieve my ultimate goal.

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LIST OF ABBREVATIONS

Ab	Antibody
AP-1	Activating protein-1
APC	Antigen presenting cell
ASK1	Apoptosis signal-regulating kinase-1
B7-1/2	Co-stimulatory molecules
BAFF	B cell-activating factor of tumor necrosis factor family
BCG	Bacillus Calmette-Guerin
BMD	Bone marrow-derived macrophage
BMDDC	Bone marrow-derived dendritic cells
BSA	Bovine serum albumin
C3bi	Subunits of complement complex (inactive subunits)
CD	Cluster of Differentiation
c-Fos	Cellular FBJ murine osteosarcoma viral oncogene homolog
CIS	Cytokine inducible SH2-containing protein
CL	Cutaneous leishmaniasis
COX	Cyclooxygenase
CPB	Cysteine peptidase B
CR1	Complement receptor1
CRP	C-reactive protein
CXCL10	C-X-C motif chemokine 10
DC	Dendritic cell
DC-ICAM-3	DC- specific ICAM-3-grabbing nonintegrin
DENV	Dengue virus
DLK	Dual leucine kinase
DMEM	Dulbecco's modified Eagles
DN	Dominant-negative
DTT	Dithiothreitol
DUSP	Dual-specificity phosphatase
ECL	Enhanced chemiluminescence
EDRF	Endothelium-derived relaxing factor
ELAM	Endothelial-leukocyte adhesion molecule

ELISA	Enzyme-linked immunosorbent assay
Elk-1	Ets like gene1
ERK	Extracellular-regulated kinase
FcR Fc	Fragment crystallizable region receptor
FCS	Fetal calf serum
FC-γR	Fragment of crystallization gamma receptor
g	Unit of gravity
GM-CSF	Granulocyte/macrophage colony-stimulating factor
Gp63	Glycoprotein-63
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HSF-1	Heat shock transcription factor-1
HSP	Heat shock protein
ICAM	Intracellular adhesion molecule
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IKK	Inhibitory kappa-B kinase
IL	Interleukin
IL-12R	IL-12 receptor
IL-1β	Interleukin-1 β
iNOS	Inducible nitric oxide syntheses
IP	Inositol phosphates
IP-10	Interferon gamma-inducible protein-10
IRAK	IL-1R-associated kinases
IRF	Interferon regulatory factor
IκB	Inhibitory kappa-B
JAK	Janus-associated tyrosine kinases
JAP	JAK-binding protein
JNK	c-Jun N-terminal kinase
kDa	kilo-Dalton
LBP	LPS-binding protein
L-NAME	N^{ω}-nitro-L-arginine methyl ester
LPG	Lipophosphoglycan

LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
L-SIGN	C-type lectin - specific ICAM-3-grabbing nonintegrin
LTA	Lipoteichoic acid
LTbR	Lymphotoxin beta receptor
MAPK	Mitogen-activated protein kinase
MAPK/ERK	Extracellular signal-regulated kinases
MAPK/JNK	c-Jun NH2-terminal kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MCL	Mucocutaneous leishmaniasis
MCP-1	Monocyte chemotactic protein-1
M-CSF	Macrophage colony stimulating factor
MD-2	Myeloid Differentiation protein-2
MEK	MAP kinase kinase
MEKK	MAP kinase kinase kinase
MFR	Mannose-Fucose receptor
MHCII	Major histocompatibility complex class II
MIP-1	Macrophage inflammatory protein-1
MIP-1α/β	Macrophage inflammatory protein-1α/β
MLK3	Mixed lineage kinase 3
MOI	Multiplicity of infection
MR	Mannose receptor
MyD88	Myeloid Differentiation primary response gene 88
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor kappa-B
NK	Natural killer cells
NLS	Nuclear localization sequence
NO	Nitric oxide
nor-NOHA	Nω-hydroxy-nor-Arginine
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PGE_2	Prostaglandin E_2
PI3K	Phosphatidyl inositol-3 kinase

PKA	Protein kinase A
PKC	Protein kinase C
PMA	Phorbol myristate acetate
PMN	Polymorphonuclear
PRAK	The MAPK/p38-regulated/activated protein kinases
PRR	Pattern recognition receptors
PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatase
pV(phen)	Peroxovanadium
PVs	Parasitophorous vacuoles
RANTES	Regulated upon activation normal T cell expressed and presumably secreted
rpm	Revolutions per minute
SAPK	Stress-activated protein kinase
SH2	Src homology 2
SHP	Src-homology 2 domain –containing tyrosine phosphatase
siRNA	Small interfering RNA
SOCS3	Suppressor of cytokine signaling 3
Sp1	Specificity protein 1
SR	Scavenger receptor
STAT	Signal transducer and activator of transcription
TAB	Transforming TAK-1 Binding protein
TAK1	Transforming growth factor-β-activated protein kinase1
TAO1	Thousand and one amino acid
TBK1	TAK binding kinase1
TCR	T-cell Receptor
TEMED	N, N, N', N'-tetramethylenediamine
TGF	Tumor growth factor
TGF-β	Transforming growth factor-β
Th	T- helper cells
TICAM	TIR domain-containing adaptor molecule
TIR	Toll/IL-1 receptor
TIRAP	TIR domain-containing adaptor protein

TLR	Toll-Like Receptor
TNF-α	Tumor necrosis factor-α
Tpl-2	Tumor progression locus-2
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adaptor molecule
Treg	Regulatory T-cell
TRIF	TIR-domain-containing adaptor-inducing interferon-b
TSG-6	TNF-α stimulated gene-6
Tyk2	Tyrosine kinase 2
UV	Ultraviolet
VL	Visceral leishmaniasis
YopJ	*Yersinia* outer membrane protein-J

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CHAPTER 1

GENERAL INTRODUCTION

1.1 *LEISHMANIA*

1.1.1 Overview of *Leishmania*

Leishmania is a member of the protozoan flagellate group, which also includes the family Trypanosomatidae and order Kinetoplastida (Hawn et al., 2002). It was first described in 1903 by William Leishman and Charles Donovan assessing clinical features of patients with splenomegaly (Awasthi et al., 2004). This obligate intracellular parasite is now responsible for one of the most common human infections throughout the world and is of great medical importance. *Leishmania* infection leading to leishmaniasis represents a major public health problem and the burden is rising (Goto and Lindoso, 2010, Desjeux, 2001, Desjeux, 2004). The distribution of human leishmaniasis is widespread, but occurs mostly in the tropics and subtropics, with an incidence of 12 million cases overall including approximately 500 thousand cases of visceral leishmaniasis (VL) and 1.5 million cases of cutaneous leishmaniasis (CL) (Awasthi et al., 2004). In addition, *Leishmania* infection is primarily dependent on the general health and genetic make-up of the infected individual (Alexander et al., 1999). Thus, the problem of leishmaniasis is exacerbated by co-infection with human immunodeficiency virus and is becoming more and more complex in developing and industrialized countries (Southern Europe) (Desjeux, 2001, Desjeux, 2004).

To date there are over 30 species of the genus *Leishmania* and at least 10 are of medical and veterinary significance (Bates, 2006, Lainson, 2006). The *Leishmania* parasite has two morphological forms, one as a dimorphic organism that lives and multiplies in the gut of sand flies and the other as either a flagellated motile form (promastigote) or a non-motile aflagellated form (amastigote) in mammalian hosts (Laskay et al., 2003, Bogdan et al., 2000a). There are three types of leishmaniasis, each type associated with major clinical syndromes found in infected human beings. These are cutaneous, mucocutaneous and visceral leishmaniasis and can present in a wide variety of forms, depending on the parasite species. For example, members of the subgenus *Leishmania*, including *L. major, L. tropica, L. mexicana*, and members of the subgenus Viannia, (*L. braziliensis*) elicit cutaneous leishmaniasis. Mucocutaneous leishmaniasis (MCL) is caused by *L. braziliensis* and visceral

leishmaniasis by a diverse group including *L. infantum* (identical to *L. chagasi)* and *L. donovani* (Chappuis et al., 2007, Lukes et al., 2007). The demonstrated route of infection is caused by the bite of the female phlebotomine sand fly (Bates and Rogers, 2004).

1.1.2 Life cycle and pathogenesis

The life cycle of *Leishmania* **(Figure 1.1)** requires both an intermediate host and a definitive host. The parasite has two basic life cycle stages, one extracellular stage within the intermediate host, the sand fly and one intracellular stage within the definitive mammalian host (Banuls et al., 2007). Both *Phlebotomus* species in the Old World and *Lutzomyia* species in the New World can be considered as potential intermediate hosts, with all members of the animal kingdom being potentially the definitive host. Initial infection is achieved by injection of promastigotes by the sand fly bite during a blood meal, which then transforms into amastigotes, a stage which is very long-lived. When amastigotes multiply and disrupt host immune cells and infect other new cells and different tissues, dependent on the *Leishmania* species, the absence of an effective immune response can cause chronic disease.

1.1.2.1 Infection within the invertebrate host

As mentioned above, the sand fly vectors are divided into two types *Phlebotomus* of the 'Old World' and *Lutzomyia* of the 'New World' further sub-divided into 12 and 25 subgenera and species groups respectively (Killick-Kendrick, 1990a, Killick-Kendrick, 1999). Upon feeding on the blood of an infected individual or an animal source, the sand fly vector will be infected. *Leishmania* parasites are present in the infected individual living inside the macrophage in a defined stage as round, non-motile amastigotes approximately 3-7 μm in diameter, which are particularly rich in cysteine peptidases (CPs) (Mottram et al., 2004). During the blood meal, the sand fly takes up the infected macrophages and in the mid gut the amastigotes are released from the infected macrophages. The amastigotes convert rapidly into motile, elongated flagellate promastigotes of approximately 10-20μm (Killick-Kendrick, 1990b). Inside the gut of the fly promastigotes live extracellularly and multiply by simple binary fission (Guevara et al., 2001).

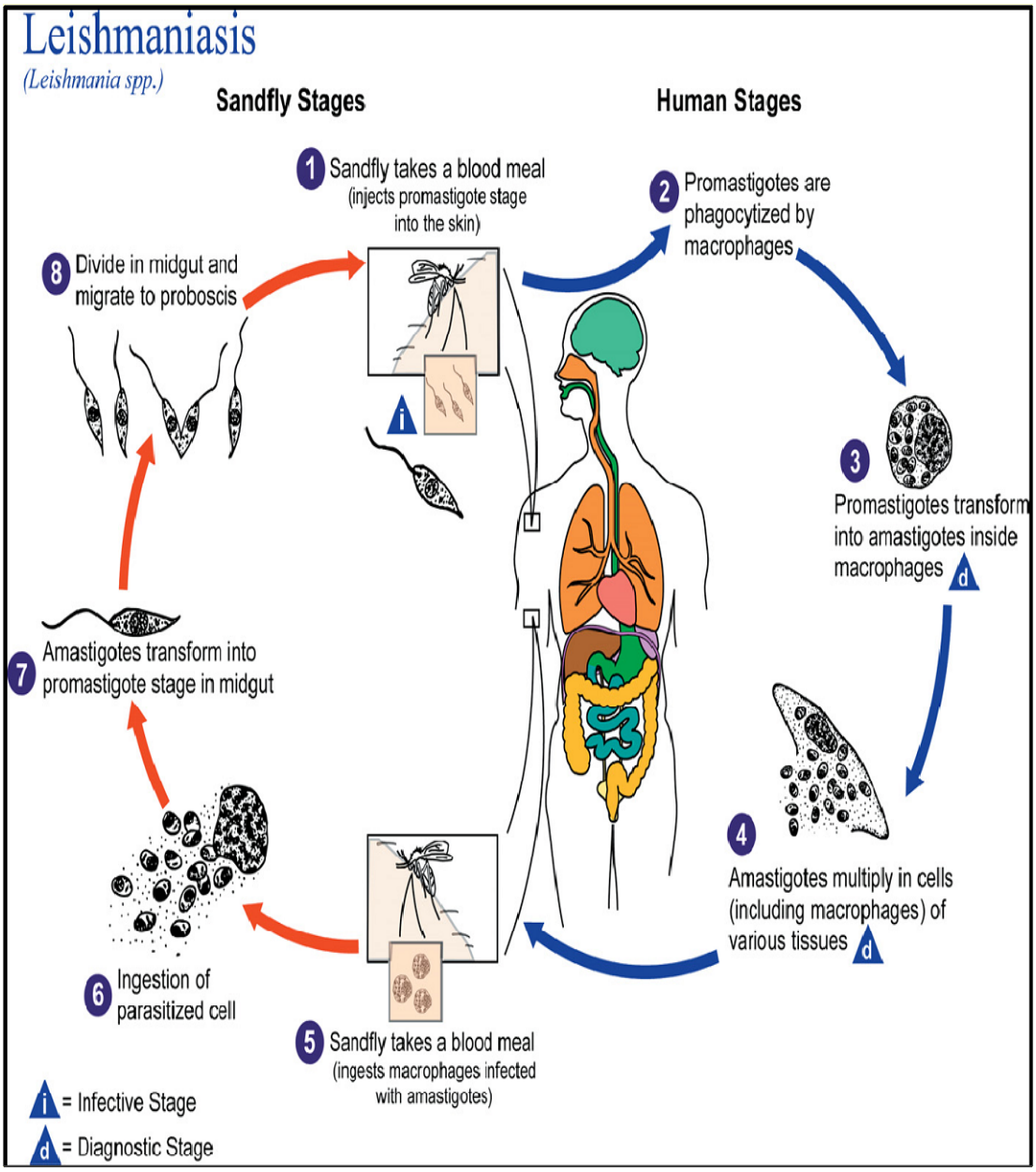


Figure 1.1: The Life Cycle of *Leishmania* species, the Causal Agents of Leishmaniasis. Adapted from (Hailu et al., 2005).

Promastigotes inside the sand fly mid gut are protected by the saliva which also is able to inhibit parasite killing by macrophages (Hall and Titus, 1995). Promastigotes themselves are protected by a glycoconjugate, lipophosphoglycan (LPG), which forms a dense glycocalyx that covers the entire surface of the parasite including the flagellum. There are two types of promastigote stages, one consisting of immature promastigotes, termed procyclics, which have shorter LPG molecules. The second stage is comprised of mature promastigotes, termed metacyclics, which have a complete LPG supported by both β-galactose and α-arabinose residues elongated by repeating disaccharide units. The mature metacyclic form is elaborated from the midgut and transfers to the proboscis. Metacyclic forms of the organism have the ability to activate the classical complement pathway, but are not lysed. Conversely, the procyclic form from log phase culture is tremendously sensitive to complement-mediated lysis through the alternative pathway (Sacks and Kamhawi, 2001).

1.1.2.2 Infection within the vertebrate (mammalian) host

Infection by *Leishmania* can be induced by promastigotes. When the individual is exposed to *Leishmania* by the sand fly bite, the infection will be initiated by promastigotes. These organisms establish parasitophorous vacuoles (PVs) within phagocytic cells such as macrophages and directly transform into amastigotes over a period of 24-72 hours. Subsequently, in infected cells or organs, the amastigote form is sustained during infection and can persist within cells for many days or weeks (Kima, 2007). More interestingly, every organ containing macrophages and phagocytic cells is infected, mainly the liver, spleen and bone marrow.

When the sand fly feeds on a mammalian host its proboscis pierces the skin. The sand fly saliva contains anti-coagulant, anti-platelet and vasodilatory components to increase the haemorrhagic pool and prevent the blood from clotting. This facilitates the transfer of *Leishmania* promastigotes into the host along with the saliva (Valenzuela et al., 2001). Once internalized, promastigotes transform into intracellular amastigotes within the macrophages, where they shrink their flagella and become oval, non-motile amastigotes. The parasite replicates in the macrophage and ultimately bursts free, the released amastigotes are taken up by new macrophages and

therefore the life cycle continues. Once a new insect bites the infected vertebrate host, it ingests the infected macrophage, the parasites differentiate into promastigotes and the sand fly is ready to infect another vertebrate host (Chang et al., 2003)

1.2 INTERACTION BETWEEN *LEISHMANIA* AND THE IMMUNE SYSTEM

Following systemic access *Leishmania* interacts with a number of cell types in addition to the host macrophage. Therefore, there are a host of cell specific events through which *Leishmania* mediates its actions. Infection impinges on the interactions between different cell types activated as part of both the innate and adaptive immune system. With regard to *Leishmania* infection three main cell types interact, the macrophage, the dendritic cell and the T cell (**see Figure 1.2**). Therefore, prior to understanding the signalling mechanisms involved in regulating the effects of *Leishmania* it is first of all essential to present a brief overview of how *Leishmania* interacts with each cell type.

1.2.1 Interaction between *Leishmania* and macrophages

As indicated above the main cell type with which *Leishmania* interacts is the macrophage and the pathogen uses a wide variety of sophisticated mechanisms to access the macrophages and to modulate immune responses. Firstly, complement component 3 (C3b) and complement receptor type 3 (C3bi) molecules found on the cell surface of *Leishmania* permit the binding with macrophage complement receptor 1 (CR1) and CR3, respectively. However, since C3b is rapidly converted to C3bi by surface glycoprotein-63 (gp63), it appears that CR3 is the more important receptor, and interaction with CR1 is only transient (Kane and Mosser, 2000, Guy and Belosevic, 1993). Also, promastigotes can attach to the macrophage via the Mannose-fucose receptor (MFR), which binds to mannose residues of *Leishmania* LPG. LPG can interact with C-reactive protein (CRP), an early inflammatory product and thus it might trigger phagocytosis via this receptor without leading to macrophage activation that is usually observed following CRP receptor-mediated phagocytosis (Bodman-

Smith et al., 2002). Since Toll like receptors (TLRs) are present on the surface of macrophages it is likely that *Leishmania* may also bind to TLRs to regulate parasite and host interactions but the mechanisms involved are not yet fully described (Awasthi et al., 2004). Recently, upregulation and stimulation of TLR-2 by *L. major* lipophosphoglycan has been observed in human NK cells (Becker et al., 2003). The interactions between the pathogens and TLRs will be discussed further in section 1.3.3.2.

Once *Leishmania* is inside the macrophage within the parasitophorous vacuole, it tries to escape the host immune defense and survive by developing different strategies to inhibit several macrophage responses which function to destroy the pathogen. This can be linked to the life cycle of the parasite. As mentioned above amastigotes express a number of cysteine peptidases (CPA, CPB and CPC) which play an essential role in the intracellular interactions between host and parasite and are directly linked to virulence of infection (Mottram et al., 2004). However, other species lack these enzymes but are still able to subvert the macrophage response. This includes modulation of phagocytosis, lack of nitric oxide (NO) generation and interleukin-12 (IL-12) production and inhibition of expression of major histocompatibility complex class II (MHC II) (Belkaid et al., 1998, De Souza Leao et al., 1995, von Stebut et al., 1998, Antoine et al., 1999, Kima et al., 1996), which normally links macrophage activation to the cell mediated immune response involving T cells (**see Figure 1.2**). These effects are mediated by interactions with key intracellular signalling pathways such as nuclear factor κ B, the MAP kinases and the JAK/STATs. The interaction of *Leishmania* species with these pathways will be discussed further in sections 1.4.1, 1.4.2. and 1.7.1.

In addition to inhibiting the functions of the host macrophage, *Leishmania,* again through regulation of intracellular signalling, can induce the production and/or secretion of various immunosuppressive signalling molecules such as the cytokines TGF-β and IL-10 which distorts the normal immune response, favoring parasite survival. *Leishmania*-infected macrophages produce IL-10 via interaction with the FC gamma receptor. Its production is responsible for the suppression of macrophage microbicidal activity via suppression of IL-1β, IL-12, and TNF-α release and also inhibition of co-stimulatory molecule expression, such as B7-1/2 reviewed by

(Cunningham, 2002). Other intermediates such as prostaglandin E_2 (PGE_2) are also generated by *Leishmania* and have been reported to inhibit macrophage proliferation in addition to suppression of TNF-α, IL-1 and reactive oxygen intermediate release (Belley and Chadee, 1995). Overall, it is clear that *Leishmania* parasites are capable of modulating numerous macrophage functions in order to promote survival (Matte et al., 2001).

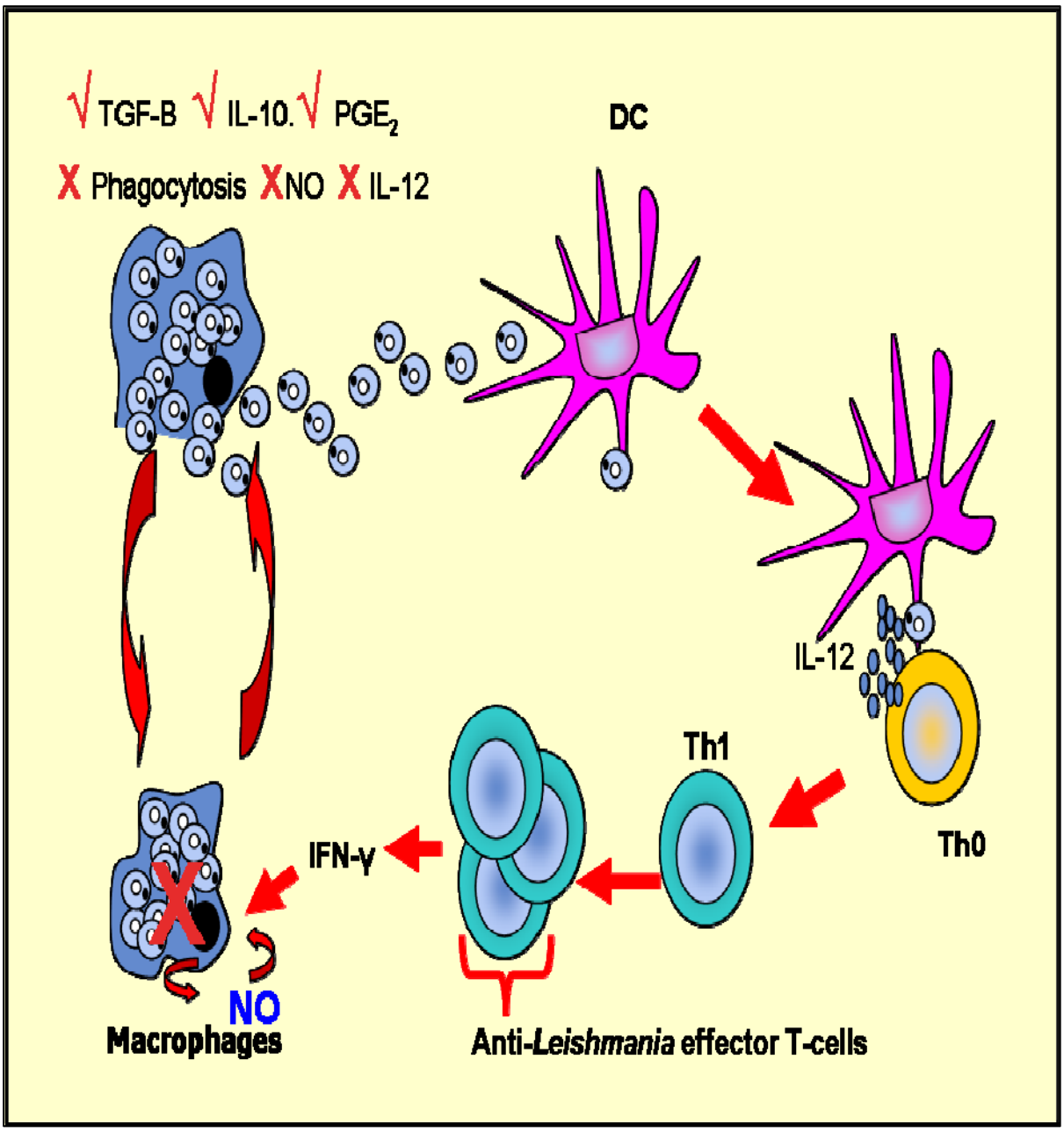


Figure 1.2: The cells and cytokines involved in inducing protective immunity against *Leishmania* infection. Macrophage and dendritic cells play a crucial role in innate immunity which happens through the infection of these cells with a pathogen. Classical activation requires IFN-γ. MΦ and DC produce IL-12 which results in induction of adaptive immunity by driving naïve T cells towards Th1. This results in IFN-γ production. In macrophages IFN-γ induces the production of NO, which leads to destruction of the parasites.

1.2.2 Interaction between *Leishmania* and dendritic cells

Dendritic cells (DCs) are another important host cell for *Leishmania* and are implicated in parasite distribution (Moll, 1993, Moll et al., 1993, Moll et al., 1995). DCs are derived from different origins such as lymphoid, myeloid and plasmacytoid (Shortman and Liu, 2002) and form a family of leukocytes that play critical roles in the innate and adaptive immune systems dependent upon their stage of maturity. As such, they are specialised in the recognition of pathogens through pattern recognition receptors (see sections 1.3.3.1 and 1.3.3.2), a process that can lead to their activation. Also they ensure the transport of the antigens they capture from the peripheral tissues to the draining secondary lymphoid organs and they are the only antigen-presenting cells able to prime naive specific T lymphocytes (Banchereau et al., 2000).

Leishmania amastigotes enter dendritic cells irrespective of their maturation state, characterized as silent entry. Interestingly, after *L. major* infection of C57BL/6 resistant mice with a low parasite dose into a dermal site, a prolonged silent phase of parasite amplification in the skin, lasting 4–5 weeks, is observed before the onset of lesions and immunity (Belkaid et al., 2000). Remaining in the immature stage means that DCs are unable to elicit an efficient anti-*Leishmania* T cell response. Delayed, incomplete or even the absence of DC maturation may therefore be beneficial for parasites, allowing their establishment and amplification before the onset of the immune response. However, infected dendritic cells can be induced to undergo maturation in response to exogenous stimuli and this would suggest that the parasite effect on the cell is mostly localised (Bennett et al., 2001, Brandonisio et al., 2004, Prina et al., 2004). It has been shown that the uptake of antibody-opsonised amastigotes results in the induction of dendritic cell maturation (Colmenares et al., 2004).

Amastigotes avoid the activation of dendritic cells, not by releasing virulence factors that deactivate host cell processes, but by selective interaction with a surface receptor that does not result in cell activation. C-type lectin and ICAM-3-grabbing non-integrin (DC-SIGN) are recognised as putative receptors on dendritic cells for *L. pifanoi* and *L. infantum* amastigotes. These molecules are parasite species-restricted to

some extent, since another C-type lectin L-SIGN has been shown to be a receptor for *L. infantum* but not *L. pifanoi* (Colmenares et al., 2002). DCs undergo dramatic phenotypic changes as a result of *Leishmania* uptake, resulting in the redistribution of peptide-loaded MHC II molecules to the cell surface, up-regulation of co-stimulatory molecules, expression of IL-12 and downregulation of phagocytosis (McDowell et al., 2002, Bennett et al., 2001).

Once inside the DC, *Leishmania* again avoids the generation of an effective immune response. This is primarily through the regulation of IL-12 release and function, which may vary according to DC subtype and to *Leishmania* species. Murine C57BL/6 skin-derived DCs were recognised as the main source of IL-12p40 immediately after dermotropic *L. major* infection (von Stebut et al., 1998). Also, after viscerotropic *Leishmania donovani* infection in BALB/c mice, splenic DCs that are localised in the periarteriolar lymphoid sheath were found to be the critical source of early IL12p40 production (Gorak et al., 1998). In contrast, infection of murine BMD DCs with *L. mexicana* promastigotes failed not only to induce IL-12 release, but also to activate immature DCs. Moreover, it was found that resting murine and human myeloid cells, including DCs, contain preformed, membrane-associated IL-12p70 stores, which are released within minutes after contact with *L. donovani* either *in vitro* or *in vivo* (Quinones et al., 2000).

1.2.3 T cell responses & interaction with *Leishmania*

During the generation of an adaptive response, immunological information is passed from DCs to naïve $CD4^+$ T helper cells in draining lymph nodes through DC- specific ICAM-3-grabbing nonintegrin (DC-SIGN) (Steinman, 1998, Moll, 2003, van Kooyk and Geijtenbeek, 2003). T cells play a major role in generating specific and memory T-cell responses to *Leishmania* infection. Th1 and Th2 cells can be distinguished by the cytokines they secrete; Th1 cells secrete activators of cell-mediated immunity such as IFN-γ, while Th2 cells secrete cytokines, such as IL-4, which promote antibody responses. The Th1/Th2 paradigm of resistance or susceptibility to intracellular infection is largely based on investigations using *L. major*. T-cell differentiation either to Th1- or Th2-type effector cells depends chiefly on priming

during differentiation. IL-4 induces Th2 whereas IL-12 induces Th1 differentiation (Tripathi et al., 2007b, Tripathi et al., 2007a). Resistant mice depleted of IL-12 by genetic means or antibody neutralization become susceptible to *L. major*, while BALB/c mice treated with IL-12 develop a Th1 response and resistance (Heinzel et al., 1993, Mattner et al., 1996). Also, it has been shown that upon infection with *L. major*, mice of the resistant phenotype develop a dominant Th1 phenotype of immune response to the parasite antigens, whereas BALB/c mice develop a typical Th2 response (Tripathi et al., 2007b).

It should be noted that most strains of mice (C57BL/6, C3H, CBA) develop a self-limiting cutaneous disease when infected with *L. major*, driven principally by a Th1 response. In these mice, resolution of infection is mediated by Th1 cells that produce IFN-γ (Tripathi et al., 2007b). In macrophages that harbor *L. major* IFN-γ induces the production of nitric oxide (NO), which leads to destruction of the parasite (Bogdan et al., 2000b). In contrast, treatment of non-healing cutaneous leishmaniasis, for example *L. mexicana* with IFN-γ, results in a rapid and complete resolution of lesions compensating for the lack of IL-12 production by macrophages and an inability to activate Th1 differentiation (Kolde et al., 1996). Ultimately, Th1 cells are responsible for the elimination of *L. major* from the host organism.

Overall these studies have clearly identified the potential of *Leishmania* species to interrupt the normal functioning of macrophages, dendritic and T cells. These effects are by and large mediated through interaction with a number of intracellular signalling pathways, although understanding of such interactions is at present incomplete. However, a framework to enhance the knowledge of mechanisms by which *Leishmania* regulates major signalling events, can be achieved by examining signalling events in macrophages stimulated by a wide range of infectious agents.

1.3 MACROPHAGE ACTIVATION PATHWAYS

1.3.1 Types of macrophage activation

Recently there has been much interest in determining macrophage heterogeneity following activation during infection (Gordon, 2007, Gordon and Taylor, 2005, Edwards et al., 2006). Basically, there are four types of macrophage activation, each defined by different cellular activators and functional properties. These are innate activated macrophages, classical activated macrophages, alternatively activated macrophages and Type-II-activated macrophages. The formation of classically activated macrophages is dependent on participation of two signals, priming by the Th1 cytokine, interferon gamma (IFN-γ) followed by synergistic activation with LPS (via the TLRs) or TNF-α (Ma et al., 2003, Chen et al., 2003, Mosser and Zhang, 2008, Battistini et al., 2002). Markers of activation include enhanced NO production, upregulation of MHC II, expression of co-stimulatory molecules and increased inflammatory cytokine and inflammatory mediator production (Mosser and Zhang, 2008).

In comparison, Type-II-activated macrophage cells are pretreated with IFN-γ and then stimulated with both LPS and immune complexes (Anderson and Mosser, 2002b, Edwards et al., 2006). This results in the increased ability to regulate cytokine production, immune complexes engage the Fcγ receptor (FcγR) leading to inhibition of IL-12 production and strong induction of IL-10. Consequently, when these cells link with naïve T cells for presenting the antigen, they respond to the secretion of Th2 cytokines by producing high levels of IL-4 (Gerber and Mosser, 2001, Anderson and Mosser, 2002a).

Stein et al. was the first person to describe the third type of macrophage activation following stimulation with IL-4, termed the alternative activated pathway by Gordon and colleagues (Stein et al., 1992). The activation of the alternative pathway is associated with the stimulation of macrophages by Th2 related cytokines such as IL- 4 or 13 (Wynn, 2003, Gordon, 2003). Recently it was reported that this type of activation is related to increased susceptibility to intracellular infections (Holscher et al., 2006). Alterations in arginine metabolism is considered a major feature of this

activation and exposure of cells to IL-4 induces arginase enzyme expression which converts arginine to ornithine, a precursor of polyamines and collagen (Hesse et al., 2001).

In addition to the key cytokines outlined above, there are several other agents which regulate the activity status of the macrophage. These include, IL-10 (Bogdan et al., 1991), Prostaglandins E_2 (PGE_2) (Matte et al., 2001) and Transforming Growth Factor (TGF)-β (Werner et al., 2000). For example, overproduction of IL-10 blocks off the production of the p40 subunit of IL-12/23. Consequently, delineating regulatory macrophage responses is dependent on the measurement of these cytokines (Mosser and Zhang, 2008). **(Figure 1.3)**. Prostaglandin E_2 (PGE_2) plays a crucial role in the regulation of both pro-inflammatory and anti-inflammatory cytokines in macrophages. For example, IL-10 and IL-6 levels are upregulated by PGE_2 and this response is mediated by p38 MAP kinase activity (Williams et al., 2000). PGE_2 also abrogates the production of Th1 cytokines such as IL-2, IL-12 and IFN-γ, whilst enhancing secretion of Th2 cytokines including IL-4 and IL-5 (Betz and Fox, 1991). Consequently, a more recent study by Shibata et al, has demonstrated that PGE_2 formation from activated splenic macrophages treated with heat-killed BCG shows a shift in the immune response from Th1 to Th2 activation (Shibata et al., 2005).

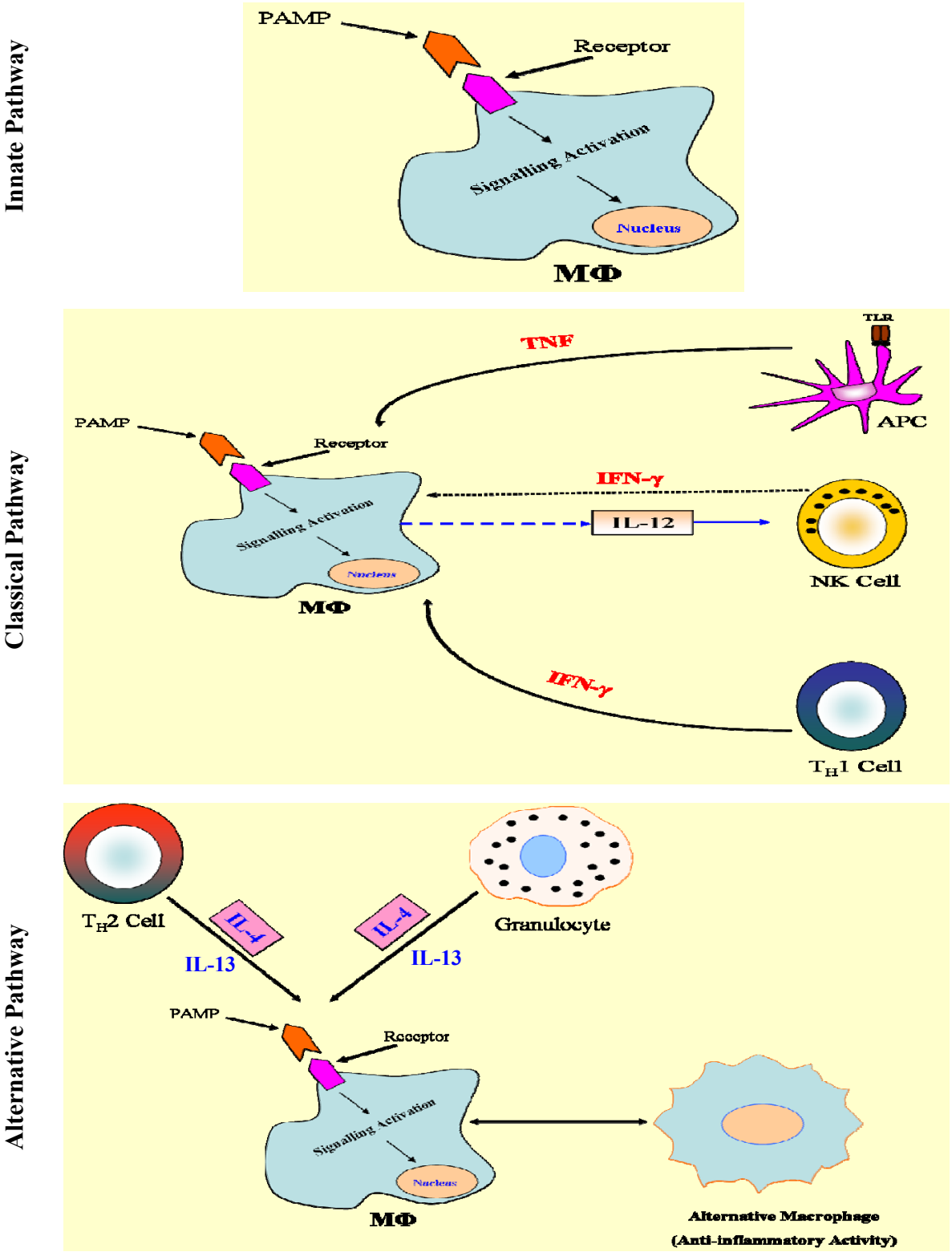


Figure 1.3: Types of macrophages activity. Innate activation happens through stimulation of macrophage with a pathogen-associated molecular patterns (PAMP), such as LPS. In cooperation with a PAMP or a TNF-α inducing mediator, classical activation requires IFN-γ. Macrophage exposure to a Th2-associated cytokines, such as IL-4, results in induction of alternative activation.

1.3.2 The innate immune response and macrophage activation

1.3.2.1 Recognition of pathogen

Macrophages are key cells in recognition of pathogens as a part of the innate immune response (Taylor et al., 2005). They have the ability to recognize a wide variety of bacteria, viruses, parasites and fungi (Buckland, 2001), through specific structures located on the cell membrane of pathogens called pathogen-associated molecular patterns (PAMPs). These recognition structures trigger innate immune responses in macrophages and dendritic cells (DCs) through the expression of a number of pattern recognition receptors (PRRs) on their cell surface. Specific intracellular signalling pathways are initiated directly after engagement of pattern recognition receptors (PRRs) with their microbial ligands (PAMPs), and thus enhance direct protection from pathogens by the host cell. There are a number of cellular systems which mediate such a defense including activation of the complement pathway, phagocytosis of microbes, the release of direct anti-microbial mediators such as NO through iNOS (Ando et al., 2000) and production of cytokines and chemokines, including type I interferons (IFN-α or -β) (Rakoff-Nahoum and Medzhitov, 2009). Additional features also include enhanced antigen presentation, upregulation of co-stimulatory molecules on the surface of antigen presenting cells (APC) and maturation of DCs that leads to activation of adaptive immunity (Werling et al., 2009, Pasare and Medzhitov, 2005, Shimura et al., 2005, Kawai and Akira, 2005). PRRs that mediate pathogen ligand recognition (Gordon, 2002), include the TLRs, the type 3 complement receptor (CR3) (Ross, 2000), the scavenger receptors (SR) (Peiser et al., 2002), dectin-1 (Brown et al., 2002) and the mannose receptor (MR) (Zamze et al., 2002).

1.3.2.2 Toll Like Receptors (TLRs)

TLRs are a major group of cell surface receptors which contribute to the activation of the innate immune response (Liu and Zhao, 2007, Ivashkiv, 2008). TLRs are able to recognize a number of pathogens including Gram positive and negative bacteria, double stranded DNA and RNA **(Table 1.1)**. To date there are 11 TLRs in humans, whilst some 13 TLRs have been recognized in mice (Akira and Takeda, 2004). There

are two major structures in TLRs which allow the receptor to respond to invading pathogens. The first one responsible for the direct recognition of PAMPs is an extracellular domain composed of amino-terminal leucine-rich repeats, whilst the second is the intracellular carboxy-terminal Toll–interleukin-1 (IL-1) receptor (TIR) domain. Transduction of the receptor signal initiated after binding of the ligand, leads to the recruitment of adaptor molecules followed by activation of key intracellular signalling pathways and the regulation of transcriptional activity (Takeda et al., 2003). This results in the expression of pro-inflammatory cytokines as well as the functional maturation of antigen presenting cells (Akira et al., 2006, Lee and Iwasaki, 2007). However, recent studies have shown that TLRs also play a crucial role in the adaptive immune response and activate T cells through their function as co-stimulatory receptors that complement T cell receptor activation to enhance proliferation, survival and cytokine production (Cottalorda et al., 2009, Liu and Zhao, 2007).

Whilst normally a single PRR recognises a single PAMP specifically, a number of studies have demonstrated that several PAMPs can activate TLR4. These include; lipopolysaccharide (LPS) from Gram-negative bacteria (see below), fusion (F) protein from respiratory syncytial virus, the envelope protein from mouse mammary tumor virus (Kurt-Jones et al., 2000, Rassa et al., 2002), heat-shock proteins, hyaluronic acid and β-defensin 2 (Ohashi et al., 2000, Biragyn et al., 2002). Lipopolysaccharide (LPS), one of the best studied immunostimulatory components, is an important structural component of the outer membrane of Gram-negative bacteria (Freudenberg et al., 2008). LPS stimulation promotes the induction of a strong pro-inflammatory response through interaction with TLR4, resulting in systemic inflammation and sepsis (Beutler and Rietschel, 2003, Beutler, 2000). The structure of LPS consists of three parts: lipid A, a core oligosaccharide, and an O side chain (Raetz and Whitfield, 2002, Miller et al., 2005). The main part that induces immunological responses following engagement of LPS with TLR4 is lipid A. It represents the preserved molecular pattern of LPS (Park et al., 2009).

Receptors	Location	Ligand	Origin of Ligand
TLR1	Cell surface	-Triacyl lipopeptides - Soluble factors	-Bacteria & mycobacteria -*Neisseria eningitidis*
TLR2	Cell surface	-Lipoprotein/lipopeptides -Peptidoglycan -Lipoteichoic acid -Phenol-soluble modulin -Glycoinositolphosp -Glycoinositolphospholipids -Glycolipids -Porins -Atypical lipopolysaccharide -Atypical lipopolysaccharide -Zymosan - Heat-shock protein 70*	-Various pathogens -Gram-positive bacteria -Gram-positive bacteria - Mycobacteria -*Staphylococcus epidermidis* - *Trypanosoma cruzi* -*Treponema maltophilum,* - *Neisseria* -*Leptospira interrogans* *Porphyromonas gingivalis* -Fungi -Host
TLR3	Cell compartment	-Double-strand RNA	-Viruses
TLR4	Cell surface	-Lipopolysaccharide -Taxol -Fusion protein Respiratory syncytial -Envelope protein -Heat-shock protein 60* -Heat-shock protein 70* -Type III repeat extra domain A of fibronectin* -Oligosaccharides of hyaluronic acid* -Polysaccharide fragments of heparan sulphate* -Fibrinogen*	-Gram-negative bacteria -Plants -Respiratory syncytial virus -Mouse mammary-tumour virus -*Chlamydia pneumoniae* -Host -Host -Host -Host -Host
TLR5	Cell surface	-Flagellin	-Bacteria
TLR6	Cell surface	-Diacyl lipopeptides -Lipoteichoic acid -Zymosan	-*Mycoplasma* -Gram-positive bacteria -Fungi
TLR7	Cell compartment	-Imidazoquinoline -Loxoribine -Bropirimine -Single-stranded RNA	-Synthetic compounds -Synthetic compounds -Synthetic compounds -Viruses
TLR8	Cell compartment	-Imidazoquinoline -Single-stranded RNA	-Synthetic compounds -Viruses
TLR9	Cell compartment	-CpG DNA -dsDNA -hemozoin	-bacteria and vial -viruses -Plasmodium
TLR10	Cell surface	Unknown (only in human)	Unknown
TLR11	Cell surface	-Profilin-like protein -Uropathogenic bacteria	-*Toxoplasma gondi* -Unknown
TLR12	Unknown	Unknown (only in mice)	Unknown
TLR13	Unknown	Unknown (only in mice)	Unknown

Table 1.1: Toll like receptors (TLRs) and their ligands Adapted from (Akira and Takeda, 2004, Beutler, 2009).

As mentioned above the recognition of LPS by TLR4 leads to dimerization and recruitment of downstream adaptors through interaction with the TIR (Toll-interleukin-1 receptor) domain. A previous study has shown the importance of TIR for TLR4 mediated signal transduction, because mutation of the TIR domain can prevent the signalling response to LPS (Poltorak et al., 1998). To date, there are five types of TIR domain-containing adaptor proteins identified reviewed in; (O'Neill and Bowie, 2007) and TLR4 is unique in function as the only one which has the ability to utilize all these adaptors. Conversely, other TLRs use different combinations of adaptor proteins to mediate downstream signalling reviewed in; (Lu et al., 2008). There are a series of interactions leading to stimulation of mammalian cells with LPS, involving several interacting proteins including the LPS binding protein (LBP), CD14, MD-2 and TLR4 itself (Gioannini and Weiss, 2007, Miyake, 2007). LPS firstly binds to LBP, since LPS does not bind directly with TLR4. The LBP works as a soluble shuttle protein connecting LPS and CD14. CD14 itself lacks the ability to transfer the signal from the cell surface to the nucleus as it is without a transmembrane domain (Akira, 2003). Consequently, for pro-inflammatory responses to be activated, CD14 associates with TLR4 in conjunction with the extracellular adaptor protein MD-2 (Palsson-McDermott and O'Neill, 2004), which is unique to TLR4 signalling (Shimazu et al., 1999).

The binding of TLR4 by LPS triggers each of the major MAP kinases, extracellular signal-regulated protein kinase (ERK), c-Jun amino-terminal kinases (JNK) and p38 MAP kinases (p38 MAP) and also NF-κB; both pathways are crucial for cell survival and controlling the expression of immune mediators (Symons et al., 2006). There are two main signalling cascades related to TLR4, the Myeloid Differentiation Factor 88 (MyD88)-dependent and MyD88-independent pathways, which couple to MAP kinases and NF-κB, linking TLR4 to gene production (as summarised in **Figure 1.4**). Following the phosphorylation and activation of TNF Receptor Associated Factor (TRAF)-6, in the MyD88-dependent pathway, TRAF6 creates a link between IRAK-1 and Transforming Growth Factor-β-Activated Kinase (TAK)-1, Transforming TAK1-Binding Protein (TAB)-1, TAB2 and TAB3 (Lomaga et al., 1999, Gohda et al., 2004). Upon phosphorylation of TAK1 and TAB2, IRAK1 dissociates from the complex, allowing the activation of two distinct downstream signalling pathways, one involving

the mitogen-activated protein kinases and the other involving the IκB kinase (IKK) complex (Sato et al., 2005, Chau et al., 2008, Karin and Ben-Neriah, 2000). Degradation of the complex upon the subsequent phosphorylation of a set of inhibitory binding proteins (IκB-α), leads to translocation of nuclear factor-κB (NF-κB) to the nucleus. However, activation of both transcription factors activation NF-κB and (AP)-1, in addition to MAPK is critical to activate pro-inflammatory cytokine production (Lu et al., 2008, Kawai and Akira, 2007).

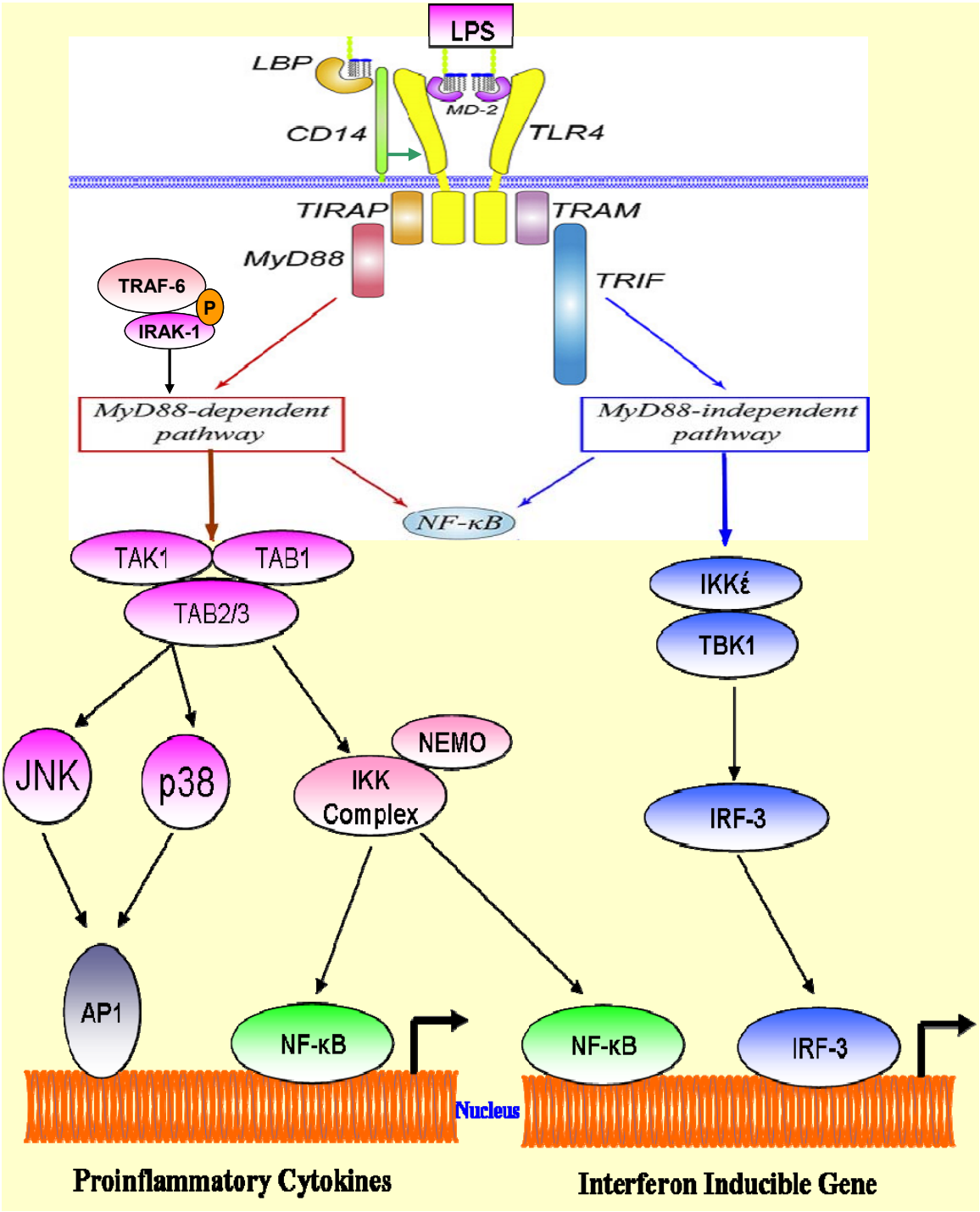


Figure 1.4: LPS and TLR4 signalling pathway. LPS recognition is facilitated by LBP and CD14, and is mediated by the TLR4/MD-2 receptor complex. LPS/TLR4 signalling can be separated into MyD88-dependent and MyD88-independent pathways, which mediate the activation of pro-inflammatory cytokine and Type I interferon genes. Modified from (Lu et al., 2008).

1.4 NF-κB and MAP Kinases as Intermediates of classical activation in macrophages

1.4.1 NF-κB pathway

The NF-κB-Rel family is composed of five different members, NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and C-Rel. They all share an approximately 300–amino acid NH_2-terminal Rel homology domain that contains sequences essential for dimerization, DNA binding, and nuclear transport (Ghosh et al., 1998). In resting cells these proteins are present as inactive complexes sequestered in the cytoplasm by binding to the protein, inhibitory κB (IκB-α). Following stimulation NF-κB dissociates from IκB-α and translocates to the nucleus whereupon it binds to specific transcription factor binding sites and regulates transcription. There are two major pathways involved in NF-κB activation, the classical (or canonical) pathway and the alternative pathway. Classical activation is mediated by a variety of inflammatory agents, including LPS and TNF-α and is dependent upon the activation of inhibitory κB kinases (IKK), in particular IKKβ. The alternative pathway is activated by LTbR, BAFF and CD40L and involves the nuclear translocation of p52–RelB dimers, with IKKα playing a central role. The mechanism of the activation of the alternative pathway is linked to the expression of genes involved in development and maintenance of secondary lymphoid organs (Ghosh and Karin, 2002). (**Figure 1.5**)

1.4.1.1 The function of the NF-κB pathway

The generation of an innate immune response through activation of NF-κB is important in mediating a largely non-specific resistance to many different viral, bacterial and parasitic infections, but is also essential in the initiation of the adaptive immune response. As part of this response a number of key molecules are induced in a NF-κB dependent manner. These include chemokines such as IL-8, macrophage inflammatory protein alpha (MIP-1alpha), regulated upon activation normal T cell expressed and presumably secreted (RANTES), monokine induced by interferon-γ (MIG), monocyte chemoattractant protein-1 (MCP-1), interferon-inducible protein-10

(IP-10) and CXCL10 (Wong et al., 2005, Yeruva et al., 2008) , cytokines such IL-1β, IFN-γ, IL-12 and TNF-α (Cardozo et al., 2001, Al-Mohanna et al., 2002, Baumann et al., 2005, Kang et al., 2005, Malmgaard et al., 2000), the adhesion molecules, intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and endothelial-leukocyte adhesion molecule-1(ELAM) and enzymes that produce secondary inflammatory mediators and inhibitors of apoptosis (Ghosh et al., 1998). Studies using an NF-κB antagonist in human B lymphocytes and plasma cells demonstrated that the activation of NF-κB is an important molecular mechanism for constitutive expression of VCAM-1 and ICAM-1 (Xia et al., 2001). Recently it has been reported that the NF-κB dependent pathway is more critical for the expression of VCAM-1 in LPS- stimulated lymphatic endothelial cells (Sawa et al., 2008). These molecules play a crucial role in enhancing the innate immune response against invading pathogens and are required for migration of inflammatory and phagocytic cells into tissues.

The NF-κB pathway is activated within different cell types dependent on the infectious agent. For example, NF-κB is activated by exposure of intestinal epithelial cells to entero-invasive bacteria, resulting in the production of (MCP-1), IL-8, TNF-α, ICAM and cyclooxygenase 2 (COX-2) (Elewaut et al., 1999). Similarly following infection with *Shigella* the causative agent of diarrhea, NF-κB activity is rapidly enhanced and may play a role in the pathogenesis of the condition (Dyer et al., 1993). In a model of intracellular protozoan infection, *Theileria parva* induces activation of NF-κB via the degradation of IκB-α in T cells. This activation is required to prevent apoptosis in *T. parva*-transformed T cells, by conveying protection against an apoptotic signal that accompanies parasite-mediated transformation (Heussler et al., 1999).

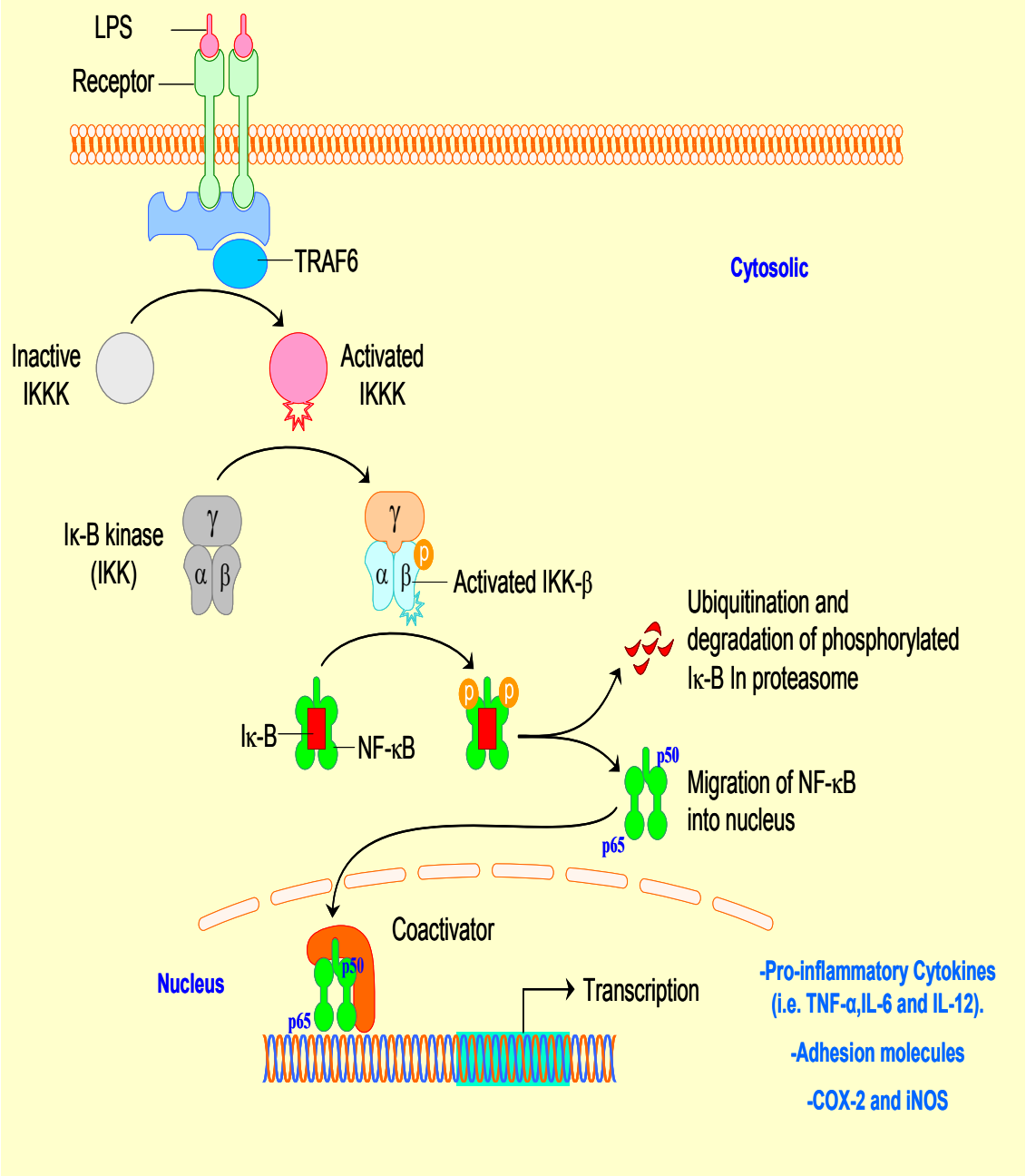


Figure 1.5: Activation of NF-κB by LPS: The classical NF-κB pathway is activated by a variety of inflammatory signals like (LPS) resulting in direct expression of multiple inflammatory and innate immune genes.

The protozoan parasite *Trypanosoma cruzi* in endothelial cells also activates NF-κB, resulting in the increased expression of adhesion molecules (Huang et al., 1999). Another parasite, *Toxoplasma gondii* has the ability to induce NF-κB activation and plays a crucial role in protection against apoptosis through the induction of antiapoptotic gene expression (Molestina et al., 2003). In contrast, a previous study by Shapira et al., demonstrates that infection of macrophages and fibroblasts with *Toxoplasma gondii* did not induce NF-κB activation but inhibits LPS induced NF-κB activation (Shapira et al., 2002). The infection of pulmonary epithelial cells by *Mycobacterium tuberculosis* leads to the activation of the non-canonical pathway, which results in the release of IL-1β and the subsequent activation of the classical NF-κB pathway in neighboring cells (Wickremasinghe et al., 1999). These studies clearly indicate that NF-κB is part of a central pathway responding to virtually any infectious agent.

A number of studies have revealed the importance of NF-κB subunits in mediating innate immune responses. Analysis of macrophages from Rel $B^{-/-}$ mice demonstrates a lack of ability to produce TNF-α but show normal levels of IL-10 and IL-12 (Caamano et al., 1999). However, a compensatory overproduction of granulocyte–macrophage colony-stimulating factor (GM-CSF) and IL-6 is observed. Similarly, there is a reduced capacity to produce TNF-α from macrophages isolated from c-$Rel^{-/-}$ mice (Grigoriadis et al., 1996). Mice deficient in p52 ($p52^{-/-}$) exhibit a specific impairment in CD40-induced IL-12 production (Speirs et al., 2002). Macrophages from c-Rel-deficient mice also exhibit a marked reduction in NO and TNF-α production and parasite killing compared to macrophages from wild-type mice (Grigoriadis et al., 1996), which correlates with a study by Gerondakis et al, showing increased susceptibility to *L. major* and enhanced parasite replication (Gerondakis et al., 1996). Also, c-Rel has been shown to play a significant role in the regulation of other cytokine genes in macrophages, especially IL-12p40, in response to infection (Mason et al., 2002, Sanjabi et al., 2000, Gri et al., 1998).

Infectious agents negatively target the NF-κB pathway in a diverse number of ways. The pathogen *Yersinia pestis* avoids detection by the innate immune system by inducing macrophage apoptosis through injection of YopJ, a protein that prevents IKK activation (Orth et al., 2000). It has been reported that infection of macrophages

by *Toxoplasma gondii* results in degradation of IκB-α but does not lead to nuclear translocation of NF-κB (Shapira et al., 2002). In addition, *Mycobacterium ulcerans*, which also inhibits NF-κB activation, may act either by blocking phosphorylation of RelA and subsequent nuclear translocation or by directly interfering with NF-κB DNA binding itself (Pahlevan et al., 1999). A recent study by Cameron et al, has also shown that infecting mouse bone marrow derived macrophages with *Leishmania mexicana* amastigotes inhibits LPS-induced IL-12 production consistent with a previous study (Cameron et al., 2004, Weinheber et al., 1998). This effect is associated with a time-dependent degradation of IκB-α, IκB-β and NF-κB. Interestingly, the same study has shown that the effect of the amastigotes is mediated by the expression of cysteine peptidase B (CPB). Similarly, another study has shown that *L. major* amastigotes induces the degradation of NF-κB in phorbol myristate acetate (PMA)-activated human cells (Guizani-Tabbane et al., 2004). However, *L. donovani* infection does not result in IκBα degradation (Prive and Descoteaux, 2000) indicating that different *Leishmania* species do not necessarily have the same effect on the NF-κB pathway. Overall infectious agents both activate the NF-κB pathway and also have the capacity to subvert such a pathway both depending on the survival advantage.

1.4.2 The mitogen-activated protein kinase (MAPK) pathway

The MAPKs are a family of serine/threonine protein kinases that play an important role in a number of pathophysiological responses including proliferation, differentiation, apoptosis, inflammation and immunity see review by (Dong et al., 2002) **(Figure 1.6).** They are activated by phosphorylation on threonine (Thr) and tyrosine (Tyr) residues within the activation loop by dual-specificity MAP kinase kinases (MKKs). MKKs are in turn activated by phosphorylation on their Ser / Thr residues by MAP kinase kinase kinases (MKKKs). To date, 7 MKKs and 14 MKKKs have been identified (Dong et al., 2002). On the basis of the differences in structure and function, the MAPK family can be divided into three groups: extracellular signal-regulated protein kinase (ERK), p38 MAP kinase and Jun N-terminus kinase (JNK) (Johnson and Lapadat, 2002).

The first MAP kinase to be an identified and characterised as a vital mediator of a number of cellular fates including growth, proliferation, and survival is ERK (Ray and Sturgill, 1988, Yoon and Seger, 2006). There are two ERK isoforms that are ubiquitously expressed, ERK1 and ERK2 (Boulton et al., 1991), encoding proteins of 44 and 42 kDa, which have 85% amino acid identity (Chen et al., 2001). A number of ligands, such as serum, growth factors, cytokines, certain stresses, transforming agents and cellular perturbations, are capable of stimulating their phosphorylation and activation within numerous cell types (Lewis et al., 1998). In this system the Raf protein, which functions as a MAPKKKs activates MEK1 and MEK2. ERK1/2 is activated by dual phosphorylation of Tyr and Thr residues, of the Thr-Glu-Tyr motif, within the activation loop (Payne et al., 1991). Activated ERK1/2 exert its effect through a family of transcription factors known as activating protein (AP-1) which includes c-Jun and c-Fos (Todd et al., 1999, Pearson et al., 2001).

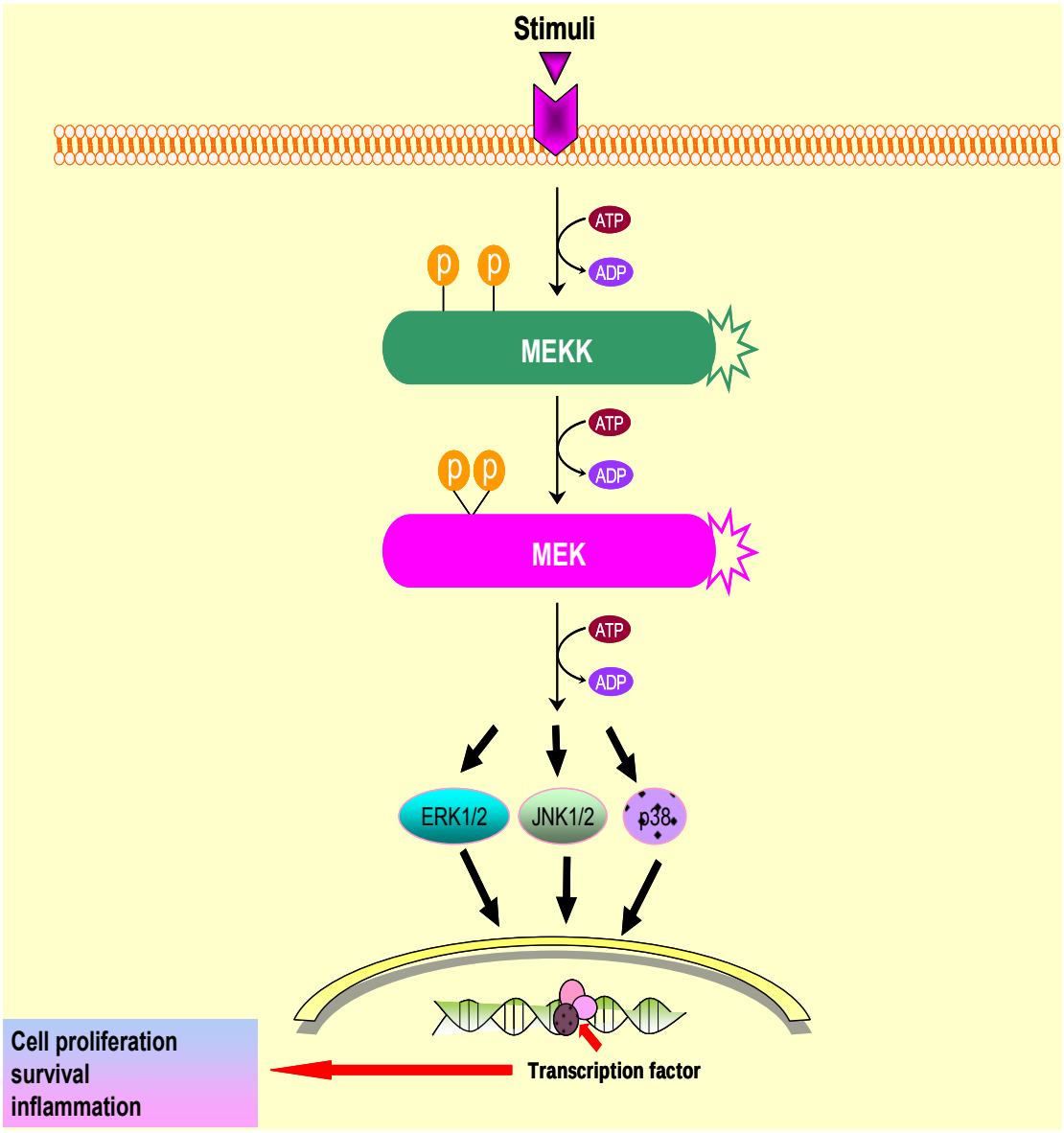


Figure 1.6: Mitogen-activated protein kinase pathway.

A second group of MAP kinases was discovered and cloned while studying intracellular signalling pathways in response to inflammation and stress responses (Han et al., 1994, Lee et al., 1994, Rouse et al., 1994). To date, four isomers of p38 MAP kinase have been identified sharing 60% sequence identity with one another and 40-45% with other MAP kinase family members reviewed in (Kyriakis and Avruch, 2001). The expression of p38 isoforms varies, whilst p38α and p38β are ubiquitously expressed p38γ is predominant in skeletal muscle, whereas p38δ gene expression is highest in lungs, kidneys, testis, pancreas, and small intestine (Hommes et al., 2003).

