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*Leishmania donovani*  
**Lipophosphoglycan**

Modulation of Macrophage and  
Dendritic Cell Function

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*Cover illustrations*

Front cover: a scanning electron micrograph of an immature human monocyte-derived dendritic cell (Mo-DC) interacting with wild-type *Leishmania donovani*.

Back cover: a Mo-DC.

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*Till Jenny och Niclas*



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## LIST OF PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

- I. Holm Å, **Tejle K**, Magnusson K-E, Descoteaux A, and Rasmusson B. *Leishmania donovani* lipophosphoglycan causes periphagosomal actin accumulation: correlation with impaired translocation of PKC $\alpha$  and defective phagosome maturation.  
*Cellular Microbiology* (2001) **3**(7), 439-447
- II. **Tejle K**, Magnusson K-E, and Rasmusson B. Phagocytosis and phagosome maturation are regulated by calcium in J774 macrophages interacting with un-opsonized prey.  
*Bioscience Reports* (2002) **22**(5-6), 529-40
- III. Holm Å, **Tejle K**, Gunnarsson T, Magnusson K-E, Descoteaux A, and Rasmusson B. Role of protein kinase C  $\alpha$  for uptake of unopsonized prey and phagosomal maturation in macrophages.  
*Biophysical and Biochemical Research Communications* (2003) **302**(4), 653-658
- IV. **Tejle K**, Lindroth, M, Magnusson K-E, and Rasmusson B. *Leishmania donovani* promastigotes block maturation, increase integrin expression and inhibit detachment of human monocyte-derived dendritic cells – a role for lipophosphoglycan (LPG).  
*Manuscript*.



# ABSTRACT

*Leishmania donovani* is a blood-borne tropical parasite, which infects humans through bites by *Phlebotomus* sandflies. The parasite survives and multiplies inside macrophages in inner organs, and causes the deadly disease visceral leishmaniasis (Kala-Azar).

Macrophages and dendritic cells (DC) are professional antigen-presenting cells involved in the initiation of immune responses. Immature DC are present in all tissues where they internalise and process antigen, in response to which they migrate from tissue, into draining lymphoid organs, undergo maturation and present antigens to lymphocytes. Control measures for leishmaniasis include testing of new diagnostics and development of affordable and effective vaccines for humans.

Lipophosphoglycan (LPG) is the major surface component of *Leishmania donovani* promastigotes. LPG comprises a membrane-anchoring lysophosphatidylinositol part and an extracellular chain of disaccharide phosphates. These repetitions are crucial for parasite survival inside macrophages following phagocytosis. LPG has several specific effects on the host cell including inhibition of protein kinase C (PKC) activity, and inhibition of phagosomal maturation, a process requiring depolymerization of periphagosomal F-actin.

Confocal microscopy and image analysis were used to follow F-actin dynamics in single macrophages during phagocytosis of *L. donovani* promastigotes and LPG-coated particles. F-actin did not depolymerize, but instead progressively polymerized around phagosomes with LPG-

containing prey. This correlated with reduced translocation of PKC $\alpha$  to the phagosome and blocked phagosomal maturation. LPG also inhibited cortical actin turnover, which could be the underlying cause of the reduced uptake of LPG-containing prey. Extracellular- and intracellular calcium was necessary for phagocytosis, periphagosomal F-actin breakdown and phagosomal maturation in macrophages interacting with unopsonized prey, and for the action of LPG.

We also studied F-actin turnover in macrophages overexpressing dominant-negative (DN) PKC $\alpha$ . DN PKC $\alpha$  macrophages showed increased amounts of cortical F-actin, decreased phagocytic capacity, inhibition of periphagosomal F-actin breakdown and defective phagosomal maturation. When DN PKC $\alpha$  macrophages interacted with LPG-containing prey, phagocytosis was almost completely blocked.

Moreover, we found that *Leishmania* promastigotes and particularly LPG inhibit DC maturation and detachment from distinct surfaces. Thus, LPG from *Leishmania donovani* could directly inhibit DC migration to lymphoid organs, antigen-presentation and development of immunity.

# POPULÄRVETENSKAPLIG

## SAMMANFATTNING

Sjukdomen visceral leishmaniasis (VL) beskrevs första gången 1903. Den fick sitt namn efter upptäckarna, William Leishman och Charles Donovan, som påvisade parasiter i mjälten hos en patient med malaria-liknande symptom.

Leishmaniasis betecknar idag en grupp av sjukdomar orsakade av insektsburna encelliga mikroorganismer (protozoer) som tillhör släktet *Leishmania*. Parasiterna förekommer både hos djur (t.ex. gnagare, räv, sjakal och hund) och människa. Drygt 20 arter kan infektera människa. Sjukdomen förekommer idag inte sällan tillsammans med HIV/AIDS.

Fler än 12 miljoner människor fördelade på 88 länder beräknas vara leishmaniainfekterade. Ungefär en halv miljon fall av visceral leishmaniasis (VL), som drabbar inre organ, och en och en halv miljon fall av kutan leishmaniasis (CL) diagnostiseras varje år. VL förekommer framförallt i Indien, Bangladesh, Nepal, Sudan och i Brasilien. CL finns främst i Afghanistan, Iran, Syrien, Saudiarabien, Algeriet, Brasilien och Peru. Förekomsten avspeglar utbredningen av de sandflugor som sprider infektionen via sina bett.

VL kallas även Kala-azar, som på hindu betyder svarta febern. I "den gamla världen" (Afrika, Asien) orsakas denna sjukdom oftast av *Leishmania donovani* (LD). LD infekterar vita blodkroppar i lever, mjälte och benmärg. Infektionen är kronisk och utan behandling är dödligheten

hög. Ofta avlider patienten av andra infektioner som lunginflammation, tuberkulos och mag-tarminfektioner.

CL är en relativt godartad infektion som självläker och ger livslång immunitet. Det klassiska symptomet är ett "torrt" sår i ansiktet, på en arm eller ett ben. Mukutan leishmaniasis (MCL) är en destruktiv form av CL som kan försvåras av samtidiga bakterieinfektioner. Diffus kutan leishmaniasis (DCL) innefattar olika kroniska hud- och organskador. Symptomen liknar lepromatös lepra. DCL är svårbehandlad.

Parasiten *Leishmania* existerar i två olika former: amastigoter och promastigoter. Sandflugan får genom att bita och dricka blod från en infekterad värd i sig amastigoter, vilka utvecklas till rörliga promastigoter i den främre delen av sandflugans tarm. Vid nästa "måltid", kan promastigoter överföras till ett friskt djur eller människa. Inuti den nya värden tas promastigoterna upp av makrofager via s.k. fagocytos, i syfte att döda parasiten. LD överlever dock detta, omvandlas till amastigoter och förökar sig inuti makrofager. Till slut sprängs den parasitfyllda makrofagen sönder.

Lipofosfoglykan (LPG) är den dominerande ytmolekylen hos LD-promastigoter. Förekomsten av LPG är avgörande för parasitens överlevnad i makrofager.

I avhandlingen studeras effekten av LPG på upptag och avdödning av LD i makrofager, särskilt påverkan av LPG på cellskelettproteinet F-aktin och betydelsen av enzymet protein kinas C ( $PKC\alpha$ ) och fritt calcium studerades. Därutöver undersöks hur LPG påverkar mognaden av en annan typ av vit blodkropp, den dendritiska cellen (DC).

Konfokal mikroskopi och bildanalys används för att följa F-aktin-nivåerna i enskilda celler under fagocytos. Normalt bryts F-aktin ned runt den membranblåsa (fagosom) i vilken bytet hamnar inuti makrofagen. Vi fann att LPG hindrade denna nedbrytning och istället orsakade en uppbyggnad av F-aktin runt fagosomen. Samtidigt observerade vi en minskad mängd PKC $\alpha$  runt fagosomen och hämmad fagosomal mognad, vilket kan medföra ökad överlevnad av bytet. Resultaten styrks av att en ”konstlad” hämning av PKC $\alpha$  också orsakade en uppbyggnad av F-aktin runt fagosomen och hämmad fagosomal mognad. LPG orsakade även förhöjda mängder av F-aktin nära cellmembranet, vilket skulle kunna förklara varför LPG-klätt byte är svårare att fagocytera för makrofager jämfört med ”naket” byte.

Vi fann också att fritt kalcium var nödvändigt både för nedbrytningen av F-aktin runt makrofagers fagosomen och för den fagosomala mognaden. Fritt kalcium behövdes även för att LPG skulle kunna påverka dessa processer.

Vi studerade också hur LD promastigoter påverkar utmognaden av dendritiska celler. DC presenterar mikrobiella molekyler och andra antigen för immunförsvaret och är aktiva redan tidigt vid en infektion. Omogna DC finns i alla vävnader. Efter upptag av antigen, börjar DC mogna, lossnar från vävnaden och transporteras till olika lymforgan där de visar upp antigen för andra immunceller.

Vi odlade fram omogna DC från monocyter från blodgivare genom att inkubera monocyterna med cytokinerna GM-CSF och IL-4 i 6 dagar. Mogna DC framställdes genom att tillsätta cytokinet TNF- $\alpha$  i ytterligare 3 dagar.

Vi fann att omogna humana DC kan fagocytera LD-promastigoter och att LPG-defekta mutanter stimulerar mognad av DC. Virulenta LD påverkade uttrycket av adhesionsmolekylerna CD11b och CD51 och hindrade DC från att lossna från ytor. Detta tyder på att LPG kan hämma DC utmognad och transport till lymforgan, och därmed hämma immunsvaret mot parasiten.

# TACK

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# *ABBREVIATIONS*

Arp2/3	actin-related protein 2/3
BSA	bovine serum albumin
C3b	fragment of the third complement factor
CL	cutaneous leishmaniasis
CLR	C-type lectin receptor
CLSM	confocal laser scanning microscopy
CR	complement receptor
DAT	direct agglutination test
DC	dendritic cell
DCL	diffuse (disseminated) cutaneous leishmaniasis
DN	dominant negative
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide
ECL	enhanced chemiluminescence
EGTA	ethylene glycol-bis ( $\beta$ -aminoethylether)-N,N,N',N'- tetraacetic acid
F-actin	filamentous actin
Fc $\gamma$ -R	Fc gamma-receptor
FCS	fetal calf serum

FITC	fluorescein isothiocyanate
G-actin	globular actin
Gal	galactose
GFP	green fluorescent protein
GM-CSF	granulocyte macrophage-colony stimulating factor
gp63	glycoprotein 63
GTP	guanosine triphosphate
HP	heat-inactivated human plasma
IFN	interferon
IgG	immunoglobulin G
IL	interleukin
IMDM	Iscoe's modified Dulbecco's medium
KB	Krebs-Ringer's glucose phosphate buffer with 1% bovine serum albumin
KRG	Krebs-Ringer's glucose phosphate buffer
LAMP	lysosome-associated membrane protein
IL	interleukin
LPG	lipophosphoglycan
LPS	lipopolysaccharide
<i>lpg1</i> -KO	<i>L. donovani</i> promastigote, lipophosphoglycan 1- knock out
<i>lpg2</i> -KO	<i>L. donovani</i> promastigote, lipophosphoglycan 2- knock out
MARCKS	myristoylated alanine-rich protein kinase C substrate

MAPT/AM	bis-(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetoxymethyl ester
MCL	mucocutaneous leishmaniasis
Man	mannose
MHC	major histocompatibility complex
PBS	phosphate-buffered saline
PB	phosphate-buffered saline with 1% bovine serum albumin
PBMC	peripheral blood mononuclear cells
PE	phycoerythrin
PFA	paraformaldehyde
PG	phosphoglycan
PKC	protein kinase C
PKDL	post Kala-azar dermal leishmaniasis
PRR	pattern-recognition receptor
PVDF	polyvinylidene difluoride
TLR	Toll-like receptor
TDR	Tropical Disease Research
TNF	tumor necrosis factor
VL	visceral leishmaniasis
WASP	Wiscott-Aldrich syndrome protein
WHO	World Health Organization
WT	wild-type



# INTRODUCTION

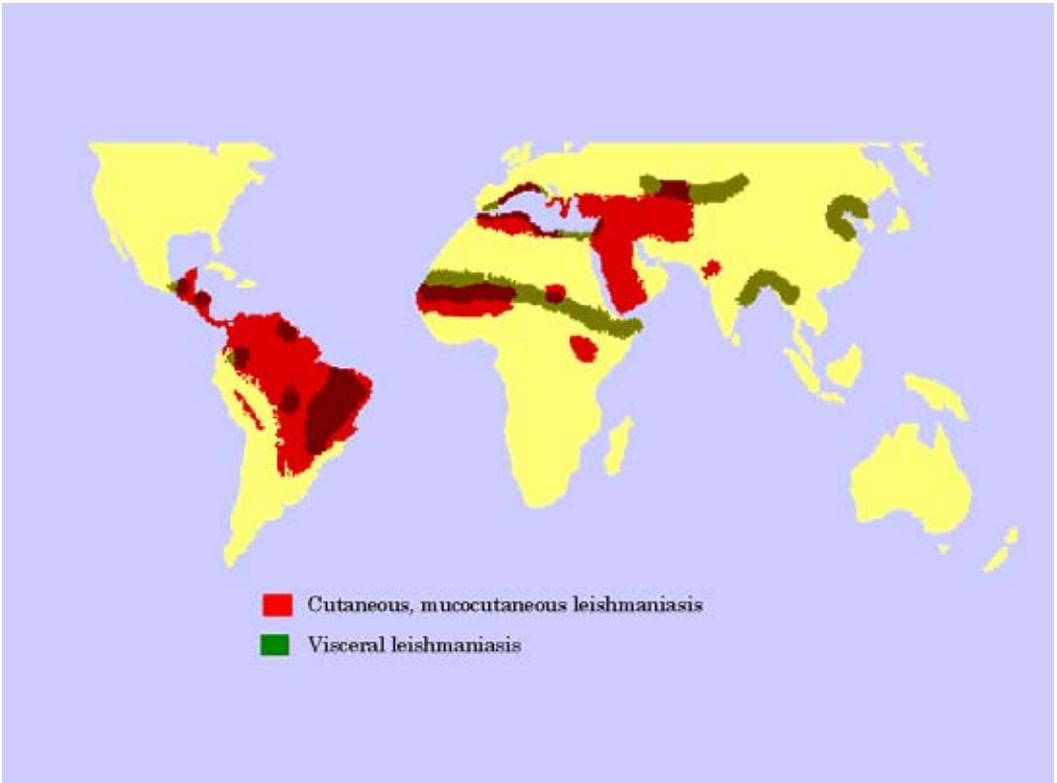
## ***Leishmania***

Protozoan parasites of the genus *Leishmania* are members of the family Trypanosomatidae, which comprises unicellular organisms characterized by the presence of a single flagellum and a DNA-rich, mitochondria-like organelle, the kinetoplast (*Lainson and Shaw, 1978; Orlandi and Turco, 1987; Pearson and Sousa, 1985*). The various species of the genus *Leishmania* infect millions of people world-wide, causing a wide spectrum of diseases from self-healing ulcers to fatal visceral infection.

More than 20 species of *Leishmania* can infect humans and other species are emerging, especially in association with HIV/AIDS (*Desjeux, 1999*). In some areas, leishmaniasis is a zoonotic infection involving various animal reservoirs, while in others, humans are the sole reservoir of infection.

## ***Leishmaniasis***

Leishmaniasis is endemic in areas of the tropics, subtropics, and southern Europe, in settings ranging from rain forests in the Americas to deserts in west Asia (*Herwaldt, 1999*) (Figure 1). The geographic distribution of leishmaniasis is limited by the distribution of the sand fly, the carrier of the disease, its tendency to draw blood from humans or animal reservoir hosts, and its capacity to support the internal development of specific species of *Leishmania* (*Sacks, 2001*).



*Figure 1*

*Distribution of Leishmaniasis in 88 countries on 4 continents (from WHO, 2003)*

More than 12 million people in 88 countries are infected with leishmaniasis. There is an approximate incidence of 0.5 million cases of VL per year and 1.5 million cases of CL (*World Health Organization (WHO) (Awasthi et al., 2004)*).

The visceral disease is particularly prevalent in India, Bangladesh, Nepal, Sudan and Brasil. These countries together account for ca 90% of the cases. The cutaneous disease is prevalent in Afghanistan, Iran, Syria, Saudi Arabia, Algeria, Brazil and Peru. Mucocutaneous leishmaniasis is endemic in Mexico, Central and South America (*WHO, Tropical Disease Research (TDR) 2005, Seventeenth Programme Report, Progress 2003-2004*).

*Leishmaniasis can present itself in man into four different forms:*

1. Visceral leishmaniasis (VL), also known as Kala-azar, is (in the Old world) caused by the protozoan parasite *L. donovani* (*Lainson and Shaw, 1978; Pearson and Sousa, 1985*). *L. donovani* infects macrophages of the liver, spleen and bone marrow. The infection is chronic and may be fatal in untreated cases. After successful treatment, 3 to 10 % of the patients develop post Kala-azar dermal leishmaniasis (PKDL), wart like nodules over the face and extensor surface of the limbs (*Awasthi et al., 2004*). Epidemics of Kala-azar are often associated with natural disasters or social upheavals.

2. Cutaneous leishmaniasis (CL) is the most common form, an infection characterized by ulcerative skin lesion in the face, on arms or legs. CL is principally caused by *L. major*, *L. tropica* and *L. mexicana*. Although the lesions will persist and disseminate, cutaneous leishmaniasis is generally a self-healing disease.

