NOTE TO USERS

Role of Toll-Like receptor 4 in *Leishmania*-induced chemokine gene expression and inflammatory response

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ABSTRACT

Modulation of the innate immune response by *Leishmania* has been extensively studied; however, some questions still need to be answered. In the present study, we demonstrated, *in vitro* and *in vivo*, that *Leishmania* interacts with TLR4 in order to induce chemokine mRNA expression. That TLR4-dependent macrophage (Mϕ) activation was shown to be MyD88-independent *in vitro*, and results in the activation of the transcription factors NF-κB and CREB. Their role in chemokine mRNA expression was further demonstrated using specific inhibitors toward these transcription factors. Moreover, using TLR4-deficient mice we confirmed the role of TLR4 in *Leishmania*-induced chemokine gene expression and in the subsequent recruitment of inflammatory cells. Collectively, our results bring new insights for understanding the interaction of *Leishmania* with its host cell, the Mϕ.

RÉSUMÉ

La modulation de la réponse immune innée par *Leishmania* a été intensivement étudiée ; néanmoins, certaines questions sont encore sans réponse. Dans cette étude, nous démontrons que *Leishmania* interagit avec TLR4 afin d'induire la transcription de chimiokines *in vitro* et *in vivo*. Cette activation des Mϕ dépendente de TLR4 est indépendente de MyD88 et entraîne l'activation des facteurs transcriptionel NF-κB et CREB. Leur rôle dans l'expression de l'ARNm de chimiokines a été confirmé en employant des inhibiteurs spécifics de ces facteurs. De plus, par l'utilisation de souris déficientes en TLR4, nous avons confirmé le rôle de ce récepteur dans la transcription de chimiokines induite par *Leishmania*, ainsi que le recrutement de cellules inflammatoires qui en résulte. Collectivement, nos résultats apportent des éléments important pour la compréhension de l'interaction de *Leishmania* avec sa cellule hôte, le Mϕ.

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CONTRIBUTION

This thesis has been presented under the format of manuscript-based thesis. Therefore, the contribution of the author of this thesis is provided.

CHAPTER IV

Godbout, M., D. Malo, D. Radzioch, and M. Olivier. Leishmania-induced chemokine gene expression is TLR4-dependent, but MyD88-independent. (Submitted to *The Journal of Immunology*).

Contribution: experimental 100%, conceptual 85%

Dr Malo and Dr Radzioch have provided the transgenic mice and the macrophage cell line, respectively.

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ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
ATAC	activation-induced, chemokine-related $CD8^+$ T cell-expressed
BCA	B cell activating chemokine
BLC	B lymphocyte chemoattractant
BRAK	Breast and kidney chemokine
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CMV	Cytomegalovirus
CR	Complement receptor
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
DARC	Duffy antigen/receptor chemokine
DC	Dendritic cell
DD	Death domain
EMSA	Electrophoretic mobility shift assay
ENA	Epithelial cell-derived Nϕ-activating factor
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GIPL	Glycoinositolphospholipid
gp	Glycoprotein
GPCR	heterotrimeric G protein-coupled receptor
GRO	Growth-related oncogene
HCC	Hemofiltrate CC chemokine
HIV-1	Human immunodeficiency virus type 1
HSP	Heat shock protein
IFN	Interferon
IL	Interleukin
ILC	IL-11 receptor α-locus chemokine
iNOS	Inducible nitric oxide synthase
IP	IFN-γ-inducible protein
IRAK	IL-1R-associated kinase
IRF	Interferon regulated factor
Jak	Janus kinase
JNK	c-Jun N-terminal kinase
LARC	Liver- and activation-related chemokine
Ld	*Leishmania donovani*
LEC	Liver-expressed chemokine
Lkn	Leukotactin
Lm	*Leishmania major*
LPG	Lipophosphoglycan
LPK	*Leishmania* protein kinase
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
Mal	MyD88-adaptor-like

MCAF	Monocyte chemoattractant and activating factor
MCP	Monocyte chemoattractant protein
MGSA	Melanoma growth-stimulatory activity
MDC	Macrophage-derived chemokine
MHC	Major histocompatibility complexe
Mig	Monokine induced by IFN-γ
MIP	Macrophage inflammatory protein
MRP	MARCKs-Related Protein
MyD88	Myeloid differentiation factor 88
Mϕ	Macrophage
Nϕ	Neutrophil
NF-κB	Nuclear factor κB
NK	Natural killer cell
NO	Nitric oxide
PARC	Pulmonary- and activation-regulated chemokine
PBMC	Peripheral blood mononuclear cell
PF	Platelet factor
PKC	Protein kinase C
PTP	Phosphotyrosine phosphatase
RAGE	Receptor for advanced glycosylation end products
RANTES	Regulated on activation normal T cell expressed and secreted
RPA	RNase protection assay
RSV	Respiratory syncitial virus
SARM	SAM and ARM- containing protein
SDF	Stromal cell-derived factor
SOCS	Suppressor of cytokine signalling
STAT	Signal transducers and activators of transcription
STCP	Stimulated T cell chemoattractant protein
TARC	Thymus- and activation-related chemokine
TCA	T-cell activated protein
TECK	Thymus-expressed chemokine
TGF	Tumor growth factor
Th	T helper lymphocyte
TICAM	TIR-containing adaptor molecule
TIRAP	TIR-domain-containing adaptor protein
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF	TNF-associated factor
TRAM	Trif-related adaptor molecule
TRIF	TIR-domain-containing adaptor inducing interferon-β

CHAPTER I

LEISHMANIA; THE PARASITE AND THE INFECTION

1.1 *Leishmania*

1.1.1 History and Taxonomy

Long before the first description of the cause of black sickness, also called Kala-Azar, by Dr. Leishman, images of people harbouring facial lesions of what could very well be the mucocutaneous form of the disease, were found on 500-year-old Inca's pottery (1). Nevertheless, it is only in 1900 that Leishman observed the presence of ovoid-shaped microorganisms in spleen phagocytes from a deceased of Kala-Azar. Later, Donovan supplemented this observation and developed the first and still used diagnostic procedure, which consist in the identification of "Leishman-Donovan" bodies in splenic macrophages of patient with Kala-Azar symptoms (1). From this, the species responsible for Kala-Azar was given the name of *Leishmania donovani.*

Leishmania are protozoan parasites of the order kinetoplastida, and part of the Trypanosomatidae family. Of the genus *Leishmania*, about 28 species have been identified, and reported so far, and are divided in two subgenera, *L. (Leishmania)* spp. and *L. (Viannia)* spp. (2, 3).

1.1.2 Geographic Distribution and Prevalence

Leishmaniases, the group of diseases caused by *Leishmania*, are endemic in 88 countries of tropical and subtropical regions worldwide, with the exception of Australia and Antarctica (Figure 1) (3-5). A 10^{th} of the world population is at risk, with an overall prevalence of 12 million people infected (4, 6, 7). Each year, two million cases are reported, of which, 1.5 million cases of cutaneous leishmaniasis and 500,000 cases of visceral leishmaniasis (4, 5, 7, 8). Leishmaniases are a growing public health concern for several countries, due to the increasing number of overseas travellers, Gulf War veterans, and also with the emerging AIDS-leishmaniasis co infection (4, 7). Other risk factors affecting the spread of the disease are the economic development, environmental changes, and deforestation (5).

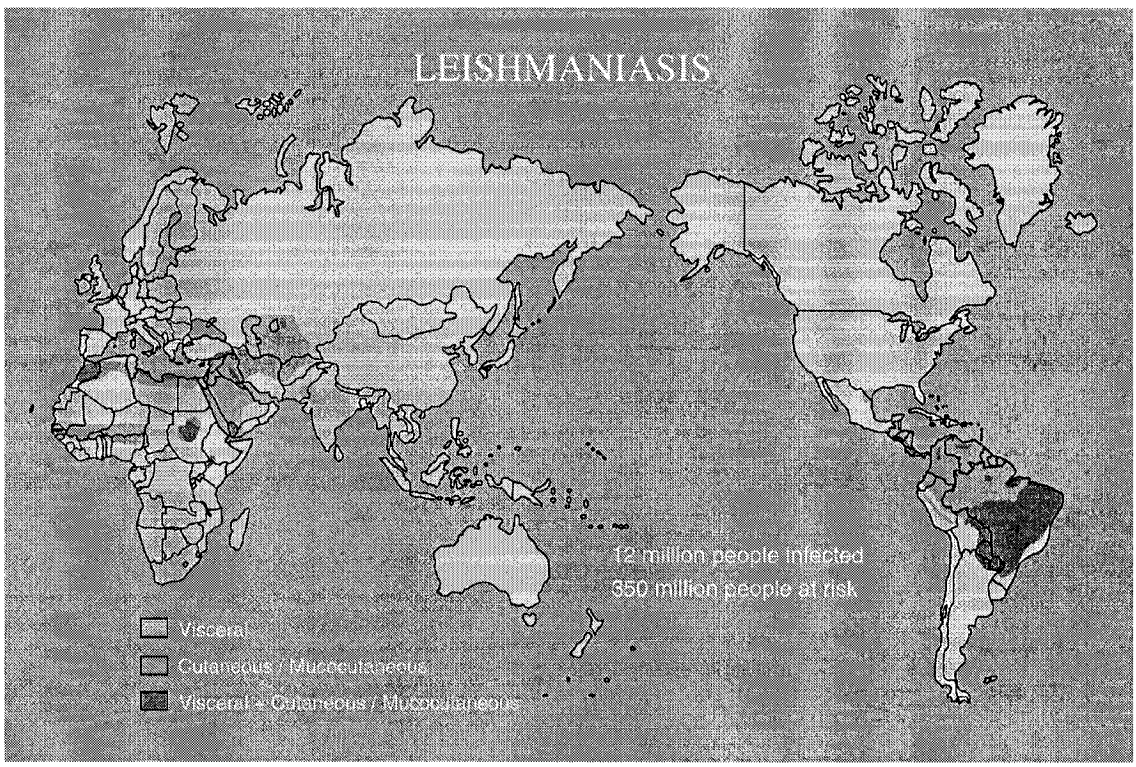

Figure 1. Geographical distribution of Leishmaniasis. *Taken from* (6)

1.1.3 Morphology and Life Cycle

Leishmania parasites are characterized by a dimorphic life cycle, the two developmental stages being the amastigote and the promastigote forms (Figure 2). The promastigote form is defined by its slender shape, 4 by 15 to 20 μm, and the presence of a flagella. This form is the extracellular stage found in the midgut of the vector (7, 9-13). The second stage is the intracellular amastigote, characterized by its round or oval shape, 2 to 4 μm, and the absence of a flagella, which renders them non-motile (7, 9-13).

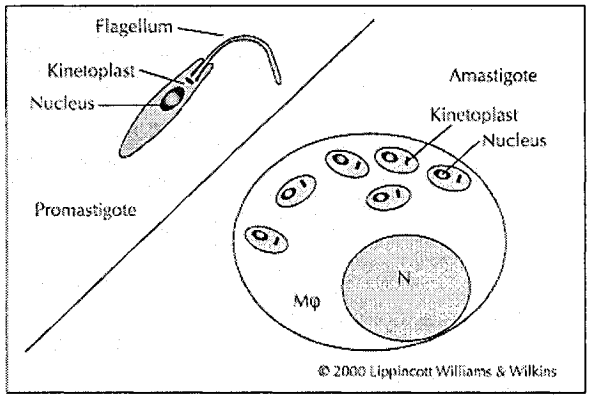

Figure 2. Promastigote and amastigote forms. *Taken from* (10).

The cycle (Figure 3) begins when the vector, a sandfly of the genera *Phlebotomus* or *Lutzomia*, takes a blood meal from an infected mammal and ingests infected phagocytes (7). In the midgut of the sandfly, the amastigotes then transform in promastigotes. These immature promastigotes, called procyclic, multiply by binary fission (11, 14). Then, the lipophosphoglycan (LPG; the major surface molecule of *Leishmania* parasites) terminal β-galactose residues bind to the epithelium lectin-like molecules of the midgut (7, 12).

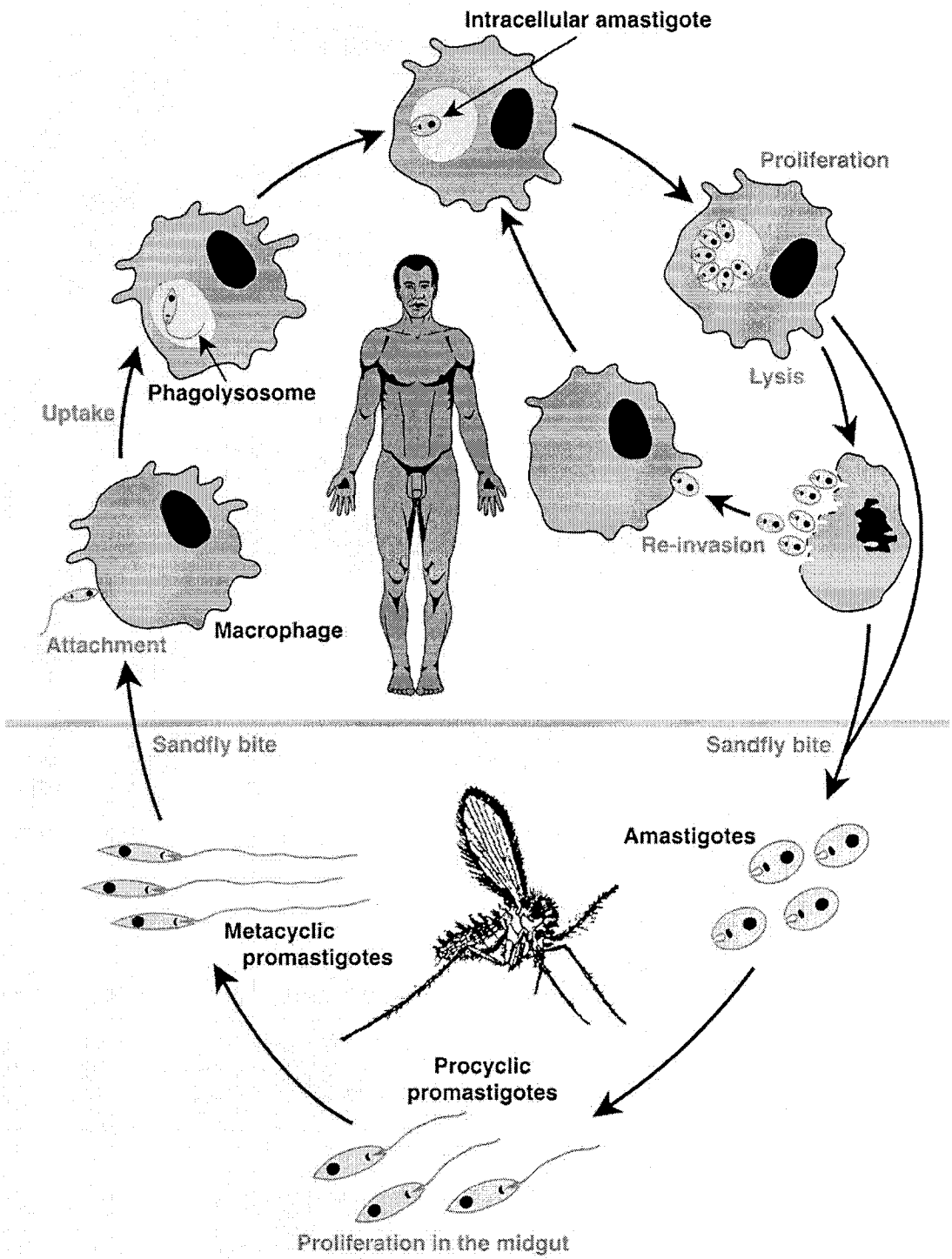

Figure 3. The life cycle of Leishmania. *Taken from* (6)

After attachment, the procyclic promastigotes stop replicating, and go through different maturation stages, to finally differentiate into metacyclic promastigotes (6, 12, 15). By going through this metacyclogenesis, promastigotes acquire virulence capabilities and become more motile (2, 7). The resulting metacyclic promastigotes possess longer LPGs, and the terminal β-galactose have been capped with α-arabinose. Thereafter, the metacyclic promastigotes detach from the epithelium and migrate to the pharynx and buccal cavity (12, 16). During the next blood meal, the infected sandfly vector will regurgitate metacyclic promastigotes in the small blood pool formed at the bite site in the host (10).

In the mammalian host (rodent, dog or human), the parasites attach to receptors on mononuclear phagocytes, and are phagocytosed (10, 13). Many macrophage (Mϕ) surface receptors are thought to be involved in this host-parasite interaction. Among them are the complement receptors CR1 and CR3, mannose-fucose receptor, fibronectin receptor, and RAGE (10, 13, 17). Phagocytosis can occur by classical "zipper" phagocytosis, as well as by "coiling" phagocytosis. "Zipper" phagocytosis is seen where the parasite interact with one, or more, receptor which induces the recruitment of more receptors, formation of a pseudopode and then engulfment of the parasite (13). "Coiling" phagocytosis which is characterized by the asymmetrical occurrence of pseudopodia coils and multi layered pseudopode stacks (13). Once engulfed in phagolysosome, the promastigotes transform in non-motile amastigotes, which then replicate. Proliferation of these intracellular amastigotes lead to the lysis of the phagocytic cell, resulting in the liberation of the amastigotes which then infect neighbouring cells, and propagate the infection (2, 7, 10).

1.2 Leishmaniases

Of the 28 species of *Leishmania*, only six species have been formally identified as human pathogens (Table 1) (6). Although their morphology is very similar, they can cause strikingly different pathological responses. The disease forms can range from a self-resorbing cutaneous ulcer, to a mucocutaneous disease with severe disfigurement, or a life-threatening visceral infection.

TABLE 1. *Leishmania* species pathogenic for humans, their vectors, host range and disease manifestations (6)

Species	**Host range**	**Main vector**	**Disease manifestations**
L. donovani	Dogs, savannah rodents, humans	*P. argentipes, L. longipalpis*	Visceral leishmaniasis (kala azar), PKDL
L. major	Desert and savannah rodents; *Rhombomys, Psammomys, Arvicanthis*	*P. papatasi*	Cutaneous leishmaniasis, (rural, wet Oriental sore)
L. tropica	Humans	*P. sergenti*	Cutaneous leishmaniasis (urban, dry Oriental sore), visceral leishmaniasis
L. aethiopica	Rock hyrax	*P. longipes*	Cutaneous leishmaniasis, diffuse cutaneous leishmaniasis
L. braziliensis	Sloth, dog	*L. umbratilis* and many others	Cutaneous leishmaniasis, mucocutaneous leishmaniasis
L. mexicana	Forest rodents	*L. flaviscutellata, L. olmeca*	Cutaneous leishmaniasis, diffuse cutaneous leishmaniasis

1.2.1 Cutaneous Leishmaniasis

The infection by *Leishmania* species causing cutaneous leishmaniasis can remain undetected (asymptomatic) or become clinically apparent after an incubation period of a few days to several months in some cases (7, 18). Skin lesions are usually localized at the site of the sandfly bite (Figure 4), although some rare diffuse forms may result in the dissemination of the lesions (4, 7, 18). These lesions can evolve from small, red papule (indicating the infiltration of plasma cells, lymphocytes and macrophages), to nodule and ulcerative lesions. Ultimately spontaneous healing with atrophic scars occurs within 3 to 6 months (4, 7, 18). Even though lesions disappear, some parasites still remain in the host. These remaining parasites are thought to provide a strong immunity and resistance to a reinfection, though in some cases these can result in the reactivation of the disease (7).

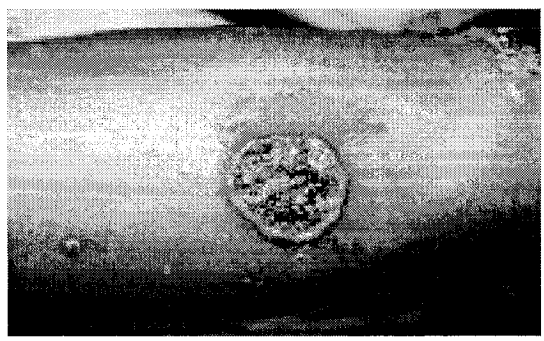

Figure 4. Cutaneous leishmaniasis. *Taken from* (19)

1.2.2 Mucocutaneous Leishmaniasis

The mucocutaneous form is caused by *Leishmania braziliensis*. Months to years after the initial skin lesion has healed, metastatic lesions develop in the mucosal system of the nasal and buccal cavity (4, 6, 18). Following dissemination of amastigotes in the naso-oropharyngeal mucosa, progressive destruction of the lips, nose, hard and soft palates and vocal cords can be observed, which result in major disfiguration (Figure 5) (7, 18, 20).

