ABSTRACT

Title of Dissertation: CHARACTERIZATION OF THE ROLE OF MAPKS IN *LEISHMANIA* INFECTED MACROPHAGES.

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In the current study, we examined the role of the Mitogen Activated Protein Kinases (MAPKs) on the biological responses of macrophages infected with *Leishmania*. The first section examined the role of MAPK/ERK in IL-10 production by *Leishmania*-infected macrophages. The macrophage-derived IL-10 has been shown to exacerbate Leishmaniasis. However, the molecular mechanisms whereby Leishmaniasis prompts IL-10 induction are poorly understood. A combination of two signals was necessary for IL-10 induction by the *Leishmania* amastigotes-infected macrophages. The first signal is mediated by TLR ligation whereas the second signal is mediated by FcγR ligation, which yields a population of regulatory macrophages that produce high levels of IL-10. Infection of macrophages with *L. amazonensis* amastigotes from the lesion sites sparked MAPK/ERK activation, which was required, but not sufficient for IL-10 induction. In combination with an inflammatory stimulus, LMW-HA from the extracellular matrix, these parasites triggered the macrophages to highly produce IL-10. MAPK/ERK

activation initiated an epigenetic modification of chromatin at the IL-10 locus, which allowed for transcription factor Sp1 binding to drive IL-10 transcription and subsequent production. U0126, an inhibitor of MAPK/ERK activation, decreased lesion progression in *Leishmania* infected mice.

The second section examined the role of MAPK/p38 in cytokine production and vaccination against Leishmaniasis. TLR agonists activate macrophages to produce pro-inflammatory cytokines and reactive oxygen intermediates. Inhibition of MAPK/p38 reciprocally increased IL-12 but decreased TNFα production from LPS-stimulated macrophages, which also occurred following stimulation by a variety of other TLR agonists, and using different APCs. MAPK/p38 inhibition induced IL-12p40 mRNA accumulation mainly due to enhanced mRNA stability, which was independent of IL-10. Similar results were observed by knocking down MAPK/p38 using specific siRNAs or by targeted deletion of MKK3. IL-12 production following the inhibition of MAPK/p38 skewed antigen-specific T cells to produce more IFN-γ and less IL-4 in vitro. A MAPK/p38 inhibitor was applied as an adjuvant to vaccine mice against *L. major*, which resulted in smaller lesions with fewer parasites.

Our findings reveal an important role of MAPKs in the *Leishmania* pathogenesis, and suggest that the manipulation of these kinases may provide novel therapeutics for potential clinical applications.

CHARACTERIZATION OF THE ROLE OF MAPKS IN *LEISHMANIA* INFECTED MACROPHAGES

By

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DEDICATION

This dissertation is dedicated to the people who love me, care about me and have always been there for me!

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LIST OF ABBREVIATIONS

ADCC	Antibody dependant cell-mediated cytotoxicity
AP-1	Activator protein-1
APC	Antigen presenting cell
ATF	The transcription factors activating transcription factor
ARE	AU-rich element
AUF1	AU-rich element binding factor 1
BCR	B-cell receptor
BMDC	Bone marrow derived dendritic cells
BMMϕ	Bone marrow derived macrophages
cAMP	Cyclic adenosine monophosphate
Calcr	calcitonin receptor
C/EBP	CCAAAT/enhancer-binding protein
c-Fos	Cellular FBJ murine osteosarcoma viral oncogene homolog
ChIP	Chromatin immunoprecipitation
CHOP	cAMP response element-binding protein-homologous protein
CK2	Casein kinase 2
CL	Cutaneous leishmaniasis
c-Maf	Musculoaponeurotic fibrosarcoma oncogene homolog
c-Myc	Cellular v-myc myelocytomatosis viral oncogene homolog
Cox-2	Cyclooxygenase-2
CREB	cAMP responsive element binding protein

CTL	Cytotoxic T-lymphocytes
DC	Dendritic cells
dsRNA	double-stranded ribonucleic acid
4E-BP1	elF4E-binding protein-1
eIF4E	Eukaryotic translation initiation factor 4E
ELISA	Enzyme-linked immunosorbent assay
Elk-1	Ets like gene1
ETS	E26 transformation-specific
FcR	Fc (fragment crystallizable region) receptor
FITC	Fluorescein isothiocyanate
FIZZ1	Found in inflammatory zone-1
Foxp3	Forkhead box P3
GA12	GATA sequence in the IL-12 promoter
GADD153	Growth arrest DNA damage 153
GAP12	GA12-binding protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GATA-3	GATA binding protein 3
GM-CSF	Granulocyte/macrophage colony-stimulating factor
GPCRs	G-protein-coupled receptors
GPI	glycosylphosphatidylinositol
HMG-14	High-mobility group protein 14
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HRP	Horseradish peroxidase

HSP	Heat shock protein
HuA-D/R	RNA-binding protein homologous to human A-D or R antigen
ICSBP	IFN-consensus sequence-binding protein
IFN	Interferon
γ-IRE	IFN-γ-response element
Ig	Immunoglobulin
IκB	Inhibitor of kappa B
IKK	IκB kinase
IL	Interleukin
IL-10R	IL-10 receptor
IL-12R	IL-12 receptor
iNOS	Inducible nitric oxide synthase
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factor
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
Jak	Janus kinase
KSRP	K homology-type splicing regulatory protein
LIGHT	Homologous to lymphotoxins, shows inducible expression and competes with herpes simplex virus glycoprotein D for herpes virus entry mediator (HVEM)/TNF-related 2.
LMW-HA	Low-molecular weight hyaluronic acid

LRR	Leucine-rich repeat
LPG	Lipophosphoglycan
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
Ly6C	Lymphocyte antigen 6 complex
MAL	MyD88-adaptor like
MAPK	Mitogen activated protein kinase
MAPKAPK (MK)	MAPK activated protein kinase
MAPK/ERK	Extracellular signal-regulated kinases
MAPK/JNK	c-Jun NH2-terminal kinase
MCL	Mucocutaneous leishmaniasis
MCP-1	Monocyte chemotactic protein-1
M-CSF	Macrophage colony stimulating factor
MEF	Myocyte enhancer factor
MEK	MAP/MAPK/ERK kinase kinase
MHC class-II	Major histocompatibility complex class-II
MNK	MAPK-intergrating kinase
MOI	Multiplicities of infection
MSK	Mitogen- and stress- activated protein kinase
mTOR	Mammalian target of rapamycin
MyD88	Myeloid differentiation primary response gene 88
NES	Nuclear export signal
NF-AT	Nuclear factor of activated T- cells

NF-κB	Nuclear factor-κ B
NK Cells	Natural killer cells
NKT Cells	Natural killer T cells
NLS	Nucleus localization signal
NO	Nitric oxide
OVA	Ovalbumin
PAMP	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PGE2	Prostaglandin E2
PI3K	Phosphoinositide 3-kinases
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PMN	Polymorphonuclear neutrophil granulocytes
PRAK	The MAPK/p38-regulated/activated protein kinases
PRR	Pattern recognition receptors
qRT-PCR	Quantitative-real-time PCR
RELMα	Resistin-like molecule-α
RIG-I	Retinoic acid inducible gene-1
ROI	Reactive oxygen intermediates
RSK	Ribosomal protein S6 kinase
S10	Serine 10

SCM	Schneider’s complete medium
SH2	Src homology 2
SHIP	SH2-containing inositol phosphatase
siRNA	Small interfering RNA
SLA	Soluble *leishmania* antigen
SOCS3	Suppressor of cytokine signaling 3
Sp1	Specificity protein 1
SRF	Serum response factor
STAT	Transducers and activators of transcription
SYK	Spleen tyrosine kinase
TAB	Transforming growth factor-β-activated protein kinase- 1 binding protein (TAK1 binding protein)
TAK1	Transforming growth factor-β-activated protein kinase 1
TCR	T-cell Receptor
TGFβ	Transforming growth factor β
Th	T-helper lymphocyte
TIA-1	T-cell restricted intracellular antigen-1
TIAR	TIA-1-related protein
TIR	Toll/Interleukin-1 receptor
TICAM	TIR domain-containing adaptor molecule
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α

TRAF	TNF receptor-associated factor
TRAM	TRIF-related adaptor molecule
Treg	Regulatory T-cell
TRIF	TIR domain-containing adaptor inducing IFN-β
TSC1	Tumor-suppressor proteins hamartin
TTP	Tristetraprolin
Tyk2	Tyrosine kinase 2
UTR	Untranslated region
VL	Visceral leishmaniasis
WHO	World Health Organization
Zc3h12a	Zinc finger

CHAPTER 1: INTRODUCTION

Part 1: *Leishmania* Infection and Host Defense

I. *Leishmania*

Leishmaniasis is an infectious disease with worldwide prevalence. The World Health Organization (WHO) estimates that about 12 million symptomatic and asymptomatic humans are infected by *Leishmania* in 88 countries. Most of the affected countries are in the tropics or subtropics. Leishmaniasis is found in the areas of America - from Mexico to northern Argentina (not in Uruguay, Chile, or Canada), southern Europe, Asia (not in southeast part of Asia), the Middle East, and Africa (particularly East and North Africa). More than 90 percent of visceral leishmaniasis (VL) cases are found in India, Bangladesh, Nepal, Sudan, and Brazil.

Leishmania Life Cycle

Leishmania exists in two developmental forms: promastigote and amastigote. When a sandfly takes a blood meal from an infected patient, parasites are released from infected macrophages and differentiate from intracellular amastigotes to extracellular procyclic promastigotes that resides in the digestive tract of the sandfly (Sacks *et al.*, 1993). Then in the sandfly's gut the immature procyclic promastigote undergoes a transformation to a form that is infectious to mammalian macrophages, called the metacyclic promastigote. This form is morphologically distinct from non-infective form, with an elongated, narrow body with a long flagellum more than twice the body size. The promastigote surface including the flagellum is covered with a dense surface glycocalyx,

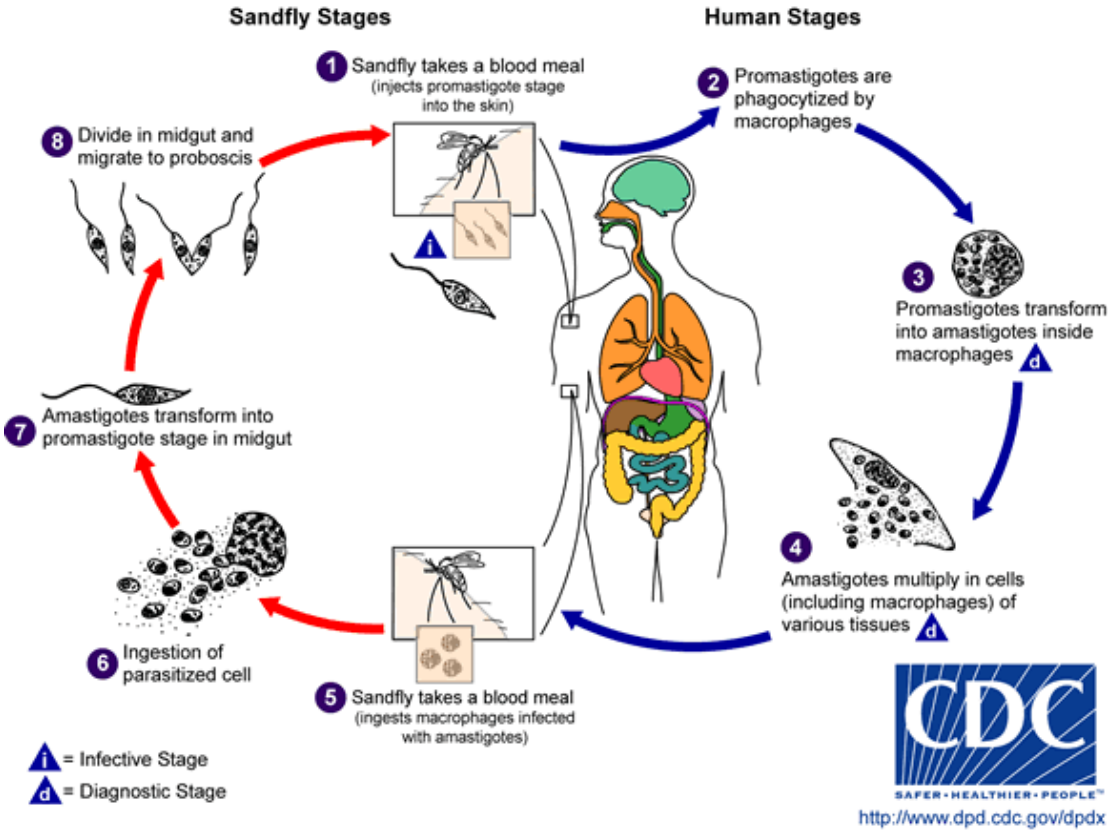

Figure 1. *Leishmania* life cycle. (1) *Leishmania* infected, female sandfly takes a blood meal and transfers metacyclic promastigotes to mammalian host. (2) Macrophage takes up promastigote. (3) Promastigote is exposed to macrophage phagolysosome. (4) Promastigote transforms to amastigote form permitting survival in phagolysosome. (5) Amastigote replicates. (6) Amastigote replication leads to macrophage lysis. (7) Uninfected sandfly takes a blood meal and ingests amastigotes. (8) In the sandfly gut, each amastigote differentiates into promastigote form preparing for eventual transfer. Adapted from the Centers for Disease Control, www.cdc.gov.

composed of molecules attached by glycosylphosphatidylinositol (GPI) anchors. The most abundant constituent is lipophosphoglycan (LPG). Procyclic promastigotes express shorter LPG and metacyclic promastigotes express longer LPG (Awasthi *et al.*, 2004). When an infected sandfly takes a blood meal, these promastigotes are injected into the mammalian host. The infective metacyclic promastigotes are taken up by phagocytic cells to rapidly transform into the amastigote form, which is non-motile and oval-shaped. Amastigotes live and replicate in the phagolysosomes of mononuclear phagocytes. The infection spreads when infected mononuclear phagocytes burst to release amastigotes into the surrounding tissue where they enter neighboring macrophages.

Leishmania are intracellular parasites that reside primarily within host tissue macrophages. Although polymorphonuclear neutrophil granulocytes (PMN) are able to internalize *leishmania* promastigotes, these cells are not considered to be host cells for the parasites due to their short life span. *Leishmania* does not enter dendritic cells (DCs) as efficiently as macrophages (Henri *et al.*, 2002), possibly due to the fact that macrophages express higher levels of surface receptors that mediate phagocytosis than the splenic DCs.

Disease Manifestation

There are three main clinical categories of Leishmaniasis: cutaneous (*L. major, L. amazoneisis, L. mexicana and L. tropica*), mucocutaneous (*L. braziliensis and L. guyanensis*) and visceral (*L. donovani, L. chagasi, and L. infantum*). According to the U.S. Centers for Disease Control and Prevention, every year there is an estimated

incidence of 1.5 million cases of cutaneous leishmaniasis (CL) and 500,000 cases of visceral leishmaniasis (VL) (www.cdc.gov/ncidod/dpd/parasites/leishmania/default.htm).

CL patients develop small skin lesions after being bitten by sand flies. The lesions will eventually heal with scarring. Mucocutaneous leishmaniasis (MCL) commences with skin ulcers, but eventually leads to extensive and disfiguring destruction of mucous membranes of the nose, mouth and throat. VL (also known as kala-azar) is the most severe form and potentially fatal if untreated. Many patients do not have symptoms for a few months after infection. Later they can manifest symptoms of fever, weight loss, damage to the spleen and liver, and anaemia.

Diagnosis, Treatment and Vaccination

The best way to identify a cutaneous infection is to take a biopsy from the edge of the lesion and analyze it microscopically for the presence of parasites. Alternatively parasites from the biopsy can be grown in culture. In addition, PCR-based methods that amplify mitochondrial (kinetoplast) DNA common to all *Leishmania* species can be used to accurately diagnose leishmaniasis. For visceral patients, blood antibodies levels can be used as diagnostic markers (Murray *et al*., 2005).

Cutaneous leishmaniasis can usually heal without any treatment. For visceral leishmaniasis, several drugs have been used in clinics. The most commonly used drug is antimony (pentavalent antimonials). Two other drugs that are more expensive include amphotericin B and pentamidine. Miltefosin, a newly developed drug for both visceral and cutaneous leishmaniasis, is more affordable. All the treatments have side effects

including nausea, pain, vomiting, anorexia, myalgia, arthralgia, headache and malaise. Unfortunately, in some patients, the parasites start to become resistant to antimony. Because of side effects and drug resistance, it is becoming urgent to develop a *leishmania* vaccine. There is no effective vaccine currently available (Schwartz *et al*., 2006).

Since *Leishmania* is intracellular parasite, the development of vaccines is mainly focused on the generation of T helper cells (Th1) immunity. Some *Leishmania* vaccine studies have shown that immunization with defined parasite antigens in combination with Th1-inducing adjuvants provides protection against several *Leishmania* species in various strains of inbred mice. Although the generation of a vaccine-induced Th1 response is necessary for protection, it may not be sufficient. Control of deactivating responses during infection has been proposed to improve vaccine success (Wilson *et al.,* 2005). Thus understanding of how host responds to *Leishmania* is helpful to design effective vaccines.

II. Host Defense against *Leishmania*

Host Defense Overview

The host immune system is composed of innate and adaptive immunity. Innate immunity provides the first lines of defense against microbes and other pathogens. This is mediated primarily by neutrophils, macrophages, DCs and natural killer cells (NK cells). Adaptive immune responses develop later, and are mediated by T and B lymphocytes as well as their products. Antigen-specific lymphocytes become activated and proliferate upon recognizing antigens. B cells produce antibodies to neutralize and

eliminate extracellular microbes. $CD4^+$ T and $CD8^+$ T cells are two major groups of T cells. Under the influence of cytokines produced from innate immune cells, naïve $CD4^+$ Th cells differentiate into Th1, Th2, Th17, or regulatory T cells (Treg). In the simplified terms, Th1 cells produce interferon-γ (IFN-γ) to promote cell-mediated immunity whereas Th2 cells are associated with humoral immunity by producing IL-4 to promote class-switching of immunoglobulin genes in B cells. CD8+ T cells can contribute to Th1 responses by producing IFN-γ, and they can destroy the cells infected with intracellular microorganisms including viruses and some bacteria. Mammalian host protection against *Leishmania* infection is mainly dependent on the development of IFN-γ producing $CD4^+$ Th1 and $CD8^+$ T cells, which trigger enhanced leishmanicidal activity by macrophages. In *Leishmania* infected mouse models, susceptible BALB/c mice are unable to control infection because of an aberrant Th2 response, while resistant C57BL/6 mice control infections by developing protective Th1 responses (Scott *et al.*, 1989; Wakil *et al.*, 1998; Fowell *et al.*, 1998; Scharton-Kersten and Scott, 1995).

T cell Responses

T cell-mediated immunity has a dominant role in human and experimental leishmaniasis. During *Leishmania* infection, specific Th cells are generated to establish an immune response that is essential to determine the outcome of the infection. Th cells develop into two major subtypes, Th1 and Th2, which mainly depend on the nature of priming during differentiation. IL-4 induces Th2 cells, whereas IL-12 induces Th1 differentiation. Th1 and Th2 cells can be distinguished by the cytokines that they secrete. Th1 cells secrete IFN-γ to activate cell-mediated immunity, whereas Th2 cells secrete

cytokines such as IL-4 that promote antibody responses. The Th1/Th2 paradigm of resistance/susceptibility to *Leishmania* mainly relates to murine infections using *L. major*. Most strains of mice (C57BL/6, C3H, and CBA) develop a self-limiting cutaneous disease when they are inoculated with *L. major*. In these mice, the infection is resolved *via* Th1 cells-mediated immunity characterized by the production of IFN-γ. IFN-γ induces the production of nitric oxide (NO) from infected macrophages to destroy the intracellular parasite. BALB/c mice are extremely sensitive to *L. major* infection. The resulting immune response is characterized by an expansion of Th2 cells and the production of IL-4, a hallmark of Th2 immunity. It has been proposed that antibody has no protective role for infection initiated by *Leishmania* (Kima *et al.*, 2000; Miles *et al.*, 2005). Previous studies suggested that antibody generated during leishmaniasis could contribute to disease progression by their activation of Fcγ receptors on the macrophages to produce a large amount of IL-10 (Miles *et al.*, 2005). IL-10 has been known to render macrophages refractory to IFN-γ activation for intracellular killing of *Leishmania*. BALB/c IL-10-deficient mice are relatively resistant to *L. major* infection (Kane and Mosser, 2001), although this resistance is significantly improved when both IL-4 receptor and IL-10 are deleted (Noben-Trauth *et al.*, 2003). The role of Th17 cells in the development of leishmaniasis has recently been assessed (Lopez *et al.*, 2009). It has been found that *L. major* infected BALB/c mice produced increased levels of IL-17 and IL-17-deficient BALB/c mice exhibited attenuated disease progress despite of typical Th2 phenotype. The role of T cells in leishmaniasis is more complicated than what we thought, and some are beneficial whereas others are detrimental.

Cytokines IL-12 and IL-10

During *Leishmania* infection, the cells of the innate and adaptive immune response will secrete a variety of cytokines, including IL-1, IL-4, IL-6, IL-10, IL-12, IL-13, IL-23, IFN-γ, and tumor necrosis factor-α (TNF-α). IL-12 and IL-10 are two important cytokines with opposite functions in maintaining immune homeostasis. IL-12 primarily drives the pro-inflammatory cell-mediated immune response whereas IL-10 mainly antagonizes inflammation.

IL-12 is produced by activated antigen presenting cells (APCs) such as DCs and macrophages (Trinchieri, 2003). IL-12p70 is a heterodimeric cytokine composed of two subunits, p35 and p40. They are encoded by two separate genes, and their expression is independently regulated at the transcriptional and posttranscriptional levels. IL-12p70 uses IL-12 receptor (IL-12R) β1 (p40) and IL-12R β2 (p35) chain for signaling. Once activated by the binding of IL-12p70, signal transduction through IL-12R induces tyrosine phosphorylation of the Janus tyrosine kinases Jak2 and Tyk2 that are respectively associated with IL-12Rβ1 and IL-12Rβ2. Activated Jak2/Tyk2 causes the phosphorylation of the cytoplasmic tails of the receptors and the recruitment of a number of the signal transducer and activator of transcription (STATs), primarily STAT4 that drives most of the effects of IL-12. STAT4 homodimers translocate into the nucleus and binds to STAT elements to induce the production of the immune response genes. IL-23, another heterodimeric cytokine from IL-12 family, has similar biological function as IL-12p70. IL-23 is composed of p19 and p40. IL-23 receptor (IL-23R) consists of the IL-12Rβ1 and the IL-23R chain.

Both of IL-12p70 and IL-23 can drive Th1 responses during T-cell activation and IFN-γ induction (Hunter, 2005). While IL-23 acts on memory CD4+T cells, IL-12p70 preferentially acts on naïve CD4+T cells. IL-12/23-induced IFN-γ in turn activates macrophages to exert enhanced microbial killing ability by forming a proinflammatory loop to increase other proinflammatory cytokine production. Recently, IL-23 is found as a potent inducer for IL-17 but not IFN-γ in murine T cells (Stumhofer *et al.*, 2007). IL-17 has been suggested as a cytokine to promote leishmaniasis (Lopez *et al.*, 2009). Thus, in contrast to IL-12p70, IL-23 is implicated in disease progression rather than eradication (Lopez *et al.*, 2009). IL-12p40 can exist in a monomeric or homodimeric forms. Monomeric p40 is induced in excess of several to 1000-fold over the other heterodimers subunits, p35 of IL-12p70 and p19 of IL-23. Redundant p40 subunits can form p80 homodimers that may work as antagonists of IL-12p70 and IL-23 in murine models because they competitively bind to the IL-12Rβ1 (Cooper and Khader, 2007). The proper balance between these IL-12p40-containing cytokines can control the development of normal or pathological Th1 immune responses. IL-12p40$^{-/-}$ mice lack of both IL-12p70 and IL-23 show deficient Th1 development, reduced DTH responses, and NK cell responses (Trinchieri, 2003). However IL-12p35$^{-/-}$ mice only have some phenotypes of IL-12p40$^{-/-}$ mice, but not all (Hunter, 2005).

Effective primary immunity against *L. major* requires IL-12-dependent production of IFN-γ from CD4^{+} T cells (Sypek *et al.*, 1993; Scharton-Kersten *et al.*, 1995). IL-12 by APCs and IFN-γ by T cells play important roles in the intracellular killing of the parasites. IL-12p40$^{-/-}$ mice were more susceptible to *L. amazoneisis* than the wild type C57BL/6 (Hernández *et al.*, 2006) At the time of infection, treatment with

recombinant IL-12 or with antibodies to IL-4, CD4, or TGF-ß has been shown to shift the immune response to Th1 profile and enable BALB/c mice to resolve *L. major* or *L. amazoneisis* infection. (Barral-Netto *et al.*, 1992; Chatelain *et al.*, 1992; Hilkens *et al.*, 1996; Sadick *et al.*, 1990).

IL-10 can be produced as a non-covalent homodimer with an apparent molecular weight of 37 kDa (Moore *et al*., 2001; Mosser and Zhang, 2008). This anti-inflammatory cytokine is produced from Th cells, Treg cells, macrophages, B cells and non-immune cells including keratinocytes, epithelial cells, and tumor cells. IL-10 receptor (IL-10R) composes of two subunits. IL-10 binds to IL-10R1 to trigger its association with IL-10R2 and subsequent activation of Jak1 and Tyk2 that are associated with IL-10R1 and IL-10R2 respectively. Activated Jak1 and Tyk2 induce phosphorylation of STAT3. Phosphorylated STAT3 forms homodimers that translocate into the nucleus and bind to corresponding *cis*-elements to regulate expression of the immune response genes, such as IL-10 itself and suppressor of cytokine signaling 3 (SOCS3) that controls the quality and quantity of STAT activation.