Like ERK, p38 MAP kinases are primarily activated by a kinase cascade which includes firstly a MAPKKK, such as TAK1, ASK1 and MLK3, followed by a MKK, either MKK3 and MKK6 (Derijard et al., 1995, Enslen et al., 2000). There are a vast number of p38 substrates that have been found including other protein kinases, transcription factors such as ATF1/2, Elk-1, Sap-1, Est-1 and p53, and other proteins (Kyriakis and Avruch, 2001). Phosphorylation of these substrates is essential for executing the biological functions of p38 MAP kinases, including the regulation of the cell cycle, cellular maturation, differentiation, senescence, tumorigenesis, apoptosis, and immune responses (see review by (Ono and Han, 2000).

The third member of the family JNK, was originally identified as stress-activated protein kinases (SAPKs) in the livers of cycloheximide-challenged rats (Kyriakis and Avruch, 1990). The mammalian JNKs are encoded by three distinct genes, *Jnk1*, *Jnk2*, and *Jnk3,* and vary in size from 46 kDa to 55 kDa (Barr and Bogoyevitch, 2001). While JNK1 and JNK2 are expressed in a variety of tissues, JNK3 expression is restricted primarily to the brain, heart, and testes (Martin et al., 1996). They are strongly activated by a variety of stress stimuli such as cytokines, protein synthesis inhibitors, UV irradiation, growth factor deprivation, DNA-damaging agents, serum, and growth factors (Kyriakis and Avruch, 2001).

JNK is also activated upon dual phosphorylation on Thr and Tyr residues separated by a proline residue, within the activation loop by either MKK4 or MKK7 (Holland et al., 1997, Yamauchi et al., 1999). MKKs are themselves activated by dual phosphorylation, by MEKKs 1-4, MLKs 2 and 3, Tpl-2, DLK, TAO1 and 2, TAK1,

and ASKs 1 and 2 reviewed in (Kyriakis and Avruch, 2001, Davis, 2000). JNK-mediated phosphorylation of the transcription factors ATF-2, NF-ATc1, HSF-1, and STAT3 (Chen et al., 2001, Kyriakis and Avruch, 2001) regulates processes such as inflammation, differentiation, apoptosis and insulin resistance (Barr and Bogoyevitch, 2001).

Evidence accumulated over the last two decades has demonstrated important roles for the MAP kinases in the function of immune cells, particularly in response to infection. Originally, it was shown that LPS stimulated, with differential kinetics, the activation of ERK, JNK, and p38 MAP kinases in macrophages (Pang et al., 1995, Cuenda et al., 1995). More recent studies have demonstrated that MAP kinases, in particular ERK1/2 and p38, play a crucial role in maturation of dendritic cells. For example, bone marrow-derived DCs infected with parasites of *Rhipicephalus sanguineus* resulted in impaired maturation of DCs stimulated with LPS and down-modulation of the host immune response due to inhibition of ERK1/2 and p38 MAP kinase (Oliveira et al., 2010).

Furthermore, MAP Kinases have been documented as having an important role in T helper cell regulation, activation, differentiation and cytotoxicity function (reviewed by (Dong et al., 2002). Using isoform specific JNK knockout mice, Constant et al., showed that the negative regulation of Th2 cytokine production by JNK1 was essential for generating Th1 polarized immunity against intracellular pathogens (Constant et al., 2000). Furthermore, loss of JNK1 activity has been shown to drive insensitivity of myeloid cells to certain microbes and reduced ability to generate IL-17 production. Moreover, JNK1-deficiency prevented the response between central nervous system myeloid cells and effector T cells, leading to progression to neuroinflammation (Tran et al., 2006). In contrast, the activation of ERK pathway has been shown to be important in Th2 differentiation and IL-4 receptor function. Jorritsma et al., found that reducing ERK activity, either by compromising the TCR signal or by using a pharmacological inhibitor, induced early IL-4 expression in naïve CD4+ T cells and subsequent Th2 differentiation. In contrast, in dominant-negative ERK transgenic T cells Th2 differentiation was impaired (Jorritsma et al., 2003, Yamashita et al., 1999). Moreover, activation of p38 MAP kinase has been implicated

in both Th1 and Th2 cell differentiation, p38 MAP kinase is selectively activated in Th1 effector cells but not in Th2 cells. This selective activation plays a significant role in IFN-γ production, which is one of the features that define Th1 cells (Rincon et al., 1998, Cook et al., 2007). T cells from mice deficient in MKK3, an upstream regulator of p38 MAP kinase, have a defect in IFN-γ production (Lu et al., 1999). Overall these and other studies indicate an essential requirement for the MAP kinases in both innate and adaptive immune responses.

1.4.3 Targeting of MAP Kinases by intracellular pathogens to subvert macrophage function

As with the NF-κB pathway there are several strategies developed by invading pathogens to either activate or subvert the MAP kinase pathway, depending on the circumstances, to allow escape from macrophage microbicidal effector function and disruption of the innate inflammatory response (Olivier et al., 2005). Martiny et al., demonstrated that *L. amazonensis* amastigote infection resulted in the dephosphorylation of ERK1, however it was not determined whether the loss in phosphorylation was the result of selective degradation of ERK or the action of a phosphatase activated by infection (Martiny et al., 1999). Similarly, another study using amastigotes from *L. donovani* showed that infection attenuated ERK in macrophages, this deactivation was mediated by activating cellular phosphotyrosine phosphatases (Nandan et al., 1999). Feng et al., also found that infection of macrophages with *L. donovani* lipophosphoglycans abolished LPS induced IL-12 p40, this inhibition was mediated by ERK activation (Feng et al., 1999). It was also demonstrated that *Leishmania major* acted by inhibiting p38 MAP kinase to inhibit macrophage iNOS induction and NO production, thus promoting parasite survival (Awasthi et al., 2003). More recently, *L. mexicana* amastigotes have been shown to cause degradation of both JNK and ERK in macrophages (Cameron et al., 2004). The same study demonstrated, using deletion mutant parasites for CPB as well as inhibitors, that cysteine peptidase B was responsible. In contrast, Yang et al., has reported that infection with *L. amazonensis* amastigotes resulted in the activation of ERK which enabled the parasite to induce secretion of IL-10, an anti-inflammatory

cytokine (Yang et al., 2007). The importance of MAPK kinases in *Leismania* infection was also illustrated in JNK1-deficient mice; they exhibited an exaggerated Th2 response which led to greatly exacerbated disease and failure to heal skin lesions following *Leishmania* infection (Constant et al., 2000). In addition, it was shown using JNK2$^{-/-}$ mice, that this isoform was required for IFN-γ production known to be important for eliminating *Leismania* infection (Dong et al., 2001).

Additional studies aimed at evaluating the effect of *Leishmania* infection on macrophage signalling showed divergent effects on tyrosine phosphorylation of different proteins (Martiny et al., 1999, Love et al., 1998, Martiny et al., 1996). Data from the study of *Yersinia,* has revealed rapid negative modulation of host cell tyrosine phosphorylation through bacteria-encoded YopH PTP (Bliska et al., 1991). This early finding suggested that enhanced tyrosine phosphatase activity mediated by members of the PTP family was one of the main contributors to the effects of *Leishmania* upon its host. In support of this idea, the PTP inhibitor, peroxovanadium (Posner et al., 1994), has been found to have the ability to arrest the progression of murine visceral and cutaneous leishmaniasis *in vitro* and *in vivo,* through the up-regulation of nitric oxide production (Olivier et al., 1998). These data suggested that induction of one or more PTP activities is an important factor in the pathogenicity of this parasite.

More recent studies have implicated the cytosolic SH-2 domain-containing PTP, SHP-1, an enzyme which is abundantly expressed in macrophages, in the negative regulation of many activation and growth-promoting hemopoietic signalling cascades (Kozlowski et al., 1998, Pani et al., 1996, Klingmuller et al., 1995). A study conducted by Blanchette et al., has shown that *L. donovani* promastigotes rapidly triggered host SHP-1 activity simultaneous with dephosphorylation of macrophage JAK2 and inhibition of protein tyrosine kinase (PTK) activity (Blanchette et al., 1999). Furthermore in SHP-1$^{-/-}$ macrophages ERK and AP-1 activation were enhanced and this resulted increased NO production. The consequence of these findings have been addressed *in vivo*, increased NO generation and a reduction in parasite load was revealed in both SHP-1-deficient mice and wild type mice treated with PTP inhibitors (Blanchette et al., 2007, Forget et al., 2006, Matte et al., 2000). In

contrast to these studies, more recent work by Spath et al., demonstrated that SHP-1 deficiency did not alter intracellular survival of *L. major in vitro* or *in vivo* (Spath et al., 2008). These studies show the potential for *Leishmania* to negatively regulate PTP upstream in both the MAP kinase and JAK/STAT pathways (see sections 1.4.3 and 1.7.2 for JAK/STAT signalling), but the outcomes for infection are not necessarily consistent.

1.5 The Cyclooxygenase enzyme and prostaglandin production

Macrophages are a major source of bio-active lipids released during inflammation. Of these the prostaglandins are amongst the best-studied mediators, produced by arachidonic acid metabolism (Wilborn et al., 1995). This pathway is regulated by the enzyme cyclooxygenase (COX) of which there are three known isoforms COX-1, COX-2, and COX-3 (Chandrasekharan et al., 2002, Houliston et al., 2001). COX-1 is ubiquitously and constitutively expressed in mammalian tissues and cells, whereas COX-2 expression is induced, from very low background levels, by many types of stimuli such as LPS and cytokines (Bozza et al., 1997, Liou et al., 2000). Both classes of COX are bifunctional enzymes with two distinct catalytic activities: cyclooxygenase (or bis-dioxygenase) activity and peroxidase activity. COX-1 is responsible for regulating normal physiological functions such as platelet aggregation and vascular tone (Dubois et al., 1998, Langenbach et al., 1999), whereas COX-2 mediates in inflammatory responses such as macrophages, fibroblasts, and endothelial cells, through the production of large amount of PGs such as PGE_2 (Morham et al., 1995, Barrios-Rodiles and Chadee, 1998). PGs interact with a family of receptors which preferentially bind each of the main prostanoids. PGE_2 binds to at least four sub types of the EP receptor which also have a number of isoform and splice variants (Breyer et al., 2001, Bhattacharya et al., 1999).

The essential physiological importance of COX-2 in inflammation and infection was confirmed through studies on COX-2 deficient mice (Langenbach et al., 1999, Loftin et al., 2002, Lee et al., 1992, Tiano et al., 2002, Bonner et al., 2002). COX-2 knockout mice were found to be susceptible to peritonitis; they developed congestive heart failure from cardiac fibrosis, (Langenbach et al., 1999, Loftin et al., 2002). These

studies also demonstrated that COX-2 deficiency was beneficial to the host during influenza viral infection. Infection induced less severe sickness in COX-$2^{-/-}$ mice in comparison to WT and COX-$1^{-/-}$ mice, mortality was significantly reduced in COX-$2^{-/-}$ mice. These differences were related to immune function, the inflammatory and cytokine responses were blunted in COX-$2^{-/-}$ mice, whereas in COX-$1^{-/-}$ mice inflammation and earlier appearance of pro-inflammatory cytokines was enhanced (Carey et al., 2005). Despite this evidence however, recent studies have suggested that COX-2 may play an anti-inflammatory role (Lawrence et al., 2002). Indeed, TNF-α stimulated gene-6 (TSG-6) upregulated COX-2 synthesis in macrophages that resulted in preferential synthesis of prostaglandin D_2 and its metabolite 15-deoxy-12, 14-prostaglandin J_2 ($15dPGJ_2$), both of which can act as negative regulators of inflammation (Mindrescu et al., 2005).

Within macrophages and other related cell types MAP kinases and NF-κB have been found to be key regulators of COX-2 induction, however there appears to be a large variation in the contribution of each MAP kinase family member. In several studies p38MAPK plays a role whereas ERK1/2 does not contribute (Caivano and Cohen, 2000, Wu et al., 2005). On the contrary, in cardiac myocytes TNF-α-induced COX-2 expression depends greatly on activation of ERK1/2 and NF-κB. Similarly, IL-1β-induced cardiomyocyte COX-2 gene expression and PGE_2 synthesis has been shown to be regulated by both p38 and ERK1/2 as judged by sensitivity to pharmacological inhibitors (Degousee et al., 2003).

1.5.1 Effect of prostaglandins on macrophage function

PGs play a crucial role in the immune system. PGE_2 is thought to suppress cell-mediated immune responses whilst enhancing humoral responses (Phipps et al., 1991, Snijdewint et al., 1993). PGE_2 also modulates the activities of APCs, such as macrophages and dendritic cells (DCs), in a variety of ways, including the suppression of cytokine production (Harris et al., 2002, Harizi et al., 2001). Pre-treatment with PGE_2 down-regulates the expression of the IL-12 receptor and inhibits the production of tumor necrosis factor α (TNF-α), IL-1β, IL-8 and IL-12 in macrophages (Hinz et al., 2000a). In a similar study, peritoneal macrophages treated

with LPS, induced PGE_2 formation but subsequently decreased TNF-α production (Ikegami et al., 2001). Macrophages treated with zymosan demonstrated that PGE_2 caused simultaneous down-regulation of TNF-α production and up-regulation of IL-10, through activation of EP2 and EP4 receptors (Shinomiya et al., 2001). As a consequence, PGE_2 acts to up-regulate Th2 responses in macrophages. Therefore, PGE_2 may act as an autocrine feedback regulator being released as a part of an initial inflammatory response but subsequently functioning to dampen down inflammation.

1.5.2 Relationship between prostaglandin production and *Leishmania* infection

PGE_2 production is relevant to immune responses elicited in response to *Leishmania* infection. An early study demonstrated that the severity of *Leishmania* infection and the exacerbated Th2-cell response was mediated by parasite-derived PGE_2 (Wilborn et al., 1995). However, other work has reported that *Leishmania* infection initiated endogenous PGE_2 production both *in vitro* and *in vivo* and that this may favour *Leishmania* persistence and progression inside the host immune cell (Farrell and Kirkpatrick, 1987, Reiner and Malemud, 1984). Furthermore, *L. donovani* has been shown to increase the synthesis of PGE_2, possibly by inducing alterations in COX expression or activity (Reiner and Malemud, 1985, Reiner and Malemud, 1984).

Leishmania infected BALB/c mice were found to be characterized by an excessive production of prostaglandins (PGs) (Scott and Farrell, 1981, Farrell and Kirkpatrick, 1987), however the functions of the prostaglandins were not considered. In another study BALB/c mice were treated with indomethacin (INDO), a non-selective inhibitor of COX activity, which resulted in enhanced IFN-γ and NO and increased resistance to infection by *L. major* (De Freitas et al., 1999). This is consistent with the notion that successful elimination of the parasite required IL-12-driven activation of Th1 cells for production of IFN-γ and NO, and that PGE_2 was a potent inhibitor of IL-12 production and IL-12R expression by human monocytes (van der Pouw Kraan et al., 1995, Wu et al., 1998). Thus, the addition of INDO to macrophages and dendritic cell cultures from BALB/c mice infected with *L. mexicana*, resulted in increased IL-12

production and polarisation of the immune response towards a Th1 phenotype (Perez-Santos and Talamas-Rohana, 2001).

1.6 The iNOS enzyme and nitric oxide production

Nitric oxide (NO) is a minute, relatively stable gas that willingly diffuses into cells and cell membranes and reacts with its molecular targets. NO was discovered as "endothelium-derived relaxing factor" (EDRF) by (Furchgott and Zawadzki, 1980). In animal tissues NO is generated enzymatically by synthases (NOS), which oxidise L-arginine to L-citrulline (Michel and Feron, 1997, Ignarro, 2002). There are three isoforms of NOS; NOS I or nNOS, the neuronal form, NOS II known as inducible nitric oxide synthase (iNOS), which is induced in various cell types upon inflammatory stimulation (e.g. macrophages) and NOSIII or eNOS, a constitutive enzyme expressed primarily in the endothelium. All three isoforms have a similar molecular structure and require multiple cofactors (West et al., 2001).

In macrophages, NO is generated by inducible NO synthase (iNOS) and modulates immune responses as well as host defense against pathogens and tumor cells (Gross and Wolin, 1995, Nussler and Billiar, 1993). The induction of iNOS can be initiated by inflammatory cytokines such as IFN-γ, TNF-α or IL-1β (Heba et al., 2001, Nussler et al., 1992, Guo et al., 2007). Nevertheless, the most well recognised inducer is lipopolysaccharide (LPS), which through the activation of TLR4 (Matsuno et al., 1998), is known to be responsible for the initiation of systemic inflammatory response syndrome (SIRS) and resultant sepsis following exposure to Gram-negative bacteria (Xie and Nathan, 1994).

It is now recognised however that several other infectious agents are able to mediate induction of iNOS through other mechanisms and via different receptors. In response to the Gram-positive bacterium *Staphylococcus aureus*, astrocytes induced iNOS expression leading to the production of a high level of NO, mediated through the TLR2 pathway (Phulwani et al., 2008, Esen et al., 2004). In addition, infection by *Plasmodium* sporozoites in primary hepatocytes resulted in induction of iNOS protein expression (Torgler et al., 2008). Viruses have also been shown to have the ability to

induce iNOS in macrophages, including the encephalomyocarditis virus (EMCV), (Freudenburg et al., 2010, Maggi et al., 2002). Infection of monocytes with dengue virus (DENV) *in vitro* resulted in cellular activation and increased iNOS expression, which led to the formation of NO and its release (Neves-Souza et al., 2005). It has also been reported that infection of monocyte-derived macrophages (MDM) with human immunodeficiency virus type one (HIV-1) or HIV envelope proteins caused induction of iNOS protein expression leading to NO production (Mollace et al., 1993, Adamson et al., 1996, Ouaaz et al., 1996).

1.6.1 iNOS and NO in inflammation

There are multiple intracellular mechanisms through which nitric oxide may act as a pro-inflammatory mediator. Inducible NOS produces very large, toxic amounts of NO which are sustained at high levels and play a crucial role in inflammation, whereas constitutive NOS isoforms produce moderate amounts of NO within seconds which is direct and short acting and are not relevant to immunity (Salvemini et al., 2003). There are some exceptions, a recent study has shown that NO produced by constitutive NOS enzyme may be involved in immune regulation of T helper cell proliferation and cytokine production (Coleman, 2001). Moreover, NO generated by eNOS is essential to maintain tissue perfusion, confers cyto-protection in the pulmonary and coronary circulation against toxic lipids, and preserves red cell deformability which becomes reduced in septicemia (Korbut et al., 1989, Korbut et al., 2002, Gryglewski et al., 1998). However, once NO is produced through iNOS induction, this leads to the onset of pathophysiological responses reviewed in; (Dawson, 1995). For example, iNOS is linked to optic nerve degeneration and posterior retinal degeneration, which in turn results in glaucoma, retinopathy, myopia, cataracts and uveitis reviewed by (Chiou, 2001). Several studies also demonstrate NO produced by iNOS is an important factor in the progression of the chronic inflammatory diseases in the gastrointestinal tract (Nguyen et al., 1992, Kimura et al., 1997). This may also include the subsequent development of colon cancer (Erdman et al., 2009).

1.6.2 The iNOS and NO in immune regulation

As outlined above nitric oxide derived from iNOS serves as a potent inflammatory factor however, its overall role in the immune system remains to be clarified. In some cell types NO is essential to inhibit the expression of genes involved in cellular proliferation and growth. Additionally, NO has also been shown to have anti-apoptotic effects (Kroncke et al., 2001). NO produced by antigen presenting cells and monocytes have shown to inhibit T cell proliferation particularly that of the Th1 subsets of T helper cells (Becherel et al., 1995). Interestingly, mouse Th1 cells were also found to produce NO, and several studies in iNOS knockout animals indicated a role for NO in the inhibition of Th1 cytokine (IFN-γ) production (Taylor-Robinson et al., 1994). iNOS knockout mice also showed enhanced Th1 responses following infection (Wei et al., 1995). These studies above also suggested involvement of a negative feedback mechanism (Barnes and Liew, 1995); in which NO promoted Th2 type cytokine release leading to humoral and allergic responses that would inhibit Th1 response. Interestingly, both Th1 and Th2 cells produce similar amounts of NO and both subsets responded similarly to nitric oxide (van der Veen, 2001). Many researchers have also shown that NO inhibited expression of numerous cytokines in lymphocytes, eosinophils and monocytes (Marcinkiewicz and Chain, 1993, Giustizieri et al., 2002). These effects lead to the down-regulation of the inflammatory response through the inhibition of a number of cytokines which have a critical role in developing the innate inflammatory response, such as IL-1β, TNF-α, IL-6 and IFN-γ (Bogdan, 2001). An additional pathway which may impact on iNOS, its activity and NO production is that of arginase activity. This pathway utilises L-arginine as a substrate for production of polyamines that is competitive with iNOS activity. In contrast to the Th1 response, there are different cytokines which have the ability to induce the production of polyamines. These cytokines, such as IL-4, IL-10, IL-13 and transforming growth factor-*β* (TGF-*β*) (Iniesta et al., 2002, Barksdale et al., 2004), are termed Th2 cytokines. As polyamines have been shown to play an essential role as nutrients utilised directly by intracellular pathogens like *Leishmania*, the exposure to Th2 cytokines may therefore provide support to pathogen growth (Iniesta et al., 2002, Kane and Mosser, 2001).

1.6.3 Role of MAP kinases in modulating of iNOS induction

The regulation of iNOS induction by MAP kinases was largely inferred by studies of the promoter of the iNOS gene itself. It contains several binding sites for transcription factors, such as nuclear factor-κB (NF-κB), activator protein-1 (AP-1) and the members of the STAT family (Lowenstein et al., 1993, Xie et al., 1993). However, the vast majority of studies that have confirmed their involvement have used either dominant-negative mutant variants of pathway intermediates, pharmacological inhibitors or knockout mice. For example, in RAW 264.7 pre-treatment with the p38 inhibitor SB2003580 inhibited iNOS expression and NO production due to an indirect effect upon NF-κB DNA binding (Chen and Wang, 1999). Regarding JNK, studies have demonstrated JNK mediates an AP-1 response essential for iNOS expression by cytokines (Karin, 1995, Marks-Konczalik et al., 1998).

TNF-α and other stimuli that lead to activation of MAPKs have also been shown to regulate the induction of iNOS; these findings come from studies using pharmacological inhibitors of JNK/SAPK, ERK and p38 and indicated to have direct effects on the transcriptional and post-transcriptional regulation of iNOS (Bhat et al., 1998, Chan and Riches, 1998, Da Silva et al., 1997). In addition, the MEKK1-MKK4-JNK pathway has been shown to play an essential role in the transcriptional regulation of iNOS by TNF-α in macrophages (Chan et al., 1999). These findings strongly suggest a role for these MAP kinase pathways as important signalling mechanisms underlying the inflammatory processes involved in iNOS expression. In human airways, Gram-negative bacterial infection, caused inflammation in part by initiating NO production through iNOS, and by production of tumor necrosis factor-α, interferon-γ (IFN-γ), and interleukin-1β (IL-1β) (Thiemermann, 1997). Consistent with these findings is a study in the macrophage cell line A549 that links the MAP kinase pathway to iNOS induction via AP-1 (Yuhas et al., 2009).

These findings in macrophages are consistent with studies in other cell types. For example, an earlier study has shown in renal mesangial cells treated with IL-1β the overexpression of the dominant-negative form of JNK1 or p54 SAPKβ/JNK2 significantly reduced the iNOS protein expression and NO production. This study and

others suggested that activation of both SAPK/JNK and p38α MAPK signalling cascades are necessary for the IL-1β -induced expression of iNOS and production of NO in renal mesangial cells (Guan et al., 1999, Guan et al., 1997, Kunz et al., 1996). In addition, another study on different cell types demonstrated that p38MAPK and ERK are essential in IL-1β -induced rat pancreatic islet nitric oxide synthesis (Larsen et al., 1998). Furthermore, a recent study demonstrated that JNK plays a crucial role in LPS and IFN-γ induced iNOS protein expression in astrocytes. This was illustrated by using the pharmacological inhibitor SP600125 and RNA knockdown studies with JNK subtype-specific short-interfering RNA (siRNA), and all inhibited iNOS expression. Interestingly, the same study showed that ectopic expression of a constitutively active form of MEKK1 (MAPK/ERK kinase kinase- 1), an upstream activator of JNK, led to an induction of a co-transfected iNOS promoter and, in the presence of LPS, to an enhanced expression of iNOS (Pawate and Bhat, 2006).

Moreover, Chan and Riches, showed that the regulation of iNOS induction by a combination of IFN-γ and TNF-α was not dependent on ERK and p38 MAPK. The group also sought to determine the role of the MAPKs in the regulation of iNOS function by IFN-γ in combination with LPS (Chan and Riches, 2001) **(Figure 1.7)**. This and other studies often use pharmacological inhibitors or molecular inhibition such as dominant-negative (DN) inhibitors of the MAP kinasese. However in many instances both LPS + IFN-γ are used as stimulants despite the inhibitors being only effective against the LPS component. This makes the interpretation of the relevant involvement of each MAP kinase pathway difficult (Morris et al., 2003).

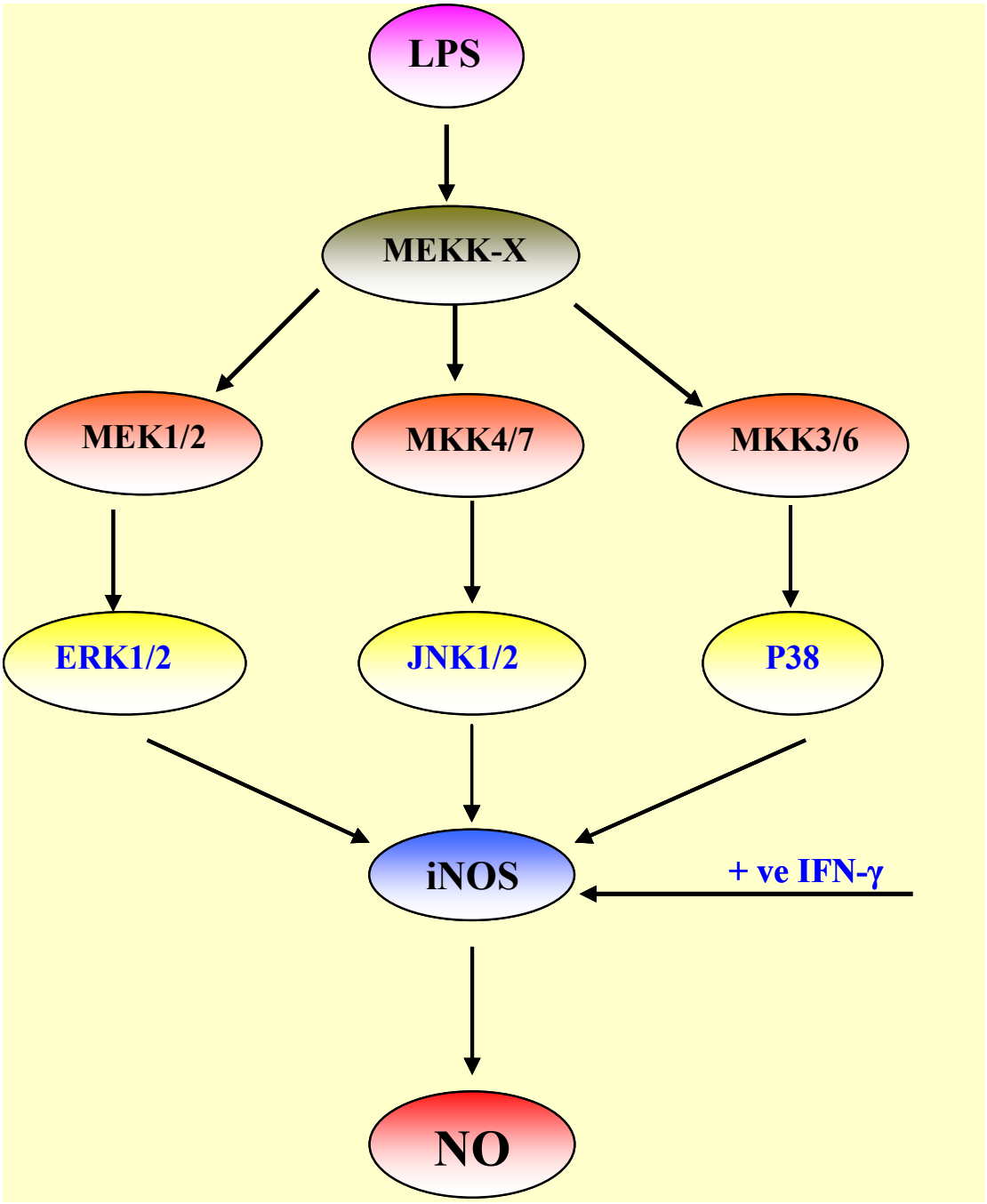


Figure 1.7: iNOS and NO production: hypothesized mitogen-activated protein kinase (MAPK) signalling pathways in lipopolysaccharide (LPS) induction of inducible nitric oxide synthase (iNOS). Adapted from (Chan and Riches, 2001).

Studies show that p38 can act indirectly to regulate iNOS production via NF-κB. In RAW 264.7 macrophages NO production was inhibited by the specific inhibitor SB203580 resulting in the inhibition of LPS-induced NF-κB DNA-protein binding. Consistent with this, p38 MAPK activation following exposure to LPS resulted in the stimulation of NF-κB-specific DNA-protein binding and the consequent expression of inducible form of NO synthase (Chen and Wang, 1999) and other pro-inflammatory mediators such as tumor necrosis factor -α (TNF-α) (Brook et al., 2000), interleukin 1-β (IL-1-β) (Caivano and Cohen, 2000), IL-6 (Beyaert et al., 1996), and IL-12 (Zhang et al., 2000) in murine macrophages.

ERK is recognised to regulate LPS-induced production of NO (Lahti et al., 2000) and pro-inflammatory cytokines (Chan and Riches, 2001) in mouse macrophages. This adds to the great number of studies that have indicated the relative roles of p38 and ERK on LPS-induced iNOS expression in murine cells. For example, interestingly in human (hiNOS) the extracellular signal-regulated kinases (ERKs), c-Jun N-terminal Kinase/stress-activated protein kinases (JNK/SAPKs), and p38 MAPK pathways have been implicated in the activation of AP-1 (Karin, 1995), which were involved in hiNOS expression by cytokines (Marks-Konczalik et al., 1998).

1.6.4 Nitric oxide and inducible nitric oxide synthase (iNOS) in *Leishmania* infection

NO production is important when assessing interaction of *Leishmania* with the immune system. During experimental infection of mice with *Leishmania* spp. the parasiticidal activity of infected macrophages is dependent on upregulation of inducible nitric oxide synthase (iNOS) and subsequent nitric oxide production (NO) (Wei et al., 1995, Stenger et al., 1996). Confirmatory studies indicate that the anti-parasitic activity of this enzyme involves generation of reactive nitrogen intermediates, such as nitric oxide as well as reactive oxygen intermediates such as superoxide (Linares et al., 2001). A recent report proposed that the contribution of NADPH-oxidase-dependent superoxide production to a leishmanicidal effect may differ between different cell types and stage of infection (Murray and Nathan, 1999, Blos et al., 2003). It has been demonstrated that lymphocyte-induced killing of *L.*

amazonensis amastigotes *in vitro* required in addition to NO both antibody and superoxide production (Mukbel et al., 2006), in comparison to *L. major* which was highly sensitive to NO. Indeed, more recent work by Mukbel et al., has shown that in macrophages *L.amazonensis* amastigotes were largely unaffected by NO induced by LPS and IFN-γ and not killed as proficiently as *L. major* amastigotes at equivalent iNOS and nitric oxide levels (Mukbel et al., 2007). Also a recent study by Murray et al., used mice deficient in phagocyte NADPH oxidase (phagocyte oxidase [phox]) (gp91*phox*$^{-/-}$) or inducible nitric oxide synthase (iNOS$^{-/-}$) to gauge the contributions of these intracellular mechanisms to host antileishmanial defense (Murray et al., 2006). Phox KOs reduced parasite burdens as efficiently as selfcuring wild-type (WT) mice, while iNOS KOs developed progressive infection. Since the latter expressed phox protein normally (Murray and Nathan, 1999, Blos et al., 2003), this study concluded that iNOS was necessary and sufficient to control *L. donovani* and convert infection in the liver to a chronic, low-level state (Murray and Nathan, 1999).

1.7 IFN-γ and IL-12 as mediators of classical activation

The first part of this introduction centered upon the effect of *Leishmania* on intracellular signalling pathways and inflammatory mediator productions in response to activation of the innate immune system, principally involving LPS stimulation. However, two important additional mediators need to be considered in relation to classical activation, IFN-γ and IL-12. Upon stimulation of APCs (macrophages and dendritic cells) with a PAMP ligand such as LPS, IL-12 is produced which can in turn act upon other cells of the immune system to further potentiate the response through the production of IFN-γ. Thus, IL-12 can be described as a marker of innate and classical activation. IL-12 exists as a heterodimeric component of 70 kDa, comprising two disulphide-bonded subunits, p40 and p35, which are controlled by separate genes (Trinchieri, 1994, Kang et al., 2005a). Transcriptional regulation of IL-12 is mediated principally through the induction of the p40 subunit which has binding sites within its promoter for NF-κB (Murphy et al., 1995) and AP-1 (Zhu et al., 2001).

Cytokines such as IL-12 serve as a bridge between infection and IFN-γ production as part of the innate immune response (Golab et al., 2000, Dinarello, 1999, Lauw et al., 2001). IL-12 and chemokines such as MIP-1α, MIP-1β and MCP-1, attract NK cells to the site of inflammation, and it is here that IL-12 promotes IFN- γ synthesis in these cells (Salazar-Mather et al., 2000, Pien et al., 2000, Flesch et al., 1998). IL-12 binds the high affinity IL-12 receptor (IL-12R), comprised of a β1 and a β2 subunit (Gately et al., 1998), which signals through the recruitment of the tyrosine kinase, TYK-2 and janus kinase (JAK)-2, respectively (Zou et al., 1997). A number of proteins called Signal Transducer and Activator of Transcription (STATs) are associated with IL-12 signalling, and of these STAT4 is required to induce IFN-γ production (Lawless et al., 2000, Morinobu et al., 2002). In combination with IL-18, IL-12 acts synergistically to further increase IFN- γ production in macrophages, NK cells and T cells (Akira, 2000, Schindler et al., 2001, Frucht et al., 2001). A number of studies have shown negative regulation of IFN-γ production by Th2 cytokines including IL-4, IL-10, IL-13 and transforming growth factor-β (TGF-β) (Fukao et al., 2000, Hochrein et al., 2001).

1.7.1 IFN-γ and macrophage cell signalling pathways

A main target of IFN-γ is the macrophage. IFN-γ has been shown to regulate iNOS expression at both transcriptional and post-transcriptional level which is an essential component in parasite killing as well as favoring Th1 development (Kleinert et al., 2004, Korhonen et al., 2005). As with IL-12, IFN-γ mediates induction of iNOS through activation of the JAK/STAT pathway (Platanias, 2005). In this pathway IFN-γ binds to its cognate receptor IFN-γR (Interferon gamma receptor). Following oligomerization, JAK proteins are targeted to the cytoplasmic portion of the receptor where they phosphorylate receptor associated STAT proteins tyrosine residues. Phosphorylation promotes the formation of STAT homo or heterodimers which translocate to the nucleus and bind specific response elements to induce transcription **(Figure 1.8)**. For IFN-γ signalling STAT1α is the principle mediator involved (Durbin et al., 1996, Meraz et al., 1996), whereas for type one interferons, STAT2 is implicated (Levy, 1999). For IL-4 and IL-12 responses, STAT6 and STAT4 are

required (Takeda et al., 1996, Kaplan et al., 1996a, Kaplan et al., 1996b, Thierfelder et al., 1996). Signalling is mediated through direct engagement with the STAT-binding GAS site (γ-activation site) within the mouse iNOS gene promoter, which is considered necessary for full expression of iNOS in IFNγ-stimulated RAW 264.7 macrophages (Gao et al., 1997). Several studies using pharmacological inhibitors demonstrate a critical role for JAK/STAT in the induction of iNOS protein expression. For example, in human epithelial-like colon carcinoma DLD-1 cells, cytokine induction of iNOS is blocked by a JAK2-specific inhibitor (Kleinert et al., 1998). Furthermore, in STAT-$1^{-/-}$ mice complete inhibition of cytokine induced up-regulation of iNOS transcription is observed (Gysemans et al., 2005).

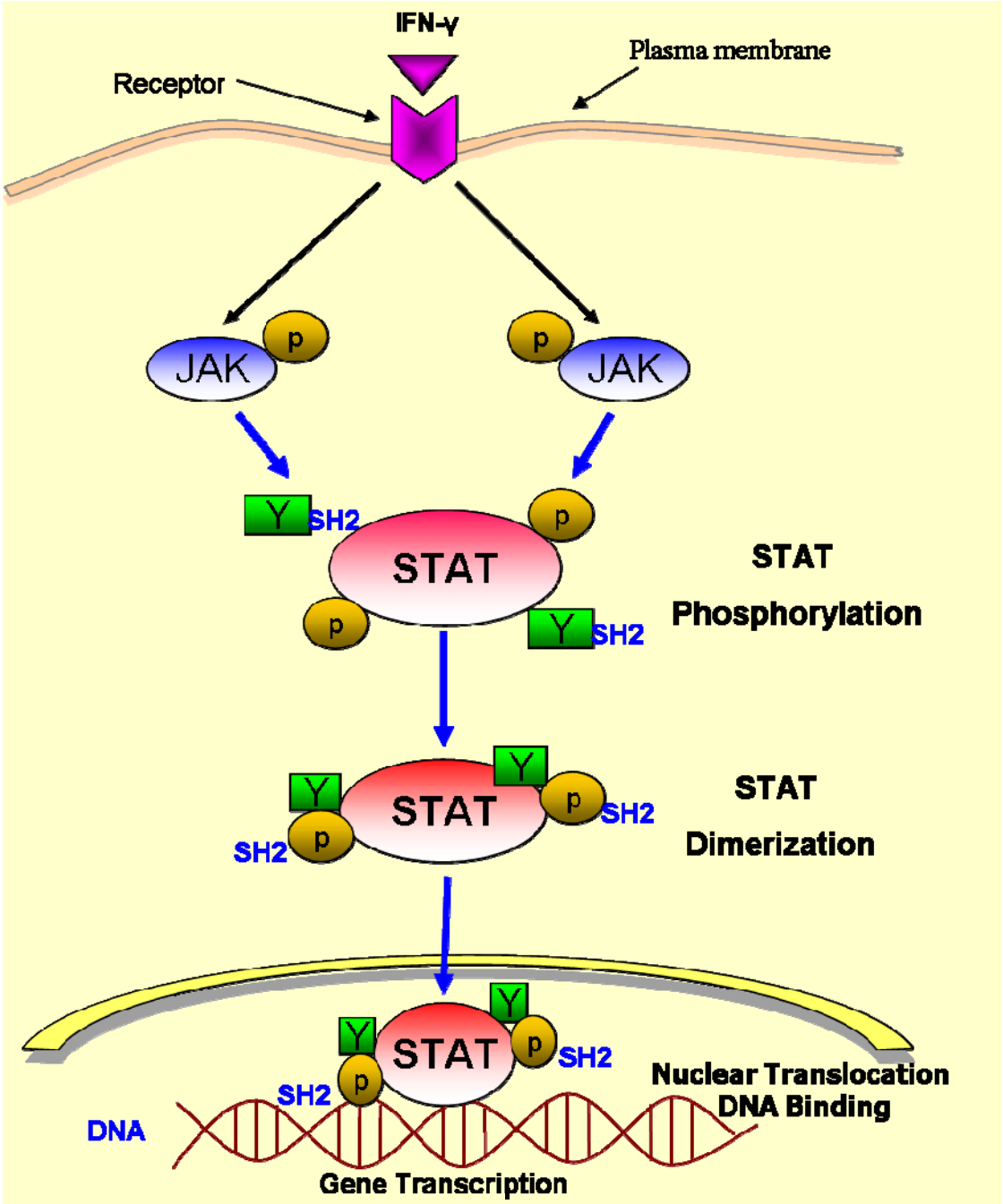


Figure 1.8: IFN-γ induced JAK-STAT signal transduction pathway: STATs are phosphorylated by the JAKs on a conserved tyrosine residue in the c-terminal domain to form STAT homodimers or heterodimers. STATs dissociate from the receptor after dimerisation and translocate into the nucleus. In the nucleus, STATs bind to specific response elements and induce gene transcription. Modified from (Benekli et al., 2003).

1.7.2 Modulation of IFN-γ signalling pathways by *Leishmania* infection

As with NF-κB and MAP kinase pathways, pathogens including *Leishmania* have developed numerous strategies to modify IFN-γ mediated signalling which is essential for protection in response to pathogens. IFN-$\gamma^{-/-}$ mice are extremely susceptible to intracellular agents such as *Mycobacterium avium* (Alvarez et al., 2003), *Listeria monocytogenes* (Harty and Bevan, 1995), *Candida albicans* (Balish et al., 1998) and *Plasmodium berghei* (Amani et al., 2000). It is therefore not surprising that several different pathogens have evolved effective avoidance strategies. For example, viral mediated mechanisms for inhibiting IFN-γ signalling can involve decoy receptors, production of regulatory intermediates and expression of dominant negative proteins (Gotoh et al., 2002). For example, paramyxovirus has been shown to cause specific degradation of STAT1α by the proteasome through the expression of a cellular E3-ubiquitin-protein isopeptide ligase (Garcin et al., 2004, Ulane and Horvath, 2002).

Leishmania is also able to prevent the activation of JAK/STAT pathways by targeting the IFN-γ receptor complex (Ihle et al., 1995, Taniguchi, 1995). For example, *L. mexicana* and *L. major* have shown the ability to inhibit STAT1 phosphorylation by reducing levels of IFN-γRα and IFN-γRβ (Bhardwaj et al., 2005). In human monocytes attenuation of IFN-γR expression by *L. donovani* has also been observed (Nandan and Reiner, 1995). An additional study has also demonstrated the potential for *Leishmania* to interact directly with the receptor to prevent IFN-γ binding (Blanchette et al., 1999). Furthermore, a recent study by Forget and co-workers has demonstrated that infection of macrophages with *L. donovani* resulted in STAT1α degradation mediated through the proteasomal pathway (Forget et al., 2005).

In addition, *Leishmania* infection has been shown to regulate intermediates of the JAK/STAT signalling pathways. Infection of macrophages with *L. donovani* promastigotes down-regulates IFN-γ signalling by attenuating tyrosine phosphorylation of JAK1, JAK2 and STAT1α. This was found to be due to the ability of the parasite to activate SHP-1 which in turn dephosphorylates JAK2 (Nandan and Reiner, 1995, Blanchette et al., 1999, Nandan et al., 1999). In corroboration with

these findings, another study has shown that inhibition of iNOS by *L. donovani* was due to an activation of SHP-1 and reduction in JAK signalling (Nandan et al., 1999). Furthermore, in macrophages *L. donovani* has been shown to enhance the expression of both SOCS1 and SOCS3 which bind to STAT1α resulting in their degradation (Bertholet et al., 2003, Forget et al., 2005). These findings are supported by studies using either PTP inhibitors or SHP-1-deficient macrophages which showed enhanced IFN-γ stimulated STAT1α phosphorylation, nuclear translocation, increased iNOS expression and NO production (Blanchette et al., 2009, Blanchette et al., 2007).

These studies correlate with observations *in vivo*. Indeed, mice which had been injected with SHP inhibitors, prior to infection with, *L. major and L. donovani* were able to control infection through reduced lesion size and parasitic load (Olivier et al., 1998). In addition, Matte and co-workers have demonstrated that the inhibitory effect of bis-peroxovanadium, which mediated protection to infection, was dependent upon enhanced iNOS protein expression and NO release (Matte et al., 2000). Infection of SHP-$1^{-/-}$ mice with *L. major* resulted in a lack of lesion development and a significantly reduced parasitic load. This protection was mediated through enhanced expression of iNOS gene involving amplified STAT1α and NF-κB activity. Correspondingly, infection of SHP-$1^{-/-}$ mice with an iNOS inhibitor resulted in complete reversal of protection from *L.major* infection (Forget et al., 2001).

1.8 AIM OF THE STUDY

Numerous studies outlined in this section exemplify the potential for *Leishmania* species to regulate macrophage intracellular signalling pathways in order to subvert immune responses. Relative to other *Leishmania* species, such as *L. donovani* and *L. major*, there is relatively little information regarding the effects of *L. mexicana*. Therefore, this thesis focused on the examination of the effects of both amastigotes and promastigotes from *L. mexicana* on NF-κB, MAP kinase and JAK/STAT signalling to better understand how these events could influence macrophage activation and the potential consequences for the immune system. The following objectives were undertaken:

I. To assess the effect of *L. mexicana* amastigotes on the components of the MAP kinase and NF-κB pathways stimulated by LPS and the JAK/STAT pathways activated by IFN-γ.

II. To study the effects of amastigotes on the induction of the inflammatory proteins COX-2 and iNOS, and the cytokines IL-12, IL-6 and IL-10.

III. To compare the activation of macrophages by *L. mexicana* promastigotes and amastigotes, determine the involvement of the amastigote specific CPB enzyme and to assess the role of macrophage TLR-2 and TLR-4 in mediating MAP kinase signaling, COX-2 and iNOS protein expression.

IV. To determine the ability of promastigotes to regulate the arginase pathway in macrophages through the activation of TLRs.

V. To determine the influence of COX-2, iNOS and arginase pathways on the regulation of both pro- and anti-inflammatory cytokines, IL-12, IL-6 and IL-10, mediated by *L. mexicana* promastigotes.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 General Reagents

All materials used were of highest commercial purity available and were supplied by Sigma Aldrich Chemical Company Ltd. (Poole, Dorset, UK) unless otherwise stated.

Bio-Rad Laboratories (Hertfordshire, UK).

Bio-Rad DC™ Protein Assay kit

Pre-stained SDS-Page molecular weight markers.

Boehringer Mannheim (East Sussex, UK).

Bovine serum albumin (BSA, Fraction V).

Corning B.V. (Netherlands).

Tissue culture plasticware.

Gibco Life Technologies Ltd (Renfrewshire, UK).

Modified Eagle's Medium (DMEM) containing glucose and sodium pyruvate-L-Glutamine.

Invitrogen , Paisley, UK.

Fetal calf serum (FCS).

Antibiotics ; penicillin, streptomycin, Geneticin (G418).

Roche diagnostics GmbH.

Dithiothreitol (DTT).

Amersham International Plc (Aylsbury, Buckinghamshire, UK)

ECL detection reagents

Sigma GmbH.

Lipopolysaccharides (LPS) from *Salmonella abortus* (L1887-5MG).

Sigma-Aldrich Co Ltd (Poole, Dorset, UK)

Acrylamide, Ammonium Persulfate (APS), Glycerol, Glycine, Kodak X-OMAT LS X-ray film, Methanol, Sodium Chloride, Sodium Dodecyl Sulphate (SDS), N,N,N',N'-tetramethylenediamine (TEMED), TWEEN-20, and Trizma Base.

BD Pharmingen ™, (USA)

Recombinant murine IL-4

R & D Systems Europe, Ltd, (UK)

Recombinant murine IFN-γ

Whatman (Kent, UK).

Nitrocellulose membrane.

2.1.2 Antibodies

Cell Signalling Technology, Inc (USA)

Rabbit polyclonal p-p-65 (Ser-536)

Rabbit polyclonal p-JAK2 (Tyr 1007/1008)

Rabbit polyclonal p-STAT1α (Tyr 701)

Rabbit polyclonal T-JAK2 (D2 E12)

Santa Cruz Biotechnology Inc. (CA, USA).

Mouse monoclonal anti-p-ERK (E-4)

Rabbit polyclonal anti- IκBα (C-21)

Rabbit polyclonal anti-p38 (N-20)

Rabbit polyclonal anti-JNK-1 (FL)

Rabbit polyclonal anti-ERK-1 (K-23)

Rabbit polyclonal NF-κB p65 (C-20)

Rabbit polyclonal T-STAT1α (E-23)

Biosource Europe SA, Invitrogen Ltd (Paisley, UK)

Anti-phospho-p38 MAP kinase (rabbit polyclonal)

Rabbit polyclonal anti-p- JNK 1 & 2

Cayman chemical, Michigan, USA.

Rabbit polyclonal anti-COX-2

Rabbit polyclonal anti-iNOS

The following iNOS antibody was kindly provided by:

Dr. Clare Bryant (Department of Veterinary Medicine, Cambridge University, UK)

Rabbit polyclonal anti-iNOS

Jackson ImmunoResearch Laboratories Inc., (PA, USA)

Horseradish peroxidase (HRP)-conjugated AffiniPure goat anti-rabbit IgG (111-035-144)

HRP-conjugated AffiniPure donkey anti-mouse IgG (715-035-150)

BD Transduction Laboratories ™, (USA)

Mouse monoclonal anti-arginase-I IgG1 (610708)

2.1.3 Enzyme-Linked Immunosorbant Assay (ELISA)

BD-Pharmingen, San Diego, CA (Biotin-labelled)

Anti mouse-IL-12 (p40/p70) IgG1-551219- clone C15.6

Anti mouse-IL-6 IgG2a- clone MP-5-32 C11

Anti mouse-IL-10 IgG1- clone JES5-2A5

R & D Systems, INC, (USA)

Anti mouse- PGE_2- catalog number PKGE004B

2.1.4 Inhibitors

Calbiochem, Merck KGaA, Darmstadt, Germany

N^{ω}- Hydroxy-nor-L-arginine (399275)

Sigma-Aldrich Co Ltd (Poole, Dorset, UK)

Indomethacin -COX-2 inhibitor (17378)

L-NAME -NO inhibitor (5751)

2.1.5 Microscopy

Merck-Calbiochem (Nottingham, UK)

Mowiol

VWR International Ltd (Leicestershire, UK)

No. 0, (0.09-0.13 mm thick), circular glass 13 mm diameter coverslips.

0.8-1.0mm thick glass microscopy slides

2.2 METHODS

2.2.1 Cell culture

All isolated macrophages were grown in 10 cm^2 plastic Petridishes (Sterlin). All isolation procedures and subsequent cell culture work was carried out in a class II cell culture hood (Heraeus) under aseptic conditions.

2.2.1.1 Procedure for L-cells conditional medium

Large quantities of macrophage-colony stimulating factor (M-CSF), required for the culture of bone marrow- derived macrophages (BMM), was derived using L929 cells (ECACC). An aliquot of L929 cells was dispersed at 37^oC and centrifuged at 200*g* for 5 minutes. The pellet was resuspended in 13ml of D10 medium, consisting of DMEM containing sodium pyruvate, pyridoxine hydrochloride and high glucose (Invitrogen, Paisley, UK), 10% HI-FCS (Invitrogen, Paisley, UK), 2mM L-glutamine (Cambrex BioScience, Veniers, Belgium), 100U/ml penicillin (Invitrogen, Paisley, UK) and 100µg/ml streptomycin (Invitrogen, Paisley, UK). The cells were cultured in a 75 cm^3 cell culture flask (Greiner Bio-One, Stonehouse, UK) for approximately 4-5 days until confluent. Media was aspirated from the flask and replaced with 5ml cold PBS. After 10 minutes at 4^oC, cells were gently scraped using a 30cm cell scraper (TPP, Switzerland). The resulting cell suspension was centrifuged at 200*g* for 5 minutes and subsequently the supernatant was removed and the pellet was resuspended in 10ml D10 medium. To each of ten 160 cm^3 cell culture flasks, 1ml of cell suspension was added. A further 30 ml D10 medium was added to each flask, which were then incubated in a CO_2 incubator at 37^oC / 5% CO_2 until 80-90% confluent. Nine of the ten flasks were scraped, centrifuged at 200*g* for 5 minutes and resuspended in 36 ml D10 medium. 180 ml of D10 medium and 1ml of cells was added to each triple layer flask. The remaining flask was used to seed a further ten 160 cm^3 cell culture flasks. After 7 days, the supernatant was collected from the flasks, aliquoted into 50ml tubes and stored at -20^oC for later use in the differentiation of macrophages from bone marrow stem cells.

2.2.1.2 Bone marrow isolation and the generation of macrophages

Male C57B/L6 mice, aged between 3-4 months were dissected and the femurs and tibia bones were removed and washed with alcohol (70%) and sterile PBS (pH = 7.4). Five ml BMD-macrophage medium per bone was used to wash the bone marrow stem cells from within the bone into a 50 ml tube using a 25 gauge needle (BD, Drogheda, Ireland). BMD-macrophage medium consisted of DMEM containing sodium pyruvate, pyridoxine hydrochloride and high glucose (Invitrogen, Paisley, UK) supplemented with 10% HI-FCS, 30% L-cell-conditioned medium, 5mM L-glutamine, 100U/ml penicillin (Invitrogen, Paisley, UK) and 1μg/ml streptomycin. The cell suspension was then flushed through a 21 gauge needle three times in order to produce a single cell suspension. The cell suspension was then transferred into petri dishes with each one supplemented with a further 10 ml BMD-macrophage medium. The cells were incubated at 37^{o}C / 5% CO_2 for 10 days. After 3 days incubation, dishes were supplemented with 20 ml BMD-macrophage media. Furthermore, on days 5 and 7, medium was replaced with 20 ml of warm fresh medium. Once cells had become confluent after 10 days, they were plated out for use in experiments. The medium was removed from each petri dish and replaced with 5ml of cold, sterile RPMI 1640 medium. Using a 30 cm cell scraper, cells were removed from the bottom of the petri dish and collected in a 50 ml tube. The cell suspension was then centrifuged at 1000 rpm (350g) for 5 min and subsequently the cells were washed twice with sterile RPMI 1640 medium and complete RPMI 1640 to remove any traces of DMEM. Finally, the resulting pellet was resuspended in 10 ml of complete medium consisting of RPMI 1640 supplemented with 5mM L-glutamine, 100U/ml penicillin and 1μg/ml streptomycin. A dilution of the cell suspension was made in complete RPMI and cells were counted using a haemocytometer (Hausser Scientific, USA), cells were then diluted to the desired concentration.

2.2.2 *In vitro* and *in vivo* maintenance of parasites

2.2.2.1 Culture of *Leishmania mexicana* promastigotes *in vitro*

In this study two forms of *Leishmania mexicana* were used for infection, the amastigote and promastigote stages. TC100 insect medium (Sigma, St. Louis, USA)

supplemented with v/v 10% FCS was used for the culture and maintenance of promastigotes *in vitro*. For this purpose, *L. mexicana* (MNYC/BZ/62/M379) promastigotes were cultured in 25 cm^3 culture flasks in 10 ml of insect medium and incubated at 20°C for seven days, until the metacyclic stage was achieved. Parasites were then harvested and 1 ml of the parasite culture was then supplemented with 10 ml of TC100 insect medium containing 10% FCS to maintain the parasites in culture. The remaining parasite culture was used for infections. Parasites were passaged after 10 weeks of culture.

2.2.2.2 Maintenance of *Leishmania mexicana* amastigotes *in vivo*

BALB/c mice were infected subcutaneously in the rump or footpad with late stationary-phase promastigotes to obtain lesions of *L. mexicana* amastigotes. Lesions developed 6-8 weeks post infection and subsequently amastigotes were harvested from lesions as described below.

2.2.2.2.1 Harvesting of *Leishmania mexicana* amastigotes

Lesions were removed and pushed through a metal mesh with 5 ml of RPMI 1640 supplemented with v/v 1% penicillin-streptomycin and 1% L-glutamine. The parasites were suspended in 5 ml RPMI 1640 and then centrifuged at 500g for 5 min. The resultant pellet was resuspended in 3 ml of Boyles solution (1:9 Tris: NH_4Cl) to remove red blood cells and once again centrifuged for 5 min at 1428.5 rpm (500g). The resultant pellet was then washed again with RPMI 1640 containing supplements, resuspended and centrifuged again at 1428.5 rpm (500g) for 5 min. This washing stage was repeated twice more and the final pellet resuspended in 5 ml of RPMI 1640 containing supplements. The parasites were then counted using a haemocytometer and diluted appropriately with RPMI 1640 containing supplements.

2.2.2.2.2 *Leishmania mexicana* infection at the base of the tail

BALB/c mice, aged 6-8 weeks, were infected by inoculation subcutaneously with $1x10^7$ *L. mexicana* amastigotes in 50μL of RPMI 1640 at the base of the tail. As amastigotes represent the life cycle stage of an established mammalian infection, inoculation with amastigotes results in a more rapid progression of lesion development than inoculation with metacyclic promastigotes. Therefore, amastigotes were used to ensure all parasites were infective, removing the possibility of an inoculum containing promastigotes at various stages of differentiation and therefore an inoculation with an undefined number of infective metacyclic promastigotes.

2.2.2.2.3 *Leishmania mexicana* infection of the hind footpad

The maintenance of a *L. mexicana* lesion in the footpad was achieved in the same manner as previously described for the rump lesion with the following differences. BALB/c mice aged 6-8 weeks old were inoculated with $2x10^5$ *L. mexicana* amastigotes in 25μL with RPMI 1640. Again, based on the same principle as above, amastigotes were used to ensure all parasites were infective.

2.2.2.2.4 Isolation and enumeration of parasites from mouse lesions

The rump or hind foot lesions of infected mice were excised and passed through a metal mesh with 5ml of RPMI 1640 as previously described in section (2.2.2.2.1). The parasites were then enumerated using a haemocytometer and the total number of parasites per lesion was calculated.

2.2.2.2.5 Infection of macrophages with *L. mexicana* amastigote or promastigote

BMD-macrophages were grown to ($1x10^6$/ml) in 12-well plates in complete RPMI 1640 supplemented with 10% HI-FCS, 5mM L-glutamine, 100U/ml penicillin and 1μg/ml streptomycin. The cell number was then determined by using a haemocytometer. The cells were then infected with amastigotes or stationary phase promastigotes following harvesting as previously described, using the appropriate multiplicity of infection (MOI) required (0, 0.5, 1, 2 and 5 parasite/cell) and for the indicated times. Cells were infected by adding 500 μL *Leishmania* and incubating the cells for the indicated times at 33ºC and 5% CO_2 . Subsequently, cells were stimulated with agents as required for the experimental design. Following infection and stimulation, cultures were rapidly cooled on an ice tray and cell supernatants were collected in fresh microcentrifuge tubes in order to determine the production of cytokines or NO. After collection of supernatants, cells were washed twice in 0.5 ml ice cold PBS (pH= 7.4) and the cells were scraped on ice. Whole cell extracts were then prepared for analysis (see section 2.2.3).

2.2.3 WESTERN BLOTTING

2.2.3.1 Preparation of whole cell extracts

Cells were grown to ($1x10^6$/ml) in 12-well plates and then exposed to *Leishmania* and/or appropriate agonists for the required time points. Monolayers were then washed twice with ice cold PBS before adding 200μl of pre-heated (~ 85 °C) Laemmli's sample buffer [63mM Tris-HCl, (pH 6.8), 2mM Na-$4P_2O_7$, 5mM EDTA, 10% (v/v) glycerol, 2% (w/v) SDS, 50mM DTT, 0.007% (w/v) bromophenol blue] and protease inhibitor cocktail (Sigma). Cells were then scraped from wells on ice and the chromosomal DNA was sheared by repeated passage through a 23 gauge needle. Samples were then transferred to microcentrifuge tubes. The lids of the tubes were pierced and the tubes were then boiled for 3-5 min to denature proteins and then stored at -20 °C until use.

2.2.3.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Resolving gels were prepared containing an appropriate amount (7.5% (w/v), 9% (w/v), 10% (w/v), 11% (w/v) acrylamide: [N, N'-methylenebis-acrylamide (30:0.8), 0.375M Tris (pH 8.8), 0.1% (w/v) SDS and 10% (w/v) ammonium persulfate (APS)]. Polymerisation was initiated by the addition of 0.05% (v/v) N, N, N', N'-tetramethylethylenediamine (TEMED). The solution was poured between two glass plates assembled in a vertical slab configuration according to the manufacturers instructions (Bio-Rad) and overlaid with 200 µl 0.1% (w/v) SDS. Following gel polymerization, the layer of 0.1% SDS (w/v) was removed and a stacking gel containing [10% (v/v) acrylamide: N,-methylenebis-acrylamide (30:0.8) in 125mM Tris (pH 6.8), 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulfate and 0.05% (v/v) TEMED] was poured directly on top of the resolving gel. A teflon comb was immediately inserted into the stacking gel solution. After polymerisation was then complete, the comb was removed and the polyacrylamide gels were assembled in a Bio-Rad Mini-PROTEAN II^{TM} electrophoresis tank, with both reservoirs filled with electrophoresis buffer [25mM Tris, 129mM glycine, 0.1% (w/v) SDS]. Aliquots of samples (20-30µg/ml) were then loaded into the wells using a microsyringe. A pre-stained SDS-PAGE molecular weight marker of known molecular weights was run concurrently in order to identify the polypeptide of interest. Samples were electrophoresed at a constant voltage of 120 V, until the bromophenol dye had reached the bottom of the gel.

2.2.3.3 Electrophoretic transfer of proteins to nitrocellulose membrane

The proteins separated by SDS-PAGE were transferred to nitrocellulose membranes by electrophoretic blotting following a standard protocol (Towbin et al., 1979). The gel was pressed firmly against a nitrocellulose sheet and assembled in transfer cassette, sandwiched between two Whatman 3MM paper and two sponge pads. The cassette was immersed in blotting buffer [25M Tris, 19mM glycine, 20% (v/v) methanol] in a Bio-Rad Mini Trans-Blot ^{TM} tank and a constant current of 300 mA was applied for 105 min, whilst the tank was cooled by inclusion of an ice reservoir.

2.2.3.4 Immunological detection of protein

Following transfer of the proteins to the nitrocellulose membrane, the membrane was removed and any remaining protein blocked by incubation in a solution of 2% (w/v) BSA in NaTT [150mM NaCl, 20mM Tris (pH 7.4), 0.2% (v/v) Tween-20] for 2h with gentle agitation on a platform shaker. The blocking buffer was removed and membranes incubated overnight with antiserum specific to the target protein appropriately diluted in NaTT buffer containing 0.2% (w/v) BSA. The following day, membranes were washed in NaTT every 15 min for 90 min with gentle agitation. The membranes were then incubated for a further 2 hrs at room temperature with secondary horseradish peroxidase-conjugated IgG directed against the first immunoglobulin diluted to approximately 1:10000 in NaTT buffer containing 0.2% BSA. After six additional washes in NaTT as described before, immunoreactive protein bands were detected by incubation in enhanced chemiluminescence (ECL) for 2 min. The membranes were blotted on a paper towel and mounted in an exposure cassette and covered with cling film, then exposed to Kodak X-OMAT LS film for the appropriate time under darkroom conditions and developed by a KODAK M35-M X-OMAT processor.

2.2.3.5 Stripping for reprobing of nitrocellulose membrane protocol

Used nitrocellulose membranes were stored in a sealed container containing NATT buffer, until reprobing was desired. Antibodies were then stripped from the nitrocellulose membrane by incubating in 15 ml stripping buffer containing [0.05 M of Tris HCL, 2% of SDS and 0.1 M of β-mercaptoethanol in ultra pure water (pH= 6.7)]. Blots were incubated for 1 hr at 70°C on a shaker (Stuart Science Equipment). After the incubation period, the stripping buffer was discarded and membranes rinsed with NaTT buffer (pH=7.4) three times at 15 min intervals to remove residual stripping buffer. Finally membranes were incubated with primary antibody in 0.2 % BSA/NaTT buffer (pH=7.4) and left overnight on the shaker at room temperature. The following morning, the membrane was washed with NaTT buffer for six times over 90 min. The same procedure was used for protein detection as outlined previously.

2.2.4 (ELISA) Enzyme-linked immunosorbent assay

2.2.4.1 Measurement of cytokine production

Cell supernatants were collected in fresh microcentrifuge tubes and used to measure IL-12, IL-6, IL-10 and PGE_2 levels by ELISA. 96 well plates were coated with the appropriate capture antibody (rat anti mouse IL-12p40 mAb, 2µg/ml IgG1, clone C15.6), anti- IL-6 mAb anti-mouse (IgG1 clone MP5-20F3) mAbs 2µg/ml and capture anti IL-10 was anti-mouse monoclonal prepared from (IgG1 clone JES5-2A5) (Pharmingen) at 2µg/ml), using coating buffer (PBS pH=9.0) at a volume of 50µl in each well and incubated overnight at 4°C. The following morning, excess capture antibodies were washed three times with wash buffer (PBS and 0.05% Tween-20, pH= 7.5) then dried by beating against paper towels to discard remaining wash buffer from the wells. Wells were incubated with 200µL blocking buffer [PBS (pH=7.0) and FCS (10%)] and the plates were incubated at 37°C for 1 hour. After blocking incubation, the plates were washed 3 times as mentioned before. Supernatants sample were then added to the wells in a 50µL volume along with the relevant recombinant protein (Pharmingen) for standard curve production which was titrated in doubling dilutions from 20ng/ml-387.5 pg/ml in 50µL volumes. Plates were then incubated at 37°C for two hours. Following incubation, the wells were washed 4 times in wash buffer. Biotin labelled rat anti-mouse antibody for each cytokine from (Pharmingen) was added at 1µg/ml, in a 50µL volume. Plates were then incubated at 37°C for one hour. Plates were then washed four times before the addition of 100µL streptavidin-alkaline phosphatase (AKP) (Pharmingen), diluted in 2000 in blocking buffer. Plates were then incubated at 37°C for 30 minutes. Next, plates were washed five times before the addition with 100µL of p-Nitrophenyl phosphate (p-NPP, Sigma) prepared in glycine buffer at 1mg/ml. Absorbances were measured at 450 nm using a SPECTRAmax 190 microtitre plate spectrophotometer reader and Softmax PRO 3.0 software.

2.2.4.2 Measurement of PGE_2 production protocol

This assay is based on the forward sequential competitive binding reaction in which PGE_2 present in supernatants samples were measured by ELISA by using a PharmPak (R&D Systems, Catalog # PKGE004B) Enzyme immunoassay kit according to the manufacturer's protocol. The procedure was essentially similar to that conducted for cytokine measurement with specific modifications as indicated in the protocol.

2.2.5 Arginase activity determination in BMD-macrophages

Arginase activity in murine BMD-macrophages was determined as described by (Corraliza et al., 1994). Following the designated time, for each treatment the supernatant was collected in fresh microcentrifuge tubes for later analysis of NO production levels. Then cells were scraped in 500µL sterile ice cold PBS (pH=7.4) and collected in microcentrifuge tubes. An additional 500µL PBS was added to the wells and remaining cells collected. Samples were centrifuged for 13000 rpm (4550g) for 5 min. The pellets were resuspended in 50µL of a 0.1% solution of Triton X-100 containing 5mg/ml Pepstatin A (Calbiochem, UK), 5mg/ml Bovine Lung Aprotinin (Calbiochem, UK) and 5mg/ml Antipain hydrochloride (Calbiochem, UK). Samples were then incubated for 30 min at room temperature on a shaker at 220 rpm before the addition of 50µL of a solution containing 10 mM $MnCl_2$ and 50mM Tris (pH 7.5). After incubation at 55^{o}C for 10 min to activate the arginase enzyme, a 25µL aliquot of each sample was added to 25µL of 0.5M arginine (pH 9.7) and incubated at 37^{o}C for 1 hour. To terminate the reaction, 400µL of an acid solution containing H_2SO_4, H_3PO_4 and H_2O in a ratio of 1:3:7 was added, along with 25µL of a 9% solution of ISPF. The acid mixture and ISPF were also added to 100µL aliquots of urea standards. Samples along with standards were incubated at 95^{o}C for 45 minutes, and then allowed to cool for 10 min in darkness. Aliquots (200µL) were added to wells of a 96 well plate and absorbance read at 540 nm on a Spectromax 190 plate reader. Arginase activity of the samples was determined relative to a standard curve constructed with solutions of urea. The enzyme activity which catalysed the production of 1 µMol urea/min was defined as one unit of arginase activity.