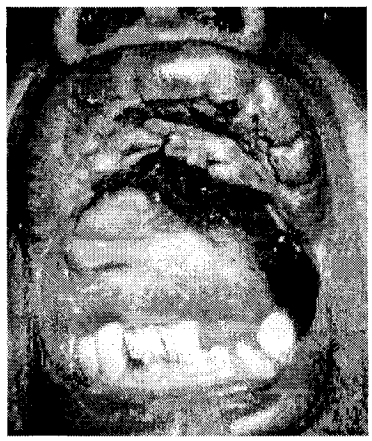

Figure 5. Mucocutaneous leishmaniasis. *Taken from* (19)

1.2.3 Visceral Leishmaniasis

Also known as Kala-Azar (Hindi for black sickness) or Dum-Dum fever, this form of leishmaniasis is caused by *Leishmania* species targeting the visceral organs. Although the majority of individuals remain subclinically infected, a small percentage develop the active form of the disease after an incubation period of 2 to 4 months (7, 21). The symptoms associated with an active visceral leishmaniasis are fever, general malaise, wasting, anemia, massive hepatosplenomegaly (splenomegaly generally predominant), thrombopenia, hypergammaglobulinemia, and ultimately, if untreated and sometimes despite treatment, death (4, 6, 7, 18, 21-23). Activation of sub clinical infection can also occur upon immunosuppression, and this phenomenon is increasing with the spread of AIDS. An interesting observation is that *Leishmania* species usually causing cutaneous leishmaniasis can, in co infection with AIDS, cause visceral leishmaniasis, These co infections are often resistant to treatment and accelerate AIDS (4, 6).

1.3 Host-parasite interaction

Normally, the encounter of a pathogen by the immune system results in the death of this invader. The killing of an outsider organism can occur by different mechanisms; the complement cascade can directly lyse the pathogen, or the fixation of the complement can opsonized the pathogen. Opsonization will result in the phagocytosis of the microorganism by phagocytic cells, like Mϕ. When an invader is phagocytosed, it is degraded by exposure to several harsh elements, such as oxygen and nitrogen radicals, acidic pH, lysosomal enzymes, nutrient deprivation, and many antimicrobial proteins (24). In order to gain entry into the phagocytic cells and survive the harsh environment of the phagolysosome, *Leishmania* parasites have evolved numerous strategies (Table 2). These strategies can involve the modulation of extracellular pathways, as well as of the intracellular signalling of their host cells. Of all the signalling alteration associated with an infection by *Leishmania*, several have been reported to be a direct or indirect cause of two parasite surface molecules, lipophosphoglycan (LPG) and glycoprotein 63 (gp63).

1.3.1 LPG modulated pathways

LPG is a major constituent of the surface of all *Leishmania* parasites. Several studies have linked this molecule to the impairment of a wide variety of Mϕ functions, inhibition of PKC being the best known (25). In other studies LPG has been associated with resistance against complement-mediated lysis, interference with phagosome-endosome fusion, and phagolysosome maturation, modulation of immunomodulatory effector molecules, such as iNOS and cytokines, and scavenging of hydroxyl radicals and superoxide anions (2, 17, 26-29). By doing so, *Leishmania* assure its survival during its short passage in the blood, increases its chances of being phagocytosed, and most importantly, ensures its survival inside the phagolysosome by providing protection against oxidative burst/damage and digestion within the phagolysosome (2, 17, 26-29).

TABLE 2. Evasion strategies of *Leishmania* parasites (30)

Strategy	Mechanism	Example	Reference
Alteration of the host complement system	Inactivation of complement components by phosphorylation Shedding of C5b-C9 Protease-catalysed conversion of C3b to C3bi on parasite surface →parasite uptake by Mϕ via CR3	Leishmanial protein kinase (LPK-1, c-lpk2) *L. major* promastigotes Gp63 metalloproteinase	(31, 32) (33) (34)
Protection against anti-leishmanial products	Invasion of cells lacking leishmanicidal mechanisms Inhibition of phagolysosomal fusion Inhibition of degrading phagolysosomal enzymes Scavenging of reactive oxygen intermediates Transformation into amastigotes	Immature or stromal Mϕ, Langerhans cells *L. donovani* LPG gp63 LPG Enhanced resistance	(35, 36) (26) (37) (27) (30)
Suppression of the synthesis of anti-leishmanial products	Inhibition of oxidative burst (abnormal PKC activation) Inhibition of iNOS expression or activity	All *Leishmania* spp.; LPG; gp63 *L. major, L. amazonensis*; LPG; GIPLs	(25, 38-40) (41, 42)
Cytokine modulation	Induction of cytokines inhibiting/deactivating Mϕ Suppression or lack of induction of activating cytokines	Upregulation of TGF-β, IL-10 Impaired IL-1/TNF-α production; Lack/downmodulation of IL-12 expression by promastigotes, amastigotes, and LPG	(10, 21, 30) (30, 43) (28, 44-47)
Inhibition of antigen-presentation and T cell stimulation	Suppression of MHC class II expression Internalization and degradation of MHC class II Inhibition/sequestration of antigen processing/peptide loading of MHC molecules	*L. donovani* *L. amazonensis* *L. donovani, L. major,L. amazonensis,L. mexicana*	(24, 30) (48) (30, 49, 50)
Alteration of T cell differentiation/function	Induction of a disease exacerbating T cell response (Th2)	Induction of an early IL-4 peak	(10, 21, 51, 52)

1.3.2 gp63 modulated pathways

Gp 63, the most abundant protein expressed on the surface of promastigotes, has also been associated with resistance to complement-mediated lysis (reported to facilitate and control complement inactivation in the serum), and protection against intraphagolysosomal killing (2, 10, 53, 54). Although gp63 does not appear to be essential for establishment of infection, its presence enhances phagocytosis and survival inside phagolysosome. Moreover, it has been demonstrated that in absence of gp63, the lesions observed during the infection are smaller than in the presence of this glycoprotein (54).

1.3.3 Other modulated pathways

Beside these alterations caused by the surface molecules, other pathways are also known to be modulated in the presence of *Leishmania*, but the precise molecule/ligand still remains to be identified. Among these alterations, it has been shown that *Leishmania* can induce the production of inhibitory molecules such as TGF-β and PGE2, therefore preventing the activation and proliferation of Mϕ, respectively (55-59). Others have demonstrated that the parasite is responsible for the inhibition of MRP, a PKC substrate, and inhibition of c-fos, a PKC-inducible protein (60, 61). It has been reported that *Leishmania* is able to down-regulate γ-IFN-activated Jak-Stat1 signalling pathway, through the activation of PTPs such as SHP-1 (10, 28, 62-64). Other molecules also found to be affected during infection are MAPKs. Different studies have found that ERK1/2 phosphorylation in response to LPS or PMA was abrogated (65, 66). Many hypothesis were explored in hope of finding the mechanism underlying this inhibition. One suggestion was that this could be the result of ceramide induction by the parasite (67). Other studies have reported the ability of *Leishmania* to modulate the expression of chemokines at different periods post-infection. The up- or down-regulation of Mϕ chemokine mRNA has, in some cases, been linked to susceptibility or resistance to infection by *Leishmania* (68, 69).

CHAPTER II

CHEMOKINES

2.1 Generalities on chemokines

2.1.1 Chemokine Structure

Chemokines were discovered 16 years ago with the identification of the first leukocyte subtype selective chemoattractant molecule, IL-8 (70, 71). However, it is only four years later, at the Third International Symposium on Chemotactic Cytokines in Baden, that they were officially given the name "chemokine", derived from chemotactic cytokine (70). With close to 50 distinct chemokines, they represent the largest family of cytokines in charge of the regulation of all white blood cell trafficking, from hematopoietic stem cells to mature Nϕ and lymphocytes (72, 73).

Chemokines are small basic proteins of 70 to 125 amino acids, with a molecular weight range of 6 to 17 kDa (74, 75). Chemokines are usually act as monomers, and most are secreted, although some are expressed on the cell surface (76). These heparin-binding proteins have a β-sheet structure with a short loop in a Greek key, and also have an exposed loop in the backbone between the second and third cysteine where the regions that interact with the receptor can be found (76).

2.1.2 Chemokine Classification

Based on the positioning of the N-terminal cysteine residues, chemokine can be classified into four families, C, CC, CXC, and CX_3C. CC, CXC, and CX_3C have four conserved cysteine, although some CC chemokines have six, whereas C chemokines have only two (70). CXC chemokines can be further separated into two groups, ELR+ and ELR-, based on the presence or absence of a tripeptide motif glutamic acid-leucine-arginine (ELR) N-terminal of the first cysteine (77, 78). The presence of the ELR motif is associated with the specificity for chemotaxis and activation of neutrophils (Nϕ), while the absence of this motif is associated with the specificity for lymphocytes and cells outside the hematopoietic compartment (77, 79). CC chemokines attract monocytes, lymphocytes, basophils, and eosinophils, but do not affect Nϕ (75, 80). Together, CXC and CC chemokines represent more than 85% of all known chemokines (77). Chemokines can also be subdivided in two categories depending on their expression; some chemokines are constitutively expressed, while others are inducible. Constitutive chemokines are said to be responsible for the basal leukocyte trafficking and to also be involved in the architecture of secondary lymphoid organs. Inducible chemokines are responsible for the recruitment of leukocytes in response to different stimuli (72). Table 3 summarizes the members of all four families of chemokines, including their systematic names (nomenclature developed by Zlotnik and Yoshie (81)), their common human and mouse names, the receptors they bound to and their function (inflammation or homeostasis).

2.2 Chemokine receptors

Chemokines can interact with the negatively glycosaminoglycans (GAGs). The binding on the different GAGs is chemokine specific and its affinity is variable (76). The creation of the chemokine gradient needed for the proper chemotaxis is thought to occur by oligomerization of chemokines on GAGs (82, 83).

However, the biological signals are produced when chemokines bind to their receptors (82). These receptors are a group of about 20 heterotrimeric G_i protein-coupled receptor (GPCR) that possess seven hydrophobic transmembrane domains, three intracellular and three extracellular hydrophilic loops (71, 74). Chemokine GPCRs are normally constituted of 340 to 370 amino acids, an acidic NH_2 terminus, 10 conserved amino acids in the second intracellular domain and one conserved cysteine in each extracellular loop (76). An intracellular C-terminus containing serine and threonine residues that act as phosphorylation sites for receptor regulation is also present (Figure 6) (70, 84). Chemokine recptors were first found to be expressed on the surface of leukocytes. Nevertheless, it is now known that they can also be found on endodermal, mesenchymal, ectodermal and neuroectodermal cells (74).

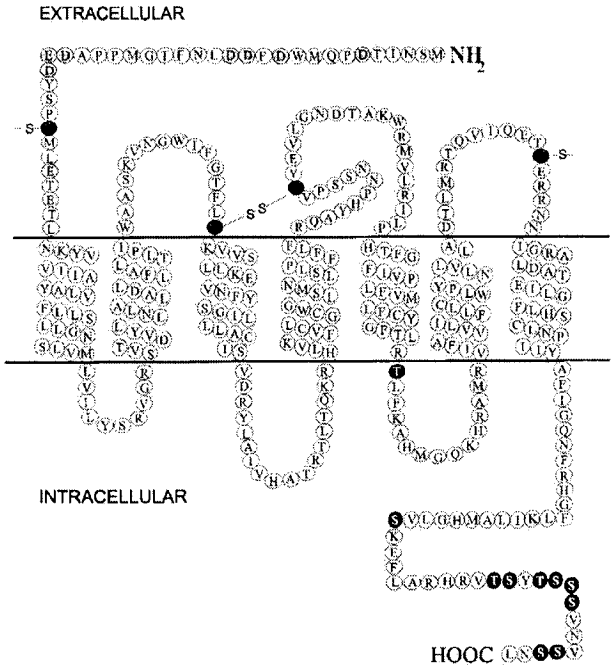

Figure 6. Schematic Representation of Chemokine Receptors. Extracellular N-terminal acidic residues are shaded. C-terminal potential phosphorylation residues (serine and threonine) are black, and conserved cysteines are hatched. *Taken from (84).*

Table 3. The chemokine families

Systematic Name	Human Common Names	Mouse Common Names	Receptors Bound	Function
CC chemokine				
CCL1	I-309	TCA-3	CCR8	Inflamm
CCL2	MCP-1, MCAF	JE/MCP-1	CCR2	Inflamm
CCL3	MIP-1α, LD78α	MIP-1α	CCR1,5	Inflamm
CCL4	MIP-1β	MIP-1β	CCR5,8	Inflamm
CCL5	RANTES	RANTES	CCR1,3,5	Inflamm
CCL6	?	C10, MRP-1	?	?
CCL7	MCP-3	MCP-3/FIC, MARC	CCR1,2,3	Inflamm
CCL8	MCP-2	MCP-2	CCR3	Inflamm
CCL9	?	MRP-2, MIP-1γ	?	?
CCL10	?	?	?	?
CCL11	Eotaxin	Eotaxin	CCR3	Inflamm
CCL12	?	MCP-5	CCR2	Inflamm
CCL13	MCP-4	?	CCR2,3	Inflamm
CCL14	CC-1, HCC-1, CKβ1, MCIF	?	CCR1	?
CCL15	HCC-1, Lkn-1, MIP-5,	?	CCR1,3	?
CCL16	HCC-4, LEC, Mtn-1	LCC-1	CCR1	?
CCL17	TARC	TARC	CCR4	Inflamm, Homeo
CCL18	DC-CK-1, PARC, MIP-4	?	?	Homeo
CCL19	MIP-3β, ELC, exodus-3, ckβ11	MIP-3β, ELC	CCR7	Homeo
CCL20	MIP-3α, LARC, exodus-1	ST38, MIP-3α, LARC	CCR6	Inflamm, Homeo
CCL21	6Ckine, SLC, ckβ9, TCA-4, exodus-2	SLC, TCA-4	CCR7	Homeo
CCL22	MDC, STCP1	Abcd-1, dc/β-ck	CCR4	Inflamm, Homeo
CCL23	MPIF-1, ckβ8-1, MIP-3	?	CCR1	?
CCL24	MPIF-2, eotaxin-2, ckβ6	?	CCR3	Inflamm
CCL25	TECK, ckβ5	TECK	CCR9	Homeo
CCL26	Eotaxin-3, MIP-4α	?	CCR3	Inflamm
CCL27	Eskine, CTACK, ILC	ALP, skinkine	CCR10	Homeo
CCL28	MEC	?	CCR10	Inflamm, Homeo
C chemokine				
XCL1	Lymphotactin α, SCM-1α, ATAC	Lymphotactin	XCR1	?
XCL2	Lymphotactin β, SCM-1β, ATAC	?	XCR1	?
CXC chemokine				
CXCL1*	GROα, MGSA-α	MIP-2, KC	CXCR1,2	Inflamm
CXCL2*	GROβ, MIP-2α, MGSA-β	KC	CXCR2	Inflamm
CXCL3*	GROγ, MIP-2β, MGSA-γ	KC	CXCR2	Inflamm
CXCL4	PF4	PF-4	?	Inflamm
CXCL5*	ENA-78	LIX ?	CXCR2	Inflamm
CXCL6*	GCP-2	GCP, CKα-3	CXCR1,2	Inflamm
CXCL7*	NAP-2	?	CXCR2	Inflamm
CXCL8*	IL-8	?	CXCR1,2	Inflamm
CXCL9	Mig	MIG	CXCR3	Inflamm
CXCL10	IP-10	IP-10, CRG-2	CXCR3	Inflamm
CXCL11	I-TAC, IP9, H174	?	CXCR3	Inflamm
CXCL12	SDF-1α/β, PBSF	SDF-1	CXCR4	Homeo
CXCL13	BLC, BCA-1	BLC, BCA-1	CXCR5	Homeo
CXCL14	BRAK, bolekine	BRAK	?	Homeo
CXCL15	?	Lungkine	?	?
CXCL16	CXCL16	CXCL16	CXCR6	Inflamm
CX_3C chemokine				
CX3CL1	Fractalkine	Fractalkine, Neurotactin	CX3CL1	Inflamm

This table is an adaptation of those presented in (70, 74, 76, 85)
The terms Inflamm and Homeo refer to inflammation and homeostasis, respectively.
*, ELR+
?, unknown.

2.3 Biological Effects of Chemokines

With the discovery of the existence of about 50 chemokines and 20 chemokine receptors; and that virtually all cell types and tissues tested can be induced to produce chemokines, it was expected that chemokines would have many roles. These data in mind, along with the fact that one cell can produce a variety of chemokines and chemokine receptors, more research was done and it was rapidly found that the biological effects of the ligation of the chemokine to its receptor can go further then the regulation of leukocyte migration (74). Apart from being the most important regulators of leukocyte trafficking and activation, chemokines have also been shown to be involved in tissue repair processes, angiogenesis, hematopoiesis, antigen sampling in secondary lymphoid tissue, and immune surveillance (71, 78, 85-88). Other studies have reported that chemokines also play a role in organogenesis and in neuronal communication with microglia (89, 90). An appropriate activation of chemokines is necessary to mount an immune response and initiate wound healing; however, inappropriate activation of the chemokine network can cause tissue destruction in diverse diseases such as rheumatoid arthritis, myocardial infarction, and adult respiratory distress syndrome. (72, 73). Inappropriate production of chemokines has also been reported to play a role in asthma, neurological diseases, carcinogenesis, pathogenesis of HIV infection, and autoimmune infections (78, 91).

2.3.1 Chemokines in Microbial Infections

Chemokines are known to be involved in antimicrobial mechanisms. The production of chemokines can be directly increased by the encounter of a pathogen. Nevertheless, depending on the chemokines induced in response to this specific pathogen (bacteria, nematode, virus, or parasite), the effect of certain chemokines can be detrimental for the host.

2.3.1.1 Chemokines and bacteria

The interaction of a bacteria with the immune system can trigger the expression of chemokines. For instance, during an infection with *Streptococcus pneumoniae* the predominance of Nϕ can be observed and is thought to be responsible for the acute inflammation associated with this infection (75). Other studies have demonstrated that the induction of MCP-1 and MIPs following infection with *Mycobacterium tuberculosis* results in acute and chronic inflammation, and that high secretion levels of IL-8 in presence of *Helicobacter pylori* is associated with a high degree of gastritis (75, 92, 93). Moreover, the increase of IL-8 during an infection with either *Escherichia coli, Pseudomonas aeruginosa* or *Staphylococcus aureus*, is also associated with an increased infiltration of Nϕ and causes a severe acute illness (75).

2.3.1.2 Chemokines and viruses

Viruses have also been reported to be able to modulate the expression of chemokines. Indeed, during an infection with the CMV virus, activated NK cells have been reported to express increased levels of MIP-1α, MIP-1β and RANTES (94). In human respiratory tract, respiratory syncytial virus (RSV) induces the expression of several chemokines such as IL-8, RANTES, MIP-1α and MCP-1, all of which were reported to contribute to symptoms severity (95-99). A great deal of research has focused on the interaction between HIV-1 and the chemokine system (75, 100). Numerous studies have demonstrated the essential use of CCR5 and CXCR4, in addition to CD4, by HIV-1 as primary co-receptors in order to gain entry in their host cells (101-103). By contrast, infection by HIV-1 down-regulates the expression of CXCR5 on the surface of activated naïve B cells, which may account for the induction of immunological deficiencies by the virus (104). In the brain of HIV-1-infected individuals, the Tat protein of the virus was shown to cause a drastic increase in the expression of several chemokines; MCP-1, IL-8, IP-10, MIP-1α, MIP-1β and RANTES, all of which are thought to contribute to the neuropathogenesis of HIV-1 infection (105, 106). Interestingly, it was recently found that some chemokines could also inhibit the replication of HIV-1 (107). Indeed, a recent study demonstrated that increased expression of RANTES, MIP-1α, MIP-1β and SDF-1 could decrease the replication of HIV-1 in peripheral-blood mononuclear cells (PBMC) (107).