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3. Mucocutaneous leishmaniasis (MCL) is a variant form of cutaneous leishmaniasis caused by *L. braziliensis*. This parasite has a tropism for macrophages of the oro-naso-pharyngeal region, where it produces a mucosal granuloma that eventually destroys the nose and mouth.

4. Diffuse cutaneous leishmaniasis (DCL) is a mutilating disease caused by *L. mexicana*. The infection disseminates with chronic skin lesions resembling those of lepromatous leprosy, and is difficult to treat (Table 1).

Tabel 1.

*Epidemiological traits of leishmaniasis: distribution, reservoirs and clinical syndromes*

Species	Geographic distribution	Reservoir	Clinical syndrome
<i>L. donovani</i> (Old world)	Africa, India, Bangladesh, East Asia, China	Humans, rodents	VL, PKDL
<i>L. infantum</i> (Old world)	Southern Europe, Mediterranean, Eastern China, Central Asia	Dogs, foxes, jackals	VL, CL
<i>L. major</i> (Old world)	Middle East, Indian, Northwestern China, Africa	Humans, rodents	CL
<i>L. tropica</i> (Old world)	Middle East, Indian, Western Asia	Humans rodents	CL,
<i>L. aethiopica</i> (Old world)	East Africa	Hyraxes	DCL, MCL
<i>L. chagasi</i> (New world)	Central and South America,	Dogs, foxes	VL, CL, PKDL
<i>L. amazonensis</i> (New world)	South America	Forest rodents	CL, VL, PKDL MCL, DCL
<i>L. braziliensis</i> (New world)	Central and South America	Forest rodents	CL, MCL
<i>L. mexicana</i> (New world)	Central America	Forest rodents	CL, DCL

VL, visceral leishmaniasis; CL, cutaneous leishmaniasis; PKDL, post Kala-azar dermal leishmaniasis; MCL, mucocutaneous leishmaniasis; DCL, diffuse cutaneous leishmaniasis (adapted from Awasthi et al, 2004; Bryceson, 1996; Sacks, 2001).

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## ***Diagnostic tests of Leishmaniasis***

VL is usually diagnosed by demonstrating the parasite in aspirates from the spleen, bone marrow or lymph nodes, but this method is unsuitable in field settings. Quick differential diagnosis of leishmaniasis (Sundar and Rai, 2002) can be achieved with the direct agglutination test (DAT), which is quantitative, and uses a freeze-dried antigen (Oskam et al., 1999), the urine antigen-detection test (dipstick K39) that is particularly useful in immunocompromised patients and to evaluate treatment efficacy (Attar et al., 2001; Sundar and Rai, 2002).

For CL, parasitological diagnosis is based on skin smears. For MCL cases, diagnosis relies on serological tests. This is however, not helpful in most cases because the antibody levels are too low, whereas manifestations of cell-mediated immunity e.g. Skin-test reactivity, usually develop during an active infection (Herwaldt, 1999).

## ***Treatment of Leishmaniasis***

Medically, there are good reasons to regard leishmaniasis second in importance to Malaria among the protozoal diseases. Epidemiological studies furthermore indicate that *Leishmania* is an opportunistic pathogen in immunocompromised, HIV-1-infected individuals (Desjeux, 1999; Orsini et al., 2002).

VL is an intracellular infection in a patient who has failed to express cellular immunity. *Leishmania* show different sensitivities to drugs between and within species, and individual patients respond differently to treatment. The chemotherapeutic treatment of VL is threatened by the spread of drug resistance, e. g. in India and Sudan (Bryceson, 1996). Ongoing

research on drugs for treating leishmaniasis include Miltefosine, an oral drug and potential first-line treatment for elimination of Kala-azar and paromomycin as a potential alternative or second-line therapy for elimination of VL on the Indian subcontinent (*Bhattacharya et al., 2004; Herwaldt, 1999*).

HIV co-infected individuals often relapse after traditional antimonial treatment. Pentavalent antimonials still remain the drug of choice because of cost, availability, efficacy and familiarity. Amphotericin B is a useful but costly and toxic alternative (*Bryceson, 1996; Herwaldt, 1999*).

Efficient prophylactic measures, including safe vaccines, are currently not available. However, alum-precipitated autoclaved *Leishmania major* plus BCG (*Bacillus Calmette Guérin*) has been tested as a therapeutic vaccine against human leishmaniasis in Colombia, Iran and Sudan. Preliminary results suggest that the vaccine is safe and efficacious (*TDR, 2005, Seventeenth Programme Report, Progress 2003-2004*).

CL and MCL are very common problems among people working in heavily forested areas, such as the Amazonas region of Brazil (*Lainson and Shaw, 1978*). Protection against CL could be achieved by elimination of the infected sand flies (*Asilian et al., 2003*). Heat treatment may accelerate the cure. Chemotherapy, using aminosidine ointment, cures up to 80% of the CL cases, but needs to be applied for up to 12 weeks. Pentavalent antimonials remain the most generally useful and available treatment. Local injections of interferon- $\gamma$  can increase cellular immunity, but immunotherapy has so far proved unsuccessful (*Awasthi et al., 2004; Bryceson, 1996; Herwaldt, 1999*).

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## ***The life cycle of Leishmania donovani***

The parasite *Leishmania* alternates between two developmental stages (Figure 2). In the mammalian host, it exists as a non-motile amastigote form (3-7  $\mu\text{m}$ ), which proliferates within the acidic and hydrolase-rich phagolysosomal compartment of host macrophages (Alexander and Russell, 1992). The 2-3 mm long sandfly vector of the genus *Phlebotomus* (old world) or *Lutzomyia* (new world) becomes infected, when feeding on the blood of an infected mammal or an animal reservoir (Awasthi et al., 2004; Lainson and Shaw, 1978; Pearson and Sousa, 1985; Pimenta et al., 1992).

Sand flies are “pool-feeders”, which means that they first make a small wound in the skin, then feed on the blood accumulating in the wound (Ashford and Bates, 1998).

During the digestion of the blood-meal, amastigotes differentiate into elongated flagellate promastigotes (10-20  $\mu\text{m}$ ) which become motile in the sandfly midgut. Virulence is acquired during metacyclogenesis, a process by which dividing, non-infective procyclic promastigotes transform into a non-dividing infective form (Sacks, 1989). The metacyclic promastigotes detach from the gut epithelial cells and migrate towards the anterior end of the digestive tract of the sand fly. During the next blood meal, the promastigotes are inoculated into the mammalian host (Figure 2).

Sand fly saliva selectively inhibits nitric oxide production and parasite killing by host macrophages (Awasthi et al., 2004; Hall and Titus, 1995). Inside the host, the promastigotes must evade and resist host defense mechanisms such as complement-mediated lyses, before ultimately binding and entering into macrophages through a receptor-mediated process (Descoteaux and Turco, 2002).

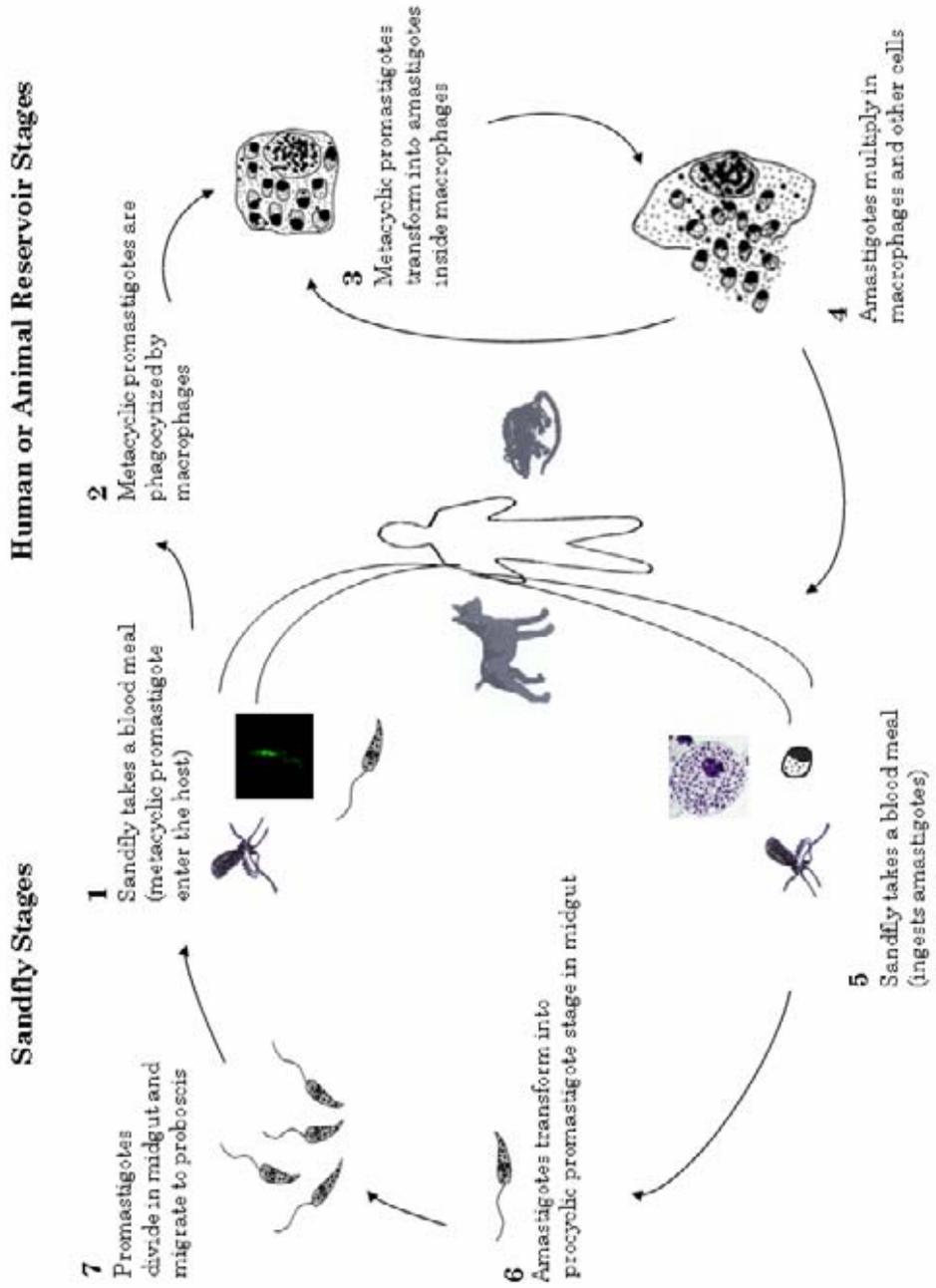


Figure 2  
Life cycle of *Leishmania*

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Once inside a phagosome, the promastigotes avoid degradation primarily through inhibition of phagolysosome formation. The combination of low pH and high temperature (37°C) induces the transformation of the promastigotes to amastigotes in all *Leishmania* species (Garlapati et al., 1999; Zilberstein and Shapira, 1994). Promastigotes also affect the signalling capacity of the macrophage by inhibition of cytokine production (Bogdan and Rollinghoff, 1999; Gorak et al., 1998).

Finally, infected macrophages rupture and release the amastigotes into the surrounding tissue, where they may be taken up by neighbouring macrophages and continue to multiply (Chang and Dwyer, 1976; Descoteaux and Turco, 2002; Lainson and Shaw, 1978; Pimenta et al., 1992; Turco and Descoteaux, 1992).

### ***Parasite surface molecules***

The major surface glycoconjugate of all *Leishmania* promastigotes is lipophosphoglycan (LPG) (Homans et al., 1992; McConville and Blackwell, 1991). The unique structure of LPG from *L. donovani* is shown in Figure 3. It has four domains: (i) a 1-*O*-alkyl-2-lyso-phosphatidyl(*myo*)inositol anchor, (ii) a glycan core, (iii) repeating disaccharide phosphate units (n = 16-30 units per molecule), and (iv) a small oligosaccharide cap (Descoteaux and Turco, 1999; Mengeling et al., 1997; Turco and Orlandi, 1989; Turco and Descoteaux, 1992; Turco et al., 2001; Turco et al., 1987). LPG from different species of *Leishmania* have an identical lipid anchor and glycan core, but vary in the repeating sugars (Barron and Turco, 2005; McConville, 1995).

LPG undergoes several modifications during the life cycle of the parasite, which is characteristic for each *Leishmania* species. During the metacyclogenesis process, the number of repeating units in LPG

increases. This is important for binding and release of the parasite from the sand fly midgut (Beverley and Turco, 1998; Descoteaux and Turco, 2002; Sacks, 1989).

LPG has several functions once the promastigote has entered the human host. It activates complement, which aids the uptake of the promastigotes by macrophages. However, at the same time LPG protects against complement-induced lysis of the parasite in several ways (Descoteaux and Turco, 2002; Sacks, 1992).

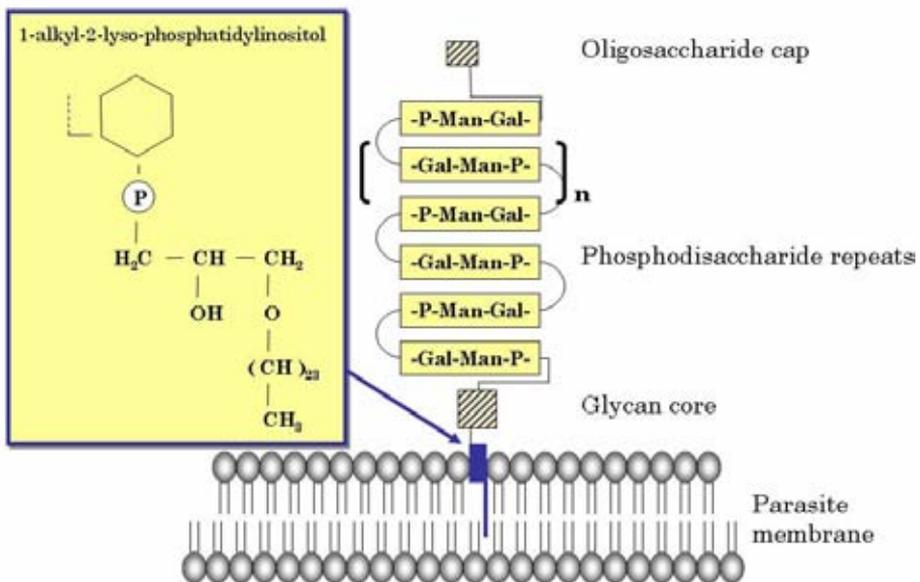
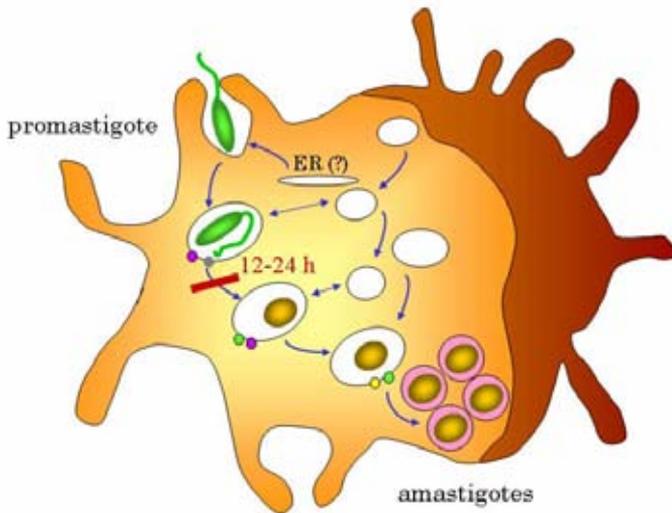


Figure 3

The structure of lipophosphoglycan (LPG) from *L. donovani*. LPG consists of a lyso-phosphatidyl inositol lipid anchor, a phosphosaccharide glycan core, multiple repeating disaccharide-phosphate units and a small oligosaccharide cap. Abbreviations: Gal = galactose, Man = mannose, P = phosphate.

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The most important aspect of LPG is, however that the molecule is absolutely necessary for promastigote survival inside the macrophage phagosome, since it blocks phagosome maturation (Descoteaux and Turco, 2002; Desjardins and Descoteaux, 1998; Handman et al., 1986; McNeely and Turco, 1990). The blockage takes place after the acquisition of the G-protein Rab5, but before the acquisition of Rab7 (Scianimanico et al., 1999). Inside this protected environment the metacyclic promastigotes convert to amastigotes, a process which takes 1-2 days (Descoteaux and Turco, 2002) (Figure 4).



*Figure 4*

*Proliferation of Leishmania donovani amastigotes. The expression of LPG is down-regulated when the promastigote transforms into an amastigote. After transformation, phagosome maturation continues. Amastigotes proliferate inside mature phagosomes. ER, endoplasmic reticulum.*

While LPG is abundant on the surface of promastigotes, it is virtually absent from amastigotes (*Turco and Sacks, 1991*). The lack of LPG allows progression of the phagosome to a fully mature phagolysosome (*Desjardins and Descoteaux, 1997*). The importance of LPG is further stressed by the fact that promastigotes would be instantly killed in the hostile environment of a mature phagolysosome. By contrast, amastigotes are resistant to lysosomal enzymes and have effective means of regulating their internal pH in an acidic environment. Amastigotes are very active at a low pH and soon start dividing inside the phagolysosome (*Desjardins and Descoteaux, 1998; Zilberstein and Shapira, 1994*).

