A *Leishmania* Ortholog of Macrophage Migration Inhibitory Factor Modulates Host Macrophage Responses

Von der Fakultät für Mathematik, Informatik und Naturwissenschaften der RWTH Aachen University zur Erlangung des akademischen Grades einer Doktorin der Naturwissenschaften genehmigte Dissertation

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Albert Einstein: "No amount of experimentation can ever prove me right; a single experiment can prove me wrong."

In memory of my grandfather Dr. med. Philipp Sachter who didn't live long enough to see any of my graduations but whose memories will never be forgotten.

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List of abbreviations:

Å	Ångström
Ab	Antibody
Fig.	Figure
ABTS	2,2'-azino-di(3-ethylbenzthiazoline sulfonate)
AIDS	Acquired immune deficiency syndrome
BMM	Bone-marrow macrophages
Bp	Basepair
BSA	Bovine Serum Albumine
C-terminal	Carboxy-terminal
CHMI	5-carboxymethyl-2-hydroxymuconate isomerase
COX2	Cyclooxygenase 2
DDT	D-Dopachrome tautomerase
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagles's medium
DNA	Desoxyribonucleic acid
Ds	Double-strand
EDTA	Ethylendiamintetraacetat
ELISA	Enzyme-linked immunosorbant assay
ERK	Extracellular signal-regulated kinase
EtBr	Ethidium bromide
FBS	Fetal bovine serum
hr(s)	*Hour(s)*
HIV	Human immunodeficiency virus
hMIF	Human MIF
HRP	horseradish peroxidase
IFN-γ	Interferon-γ
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IPTG	Isopropyl-β-D-thiogalactopyranosid

ISO-1	(S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester
JNK	Jun N-terminale kinase
Kb	Kilo base pairs
kDa	Kilo Dalton
LDS	Lithium dodecyl sulphate sample buffer
LmjF	*L. major* Friedlin
LmMIF	*L. major* MIF
LPS	Lipopolysaccharid
MAP	Mitogen-activated protein
Mb	Mega base pairs
MIF	Macrophage migration inhibitory factor
mMIF	Mouse MIF
MP	Microplate
Min	Minute(s)
N-terminal	Amino-terminal
NO	Nitric oxide
4-OT	4-oxalocrotonate tautomerase
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PGE_2	Prostaglandin E2
PLA2	Phospholipase A2
PMNs	Polymorphonuclear neutrophil granulocytes
PnP	p-Nitrophenole
PnPP	p-Nitrophenyl phosphate
PVDF	Polyvinylidene difluoride
qPCR	Quantitative PCR
RNA	Ribonucleic acid
Rpm	Rotation per minute
ROS	Reactive oxygen species
sCD74	Soluble CD74
SDS-PAGE	sodium dodecyl sulfate polyacrylamide

	gel electrophoresis
SNP	Sodium nitroprusside
sCD74	Soluble CD74
TAE	Tris-Acetat-EDTA-Buffer
Taq	Thermus aquaticus
TE	Tris-EDTA-buffer
Th1	T-helper 1 lymphocytes
Th2	T-helper 2 lymphocytes
TLR4	Toll-like receptor 4
Tm	Melting temperature
TNF-α	Tumor necrosis factor-α
TPOR	Thiol-protein oxidoreductase
Tris	Tris(hydroxymethyl)aminomethan
Trx	Thioredoxin
TTBS	Tris-buffered salt solution with Tween 20
U	Specific enzyme activity
UV	Ultraviolet

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1 Introduction

1.1 Host-parasite relationships between man and protozoa

Every infection is a battle between the parasite and the host (Playfair 1995). Occasionally, a parasite becomes extremely dangerous to humans (e.g., plague and influenza). The Antonine Plague, from 165–180 AD, also known as the Plague of Galen, was an ancient pandemic of either smallpox or measles. It caused up to 2,000 deaths per day in Rome. Total deaths were estimated at five million individuals. The Spanish pandemic of influenza between 1918 and 1920 killed at least 25 million people worldwide. The various stages and outcomes of infection as well as the main strategies of parasitic attack and host counter-attack are shown in the graph below.

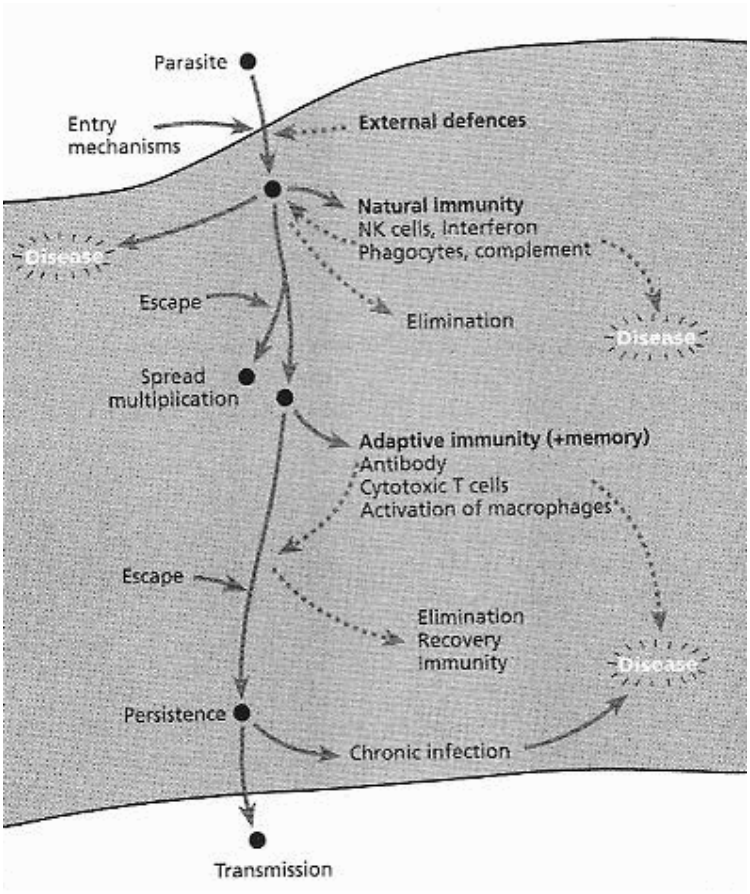


Fig. 1. Various stages and outcomes of infection. Shown are the main strategies of the parasite (left) and host (right). Taken from: (Playfair 1995).

1.2 Immunity

The body surface is the first line of defense against a large number of microorganisms. When this defense mechanism is penetrated, the innate immune system becomes activated. Innate immunity, which involves dendritic cells as well as monocytes/macrophages, refers to non-specific defense mechanisms that appear immediately or within hours of the appearance of an antigen and are based on the non-clonal recognition of microbial components. Innate immunity is evolutionary conserved and essential for multicellular organisms; it can be traced back to the earliest forms of life. Innate immunity, also called non-specific, non-adaptive, natural, or native immunity, is particularly important in immuno-compromised patients who lack activated, adaptive immune responses. Non-specific immune defenses are primed prior to the occurrence of infection and consist of phagocytic cells, mast cells, complement, natural killer (NK) cells, and cytokines. Responses generated by the innate immune system are rapid and are often capable of eliminating infectious agents. If the innate immune system fails, an adaptive (also known as acquired or specific) immune response will ensue, distinguishing between different microbes and molecules. The components of this immune response include T and B lymphocytes and their products.

1.3 Aleppo Evil

1.3.1 Leishmaniasis

The World Health Organization considers leishmaniasis to be one of the major tropical diseases and public health problems of developing countries (WorldHealthOrganization). Additionally, leishmaniasis may occur in patients who have traveled to endemic areas (Harms, Schonian *et al.* 2003) as well as in people who have not (Bogdan, Schonian *et al.* 2001).

Leishmaniasis is caused by an obligate, intracellular infection of macrophages with species of the eukaryotic genus *Leishmania*. This protozoan haemoflagelate belongs to the order *Kinetoplastida* and the family *Trypanosomatidae. Leishmania spp.* infect their mammalian hosts, including humans, in which they elicit a spectrum of diseases from cutaneous to mucocutaneous and visceral forms in diverse locations of the world (Pearson and Sousa 1996). At present, leishmaniasis threatens 350 million people in

88 countries (Desjeux 2001). The variability of the clinical features results from both the diversity of the *Leishmania* species as well as the immune responses of the host.

Approximately 1 to 1.5 million people suffer from local, self-healing, cutaneous leishmaniasis, which is caused by *Leishmania major* (*L. major)* and *L. tropica* in Europe and the Near and Far East and by *L. viannia braziliensis*, *L. viannia guyanensis*, and *L. mexicana* in the Americas. Cutaneous leishmaniasis causes skin lesions known as Oriental sores, Jericho Buttons, Baghdad boils, Delhi boils, as well as other common names. *L. major* and *L. tropica* are biochemically distinct, cause slightly different skin lesions, and do not overlap geographically. In cutaneous leishmaniasis, the replicating amastigotes are limited to tissue surrounding the initial bite site.

Approximately 500,000 people contract visceral leishmaniasis each year, known in India as Dum-Dum fever or Kala-azar. Although not restricted to India, visceral leishmaniasis, caused by *L. donavani*, *L. infantum*, and *L. chagasi* affect primarily the spleen and liver, but also the bone marrow. In southern Sudan between 1989 and 1994, Kala-azar reached epidemic and epizootics proportions, leaving approximately 100,000 people dead (Dedet 2002). Individuals with chronic viscerotropic infection suffer from fever, fatigue, weight loss, peripheral edema, splenomegaly, hepatomegaly, anemia, and bleeding disorders; such infection results in immunosuppression and secondary infections, which is the usual cause of death.

Mucocutaneous leishmaniasis is caused by *L. braziliensis*. Skin lesions may be open and oozing, potentially spreading amastigotes to other parts of the body or to other individuals. Secondary infections may occur coincidentally with primary infections or may occur many years after the primary infection has healed. In some parts of South America, the disease is known as espundia or uta and refers to secondary lesions that occur in the mucous membranes, particularly in the nasal and oral regions, resulting in grossly destructive anomalies caused by disfiguring sores and gaping holes. The nasal septum and other nasal/oral tissues appear to be "eaten away." Espundia may develop metastatically from sores originally found elsewhere on the body. Commonly, this form of leishmaniasis leads to the host's death due to a secondary bacterial infection, pharyngeal obstruction, and/or malnutrition. Invasion of the larynx may result in a loss of speech.

Rarely, leishmaniasis is spread from a pregnant woman to her baby. Additionally, leishmaniasis can be spread by blood transfusions and contaminated needles. Due to

its pathology, leishmaniasis leaves its victims scarred and disfigured, causing severe stigmatization among affected individuals, particularly children and women who are often deemed unsuitable for marriage or for raising children.

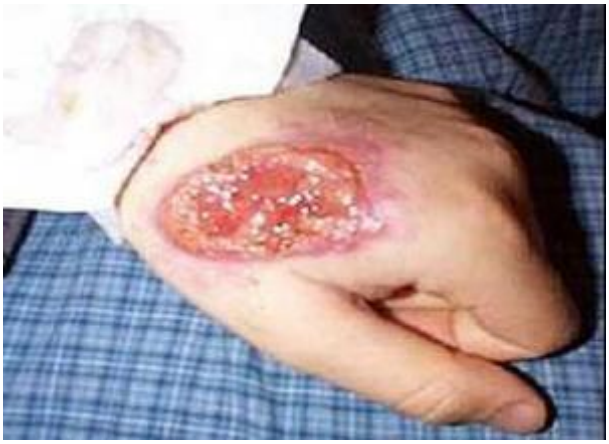


Fig. 2. Cutaneous leishmaniasis. Classification is often obscured by the existence of a spectrum of intermediate forms.
From Science Daily (Aug. 16, 2007), Dr Ross Boyle, University of Hull, United Kingdom — *Flesh-eating disease appears to be on the rise due to global warming. Should global warming continue to ravage our planet at current rates, the numbers of people suffering Leishmaniasis, a flesh-eating and sometimes fatal disease will increase dramatically, experts warn. Leishmaniasis is caused by a parasite transmitted via sand fly bites usually found only in tropical climates. Rising temperatures will increase the number of countries the sand fly colonises, moving further north and through Europe.*

The *Leishmania* vector is the female sand fly of the genera *Phlebotomus* in the Old World and *Lutzomya* in the New World. Sand flies are small blood-sucking insects that are capable of passing through certain kinds of mesh screens in windows and doors that are used in the fight against malaria; a very fine-mesh netting is needed for an effective barrier against sand flies as they are only about one-third the size of a typical mosquito.

1.3.2 Leishmania genome

The size of the *Leishmania* genome is approximately 3.55×10^7 bp and is organized into 34–36 chromosomes. The parasite is a diploid organism but is functionally asexual. It exhibits genomic plasticity that implies that certain genes, most likely vital or highly expressed genes, are variable in both size and number. Of particular importance is that *Leishmania spp.*, in contrast to most protozoa, do not undergo

chromosome condensation during mitosis (Stiles, Hicock *et al.* 1999). *Leishmania* parasites multiply by binary fission though assumptions of sexual reproduction due to their diploid genome have been made (Bastien, Blaineau *et al.* 1992; Gibson and Stevens 1999). Homologous chromosomes vary in size not only between species but within clones of a single species (Britto, Ravel *et al.* 1998). Chromosomal alterability in *Leishmania* is probably not caused by interchromosomal rearrangements, but rather by amplification/deletion events in sub-telomeric regions, accounting for the variability in chromosomal size (Victoir, Dujardin *et al.* 1995; Stiles, Hicock *et al.* 1999). In 1992, Bastien *et al.* suggested a 'mosaic structure' for *Leishmania* genomes with different cells that contain differently sized chromosomal homologs due to frequent amplification/deletion events (Bastien, Blaineau *et al.* 1992). The open reading frames are arranged in long, polycistronic arrays. Monocistronic mRNAs are created by post-transcriptional processing events. The *Leishmania* genome contains repetitive DNA sequences with a 58–60% G/C content; its coding regions have a higher G/C content than the non-coding regions, with 5'-coding regions having a lower G/C content than the 3'-coding regions. Alonso *et al.* suggested that the high G/C content might reflect a more primitive nature (Alonso, Guevara *et al.* 1992). The genes of *Leishmania* lack introns, but occur as single genes, paired genes, or repeated genes in tandem arrays, which vary in their flanking sequences, even between closely related genes. Cis-splicing mechanisms have not been detected in any of the *Leishmania* genomes. The most common microsatellite DNA motifs, among di-, tri-, and tetra-nucleotide repeat motifs, are $(CA)_n$ repeats, present on the chromosomes of *Leishmania spp*. (Rossi, Wincker *et al.* 1994). Repetitive motifs are common to molecules exposed on the membranes of *Leishmania*. Parasitic protozoa contain many peptides with repeating amino-acid domains, a structure often not present in their higher eukaryotic homologues, provoking elevated antibody responses (Kemp, Coppel *et al.* 1987; McCutchan, de la Cruz *et al.* 1988; Stiles, Hicock *et al.* 1999).

1.3.3 History

Leishmania infections can be traced back to Burmese amber from the Albians of the Lower Cretaceous period (105–100 million years ago). Sand flies probably introduced trypanosomatids to dinosaurs. This idea is based on the discovery of a blood-filled sand fly fossil (*Palaeomyia burmitis*) in Burmese amber that contained reptilian blood cells infected with the trypanosomatid, *Paleoleishmania proterus*. The

sand fly contained stages of a *Leishmania* trypanosomatid in both its proboscis and abdominal midgut (Poinar and Poinar 2004). Some of the discovered blood cells, which were ascribed to amphibians, contained developing parasites. The extinction of dinosaurs via sand fly-transmitted visceral *Leishmania* parasites was suggested in 1991 by Desowitz (Desowitz 1991). Based on the discovered fossils, it is estimated that sand flies fed on vertebrate blood 130 million years ago and became vectors for trypanosomatids approximately 115–120 million years ago (early Cretaceous) when the co-evolution of sand flies, trypanosomatids, and reptiles is assumed to have begun. Furthermore, it is assumed that the parasite-host-vector relationship evolved in species, such as dinosaurs, where it became lethal and survived in hosts that had immunity against the parasites. Descendents of these hosts appear to have survived, and present-day reptilian leishmaniasis appears to have little effect on the hosts (Telford 1995). At present, sand flies of the genus *Sergentomyia* feed and transmit the related *Sauroleishmania* to snakes and lizards (Telford 1995). The effects of *Sauroleishmania* infections have not been well studied and, therefore, are not well understood. However, studies in which chameleons were infected with *Sauroleishmania* showed that none of the animals survived infection (Brygoo 1963). Inside the sand fly fossil, blood cell vacuoles were detected that bore a striking resemblance to parasitophorous vacuoles containing *Sauroleishmania* amastigotes, transmitted by the Old World sand fly *Sergentomyia* of present-day lizards (Telford 1995).

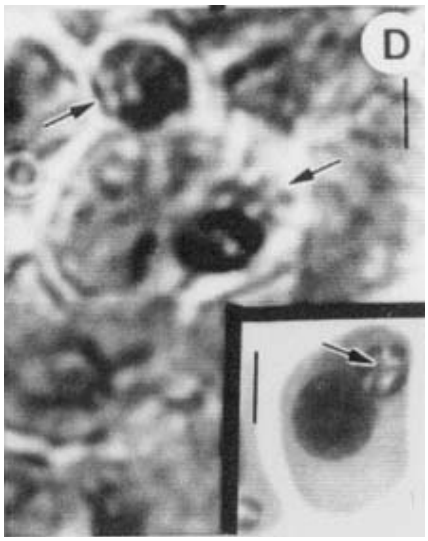


Fig. 3. Two blood cell nuclei with putative parasitophorous vacuole-containing amastigotes (arrow) from the midgut of the fossil sand fly. Bar = 4 µm. High similarity to a parasitophorous vacuole containing amastigotes of *Sauroleishmania* in an extent lizard (insert). From: (Poinar and Poinar 2004).

Representations of skin lesions and facial deformities, as seen in cutaneous leishmaniasis, have been found on pre-Incan pottery from Ecuador and Peru dating back to the first century AD. This evidence shows that cutaneous and mucocutaneous forms of leishmaniasis prevailed in the New World as early as this period.

One of the first and most important clinical descriptions of leishmaniasis was made in 1756 by the Englishman Alexander Russell following the examination of a Turkish patient. Russell resided at this time in Aleppo of the Ottoman Empire (now Syria). The city was as large as other major European cities during this period with a population of 233,000 people. During his 13 years of residence in Aleppo, Russell collected material on mores, climatology, flora, fauna, and various endemic diseases (Gelpi 1987). Ultimately, he published his gathered information (Alex. Russell 1756) in which he described a cutaneous disease:

"Cutaneous disease, thought by some to be peculiar to this place, has acquired the name of Il mal d'Aleppo, or Aleppo evil, among the Europeans. The natives call it Habt is Senne, or Botch of a Year, from the supposed time of its duration. In Turkish, Haleb Choban, or the Aleppo ulcer. This disease is not, however, peculiar to this place, being almost as common at Antab, and all the other villages on the banks

of the rivers Sejour and Coik, as at this place; which favours the opinion of its being occasioned by the water."