2.3.1.3 Chemokines and parasites

Numerous parasites (*Toxoplasma*, *Plasmodium*, *Trypanosoma*, and *Leishmania*) can also interact with the immune system and affect the expression of chemokines. Several studies have demonstrated that during an infection by *Toxoplasma gondii* tachyzoites, the expression of MCP-1, RANTES, and IP-10 by astrocytes was found to be increased in human brain (108-110). These chemokines were found to participate in the control of the infection by recruiting monocytes and T lymphocytes (111, 112). Others have reported that *T. gondii* induced Nϕ to express MIP-1α, MIP-1β, RANTES, and CCL20, four strong chemoattractants of dendritic cells (DC), which are necessary to initiate type 1 immunity against the parasites (113, 114). It should be noted that tachyzoites can interact with the immune system in an unconventional way; molecular mimicry was recently shown between *Toxoplasma* cyclophilin and the host CCR5-binding ligands (115).

Chemokines have also been implicated in the infection by *Plasmodium*. This parasite uses DARC (Duffy antigen/receptor for chemokine), a chemokine receptor for IL-8 and RANTES, to gain entry in erythrocytes (116-118). Infected erythrocytes then adhere to vascular endothelial cells by binding to the membrane-bound chemokine, fractalkine, on the surface on those cells. This mechanism demonstrates a second mode of exploitation of the chemokine-receptor system (119). During infection, *Plasmodium* was also found to modulate the expression of chemokines, and different groups have shown that hemozoin, a parasite metabolite, is responsible for the induction of MIP-1α, MIP-1β, MIP-2, and MCP-1 (120, 121). Induction of these chemokines demonstrated the proinflammatory role of hemozoin and that it may contribute to the immunopathology of the disease (120).

An other protozoan parasite capable of modulating the expression of chemokine is *Trypanosoma*. It was indeed demonstrated that *Trypanosoma cruzi* can increase the expression of RANTES and MIP-1α in the heart of infected patients, giving rise to cardiac dysfunction (122). *In vitro* experiments using murine embryonic cardiomyocytes have also demonstrated elevated expression of chemokines, along with other chemokines

(GRO, MIG, MIP-2, IP-10, RANTES, and MCP-1) (123). Other studies have shown that an increase in the expression of MIP-1α, RANTES and MCP-1 could be measured in the peritoneal exudate of *T. cruzi*-infected mice (124). By contrast to what was found in the heart, expression of these chemokines may promote parasite uptake and control of the replication (124). Experiments using *Trypanosoma brucei brucei* have shown that this specie can increase the expression of MIP-2, RANTES, and MIP-1α in astrocytes, microglia, Mϕ and T lymphocytes, and are thought to contribute to the CNS immunopathogenesis (125). These chemokines, along with MCP-1 were also found to be induced in splenocytes in *T. b. brucei*-infected mice (126).

Chemokines also play an important role in *Leishmania* infection. Indeed, in presence of *Leishmania major*, phagocytic cells were shown to produce RANTES, MIP-1α, IP-10, IL-8/mMIP-2 and MCP-1 (68, 127, 128). By their chemotactic action on monocytes, T cells, NK cells, Nϕ and DC, these chemokines participate in the efficient control of the infection (68, 129). The pattern of chemokine expression has also been associated with different forms of cutaneous leishmaniasis. High expression of MCP-1 and low expression of MIP-1α has been associated with self-healing cutaneous leishmaniasis (LCL). On the other hand, predominant expression of MIP-1α and low MCP-1 have been reported in progressive diffuse cutaneous leishmaniasis (DCL) (130, 131). These findings, along with the discovery that MCP-1 could synergize with IFN-γ to activate monocytes, suggest that MCP-1 may contribute to the killing of the parasite, whereas its absence leads to progression of the infection (132). Furthermore, different studies have demonstrated that *L. donovani* could induce the same chemokines as *L. major* (*e.g.* MIP-1α, MIP-1β, MCP-1, MIP-2, IP-10, RANTES); however, in the comparative study of the two species done by our laboratory, *L. major* was shown to be a more potent inducer of those chemokines (69, 133, 134). Overall, these observations suggest that the difference in induction intensity, and therefore in the inflammation response, could influence the development of different pathologies (69).

CHAPTER III

TOLL-LIKE RECEPTORS

3.1 TLR structure and signalling

Exposition to microorganisms present in the environment is a fact that every living organism has to deal with and need to somehow survive invasion by these microorganisms (135). In order to mount the proper immune reaction, the organism has to be able to recognize the invading microorganisms. Since their discovery, Toll-like receptors (TLRs) have been demonstrated to be important in the detection of such invading pathogens (136).

The involvement of Toll receptors in innate immunity was first described in *Drosophila* (136). However, Toll receptors were first described as an important pathway for the establishment of the embryonic dorsoventral polarity in *Drosophila* (137, 138). It is only after the discovery of the similarity between the *Drosophila* Toll and the mammalian IL-1 receptor that an involvement in immunity was proposed (136). Both receptors possess a cytoplasmic domain now known as the Toll receptor-IL-1R (TIR) domain, which is characterized by the presence of three highly homologous regions known as boxes 1, 2 and 3 (135, 139). Furthermore, observations demonstrated that TLRs could interact with the same downstream signalling molecules used by IL-1R (140). The first mammalian homologue was found and named Toll-like receptor (TLR) 4, a year after the discovery of Toll in *Drosophila*, (136). As of now, 10 members of the TLR family have formally been identified (Table 4), and very recently, some experiments have suggested the existence of TLR11 in humans (141).

In general, upon activation of TLR by its ligand, the adapter molecule Myd88 associates to the TIR domain of the TLR. Myd88 first recruits IRAK4, then IRAK1. After phosphorylation of IRAK1 by IRAK4, TRAF6 is recruited to the receptor complex and binds to the phosphorylated IRAK1 (135, 142). After dissociation of the IRAK1-TRAF6 complex, and association to other signalling molecules, the IKK complex is ultimately activated and will later lead to the phosphorylation of I-κB, and therefore to the activation and translocation of NF-κB to the nucleus (Figure 7). For the majority of TLRs, activation of the signalling cascade will subsequently lead to the activation of different transcription factors such as NF-κB and AP-1, which will ultimately lead to the production of cytokines and chemokines (143). Although this pathway is used by most TLRs, this is not the only one and the signalling mechanism is much more complex then researchers first thought. Indeed, it has rapidly become evident that given the limited number of receptors and the large number and variety of ligands (Table 4), there would also have to be a certain variability in the signalling pathway used by each receptor in order to produce the proper response to a given pathogen/ligand. This variability can, in part, be associated with the different adaptor molecules that can be recruited by the TLRs.

TABLE 4. Toll-like Receptors *adapted from Akira 2004 (135)*

Receptor	Localization	Ligand	Ligand origin	References
TLR1	Cell membrane	Tri-acyl lipopeptide	Mycobacteria, bacteria	(144)
		Soluble factors	*Neisseria meningitidis*	(145)
TLR2	Cell membrane	Lipoprotein/lipopeptide	Many pathogen	(146, 147)
		Peptidoglycan	Gram-positive bacteria	(148)
		Lipoteichoic acid	Gram-positive bacteria	(148)
		Lipoarabinomannan	Mycobacteria	(149)
		Phenol-soluble modulin	*Staphylococcus epidermidis*	(150)
		Glycoinositolphospholipids	*Trypanosoma cruzi*	(151)
		Glycolipids	*Treponema maltophilum*	(152)
		Porins	*Neisseria*	(153)
		Atypical LPS	*Leptospira interrogans, Porphiromonas gingivalis*	(154, 155)
		HSP70	Host	(156)
		Zymosan	Fungi	(157)
TLR3	Endosome ?	Double-stranded RNA	Viruses	(158)
TLR4	Cell membrane	LPS	Gram-negative bacteria	(159)
		Taxol	Plant	(160)
		Fusion protein	RSV	(161)
		Envelope protein	MMTV (mouse mammary-tumor virus)	(162)
		HSP60	*Chlamydia pneumoniae*, host	(136, 163, 164)
		HSP70	Host	(165)
		Fibronectin (Type III repeat extra domain A)	Host	(166)
		Hyaluronic acid (Oligosaccharides)	Host	(167)
		Heparan sulfate (Plysaccharide fragments)	Host	(168)
		Fibrinogen	Host	(169)
TLR5	Cell membrane	Flagellin	Bacteria	(170)
TLR6	Cell membrane	Di-acyl lipopeptides	Mycoplasma	(171)
		Lipoteichoic acid	Gram-negative bacteria	(148)
		Zymosan	Fungi	(172)
TLR7	Endosome	Imidazoquinoline	Synthetic compounds	(173)
		Loxoribine	Synthetic compounds	(174)
		Bropirimine	Synthetic compounds	(174)
		Single-stranded RNA	Viruses	(175, 176)
TLR8	Endosome	Imidazoquinoline	Synthetic compound	(177)
		Single-stranded RNA	Viruses	(175)
TLR9	Endosome ?	CpG DNA	Bacteria	(178)
TLR10	Cell membrane	?	?	
TLR11	Cell membrane ?	?	Uropathogenic bacteria	(141)

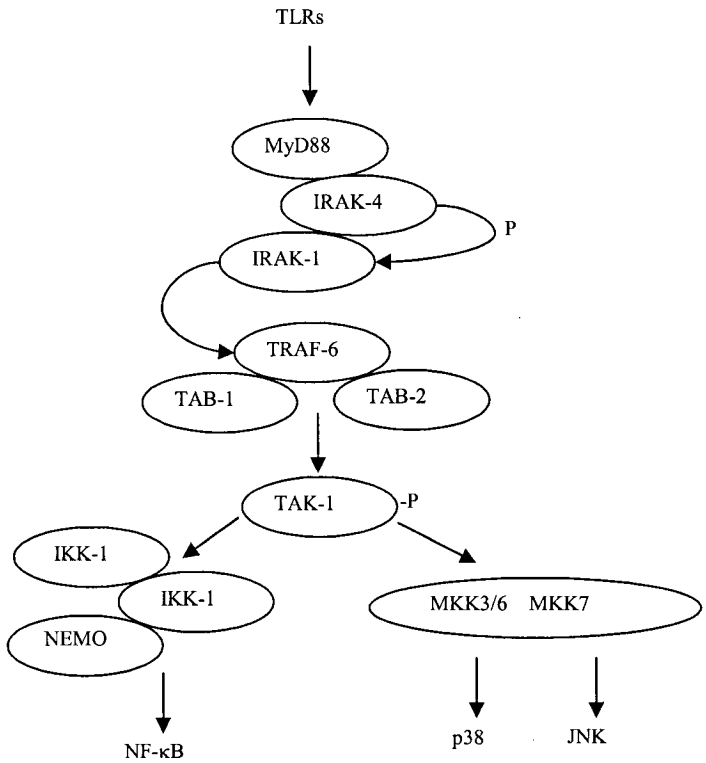

Figure 7. TLR signalling. *Adapted from (179).*

3.1.1 The adaptor family

Following the discovery of the Myd88-independent pathway, the study of the signalling pathways of each TLRs has revealed the existence of other adaptor molecules. Up to date, there is five different adaptors that have been identified to be used by TLRs (180).

MyD88, like all five adaptors molecules has a TIR domain, but is the only adaptor protein to possess a N-terminal death domain (DD) (181). Upon its activation, MyD88 functions as a link between the TLR/IL-1R and downstream signalling molecules that possess a DD like IRAK (135). MyD88 can be used by all TLRs, with the exception of TLR3, and appears to be essential for the recognition of the different ligands of TLR1, TLR2 and TLR5-9 (135, 136, 179).

TIRAP/Mal, was first thought to be the adaptor mediating the TLR4 MyD88-independent signalling pathway (182, 183). However, Mal knockout mice experiments have demonstrated that this adaptor was not involved in MyD88-independent signalling but was actually an adaptor of the Myd88-dependent pathways used by both TLR2 and TLR4 (184, 185). Indeed, it was shown that the signalling activated by TLR2 ligands (using TLR1 and TLR6 as co-receptors) is entirely abrogated by the deletion of Mal. Furthermore, TLR2 signalling is also entirely abolished in MyD88-deficient mice, which suggests that this later adaptor and Mal act together and are both essential (179, 186). Other studies have demonstrated that TLR3, TLR5, TLR7 and TLR9 do not signal through Mal, suggesting that the last three receptors only use MyD88 as an adaptor molecule, and that TLR3 does not require the action of neither Mal nor MyD88 (179).

Trif/TICAM-1, was identified when the search for novel adaptor molecules possibly involved in TLR signalling was conducted (184, 187). Studies have shown Trif (TIR-domain-containing adaptor inducing interferon-β) to be an essential element in the signalling mechanistic of TLR2, TLR3, TLR4 and TLR7 for the activation of NF-κB, via TRAF 6, and of TLR3 and TLR4 for the induction of IRF3 and IFN-β (188, 189).This activation of NF-κB by Trif was shown to be at a lower extent than what can be observed with MyD88. Nevertheless, the use of a double knockout, were the activation of NF-κB is entirely abolished, suggests that Trif would actually be responsible for this delayed activation of the transcription factor seen in MyD88-deficient mice (187). Furthermore, these studies also demonstrate that Trif is the main adaptor used by TLR3 (179).

TRAM/TIRP, (Trif-related adaptor molecule/TIR domain-containing protein) the fourth adaptor, has been identified by sequence homology in database searches (190). Interaction studies have demonstrated that TRAM can interact with Trif, Mal and TLR4; however, inhibition of TRAM showed impaired activation of IRF3 only for TLR4 signalling and not for TLR3 (190-192). Two groups have demonstrated that TRAM is actually acting as a bridging molecule between TLR4 and Trif (191, 192). The

involvement of TRAM in the MyD88-independent TLR4 induction of IRF3 has been further supported by the results obtained using TRAM-deficient mice; the activation of cytokine production was impaired in response to LPS (only the MyD88-independent response) but not in response to the ligands for TLR2, TLR3, TLR7 and TLR9 (193).

SARM, (SAM and ARM-containing protein) is the fifth adaptor protein of the TIR signalling to be described. Like the other adaptors, SARM also contain a TIR domain, but is the only adaptor to possess sterile α (SAM) and HEAT/Armadillo (ARM) motifs (179, 194). The fact that the SAM domain is known to be involved in signalling and is widespread in nuclear proteins has made SARM a protein likely to be part of the TLR signalling (195). Nevertheless, besides that SARM contains a TIR domain and that it is closely related to two TIR domain-containing proteins of *C. elegans*, no experimental data have demonstrated its exact role in the TLR signalling (179, 180).

3.1.2 Negative regulator of TLR pathways

Excessive immune responses can induce serious systemic disorders that are detrimental to the host, and therefore a negative regulation of the TLR signalling is crucial for the immune system integrity (135, 196). Study of the TLR signalling has identified several molecules thought to be negative regulators;such as PI3K, IRAK-M, MyD88s, SOCS1, SIGIRR and ST2 (135, 196).

PI3K, Recent evidence suggest a role for phosphatidylinositol (PI) 3-kinase in different inflammatory response and it seems to play numerous roles in TLR signalling (196). Fukao et al. have demonstrated the ability of PI3K to suppress the production of IL-12 by TLR2, TLR4 and TLR9 (197). More recent observations by this team showed that PI3K would function at an earlier time than IRAK-M in TLR signalling, and therefore would play a role in the modulation of the intensity of the primary activation (196). Interestingly, negative regulation is not the only role played by PI3K in TLR signalling. Indeed, it was demonstrated by an other team that PI3K is involved in the induction of

cytokines downstream of TLR4 and TLR2, as well as NF-κB activation by TLR2 and induction of chemotaxis in response to TLR9 ligand, which point out even more the complexity of TLR signalling (198-201).

IRAK-M, is part of the interleukin-1 receptor associated kinases (IRAKs) which has four human members, but unlike the other members of the family, IRAK-M is not ubiquitous and is expressed mainly by monocytes/Mϕ (202). Kobayashi et al., have demonstrated that IRAK-M negatively regulate TLR signalling by preventing the dissociation of IRAK-1 and IRAK-4 from MyD88, and therefore inhibits the formation of the IRAK-TRAF6 complexes (203). IRAK-M seems to be induced following peptidoglycan (PNG) and LPS stimulation, and its induction has been reported to be an essential element for the PNG- and LPS-induced tolerance (204, 205). Furthermore, it was recently shown that this induction of IRAK-M could be triggered by NO via TNF-α in monocytes (206).

MyD88s (MyD88short), is a splice variant of MyD88 that lacks a short domain between the DD and TIR domain (142). MyD88s was shown to down regulate TLR signalling by interfering with the phosphorylation of IRAK-1, as well as with the activation and ubiquitination of NF-κB (142, 207). It should be noted that MyD88s inhibits only LPS-induced NF-κB activation and not TNF-induced, nor does it interfere with the activation of AP-1 (207, 208).

SOCS1, is part of the suppressor of cytokine signalling (SOCS). Following LPS stimulation, SOCS1 was shown to be rapidly induced and to negatively regulate LPS signalling (209). Kinjyo et al. have concluded from their results that SOCS1 was directly suppressing TLR4 signalling; however, a recent study demonstrates that the effect was actually indirect. In this recent study it was shown that SOCS1 would in fact act on TLR signalling through the regulation of IFNα/β signalling (210). Nevertheless, even if the effect is indirect, SOCS1 still is responsible for a negative feedback on TLR signalling.

SIGIRR (single Ig IL-1R-related molecule), also known as TIR8, is part of the IL-1 receptor family and functions as a modulator of TLR-IL-1R signalling (211, 212). The exact mechanism of action of SIGIRR is more or less clear; however, a recent study has revealed that it would act as a decoy by transiently interacting with TLR4, IRAK and TRAF6, and therefore negatively regulating TLR signalling (211, 213).

ST2, is an orphan receptor member of the TIR family (214, 215). However, ST2 does not activate NF-κB like the other members of the family (215). Very few studies have been conducted on this receptor, but a group recently demonstrated that the membrane-bound form of ST2 was involved in the negative regulation of IL-1R and TLR4 by interacting with MyD88 and Mal (215).

3.2 TLR2

Of all the TLRs, only two have been shown to be involved in the recognition of *Leishmania*, TLR2 and TLR4. For this reason these two receptors will be discussed in more details in these next two sections.

TLR2 can recognize a broad range of pathogen associated molecular patterns (PAMPs) from a variety of microorganisms. Activators of TLR2 signalling include: peptidoglycan from Gram-positive bacteria (148), bacterial lipoproteins (147), mycobacterial cell-wall lipoarabinomannan (149), glycophosphatidylinositol lipid from *T. cruzi* (151), a phenol-soluble modulin produced by *S. epidermidis* (150), and yeast cell walls (157). Furthermore, TLR2 has been shown to be involved in the recognition of atypical LPS such as LPS from *Leptospira interrogans* and *Porphiromonas gingivalis* (154, 155). These LPS are different from the one found on the surface of enteric bacteria like *E. coli* and *Salmonella* spp.; these two types of LPS have a different number of acyl chains in the lipid A component (216). In addition, Becker *et al.* have also demonstrated that TLR2 can be activated by *Leishmania* LPG and lead to the activation of human NK cells *in vitro* (217). The vast number of TLR2 ligands may be explained, in part, by the discovery that it can heterodimerized with at least two other TLRs, TLR1 and TLR6 (171, 172). Cooperation between TLR2 and either TLR1 or TLR6 seems to dictate the specificity of the ligand recognition (218). For example, it was found that TLR2-deficient mice were unresponsive to both bacterial and mycoplasmal lipoprotein. However, TLR6-deficient mice were unresponsive to only mycoplasmal lipoprotein (171). These findings demonstrate that TLR2 cooperates with TLR6 for the recognition of mycoplasmal lipoprotein, but may use another TLR for the recognition of bacterial lipoprotein. The signal triggering the heterodimerization has not been identified yet; it is still unclear whether it happens upon ligation of the ligand or prior. Nevertheless, it has been demonstrated that once activated, both heterodimers (TLR2-TLR1 and TLR2-TLR6) uses the same adaptor proteins, MyD88 and MAL, and that the signalling pathways appear to

be very similar (Figure 8). It is worth mentioning that up to date no MyD88-independent pathway has been known to be used by TLR2.

3.3 TLR4

TLR4 was the first *Drosophila* Toll homologue identified in human (219). Soon after its discovery, its first ligand was identified using LPS-hyporesponsive C3H/HeJ mice (220). The hyporesponsiveness of these mice was first found to be the cause of the *Lps* locus (endotoxin unresponsive gene locus) located on the same region of the chromosome 4 as TLR4 gene (221). The genetic comparison of C3H/HeJ mice with TLR4-deficient mice demonstrated that TLR4 was the gene product of this *Lps* locus (220).