LPG can be said to “buy time” for the promastigote to evolve into a resistant amastigote, and LPG is therefore crucial for establishment of infection in the human host. The mechanism by which it inhibits phagolysosome fusion is still unclear. LPG has several effects on the host macrophage, including a strong inhibitory effect on macrophage PKC activity. This in turn may affect several macrophage functions, including phagolysosome fusion (*Dermine et al., 2005; Descoteaux and Turco, 2002; Holm et al., 2001*), cytokine production and generation of nitric oxide (NO) (*Basu et al., 1997; Bouvier et al., 1995; Descoteaux and Turco, 2002; MacMicking et al., 1997; Proudfoot et al., 1996*).

LPG domains also appear as components of other parasite glycoconjugates (*Mengeling et al., 1997; Sacks and Kamhawi, 2001*). Secreted acid phosphatase (sAP), phosphoglycan (PG), and proteophosphoglycan (PPG) all possess disaccharide-phosphate repeating units (*Descoteaux and Turco, 1999; Descoteaux et al., 1998; Ilg, 2000*). That the repeats are found on several molecules suggests that they may play a significant role during the intracellular life cycle of the parasite.

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Another abundant molecule on the promastigote surface is the 63-kDa glycoprotein protease (gp63), also called leishmanolysin (*Bouvier et al., 1995; Sacks and Kamhawi, 2001*). Like LPG, this glycoprotein is retained in the membrane by a phosphatidylinositol anchor (*Schneider et al., 1990*). Glycosylation of gp63 generates a considerable heterogeneity at the level of the mature protein.

### ***The macrophage***

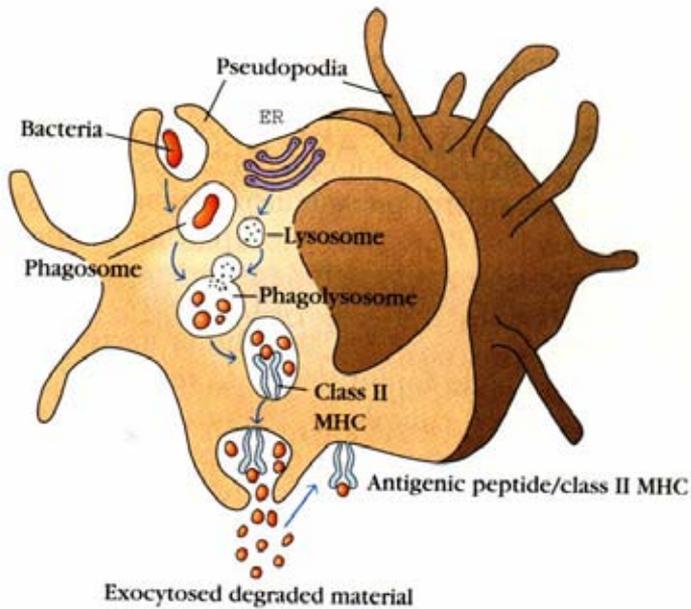
Macrophages are phagocytic cells capable of ingesting and digesting exogenous material, such as whole microorganisms, insoluble particles, injured and dead cells, cellular debris and other antigens (*Brown, 1995; Gold et al., 1999; Silverstein, 1995*). In the first step of phagocytosis, macrophages sense and actively move towards a variety of substances generated at the infections site. This process is called chemotaxis (*Kuby, 1996*). The next step involves adhesion of the antigen to the macrophage cell membrane. Complex antigens, such as whole bacterial cells or viral particles, tend to adhere well and are readily phagocytosed (*Aderem, 2002; May and Machesky, 2001; Underhill and Ozinsky, 2002*).

Macrophages have specific phagocytosis-promoting receptors on their surface (*Gold et al., 1999*). The mannose receptor recognizes conserved motifs on pathogens, e. g. the yeast cell wall, which consists mainly of repeating units of mannan and  $\beta$ -glycan (*Bos and de Souza, 2000; Brown et al., 2002; Handman and Bullen, 2002*). This receptor is a single chain receptor with a short cytoplasmic tail and an extracellular domain including eight lectin-like carbohydrate-binding domains (*Allen and Aderem, 1996; Blackwell, 1985; Mullin et al., 1997*). These domains share homology with other C-type lectins

(*Haltiwanger and Hill, 1986; Horn et al., 1990; Wu et al., 1996*). The cytoplasmic tail is crucial for both the endocytic and phagocytic traits of the receptor, but little is known about the signals that lead to phagocytosis (*Taylor et al., 1990*). Toll-like receptors (TLRs) are a family of innate immune recognition receptors that mediate detection of a broad range of microbial products, including lipopolysaccharide (LPS), peptidoglycan, and bacterial lipopeptides (*Dobrovolskaia and Vogel, 2002; Flandin et al., 2006; Takeda and Akira, 2004; Takeuchi and Akira, 2002*).

Other receptors recognize attached opsonins, mainly immunoglobulins and the complement factor C3b (*Carroll, 1998; Colucci-Guyon et al., 2005; Ravetch and Clynes, 1998*). Best-characterized among these are the various receptors for the Fc portion of IgG (Fc $\gamma$ R) and the complement receptor 3 (CR3) (*Aderem and Underhill, 1999; Colucci-Guyon et al., 2005; Kwiatkowska and Sobota, 1999; Takai, 2002; Tjelle et al., 2000; Tuyaerts et al., 2002*). Binding of a particulate prey to phagocytic receptors on the macrophage induces the formation of membrane protrusions, pseudopodia, that extend around the attached material (*Kuby, 1996*).

After fusion, the pseudopodia encloses the material within a membrane-bound vesicle, the phagosome (*Aderem and Underhill, 1999; Kwiatkowska and Sobota, 1999; Tjelle et al., 2000*). The phagosome enters the endocytic-processing pathway, where it moves toward the cell interior, sequentially fuses with other endosomes and lysosomes, and finally form a mature phagolysosome (*Desjardins, 1995; Desjardins, 2003; Desjardins et al., 1994a; Desjardins et al., 1994b; Touret et al., 2005*) (Figure 5).



*Figure 5*

*Macrophages can ingest and degrade particulate antigens, including bacteria. The figure shows phagocytosis and the processing of exogenous antigens by macrophages. Most of the products resulting from digestion of ingested material are exocytosed, but some peptide products interact with MHC class II molecules, forming complexes that move to the cell surface and become presented to T helper ( $T_H$ ) cells (from Kubly, 1996). ER, endoplasmatic reticulum; MHC, major histocompatibility complex.*

Lysosomes contain enzymes (NADPH-oxidase) that generate hydrogen peroxide and oxygen free radicals, peroxidase, lysozyme and various hydrolytic enzymes, which digest the ingested material (*Descoteaux and Turco, 2002; Desjardins et al., 1994a; Méresse et al., 1999; Tjelle et al., 2000*). Most microorganisms are killed by the lysosomal content that is released into phagosomes. Some microorganisms, however, can survive and multiply within macrophages, such as *Leishmania*, *Salmonella*, *Mycobacterium* and *Chlamydia* (*Descoteaux and Turco, 2002; Desjardins and Descoteaux, 1998; Finlay and Falkow, 1997; Scott et al., 2003*).

### ***Phagolysosome fusion***

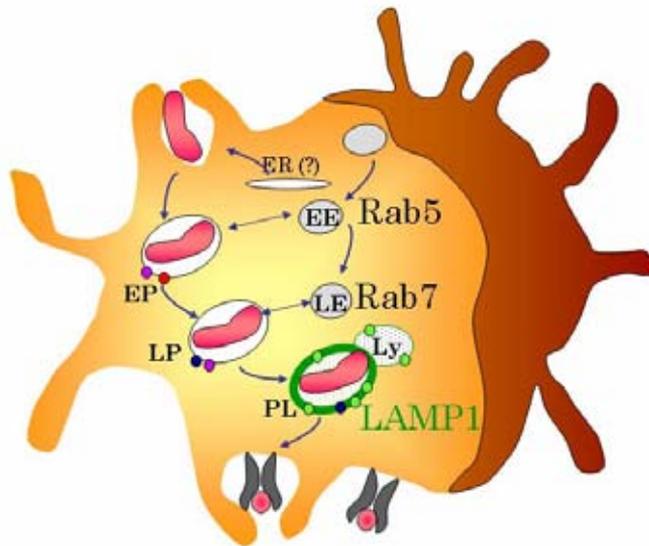
Inside a macrophage, the phagosome subsequently undergoes a series of fusions and fissions with other intracellular vesicles in a process called phagosomal maturation. This usually leads to phagolysosome formation and killing of the ingested pathogen (*Desjardins et al., 1994b; Méresse et al., 1999; Tjelle et al., 2000*).

The regulators of phagosome-endosome fusion include small GTP-binding proteins, such as Rab5 and Rab7, and possibly annexins (*Burkhardt et al., 1995; Gold et al., 1999; Miaczynska and Zerial, 2002; Rabinovitch, 1995*). The maturing phagosome also experiences a complex series of biochemical changes. It acquires markers of late endocytic organelles, such as Rab7, LAMP1 and LAMP2, and loses markers of early endocytic organelles, including Rab5 (*Desjardins et al., 1994a; Garin et al., 2001; Rabinovitch, 1995*) (Figure 6).

Tyrosine phosphorylations and dephosphorylations of phagosomal proteins may also be important events in phagolysosomal fusion (*Emans et al., 1996*). Microorganisms that replicate inside phagocytes generally employ distinct strategies to evade or resist the hostile environment of the

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phagolysosome, including inhibition of phagolysosome formation. The molecular reactions behind the arrest of phagolysosome biogenesis are largely unknown for most microorganisms (*Descoteaux and Turco, 2002; Desjardins and Descoteaux, 1998; Finlay and Falkow, 1997*).



*Figure 6*

*The phagocytic pathway. Inside a macrophage, the phagosome undergoes a series of changes including fusion with endocytic organelles. Complete degradation of microorganisms occurs in phagolysosomes. EE, early endosome; EP, early phagosome LP, late phagosome; Ly, lysosome; Rab5 and Rab7, small GTPases; LAMP1, lysosome-associated membrane protein.*

***The cytoskeleton and the role of actin***

The cytoskeleton is a complex structure without which eukaryotic cells would not be able to change shape or move. Three major types of protein families form the basis of the cytoskeleton; (i) microfilaments (3-6 nm thick), (ii) filamentous (F-) actin which is a polymer of globular (G-) actin and (iii) microtubules (20-25 nm) which are polymers of tubulin and intermediate filaments e.g. vimentin and various keratins (*Chou et al., 1997; Geiger and Karsenti, 1997; Southwick and Stossel, 1983; Wade and Hyman, 1997*).

F-actin is a dynamic structure, where monomers associate and dissociate at the two ends of the filament. These ends are called the barbed (fast-growing) and the pointed end (slow-growing). Actin filaments are often linked together by different cross-linking proteins, forming three-dimensional networks (*Lambrechts et al., 2004; Welch et al., 1997*).

F-actin polymerization and depolymerization are regulated by several actin-binding proteins, e. g. WASP (Wiskott-Aldrich Syndrome Protein), Arp2/3, gelsolin and MARCKS (myristoylated alanine-rich protein kinase C substrate). The activity of these is regulated by intracellular signals, which include changes in protein phosphorylations and the level of intracellular calcium (*Alberts et al., 2002; Schmidt and Hall, 1998; Silverstein et al., 1989*).

Members of the Rho family of small (molecular weight) GTPases participates in the regulation of the actin cytoskeleton in response to a variety of extracellular signals (*Boquet and Lemichez, 2003*). They act hierarchically during cell spreading: Cdc42 participates in the formation of filopodia and in the activation of Rac. Rac stimulates membrane ruffling and activates Rho, which in its turn stimulates the formation of focal

adhesions and stress fibers (Aspenström, 1999; Chimini and Chavrier, 2000; Hall, 1998; Underhill and Ozinsky, 2002) (Figure 7).

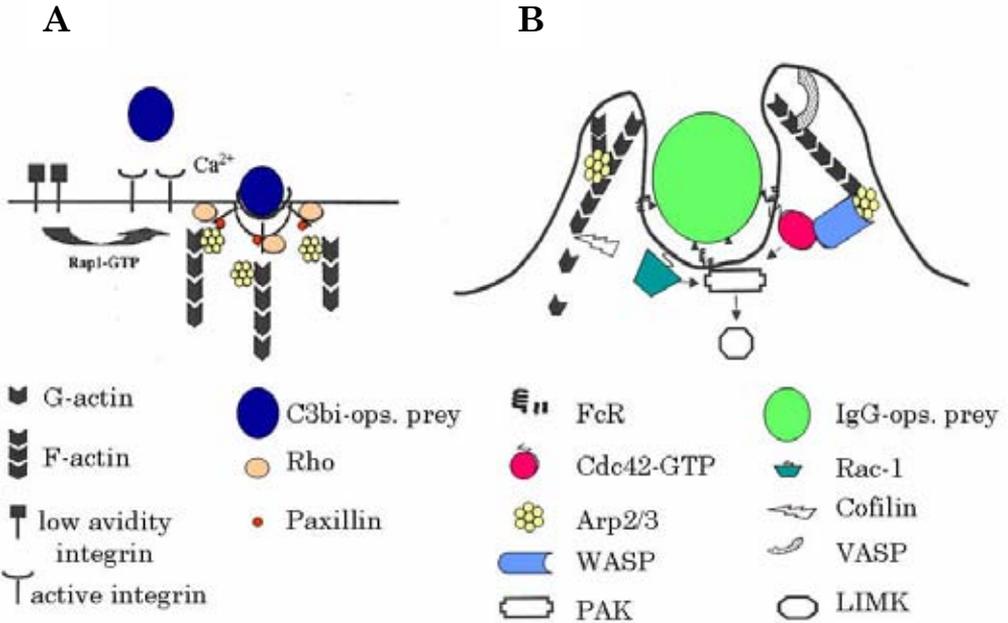


Figure 7

A, CR3-mediated phagocytosis and B, FcR-mediated phagocytosis (from Castellano et al, 2001). G-actin, globular actin; F-actin, filamentous actin; C3b, fragment of the third complement factor; Fc-R, Fc receptor; GTP, guanosine triphosphate; Arp, actin-related protein; WASP, Wiscott-Aldrich syndrome protein; PAK, p21-activating kinase 1; VASP, vasodilator-stimulated phosphoprotein; LIMK, light chain myosin kinase.

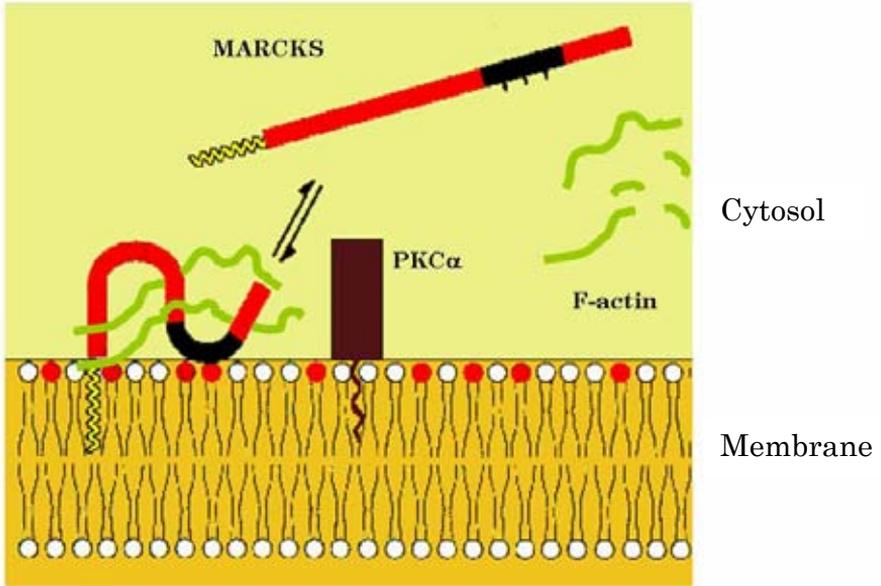
The WASP proteins are the key regulators of actin polymerization downstream of Cdc42 and Rac and are thought to control actin nucleation at the leading edge of a moving cell. WASP family proteins bind to and stimulate the nucleating activity of the Arp2/3 complex (*Castellano et al., 2001; Lorenzi et al., 2000; Machesky and Insall, 1998*), which results in the formation of a network of filaments that is essential for lamellipodial extension (*Borisy and Svitkina, 2000; Danuser, 2005*). WASP is also necessary for efficient IgG-mediated phagocytosis (*Lorenzi et al., 2000*). Here the Arp2/3 complex translocates to phagocytic cups and co-localizes with actin during phagocytosis of Ig-opsonized particles (*Castellano et al., 2001*).

The Rho family of proteins also participates in phagocytosis, where Cdc42 and Rac1 seem to control different steps in the phagocytic process. Cdc42 controls pseudopod formation and Rac1 phagosome closure during Fc $\epsilon$ RI-mediated phagocytosis (*Castellano et al., 2001; Hackam et al., 1997; Massol et al., 1998; Ng Yan Hing et al., 2004*).

CR3-mediated phagocytosis is dependent on RhoA activation, which promotes myosin filament formation and contraction. This makes the particle “sink” into the cell (*Castellano et al., 2001; Chimini and Chavrier, 2000*).

F-actin re-arrangements are thus crucial for the uptake of most phagocytic prey by leukocytes. Early phagosomes are surrounded by a rim of F-actin, called periphagosomal F-actin, which needs to be disassembled for phagolysosome formation (*Bengtsson et al., 1993; Holm et al., 2001; Jaconi et al., 1990*) (Figure 7).

The breakdown of periphagosomal F-actin is regulated by the activities of actin-binding proteins, but the exact mechanisms and signalling pathways involved are yet not fully delineated (Figure 8).