Furthermore, he described both a female species (assumed to be *L. major*) and a male species (assumed to be *L. tropica*) as the origins of the above-described cutaneous disease:

"What is called the female species begins like the former; but after a month or two it becomes somewhat painful, encreases often to double the extent of the male, discharges a good deal of the ichorous matter from under the scab, and by degrees comes to have the appearance of an indigested scorbutic ulcer, with a livid circle round it; but seems to be no deeper than the tunica cellulosa. In this condition it remains for several months, and is in general about a year from its first appearance before it is cured; but this is not a thing certain, many getting well some months sooner, while others remain several months longer. After it is cicatrized, it leaves an ugly scar, which remains thro' life, and for many months has a livid colour. When they are not irritated, they seldom give much pain."

Independently, Sir William Boog Leishman (1865–1926) and Charles Donovan (1863–1951) discovered the cause of Kala-azar at the beginning of the 20th century. The parasite was named *L. donovani* in memory of these two researchers. In 1921, brothers Edouard and Etienne Sergent succeeded in proving that the vector for transmission of *Leishmania* parasites to mammals is the sand fly of the genus *Phlebotomus* (Sergent, Sergent *et al.* 1921).

1.3.4 Leishmaniasis: disease and parasitic life-cycle

There are two morphological forms of *Leishmania*, the promastigote and the amastigote. The promastigote form of the parasite is found in female sand fly vectors of the genera *Lutzomyia* and *Phlebotomus*.

The infection process begins with the bite of an infected host by a sand fly, resulting in the ingestion of amastigotes by the fly. *Leishmania* parasites undergo metacyclogenesis in the vector, a process in which the parasites are transformed from poorly infective flagellated procyclic promastigotes into highly infective metacyclics

that move up the proboscis and clog the esophagus of the fly. Feeding sand flies then disgorge promastigotes (6–8 µm) on the skin of the mammalian host where they may enter the bite wound. Within their vertebrate host, the promastigotes are quickly taken up by tissue phagocytes (monocytes/macrophages and neutrophils) that accumulate at the infection site due to the damage created by sand fly feeding (Wilson, Innes *et al.* 1987). The only cells in which *Leishmania* amastigotes are able to proliferate in the mammalian host are macrophages. Within these cells, promastigotes lose their flagella and transform into non-motile amastigotes. Upon entry into the mammalian host, promastigotes are immediately faced with the host's innate immune system, lysis by complement, and destruction by macrophages. *Leishmania* promastigotes not only avoid destruction by complement and phagocytes but also actually dominate macrophages that are functionally arrested within the innate immune system. Therefore, after the bite of an infected sand fly and the inoculation of promastigotes into the dermis, the promastigotes adhere to the macrophages via both type-I and type-III complement receptors (Mosser and Rosenthal 1993), the major cell surface protein gp63, and the glycoconjugate LPG (lipophosphoglycan), followed by their internalization through phagocytosis (Brittingham and Mosser 1996).

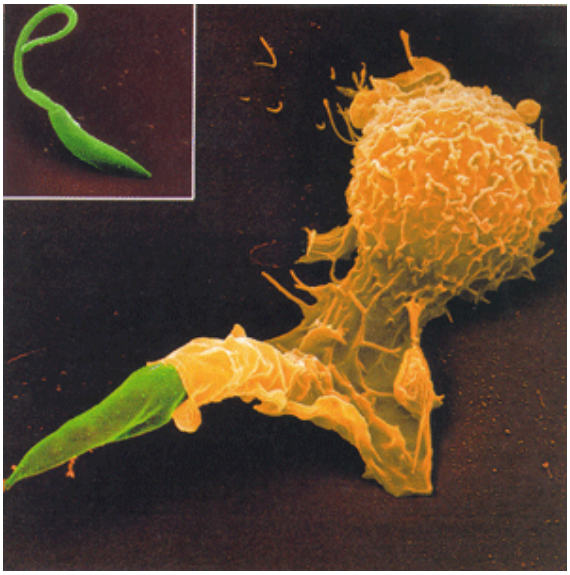


Fig. 4. A macrophage (orange) stretches its pseudopodia to engulf/endocytose an invading flagellated *L. major* promastigote.
Photograph taken from the Web page of the Christian de Duve Institute of Cellular Pathology; Research Unit of Tropical diseases TROP. http://www.icp.be/trop/about/subject2.htm

The newly formed phagosome, a vacuole around the promastigote, undergoes maturation via fusion with endocytic organelles (i.e., endosomes and lysosomes), leading to the formation of the mature phagolysosome (Antoine, Prina *et al.* 1998). The maturation of the phagolysosome is important, as it becomes the microbicidal site within macrophages because of its acidic pH, lysosomal hydrolyzing enzymes, nitric oxide (NO), and other reactive oxygen intermediates. The results of Desjardin *et al.* suggest that inhibition of phagolysosomal biogenesis by *Leishmania* represents an intramacrophage survival strategy used by promastigotes to establish infection (Desjardins and Descoteaux 1997) as well as protection against an acidic environment due to their sensitivity to low pH. Delaying the above-described process extends the time during which promastigotes are able to differentiate into acidic pH-resistant amastigotes, normally a 48-hour process, subsequent to phagocytosis. The cytodifferentiation from the promastigote to the amastigote that occurs in the phagolysosomal compartment of the macrophage is a transformation process that is a prerequisite for parasite survival. Astonishingly, amastigotes do not merely tolerate low pH in the phagolysosome but are established within these compartments because an acidic pH is required for proliferation and expression of amastigote-specific genes (Chang and Dwyer 1976; Charest, Zhang *et al.* 1996). Once the amastigotes proliferate, macrophages ultimately lyse, release new amastigotes, and are endocytosed by uninfected macrophages. Phagocytosis of amastigotes appears to occur primarily through Fc receptors (FcRs) and complement receptor-type CR3; however, other unidentified receptors may participate in this process (Guy and Belosevic 1993). While resident in macrophages, the parasite avoids triggering antimicrobial responses; however, it may be eliminated over time with the development of an effective adaptive immune response.

Rogers *et al.* showed recently in an experimental study using mice that *Leishmania* parasites could influence the feeding behavior of sand flies so that infected flies become more persistent in feeding on their host if interrupted. Furthermore, the parasites in the sand fly produce a filamentous proteophosphoglycan (fPPG) gel that impedes the flies gut and mouthparts and, consequently, interferes with feeding, leading to additional feeding on more hosts. Hence, this behavior leads to a selective advantage of the parasite by increasing transmission (Rogers and Bates 2007).

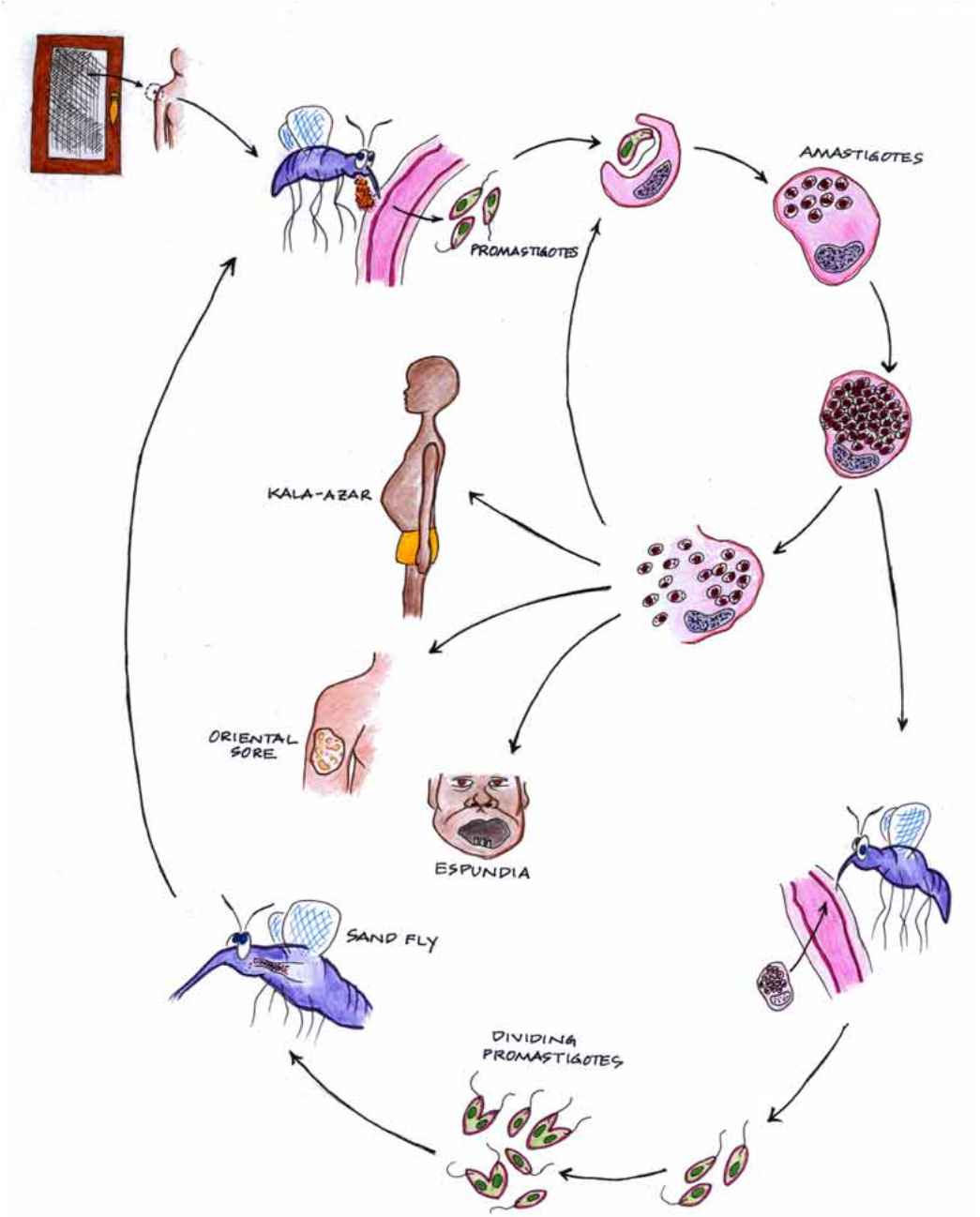


Fig. 5. ***Leishmania* life cycle.** Leishmaniasis is transmitted by the bite of female sand flies. The sand flies inject the infective metacyclic promastigotes during blood meals into their hosts. Metacyclic promastigotes are phagocytosed by macrophages, transform into amastigotes, and multiply in macrophages. Sand flies feed on *Leishmania*-infected mammalian species, and ingest macrophages infected with amastigotes. In the sand fly's midgut, the parasites differentiate into promastigotes, which multiply, differentiate into metacyclic promastigotes, and migrate to the sand fly's proboscis. Here, the cycle begins again. Depending on the parasitic strain, it is possible to distinguish between a spectrum of clinical diseases: visceral Leishmaniasis, Kala-Azar, *L. donovani*; cutaneous Leishmaniasis, Oriental Sore, *L. major*; mucocutaneous leishmaniasis, Espundia, L. braziliensis. Drawing taken from the Web page of Thomas P. Buckelew, Ph.D., Professor Emeritus. Drawn by Meghan Petrucci. http://workforce.cup.edu/Buckelew/

1.3.5 *Leishmania* RNA virus

The *Leishmania* RNA virus (LRV) is an ancient virus that co-evolved with its protozoan host. Virus-like particles (VLP) have been found in lower eukaryotes, such as *Leishmania spp.*, *Entamoeba histolytica*, and *Trypanosoma spp*. The family *Totiviridea* includes three genera of dsRNA viruses: *Totivirus*, *Giardiavirus*, and *Leishmaniavirus*. Two types of *Leishmaniaviruses*, which infect only protozoa, exist (types LRV1 and LRV2). Viruses with non-segmented dsRNA genomes of approximately 5,200 bp in length have been identified in both *L. braziliensis* and *L. major*. The lack of an infectious phase for these viruses suggests that LRV arose prior to the divergence of Old and New World parasites. Genetic recombination is considered unlikely because of the asexual reproduction of *Leishmania* parasites. Patterson *et al.* showed that *Leishmaniavirus* exists in human biopsy material of *Leishmania*-infected patients (Saiz, Llanos-Cuentas *et al.* 1998). Whether the presence of *Leishmaniavirus* alters parasitic phenotype and affects disease pathogenesis has yet to be addressed (Gupta and Deep 2007). The study of *Leishmaniaviruses* is still in its infancy and is neither well reported nor understood.

1.3.6 *Leishmania*/HIV co-infection

Infection by two different intracellular pathogens of the same target cell will affect immune responses and might influence the expression and multiplication of either one or both pathogens.

Leishmaniasis is an opportunistic infection that is particularly troublesome for patients with human immunodeficiency virus (HIV)/acquired immune deficiency syndrome (AIDS) in that both diseases attack the immune system and thereby worsen a patient's already compromised ability to resist other infections. Leishmaniasis accelerates the onset of AIDS by encouraging further opportunistic infections (e.g., pneumonia and/or tuberculosis) and, thus, reduces life expectancies of HIV patients (Anema and Ritmeijer 2005). Leishmaniasis can be transmitted directly from person to person through the sharing of needles, as is often the case among intravenous drug users, the main population at risk for co-infection. Although people are often bitten by sand flies infected with *Leishmania*, most do not develop leishmaniasis. However, immunosuppressed people (e.g., those under immunosuppression for organ transplants, cancer patients, those with auto-immune diseases, and HIV-infected

people) quickly develop severe leishmaniasis (World Health Organization, Fact sheet N° 116, revised May 2000).

Leishmania and HIV infect and multiply in macrophages that can lead to a dysregulation of the immune system. Both infections mutually affect each other. *Leishmania* can induce HIV activation in both macrophages and T cells; in contrast, HIV can enhance intracellular growth of *Leishmania* in macrophages. HIV destroys $CD4^+$ T helper cells and gains access to all other cell types that express CD4, such as monocytes/macrophages and dendritic cells. Progression to AIDS among HIV-infected patients has been linked to active replication of HIV, induced by stimuli such as those caused by parasitic activation of immune cells (Wolday, Berhe *et al.* 1999).

1.3.7 The Trojan horse of *Leishmania*

Leishmania parasites exhibit a pronounced preference for macrophages although they have the capacity to infect a variety of other phagocytic and non-phagocytic mammalian cells. *L. major* promastigotes secrete a *Leishmania* chemotactic factor, (LCF) that recruits solely PMNs (polymorphonuclear neutrophil granulocytes). Production of the chemokine IL-8 by inflammatory granulocytes appears to serve as an amplifying loop and attracts more PMNs to the site of inflammation. Infection of PMNs with *L. major* leads to longevity of PMNs and apoptosis is delayed by approximately 24 hours. IL-8 can prolong the life span of PMNs although it is assumed that *L. major* uses a non-IL-8 strategy as its anti-apoptosis mechanism. Neutrophils exposed to a chemoattractant (IL-8) extend the first pseudopod in the correct direction and sequentially measure the concentration of attractant at two points in the gradient. As long as the gradient is maintained the chemotactic response persists. Only when the gradient is compromised, either by removal of the chemoattractant or by uniform distribution across the cell, does the response subside. PMNs phagocytose *L. major* but the promastigotes cannot multiply within these cells. The secretion of the chemokine IP-10 by PMNs is inhibited by *L. major*, which leads to a blockade of both NK- and Th1-cell recruitment. Instead, *L. major* induces the gene expression and secretion of the chemokines MIP-1α and MIP-1β that attract monocytes/macrophages to the site of infection. An early hallmark of apoptosis is the appearance of phosphatidylserine on the outer leaflet of the plasma membrane, a process called membrane flip. In the case of PMNs, the cell membrane sends an “eat me” signal to macrophages. The now-apoptotic PMNs do not represent a threat to

phagocytic macrophages and do not activate macrophage antimicrobial effector functions. Therefore, the function of these phagocytes is silenced. PMNs become completely engulfed by macrophage phagosomes without any direct physical interaction with macrophage surface receptors. This anti-inflammatory milieu in the macrophage is beneficial for the survival of *L. major*. The anti-inflammatory cytokine expression of TGF-β becomes up-regulated and TNF-α secretion was suggested as a possible mediator of delayed macrophage apoptosis (Moore and Matlashewski 1994; Muller, van Zandbergen *et al.* 2001; Aga, Katschinski *et al.* 2002; van Zandbergen, Hermann *et al.* 2002; van Zandbergen, Klinger *et al.* 2004).

1.3.8 Th1 versus Th2

Like all parasites, *Leishmania* protozoa have evolved specialized strategies to evade immune destruction and to complete their life cycles (Bogdan and Rollinghoff 1998). Several host- and parasite-specific factors play roles in the persistence of *Leishmania* within infected cells and influence the clinical manifestations of disease, including non-healing cutaneous ulcers and visceral forms. Among these factors, the host cytokine response plays a key role by affecting innate, antimicrobial responses and by promoting the differentiation of a protective T-cell response (Scharton-Kersten and Scott 1995). Following the survival of the innate immune response, amastigotes must evade the host's adaptive immune response in order to establish a chronic infection. The role of macrophages in this process is the presentation of antigen and release of cytokines to T lymphocytes. *Leishmania*-infected cells are defective in the expression of MHC class-II molecules on the cell surface (Reiner, Ng *et al.* 1987). Therefore, macrophages have a reduced capacity to directly interact with T cells, thus impairing the initiation of the acquired immune response (Farrell 2002). The outcome of this combat between amastigotes and the adaptive immune system does not solely depend on *Leishmania* but also on the immunological competence of the host to combat parasite growth . However, early infection determines outcome and is regulated by cytokine production (Chatelain, Varkila *et al.* 1992; Sypek, Chung *et al.* 1993). Resistance to *Leishmania* is associated with a IL-12-driven Th1-cytokine profile (IL-2, IFN-γ, and TNF-α), whereas non-healing leishmaniasis forms are associated with an IL-4-driven Th2 response (IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, and TGF-β) (Pirmez, Yamamura *et al.* 1993; Sacks and Noben-Trauth 2002). The inability of *L. major* parasites to drive early IL-12 production in either resistant (C57BL/6) or

susceptible (BALB/c) mouse strains is the reason for the initial Th2 response to *L. major* infection.

When activated, Th2 cytokines inhibit the IL-12-driven, interferon-γ-dominated Th1 response that promotes healing and parasite clearance by the down-regulation of IL-12 and IFN-γ, as well as NO production of macrophages that is critical for macrophage leishmanicidal activity (Heinzel, Sadick *et al.* 1989; Chatelain, Varkila *et al.* 1992; Vouldoukis, Becherel *et al.* 1997). In contrast, IL-12 production leads to a Th1 response in conjunction with IFN-γ production. The activation of macrophages is essential for their leishmanicidal activity (Reed and Scott 1993; Sypek, Chung *et al.* 1993; Gallin, Farber *et al.* 1995) and refers to a stage during which macrophages acquire functional changes compared with their resting state, including the activation of hydrolytic lysosomal enzymes, the release of NO and reactive oxygen intermediates (Hughes 1988; Alexander, Satoskar *et al.* 1999; Mossalayi, Arock *et al.* 1999). The latter two compounds are toxic to amastigotes; therefore, macrophages should be able to destroy and resolve the cause of infection and the infection itself over time.