Recognition of LPS involves several accessory molecules. First, LPS binds to serum LBP (LPS-binding protein), and is then transferred to CD14, which is thought to interact with TLR4 (222). Another molecule that was shown to be important in the recognition of LPS using deficient mice is MD-2, which is a small protein expressed on the cell surface in association with the ectodomain of TLR4 (223, 224). However, its exact function is still unclear (223, 224). Some studies suggest that LPS might interact directly with TLR4; however, this interaction would also be enhanced by CD14 and MD-2 (225).

In addition to LPS, TLR4 is involved in the recognition of several other ligands from plant, bacteria, viruses and host (Table 4). Moreover, recent *in vivo* studies have brought evidences that TLR4 would also play a role in the recognition of *Leishmania* since the absence of this receptor has resulted in enhanced replication of this protozoan parasite (226). The results obtained by this team suggest a role for TLR4 both in the late innate and adaptive immunity against *Leishmania*. The exact mode of interaction between *Leishmania* and TLR4 still remains to be identified; however, once TLR4 is activated, several observations, *in vitro* in transfected peritoneal Mϕ as well as *in vivo*, have involved MyD88 in the signalling pathway induced (227, 228).

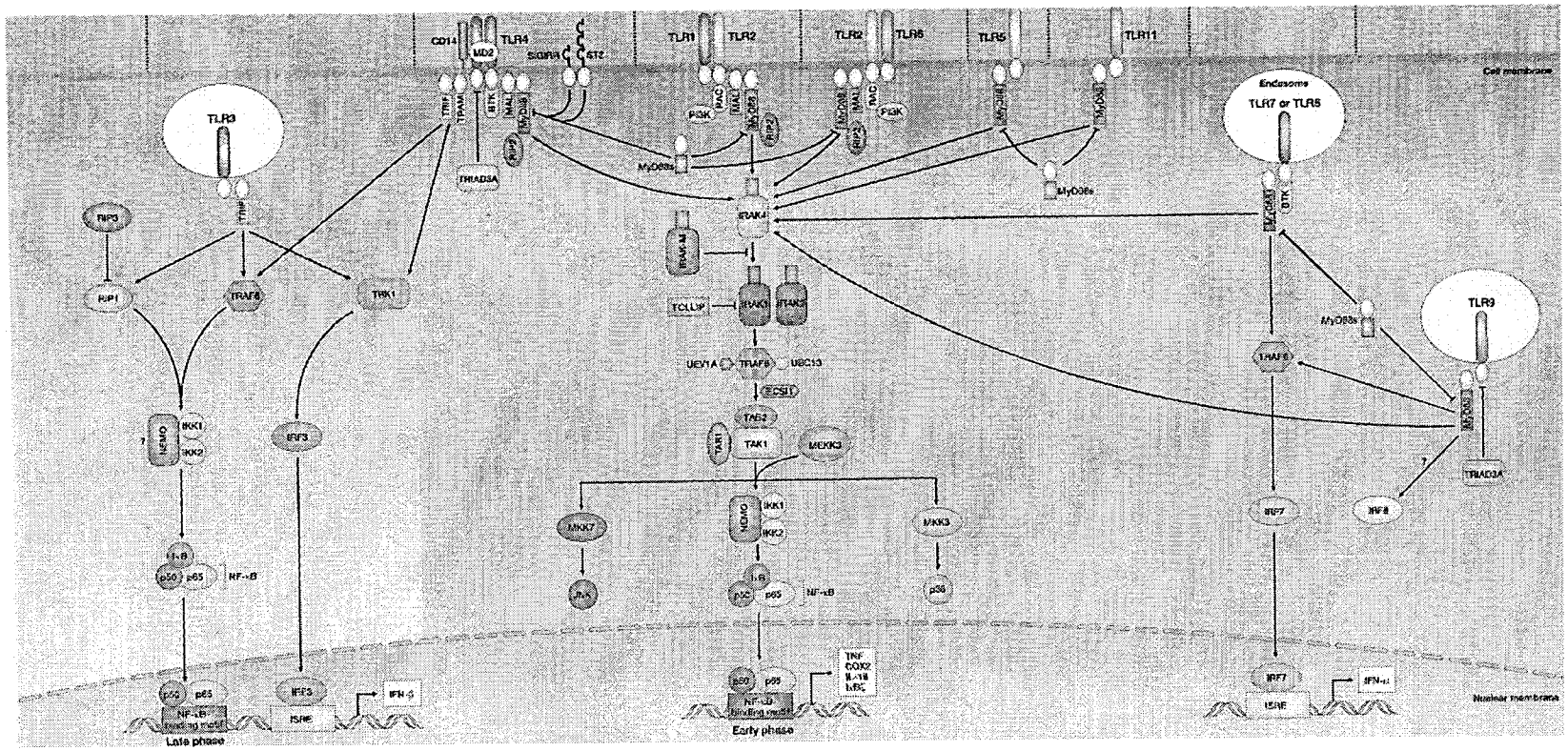

Figure 8. TLR2 and TLR4 signalling. *Taken from* (229)

Once activated, it was demonstrated that TLR4 can signal through MyD88 and Mal, and start a signalling pathway similar to the one activated by TLR2, leading to the activation of NF-κB, p38 and JNK (Figure 8) (230). In contrast with TLR2, TLR4 can also signal through MyD88-independent pathways. The observation that MyD88-deficient mice stimulated with LPS would still activate NF-κB and JNK/p38, although in delayed time compared to wild type mice, led to the identification of MyD88-independent pathways used by TLR4 (231). As said previously in this chapter, TRIF and TRAM are the two adaptor molecules used by TLR for the MyD88- and Mal-independent signalling. The use of TRAM-deficient mice indeed demonstrated that TRAM is used by TLR4 for the activation of IRF3 (193). Moreover, studies using TRIF-deficient mice led to the same observations, meaning that this adaptor was also part of the MyD88-independent signalling of TLR4 leading to the late activation of NF-κB (187). Of interest, it was demonstrated that TLR4 and TLR3 are the only TLRs known to activate the IRF3 pathway leading to the early up regulation of IFN-β, IP10 and RANTES (232).

RATIONALE AND AIMS OF THE STUDY

Exposition of the immune system to *Leishmania* results in the modulation of several signalling pathways leading to the induction of cytokines and chemokines. This phenomenon is well documented; however, the exact mechanistm underlying the induction of these pro-inflammatory molecule still remains to be fully discovered.

Specific aims

To evaluate the possible involvement of TLR4 in the induction of chemokine mRNA by Leishmania and to define the transductional events involved in this signalling pathway

TLR4 has been shown to be involved in the recognition of a variety of PAMPs, from bacteria, plant and virus. Therefore we were interested to determine whether TLR4 was necessary for the recognition of *Leishmania* leading to the induction of chemokine mRNA expression by Mϕ. We also seek to identify the possible transcription factors involved. Furthermore, in vivo experiments were done to confirm the TLR4-mediated chemokine mRNA expression following *Leishmania* inoculation and its role in the subsequent chemokine-mediated inflammatory cell recruitment.

CHAPTER IV

LEISHMANIA-INDUCED CHEMOKINE mRNA EXPRESSION IS TLR4-DEPENDENT, BUT MyD88-INDEPENDENT

LEISHMANIA-INDUCED CHEMOKINE GENE EXPRESSION IS TLR4-DEPENDENT, BUT MYD88-INDEPENDENT

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Abstract

Leishmania's ability to exploit the innate immune response has been extensively studied. One of the strategies through which *Leishmania* assures its survival is by modulating the pattern of macrophage (Mϕ) chemokine expression, which will ultimately favor the recruitment of selected inflammatory cells. In the present study, we investigated the role of Toll-Like Receptors (TLR) and the signaling mechanism underlying this chemokine mRNA induction *in vitro*, using different murine bone marrow-derived Mϕ cell lines (TLR4-deficient, TLR4 KO, TLR2-/-, MyD88-/- and their wild type counterparts), and *in vivo*, using mice that were TLR4-competent, TLR4-deficient, and overexpressing TLR4. Our results revealed that both *Leishmania major* (*L. major*) *and L. donovani* have the capacity to induce chemokine gene expression (MIP-1α/β, MCP-1, MIP-2) in a TLR4-dependent and MyD88-independent manner. Analyze of different transcription factors revealed that NF-κB and CREB nuclear translocation is an essential element to this activation process, whereas AP-1 is not involved. Specific blockage of NF-κB resulted in reduction of its translocation, DNA binding activity and inhibition of MIP-1β and MCP-1 transcripts induction. By contrast, inhibition of cAMP-dependent CREB phosphorylation reduced only slightly the translocation of CREB and the *Leishmania*-induced chemokine gene expression. The in vivo results demonstrate that TLR4 is essential for the proper leukocyte recruitment upon *Leishmania* infection and chemokine gene expression by recruited cells. Collectively, our study provides important information concerning the mechanisms whereby *Leishmania*-parasite can modulate host chemokine gene expression by exploiting TLR-dependent signaling.

Introduction

Leishmania are obligate intracellular parasites that reside almost exclusively in mononuclear phagocytic cells of their mammalian host (1, 2). These protozoan parasites are the causative agent of leishmaniasis, a group of diseases that affect more than 12 million people distributed on all continents, with the exception of Antarctica and Australia (3). The clinical manifestations and severity of the disease depend both on the infecting species of *Leishmania*, of which more than 20 have been identified so far (4), and the immune response of the host (5-7). The pathology can range from asymptomatic to self-resorbing nodules to disfiguring mucocutaneous lesions or a severe visceral form of the disease, that is lethal in some cases (3, 5, 7).

In order to survive and establish infection in its mammalian host, *Leishmania* has evolved several strategies through out its life cycle. Numerous studies have reported the important role played by the parasite surface molecule lipophosphoglycan (LPG) and the 63 kDa glycoprotein (gp63) in the evasion of the immune response, by evading the complement-mediated lysis, protecting against or suppressing the synthesis of antileishmanial products (8-11). Extensive research has also demonstrated the ability of *Leishmania* to regulate the expression of a great number of cytokines, which include IL-1, IL-4, IL-12, and TNF-α (12-15). Other reports have also described the capacity of *Leishmania* to modulate the expression of chemokines by infected macrophages (Mϕ). It was indeed demonstrated that during infection, the monocyte chemotactic protein-1 (MCP)-1, the macrophage inflammatory protein (MIP)-1-α and MIP-1β as well as MIP-2 were differentially expressed (16-18).

Chemokines are important molecules involved in activation of leukocytes, inflammatory diseases, anti-microbial mechanisms, and also act as effectors of the innate immune response in regard of the leukocyte trafficking (19, 20). By their actions on adaptive immune cells, chemokines are crucial for the modeling of the adaptive immune response, which will often determine the outcome of an infection (20). In leishmaniasis, the different chemokines expressed and the cellular populations present at the infection

site have suggested that chemokines would be responsible for driving the adaptive immune response toward either a Th1 or a Th2 type of response (21).

Although the innate immune response was first thought to be non-specific, it is now well established that the innate immune cells can recognize, to a certain extent, pathogen-associated molecular patterns (PAMPs) (19). Recognition of these PAMPs is achieved by their interaction with pattern-recognition receptors (PRRs), like the Toll-like receptors (TLRs), which when activated, can induce expression of certain chemokines, such as MIP-1α, MIP-1β, MIP-3α, IFN-γ-inducible protein (IP)-10, IL-8 and regulated upon activation, normal T-cell expressed and activated (RANTES) (19, 22).

In the present study, we were interested to determine whether *Leishmania*-induced macrophage chemokine gene expression was trigger upon parasite/TLR interaction. We obtained results demonstrating that induction of chemokine mRNA expression by *Leishmania*-infected macrophages is mediated through TLR4, but of interest, is independent of the myeloid differentiation factor 88 (MyD88). Characterization of the signaling pathways revealed the requirement for the nuclear translocation of NF-κB and cAMP-response element binding (CREB), but not of AP-1, in these TLR4-dependent events. Although the nature of this interaction remains to be further elucidated, our data suggest that it does not involve the parasite surface molecules LPG or gp63.

The *in vivo* experiments demonstrate the essential role of TLR4 in the recruitment of leukocytes in response to *L. major* infection, and consequently confirmed TLR4 involvement in *Leishmania*-induced chemokine mRNA expression.

Materials and Methods

Materials. Lipopolysaccharide (LPS ;*Escherichia coli*, serotype R515) and MALP-2 (synthetic) were purchased form Alexis (San Diego, CA). Phorbol myristate acetate (PMA) was obtained from Sigma-Aldrich (St. Louis, MO). Endotoxin-free PBS was purchased from Gibco BRL (Burlington, Canada). Isotopes [α-^{32}P]dUTP (3000 Ci/mmol) and [γ-^{32}P]dATP (3000 Ci/mmol) were obtained from Perkin Elmer (Boston, MA). Specific inhibitors MDL-12,330A hydrochloride and BAY 11-7082 were purchased from Biomol Research Laboratories (Plymouth Meeting, PA).

Cell and Culture Conditions. The murine bone marrow derived Mϕ cell lines B10R (derived from B10A.Bcgr [B10R] mice (23)), TLR-4 deletion (derived from C57Bl/10ScCr mice), TLR-4 KO (*TLR2* gene knockout mice backcrossed to C57BL/6 strain), ANA-1 (derived from C57Bl/6 mice), TLR-2 deletion (*TLR2* gene knockout mice backcrossed to C57BL/6 strain), MyD88 Control (derived from a littermate control mice from the last backcrosses to C57BL/6 strain) and MyD88 deletion (*MyD88* gene knockout mice backcrossed to C57BL/6 strain), were generated in Dr D. Radzioch laboratory and immortalized (McGill University, Montreal, Canada). Cells were maintained at 37°C and 5% CO_2, in Dulbecco's DMEM (Life Technologies, Rockville, MD) supplemented with 10% heat-inactivated FBS (Gibco BRL), 100 μg/ml penicillin-streptomycin and 2 mM L-glutamine (Gibco BRL).

Parasite cultures. Promastigotes of *L. donovani* 2211, *L. major* Friedlin, *L. major* A2, *L. major* A2 KO gp63 (24), *L. donovani* 1S2D and *L. donovani* R2D2 (Dr. S. Turco, University of Kentucky, USA) were grown at 25°C, and transferred biweekly in SDM-79 culture medium (Gibco BRL) supplemented with 10% FBS as described elsewhere (25, 26). Macrophages were infected in vitro at a parasite:cell ratio of 20:1.

RNase protection assay (RPA). Chemokine mRNA expression was monitored using an RPA kit (mCK-5 RiboQuant; BD PharMingen, San Diego CA), as we previously described (27), to enable simultaneous detection of a large number of these proinflammatory molecules (Lymphotactin, RANTES, MIP-1β, MIP-1α, MIP-2, IP-10, T cell activation protein [TCA]-3, and eotaxin). Total RNA was extracted from the stimulated, and non-stimulated cells with TRizol (Life Technologies) following the manufacturer's protocol. The commercial multiprobe was labelled with [α-^{32}P]dUTP using T7 RNA polymerase. Labelled probe (3×10^5 cpm) was added to 10 μg of total RNA, and allowed to hybridize for 16 h at 56°C. Resulting mRNA probe hybrids were subjected to an RNase A treatment, and extracted with phenol-chlorophorm. Protected hybrids were loaded on a 5% denaturing polyacrylamide sequencing gel. Once dried, the gel was exposed to a radiographic film at -80°C, and also subjected to densitometry analysis using a Molecular Imager FX and the analysis software Quantity One 1D version 4.4 (Bio-Rad). Chemokine density values were normalized to the housekeeping gene mL32, also present in the multiprobe template.

Preparation of nuclear extracts. Cell stimulation and infection were terminated by the addition of ice-cold PBS, and washed 3 times in order to remove all non-ingested parasites. Nuclear extraction was performed according to the microscale preparation protocol (28). Briefly, sedimented cells were resuspended in buffer A (10 mM HEPES pH7.9, 10 mM KCl, 1 mM DTT, and 0.5 PMSF), and incubated on ice for 15 minutes. Twenty-five igepal (Sigma-Aldrich) were added to the mixture, which was then vortexed for 10 s, and centrifuged for 30 s at 12,000 × *g*. The supernatant was discarded, and the pellet resuspended in 50 μl of buffer C (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF). The samples were then rocked at 4°C for 15 min. Cellular debris were removed by a 5 min centrifugation at 12,000 × *g* at 4°C, and the supernatant was kept at -80°C until used.

Electrophoretic Mobility Shift Assay (EMSA). EMSA was performed using 6 μg of nuclear extract. Protein concentrations were determined using the commercial BCA Protein Assay Reagent (Pierce, Rockfort, IL). The dsDNA oligonucleotides (Santa Cruz

Biotechnology, Santa Cruz, CA), used either as probes or competitors were as follows: consensus site for AP-1 c-jun homodimer and Jun/Fos heterodimeric complexes, 5'-CGCTTGATGACTCAGCCGGAA-3'; consensus binding site for CREB of the CREB/activating transcription factor family, 5'-GAGATTGCCTGACGTCAGAGAGCTAG-3'; and consensus binding site for the NF-κB/c-Rel homodimeric and heterodimeric complexes, 5'-AGTTGAGGGGACTTTCCCAGGC-3'. The oligonucleotides containing NF-κB or CREB binding sites of the murine chemokine promoters were synthesized in our laboratory as follows: NF-κB/MIP-2 5'-GAGCTCAGGGAATTTCCCTGGTCC-3' (29); and CREB/MIP-1β 5'-CTCGATGCCATGACATCATCTTTAC-3' (30). The non-specific probe Oct-2A 5'-GGAGTATCCAGCTCCGTAGCATGCAAATCCTCTGG-3' was used to confirm specificity of the DNA/nuclear protein reaction and was also synthesized in our laboratory. As we previously described (31), nuclear extracts were incubated at room temperature for 20 min in 1.0 μl of binding buffer (100 mM HEPES, pH 7.9, 40% glycerol, 10% ficoll, 250 mM KCl, 10 mM DTT, 5 mM EDTA, 250 mM NaCl), 2 μg of poly(dI-dC), and 10 μg of nuclease-free BSA (fraction V) (Sigma-Aldrich) containing 1.0 ng of radio-labelled dsDNA oligonucleotide. dsDNA (100 ng) was end-labeled using [γ-^{32}P]dATP and T4 polynucleotide kinase (New England Biolabs, Beverley, MA). After a 20-min incubation, the reaction was stopped with the addition of 5 μl of 0.2 M EDTA. The DNA/protein mixture was extracted with phenol/chlorophorm and passed through a G-50 spin column. DNA-protein complexes were resolved from free-labelled DNA by electrophoresis in native 4% (w/v) polyacrylamide gels containing 50 mM Tris-HCl, pH 8.5, 200 mM glycine, and 1 mM EDTA. The gels were subsequently dried and autoradiographed. Cold competitor assays were conducted by adding a 100-fold molar excess of unlabeled oligonucleotides homologous of the labelled dsDNA probes.

Air pouch and leukocyte migration. Air pouches were raised on the dorsum of 3 to 6 months C57BL/10J (TLR4 competent), C57BL/10ScNcr (TLR4 deficient) and C57BL/10ScNcr transgenic mice containing 6 copies of *TLR4* gene, as described elsewhere (27, 32). *L. major* stationary phase promastigotes (1×10^7 in 1 ml of

endotoxin-free PBS) were injected in the air pouches. Control animals were injected with endotoxin-free PBS (negative control) and LPS (10 μg/ml; positive and *toll4* gene deletion controls). After 6 h of stimulation, mice were lethally exposed to CO_2, and the pouches were washed with a total volume of 5 ml of endotoxin-free PBS/1 mM EDTA. Leukocytes recruited to the pouch exudates were counted directly with a hemacytometer following acetic blue staining. Cytospin preparations of pouch exudates of each animal were stained using Diff-Quick (Baxter Healthcare; Deerfield, IL) in order to perform the differential cell counts for the various stimulations. Cell exudates from each experimental group were pooled and centrifuged at 1200 rpm × 10 min at room temperature. Total RNA was extracted from the recruited cells with TRizol reagent (Life Technologies), according to the manufacturer's protocol, for further analysis of chemokine mRNA expression.