2.2.6 Measurement of NO production in BMD-macrophages

Frozen cell supernatants were thawed and 50µL aliquots of samples were added into wells of a 96 well plate. Greiss Reagents were then mixed in a ratio of 1:1 [2% (w/v) sulphanilamide in 5% (v/v) H_3PO_4 and 0.2% (w/v) naphylethylenediamine HCl in water] and 50µL added to each well. After incubation for 10 min at room temperature in the dark, absorbance was read on a Spectromax 190 plate reader at 540 nm. Nitrite production was determined relative to a standard curve constructed with solutions of sodium nitrite ($NaNO_2$) as described by (Griess, 1879, Tsai et al., 1999), from a 10 mM stock solution of $NaNO_2$ prepared in complete RPMI 1640 cell medium.

2.2.7 Scanning densitometry

Western blot films were scanned on an Epson perfection 164054 Scanjet using Epson twain 55.52 (32.32) Scan jet Picture software. All scanned images were normalized to a control and results were quantified using Scion Image Program (Scion Corp., Maryl and, USA).

2.2.8 Data analysis

All data, including densitometry data, were generated from immunoblots was expressed as mean ± S.E.M. for at least two separate observations. The statistical significance of differences between mean values from control and treated groups were determined by the one-way analysis of variance (ANOVA) using GraphPad Prism® Version 4.0 software or one tailed Students Unpaired t-test. $P < 0.05$ was taken as being significant.

CHAPTER 3

THE EFFECT OF *LEISHMANIA MEXICANA* AMASTIGOTES ON MACROPHAGE INTRACELLULAR SIGNALLING AND IMMUNE RESPONSES

3.1 INTRODUCTION

The signalling pathways involved in regulating inflammatory processes include signals that both start and maintain inflammation and signals that promote a return to normal conditions. Macrophages are a major component of the mononuclear phagocyte system that consists of closely related cells of bone marrow origin, including blood monocytes and tissue macrophages. There are three important functions of macrophages within the inflammatory process; phagocytosis, antigen presentation, and immunomodulation through production of various cytokines and growth factors. These functions are mediated through activation of a number of important conserved signalling pathways such as the MAP kinases, including p38 MAP kinase, ERK and JNK, and also the NF-κB cascade, activated in response to LPS. Another important cascade involves the JAK/STAT pathway activated in response to IFN-γ. Activation of these cascades leads to the production of inflammatory cytokines such as tumor necrosis factor (TNF-α), IL-1 and IL-12 see review by (Dong et al., 2002).

In the mammalian host, *Leishmania* is an obligate intracellular pathogen infecting the hematopoietic cells of the monocyte/macrophage lineage, which it enters by phagocytosis. Since the macrophage is expert in the destruction of entering pathogens and triggering of the host immune response, *Leishmania* has had to develop a range of complicated mechanisms to subvert the normal function of macrophages (Olivier et al., 2005). For example, it has been demonstrated that treatment of LPS/IFN-γ stimulated RAW 264.7 macrophages with *Leishmania* results in macrophages becoming unresponsive to subsequent parasite infection due to MAP kinase deactivation and impairment of NF-κB transcriptional activity (Ben-Othman et al., 2009). It has also been shown that NF-κB activity of macrophages is inhibited by *Leishmania mexicana* amastigote infection, apparently through the activity of the CPB virulence factor which limits NF-κB activity and thus decreases cytokine gene transcription leading to reduced IL-12 production (Cameron et al., 2004). However, there is a relative lack of information regarding the effect of *L. mexicana* on macrophage signalling events.

In this chapter the effect of *L. mexicana* amastigotes on the expression and activation of intermediates of both the MAP kinase and NF-κB pathways stimulated with LPS were examined using BMD-macrophages. These effects were correlated with 2 inflammatory protein markers, COX-2 and iNOS, as well as the release of the cytokines IL-12, IL-6 and IL-10. Later on in the chapter the corresponding effect of amastigotes on IFN-γ stimulated JAK/STAT phosphorylation was also assessed together with selected inflammatory markers.

3.2 THE EFFECT OF *LEISHMANIA MEXICANA* AMASTIGOTES ON SIGNALLING PATHWAYS ACTIVATED BY LPS AND IFN-γ INVOLVED IN INFLAMMATION IN MACROPHAGES

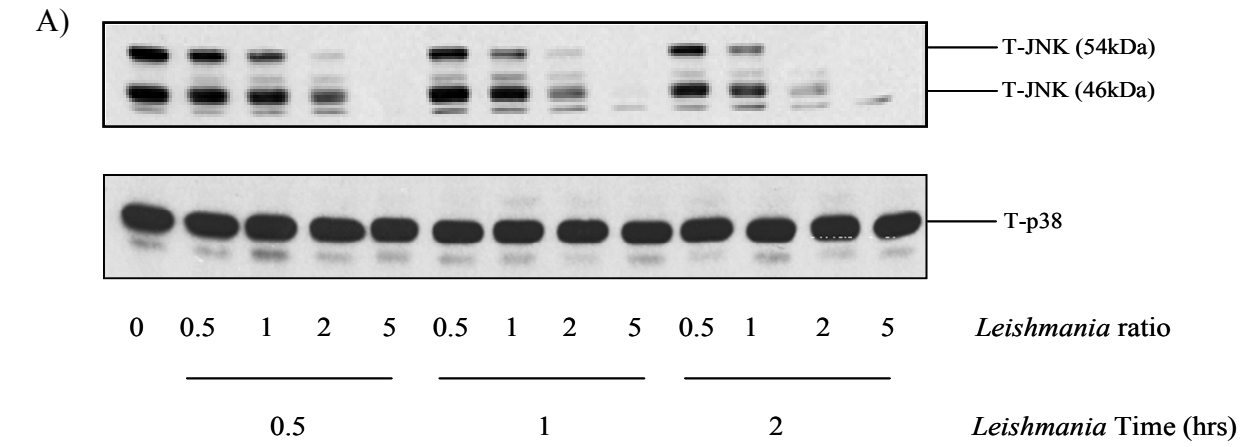
3.2.1 The effect of *Leishmania* infection on MAP Kinase protein expression in BMD- macrophages

Macrophages were infected with increasing concentrations of parasites (0.5-5:1 parasite /macrophage) for different times to assess the effect upon the expression and phosphorylation of MAP kinases. These effects were correlated with changes in the induction of the inflammatory proteins COX-2 and iNOS and cytokines IL-12, IL-6, and IL-10.

3.2.1.1 The effect of *Leishmania mexicana* infection on JNK protein expression and phosphorylation

Initially the kinetics and concentration-dependent effects of amastigotes upon JNK expression in macrophages were examined. Uninfected cells showed constitutive expression of both 46 and 54 kDa isoforms of JNK1/2, whereas macrophages infected with increasing concentration of parasites (0.5-5x10^6 parasites /macrophage) for different times caused a marked decrease in protein expression (Figure 3.1). At a ratio of 0.5:1 there was little degradation over the 2 hrs time course, however at ratios between 1:1 and 5:1 there was substantial degradation of both JNK isoforms. At a maximum ratio of 5:1 the JNK was abolished as early as 30 min (91.12 ± 3.82 1:1, 0.67 ± 0.33 5:1, for 30 min, ***$p<0.001$).

Phosphorylation of JNK in LPS stimulated macrophages infected with amastigotes at a ratio of 5:1 was then examined (Figure 3.2). LPS alone stimulated JNK phosphorylation in BMD-macrophages, reaching maximal levels between 15 and 30 min post stimulation (fold stim. at 15 and 30 min= 22.34 ± 2.87 and 23.56 ± 2.29). Following infection with amastigotes, for 2 hours prior to LPS, JNK phosphorylation was essentially abolished. This effect correlated with complete degradation of JNK similar to that observed above.



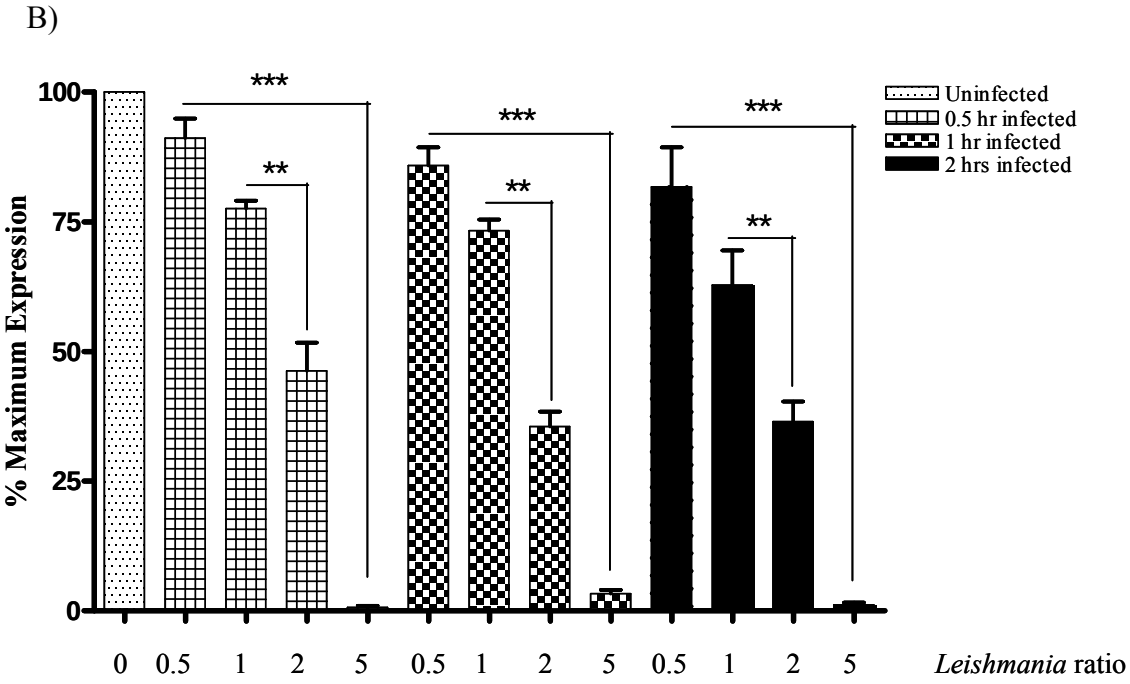
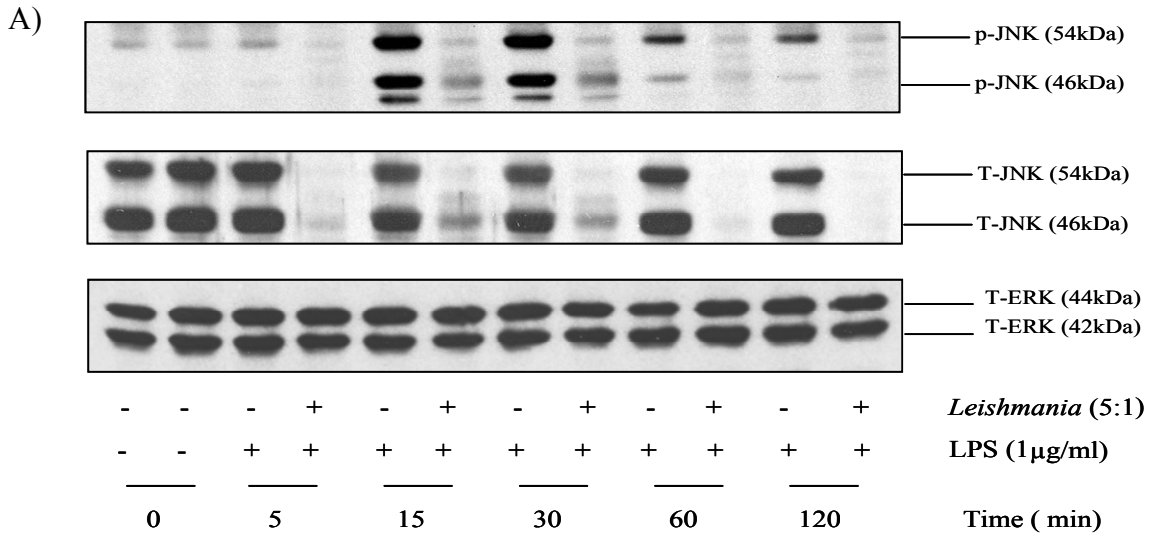


Figure 3.1: The effect of *Leishmania mexicana* infection on T-JNK protein expression in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* amastigotes at different concentrations as indicated for different times. Whole cell lysates were prepared and then assessed for A) JNK (as outlined in Section 2.2.3). Blots were quantified in B) by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings. **p<0.01 and ***p<0.001 compared with agonist-stimulated. T-p38 was used as a loading control.



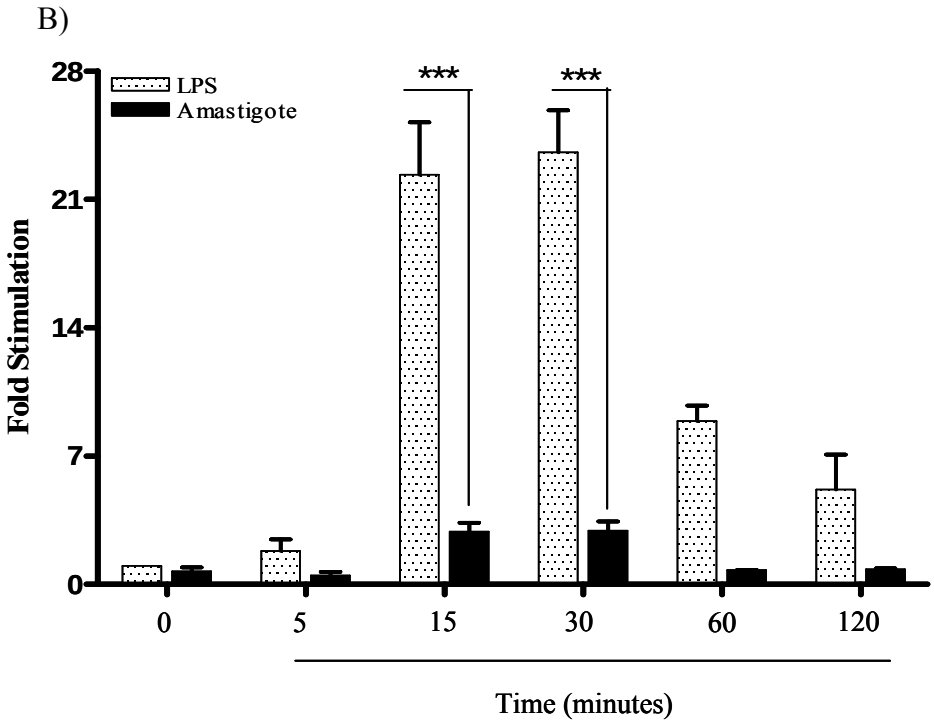


Figure 3.2: The effect of *Leishmania mexicana* infection on LPS-induced JNK phosphorylation and endogenous JNK protein expression. Cells ($1x10^6$/well) were infected with *Leishmania* amastigotes at a ratio of (5:1) for 2 hrs and then stimulated with LPS (1µg/ml) for 2 hrs. Whole cell lysates were prepared and then assessed for A) p-JNK (46/55 kDa), T-JNK and T-ERK as outlined in Section 2.2.3. Blots were quantified in B) by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings. ***$p<0.001$ compared with LPS-stimulated.

3.2.1.2 The effect of *Leishmania* infection on p38 MAP kinase protein expression and phosphorylation

Having established that *Leishmania* infection resulted in degradation of JNK, it was necessary to determine the effect upon p38 MAP kinase. Surprisingly, results demonstrated no change in the expression of this protein following amastigote infection (Figure 3.3). Similarly amastigote pre-treatment had no significant effect upon LPS induced p38 phosphorylation (Figure 3.4). Even in the presence of *Leishmania*, the LPS response was typical for p38 MAP kinase phosphorylation reaching a maximum by 30 min before returning to basal values by 120 min (fold stim. *Leishmania* plus LPS = 15.03 ± 0.78 at 30 min).

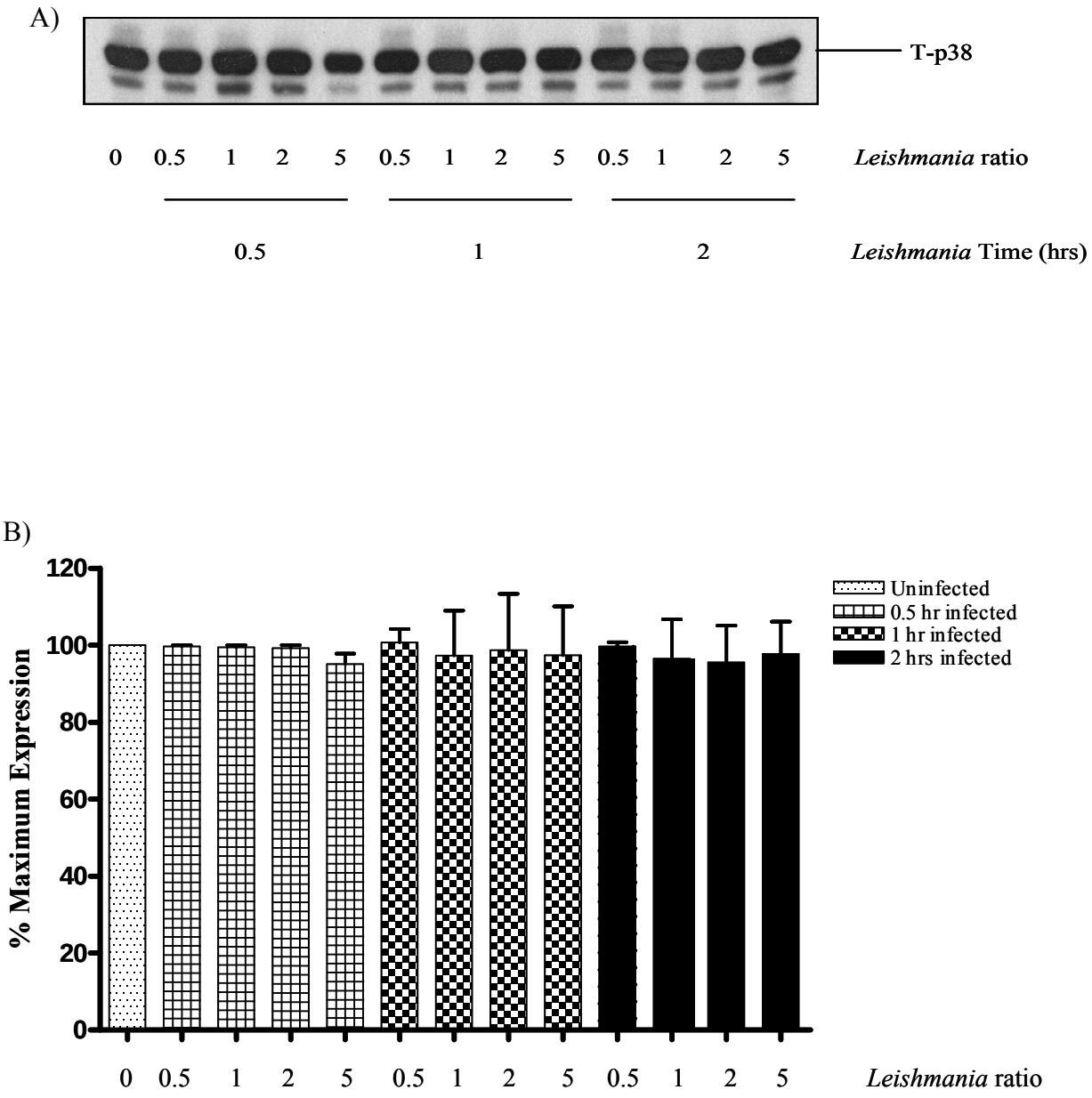


Figure 3.3: The effect of *Leishmania* infection on T-p38 protein expression in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* amastigotes at different concentrations as indicated for different times. Whole cell lysates were prepared and then assessed for A) p38 MAP as outlined in Section 2.2.3. Blots were quantified in B) by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings.

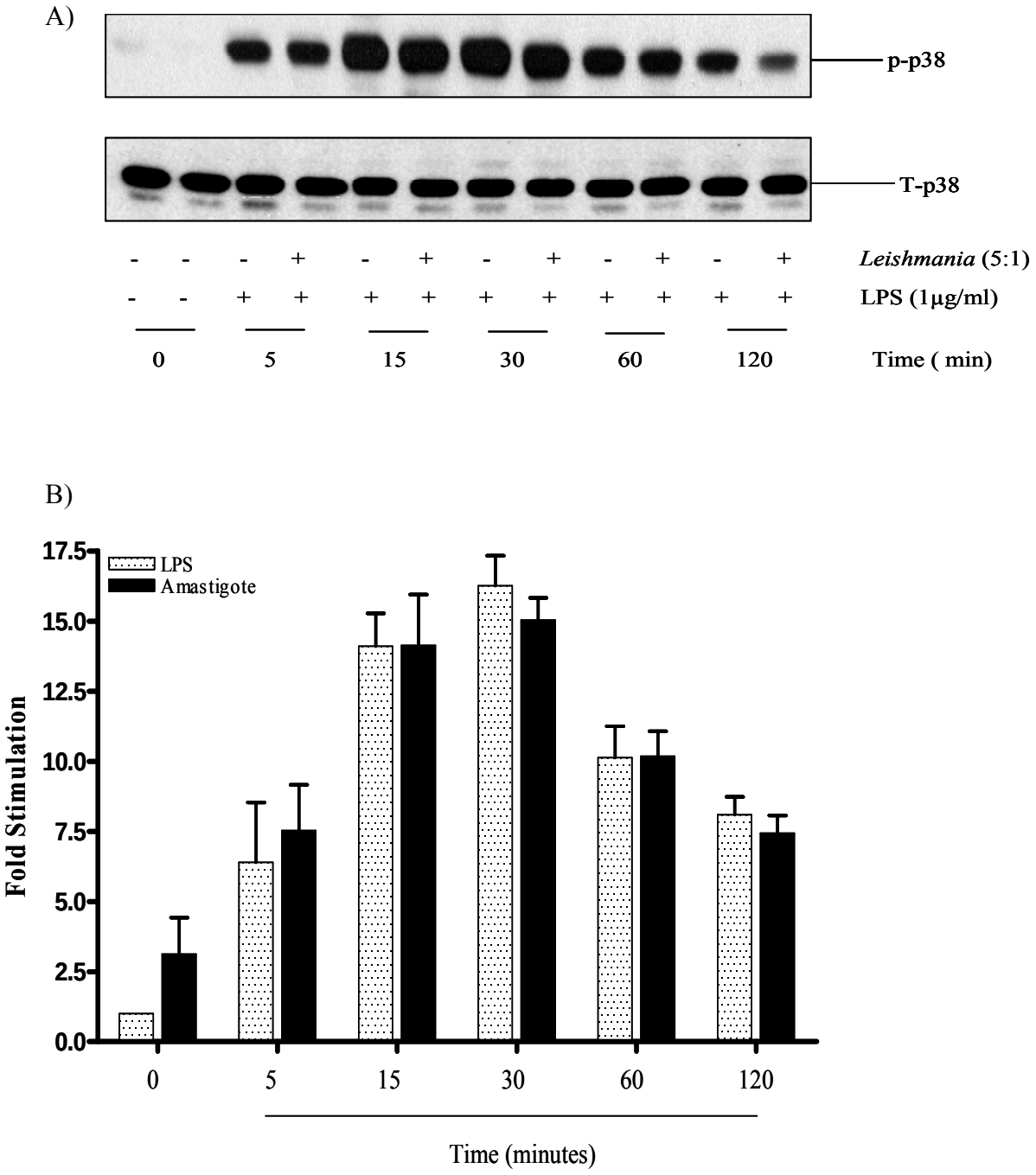


Figure 3.4: The effect of *Leishmania* infection on LPS-induced p38 phosphorylation and endogenous p38 protein expression. Cells ($1x10^6$/well) were infected with *Leishmania* amastigotes at ratio of (5:1) for 2 hours and then stimulated with LPS (1µg/ml) for 2 hrs. Whole cell lysates were prepared and then assessed for (A) p-p38 as outlined in Section 2.2.3. Blots were quantified in (B) by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings.

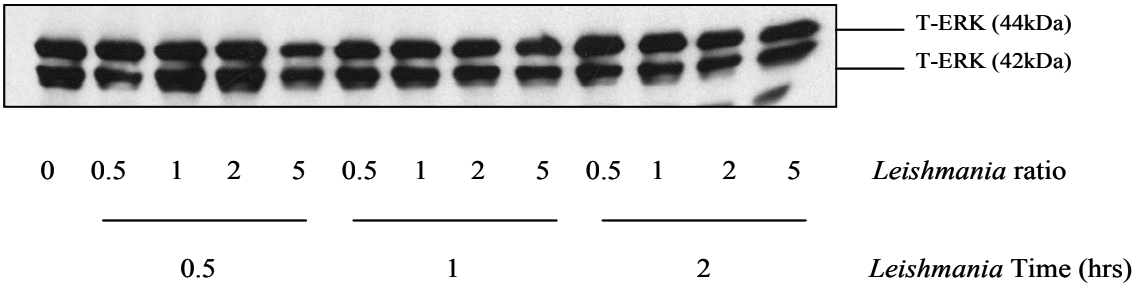
3.2.1.3 The effect of *Leishmania* infection on ERK protein expression and phosphorylation

Western blot analysis was carried out to examine ERK levels and phosphorylation status following amastigote infection. Macrophages were inoculated with different concentrations of amastigotes for a period up to 120 min. Surprisingly, results showed no alteration of endogenous ERK protein in the cells following infection (Figure 3.5), whereby levels remained similar throughout.

The effect of amastigotes on the LPS response was then examined (Figure 3.6). As with JNK and p38, LPS was found to be a strong inducer of ERK phosphorylation, reaching maximal levels between 15 and 30 min post stimulation (fold stim. LPS at 15 min, 10.85 ± 1.05). By contrast, in macrophages that were infected with *Leishmania* for 2 hours, the phosphorylation of ERK was abolished. (fold stim. *Leishmania*+LPS = 0.15 ± 0.03 at 15 min, ***$p<0.001$). Again, cellular ERK expression was not affected by *Leishmania* infection.

Surprisingly, as shown from results in Figure 3.7, ERK phosphorylation is affected but with no effect on cellular ERK levels. Therefore the effect of *Leishmania* on MEK-1 expression was examined in the equivalent samples for ERK phosphorylation. Amastigote infection caused a rapid and complete degradation of MEK1 which would adequately explain the inhibitory effect on ERK phosphorylation.

A)



B)

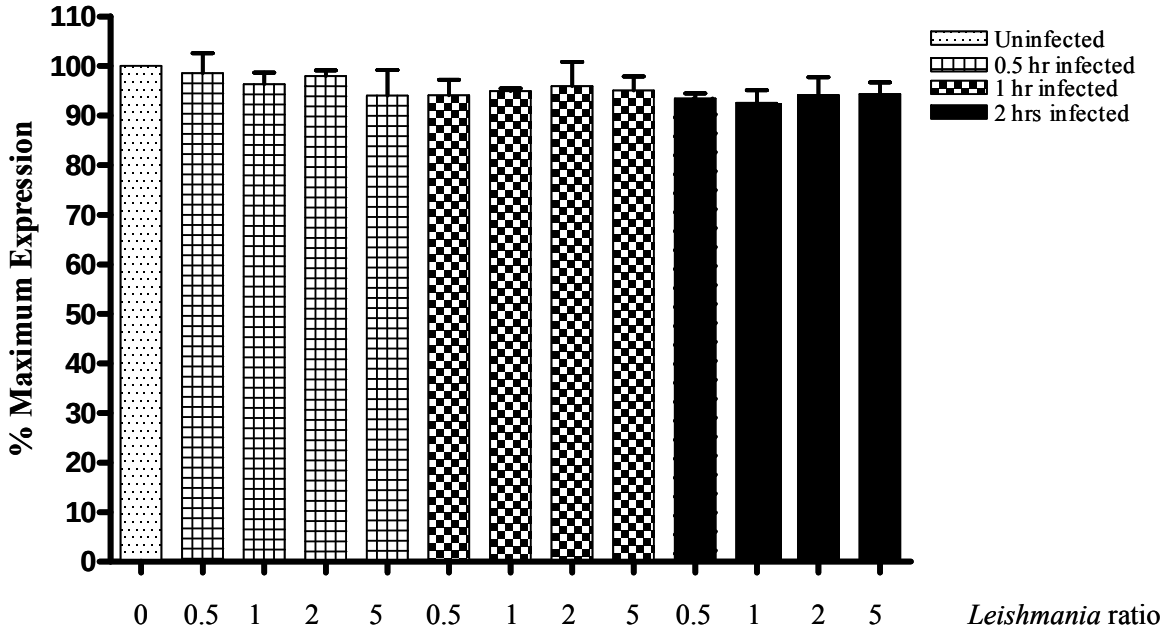


Figure 3.5: The effect of *Leishmania* infection on T-ERK1/2 protein expression in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* amastigotes at different concentration as indicated for different times. Whole cell lysates were prepared and then assessed for A) ERK1/2 (44/42 kDa) as outlined in Section 2.2.3. Blots were quantified in B) by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings.

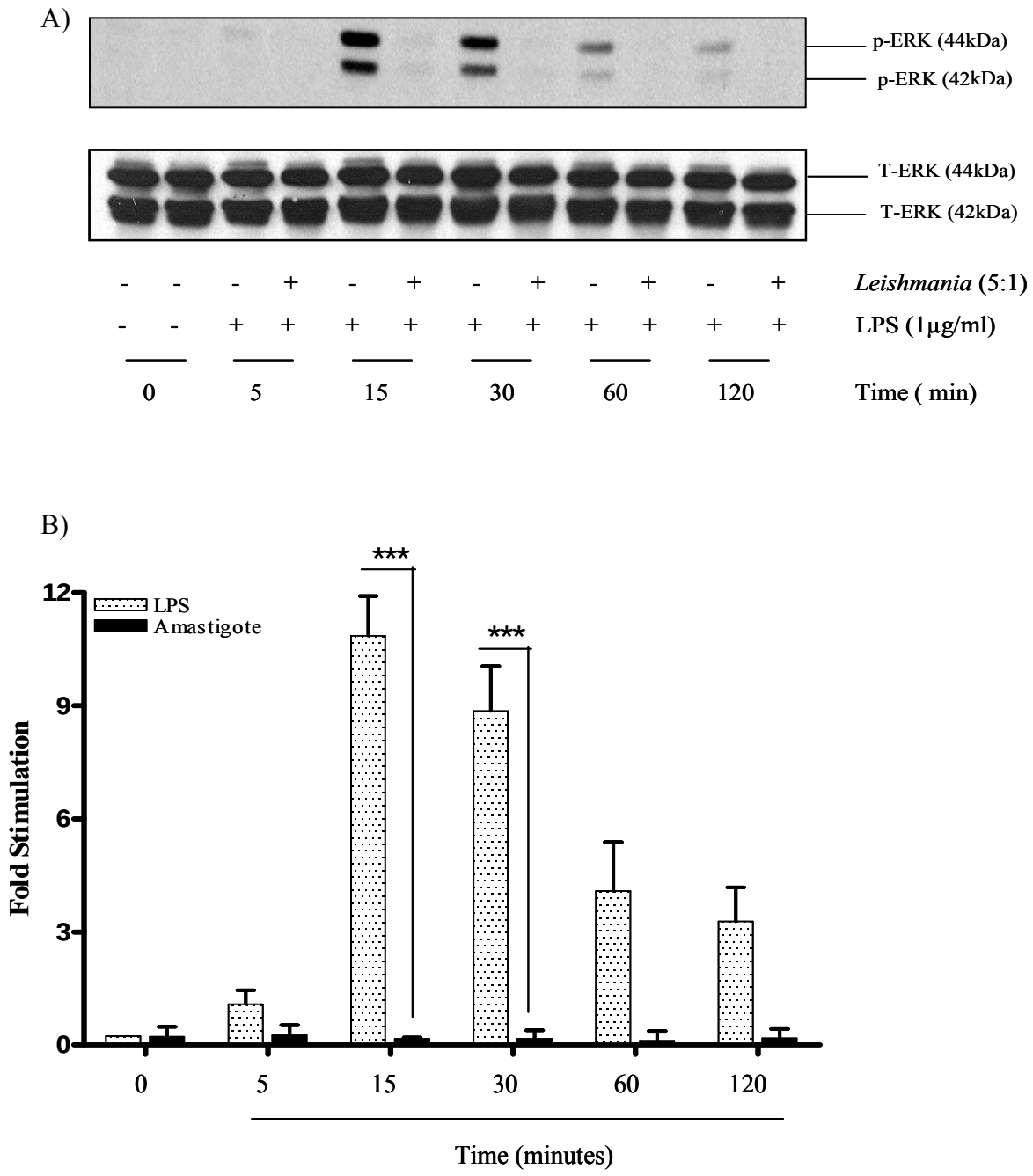


Figure 3.6: The effect of *Leishmania mexicana* infection on LPS-induced ERK1/2 phosphorylation. Cells ($1x10^6$/well) were infected with *Leishmania* amastigotes at a ratio of (5:1) for 2 hrs and then stimulated with LPS (1µg/ml) for a period of up to 2 hrs. Whole cell lysates were prepared and then assessed for (A) p-ERK1/2 (44/42 kDa) as outlined in Section 2.2.3. Blots were quantified in (B) by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings. ***$p<0.001$ compared with agonist-stimulated.

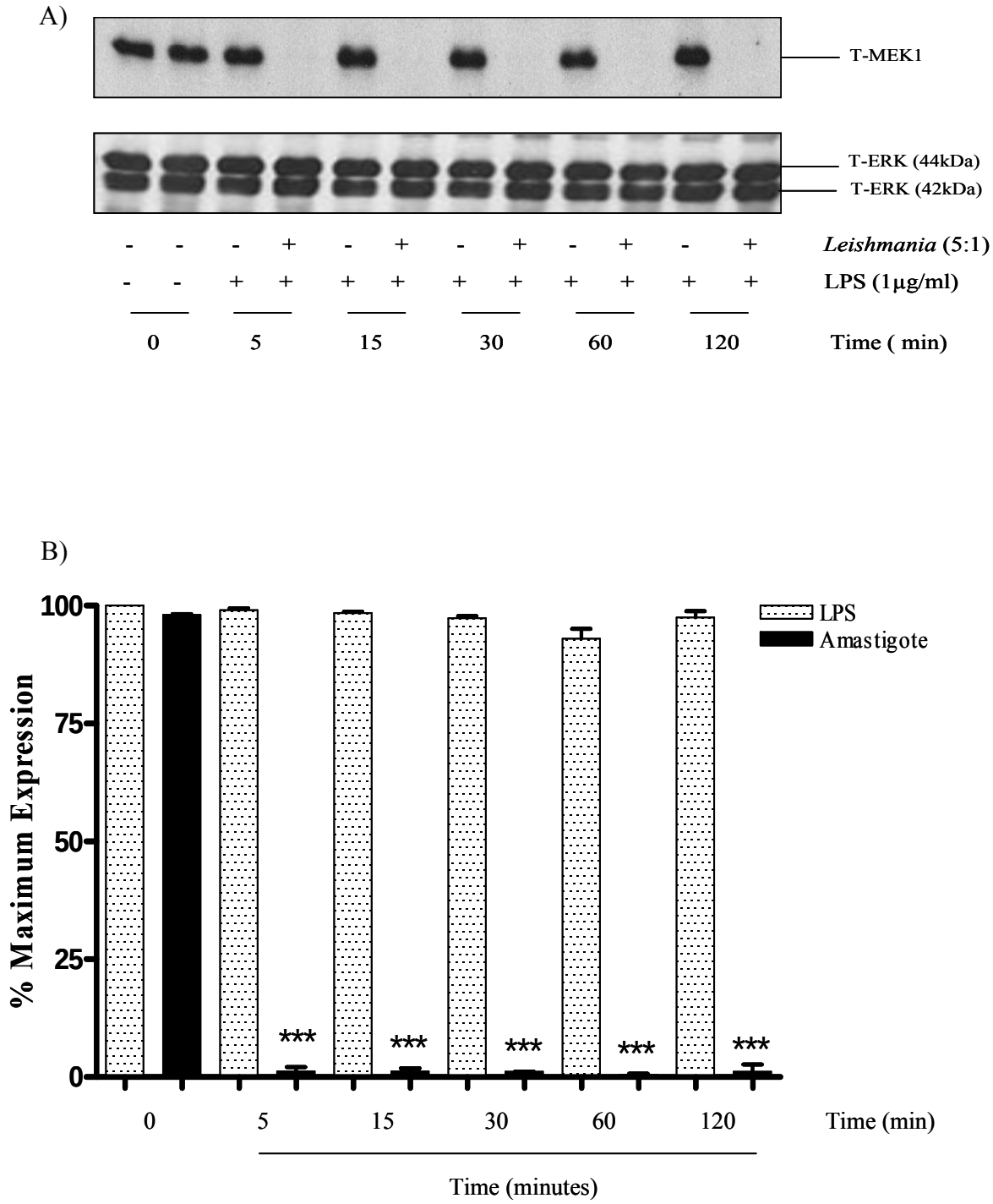


Figure 3.7: The effect of *Leishmania* infection on endogenous MEK-1 protein expression. Cells ($1x10^6$/well) were infected with *Leishmania* amastigotes at a ratio of (5:1) for 2 hrs and then stimulated with LPS (1µg/ml) for a period of up to 2 hrs. Whole cell lysates were prepared and then assessed for (A) T-MEK-1 (66 kDa) as outlined in Section 2.2.3. Blots were quantified in (B) by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings. ***$p<0.001$ compared with agonist-stimulated.

3.2.2 The effect of *Leishmania* infection on intermediates of the NF-κB pathway in BMD- macrophages

To confirm the actions of amastigotes were consistent with previous results from the laboratory (Cameron et al., 2004), the effect of *Leishmania* amastigotes on intermediates of the NF-κB pathway were examined. Initially the time and concentration dependent effect of amastigote infection was examined (Figure 3.8). As with JNK, p65 NF-κB was rapidly degraded but only in response to parasites at a ratio of 5:1. At lower ratios degradation was much slower and incomplete even after 2 hrs. (88.89 ± 4.00 for 1:1, 4.43 ± 0.98 for 5:5, at 30 min). The effect of amastigotes on phosphorylation of p65 NF-κB was then examined. Pre-treatment at a ratio of 5:1 amastigotes effectively abolished the subsequent phosphorylation of p65 NF-κB - stimulated in response to LPS (Figure 3.9).

The degradation of IκB-α was also examined following incubation with amastigotes over a time period of 120 min and different concentrations. In a manner similar to p65 NF-κB, IκB-α was rapidly degraded over time such that protein levels were abolished by 2 hrs (82.19 ± 0.96 for 1:1, 2.05 ± 0.50 for 5:1 at 2 hrs). (Figure 3.10).

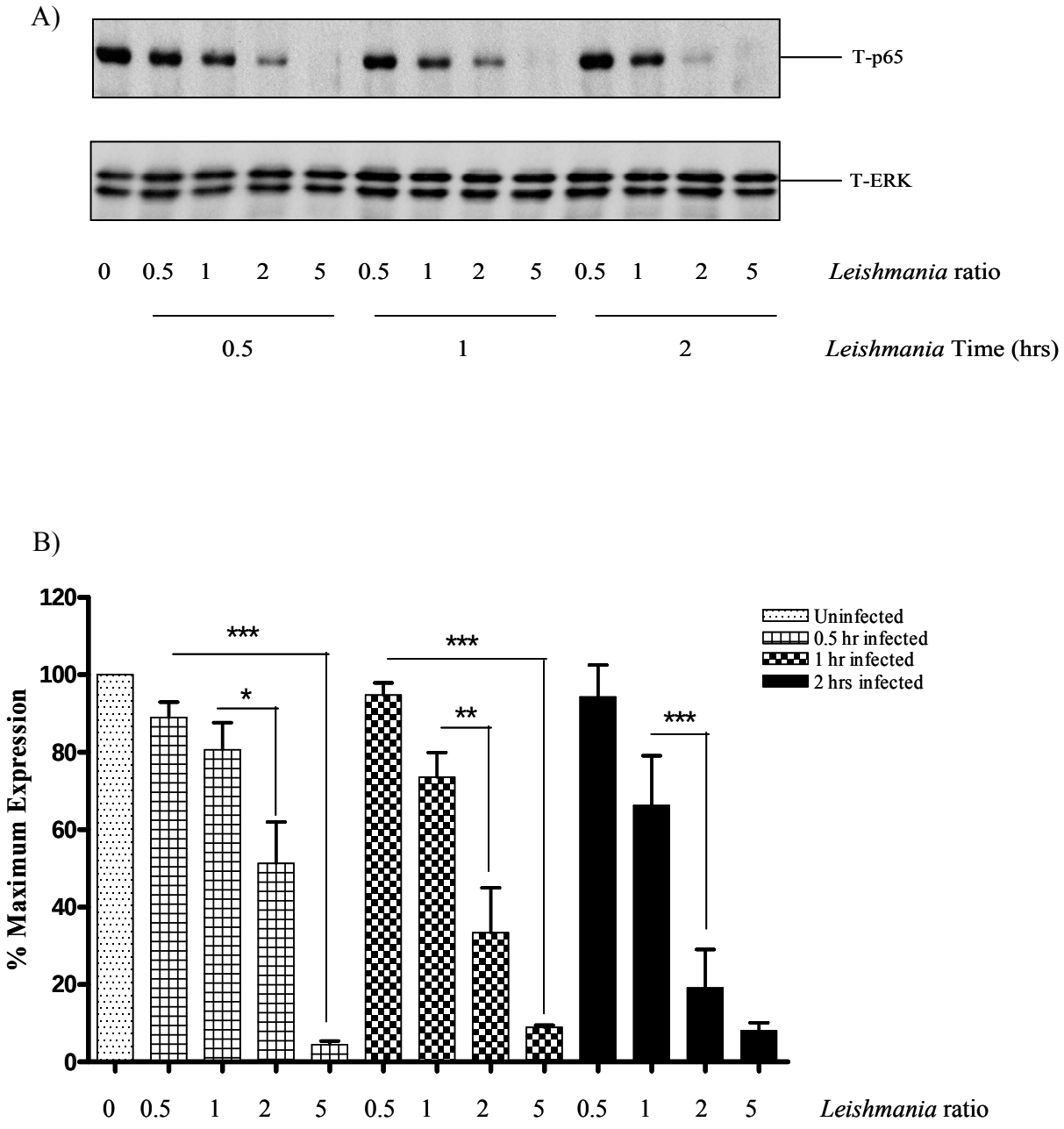


Figure 3.8: The effect of *Leishmania mexicana* infection on T-p65 protein expression in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* amastigotes at different concentrations as indicated for different times. Whole cell lysates were prepared and then assessed for A) NF-κB (p65) and T-ERK as outlined in Section 2.2.3. Blots were quantified in B) by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared with agonist-stimulated.

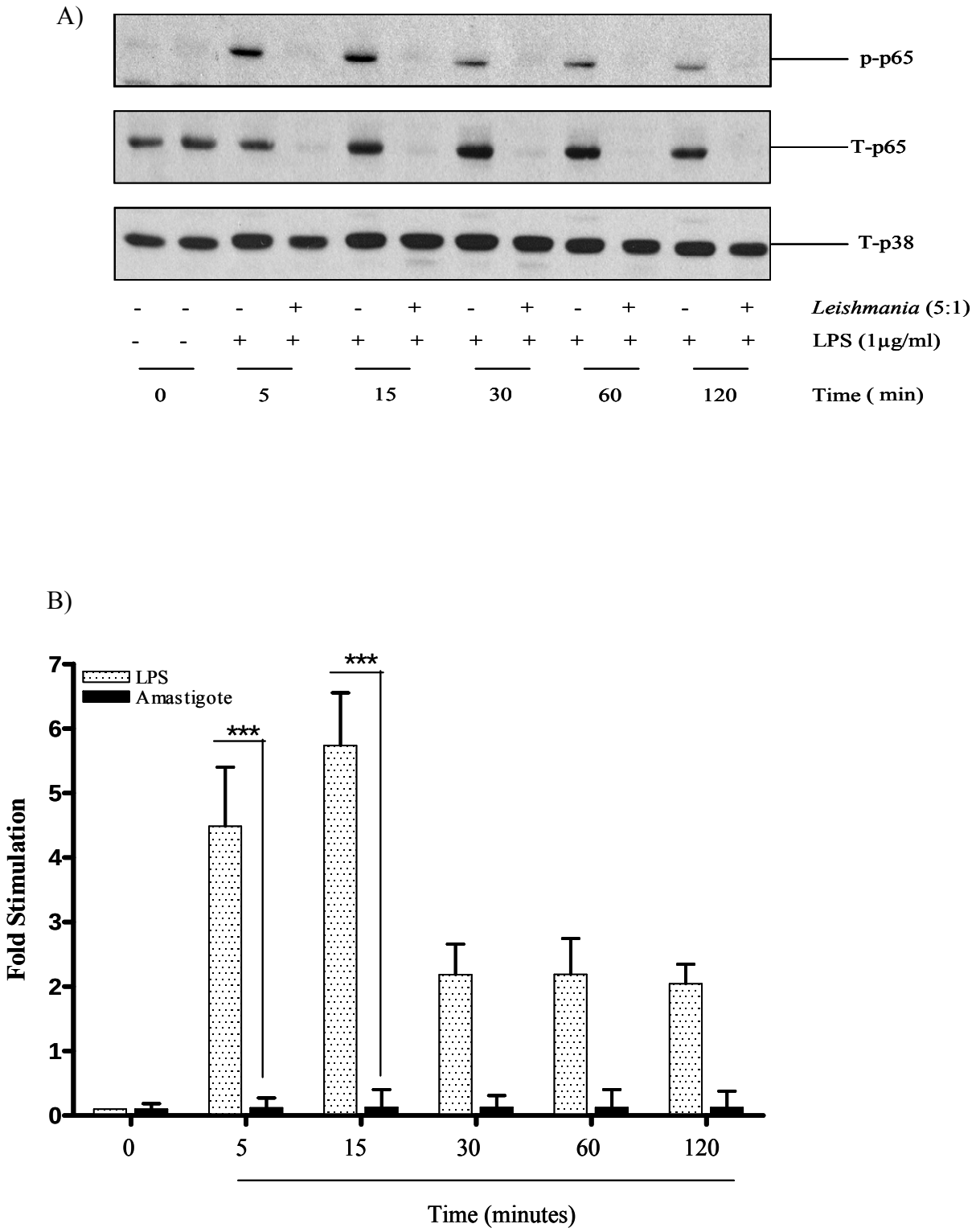


Figure 3.9: The effect of *Leishmania mexicana* infection on LPS-induced NF-κB phosphorylation and endogenous p65 protein expression. Cells ($1x10^6$/well) were infected with *Leishmania* amastigotes at a ratio of (5:1) for 2 hrs and then stimulated with LPS (1µg/ml) for a period of up to 2 hrs. Whole cell lysates were prepared and then assessed for A) for p-p65 (NF-κB) as outlined in Section 2.2.3. Blots were quantified in B) by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings. ***$p<0.001$ compared with agonist-stimulated.

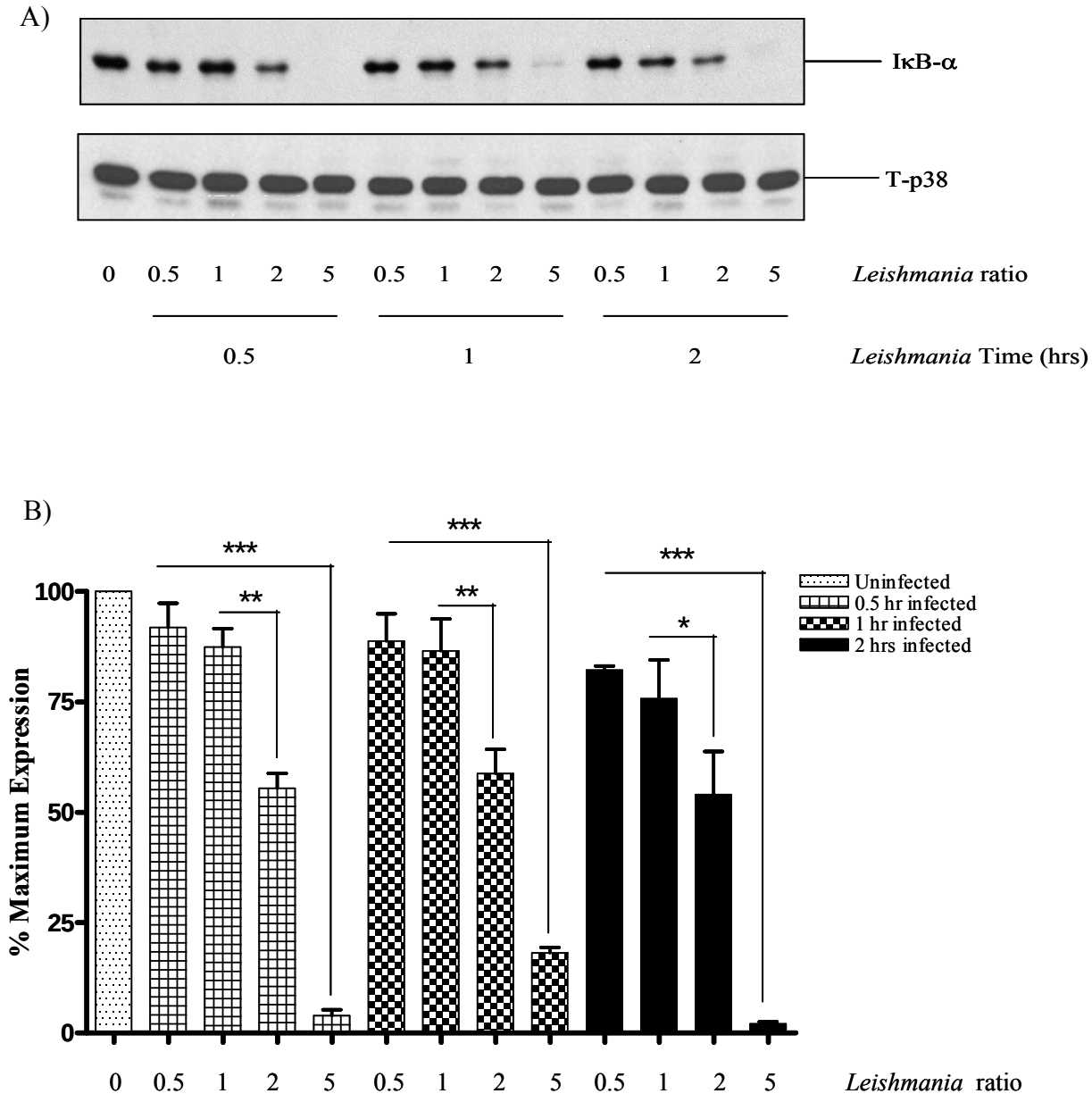


Figure 3.10: The effect of *Leishmania mexicana* infection on IκB-α protein expression in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* amastigotes at different concentrations as indicated for different times. Whole cell lysates were prepared and then assessed for A) for IκB-α (38 kDa) and T-p38 as outlined in Section 2.2.3. Blots were quantified in B) by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared with agonist-stimulated.

3.3 THE EFFECT OF *LEISHMANIA MEXICANA* AMASTIGOTE INFECTION ON LPS IN INFLAMMATORY PROTEIN EXPRESSION IN MACROPHAGES

3.3.1 LPS-stimulated COX-2 protein expression in BMD-macrophages

Having established that amastigote pre-treatment degraded key intermediates of the MAP kinase and NF-κB pathways, the subsequent effects upon the expression of the inflammatory proteins COX-2 and iNOS were examined. Initially, COX-2 was studied and Figure 3.11 illustrates the kinetics of protein expression in response to LPS stimulation over a period of 24 hrs. COX-2 protein expression was time-dependent and started to increase 4 hrs post stimulation (fold stim. 40.11 ± 5.34), gradually increasing to reach maximum expression after 6-8 hrs (fold stim. at 8 hrs, 95.61 ± 14.66 respectively) and was essentially maintained for up to 24 hrs.

3.3.2 The effect of *Leishmania* infection on COX-2 protein expression in BMD- macrophages

The effect of amastigotes on COX-2 induction stimulated by LPS was then examined. Two approaches were undertaken. First of all cells were pre-treated with amastigotes prior to stimulation with LPS for a further 6 hrs (Figure 3.12), or alternatively amastigotes were added subsequent to LPS stimulation (Figure 3.13). When macrophages were pre-treated with amastigotes, LPS induced COX-2 expression was significantly reduced by approximately 60 % (% inhibition= 61.02 ± 5.87, **$p<0.01$). When amastigotes were added subsequent to LPS stimulation there was a time-dependent decrease in COX-2 levels which was significant by 2 hrs, approximately 50 % of the control LPS values (% inhibition = 44 .267 ± 4.68, *$p<0.05$).

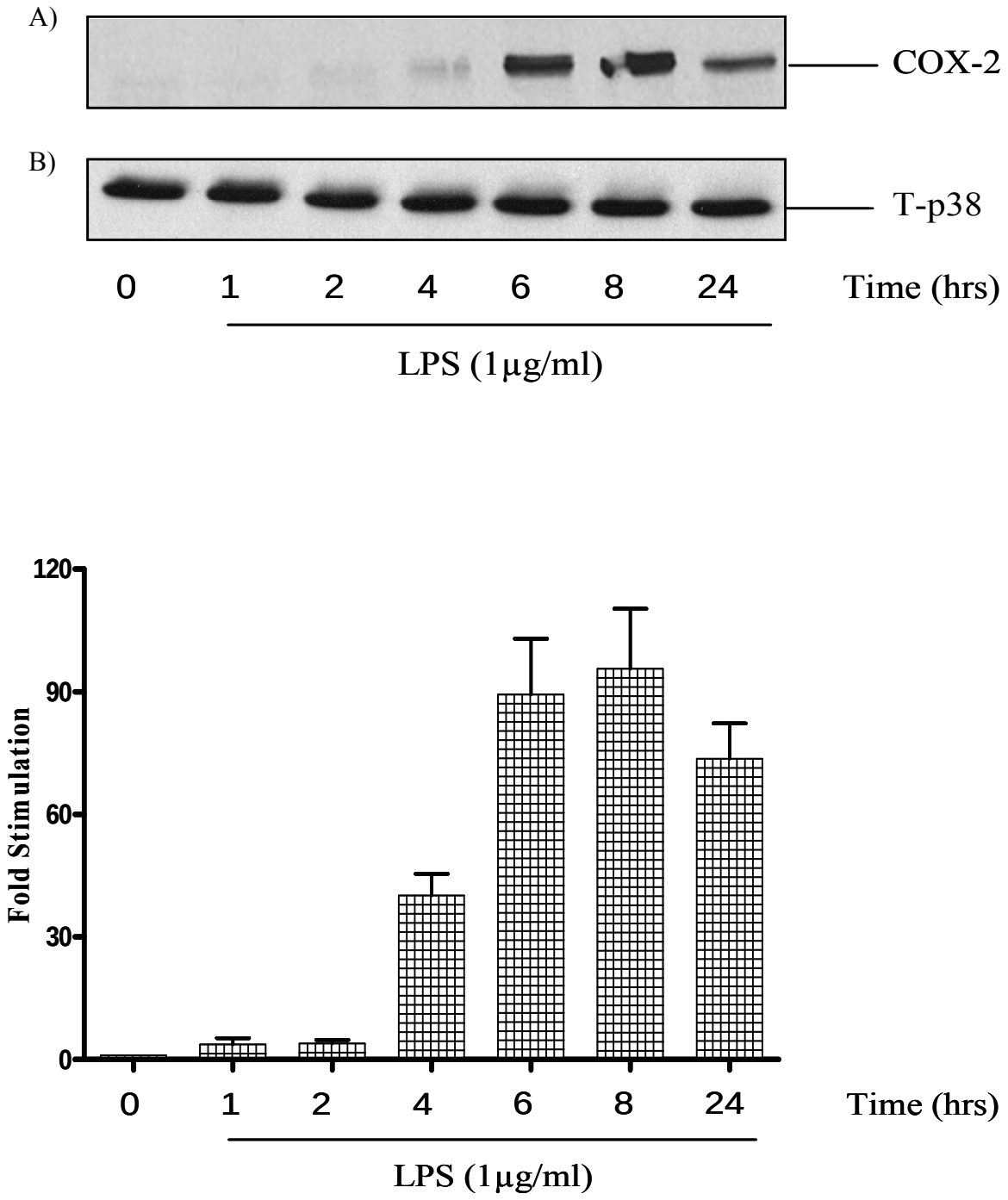


Figure 3.11: LPS-induced COX-2 protein expression in bone-marrow derived macrophages. Cells ($1x10^6$/well) were stimulated with LPS (1µg/ml) for indicated time. Whole cell lysates were prepared and then assessed for A) COX-2 (72 kDa) and B) T-p38 as outlined in Section 2.2.3. Blots were quantified by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings.

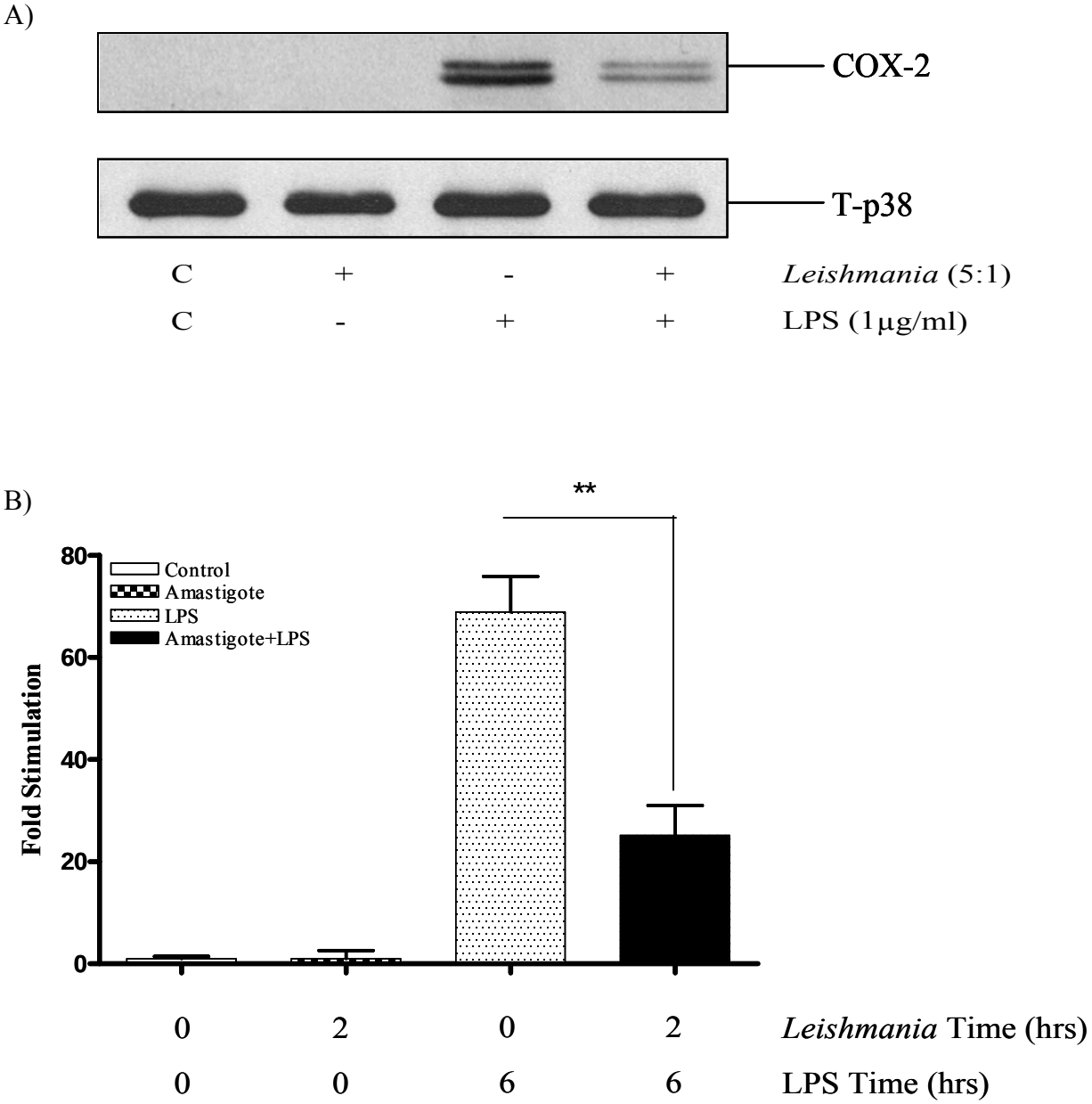


Figure 3.12: The effect of *Leishmania mexicana* infection on COX-2 protein expression in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* amastigotes at the ratio of (5:1) for 2 hrs and then stimulated with LPS (1μg/ml) for 6 hrs. Whole cell lysates were prepared and then assessed in A) for COX-2 (72 kDa) and T-p38 as outlined in Section 2.2.3, and B) Fold stimulation by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings. **$p<0.01$ compared with LPS-stimulated.

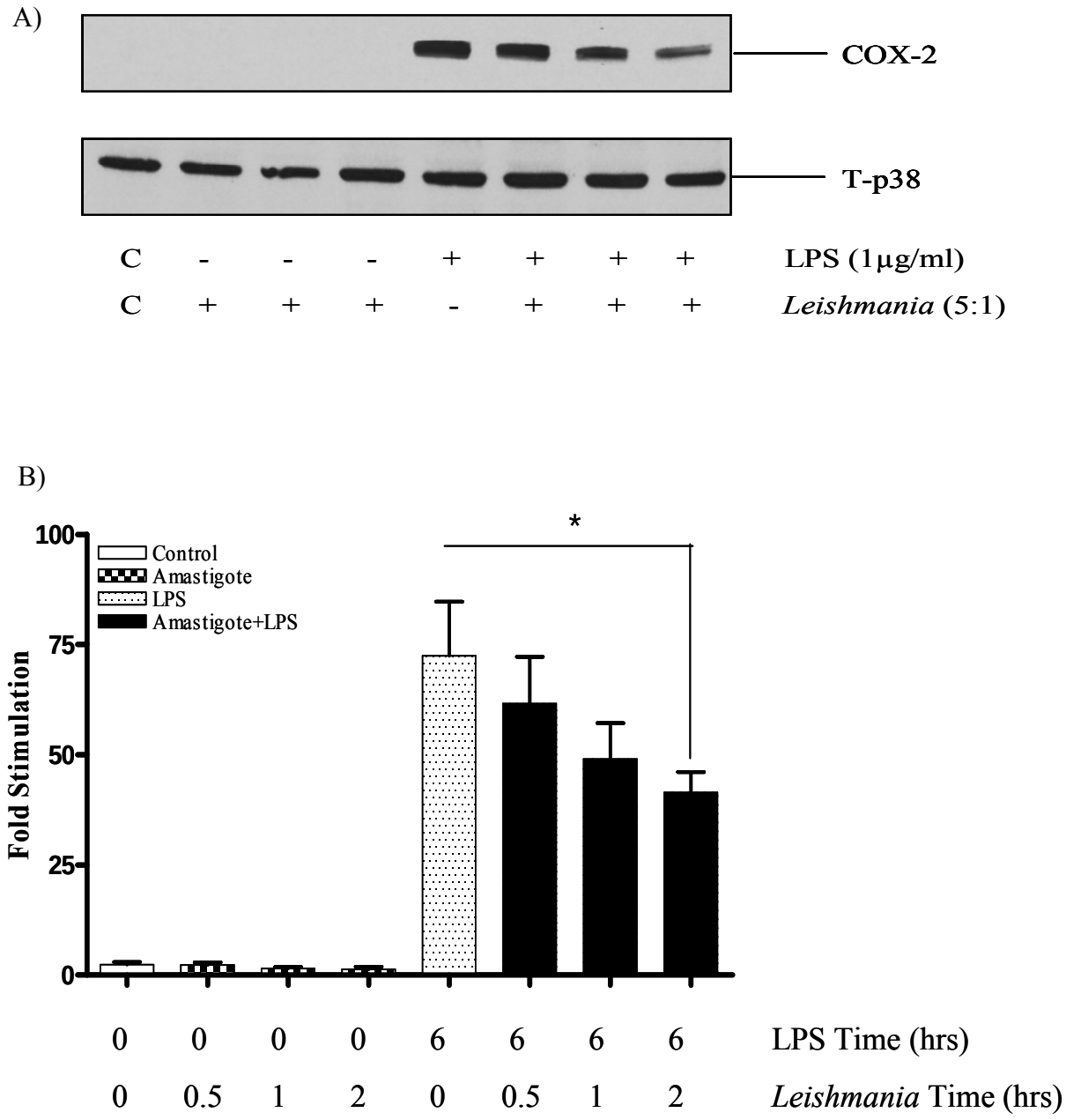


Figure 3.13: The effect of *Leishmania mexicana* infection on COX-2 protein expression in macrophages. Cells ($1x10^6$/well) were stimulated with LPS (1μg/ml) for 6 hours, and then infected with *Leishmania* amastigotes at the ratio of (5:1) for different times. Whole cell lysates were prepared and then assessed for A) COX-2 (72 kDa) and T-p38 as outlined in Section 2.2.3. B) Fold stimulation by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings. *$p<0.05$ compared with LPS-stimulated.

3.3.3 LPS-stimulated induction of iNOS protein expression in BMD-macrophages

A similar approach was used to examine the effect of amastigotes on iNOS expression. Initially the kinetics of iNOS induction was established as shown in Figure 3.14. Following a delay of 2 to 4 hrs, iNOS expression increased gradually over a 24 hrs time course. Maximum levels were obtained after 24 hrs, approximately 80 fold of basal values (fold stim. at 24 hrs = 81.05 ± 11.10). The 6 hr time point was used for subsequent experiments.

3.3.4 The effect of *Leishmania* infection on iNOS protein expression in BMD- macrophages

The effect of amastigotes pre-treatment on iNOS expression induced in response to LPS is shown in Figure 3.15. Even as early as 6 hrs, LPS gave a large increase in iNOS expression, almost 100 fold of basal values (94.16 ± 3.60). Amastigote pre-treatment effectively abolished iNOS protein expression (% inhibition= 97.241 ± 0.88, ***$p<0.001$). Furthermore, when cells were incubated with amastigotes subsequent to LPS induction, the degradation of iNOS was rapid and apparent as early as 30 minutes. However, degradation was not fully complete by the 2 hrs time point (% inhibition= 76.260 ± 4.42, **$p<0.01$). (Figure 3.16). These data show that iNOS is more susceptible to amastigote-mediated degradation compared to COX-2.