Nevertheless, one should keep clearly in mind that the Th1/Th2 response to *Leishmania* infection is always an interplay between both Th1 and Th2 responses. It is not yet understood why in certain patients or mouse models one but not the other T-cell response is primarily activated.

The recent elucidation of the *L. major* genome revealed two genes (LmjF33.1740 and LmjF33.1750) that exhibit significant sequence identity with the mammalian cytokine, macrophage migration inhibitory factor (MIF) (Ivens, Peacock *et al.* 2005). The finding of these two genes led to the hypotheses that *L. major* might mimic its mammalian ortholog in a manner that serves to evade the host immune response.

1.4 The Cytokine MIF

1.4.1 Introduction

In 1932, Rich and Lewis reported that a factor inhibited immune cell migration *in vitro*; this factor turned out to be MIF, which belongs to one of the first-described cytokines. MIF was discovered, in context with delayed-type hypersensitivity, as a lymphocyte mediator that inhibited the random migration of macrophages (George and Vaughan 1962; Bloom and Bennett 1966; David 1966). In 1989, MIF was cloned

for the first time (Weiser, Temple *et al.* 1989). MIF, which exists as a single copy in mammals, is also present in a variety of infectious protozoa, such as *L. major* and *Toxoplasma gondii*. It is hypothesized that MIF plays a role in these organisms by interfering with the immune response of their mammalian hosts. Also is MIF present in non-infectious organisms such as *Caenorhabditis elegans* and *Arabidopsis thaliana*. However, its function in these organisms is not well understood.

MIF mRNA and protein are constitutively expressed and found in immune cells as well as in non-immune cells, including T cells, monocytes/macrophages, and parenchymal cells within the heart, kidney, liver, brain, and lungs, as well as the differentiating cells of the eye lens (Wistow, Shaughnessy *et al.* 1993). MIF is found in preformed cytoplasmic pools, and at serum concentrations of 2 to 6 ng/ml in healthy individuals (Calandra, Echtenacher *et al.* 2000), with peak levels in the morning (circadian rhythm) (Petrovsky, Socha *et al.* 2003) and is ready to be released during an immune response, unlike other cytokines that require transcriptional activation and mRNA translation prior to release. MIF also has a central role in the autocrine/paracrine activation of macrophages.

1.4.2 Structure and tautomerase/isomerase activity of MIF

In 1996, MIF, a 12.5-kDa protein, was successfully crystallized by three separate research groups (Kato, Muto *et al.* 1996; Sun, Bernhagen *et al.* 1996; Suzuki, Sugimoto *et al.* 1996), forming a new protein class, the tautomerase/MIF-fold and superfamily (Rosengren, Bucala *et al.* 1996). MIF, a trimer composed of identical subunits, consists of three β-sheets that form a barrel surrounded by six α-helices. Each monomer consists of a βαββββαββ motif.

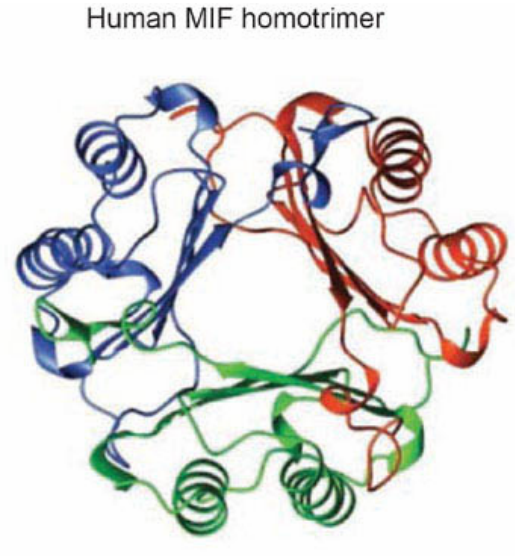


Fig. 6 Three-dimensional ribbon diagram of human MIF, revealing its homotrimeric subunit structure. Each color resembles one monomer.

MIF has the same three-dimensional structure as the microbial enzymes, CHMI (5-carboxymethyl-2-hydroxymuconate isomerase) and 4-OT (4-oxalcrotonate tautomerase), although there is no sequence homology among them . MIF possesses catalytic activity, including tautomerase and oxido-reductase activity. After the post-translational removal of the initiating methionine, Pro-1 is the catalytic residue. To date, no physiological substrate for this catalytic site has been determined. The non-physiological substrate, the D-isomer of dopachrome (2-carboxy-2,3-dihydroindole-5,6-quinone) is converted to DHICA (5,6-dihydroxyindole-2-carboxylic acid) (Rosengren, Aman *et al.* 1997). However, whether the pro-inflammatory role of MIF is based on its enzymatic activity is still under discussion. MIF also effects the enol-keto tautomerization of the substrates phenylpyruvate and hydroxyphenylpyruvate. ISO-1 [(S,R)-3-4(-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester], an inhibitor of MIF N-terminal tautomerase activity (Lubetsky, Dios *et al.* 2002; Al-Abed, Dabideen *et al.* 2005), was shown to inhibit the catalytic activity and some of the biologic activities of MIF.

1.4.3 The role of TPOR activity in MIF function

In 1996, Kleemann *et al.* demonstrated that MIF was able to mediate catalytic oxidoreductase activity (Kleemann, Kapurniotu *et al.* 1998) through its Cys-X-X-Cys (CXXC, where X is any amino acid) motif. This motif, between residues 57 and 60

of MIF, is a conserved region that is found in the thioredoxin (Trx) superfamily of thiol-protein oxidoreductases (TPORs). MIF shares this motif with other enzymes, such as Trx, protein disulfide isomerase, and glutaredoxin. In the case of MIF, the CXXC motif is a Cys-Ala-Leu-Cys (CALC). The CALC motif is conserved in all mammalian MIF orthologs; similar motifs were found in parasitic proteins of *Brugia malayi* and *Wucheria bancrofti*. MIF fulfils the requirements for the "extended TPOR motif" in having a Phe at position -7 and a Leu at position -10 of its CXXC motif. Under oxidizing conditions, MIF disulfide bridges between the Cys residues 57 and 60 are formed (Kleemann, Kapurniotu *et al.* 1998).

MIF exhibits CXXC-dependent TPOR activity with which it can undergo reversible oxidation/reduction reactions that catalyze disulfide/dithiol compounds (e.g., insulin and MHC class II molecules).

TPORs regulate protein folding and defend against oxidative and oxygen species (ROS) stress. Trx has been reported to interact with ASK-1 (apoptosis signal-regulating kinase-1), JAB1 (c-Jun activation domain binding protein-1), and Ref-1 (regulator of fusion-1) to control cellular signaling processes (Saitoh, Nishitoh *et al.* 1998; Hwang, Ryu *et al.* 2004). Trx was found to inhibit the apoptosis of immune cells through its TPOR site (Baker, Payne *et al.* 1997; Powis, Mustacich *et al.* 2000; Andoh, Chock *et al.* 2002). MIF was discovered to have certain characteristics of Trx, including intra- and extracellular activities, secretion without a leader sequence, and blockade of apoptosis, although it is not structurally homologous to the TPOR proteins. MIF is secreted upon H_2O_2 stimulation from macrophages (Kleemann, Mischke *et al.* 1998; Takahashi, Nishihira *et al.* 2001; Fukuzawa, Nishihira *et al.* 2002; Sakamoto, Fujie *et al.* 2002) and can therefore be considered an indicator of oxidative cell stress. MIF(50–65) was found to reduce cellular ROS (e.g., H_2O_2, O_2^-) within cells (Sun, Li *et al.* 2004; Matsuura, Sun *et al.* 2006), trigger mitogen-activated protein kinase (MAPK) activity (Nguyen, Beck *et al.* 2003), and bind to the MIF receptor CD74 ("MSc thesis"; Diplomarbeit). Cys60 was shown to be important in MIF leishmanicidal activity (Kleemann, Kapurniotu *et al.* 1998) as well as in CD74 interactions that were described using a C60S MIF mutant that exhibited reduced binding potency to CD74 ("MSc thesis"; Diplomarbeit). Furthermore, double mutations within the MIF CXXC motif showed altered protein folding.

1.4.4 MIF polymorphisms and expression

The genomic organization of the human MIF gene was described initially by Paralkar and Wistow in 1994 (Paralkar and Wistow 1994) who were the first to clone this gene. The human MIF gene is localized on chromosome 22, region 11 (22q11.2). The gene comprises 3 exons of 205, 173, and 183 bp separated by 2 introns of 189 and 95 bp (GeneBank Accession number: L19686; http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nuccore&id=307284). The MIF gene has a rather small 1.7 Kb size and is expressed as a single mRNA transcript in the human. Its open reading frame is 345 bp, and encodes a 115-amino acid polypeptide with a calculated molecular mass of 12,650 Da. MIF is distinct from that of conventional or secretory proteins by its lack of a hydrophobic leader sequence. This finding led to the assumption of a non-classical secretion pathway for MIF. Subsequent studies revealed a 5'-flanking sequence of 1 kb and a 3'-flanking sequence of 250 bp. Transcription of MIF was found to begin at -97, a region that is flanked by a cAMP-response element (CRE) and multiple SP1 sites. No classic eukaryotic TATA box (also called Goldberg-Hogness box) was found (Lifton, Goldberg *et al.* 1978), leading to the identification of a TATA-less promoter (Paralkar and Wistow 1994). Northern blot analysis of mRNA collected from multiple tissues and organs identified a single transcript of approximately 800 nucleotides in the human and the mouse.

Gene polymorphisms have been shown to have an impact on the pathogenesis of both autoimmune and inflammatory diseases. The MIF gene was revealed to have a polymorphism at position -794 of the promoter region, a tetranucleotide CATT-repeat (Paralkar and Wistow 1994; Baugh, Chitnis *et al.* 2002). Homozygous and heterozygous individuals for 5-, 6-, 7-, or 8-CATT repeats were subsequently identified. MIF promoter activity is proportional to the number of CATT repeats; 5-CATT repeats lead to low MIF expression levels and greater-than-5 CATT repeats lead to high MIF expression levels (Baugh, Chitnis *et al.* 2002). The latter, in conjunction with various substitutions at position -173, showed higher occurrences in individuals with inflammatory diseases. At position -173 of the human MIF promoter, a single nucleotide polymorphism (SNP) of G to C was discovered (Donn, Shelley *et al.* 2001). Other SNPs of T to C at position +254 in intron 1 and C to G- at +656 in intron 2 have been reported (Donn, Alourfi *et al.* 2002). At present, it is not known whether the intronic SNPs regulate MIF expression. Transcriptional activity

of the promoter is affected by the -794 CATT repeat and possibly by the -173 SNP in the gene's promoter. It is assumed that the transcription factor Pit-1 (pituitary-specific transcription factor 1), also known as POU1F1, might influence an individual's genetic susceptibility by enhancing MIF expression and augmenting inflammatory responses. Having a C nucleoside at position -173 leads to an AP-4 (activator protein)-binding site although its precise function is not yet known.

1.4.5 MIF as a ligand

The requirement for a cell surface receptor was debated until 1999 when Mitchell *et al.* (Mitchell, Metz *et al.* 1999) determined that MIF was able to stimulate intracellular pathways. Leng *et al.* showed that MIF mediates signal transduction through the cell surface form of the MHC class II-associated chaperone, invariant chain (Ii) or CD74 (Leng, Metz *et al.* 2003). CD74, a homotrimer, is a 32–41 kDa, type-II transmembrane protein with a cytosolic domain of 29–46 amino acids. The differences in size emerge from two, in-phase, alternative initiation codons (Arunachalam, Lamb *et al.* 1994) and N-glycosylation. MIF binding to $sCD74^{73-232}$ showed an equilibrium K_d (dissociation constant) of 9×10^{-9} to 2.3×10^{-10}. The measured K_d might differ from the *in vivo* K_d because of the CD74 transmembrane domain that requires removal prior to cloning and expression. This removal might result in altered oligomerization of native CD74. CD74 has been known to process and transport MHC class-II molecules from the endoplasmic reticulum to compartments of MHC class-II loading (Cresswell 1994; Ashman and Miller 1999). CD74 is expressed in MHC class-II-expressing cells, but, upon stimulation can also be found in MHC class-II-negative cells; this finding advanced the hypothesis that a protein like CD74 may have multiple, possibly unrelated, functions. Recent work suggested that CD44 is the signal-transducing molecule of the MIF-CD74 receptor complex (Meyer-Siegler, Leifheit *et al.* 2004; Shi, Leng *et al.* 2006).

The mechanism of MIF signaling in inflammatory atherogenic processes was discovered very recently. The MIF monomer shows three-dimensional homology to the CXCL8 dimer (Sun, Bernhagen *et al.* 1996). Consistently, MIF, which features a pseudo-ELR motif (N-terminal Glu-Leu-Arg), has the ability to bind to CXCR2, which is the CXCL8 receptor . Not only is MIF able to induce neutrophil chemotaxis through CXCR2 (Bernhagen, Krohn *et al.* 2007), but it also promotes the recruitment

of both monocytes and T cells via interactions with CXCR2 and CXCR4. Interestingly, MIF/CXCR2 binding also involved CD74 indicating that MIF signals via a functional CXCR/CD74 super-complex, demonstrating its importance in inflammatory processes and atherogenic cell recruitment. In conclusion, MIF is not only a ligand for CD74, but also directly activates the chemokine receptors, CXCR2 and CXCR4.

1.4.6 MIF function

During infection, the host responds with the activation of the hypothalamic-pituitary-adrenal (HPA) axis and, hence, the release of glucocorticoid hormones (anti-inflammatory mediators) produced in the adrenal cortex. Hormones are (bio-) chemical messengers that carry signals from one group of cells to another via the blood stream in an endocrine fashion to regulate the function of their target cells. Surprisingly, MIF production is induced after release of, as well as after treatment with, glucocorticoids. Once released, MIF antagonizes the actions of glucocorticoids by the following mechanisms: (i) MIF counteracts the inhibition of NF-κB by preventing the glucocorticoid-induced increase in cytosolic IκBα, the inhibitor of NF-κB, and/or (ii) MIF inhibits the expression of MKP-1 (MAPK phosphatase-1), the inhibitor of extracellular signal-regulated kinase (ERK1/2), p38 and JNK MAPK. After treatment with either cortisol or dexamethasone at concentrations of 10^{-6} M, the dose-response curve of macrophage MIF production is bell-shaped. This dose-response curve is also seen in MIF-producing macrophages after stimulation with pro-inflammatory stimuli, such as TNF-α, IFN-γ, endotoxin, and exotoxin. The bell-shaped curve might protect the host from the deleterious effects of exuberant concentrations of MIF. Mechanistically, our knowledge of MIF secretion is still limited, although recently it was shown that MIF is secreted via a non-classical pathway possibly involving an ABCA1 transporter (Flieger, Engling *et al.* 2003). Notably, MIF was identified as a negative regulator of the tumor suppressor p53 (Hudson, Shoaibi *et al.* 1999). p53 inhibition requires the upstream activation of ERK1/2, phospholipase A2 (cPLA2), arachidonic acid, and COX2. Furthermore, MIF not only activates the ERK1/2 pathway but also JNK (c-jun N-terminal kinase), p38 MAPK [also known as DC-2-related protein kinase or CSBP (cytokine suppressive anti-inflammatory drug binding protein)] (Roger, Chanson *et al.* 2005;

Aeberli, Yang *et al.* 2006). MIF also activates NF-κB in B cells (Starlets, Gore *et al.* 2006).

Lipopolysaccharide (LPS), the major component of the outer membrane of Gram-negative bacteria, binds to Toll-like receptor 4 (TLR4). The expression of TLR4 is up-regulated by MIF through the ETS transcription factor, PU.1 . After binding of LPS to the LPS-binding complex, composed of TLR4, CD14, and LBP (lipopolysaccharide-binding protein), NF-κB signaling followed by TNF-α expression is stimulated. This activation leads to the TNF-α-induced release of MIF from macrophages. Preformed MIF is released within six hours after stimulation with LPS (Bacher, Meinhardt *et al.* 1997). As a consequence, MIF expression is up-regulated and the cytoplasmic MIF pool is replenished within 24 hours of LPS stimulation. MIF promotes inflammatory responses by inducing TNF-α, IFNγ, IL-1β, IL-2, IL-6, IL-8, IL-12, CXCL2, NO, MMP (matrix metalloproteinase), cPLA2, and, consequently, arachidonic acid and its products, leukotrienes and prostaglandins.

1.4.7 MIF and ERK1/2

The activation of ERK has been shown to be required for inflammation, immune regulation, and cell proliferation.

MIF stimulation is associated with the protein kinase A (PKA)-dependent serine phosphorylation of both CD74 and CD44 (Shi, Leng *et al.* 2006). Upon either treatment with MIF or after the endogenous release of MIF, the p44/p42 ERK subfamily of the MAPKs is phosphorylated (Mitchell, Metz *et al.* 1999). Not only does ERK activation through MIF persist for up to 24 hours (Mitchell, Metz *et al.* 1999) but also is exogenous MIF able to stimulate the activation of the classical Raf-1/MEK/ERK MAPK pathway within a few minutes (Lue, Kapurniotu *et al.* 2006). Activation of the ERK pathway results in the phosphorylation and activation of certain cytosolic proteins, such as $P90^{rsk}$, c-myc, and $cPLA_2$ (Hayakawa, Ishida *et al.* 1993; Denhardt 1996). $cPLA_2$ mediates the production of the pro-inflammatory molecule arachidonic acid, the precursor of prostaglandins and leukotrienes, which play roles in inflammatory processes; the phosphorylation and activation of $cPLA_2$ by MIF requires ERK activation (Mitchell, Metz *et al.* 1999).

1.4.8 MIF and the tumor suppressor p53

Tumor suppressor genes slow cell division, contribute to DNA repair, and induce apoptosis. When tumor suppressor genes fail to function properly due to mutations or pathogenic regulation, cells can grow out of control, leading to cancer. In the case of neoplasia, tumor suppressor genes become disabled, allowing cells to bypass senescence and, instead of succumbing to apoptosis, continue dividing for additional generations. Therefore, the tumor suppressor protein p53 is often referred to as a "gatekeeper" for cell growth and division and "guardian of the genome" (Levine 1997; Ryan, Phillips *et al.* 2001; Fingerle-Rowson, Petrenko *et al.* 2003). The p53 protein, encoded by the TP53 gene, is mutated in over 50% of all human cancers. The p53 protein is a transcription factor that can mediate many of its downstream effects by enhancing the rate of transcription through either the activation or repression of 6–7 known genes. The human p53 protein, which plays a role in triggering apoptosis, contains 393 amino acids and is divided into four domains (Levine 1997; Ryan, Phillips *et al.* 2001). The sequence-specific, DNA-binding domain of p53 is localized between amino acid residues 102 and 292. p53 has a short half-life (~ 20 min) and is therefore present at low concentrations in cells. In response to DNA damage, the p53 half-life increases and the rate of translational initiation of p53 mRNA in cells becomes enhanced. This increase in p53 levels is proportional to the extent of DNA damage. p53 transcriptional activation is negatively regulated by the adenovirus E1B-55Kd protein, the human MDM2 protein, and MIF (Levine 1997; Hudson, Shoaibi *et al.* 1999; Ryan, Phillips *et al.* 2001). MIF inhibits p53-mediated growth arrest, gene activation, and apoptosis. Furthermore, MIF is able to inhibit the transcription of p21, cyclin G, and the pro-apoptotic protein, Bax through p53 (Hudson, Shoaibi *et al.* 1999). During inflammatory diseases, a combination of interactions between MIF, NO, and p53 can be observed. At the site of inflammation, macrophages release NO to mediate macrophage apoptosis as part of their antimicrobial repertoire (Messmer and Brune 1996; MacMicking, Xie *et al.* 1997). Hudson *et al.* could demonstrate that MIF treatment suppressed NO-induced apoptosis in a dose-dependent manner (Hudson, Shoaibi *et al.* 1999).