IL-10Rs are expressed on most cells so that a large number of diverse cells are able to respond to IL-10 (Mosser and Zhang, 2008). IL-10 exerts most of its biological function through its interactions with macrophages and DCs. IL-10 can reduce antigen presentation by down-regulation of major histocompatibility complex class-II (MHC class-II) and the co-stimulatory molecules, CD80, CD86, B7.1 and B7.2, which are required for the activation of naïve T-cells (Grütz, 2005). The other profound function of IL-10 is to inhibit the production of type-1 cytokines such as IL-1, IL-6, IL-12, INF-γ,

TNF-α and to block their actions (Donnelly *et al.*, 2004). IL-10 has been shown to rescue BALB/c mice from LPS induced endotoxemia (Howard *et al.*, 1993; Anderson and Mosser, 2002a). Being an anti-inflammatory cytokine, IL-10 is critical to maintain a balance between protecting the host from pathogen assaults and minimizing the damages caused by excessive inflammation (Reed *et al.*, 1994; Wagner *et al.*, 1994). IL-$10^{-/-}$ mice appear to be normal at birth with normal numbers and distribution of T cell, B cells and macrophages. However, IL-$10^{-/-}$ mice can develop spontaneous enterocolitis after 4–6 weeks with the appearance of adenocarcinomas at advanced stages, especially when not housed under specific pathogen-free conditions (Kuhn et al., 1993). Administration of IL-10 from birth or treatment with anti-IFN-γ or anti-IL-12 monoclonal antibody can prevent enterocolitis in IL-$10^{-/-}$ mice (Davidson *et al.*, 1998). Of note, IL-10 treatment could only ameliorate established disease without successful cure. CD4+ T cells isolated from diseased colon were found to produce high levels of IFN-γ and TNFα. Transfer of these cells could induce the host mice to develop the disease, indicating that the unbalanced interaction between enteric flora and inflammatory cells in the absence of IL-10 results in uncontrolled Th1 responses. Besides its inhibitory features, IL-10 shows stimulatory activities by co-stimulating B cells and contributes to immunoglobulin (Ig) class switching (Go *et al.*, 1990). IL-10 can enhance the expression of Fc gamma receptor (FcγR) on macrophages (Calzada-Wack *et al.*, 1996), which are correlated with enhanced phagocytosis of opsonized particles (Capsoni *et al.*, 1995) and potential pathogens.

IL-10 is important in regulating immune responses to intracellular *Leishmania* by inhibiting the production of a type 1 immune response and preventing macrophages from activation to kill the parasites. Host macrophages can also produce high amounts of IL-

10. IL-10 inhibited the activation of macrophages exposed to IFN-γ/ lipopolysaccharide (LPS) by producing less TNF-α and almost no IL-12 (Kane and Mosser, 2000). Thus, *Leishmania* parasites undergo replication within host macrophages, which eventually leads to pathology (Kane and Mosser, 2001). IL-$10^{-/-}$ BALB/c mice are relatively resistant to infection. These infected mice have reduced footpad swelling and fewer parasites in the lesions relative to wild type mice (Kane and Mosser, 2001; Buxbaum and Scott, 2005). Increase of IL-10 in the infected mice, either by the administration of exogenous IL-10 (Lang *et al.*, 2002) or the induction of endogenous IL-10 (Miles *et al.*, 2005) can exacerbate disease progression. In murine models of visceral (Murray *et al.*, 2003) and cutaneous (Chatelain *et al.*, 1999; Kane and Mosser, 2001) leishmaniasis, IL-10 contributes to disease progression. In humans, IL-10 levels were shown to directly correlate with disease severity and high levels of plasma IL-10 were found to be predictive of the development of post-kala-azar dermal leishmaniasis (Karp *et al.*, 1993). However the immune deviation is not a true predictor of disease progression in humans. For example, although ample IFN-γ is produced during *L. donovani* caused visceral leishmaniasis, infected hosts generally fail to control the disease (Kane and Mosser, 2001).

Part 2: The Role of Macrophages in *Leishmania* Infection

I. Macrophage Heterogeneity

In response to macrophage colony-stimulating factor (M-CSF) or granulocyte/ macrophage colony-stimulating factor (GM-CSF), a monocyte lineage of haematopoietic stem cells that are also the same stem cells of neutrophils, eosinophils, basophils and

mast cells generated from bone marrow undergo differentiation into monoblasts, pro-monocytes and monocytes. The differentiated mononuclear cells exit the bone marrow to enter the bloodstream. Macrophages and DCs are differentiated from those circulating peripheral blood mononuclear cells (PBMC). From the blood, they migrate into different tissues as resident cells or in response of inflammation as inflammatory cells (Zhang and Mosser, 2009).

Macrophages are phagocytic cells that are involved in the removal of apoptotic cells and cellular debris. This clearance is independent of other immune cell signaling and results in the releasing of little or no immune mediators (Kono and Rock, 2008). This daily activity is mediated by scavenger receptors, complement receptors, intergrins, phosphatidyl serine receptors and thrombospondin receptors (Erwig and Henson, 2007).

Macrophages are important immune effector cells that participate in both the innate and adaptive immune responses to eliminate pathogens. In response to environment cues, macrophages change their phenotype and physiology including alterations in the surface proteins expression and the cytokines production. Therefore, macrophages are heterogeneous cells not only with respect to biochemical marker expression but also with respect to different physiological functions. Macrophages are strongly influenced by the tissue microenvironment. Our laboratory had developed a classification of macrophage phenotypes based on the three major macrophage functions: host defense, wound healing and immune regulation (Mosser and Edwards, 2008). The activated macrophages corresponding to these three activities are: classically activated macrophages, wound-healing macrophages and regulatory macrophages (Figure 2).

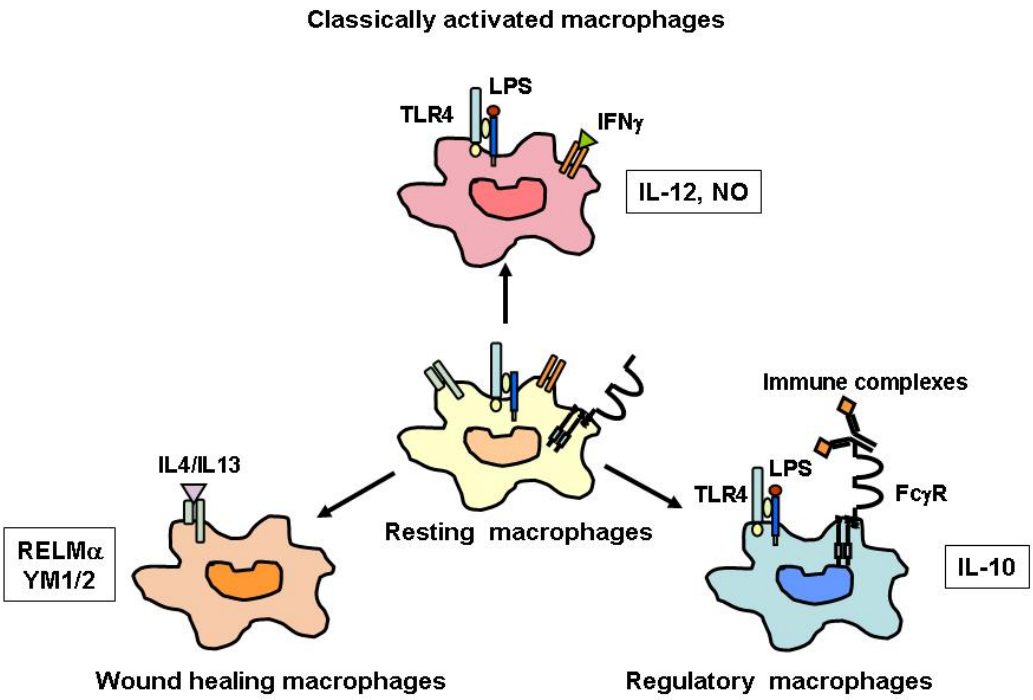

Figure 2. The classification of activated macrophages. Resting macrophages can respond to different signals and give rise to three populations. Each of these three populations has a distinct physiology. In response to IFNγ and TLR4, classically activated macrophages arise to produce high amount of IL-12 and NO. They have microbicidal activity. Wound-healing macrophages arise in response to IL-4/IL-13 and they increase RELMα, YM expression. They have a role in tissue repair. Regulatory macrophages are generated in response to LPS induced TLR4 and immune complexes mediated FcγR to produce high levels of IL-10 to suppress immune responses.

Classically Activated Macrophages

Classically activated macrophages are generated in response to stimulation with IFN-γ plus any TNFα inducer, which includes all TLR ligands (Mosser and Zhang, 2009). NK cells are important early source of innate IFN-γ production. Th1 cells and CD8+ T cells are a more stable source of IFN-γ during adaptive immune responses. In some settings, IFN-β can replace IFN-γ to activate classically activated macrophages. The role of classically activated macrophages in host defense has been well studied. Upon activation, they become effective APCs due to highly induced expression of surface molecules, such as MHC class-II and B7. These activated macrophages increase the production of superoxide anions, and nitrogen radicals, which increase their killing ability (Dale *et al*., 2008). Macrophages activated with IFN-γ were shown to be able to restrict the parasites intracellular growth. Most importantly, these macrophages produce large amounts of pro-inflammatory cytokines, IL-12 and IL-23 for Th1 differentiation. However, the excessive and uncontrolled inflammation by classically activated macrophages can lead to host–tissue damage, such as tissue destruction, chronic granuloma formation and autoimmune diseases, including inflammatory bowel disease and rheumatoid arthritis (Langrish *et al*., 2005; Szekanecz and Koch, 2007).

Wound-healing Macrophages

In contrast to the role of Th1 cells-produced IFN-γ on macrophages, Th2 cells-produced IL-4 and/or IL-13 act on macrophages to up-regulate mannose receptor that was classified as a distinctive marker of IL-4-activated macrophages together with the induction of MHC class II antigens, and those macrophages have been termed as

alternative activated macrophages (Stein *et al*., 1992) or wound-healing macrophages (Mosser and Edwards, 2008). In the innate immunity, granulocytes, such as basophils and mast cells, are the important early sources of IL-4. Th2 cells are major source of IL-4 and IL-13 in adaptive immune responses (Paul, 1991). These wound-healing macrophages increase arginase activity to convert arginine to ornithine that is a precursor of polyamines and collagen for extracellular matrix, thereby contributing to the tissue repair. These cells exhibit high expressions of mannose receptors, chitinase and chitinase-like molecules such as YM1, YM2 and resistin-like molecule-α (RELMα). Although expression of MHC class-II molecules can be found to some extent, these cells are not efficient for antigen presentation, and they may even inhibit T cell proliferation (Edwards *et al.*, 2006). These macrophages produce much less pro-inflammatory cytokines, toxic oxygen and nitrogen radicals and are ineffective at killing intracellular microbes (Munder *et al.*, 1998; Gordon, 2003). This population of macrophages is more susceptible to some intracellular infections. For example, IL-4 induced polyamine production can contribute to the intracellular growth of *Leishmania major* in macrophages (Kropf *et al*., 2005.). The uncontrolled activation of wound-healing macrophages can also lead to host tissue damage due to dysregulated fibrosis. This pathology has been observed in helminthic infections, such as chronic schistosomiasis (Hesse *et al.*, 2001).

Regulatory Macrophages

Regulatory macrophages are generated by stimulation of cells in the presence of a TLR ligand along with a second stimulus, including immune complexes, prostaglandins, G-protein-coupled receptor (GPCR) ligands, IL-10 and perhaps the phagocytosis of apoptotic cells (Mosser and Edwards, 2008). Our lab first discovered and characterized a

subpopulation of regulatory macrophages *in vitro* following their activation by two signals: LPS plus immune complexes (Mosser DM, 2003). The primary stimulus signals through one of the TLRs, whereas the second stimulus signals through the FcγRs. The second signal is accomplished by the binding of many different IgG-opsonized antigens (Anderson and Mosser, 2002a; Anderson and Mosser, 2002b). These regulatory macrophages produce high amounts of anti-inflammatory cytokine IL-10, and diminished production of the pro-inflammatory cytokine IL-12. The ratio of IL-10 to IL-12 can define regulatory macrophages. The high IL-10 production is dependent on the activation of the mitogen-activated protein kinase (MAPK)-extracellular signal-regulated kinase (ERK) signaling pathway (Lucas *et al.*, 2005). Regulatory macrophages can resolve inflammation because IL-10 can inhibit various pro-inflammatory cytokines production and activity. We hypothesize that minimizing the population of regulatory macrophages during a vaccination may be helpful for vaccine development where the induction of pro-inflammatory cytokines is required for immunity. Similar to classically activated macrophages, regulatory macrophages are effective APCs with the high expression of MHC class-II and co-stimulatory molecules CD80, CD86 and B7 (Edwards *et al.*, 2006). Previous work from this laboratory has shown that in chronic leishmaniasis, amastigotes bind host IgG to form immune complexes (Kane and Mosser, 2001; Miles *et al*, 2005). These amastigotes bind to FcγR on macrophages and induce IL-10 production. The high levels of IL-10 by these macrophages can render nearby cells refractory to the activating effects of IFN-γ preventing them from making reactive nitrogen species and killing intracellular *Leishmania* spp. (Kane and Mosser, 2001).

Both classically activated macrophages and regulatory macrophages can function as APCs to influence Th cell differentiation (Anderson and Mosser, 2002a). Classically activated macrophages stimulate T cells to primarily produce IFN-γ in response to antigen, whereas regulatory macrophages induce T cells to produce high levels of IL-4, which in turn induce B cells to produce antibody to that antigen. IL-12 secretion by classically activated macrophages induced IFN-γ production by T cells, whereas IL-10 secretion by regulatory macrophages results in IL-4 production by T cells.

In summary, macrophages exhibit remarkable plasticity in response to both innate and adaptive signals. They are functionally specialized to promote distinct physiology in host defense, wound-healing and immune regulation. Each macrophage population has the potential to be dangerous if not appropriately regulated. By manipulating different population of macrophages, we can develop therapeutic strategies to control various diseases and design better vaccines.

II. Receptors

The host is equipped with a number of pattern recognition receptors (PRRs) that are expressed on the immune cells. These receptors recognize molecules with highly conserved pathogen associated molecular patterns (PAMPs) (Janeway and Medzhitov, 2002). PAMPs are mainly identified as essential microbial components with distinct features that are not associated with host molecules. Depending on the recognition of microbes and resultant activation of intracellular signaling pathways, the innate immune system triggers and controls the major aspects of the adaptive immune response. Macrophages express various PRRs, including Toll-like receptors (TLRs), scavenger

receptors, complement receptors, C-type lectin receptors and NOD-like receptors (Zhang and Mosser, 2008). TLRs are the most well-characterized among these receptors.

Toll-like Receptors

TLRs play an important role both in innate immunity and in the development of adaptive immune responses (Janeway and Medzhitov, 2002; Akira and Takeda, 2004). TLRs have broad specificity for conserved molecular patterns shared by bacteria, viruses and parasites. TLR activation leads to signal transduction cascades for cells to trigger the production of inflammatory cytokines and the induction of antimicrobial genes. They also induce the up-regulation of co-stimulatory molecules for the adaptive immune response.

Some 13 TLRs in mice and 11 TLRs in humans have been identified to date (Akira and Takeda, 2004) (Table 1). TLRs have an extracellular region consisting of a leucine-rich repeat (LRR) motif, and a cytoplasmic tail of a Toll/IL-1 receptor (TIR) domain. The TIR domain is conserved in all TLRs except TLR3 and this domain binds to adaptor proteins to mediate TLR signaling pathways. These activating adaptors include myeloid differentiation primary response gene 88 (MyD88), MyD88-adaptor-like (MAL)/TIR domain-containing adaptor protein (TIRAP), TIR domain-containing adaptor inducing IFN-β (TRIF)/TIR domain-containing adaptor molecule-1 (TICAM-1) and TRIF-related adaptor molecule (TRAM). MyD88 is the key adaptor protein in almost all the TLRs signaling except TLR3. TLR3 mediates through TRIF/TICAM-1 adaptors. LPS induced TLR4 signaling can be transmitted through MyD88-dependent and -independent pathways (Figure 3). TRAM and TRIF are involved for TLR4-mediated MyD88 independent response.

Locations	Receptors	Ligands	Adapters
Cell surface	TLR1	Triacyl lipoprotein (bacteria and mycobacteria)	MyD88/MAL
Cell surface	TLR2	Peptidoglycan (Gram-positive bacteria); lipoarabinomannan (Mycobacteria), hemagglutinin (Measles virus), phospholipomannan (*Candida*), glycosylphosphophatidyl inositol mucin (*Trypanosoma*); lipoproteins; lipoteichoic acid; porins(*Neisseria*); fungi zymosan	MyD88/MAL
Cell compartment	TLR3	Virus double-standed RNA;poly I:C	TRIF
Cell surface	TLR4	LPS (Gram-negative bacteria); vial glycoproteins; mannan (*Candida*); hyaluronic acid; gp96; heparin sulfate; heat shock proteins; surficant protein-A; glycoinositolphospholipids; fibrinogens; β-defensin 2; HMGB1	MyD88/MAL TRIF/TRAM
Cell surface	TLR5	Flagellin	MyD88
Cell surface	TLR6	Diacyl lipoprotein(Mycoplasma), lipoteichoic acid(Streptococcus); zymosan(*Saccharomyces*)	MyD88/MAL
Cell compartment	TLR7	Virus single-standed RNA; bropirimine, imidazoquinoline and loxoribine (small synthetic compounds)	MyD88
Cell compartment	TLR8	Virus single-standed RNA	MyD88
Cell compartment	TLR9	CpG DNA(bacteria and vial); dsDNA viruses; hemozoin (Plasmodium)	MyD88
Cell surface	TLR10	Unknown (only in human)	Unknown
Cell surface	TLR11	profilin-like protein(*Toxoplasma gondi)*; uropathogenic bacteria	MyD88
Unknown	TLR12	Unknown (only in mice)	Unknown
Unknown	TLR13	Unknown (only in mice)	Unknown

Table 1: Toll like receptors (TLRs) and their ligands.

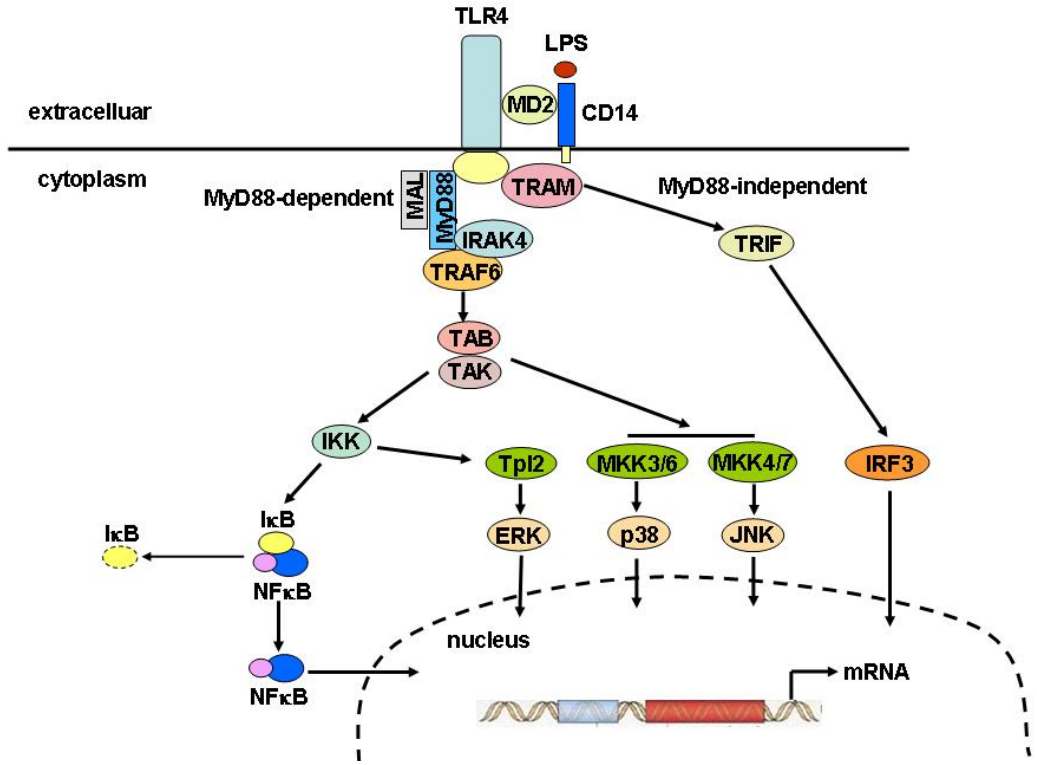

Figure 3: TLR4 mediated signaling pathways. LPS binds to the LPS coreceptors CD14 and MD2 to trigger TLR4 activation. Activation of TLR4 initiates MyD88-dependant and MyD88-independent (TRIF-dependent) signaling pathways and results in the activation of NF-κB, MAPKs and the interferon response factors (IRFs). This drives the inflammatory cytokine production.

Fcγ Receptors

The FcγRs are expressed on numerous cells of hematopoietic origin. FcγRs play critical roles in phagocytosis, clearance of immune complexes, cytokine production and the respiratory burst (Unkeless *et al.*, 1988). The FcγR is composed of an α chain that binds the Fc portion of IgG, and γ chains that are associated with one or more signal transduction regions (Nimmerjahn and Ravetch. 2008). The γ chain is a 7 kD polypeptide and is homologous to the T cell receptor's ζ chain. Four different classes of FcγRs are identified in mice with slightly different biological functions (Figure 4). Macrophages express all the four forms of FcγR on their surfaces. The FcγRI (CD64), FcγRIII (CD16) and FcγRIV, consist of a α chain, and a γ signaling chain that contains the immuno-receptor tyrosine-based activation motif (ITAM). Signaling mediated by γ chain is required for the induction of IL-10 following FcγR ligation on macrophages (Capsoni *et al.*, 1995; Sutterwala *et al.*, 1998). Mice express only the FcγRIIb, a receptor that contains a α chain with an immuno-receptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic region, while humans have two FcγRII: FcγRIIa and FcγRIIb. FcγRIIa has an ITAM containing α chain whereas FcγRIIb has an ITIM containing α chain.

FcγRI is the high affinity receptor specific for monomeric IgG. In human cells, this receptor binds IgG1 and IgG3 tightly, while in the mouse, the preferred monomeric IgG is IgG2a. Both FcγRII and FcγRIII are low affinity receptors that bind poorly to monomeric IgG, but can bind IgG coated antigens. FcγRIV is a newly identified receptor and its expression is restricted to myeloid cells. It exclusively binds IgG2a and IgG2b

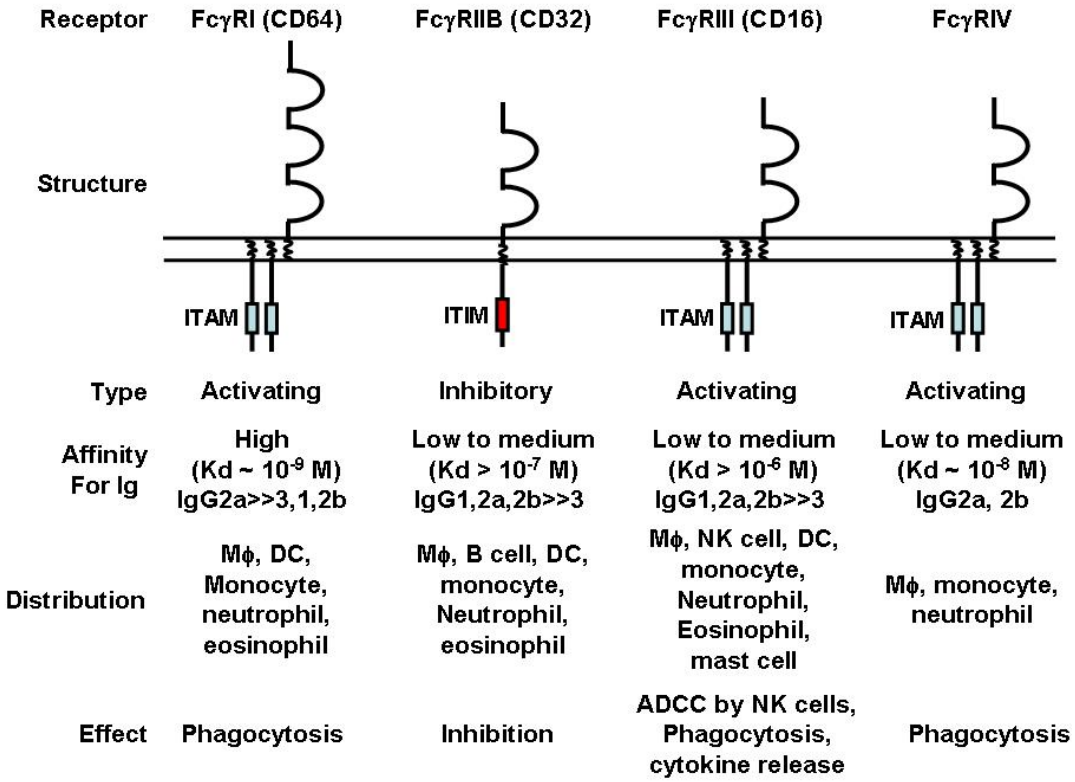

Figure 4: Schematic Presentation of Murine FcγRs.

monomers or immune complex less effectively than FcγRI but stronger than both FcγRII and FcγRIII (Nimmerjahn *et al.*, 2005).

Antibodies specific to *Leishmania* are prominent in human visceral leishmaniasis and they are also produced in BALB/c mice during infection (Junqueira *et al.*, 2003; Ghosh *et al.*, 1995; Casato *et al.*, 1999; Jeronimo *et al.*, 2000; Galvão-Castro *et al.*, 1984; Elassad *et al.*, 1994). In humans and mice, the levels of IgG increase as disease progresses and parasites disseminate (Iniesta *et al.*, 2008). Instead of providing protection, IgG antibodies to *Leishmania* actually made *L. major* infections worse in BALB/c mice (Miles *et al.*, 2005). Mice lacking B cells actually had smaller lesions with fewer parasites (Smelt *et al.*, 2000). The increase in disease severity is mainly due to an increase in IL-10 production. It has been shown that *Leishmania* amastigotes derived from infected footpads have host-derived IgG on their surface (Pearson and Roberts, 1990; Kane and Mosser, 2001). In the presence of TLR ligand, the interaction of FcγR with opsonized parasites shuts off the production of IL-12 and induces the production of IL-10 from macrophages. This reversal in cytokine production blocks the Th1 inflammatory response and biases toward a Th2 anti-inflammatory response.