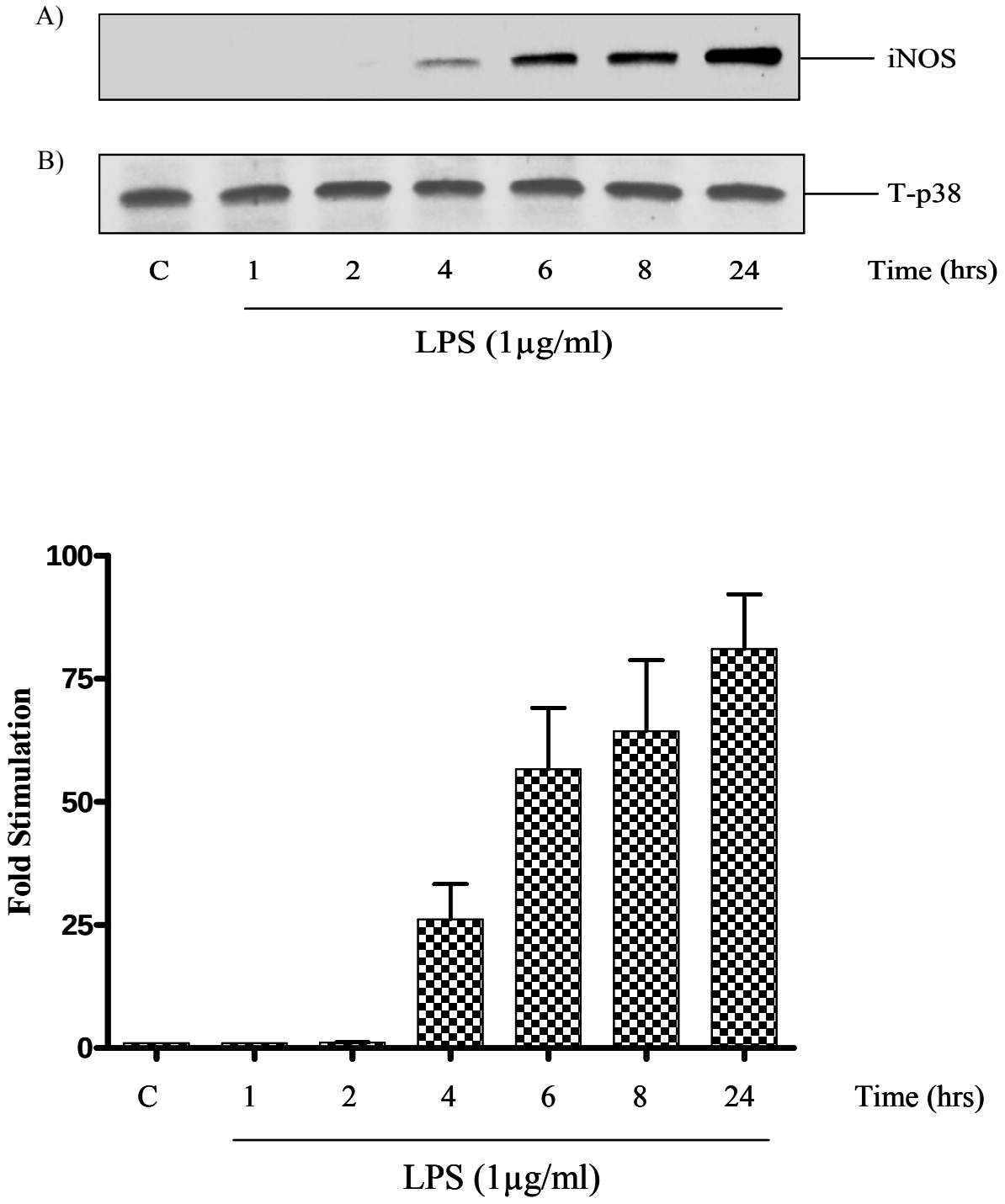


Figure 3.14: LPS-induced iNOS protein expression in bone-marrow derived macrophages. Cells ($1x10^6$/well) were stimulated with LPS (1µg/ml) for the times indicated. Whole cell lysates were prepared and then assessed for A) iNOS (130 kDa) and B) T-p38 as outlined in Section 2.2.3. Blots were quantified by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings.

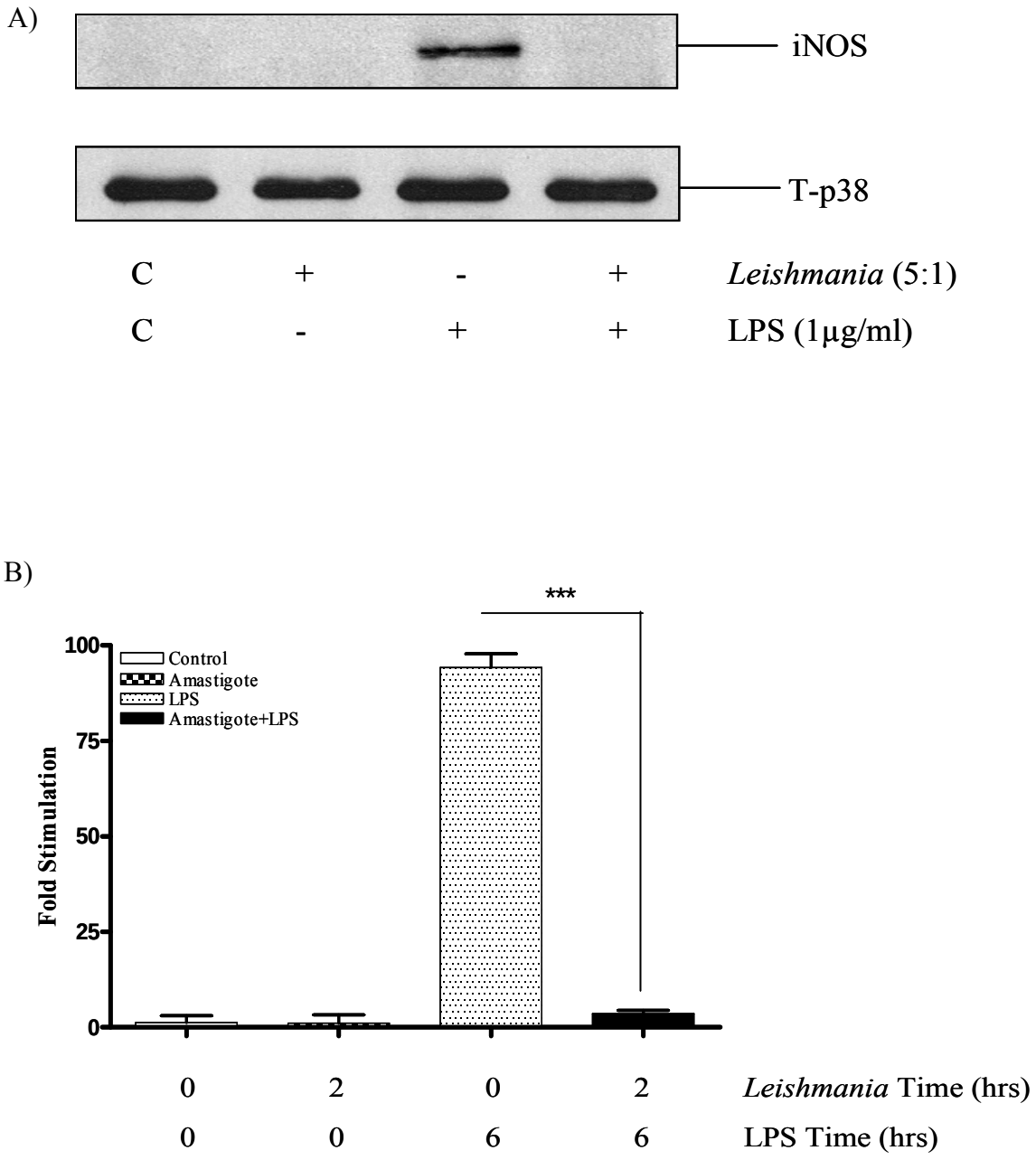


Figure 3.15: The effect of *Leishmania mecicana* infection on iNOS protein expression in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* amastigotes at the ratio of (5:1) for 2 hrs and then stimulated with LPS (1µg/ml) for 6 hrs. Whole cell lysates were prepared and then assessed for A) iNOS (130 kDa) as outlined in Section 2.2.3. B) Fold stimulation by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings. ***$p<0.001$ compared with agonist-stimulated.

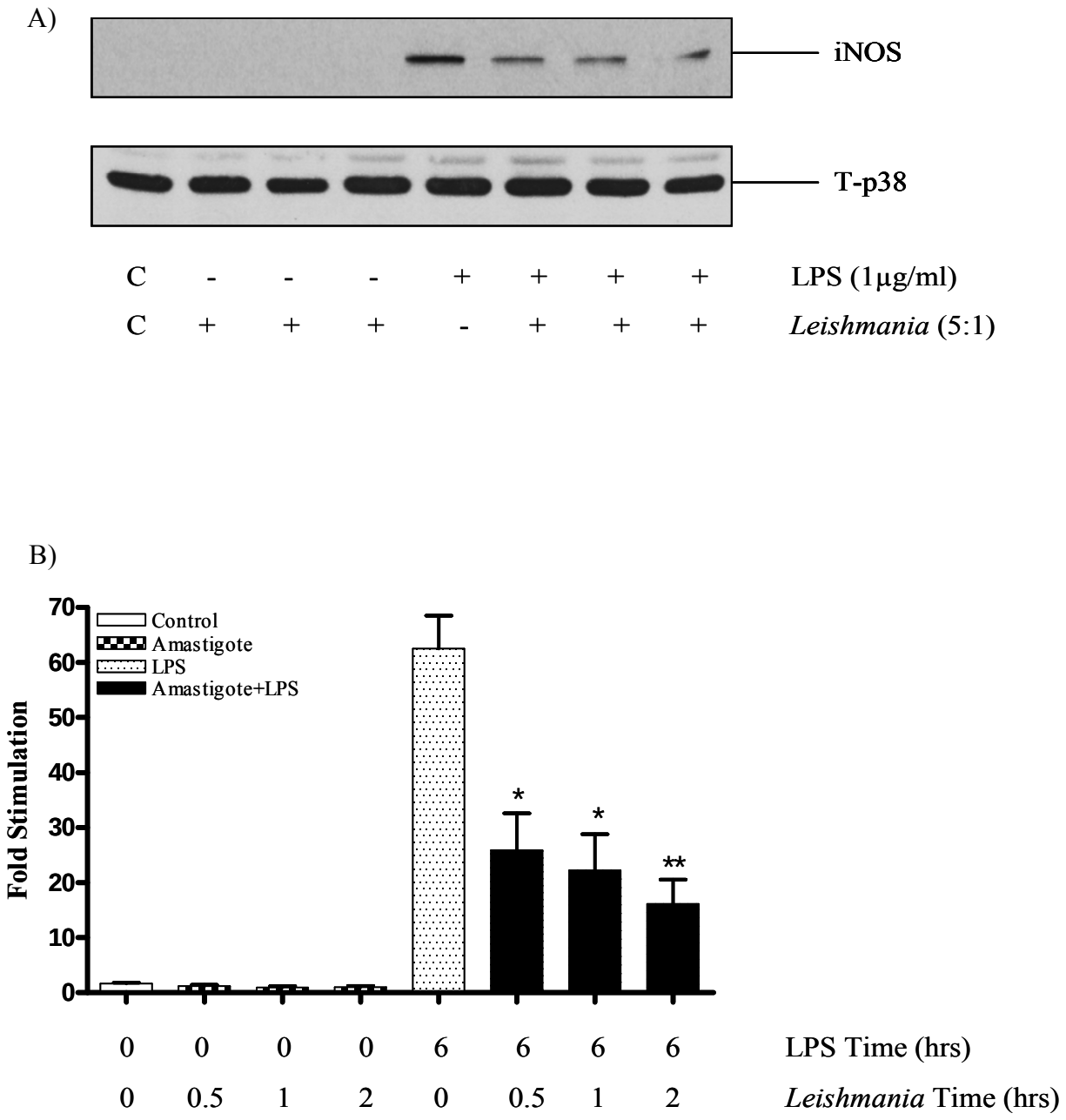


Figure 3.16: The effect of *Leishmania* infection on iNOS protein expression in macrophages. Cells ($1x10^6$/well) were stimulated with LPS (1µg/ml) for 6 hours, and then infected with *Leishmania* at the ratio of (5:1) for different times. Whole cell lysates were prepared and then assessed for A) iNOS (130 kDa- or T-p38) as a loading control as outlined in Section 2.2.3. In B) blots were quantified by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings. *$p<0.05$ and **$p<0.01$ compared with agonist-stimulated.

3.4 AMASTIGOTES MODULATION OF INFLAMMATORY CYTOKINE PRODUCTION

3.4.1 Time course for cytokine production by BMD-macrophages following stimulation with LPS

The effect of amastigotes on LPS-induced cytokine production was examined. The production of IL-12, IL-6 and IL-10 over the time course were determined from supernatants by ELISA. LPS stimulated induction of all three cytokines over 24 to 72 hrs. IL-6 and IL-10 production was maximal at 24 hrs and IL-12 at 48 hrs. Generally cytokine levels stayed to high up to 72 hrs (Figure 3.17).

3.4.2 Effect of amastigotes on LPS-induced cytokine production by BMD- macrophages

Figure 3.18 shows the effect of amastigotes on IL-12, IL-6 and IL-10 production assayed over 24 hours. Stimulation with LPS alone resulted in a 30-fold increase in IL-12 production however, this response was abolished following amastigote pre-treatment (panel A) (LPS = 16.77 ± 3.29, *Leishmania* plus LPS = 1.74 ± 0.76, ***$p<0.001$). By contrast, IL-6 production was not significantly affected by amastigote pre-treatment and levels were similar to LPS stimulated (panel B). The opposite trend was observed for IL-10 production (panel C). LPS stimulated a small 2-3 fold induction of IL-10 which was significantly potentiated in the presence of amastigotes (LPS = 0.79 ± 0.15, *Leishmania*+LPS = 4.65 ± 1.02, ***$p<0.001$).

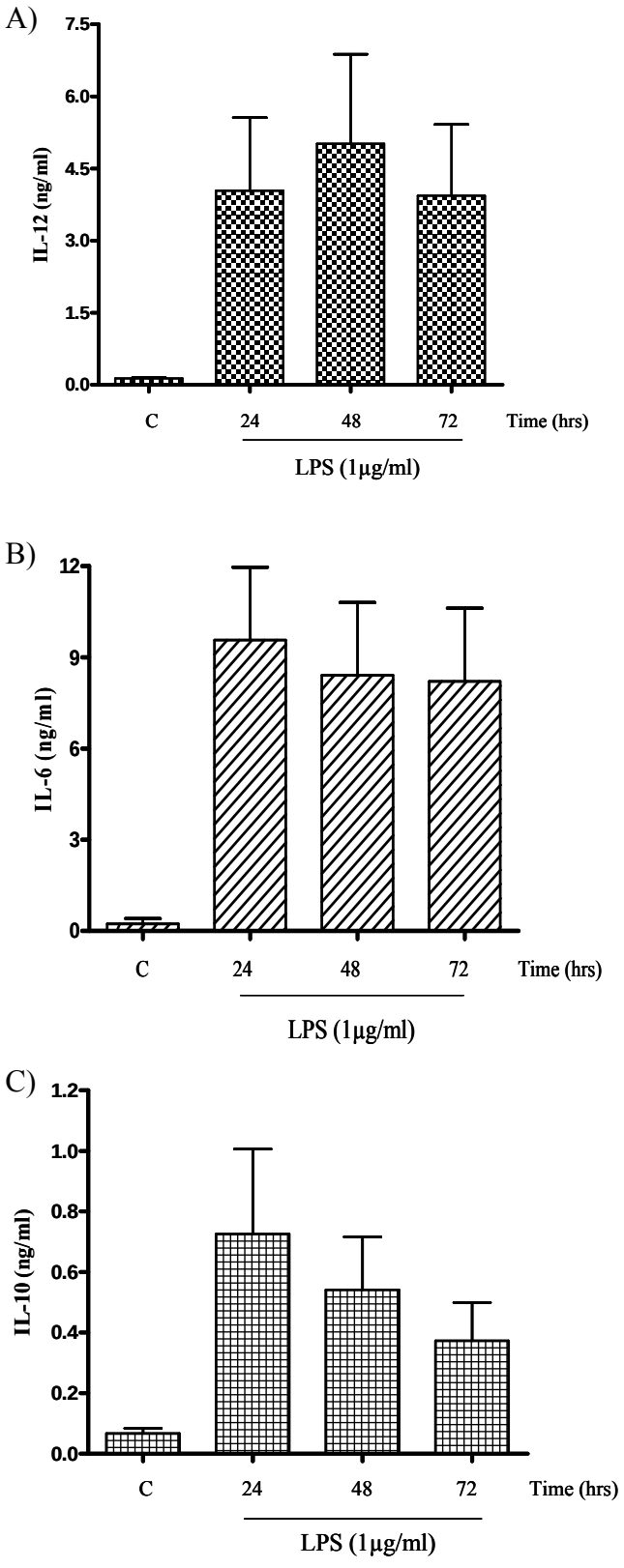


Figure 3.17: LPS-induced cytokine production in bone marrow-derived macrophages. Cells ($1x10^6$/well) were stimulated with LPS (1µg/ml) for the indicated times. Following stimulation, supernatants were assessed for **A)** IL-12, **B)** IL-6, and **C)** IL-10 as outlined in section 2.1.5. Quantification was expressed as the mean ± S.E.M. The results are representative of 3 individual experiments with similar findings.

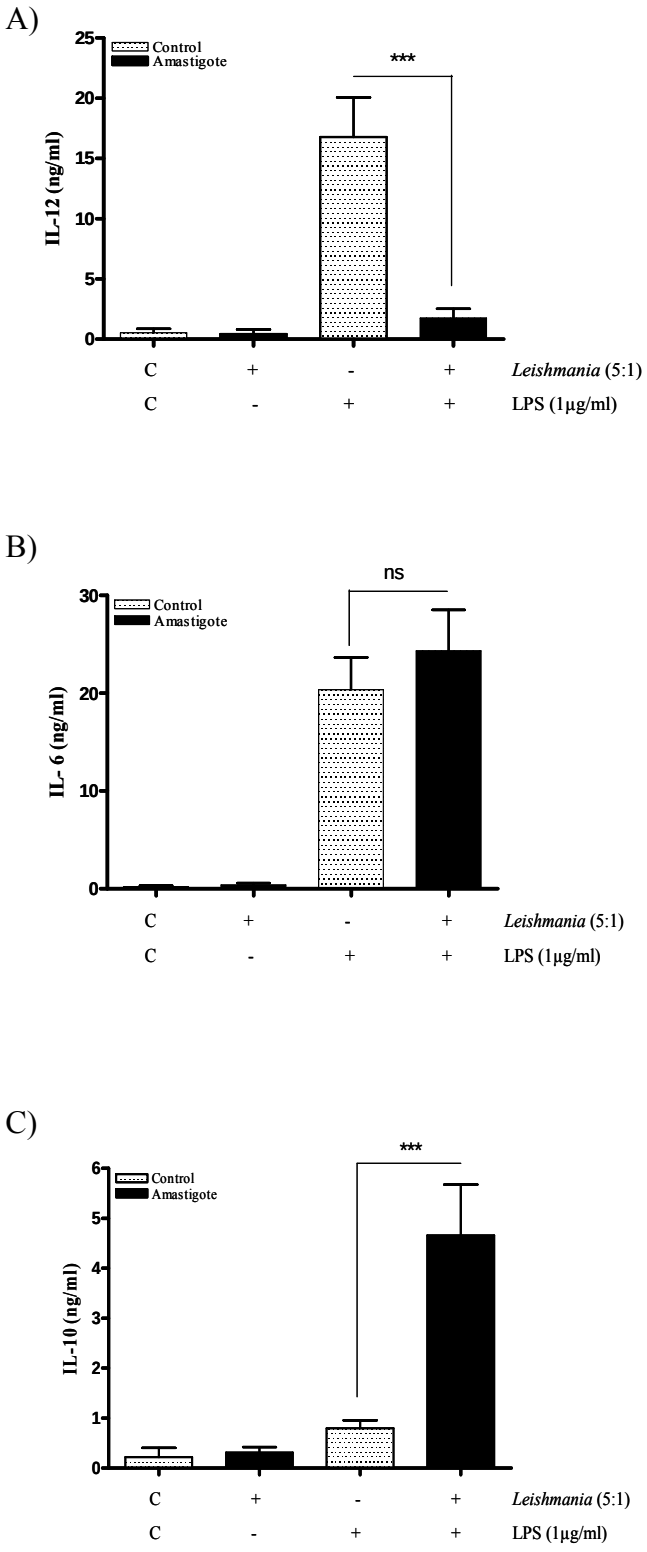


Figure 3.18: Effect of amastigotes on LPS-induced cytokine production in bone marrow-derived macrophages. Cells ($1x10^6$/well) were incubated with *L. mexicana* amastigotes at a ratio of (5:1) prior to stimulation with LPS (1µg/ml) for 24 hrs. Supernatants were collected and cytokine levels assessed by ELISA as outlined in section 2.1.5. Values are quantified and expressed as mean ± S.E.M. from 3 separate experiments (A) IL-12, (B) IL-6 and (C) IL-10 ng/ml. ***$p<0.001$ as compared with LPS stimulation.

3.5 THE EFFECT OF *LEISHMANIA MEXICANA* AMASTIGOTES ON IFN-γ SIGNALLING PATHWAYS IN MACROPHAGES

The effect of amastigotes on pathways linked to IFN-γ activation of macrophages was examined in a manner similar to that studied for LPS signalling. In particular we assessed the components of the JAK/STAT pathway. As mentioned previously, IFN-γ regulates Th1 responses therefore the effect on IFN-γ signalling is likely to be highly relevant to the infection state.

3.5.1 IFN-γ induced activation of the JAK/STAT signalling pathway in BMD-macrophages

Initially the kinetics of JAK/STAT activation in response to IFN-γ was established. IFN-γ stimulated a rapid increase in JAK2 phosphorylation which reached a maximum as early as 5 min and remained at this level for 120 min before returning to near basal values by 240 min (Figure 3.19). A similar profile was observed for phosphorylation of STAT1α. Increased phosphorylation was also apparent as early as 5 min and maximal between 5 and 15 min (Figure 3.20).

3.5.2 The effect of *Leishmania* infection on Janus kinase 2 (JAK2) and STAT1α expression and phosphorylation in BMD-macrophages

Initially the effect upon JAK2 protein expression in macrophages treated with *Leishmania* was examined. Uninfected cells showed constitutive expression of JAK2, whereas macrophages infected with increasing concentration of parasites (0.5-5:1 parasite /macrophage) for different times caused a marked inhibitory effect on JAK2 protein expression (Figure 3.21). JAK2 was very sensitive, degradation was apparent at the lowest number of parasites tested ($0.5x10^6$parasite/macrophage) and more rapid than observed for JNK and NF-κB (see section 3.2.1, Figure 3.1 and section 3.2.2,

Figure 3.8). JAK2 protein expression after 30 minutes was virtually abolished (% inhibition of control expression = 85.308 ± 0.29 at 30 min, 0.5-5x10^6 parasite /macrophage, ***$p<0.001$).

The marked effect of amastigotes on JAK2 protein levels was reflected at the level of phosphorylation (Figure 3.22). Whilst alone IFN-γ stimulated a marked increase in JAK2 phosphorylation, this response was essentially abolished following amastigote pre-treatment (fold stim. at 15 min, IFN-γ = 16.38 ± 2.70, *Leishmania*+IFN-γ = 0.86 ± 0.21). All stimulation time points were similarly affected. Inhibition of phosphorylation unsurprisingly, correlated with almost complete degradation of JAK2 in these samples.

A similar effect was observed when STAT1α was examined. Figure 3.23 shows the time and concentration-dependent effect of amastigote treatment. Whilst 0.5 and 1 amastigotes caused a slow and incomplete degradation over the 2 hrs time period, STAT1α protein was virtually abolished at higher concentrations (2:1 or 5:1). The effect of a high concentration of amastigotes (5:1) on IFN-γ mediated STAT1α phosphorylation was also examined (Figure 3.24). Again pre-treatment of cells with amastigotes abolished STAT1α phosphorylation at all time points examined up to 120 min. Once again this inhibition correlated with the complete abolition of STAT1α protein levels. (fold stim. at 30 min, IFN-γ = 27.26 ± 4.46, *Leishmania*+IFN-γ = 1.04 ± 0.06).

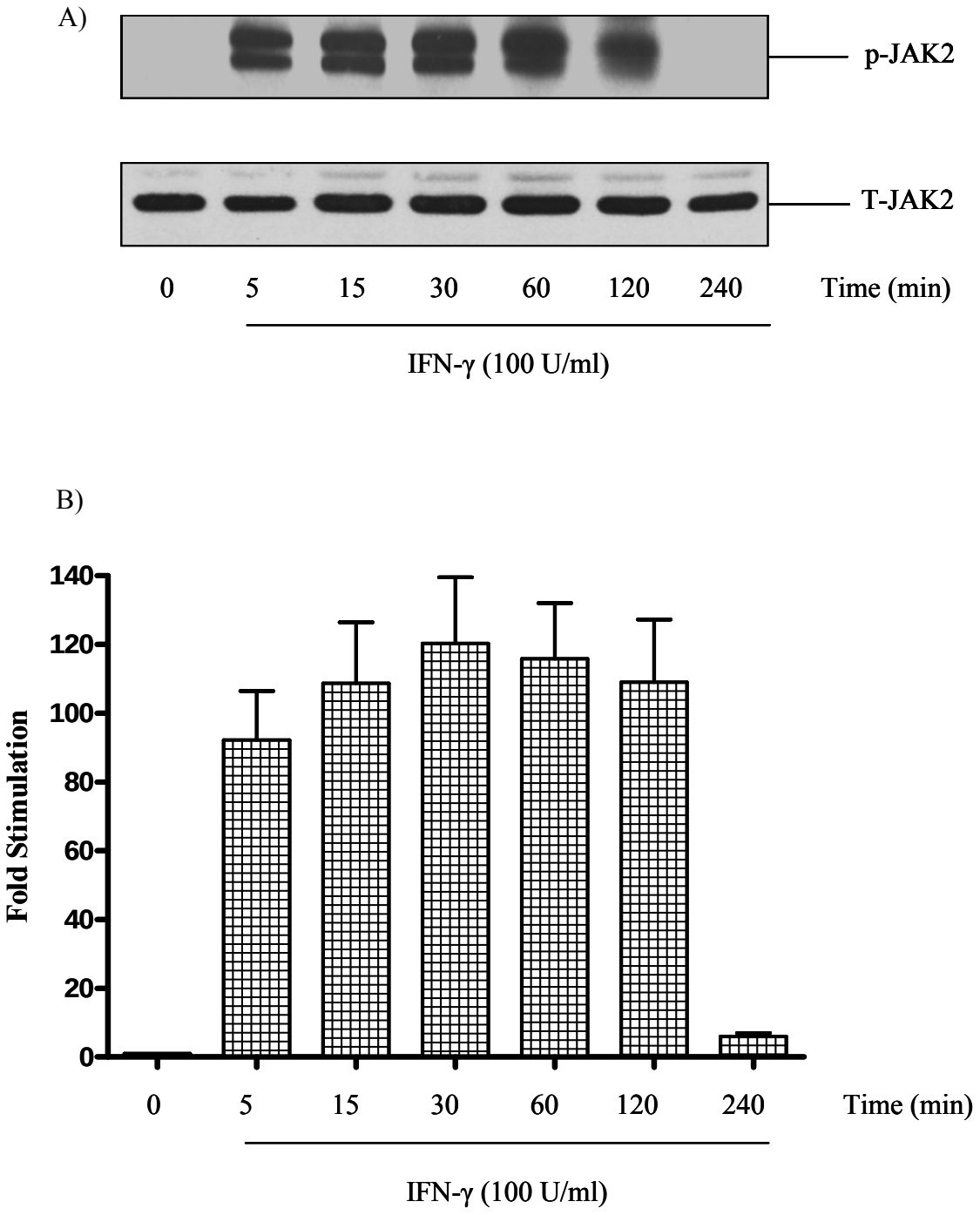


Figure 3.19: Time-dependent phosphorylation of Janus kinase (JAK2) by interferon-γ (IFN-γ). Macrophages were treated with IFN-*γ* (100U/ml) for different times as indicated. Whole cell lysates were prepared and then assessed for A) p-JAK2 (125 kDa) and T-JAK2 (125 kDa) as outlined in Section 2.2.3. Blots were quantified in B) by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings.

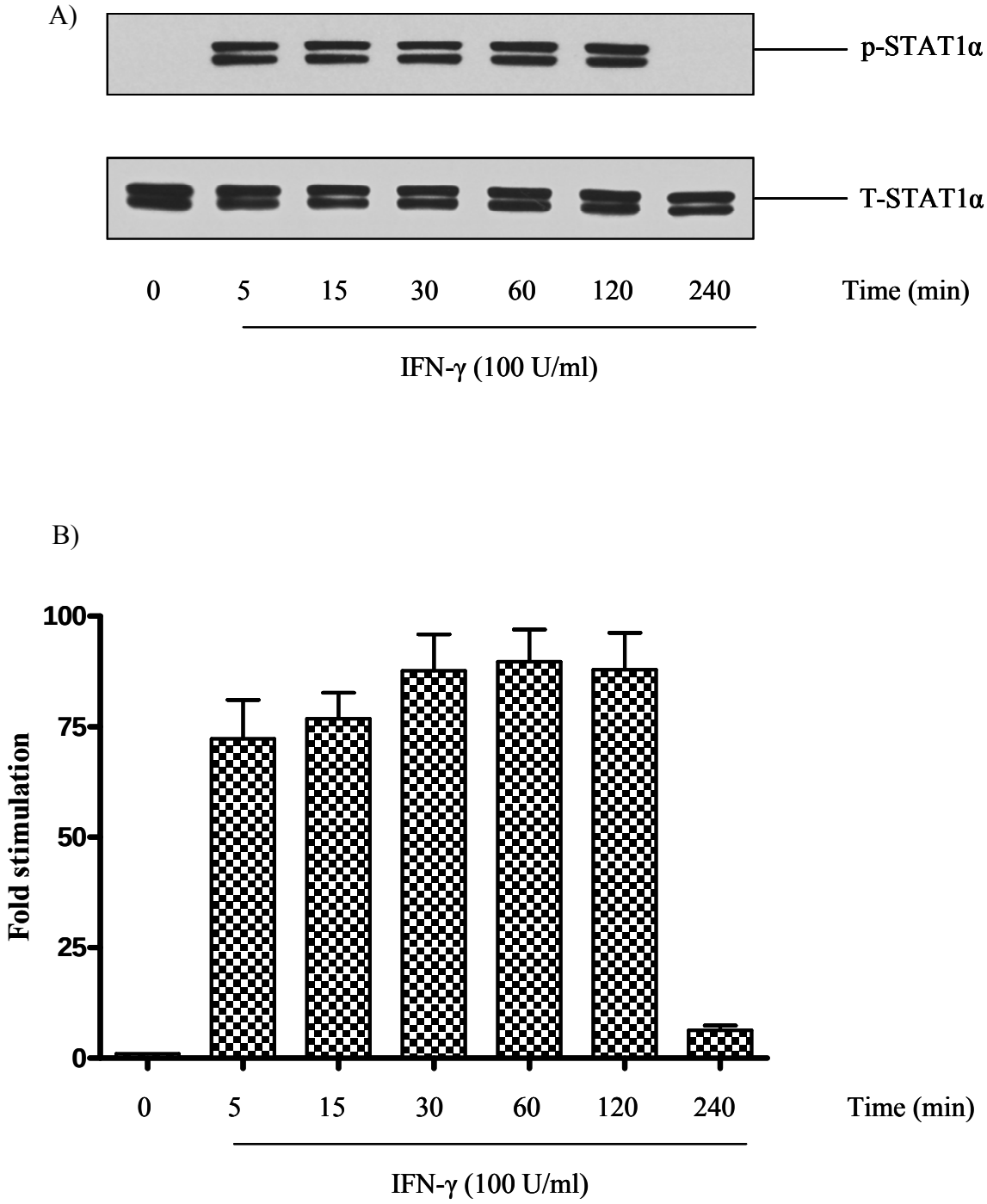


Figure 3.20: Time-dependent phosphorylation of STAT1α by interferon-γ (IFN-γ). Macrophages were treated with IFN-γ (100U/ml) for different times as indicated. Whole cell lysates were prepared and then assessed for A) p-STAT1α (91 kDa) and T-STAT1α (91 kDa) as outlined in Section 2.2.3. Blots were quantified in B) by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings.

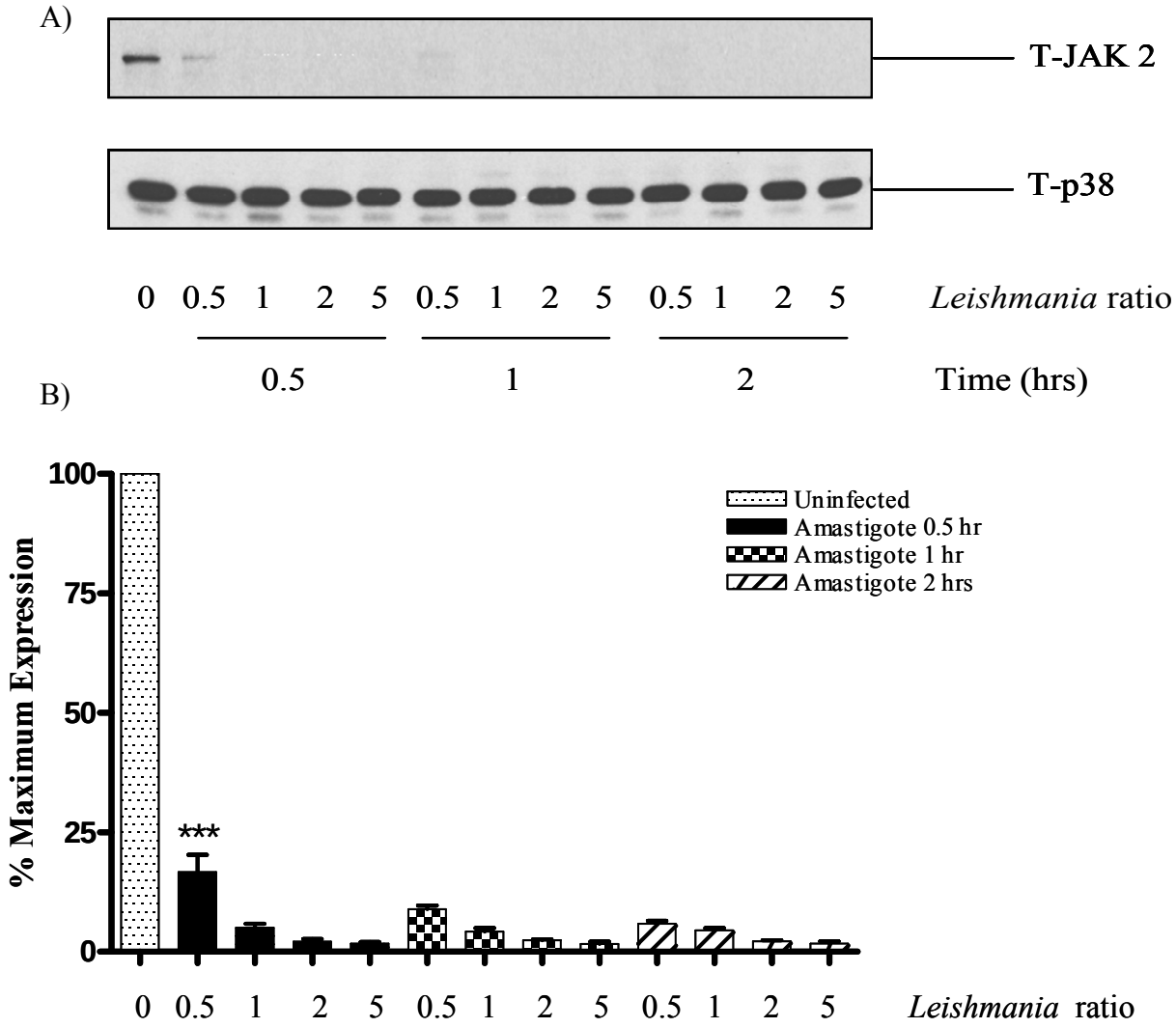


Figure 3.21: The effect of *Leishmania mexicana* infection on Janus kinase (JAK2) expression in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* amastigotes at different concentrations as indicated for different times. Whole cell lysates were prepared and then assessed for A) T-JAK2 (125 kDa) and T-p38 (38 kDa) as outlined in Section 2.2.3. Blots were quantified in B) by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings. ***$p<0.001$, compared to agonist-stimulated.

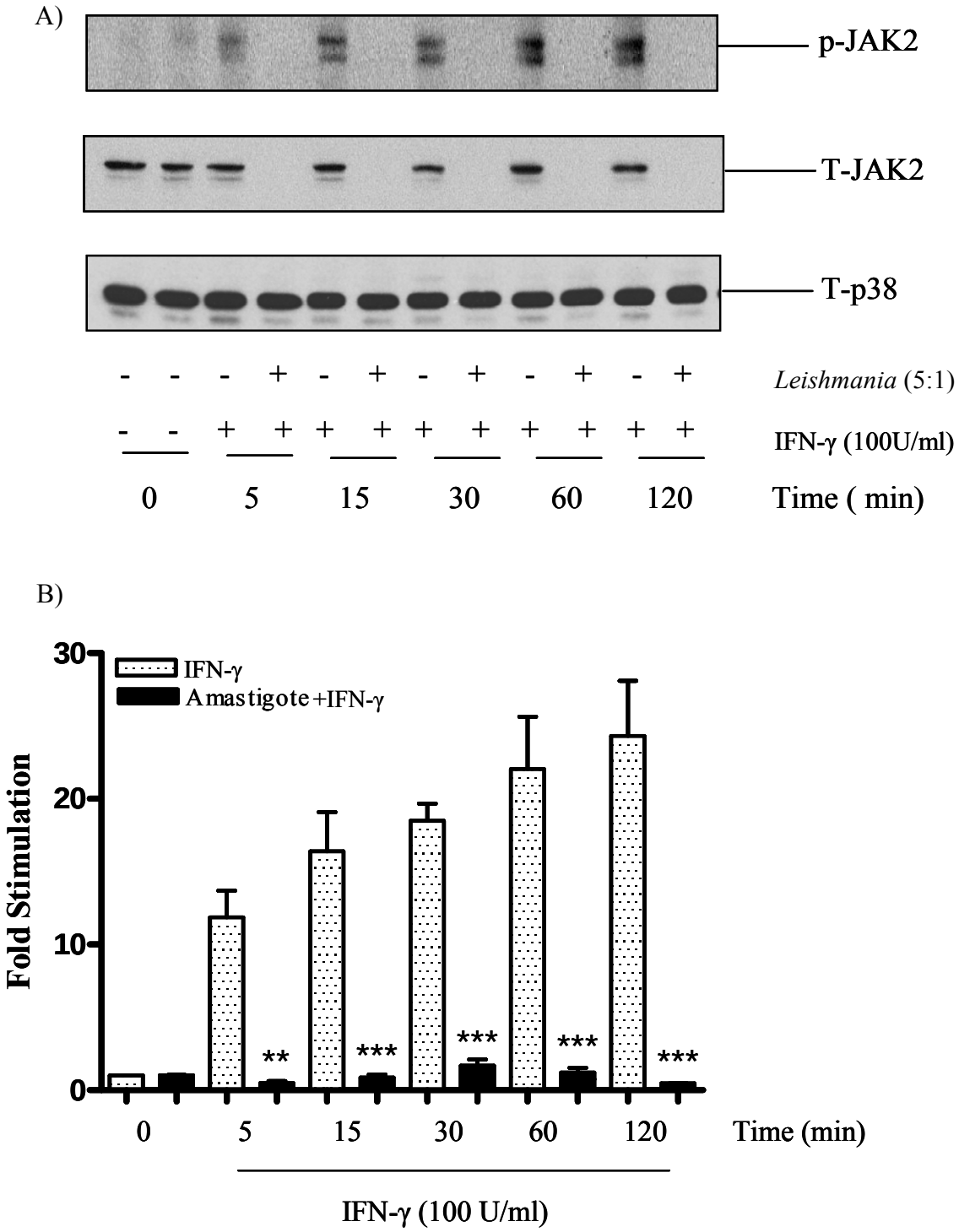


Figure 3.22: The effect of *Leishmania mexicana* infection on IFN-γ-induced JAK2 phosphorylation and protein expression in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* amastigotes at a ratio of (5:1) for 2 hrs and then stimulated with IFN-γ (100 U/ml) for a further 2 hrs. Whole cell lysates were prepared and then assessed for A) p-JAK2 (125 kDa), T-JAK2 (125 kDa) and T-p38 as outlined in Section 2.2.3. Blots were quantified in B) by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings. **p<0.01 and ***p<0.001, compared with agonist-stimulated.

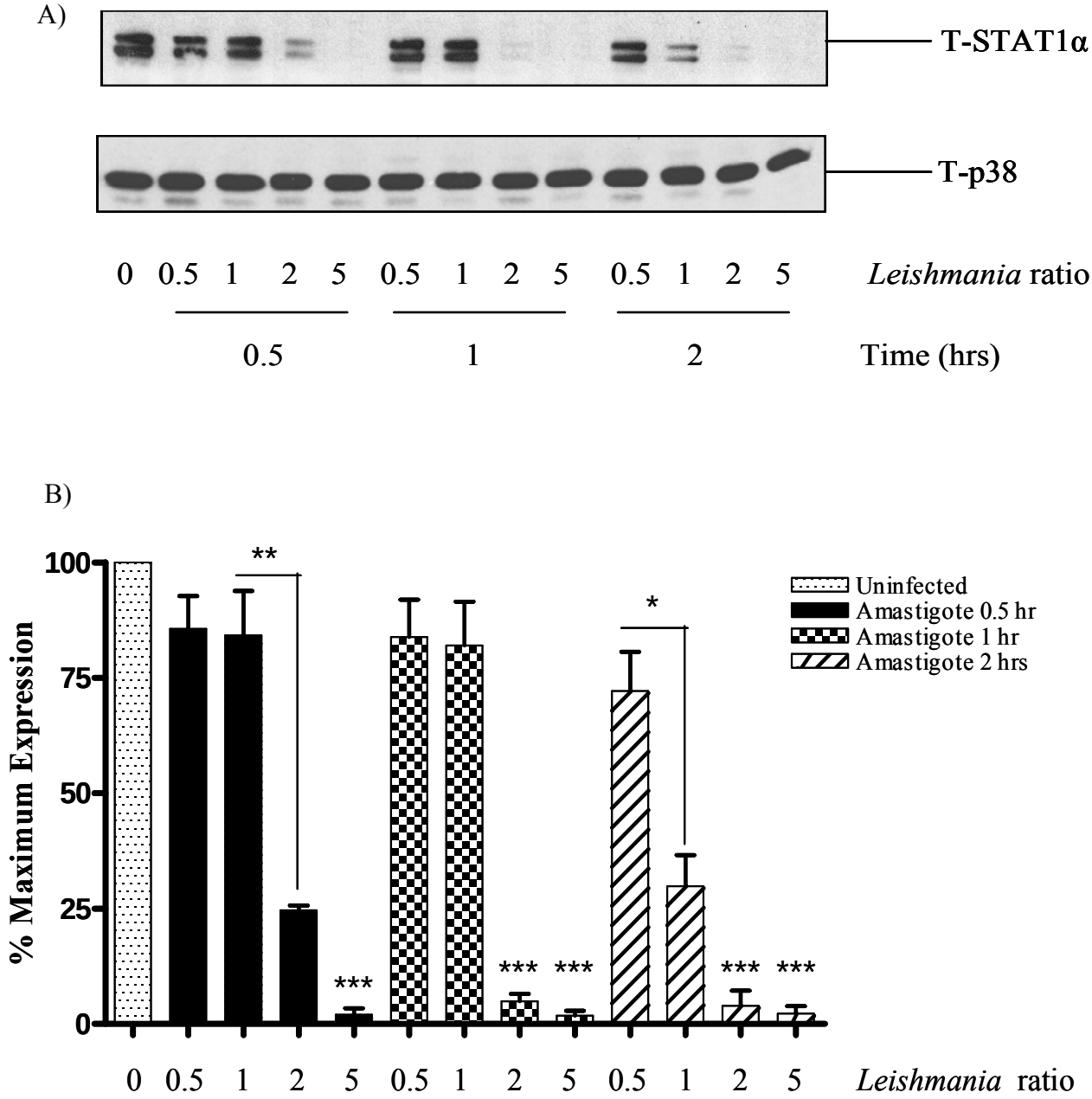


Figure 3.23: The effect of *Leishmania mexicana* infection on STAT1α expression in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* amastigotes in different concentrations as indicated for different times. Whole cell lysates were prepared and then assessed in A) T-STAT1 (91 kDa) and T-p38 as outlined in Section 2.2.3. Blots were quantified in B) by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings.*$p<0.05$, **$p<0.01$and ***$p<0.001$, compared with agonist-stimulated.

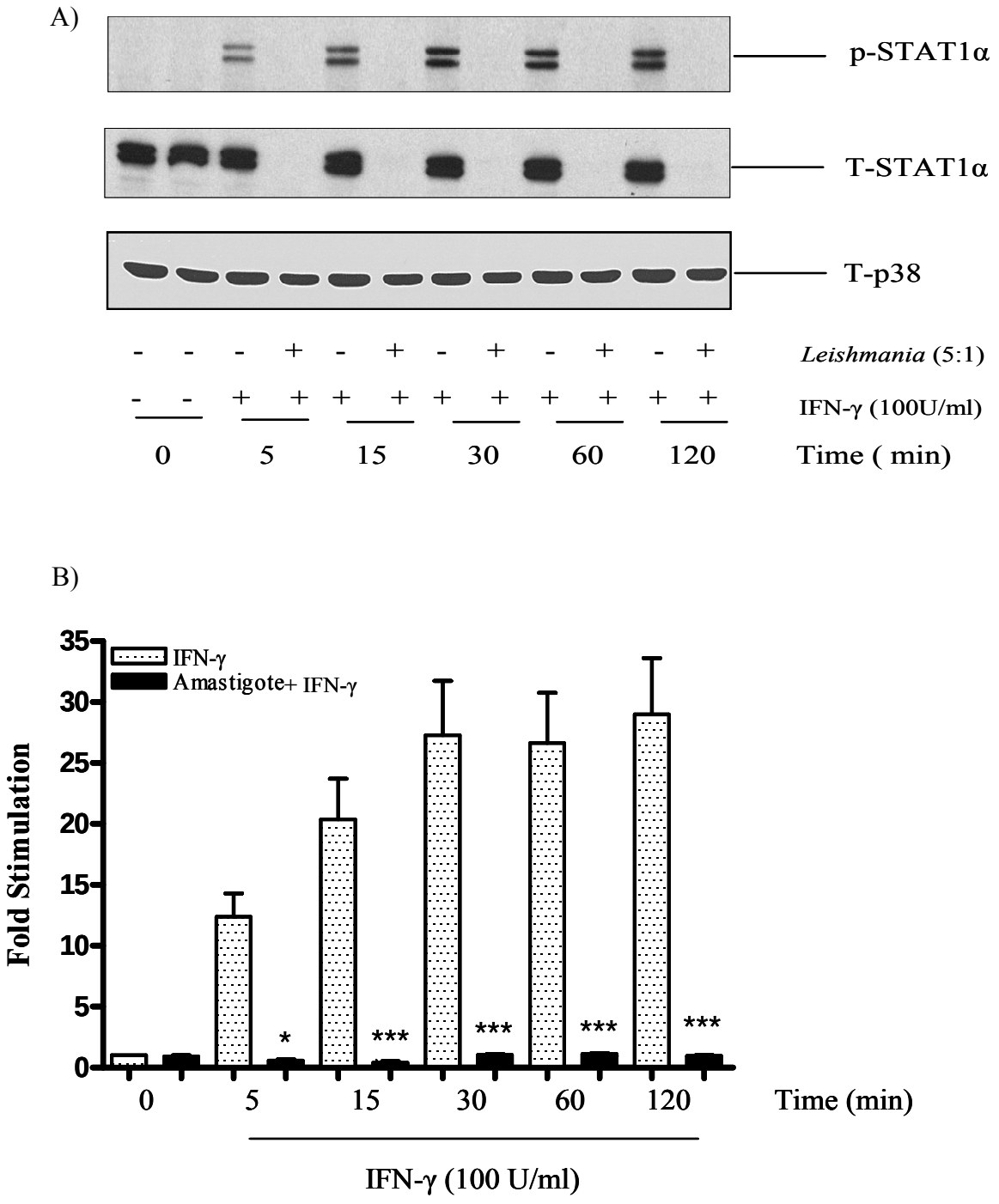


Figure 3.24: The effect of *Leishmania mexicana* infection on IFN-γ-induced STAT1 phosphorylation and protein expression in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* amastigotes at a ratio of (5:1) for 2 hrs and then stimulated with IFN-γ (100 U /ml) for a period of 2 hours. Whole cell lysates were prepared and then assessed in A) p-STAT1α (91 kDa), T-STAT1α and T-p38 as outlined in Section 2.2.3. Blots were quantified in B) by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings. *$p<0.05$ and ***$p<0.001$, compared with agonist-stimulated.

3.5.3 IFN-γ induced iNOS protein expression in BMD-macrophages

Having established that amastigotes abolished JAK/STAT signalling, the subsequent effect upon iNOS protein expression was examined. Figure 3.25 illustrates the kinetics of iNOS protein expression in response to IFN-γ stimulation over a period of 24 hrs. iNOS protein expression was time-dependent and following a delay started to increase 4 hrs post stimulation (18.81 ± 2.91 fold of basal values), gradually increasing to reach a maximum after 24 hrs (46.695 ± 8.231). COX-2 expression was not increased by IFN-γ treatment (data not shown).

3.5.4 The effect of *Leishmania* infection on IFN-γ-induced iNOS protein expression in BMD-macrophages

Next, the effect of *Leishmania* upon iNOS protein expression in macrophages was examined. Again two approaches were used, pre and post-incubation of amastigotes relative to IFN-γ stimulation. Figure 3.26 shows the effect of *Leishmania* pre-treatment on IFN-γ-induced iNOS induction. As expected, IFN-γ gave a peak expression of iNOS following stimulation with IFN-γ for 6 hrs (fold stim. = 88.31 ± 7.05). Pre-treatment with amastigotes for 30, 60, and 120 min abolished IFN-γ induced iNOS induction, an effect which correlated with rapid degradation of endogenous JAK2 during the infection period.

When cells were incubated with amastigotes following IFN-γ induction of iNOS the effect was equally as marked. Alone IFN-γ induced a marked increase in iNOS protein expression of approximately 75 fold. This response was substantially reduced following 30 min incubation with amastigotes with almost complete inhibition at 2 hrs (Figure 3.27). This again correlated with the complete loss in JAK2 expression.

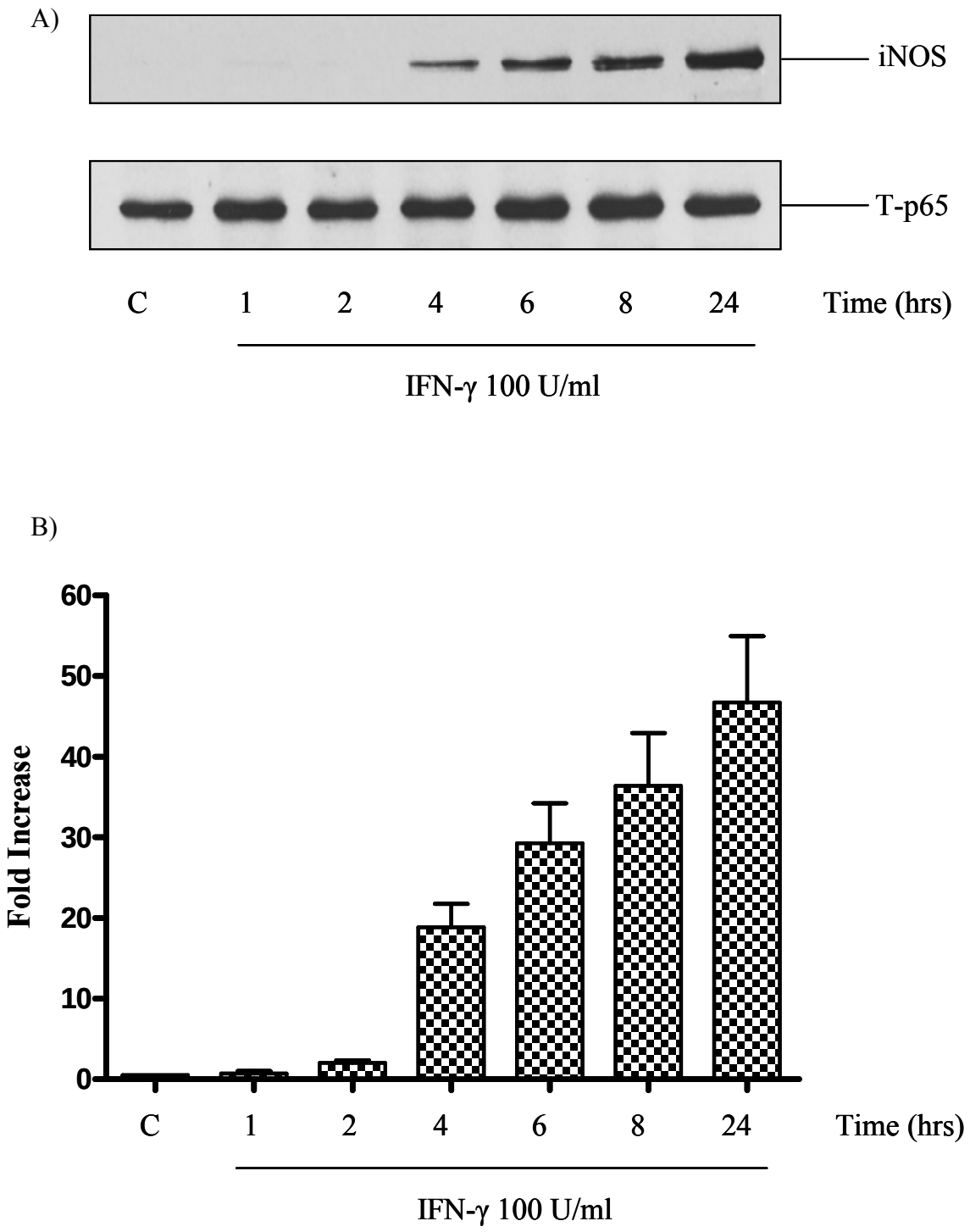


Figure 3.25: Time-dependent expressions of iNOS by interferon-γ (IFN-γ) in bone marrow-derived macrophages. Cells were treated with IFN-γ (100U/ml) for the times indicated. Whole cell lysates were prepared and then assessed for A) iNOS (130 kDa) and T-p-65 (65 kDa) as outlined in Section 2.2.3. Blots were quantified in B) by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings.

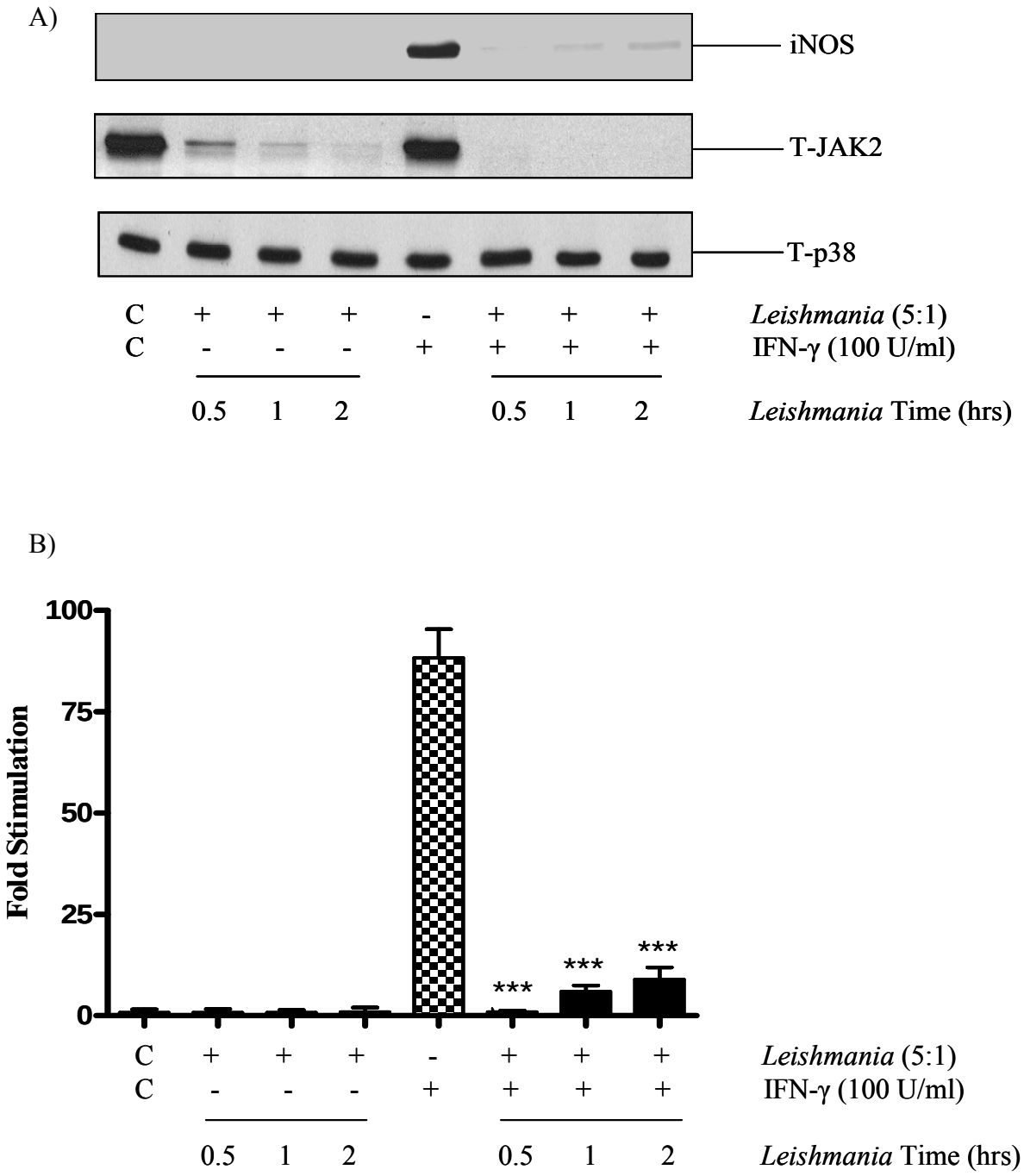


Figure 3.26: The effect of pre-treatment with *Leishmania mexicana* on iNOS protein expression in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* amastigotes at a ratio of (5:1) for different times and then stimulated with IFN-γ (100 U/ml) for 6 hrs. Whole cell lysates were prepared and then assessed for A) iNOS (130 kDa), T-JAK2 (125 kDa) and T-p38 as outlined in Section 2.2.3. Blots were quantified in B) by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar finding. ***$p<0.001$, compared with agonist-stimulated.

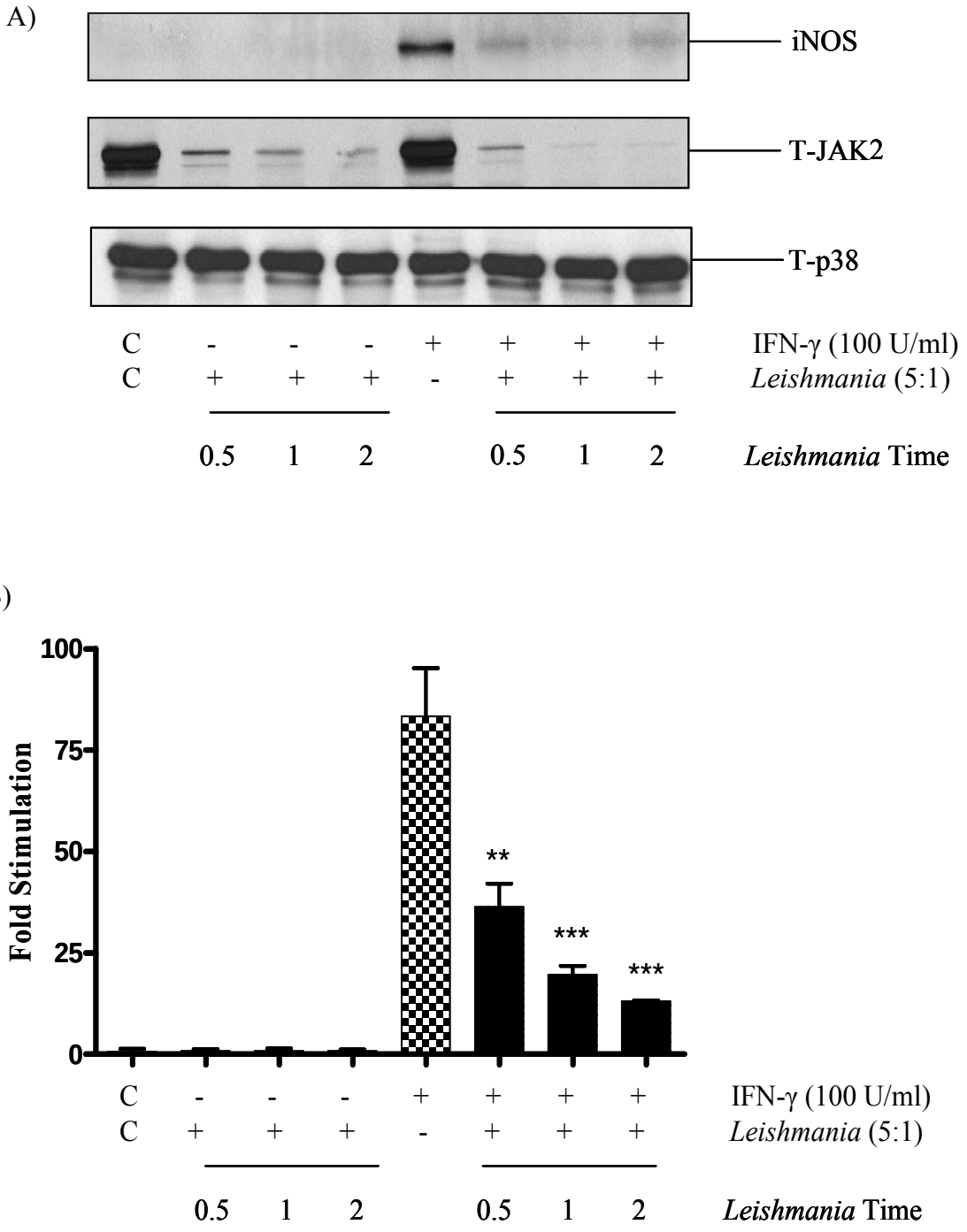


Figure 3.27: The effect of *Leishmania mexicana* infection on iNOS protein expression in macrophages following IFN-γ stimulation. Cells ($1x10^6$/well) were stimulated with IFN-γ (100 U/ml) for 6 hrs and then following infected with *Leishmania* amastigotes at a ratio of (5:1) for different times. Whole cell lysates were prepared and then assessed for A) iNOS (130 kDa) and as outlined in Section 2.2.3. Blots were quantified in B) by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings. **$p<0.01$ and ***$p<0.001$, compared with agonist-stimulated.

3.6 DISCUSSION

Inhibition of macrophage activation during systemic invasion is essential for the establishment of *Leishmania* infection within the mammalian host (Carrera et al., 1996, Reiner et al., 1994). This interaction between *Leishmania* parasites and host macrophages can result in misdirected intracellular signalling pathways, leading to survival of the parasite inside infected macrophages. However, our understanding of the macrophage signals affected by *L. mexicana* in the infected cells and the mechanisms used by this parasite to modulate the cell response is still limited. This chapter examined the effect of *L. mexicana* amastigotes (lesion-derived footpad amastigotes or rump), on inflammatory proteins expressed in response to LPS stimulation in macrophages, and investigated which signalling pathways were involved. This included IκB-α, NF-κB and the three major MAP Kinases, ERK, JNK, and p38 MAP kinase. Intermediates of the JAK/STAT pathway were also examined, stimulated in response to IFN-γ. Results presented in this section demonstrated that the expression and kinetics of activation of selected kinases are inhibited in the presence of *Leishmania* infection. It was found that JNK was rapidly degraded, p38 and ERK were not affected although ERK phosphorylation was abolished due to degradation of MEK1. Both IκB-α and NF-κB were similarly degraded in agreement with previous results obtained in the laboratory (Cameron et al., 2004). Furthermore, intermediates of the JAK/STAT pathway were also rapidly broken down. These degradatory effects of amastigotes have profound consequences for the subsequent induction of pro-inflammatory proteins and production of cytokines relevant to innate immune response in macrophages.

Initially it was demonstrated that *Leishmania* affected MAPKs and NF-κB pathways through the degradation of key intermediates. However, there is a clear difference in the sensitivity of each kinase to the effects of the amastigotes, with some kinases being highly susceptible. At concentrations of (5:1) *Leishmania* caused total degradation of cellular JNK, JAK2 was even more sensitive. In contrast p38 MAP kinase levels were not affected and LPS induced phosphorylation was not altered. Interestingly, ERK phosphorylation was strongly inhibited despite no apparent effect on ERK levels. This finding contrasts with a previous study which showed degradation of ERK by amastigotes (Cameron et al., 2004) and another using

Leishmania amazonensis amastigotes, a related species, which stimulated MAP kinases in particular, ERK1/2 phosphorylation (Yang et al., 2007). Another species, *L. major,* was found to induce ERK1/2 activation in macrophages by skewing CD40 signalling toward ERK1/2, and away from p38 MAP kinase (Mathur et al., 2004). However, the present study also showed for the first time strong degradation of MEK1 as a mechanism for inhibition of ERK phosphorylation. Other species for example, *L. donovani* have also been found to inhibit ERK activation indirectly through activation of SHP-1 (Forget et al., 2006). Furthermore, a recent study by Abu-Dayyeh et al., demonstrated that infection of macrophages with *L. donovani* parasites rapidly inactivated IRAK-1 kinase activity via stimulation of SHP-1, resulting in inhibition of TLR signalling and downregulation of macrophage function (Abu-Dayyeh et al., 2008). However, in preliminary studies amastigotes of *L. mexicana* were found not to degrade SHP-1 but SHP-2 (results not shown). Taken together, these studies highlight the variety and diversity of outcomes for kinase signalling in response to each *Leishmania* species.

A number of factors may dictate the differential degradation of signalling intermediates by *Leishmania* species. As mentioned previously, *Leishmania* amastigotes express a number of cysteine peptidase enzymes (Mottram et al., 2004). One of these, CPB, has a high enzymatic activity and is associated with parasite virulence. Again, a previous study in our laboratory has established that CPB is responsible for the degradation of IκB-α and NF-κB (Cameron et al., 2004). Whilst, CPB mediates degradation of several substrates (Alves et al., 2001), a consensus sequence remains to be fully characterized making it impossible to use bioinformatics to identify the presence or absence of a consensus sequence in MAP kinases, NF-κB or JAK/STATs substrates. Another consideration could be the subcellular distribution of each kinase. In resting cells many of the intermediates involved are cytosolic including p38 MAP kinase, although JNK in many cell types is usually located in the nucleus. Amastigotes are found in parasitophorous vacuoles but evidence suggests that there are cytolytic products associated with the mexicana complex inducing parasitophorous vacuole permeability which could allow access of CPB to the cytosol (Castro-Gomes et al., 2009, Almeida-Campos and Horta, 2000, Noronha et al., 2000). It would therefore have been of interest, if time had permitted, to stimulate cells, for example with LPS and assess NF-κB within the nuclear compartment to determine if

CPB from amastigotes could access the nucleus. Additional possibilities relate to the concentration of amastigotes employed in this chapter relative to other studies. A number of different protocols have been adapted by other groups. For example, (Tempone et al., 2004) used a ratio of (10:1) over 24 hours. These different infection protocols clearly, will give different outcomes.

Under normal conditions, LPS is able to stimulate several major signalling cascades in macrophages including MAP kinases and the NF-κB pathway. Activation is mediated through the interaction with Toll-like receptor 4 (TLR-4) through adaptor proteins such as MyD88. These pathways in turn play a role in initiating components of the innate immune system including the induction of iNOS and COX-2 as well as production of the pro-inflammatory cytokines, such as TNF-α, IL-1β, IL6 and chemoattractant cytokines, such as IL-8. Considerable evidence has been accumulated identifying the role of each kinase pathway in the regulation of these inflammatory molecules. Therefore, assessing amastigote-mediated degradation of the kinase pathways would allow prediction of the subsequent inhibition of COX-2 and iNOS expression.

A large body of evidence has accumulated to establish that iNOS and COX-2 induction is directly dependent on the MAP kinase and NF-κB pathways (D'Acquisto et al., 1997, D'Acquisto et al., 1998) and also PI-3K/Akt (Chan and Riches, 2001, Uto et al., 2005) in both macrophages and other systems (see Introduction). More recently, TLR-4-mediated expression of COX-2 and iNOS in bone marrow-derived and RAW 264.7 macrophages, was found to be reduced by the JNK inhibitor, SP600125 (Hambleton et al., 1996, Lin et al., 2010). Both SP600125 and the NF-κB inhibitors Bay 11-7082 and Ro 106-9920 also decreased COX-2 and PGE_2 production in response to TNF-α stimulation (Bage et al., 2010). Pretreatment with SP600125 blocked iNOS mediated NO release and TNF-α production following infection with *Edwardsiella tarda*, a Gram-negative bacterium (Wang et al., 2010). In addition, in vascular smooth muscle cells (SMCs) COX-2 induction was associated with activation of all three MAP kinases (Lamon et al., 2010).