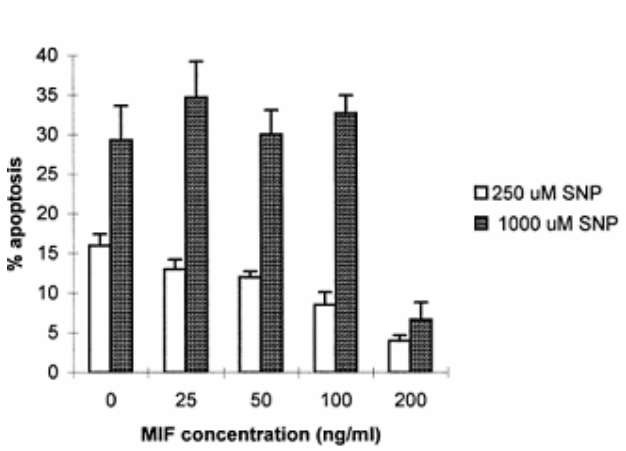


Fig. 7. RAW264 macrophages were first treated for 24 hours with 25, 50, 100, or 200 ng/ml MIF, and then with 250 µM or 1 mM SNP (NO-releaser). After 2 days, cells containing condensed or fragmented DNA were scored as apoptotic after fixation with paraformaldehyde and staining with Hoechst 33258. MIF treatment was capable of suppressing NO-induced apoptosis in a dose-dependent manner. Taken from: (Hudson, Shoaibi *et al.* 1999).

MIF interference with the transcriptional activity of p53, leading to p53 inhibition and prevention of apoptosis, may represent a cancer risk factor during certain chronic inflammatory diseases (Hudson, Shoaibi *et al.* 1999; Mitchell and Bucala 2000). Such diseases include colon cancer in ulcerative colitis or Crohn's disease (Ekbom, Helmick *et al.* 1990; Ekbom 1998), gastric cancer in *Heliobacter pylori* infection (Graham 2000), and urinary bladder cancer in the parasitic disease, Schistosomiasis (Mostafa, Sheweita *et al.* 1999).

1.4.9 Role of MIF in infectious diseases

The main virulence factor of Gram-negative bacteria (such as *E. coli*), is endotoxin, or LPS. After LPS challenge in infected mice, an increase in MIF serum levels with a peak at 20 hours was detected. Protection from lethal endotoxemia in mice was discovered after treatment with anti-MIF (Bernhagen, Calandra *et al.* 1993). Calandra *et al.* hypothesized that anti-MIF therapy might be useful to protect the host from harmful cytokine overburden, and *in vivo* experiments clearly showed that immunoneutralization of MIF protects from the development of otherwise lethal Gram-negative infection (Calandra, Echtenacher *et al.* 2000).

Studies with *Salmonella typhimurium* (Koebernick, Grode *et al.* 2002) on the other hand revealed that MIF-/- mice possessed a weakened Th1 response and incomplete macrophage activation, which led to hindered killing of intracellular *S. typhimurium*.

MIF release by macrophages also is induced by Gram-positive exotoxins, such as staphylococcal and streptococcal exotoxins. Interestingly, MIF appears to be an indicator of patient outcome during septic shock. High MIF levels have been a symptom of impending, fatal septic shock whereas lower concentrations are correlated with a high patient survival rate (Bozza, Gomes *et al.* 2004). New treatment possibilities exist for septic shock patients as MIF neutralization has a larger therapeutic window for its administration than either anti-TNF or anti-IL-1 therapies (Abraham 1999). Experiments with *Mycobacterium tuberculosis* showed that MIF neutralization in macrophages advances bacterial growth, whereas treatment with MIF leads to growth inhibition (Oddo, Calandra *et al.* 2005). One could argue that MIF is essential for host defenses against infections; however, elevated MIF levels are dangerous and harmful as seen in chronic inflammatory diseases that are linked to development of cancer. MIF is expressed in different human tumors at levels that may correlate with tumor aggressiveness (Tomiyasu, Yoshino *et al.* 2002).

Several primitive eukaryotes encode MIF-like genes that show remarkable similarity to their mammalian counterparts, such as the human parasitic nematodes, *Brugia malayi* (Pastrana, Raghavan *et al.* 1998) and *Ancylostoma ceylonicum* (Cho, Jones *et al.* 2007). The tick vector responsible for the transmission of anaplasmosis, *Amblyomma americanum*, produces an MIF ortholog that is expressed in the salivary gland (Jaworski, Jasinskas *et al.* 2001). MIF-like proteins also have been reported recently in pathogenic species of *Eimeria* (Miska, Fetterer *et al.* 2007), *Trichinella* (Wu, Boonmars *et al.* 2003), and *Plasmodium* (Augustijn, Kleemann *et al.* 2007; Cordery, Kishore *et al.* 2007); whether these proteins play a role in parasite-host interactions has not been determined.

1.4.10 Mammalian MIF and *L. major* infection

MIF is not only involved in the recruitment of pro-inflammatory cells during infection but also in the regulation of their microbicidal activities.

Juttner *et al.* showed that MIF affected *Leishmania*-infected macrophages by reducing the number of parasites in a dose-dependent manner (Juttner, Bernhagen *et al.* 1998). This effect was particularly notable late after infection, between 72 and 96 hours. The simultaneous addition of IFN-γ together with MIF resulted in intensified macrophage activation. One might argue that MIF and IFN-γ had a synergistic effect on these cells, but the work of Juttner *et al.* showed that they acted independently of each

other. The leishmanicidal effect of MIF is induced by TNF-α macrophage activation and macrophage production of endogenous NO. The cytokines IL-10 and IL-13 (secreted by Th2 cells), in addition to TGF-β (secreted by macrophages), have been shown to impair the intracellular killing of *Leishmania in vitro* (Doherty, Kastelein *et al.* 1993; Vieth, Will *et al.* 1994; Barral, Teixeira *et al.* 1995; Juttner, Bernhagen *et al.* 1998) by inhibiting the production of reactive nitrogen intermediates by IFN-γ-activated macrophages . Of note, lymph nodes draining the site of infection showed an up-regulation of MIF mRNA compared with the cytokines IL-12 (p35), IL-12 (p40), IL-10, IL-1α, and IL-1β mRNA (Juttner, Bernhagen *et al.* 1998). With the discovery of MIF functioning as a noncognate CXCR ligand (Bernhagen, Krohn *et al.* 2007), its chemokine-like functions in inflammatory monocyte and T cell recruitment in respect to *L. major* infections probably will be needed to be interpreted from another perspective.

In conclusion, MIF mRNA and protein expression both become up-regulated after *L. major* infection and contribute to the struggle of the host against the parasite.

1.4.11 Mouse models

Human and mouse MIF share 88% DNA sequence identity (Weiser, Temple *et al.* 1989; Bozza, Kolakowski *et al.* 1995); therefore, it is customary to use mouse models in MIF research studies.

The mouse genome consists of 19 autosomes plus 2 sex chromosomes with the MIF gene located in the middle of chromosome 10. The mouse MIF gene consists of 3 exons, separated by 2 introns of 201 and 144 base pairs, spanning 0.67 kb of the genome (Bozza, Kolakowski *et al.* 1995). The mouse MIF gene maps to the middle region of chromosome 10; however, pseudogenes were discovered, with three mapping to chromosomes 1, 9, and 17. These pseudogenes are highly homologous to mouse MIF but contain a variable number of mutations that would produce either truncated or mutated MIF-like proteins. The pseudogenes are assumed to have evolved from retrotranspositional events as they display characteristic features of retrotransposed pseudogenes, including a lack of introns and the presence of a poly(A) tail in both the 3' and 5'-untranslated regions (Bozza, Kolakowski *et al.* 1995). In the immediate 5'-flanking region of mouse MIF, the classical consensus TATA box is lacking as it is for human MIF.

To study the role of endogenous MIF, $MIF^{-/-}$ mice in a C57BL/6 × 129/Sv mice background were produced in 1999 by Bozza *et al.* (Bozza, Satoskar *et al.* 1999). The difference between wild-type and $MIF^{-/-}$ mice with respect to *L. major* infections was studied in 2001 by Satoskar *et al.* (Satoskar, Bozza *et al.* 2001). Infected *L. major* $MIF^{-/-}$ mice were susceptible to leishmaniasis and showed significantly larger lesions and greater parasitic burdens than $MIF^{+/+}$ mice. Satoskar *et al.* showed that this observation was due to impaired macrophage leishmanicidal activity rather than to an imbalance of Th1/Th2 responses. As discussed above, the protective role of MIF in murine leishmaniasis can be attributed to its ability to induce macrophage NO production through IFN-γ. Resting macrophages were tested for their leishmanicidal activity after IFN-γ stimulation. Seventy-two hours after stimulation with IFN-γ, a significant difference between the parasite killing levels of $MIF^{+/+}$ and $MIF^{-/-}$ mice was observed. In the course of these studies, this result was attributed to lower O_2^- levels and NO production in $MIF^{-/-}$ mice. Therefore, MIF is believed to play a significant role in the production of pro-inflammatory cytokines and anti-parasital agents to fight *L. major* infection.

2 Specific Aims

Parasites must overcome external defenses to gain host entry, but by doing so, they must escape the powerful defense mechanisms of the host immune system. One way to overcome the host defense system is to interfere with host cytokines. Given the importance of cytokines in both natural and adaptive immunity, it is not surprising that parasites often interfere with cytokine function. Parasites have been shown to possess genes that encode molecules that are virtually identical to host genes; they are called 'orthologs'. Macrophages are an important component of the host first line of defense system in the response to pathogenic infections. Infection of a macrophage leads to the induction of numerous cellular genes, several of which encode cytokines that stimulate inflammatory responses and resistance to pathogens. Hence, the control of *Leishmania* infection requires activation of macrophages that in turn leads to their leishmanicidal activity. *Leishmania* parasites have developed strategies to escape host immune defenses and to survive in their hosts.

We hypothesized that by possessing orthologs of human MIF, *L. major* has found a way to escape the host immune system, attack it, and spread infection, causing a dreadful disease in both humans and animals. The very first goal of this research was therefore to confirm the expression of *L. major* MIF *in vivo.* The next goal was to functionally characterize the identified ortholog, Lm1740MIF, which shares a 22% sequence identity with human MIF. While host MIF appears to play an important role in the immune response against infections, there is no information about the role of *L. major* MIF during either infection or with respect to immune evasion mechanisms and in innate immunity. The identification of the interaction between *L. major* MIF and macrophages will allow further studies of whether *L. major* MIF affects signal transduction. Therefore, an additional goal of this thesis was to study and characterize the interaction of MIF with the macrophage surface receptor CD74. During this work, *L. major* MIF was discovered to interact functionally with the MIF receptor, CD74. Since interactions between MIF and CD74 are known to lead to the induction of signal transduction processes, the next major aim was to investigate whether the interaction of *L. major* MIF with CD74 affected either ERK1/2 or p53 activity, which may facilitate the intracellular persistence of *L. major* in macrophages.

3 Materials and Methods

3.1 Materials

3.1.1 Mice

Mice **(**BALB/c, C3H/HeJ, and C3H/HeN) were purchased from Charles River Laboratories (Wilmington, MA). BALB/c mice deficient in the MIF receptor CD74 (CD74-KO) were originally provided by Dr. Idit Shachar (Weizmann Institute, Rehovot, Israel) (Starlets, Gore *et al.* 2006). C3H/HeJ mice are defective in their lipopopolysaccharide response allele TLR4.

3.1.2 Material lists

Source of supply	**Internet adresse**	**Headquarters**
American Bioanalytical	www.americanbio.com	Natick, MA 01760, USA
American National Can™	www.2spi.com	West Chester, PA 19381, USA
Amersham Biosciences	www.amershambiosciences.com	Piscataway, NJ 08855, USA
BD Biosciences	www.bdbiosciences.com	Franklin Lakes, NJ 07417, USA
Beckman Coulter™	www.beckmancoulter.com	Fullerton, CA 92834, USA
Bio101	siehe Qbiogene	
Biocompare	www.biocompare.com	South San Francisco, CA 94080, USA
BioRad	www.bio-rad.com	Hercules, CA 94547, USA
Carnation	www.carnation.ca	
Cell Signalling	www.cellsignal.com	Danvers, MA 01923
Corning Incorporated	www.corning.com	Big Flats, NY 14814,

		USA
Cryostar Industries, Inc.	www.sanyobiomedical.com	Bensenville, IL 60106, USA
Denville	www.denvillescientific.com	Metuchen, NJ 08840, USA
DIFCO	www.difco.com	Franklin Lakes, NJ 07417, USA
DotScientific Inc.	www.dotscientific.com	Burton, MI 48519, USA
DuPont®	www.dupont.com	Wilmington, DE 19898, USA
EmbiTec	www.embitec.com	Carlsbad, CA 92008, USA
Eppendorf AG	www.eppendorf.com	22339 Hamburg, Germany
Fisher Scientific	www.thermofisher.com	Waltham, MA 02454, USA
Frigidair Commercial	www.biobank.co.kr	Conway,AR 72032, USA
General Electrics GE	www.ge.com	
GE Healthcare	www.gehealthcare.com/lifesciences	Piscataway, NJ 08855, USA
GIBCO®	siehe Invitrogen	
GIBCO BRL®	siehe Invitrogen	
Hausser Scientific	www.hausserscientific.com	Horsham, PA 19044, USA
Hoefer Inc.	www.hoeferinc.com	San Francisco, CA 94107, USA
Invitrogen™	www.invitrogen.com	Carlsbad, CA 92008, USA
J.T.Baker	www.jtbaker.com	Phillipsburg, NJ 08865, USA
Kendro	www.kendro.com	Asheville, NC 28806,

		USA
Kodak	www.kodak.com	
Kysor Panel Systems	www.kysorpanel.com	Fort Worth, TX 76137, USA
Leica	www.leica-microsystems.com	Bannockburn, IL 60015, USA
Millipore	www.millipore.com	Billerica, MA 01821, USA
Micro-Tech Optical NE Inc.	MT01@MSN.COM	Bloomfield, CT 6002, USA
MJ Research	www.mjr.com	Waltham, MA 02451, USA
Ohaus Corporation	www.ohaus.com	Pine Brook, NJ 07058, USA
Pierce	www.piercenet.com	Rockford, IL 61105, USA
Qbiogene	www.qbiogene.com	Irvine, CA 92618, USA
Roche Diagnostics Corporation	www.roche-applied-science.com	Indianapolis, IN 46250, USA
Santa Cruz Biotechnology	www.scbt.com	Santa Cruz, CA. 95060, USA
Scotsman	www.scotsman-ice.com	Vernon Hills, IL 60061, USA
Sigma-Aldrich Co.	www.sigmaaldrich.com	St.Louis, MO 63103, USA
Steris®	www.steris.com	Mentor, OH 44060, USA
The Baker Company	www.bakerco.com	Sanford, ME 04073, USA
Thermo Scientific	www.thermo.com	Waltham, MA 02454,USA
USA Scientific, Inc.	www.usascientific.com	Ocala, FL 34478, USA

VWR Scientific	www.vwrsp.com	West Chester, PA 19380, USA

Equipment/instruments	**Source of supply**
Autoradiography cassettes	Fisher Scientific
Centrifuge 5415C	Eppendorf
+ 37ºC chamber	Kysor Panel Systems
Digi-Block™ Jr.	Laboratory Devices
DU® 530 Life Science UV/VIS Spectrophotometer	Beckman Coulter™
Microscope, Leica DM1RB	Leica
ELISA-Spectramax PLUS ^{384}	Molecular Devices
- 20ºC freezer	Frigidair Commercial
+ 4ºC fridge	National Brokar Inc.
+ 22°C incubator	Fisher Scientific
+37°C incubator	Kysor Panel Systems
GelDoc 1000	BioRad
HeraCell 240	Kendro
Ice machine	Scotsman
X-Omat 2000A processor	Kodak
MacroVue UV-25	Hoefer
Mini Cycler™ PTC-150	MJ Research
MR5000	Dynatech
Olympus CK2 microscope	Micro-Tech Optical (NE), Inc.
Peltier Thermal Cycler PTC-100	BioRad
PowerPac 300	BioRad
Programmable thermal controller	MJ Research
Run One™ electrophoresis cell	EmbiTec
Scale Scout™ Pro SP402	Ohaus Corporation
Scientific Prevacuum Sterilizer SV-120	Steris
Sorvall® RC-5B refrigerated superspeed centrifuge	DuPont Instruments

SterilGard® III Advance	The Baker Company
Turntable microwave oven	General Electrics
Vortex-Genie 2™	VWR Scientific
XCell II™	Invitrogen

Materials	**Source of supply**
2 ml aspirating pipet	BD Labware Falcon®
1-20 µl bevelled filter tips	USA Scientific
Blotting paper, 703	VWR
BlueMax™ 50 ml polypropylene conical tube, 30 x 115 mm style, nonpyrogenic	BD Labware Falcon®
BlueMax™ Jr. 15 ml polypropylene conical tube	BD Labware Falcon®
Cell culture Flask, 25cm^2, sterile, non-pyrogenic, tissue culture treated, canted neck	Corning Incorporated
Cellcounter	Hausser Scientific
Cell scraper	BD Labware
Costar stripette 5 ml, non pyrogenic, serological pipet	Corning Incorporated
Costar stripette 10 ml, non pyrogenic, serological pipet	Corning Incorporated
Costar stripette 25 ml, non pyrogenic, serological pipet	Corning Incorporated
500 ml filter system, 0.22 µm cellulose acetate, sterilizing, low protein binding membrane, non-pyrogenic, polystyrene	Corning Incorporated
0.1-10 µl filter tips	USA Scientific
Immobilion-P transfer membrane; pore size: 0.45 µm	Millipore
Microseal® 'B' Film, PCR Sealers™	BioRad
Multiplate® PCR Plates™, 96-well, clear	BioRad