Cross-linking of FcγRs on macrophages with IgG-opsonized particles triggers a downstream signaling cascade (Nimmerjahn and Ravetch, 2008). Previous studies suggested that phosphorylation of two ITAM tyrosines of the FcγRs by tyrosine kinases of Src-family leads to recruitment and activation of the spleen tyrosine kinase (Syk). ITAMs consist of evolutionarily conserved amino-acid sequence motifs of D/ExYxxLx(6-8)YxxL. Syk belongs to the Syk/ZAP-70 family of non-receptor kinases

and is characterized by two N-terminal Src homology 2 (SH2) domains and a C-terminal kinase domain with a flexible linker between them. Syk is activated through: (1) binding of its SH2 domains to phosphorylated ITAMs in the cytoplasmic tails of Ig-α and -β, (2) phosphorylation by Src family kinases, and (3) auto-phosphrylation (Wossning *et al.*, 2006). Syk plays a key role in immuno-receptor-mediated phagocytosis (Shi *et al.*, 2006).

Syk can activate phospholipase C (PLC)-γ1, phophatidylinositol 1-kinase (PI 3-kinase), and regulators of Ras and other Ras-like G proteins such as Shc and Vav. Syk can be associated with FcγRIIa and the tyrosine phosphorylated γ chain of FcγRI and FcγRIII (Crowley *et al.*, 1997). Syk is required for FcγR-induced phagocytosis of opsonized particles, but not for the phagocytosis of latex beads or unopsonized microorganisms. Syk is important for many FcγR-induced signaling events, but not for LPS-induced signaling events (Crowley *et al.*, 1997). The response of MAPK/ERK or MAPK/p38 activation to FcγR engagement was largely void in $Syk^{-/-}$ macrophages (Kiefer *et al*., 1998). In $Syk^{-/-}$ cells, FcγR-induced signaling events mediated through p85 PI 3-kinase were attenuated, whereas they were essentially normal for signaling through Vav (Crowley *et al.*, 1997).

III. MAPKs Signaling Pathways

The MAPK signaling pathways are evolutionally well conserved in eukaryotes. They play important roles in many cellular processes including cell growth, differentiation, and apoptosis. They are essential modulators of both the innate and the adaptive immune response. So far, four major MAPK pathways have been identified in

mammalian cells, *i.e*., MAPK/ERK, MAPK/p38, c-Jun NH_2-terminal kinase (MAPK/JNK) and MAPK/ERK5 (Imajo *et al.*, 2006).

The activation of all MAPK pathways is achieved through a core triple kinase cascade: MAPK kinase kinase (MKKK), MAPK kinase (MKK), and MAPK (Figure 5). MAPKs are activated upon phosphorylation on Thr and Tyr by MKKs. Activation of MKKs is initiated after phosphorylation on their Ser/Thr residues by MKKKs. To date, seven MKKs and 14 MKKK s have been identified. MKKKs are more diverse in their structures, and they can be differentially regulated by a variety of upstream stimuli that specifically regulate downstream MKKs. MKKs are highly specific for their substrates, which limits the variability of the MKK-MAPK part of the cascade. All the MAPKs consist of a Thr-X-Tyr (TXY) motif within their activation loop, and the phosphorylation of both threonine and tyrosine within the activation loop is essential and sufficient for their activation (Zhang and Dong, 2005).

MAPKs are the key mediators of eukaryotic transcriptional responses to extracellular signals as they can phosphorylate transcription factors, co-regulatory proteins and chromatin proteins. In this way MAPKs can influence DNA binding, protein stability, protein localization, transcription or repression, and nucleosome remodeling (Whitmarsh, 2007).

MAPK/ERK

MAPK/ERK1 and 2 share 83% identity in their protein sequences, and they are ubiquitously expressed protein kinases in all tissue. MAPK/ERK1 is also known as MAPK3 or p44 MAP kinase, while MAPK/ERK2 is sometimes referred to as MAPK1 or

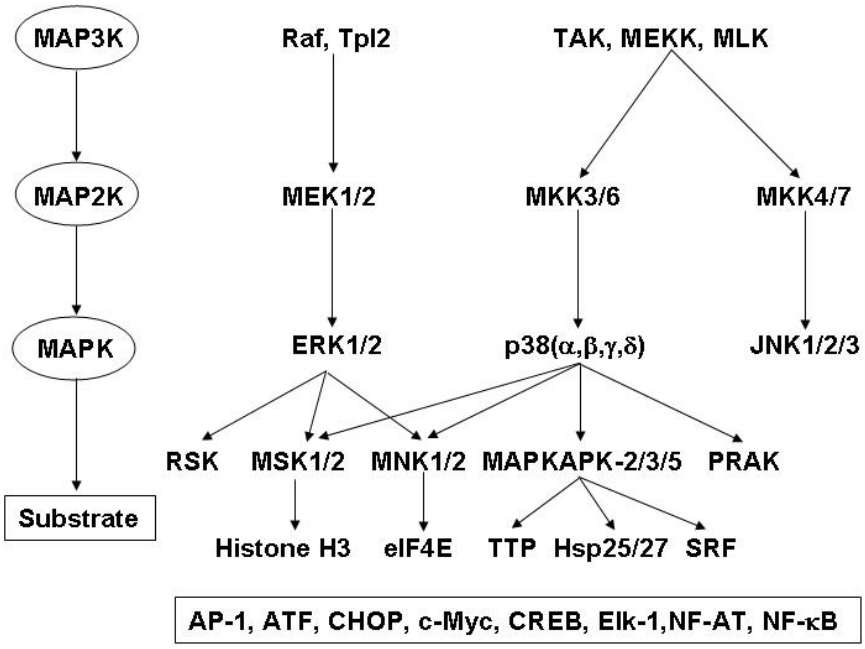

Figure 5. Mammalian MAPK signaling pathway. Mammalian MAPK activation is achieved through MAP3K-MAP2K-MAPK cascade. Phosphorylated MAPK, MAPK/ERK, MAPK/p38 and MAPK/JNK can activate a number of nuclear and cytoplasm kinases (RSK, MSK, MNK, MAPKAPK, PRAK) and transcription factors (AP-1, ATF, CHOP, c-Myc, CREB, Elk-1, NF-AT and NF-κB).

p42 MAP kinase. They are essential in the regulation of cell growth, differentiation and proliferation. Mice lacking MAPK/ERK1 are viable, and it has been suggested that the function of MAPK/ERK1 can be fulfilled by MAPK/ERK2 in most cells except for T cells (Pages *et al.*, 1999). T cell development after the $CD4^{+}CD8^{+}$ stage is retarded in MAPK/$ERK1^{-/-}$ mice. MAPK/ERK2 is essential for mesoderm differentiation and MAPK/$ERK2^{-/-}$ mice have major defects in early development (Yao *et al.*, 2003). The MAPK/ERK pathway can be activated by many stimuli including carcinogen agents, cytokines, growth factors, GPCR ligands, transforming compounds, and viruses. The Ras-Raf-MEK1/2-MAPK/ERK1/2 sequential activation is a typical process for activation of MAPK/ERK signaling pathway. Briefly, Ras is activated by the interaction of membrane-bound receptors with their corresponding ligands, allowing it to interact with downstream effector proteins such as the serine/threonine MKKK/Raf. Activated Raf binds to and phosphorylates the dual specificity MKK/MEK1/2. MEK1/2 in turns phosphorylates MAPK/ERK1/2 at the conserved Thr-Glu-Tyr (TEY) motif within the activation loop (Chong *et al.*, 2003). Two structurally unrelated compounds are commonly used to specifically block the MAPK/ERK1/2 pathway: PD98059 and U0126 (Cuenda and Alessi, 2000). They are noncompetitive inhibitors of MEK1/2 and prevent activation of MAPK/ERK1/2.

MAPK/ERK1/2 proteins are present throughout quiescent cells. Upon stimulation, a significant population of phosphorylated MAPK/ERK1/2 accumulates in the nucleus. The mechanisms of nuclear accumulation of MAPK/ERK1/2 remain largely unknown. It has been suggested that nuclear retention, dimerization, phosphorylation and release from cytoplasmic anchors are involved (Pouysségur *et al*., 2002). Nuclear localization of

activated MAPK/ERK1/2 indicates their important roles as “nuclear kinases” to act on nuclear substrates (Denhardt, 1996). Indeed, many nuclear proteins are identified as the substrates of MAPK/ERK, including nuclear factor of activated T- cells (NF-AT), Ets like gene1 (Elk-1), cellular FBJ murine osteosarcoma viral oncogene homolog (c-Fos), cellular v-myc myelocytomatosis viral oncogene homolog (c-Myc), cAMP response element binding protein (CREB) and STAT3. More importantly, three nuclear kinases, mitogen- and stress-activated protein kinase (MSKs), MAPK-intergrating kinase (MNKs) and ribosomal protein S6 kinases (RSKs) are the substrates for MAPK/ERK1/2. Both MSKs and MNKs can be activated by both MAPK/ERK1/2 and MAPK/p38, while RSK family members are exclusively activated by MAPK/ERKs. Both MSKs and RSKs contribute to gene transcriptional regulation by phosphorylation of their corresponding substrates, whereas MNKs are mainly involved in the regulation of translation.

MAPK/p38

The MAPK/p38 MAPK pathway is strongly activated by inflammatory cytokines such as IL-1 and TNF-α and also by environmental stress (Johnson and Lapadat, 2002). This pathway is associated with gene expression, cytokine production, inflammation, cell growth, cell differentiation and cell death. The MAPK/p38 pathway consists of several MKKKs, including MKKKs 1 to 4; the two MKKs, MKK3 and MKK6; and the four MAPK/p38 isoforms: α (MAPK14), β (MAPK11), γ (MAPK12) and δ (MAPK13). MAPK/p38α and MAPK/p38β are ubiquitously expressed. MAPK/p38γ is mainly expressed in skeletal muscle whereas MAPK/p38δ gene expression is found in the lung, kidney, testis, pancreas and small intestine. MKK3/6 exhibit high specificity towards

MAPK/p38. They do not activate other MAPKs such as MAPK/ERK1/2 and MAPK/JNKs. MKK3 is more selective than MKK6. MKK3 preferentially targets the MAPK/p38α and MAPK/p38β, while MKK6 can activate all MAPK/p38 isoforms. MKK3/6 specifically recognizes the activation loop of MAPK/p38 MAP kinases and phosphorylates a conserved Thr-Gly-Try (TGY) motif within the loop. The phosphorylated TGY motif and the length of the activation loop are two critical factors that contribute to the substrate specificity of MAPK/p38. Non-activated MAPK/p38 are found both in the nucleus and the cytoplasm of quiescent cells. MAPK/p38 can move from the cytoplasm to the nucleus after activation. Substrates for activated MAPK/p38 include the protein kinases, MAPK-activated protein kinases 2/3/5 (MAPKAPK or MK 2/3/5), MSK1/2, MNK1/2, casein kinase 2 (CK2) and the MAPK/p38-regulated/activated protein kinases (PRAK) as well as the transcription factors activating transcription factor 2 (ATF-1/2), cAMP response element-binding protein-homologous protein (CHOP)/ growth arrest DNA damage 153 (GADD153), myocyte enhancer factor (MEF), Elk-1, NF-κB and p53 (Dunn *et al.*, 2005).

Although MAPK/p38 MAP kinase is regulated by MKK3, MKK4 and MKK6, MKK3 and MKK6 are specific for MAPK/p38 activation whereas MKK4 can activate both MAPK/p38 and MAPK/JNK. Thus MKK4 represents an integration point of stress-activated MAPK pathways. The contribution of these MKKs to MAPK/p38 activation may vary among different cell types. It has been demonstrated that T-cell-receptor mediated MAPK/p38 activation is selectively defective in $MKK3^{-/-}$ CD4+ peripheral T cells and $MKK6^{-/-}$ thymocytes respectively due to the differences in the expression of MKK3 and MKK6 in these two cell types. Furthermore, the pool of protein kinases that

contributes to MAPK/p38 activation can be dependent on the specific stimulus that is applied. Activation of MAPK/p38 caused by UV radiation needs all of three MKKs, *i.e*., MKK3, MKK4, and MKK6 whereas TNFα-initiated MAPK/p38 activation requires both MKK3 and MKK6 in fibroblasts, and MKK3 appears to be critical for LPS-mediated MAPK/p38 activation (Lu *et al.*, 1999.). Activation of MAPK/p38 can also occur independent of MKKs as demonstrated by the studies of TAB1 (transforming growth factor-β-activated protein kinase-1 binding protein 1) (Ge *et al.*, 2002; Ge *et al.*, 2003). TAB1 binds and activates a MKKK, transforming growth factor-β-activated protein kinase 1 (TAK1), to subsequently activate both MAPK/p38 and MAPK/JNK MAPK pathway. TAB1 can also directly bind to MAPK/p38α to initiate MKK-independent autophosphorylation and activation of MAPK/p38α, particularly the splicing variant of TAB1, TAB1β that only binds to MAPK/p38α but not TAK1 to directly activate MAPK/p38α MAP kinase. It has been proposed that the MKK-independent activation of MAPK/p38 kinase by TAB1β contributes to the regulation of the basal level of MAPK/p38 activities and the activation of MAPK/p38 pathways mediated via membrane-bound receptors.

MAPK/p38$\alpha^{-/-}$ mice are embryonic lethal (Mudgett *et al*. 2000). Studies have shown that in MAPK/p38$\alpha^{-/-}$ mice embryonic fibroblasts, MK2 expression is decreased and in MK2$^{-/-}$ mice MAPK/p38α expression is significantly reduced. This implies that MAPK/p38α may form a complex with MK2 to stabilize each other. Due to its C-terminal nuclear localization signal (NLS) and nuclear export signal (NES), MK2 can shuttle between nucleus and the cytoplasm. MK2 locates in the nucleus of non-stimulated

cells because its NES is masked. MK2 can be activated by MAPK/p38α and β to exposes its NES, which promotes MK2 translocation from nucleus to cytoplasm in a complex form together with MAPK/p38α. Phospho-MAPK/p38α and phospho-MK2 as a complex remains stable in the cytoplasm (Engel *et al.*, 1998). Besides the co-exportation feature of MAPK/p38, MK2 also plays a role in actin remodeling, cell migration and development, regulation of the cell cycle (Manke *et al.*, 2005), chromatin remodeling (Voncken *et al.*, 2005), and post-transcriptional regulation (see below for details). MK2 can activate downstream cytoplasm substrates, such as heat shock protein (HSP) 25/27, lymphocyte-specific protein 1, tyrosine hydroxylase and nuclear substrate, serum response factor (SRF) and mRNA-binding proteins.

MAPK/JNK

MAPK/JNK1, 2 and 3, also known as MAPK8, 9, and 10 respectively, are characterized as stress-activated protein kinases because of their activation in response to inhibition of protein synthesis (Johnson and Lapadat, 2002). The expression of MAPK/JNK1 and MAPK/JNK2 was found to be ubiquitous, while the expression of MAPK/JNK3 is brain-specific. Although the canonical MAPK/JNK signaling pathway has been extensively characterized, the specific role of MKKK for MAPK/JNKs in response to various cues still remains elusive (Weston and Davis, 2007). The MKKK isoform TAK1 is a critical kinase for MAPK/JNK activation in response to stimuli via TLR-3, -4 and -9. TAK1 is also important for MAPK/JNK activation mediated by inflammatory cytokines such as IL-1, transforming growth factor β (TGFβ), lymphotoxin B and TNFα. The B-cell receptor and the T-cell receptor-initiated activation of

MAPK/JNKs is also mediated via TAK1. It has been reported that the MKKK isoforms TPL2 and MLK3 are other upstream kinases activated by TNFα to initiate MAPK/JNK activation. TLR-8-triggered MAPK/JNK activation appears to be mediated via the MKKK isoform MEKK3. A major target for MAPK/JNKs is the transcription factor activator protein-1 (AP-1), which is composed of Fos and Jun family members. MAPK/JNKs are found to bind and phosphorylate c-Jun thereby increasing its transcriptional activity. c-Jun protein is a component of the AP-1 transcription complex. AP-1 is activated in response to all stimuli that activates MAPK/JNKs and contributes to the regulation of many cytokine genes. Regulation of the MAPK/JNK pathway is extremely complex, and it is influenced by at least 13 MKKKs. After activation by MKKKs, both of MEK4 and MEK7 activate MAPK/JNKs by phosphorylation on tyrosine and threonine residues in a conserved Thr-Pro-Tyr (TPY) motif within the activation loop.

Some experiments suggested that MAPK/ERK activation is required for Th2 differentiation while MAPK/p38 protein kinase is required for both Th1 and Th2 differentiation, and IFN-γ production (Rincón and Pedraza-Alva, 2003). The MAPK/p38 protein kinase participates in macrophage and neutropil functional responses and mediates T-cell differentiation and apoptosis by regulating IFN-γ production. MAPK/p38 protein kinase can also regulate the immune response by stabilizing mRNAs such as TNF-α. MAPK/JNK may reduce the proliferative response of activated CD4+T cells and polarize T cell differentiation to Th1 lineage. MAPK/JNK1$^{-/-}$ mice exhibited an exaggerated Th2 response which exacerbated Leishmaniasis with non-healing skin lesions (Constant *et al.*, 2000). MAPK/JNK2$^{-/-}$ mice had low or no IFN-γ production. It

has been suggested that MAPK/JNK1 and MAPK/JNK2 may play different roles in CD8+T cell proliferation (Zhang and Dong, 2005).

IV. Cytokine Gene Regulation

Cytokine production from immune cells following a stimulus is controlled at different levels. The regulation of gene transcription is achieved at the initiation of transcription by *cis*-elements within the regulatory regions of the DNA and trans-acting factors that include transcription factors and the basal transcription complex. Regulation can occur at the epigenetics level where alterations in chromatin accessibility can expose *cis*-elements. Regulation can also occur at the post-transcriptional level, which includes mechanisms such as splicing to convert precursor mRNA into mature mRNA; regulation of transportation of mature mRNA into cytoplasm for cytokine synthesis (translation); control at mRNA degradation; regulation of posttranslational modification for maximum biological activity; and secretion of biological cytokines into extracellular space.

Transcriptional Regulation of Gene Expression

The *cis*-elements of the IL-10 promoter and the corresponding transcription factors that bind to it are conserved among all the cells that produce IL-10 (Mosser and Zhang, 2008). In the murine IL-10 gene promoter, a TATA box is located between 98 and 95 bp upstream of the first methionine codon - the translational initiation site ATG. The transcriptional initiation site for murine IL-10 gene -ACA is located between 68 and 66 bp upstream of ATG. A CCAAT box is found between -244 and -240 bp (CCAGT). Transcription factors, such as STAT3, specificity protein 1 (Sp1), IRF1, CREB, NF-κB and CCATT/enhancer-binding protein (C/EBP), all appear to be involved in IL-10

transcriptional regulation. Sp1 was originally identified to bind to GC-rich motifs in the SV-40 promoter and activate transcription *in vitro* in Hela cells (Jones and Tjian, 1985). Sp1 DNA binding domain is composed of 3C2H2 type zinc fingers. Sp1 is expressed ubiquitously and it can regulate many genes, including IL-1β, α2 collagen and TNF receptor expression. Using Sp factor-deficient Drosophila SL2 cells, the requirement for Sp1 in transcription of murine IL-10 gene was demonstrated (Tone *et al.*, 2000; Brightbill *et al.*, 2000). In addition to Sp1, the role of other transcription factors that have been shown to regulate IL-10 gene has recently been reviewed in details (Mosser and Zhang, 2008). Table 2 is a brief summary of the sequence of their binding element(s) and the immune cells that are studied.

Biosynthesis of the functional IL-12p70 heterodimer is mainly controlled at the regulation of IL-12p40 gene expression. The p40 gene is only expressed in IL-12-secreting cells and is highly inducible. On the other hand, the p35 gene is expressed ubiquitously. The IL-12p40 promoter has been well characterized (Trinchieri G, 2003). It contains several elements that are functionally important for its inducible expression. Several transcription factors have been identified to be essential for IL-12p40 gene expression in response to different stimuli. The ability of LPS-induced transcription of p40 gene is mainly via a nonconsensus NF-κB half-site in the proximal promoter region, and this NF-κB half-site is functionally synergistic with a downstream C/EBP site, although cooperative binding of Rel A/p65 and C/EBP proteins was not observed (Trinchieri G, 2003). Two E26 transformation-specific (ETS) family members, PU.1 and ETS2, bind to an ETS consensus elements upstream of the NF-κB site, and then this

TF	Binding sites	Cell distribution
Sp1	AGGAGG	Macrophage, monocyte, T cell, B cell
STAT	TCATGCTGGGATCTG ACCTTTGCCAGGAAGGCCCC	NK, monocyte, T cell
C/EBP	TGGAGGAAACAATTATTTCTCAATCC TTATTTCTCAATCC	Macrophage, monocyte, T cell
IRF	GCTAAAAAGAAAAAA AAAAAAGGGAAAGGAAAAAA AAAAGAAAGAAATTA	Macrophage, monocyte, T cell
AP-1	TGACTCA	Macrophage, monocyte, T cell
CREB	TGATGTCA; TGACTTCT; TGATGTAA; CCACGTCA	Macrophage, monocyte, T cell
c-Maf	TGCCTGGCTCAGCA	Macrophage, monocyte T cell
NF-κB	GGGGAATTCC; GAGAAGTCCC; GCCAGGAAGGCCCCACTGAGC	Macrophage, monocyte, T cell

Table 2: Murine IL-10 promoter TF binding sites and cell distribution.

complex can form a larger complex with c-REL and several IRF family members, including IRF1, IRF2 and IFN-consensus sequence-binding protein (ICSBP also known as IRF8). The IEF family members are essential for the transcription of IL-12p40 gene initiated by IFN-γ stimulation. Several inhibitors of IL-12 production have been shown to cast their effects via transcriptional repressors. GA12-binding protein (GAP12) is a repressor that interacts with GATA sequence in the IL-12 promoter (GA12) element in unstimulated cells. This GA12 element is located between the ETS and NF-κB sites, and IL-4 or prostaglandin E2 (PGE2) enhances the binding of GAP12 to GA12 elements to suppress IL-12p40 expression (Becker *et al.*, 2001).

Like the IL-12p40 promoter, the IL-12p35 promoter also contains binding sites for PU.1, C/EBP and IFN-γ-response element (γ-IRE). IFN-γ signaling induces the expression of IRF-family members such as IRF-1 and IRF-8 to induce the transcription of IL-12p35 gene (Liu *et al.*, 2004). There are multiple transcription initiation sites in IL-12p35 promoters, for example the human p35 gene has one site only for its expression in B cells whereas the other one is for its transcription in monocytes, indicating that differential cell-type may use different ones (Hayes *et al.*, 1998).

Epigenetic Regulation of Gene Expression

Histone are small, highly conserved basic proteins, found in the chromatin of all eukaryotic cells. They are associated with DNA to form nucleosomes. Core histones are histones H2A, H2B, H3 and H4. A nucleosome contains two copies of the core histones wrapped by 146-bp DNA. The DNA helix is wrapped around core histones to form a simple chromosome structure-"beads on a string" and then these fold into higher-order

chromatin. Chromatin is organized into two conformation domains, euchromatin (active) and heterochromatin (inactive), which have different chromosomal architecture, transcriptional activity and replication timing (Bernstein *et al.*, 2007). How chromatin maintains its structure and how transcription factors find their target sequences remain largely unknown. The distinction between active and inactive chromatin in eukaryotic organisms can be made by epigenetic marking via DNA methylation and post-translational modifications of core histones, which regulate transcription of the protein-encoding genome.

Most post-translational modifications of core histones occur at the amino- and carboxy-terminal histone "tails", and a few localize to the histone globular domains. These "tails" stick out of the nucleosome and are easily exposed for a variety of modifications. The resultant modifications account for a "histone code" and generate specific docking sites for other proteins to regulate chromatin structure and gene transcription (Strahl and Allis, 2000). Lysine is a key residue subjected to many modifications, including acetylation, methylation, ubiquitination and SUMOylation. Acetylation and methylation result in attachment of chemical groups with small molecular weight, whereas large moieties are attached by ubiquitination and SUMOylation, usually about two-thirds the size of histone itself. In addition, methylation can occur several times on one lysine side chain, such as mono-, di-, or trimethylation.

Phosphrylation at serine and /or threonine residues is also critical for regulation of gene transcription. Histone H3 phosphorylation, particularly phosphorylation at serine10, has been extensively characterized. Histone H3S10 phosphorylation is correlated with either activated transcription (open chromatin) or mitotic chromosome condensation

(closed chromatin), i.e., in a genomic context-dependent fashion. Activation of MAPK/ERK or MAPK/p38 MAPK signaling pathways leads to a rapid and transient histone H3 phosphorylation at serine 10 (Thomson *et al.*, 1999; Dyson *et al.*, 2005). It occurs frequently in a minute fraction of total histone H3 that is associated with the promoter region of immediate early genes (Thomson *et al.*, 1999; Dyson *et al.*, 2005) and a subset of cytokines and chemokines (Saccani *et al.*, 2001). Histone H3 phosphorylation increases the accessibility of the promoter region to transcription factors as the result of chromatin remodeling (Arbibe *et al.*, 2007; Zhang *et al*., 2006). Macrophages stimulated with TLR ligands together with Fcγ receptor ligation display a rapid MAPK/ERK activation, resulting in a transient H3S10 phosphorylation on the nucleosomes that are associated with the IL-10 promoter to promote the rapid binding Sp1 to the IL-10 promoter and subsequent IL-10 gene transcription (Lucas *et al.*, 2005).

MAPK/ERK or MAPK/p38 MAPKs-induced histone H3 phosphorylation may be mediated through downstream histone H3 kinases, such as MSK1/2. MSK1/2 activation directly elicits histone H3 phosphorylation at Ser10 and Ser28 because MSK1/2 recognizes a serine residue preceded by Ala-Arg-Lys. Phosphorylation at Ser10 of histone H3 is exceptionally sensitive to hyperacetylation caused by histone deacetylase inhibitors such as TSA (Dyson *et al.*, 2005). Histone H3 Ser10 phosphorylation recruits HAT to acetylate Lysine 14 of histone H3 (Meyer, 2001). Histone phosphorylation and/or acetylation not only lead to conformational changes making proteins easily accessible to the promoter region, but also enhance their interactions with chromatin (Meyer, 2001).

Post-transcriptional Regulation of Gene Expression

Precursor RNAs must undergo post-transcriptional processing to become mature mRNAs. This involves splicing, pre-mRNA 5' capping, and 3' polyadenylation. The concentration of mature mRNA in the cytoplasm, which depends on mRNA transport, storage and degradation, is critical for the translation. Elucidating the basic mechanisms of post-transcriptional regulation of gene expression is essential to gain a full understanding of how gene expression is regulated at different levels.