*Figure 8*

*MARCKS both crosslink and anchor F-actin filaments to membranes (plasma membrane and phagosome membrane). PKC $\alpha$  translocates to the membrane and phosphorylates MARCKS. MARCKS-PPP releases both from F-actin and the membrane and is displaced into the cytosol. This release of F-actin is probably a necessity for F-actin breakdown.*

An actin-binding protein family of particular interest is MARCKS, which cross-links F-actin. This activity is inhibited by PKC-mediated phosphorylations, and is influenced by calcium-calmodulin (Aderem and Underhill, 1999; Hartwig et al., 1992). MacMARCKS is known to translocate to

phagosomes in macrophages, and its activation by PKC  $\alpha$  has been suggested to be required for F-actin breakdown around phagosomes (Allen and Aderem, 1995a; Allen and Aderem, 1995b; Holm et al., 2003; Underhill et al., 1998; Zhu et al., 1995).

### ***Effects of calcium on leukocyte phagocytosis and periphagosomal F-actin***

It is known that the activities of many actin-binding proteins found in macrophages, such as gelsolin (Chaponnier et al., 1987; May and Machesky, 2001; Yin et al., 1981) and the calcium/calmodulin-binding protein family MARCKS, are regulated by calcium (Hartwig et al., 1992; Li and Aderem, 1992). The activity of MARCKS is for instant controlled by the calcium-dependent enzyme PKC $\alpha$ , which is recruited to early phagosomes in macrophages (Allen and Aderem, 1995a; Allen and Aderem, 1995b; Holm et al., 2003; Underhill et al., 1998; Zhu et al., 1995).

Neutrophils are the most numerous white cells in blood. The main function of neutrophils is to phagocytose and kill invading microorganisms (Jaconi et al., 1990). When human neutrophils interact with a serum-opsonized prey, actin polymerization is not calcium-dependent neither during adhesion nor phagocytosis (Bengtsson et al., 1993). However, calcium appears to trigger F-actin depolymerisation at phagosomes, a step that may be necessary for phagosome-lysosome fusion (Bengtsson et al., 1993; Jaconi et al., 1990).

In contrast, the ingestion of IgG-opsonized yeast by neutrophils is calcium-dependent (Lew et al., 1985). Disassembly of periphagosomal F-actin also occur in macrophages (Greenberg et al., 1991). But neither this process,

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nor phagosome maturation requires calcium mobilization in these cells when the prey is opsonized with serum or IgG (*Zimmerli et al., 1996*).

### ***The dendritic cell***

There are three primary types of antigen-presenting cells in peripheral lymphoid organs which can activate T cells, namely dendritic cells (DC), macrophages and B-cells. It is presently believed that the most potent of these are the DC, whose major known function is antigen presentation, while the other cell types also have other tasks in the immune system.

The nomenclature of DC is dependent on the organs to which the DC localizes. In lymphoid tissue DC are known as interdigitating cells and they are prevalent in T cell-rich areas. In the skin they are recognized as Langerhans cells (*Palucka and Banchereau, 2002*) and in other organs, e. g. in the heart, lungs and gastrointestinal tract, as interstitial DC. Circulating DC constitute 0,1% of the blood leukocyte counts; in the lymph they are known as veiled cells (*Fay, 1999*).

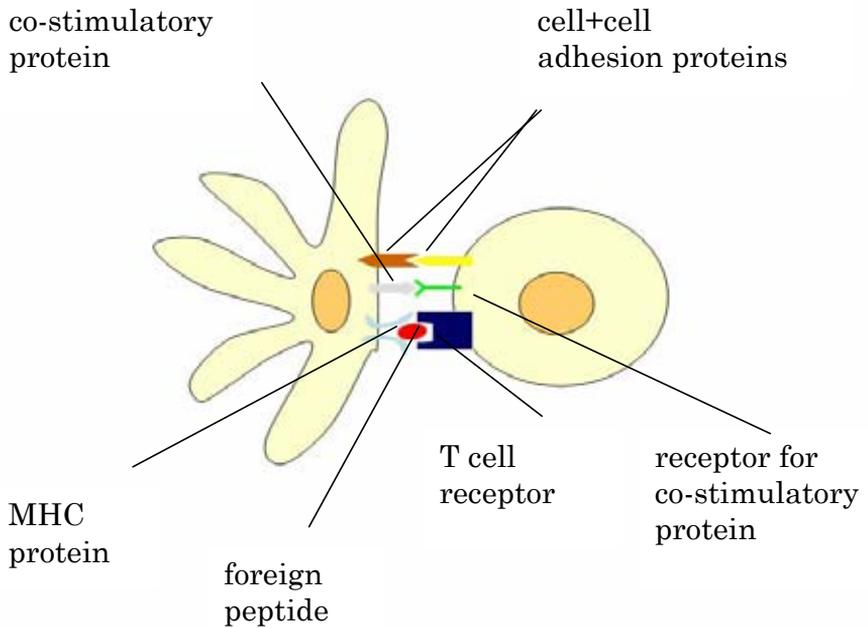
DC are described as professional antigen-presenting cells (APC) involved in the initiation of a primary immune response, a prerequisite for the establishment of an immunological memory (*Banchereau et al., 2000; Hart, 1997; Steinman and Young, 1991*). Moreover, the DC effectively prime cytotoxic T cells for immune responses against virus, bacteria and several tumors (*Hart, 1997; Lee et al., 2002; Sallusto and Lanzavecchia, 2002*).

DC progenitors in bone marrow give rise to circulating precursors finding their way to tissues, where they reside as immature DC with high phagocytic capacity, but with a relatively low ability to stimulate T cell responses.

Upon exposure by inflammatory signals, such as lipopolysaccharide (LPS) and tumor necrosis factor alpha (TNF- $\alpha$ ), and after antigen capture, immature DC migrate to nearly all lymphoid organs. Here, they undergo a process of maturation, become mature DC and acquire the ability to activate naive T cells (*Banchereau and Steinman, 1998*). DC initiate both CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses.

*The characteristics of DC that contribute to their ability to activate a T cell to become an effector cell include surface expression of:*

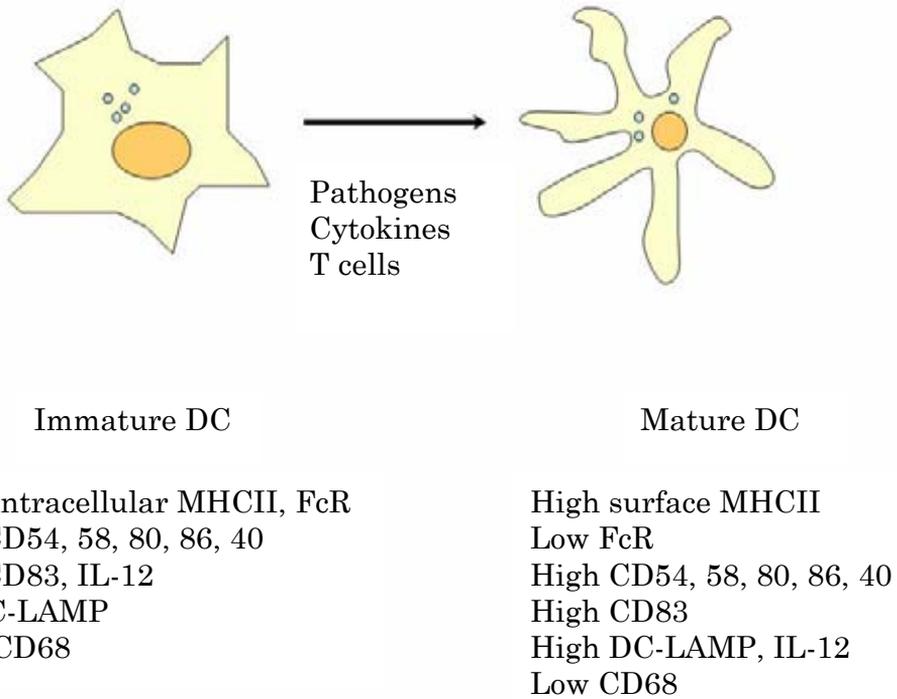
1. MHC protein (high levels of MHC class II), which helps present foreign antigen to the T cell receptor;
2. Co-stimulatory proteins (CD80/B7-1, CD86/B7-2), which bind to complementary receptors on the T cell surface;
3. Cell-cell adhesion molecules (CD11b/Mac-1, CD51/Integrin  $\alpha$ v), which enable binding to the antigen-presenting cell strongly enough for it to be activated (*Banchereau and Steinman, 1998; Lee et al., 2002*) (Figure 9).



*Figure 9*

*Proteins on the surface of an antigen-presenting cell (left) involved in activating a T cell (right).*

Mature DC are characterized by a high expression of co-stimulatory molecules, as well as other cellular markers like CD83 and DC-LAMP. DC have a well established role in antigen presentation as described earlier, but less is known about their ability to phagocytose particulate antigen. Mature DC show a significantly reduced phagocytic activity compared to macrophages (*Kiama et al., 2001; Moll, 2003; Thiele et al., 2001*) (Figure 10).



*Figure 10*

*Maturation of dendritic cells (DC) involves changes in surface expression of antigen presentation and stimulation molecules (figure modified from Banchereau and Steinman, 1998). MHC, major histocompatibility complex; FcR, Fc receptor; IL, interleukin; LAMP, lysosome-associated membrane protein.*

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DC recognize a variety of microbial antigens via activation of different receptors, e. g. Fc-receptors, C-type lectin receptors (CLRs) and pattern-recognition receptors (PRRs) like Toll-like receptors (TLRs) (*Aderem and Ulevitch, 2000; Reis e Sousa, 2001; Takeda and Akira, 2004; Wilson et al., 2006*).

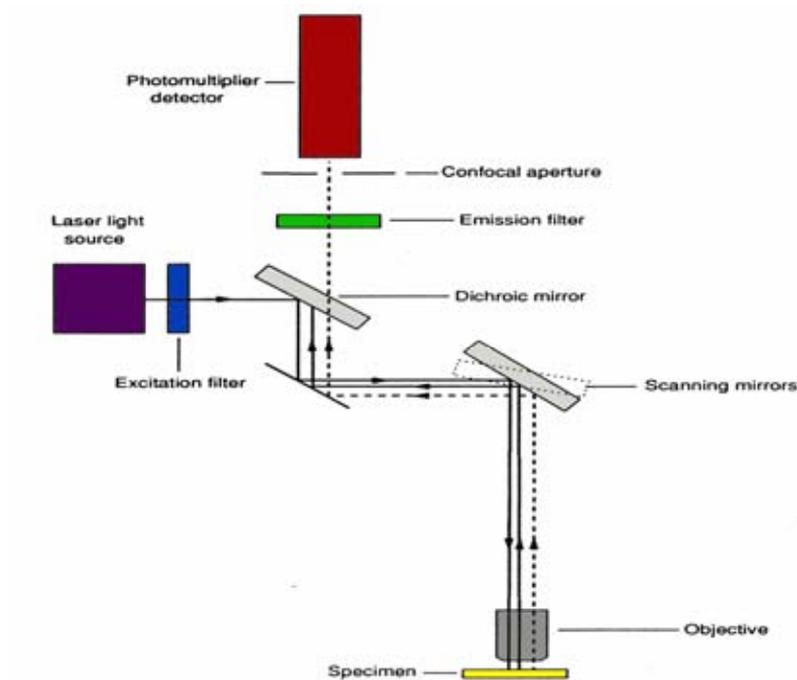
Immature DC internalize antigen via macropinocytosis, receptor-mediated endocytosis and phagocytosis (*Nagl et al., 2002; Rescigno, 2002*). Macropinocytosis and phagocytosis are related processes, both depend on regulated F-actin remodelling and thus on the activity of the Rho family of GTPases, i.e. Cdc42 and Rac (*Garrett et al., 2000; West et al., 2000*). From an evolutionary perspective, bacterial and other particles ingested via phagocytosis is probably the basic form of antigen handling by DC, while the maturation process is essential for DC to become fully capable of stimulating T cells (*Mellman and Steinman, 2001*).

### ***Confocal Laser Scanning Microscopy (CLSM)***

CLSM is a useful technique for visualizing intracellular structures labelled with fluorescent molecules (*Rawlins, 1995*). In conventional fluorescence microscopy of whole cells, the emitted fluorescent light arises not only from molecules in focus, but also from molecules above and below the plane of focus. This makes it difficult to determine the actual three-dimensional molecular arrangement. CLSM only detects fluorescent molecules in the plane of focus (Figure 11).

The confocal microscope generates thin optical sections (0-6  $\mu\text{m}$ ) with negligible out-of focus fluorescence. Usually, a focused laser beam is used as excitation light, which then illuminates a small volume of the specimen at a time. The intensity of the emitted light from the volume is detected,

stored in a computer, and displayed for each pixel (picture element) on a monitor (*Carlsson and Åslund, 1987; Holmgren Peterson et al., 1995*).



*Figure 11*

*The components of a Confocal Laser Scanning Microscope (CLSM) (from Rawlins, 1995).*

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The emitted fluorescence light is detected only from the small volume owing to a pinhole, a small aperture, in front of the detector. A horizontal x-y plane in the specimen is scanned by the laser beam, moved by a galvanometer-driven mirror. In the z direction, a stepping motor allows focusing on different x-y planes of the sample. As well as x-y planes, vertical x-z planes can be scanned. The lateral resolution (x-y) is better than the vertical (z), around 0.2 and 0.6  $\mu\text{m}$ , respectively, using a high numerical aperture (x60; N.A. = 1.4) objective (*Carlsson and Åslund, 1987; Holmgren Peterson et al., 1995*).

The overall aim of the thesis was to investigate the molecular mechanisms operating in the establishment of an infection with *Leishmania donovani*.

**The work was focused on:**

- the effects of LPG on phagocytosis and phagosome maturation in relation to effects on F-actin dynamics in the host macrophage.
- the role of free calcium for phagocytosis, phagosome maturation and the actions of LPG, with special emphasis on F-actin dynamics in the host macrophage.
- the importance of protein kinase C alpha (PKC $\alpha$ ) as a regulator of F-actin dynamics during phagocytosis, and as a possible target for LPG.
- the effects of LPG on dendritic cells, particularly on maturation and detachment to distinct surfaces.



# *MATERIALS AND METHODS*

## *Culturing of J774 cells.*

The murine macrophage cell line J774.A1 was cultured at 37°C in Ham's F-10 medium with Gluta-MAX, 10% heat-inactivated (30 min, 56°C), sterile-filtered fetal calf serum (FCS), 100 U/ml penicillin, and 100 µg/ml streptomycin (all from Gibco). The cells were passaged weekly, and cells older than 15 passages were not used. For phagocytosis experiments, the cells were grown on sterile glass coverslips for two days to approximately 50% confluence (paper I and II).

## *Culturing of RAW 264 cells.*

Four types of RAW 264.7 cells (murine macrophage cell line) were used: untransfected cells, cells stably transfected with the empty expression vector pCIN-4 (control), and two independent clones (A2 and B1), overexpressing dominant-negative (DN) PKC $\alpha$  (K368D) (*St-Denis et al., 1998*). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4 mM L-glutamine, 10% heat-inactivated FCS, 10 mM HEPES, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Gibco). The culture medium for the transfected clones was further supplemented with 500 µg/ml G418 (Gibco). Cells older than 10 passages were not used. For experiments the cells were cultured on glass coverslips over-night to approximately 75% confluence (paper III).

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### ***Monocyte isolation***

Human peripheral blood mononuclear cells (PBMC) were isolated from buffy coats from healthy donors and separated according to Böyum, 1968. After sedimentation on a dextran gradient (Lymphoprep), the cells were spun down in a swing-out rotor for 30 min at 400 x g at 4°C followed by brief hypotonic lyses. After that the cells were harvested and washed repeatedly in ice-cold calcium-free KRG (120 mM NaCl, 4.9 mM KCl, 1.2 mM MgSO<sub>4</sub>, 8.3 mM KH<sub>2</sub>PO<sub>4</sub> and 10 mM glucose) to remove density gradient residues and platelets.

After the final wash, PBMCs were isolated by negative selection using a cocktail of biotin-conjugated antibodies to CD3, CD7, CD19, CD45RA, CD56 and IgE, respectively, and MACS CD14 microbeads coupled to anti-biotin monoclonal antibodies (Miltenyi Biotec). The resulting monocyte-enriched fractions were used to generate DC (paper IV).

### ***Human monocyte-derived dendritic cells (Mo-DC)***

Cells from the monocyte-enriched fractions were grown in Iscove's modified Dulbecco's medium (IMDM) supplemented with 4 mM L-glutamine, 10% inactivated FCS, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco).

The cells were seeded onto sterile glass cover slips in cell culture plates (Nunc) at 4 x 10<sup>6</sup> cells/well and left to adhere for 2 h at 37°C in 5% CO<sub>2</sub>. Non-adherent cells were removed by washing with 1 mM CaCl<sub>2</sub> KRG, at 37°C. Mo-DC were generated by culturing the cells in 1 ml of IMDM containing 1000 U/ml recombinant human GM-CSF and 500 U/ml recombinant human IL-4 (R&D Systems). The cytokines were added to

the cultures at day 0 and day 3 to generate immature DC at day 5-6. 1000 U/ml recombinant human TNF- $\alpha$  (R&D Systems) was added at day 6 for progression of the cells into mature Mo-DC at day 9-10. (paper IV) (*Banchereau et al., 2000; Bender et al., 1998; Fonteneau et al., 2001; Romani et al., 1996; Sallusto et al., 1995*).