1.5 ml natural microcentrifuge tubes, Rnase, Dnase, DNA and pyrogen free	USA Scientific
Parafilm	American National Can™
PetriDish, 100x15mm	BD Labware Falcon®
Pipette Tips, Universal Fit, 1-200 µl, tip round	DotScientific Inc.
200 µl precision barrier tips	Denville
Scientific imaging film	Kodak
Tissue culture dish Falcon®Tissue Culture treated by vacuum gas plasma, 100 x 20 mm style, polystyrene, non-pyrogenic	BD Labware Falcon®
1000 µl ultra sharp filter tips	Denville
0.2 ml thin-wall tubes with attached domed caps	BioRad

Commercially available buffers and solutions	**source of supply**
Distilled Water 0.1 micron filtered	GIBCO®
DMEM, 1 x 0.1 micron filtered, high glucose with L-glutamine, with pyridoxine Hydrochloride, without sodium pyruvat	GIBCO®
Dulbecco's PBS, 1 x 0.1 micron filtered, without calciumchloride, without magnesium chloride	GIBCO®
ECL™Western Blotting Detection reagents	GE Healthcare
Ethidium bromide solution 10m (10 g/ml)	Invitrogen
FBS	GIBCO®
Glycerol (synthetic) $C_3H_8O_3$ 500 ml ultra pure ACS/USP grad	American Bioanalytical

Methanol	J.T. Baker
NuPage® antioxidant	Invitrogen
NuPage® LDS sample buffer 4x	Invitrogen
NuPage® MES SDS running buffer (20x)	Invitrogen
NuPage® MOPS SDS running buffer (20x)	Invitrogen
NuPage® transfer buffer (20x)	Invitrogen
Percoll	Sigma
Restore™ Western Blot stripping buffer	ThermoScientific
RPMI 1640 (1x) ; with L-glutamine	GIBCO®
Schneider's Insect Medium with L-glutamine without calcium chloride and sodium bicarbonate	Sigma-Aldrich
Thioglycollate	Sigma-Aldrich
Tris-HCl	Sigma-Aldrich
Trizol	Gibco
Tween-20	Pierce
Ultra Pure 10 x TAE buffer concentration of 10 x solution: 400 mM Tris acetate and 10 mM EDTA	Invitrogen
Versene 1:5000 (1x) contains 0.2 g/l EDTA·4Na in Phosphate-buffered saline	GIBCO®
Water, RNase free, Endotoxin tested, treated with DEPC to eliminate RNase	American Bioanalytical

Chemicals, enzymes and molecular biology materials and reagents	**Source of supply**
Agarose GPG/LE	American Bioanalytical
anti-rabbit IgG, HRP-linked Antibody	Cell Signaling
Blotting Greade Blocker Non-Fat Dry Milk	BioRad
BSA	BioRad

Cell Death Detection ELISA^{PLUS}	Roche
Dithiothreitol (DTT)	Sigma-Aldrich
100bp DNA ladder 50 µg (0.1 µg/µl)	Invitrogen
EDTA	Biocompare
EtBr	Sigma-Aldrich
Gene Clean III kit	Bio101
Gentamicin	Invitrogen
NaCl	J.T.Baker
non-fat dry milk	Carnation
NuPage® 4-12% Bis-Tris Gel, 1.0x10well	Invitrogen
NuPage® 4-12% Bis-Tris Gel, 1.0x12well	Invitrogen
penicillin/streptomycin	GIBCO®
p44/42 MAP kinase antibody	Santa Cruz Biotechnology
phopho- p44/42 MAP kinase antibody	Cell Signaling
p53 antibody	Cell Signaling
phospho- p53 antibody	Cell Signaling
PBS	BD Biosciences
PCR-Supermix	Invitrogen
Methanol	J.T.Baker
SDS-Polyacrylamid gel	Invitrogen
SeeBlue® Plus2 prestained standard (1x)	Invitrogen
SNP	Amersham Biosciences

Buffer and solutions	**Composition**
100 bp ladder	10 µl 100 bp stock solution, 50 µl 6 x loading buffer, 440 µl 0.5 x TAE + EtBr
1 kb ladder	10 µl 1 kb stock solution, 50 µl 6 x loading buffer, 440 µl 0.5 x TAE + EtBr
6 x loading buffer	0.25 % Bromphenolblue, 0.25 % Xylene Cyanole FF, 30 % Glycerol

Electrophoresis buffer	50 ml TAE, 50 µl EtBr, 950 ml dH_2O
Lysis buffer	20 mM HEPES, pH 7.4; 50 mM β-Glycerolphosphate ; 2 mM EGTA (Acetic Acid) ; 1 mM DTT (Dithiothreitol) ; 10 mM NaF ; 1 mM NaVO4 ; 1% Triton x-100 ; 10% Glycerol
MES	50 ml MES (20X); 950 ml deionized water
MOPS	50 ml MOPS (20X); 950 ml deionized water
Schneider's complete media	30 ml FCS, 4 ml L-glutamine, 166 ml Schneiders, 120 µl Gentamycin
TE buffer	10 mM Tris HCl, pH 7.4; 1 mM EDTA, pH 8.0
TBST	20 mM Tris-HCl, pH7.4; 150 mM NaCl; 0.05% Tween-20; 950 ml dH_2O
1X NuPage® transfer buffer	50ml 20X NuPage® transfer buffer; 100 ml methanol; 850ml deionized water

Primers

All primers were ordered from the W.M.Keck Oligonucleotide Synthesis Facility at Yale University, New Haven, CT, USA.

Primer name	Primer sequence	Used for
L1740 forward	5'-ATG CCG GTC ATT CAA ACG-3'	amplification of LmjF33.1740 DNA
L1740 backward	5'-TTA GAA GTT TGT GCC ATT CC-3'	amplification of LmjF33.1740 DNA
L1740 backward short	5'-CTC TGG TTT GCC GAG TAC A-3	Amplification of LmjF33.1740 DNA for qPCR analysis

L1750 forward	5'-ATG CCG TTT CTG CAG AC-3'	amplification of LmjF33.1750 DNA
L1750 backward	5'-TCA AAA GTT AGT GCC GTT-3'	amplification of LmjF33.1750 DNA
L1750 backward short	5'-AGT CAT CAC GAA GTC CTC-3'	Amplification of LmjF33.1750 DNA for qPCR analysis
ADP/ATP carrier forward	5'-ATC TCA TAC CCG CTG GAC AC-3'	amplification of the ADP/ATP carrier as a housekeeping gene for qPCR analysis
ADP/ATP carrier backward	5'-TCA AGC GAG TTG CGG TAG TT-3'	amplification of the ADP/ATP carrier as a housekeeping gene for qPCR analysis
rRNA45 forward	5'-CCT ACC ATG CCG TGT CCT TCT A-3'	amplification of rRNA45 as a housekeeping gene for qPCR analysis
rRNA45 backward	5'-AAC GAC CCC TGC AGC AAT AC-3'	amplification of the rRNA45 as a housekeeping gene for qPCR analysis

Methods

3.1.3 Sequence and phylogenetic analyses

Phylogenetic analyses were carried out using sequences from the ClustalW multiple alignment output using the Neighbor-Joining method (Saitou and Nei 1987) via the MEGA3.1 (Kumar, Tamura *et al.* 2004) interface with the following settings for DNA phylogenetic analysis: pairwise alignement parameters: gap opening penalty 15;

gap extension penalty 6.66; multiple alignment: gap opening penalty 15; gap extension penalty 6.66; delay divergent cutoff 30%; DNA transition weight 30%; weight matrix IUB with 5000 bootstrap replicates. For protein phylogenetic analysis, the following settings were chosen: pair-wise alignment parameters: gap opening penalty 10; gap extension penalty 0.1; multiple alignment: gap opening penalty 10; gap extension penalty 0.2; Gonnet protein weight matrix and delay divergent cutoff 30%. To compare identity, the Expert Protein Analysis System (ExPASy) proteomics server of the Swiss Institute of Bioinformatics was employed. The interface T-Coffee (Poirot, O'Toole *et al.* 2003) was used for sequence alignment and the residues are numbered in accordance with the alignment; gaps are counted.

3.1.4 Mice

All mice were used at 6-8 weeks of age, and experiments were performed in accordance with the Yale University's IACUC (Institutional Animal Care and Use Committee).

3.1.5 Parasite and cell cultures

Bone marrow macrophages (BMMs) were harvested from mouse femurs and cultured for 4 days in 10 cm plates with complete medium containing RPMI 1640 (GibcoBRL, Gaithersburg MD), 20% fetal bovine serum (FBS), 30% L929-conditioned medium (LCM), and 1% penicillin/streptomycin. On day 5, cells were re-plated at a density of $4x10^6$/ml and treated according to the intended experiments. Peritoneal exudate cells were obtained from mice that were injected 3-4 days previously with 2 ml of 4% sterile thioglycollate broth (Calandra, Bernhagen *et al.* 1994). *Leishmania major* (MHOM/IL/79/LRC-L251) (Beverley, Ismach *et al.* 1987) promastigotes were cultured at 23ºC in complete Schneider's medium supplemented with 20% heat-inactivated FBS and gentamicin (10 µg/ml).

3.1.6 Reverse transcriptase-PCR analyses and quantitative PCR

Total RNA from *L. major* was isolated from procyclic and metacyclic cultures, and from amastigotes isolated from homogenized, infected mouse lymph nodes using the Trizol reagent (Invitrogen). As the presence of dead or dying organisms might lead to a mixture of procyclic/metacyclic cultures and contribute to false data, live metacyclic organisms were separated from the cellular debris present in stationary-phase cultures

via a Percoll step gradient. The gradient consisted of 90% Percoll in phosphate-buffered saline (PBS) that was overlaid with parasites that had been resuspended in 45% Percoll (in PBS); the gradient was then overlaid with solutions of 30% and 20% Percoll (in PBS) and centrifuged at 6000 rpm for 45 min at 4 °C. Live parasites were isolated at the 20–30% interface. After isolation from the Percoll gradient, parasites were washed in 2 ml of 1× PBS and centrifuged at 1,500 rpm for 15 min. The Trizol reagent was used subsequently for the metacyclic pellet. For gene expression quantification, the two-step quantitative, reverse-transcriptase PCR method was applied. First, the isolated total RNA was reverse-transcribed using the AccuScript™ Kit from Stratagene according to the manufacturer's instructions. The principle for reverse-transcriptase PCR involves the extraction of all tissue or cellular RNA under study and their transcription *in vitro* into single-stranded cDNAs. The cDNA molecules obtained then serve as a template for PCR using specific primers. PCR of the cDNA products consisted 32 cycles of a denaturation step (94 ºC, 1 min), an annealing step (50 ºC, 2 min), and an elongation step (72 ºC, 3 min). For the last cycle, the elongation step was extended to 10 min at 72 ºC. The *Taq* DNA polymerase was used for the amplification steps. Specific primers used are listed in the *Material and Methods*, *Primers.* PCR products were purified from unspecific products on a 2% agarose gel. The bands at the specific fragment sizes were excised and purified with the Gene Clean III Kit. An aliquot of the resulting cDNA was used as the template for quantitative PCR (qPCR).

To evaluate the quantity of RNA present in the different developmental stages of *L. major*, qPCR was performed using SYBR Green, which intercalates into the DNA helix. The fluorescence increases as the amount of the PCR product increases and was quantified after each completed PCR cycle. The results are displayed as relative expression values of the gene of interest (GOI). Therefore, the results are related to the internal controls (control genes or housekeeping genes) that are present at constant expression levels in all samples.

Both the *L. major* ADP/ATP carrier (LmjF19.0210) (Leifso, Cohen-Freue *et al.* 2007) and rRNA45 (CC144545) (Ouakad, Bahi-Jaber *et al.* 2007) transcripts served as internal controls. The amplification primers were Lm1740FWD: 5'-ATG CCG GTC ATT CAA ACG-3'; Lm1740BWD2: 5'-CTC TGG TTT GCC GAG TAC A-3'; Lm1750 FWD: 5'-ATG CCG TTT CTG CAG AC-3'; Lm1750BWD2: 5'-AGT CAT CAC GAA GTC CTC-3'; CarrierFWD: 5'-ATC TCA TAC CCG CTG GAC AC-3';

CarrierBWD: 5'-TCA AGC GAG TTG CGG TAG TT-3'; rRNA45FWD: 5'-CCT ACC ATG CCG TGT CCT TCT A-3'; and rRNA45BWD: 5'-AAC GAC CCC TGC AGC AAT AC-3'. qPCR was performed using a DNA SYBR Green kit according to the manufacturer's instructions (Roche). Amplification of both Lm1740MIF and Lm1750MIF was carried out for 3 min at 95 °C, and 30 sec at 95 °C followed by 30 sec at 60 °C for 40 cycles. Amplification of both the ADP/ATP carrier and rRNA45 was carried out for 3 min at 95 °C, and 30 sec at 95 °C followed by 30 sec at 62 °C for 40 cycles. The linearity and optimization of the assays were ensured by performing serial dilutions of the templates for each primer set. The results were used to generate standard curves. Normalization of real-time qPCR data was performed by calculating the arithmetic mean of the internal control genes using the qgene software (Muller, Janovjak *et al.* 2002).

3.1.7 Agarose gel electrophoresis

Agarose gel electrophoresis is a method used to separate DNA molecules by size. Samples that consisted of 10 µl DNA and 2 µl loading buffer (6×) were loaded onto 2% agarose gels containing 10 mg/ml ethidium bromide (EtBr) and run at 100 V in 250 ml electrophoresis buffer. The loading buffer increases the density of the sample, making it easier to pipette into the agarose gel slots. Gels with positive results were photographed under 260-nm UV light; excision of DNA bands was performed under 312-nm UV light.

3.1.8 Purification of DNA products after PCR

After electrophoresis, the PCR products were purified and isolated with the Gene Clean III Kit (Bio101) according to the manufacturer's instructions. Purified DNA was eluted in 40 µl dH_2O and stored at -20 °C.

3.1.9 DNA and RNA concentration

Concentration and quality of DNA and RNA samples were measured with a UV spectrophotometer. After dilution in water, DNA and RNA concentrations were measured at a wavelength of 260 nm. DNA or RNA concentrations were calculated using following formula:

$$c\ (\mu g/ml) = OD_{260} * V * F$$

where c = concentration of the starting material

V = dilution factor

F = multiplication factor = for dsDNA: 50; for RNA: 40

The ratio of OD_{260}/OD_{280} provides an estimate of the purity of the sample.

3.1.10 MIF receptor binding studies

The real-time binding interaction of Lm1740MIF with CD74 was measured by surface plasmon resonance using a BIAcore 2000 optical biosensor (BIAcore AB, Piscataway, NJ) as previously described (Leng, Metz *et al.* 2003). The CM5 sensor chips and the BIA Evaluation software were from GE Healthcare (Buckinghamshire, UK). The MIF receptor ectodomain ($sCD74^{73-232}$) was immobilized according to the manufacturer's instructions using the Biacore Amine Coupling Kit. Briefly, $sCD74^{73-232}$ was diluted in 10mM sodium acetate (pH 5.2) at a concentration of 1 µM. 50 µl of an N-hyrdoxysuccinimide (NHS) and N-ethyl-N-(dimethyaminopropyl)-carbodiimide (EDC) mixture was injected at a speed of 2 µl /min for 25 min, followed by injection of 50 µl of 1 µM of purified $sCD74^{73-232}$. Once the surface plasmon resonance reached 10,000 U, the injection was stopped and the active amine sites blocked with 35 µl of 1 M ethanolamine (pH 8.5). The immobilized CM5 chip was washed overnight with 1xPBS at 20 µl/min. The derived sensor chips were washed and equilibrated in HEPES or PBS pH 8.0 (20 µl/min), and the ligand (Lm1740MIF) was introduced at five serial dilutions in BIAcore buffer (1 mM DTT, 2.5 mM $MgCl_2$, 20 mM HEPES, 1 mM EDTA, 150 mM NaCl, 0.005% P20) in 60-100 µl injection volumes at a flow rate of 20 µl/min. Binding was measured at 25°C for 5 min, followed by 15 min of dissociation. One min of sensor chip regeneration was then performed with 1 M NaCl/50mM NaOH. The whole process was repeated 3 times for each sample dilution. Sensorgram response data was analyzed in the BIA evaluation kinetics package and the equilibrium binding constants calculated.

3.1.11 Protein concentration

Protein concentration was determined using the Bradford method (Bradford 1976). A standard curve was generated using bovine serum albumin (BSA). Absorption was measured at 595 nm with a spectrophotometer.

3.1.12 Western blotting

Western blotting is a technique used to detect a specific protein in a complex mixture based on its molecular weight and ability to bind to specific antibodies. Western blotting can also be used to measure the relative amounts of the protein present in different samples as described by Laemmli (Laemmli 1970). The proteins are first separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a membrane such as polyvinylidene difluoride (PVDF), and incubated with a primary antibody raised against a specific antigen, the protein of interest. The primary antibody is then incubated with a secondary antibody, which is conjugated to a reagent for detection, such as horseradish peroxidase. Because the strength of the Western blot signal depends on two factors, that is, the affinity of the antibody for the antigen and the concentration of antigen, the described technique is not considered a quantitative measurement of antigen concentration.

Cell lysates from bone marrow-derived macrophages (BMMs) were lysed with lysis buffer and mixed with reducing 4× loading buffer and boiled for 10 min at 100 °C. After the samples were cooled to room temperature, 50 mM dithiothreitol (DTT) was added. Samples were loaded onto mini-gels (NuPage®, 4-12%, Invitrogen) and separated at 120 V for one hour. SDS-PAGE was performed at room temperature in either MES or MOPS buffer. After separation, proteins were transferred to a PVDF membrane. The wet protein-transfer method was performed using Invitrogen's XCell II™ Blot Module at 30 V for 1 hour at room temperature. The blots were subsequently blocked in 5% non-fat dry milk in TBS/0.1% Tween-20 (TBST) for 30 min and then washed three times with TBST buffer for 10 min each wash. The membranes were incubated at room temperature for 2 hours or overnight at 4 °C with different primary antibodies. After washing the membranes with TBST buffer as described above, the membranes were incubated with horseradish peroxidase-linked secondary antibodies for 1 hour at room temperature. Blots were then washed three times for 10 min each in TBST. The horseradish peroxidase-linked secondary antibody is used in conjunction with an enhanced chemiluminescence reagent (Amersham Biosciences), and the reaction product produces luminescence proportional to the amount of protein. Photographic film (Kodak) was placed against the PVDF membrane inside an autoradiography cassette, and exposure to the light

emitted by the reaction products created an image of the antibodies bound to the blot. Films were developed using an X-Omat 2000A.

3.1.13 ELISA (enzyme-linked immunosorbent assay)

The specific and highly sensitive ELISA method can be used as a quantitative measurement of proteins in solution.

A specific monoclonal antibody that binds the cytokine of interest is coated on a microtiter plate. The sample and standards are then loaded onto the microtiter plate. A second monoclonal antibody that binds a different epitope on the cytokine is applied and used for detection purposes. Any unbound reagents are washed away. The secondary antibody is labeled with biotin, which allows subsequent binding of a streptavidin-conjugated enzyme. Samples are incubated with the peroxidase substrate, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid), (ABTS**)**. Horseradish peroxidase catalyzes the conversion of ABTS in the presence of hydrogen peroxide into a green end-product. Its absorbance maximum of 405 nm can easily be detected with a spectrophotometer. The color of the reaction is proportional to the amount of bound cytokine. The concentration of cytokine is determined from comparison with a standard curve. The sensitivity of the ELISA depends primarily on the affinity of the antibodies for the protein. The detection limits for cytokine ELISAs are commonly in the lower picogram/ml range.