Statistical analysis. Statistically significant differences were determined by using EXCEL (Microsoft Office 2000). Values of $p < 0.05$ were deemed statistically significant. All data are presented as mean ± SD.

Results

In vitro modulation of chemokine mRNA expression by Leishmania. We first investigated whether both *L. major* and *L. donovani* could modulate the induction of chemokine mRNA in a similar manner, regarding the amplitude of the induction and the time-dependent response. Measurements of chemokine mRNA levels were therefore conducted by RPA after various times of infection (0.5 to 6 h) of the Mϕ cell line B10R. As shown in Fig. 1, infection by *L. major* or *L. donovani* caused an increase in the expression of several Mϕ chemokine genes, MIP-1α, MIP-1β, MIP-2 and MCP-1. The induction can be observed as early as half an hour after infection, and reached its maximum at 2 h post-infection for both strains of parasites, although *L. major* seemed to induce the expression of MIP-2 mRNA at a greater extent than *L. donovani*. These data are in correlation with previous results (18). Subsequent experiments presented in this paper have all been conducted with both *L. major* and *L. donovani* and showed significant similarity; therefore, the term *Leishmania* will be used from now on and will refer to both strains, unless otherwise mentioned. RPA experiments were also performed using metacyclic parasites, extracted with peanut agglutinine (PNA) (as described (33), in order to compare the ability of these highly infectious, non-dividing, mature promastigotes, to modulate Mϕ chemokine gene expression with the stationary phase parasites. The results from this experiment demonstrated that both types are able to induce chemokine mRNA expression. Although metacyclic showed a slightly higher capacity to up-regulate it, this difference was not statistically significant (data not shown).

Leishmania-induced chemokine gene expression does not involve toll-like receptor 2 (TLR2). Given that previous studies have reported that interaction of the LPG of *Leishmania* with TLR2 could cause the activation of the nuclear factor NF-κB (34), as well as the activation of natural killer (NK) cells (35), we sought to determine if TLR2 could also be responsible for the induction of chemokine mRNA expression observed during infection of macrophages by *Leishmania*. To this end, TLR2 DEL Mϕ (derived

from C57BL/6 mice with *TLR2* gene knockout) and ANA-1 Mϕ (wild type) were infected with *Leishmania* for 2 h, total RNA was extracted, and chemokine mRNA was monitored by RPA. An increase can be seen in both TLR2 DEL Mϕ and ANA-1Mϕ (control cell), demonstrating that TLR2 does not seem to play a role in the *Leishmania*-induced chemokine mRNA (data not shown).

Leishmania-induced chemokine mRNA expression is TLR4-dependent. We then investigated the possible role of TLR4 in the induction of Mϕ chemokine mRNA. In order to do so, we infected two different cell lines deficient in TLR4; TLR4 DEL Mϕ (derived from C57Bl/10ScCr mice) and TLR4 KO Mϕ (derived from B6 mice with a *toll4* gene disruption), in parallel with the wild type B10R Mϕ. After 2 h of infection, total RNA was extracted and subjected to RPA for chemokine mRNA measurements. The results obtained were the same for both types of deficient cell lines; therefore, only the results for TLR4 KO Mϕ are shown. As can be seen in Fig. 2, when stimulated with 100 ng/ml of LPS, a pronounced increase in the level of mRNA expression can be observed in the wild type Mϕ, but is totally abrogated in the TLR4 KO Mϕ. In a similar manner, *Leishmania*-induced chemokine transcripts expression can be observed in the wild type Mϕ (B10R), but this induction is significantly reduced, almost back to a basal level, in the TLR4 KO Mϕ. Thus, our results strongly suggest that TLR4 plays an essential role in *Leishmania*-induced chemokine mRNA expression *in vitro*.

Leishmania-induced chemokine mRNA expression is MyD88-independent. We next sought to determine if the TLR4-dependent pathway, used by *Leishmania* to induce the expression of chemokine mRNA in Mϕ, was dependent of the myeloid differentiation factor 88 (MyD88). For this purpose, we infected BMDM cell line generated from C57BL/6 deficient in the MyD88 adapter molecule, in parallel with wild type Mϕ. As a control, we stimulated with LPS at a concentration of 100 ng/ml for 2 h and, as depicted in Fig. 3, a strong induction of chemokine mRNA expression can be observed in the wild type Mϕ, but is abrogated in the MyD88 deleted cells. On the other hand, after stimulation with *Leishmania*, an increase in chemokine gene expression can be noticed in

both wild type and MyD88 deficient Mϕ cell lines. These results clearly demonstrate that increase in the expression of chemokine mRNA induced by *Leishmania* is independent of the MyD88 adaptor protein.

Identification of transcription factors involved in Leishmania-induced Mϕ chemokine gene expression. In order to elucidate the signalling pathway used by *Leishmania* to modulate macrophage chemokine gene expression, we next attempted to identify potential transcription factors involved. NF-κB is an important transcription factor controlling the expression of genes involved in inflammation (36), and is associated with the transcription of various genes, including those encoding for cytokines (IL-1, IL-2, TNF-α, and IL-12) (37), adhesion molecules, and chemokines (MCP-1, MIP-2 and IL-8 (38-41). There is an extensive list of bacteria and bacterial products that can lead to the activation of NF-κB, as either a result of inflammation or infection (37). NF-κB can also be activated by protozoan parasites, it was indeed demonstrated that *Trypanosoma cruzi* (42) as well as *Leishmania* promastigotes (43) and amastigotes (44) can lead to the activation of this transcription factor.

To define whether *Leishmania* in TLR4 KO and wild type Mϕ differentially affect NF-κB translocation, both cell lines were infected for various periods of time (0.5 to 4 h), and extracted nuclear proteins were subjected to EMSA. As shown in Fig. 4A, *Leishmania* induces a rapid, as early as 30 min, and transient translocation of NF-κB in wild type Mϕ. However, such induction by the parasites or LPS could not be observed in TLR4 KO Mϕ. To determine if the binding of NF-κB to the *MIP-2* gene was also disrupted in TLR4KO Mϕ, nuclear proteins were incubated with an oligonucleotide specific for the NF-κB binding site present in the murine MIP-2 promoter. Of interest, we found that the binding to this chemokine promoter was also inhibited in the absence of TLR4, whereas an increase in binding could be seen in B10R Mϕ (Fig. 4B). To further demonstrate the involvement of NF-κB on the *Leishmania*-induced chemokine modulation, B10R Mϕ were treated for 1 h with increasing concentrations of BAY 11-7082 (1, 3 and 5 μM), which is known to inhibit IκB phosphorylation, resulting in decrease expression of NF-κB (45). Mϕ were then stimulated for 2 h with *Leishmania* and

EMSA were performed to study NF-κB translocation and binding to the MIP-2 promoter. As expected, a concentration-dependent inhibition of *Leishmania*-induced translocation and binding could be observed for both the consensus NF-κB sequence (data not shown) and NF-κB/MIP-2 (Fig. 5A). We next tested the effect of this inhibitor on the induction of chemokine mRNA by *Leishmania*, by subjecting extracted RNA to RPA after treating the cells as it was done for the EMSA analysis. As demonstrated in Fig. 5B, treating the Mϕ with BAY 11-7082 resulted a the partial reduction of MIP-1α and MIP-2 mRNA expression, and a total inhibition of *Leishmania*-induced MIP-1β and MCP-1 transcripts.

Given that the treatment with the IκB inhibitor did not result in the absolute inhibition of all chemokine induced during infection, we studied another transcription factor known to play an important role in the regulation of chemokine expression, and like NF-κB, AP-1 possesses a binding sequence in MIP-2 promoter (40). Since previous studies have demonstrated that *Leishmania* can down-modulate the activation of AP-1 in Mϕ (43), we were interested to see whether this transcription could be involved, or not, in the TLR4-dependent signalling pathway used by the parasite to induce chemokine mRNA expression. In this optic, we studied the nuclear translocation of AP-1 as it was done previously for NF-κB. In contrast to the results obtained for NF-κB, *Leishmania* was able to modulate the translocation of AP-1, both in the wild type and the TLR4 KO Mϕ. As shown in Fig. 6, in both cell types we can observe an inhibition of AP-1 translocation following infection by *Leishmania*, demonstrating that AP-1 would not be part of this particular signalling pathway.

In addition to the study of the transcription factors NF-κB and AP-1, we evaluated the potential contribution of CREB. Although its link with any TLR signalling has not yet been clearly defined, it is well established that CREB is involved in the regulation of MIP-1β chemokine (30). EMSA were performed as previously described; in B10R Mϕ, *Leishmania* induced an increase in the nuclear translocation of CREB; however, in the TLR4 KO Mϕ, this translocation was dramatically reduced (Fig. 7A), demonstrating that CREB is potentially an essential part of this signalling mechanism. To better define the contribution of this transcription factor in the modulation of chemokine gene expression by *Leishmania*, we treated the Mϕ with an inhibitor of adenylate cyclase, MDL-12,330A

(1, 5, 10 μM), which blocks cAMP-dependent CREB phosphorylation (46), before *Leishmania* infection, and RPA were performed. As demonstrated in Fig. 7B, the treatment with MDL-12,330A, reduced only partially the induction of MIP-1β, MIP-2 and MCP-1, but did not affect MIP-1α, which suggest that this transcription factor does contribute to the induction by *Leishmania*, but only to a small part.

Evaluation of the potential role of Leishmania surface molecule in the induction of chemokine mRNA. In an attempt to identify surface molecule(s) of the parasite that might interact with TLR4 to induce chemokine mRNA expression, we used parasites deficient for various surface molecules. One of the components that we tested was the major surface molecule LPG. In order to determine its effect, we infected B10R Mϕ with a strain of parasites deficient in LPG, Ld R2D2, in parallel with its wild type counterpart, Ld 1S2D, and subjected the Mϕ RNA to RPA analysis. As shown in Fig. 8A, the absence of LPG did not significantly reduce the induction of chemokine gene expression, with the exception of MIP-2 which is slightly decreased. The second molecule tested was gp63, using the knock-out parasite strain Lm A2 KO gp63 and its wild type counterpart Lm A2, as shown in Fig. 8B, no significant change in the induction of chemokines by *Leishmania* can be seen between both strains of parasites. These results strongly suggest that neither LPG nor gp63 plays a significant part in the induction of chemokine mRNA expression by *Leishmania*.

Leishmania-induced leukocyte recruitment and chemokine mRNA expression in the air pouch are TLR4-dependent. To confirm the role of TLR4 in *Leishmania*-induced chemokine gene expression and early pro-inflammatory events in vivo, we used a murine air pouch model to monitor innate inflammatory response. After a 6 h treatment (*Leishmania*, PBS or LPS), pouch exudates were collected and the total number of leukocytes recruited in response to the different stimuli was determined. As depicted in Fig. 9, stimulation with *L. major* led to a significant increase in the number of leukocytes recruited in the pouches of wild type mice; however, this increase is dramatically reduced in TLR4 DEL mice, which demonstrates the importance of this receptor for the recruitment of leukocytes in response to *L. major*. This observation was further confirmed

using transgenic TLR4 6X, in which the elevation of leukocyte recruitment is even greater than what can be noticed in the wild type mice. By performing differential cell counts of leukocyte subpopulations for the various stimulations of the three strains of mice, we could observe that PBS-stimulated wild type mice already presented a diversity of subpopulations a high proportion of which (50%) was constituted of neutrophils (Fig. 10). When stimulated with *L. major*, the same proportions of subpopulations, as for the PBS-stimulated, were observed in the wild type mice (50% neutrophils, 25% monocytes, 20% eosinophils, and 5% lymphocytes); however, the number of cells of each subpopulation is increased (5-fold for the monocytes and neutrophils, 3-fold for the lymphocytes, and 7-fold for the eosinophils). In the TLR4 DEL mice, the subpopulations of leukocytes recruited in the presence of PBS show different proportions, 45% monocytes, 25% neutrophils, 30% eosinophils and 5% lymphocytes. Although a small increase of certain cell populations can be observe in presence of *L. major*, the proportions as well as the number of cells recruited still remain lower than what was observed in the wild type animals. The role of TLR4 can be further assessed when comparing the response of the transgenic mice TLR4 6X with the resistant wild type. As shown in Fig. 10B (*right graphic*), in presence of *L. major*, there is a recruitment of $27{,}44\times10^4$/ml monocytes and $95{,}69\times10^4$/ml neutrophils, which represent a 2,4-fold and a 4-fold increase, respectively, over the results obtained in the wild type mice infected with *L. major*. These data indicate the involvement of TLR4 in the recruitment of leukocytes, particularly of neutrophils, to the site of infection.

The examination of the induction of chemokine genes in vivo was conducted by pooling the pouch exudates of each experimental group, and total RNA was extracted from the recruited leukocytes to be subjected to RPA. In correlation with leukocyte recruitment and the data obtained in vitro, an increase in the four chemokines: MIP-1β, MIP-1α, and MCP-1 (Monocytes (47)), and MIP-2 (Neutrophils (48)) could be observed in the wild type mice, in the presence of *L. major* (Fig 11). However no such induction could be observed in the TLR4 DEL mice, where no increase was visible for MCP-1 and only a slight up-regulation was induced in the presence of *L. major* for the other three chemokines. These results, once again, support the role played by TLR4 in the

Leishmania-induced chemokine mRNA expression and consequent inflammatory cell recruitment.

Discussion

The innate immune response plays a crucial role in the recognition of different pathogens. Toll-like receptors, since their discovery, have been associated with the recognition of several pathogen-associated molecular patterns (PAMPs), first found to be mainly of bacterial origin, now known to be of a great diversity (review in (49)). In our study we found that *Leishmania* can interact with TLR4, conducting to the induction of several chemokine genes expression *in vitro*, as well as *in vivo*. *In vitro*, this chemokine mRNA induction was shown to be independent of the adaptor molecule MyD88. Nevertheless, this TLR4-dependent *Leishmania*-induced chemokine transcripts expression was shown to involve the activation of the transcription factors, NF-κB and CREB. *In vivo* experiments, have permitted to demonstrate that leukocyte recruitment was significantly reduced in the absence of TLR4, which was in correlation with the reduction of chemokine gene expression.

Chemokines are an integral part of the innate immune response, and their expression can be modulated in different ways by pathogens. As we previously reported (18), an up-regulation of chemokine gene expression was also observed in vitro, when murine BMDM were stimulated with *Leishmania*. Indeed, RPA analysis demonstrated the induction of MIP-1β, MIP-1α, MIP-2 and MCP-1, as seen in vivo. Furthermore, we demonstrated that this chemokine mRNA induction was also abrogated when macrophages deficient in TLR4 were infected with *Leishmania*, demonstrating the importance of this particular receptor for the up-regulation of those chemokines.

Previous *in vivo* experiments by Kropf *et al.* (4, 50) have revealed the importance of TLR4 for the development of efficient immune control over *Leishmania* infection. However, these experiments have not studied the role of this TLR in the initial recognition of *Leishmania* by macrophage at which time innate immunity plays a crucial role in the local containment of *Leishmania* infection (51, 52). One of the key effector cells that have been identify to reduce parasite numbers at the site of inoculation, and therefore control early systemic spreading are the neutrophils (52, 53). In agreement with the observation of Lima *et al.* (52) that neutrophils are the major cell type present at the

site of infection in the first 3 days after inoculation, we also observed that these cells were the predominant cell type (around 50% of recruited cells) present in the air pouches of the wild type mice. These data are also in agreement with previous results obtained by our team in the air pouch model, as well as in footpad infection (54, 55). However, in the TLR4 DEL mice the major cell type of the few recruited cells was found to be monocyte, and both these cells and the neutrophils were found to be present in significantly lower numbers than in the TLR4-competent mice. This inability to recruit neutrophils may be in part an indicator as to why these mice were shown to be less efficient to control *L. major* infection. In the same line of thinking, it is possible to speculate that the TLR4 6X, where a higher leukocyte recruitment was measured, and in particular of neutrophils, could be even more efficient to control *Leishmania* infection, but this hypothesis remains to be tested.

In correlation with these results, and the in vitro experiments, modulation of several chemokine transcripts; such as MIP-1β, MIP-1α, MIP-2 and MCP-1; was observed when total RNA was extracted from the recruited cells of the pouch exudates from wild type mice. Most importantly, MIP-2, a powerful neutrophil chemoattractant and activator (48), was found to be highly up-regulated and to be the chemokine that has the highest expression. This difference with the in vitro results, where MIP-2 mRNA was not as highly expressed, can be explained by the fact that, in vitro, we only studied Mϕ chemokine expression, whereas in vivo the chemokine gene expression was the one observed for the recruited cells, which is constituted be a mixed population of leukocytes, such as monocytes, neutrophils, eosinophils and lymphocytes. Meaning that the expression observed in vivo could be the one of monocytes as well as the one of neutrophils, which can also produce MIP-2 (56). This data reflect very well why neutrophils constitute the majority of the cells recruited in the pouch exudates. Moreover, the induction of these chemokines was found to be significantly reduced in the absence of TLR4, as reported by the results obtained in TLR4 DEL mice.

Given the knowledge that TLR4 can react with the adaptor molecule MyD88, and previous studies have shown that MyD88 might be essential for the clearance of *Leishmania* (34), we then sought to determine if the chemokine induction would also be dependent of MyD88. According to the results obtained from the RPA experiments using

MyD88-deficient Mϕ, this particular signalling mechanism was shown to be MyD88-independent. Of interest, in the absence of MyD88 the chemokine gene up-regulation was found to be, for some of the chemokines (MIP-1β and MIP-2 mainly), even stronger. This observation could be explained by the possibility that deletion of MyD88 also resulted in deletion of MyD88s, a splice variant of MyD88. MyD88s was found to act as a negative regulator of the TLR response, in order to prevent over activation of the cells, which could be deleterious for the organism (49, 57). Taken together, this would mean that the deletion of MyD88s could result in the over expression that we observed in the RPA experiments; however this hypothesis remained to be confirmed.

Although these data demonstrate the pivotal role played by TLR4 in *Leishmania*-induced chemokine gene expression, the transductional mechanisms involved in this modulation still remained to be studied. Thus, the possible link between TLR4 and the activation of different transcription factors (NF-κB, AP-1 and CREB) have been investigated.

For instance, we found that *Leishmania* can modulate NF-κB activity, which is in accordance with previous results obtained by others (44, 54). Of importance, results demonstrate that the early activation of NF-κB by *Leishmania*, is TLR4-dependent, since the absence of this receptor resulted in the inhibition of the activation of this transcription factor. In these experiments, we noticed that the basal level of NF-κB in the nucleus is lower in the TLR4 KO Mϕ, than in the wild type Mϕ. This difference seems to be related to a defect in the translocation rather than in the amount of NF-κB in the cell, since Western analysis of the subunits p65 and p50 revealed that these subunits are present in the same amount in both cell types (data not shown). In order to further demonstrate the need for NF-κB in chemokine gene induction by *Leishmania*, we used the inhibitor BAY 11-7082. The inhibition of NF-κB activation resulted in the inhibition of the induction of MIP-1β and MCP-1, as well as a reduction in the expression of MIP-1α and MIP-2. These results clearly demonstrate the essential role played by NF-κB in the up-regulation of Mϕ chemokine mRNA in presence of *Leishmania*.

AP-1 has previously been shown to be modulated in presence of *Leishmania* (43), in our experiments, we indeed observed a time-dependent down-modulation of its

activity. However, this ability of *Leishmania* to modulate the activity of AP-1 does not appear to be TLR4-dependent, given that the same effect of the parasite on the translocation could be observed in presence, or in absence of the receptor.

Another transcription factor studied in this paper was CREB. Although no previous report of its modulation by *Leishmania* has been published, it has been reported that another protozoan parasite, *Theileria parva*, is responsible for an increase in the binding activity of CREB in infected bovine T cells (58). In addition, it was demonstrated that CpG DNA could interfere with the binding activity of CREB (59), and since CpG is known to be a TLR9 ligand, a role for TLR in this modulation is probable. The same observation can be made about previous results showing that HSP60, a TLR4 ligand, can induce the activation of CREB (60).However, none of these studies used knock out cells or mice in order to confirmed more directly the link between TLR and CREB. In our study, however, we have demonstrated that *Leishmania* can induce CREB activation in murine Mϕ during the early time of infection, and that this activation is significantly reduced when the same experiment was carried out in Mϕ deficient in TLR4, which clearly suggest a role of TLR4 in the activation of CREB.