### ***Characterization of Mo-DC with flow cytometry***

Flow cytometry (FACSCalibur, Becton-Dickinson) was used to analyze cell-surface antigen expression of a variety of leukocyte markers. Usually,  $1 \times 10^6$  cells were incubated for 30 min on ice with either fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-human antibodies to CD68 or CD86 (Dakopatts), or phyco-erythrin (PE)-conjugated monoclonal anti-human antibody to CD80 (Becton-Dickinson). Control cells were processed similarly using FITC- or PE-matched mouse isotype control IgG<sub>1</sub> or IgG<sub>2</sub>. The cells were pelleted and resuspended in 2% (w/v) paraformaldehyde (PFA; Sigma-Aldrich).

Gates were set to discern Mo-DC. The mean fluorescence of at least 1000 gated cells was determined (*Duperrier et al., 2000; Ebner et al., 2001; Rouard et al., 2000; Strobl et al., 1998*). The results were analyzed using the WinMDI-Program (Version 2.8, 227 Joseph Trotter, Scripps Research Institute, La Jolla, CA) (paper IV).

### ***Characterization of Mo-DC by immunofluorescence***

Fixed immature and mature Mo-DC were permeabilized, and incubated for 45 min on ice with a FITC-conjugated monoclonal anti-human antibody to CD86 (Dakopatts) diluted 1:100 in PBS, pH 7.6 with 1%

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bovine serum albumin (BSA; Boehringer-Mannheim) and 10% normal goat serum (Dakopatts). Control cells were processed using mouse isotype control IgG<sub>1</sub>.

After washing, incubation was continued for 45 min on ice with Alexa594Fluor-conjugated goat anti-mouse antibody (Molecular Probes) diluted 1:200 in PBS with 1% BSA (200  $\mu$ l/cover slip). The labeled cells were further washed three times in PBS and mounted in an anti-fading medium: 20% Airvol 203 (Air Products, Utrecht, The Netherlands) and 4% Citifluor-Glycerol (Citifluor Ltd, London, UK) in 20 mM Tris buffer, pH 8.5 (paper IV).

### ***Manipulation of extra- and intracellular calcium***

To chelate intracellular calcium, J774 cells were washed in KRG (pH 7.3) and then loaded with MAPT/AM (Calbiochem) diluted 1:1000 in KRG (final volume 1ml) for 30 min at 37°C, with 5% CO<sub>2</sub>. Control cells were washed and incubated in KRG for the same period of time. After the incubation, the cells were washed in J774 culture medium supplemented with 1 mM ethylene glycol-bis( $\beta$ -aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA, pH 7.3), designated J774/EGTA, to remove extracellular calcium (paper II).

### ***Parasites***

*Leishmania donovani* promastigotes (Sudanese strain 1S), derived from amastigotes isolated from the spleen of an infected hamster were grown at 26°C in modified M199 medium (Gibco), as described previously (Holm *et al.*, 2001). WT promastigotes and the mutant strains *lpg1*-KO and *lpg2*-KO

were transfected with green fluorescent protein (GFP) by electroporation of the plasmid pXG-GFP<sup>+</sup> (Holm *et al.*, 2001; Sean Ha *et al.*, 1996). The promastigotes were cultured to stationary phase before use (paper I and IV) (Figure 12).

### ***LPG-coating of yeast***

Heat-killed yeast cells (*Saccharomyces cerevisiae*) were labeled with FITC in 0.2 M carbonate buffer, pH 9.5 for 60 min, washed and resuspended in PBS. The FITC-labeled yeast cells were coated with purified *L. donovani* LPG at a final concentration of 25 mM in PBS pH 7.3 by incubation at 37°C for 30 min. The efficiency of the LPG-coating was confirmed by immunofluorescence using the anti-LPG antibody CA7AE (Cedarlane Laboratories, Hornby, Ontario, Canada) (paper I, II, III and IV).

### ***Serum- and IgG -opsonisation of yeast***

Heat-killed yeast cells labeled with FITC were opsonised with 25% normal human serum (NHS) or with rabbit anti-yeast antibody (20 µg/ml) in 25% heat-inactivated normal human serum (IgG opsonization), and incubated at 37°C for 30 min (paper I, II and III).

### ***Type I collagen-coated surfaces***

Collagen R (type I from rat) was diluted 1:10 to 2 mg/ml in distilled water, was added to sterile glass cover slips in cell culture plates (Nunc) and left to adsorb over night at 4°C, followed by washing in PBS (paper IV).

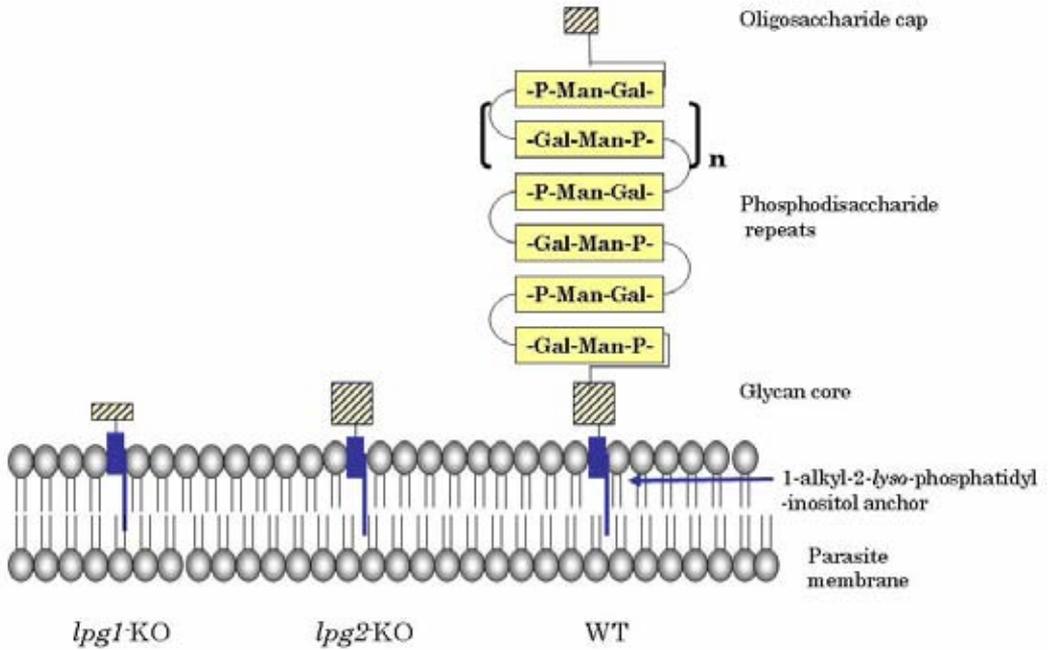


Figure 12

Structure of WT *L. donovani* LPG and truncated LPG of the *lpg1-KO* and *lpg2-KO* mutants. The *lpg1-KO* mutant expresses the truncated LPG,  $[Glc(\alpha 1-P)]Man(\alpha 1,3)Man(\alpha 1,4)GN(\alpha 1,6)-PI$ , and synthesizes repeating units. The *lpg2-KO* mutant is unable to synthesize repeating units and expresses the truncated LPG,  $Gal(\alpha 1,6)Gal(\alpha 1,3)Gal(\beta 1,3)[Glc(\alpha 1-P)]Man(\alpha 1,3)Man(\alpha 1,4)GN(\alpha 1,6)-PI$ . Gal, galactose; Glc, glucose; GN, glucosamine; Man, mannose; P, phosphate.

### ***Phagocytosis***

In paper I, *L. donovani* promastigotes were used for phagocytosis experiments. Stationary phase promastigotes were pelleted and resuspended in the same volume of fresh promastigote culture medium the day before the experiment.

On the day of the experiment, the promastigotes were again pelleted, resuspended in fresh macrophage culture medium at 37°C, and added to the macrophages at a parasite-cell ratio of 10:1.

In paper IV, on the day of the experiment, the promastigotes were pelleted, resuspended in fresh DC culture medium at 37°C and added to the Mo-DC at a parasite-cell ratio of 10:1 for WT, and 5:1 for *lpg2*-KO promastigotes to compensate for the reduced uptake of WT promastigotes compared to the mutant.

In paper I, II, III and IV, FITC-labelled heat-killed yeast, uncoated or coated with LPG, was added to the cells at a ratio of 1:1. The J774 cells were initially exposed to the promastigotes or to the yeasts at 37°C for 20 min (pulse). For RAW264.7 cells and Mo-DC cells a 30-min pulse was used. This incubation was followed by careful washing to remove unbound prey, and incubation was continued at 37°C and 5% CO<sub>2</sub> for 0, 10, 30 or 60 min for promastigotes, and 0 or 10 min for yeast, respectively (chase).

In paper II, for the calcium-depletion experiments, the yeast cells were suspended in J774 medium, or J774 medium with EGTA, and added to the macrophages at a ratio of 1:1. The J774 cells were exposed to the yeasts at 37°C for 20 min for unopsonized yeasts or 10 min for opsonized

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yeasts (pulse), and then washed with J774 medium or J774 medium/EGTA to remove unbound yeast particles. Incubation was then continued at 37°C for 0 or 10 min, respectively (chase).

### ***Labeling of F-actin***

The macrophages were fixed for 15 min at 4°C in freshly made 2% (w/v) PFA in KRG. The fixed cells were washed in KRG with 1% BSA designated KB, and incubated in KB for 30 min at room temperature to block unspecific binding of phalloidin (paper I and III).

For the calcium-depletion experiments, the macrophages were fixed for 15 min at 4°C in freshly made 2% PFA diluted in KRG or Ca<sup>2+</sup>-free KRG (PFA/KRG0). The fixed cells were washed in PBS supplemented with 1% BSA, designated PB, and then incubated in PB for 30 min at room temperature (paper II).

The Mo-DC were fixed for 15 min at 4°C in freshly made 2% PFA diluted in KRG. The fixed cells were washed in PB, and then incubated in PB for 30 min at room temperature (paper IV).

All cells were subsequently incubated for 30 min at room temperature with Alexa594 Fluor-phalloidin (Molecular Probes, Eugene, OR) to stain F-actin. Phalloidin from a stock solution (200 U/ml in methanol, kept at -20°C) was diluted 1:40 in PBS supplemented with 100 µg/ml lysophosphatidylcholine (Sigma-Aldrich) as a membrane permeabilizing agent.

After labeling, the cells were washed three times in PB and mounted in anti-fading medium as described below (paper I, II, III and IV).

***Labeling of PKC $\alpha$  and LAMP1***

Fixed macrophages were permeabilized, and unspecific binding of the primary antibody was blocked by incubating for 30 min at room temperature in PBS, pH 7.6 supplemented with 2% BSA, 1 mM EGTA, 10% normal goat serum (Dakopatts), and 0.1% saponin (Sigma-Aldrich). This was followed by washing in PBS supplemented with 2% BSA and 1 mM EGTA (buffer A). The preparations were labeled for 45 min at room temperature with monoclonal mouse antibodies against PKC $\alpha$  (P16520; Transduction Laboratories) diluted 1:100 in A supplemented with 10% normal goat serum or monoclonal rat antibodies against LAMP1 (1DB4; Developmental Studies Hybridoma Bank, Iowa City, IA) diluted 1:200 (paper I, II and III).

The cells were then washed three times in buffer A and incubated for 45 min at room temperature with 200  $\mu$ l/cover slip of Alexa594 Fluor-conjugated or Alexa488 Fluor-conjugated goat anti-rat or anti-mouse antibodies (Molecular Probes) diluted 1:400 in A, followed by washing and mounting as described above.

***Labeling of Cd86, CD11b and CD51***

Mo-DC were fixed, washed three times in PBS (pH 7.6), labeled for 45 min on ice with monoclonal mouse antibodies against CD11b and CD51 (both from Boehringer-Mannheim) diluted 1:50 in PBS, pH 7.6 with 1% BSA and 10% normal goat serum. The cells were then washed three times in PBS and incubated for 45 min on ice with Alexa594 Fluor-conjugated goat anti-mouse antibodies diluted 1:200 in PBS with 1% BSA, washed three times in PBS and mounted as described above (paper IV).

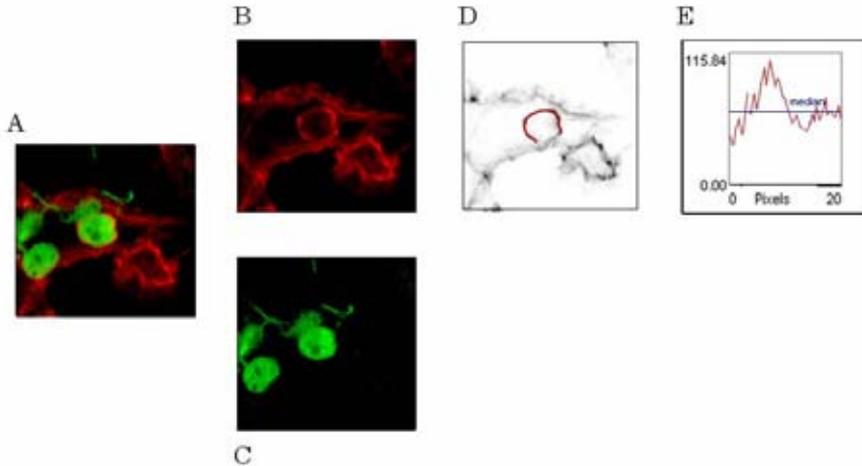
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### ***Confocal Laser Scanning Microscopy (CLSM)***

Imaging was performed with a Sarastro 2000 confocal laser scanning microscope (CLSM; Molecular Dynamics, Sunnyvale, CA), equipped with a Nikon microscope and a x60, high numerical aperture (NA 1.4) oil immersion objective. The 514-nm line of the Argon laser was used for simultaneous excitation of FITC/Alexa488 Fluor and Alexa594 Fluor. Dichroic mirrors with cut-off wavelengths of 535 and 595 nm were employed for the excited and emitted light, respectively. A 540DF30-nm band-pass filter was used to detect the green signal (FITC/Alexa488 Fluor) and a 600-nm long-pass emission filter for the red signal (Alexa594 Fluor). This filter set-up ensured negligible detection of the red signal in the green channel and vice versa (paper I, II, III and IV) (Figure 13).

### ***Quantification of phagocytosis***

Phagocytic capacity was assessed after a 10-min or a 20-min chase to allow maximal internalization of the prey. Randomly scanned confocal sections of cells labeled with Alexa594-phalloidin were scanned and the number of fluorescent prey ingested per macrophage or Mo-DC was counted. In paper I, II and III, double-blind measurements were done to ensure unbiased assessment.



*Figure 13*

*Image analysis for quantification of periphagosomal F-actin.*

*A. Dual detector confocal images of J774 cells interacting with GFP-transfected WT promastigotes. B. Red channel, F-actin. C. Green channel, WT promastigotes. D. Tracing of the F-actin surrounding the part of the phagosomes facing the cytosol. E. The fluorescence intensity profile and median intensity of the tracing shown in D.*

### ***Quantification of periphagosomal F-actin***

Periphagosomal F-actin was measured on randomly scanned confocal images of Alexa594 Fluor-phalloidin-labeled macrophages containing fluorescent prey. The F-actin rim around a phagosome was traced manually in the red channel of the confocal image, the fluorescence

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intensity profile was recorded, and the median intensity of the profile was calculated.

To avoid overlaps with cortical F-actin, only the F-actin on the part of the phagosome facing the cytosol was studied. Double-blind measurements were carried out to ensure unbiased assessments. Cortical F-actin was assessed in the same way, except that the cortical actin was traced.

All confocal images were recorded with the exact same instrument settings and acquisition parameters. The cells were focused in white light to avoid bleaching of the fluorophores and biased selection of certain sections of the preparations. Image analysis was performed on a Silicon Graphics OS2 workstation using ImageSpace v3.2 software (Molecular Dynamics, Sunnyvale, CA).

Statistical analysis was done with Student's t-test (paper I, II, III and IV).

### ***Quantification of total F-actin and expression of CD86, CD11b and CD51***

Analyses of total F-actin and expression of integrins were made in a standard fluorescence microscope (Zeiss Axioskop) using a x63 oil immersion objective with a numerical aperture of 1.4. Light microscopy images were captured with a CCD camera with a ZVS-47E amplifier (Zeiss), visualised by Easy Image Analysis 2000 (Ver. 2.7.2.2, Tekno Optik Ab, Stockholm, Sweden) and saved in Tiff format. A 496DF10nm (Texas Red, Chroma) band-pass filter was used for detection of the red signal (Alexa594 Fluor). All images were recorded with exactly the same instrument settings and acquisition parameters.

Images were digitally analysed using Scion Image Software for Windows (Ver. Beta 4.0.2, Scion Corp.). The rim around each Mo-DC was traced

manually with an Intuos3 Pen Tablet (WACOM) to acquire the cell area and mean staining intensity. The area of the Mo-DC was multiplied with the mean F-actin or integrin staining intensity. The calculations were carried out on Mo-DC from duplicate preparations. The results were compiled from data on samples from three separate experiments performed on different days. To compensate for possible day-to-day variation in instrument performance, all results were normalized against parallel data from controls (paper IV).

### ***Quantification of Mo-DC adhesion to different surfaces***

The adhesion of Mo-DC which had engulfed WT or *lpg2*-KO promastigotes was investigated after a 24 h incubation using a standard fluorescence microscope. The preparations were fixed and labelled with fluorescent phalloidin to visualize the cells. The mean number of adherent Mo-DC in 20 random fields of view was assessed. The experiment was repeated three times on separate days (Paper IV).