In the past decades, AU-rich elements (AREs) in the 3′-untranslated regions (3'-UTR) have been demonstrated to affect mRNA stability and translation. It has been shown that the MAPK/p38 pathway is involved in regulating mRNA stability of many cytokine genes via their 3′-UTRs, including TNFα, IL-8, IL-6, and GM-CSF mRNAs (Brook *et al.*, 2000; Winzen *et al.*, 1999). The MAPK/p38 pathway is also implied to have a regulatory role on TNFα biosynthesis at the translational level via AREs in the 3'-UTR of its mRNA (Winzen *et al.*, 1999).

MAPK/p38 pathway exerts its action mainly through its downstream kinase, MK2. MK2 plays important roles to affect post-transcriptional regulation rather than gene transcription (Gaestel, 2006). Gaestel et al showed that $MK2^{-/-}$ mice were resistant to LPS-induced endotoxic shock due to a 90% reduction of TNFα (Kotlyarov *et al.*, 1999) while deletion of the ARE in the 3'-UTR of the TNFα mRNA restored LPS-induced TNFα production (Neininger *et al.*, 2002). The levels of IFN-γ, IL-1, IL-6 and nitirc oxide were also lower in MK2 knockout mice (Kotlyarov *et al.*, 1999).

How MK2 regulates mRNA stability and translation remains elusive. A number of proteins are able to bind to mRNA and stabilize mRNA such as HuA-D/R (RNA-

binding protein homologous to human A-D or R antigen), or destabilize mRNA such as AUF1 (AU-rich element binding factor), TTP (tristetraprolin), KSRP (K homology-type splicing regulatory protein), TIA-1 (T-cell restricted intracellular antigen-1) and TIAR (TIA-1-related protein). The MAPK/p38-MK2-TTP axis has been shown to control post-transcription regulation of TNFα mRNA in LPS-stimulated cells. In quiescent cells, TTP binds to the ARE of TNFα mRNA in a non-phosphorylated form and destabilizes TNFα mRNA. After stimulation, activated MK2 phosphorylates TTP (Mahtani *et al.*, 2001), and the phosphorylated TTP binds to 14-3-3 proteins to prevent the target mRNA from degradation. Macrophages derived from $TTP^{-/-}$ mice tend to produce more TNFα. Due to high secretion of TNFα and GM-CSF, $TTP^{-/-}$ mice have severe inflammatory syndromes, polyarticular arthritis, myeloid hyperplasia, and cachexia (Carballo *et al.*, 1998). Hence, MK2 has a positive role to regulate TNFα mRNA stability by phosphorylation of TTP.

MK2 can also regulate TNFα biosynthesis at the translational level. Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that regulates protein synthesis and transcription. mTOR function as the catalytic unit of two distinct complexes: mTORC1 and mTORC2. mTORC1 phosphorylates at least four residues of eukaryotic translation initiation factor 4E (elF4E)-binding protein-1 (4E-BP1). Phosphorylated 4E-BP1 releases eIF4E, allowing it to bind to 5'-capped mRNAs and recruit them to the ribosomal initiation complex for translation. TSC1 (tumor-suppressor proteins hamartin, also known as tuberous sclerosis)-TSC2 (tuberin) complex inhibits translation by inhibiting mTOR mediated 4E-BP1 phosphorylation. MK2 can phosphorylate TSC2 and the phosphorylated TSC2 binds to 14-3-3 proteins, which

reverses the inhibitory function of the TSC1-TSC2 complex on 4E-BP1 phosphorylation (Li *et al.*, 2003).

IL-10 mRNA contains a long segment of 3'-UTR: 702 bp for the murine IL-10. Deletion of its 3'-UTR makes IL-10 mRNA refractory to degradation (Powell *et al*., 2000). The 3'-UTR of IL-10 mRNA contains six AUUUA pentamers, four of which are surrounded by U residues to form octameric motifs that are very similar to the class II AREs. TTP is known to bind to these AREs and trigger the rapid degradation of mRNA. In the primary macrophages obtained from $TTP^{-/-}$ mice, IL-10 mRNA decay rate is significantly reduced (Stoecklin *et al.*, 2008). IL-10 production can also be regulated at the translational level. In LPS-stimulated macrophages, adenosine-mediated signals specifically relieve the translational repressive effects of the IL-10 mRNA 3'-UTR, without affecting *IL-10* gene transcription and IL-10 mRNA stability (Németh *et al.*, 2005).

It remains elusive how IL-12p40 or p35 gene expression is regulated at the level of mRNA stability. In a very recent study led by Akira and his colleagues, they identified an RNase, Zc3h12a, as a destabilization factor to control the stability of IL-12p40 mRNA (Matsushita *et al.*, 2009). In response to TLR ligands, macrophages from Zc3h12a deficient mice had increased production of IL-6 and IL-12p40 but not TNFα as compared to the control littermates. The regulation of IL-12 biosynthesis is also controlled at the post-translational level. The p40 and p35 protein are processed by different mechanisms. Translocation of the p40 pre-protein into the ER is accompanied by cleavage of the signal peptide while removal of the p35 signal peptide occurs through two sequential cleavages

(Carra *et al.*, 2000; Trinchieri, 2003). The first cleavage takes place in the ER at a site in the hydrophobic region of the signal peptide that is not affected by glycosylation. The second cleavage may involve a metalloprotease after additional glycosylation and secretion of the p35 protein. This indicates that processing of p35 controls the output of biologically active IL-12 p70 heterodimer.

In summary, the production of IL-12 and/or IL-10 from immune cells is regulated at multiple levels. In the past, many studies were more focused on their regulation at transcription level particularly the interaction of *cis*-elements within their promoter regions with trans-factors. More recent studies indicated that the regulation of the production of these cytokines could be achieved at the levels beyond transcription. Thus, thorough exploration of the underlying mechanisms responsible for the optimal production of these cytokines under different microenvironmental cues and the expected information would shed light on the understanding how innate and adaptive immunity is modulated in response to pathogens and would be beneficial for novel therapy to counter the diseases.

CHAPTER 2: MATERIALS AND METHODS

Mice

BALB/c, J_H, and FcγR knockout female mice on the BALB/c background were purchased from the National Cancer Institute Charles River Laboratories and Taconic Farms. DO11.10 mice, which have a transgenic T cell receptor (TCRαß) for $OVA_{323-339}$, were purchased from the Jackson Laboratory and used as a source of antigen-specific $CD4^+$ T cells. IL-10 knockout mice on the BALB/c background and MKK3 knockout mice on C57BL/6 background were purchased from the Jackson Laboratory. All mice were maintained in high efficiency particle air-filtered Thoren units (Thoren Caging Systems, Hazleton, PA) at the University of Maryland. All animal studies were reviewed and approved by the University of Maryland Institutional Animal Care and Use Committee.

Reagents

All TLR ligands were obtained from InVivoGen (San Diego, CA). The MEK/MAPK/ERK inhibitors, U0126 and PD98059; the spleen tyrosine kinase (Syk) inhibitor (3-(1-methyl-1*H*-indol-3-yl-methylene)-2-oxo-2,3-dihydro-1*H*-indole-5-sulfonamide) and another Syk inhibitor piceatannol; the Src family kinase inhibitor PP2; c-Raf inhibitor ZM336372 and the MAPK/p38 MAPK inhibitor, SB203580 and the structurally-related control compound SB202474 were all purchased from Calbiochem (EMD Biosciences, San Diego, CA). Low molecular weight hyaluronic acid (LMW-HA) (MP Biomedicals, Irvine, CA), Ultra-Pure LPS (*Escherichia coli* K12, InVivoGen, San

Diego, CA) and OVA (Worthington, Lakewood, NJ) were used to stimulate cells. Anti-MAPK/ERK1/2 (total and phospho-T202/Y204) Abs, anti-MAPK/p38 (total and phospho-Thr180/Tyr182) Abs, and anti-MAPKAPK-2 Abs were obtained from Cell Signaling Technology. Anti-phosphorylated histone H3 (Ser^{10}) Ab, anti-Sp1 Ab, and chromatin immunoprecipitation (ChIP) kits were purchased from Upstate Biotechnology. TRIzol reagent was purchased from Invitrogen Life Technologies. RNase-free DNase I was obtained from Roche Diagnostics.

Cells

Bone marrow-derived macrophages (BMMϕ) were prepared as previously described (Zhang *et al.*, 2008). Briefly, bone marrow was flushed from the femurs and tibias of mice at 6–10 week of age. The cells were plated in petri dishes in DMEM/F12 supplemented with 10% FBS, 1% glutamine, 1% penicillin/streptomycin, and 10% conditioned medium from the supernatants of M-CSF secreting L929 (LC14) fibroblasts (LCCM). Cells were fed on days 4. On day 7, cells were removed from petri dishes and cultured on tissue culture dishes in complete medium without LCCM. On the next day, cells were subjected to experiments.

Bone marrow-derived dendritic cells (BMDCs) were prepared similarly to the BMMϕ as described above. However, the bone marrow cells were cultured in DMEM/F12 supplemented with 10% FBS, 1% glutamine, 1% penicillin/streptomycin, and 20% J588L cell conditioned medium that contains GM-CSF. Cells were fed on days 2 and 5. On day 8, cells were collected from the medium and cultured on tissue culture dishes in complete medium without J588L cell conditioned medium.

Peritoneal macrophages were collected by injecting 10ml PBS into mouse peritoneum as previously described (Zhang *et al.,* 2008). Fluid was withdrawn from the peritoneum slowly. The peritoneal exudate cells were centrifuged for 10 min at 300 × *g* 4°C. Supernatants were discarded and the cell pellet was resuspended in DMEM/F12-10 supplemented with 10% FBS, 1% glutamine and 1% penicillin/streptomycin.

The RAW264.7 macrophage cell lines were obtained from the American Type Culture Collection (Manassas, VA). RAW264.7 cells were maintained in RPMI supplemented with 10% FBS, 1% glutamine and 1% penicillin/streptomycin.

Parasites

Leishmania amazonensis (RAT/BA/72/LV78) (LA) and *Leishmania major* Friedlin strain, clone V1 (WHO MHOM/IL/80/Friedlin) were used. Parasites were maintained in BALB/c mice and cultured in vitro. Amastigotes are isolated from footpads of BALB/c mice that are infected for 6 to 8 weeks. Infected footpads were harvested in cold PBS containing 200 U of penicillin-6-potassium per ml and 200μg of streptomycin sulfate per ml (PBS-pen/strep), after removal. Footpads were ground by the plunger of a 10 ml syringe to pass through a cell strainer of 100μm nylon (BD Biosciences, San Jose, CA) in the presence of Schneider's complete medium and resuspended in 10 ml of PBS-pen/strep. The release of amastigotes from infected cells was achieved by passing the mixture through progressively smaller, 21-, 23-, and 25- gauge needles. Footpad-derived amastigotes were obtained by centrifugation at 1000×g for 10 min at 4°C.

Stationary phase promastigotes of *L. amazonensis* were obtained by growing parasites in Schneider's complete medium [Schneider's insect medium (Sigma-Aldrich, St. Louis, MO) supplemented with 20% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine] at 25°C. IgG-free amastigotes of *L. amazonensis* were developed by axenic culturing in Schneider's insect medium with 5% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine of pH 5.6 at 32°C. *L major* promastigotes were grown in 50:50 media [50% Schneider's complete medium and 50% M199 media (Invitrogen, Rockville, MD)].

Pre-treatment of Cells with MAPK Inhibitors and Stimulation

Cells were plated overnight in complete medium without LCCM. Cells were treated with MEK/MAPK/ERK inhibitors-U0126 or PD98059; Syk inhibitor and another Syk inhibitor-piceatannol; the Src family kinase inhibitor-PP2; c-Raf inhibitor-ZM336372 or MAPK/p38 inhibitors: SB203580 or SB202474 for 30 minutes to 1 hour. Cells were then stimulated with either 10μg/ml LMW-HA or 10ng/ml LPS alone or in combination with the different forms of parasites for indicated times.

Western Blotting Analysis

A total of 2×10^6 BMMϕ per well were plated overnight in 6-well plates. Cells were activated with different stimuli in a final volume of 1ml of DMEM/F12 without L929 conditioned medium for the indicated times. Cells were then lysed in ice-cold lysis buffer (100 mM Tris (pH 8), 2 mM EDTA, 100 mM NaCl, 1% Triton X-100 containing complete EDTA-free protease inhibitors from Roche Diagnostics, which included 5 mM

sodium vanadate, 10 mM sodium fluoride, 10 mM β-glycerophosphate sodium, and 5 mM sodium pyrophosphate). Equal amounts of protein were loaded onto 10% SDS-polyacrylamide gels, and then transferred to polyvinylidene difluoride membranes. Membranes were incubated with primary Abs (1/1000 in 5% BSA in TBS-Tween 20) overnight at 4°C, washed, and incubated with secondary Ab (1/5000) with HRP conjugates. The specific protein bands were visualized by using Lumi-LightPLUS chemiluminescent substrate (Roche Diagnostics, Indianapolis, IN).

Cytokines Measurement by ELISA

Approximately 2–5 x 10^5 cells were plated per well overnight in a 48-well plate. Cells were then stimulated and supernatants were harvested at different time intervals. Cytokines were measured by a sandwich ELISA using antibody pairs (IL-12p40, IL-10, TNFα, IL-12p70, IFN-γ and IL-4, BD Pharmingen, San Diego, CA) according to the manufacturer's instructions.

Immunofluorescence Microscopic Analysis

Amastigotes were stained with 5 μM CellTracker Blue CMAC (Invitrogen Life Technologies, Carlsbad, CA). BMMϕ were infected with amastigotes in the presence or absence of LMW-HA for a period of time. After brief washing with PBS, cells were fixed in methanol at 4°C for 15 min and then washed with PBS. Monolayers were incubated with 10% FBS in PBS for 1 h at room temperature to prevent nonspecific binding. MAPK/ERK1/2 phosphorylation was stained by a phospho-p44/42 MAPK (T202/Y204) (E10) mouse mAb (Alexa Fluor 488 conjugate) (Cell Signaling Technology, Danvers,

MA). Macrophages were counterstained for 2 min with 0.5% propidium iodide (PI). Slides were examined by using a Zeiss Axioplan 2 fluorescent imaging research microscope and Zeiss KS300 imaging software.

RNA Isolation and Quantitative Real-time PCR (qRT-PCR)

TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) was used to extract RNA from BMMϕ (3-4 $x10^6$ cells per reaction). Homogenization was conducted to facilitate RNA extraction from footpad and lymph node. RNase-free DNase I (Roche Diagnostics, Indianapolis, IN) was used to remove contaminated DNA. ThermoScript RT-PCR system (Invitrogen Life Technologies, Carlsbad, CA) was used to generate cDNA from RNA by using random hexamers (for premature mRNA) or $oligo(dT)_{20}$ (for mature mRNA). Sequences of primers are shown in Table 3. qRT-PCR was used to measure both mature and premature IL-10, IL-12p40 and TNFα mRNA levels. The mRNA levels are presented as arbitrary units that are derived from normalization values of each represented mRNA by corresponding GAPDH mRNA. The levels of mRNAs at zero hour were arbitrary set at 1. qRT-PCR was performed on an ABI Prism 7700 Sequence Detection System or Roche LightCycler 480 Sequence Detection System using SYBR Green PCR reagents purchased from Bio-Rad Laboratories (Hercules, CA).

Chromatin Immunoprecipitation (ChIP) Assay

ChIP assays were conducted using the ChIP Assay kit following the manufacturer's protocol (Upstate Biotechnology-Millipore Inc., Billerica, MA) with minor modification as previously described (Zhang *et al.*, 2006). Briefly, 4 x 10^6 BMMϕ

Gene	Primers
premRNA IL-10	5'-CATTCCAGTAAGTCACACCCA-3' (intronic primer) 5'-TCTCACCCAGGGAATTCAAA-3'
premRNA IL-12p40	5'-TCTGAGCCACTCACATCTGCT-3' (intronic primer) 5'-GGCCAATGAGAGTTCCTGTT-3'
premRNA GAPDH	5'-TGTTCCTACCCCCAATGTGT-3' 5'-TCCCAAGTCACTGTCACACC-3' (intronic primer)
mRNA IL-10	5'-AAGGACCAGCTGGACAACAT-3' 5'-TCTCACCCAGGGAATTCAAA-3'
mRNA IL-12p40	5'-GGAGGTCAGCTGGGAGTACC-3' 5'-AGGAACGCACCTTTCTGGTT-3'
mRNA TNFα	5'-AAAGGGATGAGAAGTTCCCAAAT-3' 5'-GTCTTTGAGATCCATGCCGTTG-3'
nucleosomes 2	5'-GCAGAAGTTCATTCCGACCA-3' 5'-GGCTCCTCCTCCCTCTTCTA-3'
nucleosomes 11	5'-GTTGCTTCGCTGTTGGAAA-3' 5'-GGTCAGTTCCAGGCTGAGTT-3'
LA 18S rRNA	5'-AGCAGGTCTGTGATGCTCCT-3' 5'-GGACGTAATCGGCACAGTTT-3'
L.major 18S rRNA	5'-ATCGGCATCATCAGCGGCGG-3' 5'-TCGACGGGTGGCCAATGTGC-3'
murine 18S rRNA	5'-CCCAGTAAGTGCGGGTCATA-3' 5'-AGTTCGACCGTCTTCTCAGC-3'
mRNA MAPK/p38α	5'-AAGACTCGTTGGAACCCCAG-3' 5'-TCCAGTAGGTCGACAGCCAG-3'
mRNA MAPK/p38β	5'-AAGCCCAGTGTCCCTCCTAA-3' 5'-CCACAGGCAACCACAAATCT-3'
mRNA MAPK/p38γ	5'-GCTCACCCCTTCTTTGAACC-3' 5'-TTCGTCCACGCTGAGTTTCT-3'
mRNA MAPK/p38δ	5'-AGCCCTCAGGCTGTGAATCT-3' 5'-CATATTTCTGGGCCTTGGGT-3'
mRNA GAPDH	5'-TGTTCCTACCCCC AATGTGT-3' 5'-GGTCCTCAGTGTAGCCCAAG-3'
mRNA HPRT	5'-AAGCTTGCTGGTGAAAAGGA-3' 5'-TTGCGCTCATCTT AGGCTTT-3'

Table 3: Polymerase chain reaction primers pairs used in qRT-PCR analysis.

were plated overnight in six-well plates. Cells were stimulated and then fixed for 10 min at 37°C in 1% paraformaldehyde. Cells were washed on ice with ice-cold HBSS containing 1 mM PMSF, harvested and then lysed in SDS lysis buffer. DNA was sheared by ultrasonication using a High Intensity Ultrasonic Processor (Cole-Parmer, Vernon Hills, IL) for 3 x 10 s pulses at 20% amplitude that resulted in relatively uniform DNA fragment size of ~300 bp. Lysates were cleared by centrifugation and diluted in ChIP dilution buffer. Lysates were pre-cleared using salmon sperm DNA/protein A-agarose and a sample of "input DNA" was collected at this point. Protein-DNA complexes were immunoprecipitated with 5 µg of Ab overnight at 4°C. Ab-protein-DNA complexes were then captured using salmon sperm DNA/protein A-agarose for 1 h at 4°C. After washing beads with low and high salt, LiCl, and TE buffers, the protein/DNA complexes were eluted using 1% SDS, 0.1 M $NaHCO_3$ buffer and disrupted by heating at 65°C for 4 h. DNA was then extracted using phenol/chloroform extraction and ethanol precipitation. For relative quantitation of promoter levels, qRT-PCR was performed.

Infection and Parasite Quantitation

Mice were inoculated in the right hind footpad with different numbers of parasites, as indicated in the figure legends. Lesion size was measured with a digital caliper (Chicago Brand Industrial, Fremont, CA) and expressed as the difference in thickness between the infected and the contralateral (non-infected) footpad.

Parasite burdens were determined by a limiting dilution of cell suspensions obtained from excised lesions, as described previously. The parasite suspension was then 7 times serially diluted in 10-fold dilutions in 200-µl Schneider's complete medium in

96-well plates. Plates were incubated for 7 days at 25°C, and then the wells were inspected for the growth of promastigotes. Results were expressed as -log parasite titer. Parasite burdens were expressed as the negative $\log_{10}$ dilution of which parasite growth was visible.

qRT-PCR method was used to amplify parasite DNA as a second measure of parasite burdens. In brief, the homogenates of infected lesion were treated with proteinase K at 56°C for 48 hours. The DNA was obtained after phenol/chloroform extraction and NaOAC/EtOH precipitation. Primers specific for LA 18S rRNA gene and murine 18S rRNA gene were designed. The parasite burden was expressed as a fold change by using the $\Delta\Delta C_T$ (cycle threshold) methods, as described below. For quantitation of parasite burdens in the infected lesion, the C_T value for murine 18S rRNA gene is used as normalization reference gene.

Data Analysis

The relative differences among qRT-PCR samples were determined using the $\Delta\Delta C_T$ methods as described before (Zhang et al., 2006). A ΔC_T value was determined for each sample using the C_T value from input DNA to normalize ChIP assay results. The C_T value for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene was used to normalize loading in the RT-PCRs. For quantitation of parasite burden in the infected lesion, the C_T value for murine 18S rRNA gene was used as normalization reference gene. A $\Delta\Delta C_T$ value was then obtained by subtracting control ΔC_T values from the corresponding experimental ΔC_T. The $\Delta\Delta C_T$ values were converted to fold difference compared with the control by

raising 2 to the $\Delta\Delta C_T$ power. Unpaired Student's *t* test was used for statistical analysis. Values of $p < 0.05$ were considered to be statistically significant.

RNA Stability Assay

2-3×10^6 BMMϕ were stimulated for 2 hours before the addition of actinomycin D to a final concentration of 10 μg/ml. The degradation of IL-10, IL-12p40 and TNFα mRNA was subsequently measured by qRT-PCR over the following 4 hours.

Transient Transfection and IL-12p40 Promoter Luciferase Assay

A fragment that contains mouse IL-12p40 promoter region was subcloned into the pGL3-basic luciferase expression vector (Promega, Madison, WI). Transient transfection assays in RAW264.7 macrophage-like cells was conducted using Fugen™HD reagent (Roche Diagnostics, Indianapolis, IN) following the manufacture's recommendation. The pRL-TK construct (Promega, Madison, WI) was used as an internal control for normalization of transfection efficiency. Cell lysis and luciferase assays were performed using the dual luciferase assay system following the instructions of the manufacturer (Promega, Madison, WI).

Generation of Small Interfering RNA (siRNA) and Cell Transfections

SignalSilence® MAPK/p38 MAPK siRNA Kit (Cell Signaling, Danvers, MA) was used to knock down endogenous MAPK/p38 proteins. For cell transfections, 5 x 10^6 primary BMMϕ were transfected with different dose of siRNA using the Amaxa Nucleofector system (Lonza Cologne AG, Basel, Switzerland) and stimulated 48 h later

as described before (Zhang *et al.*, 2009). Gene silencing was confirmed by western blot analysis and qRT-PCR.

T cell Isolation and Stimulation

Spleen or lymph nodes were removed from mice and placed in a petri-dish filled with PBS. A single cell suspension was obtained after the spleen or lymph nodes were meshed with a plunger of 5-ml syringe through cell strainer of 100μm nylon (BD Biosciences, San Jose, CA). Pan T Cells Isolation Kit (Miltenyi Biotec Inc, Auburn, CA) was used to obtain T cells from these single cell suspension solutions following the manufacture's recommendation.

Bone marrow-derived macrophages (2×10^5/well) were seeded in a 48-well plate and primed with IFN-γ (100 U/mL, R&D Systems, Minneapolis, MN) overnight. The primed cells were then treated with different stimuli as described in the figure legends for additional 16 hrs. The supernatants were collected for ELISA measurement and the stimulated macrophages were filled with 0.5 ml of fresh medium (RPMI 1640 supplemented with 10% FCS, HEPES pH 7.4, glutamine, Pen/Strep and 50 μM 2-mercaptoethanol). The isolated T cells (5 x 105/well) were then added to each well to reach a final concentration of 0.6 ml for further incubation. After 3 days, the supernatants were collected from the T cell-macrophage incubation for ELISA measurements of released cytokines as indicated in the figure legends.

Cytokine production by cells from lymph nodes obtained from infected BALB/c mice was determined using ELISA. Lymph nodes were removed from mice on day 56

post-infection and stimulated with anti-CD3 (BD Pharmigen, San Jose, CA) and anti-CD28 (BD Pharmigen, San Jose, CA) for 48 hours. Then the cells were stimulated with PMA (Calbiochem, San Diego, CA) for 5 hours. Supernatants were harvested after 3 days and assayed for IFN-γ and IL-4 by ELISA.

CHAPTER 3: THE ROLE OF MAPK/ERK IN IL-10 PRODUCTION BY REGULATORY MACROPHAGES

Alterations of intracellular signaling pathways in host macrophages infected by *Leishmania* has been reported (Nandan *et al.*, 2000) and implicated in promoting *Leishmania* pathogenesis (Reiner, 1994). Most of the alterations pertain to signaling pathways that trigger macrophages to eliminate parasites. The super-induction of IL-10 has proven to be one of the most important factors in progressive Leishmaniasis. The surface IgG on *Leishmania* amastigotes allows them to ligate FcγRs on macrophages causing these macrophages to preferentially induce high amounts of IL-10. The IL-10 produced by infected macrophages prevents macrophages activation and diminishes the production of IL-12. It has been demonstrated that MAPK/ERK activation is a critical event in IL-10 super-induction. Therefore my hypothesis is that MAPK/ERK activation in host macrophages infected by parasites plays an essential role in Leishmaniasis. To test this hypothesis, we examine MAPK/ERK activation in macrophages infected with *L. amazonensis* (LA).