In an attempt to identify the potential TLR4 ligand of *Leishmania*, we studied the effect of the deletion of two different surface molecules of *Leishmania*, LPG and gp63, on the induction of chemokine genes. LPG is known to play a role in the modulation of a variety of signalling pathways involved, for instance, in inducible nitric oxide synthase (61) and cytokines like IL-12 (13), resistance to complement-mediated lysis (33), and many others. In our experiments, however, the deletion of LPG from the surface of *Leishmania* did not show any significant change in the induction of chemokine mRNA expression. This result is not surprising since previous studies have shown LPG to be a TLR2 ligand (35), and in the case of our study, the deletion of TLR2 did not resulted in any significant changes in the up-regulation of chemokine genes, which supports that LPG does not play a major role in the TLR4-dependent induction of chemokine mRNA. We then tried with parasites deficient in gp63, and once again, there were no significant changes in the level of expression of chemokine mRNA.

In conclusion, our study demonstrates the important role fulfilled by TLR4 in the *Leishmania*-induced Mϕ chemokine gene expression in vitro, as well as in vivo. And to involve a MyD88-independent signalling events conducting to NF-κB and CREB activation and chemokine gene transcription. Furthermore, the in vivo experiments have shown that the induction of chemokines following infection by *Leishmania* is necessary for the recruitment of specific leukocyte subpopulations, particularly of neutrophils, to the site of infection, phenomenon also dependent on the presence of TLR4. Taken together, these data bring new insights in the signalling mechanistic involved in host-pathogen interaction, which could eventually lead to the identification of potential therapeutic targets.

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Figures with legends

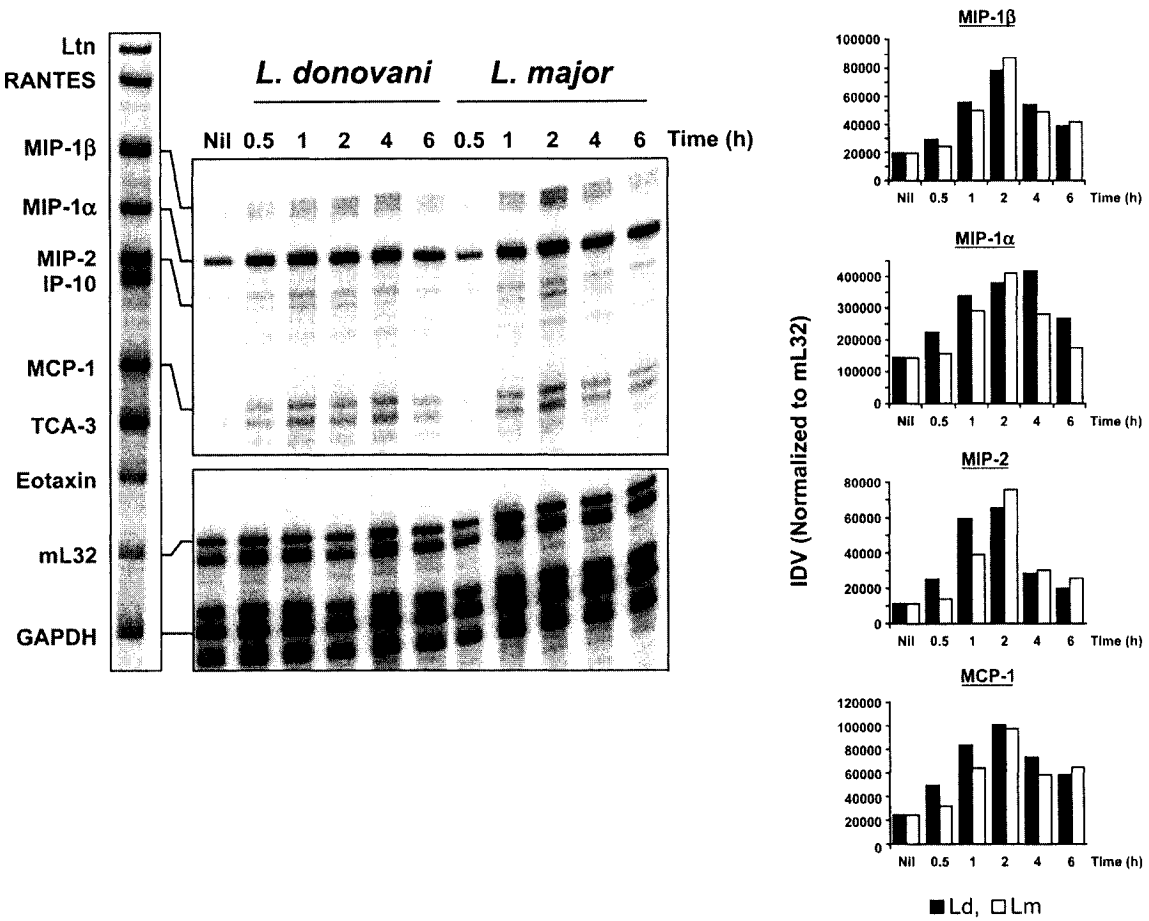


Figure 1. Kinetic analysis of *Leishmania*-induced chemokine mRNA expression. B10R Mϕ were stimulated with either *L. donovani* 2211 (*left panel*) or *L. major* Friedlin (*middle panel*), at a parasite:cell ratio of 20:1, for 0 to 6 h, and chemokine mRNA expression was monitored by using a mCK5 multiprobe RPA system. Densitometric quantification of mRNA levels over negative control after normalization to mL32 (*right panel*). Results are representative of one of three independent experiments.

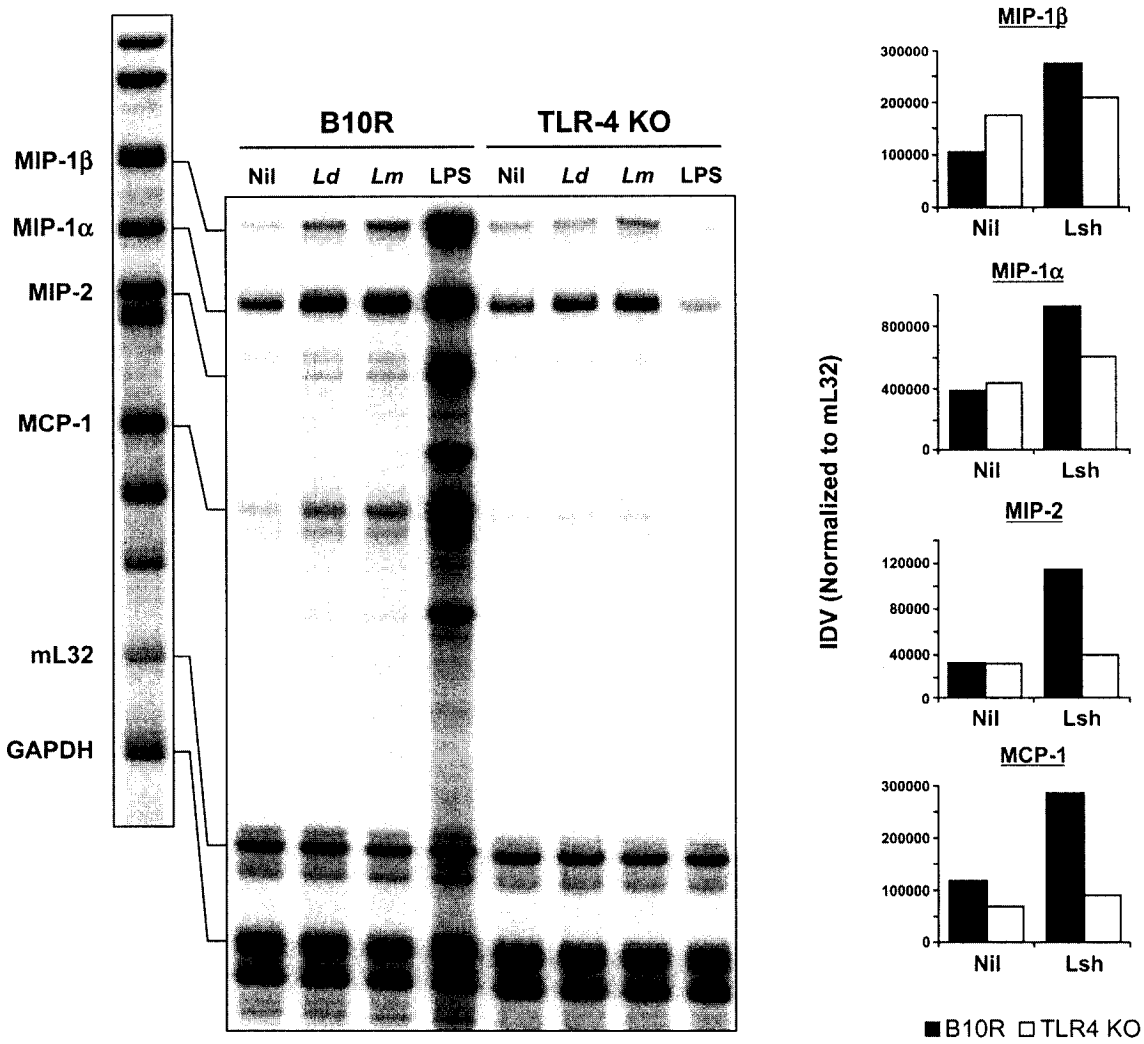


Figure 2. *Leishmania*-induced chemokine gene expression is TLR4-dependent. TLR4 KO Mϕ and B10R Mϕ were infected with either *L. donovani* (*Ld*) or *L. major* (*Lm*) (parasite:cell ratio of 20:1) for a 2 h period and chemokine mRNA expression was evaluated by RPA (*left panel*). Integrated density values of chemokine mRNA levels of both cell lines normalized to mL32 (*right panel*). Results are representative of one of three separate experiments. Nil: Untreated; Lsh: *L. major* / *L. donovani.*

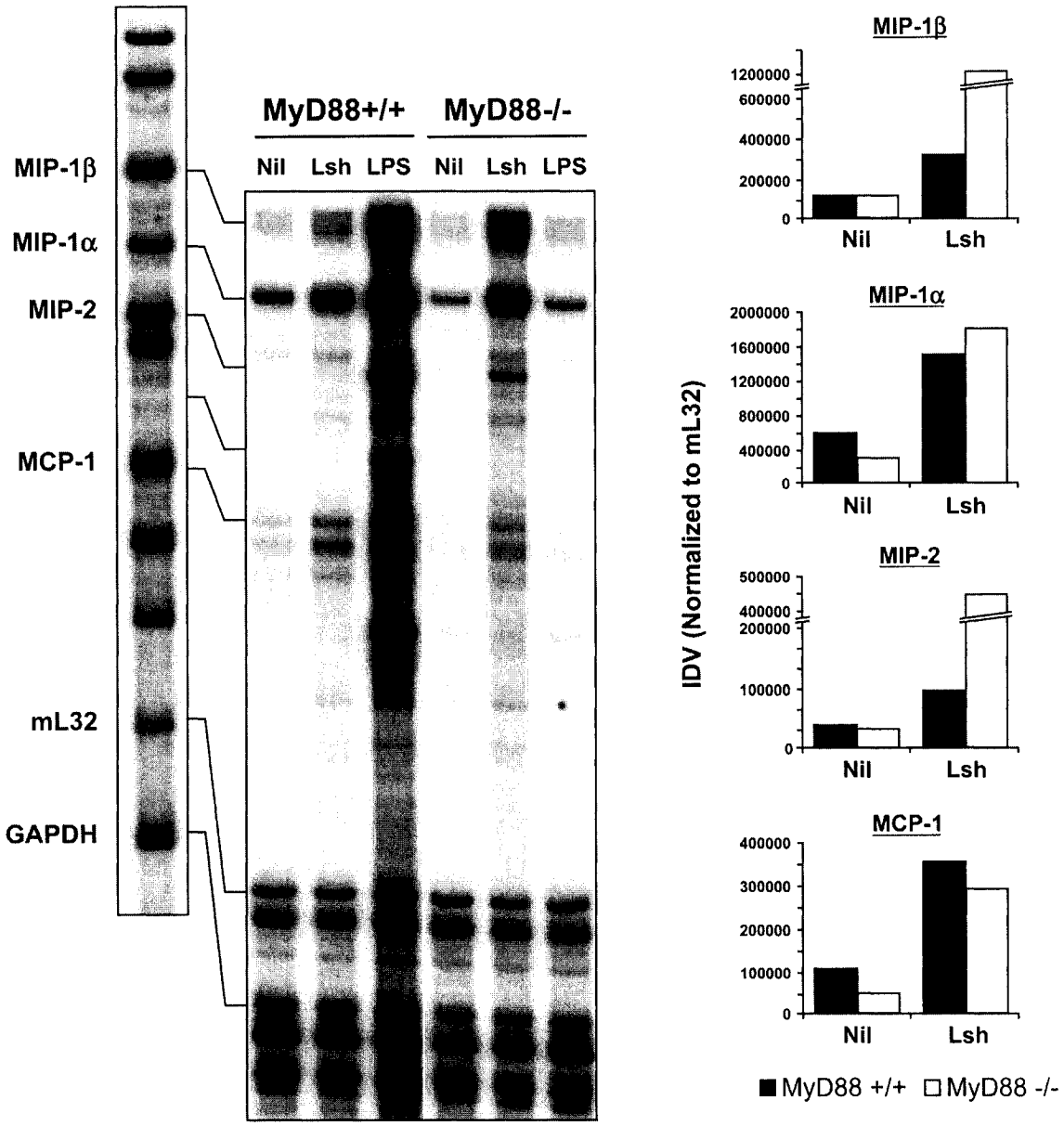


Figure 3. Induction of chemokine mRNA expression by *Leishmania* is MyD88-independent. MyD88 deficient Mϕ and their control were infected (parasite-to-cell ratio of 20:1) for 2 h and chemokine gene expression was monitored by RPA (*left panel*). Integrated density values of chemokine mRNA levels of both cell lines normalized to mL32 (*right panel*). Results are representative of one of three independent experiments.

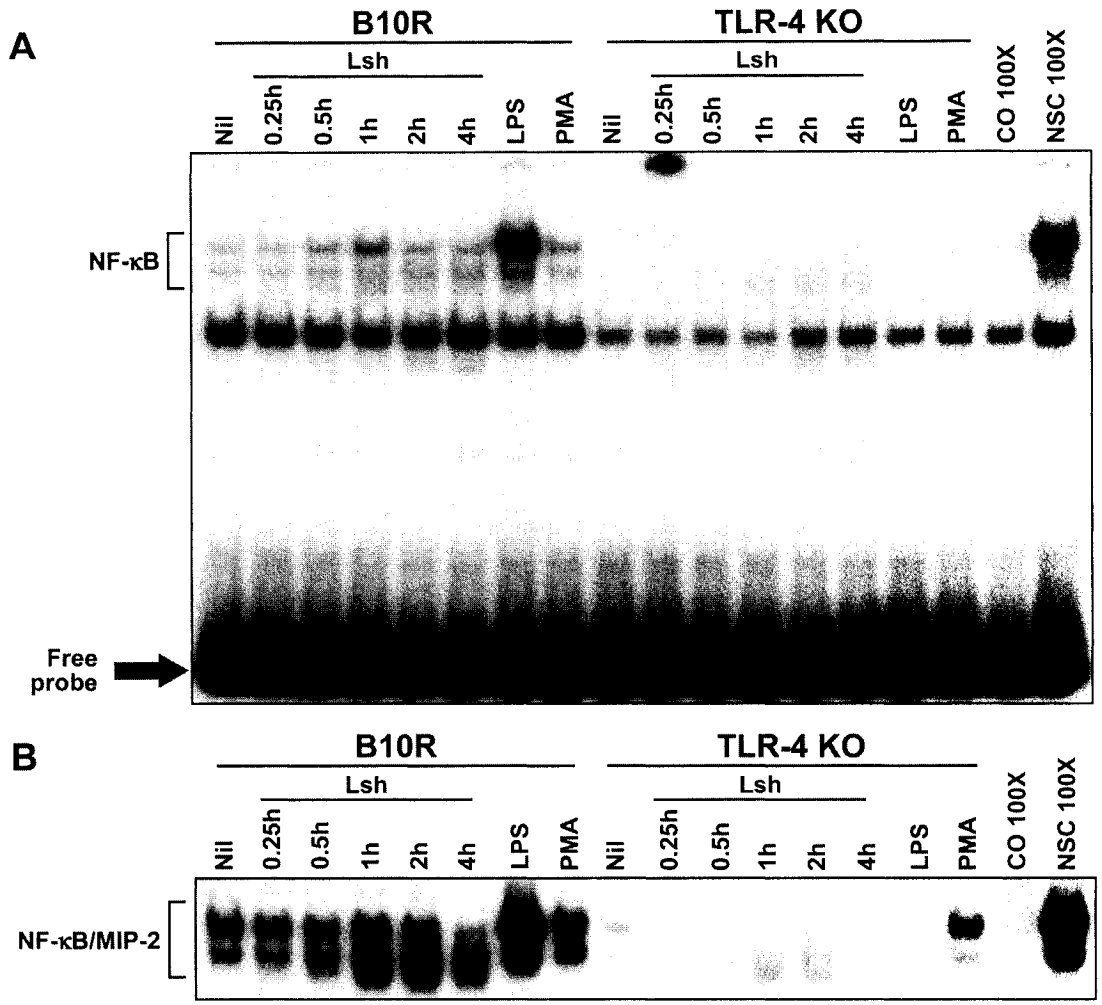


Figure 4. *Leishmania*-induced NF-κB nuclear translocation is TLR4-dependent. *A*, Nuclear extracts from TLR4 KO and B10R Mϕ stimulated for different time periods (0-4 h) were incubated with a [γ-^{32}P]-labeled NF-κB consensus probe, and subjected to EMSA. Binding specificity was tested by adding to nuclear extracts from 2-h-LPS-treated B10R Mϕ a 100-fold molar excess of either a cold NF-κB oligonucleotide (CO 100X) or a non-specific Oct2A competition (NSC). *B*, EMSA analysis was performed as described in *A*, but this time nuclear extracts were incubated with a NF-κB probe specific for the murine MIP-2 promoter. Binding specificity was tested by adding to nuclear extracts from 2-h-LPS-treated B10R Mϕ a 100-fold molar excess of cold NF-κB oligonucleotide specific for MIP-2 promoter or a non-specific Oct2A probe. Results are representative of one of three separate experiments.