3.1.13.1 Cell Death Detection ELISA^{PLUS}

BMMs from wild-type and CD74-KO mice were incubated with either Lm1740MIF or mMIF and SNP (NO donor), an inducer of apoptosis. After incubation, the cells were precipitated by centrifugation and the supernatant, which contains DNA from necrotic cells, was discarded. Cells were resuspended and incubated in lysis buffer. After lysis, intact nuclei were precipitated by centrifugation. Aliquots of the supernatant were transferred to a streptavidin-conjugated microtiter plate. Nucleosomes in the supernatant were labeled with immunoreagent, a mixture of anti-histone-biotin and anti-DNA-horseradish peroxidase. The antibody-nucleosome complexes bound to the streptavidin through the anti-histone-biotin. Cell components that were not immunoreactive were removed by washing three times with incubation buffer. Samples were then incubated with the peroxidase substrate, ABTS.

3.1.14 Apoptosis studies

BMMs were cultured for 5 days in 20-mm plates, treated with either murine MIF (Mitchell, Liao *et al.* 2002) or Lm1740MIF, and incubated overnight either with or without SNP. Apoptosis was quantified by Cell Death Detection ELISA^{PLUS} (Roche) for cytoplasmic histone-associated DNA fragments. Cytoplasmic p53 content was analyzed by immunoblotting using a pair of anti-phospho-p53 (Ser15) and anti-p53 antibodies according to the manufacturer's instructions. The secondary antibody was an anti-rabbit IgG antibody conjugated to horseradish peroxidase, and detection was by chemiluminescence. The blots displayed are representative of stimulation studies that were performed at least in triplicate.

3.1.15 Signal transduction studies

Mouse BMMs and mouse thioglycollate-elicited peritoneal macrophages (4×10^6 per plate) were rendered quiescent by incubation in 0.1% FBS prior to stimulation with MIF for 2 hours (Mitchell, Metz *et al.* 1999). Apoptosis was induced with SNP. Negative controls did not have cell death stimulated by SNP. Cells were lysed in buffer containing 20 mM HEPES (pH 7.4), 50 mM β-glycerolphosphate, 2 mM EGTA, 1 mM DTT, 10 mM NaF, 1 mM $NaVO_4$, 10% glycerol, 1% Triton X-100, and freshly added protease inhibitors (Complete, Mini, EDTA-free, Roche). For immunoblotting, cell lysates were separated via 10% SDS-PAGE and transferred to PVDF Immobilon-P membranes. Immunoblotting was carried out using antibodies directed against total ERK1/2 and phospho-ERK-1/2 according to the manufacturer's instructions. The secondary antibody was an anti-rabbit IgG antibody conjugated to horseradish peroxidase, and detection was by chemiluminescence. The blots displayed are representative of stimulation studies that were performed at least in triplicate.

4 Results

4.1 Sequence alignment and phylogenetic analyses

Two genetic loci, Lm1740 and Lm1750, were identified on chromosome 33 in the recently sequenced *Leishmania major* genome (Ivens, Peacock *et al.* 2005) to have an open reading frame (342 bp) with 10–13% identity to the human *MIF* gene. After comparison with other *Leishmania* and *Typanosomatid* genes (Landfear, Miller *et al.* 1986), both a trypanosoma-consensus TATA box and a polyadenylation signal sequence were evident in the flanking regions of these loci (Fig. 8).

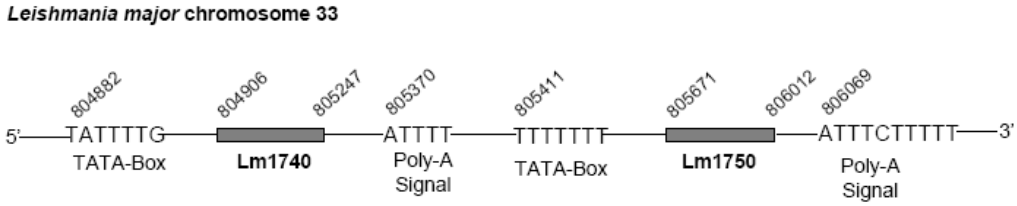


Fig. 8. **Physical map of the *Leishmania major* chromosome-33 region encompassing nucleotides 804882–806078.** The two *mif*-related genes, Lm1740 (accession number Q4Q413) and Lm1750 (accession number Q4Q413), are shown together with consensus TATA boxes and polyadenylation signal sequences.

The two putative *L. major MIF* orthologs (Lm1740MIF and Lm1750MIF) were predicted to encode a 112-amino acid protein after processing of the initiating methionine residue. Similar to other *MIF* orthologs that have been described (Pastrana, Raghavan *et al.* 1998; Jaworski, Jasinskas *et al.* 2001; Augustijn, Kleemann *et al.* 2007; Cho, Jones *et al.* 2007; Cordery, Kishore *et al.* 2007) the *Leishmania MIF*-like genes do not contain an N-terminal secretory signal sequence.

Phylogenetic analyses using the Neighbor-Joining method for both DNA and protein sequences produced similar results; results of these protein analyses are displayed in Fig. 9.

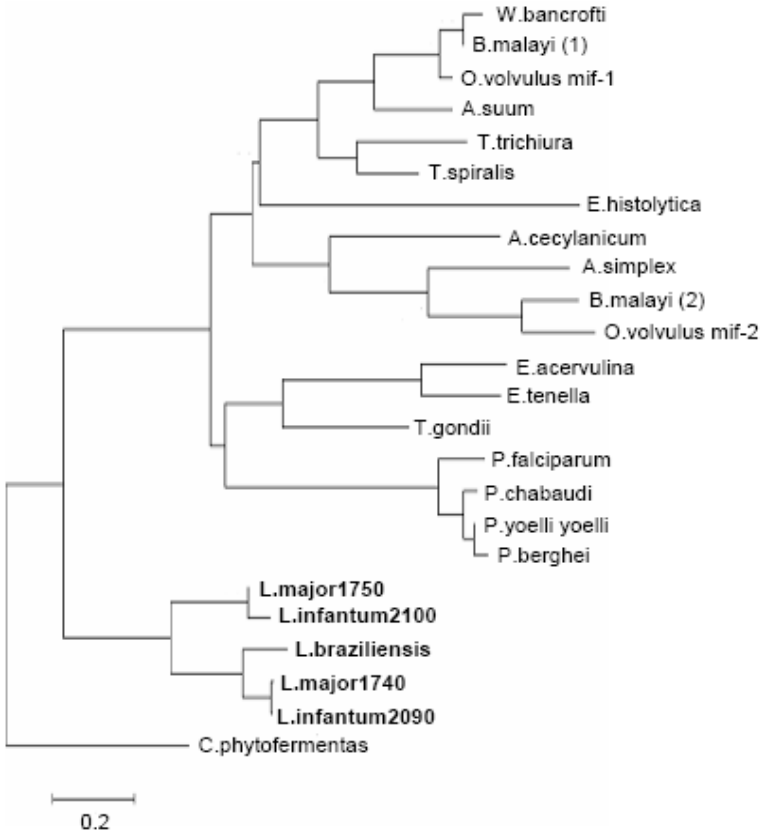


Fig. 9. **Phylogram of parasitic MIF protein sequences, including five *mif*-related proteins identified in *L. major, L infantum,* and *L. braziliensis*.** The sequence of *Clostridium phytofermentas* was used to resolve relationships among MIF-expressing parasites. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (5000 replicates) are shown next to the branches. The evolutionary distances were computed using the Poisson correction method and are presented as the number of amino acid substitutions per site. The gene accession numbers are as follows: *L. infantum* 2100 XM_001468253; *L. infantum* 2090 XM_001468252; *L. braziliensis* CAM40731; *L. major1740* Q4Q413*; L. major1750* Q4Q412; *T. gondii* DQ344450; *E. acervulina* DQ323516; *E. tenella* DQ323515; *A. simplex* EF165010; *W. bancrofti* AF040629; *P. yoelli yoelli* DQ494171; *P. falciparum* AY561832; *P. chabaudi* CAH75532; *P. berghei* CAH99597; *T. trichuria* AJ237770; *T. spiralis* AY050661; *A. suum* AB158366*; E. histolytica* XM_650516; *A. ceylanicum* EF410151*; B. malayi (1)* AF002699; *B. malayi (2)* AY004865; *O. volvulus mif-1* AF384027; and *O. volvulus mif-2* AF384028.

Gene duplication can account for paralogs within the same organism, and likely explains the origin of the two *L. major* and *L. infantum* MIF-related sequences. Lm1740MIF was 58–99% identical to MIF-like gene sequences from other *Leishmania* species and 22–31% identical to mammalian MIFs. Except for certain branch rearrangements, few differences were observed between the topology of the MIF DNA and protein phylograms (*data not shown*).

An amino acid-sequence alignment of twelve selected members of the MIF protein family was prepared using the T-Coffee multiple sequence alignment program (Fig. 10).

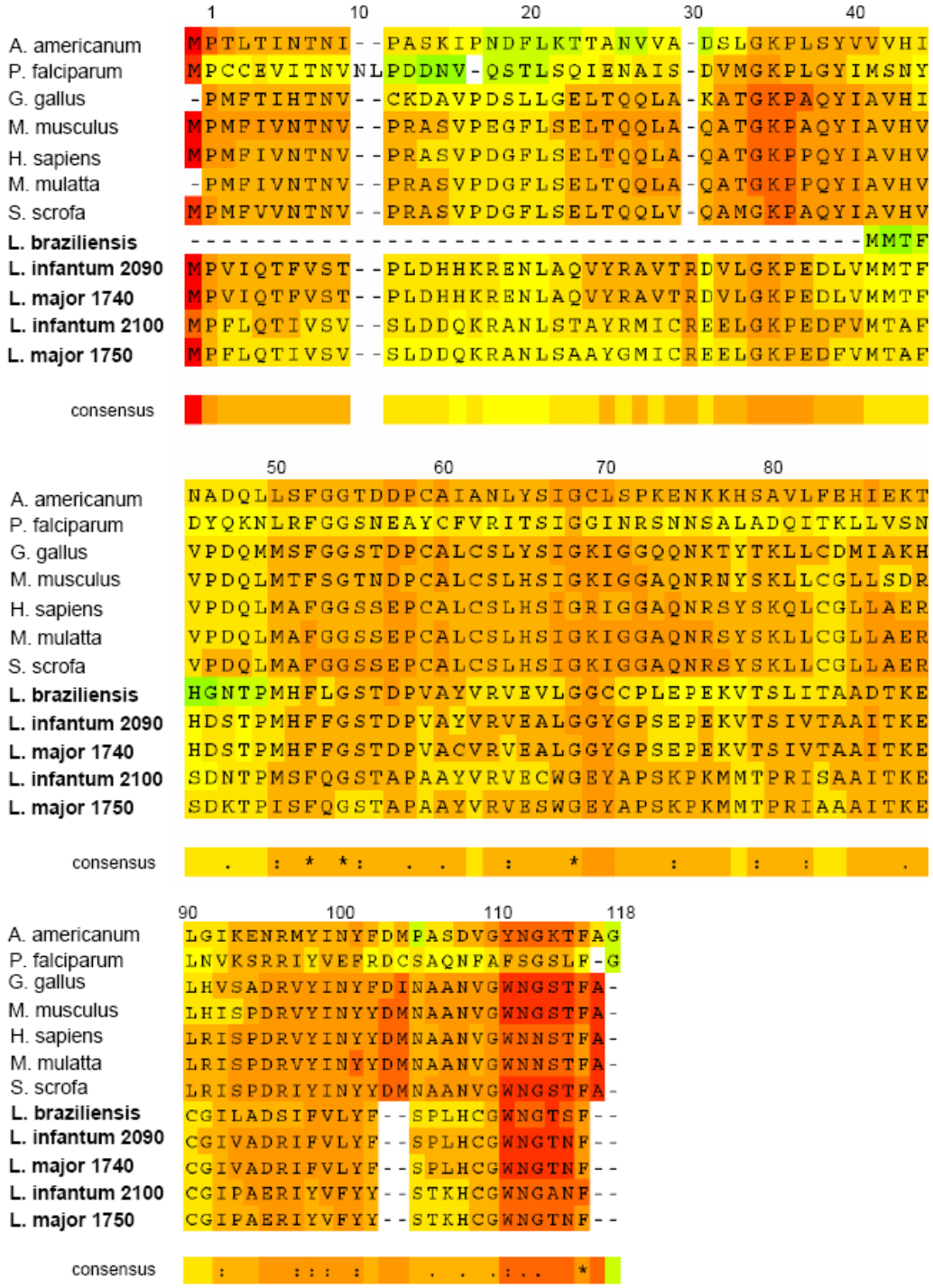


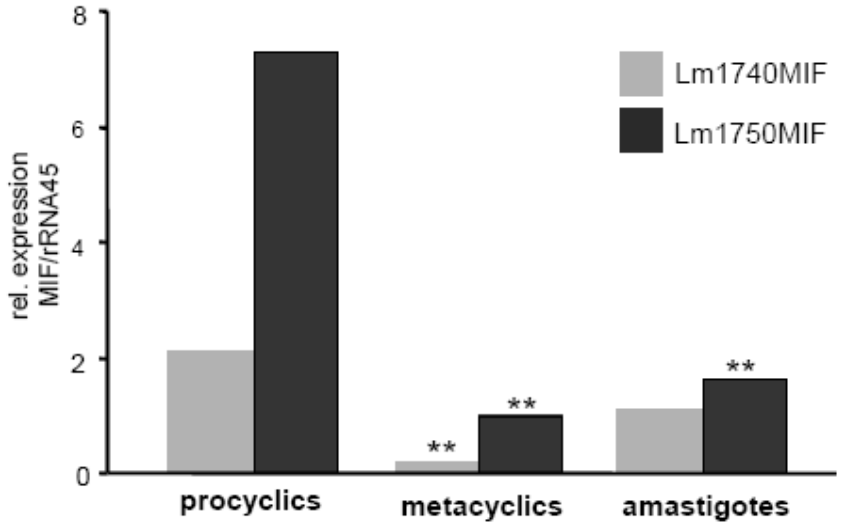
Fig. 10. Amino acid-sequence alignment of selected MIF molecules constructed with the T-Coffee program . A line marked "consensus" is shown for each residue: "*" denotes complete conservation, ":" indicates conservation of both residue size and hydropathy, and "." denotes conservation of either size or hydropathy. The color code signifies the consistency between a multiple alignment and each pair of aligned residues. : inconsistent bits, unlikely to be correctly aligned; : bits correspond to the residues more likely to be correctly aligned (with red the highest reliable portion). The consensus sequence indicates that the average reliability value for every residue column is provided. Additional gene accession numbers are as

follows: A. americanum Q9GUA9; G. gallus Q02960; M. musculus P34884; H. sapiens Q6FHV0; M. mulatta Q6DN04; and S. scrofa Q069I4.

These conserved cysteines, as well as the CXXC motif, are absent in the *L. major* sequences. The MIF N-terminal proline (Pro1) by contrast appears strictly conserved, with the exception of the grossly truncated *Leishmania braziliensis* sequence. *Leishmania* MIF does not exhibit the CXXC motif; however, it does have one of the "extended" TPOR requirements by containing the Phe of mammalian MIF at position -7 (the "extended" TPOR requirements of mammalian MIF are a Phe at position -7 and a Leu at position -10 of its CXXC motif).

4.2 Expression of Leishmania major MIF in vivo

The expression of the Lm1740MIF and Lm1750MIF genes *in vivo* was confirmed by reverse transcriptase-PCR analysis of total RNA prepared from *L. major* promastigotes (strain MHOM/IL/79/LRC-L251, (*data not shown*). Quantitative PCR analysis (qPCR) of the three life stages of the *L. major* parasite revealed that procyclic parasites expressed more LmMIF than either metacyclic or amastigote parasites, the latter isolated from within the lymph nodes of infected mice (Fig. 11). The differences in the expression levels of Lm1740MIF and Lm1750MIF were quantified using the qgene software with respect to two "housekeeping" genes, rRNA45 and ADP/ATP carrier .



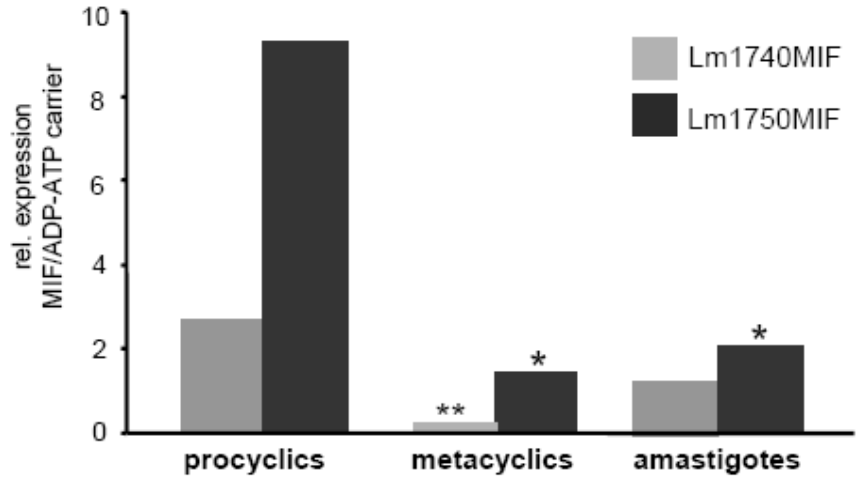


Fig. 11. Lm1740MIF and Lm1750MIF mRNA expression. Real-time qPCR analysis of total RNA from L. major procyclic and metacyclic parasites cultured in vitro, and from amastigotes present within the infected lymph nodes of mice. Results are presented for Lm1740MIF and Lm1750MIF transcripts relative to the reference genes rRNA45 and ADP/ATP carrier. All samples were run in triplicate. The p values were calculated using the Student's t test. **$p<0.01$ for procyclic versus either metacyclic or amastigote parasites. *$p<0.05$ for procyclic versus either metacyclic or amastigote parasites. The values for $p>0.05$ are not displayed.

4.3 Lm1740MIF binds to the MIF receptor CD74

After demonstrating *L. major* MIF transcription, it was determined whether Lm1740MIF could influence host immunity by engaging the human MIF receptor, CD74. To begin to achieve this goal, the equilibrium dissociation constant for Lm1740MIF binding to sCD74 (soluble CD74) was measured by surface plasmon resonance (BIAcore analysis), which measures real-time binding interactions by changes in the refractive index of a biospecific surface. BIAcore analysis revealed a K_d of $2.9\times10^{-}8$ M for the binding between Lm1740MIF and sCD74 (Fig. 12). This contrasts with a previously determined K_d for the binding of human MIF with sCD74 of $9.0\text{x}10^{-9}$ M (Leng, Metz *et al.* 2003). These data indicate that Lm1740MIF binds to the human MIF receptor with decent affinity, albeit with a 3-fold lower K_d than recombinant human MIF.