MAPK/ERK Activation Correlates with IL-10 Production in Leishmania-infected Macrophages

We examined MAPK/ERK activation in macrophages infected with different forms of LA by western blotting (Figure 6*A*). Stationary phase promastigotes and axenic amastigotes were both relatively inefficient at inducing MAPK/ERK activation in infected macrophages. In contrast, lesion-derived footpad amastigotes induced rapid but

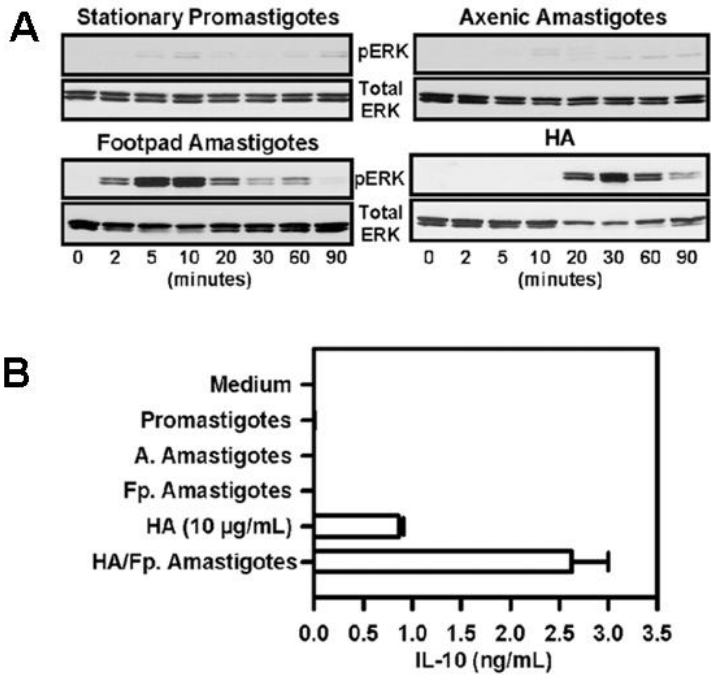

Figure 6. MAPK/ERK activation and IL-10 production in infected macrophages. *A*, Stationary-phase promastigotes, axenic cultured amastigotes, and footpad derived amastigotes were obtained, as described in *Materials and Methods*. They were added to monolayers of BMMϕ (2 x 10^6 cells/well), along with hyaluronic acid (HA) (10 µg/ml). The ratio of parasites to macrophages was 20:1. At designated times, equal amounts of whole cell lysates (15 µg) were subject to electrophoresis on 10% SDS-PAGE. Phosphorylated forms of MAPK/ERK and the corresponding total proteins were detected by Western blotting. *B*, Macrophages were treated with HA (10 µg/ml), or infected with stationary promastigotes (Promastigotes), axenic amastigotes (A. Amastigotes), footpad derived amastigotes (Fp. Amastigotes), or Fp. amastigotes and HA. Supernatants were collected after 16 h, and IL-10 was quantitated by ELISA. Data represent one of three independent experiments (mean ± SD of triplicates).

transient MAPK/ERK phosphorylation. They activated MAPK/ERK as early as 2 mins, then reached the peak between 5 to 10 mins and declined after 20 mins post-stimulation. The inflammatory extracellular matrix cleavage product, low molecular weight hyaluronic acid (LMW-HA), also induced MAPK/ERK activation, but this induction was somewhat slower than footpad amastigotes, as late as 20 mins. LMW-HA (HA), which occurs at the inflammation sites, has been identified as TLR2/4 ligands (Termeer *et al.*, 2002).

MAPK/ERK activation was next correlated with IL-10 production by macrophages (Figure 6*B*). Infection of macrophages with either promastigotes or axenic amastigotes failed to induce IL-10 production, which is consistent with their failure to activate MAPK/ERK. Either HA or footpad-derived amastigotes alone yields little or no measurable IL-10 although both of them were able to induce MAPK/ERK activation. The combination of these two signals, however, resulted in production of high amounts of IL-10.

To establish a link between MAPK/ERK activation and IL-10 production, macrophages were pretreated with U0126, an inhibitor of MEK (the upstream MAPKK which activates MAPK/ERK), and then infected with lesion-derived amastigotes plus HA. MAPK/ERK1/2 phosphorylation was strongly inhibited by U0126 at 2μM (Figure 7*A*). There were also a dose dependent inhibition of IL-10 production by U0126 with IC_{50} about 2μM (Figure 7*B*). As a control, IL-12p40 levels were also measured. There was a reciprocal increase of IL-12p40 that correlated with MAPK/ERK inhibition.

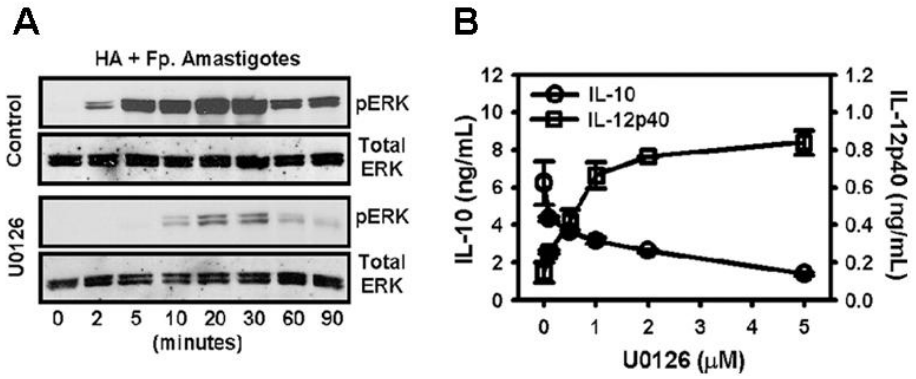

Figure 7. MAPK/ERK activation by LA amastigotes. *A*, Macrophages were pretreated with or without U0126 (2 μM) for 1 h. Cells (2 x 10^6 cells) were then stimulated with HA plus lesion-derived amastigotes for the indicated times. Electrophoresis and Western blotting were performed, as described above. *B*, Macrophages were pretreated with increasing concentrations of U0126 as indicated for 1 h and then infected with lesion-derived amastigotes in the presence of HA (10 μg/ml) for 8 h. Supernatants were harvested, and IL-10 and IL-12p40 production were determined by ELISA. Data represent one of three independent experiments (mean ± SD of triplicates).

These data indicate that MAPK/ERK was required for IL-10 production by infected macrophages and inhibition of MAPK/ERK activation prevented IL-10 production. In order to obtain decent amount of IL-10, two signals are needed: lesion-derived amastigotes and an inflammatory stimulus such as HA.

The Role of IgG in MAPK/ERK Activation by Leishmania

Axenically cultured amastigotes cannot activate MAPK/ERK but the lesion-derived amastigotes that have IgG on their surface can (Figure 6*A*). To determine the relationship between surface IgG and MAPK/ERK activation, axenic amastigotes were opsonized with antibodies against LA and then added to macrophages. MAPK/ERK activation in the absence or presence of HA was analyzed (Figure 8*A*). Opsonized axenic amastigotes induced MAPK/ERK phosphorylation to a similar degree and with similar kinetics as footpad derived amastigotes. In the presence of HA opsonized amastigotes increased the speed, magnitude and duration of MAPK/ERK phosphorylation. MAPK/ERK activation was detectable as early as 2 mins and persisted until 90 mins.

Similar to the lesion-derived footpad amastigotes, opsonized axenic amastigotes only induced IL-10 production from macrophages in the presence of HA. Under these conditions, IL-10 production was induced in a parasite number-dependent manner (Figure 8*B*, white bars). At a multiplicity of infection (MOI) between 10:1 and 20:1, a modest level of IL-10 was induced. HA was a potent inducer of IL-12, as previously reported (Hodge-Dufour *et al.*, 1997). The addition of opsonized axenic amastigotes reduced IL-12 production from macrophages (Figure 8*B*, black bars). Another MEK inhibitor PD98059

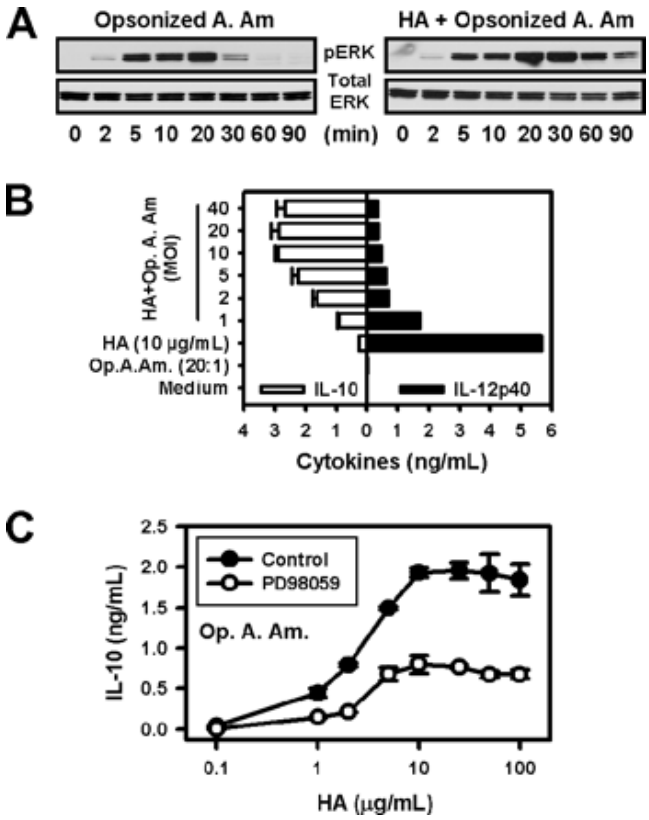

Figure 8. Opsonized axenic cultured amastigotes activate MAPK/ERK and induce IL-10 production. *A*, Macrophages (2 x 10^6 cells) were infected with LA axenic amastigotes opsonized with anti-*Leishmania* serum (Opsonized A. Am.) in the presence or absence of HA (10 µg/ml). Western blotting of phosphorylated MAPK/ERK1/2 was examined at the indicated times. Total MAPK/ERK1/2 was used as the loading control. The ratio of parasites to macrophages was 20:1. *B*, Macrophages were infected with increasing concentrations (MOI from 1:1 to 40:1) of axenic cultured amastigotes opsonized with IgG (Op. A. Am.) in the presence of HA (10 µg/ml). The supernatants were collected after 16 h, and IL-10 and IL-12p40 proteins were determined by ELISA. For controls, macrophages were treated with medium alone, opsonized axenic amastigotes (Op. A. Am.), or HA alone. Data represent one of three independent experiments (mean ± SD of triplicates). *C*, Macrophages (2 x 10^5 cells) were exposed to a 20:1 ratio of opsonized axenic cultured amastigotes (Op. A. Am.) in the presence of increasing concentrations of HA. Parallel monolayers were treated similarly, except that the MEK inhibitor, PD98059 (10 µM), was added 30 min before stimulation. The supernatants were collected 8 h later, and IL-10 concentrations were determined by ELISA. Data represent one of three independent experiments (mean ± SD of triplicates).

was administered to confirm this. Opsonized amastigotes induced IL-10 from macrophages in a HA dose-dependent manner, and this production was inhibited by PD98059 (Fig. 8*C*). These data showed that IgG is required for MAPK/ERK activation and its corresponding IL-10 production in LA infected macrophages.

MAPK/ERK phosphorylation was also monitored by fluorecence microscopy. Macrophages were infected with footpad lesion-derived amastigotes (MOI=10:1) in the presence of HA. MAPK/ERK phosphorylation was detected by a fluorescence-labeled Ab against phosphorylated MAPK/ERK 1/2 (green). Amastigotes were prestained with Cell Tracker Blue (blue) and nuclei were stained with PI (red). Similar to what have been shown by western blot (Figure 6*A*), MAPK/ERK was activated in a time dependent manner, which could be detected as early as 2 mins and reached the maximal levels by 20–30 mins post-infection (Figure 9*A*). Phosphorylated MAPK/ERK translocated to the nucleus, which is consistent with previous observations (Plows *et al.*, 2004). A similar degree of MAPK/ERK activation occurred with opsonized axenic amastigotes (Figure 9*B*). Axenic amastigotes were inefficient activators of MAPK/ERK compared to opsonized amastigotes at 15 mins post-infection.

FcγR Mediated Syk Signaling Pathways is Involved in MAPK/ERK Activation by Opsonized Parasites

Since IgG binds to FcγR, the next approach was to examine the role of FcγR in MAPK/ERK activation by using FcR $\gamma^{-/-}$ mice lacking the common γ chain through which FcγRI, III and IV signal. MAPK/ERK activation was undetectable following infection with lesion-derived amastigotes in these knockout cells compared to the wild type cells

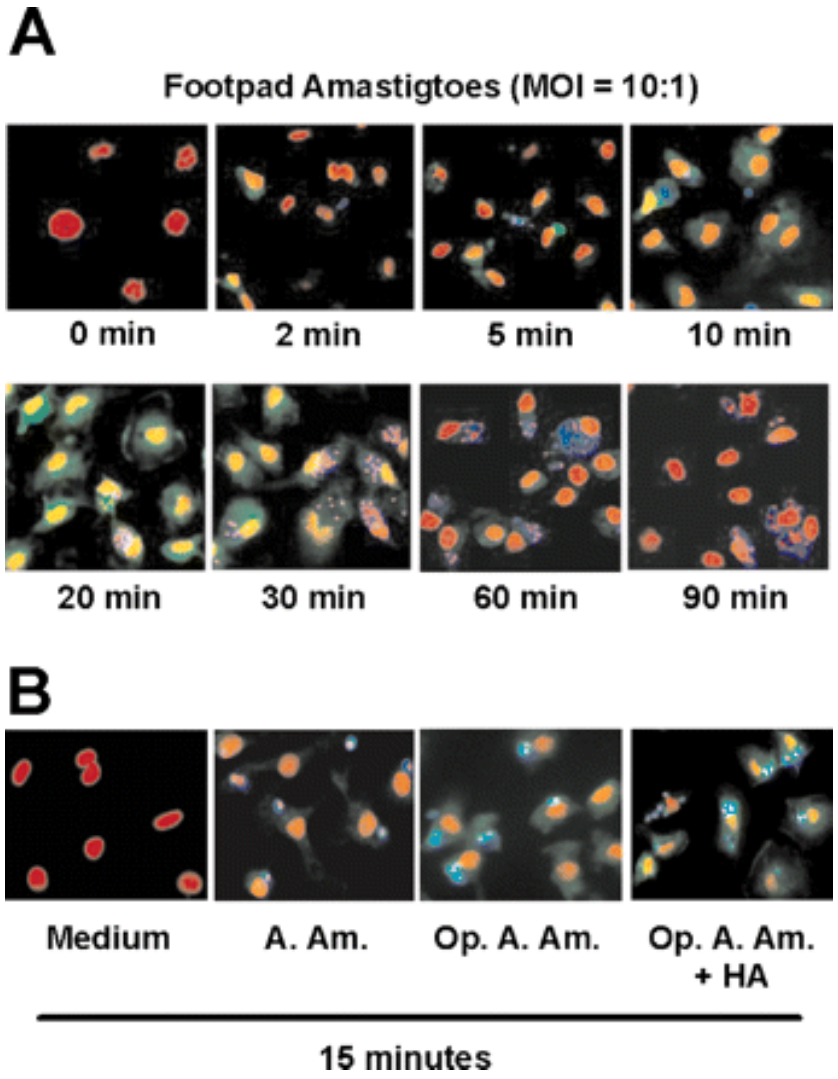

Figure 9. Immunofluorescence analysis of MAPK/ERK activation in macrophages. *A*, Macrophage monolayers (1 x 10^5 cells/coverslip) were infected with lesion-derived footpad amastigotes in the presence of HA (10 µg/ml) at MOI of 10:1 for the indicated times. Cells were fixed with cold-methanol, and visualized by fluorescence microscopy. Parasites were prestained with Cell Tracker Blue (blue). Phosphorylated MAPK/ERK was stained with mouse mAb (Alexa Fluor 488 conjugate) against phosphor-MAPK/ERK (green), and cell nuclei were stained with PI (red). *B*, Macrophages (1 x 10^5 cells) were treated with HA (10 µg/ml), and infected with unopsonized (A. Am.) or IgG-opsonized axenic cultured amastigotes (Op. A. Am.), in the presence or absence of HA for 15 min.

(Figure 10*A*). IL-10 production was significantly reduced after stimulation with varied MOI of opsonized parasites and HA in FcR$\gamma^{-/-}$ cells (Figure 10*B*). Therefore, *Leishmania*- induced MAPK/ERK activation and IL-10 production are mediated through FcγR signaling pathway.

The kinases that are upstream of MAPK/ERK activation were examined. Syk is a tyrosine kinase that has been implicated in ITAM-mediated signaling via FcγR. Hypothetically the inhibition of Syk would lead to an inhibition of MAPK/ERK activation and subsequent IL-10 induction if opsonized parasites were mediated by FcγR-Syk signaling pathway. A Syk inhibitor, 3-(1-Methyl-1H-indol-3-yl-methylene)-2-oxo-2, 3-dihydro-1H-indole-5-sulfonamide, significantly reduced lesion-derived amastigotes induced MAPK/ERK activation in a dose-dependent manner (Figure 11*A*). At 1μM Syk inhibitor concentration, IL-10 production was reduced by greater than 80% (Figure 11*B*). Another Syk inhibitor, piceatannol, and the Src family kinase inhibitor PP2 were also tested (Figure 11*B*). IC50 for Syk inhibitor is only 0.024μM while piceatannol is 10.6μM and PP2 is 23.67μM.

c-Raf has been identified as an upstream MAP3K that is responsible for MAPK/ERK activation in the well-defined Ras-Raf-MEK-MAPK/ERK signaling pathway. A c-Raf inhibitor, N-[5-(3-Dimethylaminobenzamido)-2-methylphenyl]-4-hydroxybenzamide (ZM336372, IC_{50}=70nM) was used to examine whether c-Raf was responsible for FcγR-mediated MAPK/ERK activation and IL-10 production. ZM336372 had no effect on parasite-mediated MAPK/ERK activation (Figure 12*A*) with only a minor inhibitory effect on IL-10 production (Figure 12*B*) (IC_{50}>10μM), suggesting

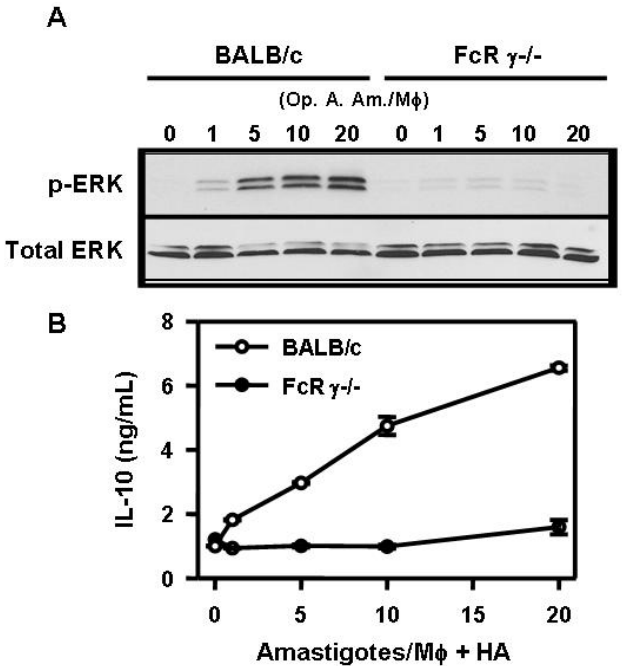

Figure 10. FcγR-mediated signaling is critical for MAPK/ERK activation. *A*, Macrophages derived either from BALB/c or FcR $\gamma^{-/-}$ mice were treated with different MOIs of opsonized axenic cultured amastigotes (Op. A. Am), as indicated. After 10-min incubation, cell lysates were collected and analyzed by Western blotting. *B*, Macrophages derived either from BALB/c or FcR $\gamma^{-/-}$ mice were infected with increasing amounts (MOI) of opsonized axenic cultured amastigotes together with HA (10 µg/ml). After 8 h, the supernatants were collected for ELISA to determine IL-10 production. Data represent one of two independent experiments (mean ± SD of triplicates).

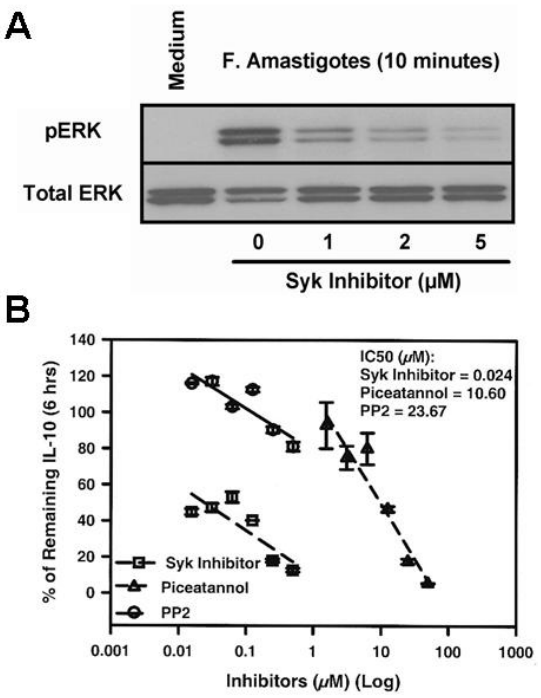

Figure 11. FcγR-mediated MAPK/ERK activation is signaled through Syk. *A*, Macrophages were pretreated with different doses of Syk inhibitor for 1 h and then stimulated with lesion-derived amastigotes (MOI = 20:1) for 10 min. Whole cell lysates were collected and analyzed by Western blotting to detect MAPK/ERK phosphorylation. *B*, Macrophages were pretreated with Syk inhibitor (squares), piceatannol (triangles) and PP2 (circles) at different concentrations 30 mins before exposed to a 20:1 ratio of opsonized amastigotes in the presence of HA. The supernatants were collected 6 hrs later, and IL-10 concentrations were determined by ELISA. Data represent one of three independent experiments (mean ± SD of triplicates).

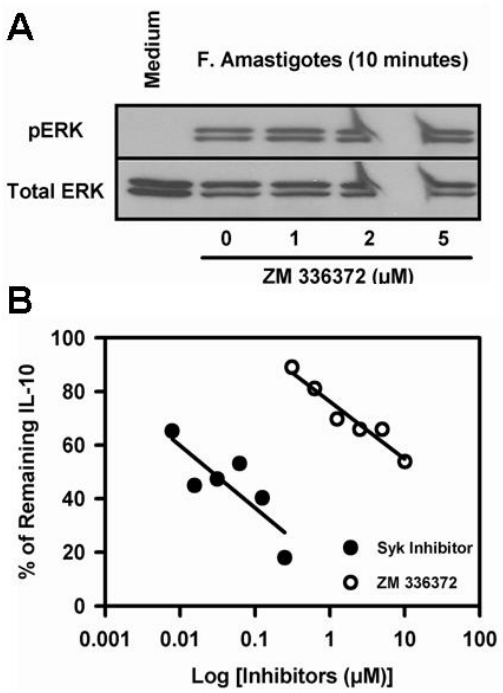

Figure 12. FcγR-mediated MAPK/ERK activation does not involve c-Raf. *A*, Macrophages were pretreated with different doses of ZM336372 for 1 h and then stimulated with lesion-derived amastigotes (MOI = 20:1) for 10 min. Whole cell lysates were collected and analyzed by Western blotting to detect MAPK/ERK phosphorylation. *B*, Macrophages were pretreated with Syk inhibitor (black circles) or ZM336372 (white circles) at different concentrations 30 mins before exposure to a 20:1 ratio of opsonized amastigotes in the presence of HA. The supernatants were collected 6 hrs later, and IL-10 concentrations were determined by ELISA. Data represent one of three independent experiments (mean ± SD of triplicates).

that opsonized parasites activated MAPK/ERK through FcγR-mediated Syk activation that is independent of the Ras-Raf pathway.

Induction of IL-10 Gene Expression by Opsonized Parasites Along with HA

The molecular mechanisms of IL-10 gene expression were studied in detail. Nuclear pre-mRNA and cytoplasmic mature mRNA was isolated from infected macrophages. Pre-mRNA formation and the accumulation of cytoplasmic mature mRNA were both examined by qRT-PCR. In the presence of HA, opsonized amastigotes induced IL-10 transcription (Figure 13*A*). Pre-mRNA transcripts were detected as early as 15 minutes post-infection and reached the peak at around 1 hour. They returned to base level by 3 hours. Mature IL-10 mRNA was induced much later than the pre-mRNA. It became detectable 1 hour after infection and reached maximal level at 3 hours. IL-12p40 gene expression was undetectable under the same conditions. A similar phenomenon was observed using footpad-derived amastigotes. MAPK/ERK inhibition by U0126 prevented both transcription of IL-10 gene (Figure 13*B*) and accumulation of mature IL-10 mRNA (Figure 13*C*) from infected macrophages. These data demonstrate that MAPK/ERK activation is required for IL-10 transcription in response to *Leishmania* infection along with HA.

MAPK/ERK Activation Results in Histone Phosphorylation at the IL-10 Promoter

Epigenetic modulation of IL-10 gene expression was investigated to further explore the molecular mechanisms of IL-10 transcription by ChIP assays. Our previous observations indicated that MAPK/ERK activation by soluble immune complexes

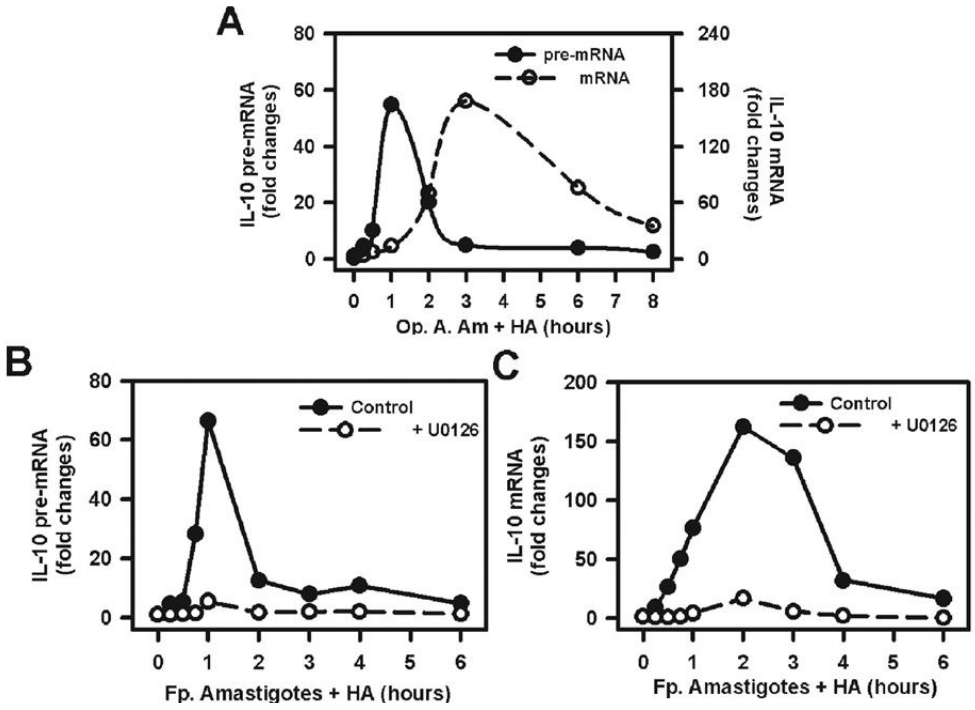

Figure 13. Parasites induce *IL-10* gene expression via MAPK/ERK activation. *A*, Macrophages (4 x 10^6 cells) were treated with opsonized axenic cultured amastigotes (Op. A. Am.) (MOI = 20:1) in the presence of HA (10 µg/ml). Cytoplasmic and nuclear RNA were isolated at different time intervals, as indicated. Real-time PCR was performed to detect the presence of *IL-10* pre-mRNA (solid line, *left axis*) and *IL-10* mRNA (dash line, *right axis*). *B* and *C*, Macrophages were pretreated with U0126 (2 µM) (white circles) or drug vehicle (black circles) for 1 h and then infected with lesion-derived amastigotes (Fp. Amastigotes) (at an MOI of 20:1) plus HA (10 µg/ml) for indicated times. Cytoplasmic and nuclear RNA were isolated, and the real-time PCR was performed to analyze the presence of *IL-10* pre-mRNA (*B*) and mature *IL-10* mRNA (*C*).

resulted in histone H3 phosphorylation (Lucas *et al.*, 2005). The nucleosome 2, as numbered from the transcriptional initiation site, contains the binding site for the transcription factor Sp1, which is important for IL-10 gene expression (Brightbill et al., 2000; Zhang *et al*. 2006). The nucleosome 11 is the control that is located about 1000bp upstream from the Sp1 binding site. 45 mins post-infection the footpad-derived amastigotes along with HA caused histone H3 phosphorylation at Ser 10 on nucleosome 2 but not distal nucleosome 11 (Figure 14*A*). There was a rapid binding of Sp1 to IL-10 promoter correlated with histone phosphorylation at nucleosome 2, but not nucleosome 11 (Figure 14*B*). PD98059 completely blocked both histone H3 phosphorylation and Sp1 binding.