### ***Analysis of translocation of LAMP1 to phagosomes***

The translocation of LAMP1 to individual phagosomes was investigated on randomly scanned confocal images of cells that had phagocytosed uncoated or LPG-coated yeast for 20 + 10 min. After identification of a phagosome in a confocal image, translocation of LAMP1 to the phagosome was classified as negative (-; no translocation), positive (+) or intermediary (+/-) (paper II and III).

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## ***Electrophoresis and immunoblotting***

The cells were grown over-night in 6-well plates (NUNC) and washed once in PBS, pH 7.3 before homogenization in 400 µl/well of boiling sample buffer containing 60 mM Tris-HCl, 2% sodium dodecyl sulphate (SDS), 5% 2-mercaptoethanol, 25% glycerol and 0.1% bromphenol blue (all from Sigma-Aldrich). The preparations were frozen in  $-20^{\circ}\text{C}$ , and then heated to  $95^{\circ}\text{C}$  for 5 min before fractionation. Samples and a positive control for PKC $\alpha$  (Transduction Laboratories) were subjected to 10% SDS-acrylamide gels (BMA) for 2 h at 20 mA. The gel and methanol-treated PVDF-membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK) were equilibrated in blotting buffert (10% Tris-glycin; Bio-Rad Laboratories), 20% methanol and 70% distilled H<sub>2</sub>O) and electroblotting was performed overnight at  $4^{\circ}\text{C}$ .

After transfer, the membranes were blocked with 1% BSA and 1% powdered milk in Tween TBS (TRIS and NaCl in distilled H<sub>2</sub>O, pH 7.3 with 0,05% Tween-20) for 1 h. They were then exposed to mouse anti-PKC $\alpha$  (Transduction Laboratories) diluted 1:1000 in Tween-TBS with 1% BSA, or goat polyclonal anti-actin (I-19, Santa Cruz Biotechnology) for 3 h on a rocking table followed by washing in Tween-TBS. The membranes were incubated with secondary antibody (rabbit-anti goat or goat anti-mouse, conjugated to horse radish peroxidase (HRP; Dakopatts) for 1 h and washed in Tween-TBS. The membranes were developed with the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech) (paper III).

***Scanning electron microscopy (SEM)***

Immature Mo-DC grown on cover slips were used on day 6. The cells were first incubated for 20 min with promastigotes at 37°C and 5% CO<sub>2</sub> (pulse) and washed three times to remove unbound prey. Incubation was continued at 37°C for 10 min or 60 min followed by rinsing twice in 0.15 M sodium cacodylate buffer (Link Nordiska). The cells were fixed in 2% glutaraldehyde (Link Nordiska), diluted in 0.15 M sodium cacodylate buffer, rinsed several times in the same buffer, and postfixed for 1 h in 1% osmium tetroxide (Link Nordiska), and diluted in 0.15 M sodium cacodylate buffer. The specimens were dehydrated in a graded series of 50-100% ethanol, critical point dried from CO<sub>2</sub>, and sputter-coated with platinum.

Digital micrographs were obtained from a LEO 1550 GEMINI FEG high-resolution scanning electron microscope (LEO Electron Microscope) operated at 5 kV and a tilt of 30° (*Lindroth et al., 1991*).



# RESULTS AND DISCUSSION

## *Paper I*

*Leishmania donovani* lipophosphoglycan causes periphagosomal actin accumulation: correlation with impaired translocation of PKC $\alpha$  and defective phagosome maturation.

Paper I is focused on the molecular mechanisms by which LPG prevents phagolysosome formation in macrophages and promotes intracellular survival of *L. donovani*. We first investigated whether LPG affected the dynamics of periphagosomal F-actin. We observed that was indeed the case, and that the accumulation correlated with an inhibited translocation of the late endosomal marker LAMP1 to the phagosomes. This suggested a causal relationship between the effects of LPG on periphagosomal F-actin and blockage of phagosome maturation. We also investigated a possible relationship between the disturbance in actin turnover and inhibited translocation of PKC $\alpha$  to the phagosomes, and whether LPG also affected the turnover of cortical F-actin.

The host cells were cultured J774 macrophages and the prey WT *L. donovani* promastigotes (expressing LPG) and two isogenic mutants defective in disaccharide-phosphate repeat synthesis (*lpg2*-KO and *lpg1*-KO). These mutants are unable to inhibit phagolysosome maturation (Holm *et al.*, 2001).

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Moreover, LPG-coated yeast particles were employed as prey and an *lpg2*-KO add-back mutant, in which LPG repeating unit synthesis was restored by transfection of the *LPG2* gene (*lpg2*-KO 1 p*LPG2*). The results show that the rim of periphagosomal F-actin initially found around all phagosomes progressively disappeared around phagosomes containing either uncoated yeast particles or the *lpg2*-KO mutant, which do not synthesize the repeating units of LPG. The *lpg1*-KO mutant, which lacks surface repeating units but secretes repeating unit-containing molecules other than LPG, induced a small transient increase in periphagosomal F-actin, which soon returned to the same low level found around *lpg2*-KO. In contrast, F-actin increased progressively over time around phagosomes containing WT promastigotes, to reach 430 % of the control (*lpg2*-KO) at 60 min. The same phenomenon was observed around phagosomes containing LPG-coated yeast and the add-back *lpg2*-KO 1 p*LPG2*. These results show that disassembly of periphagosomal F-actin normally occurs in macrophages as well as in neutrophils. Moreover LPG not only inhibits this process, but also induces an F-actin accumulation around the phagosomes.

PKC $\alpha$  normally translocates to the periphagosomal area in macrophages where it may participate in the breakdown of periphagosomal F-actin through phosphorylation of MARCKS proteins (*Allen and Aderem, 1995b*). It is also known that PKC $\alpha$  plays a role in the control of infection by *L. donovani* (*St-Denis et al., 1999*). Insertion of LPG into one membrane leaflet reduces PKC association to the opposite leaflet, blocks the activity of membrane-associated PKC $\alpha$  (*Giorgione et al., 1996*) and inhibits PKC-mediated phosphorylation of MARCKS (*Descoteaux et al., 1992*). When

investigating the translocation of PKC $\alpha$  to yeast phagosomes in J774 cells, much less PKC $\alpha$  was found around phagosomes containing LPG-coated yeasts compared to uncoated yeast. There was also an inverse correlation between the amount of PKC $\alpha$  in the phagosomal area and periphagosomal F-actin. This indicates that LPG inhibits the translocation/association of PKC $\alpha$  to phagosomes and thereby blocks F-actin breakdown.

We found a much smaller association of LAMP1 to phagosomes containing LPG-coated yeast being surrounded by a rim of F-actin, compared to normal yeast phagosomes with little or no periphagosomal F-actin. Periphagosomal F-actin might directly interfere with the recruitment of signal transducers and vesicles to the phagolysosome, thus contributing to the arrest of phagolysosome maturation. Since LPG transfers from the promastigote to the plasma membrane of the host cell upon attachment (*Duque-de-Mello et al., 1999; Tolson et al., 1990*), it could be expected that LPG also affects the turnover of cortical actin. Our results show that cortical F-actin was indeed significantly higher in cells interacting with WT promastigotes compared to *lpg2*-KO mutants.

Together, the results show that F-actin accumulates around phagosomes containing LPG-coated prey, and that this directly correlates with defective recruitment of PKC $\alpha$  and inhibited phagosomal maturation.

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## ***Paper II***

*Phagocytosis and phagosome maturation are regulated by calcium in J774 macrophages interacting with un-opsonized prey.*

Paper II focuses on the role of calcium for uptake of prey and phagosome maturation in macrophages, and on how calcium chelation influences the effects of LPG. In paper I we showed that LPG had detrimental effects on actin turnover and phagosomal maturation in J774 cells. It was suggested that the effect was mediated through inhibition of calcium-dependent enzymes, e.g. PKC $\alpha$ . In Paper II J774 macrophages were used as host cells, and unopsonized yeast, yeast coated with LPG, serum- or IgG-opsonized yeast was used as prey. Intra- and extracellular calcium was chelated with MAPTA and EGTA, respectively.

We show here that chelation of *both extra- and intracellular* calcium inhibited the uptake of unopsonized yeast, albeit to a lesser extent in the latter case. Phagocytosis of LPG-coated yeast was reduced compared to unopsonized yeast in the presence of calcium, which agrees with our other results (*Holm et al., 2001*). After chelation of extracellular calcium, uptake of LPG-coated yeast was reduced to the same level as observed with uncoated yeast. This suggests that the LPG effect is Ca<sup>2+</sup>-dependent. This is opposite to the effect of Ca<sup>2+</sup>, on phagocytosis in neutrophils (*Jaconi et al., 1990*). Removal of calcium had no impact on the ingestion of IgG-opsonized yeast, but it decreased the uptake of serum-opsonized yeast. This is likely due to reduced binding of the C3b-ligand to CR3 in the absence of calcium.

The results stress that extra- and intracellular free calcium co-operate during normal breakdown of periphagosomal F-actin around unopsonized yeast. This is interesting, since the interaction of PKC $\alpha$  with cholesterol-rich structures (e.g. caveolae, rafts) in the plasma membrane is calcium-dependent (*Mineo et al., 1998*).

To investigate a possible correlation between inhibited breakdown of periphagosomal F-actin and phagosomal maturation, we studied the translocation of LAMP1 to individual phagosomes under Ca<sup>2+</sup>-free conditions. Translocation of LAMP1 occurred to 67% of the phagosomes in control cells, but only to 36% of the phagosomes in calcium-depleted cells. This indicates that the presence of free calcium is necessary for maturation of phagosomes containing unopsonized prey. After chelation of extracellular or both extra- and intracellular calcium, there was no longer any accumulation of F-actin around phagosomes containing LPG-coated yeast. In fact, the amount of F-actin remained as high as around unopsonized yeast phagosomes. The lack of accumulation of F-actin around LPG-containing phagosomes under calcium-depleted conditions further emphasizes that extracellular free calcium is necessary for the effects of LPG.

This notion is supported by the finding that the extracellular disaccharide-phosphate repeats of the LPG molecule chelates calcium (*Turco and Descoteaux, 1992*). *Leishmania donovani* furthermore proliferates more slowly and is more efficiently cleared from host macrophages in the absence of extracellular calcium (*Mbati et al., 1994*).

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Together, the results display that calcium is important for phagocytosis mediated via lectin-like and complement receptors in macrophages. Moreover, calcium is involved in the regulation of breakdown of periphagosomal F-actin and phagosomal maturation in macrophages interacting with unopsonized prey. Calcium is also required for the effects of LPG.

***Paper III.***

*Role of protein kinase C  $\alpha$  for uptake of unopsonized prey and phagosomal maturation in macrophages.*

Paper III is focused on the role of PKC $\alpha$  in the regulation of F-actin turnover in relation to phagocytosis and phagosomal maturation. This was addressed by employing two independent clones of RAW 264.7 macrophages (A2 and B1) overexpressing a dominant-negative mutant of PKC $\alpha$ . The effect of overexpression of DN PKC $\alpha$  was compared to the influence of LPG.

In both clones overexpressing DN PKC $\alpha$  there was increased amounts of cortical F-actin compared to control cells containing the empty expression vector. The DN PKC $\alpha$  macrophages also displayed a more rounded morphology and fewer filopodia than the control cells. Clone A2, which had the highest expression of immunoreactive PKC $\alpha$ , also displayed most cortical F-actin, and the most altered morphology. The expression of total (i.e., globular + filamentous) actin was comparable in all cell lines.

The DN PKC $\alpha$ -overexpressing macrophages showed a markedly decreased phagocytic capacity compared to controls, similar to that of control cells subjected to LPG-coated yeast. Their capacity to ingest LPG-coated yeast was almost zero. Uningested prey, was frequently observed in phagocytic cups rich in F-actin. These results were further confirmed using WT and *lpg2-KO L. donovani* promastigotes as prey. One interpretation of the findings is that LPG inhibited residual endogenous PKC $\alpha$  activity, or other PKC isoenzymes involved in the regulation of phagocytosis.

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The kinetics of periphagosomal F-actin around phagosomes containing *lpg2*-KO or WT *L. donovani* promastigotes was also studied. Similar or elevated F-actin levels were seen around both types of prey in the A2 clone, which mimicked the F-actin levels observed around phagosomes containing WT promastigotes in control cells.

Thus, LPG-containing prey did not influence the phagosomal F-actin turnover in DN PKC $\alpha$  overexpressing macrophages. This is reasonable, if LPG-coated prey is phagocytosed only by macrophages expressing relatively little DN PKC $\alpha$ . The hypothesis is supported by the incidental observation that the few DN PKC $\alpha$ -overexpressing cells ingesting WT promastigotes, also displayed overall reduced staining with phalloidin compared to surrounding cells.

A defective translocation of LAMP1 to yeast-containing phagosomes in the DN PKC $\alpha$ -overexpressing clone A2 was also observed, conforming that PKC $\alpha$  is important for normal phagosomal maturation.

Together, the results show that phagocytosis of un-opsonized prey, periphagosomal actin dynamics and phagosomal maturation are controlled either directly or indirectly by PKC $\alpha$  in macrophages.

***Paper IV***

*Leishmania donovani* promastigotes block maturation, increase integrin expression and inhibit detachment of human monocyte-derived dendritic cells– a role for lipophosphoglycan (LPG).

In paper IV, the aim was to investigate whether *L. donovani* promastigotes affect Mo-DC maturation and detachment to distinct surfaces. Mo-DC maturation was examined with flow cytometry and/or indirect immunofluorescence. Maturation induced by TNF $\alpha$  resulted in increased expression of the co-stimulatory molecule CD86, an up-regulation of CD80 and concomitant down-regulation of CD68. Changes in Mo-DC morphology and F-actin were also observed.

Our results further show that immature Mo-DC differentiated *in vitro*, can be infected with WT and *lpg2*-KO promastigotes. These were found at various stages of internalization in the preparations. During engulfment of *lpg2*-KO promastigotes, a parasite sometimes appeared to be “sinking” into the Mo-DC. WT promastigotes were internalized by immature Mo-DC in at least three different ways, by formation of a narrow tube-like pseudopod around the parasite, by covering it with a smooth membrane or by wrapping the parasite in a broad pseudopod.

We have previously shown that both virulent *L. donovani* promastigotes carrying full-length LPG, or yeast particles coated with LPG, are phagocytosed to a lesser extent compared to control prey (Holm *et al.*, 2001). The LPG-positive prey induced significantly more polymerization of F-

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actin close to the plasma membrane during the uptake process (Holm et al., 2001). It is likely that hyperpolymerization of cortical F-actin renders a less flexible, and therefore less motile cell. Such a cell would, hypothetically, also have decreased phagocytic capacity. When the effect of LPG on F-actin was analyzed in immature Mo-DC, we found an increased level of F-actin compared to controls.

CD86 is a recognized marker of Mo-DC maturation (Banchereau and Steinman, 1998; Lee et al., 2002). We found a lower level of CD86 expression in cells containing WT promastigotes compared to *lpg2*-KO mutants. The inhibition of CD86 up-regulation by promastigotes may thus be affected by functional LPG.

Maturation of Mo-DC is accompanied by a loss of adhesion to surrounding tissue (Burns et al., 2004). Immature Mo-DC containing WT promastigotes showed 81 % higher degree of adhesion to glass compared to positive controls, i.e. cells induced to mature by TNF- $\alpha$ . Our results also show that Mo-DC detachment from both glass and type I collagen was reduced when the cells had been incubated with WT promastigotes for 24 h, as compared to *lpg2*-KO mutants.

Integrin-mediated adhesion and detachment are important features in cell migration. Podosome assembly is associated with  $\beta$ 2-integrin expression and the recruitment of CD11b to podosomes requires collagen I. This suggests a specific role for CD11b/CD18 and CD11c/CD18 in DC adhesion and motility (Burns et al., 2004). In this work we observed an increased expression of CD11b and CD51 on the surface of Mo-DC post interaction with WT *L. donovani* promastigotes.

Together, the results show that phagocytosis of *Leishmania donovani* promastigotes by immature Mo-DC resulted in increased expression of CD11b and CD51, and inhibition of cell detachment from distinct surfaces, and that this was dependent on the presence of LPG. LPG might thus inhibit human DC migration to lymphoid organs.



# CONCLUSIONS

The work on *Leishmania donovani* promastigotes and lipophosphoglycan (LPG) presented in this thesis can be summarized as follows:

- LPG counteracts disassembly and promotes accumulation of periphagosomal F-actin, which correlates with defective recruitment of PKC $\alpha$  to the phagosome and inhibited phagosomal maturation in macrophages.
- LPG inhibits the turnover of cortical F-actin, which in turn may control phagocytosis of promastigotes.
- Phagocytosis of un-opsonized parasites, disassembly of periphagosomal F-actin and phagosomal maturation are controlled by both PKC $\alpha$  and calcium in macrophages.
- LPG inhibits human monocyte-derived dendritic cells (Mo-DC) maturation, increases the expression of the integrins CD11b and CD51, and inhibits Mo-DC detachment from surfaces.



## *REFERENCES*

- Aderem, A. 2002. How to eat something bigger than your head. *Cell* 110:5-8.
- Aderem, A., and D.M. Underhill. 1999. Mechanisms of phagocytosis in macrophages. *Annu Rev Immunol* 17:593-623.
- Aderem, A., and R.J. Ulevitch. 2000. Toll-like receptors in the induction of the innate immune response. *Nature* 406:782-7.
- Alberts, B., A. Johnson, J. Lewis, M. Raff, K. Roberts, and P. Walter. 2002. Cell communication., p. 831-905, *In* M. S. A. a. B. Dileria, ed. *Molecular Biology of The Cell, Fourth Edition* ed. Garland Science, New York.
- Alexander, J., and D.G. Russell. 1992. The interaction of *Leishmania* species with macrophages. *Adv Parasitol* 31:175-254.
- Allen, L.A., and A. Aderem. 1996. Mechanisms of phagocytosis. *Current Opin Immunol* 8:36-40.
- Allen, L.-A.H., and A. Aderem. 1995a. Protein kinase C regulates MARCKS cycling between the plasma membrane and lysosomes in fibroblasts. *EMBO J* 14:1109-1121.
- Allen, L.H., and A. Aderem. 1995b. A role for MARCKS, the alpha isozyme of protein kinase C and myosin I in zymosan phagocytosis by macrophages. *J Exp Med* 182:829-40.