More recent work uses a variety of interventions, in particular natural products, to arrive at the same conclusion. Glucosamine (GlcN) treatment inhibited both p38

MAPK and JNK following stimulation with LPS in RAW 264.7 (Rajapakse et al., 2008) whilst 2″-hydroxy-3″-en-anhydroicaritin, isolated from medicinal plants, inhibited COX-2 and iNOS by blocking activation of all three MAP kinases consistent with other previous studies (Ci et al., 2010, Chan and Riches, 2001, Lo, 2003). In a more recent study, it has been demonstrated that inhibition of NF-κB and MAP kinase mediated COX-2 and iNOS induction in LPS stimulated RAW 264.7 cells using eupatolide was mediated through proteasomal degradation of TRAF6 (Lee et al., 2010). A natural steroid hormone, diosgenin, was also found to inhibit phosphorylation of JNK resulting in the reduction of iNOS expression and NO production (Jung et al., 2010). Taken together, these studies clearly imply that MAP kinase and NF-κB pathways regulate COX-2 and iNOS induction, however there may be some variation in the contribution of each pathway to the magnitude of gene transcription over time. In this chapter, cells were stimulated with LPS for 6 hrs and therefore at this time point a given pathway may predominate in the induction of a given gene. Since p38 MAP kinase has been implicated in the regulation of COX-2 expression and p38 was not degraded by amastigotes, this may explain why inhibition of COX-2 was only partial but iNOS induction was completely abolished. It would have been interesting to assess the sensitivity to amastigote pretreatment at different times of LPS stimulation for COX-2 and iNOS induction.

Work in this chapter investigating COX-2 and iNOS regulation demonstrated potentially an additional post-translation effect. When amastigotes were added subsequent to LPS stimulation, there was a pronounced time-dependent effect on protein expression. Both inflammatory molecules were found to be reduced after 2 hours. iNOS in particular was found to be substantially degraded by 30 minutes. No other study to date has used this strategy to assess the potential degradation of inflammatory proteins by *Leishmania* species. A key additional experiment not performed would have been to determine the turnover of both iNOS and COX-2 by treating cells with cycloheximide, a protein synthesis inhibitor, after induction and assessing protein levels over time. It has been demonstrated previously that COX-2 is degraded with a half life of 8 hrs suggesting that indeed amastigote cleavage may be direct (Shao et al., 2000). Whilst the half life of iNOS degradation has not been described, a recent study has shown involvement of the proteasome pathway in destruction of iNOS over 24 hrs (Paukkeri et al., 2007). However, it remains unclear

whether inhibition of iNOS or COX-2 by amastigotes added after LPS is due to direct degradation or inhibition of NF-κB and MAP kinase signalling.

As outlined in the introduction, both PGE_2 and NO are key mediators in a number of inflammatory and immune functions. PGE_2 has been shown to have a dual action; inhibiting the Th1 immune response through suppression of IL-12, TNF-α, IL-1β and IL-8 by macrophages (Hinz et al., 2000, Ikegami et al., 2001) whilst enhancing the production of Th2 cytokines, such as IL-4, IL-5 and IL-10 (Hilkens et al., 1996). Amastigote infection would clearly inhibit this action, however amastigotes were still able to enhance IL-10 release (see below) suggesting that PGE_2 does not stimulate the macrophage to increase IL-10 production. PGE_2 is also regarded as a virulence factor for *Leisnamia* infection. However this might not apply to amastigote infection as clearly PGE_2 was dispensable. It is also well recognized that NO is essential for parasite killing (Das et al., 2010) and dependent on induction of the inducible form of NOS, iNOS. Therefore destruction of this enzyme either through destruction of signalling pathways or perhaps the protein itself is clearly a desirable strategy for amastigote survival. Studies linking the effects of PGE_2 at a cellular level to *in vivo* immunological studies are limited and are discussed in more detail in Chapter 4 as is the link between NO and parasite survival.

The induction of the cytokines IL-12, IL-6, and IL-10 were also differentially regulated by amastigote pre-treatment. As outlined previously, IL-12 stimulates IFN-γ release from Th1 cells which in turn mediates iNOS expression in macrophages (Sutterwala and Mosser, 1999). IL-6 is an important cytokine involved in the regulation of the acute-phase response to injury and infection and subsequently plays an important role in inflammation and the immune response (Heinrich et al., 2003). On the other hand IL-10 enhances parasite survival and replication during infection due to inhibition of Th1 activity by reduction of IL-12 production, NK cell activation and macrophage function (Couper et al., 2008). In this chapter IL-12 was markedly reduced, IL-6 was unaffected whilst IL-10 was increased and again these outcomes could be correlated with effects upon signalling pathways.

It has previously been shown that NF-κB plays an important role in IL-12 production (Brereton et al., 2009, Boddupalli et al., 2007) and indeed CPB mediated degradation

of NF-κB correlates with inhibition of IL-12 production (Cameron et al., 2004). However, in this study the reduction in IL-12 was not absolute suggesting an involvement of another pathway. This is likely to be p38 MAP kinase, as SB203580 reduces IL-12 production in RAW 264.7 macrophages (Feng et al., 1999, Yi et al., 2002) and dendritic cells (Re and Strominger, 2001, Marriott et al., 2001). Interestingly, two of these publications also indicate that ERK also suppresses IL-12 activation, a result which would be at odds with the observed inhibition of ERK phosphorylation through destruction of MEK1 by amastigotes detailed in this chapter. Furthermore, some studies have shown enhanced IL-12 induction following SB203580 pre-treatment (Marriott et al., 2001), therefore results obtained using inhibitors should be viewed with caution.

The regulation of IL-6 production is also known to be mediated by MAP kinase and NF-κB signalling pathways. In particular, p38 MAP kinase has been implicated, Santos and colleagues demonstrated that MIF induced IL-6 production was inhibited following p38 MAP kinase antagonism whilst blockade of ERK was without effect (Santos et al., 2004). Other studies support a role for p38 MAP kinase (Wang et al., 2005, Sano et al., 2001, Pathak et al., 2006) although most studies utilise cell types other than macrophages. Interestingly, in some instances both ERK and NF-κB have also been implicated, thus projecting these findings to the results in this chapter would indicate a greater degree of redundancy for each pathway in the regulation of IL-6 production relative to COX-2, iNOS and IL-12. Activation of p38 MAP kinase is quite sufficient to give a full IL-6 signal. Other studies have implicated roles for PKC isoforms (Jove et al., 2005, Kwon et al., 2007), MSK-1 (Pathak et al., 2006) and STAT3 dependent signalling (Beurel and Jope, 2009) however, these pathways were not examined for sensitivity to inhibition or degradation by amastigotes in this chapter.

A similar p38 MAP kinase dependency was recorded for IL-10 production. Inhibition of p38 MAP kinase activity in LPS stimulated macrophages was found to attenuate IL-10 production, while ERK 1/2 inhibition had no effect (Schwacha et al., 2003). However, in this chapter amastigotes enhanced IL-10 production and this suggests the potential of other additional modes of regulation. Pathways such as JNK and NF-κB could negatively regulate IL-10 induction and thus degradation of these intermediates

would result in enhancement. An additional possibility is that inhibition of intermediates such as IL-12, PGE_2 or NO could facilitate IL-10 release. However, one study has shown that a high level of PGE_2 had no effect on the corresponding production of IL-10, whereas IL-12 and IFN-γ production was completely inhibited (Hilkens et al., 1996). Furthermore, Weinheber and colleagues also demonstrated that the induction of IL-10 following infection with *L. mexicana* amastigotes was independent of IL-12 production in the macrophage (Weinheber et al., 1998).

Taken together the effects of amastigotes on kinase signalling by and large predict subsequent regulation of cytokine production. The co-regulation of IL-12, IL-6, and IL-10 production in relation to *Leishmania* infection will be discussed in detail in Chapter 4.

In this chapter the effect of *L. mexicana* amastigotes on the activation of signalling pathways and iNOS expression in IFN-γ treated macrophages was also examined. As mentioned previously, *Leishmania* has several strategies to down-regulate numerous macrophage functions in response to IFN-γ, for example, NO release, MHC expression and IL-12 production (Olivier et al., 2005, Olivier, 1996, Carrera et al., 1996, Proudfoot et al., 1996). The JAK/STAT pathway is central to the regulation of these end points. The JAK-2 inhibitors AG-490 and WHI-P154 prevent IFN-γ-induced iNOS expression and NO production along with inhibition of STAT1 activation in both macrophages (Sareila et al., 2006) and glial cells (Kitamura et al., 1996). Additionally, activation of JAKs and STATs occurs not only during the innate immune response but in adaptive immune responses (Greenlund et al., 1994, Greenlund et al., 1995, Igarashi et al., 1994, Sakatsume et al., 1995).

It was found that in the presence of *L. mexicana* amastigotes a significantly reduced level of phosphorylated JAK2 was recorded, dependent on rapid JAK-2 degradation (Figure 3.22). Similar studies have been carried out by Bhardwaj et al., using *L. mexicana* and *L. major*. The effect was also observed on both phosphorylation and total protein in both species (Bhardwaj et al., 2005). A similar degradation of JAK-2 was also observed recently in dendritic cells (Xin et al., 2008). In contrast, a previous study by Blanchette et al., showed the infection with *L. donovani* attenuated JAK2

and JAK1 activation, but through the activation of the SHP1 tyrosine phosphatase, presumably through a different mechanism (Blanchette et al., 1999).

In addition, STAT1α protein levels were also inhibited in a time and dose-dependent manner in *Leishmania*-infected macrophages. Again recently, in dendritic cells, amastigotes have also been shown to degrade STAT1α as well as STATs 2 and 3 (Xin et al., 2008). These findings demonstrated that *L. mexicana* can block IFN-γ induced macrophage activation at multiple points in the same pathway which might be unexpected. However, although not properly quantified, it did seem that JAK-2 was somewhat more sensitive to degradation than STAT1α, thus it would have been interesting to record at what concentrations of parasite only JAK-2 was affected. This might relate more to infection *in vivo*, as studies examining intracellular protein degradation are hampered by the use of large amounts of parasites for prolonged periods of time (Xin et al., 2008).

As with JAK signalling, several studies have shown *Leishmania* regulation of STAT function. *L.donovani* inhibition of the IFN-γR complex formation correlated with inhibition of STAT1α binding to the IFN-γ response region (Ray et al., 2000). Also infection of J774A1 and RAW264.7 with *Leishmania mexicana* resulted in significant inhibition of STAT1α due to induction of the dominant negative STAT1β (Bhardwaj et al., 2005). Forget et al., showed STAT1α degradation following infection with *L. donovani* due to activation of the proteasome (Forget et al., 2005). Two other studies have described proteasome-mediated degradation of STATs following cytokine stimulation; one study additionally assessed STAT1α ubiquitination (Kim and Maniatis, 1996), a second study showed that STAT3 was degraded during ciliary neurotrophic factor or 12-*O*-tetradecanoylphorbol-13-acetate stimulation (Malek and Halvorsen, 1999).

Therefore, the ability of *Leishmania* to modulate IFN-γ induced macrophage iNOS activity can be explained by degradation of both JAKs and STATs. Innate macrophage activation, in this case through IFN-γR ligation by IFN-γ stimulation, results in a time-dependent balance between the iNOS and NO production, which is consistent with other studies (Blanchette et al., 2003). In our study we show that, either pre or post-treatment of macrophage with *Leishmania mexicana* amastigotes

results in a significant decrease in iNOS expression either by inhibition of signalling pathways or as discussed previously due to degradation of the iNOS protein itself. Thus IFN-γ signalling can be negatively regulated by amastigotes before, during and after IFN-γ stimulation. This may have profound consequences for immunity mediated in response to this cytokine and the interactions of the macrophage with the amastigote.

CHAPTER 4

THE EFFECT OF *LEISHMANIA MEXICANA* PROMASTIGOTES ON MACROPHAGE INTRACELLULAR SIGNALLING AND IMMUNE RESPONSES

4.1 INTRODUCTION

The previous section concentrated mainly on the use of tissue stage amastigotes to study host cell pathogen interactions. The results showed the degradation of key intermediates of the MAP kinase, NF-κB and JAK/STAT pathways. However, apart from the initiation of infection via the insect vector, it is the promastigote life cycle stage that is associated with the invasion of the macrophage. Consequently, this chapter set out to determine whether promastigotes of *L. mexicana* were capable of modifying the cell signalling in macrophages in a manner similar to amastigotes. In particular, activation of the MAP kinase and NF-κB pathways were studied and again markers of inflammation, such as COX-2, iNOS and the cytokines IL-12, IL-6 and IL-10 were assessed.

In addition, two other aspects of macrophage signalling in the context of promastigote infection were examined. First, the potential to modify the activation of the arginase pathway, which is a feature of alternative macrophage activation. This enzyme shares a common substrate with iNOS, L-arginine, and is emerging as a crucial mechanism for the regulation of immune responses (Popovic et al., 2007, Morris, 2007). It has been well documented that polyamines produced via arginase are important nutrients utilized by *Leishmania spp.*, the exposure to Th2 cytokines leads to the promotion of parasite growth via this enzyme (Iniesta et al., 2002, Kane and Mosser, 2001). *L. mexicana* (chapter three) and *L. amazonensis* infection induces the production of macrophage-derived IL-10 (Kane and Mosser, 2001, Yang et al., 2007) which can regulate arginase function (Iniesta et al., 2002) and may cooperatively contribute to T-cell hyporesponsiveness in infected hosts. In this chapter the expression and activity of arginase-1 was assessed.

Second, the attempt to define the receptors through which promastigotes mediates regulation of intracellular signalling mechanisms. As outlined in the introduction, promastigotes have the potential to interact with a large array of cell surface molecules including complement receptor 1 and 3 (CR1&3) and C3b (Da Silva et al., 1989), parasite surface glycoprotein (*e.g.*, gp63 membrane protease), the mannose fucose receptor (Wilson and Pearson, 1986, Guy and Belosevic, 1993), the Fcγ

receptor, as well as toll-like receptor (TLRs) (de Veer et al., 2003, Flandin et al., 2006). Indeed, several TLRs have been shown to mediate the effects of *Leishmania* species in the context of systemic infection (Tuon et al., 2008). Therefore, later in the chapter promastigote signalling and functional responses were investigated using macrophages from TLR deficient mice.

4.2 THE EFFECTS OF *LEISHMANIA MEXICANA* PROMASTIGOTES UPON SIGNALLING PATHWAYS ACTIVATED IN MACROPHAGES

4.2.1 *Leishmania* promastigote infection prolongs LPS-induced JNK activation in macrophages

Macrophages were infected with *L. mexicana* promastigotes (5:1) for 2 hrs prior to stimulation with LPS (1μg/ml) for a further 120 min. In response to LPS alone, JNK phosphorylation reached a maximum at 30 min with the response observed to be approximately 20 fold of basal values (fold stim. 22.18 ± 4.32). This response gradually declined after 120 min (Figure 4.1). Notably, in cells which underwent pre-incubation with promastigotes, for 2 hrs prior to LPS stimulation, JNK phosphorylation was marginally but significantly prolonged at later time points (fold stim. at 120 min LPS = 1.72 ± 0.19, *Leishmania*+ LPS = 9.33 ± 2.93) $p<0.001$. Using total JNK as a means of determining equal protein loading, showed no change.

4.2.2 *Leishmania* promastigote infection prolongs activation of LPS-induced ERK activation in macrophages

In a similar fashion to JNK, ERK activation in macrophages infected with *L. mexicana* was examined **(**Figure 4.2.). In response to LPS, phosphorylation of ERK1/2 gradually increased over time and reached a maximum between 15 and 30 min at approximately 50 fold (fold stim.; 15= 55.78 ± 9.46). Phosphorylation of ERK1/2 was transient and returned toward basal value by 120 min. By comparison, in macrophages which had been pre-incubated with promastigotes initial phosphorylation of ERK was reduced (*Leishmania*+LPS = 24.99 ± 1.57 fold of 15 min LPS values, $p<0.05$). At longer time points there was no difference between ERK phosphorylation in the presence or absence of promastigotes. Using total ERK, as a means of determining equal protein loading, showed no change.

4.2.3 *Leishmania* promastigote infection prolongs activation of LPS-induced p38 MAP kinase activation in macrophages

Finally, the effect of *Leishmania* promastigotes on p38 MAP kinase was assessed. Stimulation of macrophages with LPS increased p38 MAP kinase phosphorylation, reaching a maximum between 15 and 30 min (fold stim. 23.91 ± 1.16 and 22.09 ± 1.41 respectively). The peak response declined substantially after 60 and 120 min (15.93 ± 1.41 and 12.89 ± 2.09 fold respectively), but did not return to near basal values (Figure 4.3). In contrast to these results, the phosphorylation of p38 MAP kinase in macrophages that were infected with promastigotes (5:1) for 2 hrs, was enhanced to a small but significant extent throughout the time course (fold stim. at 60 and 120 min LPS+*Leishmania* = 21.95 ± 1.85 and 18.16 ± 1.63 respectively, $p<0.05$).

4.2.4 The effect of promastigotes on LPS-induced IκB-α degradation and NF-κB phosphorylation in macrophages

The effect of LPS on IκB-α degradation, a marker of NF-κB activation, was assessed as shown in Figure 4.4. LPS (1μg/ml) stimulated a time-dependent loss in cellular IκB-α which was maximal between 15 and 30 min post stimulation (% maximum expression = 19.38 ± 4.50 and 12.64 ± 4.89) and then gradually returned to basal levels by 120 min (Figure 4.4). The same figure shows that the pre-treatment of cells with *Leishmania* promastigotes (5:1) for 2 hrs had no effect on the maximum loss induced by LPS. However, promastigotes caused a delay in the return of cellular IκB-α to basal levels. (% basal expression = 27.71 ± 3.25 and 59.66 ± 10.93 respectively, $*p<0.05$). LPS also induced a time-dependent increase in NF-κB phosphorylation which was again maximal by 15 min before returning toward basal values by 120 min (Figure 4.5). Pre-treatment with promastigotes significantly enhanced phosphorylation at the later time point (fold stim. at 60 and 120 min = 42.26 ± 4.61 and 58.93 ± 8.80 respectively, $**p<0.01$).

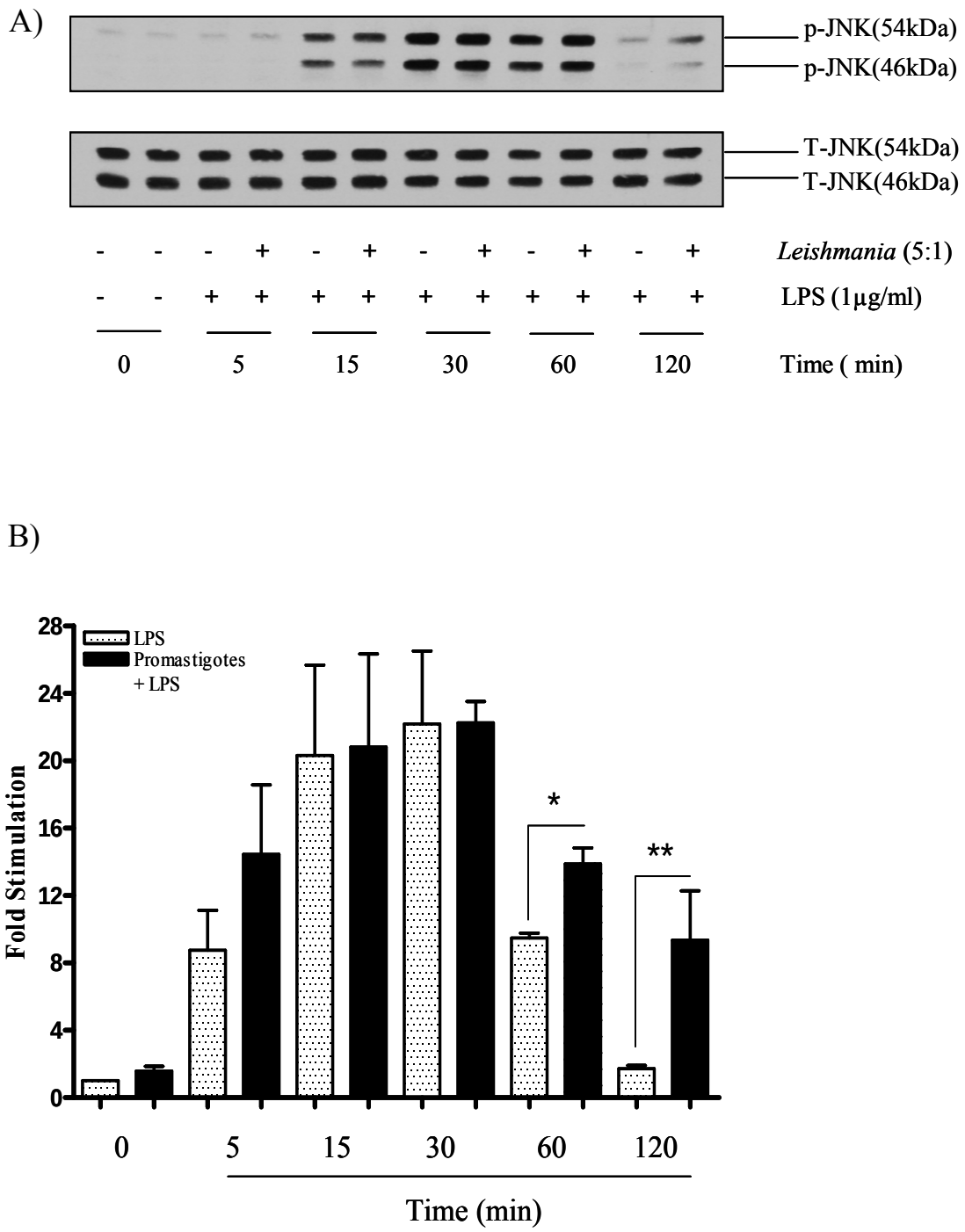


Figure 4.1: The effect of *Leishmania mexicana* promastigote infection on LPS-induced JNK1/2 phosphorylation in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* at a ratio of (5:1) for 2 hrs and then stimulated with LPS (1µg/ml) for up to a further 2 hrs. Whole cell lysates were prepared and then assessed for **A)** p-JNK (46/54 kDa) and JNK (46/55 kDa) as outlined in Section 2.2.3. Blots were quantified in **B)** by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings. *$p<0.05$ and **$p<0.001$, compared with agonist-stimulated control.

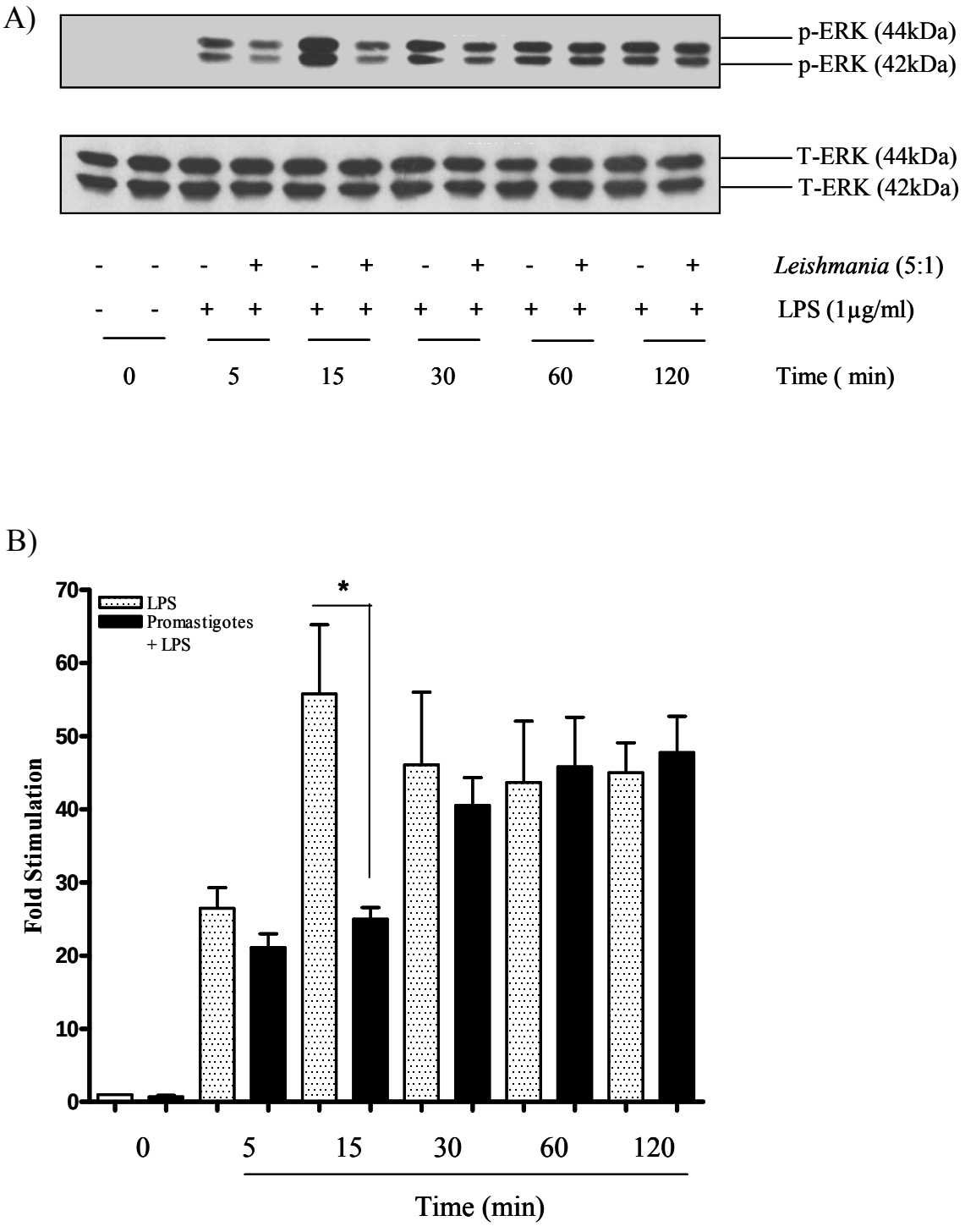


Figure 4.2: The effect of *Leishmania mexicana* promastigote infection on LPS-induced ERK1/2 phosphorylation in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* at a ratio of (5:1) for 2 hrs and then stimulated with LPS (1µg/ml) for up to a further 2 hrs. Whole cell lysates were prepared and then assessed for **A)** p-ERK1/2 (42/44 kDa) and ERK (42/44 kDa) as outlined in Section 2.2.3. Blots were quantified in **B)** by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings. *$p<0.05$, compared with agonist-stimulated control.

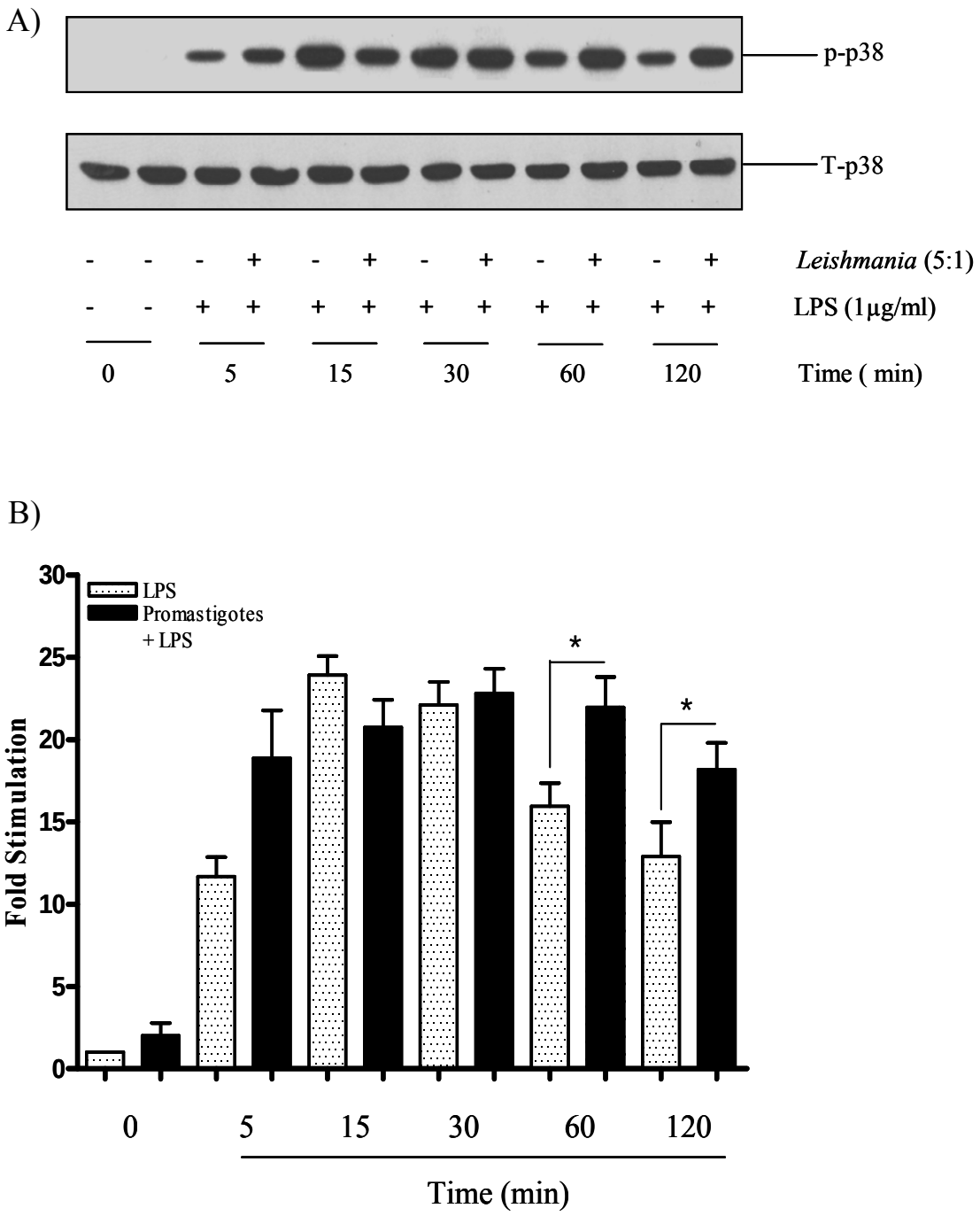
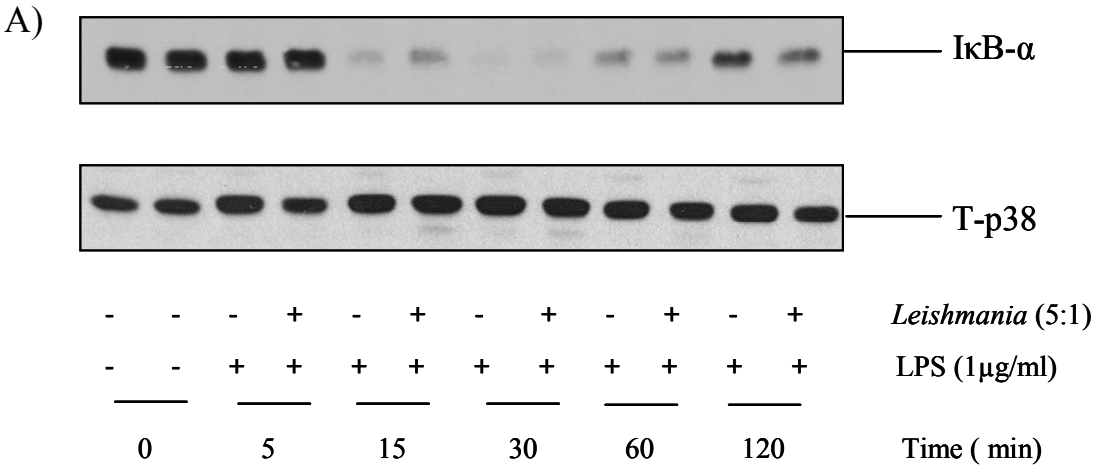


Figure 4.3: The effect of *Leishmania mexicana* promastigote infection on LPS-induced p-38 MAP kinase phosphorylation in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* at a ratio of (5:1) for 2 hrs and then stimulated with LPS (1 µg/ml) for up to a further 2 hrs. Whole cell lysates were prepared and then assessed for **A)** p-p38 and p38 as outlined in Section 2.2.3. Blots were quantified in **B)** by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings. *$p<0.05$, compared with agonist-stimulated control.



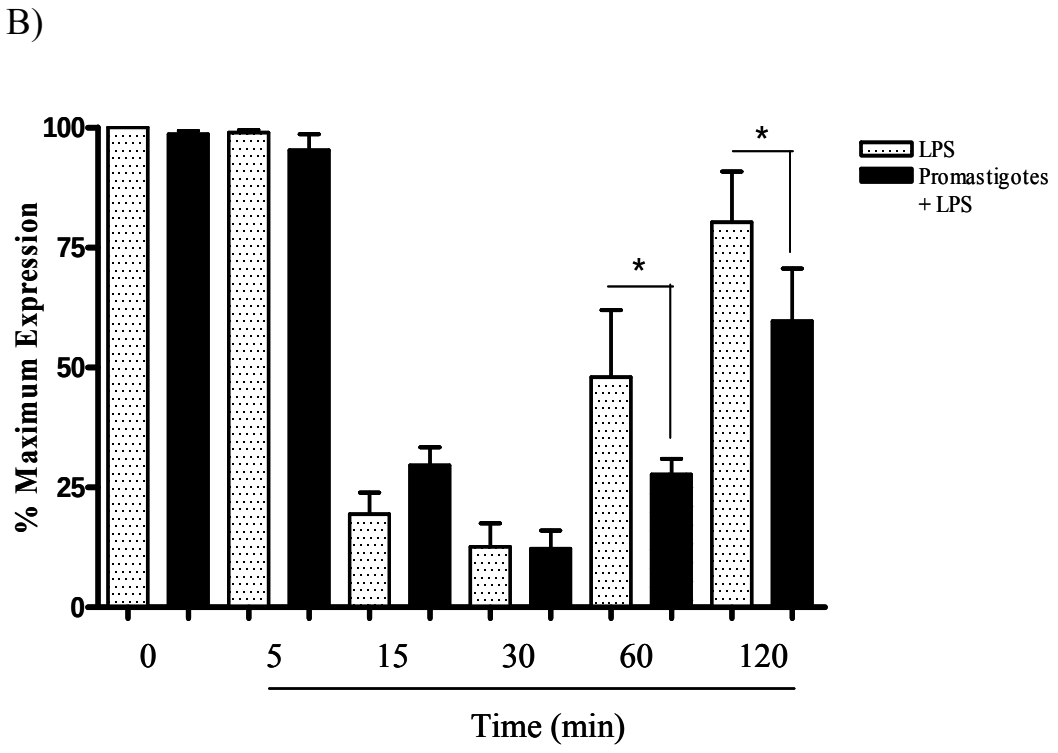


Figure 4.4: The effect of *Leishmania mexicana* promastigote infection on IκB-α loss in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* at a ratio of (5:1) for 2 hrs and then stimulated with LPS (1μg/ml) for up to a further 2 hrs. Whole cell lysates were prepared and then assessed for **A)** IκB-α (38 kDa) and T-p38 as outlined in Section 2.2.3. Blots were quantified in **B)** by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings. *$p<0.05$, compared with agonist-stimulated control.

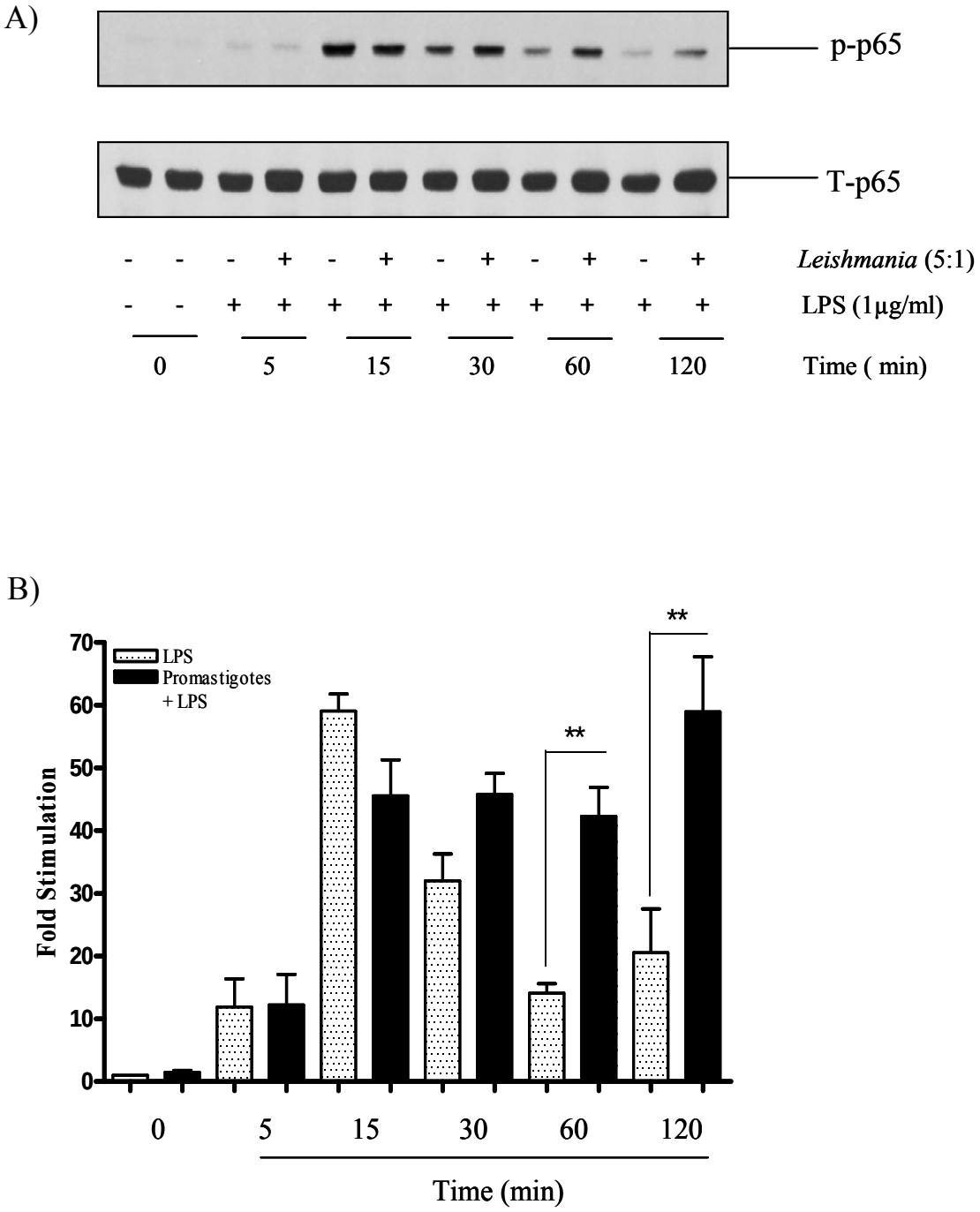


Figure 4.5: The effect of *Leishmania mexicana* promastigote infection on LPS-induced NF-κB phosphorylation in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* at a ratio of (5:1) for 2 hrs and then stimulated with LPS (1µg/ml) for up to a further 2 hrs. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for **A)** p-p65 and T-p65 as outlined in Section 2.1.3. Blots were quantified in **B)** fold stimulation by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings. **$p<0.001$, compared with agonist-stimulated control.

4.3 PROMASTIGOTES MEDIATE INCREASED MAP KINASE AND NF-κB SIGNALLING IN MACROPHAGES

4.3.1 Promastigotes induce MAP kinase phosphorylation in BMD-macrophages

Having established that promastigotes can modulate LPS-induced increases in MAP kinase and NF-κB signalling, the effect of promastigotes alone was examined. Initially, phosphorylation of JNK1/2 was assessed by Western blotting, in macrophages that had been infected with promastigotes (5:1) for a time period of 0-240 minutes. JNK phosphorylation increased as early as 15 min following promastigote addition and gave a maximum response by 30 min (fold stim. at 30 min = 40.70 ± 11.49). Phosphorylation was prolonged and remained above basal values for up to 240 min, the longest time point studied. This suggested a strong coupling of promastigotes to the JNK pathway (see figure 4.6).

Promastigotes also stimulated ERK phosphorylation (Figure 4.7). However, in contrast to JNK, ERK phosphorylation was rapid reaching a peak between 5 and 15 min at approximately 70 fold of basal values (fold stim. at 30 min = 74.03 ± 4.57). Phosphorylation was transient and returned to near basal values by 60 min. Promastigotes stimulated the phosphorylation of p38 MAP kinase with similar rapid kinetics, maximal values were reached as early as 5 min (fold stim. 53.74 ± 6.00). However, in contrast to ERK and JNK signaling p38 MAP kinase phosphorylation remained at maximum values for the rest of the time course. (see figure 4.8).

4.3.2 Promastigotes induce loss of cellular IκB-α and increase NF-κB (p65) activation in BMD-macrophage

Promastigotes also induced cellular loss in IκB-α in macrophages. However, in contrast with LPS stimulation, promastigotes alone caused a slow time-dependent decrease which was maximal by approximately 60 min (% maximum expression =

32.14 ± 10.51). Also, in contrast with LPS, there was no apparent reversal in degradation which was maintained up to 240 min with maximum degradation or loss at 120 min (% maximum expression = 16.42 ± 9.57,) (Figure 4.9). Promastigotes also stimulated the phosphorylation of p65 NF-κB which was observed by 30 min and maximal by 60 min. Again, unlike LPS, phosphorylation was maintained for 4 hours the longest time point studied (74.30 ± 16.29, fold) (Figure 4.10).

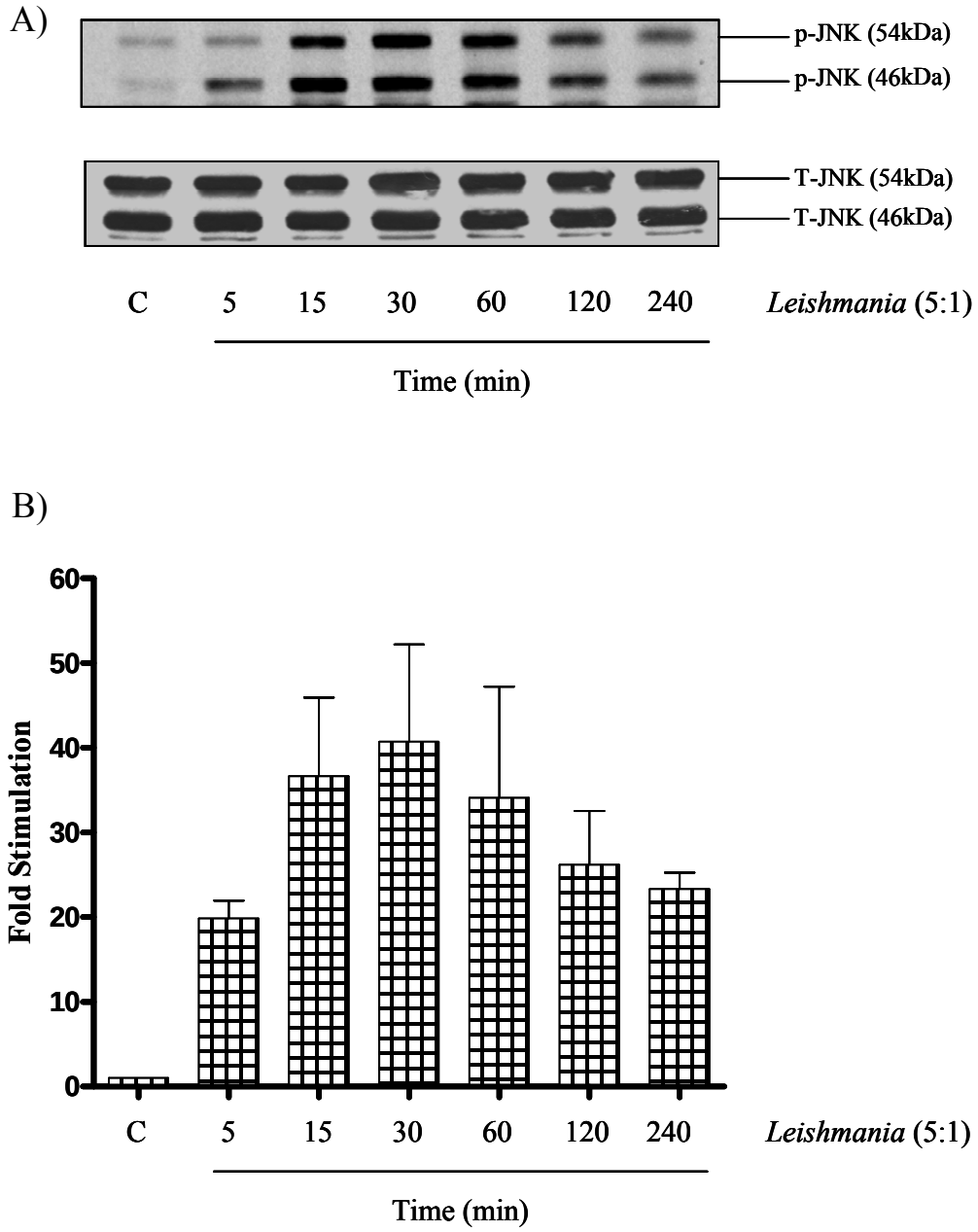


Figure 4-6: *Leishmania mexicana* promastigote infection induced JNK1/2 phosphorylation in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* at a ratio of (5:1) over a time period of 0-240 min. Whole cell lysates were prepared and then assessed for **A)** p-JNK (46/55 kDa) and JNK (46/55 kDa) as outlined in Section 2.2.3. Blots were quantified in **B)** by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings.

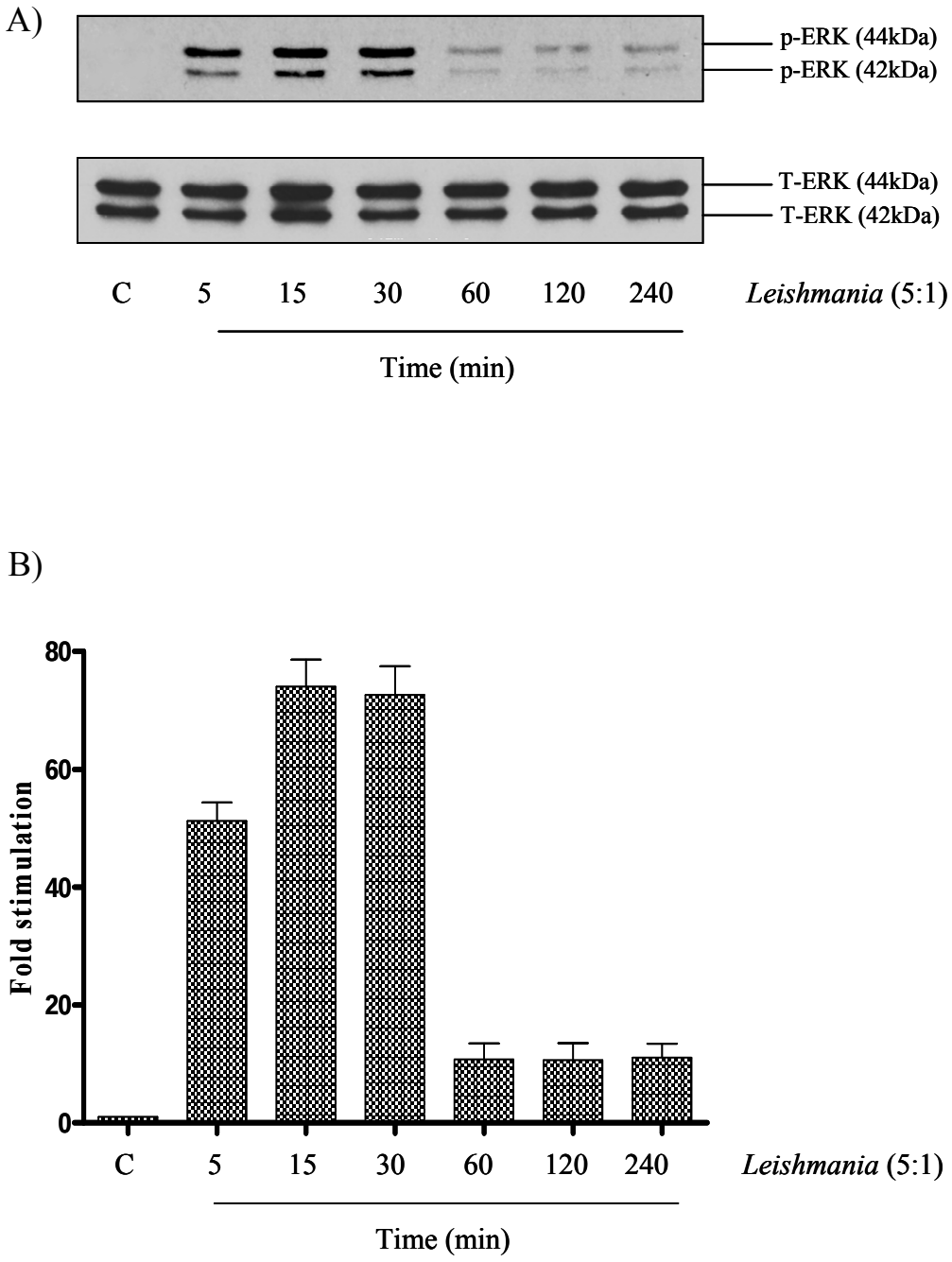


Figure 4.7: *Leishmania mexicana* promastigote infection induced ERK1/2 phosphorylation in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* at a ratio of (5:1) over a time period of 0-240 min. Whole cell lysates were prepared and then assessed for **A)** p-ERK1/2 (42/44 kDa) and ERK (42/44 kDa) as outlined in Section 2.2.3. Blots were quantified in **B)** by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings.

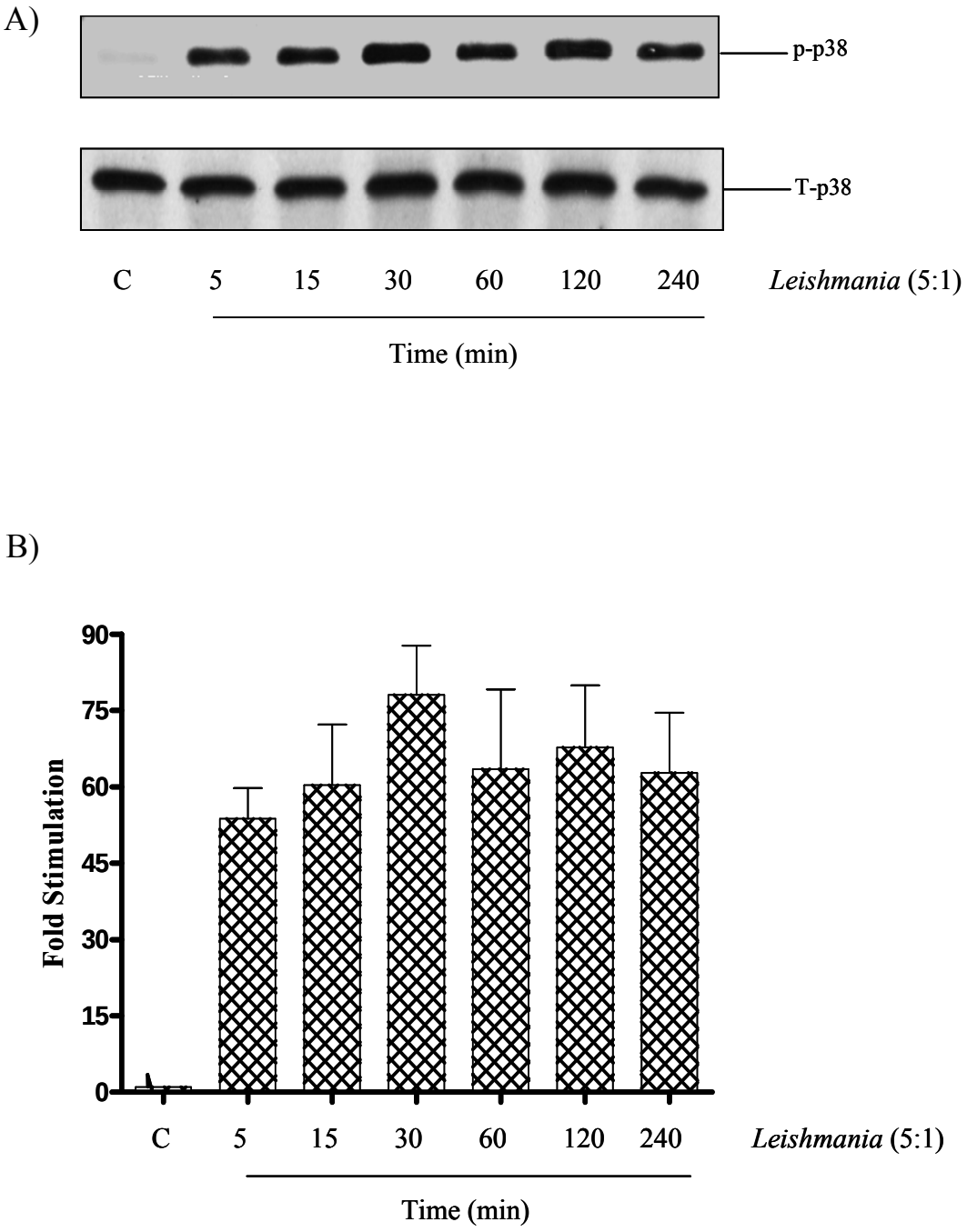


Figure 4.8: *Leishmania mexicana* promastigote infection induced p-38 MAP kinase phosphorylation in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* at a ratio of (5:1) over a time period of 0-240 minutes. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for **A)** p-p38 and T-p38 as outlined in Section 2.2.3. Blots were quantified in **B)** by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings.

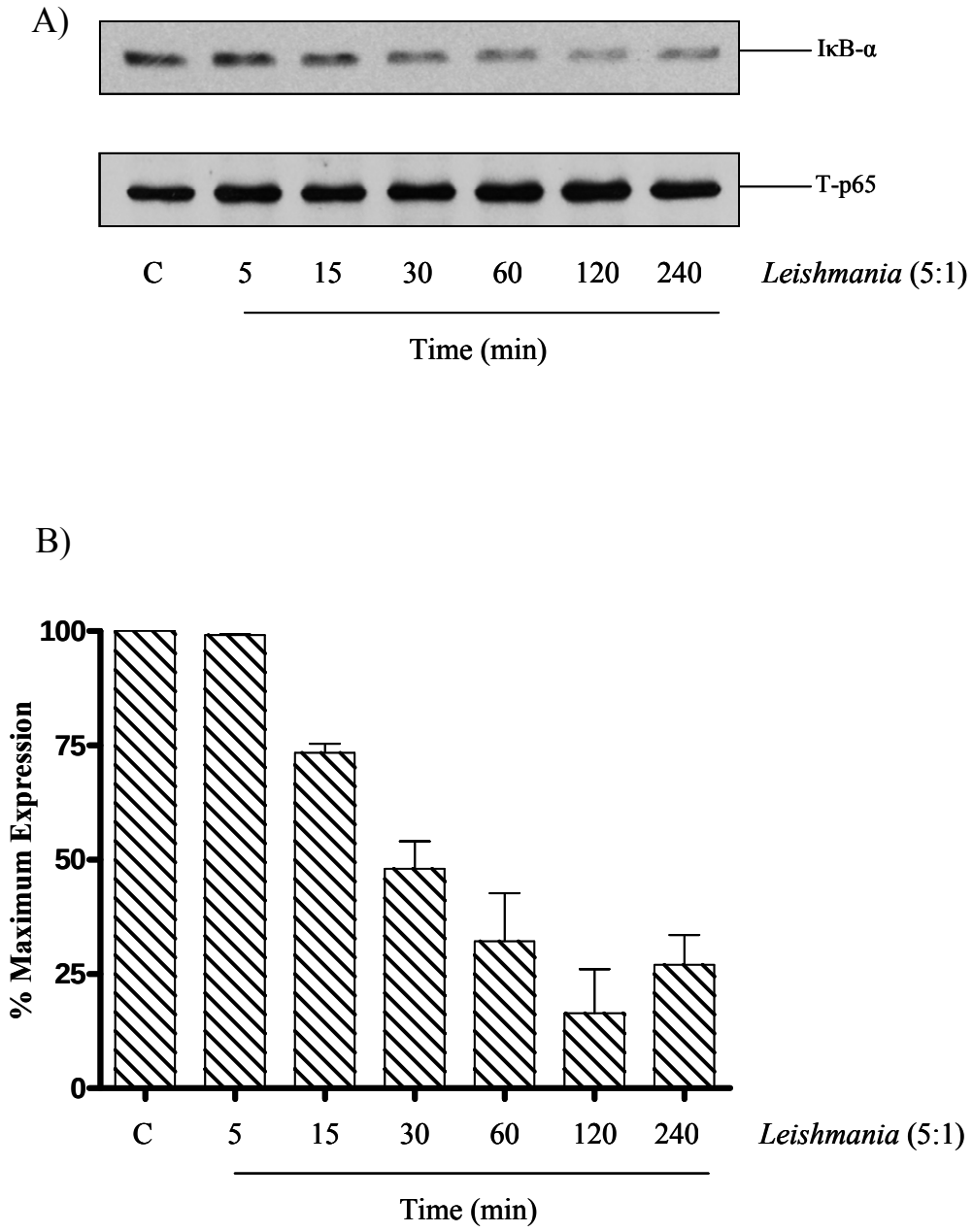
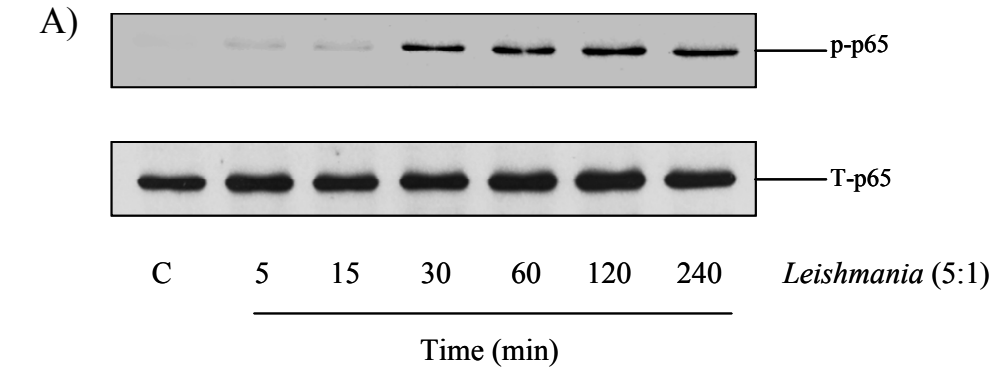


Figure 4.9: *Leishmania mexicana* promastigote infection induces IκB-α loss in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* at a ratio of (5:1) over a time period of 0-240 minutes. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for **A)** IκB-α and T-p65 as outlined in Section 2.2.3. Blots were quantified in **B)** by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings.



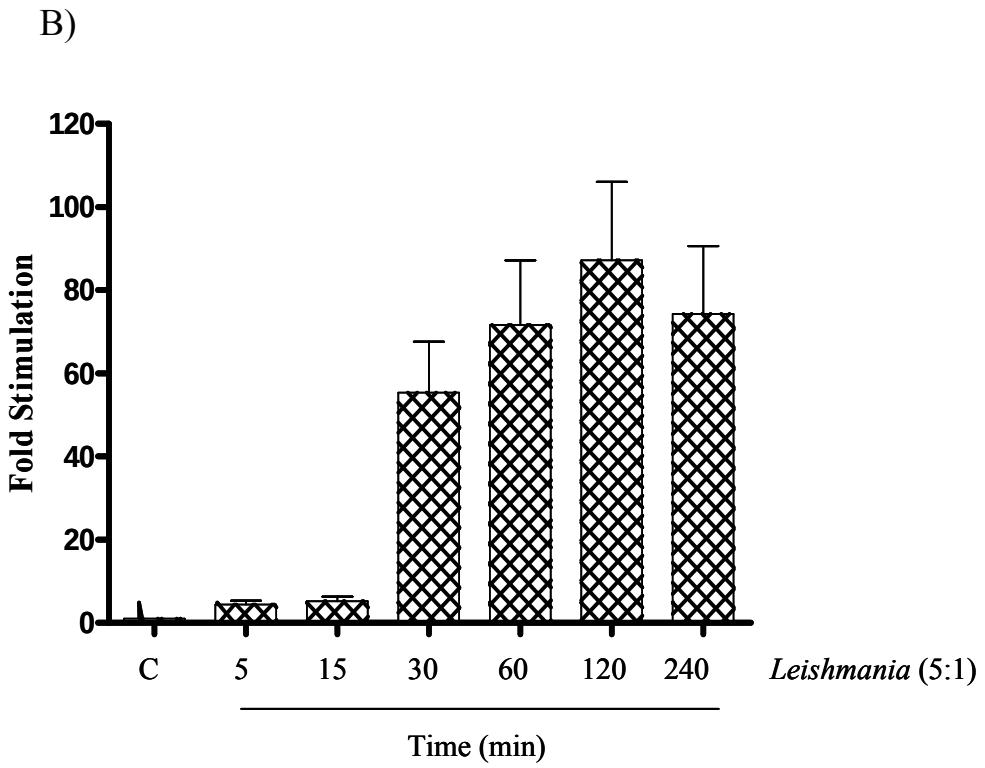


Figure 4.10: *Leishmania mexicana* promastigote infection induced NF-κB (p65) phosphorylation in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* at a ratio of (5:1) over a time period of 0-240 min. Whole cell lysates were preparedand then assessed for **A)** p-p65 and T-p65 as outlined in Section 2.2.3. Blots were quantified in **B)** by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings.

4.4 ΔCPB AMASTIGOTES ALONE MEDIATE INCREASED MAP KINASE PATHWAY SIGNALLING IN MACROPHAGES

In chapter three it had been established that amastigotes caused a largely inhibitory effect on MAP kinases signalling as well as NF-κB via a CPB enzyme-dependent mechanism. Therefore a CPB deficient form of amastigote was used to determine the effect on macrophages cellular responses in the absence of this enzyme. Macrophages were incubated with ΔCPB amastigotes under the same conditions as with the promastigotes.

4.4.1 ΔCPB amastigotes induce MAP kinase phosphorylation in BMD-macrophages

Initially, phosphorylation of JNK1/2 was assessed by Western blotting, in macrophages that had been infected with ΔCPB amastigotes (5:1) for a time period of 0-240 min. JNK phosphorylation increased as early as 5 min as observed for ΔCPB amastigotes stimulation and gave a maximum response between 30 and 60 min (fold stim. at 30 and 60 min = 39.52 ± 4.30 and 40.79 ± 4.48 fold increase). Phosphorylation was prolonged and remained above basal values for up to 240 min, the longest time point studied. This suggested a strong coupling of ΔCPB amastigotes to the JNK pathway (see figure 4.11).

ΔCPB amastigotes also stimulated ERK phosphorylation in a similar manner to promastigotes (Figure 4.12). ERK phosphorylation was rapidly reaching a peak between 5 and 15 minutes at approximately 90 fold of basal values (fold stim. at 15 min = 91.37 ± 16.46). Phosphorylation was also transient and returned to near basal values by 60 minutes. ΔCPB amastigotes stimulated the phosphorylation of p38 MAP kinase with similar rapid kinetics to that observed with promastigotes, a maximal value was reached as early as 5 min (fold stim. = 2.87 ± 0.51). However, in contrast to promastigote p38 MAP kinase phosphorylation, the response to ΔCPB amastigotes was largely transient returning to basal values within 30 min. (see figure 4.13). By and large ΔCPB amastigotes gave a similar overall MAP kinase response relative to promastigotes.

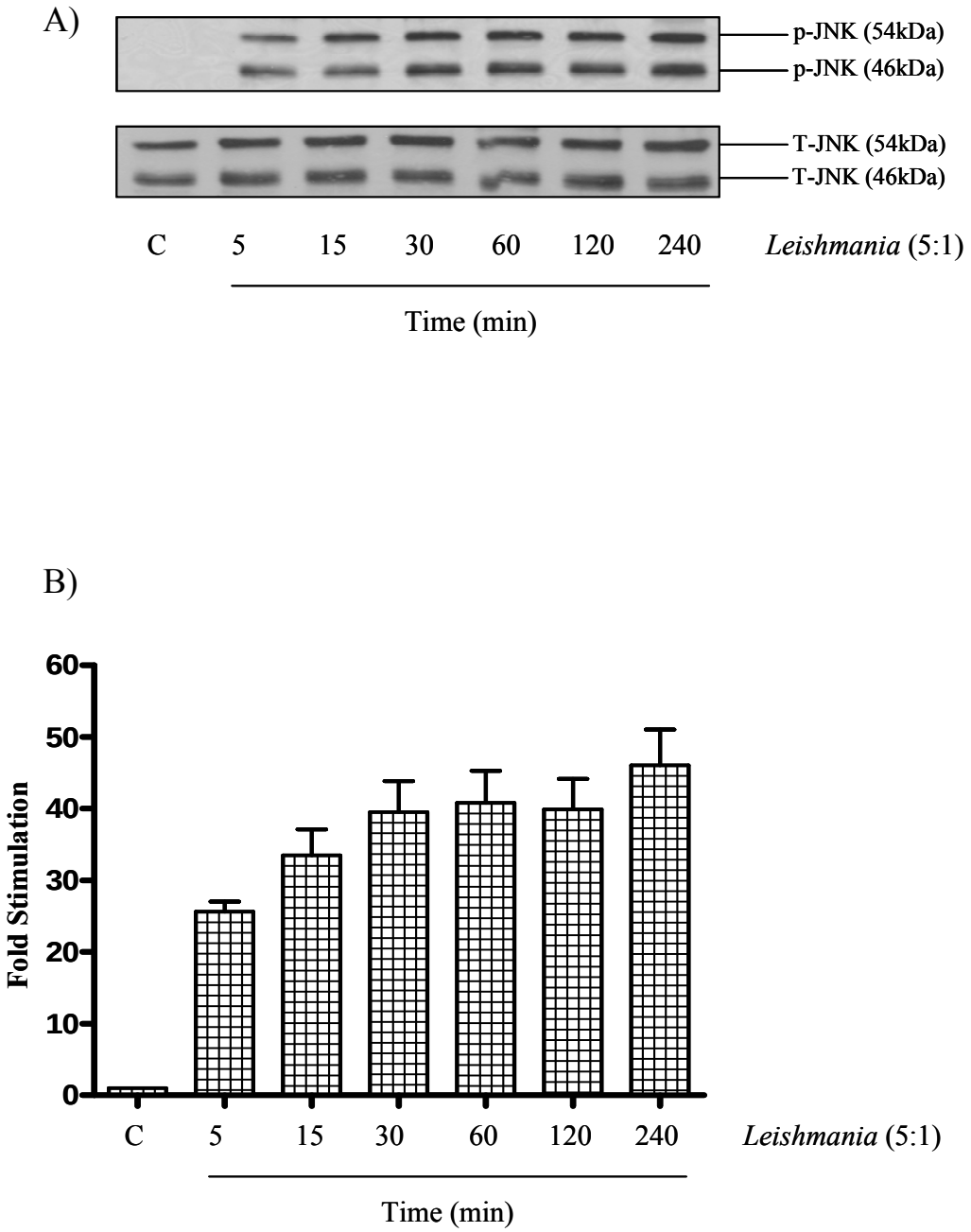


Figure 4.11: *Leishmania mexicana* ΔCPB amastigote infection induces JNK1/2 phosphorylation in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* at a ratio of (5:1) over a time period of 0-240 min. Whole cell lysates were prepared and then assessed for **A)** p-JNK (46/54 kDa) and JNK (46/54 kDa) as outlined in Section 2.2.3. Blots were quantified in **B)** by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings.