In summary, opsonized parasites along with HA activate MAPK/ERK. MAPK/ERK activation resulted in histone H3 serine-10 phosphorylation, which leads to chromatin remodeling. This remodeling allows the transcription factor Sp1 to gain access to its binding element in the IL-10 promoter and drive IL-10 gene expression.

Manipulating MAPK/ERK Activation in the Macrophages Diminishes the Severity of L. Amazonensis Infection

Modulating IL-10 levels during *Leishmania* infections can influence the course of disease progression in the host. The over-production of IL-10 can impair the immune response to many pathogens as shown in a variety of animal models of infection. In contrast, reducing IL-10 often improved the resistance of the host to infection (Kane and Mosser, 2001). When rIL-10 was administrated, mice were found to be more susceptible to intracellular pathogens such as *L. monocytogenes*, *Streptococcus pneumoniae*, *Candida*

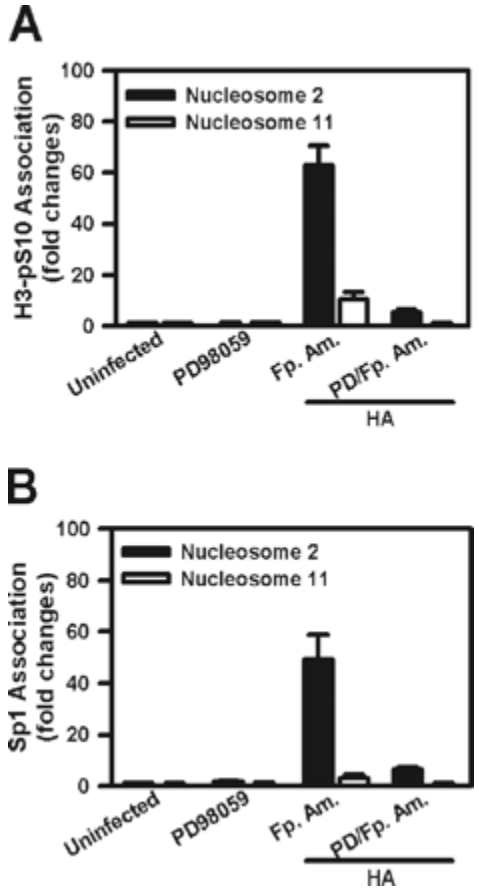

Figure 14. *IL-10* gene expression requires MAPK/ERK-mediated histone H3 Ser^{10} phosphorylation and the recruitment of Sp1. Macrophages (4 x 10^6 cells) were pretreated with or without PD098059 (10 µM) (PD) for 1 h and then infected with or without Fp. amastigotes (Fp. Am.) plus HA (10 µg/ml) for 45 min. The chromatin fragments were immunoprecipitated using a specific Ab against phosphorylated histone H3 at Ser^{10} (*A*) or an Ab to Sp1 (*B*). Real-time PCR was performed to determine the presence of DNA associated with nucleosome 2 (black bars) or nucleosome 11 (white bars), as described in *Materials and Methods*. Data represent mean ± SD with triplicates.

albicans, and *Trypanosoma cruzi* (Redpath *et al.*, 2001). However, if IL-10 levels are reduced during infection, mice are more resistant to the intracellular pathogens listed previously, as well as *T. gondii* and *L. major*. Our previous studies indicated that IL-10 contributes to leishmaniasis. IL-10 knockout mice are resistant to infection (Kane and Mosser, 2001). Because our *in vitro* studies show that MAPK/ERK activation led to the induction of IL-10 production by macrophages, we next performed *in vivo* experiments to determine whether manipulating MAPK/ERK can affect *L.amazonensis* infection in BALB/c mice.

BALB/c mice were infected with 10^5 lesion-derived amastigotes in the right hind footpad. Lesion progression was monitored twice weekly over a 7-wk period. The MAPK/ERK inhibitor, U0126 (10 mg/kg), was administrated intraperitoneally every 7-days beginning at the 18th day after infection for 5 weeks (Figure 15*A*). We selected U0126 over PD98059 for these in vivo studies because it has higher potency and solubility. Control BALB/c littermates developed measurable lesions within 3 weeks of infection and these lesions became progressively larger until the experiment was terminated by day 49. The lesions of U0126 treated mice were significantly smaller throughout the observation period ($p<0.05$) after administration of the inhibitor (Figure 15*A*). By using both serial dilution assay (left) and qRT-PCR (right) to amplify parasite DNA, the parasite burdens in the mice that received U0126 treatment were significantly lower than the untreated mice at day 49 (Figure 15*B*). After challenge with 50 µg/ml soluble *L. amazonensis* antigen (SLA), T cells of lymph nodes obtained from either control mice or U0126 treated mice produced the same amount of either IL-4 or IFN-γ,

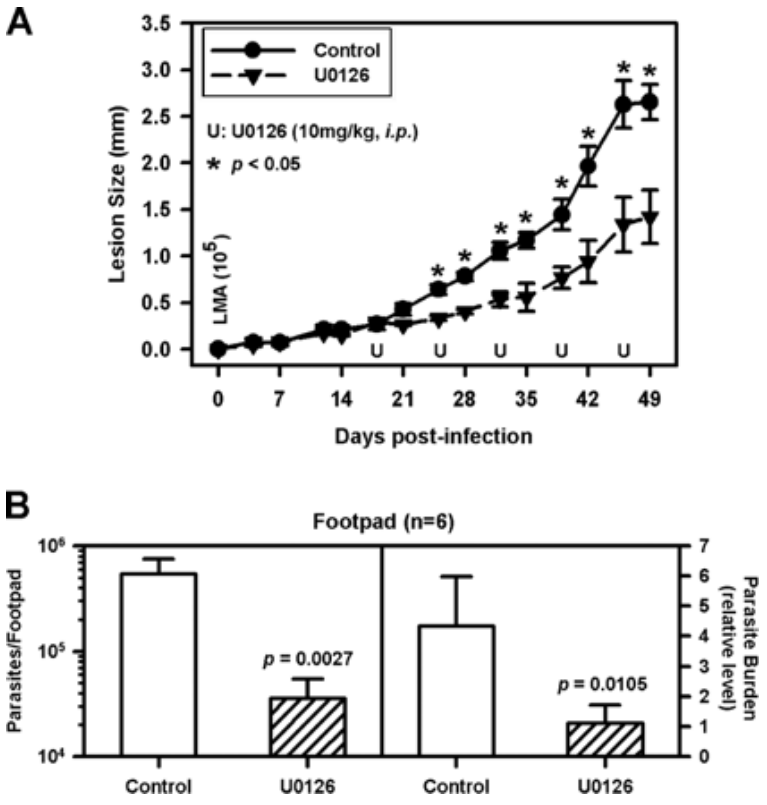

Figure 15. Inhibition of MAPK/ERK activation delays the progression of lesions in mice infected with LA in vivo. BALB/c mice control group (n = 6) (circles) and U0126-treated group (n = 6) (triangles) were injected with 1 x 10^5 lesion-derived amastigotes of LA in the hind footpad. After 18 days, weekly injections of U0126 (10 mg/kg) were administered intraperitoneally for 5 wk. The control group received the same volume of drug vehicle. *A*, Lesion size was measured on the indicated days. *B*, Parasite burdens in infected footpad were determined by limiting dilution assay (*left*) and qRT-PCR (*right*), as described in *Materials and Methods*. One representative experiment of three is shown. Data represent mean ± SD. The p values were determined by Student's t test. *, $p < 0.05$.

indicating that the inhibition of MAPK/ERK activation did not skew Th response, *i.e.*, Th1 *vs.* Th2.

To determine the correlation between the changes in lesion size and local IL-10 levels, mice were infected on day 1 with *L. amazonensis* and administered U0126 intraperitoneally at weekly intervals thereafter as indicated. At week 4, 5 and 6, mice were euthanized, and IL-10 mRNA in the feet of infected mice treated with U0126 (Figure 16, left, stripped bars) were substantially reduced relative to untreated mice (Figure 16, left, open bars). Interestingly, there was no significant difference in IL-10 levels in lymph nodes (Figure 16, right), indicating that IL-10 production in the infected footpad itself was responsible for lesion progression.

Previous studies showed that administration of anti-parasite serum to IgG-deficient JH BALB/c mice exacerbated *L. major* infection (Miles *et al.*, 2005). To further verify the inhibitory effects of U0126, the same experiments were conducted on IgG-deficient JH BALB/c mice with *L. amazonensis* infection. J_H mice were infected with 1 x 10^4 lesion-derived LA amastigotes (low dose) in the right hind footpad. Started from the 20th day post-infection, one group of mice was administrated U0126 (10 mg/kg) intraperitoneally once per week until 48 days post-infection. At the 21st day, the two groups of mice were injected intraperitoneally with 200 µl of anti-LA serum. Lesions in J_H mice became larger after being injected with anti-LA serum (Figure 17*A*, open triangles). The administration of U0126 reduced the antibody-induced increase in lesion swelling (Figure 17*A*, closed squares). These lesions were not significantly different from

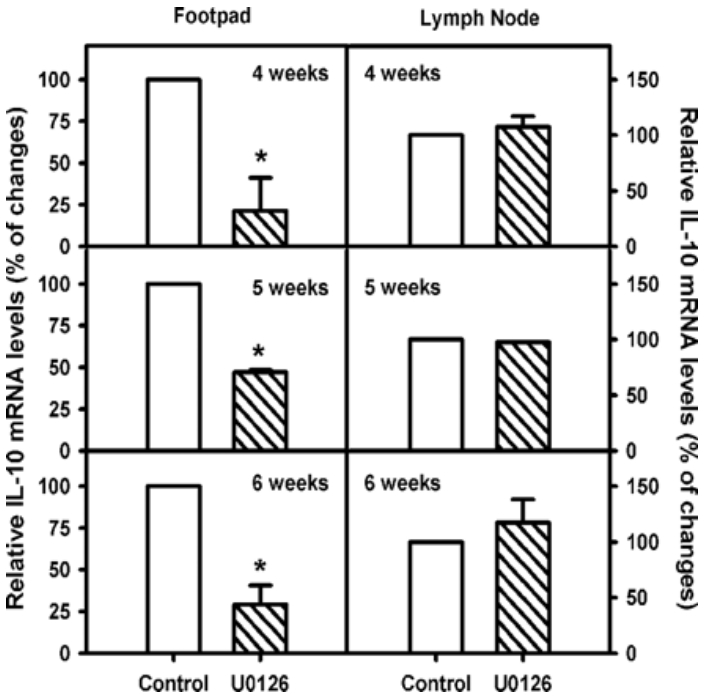

Figure 16. MAPK/ERK inhibition by U0126 reduces *IL-10* gene expression in lesions. BALB/c mice were infected with 1 x 10^5 lesion-derived amastigotes in the right hind footpad. Weekly injection of U0126 (10 mg/kg) was administrated to one group intraperitoneally for 6 wk (▨; $n = 3$). Total RNA was isolated on the day after U0126 administration for 3 wk, as indicated in the figures. IL-10 mRNA levels were determined by qRT-PCR, as described in *Materials and Methods*. After normalization by HPRT mRNA levels, IL-10 mRNA levels of infected mice without U0126 treatment (□; $n = 3$) were arbitrarily set as 100%. Relative IL-10 mRNA expression in the footpad (*left*) and the lymph node (*right*) was determined. Data represent mean ± SD with triplicates. The *p* values were determined by Student's *t* test. *, $p < 0.05$.

those in mice that didn't receive antibody (Figure 17*A*, closed circles). Parasites burdens in the infected footpads were measured by qRT-PCR (Figure 17*B*, left) and limiting dilution assay (Figure 17*B*, right). Mice that were administered anti-LA serum had significantly more parasites than untreated mice ($p \leq 0.01$) (black bars). The co-administration of U0126, however, reduced the parasite burdens back to untreated levels (stripped bars). Similar studies were also performed using high dose parasites (1×10^6), and they yielded similar results. U0126 treatment can decrease IgG-mediated increase of lesion (Figure 17*C*) that is correlated with reduced parasite burden (Figure 17*D*). These *in vivo* results are consistent with our *in vitro* data, showing that MAPK/ERK inhibition prevents IgG-mediated disease exacerbation.

Taken together, these *in vivo* observations are consistent with our *in vitro* data showing the MAPK/ERK activation is one of the critical events for parasite-immune complex-mediated leishmaniasis progression. The administration of MAPK/ERK inhibitors exhibited a significant influence on disease progression, decreasing lesion size and reducing parasite burdens. This administration also resulted in reduced IL-10 levels in the lesions, but not in the draining lymph nodes, suggesting that localized MAPK/ERK-dependent production of IL-10 in the lesions was responsible for lesion progression. Thus, pharmaceutical intervention *via* blocking MAPK/ERK activation together with other available therapeutics may provide a novel approach to the treatment of progressive visceral leishmaniasis. However MAPK's activation is not only required for IL-10 production but may also be required for macrophage microbicidal activity, such as NO production. Thus, lesion development in leishmaniasis may represent a balance between the immune activating and the immune inhibitory effects of the MAPKs.

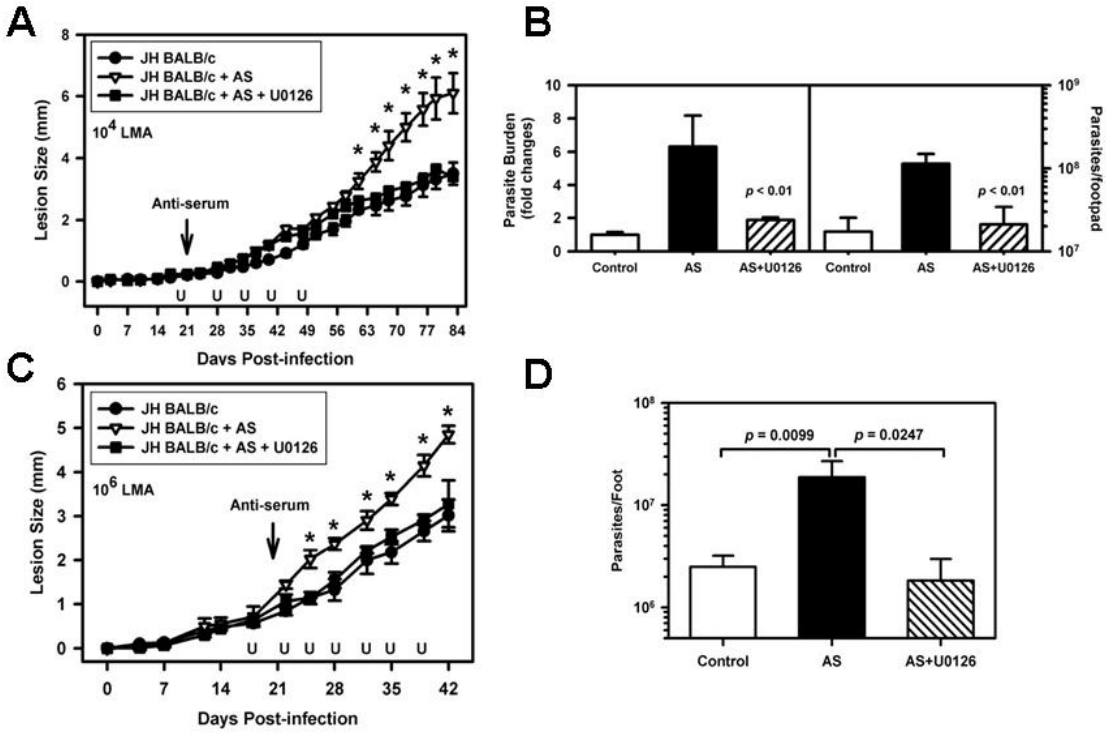

Figure 17. The inhibition of MAPK/ERK activation prevents IgG-mediated exacerbation of disease. J_H mice on the BALB/c background were infected with 1 x 10^4 (*A* and *B*) or 1 x 10^6 (*C* and *D*) lesion-derived amastigotes in the right hind footpad. Two groups of mice (n = 6) were injected intraperitoneally with 200 µl of anti-LA serum at the 21st day post-infection. One of these groups (■) was administrated with U0126 (10 mg/kg) intraperitoneally, as indicated. Another group of mice (•) (n = 6) was treated with drug vehicle as a control. Lesion size was measured on the indicated days following infection with 1 x 10^4 (*A*) or 1 x 10^6 (*C*) parasites. *B*, Parasite burdens were determined by limiting dilution assay (*right*) and qRT-PCR (*left*, and *D*), as described in Materials and Methods. Data represent mean ± SD. The p values were determined by Student's t test. *, $p < 0.05$.

Discussion

The interaction of *Leishmania* parasites with host macrophages can result in altered intracellular signaling pathways, leading to parasite survival within infected macrophages. In this study, we describe the activation of MAPK, MAPK/ERK following infection of macrophages with LA parasites. Previous studies have correlated MAPK/ERK activation with leishmaniasis. Lipophosphoglycan from *Leishmania* has been reported to subvert macrophage IL-12 production by activating MAPK/ERK (Feng *et al*., 1999). It has also been suggested that the strength of CD40 signaling may influence the specific MAPK pathway that is activated, and thereby influence cytokine production from infected cells (Mathur *et al.*, 2004). In the present work, we demonstrate that opsonized amastigotes of *Leishmania* induce MAPK-MAPK/ERK activation in macrophages. This activation results in epigenetic modifications of *il-10* gene locus, thereby causing a superinduction of IL-10 from infected macrophages.

Importantly, lesion-derived amastigotes alone are not sufficient to induce IL-10 production, despite their ability to rapidly activate MAPK/ERK. Parasites must be combined with some inflammatory stimulus to induce macrophage IL-10 production. These stimuli can be fragments of hyaluronan, called LMW-HA. Hyaluronan is a major component of extracellular matrix and exists as a high-molecular weight polymer under normal physiological conditions. After tissue injury, small fragments of hyaluronan are generated at the site of injury (Termeer *et al.*, 2002; Jiang *et. al*., 2005). Several studies suggest that these hyaluronan fragments can signal through TLR2 and 4 on endothelial cells and DCs (Hodge-Dufour et al., 1997; Termeer *et al.*, 2002; Jiang *et. al*., 2005).

LMW-HA is not the only inflammatory signal that can co-induce IL-10 production. Often leishmanial lesions are super-infected with other microorganisms, which can provide the inflammatory stimulus via any TLR, including TLR2 or 4. Alternatively, the lysis of heavily infected macrophages may release heat shock proteins (Tsan and Gao, 2004) or high mobility group protein 1 (Park *et al.*, 2004) from mammalian cells to stimulate IL-10 production.

Our findings also indicate that signaling through the macrophage FcγR is critical for IL-10 induction. Axenically grown amastigotes that lack IgG (Kane and Mosser, 2001) failed to activate MAPK/ERK and failed to induce IL-10 production (Figure 6). The opsonization of these organisms with IgG restored their ability to activate MAPK/ERK and induce IL-10 (Figure 8*B*). Furthermore, cells lacking FcR γ chains failed to activate MAPK/ERK, and they failed to produce IL-10 in response to infection (Figure 10) (Kane and Mosser, 2001).

In our model, the delayed activation of MAPK/ERK by inflammatory mediators, such as LMW-HA, is sufficient to induce only modest levels of IL-10 production from macrophages. However, the addition of IgG-opsonized amastigotes dramatically increased the speed with which MAPK/ERK was activated, and it also increased the magnitude and the duration of MAPK/ERK activation. This hyperactivation of MAPK/ERK resulted in the phosphorylation of histone H3 at Ser^{10}. The histones associated with Sp1 binding site were highly phosphorylated. The phosphorylation of histones makes this promoter region more accessible to Sp1 (Figure 14*B*), resulting in a

dramatic superinduction of *IL-10* transcription. The result is the secretion of high levels of this inhibitory cytokine by infected macrophages.

A critical component of the proposed model is that the amastigotes in the lesions have host IgG on their surface, which has been reported before (Guy and Belosevic, 1993; Peters *et al.*, 1995; Kane and Mosser, 2001). The IgG on amastigotes appears to be the result of a parasite-specific IgG response by the host. Several studies have demonstrated that high levels of parasite-specific IgG are generated during leishmaniasis (Kima *et al.*, 2000; Colmenares *et al.*, 2002). This is especially true with human visceral leishmaniasis in which rheumatoid factor (Carvalho *et al.*, 1983; Newkirk, 2000; Pearson *et al.*, 1983) and parasite-specific IgG levels are high (Junqueira *et al.*, 2003; Ghosh *et al.*, 1995; Casato *et al.*, 1999; Jeronimo *et al.*, 2000; Galvão-Castro *et al.*, 1984; Elassad *et al.*, 1994), making it more likely that amastigotes derived from lesions would be opsonized with host IgG. Our model would predict that disease exacerbation caused by immune complexes would only occur late in disease, after parasite-specific IgG was generated. We predict that the re-infection of macrophages by IgG-opsonized amastigotes would be the trigger for IL-10 production. For these reasons, we performed in vivo infection studies in which we inhibited MEK/MAPK/ERK activation relatively late in disease, after the lesions had progressed for 21 days. The administration of MAPK/ERK inhibitors at this late time still exhibited a significant influence on disease progression, decreasing lesion size and reducing parasite burdens. This administration also resulted in reduced IL-10 levels in the lesions, but not in the draining lymph nodes, suggesting that localized MAPK/ERK-dependent production of IL-10 in the lesions was responsible for lesion progression.

In summary, our current findings detail the molecular mechanisms of IL-10 production by amastigote-infected macrophages. They reveal a central role for MAPK/ERK that is required for maximal IL-10 production. These studies lead to several predictions. The first is that the activation of MAPK/ERK in any infectious disease may predispose the host to inhibitory IL-10 production. These studies confirm a role for IL-10 during disease progression, and they may lead to the development of a new class of therapeutics to treat human visceral leishmaniasis. Finally, these studies would predict that vaccines against intracellular pathogens might be more effective if administered in the presence of an MAPK/ERK inhibitor. Vaccination strategies that inhibit regulatory signaling networks in DCs by encompassing MAPK/ERK inhibitors as nanoparticles or encapsulating other regulatory proteins together with TLR ligands and relevant antigen may be worthwhile for exploration in the near future.

CHAPTER 4: THE ROLE OF MAPK/p38 IN IL-12 PRODUCTION IN CLASSICALLY ACTIVATED MACROPHAGES

Classically activated macrophages, which are activated by TLR ligands, play an important role in parasite killing by producing NO and proinflammatory cytokines, TNFα and IL-12. TLRs can trigger innate immune responses through MAPKs signaling pathways. It appears that MAPK/p38 activation may play a dominant role in TLR4-mediated signaling. It has been shown that the MAPK, MAPK/p38, plays a positive role in TNFα production (Mahtani *et al.*, 2001). Evidence of MAPKs involved in IL-12 and IL-23 production has been reported, but due to different cell types and varied experimental conditions, these results are contradictory. Thus, how MAPKs affect IL-12p40 and IL-12p70 remains elusive.