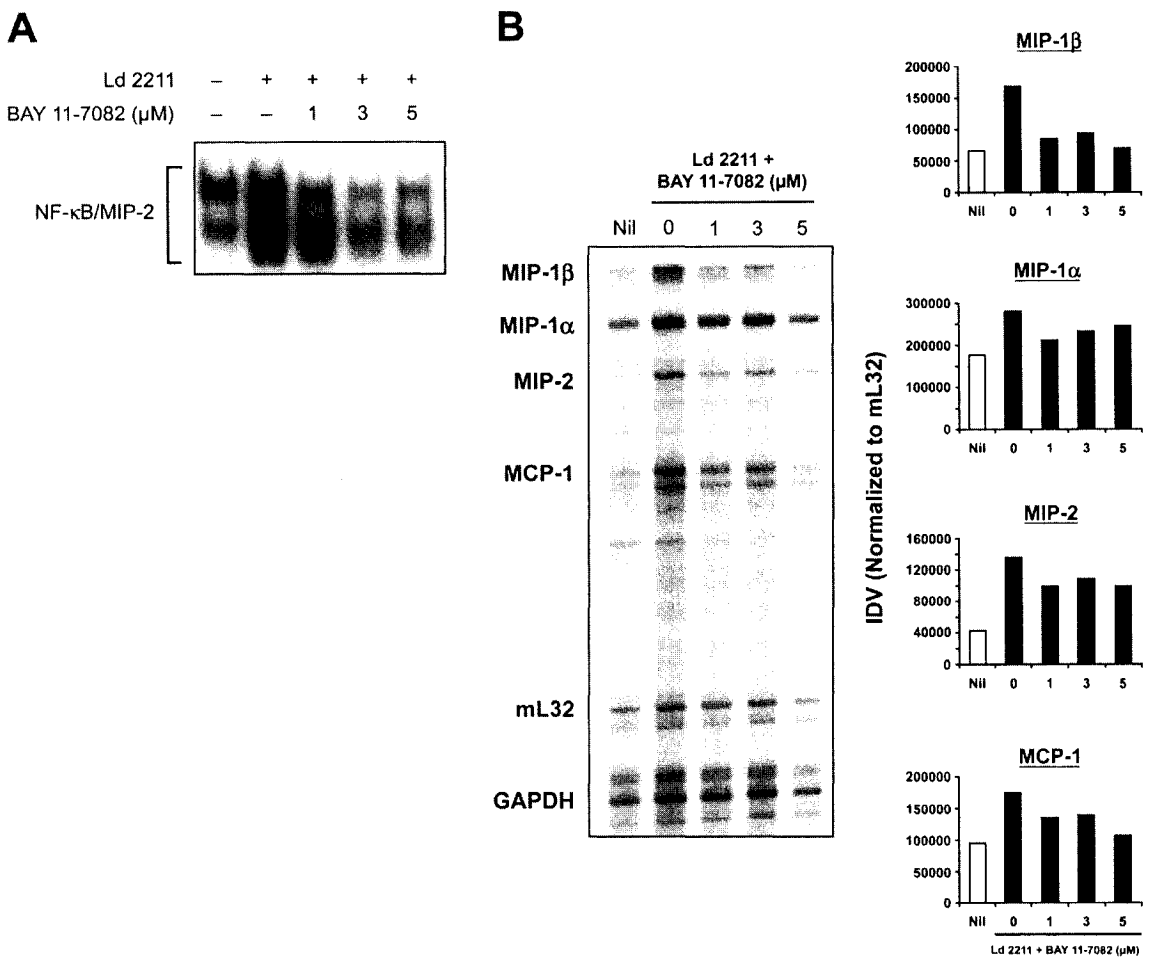


Figure 5. Role of NF-κB on Mϕ chemokine mRNA expression in response to *Leishmania*. *A*, Nuclear extracts form cells treated with BAY 11-7082 (1 h) before stimulation by *Leishmania* (2 h) were subjected to EMSA by using a probe containing a NF-κB binding site in the murine MIP-2 promoter. *B*, Total RNA was extracted from Mϕ stimulated as described in the previous experiment and chemokine mRNA modulation was monitored by RPA (*left panel*). Integrated density values of chemokine mRNA expression normalized to mL32 (*right panel*). Nil: untreated (*open bar*), *Leishmania* ± BAY 11-7082 (*solid bars*). These results are representative of one of three separate experiments.

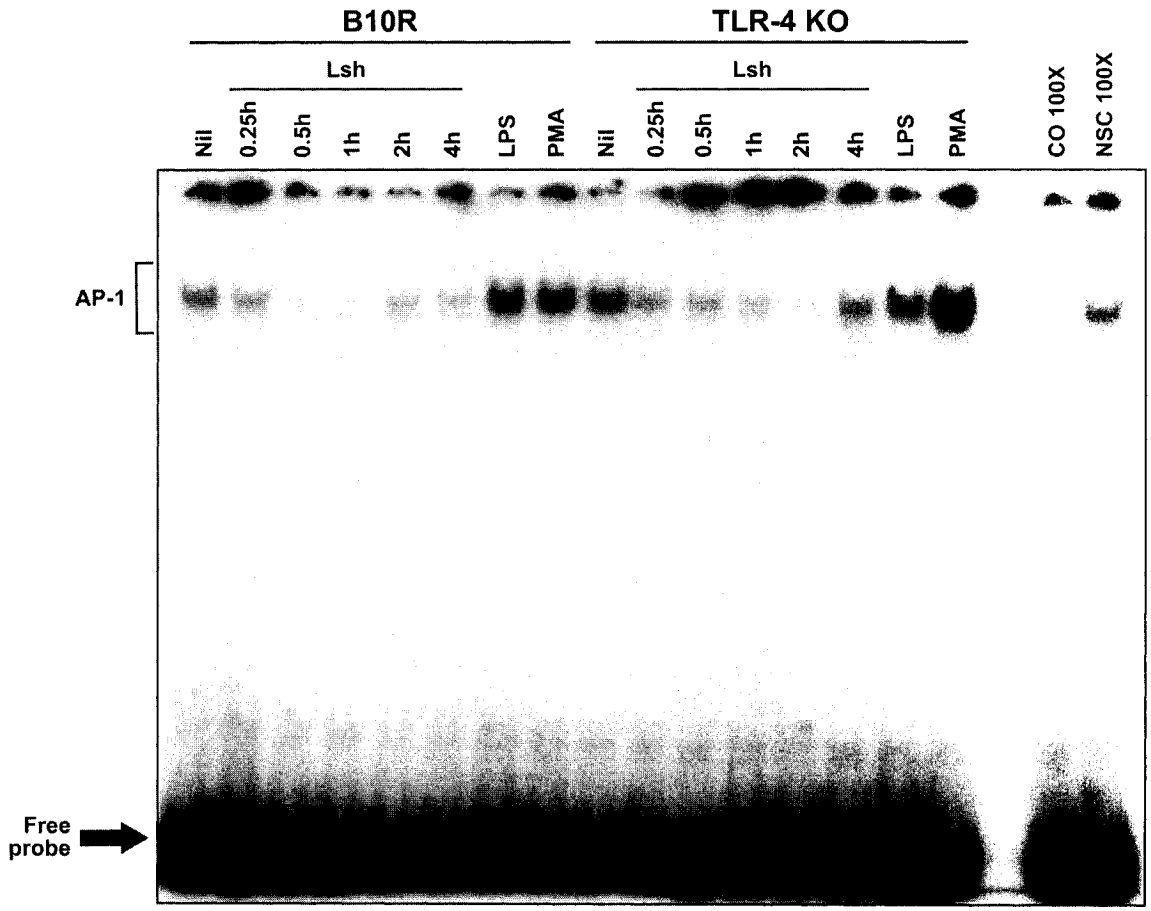
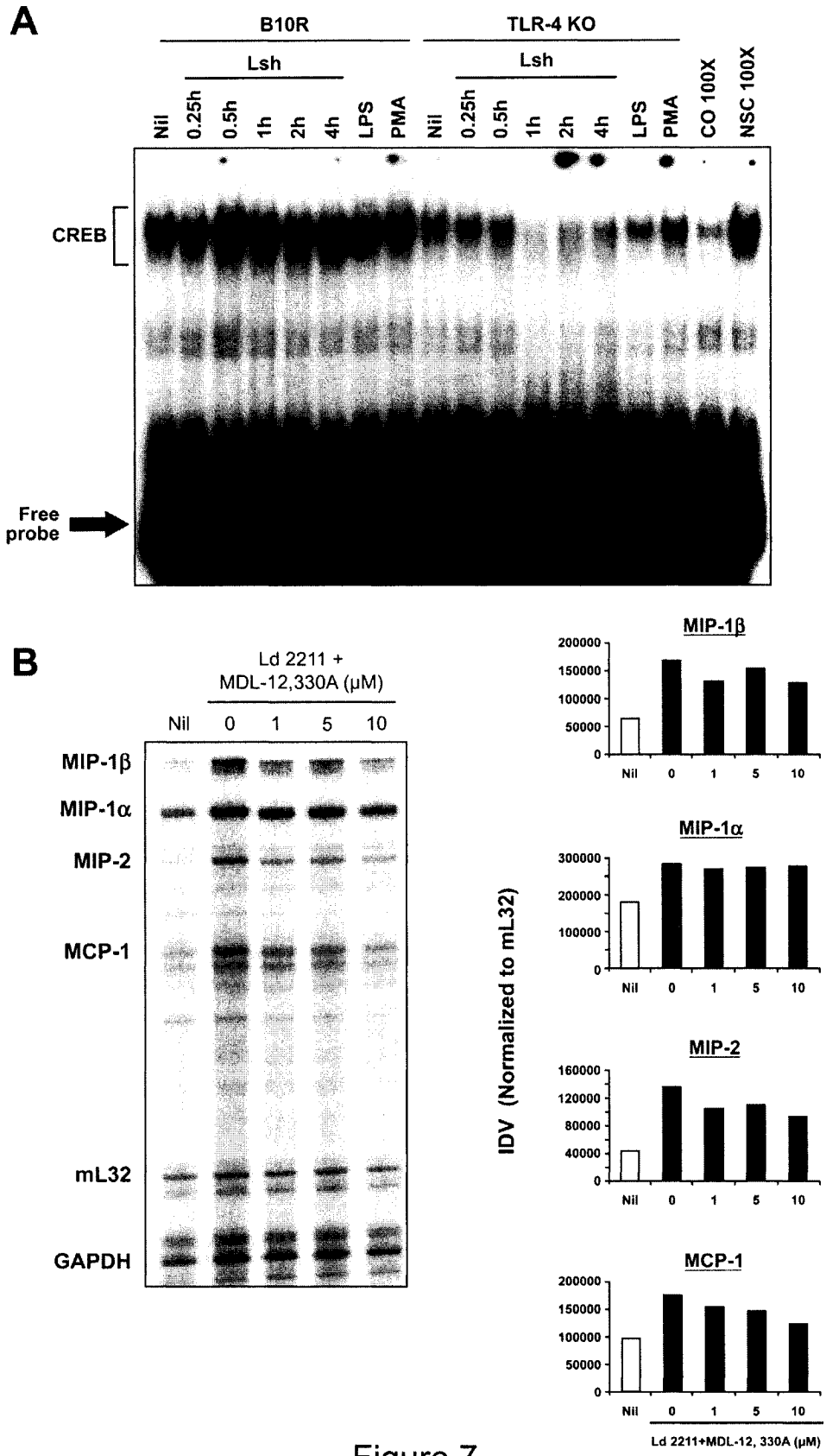


Figure 6. Down-modulation of AP-1 nuclear translocation by *Leishmania* is TLR4-independent. Nuclear extracts from TLR4 KO and B10R Mϕ, stimulated for different time periods (0-4 h), were incubated with a γ-^{32}P-labeled AP-1 probe, and were subjected to EMSA. Binding specificity was tested by adding to nuclear extracts from 2-h-LPS-treated B10R Mϕ a 100-fold molar excess of either a cold AP-1 oligonucleotide or a non-specific Oct2A probe. These results are representative of one of three independent experiments.

Figure 7. *Leishmania*-induced CREB DNA binding activity is TLR4-dependent. *A*, Time course of CREB binding in response to *Leishmania* (0-4 h), in either B10R or TLR4 KO Mϕ, was monitored by EMSA using a [γ-^{32}P]-labeled CREB probe. *B*, Following MDL-12,330A treatment (1 h) and *Leishmania* infection (2 h), total RNA was extracted from B10R Mϕ and chemokine mRNA modulation was monitored by RPA (*left panel*). Integrated density values of chemokine gene expression normalized to mL32 (*right panel*). Nil: untreated (*open bar*), *Leishmania* ± MDL-12,330A (*solid bars*). These results are representative of one of three independent experiments.


A
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TLR-4 KO
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0.25h
0.5h
1h
2h
4h
LPS
PMA
CO 100X
NSC 100X
CREB
Free probe
B
Ld 2211 + MDL-12,330A (μM)
Nil
0
1
5
10
MIP-1β
MIP-1α
MIP-2
MCP-1
mL32
GAPDH
IDV (Normalized to mL32)
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MIP-1α
MIP-2
MCP-1
Ld 2211+MDL-12, 330A (μM)

Figure 7

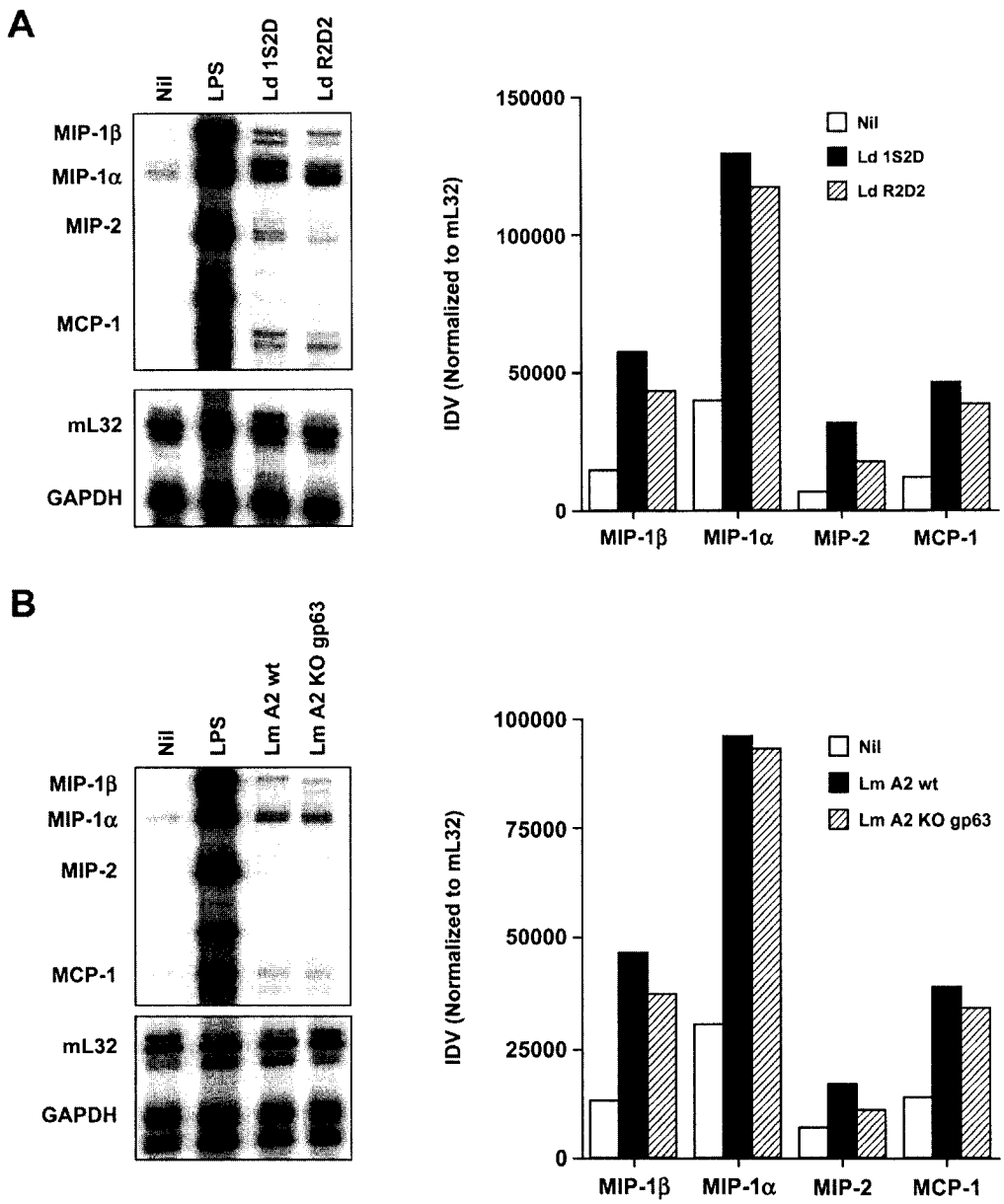

Figure 8. Role of LPG and gp63 on the induction of Mϕ chemokine gene expression by *Leishmania.* Cells were stimulated with Ld R2D2 (A) or Lm A2 KO gp63 (B), deficient in LPG and gp63 respectively, along with their respective wild type counterpart. Total RNA was extracted and chemokine mRNA expression was monitored by RPA (*left panels*). Densitometric quantification of chemokine mRNA levels over negative control after normalization to mL32 (*right panels*). Results are representative of one of three separate experiments.

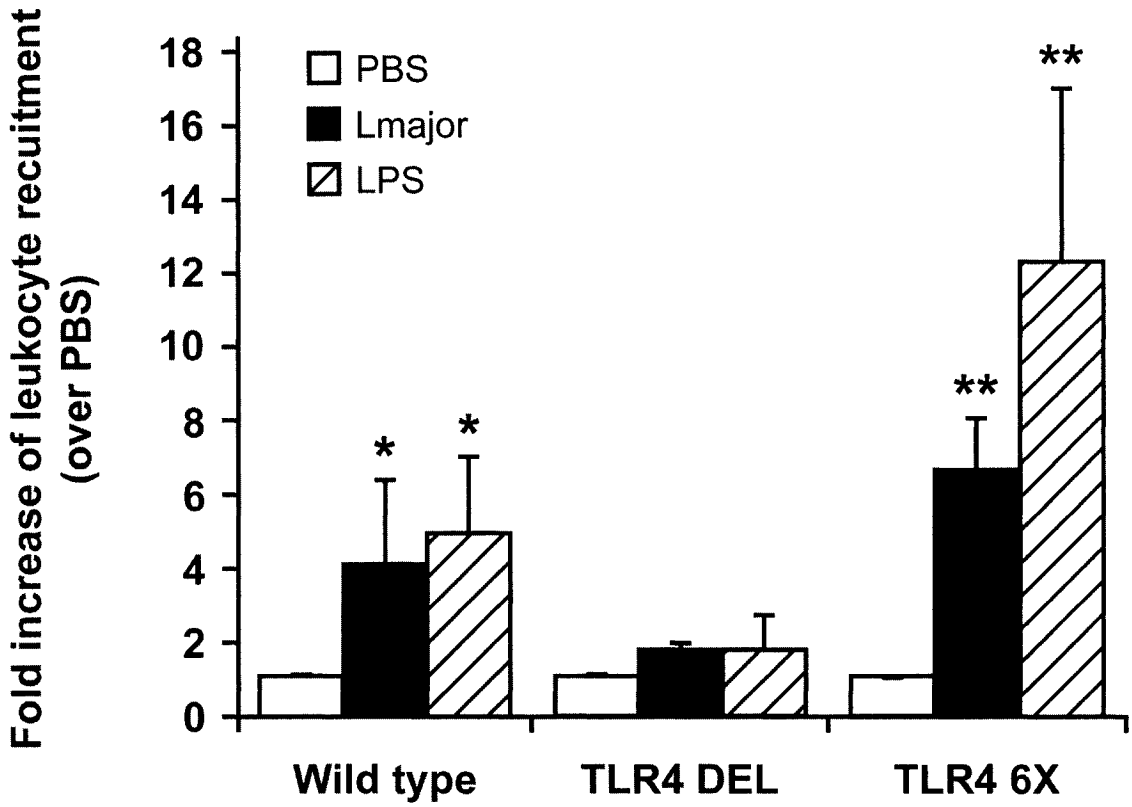

Figure 9. TLR4 involvement in *Leishmania*-induced leukocyte recruitment. Leukocyte accumulation in the air pouch of wild type, TLR4 DEL and TLR4 6X mice in response to *L. major* ($1X10^7$/ml), LPS (10 μg/ml) or endotoxin-free PBS (1 ml) after 6 h. Values are expressed in fold increase over PBS for each mouse strain. Six-hour LPS- and PBS-treated mice were used as positive and negative controls, respectively. Total cell counting was performed directly by using a hemacytometer. Results represent mean + SD of four to five mice. *, $p < 0.02$, *L. major*/LPS vs. PBS; **, $p < 0.001$, *L. major*/LPS vs. PBS. Data are representative of one of two independent experiments.