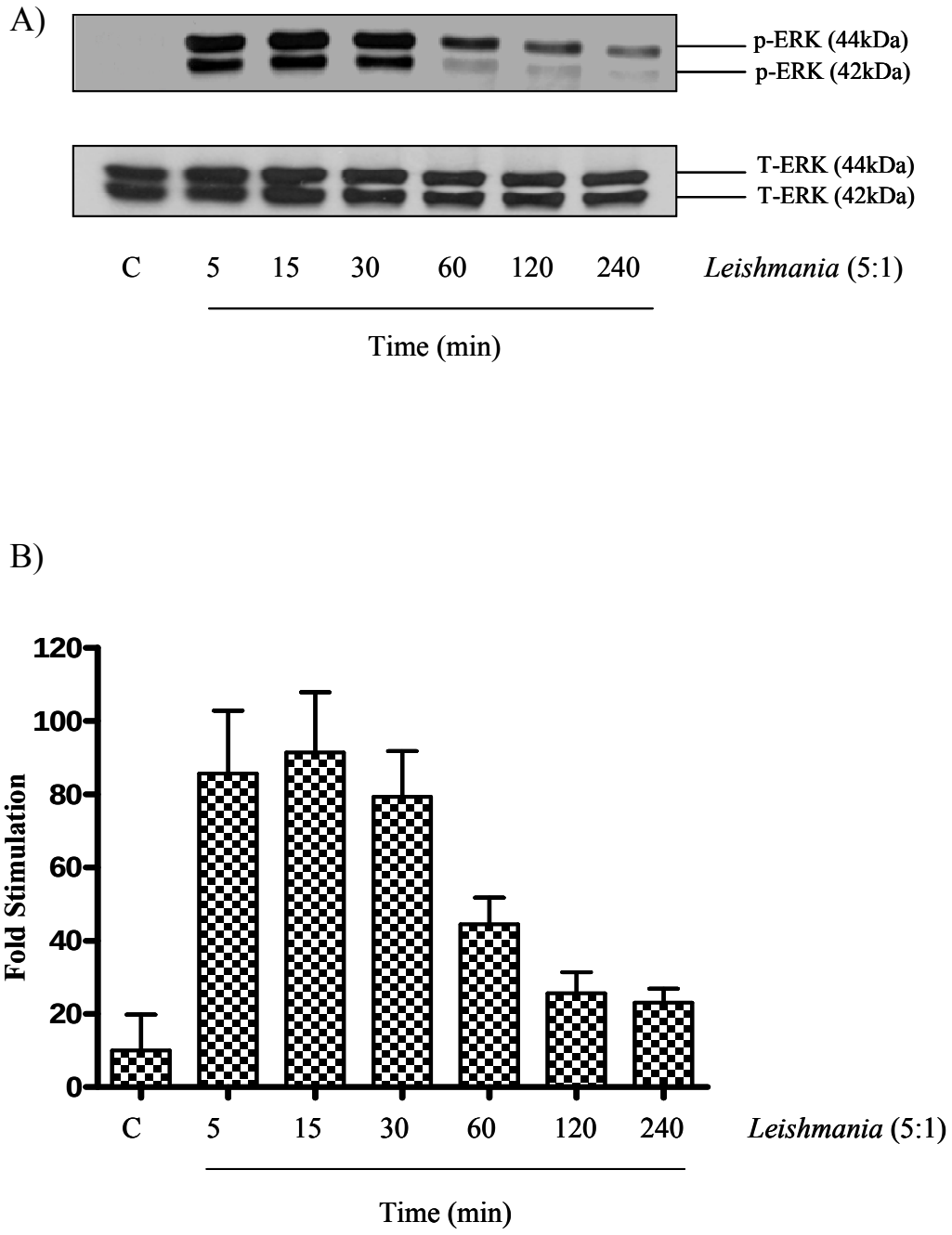


Figure 4.12: *Leishmania mexicana* ΔCPB amastigote infection induces ERK1/2 phosphorylation in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* at a ratio of (5:1) over a time period of 0-240 min. Whole cell lysates were prepared and then assessed for A) p-ERK1/2 (42/44 kDa) and ERK (42/44 kDa) as outlined in Section 2.2.3. Blots were quantified in B) by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings.

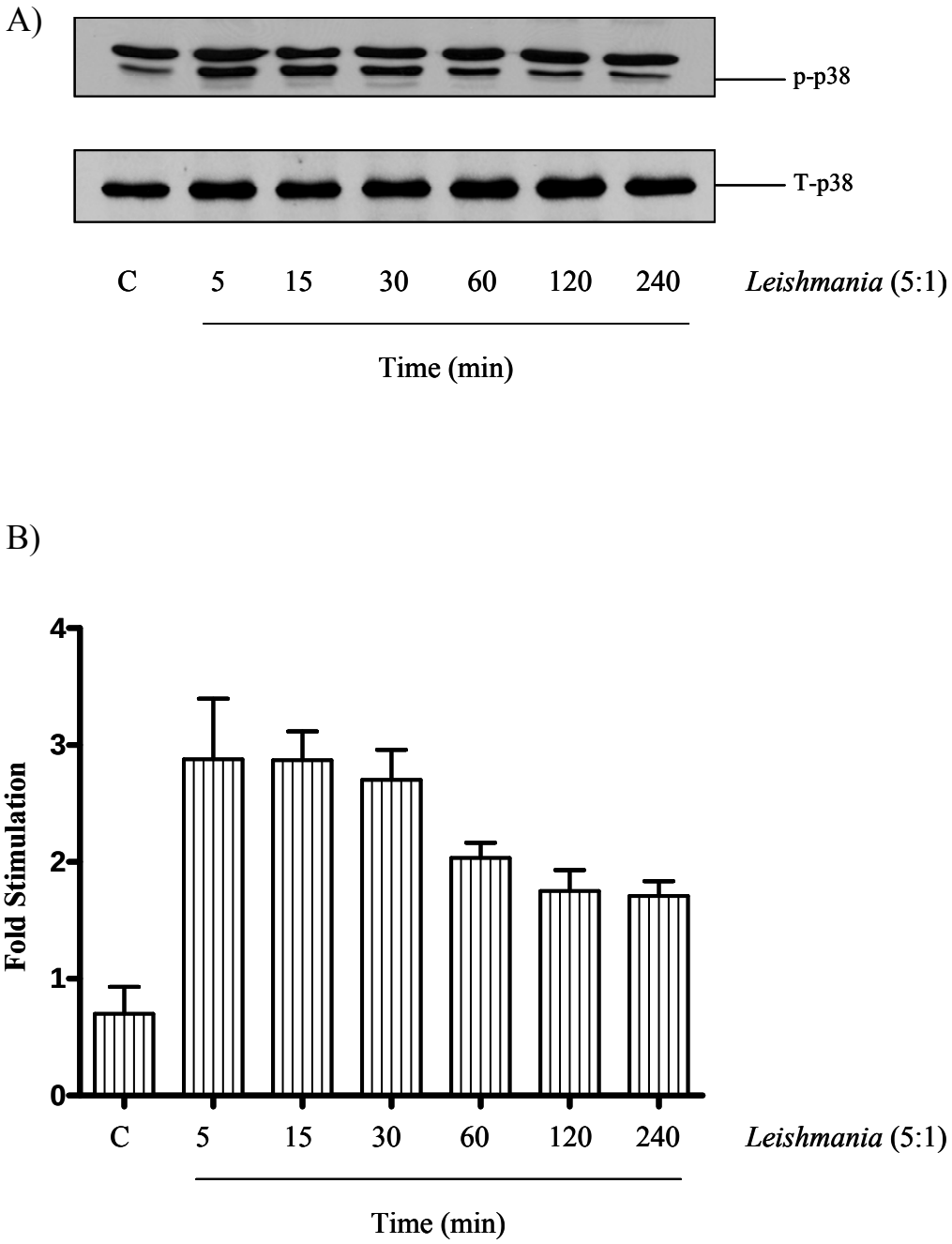


Figure 4.13: *Leishmania mexicana* ΔCPB amastigote infection induce p-38 MAP kinase phosphorylation in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* at a ratio of (5:1) over a time period of 0-240 minutes. Whole cell lysates were prepared and then assessed for **A)** p-p38 and T-p38 as outlined in Section 2.2.3. Blots were quantified in **B)** by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings.

4.5 THE EFFECT OF *LEISHMANIA MEXICANA* PROMASTIGOTES INFECTION UPON LPS MEDIATED PROTEIN EXPRESSION OF COX-2 AND iNOS

4.5.1 The effect of *Leishmania* promastigote infection on COX-2 protein expression in BMD-macrophages

Having established the ability of promastigotes to increase MAP kinase activities in infected macrophages, it was important to study the effect upon inflammatory protein expression. Initially, the induction of COX-2 was measured over 6 hrs as a direct comparison to the studies using amastigotes. LPS alone caused a substantial increase in COX-2 expression (figure 4.14). In contrast, *Leishmania* at a concentration which caused strong increase in MAP kinase activity was without effect. Furthermore, pre-incubation for 2 hrs prior to LPS stimulation failed to significantly increase cellular COX-2 levels. Nevertheless, when cells were incubated with *Leishmania* subsequent to stimulation with LPS, COX-2 expression was significantly increased (Figure 4.15). Alone LPS gave a 50 fold increased in COX-2 expression, however addition of promastigotes for a further 2 hrs increased values by almost 2 fold (fold stim., LPS= 57.19 ± 8.90, LPS+*Leishmania* = 90.93 ± 1.85). These findings prompted analysis of COX-2 expression over a time course of up to 72 hrs. Alone LPS stimulated a strong induction of COX-2 which was again maximal by 6 hrs before returning to basal values by 48 hrs. Whilst alone over a time course promastigotes failed to induce COX-2 expression over the longer time course, 2 hrs promastigotes caused a substantial enhancement of the LPS response which was apparent at 24, 48, and 72 hrs time points. At 48 hrs the expression was as high as was observed for LPS alone after 6 hrs (fold stim., LPS= 6.61 ± 2.89, *Leishmania* +LPS = 178.05 ± 35.22) (figure 4.16).

4.5.2 Pre-incubation with promastigotes prolongs LPS-induced PGE_2 production in BMD-macrophage

Previous studies have shown that macrophages and other cell type such as fibroblasts and some types of malignant cells are capable of mediating an increase in the production of PGE_2 mediated through enhanced COX-2 expression and the subsequent conversion of arachidonic acid to PGH_2 (Harris et al., 2002). Therefore, the effect of promastigotes on LPS- induced PGE_2 formation was examined.

LPS induced PGE_2 production in macrophages was time-dependent, reaching a maximum by (12-24) hrs and was maintained at this level for a further 60 hrs. As expected, pre-treatment with promastigotes enhanced LPS induced PGE_2 production as early as 12 hrs and significantly by 24 and 48 hrs (24 hrs, LPS = 640.08 ± 63.71, *Leishmania*+LPS = 1641.99 ± 201; 48 hrs, LPS = 733.76 ± 300, *Leishmania*+LPS = 1534.69 ± 370). However, in contrast to COX-2, there was no significant difference in the levels of PGE_2 production after 72 hrs (see figure 4.17).

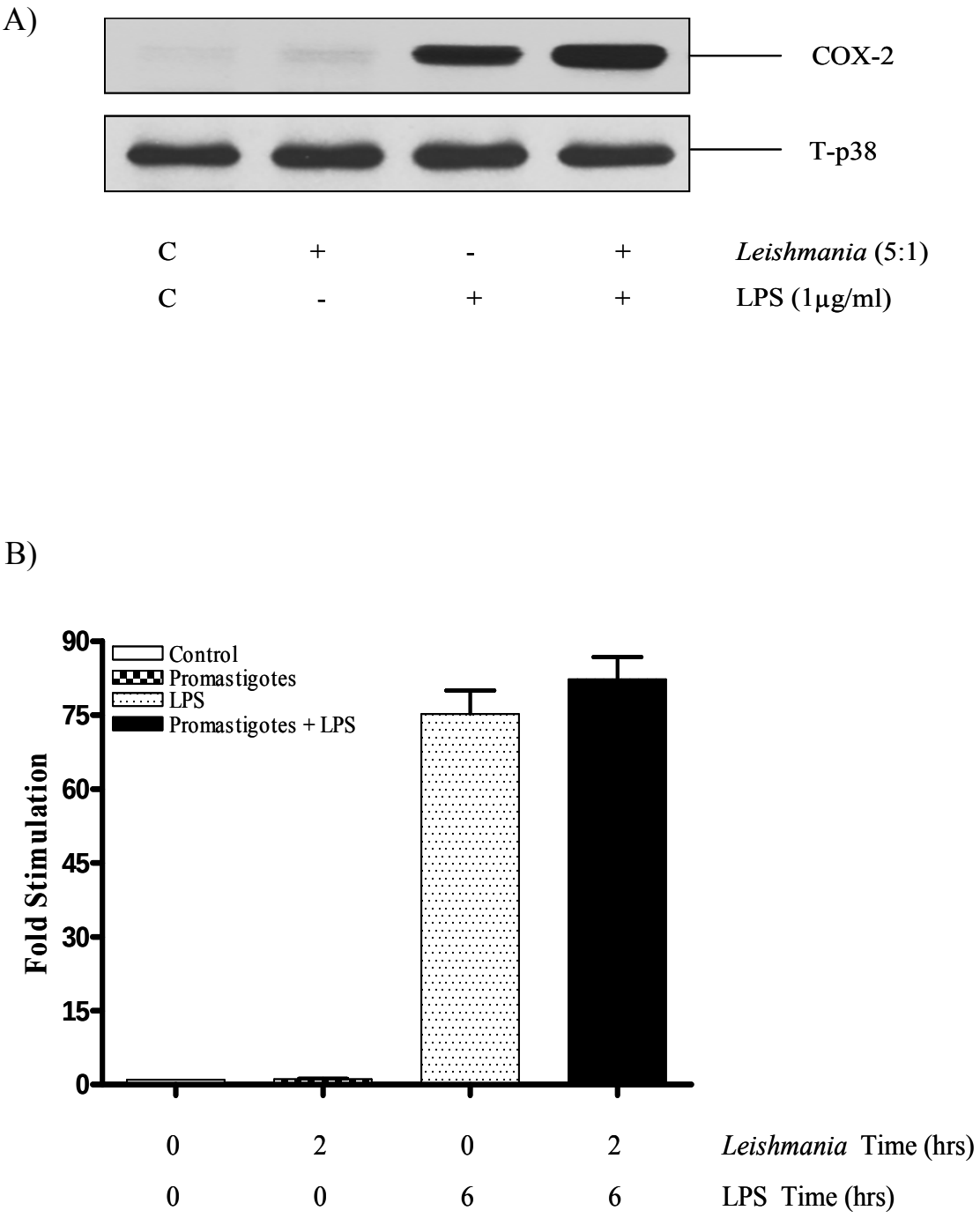


Figure 4.14: The effect of *Leishmania mexicana* promastigote infection on COX-2 protein expression in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* at a ratio of (5:1) for 2 hrs and then stimulated with LPS (1µg/ml) for 6 hrs. Whole cell lysates were prepared and then assessed for **A)** COX-2 (72 kDa) and T-p38 as outlined in Section 2.2.3. Blots were quantified in **B)** by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings.

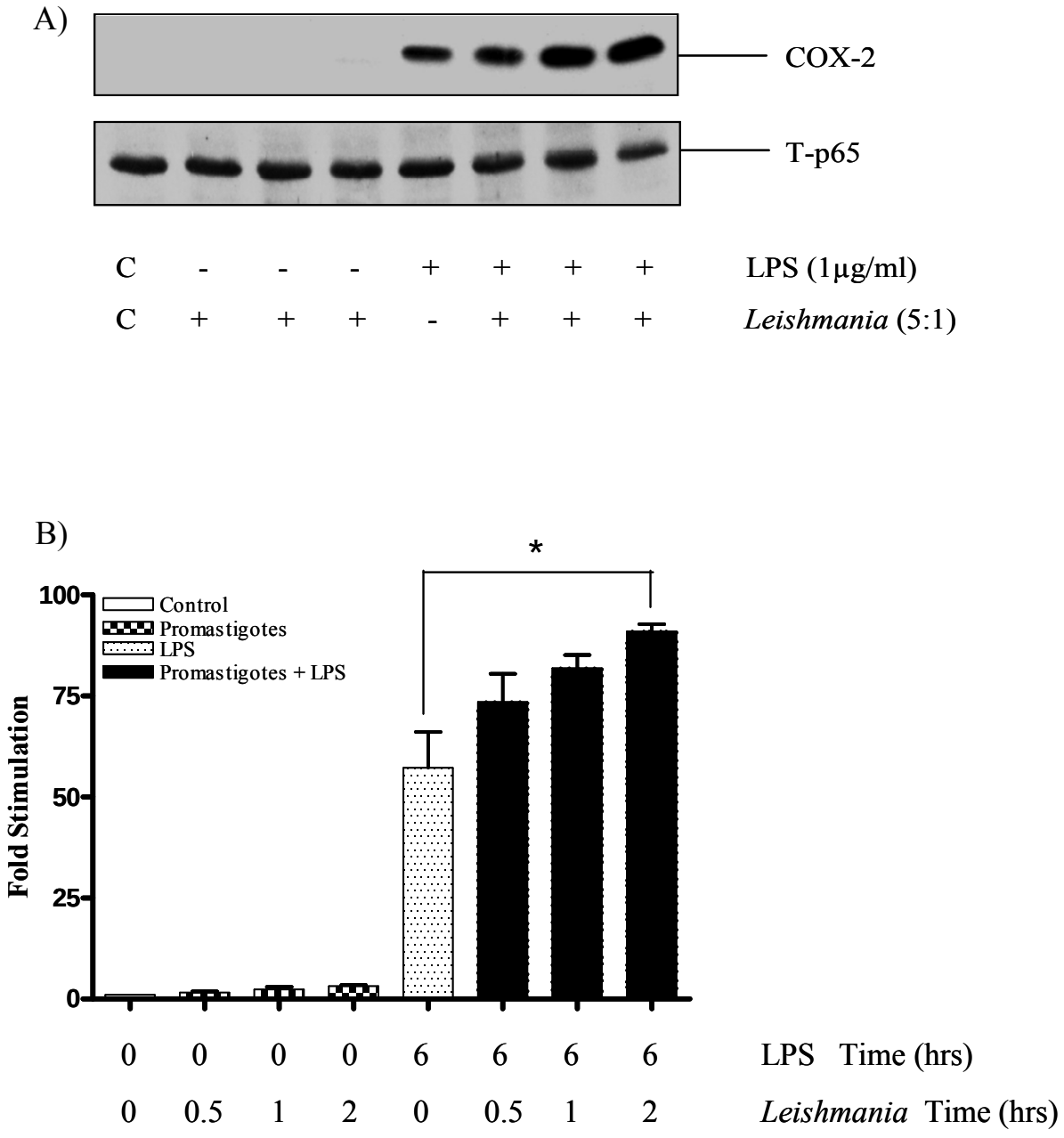


Figure 4.15: The effect of *Leishmania mexicana* promastigote infection on COX-2 protein expression in macrophages. Cells ($1x10^6$/well) were stimulated with LPS (1µg/ml) for 6 hrs and then infected with *Leishmania* at a ratio of (5:1) for different times over a period of 0.5-2 hrs. Whole cell lysates were prepared and then assessed for **A)** COX-2 (72 kDa) and T-p38 as outlined in Section 2.2.3. Blots were quantified in **B)** by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings. *$p<0.05$, compared with agonist-stimulated control.

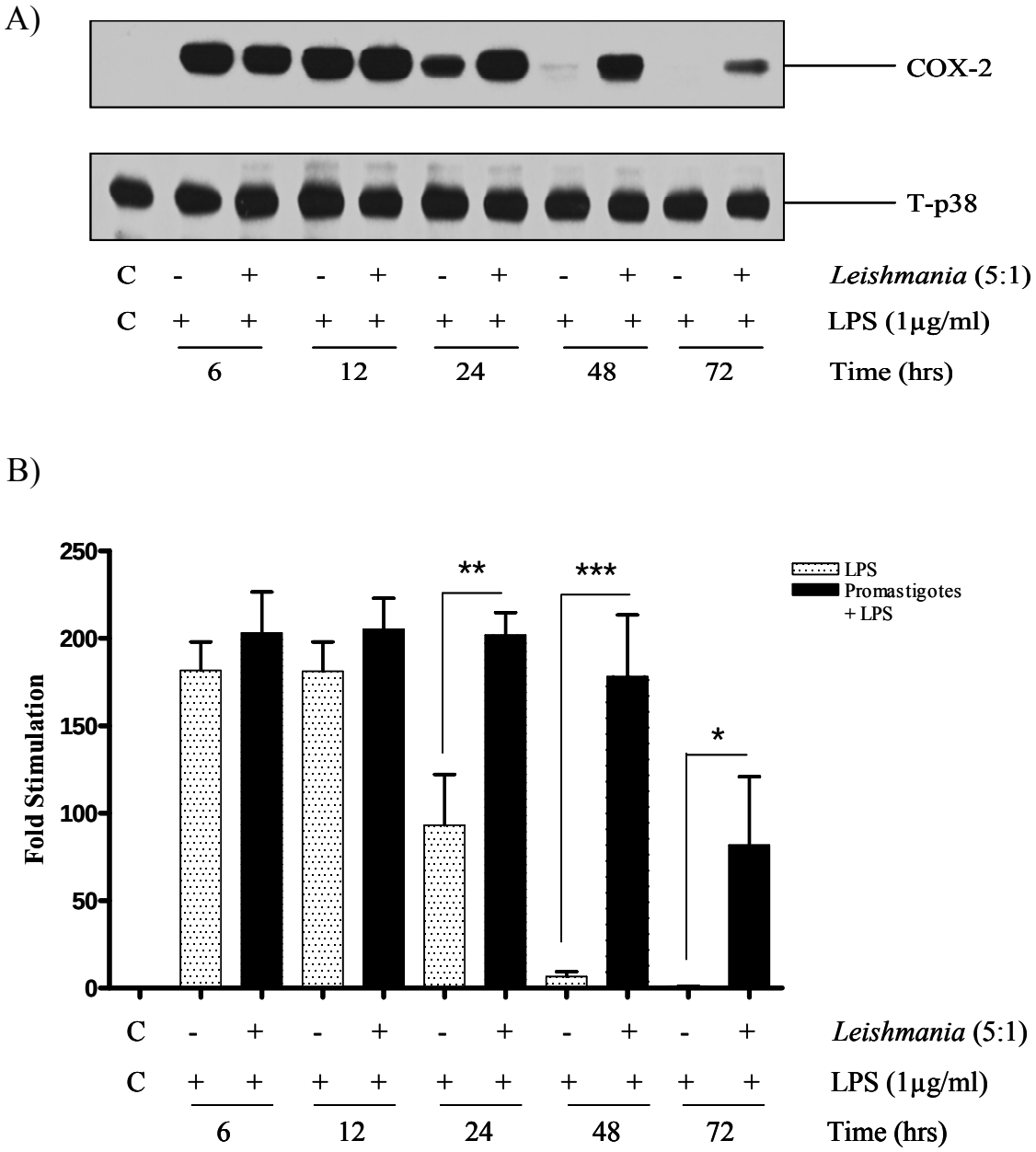


Figure 4.16: *Leishmania mexicana* promastigote infection enhanced LPS-induced COX-2 protein expression in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* at a ratio of (5:1) for 2 hrs and then stimulated with LPS (1µg/ml) over a period of 0-72 hrs. Whole cell lysates were prepared and then assessed for **A)** COX-2 (72 kDa) and T-p38 as outlined in Section 2.2.3. Blots were quantified in **B)** by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings. *p<0.05, **p<0.01 and ***p<0.001 compared with agonist-stimulated control.

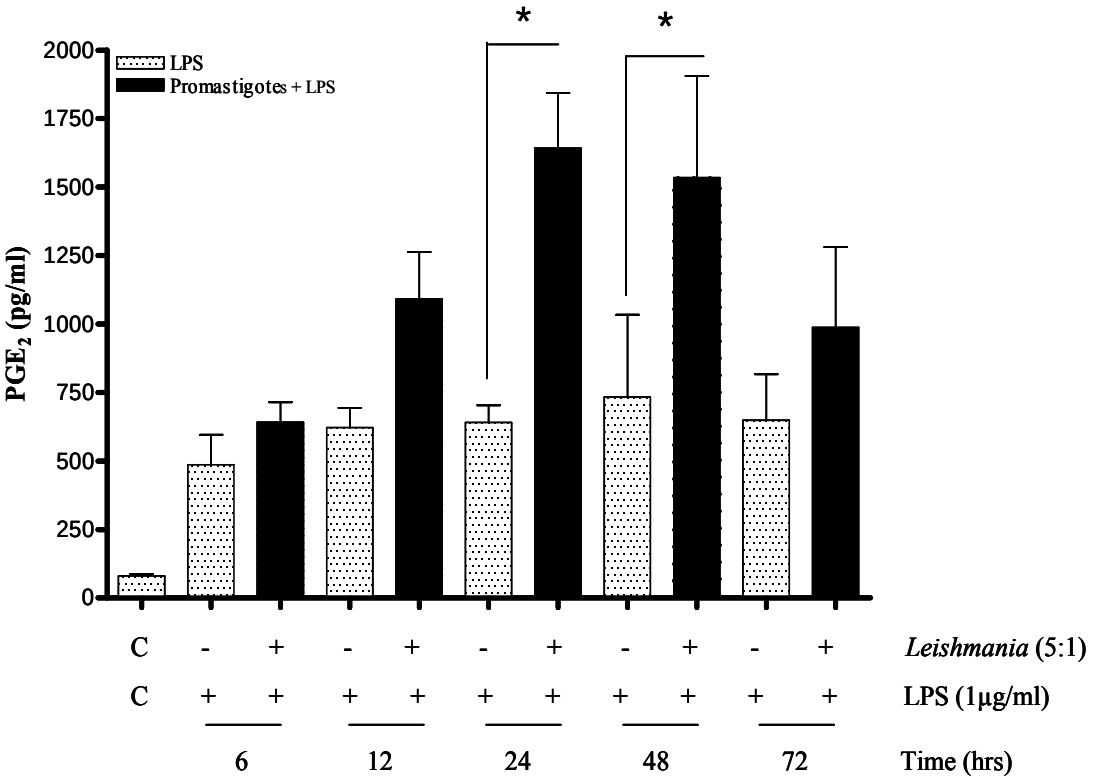


Figure 4.17: *Leishmania mexicana* promastigote infection enhanced LPS-induced PGE_2 production in macrophages. Cells ($1x10^6$/well) were grown overnight in a 12 well plate and infected with *Leishmania* at a ratio of (5:1) for 2 hrs and then stimulated with LPS (1µg/ml) over a period of 0-72 hrs. Supernatants were collected and PGE_2 levels assessed by ELISA (as outlined in section 2.2.4.2). Values were quantified and expressed as mean ± S.E.M. (PGE_2 pg/ml, *$p<0.05$ as compared with stimulated control). Each of the above columns is representative of 3 separate experiments with similar findings.

4.5.3 The effect of *Leishmania* promastigotes infection on iNOS protein expression in BMD-macrophage

The effect of *Leishmania* promastigotes on iNOS expression and NO release was compared in the equivalent samples for COX-2. Figure 4.18 shows the induction of iNOS at the 6 hr time point. Again promastigotes alone did not induce iNOS expression above basal background levels. LPS induced increased expression of the iNOS by approximately 25 fold (24.02 ± 1.59). Again, pre-incubation with promastigotes failed to enhance the LPS response. In contrast to COX-2, when promastigotes were added to the cells after LPS there was no significant increase in iNOS expression (Figure 4.19). Nevertheless, the effect of promastigotes on long term expression of iNOS in response to LPS was examined. LPS alone stimulated a strong increase in iNOS protein expression which was maximal between 12 and 24 hrs before returning to levels values by 48 hrs. Promastigotes significantly enhanced LPS induced iNOS expression at both 24 and 48 hrs, although by 72 hrs iNOS levels were no different from control (fold stim., at 48 hrs, LPS = 40.69 ± 21.00, *Leishmania*+LPS = 180.19 ± 31.72, **$p<0.01$) (see figure 4.20).

4.5.4 Pre-incubation with promastigotes prolongs LPS-induced nitric oxide (NO) production in BMD-macrophage

Previous studies have shown the ability of macrophages to mediate NO production via L-arginine through increased iNOS protein expression. Therefore the effect of promastigotes on NO release was examined (Figure 4.21). Following a delay of 6 hr, LPS caused a time-dependent induction of NO release which was maximal between 12 and 24 hrs. NO release was maintained at these levels for further 48 hrs. Pre-treatment with promastigotes caused a significant enhancement of NO production at 24 and 48 hrs. In contrast to iNOS, promastigotes caused high NO production up to the 72 hrs time point (LPS = 15.99 ± 2.45 and *Leishmania*+LPS = 28.64 ± 3.73 for 27 hrs, **$p<0.01$).

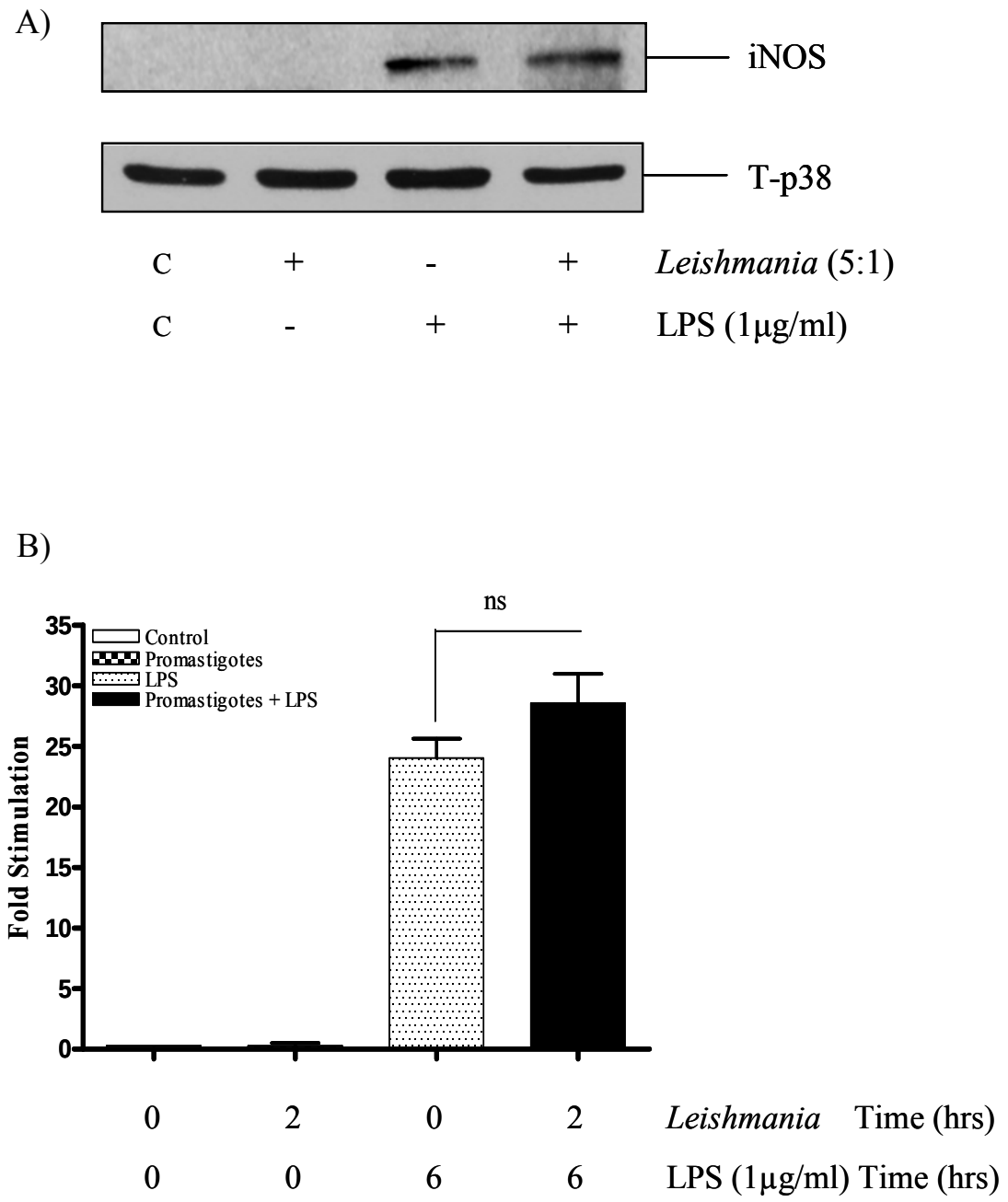


Figure 4.18: The effect of *Leishmania mexicana* promastigote infection on iNOS protein expression in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* at a ratio of (5:1) for 2 hrs and then stimulated with LPS (1µg/ml) for 6 hrs. Whole cell lysates were prepared and then assessed for **A)** iNOS (130 kDa) and T-p38 as outlined in Section 2.2.3. Blots were quantified in **B)** by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings.

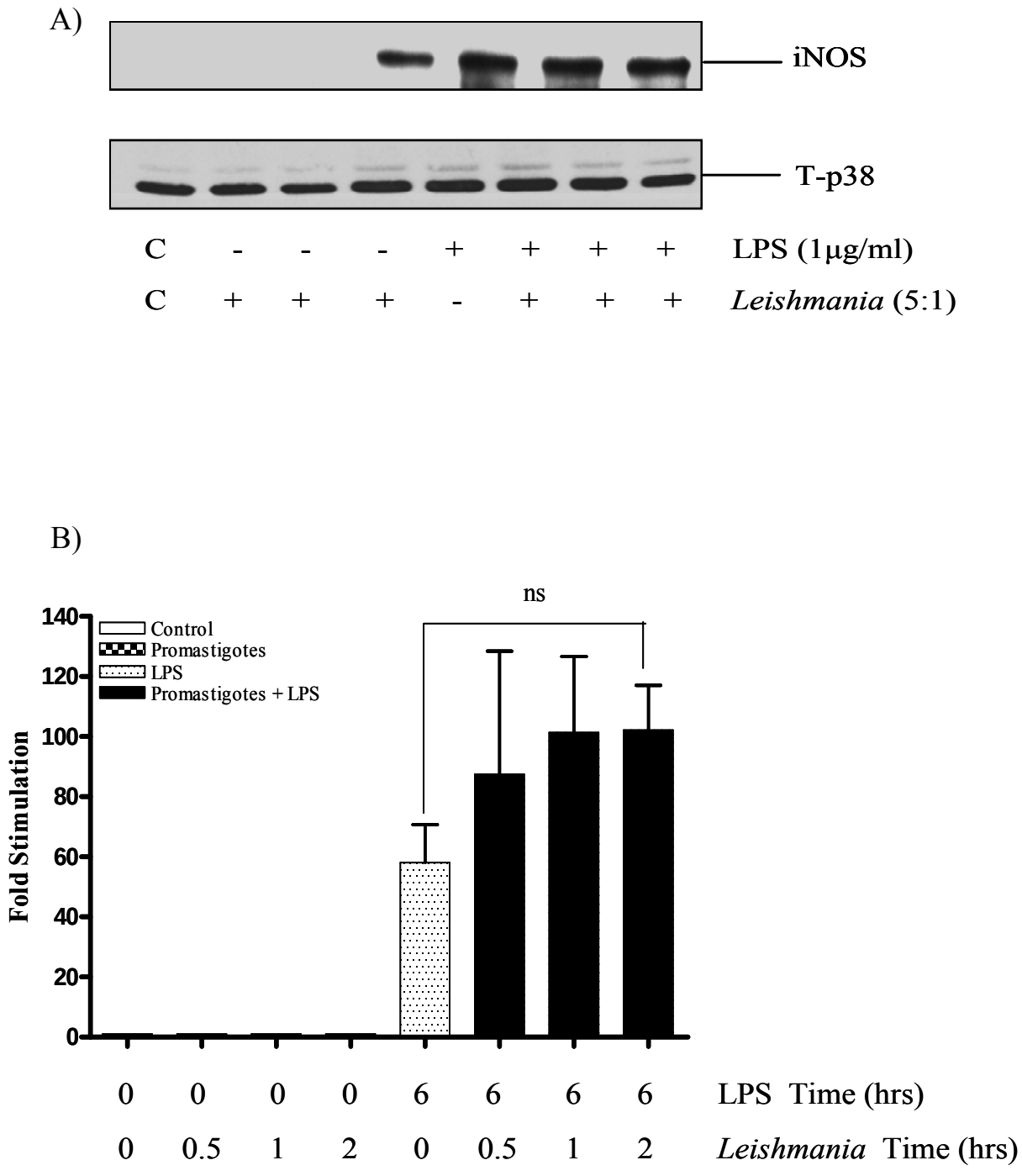


Figure 4.19: The effect of *Leishmania mexicana* promastigote infection on iNOS protein expression in macrophages. Cells ($1x10^6$/well) were stimulated with LPS (1μg/ml) for 6 hrs and then infected with *Leishmania* at a ratio of (5:1) for different times over a period of 0.5-2 hrs. Whole cell lysates were prepared and then assessed for **A)** iNOS (130 kDa) and T-p38 as outlined in Section 2.2.3. Blots were quantified in **B)** by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings.

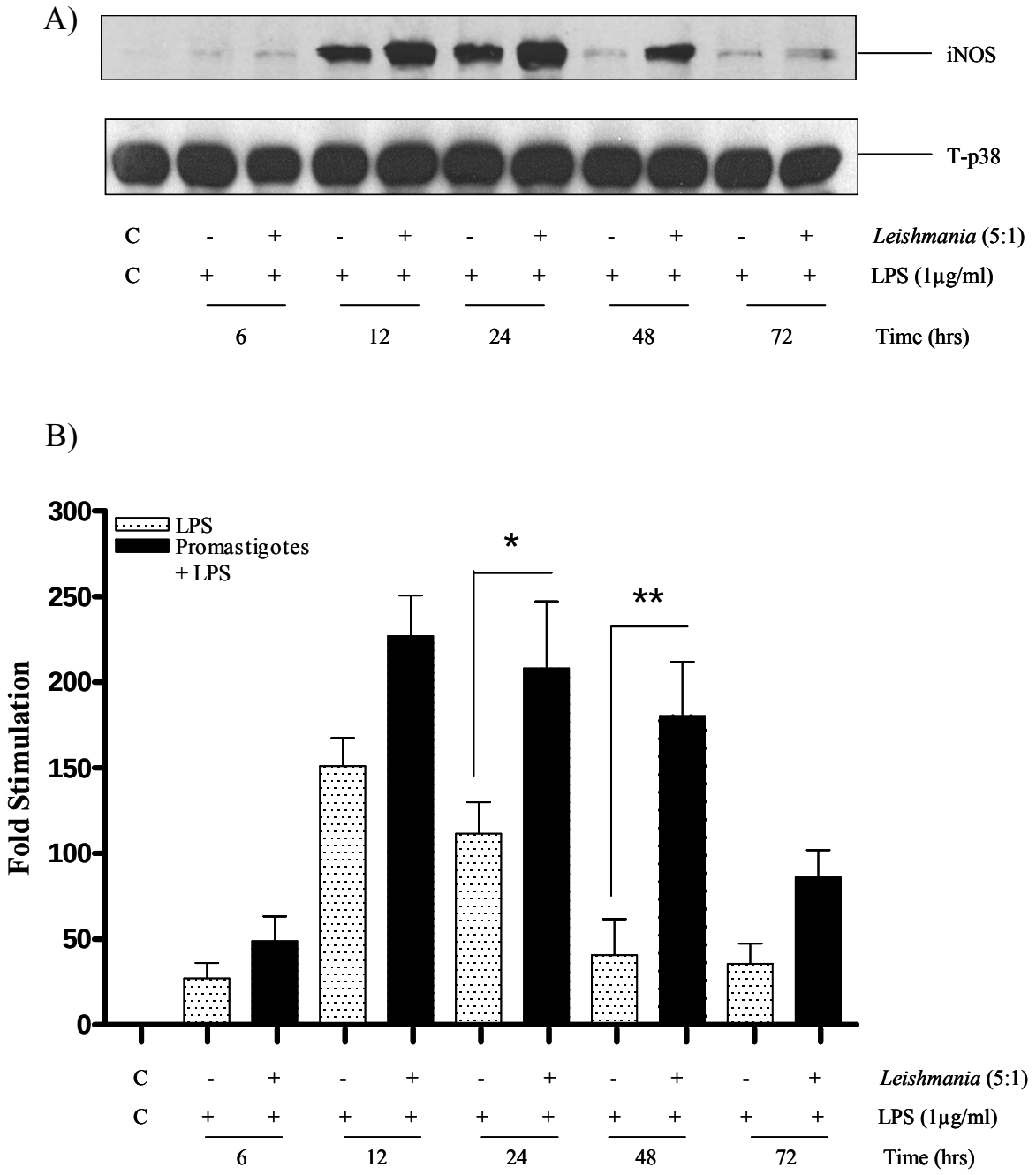


Figure 4.20: *Leishmania mexicana* promastigote infection enhanced LPS-induced iNOS protein expression in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* at a ratio of (5:1) for 2 hrs and then stimulated with LPS (1µg/ml) over a period of 0-72 hrs. Whole cell lysates were prepared and then assessed for **A)** iNOS (130 kDa) and T-p38 as outlined in Section 2.2.3. Blots were quantified in **B)** by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings. *$p<0.05$ and **$p<0.01$ compared with agonist-stimulated control.

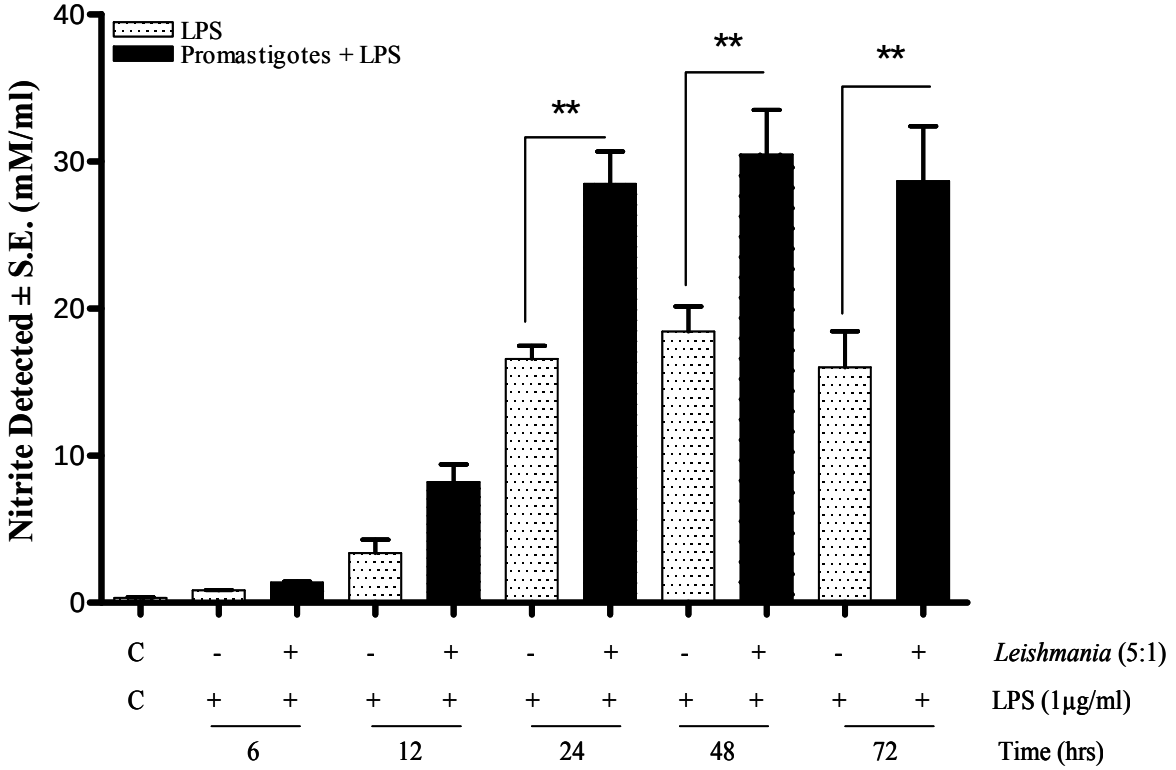


Figure 4.21: *Leishmania mexicana* promastigote infection enhanced LPS-induced NO production in macrophages. Cells ($1x10^6$/well) were grown overnight in a 12 well plate and infected with *Leishmania* at a ratio of (5:1) for 2 hrs and then stimulated with LPS (1µg/ml) over a period of 0-72 hrs. Supernatants were collected and nitrite levels assessed by Griess assay (as outlined in section 2.2.6). Values were quantified and expressed as mean ± S.E.M. (nitrite mM/ml, **$p<0.01$ as compared with stimulated control). Each of the above columns is representative of 3 separate experiments with similar findings.

4.5.5 The effect of *Leishmania* ΔCPB amastigotes infection on COX-2 protein expression in BMD-macrophages

As ΔCPB amastigotes caused a strong increase in MAP kinase activities in infected macrophages, it was important to compare the effect of this mutant on inflammatory protein expression, relative to promastigotes. Therefore, the effect of *Leishmania* ΔCPB amastigotes on LPS-induced COX-2 and iNOS expression in macrophages was examined. Initially the induction of COX-2 was measured at the 6 hr time point as a direct comparison to the studies using promastigotes in the previous section. LPS alone caused a substantial increase in COX-2 expression (Figure 4.22). In contrast *Leishmania*, at a concentration which caused strong increases in MAP kinase, was without effect. Furthermore, pre-incubation for 2 hrs prior to LPS stimulation failed to significantly increase cellular COX-2 levels. Nevertheless, when cells were incubated with *Leishmania* subsequent to stimulation with LPS, COX-2 expression was significantly increased (Figure 4.23). Alone LPS gave a 2 fold increase in COX-2 expression, however addition of ΔCPB amastigotes for a further 2 hrs increased values by almost 2 fold (fold stim., LPS = 68.09 ± 9.18, LPS+*Leishmania* = 128.40 ± 19.12, $^{**}p<0.01$).

4.5.6 The effect of *Leishmania* ΔCPB amastigotes infection on iNOS protein expression in BMD-macrophages

The effect of *Leishmania* ΔCPB amastigotes on iNOS expression was also compared in equivalent samples as for COX-2. Figure 4.24 shows the induction of iNOS at the 6 hrs time point. Again ΔCPB amastigotes alone did not induce iNOS expression above basal background levels. LPS alone induced increased expression of the protein by approximately 27 fold (fold stim., 27.97 ± 8.09). Again, pre-incubation with ΔCPB amastigotes failed to enhance the LPS response. Similar to COX-2, when ΔCPB amastigotes were added to the cells there was a significant increase in iNOS expression (fold stim., LPS = 66.48 ± 22.28 and *Leishmania*+LPS = 148.13 ± 29.04, $^{*}p<0.05$). (Figure 4.25).

4.5.7 Pre-incubation with ΔCPB amastigotes prolongs LPS-induced nitric oxide (NO) production in BMD-macrophages

Lack of an availability of iNOS antibodies did not allow the assay of iNOS expression in response to ΔCPB amastigotes over the 72 hrs as performed for promastigotes. Nevertheless, NO release was assessed in the presence of ΔCPB amastigotes as shown in (Figure 4.26). Following a delay of 6 hrs LPS caused induction of NO release which was maximal between 12 and 24 hrs. NO release was maintained at these levels for further 24 hrs. Pre-treatment with ΔCPB amastigotes caused a significant enhancement of NO production at 24 hrs, in comparison to LPS stimulation (fold stim., LPS= 23.65 ± 3.53, *Leishmania*+LPS = 33.98 ± 4.46, *$p<0.05$).

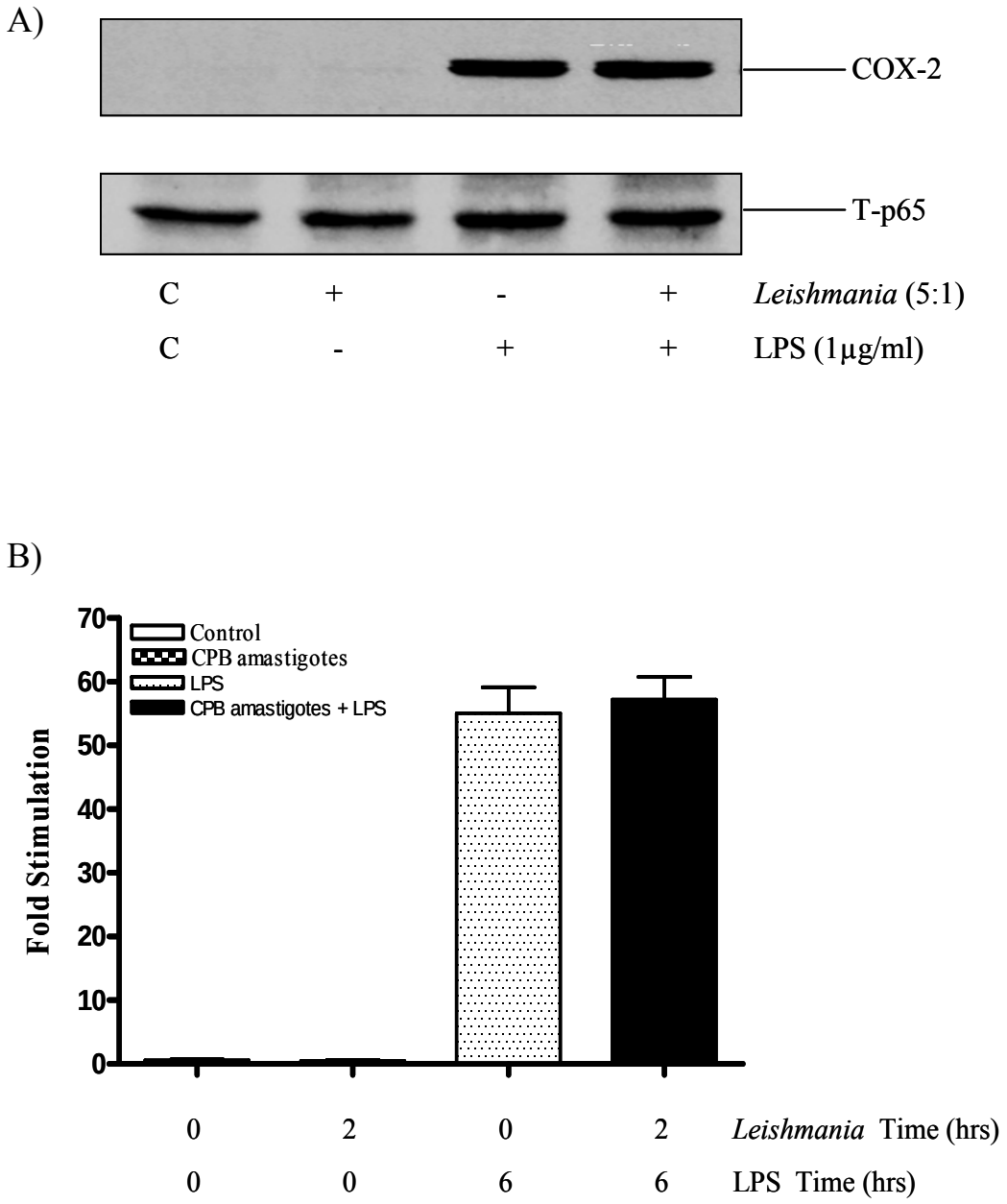


Figure 4.22: The effect of *Leishmania mexicana* ΔCPB amastigote infection on COX-2 protein expression in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* at a ratio of (5:1) for 2 hrs and then stimulated with LPS (1µg/ml) for 6 hrs. Whole cell lysates were prepared and then assessed for **A)** COX-2 (72 kDa) and T-p65 as outlined in Section 2.2.3. Blots were quantified in **B)** by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings.

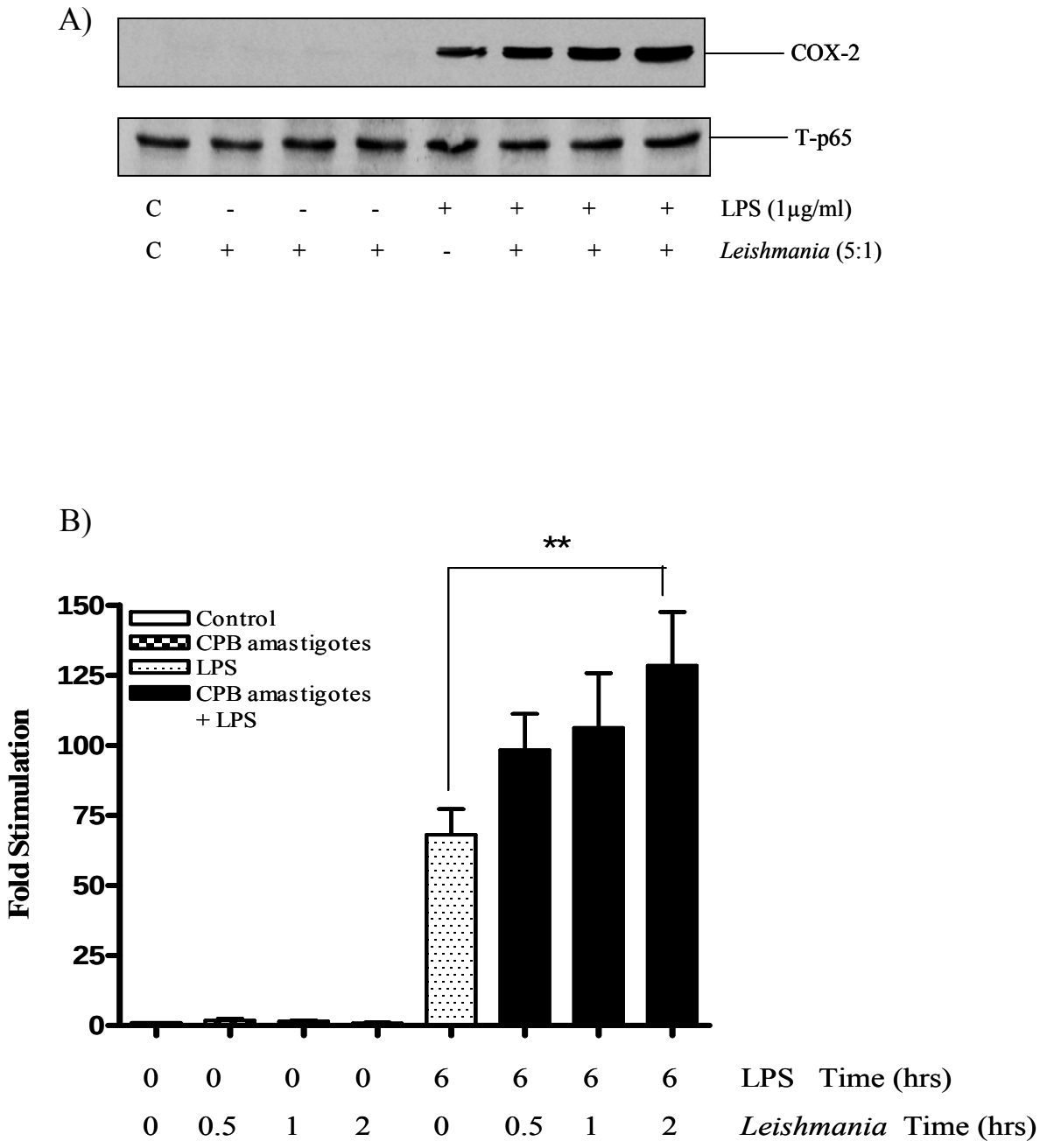


Figure 4.23: The effect of *Leishmania mexicana* ΔCPB amastigote infection on COX-2 protein expression in macrophages. Cells ($1x10^6$/well) were stimulated with LPS (1μg/ml) for 6 hrs and then infected with *Leishmania* at a ratio of (5:1) for different times over a period of 0.5-2 hrs. Whole cell lysates were prepared and then assessed for **A)** COX-2 (72 kDa) and T-p65 as outlined in Section 2.2.3. Blots were quantified in **B)** by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings. **$p<0.01$, compared with agonist-stimulated control.

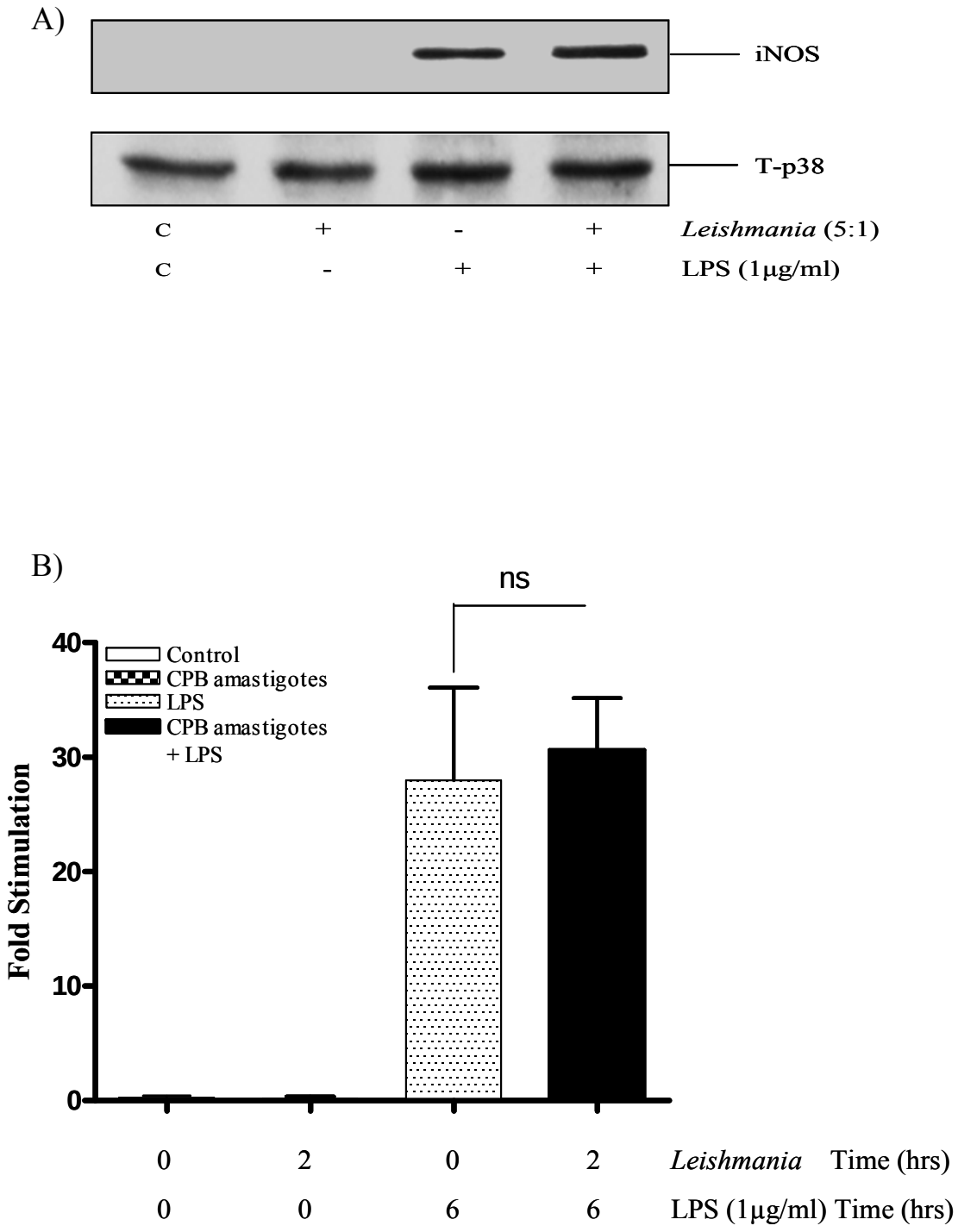


Figure 4.24: The effect of *Leishmania mexicana* ΔCPB amastigote infection on iNOS protein expression in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* at a ratio of (5:1) for 2 hrs and then stimulated with LPS (1μg/ml) for 6 hrs. Whole cell lysates were prepared and then assessed for **A)** iNOS (130 kDa) and T-p38 as outlined in Section 2.2.3. Blots were quantified in **B)** by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings.

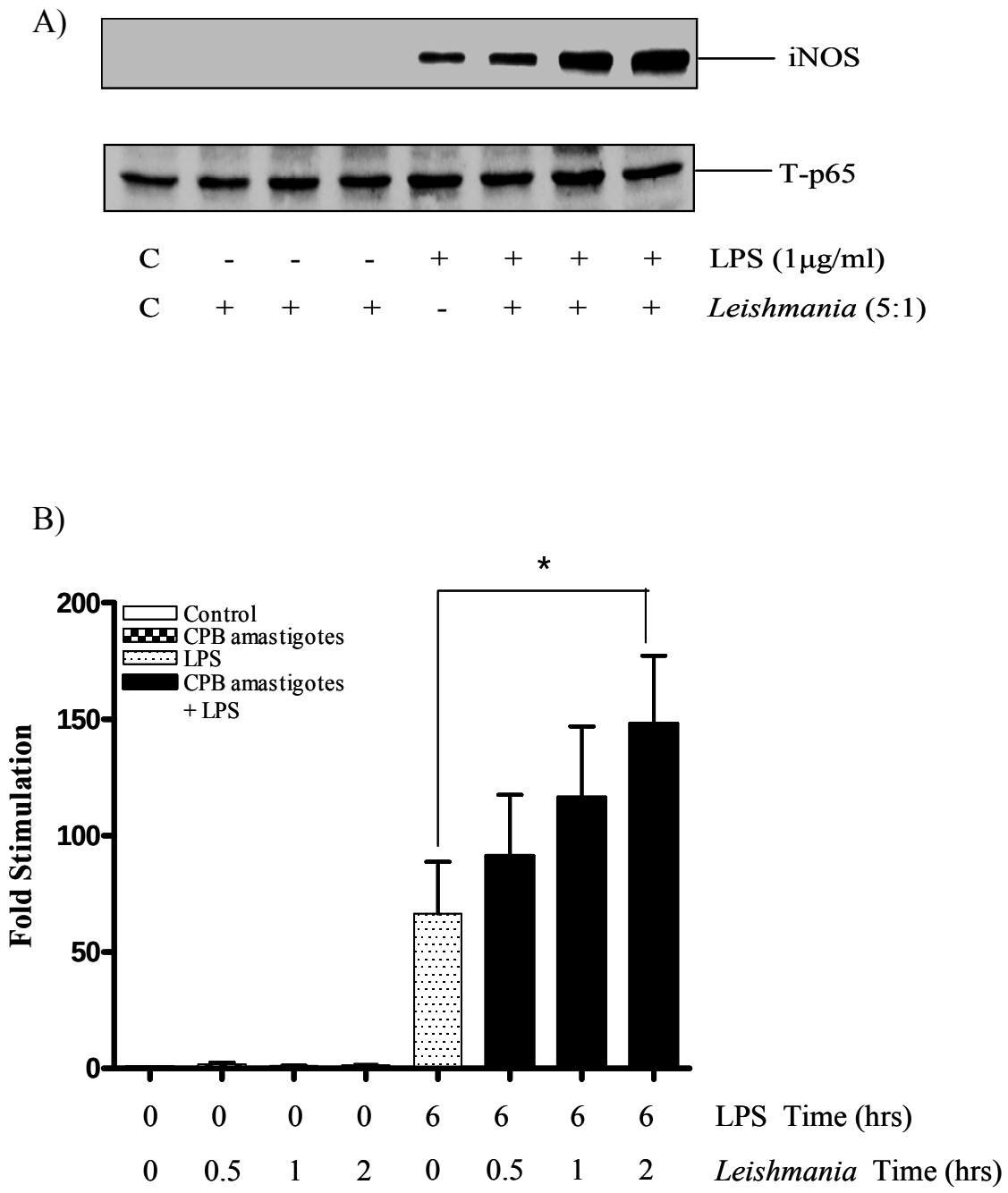


Figure 4.25: The effect of *Leishmania mexicana* ΔCPB amastigote infection on iNOS protein expression in macrophages. Cells ($1x10^6$/well) were stimulated with LPS (1µg/ml) for 6 hrs and then infected with *Leishmania* at a ratio of (5:1) for different times over a period of 0.5-2 hrs. Whole cell lysates were prepared and then assessed for A) iNOS (130 kDa) and T-p65 as outlined in Section 2.2.3. Blots were quantified in B) by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings. *$p<0.05$, compared with agonist-stimulated control.

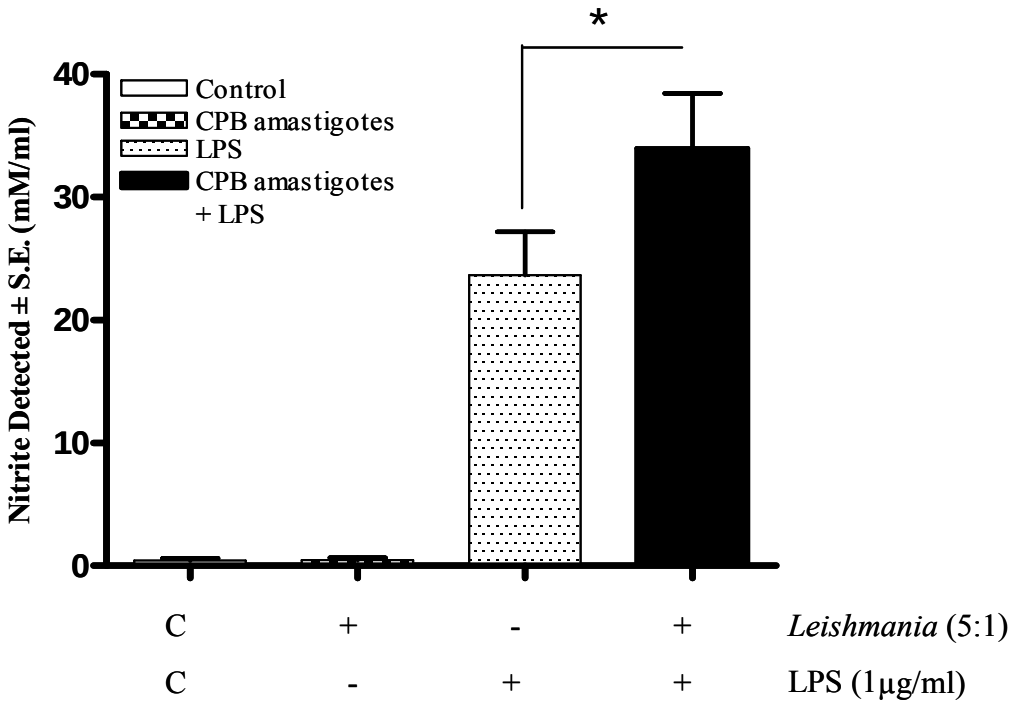


Figure 4.26: *Leishmania mexicana* ΔCPB amastigote infection enhanced LPS-induced NO production in macrophages. Cells ($1x10^6$/well) were grown overnight in a 12 well plate and infected with *Leishmania* at a ratio of (5:1) for 2 hrs and then stimulated with LPS (1μg/ml) over a period of 24 hrs. Supernatants were collected and nitrite levels assessed by Griess assay as outlined in section 2.2.6. Values are quantified and expressed as mean ± S.E.M. (nitrite mM/ml, *$p<0.05$ as compared with stimulated control). Each of the above columns is representative of 3 separate experiments with similar findings.

4.5.8 *Leishmania* promastigotes induce arginase-1 protein expression and activity in BMD-macrophage

As previous results have shown the ability of promastigotes to prolong both COX-2 and iNOS expression, Western blot analysis was carried out to further investigate the potential of promastigotes to regulate the induction of arginase-1, a potent marker in the alternative pathway activated during infection of macrophage cells. The effect was compared with LPS and IL-4 (Figure 4.27). Promastigotes of *L. mexicana* (5:1) alone induced a significant increase in arginase-1 protein expression compared to control levels (fold stim., control = 1.00 ± 2.19 and *Leishmania* = 18.61 ± 2.28, respectively, *$p<0.05$). LPS also caused a small increase in expression, which was further increased by the presence of promastigotes (8.85 ± 1.12, 45.51 ± 5.54, **$p<0.01$). IL-4 alone caused a significant increase in expression above control levels, and again, in the additional presence of promastigotes, arginase-1 induction was enhanced (fold stim., IL-4 = 17.41 ± 4.90, *Leishmania*+IL-4 = 39.23 ± 4.78, **$p<0.01$). All three agents in combination gave no additional increase over levels of expression using two agents together. Figure 4.28 shows the resultant effect upon arginase activity in cells stimulated under the same conditions as above. Again, *Leishmania* alone induced an increase in arginase activity reflective of the increase in protein expression. As expected, promastigotes enhanced both LPS and IL-4 induced activity (fold stim., LPS =11.22 ± 2.64, *Leishmania*+LPS = 28.06 ± 3.20, IL-4= 14.04 ± 1.48, *Leishmania*+IL-4 = 29.01 ± 2.30, **$p<0.01$).