MAPK/p38 Inhibition Has Reciprocal Effects on IL-12 and TNFα

MAPK/p38 inhibitors were employed to determine whether MAPK/p38 could influence macrophage IL-12p40 production. LPS induced the production of both TNFα and IL-12p40 from bone marrow derived macrophages (Figure 18*A*). Inhibition of MAPK/p38 by SB203580 reduced TNFα but increased IL-12p40 in a dose-dependent manner (Figure 18*A*). At the highest levels of MAPK/p38 inhibitor used, IL-12p40 level was increased by approximately 5-fold. It has been previously reported that SB203580 can inhibit TNFα production (Mahtani *et al.*, 2001), but the enhancement of IL-12p40 was not expected. Next MAPK/p38 activation was examined in BMMϕ using western blot analysis. MAPK/p38 phosphorylation was found in BMMϕ upon stimulation with

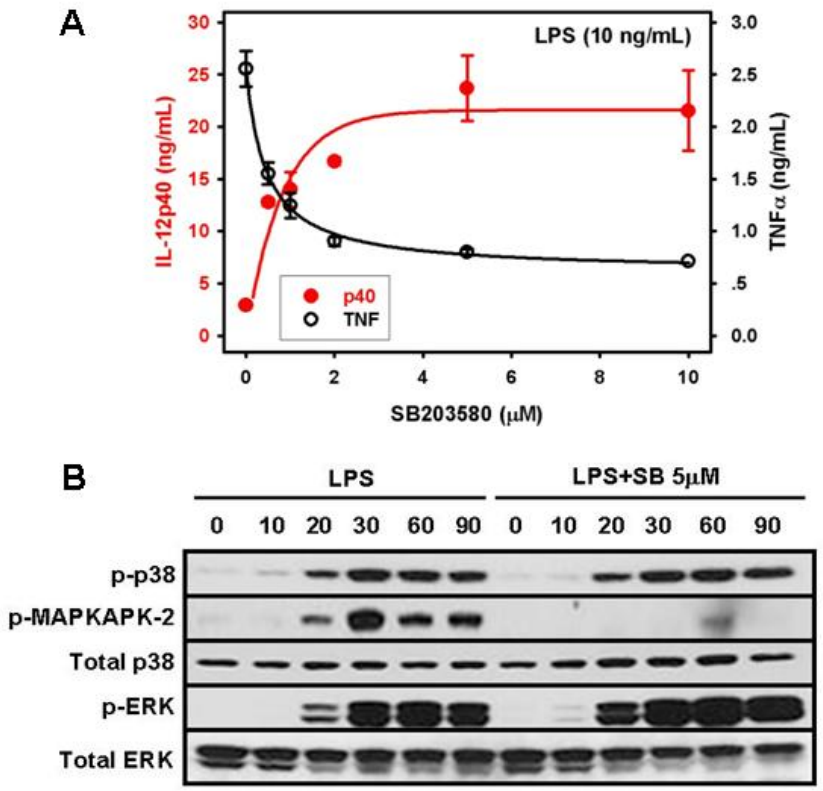

Figure 18. MAPK/p38 inhibition has reciprocal effects on IL-12 and TNFα. *A*, Macrophages (3×10^5 cells) were pretreated with increasing concentrations of SB203580 for 1 h and then stimulated with LPS (10 ng/ml) for overnight. Supernatants were harvested, and IL-12p40 and TNFα production were determined by ELISA. Values are representative of at least three independent experiments (mean ± SD of triplicates). *B*, Macrophages (2×10^6 cells) were pretreated with SB203580 (5 μM) for 1 h. Cells were then stimulated with LPS (10 ng/ml) for 0, 10, 20, 30, 60 and 90mins. Cell lysates were prepared for Western blotting analysis to detect phosphorylated MAPK/p38, phosphorylated MAPKAPK-2 and total MAPK/p38 protein.

LPS in a time-dependent manner (Figure 18*B*). Both MAPK/p38 and its downstream kinase MAPKAPK-2 activation could be detected as early as 20 min after stimulation. Activation reached a peak at 30 mins and persisted for 90 mins. SB203580 inhibits the enzymatic activity of MAPK/p38. Thus, treatment of macrophages with 5 μM SB203580 blocked the phosphorylation of its downstream MAPKAPK-2 without affecting MAPK/p38 phosphorylation (Figure 18*B*).

MAPK/p38 Inhibition Has the Similar Effects on Cytokine Production Mediated by Different TLR Agonists

In order to test whether MAPK/p38 inhibition-induced IL-12p40 production is a unique feature for TLR4 stimulation or common to all TLR activation, CpG, a ligand for TLR9, lipoprotein A, a ligand for TLR2/TLR6, and flagellin, a ligand for TLR5 were used to stimulate macrophages. All of these TLR agonists induced TNFα and IL-12p40 production from macrophages (Figure 19). SB203580 showed similar effects on cytokine production as it did for LPS stimulation. TNFα was reduced while IL-12p40 was increased in a dose-dependent manner (Figure 19). The total amount of cytokine induced by these different TLR agonists varied, but the extent of MAPK/p38-mediated cytokine enhancement and inhibition was comparable. These data indicate that MAPK/p38 inhibition-induced IL-12p40 production was a universal feature for TLR-mediated signaling pathways.

Similar Roles of MAPK/p38 on Cytokine Production in Different APCs

To determine whether this phenomenon was applicable to BMMϕs only, similar

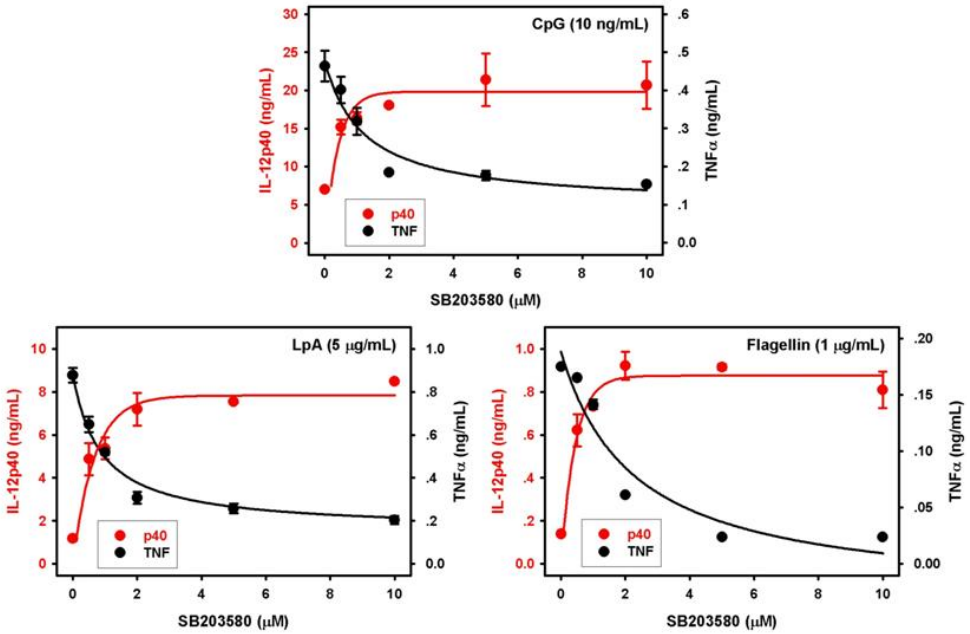

Figure 19. MAPK/p38 inhibition has the similar effects on cytokine production mediated by different TLR agonists. Macrophages (3 x 10^5 cells) pretreated with increasing concentrations of SB203580 for 1 h were stimulated with CpG (10 ng/ml), lipoprotein A (5μg/ml) and flagellin (1μg/ml) for overnight. Supernatants were harvested to detect IL-12p40 and TNFα production using ELISA. Data represent one of three independent experiments (mean ± SD of triplicates).

experiments were carried out using resident peritoneal macrophages and BMDCs in addition to BMMϕs. In the presence of LPS, SB203580 induced IL-12p40 production from all three types of cells, BMMϕs, peritoneal resident macrophages and BMDC (Figure 20). In BMDCs, MAPK/p38 inhibition also enhanced IL-12p70 production by LPS (Figure 20). A structurally related control compound, designated SB202474, had no effect on IL-12p40 and IL-12p70 production. For all of these studies, the structural control compound SB202474 failed to influence cytokine production.

These data show a similar degree of IL-12 enhancement by inhibition of MAPK/p38 with different TLR agonists not only in BMMϕ but also in peritoneal resident macrophages and BMDCs. Thus, the MAPK/p38 pathway plays a negative regulatory role in IL-12 production by antigen-presenting cells.

MAPK/p38 Inhibition Increases IL-12p40 Due to Enhanced mRNA Stability

To gain further insight into the molecular mechanisms of *IL-12p40* gene expression by MAPK/p38, nuclear pre-mRNA and cytoplasmic mature mRNA were isolated following stimulation of macrophages with LPS. Gene transcription was monitored by measuring pre-mRNA formation using real-time PCR as previously described (Zhang *et al.*, 2006). The accumulation of cytoplasmic mature spliced IL-12 mRNA was also examined. IL-12p40 pre-mRNA expression quickly reached maximal levels at 1 hour after LPS stimulation and returned to basal levels by 4 hours (Figure 21*A*, solid line). IL-12p40 mature mRNA-induced by LPS also peaked at around 1 hour and returned to basal level between 4 to 6 hours (Figure 21*B*, solid line). Interestingly, MAPK/p38 inhibition by SB203580 prevented *IL-12p40* transcription (Figure 21*A*, dash

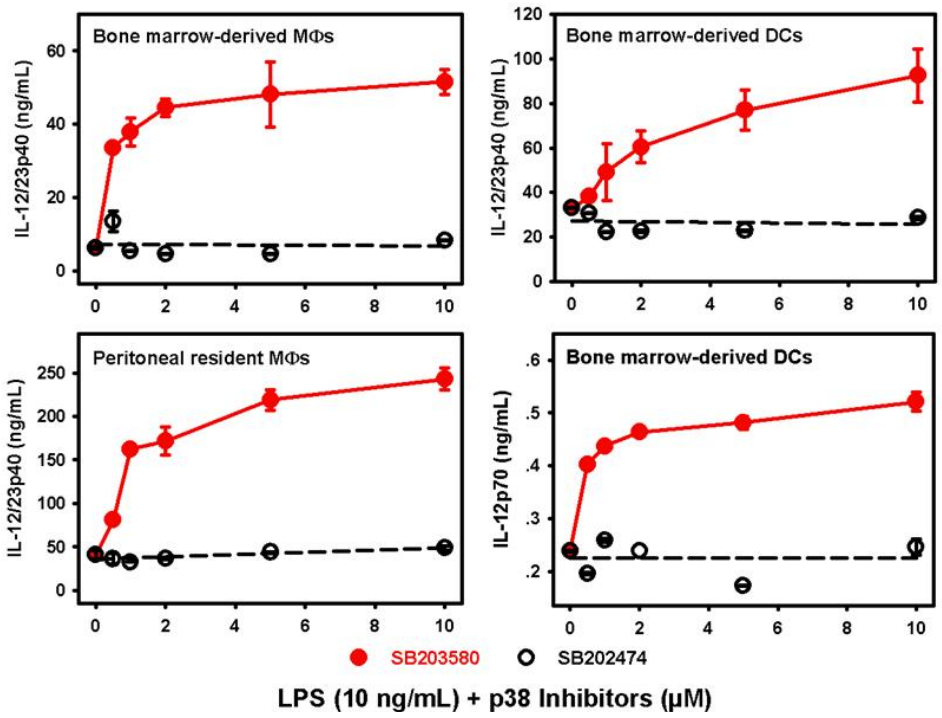

Figure 20. Similar roles of MAPK/p38 on cytokine production from BMMϕs, peritoneal resident macrophages and BMDCs. BMMϕs (top panel), peritoneal resident macrophages (middle panel) and BMDCs (bottom panel) (3 x 10^5 cells) were pretreated with increasing concentrations of SB203580 (red circle) and SB202474 (white circle) for 1 h and then stimulated with LPS (10 ng/ml) for overnight. Supernatants were harvested for ELISA analysis of IL-12p40 and IL-12p70 production. Data represent one of three independent experiments (mean ± SD of triplicates).

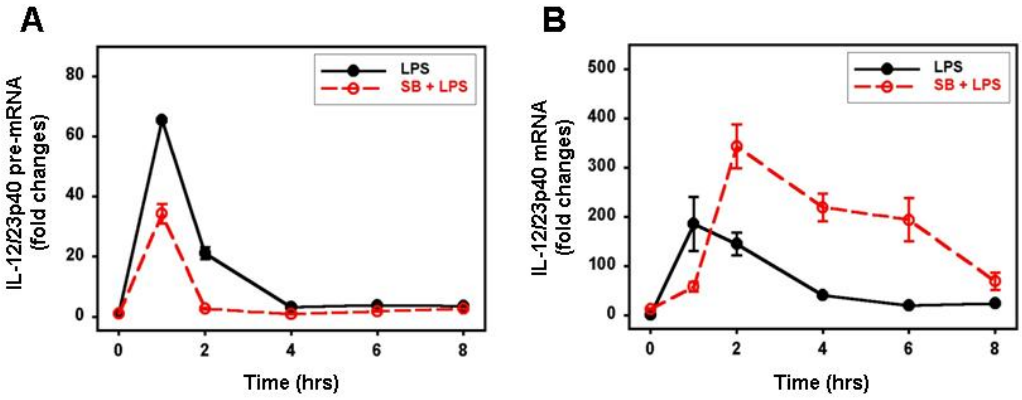

Figure 21. MAPK/p38 inhibition enhances IL-12p40 mRNA accumulation. *A*, Macrophages (4×10^6 cells) were pretreated with (dash line) or without (solid line) SB203580 (5 µM) and then stimulated with LPS (10 ng/ml). Cytoplasmic and nuclear RNA were isolated at different time intervals as indicated. Real-time PCR was performed to detect the presence of *IL-12p40* pre-mRNA (*A*) and *IL-12p40* mRNA (*B*).

line) but enhanced mature mRNA formation (Figure 21*B*, dash line).

In order to verify the effect of MAPK/p38 inhibition on IL-12p40 promoter activity, RAW264.7 cells were transfected with pGL3-IL-12p40-promoter plasmid and pRL-TK plasmid (as an internal control) to normalize for transfection and harvest efficiency. After 24 hrs, cells were pretreated for 1 hour with drug vehicle (control) or SB203580, then stimulated with LPS. SB203580 can inhibit IL-12p40 promoter activity (Figure 22), which is correlated with the results shown in Figure 21*A* that MAPK/p38 inhibition inhibited *IL-12p40* transcripts.

Since IL-12p70 is a heterodimeric cytokine comprising p35 and p40, the changes of IL-12p35 mRNA were measured in both BMMϕ (Figure 23*A*) and BMDC (Figure 23*B*). Inhibition of MAPK/p38 activation by SB203580 did not have a significant effect on IL-12p35 mRNA as compared to IL-12p40 mRNA.

As MAPK/p38 inhibition had a positive effect on IL-12p40 mRNA accumulation but not on transcription, therefore *IL-12p40* mRNA degradation was further studied in more details. Macrophages treated with SB203580 or its drug vehicle was stimulated with LPS for 2 hours, and then a transcription inhibitor actinomycin D (10 μg/ml) was added. Degradation of mRNA was measured at different time points for the following 4 hours. As shown in Figure 24, in the presence of SB203580 IL-12p40 mRNA (top panel) was more stable with its half-life increased by approximately two-fold, from 1.1 hours to 2.5 hours. In contrast, both TNFα (middle panel) and IL-10 (bottom panel) mRNA were less stable when MAPK/p38 was inhibited. The half-life of TNFα was reduced more than

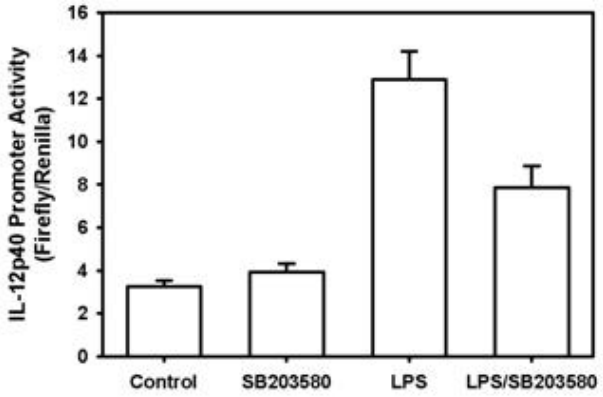

Figure 22. IL-12p40 promoter activity is down-regulated by MAPK/p38 inhibitor.
RAW264.7 macrophage cells were transfected with pGL3-IL-12p40-promoter plasmid and pRL-TK plasmid as internal control to normalize for transfection and harvest efficiency. After 24 hrs, cells were pretreated 1 hour with drug vehicle (control) or 5μM SB203580, and then stimulated with LPS (10 ng/mL). IL-12p40 promoter activity is expressed as the ratio of firefly luciferase activity and renilla luciferase activity. Results are mean±SEM for three independent experiments.

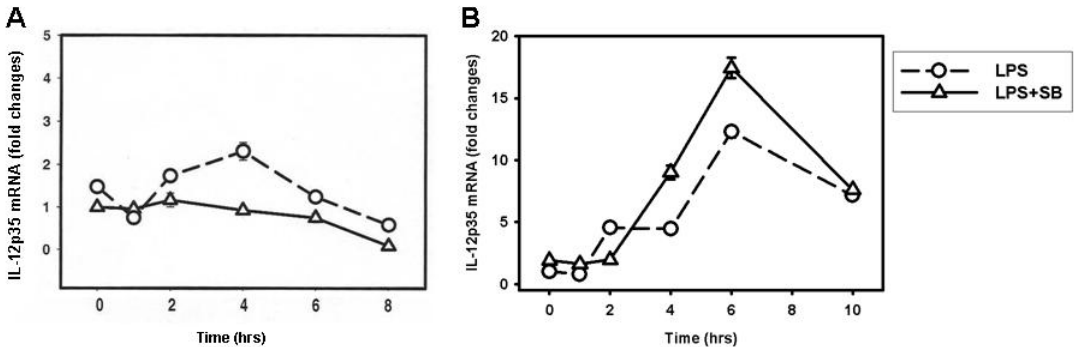

Figure 23. MAPK/p38 inhibition has no significant effect on IL-12p35 mRNA. BMMϕ (A) or BMDC (B) were pretreated without (circle and dash line) or with (triangle and solid line) SB203580 (5 μM) and then stimulated with LPS (10 ng/ml). Cytoplasmic RNA was isolated at different time intervals as indicated. Real-time PCR was performed to detect the presence of *IL-12p35* mRNA.

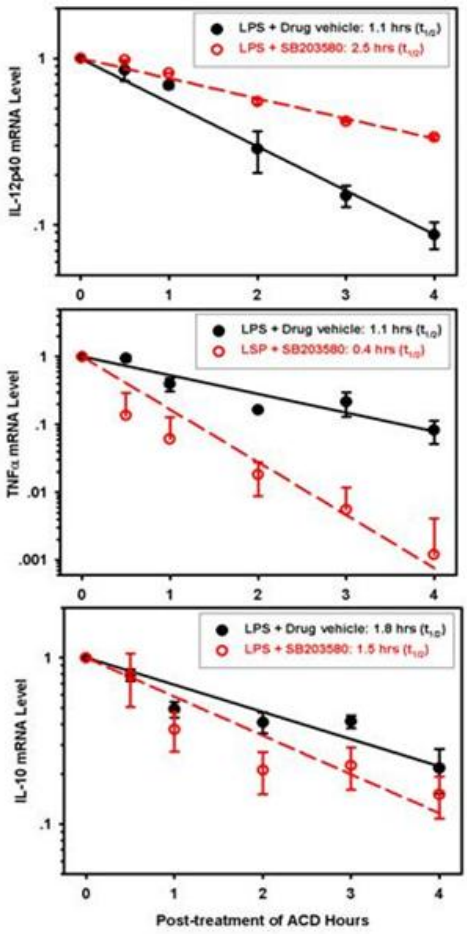

Figure 24. MAPK/p38 inhibition affects the mRNA stability of cytokines. Macrophages pretreated with drug vehicle (solid line) or SB203580 (5 μM) (dash line) for 1 h were stimulated with LPS (10 ng/ml) for 2 hours and then actinomycine D (10μg/ml) was added. RNA was isolated at the indicated time intervals. qRT-PCR was performed to analyze the mRNA levels of cytokines (top: *IL-12p40,* middle: *TNFα,* and bottom: *IL-10*) and GAPDH. Relative levels of each cytokine are normalized with GAPDH as described in Materials and Methods. The normalized mRNA levels in arbitrary units are presented on a log scale to obtain a linear relationship. Data represent one of three independent experiments (mean ± SD of triplicates).

two-fold, from 1.1 hours to 0.4 hours, which is in agreement with previous findings (Mahtani *et al.*, 2001). These data indicate that the increased IL-12p40 production mediated by MAPK/p38 inhibition is at least in part attributed to enhanced mRNA stability.

The Effect of MAPK/p38 on IL-12p40 Can be Independent of IL-10

It is well know that IL-10 can inhibit IL-12 transcription and translation (Mosser and Zhang, 2008). Therefore macrophages derived from IL-10 knockout mice were used to determine whether IL-12 enhancement caused by MAPK/p38 inhibition was dependent on IL-10 changes. MAPK/p38 inhibition by SB203580 increased IL-12p40 production by about three-fold in IL-$10^{-/-}$ macrophages (Figure 25*A*), while TNFα production was significantly reduced by SB203580 (Figure 25B). This increase occurred despite the fact that basal LPS-induced IL-12 production was much higher in IL-$10^{-/-}$ macrophages as compared to the cells derived from control littermates. Similar to what had been observed in macrophages derived from wild type mice, MAPK/p38 inhibition diminished early *IL-12p40* transcription induced by LPS but increased mature mRNA accumulation in IL-10 deficient macrophages (Figure 26) due to increase in mRNA stability. LPS-induced IL-12p40 mRNA was more stable in the presence of SB203580 (Figure 27*A*) whereas TNFα mRNA became less stable (Figure 27*B*). These data indicate that MAPK/p38 inhibition induced enhancement of IL-12 production can occur in the absence of IL-10.

Specific siRNA Knock Down on MAPK/p38 Has the Similar Effect as SB203580 Does on IL-12p40 Production

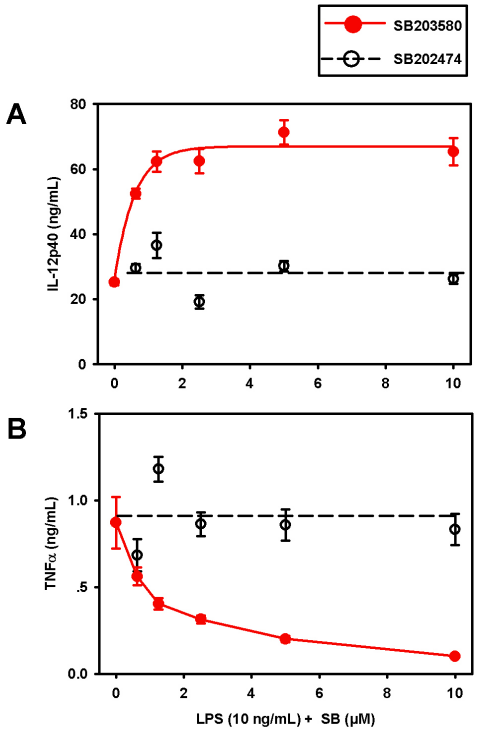

Figure 25. The effect of MAPK/p38 on IL-12 can be independent of IL-10. IL-$10^{-/-}$ macrophages were pretreated with increasing concentrations of SB203580 (solid line) or SB202474 (dash line) for 1 hour and then stimulated with LPS (10 ng/ml) for overnight. Supernatants were harvested, and IL-12p40 (A) and TNFα (B) production were determined using ELISA. Data represent one of three independent experiments (mean ± SD of triplicates).

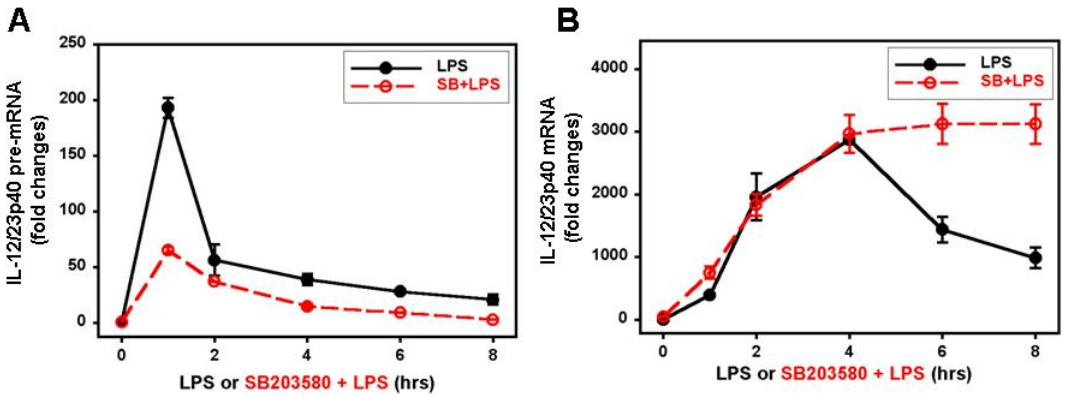

Figure 26. MAPK/p38 inhibition induced IL-12p40 mRNA accumulation is independent of IL-10. *A*, IL-10$^{-/-}$ macrophages (4 x 10^6 cells) were pretreated with (dash line) or without (solid line) SB203580 (5 µM) and then stimulated with LPS (10 ng/ml). Cytoplasmic and nuclear RNA were isolated at different time intervals as indicated. Real-time PCR was performed to detect the presence of *IL-12p40* pre-mRNA (*A*) and *IL-12p40* mRNA (*B*).

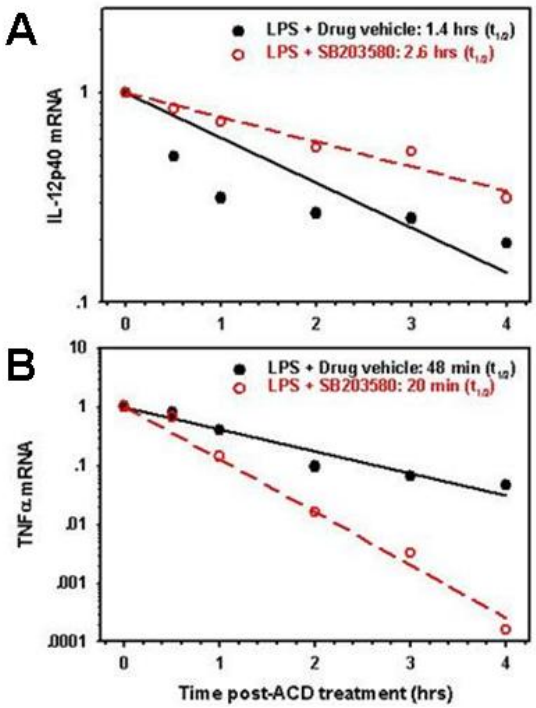

Figure 27. Regulation of cytokines mRNA stability by MAPK/p38 inhibition is IL-10 independent. IL-$10^{-/-}$ macrophages were pretreated with SB203580 (solid line) or with SB202474 (dash line) (5 µM) for 1 h were stimulated with LPS (10 ng/ml) for 2 hours and then actinomycin D (10µg/ml) was added. RNA was isolated afterwards at the indicated time intervals. qRT-PCR was performed to analyze the mRNA stability of cytokines (A, *IL-12p40*, and B, *TNFα*). The normalized mRNA levels in arbiturary units are presented on a log scale to obtain a linear relationship.