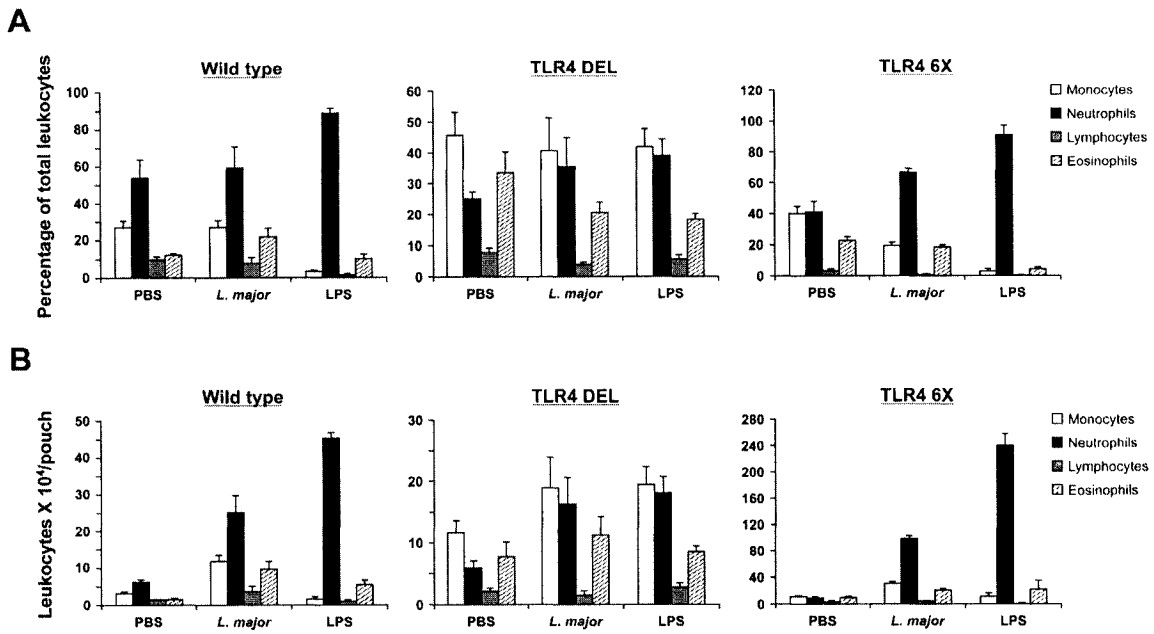

Figure 10. Differential leukocyte recruitment induced by *Leishmania*. *A*, Percentage of leukocyte subpopulations in the air pouch of wild type, TLR4 DEL and TLR4 6X mice in response to *L. major* ($1X10^7$/ml), LPS (10 μg/ml) or endotoxin-free PBS (1 ml) after 6 h. *B*, Number of monocytes, neutrophils, lymphocytes and eosinophils recruited in the pouch exudates, in response to the same stimuli as described in *A*. Differential cell count were performed on Wright-Giemsa stained cytospin preparations. Results represent mean + SD of four to five mice. Data are representative of one of two separate experiments.

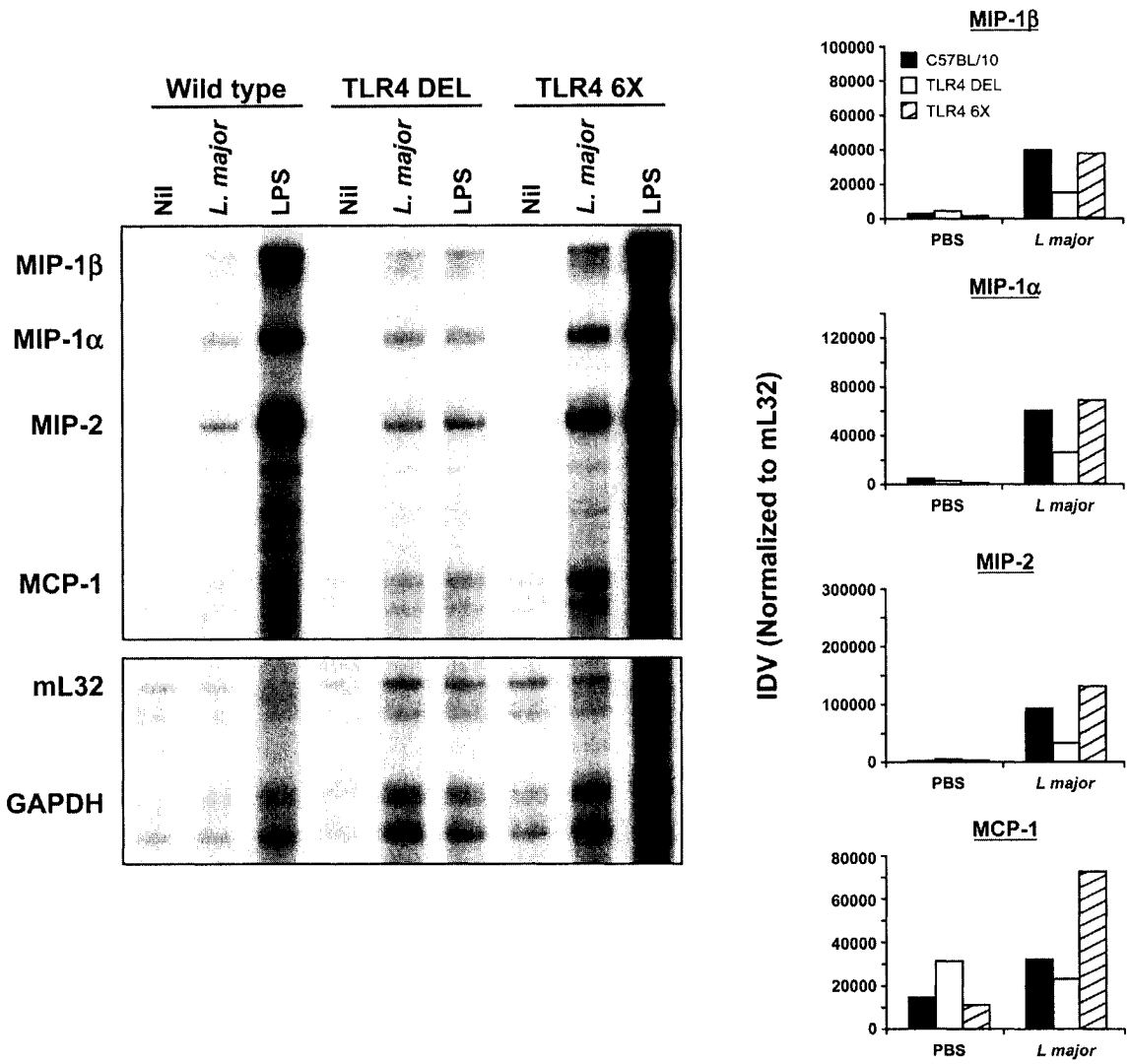

Figure 11. *Leishmania*-induced up-regulation of chemokine mRNA in the air pouch is TLR4-dependent. Following a 6-h treatment, exudates from each experimental group were pooled and total RNA was extracted from the leukocytes recruited. Chemokine mRNA levels were monitored by RPA (*left panel*). Densitometric quantification of chemokine mRNA expression over negative control after normalization to mL32 (*right panel*). Data are representative of one of two independent experiments.

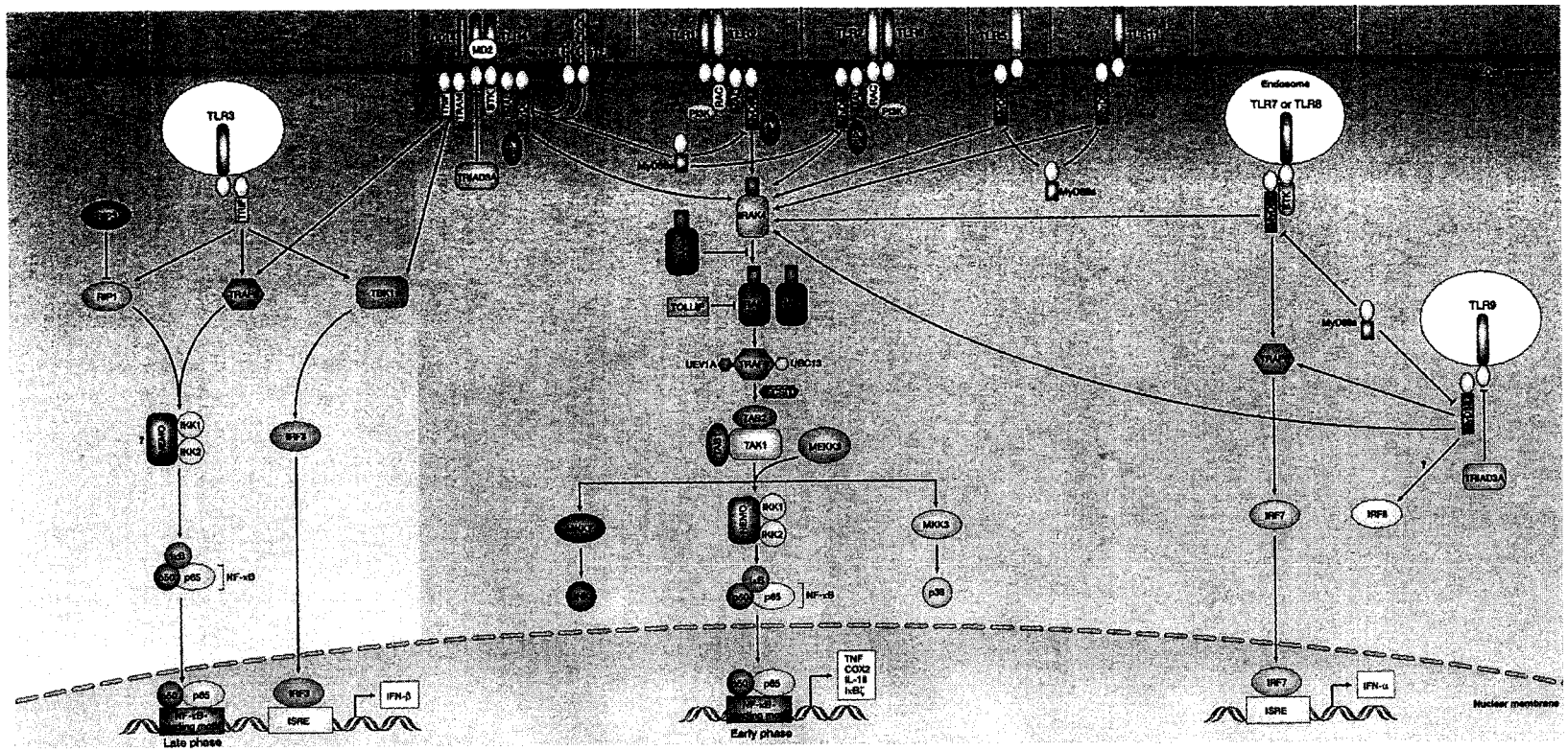

Figure 8. TLR2 and TLR4 signalling. *Taken from* (229)

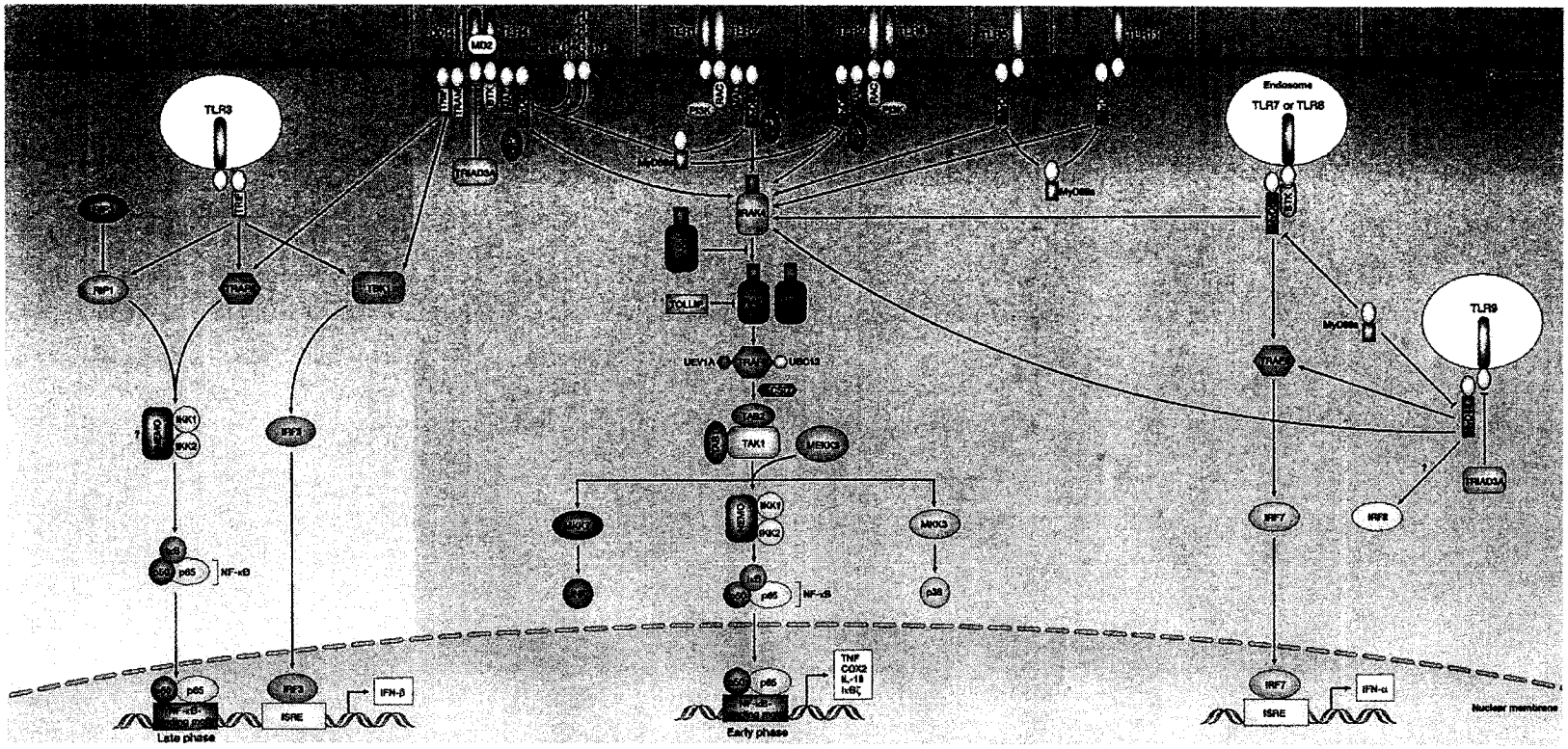

Figure 8. TLR2 and TLR4 signalling. *Taken from* (229)

CHAPTER V

GENERAL DISCUSSION AND CONCLUSION

Leishmania is a widespread parasite affecting 12 million people in a great number of country and each year new cases are reported. The increasing incidence due to wars and an increase in overseas travellers has made the need for more efficient treatment and vaccines an even bigger concern. However, before being able to find those, a better understanding of the host-parasite interact is necessary.

In order to mount the proper immune response against a pathogen, the immune system has to recognize the invader. Recognition of an outsider organism by immune cells is crucial for the activation of different signalling pathways which would eventually lead to the activation of these and other immune cells. These activated phagocytes can then phagocytose and degrade the pathogen. Previous studies have demonstrated that recognition of *Leishmania* by Mϕ can lead to the expression of different pro- and anti-inflammatory molecules such as chemokines and cytokines. Although the induction of these molecules in response to the parasite is known, the exact mechanism of recognition and signalling pathway involved in the activation of these functions is still largely unknown (10, 30, 69).

In order to identify of the possible receptors involved in the recognition of *Leishmania*, different groups have discovered that the parasite surface molecule LPG could be recognized by TLR2, leading to the activation of NF-κB, as well as the activation of NK cells (217, 228). Other groups have reported that different TLRs could be involved in the development of an immune response toward *Leishmania* infection. Using Mϕ deficient for MyD88, one group has demonstrated that *Leishmania* would activate the expression of IL-1α through the activation of the TLR adaptor protein

MyD88, which suggest the TLR involvement (227). A role for TLR was also demonstrated by Kropf et al. when they looked at the progression of *Leishmania* infection in mice deficient in TLR4. In their experiments, this group has found that TLR4 was important for the efficient control of the infection (226, 233). Although more and more evidences demonstrate the importance of TLRs in the recognition of *Leishmania*, none of these studies have yet investigated their role in the *Leishmania*-induced chemokine mRNA expression in the early time of infection.

In the present study, our goal was to determine whether TLR receptors were involved in Leishmania-induced chemokine expression and to decipher the signalling events conducting to their activation. In order to achieve this goal we used BMDM deficient in TLR2, TLR4 or in the TLR adaptor molecule MyD88 for the in vitro chemokine expression study and identification of transcription factors involved. We confirmed our findings using TLR4-deficient and TLR4-overexpressing mice.

The results from our *in vitro* study have demonstrated that the induction of chemokine mRNA expression upon *Leishmania* infection is TLR4-dependent. However, by contrast with the results for the induction of IL-1α, *Leishmania*-induced chemokine expression does not depend on the adaptor molecule MyD88. In fact, it seems that the absence of MyD88 actually lead to an increase in the level of gene expression of some of the chemokines induced by *Leishmania*. This observation could be explained by several events, one could be that recognition of *Leishmania* by Mϕ is also activating a pathway involving MyD88 who would act as a negative regulator or moderator of the response induced by the TLR4 pathway. Another possibility is that the deletion of the MyD88 gene in our cells also resulted in the deletion of MyD88s, a short spliced variant of MyD88. Research on MyD88s have demonstrated that this molecule is involved in the negative regulation of TLR signalling; however, no studies have tested if it is involved in the negative regulation of TLR-dependent but MyD88-independent pathways (142, 207). More research on these hypothesis would have to be done in order to clarify which negative regulation mechanism is involved in our case.

The results regarding the role of TLR4 in the *Leishmania*-induced chemokine mRNA expression was further demonstrated *in vivo*. The *in vivo* experiments were performed using wild type, TLR4-deficient and TLR4-overexpressing mice in the air pouch model. Comparison of the level of chemokine gene induction in the recruited cells, as well as the number of leukocytes recruited to the pouch enabled us to assess the role of TLR4. These data revealed that TLR4 was indeed playing an important part in the induction of chemokine mRNA, which is crucial for the cellular recruitment induced following infection by *Leishmania*. Analysis of the *in vivo* results has also shown that MIP-2 was the chemokine which has the highest induction following infection, and this induction was strongly inhibited in absence of TLR4. These data are very interesting because they correlate nicely with the observation that Nϕ are the major leukocyte subfamily recruited in the air pouch, and it is common knowledge that these cells are chemoattracted by MIP-2. Worth mentioning is that Lima *et al.* have demonstrated that Nϕ are very important for the control of *Leishmania* infection since they are able to ingest and degrade the parasites (234). The same important role for Nϕ was demonstrated by our team in SHP-1-deficient mice, in this study the high recruitment of Nϕ was correlated with almost total abolition of *L. major* infection and footpad swelling (63). This could mean that since the mice overexpressing TLR4 exhibit a higher recruitment of Nϕ, they would control the infection more efficiently than the wild type mice; however, other studies have also demonstrated that a high recruitment of Nϕ could sometime result in an increase of inflammation which would exacerbate the disease (128, 235). Given that recruitment of a high number of Nϕ can result in either fast clearing of the infection, or in exacerbation of the disease, it would be necessary to do the follow up of the progression of *Leishmania* infection in the three different types of mice in order to really understand the role played by the TLR4-dependent Nϕ recruitment.

We also performed EMSA experiments with the deficient Mϕ in order to identified which transcription factors could be involved in the TLR signalling. The results obtained demonstrate that NF-κB and CREB are both involved in the TLR4-dependent *Leishmania*-induced chemokine mRNA expression since their activation was significantly

reduced in absence of TLR4. These data were confirmed using inhibitors prior to *Leishmania* infection and monitoring of chemokine RNA expression by RPA. Although an inhibition of the induction of some chemokine could be seen, neither the use of the NF-κB nor the CREB inhibitor resulted in the complete inhibition of the induction of the chemokine. These data suggest that other transcription factors could be involved in this signalling mechanism, therefore more study should be performed in order to identify these transcription factors. Since previous studies have reported that one of the transcription factor that could be activated in TLR4-dependent, MyD88-independent pathways is IRF3, this could be a good starting point for our investigation (232). Furthermore, members of the STAT family and C/EBP could also be tested since different groups have reported their role in the transcription of some chemokines (236-239). After the identification of potential transcription factors, confirmation of their effect on *Leishmania*-induced chemokine mRNA expression would have to be conducted using specific inhibitors and RPA analysis would have to be done.

In conclusion, we have demonstrated that *Leishmania*-induced chemokine mRNA expression was dependent on TLR4, but not for the TLR adaptor molecule MyD88 that was found not to be involved in this cellular activation. The chemokine gene induction was found to correlate with the recruitment of specific leukocyte subpopulations to the site of infection and this particular recruitment was also dependent on the presence of TLR4. Moreover, our *in vitro* results showed that both NF-κB and CREB were involved in the signalling pathway leading to the induction of chemokine genes, and their activation was also dependent on the presence of TLR4. Although these discovery are of a great interest and bring new insights necessary for a better understanding of the *Leishmania*-host interaction, details remains to be clarified. The signalling pathway following the activation of TLR4 until the activation of the different transcription factors is still largely unclear and will have to be characterized in order to know everything that is involved and to what extent. It is also unclear what is the exact impact of the activation of TLR4; leading to the expression of chemokines and leukocyte recruitment; on the outcome of the disease, as well as on the parasite survival, and these are crucial elements that need to be understood.

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APPENDIX:

COMPLIANCE CERTIFICATES