- 
- Ashford, R.W., and P.A. Bates. 1998. p. 215-40 In Topley and Wilson Microbiology and Microbial Infections, Vol. 5. Edwards Arnold, London.
- Asilian, A., A. Sadeghinia, F. Shariati, M. Imam Jome, and A. Ghoddusi. 2003. Efficacy of permethrin-impregnated uniforms in the prevention of cutaneous leishmaniasis in Iranian soldiers. *J Clin Pharm Ther* 28:175-8.
- Aspenström, P. 1999. Effectors of the Rho GTPases. *Curr Opin Cell Biol* 11:95-102.
- Attar, Z.J., M.L. Chance, S. el-Safi, J. Carney, A. Azazy, M. El-Hadi, C. Dourado, and M. Hommel. 2001. Latex agglutination test for the detection of urinary antigens in visceral leishmaniasis. *Acta Trop* 78:11-6.
- Awasthi, A., R.K. Mathur, and B. Saha. 2004. Immune response to *Leishmania* infection. *Indian J Med Res* 119:238-58.
- Banchereau, J., and R.M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature* 392:245-52.
- Banchereau, J., F. Briere, C. Caux, J. Davoust, S. Lebecque, Y.J. Liu, B. Pulendran, and K. Palucka. 2000. Immunobiology of dendritic cells. *Annu Rev Immunol* 18:767-811.
- Barron, T.L., and S.J. Turco. 2005. Quantitation of *Leishmania* lipophosphoglycan repeat units by capillary electrophoresis. *Biochim Biophys Acta*.

- 
- Basu, N.K., L. Kole, A. Ghosh, and P.K. Das. 1997. Isolation of a nitric oxide synthase from the protozoan parasite, *Leishmania donovani*. FEMS Microbiol Lett 156:43-7.
- Bender, A., M. Albert, A. Reddy, M. Feldman, B. Sauter, G. Kaplan, W. Hellman, and N. Bhardwaj. 1998. The distinctive features of influenza virus infection of dendritic cells. Immunobiology 198:552-67.
- Bengtsson, T., M.E.E. Jaconi, M. Gustafsson, K.-E. Magnusson, J.-M. Theler, D.P. Lew, and O. Stendahl. 1993. Actin dynamics in human neutrophils during adhesion and phagocytosis is controlled by changes in intracellular free calcium. Eur J Cell Biol 62:49-58.
- Beverley, S.M., and S.J. Turco. 1998. Lipophosphoglycan (LPG) and the identification of virulence genes in the protozoan parasite *Leishmania*. Trends Microbiol 6:35-40.
- Bhattacharya, S.K., T.K. Jha, S. Sundar, C.P. Thakur, J. Engel, H. Sindermann, K. Junge, J. Karbwang, A.D. Bryceson, and J.D. Berman. 2004. Efficacy and tolerability of miltefosine for childhood visceral leishmaniasis in India. Clin Infect Dis 38:217-21.
- Blackwell, J.M. 1985. Role of macrophage complement and lectin-like receptors in binding *Leishmania* parasites to host macrophages. Immunol Lett 11:227-32.
- Bogdan, C., and M. Rollinghoff. 1999. How do protozoan parasites survive inside macrophages? Parasitol Today 15:22-8.
- Boquet, P., and E. Lemichez. 2003. Bacterial virulence factors targeting Rho GTPases: parasitism or symbiosis? Trends Cell Biol 13:238-46.

- 
- Borisy, G., and T. Svitkina. 2000. Actin machinery: pushing the envelope. *Curr Opin Cell Biol.* 12:104-12.
- Bos, H., and W. de Souza. 2000. Phagocytosis of yeast: a method for concurrent quantification of binding and internalization using differential interference contrast microscopy. *J Immunol Meth* 238:29-43.
- Bouvier, J., P. Schneider, and R. Etges. 1995. Leishmanolysin: surface metalloproteinase of *Leishmania*. *Meth Enzymol* 248:614-33.
- Brown, E.J. 1995. Phagocytosis. *Bioessays* 17:109-17.
- Brown, G.D., P.R. Taylor, D.M. Reid, J.A. Willment, D.L. Williams, L. Martinez-Pomares, S.Y. Wong, and S. Gordon. 2002. Dectin-1 is a major beta-glucan receptor on macrophages. *J Exp Med* 196:407-12.
- Bryceson, A.D.M. 1996. Leishmaniasis, p. 1213-45 In Manson's Tropical Diseases. Bath Press, London.
- Burkhardt, J., L.A. Huber, H. Dieplinger, A. Blocker, G. Griffiths, and M. Desjardins. 1995. Gaining insight into a complex organelle, the phagosome, using two-dimensional gel electrophoresis. *Electrophoresis* 16:2249-57.
- Burns, S., S.J. Hardy, J. Buddle, K.L. Yong, G.E. Jones, and A.J. Thrasher. 2004. Maturation of DC is associated with changes in motile characteristics and adherence. *Cell Motil Cytoskeleton* 57:118-32.
- Carlsson, K., and N. Åslund. 1987. Confocal imaging for 3D digital microscopy. *Appl Optics.* 26:3232-38.

- 
- Carroll, M.C. 1998. The role of complement and complement receptors in induction and regulation of immunity. *Annu Rev Immunol* 16: 545-568.
- Castellano, F., P. Chavrier, and E. Caron. 2001. Actin dynamics during phagocytosis. *Semin Immunol* 13:347-355.
- Chang, K.P., and D.M. Dwyer. 1976. Multiplication of a human parasite (*Leishmania donovani*) in phagolysosomes of hamster macrophages in vitro. *Science* 193:678-680.
- Chaponnier, C., H.L. Yin, and T.P. Stossel. 1987. Reversibility of gelsolin/actin interaction in macrophages. *J Exp Med* 165:97-106.
- Chimini, G., and P. Chavrier. 2000. Function of Rho family proteins in actin dynamics during phagocytosis and engulfment. *Nat Cell Biol* 2:E191-6.
- Chou, Y.H., O. Skalli, and R.D. Goldman. 1997. Intermediate filaments and cytoplasmic networking: new connections and more functions. *Curr Opin Cell Biol* 9:49-53.
- Colucci-Guyon, E., F. Niedergang, B.J. Wallar, J. Peng, A.S. Alberts, and P. Chavrier. 2005. A role for mammalian diaphanous-related formins in complement receptor (CR3)-mediated phagocytosis in macrophages. *Curr Biol* 15:2007-12.
- Danuser, G. 2005. Coupling the dynamics of two actin networks--new views on the mechanics of cell protrusion. *Biochem Soc Trans* 33:1250-3.

- 
- Dermine, J.F., G. Goyette, M. Houde, S.J. Turco, and M. Desjardins. 2005. *Leishmania donovani* lipophosphoglycan disrupts phagosome microdomains in J774 macrophages. *Cell Microbiol* 7:1263-70.
- Descoteaux, A., and S.J. Turco. 1999. Glycoconjugates in *Leishmania* infectivity. *Biochim Biophys Acta* 1455:341-352.
- Descoteaux, A., and S.J. Turco. 2002. Functional aspects of the *Leishmania donovani* lipophosphoglycan during macrophage infection. *Microbes Infect* 4:975-981.
- Descoteaux, A., G. Matlashewski, and S.J. Turco. 1992. Inhibition of macrophage protein kinase C-mediated protein phosphorylation by *Leishmania donovani* lipophosphoglycan. *J Immunol* 149:3008-3015.
- Descoteaux, A., B.J. Mengeling, S.M. Beverley, and S.J. Turco. 1998. *Leishmania donovani* has distinct mannosylphosphoryltransferases for the initiation and elongation phases of lipophosphoglycan repeating unit biosynthesis. *Mol Biochem Parasitol* 94:27-40.
- Desjardins, M. 1995. Biogenesis of phagolysosomes: the 'kiss and run' hypothesis. *Trends Cell Biol* 5:183-186.
- Desjardins, M. 2003. ER-mediated phagocytosis: a new membrane for new functions. *Nat Rev Immunol* 3:280-91.
- Desjardins, M., and A. Descoteaux. 1997. Inhibition of phagolysosome biogenesis by the *Leishmania* lipophosphoglycan. *J Exp Med* 185:2061-2068.
- Desjardins, M., and A. Descoteaux. 1998. Survival strategies of *Leishmania donovani* in mammalian host macrophages. *Res Immunol* 149:689-92.

- 
- Desjardins, M., L.A. Huber, R.G. Parton, and G. Griffiths. 1994a. Biogenesis of phagolysosomes proceeds through a sequential series of interactions with the endocytic apparatus. *J Cell Biol* 124:677-688.
- Desjardins, M., J.E. Celis, G. van Meer, H. Dieplinger, A. Jahraus, G. Griffiths, and L.A. Huber. 1994b. Molecular characterization of phagosomes. *J Biol Chem* 269:32194-32200.
- Desjeux, P. 1999. Global control and *Leishmania* HIV co-infection. *Clin Dermatol* 17:317-25.
- Dobrovolskaia, M.A., and S.N. Vogel. 2002. Toll receptors, CD14, and macrophage activation and deactivation by LPS. *Microbes Infect* 4:903-14.
- Duperrier, K., A. Eljaafari, C. Dezutter-Dambuyant, C. Bardin, C. Jacquet, K. Yoneda, D. Schmitt, L. Gebuhrer, and D. Rigal. 2000. Distinct subsets of dendritic cells resembling dermal DCs can be generated in vitro from monocytes, in the presence of different serum supplements. *J Immunol Meth* 238:119-31.
- Duque-de-Mello, M., J.L. Ho, M.A. Vannier-Santos, and P.F.P. Pimenta. 1999. Lipophosphoglycan traffic and degradation in *Leishmania*-infected macrophages. *Mem. Inst. Oswaldo Cruz* 94 (Suppl. II):70.
- Ebner, S., S. Neyer, S. Hofer, W. Nussbaumer, N. Romani, and C. Heufler. 2001. Generation of large numbers of human dendritic cells from whole blood passaged through leukocyte removal filters: an alternative to standard buffy coats. *J Immunol Meth* 252:93-104.

- 
- Emans, N., N.N. Nzala, and M. Desjardins. 1996. Protein phosphorylation during phagosome maturation. *FEBS Lett* 398:37-42.
- Fay, J.W. 1999. Dendritic cells in the treatment of cancer. *BUMC Proc* 11:2-6.
- Finlay, B.B., and S. Falkow. 1997. Common themes in microbial pathogenecity revisited. *Microbiol Mol Biol Rev* 61:136-169.
- Flandin, J.F., F. Chano, and A. Descoteaux. 2006. RNA interference reveals a role for TLR2 and TLR3 in the recognition of *Leishmania donovani* promastigotes by interferon-gamma-primed macrophages. *Eur J Immunol* 36:411-20.
- Fonteneau, J.F., M. Larsson, S. Somersan, C. Sanders, C. Munz, W.W. Kwok, N. Bhardwaj, and F. Jotereau. 2001. Generation of high quantities of viral and tumor-specific human CD4<sup>+</sup> and CD8<sup>+</sup> T-cell clones using peptide pulsed mature dendritic cells. *J Immunol Meth* 258:111-26.
- Garin, J., R. Diez, S. Kieffer, J.F. Dermine, S. Duclos, E. Gagnon, R. Sadoul, C. Rondeau, and M. Desjardins. 2001. The phagosome proteome. Insight into phagosome functions. *J Cell Biol* 152:165-180.
- Garlapati, S., E. Dahan, and M. Shapira. 1999. Effect of acidic pH on heat shock gene expression in *Leishmania*. *Mol Biochem Parasitol* 100:95-101.
- Garrett, W.S., L.M. Chen, R. Kroschewski, M. Ebersold, S. Turley, S. Trombetta, J.E. Galan, and I. Mellman. 2000. Developmental control of endocytosis in dendritic cells by Cdc42. *Cell* 102:325-34.
- Geiger, B., and E. Karsenti. 1997. Cytoskeleton. *Curr Opin Cell Biol* 9:1-3.

- 
- Giorgione, J.R., S.J. Turco, and R.M. Epand. 1996. Transbilayer inhibition of protein kinase C by the lipophosphoglycan from *Leishmania donovani*. Proc Natl Acad Sci USA 93:11634-11639.
- Gold, E.S., D.M. Underhill, N.S. Morrissette, J. Guo, M.A. McNiven, and A. Aderem. 1999. Dynamin 2 is required for phagocytosis in macrophages. J Exp Med 190:1849-56.
- Gorak, P.M., C.R. Engwerda, and P.M. Kaye. 1998. Dendritic cells, but not macrophages, produce IL-12 immediately following *Leishmania donovani* infection. Eur J Immunol 28:687-95.
- Greenberg, S., J. El Khoury, F. Di Virgilio, E.M. Kaplan, and S. Siverstein. 1991. Ca<sup>2+</sup>-independent F-actin assembly and disassembly during Fc receptor-mediated phagocytosis in mouse macrophages. J Cell Biol 113:757-767.
- Hackam, D.J., O.D. Rotstein, A. Schreiber, W. Zhang, and S. Grinstein. 1997. Rho is required for the initiation of calcium signaling and phagocytosis by Fcγ receptors in macrophages. J Exp Med 186:955-66.
- Hall, A. 1998. Rho GTPases and the actin cytoskeleton. Science 279:509-14.
- Hall, L.R., and R.G. Titus. 1995. Sand fly vector saliva selectively modulates macrophage functions that inhibit killing of *Leishmania major* and nitric oxide production. J Immunol 155:3501-6.
- Haltiwanger, R.S., and R.L. Hill. 1986. The isolation of a rat alveolar macrophage lectin. J Biol Chem 261:7440-7444.

- 
- Handman, E., and D.V. Bullen. 2002. Interaction of *Leishmania* with the host macrophage. *Trends Parasitol* 18:332-4.
- Handman, E., L. Schnur, T.W. Spithill, and G.F. Mitchell. 1986. Passive transfer of *Leishmania* lipopolysaccharide confers parasite survival in macrophages. *J Immunol* 137:3608-3613.
- Hart, D.N. 1997. Dendritic cells: unique leukocyte populations which control the primary immune response. *Blood* 90:3245-87.
- Hartwig, J.H., M. Thelen, A. Rosen, P.A. Janmey, A.C. Nairn, and A. Aderem. 1992. MARCKS is an actin filament crosslinking protein regulated by protein kinase C and calcium-calmodulin. *Nature* 356:618-622.
- Herwaldt, B.L. 1999. Leishmaniasis. *Lancet* 354:1191-9.
- Holm, A., K. Tejle, K.E. Magnusson, A. Descoteaux, and B. Rasmusson. 2001. *Leishmania donovani* lipophosphoglycan causes periphagosomal actin accumulation: correlation with impaired translocation of PKC alpha and defective phagosome maturation. *Cell Microbiol* 3:439-47.
- Holm, A., K. Tejle, T. Gunnarsson, K.E. Magnusson, A. Descoteaux, and B. Rasmusson. 2003. Role of protein kinase C alpha for uptake of unopsonized prey and phagosomal maturation in macrophages. *Biochem Biophys Res Commun* 302:653-8.
- Holmgren Peterson, K., K.E. Magnusson, L. Stenhammar, and K. Falth-Magnusson. 1995. Confocal laser scanning microscopy of small-intestinal mucosa in celiac disease. *Scand J Gastroenterol* 30:228-34.

- 
- Homans, S.W., A. Mehlert, and S.J. Turco. 1992. Solution structure of the lipophosphoglycan of *Leishmania donovani*. *Biochemistry* 31:654-61.
- Horn, S., J. Gopas, and N. Bashan. 1990. A lectin-like receptor on murine macrophage is involved in the recognition and phagocytosis of human red cells oxidized by phenylhydrazine. *Biochem Pharmacol* 39:775-780.
- Ilg, T. 2000. Proteophosphoglycans of *Leishmania*. *Parasitol Today* 16:489-97.
- Jaconi, M.E.E., D.P. Lew, J.-L. Carpentier, K.-E. Magnusson, M. Sjögren, and O. Stendahl. 1990. Cytosolic free calcium elevation mediates the phagosome-lysosome fusion in human neutrophils. *J Cell Biol* 110:1555-1564.
- Kiama, S.G., L. Cochand, L. Karlsson, L.P. Nicod, and P. Gehr. 2001. Evaluation of phagocytic activity in human monocyte-derived dendritic cells. *J Aerosol Med* 14:289-99.
- Kuby, J. 1996. Cells and organs of the immune system, p. 65-66, *In* W. H. F. a. Co, ed. *Immunology*, Third ed, New York.
- Kwiatkowska, K., and A. Sobota. 1999. Signaling pathways in phagocytosis. *Bioessays* 21:422-31.
- Lainson, R., and J.J. Shaw. 1978. Epidemiology and ecology of leishmaniasis in Latin-America. *Nature* 273:595-600.
- Lambrechts, A., M. Van Troys, and C. Ampe. 2004. The actin cytoskeleton in normal and pathological cell motility. *Int J Biochem Cell Biol* 36:1890-909.