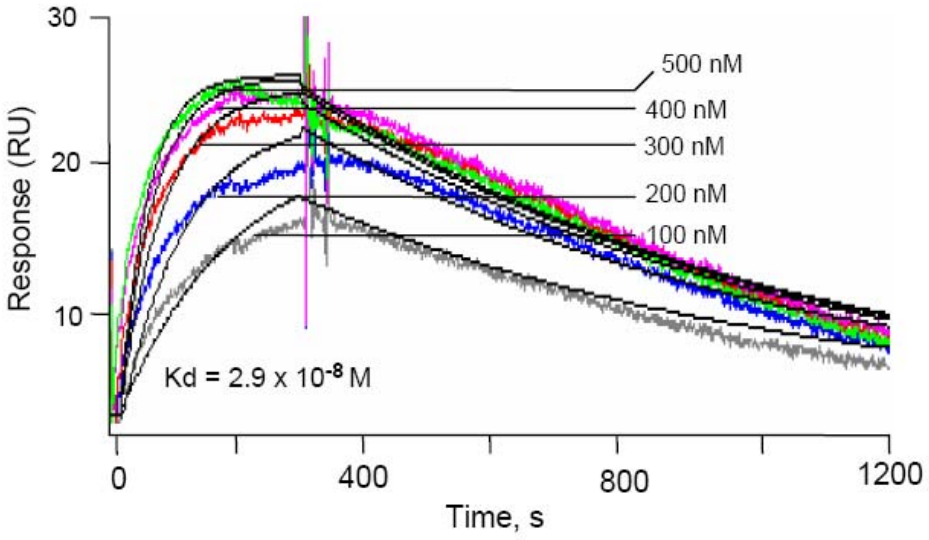


Fig. 12. Lm1740MIF binds to the MIF receptor, CD74. Real-time surface plasmon resonance analysis (BIAcore) of the interaction between recombinant Lm1740MIF and sCD74. A K_d of 2.9×10^{-8} M for the binding between Lm1740MIF and sCD74 was measured.

4.4 Lm1740MIF inhibits monocyte/macrophage apoptosis

Leishmania-infected macrophages survive longer and are more viable than uninfected macrophages (Moore and Matlashewski 1994). An important function of MIF is to activate macrophages and lead to ERK1/2 phosphorylation. MIF activates ERK and acts downstream to maintain monocyte/macrophage function by inhibiting activation induced p53-dependent apoptosis (Mitchell, Liao *et al.* 2002). It was hypothesized that Lm1740MIF may contribute to parasitism by prolonging the survival of infected macrophages through inhibition of apoptosis.

4.4.1 Signal transduction in wild-type mice

MIF signal transduction through CD74 results in the activation of the ERK1/2 mitogen-activated protein kinase (MAPK) pathway (Mitchell, Metz *et al.* 1999). Downstream of ERK1/2 activation, the antiapoptotic action of MIF is associated with a reduction in the intracytoplasmic content of Ser15-phosphorylated p53, which increases in response to NO treatment (Mitchell, Liao *et al.* 2002). It was examined whether Lm1740MIF inhibits apoptosis in bone marrow-derived macrophages, both to confirm its biological activity and to assess its ability to influence host-cell responses when present in the extracellular milieu. The effect of Lm1740MIF was

less than that observed with murine MIF (Fig. 13). Protection from apoptosis occurred after incubation of bone-marrow derived macrophages with Lm1740MIF and induction of apoptosis with SNP (an NO donor). This result was associated with a diminuition in the cellular content of Ser15-phosphorylated p53 (Fig. 14). Furthermore, it was shown that Lm1740MIF also induced ERK1/2 phosphorylation in primary murine macrophages (Fig. 14).

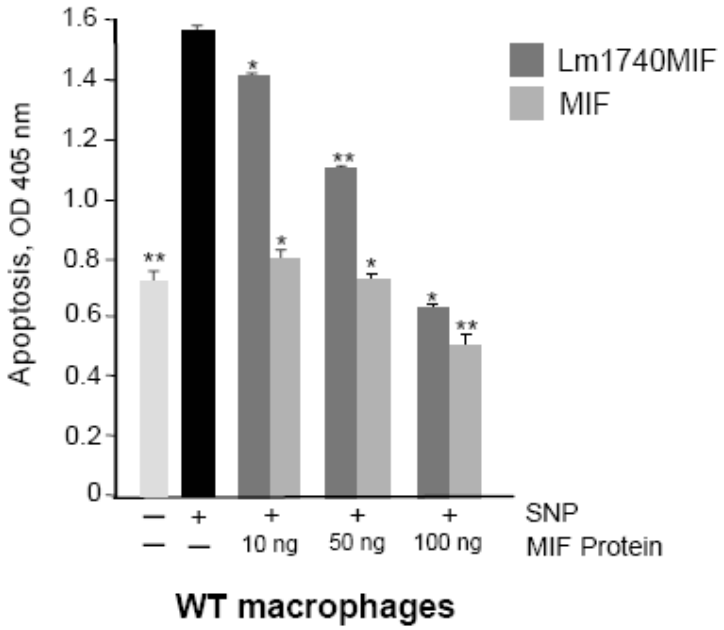
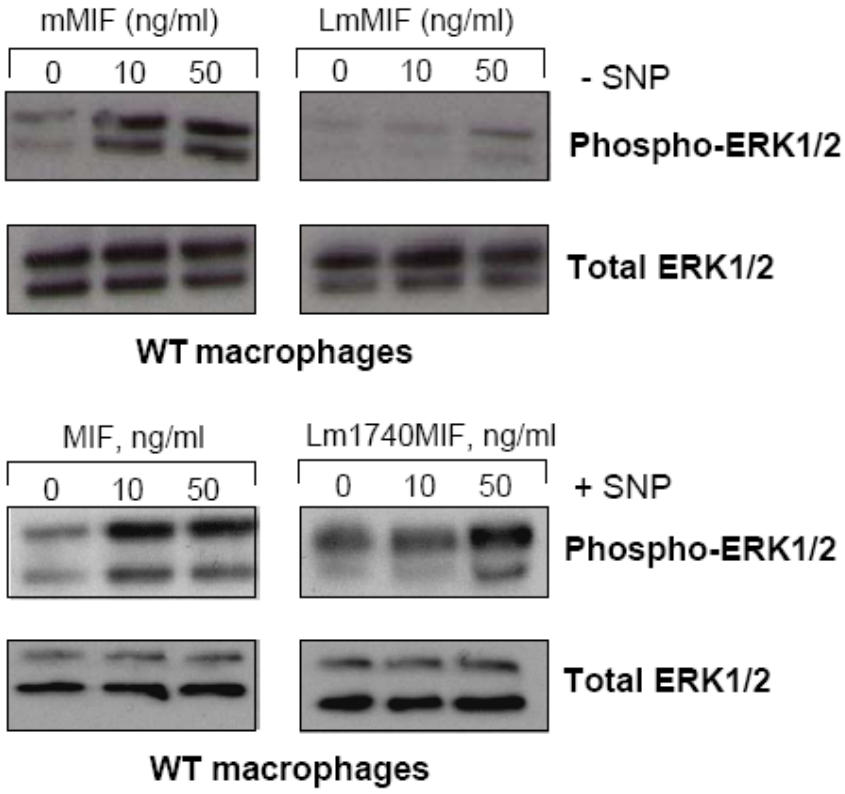


Fig. 13. Lm1740MIF protects macrophages from SNP-induced apoptosis. Wild-type (WT) bone marrow-derived macrophages were treated with either Lm1740MIF or murine MIF prior to the addition of the apoptosis inducer, SNP. Negative controls did not include SNP addition. DNA fragmentation was assessed by ELISA after 36 hrs. The p values were calculated using the Student's t test. **$p<0.01$, *$p<0.05$ for MIF versus SNP controls. **$p<0.01$, *$p<0.05$ for Lm1740MIF versus MIF. **$p<0.01$ for control versus SNP addition. The values for $p>0.05$ are not displayed. Results are representative of at least four independent experiments.


mMIF (ng/ml)
LmMIF (ng/ml)
0 10 50
0 10 50
- SNP
Phospho-ERK1/2
Total ERK1/2
WT macrophages
MIF, ng/ml
Lm1740MIF, ng/ml
0 10 50
0 10 50
+ SNP
Phospho-ERK1/2
Total ERK1/2
WT macrophages

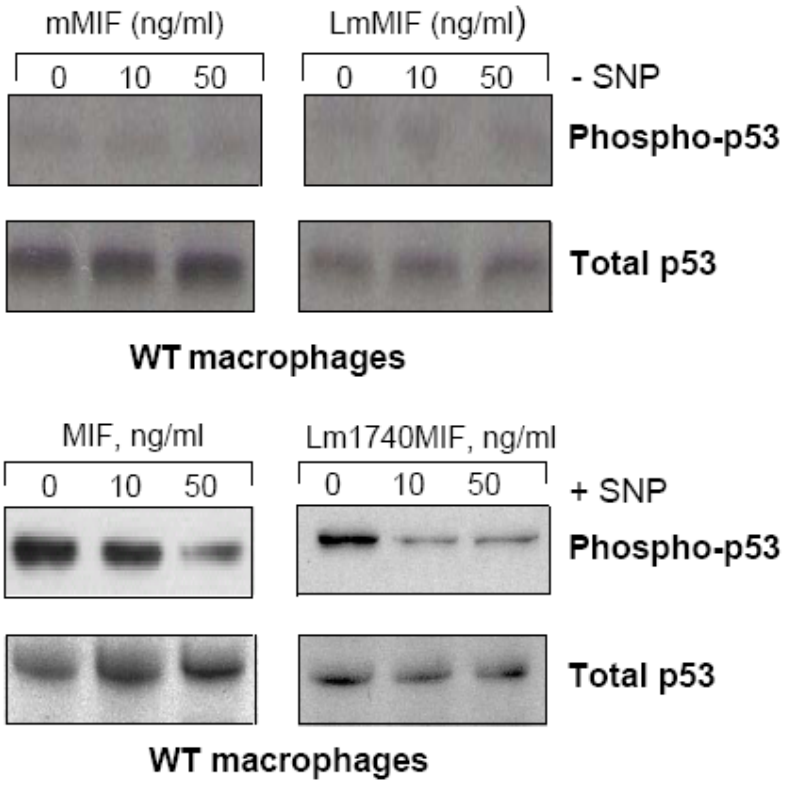


Fig.14. Lm1740MIF induces phosporylation of ERK1/2 and inhibits p53 phosphorylation. Wild-type (WT) bone marrow-derived macrophages were treated with Lm1740MIF or murine MIF at the concentrations shown prior to the addition of the apoptosis inducer, SNP, as indicated in Material and Methods. Negative controls were conducted by not stimulating cell death with SNP. The phosphorylation of ERK1/2 and p53 was assessed by Western blotting using specific phospho-ERK1/2, total ERK1/2, phospho-p53, and total p53 antibodies. The western blots are representative of at least four independently performed experiments.

4.4.2 ERK1/2 activation by endotoxin

Because ERK1/2 activation in macrophages may be induced by endotoxin (LPS), ERK1/2 activation by Lm1740MIF was additionally tested in macrophages from C3H/HeJ mice that have a loss-of-function mutation in the LPS receptor, Toll-like receptor 4 (TLR4) (Poltorak, Ricciardi-Castagnoli *et al.* 2000). A dose-dependent increase in ERK1/2 phosphorylation in response to Lm1740MIF was also observed in these cells.

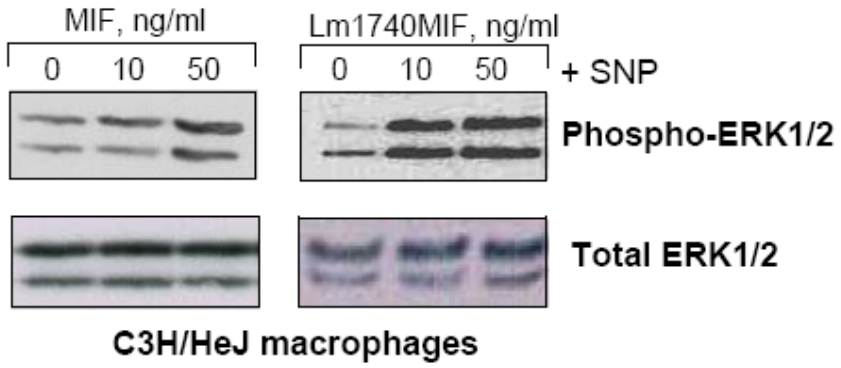


Fig. 15. ERK1/2 phosphorylation serves as a control against LPS contamination. C3H/HeJ bone marrow-derived macrophages were treated with either Lm1740MIF or murine MIF at the concentrations shown prior to the addition of the apoptosis inducer, SNP, as indicated in the Material and Methods. The phosphorylation of ERK1/2 was assessed by Western blotting using specific phospho-ERK1/2 and total ERK1/2 antibodies. Results are representative of at least four independent experiments.

4.4.3 Signal transduction in CD74-knockout mice

The functional requirement for the MIF receptor in Lm1740MIF action was further examined by studying both the signaling and apoptotic responses of macrophages obtained from mice deficient in CD74.

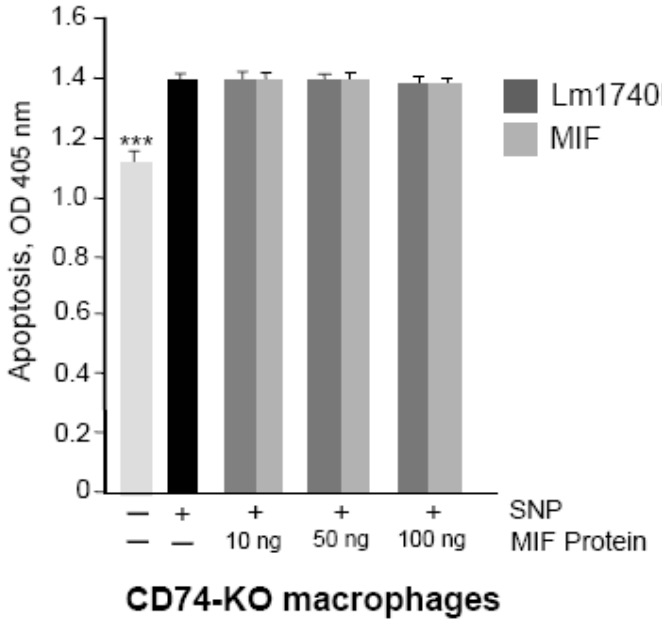


Fig. 16. Lm1740MIF did not protect CD74-KO macrophages from SNP-induced apoptosis. CD74-knockout bone marrow-derived macrophages were treated with either Lm1740MIF or murine MIF prior to the addition of the apoptosis inducer, SNP. DNA fragmentation was assessed by ELISA after 36 hrs. The p values were calculated using the Student's t test. ***$p<0.001$ for control versus SNP addition. The p values for comparisons with $p>0.05$ are not displayed. Results are representative of at least four independent experiments.

Differences in the SNP effect between WT and CD74-KO macrophages can be attributed to the indirect testing principle of the ELISA method. This method does not measure the apoptosis rate of the samples directly but the conversion of ABTS in the presence of hydrogen peroxide into a green end-product. For starting the ABTS reaction there are more than seven necessary intermediate steps which all allow a certain inaccuracy in the end result leading to the differences seen between the controls of WT and CD74-KO macrophages.

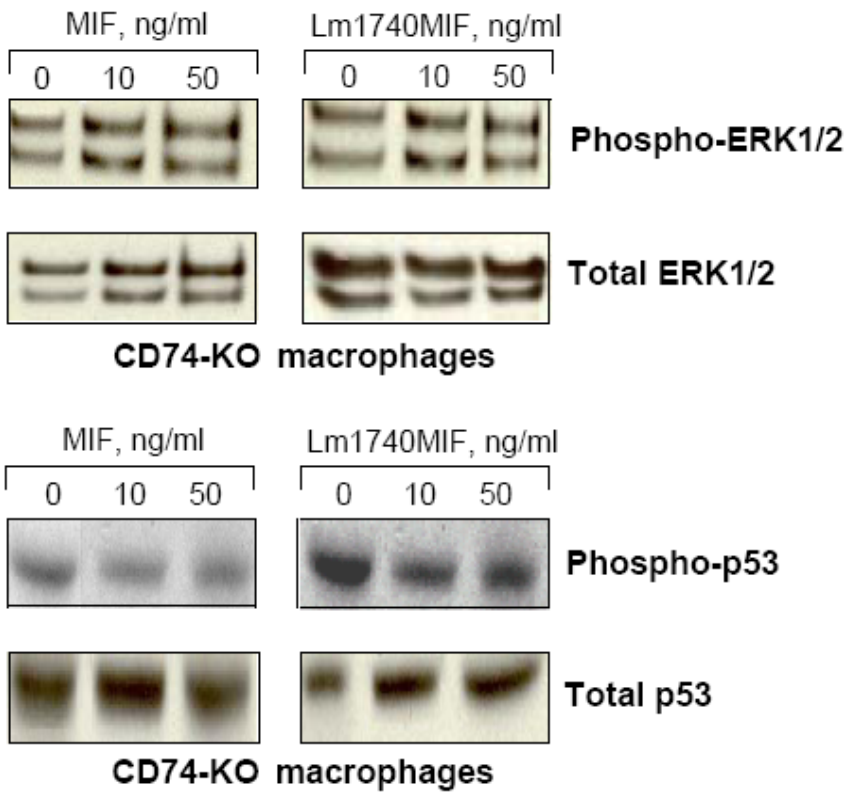


Fig. 17. Lm1740MIF did not induce phosporylation of ERK1/2 or inhibit p53 phosphorylation in CD74-KO macrophages. Bone marrow-derived macrophages from MIF receptor-deficient (CD74-KO) mice were prepared and studied for protection from apoptosis, ERK1/2 phosphorylation, and phospho-p53 content as described in Material and Methods. Results are representative of at least four independent experiments.

The treatment of bone marrow-derived macrophages from CD74 receptor-deficient (CD74-KO) mice with either mammalian MIF or Lm1740MIF did not have the same effects as treatment of cells from wild-type mice. Murine MIF was not active in these cells, which is in agreement with prior reports (Leng, Metz *et al.* 2003; Shi, Leng *et al.* 2006) and there was no effect of Lm1740MIF with respect to protection from apoptosis, intracellular phospho-p53 content or ERK1/2 phosphorylation (Figs. 16 and 17). These data show that CD74 is necessary for Lm1740MIF signaling as it is for MIF. Quantification of the blots was not carried out since inhibition of apoptosis was shown by ELISA.

5 Discussion

The increasing incidence of vector-borne diseases is thought to be a result of global warming associated with extreme weather events as well as annual changes in weather conditions. In addition, increases in infectious disease outbreaks (Sutherst 2004; Khasnis and Nettleman 2005) have resulted from the growth of international travel that elevates the risk of importing vector-borne diseases from tropical areas (Weitzel, Muhlberger *et al.* 2005). Therefore, obtaining a better understanding of vector-borne diseases will aid in the control of such outbreaks.