4.5.9 IL-4 inhibits promastigotes enhancement of NO release in BMD-macrophage

Supernatants from the above experiments were used to confirm the actions of both promastigotes and IL-4 on NO production (Figure 4.29). As shown previously LPS stimulated a strong induction of NO which was significantly increased with *Leishmania* promastigotes. IL-4 markedly reduced the response to LPS and severely ablated the potentiating effect of promastigotes. This confirms that IL-4 is functioning as an effective agent in this system (fold stim., LPS = 22.05 ± 3.23, *Leishmania*+LPS= 45.66 ± 11.94, LPS+IL-4 = 11.55, *Leishmania*+LPS+IL-4 = 19.25 ± 2.30).

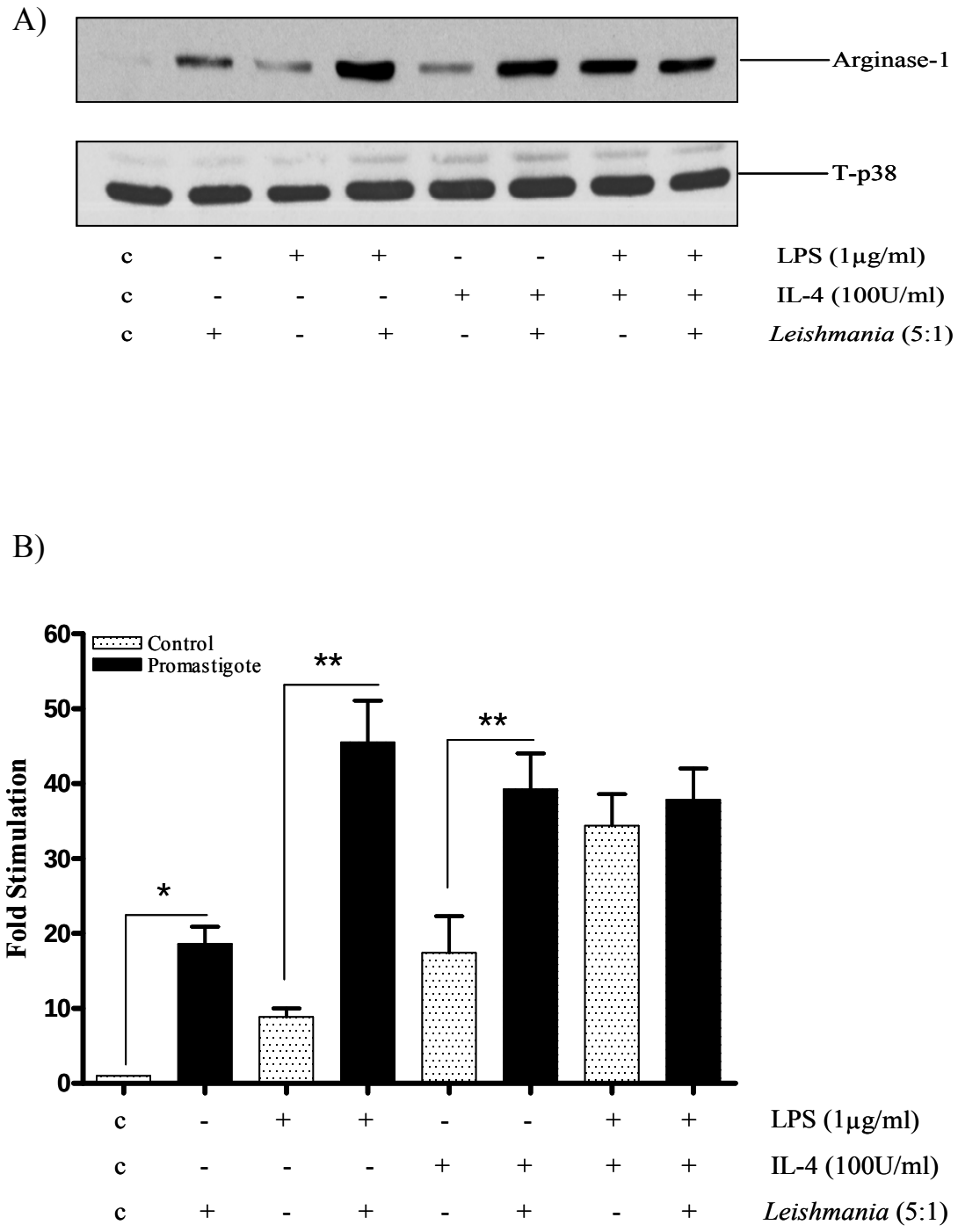


Figure 4.27: *Leishmania mexicana* promastigote-induced arginase-1 protein expression in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* at a ratio of (5:1) for 2 hrs and then stimulated with LPS (1μg/ml), IL-4 (100U/ml) and both over a period of 24 hrs. Whole cell lysates were prepared and then assessed for **A)** Arginase-1 (35 kDa) and T-p38 as outlined in Section 2.2.3. Blots were quantified in **B)** by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings. *$p<0.05$ and **$p<0.01$ compared in the absence of promastigotes.

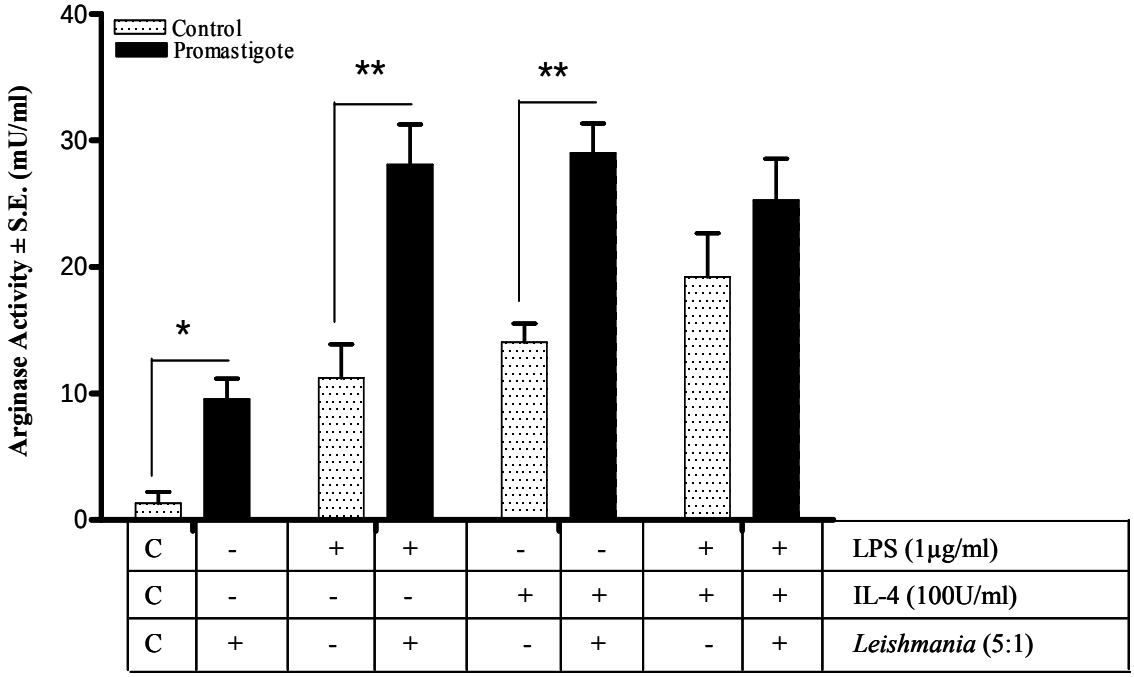


Figure 4.28: *Leishmania mexicana* promastigote infection enhanced LPS and IL-4 induced arginase activity in macrophages. Cells ($1x10^6$/well) were grown overnight in a 24 well plate and infected with *Leishmania* at a ratio of (5:1) for 2 hrs and then stimulated with LPS (1µg/ml), IL-4 (100U/ml) alone or in combination, over a period of 48 hrs. Cells samples were assayed for arginase activity (as outlined in section 2.2.5). Figure 4.22 represents quantification of arginase activity, expressed as mean ± S.E.M. (*$p<0.05$, **$p<0.01$ as compared with stimulated control). Each of the above columns is representative of 3 separate experiments with similar findings.

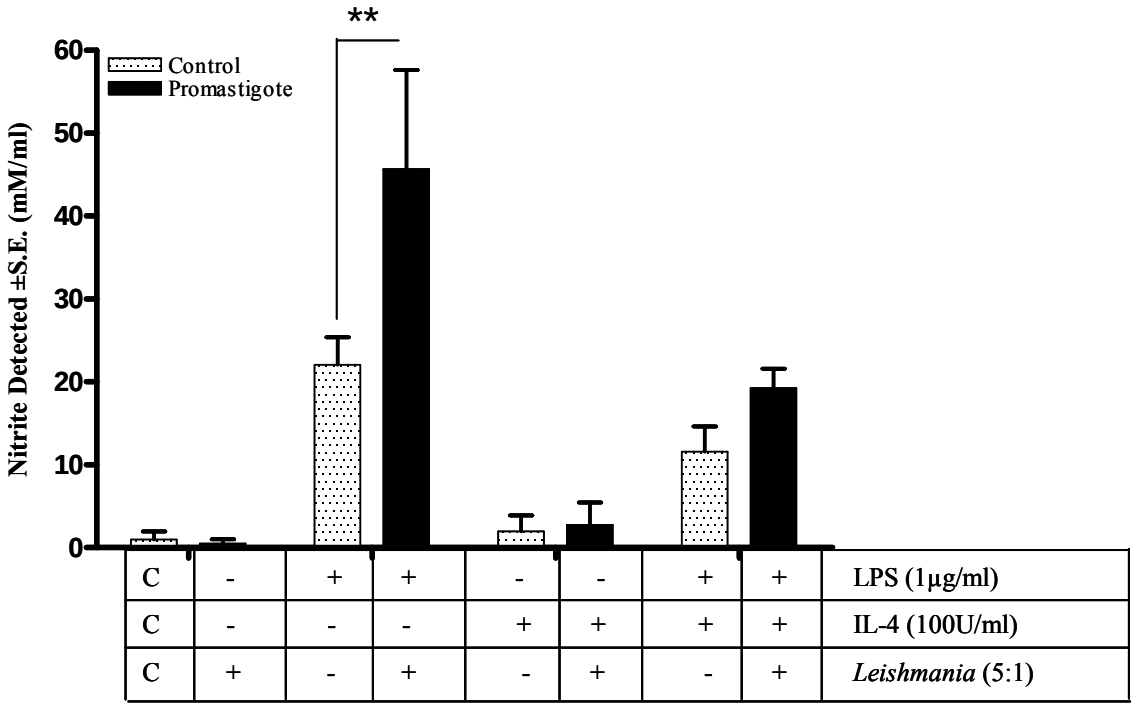


Figure 4.29: Differential effects of promastigote and IL-4 on NO production in macrophages. Cells ($1x10^6$/well) were grown overnight in a 24 well plate and infected with *Leishmania* at a ratio of (5:1) for 2 hrs and then stimulated with LPS (1µg/ml), IL-4 (100U/ml) alone or in combination, over a period of 48 hrs. Supernatants were collected and nitrite levels assessed by Griess assay (as outlined in section 2.2.6). Values are quantified and expressed as mean ± S.E.M. (**$p<0.01$ as compared with stimulated control). Each of the above columns is representative of 3 separate experiments with similar findings.

4.6 CHARACTERISATION OF *L. MEXICANA* PROMASTIGOTES AND ΔCPB AMASTIGOTES INFECTION ON PRO-INFLAMMATORY AND ANTI-INFLAMMATORY CYTOKINE PRODUCTION IN MACROPHAGE

In previous parts of the chapter it has been established that both promastigotes and ΔCPB amastigotes interact with macrophages to initiate signalling cascades such as MAP kinase and NF-κB pathway, and the subsequent induction of COX-2 and iNOS. Furthermore, it has also been demonstrated that promastigotes can upregulate arginase-1 expression. In the section below the effects of promastigotes on key cytokines IL-12, IL-6, and IL-10 were examined and compared with ΔCPB amastigotes.

4.6.1 Effect of promastigotes on LPS induction of IL-12, IL-6 and IL-10 production by BMD-macrophage

Macrophage cells were pre-incubated with promastigotes (5:1) for 2 hrs prior to stimulation with LPS (1μg/ml) for 24 hrs, the time point that gave maximum release of each cytokine. Results showed that over the 24 hrs time period, no IL-12 production was detectable in supernatants from non-stimulated (control) cells or from promastigotes alone (0.10 ± 0.02 and 0.06 ± 0.04). In contrast, IL-12 production was significantly increased in response to LPS (19.99 ± 0.36, n=3). Following pre-incubation with promastigotes, IL-12 levels were significantly reduced, by approximately 80 percent (4.70 ± 1.07, ***$p<0.001$) (Figure 4.30). Similarly, the effect of promastigotes upon IL-6 production was measured. In contrast to the effect upon IL-12 production, promastigotes gave a small but insignificant increase in LPS induced IL-6 (panel B). When IL-10 production was assessed it was found that promastigotes alone caused an increase in IL-10 which was equvilent of that observed for LPS (panel C). However, promastigotes significantly increased LPS induced IL-10 production to a level which was more than additive (LPS = 0.51 ± 0.18, *Leishmania*+LPS = 2.03 ± 0.73, respectively, *$p<0.05$).

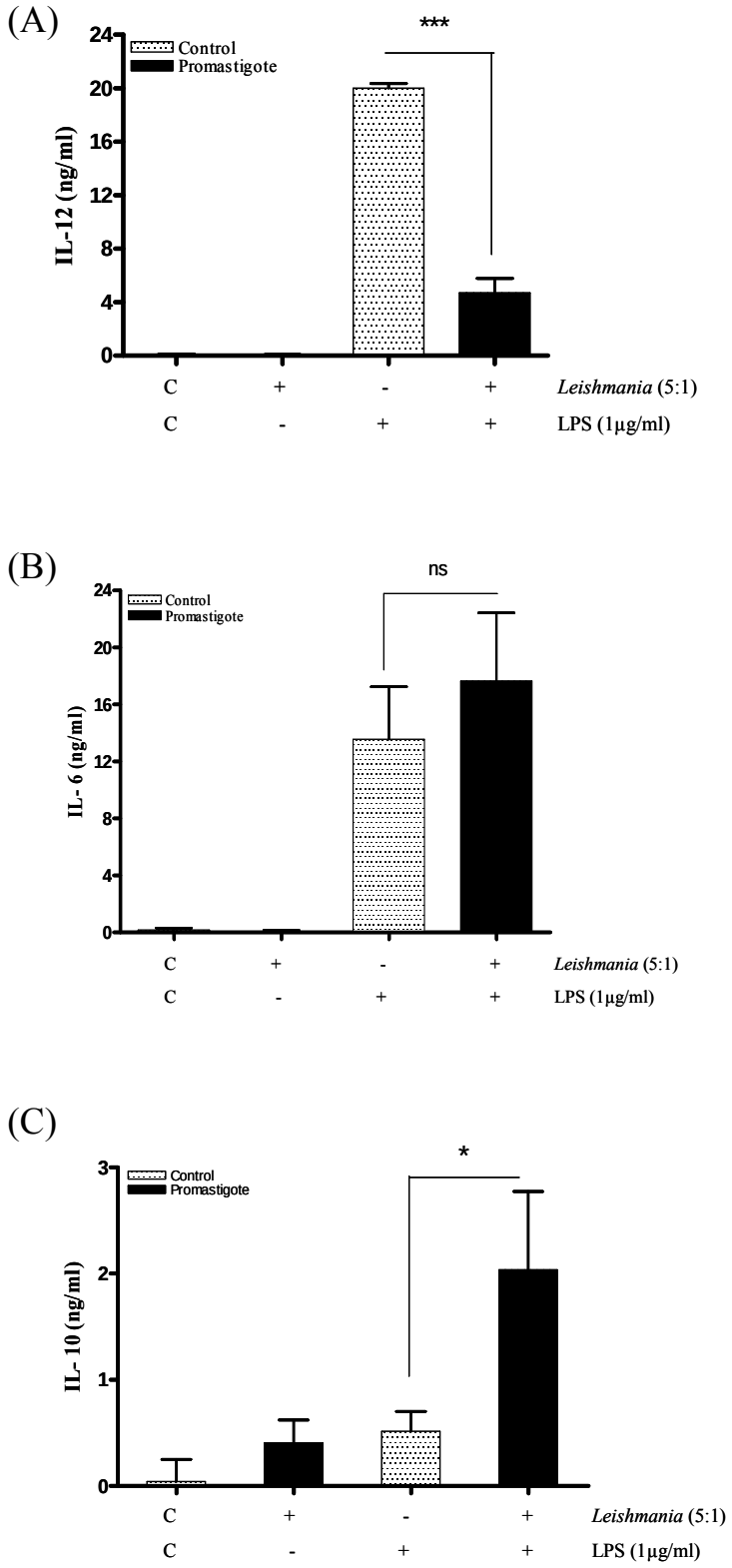


Figure 4.30: ***Leishmania mexicana*** **promastigote effect on LPS- mediated IL-12, IL-6, and IL-10 production in macrophage cells.** BMD-macrophage cells were grown to ($1x10^6$/ml) on a 12 well plate. Cells were pre-incubated with promastigotes at a ratio of (5:1) for 2 hrs prior to being stimulated with LPS (1µg/ml) for 24 hrs. Following stimulation, supernatants were collected and IL-12 (**A**), IL-6 (**B**) and IL-10 (**C**) levels assessed by ELISA as outlined in section 2.2.4. Values were quantified and expressed as mean ± S.E.M. from 3 separate experiments. *$p<0.05$, ***$p<0.001$ as compared with LPS stimulation.

4.6.2 Effect of ΔCPB amastigotes on LPS induction of IL-12, IL-6, and IL-10 by BMD-macrophage

Results showed that over the 24 hrs time period, no IL-12 production was detectable in supernatants from non-stimulated cells (control) or from ΔCPB amastigotes alone (0.38 ± 0.02 and 0.37 ± 0.02). In contrast, IL-12 production was significantly increased in response to LPS (18.01 ± 1.55). Following pre-incubation with ΔCPB amastigotes IL-12 levels were significantly reduced, by approximately 50 % (9.83 ± 1.11, **$p<0.01$), less in than that observed for promastigotes (Figure 4.31). Similarly, the effect of ΔCPB amastigotes upon IL-6 production was measured. In contrast to IL-12 production, ΔCPB amastigotes gave a significant increase in LPS induced IL-6 (12.97 ± 1.12 and 20.80 ± 2.81, **$p<0.01$) (Figure 4.26). When IL-10 production was assessed it was found that ΔCPB amastigotes significantly increased LPS induced IL-10 production to a level which was more than additive (panel C). The effect of ΔCPB amastigotes on NO release was assessed. As expected ΔCPB amastigotes significantly enhanced LPS induced NO production (panel D). Overall both promastigotes and ΔCPB amastigotes initiated similar effects on the production of all three cytokines.

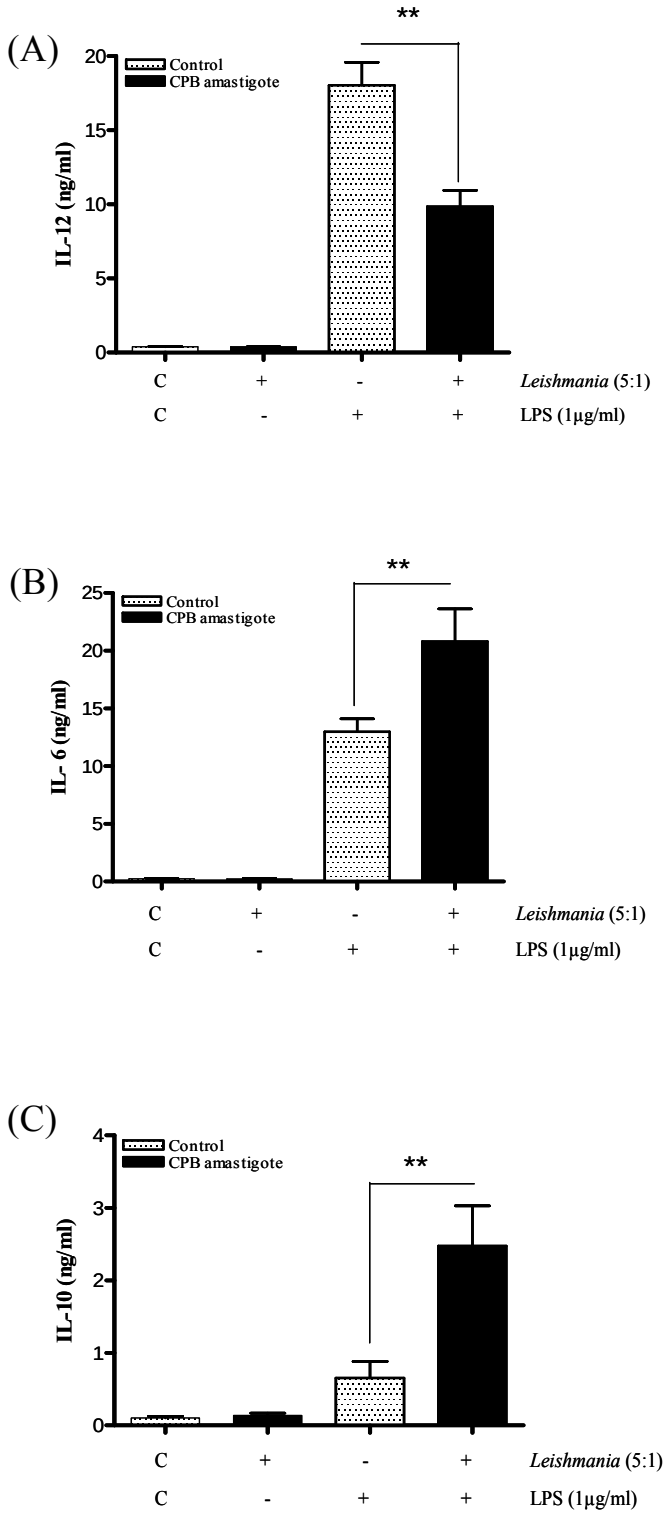


Figure 4.31: *Leishmania* ΔCPB amastigote effect on LPS- mediated IL-12, IL-6, IL-10, and NO production in macrophage cells. BMD-macrophage cells were grown to ($1x10^6$/ml) on a 12 well plate. Cells were pre-incubated with promastigotes at a ratio of (5:1) for 2 hrs prior to being stimulated with LPS (1µg/ml) for 24 hrs. Following stimulation, supernatants were collected and cytokine levels assessed by ELISA as outlined in section 2.1.5. Values are quantified and expressed as mean ± S.E.M. (**A**) IL-12, (**B**) IL-6, (**C**) IL-10 ng/ml, **$p<0.01$ as compared with stimulated control. Each of the above columns is representative of 3 separate experiments with similar findings.

4.7 THE EFFECT OF PHARMACOLOGICALLY CHANGING THE AMOUNT OF PGE_2 AND NO AND AFFECTING ARGINASE ACTIVITY ON PROMASTIGOTE-MEDIATED INHIBITION OF LPS-INDUCED IL-12 PRODUCTION

So far, previous results have demonstrated enhancement of PGE_2 and NO release and also arginase activity in response to promastigotes. Since evidence has shown that both PGE_2 and NO can modulate IL-12 production in a number of cell types (Schwacha et al., 2002, Boddupalli et al., 2007), it was necessary to determine if inhibition of these mediators could modify the effect of promastigotes on IL-12 production. Therefore, BMD-macrophage cells were co-incubated with indomethacin, a known COX inhibitor, the non-selective NOS inhibitor, L-NAME and the arginase inhibitor nor-NOHA.

4.7.1 The effect of indomethacin and L-NAME on promastigote-mediated inhibition of LPS-induced IL-12 production in macrophages

In these experiments two approaches were undertaken. Firstly, cells were pretreated with inhibitor prior to pre-incubation with promastigotes and subsequent addition of LPS. Alternatively, inhibitor was added 6 hrs after LPS stimulation (Figure 4.32). LPS stimulated a significant increase in IL-12 production over 24 hrs and as expected promastigotes treatment markedly inhibited production (LPS = 18.42 ± 1.03, *Leishmania*+LPS = 4.90 ± 1.46, ***$p<0.001$) (panel A). When indomethacin was added prior to promastigote treatment there was a small reversal in the inhibitory effect of the promastigotes. However, when indomethacin was added after LPS stimulation, the reversal was much more marked (*Leishmania*+LPS = 4.90 ± 1.46, LPS+indomethacin+*Leishmania* = 18.75 ± 1.77).

A similar result was obtained when L-NAME was used instead of indomethacin (panel B). Pre-incubation with L-NAME prior to promastigote infection gave a small reversal in the inhibitory effect of promastigotes on IL-12 production, once again adding L-NAME subsequent to LPS gave a much greater and significant reversal.

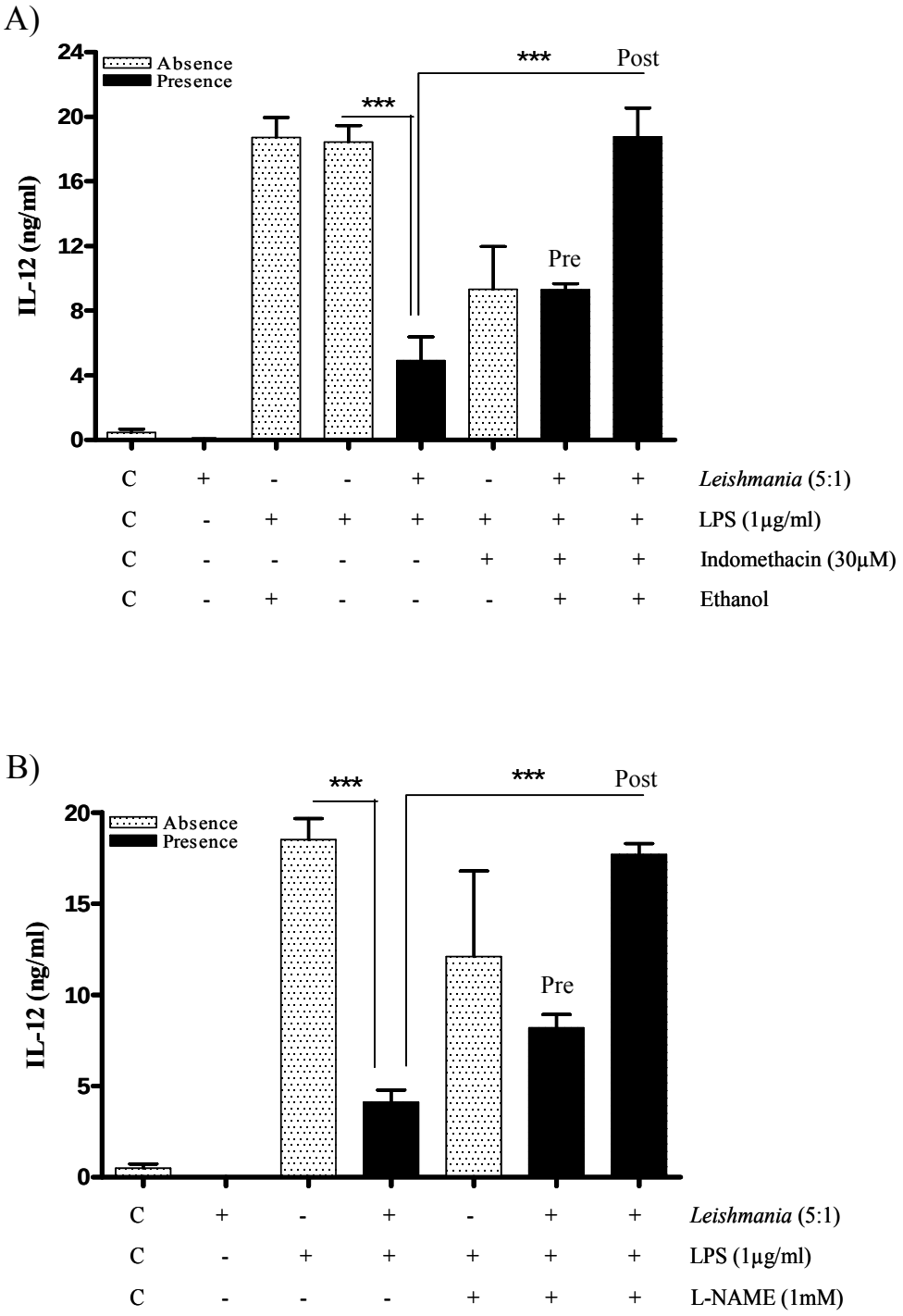


Figure 4.32: Effect of indomethacin and L-NAME on promastigote-mediated inhibition of IL-12 production in macrophages. Macrophages were grown to ($1x10^6$/ml) overnight and infected at a ratio of (5:1) with promastigotes for 2 hrs; Cells were pre-treated with indomethacin (A) and L-NAME (B) for 1 hour prior to infection with promastigotes (pre), or added following 6 hr of LPS stimulation (post). Cells were stimulated with LPS for a total of 24 hrs. Following stimulation, supernatants were collected and IL-12 levels assessed by ELISA as outlined in section 2.2.4. Values are quantified and expressed as mean ± S.E.M. from 3 separate experiments ng/ml. ***$p<0.001$ as compared with agonist-stimulated control.

4.7.2 The effect of nor-NOHA on promastigote-enhanced LPS-induced NO and IL-12 production in BMD-macrophages

The results in Figure 4.32 demonstrated that reducing NO can reverse the inhibitory effect of promastigotes on IL-12 production. Therefore, the arginase inhibitor nor-NOHA was used to confirm that changing intracellular levels of nitric oxide by inhibiting arginase-1 activity could also affect IL-12 production (Figure 4.33). Panel A shows the effect of nor-NOHA on nitric oxide production as measured by nitrite release. Nor-NOHA enhanced nitrite production by approximately 3 fold in response to LPS alone or in the additional presence of promastigotes. When IL-12 production was examined the results were not as predicted. Although, nor-NOHA alone reduced IL-12 production in response to LPS, indicating that high levels of NO *per se* are inhibitory, when cells were pre-incubated with nor-NOHA subsequent to promastigote pre-treatment, an expected and substantial reversal of IL-12 inhibition was also observed, giving values which were similar to those obtained with LPS alone. This suggests that arginase may be able to modify IL-12 production in a manner that is not related to the direct effect upon nitric oxide levels.

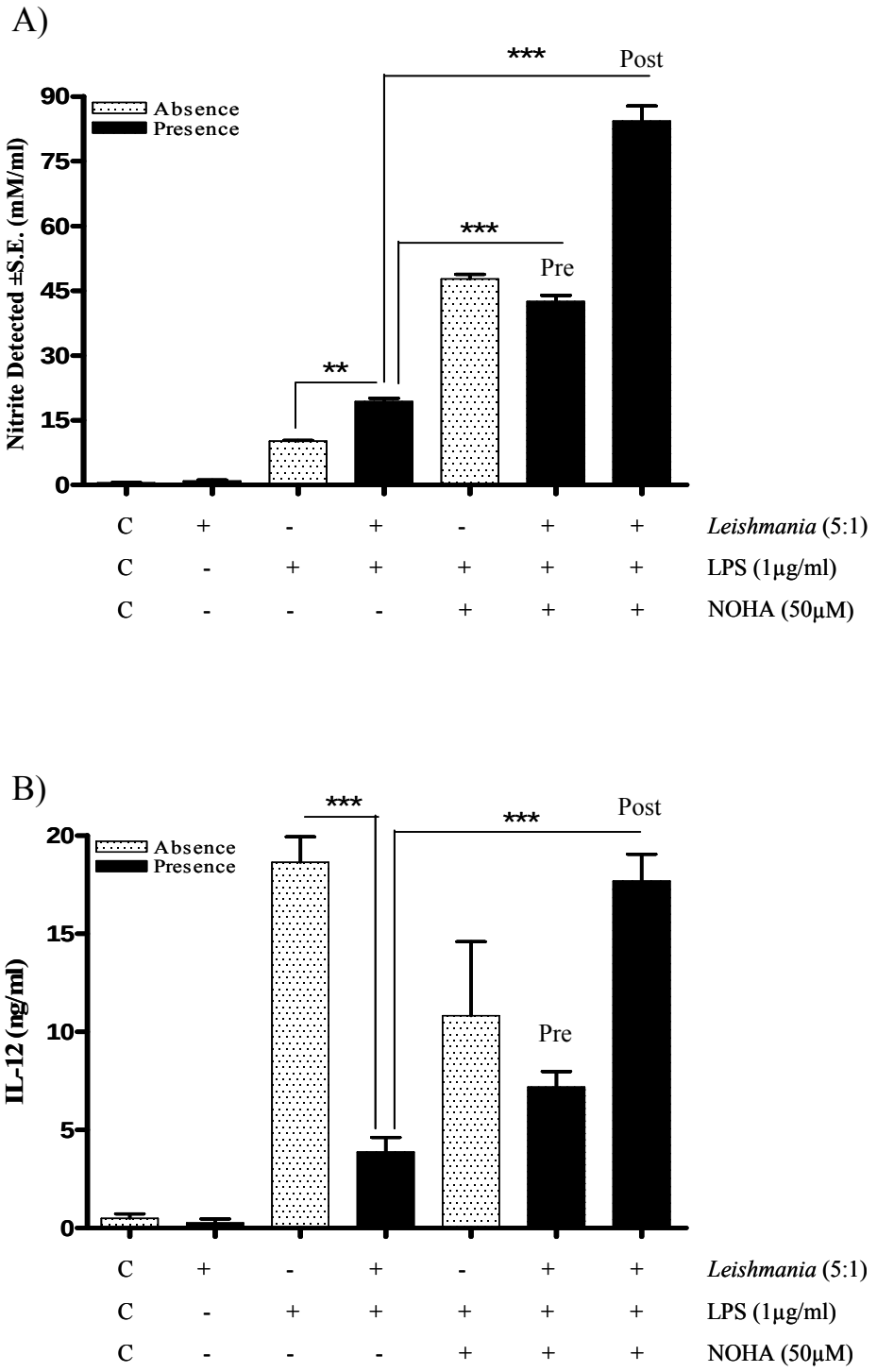


Figure 4.33: Effect of nor-NOHA on promastigote-mediated inhibition of IL-12 and NO enhancing in macrophages. Macrophages were grown to ($1x10^6$/ml) overnight and infected at a ratio of (5:1) of promastigotes for 2 hrs; cells were incubated with LPS (1µg/ml) for 24 hrs. Cells were pre-treated with nor-NOHA for 1 hour prior to infection with promastigotes (pre), or added following 6 hr of LPS stimulation (post). Following stimulation, supernatants were collected and nitrite levels were assessed by Griess assay as outlined in section 2.1.7 (**A**) and IL-12 level assessed by ELISA in (**B**) as outlined in section 2.1.5. Values are quantified and expressed as mean ± S.E.M. from 3 separate experiments ng/ml. ***$p<0.001$ as compared with agonist-stimulated control.

4.8 THE ROLE OF TLRS IN THE INFLAMMATORY RESPONSE FOLLOWING INFECTION WITH *L. MEXICANA* IN MACROPHAGES

Previous studies have shown the ability of TLRs to mediate the activation of MAP kinase pathways including, ERK, JNK and p38 MAP kinase, which are essential for cell survival and controlling the expression of immune mediators (Symons et al., 2006). In macrophages this includesTLR1, TLR2, TLR4, TLR5, TLR6, TLR7 and TLR9. Toll-like receptor 4 (TLR4) is one of the most important pattern recognition receptors for the action of LPS in macrophages (Cook et al., 2004). However, the potential for TLR4 and possibly others, to be involved in the effects of *Leishmania* promastigotes has not been elucidated. Having already established in this chapter that *L. mexicana* promastigotes and ΔCPB amastigotes enhance MAP kinase and NF-κB activity in BMD-macrophages, in a manner very similar to that observed for TLR engagement, the potential for TLR4 and/or TLR2 to mediate the effect of promastigotes was examined.

4.8.1 The effect of TLR4 deficiency on LPS and promastigote-induced MAP kinase phosphorylation in BMD-macrophages

Macrophages from four groups of mice; WT, TLR2$^{-/-}$, TLR4$^{-/-}$, and double knockout TLR2/4$^{-/-}$ were generated and grown to (1x10^6/ml) on 12 well plates. Cells were stimulated with LPS (1μg/ml) or promastigotes (5:1) for 30 min. As shown in Figure 4.34, following stimulation with both, the level of JNK phosphorylation increased significantly. The increase in JNK phosphorylation was lower in response to promastigotes compared to LPS alone. (fold stim., WT, LPS = 38.55 ± 9.53, promastigote = 23.70 ± 4.11, n=3). In the absence of TLR2 LPS and promastigotes responses were not affected, however in macrophages deficient in TLR4 the JNK response was markedly reduced. As expected, in TLR2/4$^{-/-}$ macrophages JNK phosphorylation was completely abolished. Taken together, these results suggest that TLR4 mediates activation of JNK in response to both Gram-negative bacteria and *Leishmania* promastigotes.

Similarly results in Figure 4.35 demonstrate that, in wild type and TLR2$^{-/-}$ macrophages, both LPS and promastigotes gave a similarly strong increase in ERK phosphorylation (fold stim., WT, LPS = 11.73 ± 3.33, promastigote = 9.80 ± 2.58 for WT, LPS = 9.96 ± 2.94, promastigote = 9.01 ± 2.47 for TLR2$^{-/-}$). In TLR4$^{-/-}$ macrophages ERK phosphorylation in response to LPS was substantially but not completely abolished which was probably due to lipoprotein contaminants interacting with TLR2. However, there was no activation of ERK1/2 following promastigotes infection, therefore, confirming TLR4 mediated the activation of ERK1/2. Again, results in TLR2/4$^{-/-}$ cells showed a complete inhibition of ERK1/2 signaling pathways in response to both treatments.

For p38 MAP kinase, a similar pattern was observed as demonstrated (see Figure 4.36). In both WT and TLR2$^{-/-}$ macrophages substantial increases in phosphorylation were recorded following LPS and promastigote infection, LPS gave a more sizeable response in each cell type (LPS = 97.52 ± 8.35, promastigote = 59.36 ± 7.35 for WT, LPS = 84.43 ± 10.83 , promastigote = 53.60 ± 4.63 for TLR2$^{-/-}$). Phosphorylation of p38 MAP kinase was substantially reduced but not abolished in TLR4$^{-/-}$ cells stimulated with LPS for the same reason as mentioned before for ERK. Again, in the absence of TLR4 the p38 response to promastigotes was essentially abolished. In the TLR2/4$^{-/-}$ double knockout, there was no apparent signal for either LPS or promastigotes.

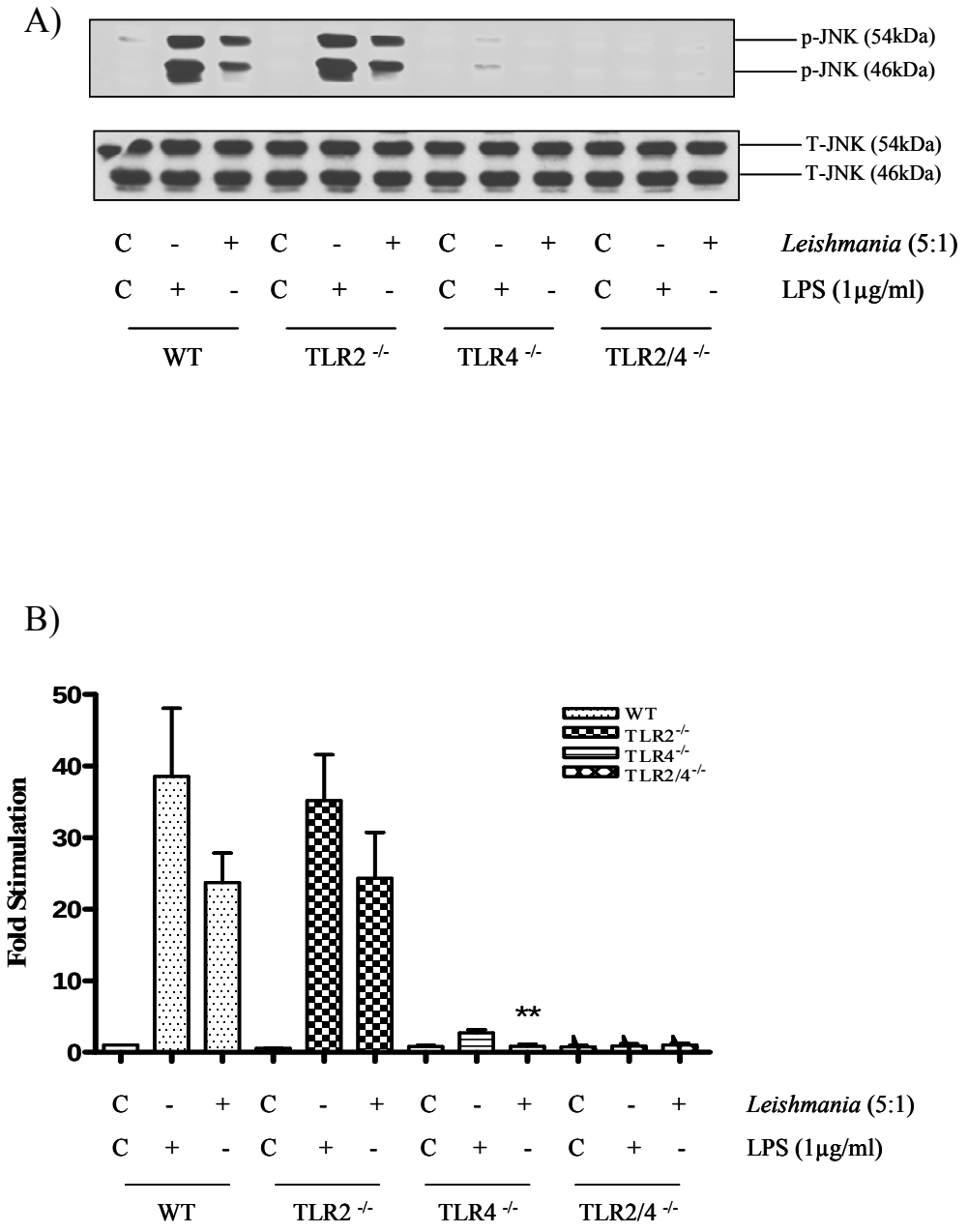


Figure 4.34: TLR4-mediated promastigote-induced JNK1/2 phosphorylation in macrophages. Cells ($1x10^6$/well) were stimulated with LPS (1µg/ml) or incubated with *Leishmania* at a ratio of (5:1) for 30 min. Whole cell lysates were prepared and then assessed for **A)** p-JNK (46/54 kDa) and T-JNK (46/54 kDa) as outlined in Section 2.2.3. Blots were quantified in **B)** by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings. **$p<0.01$ as compared with wild type stimulation.

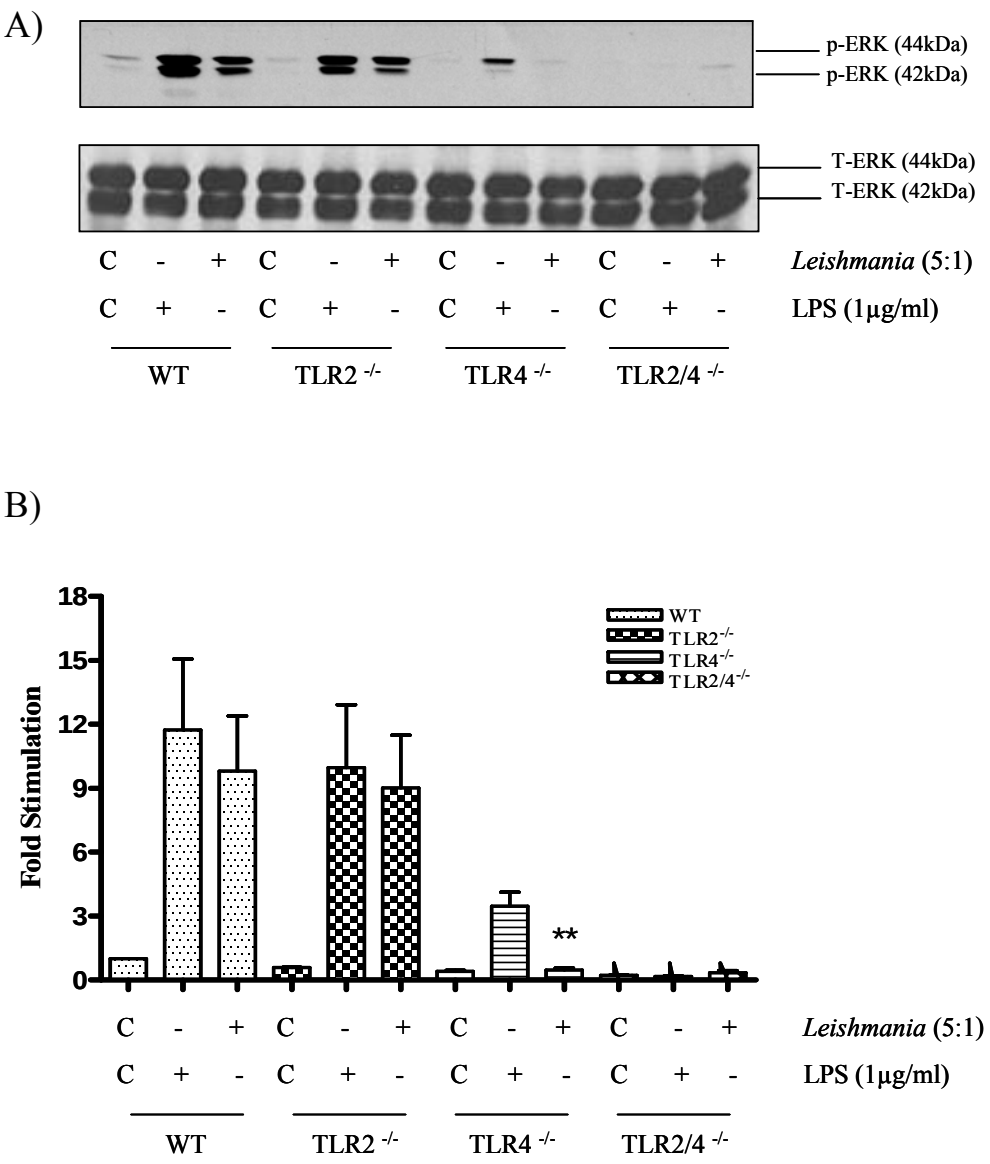
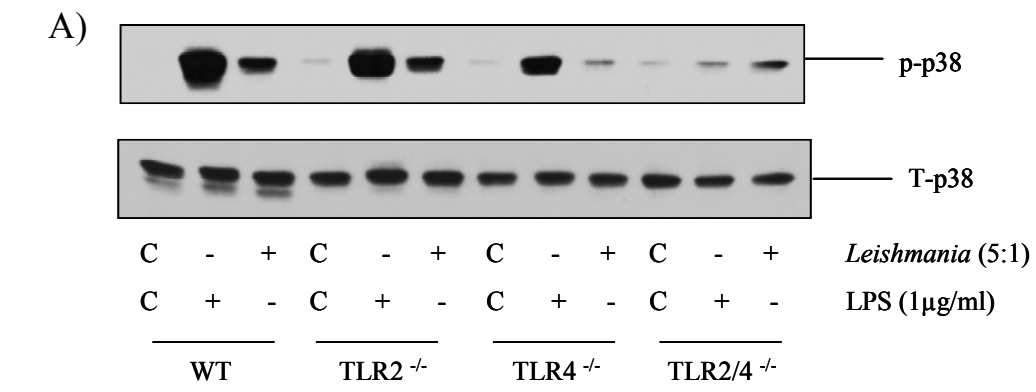


Figure 4.35: TLR4-mediated promastigote-induced ERK1/2 phosphorylation in macrophages. Cells ($1x10^6$/well) were stimulated with LPS (1µg/ml) or incubated with *Leishmania* at a ratio of (5:1) for 30 min. Whole cell lysates were prepared and then assessed for A) p-ERK1/2 (42/44 kDa) and ERK (42/44 kDa) as outlined in Section 2.2.3. Blots were quantified in B) by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings. **$p<0.01$ as compared with wild type stimulation.



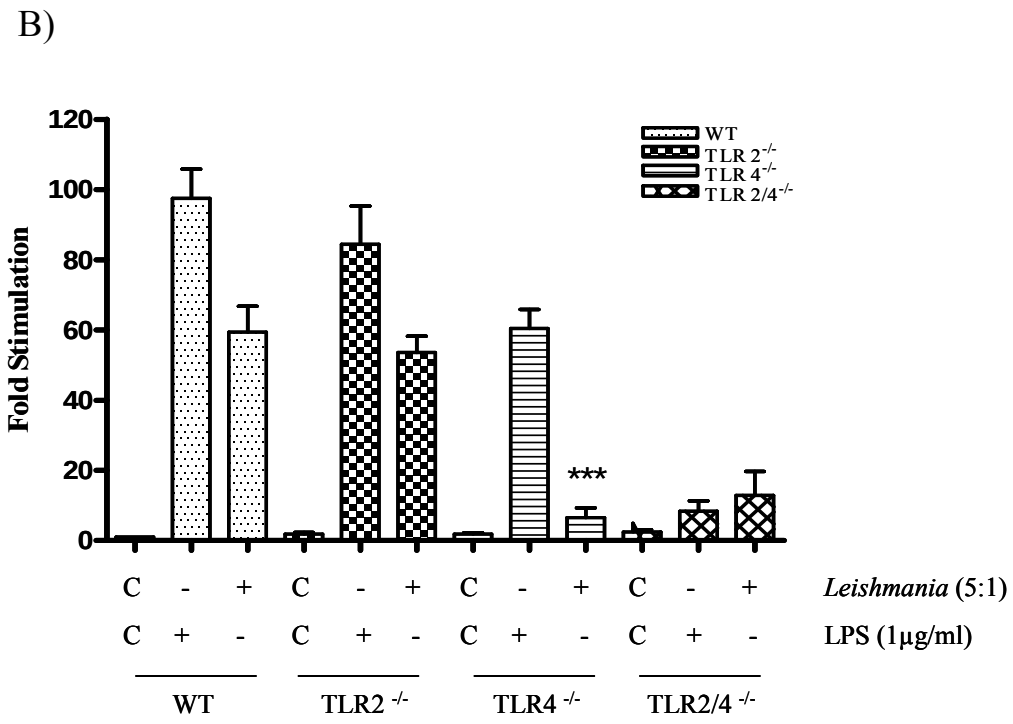


Figure 4.36: TLR4-mediated promastigote-induced p38 MAP kinase phosphorylation in macrophages. Cells ($1x10^6$/well) were stimulated with LPS (1µg/ml) or incubated with *Leishmania* at a ratio of (5:1) for 30 min. Whole cell lysates were prepared and then assessed for **A)** p-p38 and T-p38 as outlined in Section 2.2.3. Blots were quantified in **B)** by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings. ***$p<0.001$ as compared with wild type stimulation.

4.8.2 The effect of TLR4 deficiency on MAP kinase phosphorylation induced by ΔCPB amastigotes in BMD-macrophages

Initially the induction of MAP kinase was measured over 30 minutes as a direct comparison to the studies using promastigotes. As shown in (Figure 4.37), upon stimulation with LPS and ΔCPB amastigotes, the level of JNK phosphorylation increased. Again, phosphorylation was similar in both WT and TLR2$^{-/-}$ macrophages (fold stim., LPS = 7.69 ± 0.68, ΔCPB = 5.42 ± 0.67 for WT, LPS = 7.71 ± 0.83, ΔCPB = 4.26 ± 1.17 for TLR2$^{-/-}$). Also and consistent with the results for promastigotes, JNK phosphorylation in TLR4$^{-/-}$ deficient macrophages was significantly reduced in response to ΔCPB amastigotes. As expected, in TLR2/4$^{-/-}$ cells the JNK phosphorylation was completely abolished. Taken together, these results suggest that ΔCPB amastigotes mediate the activation of the JNK via a TLR4-dependent mechanism.

Figures 4.38 and 4.39 show the effect of TLR deletion on ERK and p38 MAP kinase phosphorylation. Both figures demonstrate consistent phenomena. Responses were significantly reduced in TLR4$^{-/-}$ macrophages and essentially abolished in TLR2/4$^{-/-}$ macrophages. (fold stim., ΔCPB = 8.45 ± 1.98 for WT, ΔCPB = 0.90 ± 0.17 for TLR4$^{-/-}$ p-ERK, ΔCPB = 4.34 ± 0.48 for WT, ΔCPB = 0.62 ± 0.18 for TLR4$^{-/-}$, p-p38 MAP kinase).

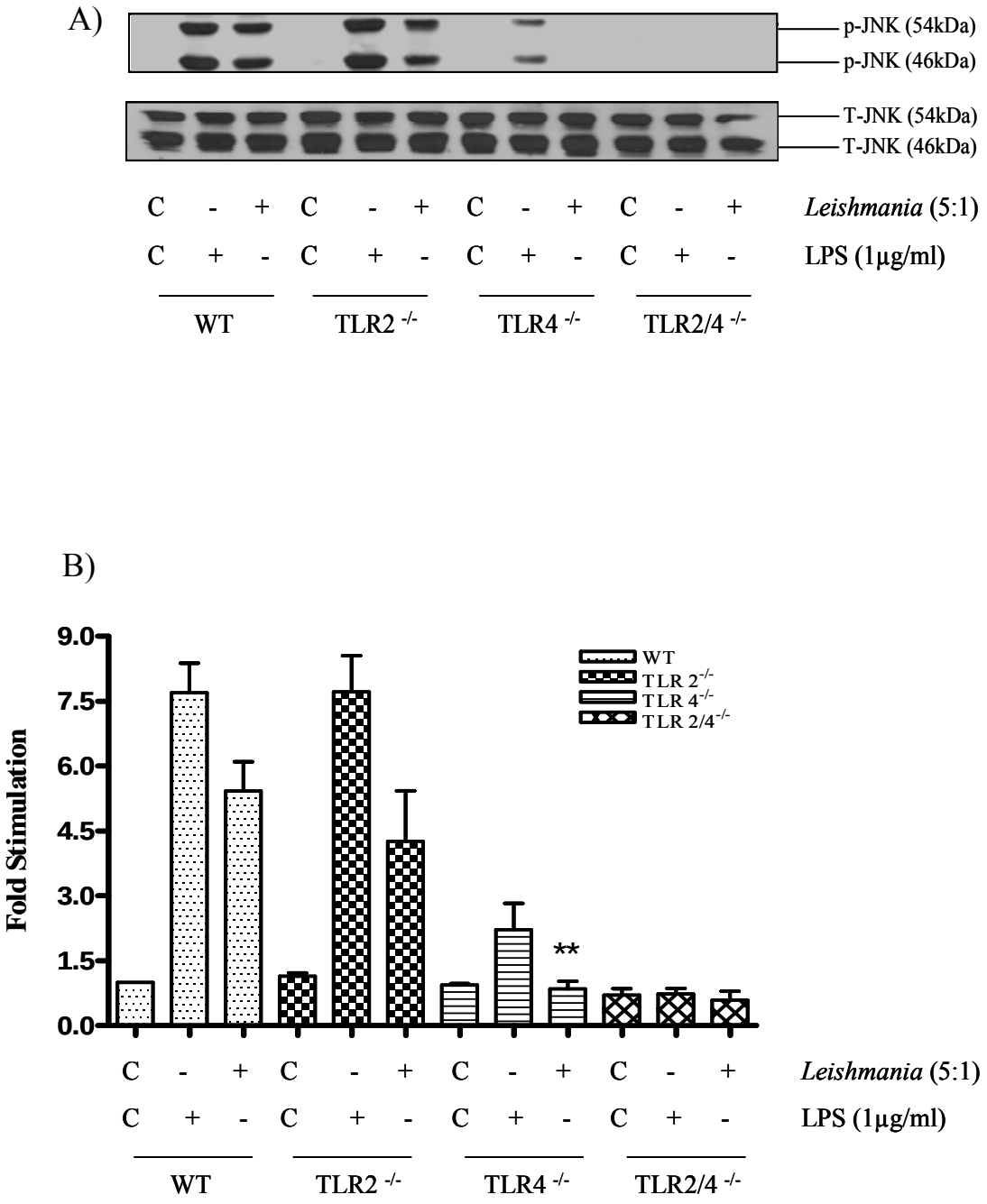


Figure 4.37: TLR4-mediated ΔCPB amastigote-induced JNK1/2 phosphorylation in macrophages. Cells ($1x10^6$/well) were stimulated with LPS (1µg/ml) or incubated with *Leishmania* at a ratio of (5:1) for 30 min. Whole cell lysates were prepared and then assessed for A) p-JNK (46/54 kDa) and T-JNK (46/54 kDa) as outlined in Section 2.2.3. Blots were quantified in B) by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings. **$p<0.001$ as compared with wild type stimulation.

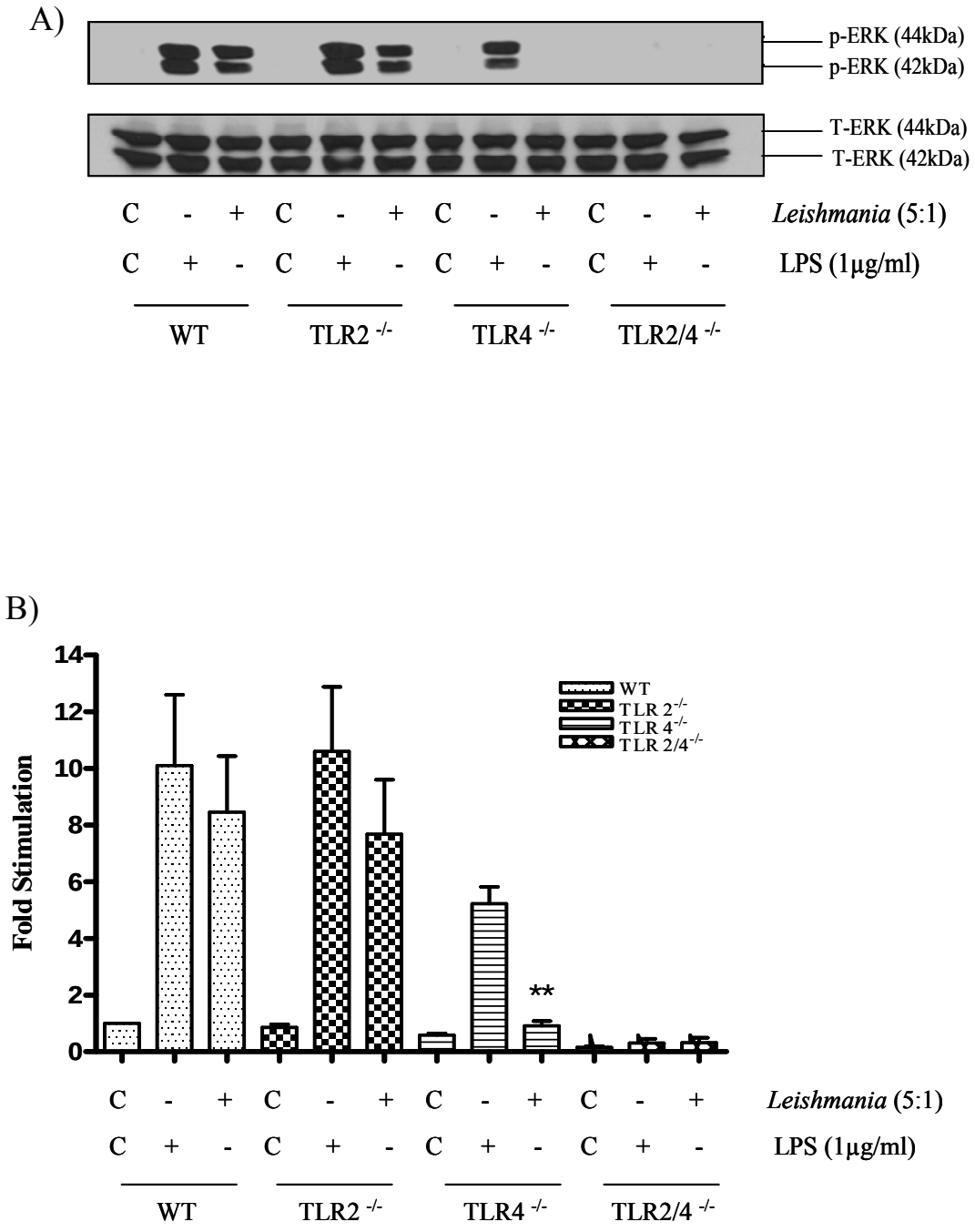


Figure 4.38: TLR4-mediated ΔCPB amastigotes-induced ERK1/2 phosphorylation in macrophages. Cells (1x106/well) were stimulated with LPS (1µg/ml) or incubated with *Leishmania* at a ratio of (5:1) for 30 min. Whole cell lysates were prepared and then assessed for A) p-ERK1/2 (42/44 kDa) and ERK (42/44 kDa) as outlined in Section 2.2.3. Blots were quantified in B) by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings. **p<0.001 as compared with wild type stimulation.

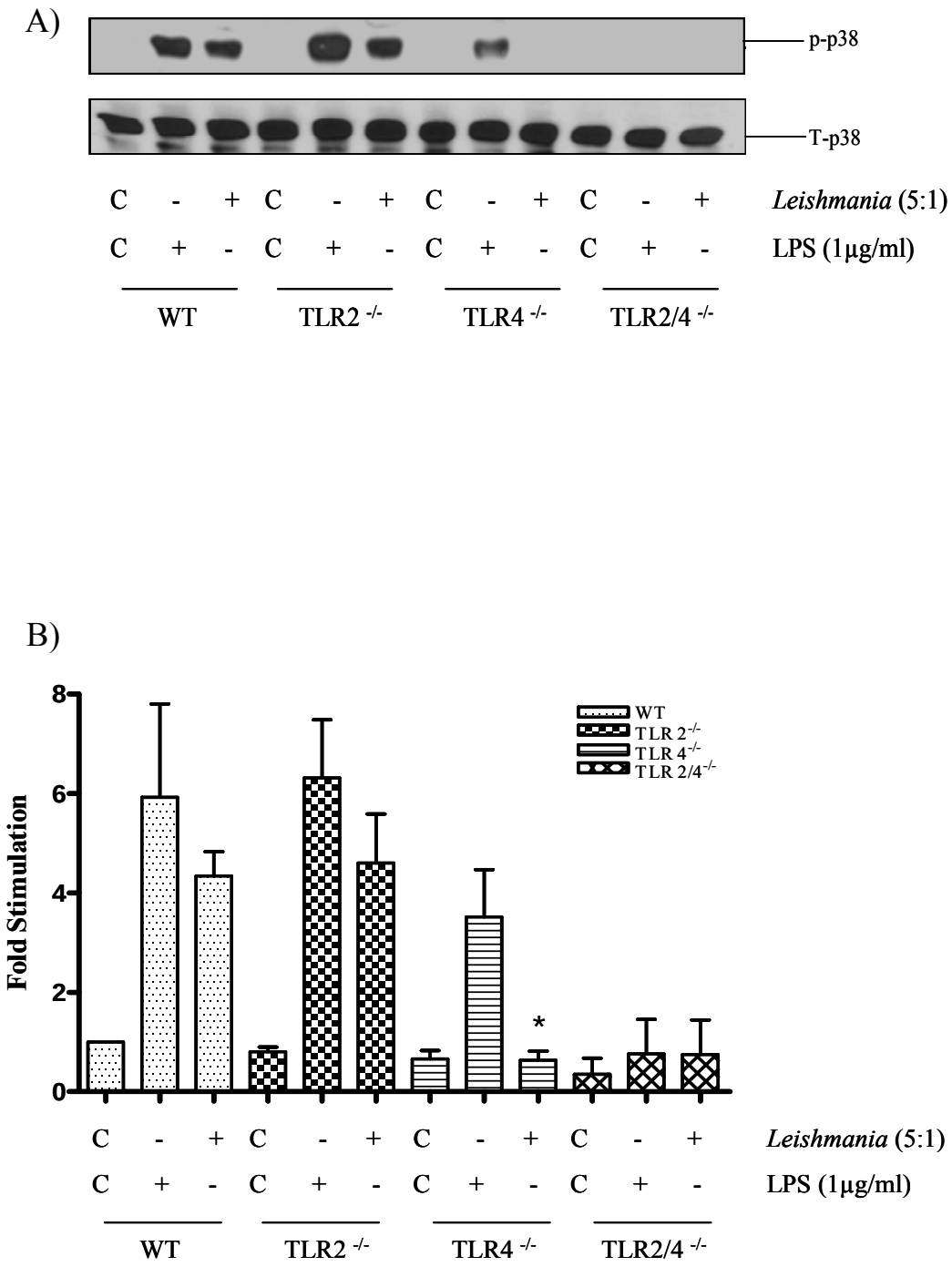


Figure 4.39: TLR4-mediated ΔCPB amastigote-induced p38 MAP kinase phosphorylation in macrophages. Cells ($1x10^6$/well) were stimulated with LPS (1μg/ml) or incubated with *Leishmania* at a ratio of (5:1) for 30 minutes. Whole cell lysates were prepared and then assessed for **A)** p-p38 and T-p38 as outlined in Section 2.2.3. Blots were quantified in **B)** by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings. *$p<0.05$ as compared with wild type stimulation.

4.9 THE ROLE OF TLR4 ON COX-2 AND iNOS PROTEIN EXPRESSION IN MACROPHAGES

To further support the hypothesis that TLR4 is an important mediator of promastigote effects on macrophages, it was necessary to assess the effect on the production of pro-inflammatory proteins, COX-2 and iNOS. Macrophages from WT and TLR deficient mice were treated as outlined previously and COX-2 and iNOS assessed, as well as NO release.

4.9.1 The effect of TLR4 deficiency on LPS and promastigote induced COX-2 protein expression in BMD-macrophages

The magnitude of TLR4-mediated COX-2 protein expression was measured over a period of 24 hours as shown in Figure 4.40. The results showed that WT macrophages responded to both LPS alone and the presence of *Leishmania* promastigotes with a marked increase in COX-2 protein expression. In $TLR2^{-/-}$ deficient macrophages this was reduced to a small extent (fold stim., LPS = 104.35 ± 17.25, promastigote = 129.82 ± 16.33 for WT, LPS = 70.40 ± 11.38, promastigote = 102.35 ± 19.16 for $TLR2^{-/-}$). In contrast, in $TLR4^{-/-}$ cells, LPS induced expression was significantly reduced but not abolished. This reduction was also observed in macrophages which were also infected with promastigotes. Furthermore, experiments in $TLR2/4^{-/-}$ macrophages showed that LPS even in the presence of promastigotes failed to induce COX-2 protein expression above background.

4.9.2 The effect of TLR4 deficiency on LPS and promastigote-induced iNOS protein expression in BMD-macrophages

In a similar fashion to COX-2, iNOS protein was examined (Figure 4.41). The results showed that WT and $TLR2^{-/-}$ macrophages responded to LPS alone or in the presence of *Leishmania* with the expected increase in iNOS protein expression as observed

previously. In TLR2$^{-/-}$ cells the response to LPS was reduced to some extent but not in macrophages that were also infected with promastigotes (fold stim., LPS = 51.31 ± 16.03, promastigote = 71.12 ± 21.52 for WT, LPS = 34.96 ± 8.26, promastigote = 63.58 ± 12.70 for TLR2$^{-/-}$). As with COX-2, in cells lacking TLR4 treated both with LPS and LPS plus promastigotes iNOS responses were completely abolished. Similar findings were observed in TLR2/4$^{-/-}$ macrophages. Supernatants from the experiments above were also used to measure NO production indirectly as nitrite formation (Figure 4.42). The results obtained were essentially the same for that observed for iNOS protein. In WT and TLR2$^{-/-}$ macrophages NO release in response to LPS in the presence of promastigotes was essentially similar. However, in either TLR4$^{-/-}$ or TLR2/4$^{-/-}$ macrophages nitrite release was essentially abolished (promastigote+LPS = 44.00 ± 12.23 for WT, 33.39 ± 0.29 for TLR2$^{-/-}$ and 1.56 ± 0.13 for TLR4$^{-/-}$).