Four isoforms of MAPK/p38, α, β, γ and δ, have been identified. Western blot analysis could only detect the presence of the α form but not the other three isoforms in the BMMϕs (Figure 28*A*, inserted), and real-time PCR analysis showed that MAPK/p38α was the dominant form, exceeding MAPK/p38β levels by approximately 10-fold (Figure 28*A*). MAPK/p38δ and γ mRNA expression was barely measurable (Figure 28*A*). To further examine the specific role for MAPK/p38 in IL-12 induction, siRNA specific for MAPK/p38α were introduced into BMMϕs 48 hours before cells were stimulated with LPS. As shown in Figure 26*B*, 10 nM of siRNA specific for MAPK/p38α were sufficient to knock down 80% of MAPK/p38α mRNA expression as measured by real-time PCR and more than 60% of its protein level as confirmed by western blot analysis. Knock-down of MAPK/p38 in primary macrophages enabled these cells to produce more IL-12p40. This increase in p40 production correlated with the dose of siRNA used, and at the concentration of 30 nM, p40 production approached the levels observed with SB203580 inhibition (Figure 28*C*). The data further confirm that MAPK/p38 is the target for its role on IL-12p40 production and down-regulation of MAPK/p38 activity increases LPS-induced IL-12p40 production.

MKK3 Plays the Same Role on LPS-induced IL-12 Production in Macrophages

MKK3 is one of the upstream kinases that directly activate MAPK/p38. In order to show a role of MAPK/p38, macrophages from the MKK3 knockout mice were employed. In the wild type macrophages, LPS induced phosphorylation of both MAPK/p38 and its downstream kinase MK2 in a time-dependent manner, whereas in

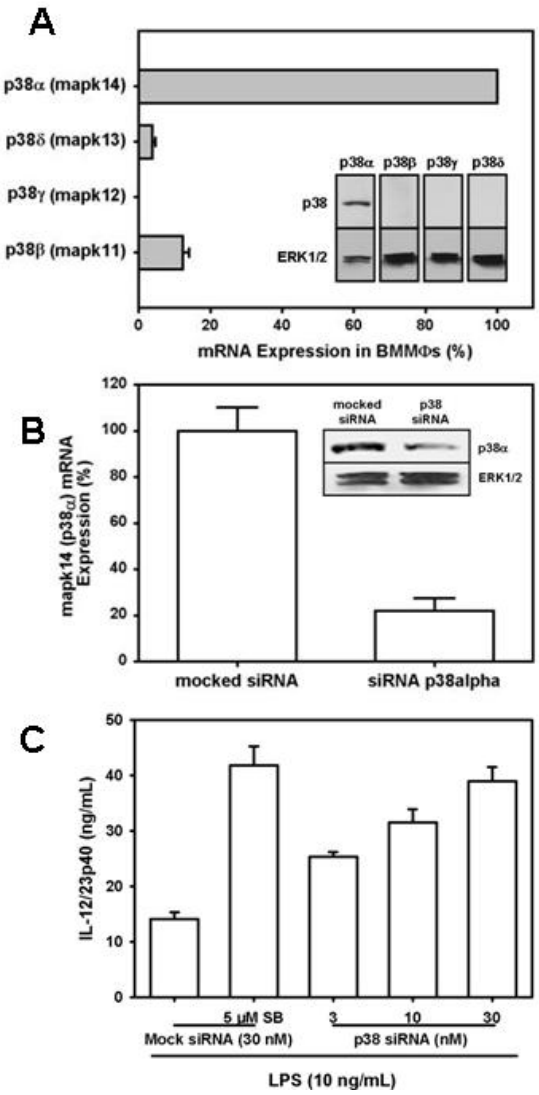

Figure 28. MAPK/p38 knock down has the same effect on IL-12p40. *A*, Expression of MAPK/p38 isoforms in BMMϕs. qRT-PCR was performed to detect the presence of MAPK/p38α, β, γ and δ mRNA. *(Insertion)* Cell lysates from macrophages were prepared for western blotting analysis of protein MAPK/p38α, β, γ or δ and MAPK/ERK. *B*, 10nM siRNA or mock siRNA was transfected into BMMϕs by nucleofection. BMMϕs were cultured for 48 hours. Total RNA was isolated and qRT-PCR was performed to analyze MAPK/p38α mRNA. *(Insertion)* Cell lysates from siRNA-transfected macrophages were prepared for western blotting analysis of protein MAPK/p38α or MAPK/ERK. *C*, BMMϕs were transfected with mock siRNA (30 nM) for 48 hours, and then treated with or without SB203580 (5 μM) for 1 hour before LPS stimulation for overnight; or BMMϕs were transfected with 3nM, 10nM and 30nM siRNA targeting MAPK/p38 for 48 hours, and then stimulated with LPS for overnight. Supernatants were harvested to detect IL-12p40 protein using ELISA.

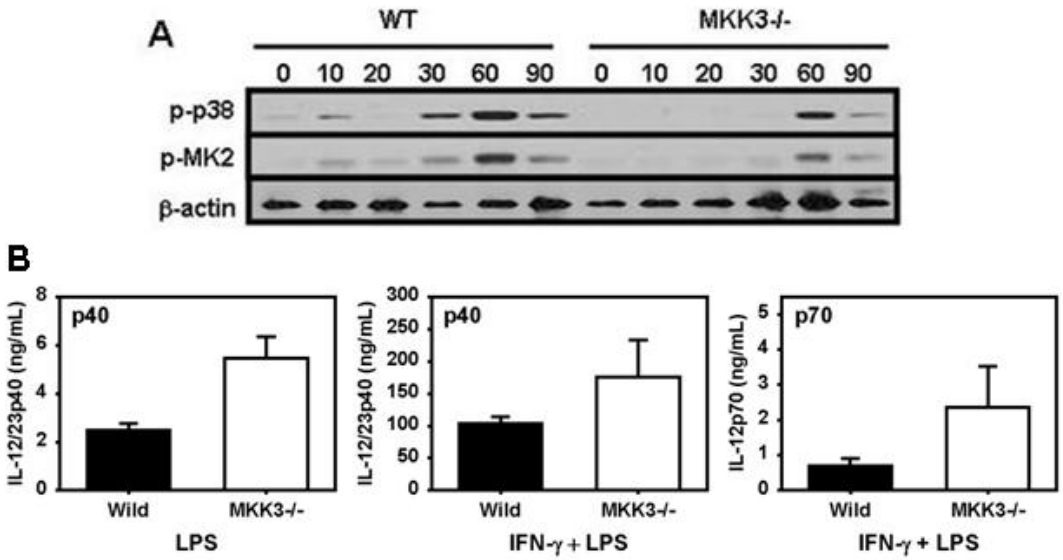

Figure 29. The role of MKK3 on cytokine production. *A*, BMMϕs from MKK3 knocked out mice and control littermates were stimulated with LPS (10ng/ml) for the indicated time interval. Cell lysates were prepared for western blotting analysis of phospho-MAPK/p38, phospho-MK2 and phospho-MAPK/ERK as well as β–actin. *B*, BMMϕs from wild type mice (black bars) and MKK3 knocked out mice (white bars) were primed with or without IFNγ (100U/ml) and then stimulated with LPS (10ng/ml) for overnight. ELISA was performed to detect the cytokine production.

MKK3$^{-/-}$ cells, the extent of LPS-induced MAPK/p38 and MK2 activation was markedly reduced (Figure 29*A*). In the absence of MKK3, these stimulated macrophages were capable of producing substantial amounts of IL-12p40 upon LPS stimulation (Figure 29*B*, left panel). Furthermore, when these macrophages were primed with IFN-γ (100U/ml) overnight and then stimulated, MKK3$^{-/-}$ macrophages produced more IL-12p40 (Figure 29*B*, middle panel) and IL-12p70 (Figure 29*B*, right panel) than wild type macrophages upon LPS stimulation. These data demonstrate again that MAPK/p38 plays a negative role on LPS-induced IL-12 production.

Inhibition of MAPK/p38 Activation Favors a Th1 Immune Response

Since MAPK/p38 inhibition could increase IL-12 production *in vitro*, we investigated whether MAPK/p38 inhibition could polarize Th1 immune response. CpG is a well-defined vaccine adjuvant for years. Macrophages were stimulated with OVA with or without CpG plus SB203580. OVA itself barely induced IL-12 production (Figure 30*A*). Addition of CpG induced some IL-12p40 (Figure 30*A*, white bar) and a measurable level of IL-12p70 (Figure 30*A*, gray bar). Inhibition of MAPK/p38 activation by SB203580 strongly facilitated CpG to induce much more IL-12p40 and IL-12p70 (Figure 30*A*). T cells derived from D011.10 mice, expressing transgenic TCRs specific for OVA peptides, were co-cultured with those treated macrophages for 3 days, and the supernatants were collected for detection of IFN-γ and IL-4. These T cells produced more IFN-γ and less IL-4 in the presence of macrophages that were pre-treated with OVA plus CpG and SB203580 (Figure 30*B*), indicating MAPK/p38 inhibition favored a Th1 response.

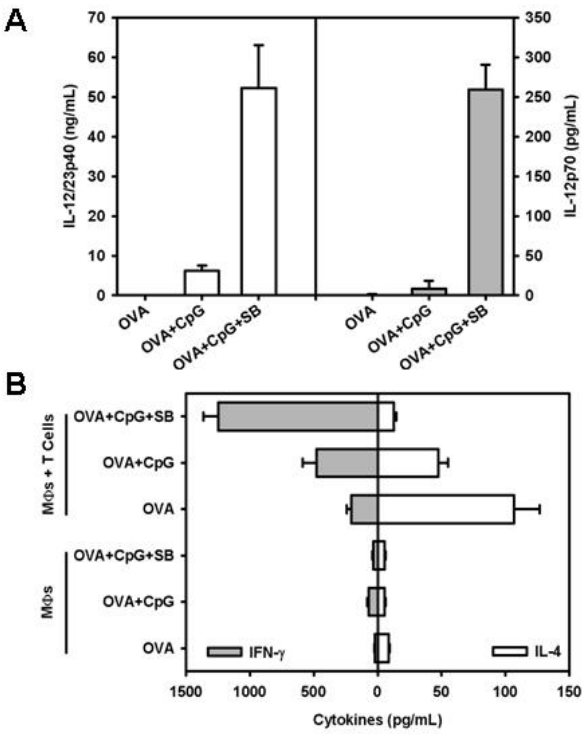

Figure 30. Inhibition of MAPK/p38 activation favors Th1 responses *in vitro*. *A*, BMMϕs were stimulated with OVA (100μg/ml), OVA+ CpG (10ng/ml) with or without SB 203580 (5μM) for overnight. Supernatant were collected to detect IL-12p40 and IL-12p70. *B*, The remaining treated BMMϕs were co-cultured with or without D011.10 T cells for 3 days. Supernatants were harvested to detect IFNγ and IL-4 production using ELISA.

To examine whether the inhibition of MAPK/p38 would improve vaccinations against leishmaniasis, whose healing is linked to Th1 immunity, heat-killed *L. major* (HKLM) with or without CpG and SB203580 were administrated to BALB/c mice in the left hind footpad. One week later the mice were boosted again. Then the mice were infected with promastigotes in the right hind footpad. Lesion progression was monitored weekly over an 8-wk period. As shown in Figure 31A, control BALB/c mice, or mice vaccinated with HKLM along with control CpG developed measurable lesions within 5 weeks of infection, and these lesions became progressively larger until the experiment was terminated on week 8. Lesions in mice vaccinated with HKLM plus a low amount (0.5 μg) of CpG were significantly smaller after 5 weeks of infection. The addition of SB203580 to the CpG/HKLM vaccine resulted in a further reduction in lesion size. Parasite burdens in the infected feet were measured by limiting dilution. SB treated mice had fewer parasites that correlated with smaller lesions (Figure 31*B*). Cytokine production was measured from the supernatant obtained from the lymphocytes derived from lymph nodes from these mice. Production of more IFN-γ accompanied by less IL-4 was observed in SB treated group (Figure 31*C*). Thus, inhibition of MAPK/p38 can enhance CpG adjuvant effects by polarizing the host immunity towards Th1 immune response.

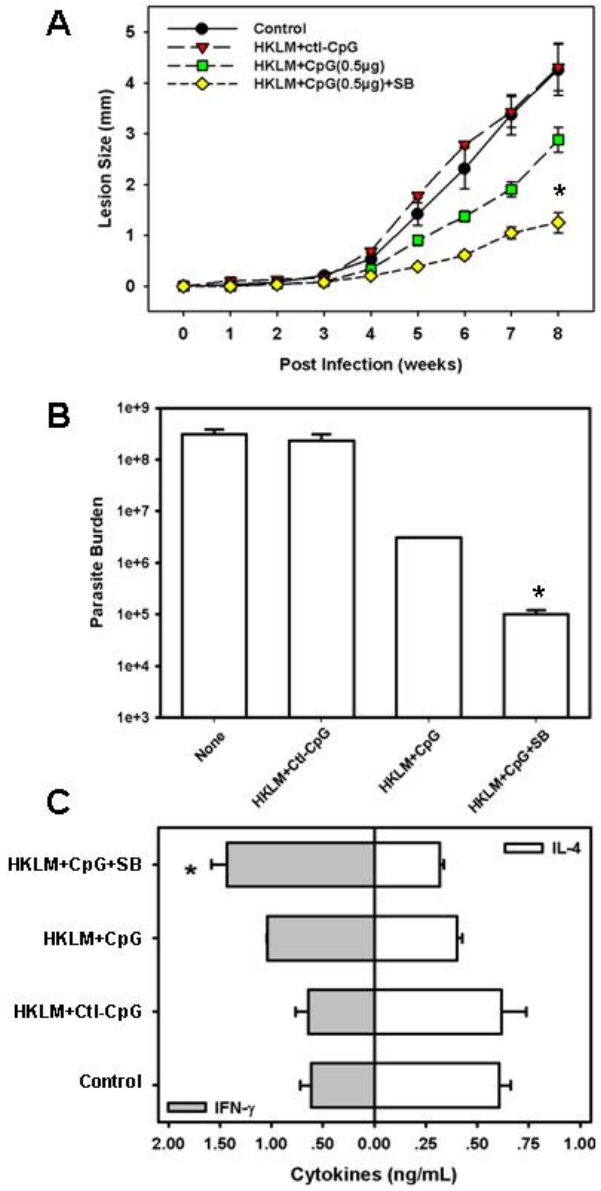

Figure 31. Inhibition of MAPK/p38 activation polarizes Th1 responses and enhances CpG adjuvant effect to protect leishmaniasis *in vivo*. *A*, BALB/c female mice were injected with PBS (black closed circle) or heat-killed *L. major* (50 μg) with or without CpG (0.5 or 2 μg/ml) and SB203580 (20μM) on their left footpad as indicated in the figure on day 0 and day 7. On day 30, mice were challenged with 1×10^5 *L. major* metacyclic promastigotes on their right footpad. Footpad lesions were monitored on the indicated days. *B*, Parasite burdens in infected footpad were determined by limiting dilution assay as described in *Materials and Methods*. One representative experiment of three is shown. *C*, Cytokine production by lymph node T cells from infected mice. Lymph nodes were removed on day 56 and stimulated with anti-CD3 and anti-CD28 for 48 hours. Then the cells were stimulated with PMA for 5 hours. Supernatants were harvested and assayed for IFN-γ and IL-4 by ELISA. Data represent mean ± SD. The *p* values were determined by Student's *t* test. The symbol of * represents $p < 0.05$.

Discussion

In this portion of the dissertation, we provide evidence to support the notion that manipulation of the MAPKs can result in profound alterations in cytokine production and bias a developing immune response. Specifically, we show that the inhibition of MAPK/p38 activation can induce the hyper-production of IL-12 by stimulated macrophages or DCs, which can polarize host immunity towards a Th1 response. We utilized several different experimental approaches to manipulate MAPK/p38 activity and increase in IL-12 production. MAPK/p38 inhibitor, SB203580, was used. It enhanced IL-12 production and reduced the production of TNFα in a dose-dependent manner. Knock-down of MAPK/p38 by specific siRNAs also resulted in increased IL-12 production, and macrophages taken from mice with a targeted deletion of the upstream kinases MKK3 had a similar effect on IL-12 and TNFα production. We demonstrated that APCs pre-treated with MAPK/p38 inhibitors skewed antigen-specific T cells to produce more IFN-γ and less IL-4. Finally, MAPK/p38 inhibitor enhanced the efficacy of CpG plus HKLM vaccination against leishmaniasis. Thus, this work suggests that MAPK/p38 inhibitor may be applied as adjuvants to improve vaccinations against intracellular pathogens.

Previous studies have indicated that MAPK/p38 can promote inflammation by targeting NF-κB to the promoters of inflammatory genes (Saccani *et al.*, 2002) and by stabilizing inflammatory gene transcripts (Mahtani *et al.*, 2001). However, the role of MAPK/p38 activation on IL-12 has remained somewhat controversial. In an early report, Salmon et al (Salmon *et al.*, 2001) reported that SB203580 could enhance LPS-initiated IL-12 production by peritoneal exudate macrophages. However, in a more recent study,

Kang et al (Kang *et al.*, 2008) showed that macrophage deletion of MAPK/p38α had a negative effect on IL-12 production. MK2 is the kinase directly downstream of MAPK/p38α/β. In $MK2^{-/-}$ macrophages LPS induced more IL-12p40 than the cells of control littermates (Kotlyarov and Gaestel, 2002). MKK3 is the dominant upstream kinase that controls activation of MAPK/p38 kinases. Lu *et al*. reported that IL-12 production was reduced in the "elicited" peritoneal macrophages from MKK3 deficient mice (Lu *et al.*, 1999). In our current study, we showed that MAPK/p38 inhibition enhanced IL-12p40 production from macrophages and IL-12p70 production from dendritic cells. Our results are in agreement with the results from the report of $MK2^{-/-}$ macrophages (Kotlyarov and Gaestel, 2002) and a more recent study in which plasmodium falciparum glycosylphosphatidylinositols (GPIs) or LPS-induced IL-12p40 production was enhanced in $MK2^{-/-}$ macrophages or SB203580-treated wild-type macrophages (Zhu *et al.*, 2009).

MK2 as a major target downstream of MAPK/p38α and plays a key role to regulate gene expression either at transcriptional or post-transcriptional level. It has been shown that MK2 is essential for LPS-induced regulation of cytokine mRNA stability and translation possibly through the modification of mRNA-binding protein by phosphorylation (Mahtani *et al.*, 2001). TNFα is one of the cytokines whose mRNA is stabilized by the activation of MAPK/p38-MK2 pathway. In the current study, MK2 activation was inhibited by MAPK/p38 inhibitor, which resulted in the reduction of TNFα production and an increase in IL-12p40 production. Our data showed that MAPK/p38 inhibition caused a decrease in TNFα mRNA half-life, and a concordant increase in the stability of IL-12p40 mRNA. It has been known that TTP is the target of

MK2 activation. Phosphorylated TTP binds to the AU-rich region of 3'-mRNA of TNFα to prevent its degradation (Sun *et al.*, 2007). How inactivation of MK2 or MAPK/p38 enhances LPS-induced *IL-12p40* gene expression *via* its mRNA stabilization needs to be addressed in future experiments. None of typical AU-rich elements has been definitively identified in the 3'-untranslated region of IL-12p40 mRNA, thus, tristetraprolin may not function on IL-12p40 mRNA as it does with TNFα or other mRNAs containing AU-rich elements. As recently reported by Akira and colleagues, zinc finger CCCH-type containing 12A (Zc3h12a) that has been identified as a TLR4 signaling-inducible RNase specifically accelerates mRNA degradation of *Il6* and other genes including *Il12p40* and calcitonin receptor (*Calcr*) (Matsushita *et al.*, 2009). The effect of MAPK/p38-MK2 pathway on the function of Zc3h12a warrants evaluation in the near future. Microarray will provide us more candidate molecules that may be involved in IL-12p40 mRNA degradation. MAPK/p38-MK2 pathway can also regulate the targets other than TTP. In the study of plasmodium falciparum glycosylphosphatidylinositols-induced IL-12p40 in $MK2^{-/-}$ macrophages, the enhanced binding of NF-κB to *IL-12p40* promoter region and the reduction in the expression of transcription repressors GAP-12 and c-Maf were attributed to the increasing IL-12p40 gene expression (Zhu *et al.*, 2009). In our study, *IL-12p40* transcription as determined by measuring pre-spliced mRNA that was moderately decreased in the macrophages treated with MAPK/p38 inhibitors. Furthermore, MAPK/p38 inhibition reduced *IL-12p40* promoter activity as determined by transient transfection of *IL-12p40* promoter reporter experiments. Bioactive IL-12 is composed of equal molar amounts of p40 and p35. It has been known that contrary to expectation, p40 is induced in as much as 100-fold excess over p35 regardless of stimuli (Trinchieri G.

1995; Liu *et al*., 2004). The current result of which IL-12p35 mRNA level was not significantly affected by MAPK/p38 inhibition in both bone marrow derived macrophages and DCs suggests that IL-12p40 mRNA is the main target for such regulation at mRNA level. However, one could not rule out the possibility of MAPK/p38 pathway may play a role to regulate IL-12p35 at translational and/or post-translational levels, which warrents for future experiments. Taken together, the effect of MAPK/p38 inhibition on IL-12 regulation is mainly at the post-transcriptional level. In addition to its role in mRNA decay, MAPK/p38 and MK2 have also been implicated in controlling mRNA translational and protein folding activity (Gaestel, 2006). Thus, it is possible that MAPK/p38-MK2 pathway may also control IL-12 production at the translational level.

In response to signals from parasite antigens or adjuvants, such as TLR ligands, resident immature DCs at the site of infection or vaccination undergo a maturation program characterized by enhanced expression of co-stimulatory molecules and inflammatory cytokines. These highly stimulated DCs migrate to T cell areas in the draining lymph nodes and present antigens to T cells, which result in increased antigen-MHC complexes, co-stimulatory molecules and pro-inflammatory cytokines. IL-12 and IL-18 secreted by DCs induce IFN-γ from T cells and favor robust Th1 immunity. The differential development of Th1- versus Th2-type responses in *Leishmania*-infected mice has provided an excellent model for the study of immune regulators that manipulate the *in vivo* immunological development to alter the disease phenotype. The ability to alter Th responses in BALB/c mice and to assess the functionality of these responses with a biologic correlation has made this a useful model for vaccine development against diseases requiring Th1 immunity.

IL-12 functions as a Th1-skewing cytokine to induce IFN-γ, thus, MAPK/p38 inhibition would be a strategy to modulate the host immunity against certain diseases. Indeed, macrophages primed with OVA antigen together with MAPK/p38 inhibitor skewed T cells to produce more IFN-γ during secondary stimulation. Thus, in theory MAPK/p38 inhibitors such as SB203580 could be used as an adjuvant to boost cell mediated immunity and improve vaccines against intracellular pathogens such as *Leishmania*. The efficacy of different anti-*leishmania* vaccine strategies has been experimentally evaluated. Among these strategies, it appears that CpG is an effective adjuvant to boost the host immunity against this disease (Rhee *et al.*, 2002). MAPK/p38 inhibition dramatically enhanced CpG-induced production of both IL-12p40 and IL-12p70 from macrophages and DCs. CpG has been known to enhance the function of APCs. CpG, *via* TLR9, augments both the activation and maturation of DCs. When added along with SB203580 to inhibit MAPK/p38 activation, CpG enhanced IL-12 production and skewed immunity towards Th1 by producing more IFN-γ (Hemmi *et al.*, 2000). In our mouse model of *L. major* infection, vaccination with autoclaved *L. major* parasites plus CpG and SB203580 was able to inhibit disease progression and had a higher efficacy than autoclaved *L. major* parasites plus CpG without SB203580.

Inhibitors for MAPK/p38 kinase have been considered for clinical applications to control inflammation and different generations of MAPK/p38 inhibitors with more potent affinity and specificity have been developed such as VX-745 and BIRB-796 by pharmaceuticals for clinical applications (Gaestel *et al.*, 2007). Our data suggests that targeting MAPK/p38 activation can not only control unwanted inflammatory response such as TNFα overproduction that may have harmful consequences on the host, but also

increase IL-12 production that leads to the beneficial immunity against invading microorganisms. Thus, it might be possible that administration of the inhibitors specific for MAPK/p38-MK2 pathway would be an alternative strategy to effectively vaccinate against certain infectious diseases such as leishmaniasis.

CONCLUSIONS

The consequence of events that occur during and after *Leishmania* invasion can be considered as macrophage defects to efficiently kill the parasites. The interaction of macrophages receptors with appropriate ligands leads to trigger downstream transduction signaling pathways to yield a variety of responses of which is characterized by their protective or hazardous role. Macrophages can be activated by both IFN-γ and TLR ligands to produce IL-12 that promotes Th1 cell differentiation and induces IFNγ production from T cells. IFN-γ in turns activates more macrophages to produce NO to kill *Leishmania*. During the chronic infection stage, host will generate large amounts of anti-*Leishmania* IgG that opsonizes parasites. High production of IL-10 initiated by the ligation of FcγR by IgG opsonized *Leishmania* and activation of TLRs plays a hazardous role for the host. Our findings reveal the molecular mechanisms of how the MAPKs regulate the cytokines production in *Leishmania* infection. MAPK/p38 inhibition induced IL-12p40 hyperinduction is mainly due to enhanced mRNA stability. MAPK/ERK activation initiated chromatin modification at the *IL-10* locus, which allowed for transcription factor Sp1 binding to drive *IL-10* expression. The metacyclic *L. major* promastigotes are poor inducers of IL-12 production. In contrast, the procyclic promastigotes collected from the logarithmic phase of the culture are capable of inducing IL-12 and other cytokines (Sartori et al., 1997). Furthermore, almost every infected macrophage lost its capability to produce IL-12 in response to IFN-γ/LPS stimulation. Hence, the question remains to be addressed in the future: do metacyclic parasite-infected macrophages utilize the same signaling pathway as procyclic parasite-infected macrophages to produce cytokines or do they acquire novel mechanisms? IL-12 has been

administrated as an adjuvant to vaccination with limited efficacy, possibly due to its compensatory induction of IL-10 (Meyaard *et al.*, 1996). Thus, circumventing the immunosuppressive effects of IL-10 could enhance the therapeutic efficacy of IL-12 as an adjuvant. Therefore, based on our current observations, *i.e.*, down-regulation of IL-10 by inhibition of MAPK/ERK and enhancement of IL-12 via inhibition of MAPK/p38, the combination of both MAPK/ERK and MAPK/p38 inhibitors could be envisaged as a novel adjuvant to vaccination in the future. By manipulating the MAPKs may provide us novel therapeutics for potential clinical applications.

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