This thesis focused on the description of two orthologs (with an emphasis on Lm1740MIF) of the pro-inflammatory cytokine, macrophage migration inhibitory factor (MIF); these orthologs are produced by the obligate intracellular parasite, *Leishmania major*. Several primitive eukaryotes were also found to encode MIF-like genes that show remarkable similarity with the mammalian gene, such as the human parasitic nematodes *Brugia malayi* (Pastrana, Raghavan *et al.* 1998) and *Ancylostoma ceylonicum* (Cho, Jones *et al.* 2007). The tick vector responsible for the transmission of anaplasmosis, *Amblyomma americanum*, produces an MIF ortholog that is expressed in the salivary gland (Jaworski, Jasinskas *et al.* 2001). MIF-like proteins also have been reported recently in the pathogenic species *Eimeria* (Miska, Fetterer *et al.* 2007), *Trichinella* (Wu, Boonmars *et al.* 2003), and *Plasmodium* (Augustijn, Kleemann *et al.* 2007; Cordery, Kishore *et al.* 2007), and the question has been raised as to whether these proteins play a role in parasite-host interactions.

MIF was demonstrated to reduce the number of *Leishmania* parasites in infected macrophages (Juttner, Bernhagen *et al.* 1998). Noteworthy is the finding that macrophages are activated by the simultaneous addition of IFN-γ and MIF even though these factors act independently of one another. Leishmanicidal effects elicited by MIF follow TNF-α-induced macrophage activation and the production of endogenous NO by these activated macrophages. MIF is assumed to inhibit monocyte/macrophage migration after entering the site of infection to protect the host from infected phagocytes. MIF levels increase in the host after *L. major* infection, resulting in an immune response.

A molecular biological approach to further elucidate the functional characteristics of the *L. major* MIF orthologs was chosen to study LmMIF in the host immune response as well as MIF's function in the parasite's immune-evasion machinery.

5.1 Computational analysis

Phylogenetics is the study of evolutionary relatedness among various groups of organisms (e.g., species, populations). Phylogenetic analysis using the Neighbor-Joining method (Saitou and Nei 1987) surprisingly revealed that *L. major* and *L. infantum* MIF genes were more closely related to one another than to MIF orthologs within the same species. This finding substantiates the hypothesis that Lm1740MIF and Lm1750MIF are paralogous genes, originating from gene duplication of the same ancestral gene. From the primary structural similarities between *Leishmania* and mammalian MIF sequences, it follows that LmMIF and MIF are orthologs, related to a common ancestor and having similar, if not the same, cellular functions. These functions were further determined during the course of this thesis. Lm1740MIF and Lm1750MIF were found to be 58% identical to one another. Compared with human MIF (hMIF), Lm1740MIF and Lm1750MIF were found to be 13% and 10% identical and 22% and 13% homologous, respectively. The majority of experiments in this thesis were carried out using Lm1740MIF, since Lm1740MIF was more structurally similar to hMIF than was Lm1750MIF.

The amino acid-sequence alignment of twelve selected members of the MIF protein family shows the existence of nine invariant residues of MIF (Pro1, Leu21, Gly34, Lys35, Pro36, Phe52, Gly54, Gly68, and Phe116). Mammalian MIF is distinguished by the presence of three conserved cysteines (Cys57, Cys60, and Cys81 or 56,59,80, depending on whether you count the Met-1), the first two of which define a CXXC motif that mediates thiol-protein oxidoreductase activity (Nguyen, Beck *et al.* 2003). These conserved cysteines, as well as the CXXC motif itself, are absent in *Leishmania* sequences, though they satisfy requirements of the "extended" TPOR sequence with a Phe at position -7 (Kleemann, Kapurniotu *et al.* 1998). The MIF N-terminal proline residue (Pro1) by contrast appears to be strictly conserved, with the exception of the grossly truncated *L. braziliensis* sequence. Pro1 functions as a catalytic base for substrate tautomerization (whether a physiological substrate exists is unknown (Swope and Lolis 1999)); this residue has been shown by X-ray crystallography to reside within a hydrophobic, substrate-binding pocket of mammalian proteins (Bendrat, Al-Abed *et al.* 1997; Stamps, Fitzgerald *et al.* 1998; Lubetsky, Swope *et al.* 1999).

The finding of trypanosoma-specific putative TATA boxes and polyadenylation signals in the flanking regions of both Lm1740MIF and Lm1750MIF led to the assumption that these two genes are expressed *in vivo* and have functional activity. Furthermore, this discovery further supports the hypothesis that Lm1740MIF and Lm1750MIF originated via gene duplication. This finding elicited the analysis of MIF expression in *L. major* parasites.

5.2 Investigation of L. major MIF expression in vivo

Both reverse transcription-PCR and qPCR are techniques highly applicable for the gene expression analysis of developmentally regulated genes. These analyses are crucial to the understanding of biological gene function (Ding and Cantor 2004). Normalization of gene levels with stably expressed reference genes is critical for the generation of reliable results with biological significance. This is particularly true for pathogens, like *Leishmania* parasites, that alternate between three different developmental stages during their life cycles. Developmental stage-specific gene expression experiments were performed on total RNA (reverse-transcribed into cDNA) isolates from all *L. major* life stages (procyclic, metacyclic and amastigote parasites) using real-time qPCR. Expression of the target genes was related to the stable expression of internal controls, also referred to as "housekeeping genes," simultaneously determined in the same sample (Huggett, Dheda *et al.* 2005). The sequences encoding rRNA45 and ADP/ATP carrier were stably expressed in all developmental stages of the parasite (Leifso, Cohen-Freue *et al.* 2007; Ouakad, Bahi-Jaber *et al.* 2007) and were thus used as reference genes in the gene expression studies of Lm1740MIF and Lm1750MIF in *L. major*.

This study revealed that both Lm1740MIF and Lm1750MIF were expressed *in vivo* throughout all *Leishmania* life stages. However, Lm1750MIF had higher expression levels than Lm1740MIF during all life-stages of the parasite; the highest expression of both LmMIF forms was in the procyclic life-stage of the parasite. This distribution pattern might point towards a yet unexplored role of LmMIF, especially of Lm1750MIF, within the sand-fly vector. A closer examination of whether these proteins function as virulence factors or in metacyclogenesis that leads to infective metacyclic parasites is imperative to undertake in future experiments.

5.3 Binding of Lm1740MIF to the human macrophage MIF receptor, CD74

The possibility that Lm1740MIF influences host immunity prompted us to examine whether Lm1740MIF binds to the human MIF receptor, CD74 in macrophages. In addition to its interaction with the CXCR chemokine receptors, MIF activates cells by engaging its cell surface-binding receptor, CD74. A high-affinity binding interaction between MIF and the CD74 ectodomain ($CD74^{73-232}$ or sCD74) had previously been demonstrated by BIAcore analysis (Leng, Metz *et al.* 2003).

BIAcore analysis measures real-time binding interactions by changes in the refractive index of a biospecific surface and generates unique data on protein-protein interactions. The extent to which a molecule interacts with a single partner immobilized on a sensor surface reveals the specificity of the interaction.

During this thesis, the equilibrium dissociation constant was measured for the binding of Lm1740MIF to sCD74 by surface plasmon resonance (BIAcore analysis). BIAcore measurements revealed a K_d of $2.9\times10^{-}8$ M for the binding between Lm1740MIF and sCD74. These data indicate that Lm1740MIF binds to the human MIF receptor with decent affinity. This value contrasts with a previously determined K_d for the binding of human MIF with sCD74 of $9.0\times10^{-}9$ M to 2.3×10^{-10} M (Leng, Metz *et al.* 2003). These data indicate that Lm1740MIF binds to the human MIF receptor CD74 with high affinity, albeit with a 3-fold lower K_d than that of human MIF. Nevertheless, there is sufficient structural homology between Lm1740MIF and human MIF to allow strong binding to CD74.

The finding that Lm1740MIF interacts with CD74 is preliminary; many more experiments, especially interaction studies in complementing assay systems such as cellular binding assays, receptor internalization assays, or CoIPs must be performed before further conclusions about this interaction can be made.

Future experiments shall also include studies of Lm1740MIF secretion by *L. major*, a pathophysiological prerequisite to enable CD74 binding to occur. The determination of the surface contacts between Lm1740MIF and CD74 and the identification of inhibitors of this interaction are additional tasks that need to be addressed.

5.4 Impact of Lm1740MIF on CD74-mediated signal transduction

Signal transduction refers to the transmission of signals from the outside of the cell to the inside of the cell that involves the coupling of a ligand to its receptor. This interaction induces many intracellular events, including phosphorylation of a variety of cellular kinases and transcription factors. Protein phosphorylation can alter protein activity and/or protein conformation with an eventual outcome of altered cellular activity.

An important biologic action of the cytokine MIF is to sustain monocyte/macrophage function by inhibiting activation-induced, p53-dependent apoptosis (Mitchell, Liao *et al.* 2002). Transient transfection of an MIF-expressing plasmid into RAW264.7 macrophages inhibits apoptosis by an autocrine/paracrine pathway involving the CD74 membrane receptor (Mitchell, Liao *et al.* 2002; Shi, Leng *et al.* 2006). The comparative ability of plasmids encoding murine MIF, Lm1740MIF, and Lm1750MIF to protect cells from NO-mediated apoptosis was tested in a model system. Both *Leishmania* MIF orthologs inhibited apoptosis in a dose-dependent fashion, but at levels that were approximately 30% lower than that of transfected murine MIF ("MSc thesis"; Diplomarbeit).

It was postulated that Lm1740MIF contributes to parasitism by prolonging the survival of the infected macrophage, leading to its longevity.

To confirm the results of previous transfection experiments ("MSc thesis"; Diplomarbeit) and to evaluate the functional significance of the binding interaction between Lm1740MIF and CD74, the ability of recombinant Lm1740MIF to inhibit apoptosis of primary macrophages was examined. Additionally, known targets of MIF were analyzed to confirm the biological activity of Lm1740MIF and to assess the ability of Lm1740MIF to influence host-cell immune responses when present in the extracellular milieu.

The effect of Lm1740MIF was decreased compared to that of murine MIF, which is consistent with both the transfection data ("MSc thesis"; Diplomarbeit) as well as its reduced receptor binding activity. MIF signal transduction through CD74 is known to result in the activation of the ERK1/2 MAPK pathway (Mitchell, Metz *et al.* 1999), and Lm1740MIF was also shown to be capable of inducing ERK1/2 phosphorylation in primary murine macrophages. Because ERK1/2 activation in macrophages may be induced by endotoxin (LPS), which is present in trace quantities in recombinant

Lm1740MIF (< 20 pg LPS/µg protein), Lm1740MIF was additionally tested in macrophages from C3H/HeJ mice, which have a loss-of-function mutation in TLR4 (Poltorak, Ricciardi-Castagnoli *et al.* 2000). A dose-dependent increase in ERK1/2 phosphorylation in response to endogenous Lm1740MIF was observed in these cells, arguing against a role for contaminating endotoxin in Lm1740MIF-induced ERK1/2 phosphorylation.

After treatment with the NO donor, SNP, macrophage life spans are prolonged through MIF, which is associated with a reduction in the intracytoplasmic content of phosphorylated Ser15 of p53 (Mitchell, Liao *et al.* 2002). Lm1740MIF was shown to induce protection from apoptosis after exposure of bone marrow-derived macrophages to NO; this protection was associated with a diminution in the cellular content of Ser15-phosphorylated p53 as well.

While differences in the relative ability of Lm1740MIF *versus* murine MIF to affect changes in phosphorylation may be evident, quantitative comparisons are difficult to make given the narrow linearity of signals that is apparent by Western blotting. These results nevertheless indicate that Lm1740MIF is biologically active when added to mammalian cells and, like mammalian MIF, stimulates ERK1/2 phosphorylation and reduces the cellular content of phospho-p53 that leads to macrophage longevity.

Finally, the functional requirement for the human MIF receptor CD74 in Lm1740MIF action was examined by studying the signaling and apoptotic responses of macrophages obtained from mice deficient in the CD74 receptor. Murine MIF was inactive in these cells in agreement with previous reports (Leng, Metz *et al.* 2003; Shi, Leng *et al.* 2006). Similar to mouse MIF, apoptosis protection by Lm1740MIF was dependent on the presence of CD74 and, therefore, Lm1740MIF had no effect on CD74-KO primary macrophages with respect to protection from apoptosis, ERK1/2 phosphorylation, or intracellular phospho-p53 content in $CD74^{-/-}$ mice.

Taken together, these data support the conclusion that the *Leishmania* ortholog, Lm1740MIF, activates the human MIF receptor CD74 and influences host cell responses, specifically monocyte/macrophage survival, by engaging CD74. The mechanism by which Lm1740MIF interacts with CD74 is not yet known. It is possible that Lm1740MIF is secreted by the parasite after infection and binds to CD74 upon interaction with macrophages. Alternatively, Lm1740MIF may be secreted after internalization of the parasite into a macrophage and be released from the cell and bind to CD74 as was reported for MIF (Flieger, Engling *et al.* 2003). One

might speculate that the interaction with CD74 may also occur intracellularly by gaining access to the endosomal compartment (Kleemann, Hausser *et al.* 2000).

5.5 Concluding remarks

Leishmania is an intracellular infection of monocytes/macrophages; therefore, one function of *Leishmania*-encoded MIF may be to sustain monocyte/macrophage longevity that contributes to the persistence of the parasite within host cells for completion of the parasitic life cycle. To measure LmMIF concentration in macrophages and to find out which other proteins interact with LmMIF, how and where LmMIF moves within the cell and how LmMIF responds to changes in cell conditions, LmMIF tagging approaches have started to be undertaken. The precise cellular pathway by which Lm1740MIF activates CD74 remains to be investigated though it appears from the herein reported results that Lm1740MIF binds to the extracellular C-terminal domain of CD74. An additional question posed by these findings is whether there exist further, functional roles for *Leishmania* MIF with respect to the host-parasite interaction. It is noteworthy that MIF binding to CD74 was shown recently to affect recruitment and activation of additional signaling proteins, including CD44 (Shi, Leng *et al.* 2006), as well as the chemokine receptors, CXCR2 and CXCR4 (Bernhagen, Krohn *et al.* 2007). Whether Lm1740MIF modulates additional pathways that are important for intracellular parasitism or immune evasion but are not activated by mammalian MIF remains to be determined/ For this purpose microarray analysis have already been started to be carried out, but their in-depth analysis, confirmation by Northern blot and quantitative PCR, and interpretation fall without of the time-frame and scope of this thesis. It is also notable that while host-derived MIF may augment the killing of *L. major*, its specific activity is low compared with cytokines, such as IFN-γ (Juttner, Bernhagen *et al.* 1998).

While our data are consistent with a role for *Leishmania* MIF in modulating the host immune response, they exclude neither the intrinsic function for Lm1740MIF in the growth or replication of the parasite nor the function of LmMIF in promastigotes within the parasitic vector.

Two *mif*-like genes were described to be transcriptionally upregulated in the dauer stage of the free-living nematode, *C. elegans*. It has been hypothesized that these

genes may have a homeostatic role during adverse conditions that cause developmental arrest (Marson, Tarr *et al.* 2001).

A physiologic role for *Leishmania* MIF in the parasitic life cycle and a closer examination of whether these proteins function as virulence factors may be attained by creating different strains of *Leishmania* that lack MIF orthologs. Such studies, as well as the identification of the surface contacts between MIF and the CD74 surface receptor, may also provide support for selective, pharmacologic targeting of *Leishmania* MIF for therapeutic benefit. Another interesting aspect of Leishmaniasis with respect to MIF is the study of the MIF promotor region to determine patient genotypes and assess whether MIF expression levels can be linked to disease severity and outcome. Unfortunately, these interesting questions have been outside the scope of this thesis.

6 Summary

Parasitic organisms have evolved specialized strategies to evade host immune defense mechanisms. This thesis describes the characterization of two *Leishmania major*-encoded orthologs of the pro-inflammatory pleiotropic cytokine, macrophage migration inhibitory factor (MIF). These two MIF-like sequences show 22% sequence identity when aligned with human MIF, and are most likely the result of gene duplication.

The Lm1740MIF and Lm1750MIF genes (58% identity) are expressed in all developmental stages of *L. major*. Interestingly, LmMIF expression levels were shown to be significantly up-regulated in the procyclic life-stage of the parasite, which exists solely in the host sand fly. Furthermore, Lm1750MIF levels were 3–4 fold higher than Lm1740MIF levels. The finding that LmMIF is expressed in procyclic parasites might reveal a new function for MIF within its vector, the sandfly.

Furthermore, the work described here postulated that Lm1740MIF might interact with the macrophage surface receptor, CD74. To evaluate this hypothesis, binding analyses using surface plasmon resonance (Biacore) were carried out. The K_d of MIF was previously approximated to be between 9×10^{-9} to 2.3×10^{-10}. Lm1740MIF showed a significant binding interaction with CD74 ($K_d = 2.9 \times 10^{-8}$ M). Thus, there is sufficient structural homology between Lm1740MIF and human MIF to permit high-affinity binding to CD74.

To investigate whether Lm1740MIF induces ERK1/2 activation in a CD74-dependent manner and to determine if it inhibits activation-induced macrophage apoptosis similar to its mammalian counterpart, the ability of Lm1740MIF to stimulate ERK1/2 phosphorylation as well as to inhibit Ser15 phosphorylation of the tumor suppressor p53 was tested. Macrophages treated with Lm1740MIF were assessed for apoptosis by the ELISA method; additionally, Western blot analyses for ERK1/2 and p53 activation were performed. The interaction between Lm1740MIF and CD74 led to a similar signal transduction response in macrophages obtained from both wild-type and C3H/HeJ mice, indicating that signaling was not due to the presence of contaminating endotoxin.

Lm1740MIF was functionally active in both ERK1/2 signaling and protection from apoptosis, although the level of activity was generally lower than for mammalian MIF, an observation that appeared consistent with the lower K_d of Lm1740MIF for

the CD74 receptor. For mouse MIF, protection from apoptosis was dependent on CD74, and the response was associated with a decrease in the cytoplasmic content of Ser15-phosphorylated p53. The ability of Lm1740MIF to inhibit apoptosis may facilitate the persistence of *Leishmania* within macrophages and contribute to its evasion from immune destruction. The precise cellular pathway by which Lm1740MIF activates CD74 and whether Lm1740MIF modulates additional pathways that are important for either intracellular parasitism or for immune evasion remains to be determined.

In conclusion, the presented results indicate that a *Leishmania* ortholog of the cytokine MIF has the ability to activate the human MIF receptor and influence the functional responses of monocytes/macrophages. Because *Leishmania* is an intracellular infection of monocytes/macrophages, *Leishmania*-encoded MIF is hypothesized to sustain monocyte/macrophage survival and contribute to the persistence of the parasite for completion of its infectious life cycle. While these data are consistent with a role for *Leishmania* MIF in modulating host-immune responses, they do not exclude the possibility that Lm1740MIF may function in the growth and/or replication of the parasite.

7 References

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