- 
- Lee, A.W., T. Truong, K. Bickham, J.F. Fonteneau, M. Larsson, I. Da Silva, S. Somersan, E.K. Thomas, and N. Bhardwaj. 2002. A clinical grade cocktail of cytokines and PGE2 results in uniform maturation of human monocyte-derived dendritic cells: implications for immunotherapy. *Vaccine* 20 (Suppl 4):A8-A22.
- Lew, D.P., T. Andersson, J. Hed, F. Di Virgilio, T. Pozzan, and O. Stendahl. 1985. Ca<sup>2+</sup>-dependent and Ca<sup>2+</sup>-independent phagocytosis in human neutrophils. *Nature* 315:509-11.
- Li, J., and A. Aderem. 1992. MacMARCKS, a novel member of the MARCKS family of protein kinase C substrates. *Cell* 70:791-801.
- Lindroth, M., B.A. Fredriksson, and P.B. Bell. 1991. Cryosputtering--a combined freeze-drying and sputtering method for high-resolution electron microscopy. *J Microsc* 161 ( Pt 2):229-39.
- Lorenzi, R., P.M. Brickell, D.R. Katz, C. Kinnon, and A.J. Thrasher. 2000. Wiskott-Aldrich syndrome protein is necessary for efficient IgG-mediated phagocytosis. *Blood* 95:2943-6.
- Machesky, L., and R. Insall. 1998. Scar1 and the related Wiskott-Aldrich syndrome protein, WASP, regulate the actin cytoskeleton through the Arp2/3 complex. *Current Biology* 8.
- MacMicking, J., Q.W. Xie, and C. Nathan. 1997. Nitric oxide and macrophage function. *Annu Rev Immunol* 15:323-50.
- Massol, P., P. Montcourrier, J.C. Guillemot, and P. Chavrier. 1998. Fc receptor-mediated phagocytosis requires CDC42 and Rac1. *EMBO J* 17:6219-29.

- 
- May, R.C., and L.M. Machesky. 2001. Phagocytosis and the actin cytoskeleton. *Journal of Cell Science* 114:1061-77.
- Mbati, P., A., K. Abok, A. Orago, S., C. Anjil, O., J. Githure, I., and D. Koech, K. 1994. Ethylenglycol-bis-( $\beta$ -aminoethyl ether)N,N,NN,N,-tetraacetic acid (EGTA) inhibits *Leishmania donovani* *in vitro*. *Afr J Health Sci* 1:160-64.
- McConville, M.J. 1995. The surface glycoconjugates of parasitic protozoa: potential targets for new drugs. *Austral N Z J Med* 25:768-76.
- McConville, M.J., and J.M. Blackwell. 1991. Developmental changes in the glycosylated phosphatidylinositols of *Leishmania donovani*. Characterization of the promastigote and amastigote glycolipids. *J Biol Chem* 266:15170-9.
- McNeely, T.B., and S.J. Turco. 1990. Requirement of lipophosphoglycan for intracellular survival of *Leishmania donovani* within human monocytes. *J Immunol* 144:2745-2750.
- Mellman, I., and R.M. Steinman. 2001. Dendritic cells: specialized and regulated antigen processing machines. *Cell* 106:255-8.
- Mengeling, B.J., S.M. Beverley, and S.J. Turco. 1997. Designing glycoconjugate biosynthesis for an insidious intent: phosphoglycan assembly in *Leishmania* parasites. *Glycobiology* 7:873-80.
- Méresse, S., O. Steele-Mortimer, E. Moreno, M. Desjardins, B. Finlay, and J.-P. Gorvel. 1999. Controlling the maturation of pathogen-containing vacuoles: a matter of life and death. *Nature Cell Biol* 1:183-188.

- 
- Miaczynska, M., and M. Zerial. 2002. Mosaic organization of the endocytic pathway. *Exp Cell Res* 272:8-14.
- Mineo, C., Y.S. Ying, C. Chapline, S. Jaken, and R.G. Anderson. 1998. Targeting of protein kinase C alpha to caveolae. *J Cell Biol* 141:601-10.
- Moll, H. 2003. Dendritic cells and host resistance to infection. *Cell Microbiol* 5:493-500.
- Mullin, N.P., P.G. Hitchen, and M.E. Taylor. 1997. Mechanism of Ca<sup>2+</sup> and monosaccharide binding to a C-type carbohydrate-recognition Domain of the macrophage mannose receptor. *J Biol Chem* 272:5668-5681.
- Nagl, M., L. Kacani, B. Mullauer, E.M. Lemberger, H. Stoiber, G.M. Sprinzl, H. Schennach, and M.P. Dierich. 2002. Phagocytosis and killing of bacteria by professional phagocytes and dendritic cells. *Clin Diagn Lab Immunol* 9:1165-8.
- Ng Yan Hing, J.D., M. Desjardins, and A. Descoteaux. 2004. Proteomic analysis reveals a role for protein kinase C-alpha in phagosome maturation. *Biochem Biophys Res Commun* 319:810-6.
- Orlandi, P.A., and S.J. Turco. 1987. Structure of the lipid moiety of the *Leishmania donovani* lipophosphoglycan. *J Biol Chem* 262:10384-10391.
- Orsini, M., M. Silva, Z.M. Profeta da Luz, J. Disch, O. Fernandes, D. Moreira, A.C.M. Guedes, and A. Rabello. 2002. Identification of *Leishmania chagasi* from skin in *Leishmania/HIV*: relato de caso. *Rev Soc Bras Med Trop* 35:1-6.

- 
- Oskam, L., J.L. Nieuwenhuijs, and A. Hailu. 1999. Evaluation of the direct agglutination test (DAT) using freeze-dried antigen for the detection of anti-*Leishmania* antibodies in stored sera from various patient groups in Ethiopia. *Trans Royal Soc Trop Med Hyg* 93:275-7.
- Palucka, K., and J. Banchereau. 2002. How dendritic cells and microbes interact to elicit or subvert protective immune responses. *Curr Opin Immunol* 14:420-31.
- Pearson, R.D., and A.Q. Sousa. 1985. *Leishmania* species (Kala-azar, cutaneous and mucocutaneous leishmaniasis), p. 1522-1531, *In* G. L. Mandell, et al., eds. *Principle and Practice of Infectious Diseases*. Wiley, New York.
- Pimenta, P.F.P., S.J. Turco, M.J. McConville, P.G. Lawyer, P.V. Perkins, and D.L. Sacks. 1992. Stage-specific adhesion of *Leishmania* promastigotes to the sandfly midgut. *Science* 256:1812-1815.
- Proudfoot, L., A.V. Nikolaev, G.J. Feng, W.Q. Wei, M.A. Ferguson, J.S. Brimacombe, and F.Y. Liew. 1996. Regulation of the expression of nitric oxide synthase and leishmanicidal activity by glycoconjugates of *Leishmania* lipophosphoglycan in murine macrophages. *Proc Natl Acad Sci USA* 93:10984-10989.
- Rabinovitch, M. 1995. Professional and non-professional phagocytes: an introduction. *Trends Cell Biol* 5:85-87.
- Ravetch, J., V., and R. Clynes, A. 1998. Divergent roles for Fc receptors and complement in vivo. *Annu Rev Immunol* 16:421-32.
- Rawlins, D., J. 1995. *Light Microscopy.*, Second Edition. ed. BIOS Sci Publ Ltd, Oxford.

- 
- Reis e Sousa, C. 2001. Dendritic cells as sensors of infection. *Immunity* 14:495-8.
- Rescigno, M. 2002. Dendritic cells and the complexity of microbial infection. *Trends Microbiol* 10:425-61.
- Romani, N., D. Reider, M. Heuer, S. Ebner, E. Kampgen, B. Eibl, D. Niederwieser, and G. Schuler. 1996. Generation of mature dendritic cells from human blood. An improved method with special regard to clinical applicability. *J Immunol Meth* 196:137-51.
- Rouard, H., A. Leon, B. Klonjowski, J. Marquet, L. Tenneze, A. Plonquet, S.G. Agrawal, J.P. Abastado, M. Eloit, J.P. Farcet, and M.H. Delfau-Larue. 2000. Adenoviral transduction of human 'clinical grade' immature dendritic cells enhances costimulatory molecule expression and T-cell stimulatory capacity. *J Immunol Meth* 241:69-81.
- Sacks, D., and S. Kamhawi. 2001. Molecular aspects of parasite-vector and vector-host interactions in leishmaniasis. *Annu Rev Microbiol* 55:453-83.
- Sacks, D.L. 1989. Metacyclogenesis in *Leishmania* promastigotes. *Exp Parasitol* 69:100-3.
- Sacks, D.L. 1992. The structure and function of the surface lipophosphoglycan on different developmental stages of *Leishmania* promastigotes. *Infect Agents Dis* 1:200-6.
- Sacks, D.L. 2001. *Leishmania*-sand fly interactions controlling species-specific vector competence. *Cell Microbiol* 3:189-96.

- 
- Sallusto, F., and A. Lanzavecchia. 2002. The instructive role of dendritic cells on T-cell responses. *Arthritis Res* 4 (Suppl 3):S127-32.
- Sallusto, F., M. Cella, C. Danieli, and A. Lanzavecchia. 1995. Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. *J Exp Med* 182:389-400.
- Schmidt, A., and M.N. Hall. 1998. Signaling to the actin cytoskeleton. *Annu Rev Cell Dev Biol* 14:305-338.
- Schneider, P., M.A. Ferguson, M.J. McConville, A. Mehlert, S.W. Homans, and C. Bordier. 1990. Structure of the glycosyl-phosphatidylinositol membrane anchor of the *Leishmania major* promastigote surface protease. *J Biol Chem* 265:16955-64.
- Scianimanico, S., M. Desrosiers, J.-F. Dermine, S. Meresse, A. Descoteaux, and M. Desjardins. 1999. Impaired recruitment of the small GTPase rab7 correlates with the inhibition of phagosome maturation by *Leishmania donovani* promastigotes. *Cell Microbiol* 1:19-32.
- Scott, C.C., R.J. Botelho, and S. Grinstein. 2003. Phagosome maturation: a few bugs in the system. *J Membr Biol* 193:137-52.
- Sean Ha, D., J.K. Scharz, S.J. Turco, and S. Beverley. 1996. Use of the green fluorescent protein as a marker in transfected *Leishmania*. *Mol and Biochem Parasitol* 77:57-64.
- Silverstein, S., C. 1995. Phagocytosis of microbes: insights and prospects. *Trends cell biol* 5:141-42.

- 
- Silverstein, S.C., S. Greenberg, F. Di Virgilio, and T.H. Steinberg. 1989. Phagocytosis, p. 703-720, *In* W. E. Paul, ed. *Fundamental Immunology*, Second ed. Raven Press Ltd., New York.
- Southwick, F.S., and T.P. Stossel. 1983. Contractile proteins in leukocyte function. *Sem Hematol* 20:305-321.
- St-Denis, A., V. Caouras, F. Gervais, and A. Descoteaux. 1999. Role of protein kinase C-alpha in the control of infection by intracellular pathogens in macrophages. *J Immunol* 163:5505-11.
- St-Denis, A., F. Chano, P. Tremblay, Y. St-Pierre, and A. Descoteaux. 1998. Protein kinase C-alpha modulates lipopolysaccharide-induced functions in a murine macrophage cell line. *J Biol Chem* 273:32787-92.
- Steinman, R.M., and J.W. Young. 1991. Signals arising from antigen-presenting cells. *Curr Opin Immunol* 3:361-72.
- Strobl, H., C. Scheinecker, E. Riedl, B. Csmarits, C. Bello-Fernandez, W.F. Pickl, O. Majdic, and W. Knapp. 1998. Identification of CD68<sup>+</sup>lin<sup>-</sup> peripheral blood cells with dendritic precursor characteristics. *J Immunol* 161:740-8.
- Sundar, S., and M. Rai. 2002. Laboratory diagnosis of visceral leishmaniasis. *Clin Diagn Lab Immunol* 9:951-8.
- Takai, T. 2002. Roles of Fc receptors in autoimmunity. *Nat Rev Immunol* 2:580-92.
- Takeda, K., and S. Akira. 2004. Microbial recognition by Toll-like receptors. *J Dermatol Sci* 34:73-82.

- 
- Takeuchi, O., and S. Akira. 2002. Genetic approaches to the study of Toll-like receptor function. *Microbes Infect* 4:887-95.
- Taylor, M., J. Conary, M. Lennartz, P. Stahl, and K. Drickamer. 1990. Primary structure of the mannose receptor contains multiple motifs resembling carbohydrate-recognition domains. *J Biol Chem* 265:12156-62.
- Thiele, L., B. Rothen-Rutishauser, S. Jilek, H. Wunderli-Allenspach, H.P. Merkle, and E. Walter. 2001. Evaluation of particle uptake in human blood monocyte-derived cells in vitro. Does phagocytosis activity of dendritic cells measure up with macrophages? *J Control Release* 76:59-71.
- Tjelle, T.E., T. Lövdahl, and T. Berg. 2000. Phagosome dynamics and function. *Bio Essays* 22:255-263.
- Tolson, D.L., S.J. Turco, and T.W. Pearson. 1990. Expression of a repeating phosphorylated disaccharide lipophosphoglycan epitope on the surface of macrophages infected with *Leishmania donovani*. *Infect Immun* 58:3500-3507.
- Touret, N., P. Paroutis, M. Terebiznik, R.E. Harrison, S. Trombetta, M. Pypaert, A. Chow, A. Jiang, J. Shaw, C. Yip, H.P. Moore, N. van der Wel, D. Houben, P.J. Peters, C. de Chastellier, I. Mellman, and S. Grinstein. 2005. Quantitative and dynamic assessment of the contribution of the ER to phagosome formation. *Cell* 123:157-70.
- Turco, S., J., and D. Sacks, L. 1991. Expression of a stage-specific lipophosphoglycan in *Leishmania major* amastigotes. *Mol Biochem Parasitol* 45:91-00.

- 
- Turco, S.J., and P.A. Orlandi. 1989. Structure of the phosphosaccharide-  
inositol core of the *Leishmania donovani* lipophosphoglycan. *J Biol  
Chem* 264:6711-6715.
- Turco, S.J., and A. Descoteaux. 1992. The lipophosphoglycan of  
*Leishmania* parasites. *Ann Rev Microbiol* 46:65-94.
- Turco, S.J., G.F. Spath, and S.M. Beverley. 2001. Is lipophosphoglycan a  
virulence factor? A surprising diversity between *Leishmania* species.  
*Trends Parasitol* 17:223-6.
- Turco, S.J., S.R. Hull, P.A. Orlandi, and S.D. Shepherd. 1987. Structure of  
the major carbohydrate fragment of the *Leishmania donovani*  
lipophosphoglycan. *Biochem* 26:6233-6238.
- Tuyaerts, S., S.M. Noppe, J. Corthals, K. Breckpot, C. Heirman, C. De  
Greef, I. Van Riet, and K. Thielemans. 2002. Generation of large  
numbers of dendritic cells in a closed system using cell factories. *J  
Immunol Meth* 264:135-51.
- Underhill, D.M., and A. Ozinsky. 2002. Phagocytosis of microbes:  
complexity in action. *Annu Rev Immunol* 20:825-52.
- Underhill, D.M., J. Chen, L.A. Allen, and A. Aderem. 1998. MacMARCKS  
is not essential for phagocytosis in macrophages. *J Biol Chem*  
273:33619-23.
- Wade, R.H., and A.A. Hyman. 1997. Microtubule structure and dynamics.  
*Curr Opin Cell Biol* 9:12-7.
- Welch, M.D., A. Mallavarapu, J. Rosenblatt, and T.J. Mitchison. 1997.  
Actin dynamics in vivo. *Curr Opin Cell Biol* 9:54-61.

- 
- West, M.A., A.R. Prescott, E.L. Eskelinen, A.J. Ridley, and C. Watts. 2000. Rac is required for constitutive macropinocytosis by dendritic cells but does not control its downregulation. *Curr Biol* 10:839-48.
- Wilson, N.S., G.M. Behrens, R.J. Lundie, C.M. Smith, J. Waithman, L. Young, S.P. Forehan, A. Mount, R.J. Steptoe, K.D. Shortman, T.F. de Koning-Ward, G.T. Belz, F.R. Carbone, B.S. Crabb, W.R. Heath, and J.A. Villadangos. 2006. Systemic activation of dendritic cells by Toll-like receptor ligands or malaria infection impairs cross-presentation and antiviral immunity. *Nat Immunol* 7:165-172.
- Wu, K., J. Yuan, and L.A. Lasky. 1996. Characterization of a novel member of the macrophage mannose receptor type C lectin family. *J Biol Chem* 271:21323-30.
- Yin, H.L., J.H. Albrecht, and A. Fattoum. 1981. Identification of gelsolin, a Ca<sup>2+</sup>-dependent regulatory protein of actin gel-sol transformation, and its intracellular distribution in a variety of cells and tissues. *J Cell Biol* 91:901-6.
- Zhu, Z., Z. Bao, and J. Li. 1995. MacMARCKS mutation blocks macrophage phagocytosis of zymosan. *J Biol Chem* 270:17652-17655.
- Zilberstein, D., and M. Shapira. 1994. The role of pH and temperature in the development of *Leishmania* parasites. *Annu Rev Microbiol* 48:449-70.
- Zimmerli, S., M. Majeed, M. Gustafson, O. Stendahl, D.A. Sanan, and J.D. Ernst. 1996. Phagosome-lysosome fusion is a calcium- independent event in macrophages. *J Cell Biol* 132:49-61.