4.9.3 The effect of TLR4 deficiency on LPS and ΔCPB amastigote induced COX-2 and iNOS protein expression in BMD-macrophages

Figures 4.43 and 4.44 shows COX-2 and iNOS protein expression measured over a period of 24 hrs. The results showed that WT and TLR2$^{-/-}$ macrophages responded to LPS in the presence of ΔCPB amastigotes with an increased in both COX-2 and iNOS expression which was markedly greater than control values. Following TLR2 deletion, COX-2 protein expression was partially decreased. In the absence of TLR4 the LPS response was significantly reduced but not abolished, however surprisingly in the additional presence of CPB amastigotes, the response was severely ablated. Only in the TLR2/4$^{-/-}$ deletion macrophages was the COX-2 expression reduced to basal values. In contrast, protein expression exquisitely sensitive to the presence of TLR4. In its abscence, iNOS expression was abolished. Similar finding was demonstrated in TLR2/4$^{-/-}$ macrophages (fold stim., LPS = 14.52 ± 1.05, *Leishmania* = 17.20 ± 2.41 for WT, LPS = 11.08 ± 0.33, *Leishmania* = 12.69 ± 0.67 for TLR2$^{-/-}$).

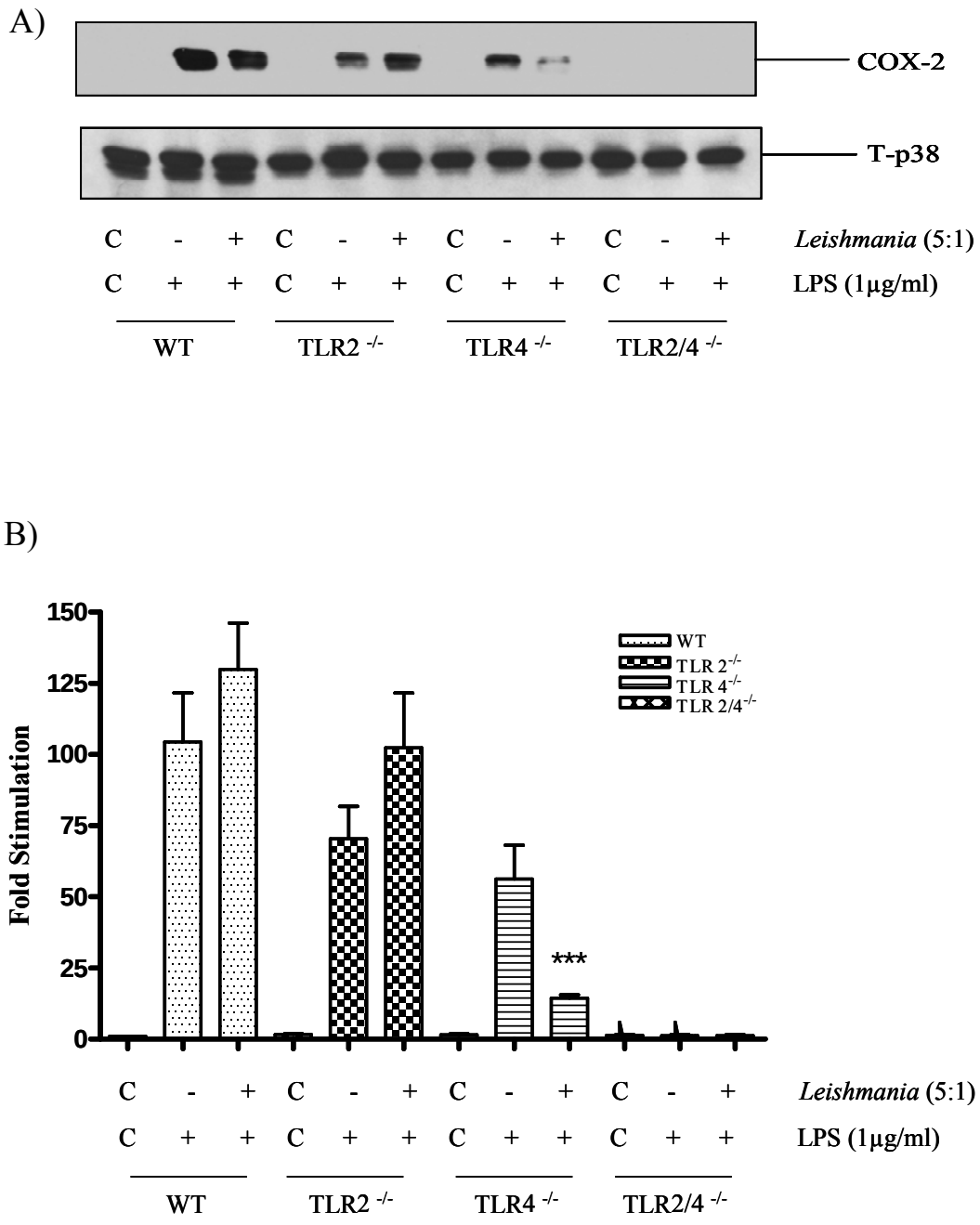


Figure 4.40: Role of TLR4 upon promastigote-mediated COX-2 protein expression in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* at a ratio of (5:1) for 2 hrs and then stimulated with LPS (1µg/ml) for 24 hrs. Whole cell lysates were prepared and then assessed for **A)** COX-2 (72 kDa) and T-p65 as outlined in Section 2.2.3. Blots were quantified in **B)** by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings. ***$p<0.001$ as compared with wild type stimulation.

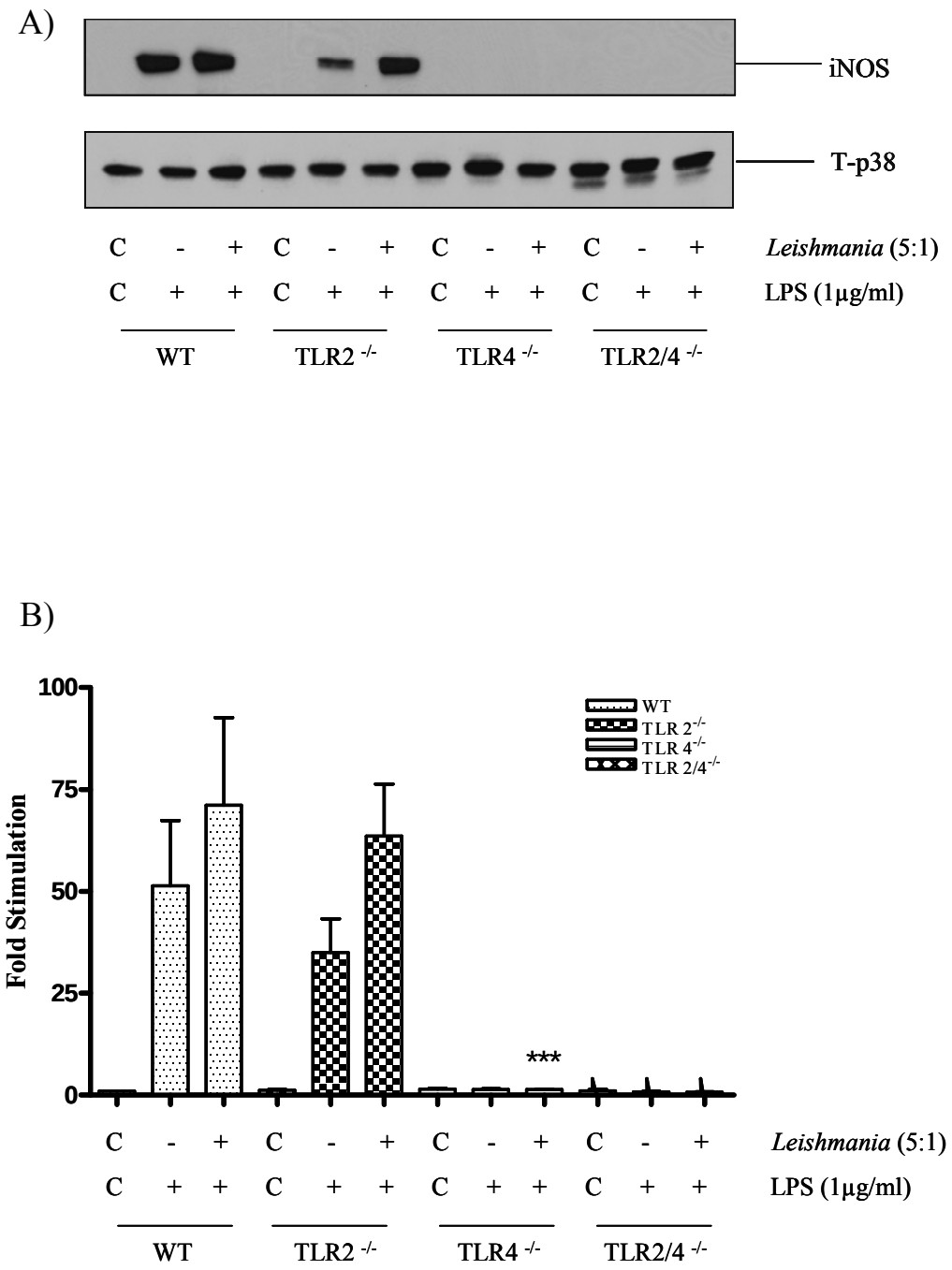


Figure 4.41: Role of TLR4 upon promastigote-mediated iNOS protein expression in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* at a ratio of (5:1) for 2 hrs and then stimulated with LPS (1µg/ml) for 24 hrs. Whole cell lysates were prepared and then assessed for A) iNOS (130 kDa) and T-p38 as outlined in Section 2.2.3. Blots were quantified in B) by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings. ***$p<0.001$ as compared with wild type stimulation.

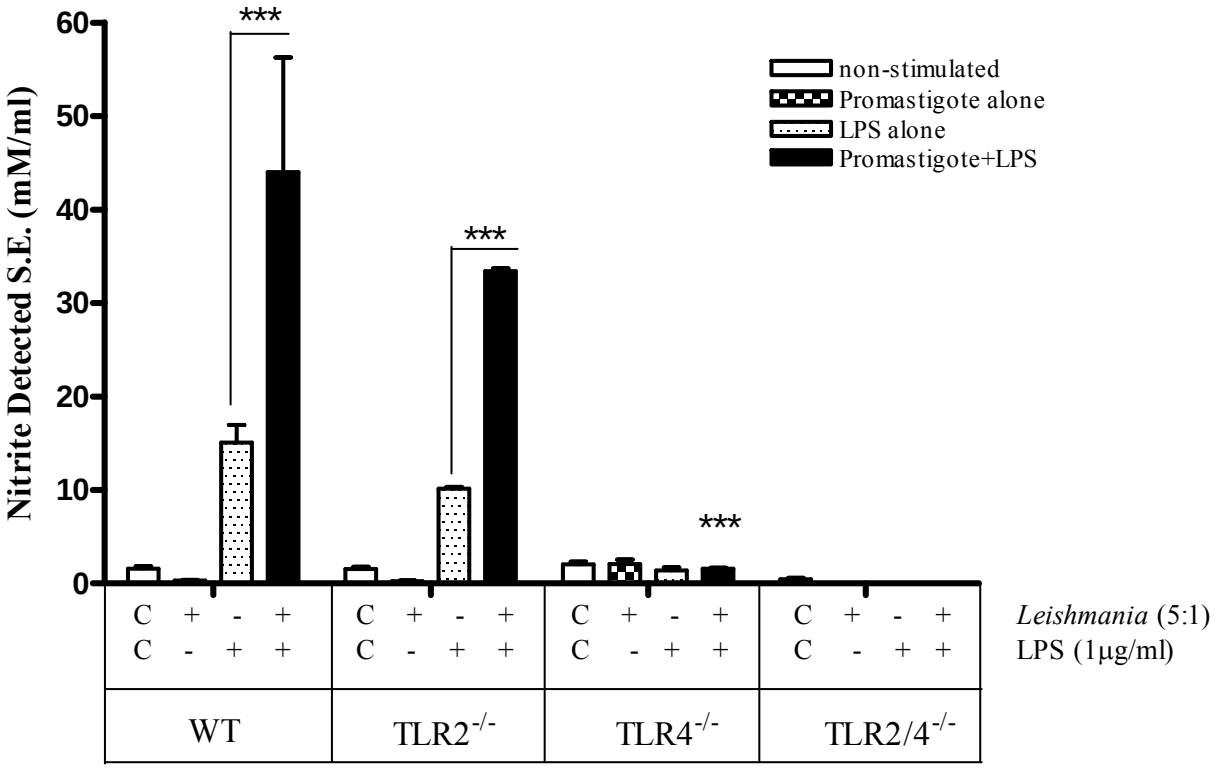


Figure 4.42: Role of TLR4 upon *Leishmania mexicana* promastigote infection induced NO production in macrophages. Cells ($1x10^6$/well) were grown overnight in a 24 well plate and infected with *Leishmania* at a ratio of (5:1) for 2 hrs and then stimulated with LPS (1µg/ml), over a period of 48 hrs. Supernatants were collected and nitrite levels assessed by Griess assay (as outlined in section 2.2.7). Values are quantified and expressed as mean ± S.E.M.(***$p<0.001$ as compared with wild type stimulation). Each of the above columns is representative of 3 separate experiments with similar findings.

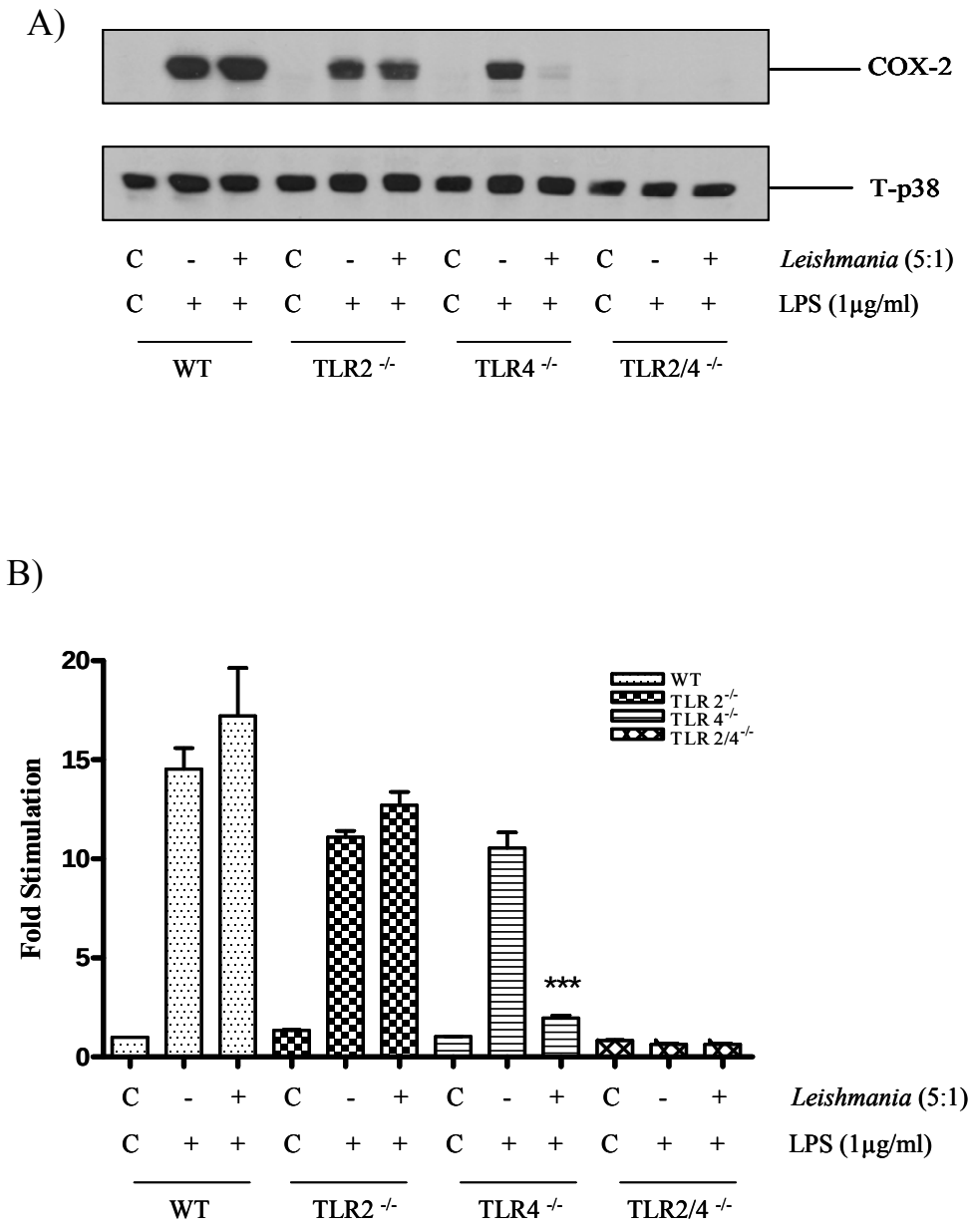


Figure 4.43: Role of TLR4 upon ΔCPB amastigote mediated COX-2 protein expression in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* at a ratio of (5:1) for 2 hrs and then stimulated with LPS (1µg/ml) for 24 hrs. Whole cell lysates were prepared and then assessed for **A)** COX-2 (72 kDa) and T-p65 as outlined in Section 2.2.3. Blots were quantified in **B)** by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings. ***p<0.001 as compared with wild type stimulation.

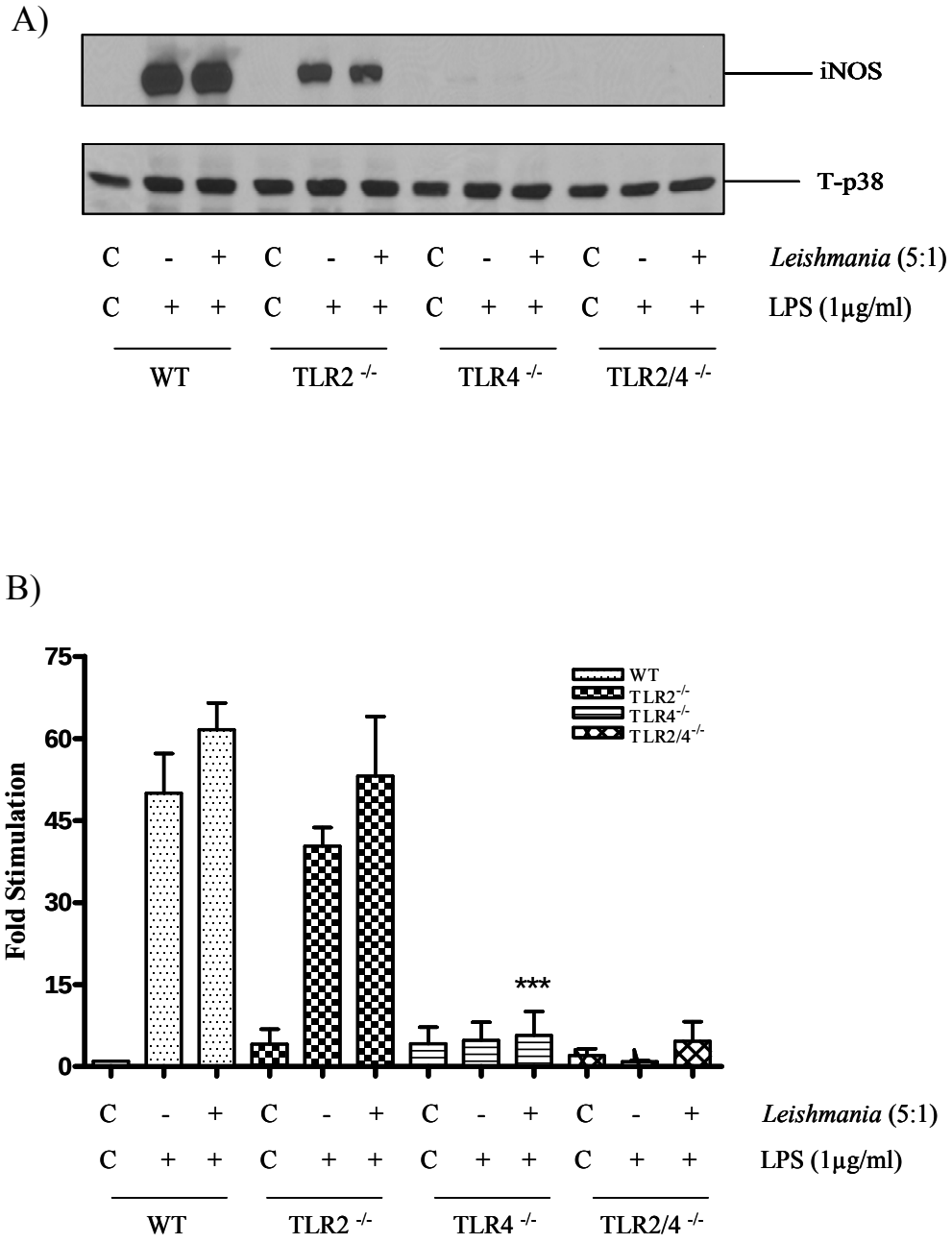


Figure 4.44: Role of TLR4 upon ΔCPB amastigote-mediated iNOS protein expression in macrophages. Cells (1x106/well) were infected with *Leishmania* at a ratio of (5:1) for 2 hrs and then stimulated with LPS (1µg/ml) for 24 hrs. Whole cell lysates were prepared and then assessed for A) iNOS (130 kDa) and T-p38 as outlined in Section 2.2.3. Blots were quantified in B) by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings. ***$p<0.001$ as compared with wild type stimulation.

4.9.4 The effect of TLR4 deficiency on LPS and promastigote-induced arginase-1 protein expression and activity in BMD-macrophages

Next arginase-1 expression was examined in LPS and *Leishmania*-infected macrophages lacking TLR2, TLR4 alone or both together. Samples which had been prepared as cellular extracts were analysed by Western blotting with a specific Arg-1 antibody and also assessed for arginase activity (Figure 4.46). The results showed that in WT macrophages both LPS and promastigotes alone caused a substantial increase in arginase-1 expression. However, unlike previous experiments there was no enhancing effect when both agents were added together. In $TLR2^{-/-}$ macrophages the response to each agent alone or together was not affected. In contrast, in $TLR4^{-/-}$ cells both LPS and promastigote induced arginase-1 expression was reduced back to near basal values (fold stim., promastigotes = 6.16 ± 1.13 for WT, promastigotes = 6.22 ± 0.73 for $TLR2^{-/-}$, promastigotes = 0.75 ± 0.35 for $TLR4^{-/-}$, ***$p < 0.001$). In $TLR2/4^{-/-}$ cells there was a further reduction in expression below the initial control value (see Figure 4.45).

Arginase activity was also assessed under similar conditions of stimulation (Figure 4.46). LPS alone caused a significant increase in arginase activity in comparison to non-stimulated cells (control) (fold stim., LPS = 35.16 ± 4.15, control = 3.98 ± 0.33). Promastigotes also caused a similar increase in activity, and there was an additional increase in the presence of both agents together. As with arginase-1 expression, there was no significant decrease in response in $TLR2^{-/-}$ macrophages, however, activity was reduced to near control values in macrophages in which $TLR4^{-/-}$ or both $TLR2^{-/-}$ and $TLR4^{-/-}$ together were deleted (fold stim., *Leishmania*+LPS = 4.87 ± 0.53 for $TLR4^{-/-}$, *Leishmania*+LPS = 2.75 ± 0.28 for $TLR2/4^{-/-}$).

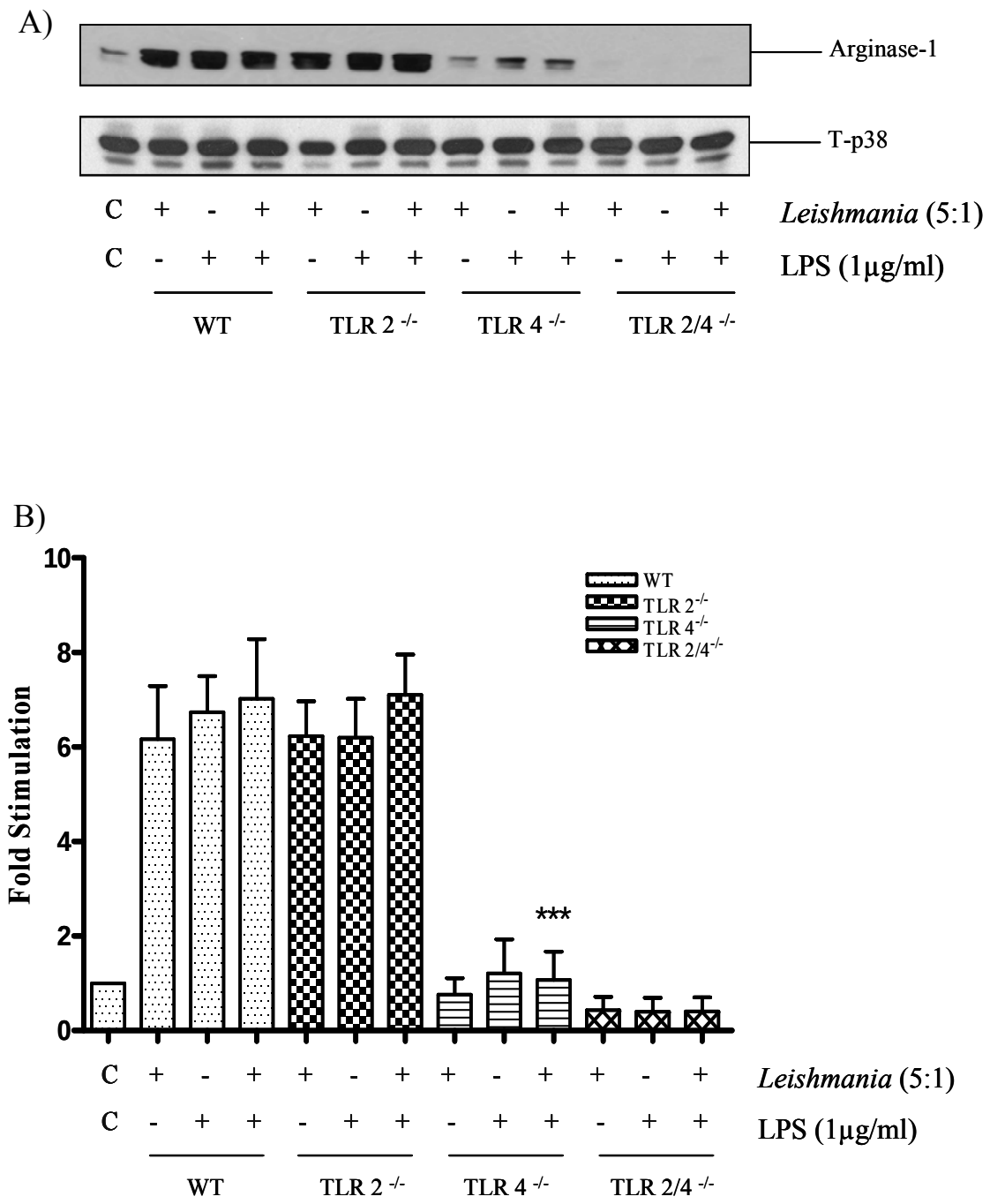


Figure 4.45: Role of TLR4 upon promastigote-mediated Arg-1 protein expression in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* at a ratio of (5:1) for 2 hrs and then stimulated with LPS (1µg/ml) for 24 hrs. Whole cell lysates were prepared and then assessed for **A)** Arg-1 (35 kDa) and T-p38 as outlined in Section 2.2.3. Blots were quantified in **B)** by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar findings. ***$p<0.001$ as compared with wild type stimulation.

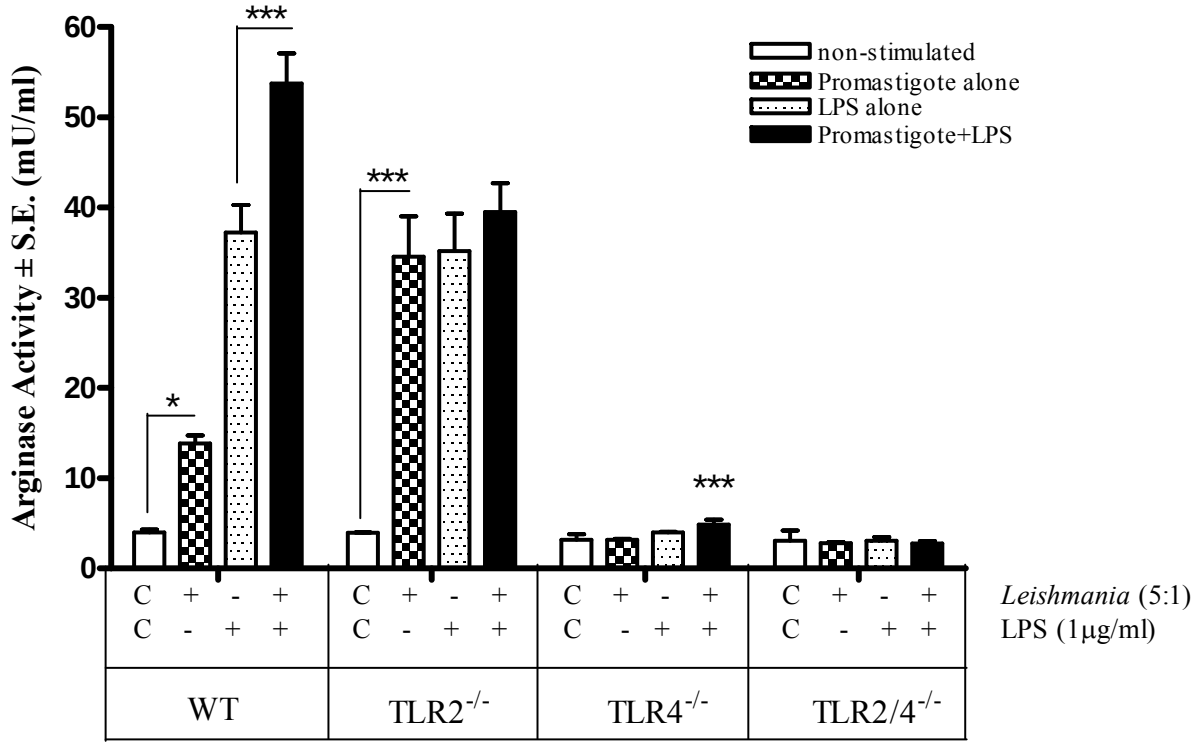


Figure 4.46: Role of TLR4 upon *Leishmania mexicana* promastigote-induced arginase activity in macrophages. Cells ($1x10^6$/well) were grown overnight in a 24 well plate and infected with *Leishmania* at a ratio of (5:1) for 2 hrs and then stimulated with LPS (1μg/ml), IL-4 (100U/ml) and both, over a period of 48 hrs. Cells were collected, lysed by specific buffer, and subject to arginase assay (as outlined in section 2.2.5). Figure 4.46 represents quantification of arginase activity, expressed as mean ± S.E.M. (*$p<0.05$, ***$p<0.001$ as compared with wild type stimulation). Each of the above columns is representative of 3 separate experiments with similar findings.

4.10 DISCUSSION

Promastigotes have been widely reported to modulate the function of a number of immune cells including macrophages (Ben-Othman et al., 2009, Olivier et al., 2005, Forget et al., 2005), by regulating intracellular signalling pathways. A greater understanding of these events is important for the future development of therapeutic targets and the development of new drugs directed against *Leishmania* infection. In this chapter the effect of promastigotes on macrophage MAP kinase and NF-κB signalling and the expression of inflammatory proteins such as COX-2 and iNOS via ligation of TLR-4 were investigated. In addition, the interaction with the arginase pathway was examined.

Initially it was found that unlike amastigotes, promastigotes had no effect on the expression of endogenous MAP kinases in BMD-macrophages (see chapter 3), nor substantial effect on LPS-induced responses. However, alone promastigotes caused a marked increased in the activation of all three major MAP kinases, JNK1/2, ERK1/2 and p38 MAP kinase and also intermediates of the NF-κB pathway. Whilst giving an insight into the actions of *L. mexicana*, these results are difficult to fit into a defined framework for the activation of MAP kinases by *Leishmania*. The results are partly consistent with another study showing that *L. donovani* promastigotes-induced ERK1/2 and p38 MAPK phosphorylation but inhibited NF-κB activity (Ben-Othman et al., 2009). However, a previous study demonstrated no effect on any MAP kinases nor NF-κB using *L. donovani* (Prive and Descoteaux, 2000). Another group used macrophages primed with IFN-γ and showed that *L. donovani* promastigotes had the ability to induce the phosphorylation of ERK1/2 and p38 both of which were together required for parasite-induced TNF-α release. That said, treatment of macrophages with promastigotes of *L. amazonensis,* closely related to *L. mexicana*, actually demonstrated rapid inactivation of ERK1/2 (Martiny et al., 1999). These and other studies (Nandan et al., 1999) indicate a vast difference in the regulation of kinase signaling, likely to be dependent not only on the type of species involved, but also the concentration and treatment conditions.

In this study ΔCPB deficient amastigotes were also utilized. Whilst this approach revealed a key difference in the mechanisms of action highlighting the role of CPB in signalling pathway destruction (see chapter 3), it also indicated that macrophage MAP kinases could be activated in a stage-dependent manner by components common to both promastigotes and amastigotes of *L. mexicana* which is responsible for activation. This could be cell surface molecules such as LPG and GP63 which have been demonstrated to regulate kinase signalling in a number of studies (Halle et al., 2009, Balaraman et al., 2005, Prive and Descoteaux, 2000, Feng et al., 1999). Here promastigotes were found to rapidly activate MAP kinases through interactions with TLR4 and recent studies have shown other *Leishmania* sub species to interact with a variety of TLRs (Ribeiro-Gomes et al., 2007, Flandin et al., 2006, Kropf et al., 2004, Muraille et al., 2003, Becker et al., 2003, Hawn et al., 2002, Mosser and Brittingham, 1997).

In this study promastigotes were found to upregulate the expression of both COX-2 and iNOS proteins, which in turn enhanced the formation of PGE_2 and nitric oxide. Enhanced COX-2 and PGE_2 formation has been previously demonstrated in response to *L. donovani* and *L. major* infection (Anstead et al., 2001, Matte et al., 2001, Farrell and Kirkpatrick, 1987), although a recent study has revealed that macrophages have the ability to express higher levels of both COX-2 protein and PGE_2 synthase mRNA in response to *L. donovani* than *L. major* (Gregory et al., 2008). For NO production, recent work has shown that *L. major* LPG promoted NO production by murine macrophages (Kavoosi et al., 2006). In agreement, Proudfoot et al., demonstrated that the larger LPG molecules in promastigotes had a higher stimulatory effect on NO synthesis than the smaller LPGs (Proudfoot et al., 1996). Furthermore, other studies have demonstrated that infection with *Leishmania* species, for example promastigotes of *L. amazonensis* and *L. donovani* caused high levels of NO production (Genestra et al., 2006b, Genestra et al., 2003a, Genestra et al., 2003b, Genestra et al., 2003c, Temporal et al., 2005, Genestra et al., 2004, Basu et al., 1997). Consistent with these data it has been demonstrated that the production of NO during *L. amazonensis* axenic amastigote infection was mediated through NOS enzyme expression suggesting that NO pathways are regulated by signalling mechanisms during the host cell-parasite interaction (Genestra et al., 2006a, Temporal et al., 2005). Indeed, Balestieri and co-workers have suggested that the mechanisms of NO production following *Leishmania*

infection may be due to the alteration of the intracellular signalling pathway by the parasite (Balestieri et al., 2002). With other types of infection models, for example *T. gondii* and *T. cruzi* both the synthesis of NO by the parasite and endogenous host cell NO may be necessary to trigger a deactivating response mechanism by the parasite. Thus NO is likely to serve as a multipurpose molecule in parasites as recent studies show it to stimulate cell motility and down regulate apoptosis in response to infection (Seabra et al., 2002, Genestra et al., 2006b).

Whilst not examined in this thesis other studies have demonstrated a causal link between MAP Kinase and NF-κB signalling and expression of COX-2 and iNOS proteins following *Leishmania* infection. For example, infection of macrophages with *L. donovani* showed upregulation of COX-2 mRNA, protein expression and PGE_2 production, mediated by a PKC-dependent pathway (Matte et al., 2001). In these cells activation of PKC and PTK signalling implies subsequent activation of a MAP kinase cascade (Matte et al., 2001). Induction of transcription factors mainly through activation of mitogen-activated protein kinases (MAPKs) and also PI3K/Akt pathways have also been shown to mediate expression of COX-2 and iNOS in macrophages (Chan and Riches, 2001, Uto et al., 2005). It has also been reported that in mouse macrophages JNK and ERK were involved in the induction of iNOS and COX-2 respectively (Nick et al., 2000, Ho et al., 2004).

Promastigote induced COX-2 and iNOS expression and the resultant increase in PGE_2 and NO release is important in regulating immune function in particular the induction of IL-12 and other cytokines, which will be discussed at a later point. However, another interesting aspect of this study was the survival of promastigotes in macrophages under conditions of high cellular NO levels. This is related to macrophage infection in two ways. Firstly, high NO levels produced following infection can enhance apoptosis in the host macrophage as demonstrated for *L. major* (Kharazi et al., 2003). However, this was not associated with the effect of *L. mexicana* promastigotes as the morphology of the macrophages was not altered in this study. The second aspect is that NO will destroy the promastigotes by oxidation. This has been demonstrated previously using exogenous NO donors and mice models deficient in iNOS (Nahrevanian et al., 2009, Salvati et al., 2001, Murray et al., 2006, Blos et al., 2003). Since *L. mexicana* promastigotes enhanced iNOS expression and

prolonged LPS-induced NO production, these findings suggest that promastigotes are in fact, largely resistant to killing by NO. Whilst this was not tested directly, a recent study by Giudice and his colleague demonstrated that promastigotes from *L. braziliensis* and *L. amazonensis* have the ability to resist nitric oxide induced killing and that these differences were related to the clinical symptoms of infection (Giudice et al., 2007). This finding is also in agreement with previous studies which demonstrated that *Leishmania* species are themselves able to produce NO, in addition to NO derived from the macrophage (Basu et al., 1997, Genestra et al., 2006b). This is a likely strategy used by the parasite to enhance survival. Alternatively, since NO can also play a role in regulating signalling pathways within the infected cell, it is possible that NO resistant promastigotes utilise either endogenous or host NO to regulate the expression of effector molecules important in immune function as discussed later.

In the present study, the potential for promastigotes to regulate arginase expression and activity was also examined. Arginase plays a crucial role in parasitic infection including *Leishmania* pathogens reviewed by; (Stempin et al., 2010). Arginase protein expression in macrophages is considered the prototypic marker for alternative activation (Martinez et al., 2009). Therefore, exposure to Th2 cytokines such as IL-4 and IL-13 provoke production of arginase-1 expression in macrophages (Munder et al., 1998). This leads to a transfer of arginine metabolism from NO production via iNOS toward production of L-ornithine, a precursor for polyamines and collagen (Goerdt and Orfanos, 1999, Hesse et al., 2001).

The results in this study indicate that promastigotes had a significant effect on arginase-1 protein expression and activity. This activity was dependent on TLR4 and was enhanced by either LPS or IL-4. The role of alternatively activated macrophages during *Leishmania* infection is at present an understudied area of parasite immunology. Nevertheless, studies examining the role of arginase-1 induction during leishmaniasis have revealed that this enzyme, induced by Th2 cytokines and others such as IL-10 and TGF-β (Munder et al., 1999, Hesse et al., 2001), plays a key role in the immune response following infection. Studies carried out by Kropf et al., demonstrated a role for arginase-1 in the pathogenesis of nonhealing leishmaniasis, a prototypic Th2 disease, and demonstrated that the activity of this enzyme promotes

pathology and uncontrolled growth of *Leishmania* parasites *in vivo* (Kropf et al., 2005). Another study *in vitro* by Iniesta et al., demonstrated a protective role for arginase-1 induction in enhanced replication of *Leishmania* inside macrophages (Iniesta et al., 2005). In the same publication, it has also been reported that infection of macrophages with *L. major* or *L. infantum* prior to treatment with IL-4 resulted in the increased synthesis of polyamines due to enhanced arginase-1 activity. Additionally, Holscher and his coworkers demonstrated that *Leishmania* susceptibility was related to the alternative macrophage pathway. In mice with targeted deletion of the macrophage and neutrophil IL-4Rα receptor, a significant delay in disease progression was associated with reduced arginase-1 activity (Holscher et al., 2006).

A key feature of this study was the simultaneous upregulation of both iNOS and arginase-1 and the balance in the production of NO. It has been suggested that the delay in arginase-1 expression relative to iNOS could be a mechanism to limit the inflammation caused by NO production in response to endotoxin challenge, allowing the healing process to begin (Salimuddin et al., 1999). Indeed, in preliminary studies it was demonstrated that iNOS was induced much earlier than arginase-1. Sonoki et al., has previously shown that both proteins were expressed in rat peritoneal macrophages following LPS stimulation, with the same priority of expression, arginase 1 mRNA was induced more slowly than iNOS mRNA (Sonoki et al., 1997). This might suggest that increased arginase-1 activity following promastigote infection will reduce arginine availability for the NOS reaction in activated macrophages and may limit overproduction of NO. A recent study has demonstrated that during co-infection with *L. major, L. mexicana* induced larger cutaneous lesions due to the induction of arginase-1 expression in macrophages (Rodriguez-Sosa et al., 2006). In addition, pre-treatment of macrophages with IL-4 and subsequent infection with *L. major* or *L. infantum* induced the synthesis of polyamines through arginase-1 leading to promote the intracellular growth of the parasite (Iniesta et al., 2001). Furthermore, following infection of mice with *L. major*, arginase-1 induction was detected during the progression of the disease. This resulted in the reduction of iNOS protein expression and a change in the balance between IL-4 and IL-12 (Iniesta et al., 2005). However, it is clear that in response to promastigotes, LPS induced NO was not markedly inhibited, suggesting that there is a balance between these two pathways with respect to *L. mexicana* infection. These results and other studies suggest that

there is a complex regulation of genes encoding enzymes and transporter proteins involved in arginine metabolism that together control NO production in cells when infected with intracellular parasites.

The mechanisms underlying the ability of *Leishmania* to induce arginase activity in BMD-macrophages are yet to be characterised. One question is whether arginase activity is induced through the actual infection of macrophages by *Leishmania*, or by the presence of cell surface immunostimulatory molecules such as LPG and gp63. In this current study it was found that the enhancing effects of *Leishmania mexicana* promastigotes on arginase-1 induction involved TLR4. It has been shown previously that TLR4 plays a role in the host defense against *L. major* (Kropf et al., 2004). However, whilst it was established that, TLR4 contributed to both innate and adaptive immune responses via an early induction of inducible nitric oxide synthase, parasite survival was increased in TLR4-deficient mice through a higher arginase activity in host cells (Kropf et al., 2004). A recent study by El Kasmi et al., has reported that some intracellular pathogens induced arginase-1 expression in mouse macrophages through the TLR pathway (El Kasmi et al., 2008). Furthermore, specific elimination of arginase-1 in macrophage favored host survival during *Toxoplasma gondii* infection and decreased lung bacterial load during tuberculosis infection (El Kasmi et al., 2008). This was likely to involve TLRs since it has been previously shown that *Toxoplasma gondii* was able to bind both TLR2 and TLR4 through immunostimulatory molecules such as GPIs and HSP70 (Debierre-Grockiego et al., 2007, Mun et al., 2005, Aosai et al., 2006). Another pathogen, *T. cruzi*, was able to induce host arginase directly in macrophages via the production of a parasite derived molecule, cruzipain (Stempin et al., 2002). Consistent with findings in this thesis a study by Freire-De-Lima et al., demonstrated that macrophages infected with *T. cruzi* showed less NO production when treated with apoptotic cells, concomitant with increased arginase-1-expression (Freire-de-Lima et al., 2000). Recent studies indicated that these agents also upregulated arginase-1 expression by utilizing signaling pathways distinct to that observed for IL-4 and IL-13 which involves STAT6 activation, both, *Mycobacterium* and *T. gondii* utilized a STAT6- independent pathway. Finally, it should be noted that *Leishmania* parasites have been shown to express their own arginase which may function to reduce NO production in the host

cell (Kropf et al., 2003, Roberts et al., 2004). This possibility was not examined directly in this thesis.

An additional aspect of increased arginase-1 expression is the potential to limit polyamine synthesis (Kepka-Lenhart et al., 2000, Fligger et al., 1999). Polyamines are not only helpful for the growth of *Leishmania*, they also have additional effects including the regulation of signal transduction pathways, modulation of the nucleic acid conformation, RNA export and their degradation, protein synthesis, cell growth and differentiation, tumor progression and the modulation of immune responses (Tabor and Tabor, 1984, Morgan, 1994). For example, they can act as negative immune regulators in lymphocytes, neutrophils and natural killer (NK) cells (Byrd et al., 1977, Ferrante et al., 1986, Quemener et al., 1994), spermine and spermidine inhibit the secretion of pro-inflammatory cytokines such as IL-12p40 and IFN-γ in macrophages and enhance IL-10 production (Perez-Cano et al., 2003, Hasko et al., 2000). Spermine also counter-regulates the innate immune response induced by the TLR4 ligand lipopolysaccharide (Zhang et al., 1997, Hasko et al., 2000). Notably, high intracellular polyamine levels have been demonstrated to shift macrophage mediated cytotoxicity, particularly decreased the capacity of macrophages to phagocytize parasites and inert particles (Tjandrawinata et al., 1994). Moreover, a recent study has documented that polyamines are essential factors in the regulation of leishmaniasis (Kropf et al., 2005). These possibilities were again not directly examined in this thesis, however because promastigotes increased both iNOS and arginase-1 it is likely that arginase activity is not just restricted to regulating NO synthesis.

A key aspect of this study was to determine the effects of PGE_2, NO and arginase on the regulation of IL-12 production. Infected macrophages release pro-inflammatory cytokines such as IL-12, IL-6 and TNF-α to support inflammation as an anti-parasitic immunity (Alexander et al., 1997). IL-12 has been shown to drive IFN-γ production which is vital in determining the outcome of *Leishmania* infection, since control of parasite growth requires activation of macrophages by IFN-γ (Sutterwala and Mosser, 1999). Results in this thesis, in agreement with several other studies, demonstrated that IL-12 production in macrophages was substantially inhibited by promastigotes. Carrera and coworkers reported inhibition of macrophage IL-12 production following

Leishmania infection leading to delayed IFN-γ production and down-regulation of NO release (Carrera et al., 1996). Lipophosphoglycan, the major macromolecule on the promastigote surface, may play a role, as previous studies have revealed promastigote LPG inhibited IL-12 production in macrophages (Piedrafita et al., 1999, Proudfoot et al., 1996). In contrast, a recent study by Aebischer and co-workers has reported cell specific effects of LPG, demonstrating increase in IL-12 production in dendritic cells (Aebischer et al., 2005). *L. mexicana* LPG-mediated inhibition of IL-12 production was demonstrated more recently in monocytes but not in dendritic cells (Argueta-Donohue et al., 2008). In this current study rather than using LPG, complete promastigotes were utilized however, it is likely that the mechanisms which resulted in inhibition of IL-12 will be mechanistically similar.

The results in this chapter indicated that PGE_2 directly contributed to regulation of IL-12 inhibition by promastigotes. Treatment with indomethacin, a non-selective COX inhibitor reversed the inhibitory effects of the promastigotes. It should be noted that indomethacin was only effective when added subsequent to LPS stimulation, pre-treatment did not reverse promastigote inhibition and non-specific effects cannot be discounted. Nevertheless, substantial previous evidence has demonstrated that PGE_2 inhibits IL-12 production. This was originally elucidated as early as 1995 by (van der Pouw Kraan et al., 1995) and several studies support these findings (Sharma and Cotlier, 1982, Schwacha et al., 2002). Several mechanisms may be involved in the effect of PGE_2. For example, PGE_2 can upregulate IL-10 production which functions to inhibit IL-12 production (Ayala et al., 1994, Demeure et al., 1997, Strassmann et al., 1994). In T cells, PGE_2 can inhibit IL-12 production and increase IL-10 but with different sensitivities, suggesting different mechanisms of regulation (Betz and Fox, 1991, Snijdewint et al., 1993, Van der Pouw-Kraan et al., 1992). PGE_2 has also been demonstrated to have a direct inhibitory effect on the NF-κB activation pathway, known to be important for IL-12 production (Suk et al., 2001, Kim et al., 2007, Arai et al., 2008). What ever the mechanisms of action at a cellular level, PGE_2 has been suggested to be an important regulatory factor in inducing a Th-2 type response (Abe et al., 1997, Kuroda et al., 2000, Monteleone et al., 1999, van der Pouw Kraan et al., 1995), functioning to negatively cross-talk in the production of Th-1 and Th-2 cytokines (Phipps et al., 1991).

There are only a few studies which have examined the function of endogenous PGE_2 preferring to administer PGE_2 *in vitro* and *in vivo*. The results in this chapter demonstrated that the production of PGE_2 by promastigotes was functionally important in the suppressive effect of promastigotes on IL-12 production. An early study by Scott and Farrell, demonstrated that cells from BALB/c mice infected with *L. major* produced large amounts of PGE_2 that suppress proliferation of parasite-specific T cells, an effect that could be reversed by culturing cells with the cyclooxygenase inhibitor indomethacin (Scott and Farrell, 1981). More recent findings consistent with this current study demonstrated that addition of indomethacin to antigen-stimulated cells from *L. mexicana*-infected mice increased production of both IL-12 and IFN-γ (Perez-Santos and Talamas-Rohana, 2001). This has implications *in vivo* since administration of indomethacin to *L. major*-infected mice was found to moderate the course of lesion development and promote an enhanced Th1-type response (De Freitas et al., 1999, Farrell and Kirkpatrick, 1987).

In this study it was found that enhancement of NO production by promastigotes inhibited IL-12 production in macrophages. This was assessed using the non selective NOS inhibitor L-NAME, which reversed the inhibitory effect of promastigotes. These findings are in agreement with several other studies which demonstrated inhibition of IL-12 production in macrophages following generation of NO (Bielawska-Pohl et al., 2010, Zhu et al., 2006, Xiong et al., 2004). Huang and co-workers also demonstrated that cells from iNOS-deficient mice made more IL-12 p40 protein than wild type mice following LPS activation and a marked augmentation IL-12 p40 mRNA accumulation in iNOS deficient splenocytes (Huang et al., 1998). Consistent with these findings a recent study has revealed that following stimulation with LPS and IFN-γ, peritoneal macrophages produced a high level of NO but low levels of IL-12, whilst the reverse was true for splenic macrophages (Zhu et al., 2006). With regard to a possible mechanism, a recent study has shown that NO inhibited LPS-induced IL-12 p40 gene expression and promoter activity by attenuating NF-κB DNA binding, an effect mediated via inhibition of IRAK activity (Xiong et al., 2004). To further understand the intracellular mechanism involved, dominant negative variants of MyD88, IRAK, and TRAF6 were used to examine LPS induction of the IL-12 p40 promoter in RAW 264.7 cells and indicated that NO inhibited IRAK activity and disrupted the

interaction between IRAK with TRAF6 in activated macrophages (Zhu et al., 2001, Xiong et al., 2004).

Interestingly, previous studies have demonstrated that NO has the ability to downregulate both p65 NF-κB and c-rel transcription factors (Boddupalli et al., 2007). This finding is important since, Sanjabi et al., found that the IL-12 p40 gene is predominantly regulated by c-rel rather than p65 NF-κB (Sanjabi et al., 2000). One study has demonstrated that inhibiting p38 MAP kinase using SB203580 increases nuclear c-rel levels in activated RAW 264.7 macrophages and this is well correlated with upregulation of IL-12 p40 (Browning et al., 1999, Jun et al., 1999, Boddupalli et al., 2007). Using a dominant negative p38 MAPK, they further confirm that activated p38 MAPK negatively influences nuclear c-rel levels and affects IL-12 p40 induction. These data indicate that NO activates p38 MAPK, which in turn regulates nuclear c-rel and this plays an important role in NO-mediated inhibition of IL-12 p40 (Boddupalli et al., 2007). Results in this chapter show both increased p38 MAP kinase and enhanced NO production. These two events could converge to negatively regulate IL-12 production. Finally, in addition to inhibition of IL-12 production, NO modulates immune responses by a number of mechanisms including potentially parasite killing (although not likely here). In addition to suppression of Th1 responses by NO there are other phenotypic effects of NO on the immune system such as disruption of signalling pathways and transcription factors, anti-inflammatory or immunosuppressive effects through upregulation of IL-10 and TGF-β, inhibition of T and B cell proliferation, inhibition of antibody production, downregulation of MHCII or pro-inflammatory cytokines and downregulation of chemokines and adhesion molecules including ICAM and VCAM reviewed by; (Bogdan, 2001).

As outlined previously the importance of arginase activity is through the effects upon growth of *Leishmania* via conversion of arginine to ornithine and urea (Das et al., 2010, Yaneth E. Osorio, 2008, Iniesta et al., 2005). In the present study, we investigated whether L-arginine availability modulated by arginase can serve as another control mechanism for *Leishmania* infection. We used nor-NOHA a potent inhibitor of arginases *in vitro* (Buga et al., 1998, Tenu et al., 1999). As expected inhibition of arginase by nor-NOHA increased NO production as indicated by the

high levels of nitrite production, however, somewhat surprisingly nor-NOHA also reversed the inhibitory effect of promastigotes on IL-12 production. This result was unexpected because high NO levels would be expected to inhibit IL-12 either in the presence or absence of promastigotes. The mechanism of reversal is currently unclear but another recent study has shown that in human monocytes treatment with another arginase antagonist induced IL-12/IL-23p40 production (Babu et al., 2009). Increased pro-inflammatory cytokine production and exacerbated colitis induced by *Citrobacter rodentium* was also observed following arginase inhibition (Gobert et al., 2004). However, in these studies it was unclear whether arginase inhibition was linked to enhanced NO production or another mechanism. A recent study has demonstrated that arginase-1 has the ability to suppress IL-12/IL-23p40 production by macrophages infected with *Schistosoma mansoni* (Herbert et al., 2010, Pesce et al., 2009) and has also demonstrated that infected organs from arginase-1 gene deletion mice have increased percentage of Th1 and Th17 cells, further suggesting a link between arginase 1, IL-12 and T cell associated inflammation. Indeed, arginase-1 expression could suppress inflammation via inhibition of macrophage and/or dendritic cell production of the p40 component of IL-12 and IL-23, which in turn, stimulates Th1 and Th17 responses, respectively (Rutitzky and Stadecker, 2006, Ivanov et al., 2006).

Therefore, arginase may play an important role in cell-mediated immunity independent of effects upon NO production and may function as an evasion strategy that dampens classically activated macrophages (El Kasmi et al., 2008). The data in this chapter therefore support a hypothesis that alternative-activated macrophage derived arginase-1 drives a critical immunoregulatory network that limits *Leishmania*-driven inflammation within the macrophage by antagonising Th1-associated immunopathology.

The results in the present study demonstrated that the effect of promastigotes varied depending on the cytokines studied. Whilst IL-12 production was markedly inhibited, IL-6 levels were unchanged and IL-10 production was significantly enhanced. IL-10 is known to be a strong anti-inflammatory cytokine which has the ability to control the overproduction of inflammatory cytokines and allows for the elaboration of a Th2 response. Numerous *Leishmania* species including, for example, *Leishmania major* are able to induced IL-10 production which enhances parasite survival (Fujiwara and

Kobayashi, 2005, Miles et al., 2005) and exacerbates the severity of infection (Vouldoukis et al., 1997). For *L. major* itself this may be mediated via FcγR ligation (Sutterwala et al., 1998, Kane and Mosser, 2001). A recent study by Bhattacharjee and co-workers demonstrates a significant role for IL-10 in the severity of visceral leishmaniasis, by using anti-IL-10 mAbs, which reveal enhancement of Th1 responses and inhibition of the Th2 response (Bhattacharjee et al., 2009). In other models of pathogen infection such as *Listeria monocytogenis* and *Mycobacterium avium* in infected mice, IL-10 is able to mediate the increase of the severity of disease (Bermudez and Champsi, 1993, Wagner et al., 1994). A recent signalling study demonstrates that IL-10 blocks nuclear c-rel levels, in addition to p65 NF-κB and these results in suppression of IL-12 production (Rahim et al., 2005). An additional experiment not performed in this current work could have examined whether pre-incubation with an IL-10 antibody was able to reverse promastigote mediated inhibition of IL-12 production. This approach would have allowed the contribution of IL-12 to be properly established.

CHAPTER 5

GENERAL DISCUSSION

During and after *Leishmania* infection there are several cellular outcomes that signify macrophage activation and destruction of invading pathogens. The interaction of pathogens leads to the activation of a series of intracellular signal transduction pathways which play a crucial role in mediating protection against these pathogens. These cascades are essential for the eventual destruction of *Leishmania* via the production of macrophage IL-12 which in turn drives naïve T cells to become differentiated Th1 cells and to produce IFN-γ. Both amastigote and promastigote infection stages use the macrophage as a host however, infection results in the regulation of intracellular signalling pathways in different ways, resulting in diverse effects upon inflammatory responses. Furthermore, whilst amastigotes and promastigotes regulate COX-2 and iNOS expression differentially, the same effect is observed on inflammatory cytokines in particular, IL-10 and IL-12. Therefore, the intracellular regulatory mechanisms regulated by these stages of *Leishmania* are of great interest and considerable medical importance.

For *L. mexicana* the relative effects of these stages, or their virulence, have been used as a means of understanding classical and alternative activation in various studies *in vitro* and in animal models of the disease. In the experiments designed in this thesis, I characterised the time-dependent stimulation or inhibition of MAP kinase, NF-κB and JAK/STAT pathways and the induction of COX-2, iNOS and arginase-1 in BMD-macrophages. In addition, I correlated these effects with the release of both pro and anti-inflammatory cytokines. I found that amastigotes caused inhibition of JNK, ERK and NF-κB pathways through degradation of key intermediates which resulted in inhibition of iNOS and COX-2 induction. However, p38 MAP kinase was unaffected and this highlights the remarkable selectivity of amastigotes in terms of the signalling pathways degraded. Other studies have shown that amastigotes of *L. donovani* and *L. major* induce the activation of p38 MAPK in macrophages, suggesting that preservation of this pathway is likely to be a desired outcome for the pathogen. Indeed, p38 MAP kinase has been identified as a hallmark of resistance to cell apoptosis (Junghae and Raynes, 2002, Ruhland et al., 2007) and maintenance of this pathway may allow the macrophage to survive long enough for amastigote replication and growth. Another recent study has demonstrated the ability of *Leishmania amazonensis* amastigotes to stimulate ERK phosphorylation in macrophages (Yang et al., 2007), whilst infection with *Leishmania major* amastigotes induced NF-κB

activation (Guizani-Tabbane et al., 2004). These and other studies, including the work in this thesis, indicate that the regulation of MAP kinase and NF-κB pathways is species-dependent. It is intriguing that over the course of *Leishmania* evolution different species have acquired such diverse mechanisms of regulation and would be useful to construct some sort of framework regarding effects on kinase signalling. However, given the large variation in approaches used by different groups regarding the concentration of *Leishmania* used and the time scale of infection make this difficult.

In contrast, this study revealed for the first time, the strong temporal activation of the MAP kinases and NF-κB in cultured murine macrophages infected alone by promastigotes. In addition, COX-2 and iNOS induction was significantly prolonged. These events represent both early and late markers of innate macrophage activation. Furthermore, it was also demonstrated that arginase-1 expression, a marker of alternative macrophage activation, was also induced by promastigotes. These three enzymes were linked to regulation of IL-12 production and were induced by the activation of TLR4, again a novel finding.

The co-regulation of all three inflammatory markers deserves some consideration. Up-regulation of COX-2 and iNOS is associated with activation of the immune response and inflammation. Therefore, paradoxically promastigotes may be seen to enhance inflammation, a response designed to fight infection. However, products of these enzymes can clearly have divergent effects. For example, PGE_2 whilst induced during inflammation can nevertheless dampen down inflammatory responses as well as inhibit IL-12 and clearly the promastigotes are able to balance these outcomes. Induction of iNOS and the resultant increase in NO production also caused inhibition of IL-12 and would be expected to promote destruction of the pathogen. Clearly however, promastigotes tolerate increased intracellular NO well and it would have been interesting to assess this further and to compare with other *Leishmania* species. Increased arginase activity would also promote promastigote survival through the formation of polyamines and again future studies examining *Leishmania* uptake and division in conditions of arginase inhibition would be revealing. Pharmacological inhibition of arginase also unexpectedly revealed a role in the regulation of IL-12 and whilst recent evidence supports this regulation (Herbert et al., 2010, Iniesta et al.,

2005), using a single inhibitor is clearly limiting. It would be essential to use other inhibitors, perhaps of a different chemical structure, to inhibit the arginase-1 and compare results. This also applies to the inhibition of both COX-2 and iNOS. Transfection of macrophages is difficult but has been achieved in some cases and therefore using siRNA or antisense directed against COX-2, iNOS or arginase-1 might be a useful strategy. Alternatively, relevant genetic knockout models may be useful. Overall it seems likely that for promastigotes these pathways operate in a balanced way to allow inhibition of cell mediated immunity through inhibition of IL-12 production, whilst at the same time facilitating survival.

A key question not answered in this thesis was to determine whether the enhancement of IL-10 formation was sufficient to contribute to promastigote-mediated inhibition of IL-12 production. Future experiments could utilize IL-10 specific antibodies, incubating cells prior to infection of the macrophage. As outlined previously consideration of the pathways that regulate IL-10 formation again exemplify how specific the intracellular effects of *Leishmania* can be. For amastigotes p38 MAP kinase is untouched allowing IL-10 to be induced. For promastigotes, activation of p38 MAP kinase and ERK, which also enhances IL-10 production (Yang et al., 2007) assures high levels of the cytokine.

The ability of *L. mexicana* to stimulate IL-10 production in macrophages could have two advantages for the parasite. Firstly, IL-10 production in macrophages could shift the activation and prevent IL-12 production. As IL-12 has been shown to activate Th1 to produce IFN-γ, the potential benefits of such a mechanism are clearly against *Leishmania* infection (Bhattacharjee et al., 2009). Secondly, *Leishmania donovani* has been demonstrated to inhibit macrophage activity as a consequence of IL-10 inhibiting NF-κB activity (Alvarez et al., 2010, Bhattacharyya et al., 2001). Consequently, IL-10 induction by *L. mexicana* allows the parasite to maintain a persistent chronic infection.

Another important area of investigation relates to the regulation of NF-κB signaling in response to promastigotes. Whilst, studies in this thesis suggested that promastigotes activated NF-κB this was only assessed by examining IκB-α loss and phosphorylation of p65 NF-κB. Preliminary studies showed that promastigotes could prevent NF-κB

translocation to the nucleus (not shown) which may represent an additional effect which is not mediated via TLR4 but is dependent on uptake of the parasite. One way to test this would be to stimulate TLR4$^{-/-}$ macrophages with another TLR-ligand such as poly-IC following pre-treatment with promastigotes. This proposed new work is supported by another recent study which has shown that LPG from *L. mexicana* diminished NF-κB translocation in monocytes (Argueta-Donohue et al., 2008). This would also be consistent with other models of pathogen infection which have demonstrated disruption of immune responses through targeting the NF-κB pathway. *Salmonella* and *Yersinia enterocolitica* abolish of ubiquitination and phosphorylation of IκB-α which are both necessary for IκB-α degradation which is required for activation of NF-κB (Neish et al., 2000, Schesser et al., 1998). Furthermore, the intracellular pathogens *Toxoplasma gondii* and *Plasmodium berghei* actively interfere with the NF-κB signalling pathways (Butcher et al., 2001, Xu et al., 2001, Shapira et al., 2002).

A number of recent studies now show the importance of TLR in the activation of immune cells in response to *Leishmania* species (Tuon et al., 2008). However, there are clear differences in the TLR utilized in each case. This may be dependent on cell surface LPG which mediates the activation of macrophages by *L. major* via TLR2 (de Veer et al., 2003). Flandin et al., also demonstrated that TLR2 and TLR3 are involved in macrophage recognition of promastigotes of *L. donovani* in IFN-γ primed mouse macrophages (Flandin et al., 2006). In this thesis it was clearly demonstrated that *L. mexicana* promastigotes interact exclusively with TLR4. Previously published studies provide evidence for the involvement of TLR2, TLR3 and TLR4 in the response of NK cells, macrophages or mice to *Leishmania* parasites (Becker et al., 2003, Flandin et al., 2006, Kropf et al., 2004). In addition, recent studies indicate a role for TLR9 in responses to *L. major*, *L. braziliensis* or *L. infantum* (Liese et al., 2007, Schleicher et al., 2007) indicating that internalization/uptake of the parasite is required for receptor activation. It remains unclear if binding to cell surface TLRs such as TLR2 and 4 promotes receptor internalization or if uptake is mediated by another mechanism. Future experiments could used TLR4 deficient macrophages to measure promastigote uptake using a parasite killing assay.

The results in this thesis suggest that TLR4 plays a role in the host defense against parasitic *Leishmania*, TLR4 was indispensible for the potentiation of both COX-2 and iNOS. Our data confirm other previous studies; TLR4 signalling contributes to the induction of the iNOS pathway (Schilling et al., 2002, Toshchakov et al., 2002) and it is tempting to speculate that the beneficial effect of TLR4 in the control of *L. mexicana* infection is in part associated with TLR4-mediated induction of iNOS protein expression. Future studies could entail infecting $TLR4^{-/-}$ deficient mice with *Leishmania* promastigotes and assessing parasite burden and immune responses. A previous study has been conducted in the same manner using *L. major*, $TLR4^{-/-}$ mice were clearly less efficient to control parasite growth and had larger parasite burdens. This was linked to the production of NO at the site of infection (Kropf et al., 2004). Similarly, other studies link TLRs to the induction of iNOS and the release of NO (Kavoosi et al., 2010, Flandin et al., 2006).

A major finding in this thesis was the demonstration that promastigotes enhanced arginase-1 expression and activity through TLR4. It has recently been established that activation of the innate immune system is linked to alternative macrophage activation via arginase-1. Recent studies indicate the involvement of TLR2 mediated induction of arginase-1 with *Mycobacterium tuberculosis and Toxoplasma gondii* (El Kasmi et al., 2008). Arginase activity mediated via TLRs is linked to the effects of other pathogens, particularly in relation to iNOS, but the findings are controversial. NO has no obligate function in the clearance of *Chlamydia* (Igietseme et al., 1998), which have evolved to parasitize various cell types in diverse anatomical niches including the eye, lungs and genital tract. Notably, MyD88-dependent Arg1 expression is found in total lung homogenates in the early phase of *Chlamydia pneumoniae* infection (Rodriguez et al., 2007). In disagreement with this finding a previous study demonstrated increased parasite proliferation and arginase activity in $TLR4^{-/-}$ deficient macrophages. In addition IL-4 acted synergistically with *L. major* in the induction of arginase activity (Kropf et al., 2004), indicating that TLR-induced Arg1 has a specific function in the control of parasitism. The results in this thesis raise the issue of why an anti-pathogen response mediated by TLR4, increased NO release would include a component that favors intracellular pathogens. We speculate that the TLR-mediated induction of macrophage arginase-1 has positive antimicrobial effector function against other types of pathogens (El Kasmi et al., 2008).

A key question that remains to be addressed can stage specific effects of *L. mexicana* be exploited therapeutically? In recent years understanding of these events have lead to the potential of vaccine generation particularly that of adjuvant. For example, Meyaard et al., used IL-12 as an adjuvant to vaccinate with limited effectiveness due to its rebound induction of IL-10 (Meyaard et al., 1996). Therefore, blocking the immunosuppressive effects of IL-10 may perhaps enhance therapeutic efficiency of IL-12 as an adjuvant. Whether or not amastigotes or promastigotes themselves could also be targets for vaccine generation remains to be determined. Other therapeutic possibilities might include the selective blocking or activation of kinase pathways using inhibitors and activating molecules. However, this is clearly based on a greater understanding of the signalling pathways regulated by amastigotes and promastigotes and will be the focus of future studies.

CHAPTER 6

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