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Stage specific interactions of *Leishmania major* with host phagocytes

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ABBREVIATIONS

ABC-transporter	ATP-binding-cassette-transporter
AnxA5	Annexin A5
APC	Antigen-presenting cell
CD	Cluster of differentiation
cDNA	Complementary DNA
CR1	Complement receptor 1
CR3	Complement receptor 3
DNA	Desoxyribonucleic acid
dsRED	Discosoma RED fluorescent protein
eGFP	Enhanced Green Fluorescent Protein
FACS	Fluorescent activated cell sorter
FCS	Fetal calf serum
FITC	Fluoresceinisothiocyanat
fMLP	Formyl-Methionin-Leucin-Phenylalanin
GM-CSF	Granulocyte-Macrophage colony stimulating factor
IgG	Immuneglobulin G
IL-10	Interleukin 10
L. major, donovani, tropica, amazonensis	*Leishmania major, donovani, tropica, amazonensis*
LCF	Leishmania chemotactic factor
LxA4	Lipoxin A4
M-CSF	Macrophage-colony stimulating factor
mRNA	Messenger RNA
MΦ	macrophage
PCR	Polymerase chain reaction
PMA	Phorbolmistrate acetat
PMN	Polymorphonuclear Granulocyte
Poly I	Polyinositylic acid
PS	Phosphatidylserine
Q	Coulomb
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal RNA

ROS	Reactive oxygen species
rRNA	Ribosomal RNA
RT	Reverse Transcriptase
SCF	Stem cell factor
SEM	Standard error of the mean
SHERP	Small hydrophilic endoplasmic reticulum associated protein
SR-A	Scavenger Receptor class A
TGF-β	Transforming Growth Factor - beta
Th1	T helper lymphocyte 1
Th2	T helper lymphocyte 2
TNF-α	Tumour Necrosis Factor - alpha

INTRODUCTION

1.1 Leishmaniasis

Currently 350 million people in 88 countries around the world are at risk of Leishmaniasis (Alvar et al., 2006). Leishmaniasis is a generic term for a group of diseases caused by the infection with the protozoan parasites of the genus *Leishmania.* The disease symptoms range from self-healing skin ulcers caused by *Leishmania major* and *Leishmania tropica* to the more severe chronic mucocutaneous infections caused by *L. braziliensis* by the spread of the parasites to the mucous membranes, especially those of the nose, mouth and throat. It leads to extensive damage and disfiguration. The most severe form is visceral Leishmaniasis or kala azar. This disease is characterised by affecting internal organs, in particular the spleen, liver, bone marrow and lymph nodes. If left untreated visceral Leishmaniasis is lethal. Kala azar is caused by parasites from the *L. donovani* complex including *L. chagasi*, *L. infantum* and *L. donovani* (Herwaldt, 1999). The incidence of cutaneous Leishmaniasis ranges from 1 to 1.5 million cases annually, wheras 0.5 million new infection of visceral Leishmaniasis are estimated (2009).

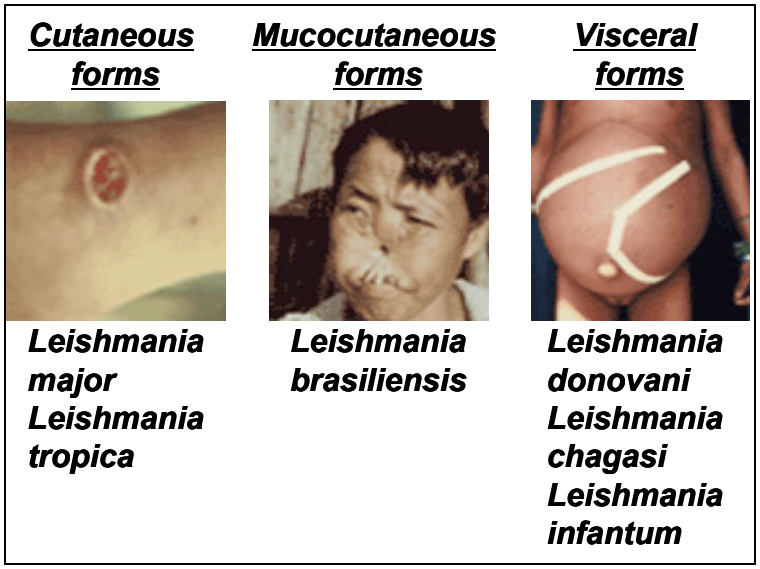

Fig. 1.1 Different manifestations of *Leishmaniasis*

1.2 *Leishmania* life cycle

Leishmania spp. are protozoan endoparasites of the family Trypanosomatidae. Around 30 species belong to the genus *Leishmania*, of which 21 are known to infect man (Lainson et al., 1987). *Leishmania* are characterised by a digenic life cycle between sand flies of the genus *Phlebotomus* or *Lutzomyia*, depending on the *Leishmania* species, and numerous mammalia. Inside the sand flies the parasites live as extracellular flagellated promastigotes in the alimentary tract. Promastigotes have long and lean bodies (about 1.5 x 15 µm) with one apical flagellum. Two developmental stages have been described for promastigotes. The avirulent procyclic promastigotes attach to epithelial cells of the midgut of the sand fly and multiply there. Upon maturation, a process called metacyclogenesis, the avirulent procyclic parasites mature to virulent metacyclic promastigotes and stop dividing. The virulent metacyclic promastigotes arrive at the saliva gland of the sand fly. Upon the bite of an infected sand fly these metacyclic promastigotes are then transmitted together with saliva into the wound of the mammalian hosts.

In mammalia, *Leishmania* spp. are obligate intracellular parasites. At the sub-cutane site of infection *Leishmania* release a chemotactic factor (LCF) that attracts polymorphonuclear granulocytes (PMN) (van Zandbergen et al., 2002). These phagocytes take up the promastigotes and secrete IL-8, amplifying the recruitment of PMN. The parasites survive inside PMN without replication, using it as an intermediate host cell (van Zandbergen et al., 2004). Besides IL-8, MIP-1β is released by the PMN, attracting macrophages (MΦ) to the site of infection. Infected, apoptotic PMN are subsequently taken up by the infiltrating MΦ and the parasites are transmitted to these phagocytes the final host cells of *Leishmania* (van Zandbergen et al., 2004). Inside MΦ promastigote *Leishmania* are located in phagosomal compartments that fuse with endocytic organelles to form the parasitophorous vacuole. Inside this compartment the promastigotes transform into elliptic shaped, a-flagellated, non-motile amastigotes of 2 x 6 µm in diameter. Inside the acidic environment of the parasitophorous vacuole the amastigotes start to replicate and are suggested to spread to surrounding phagocytes, causing Leishmaniasis. The exact mechanisms underlying the propagation of amastigotes within host phagocytes, leading to the manifestation of the disease are poorly understood. During another blood meal of a sand fly, amastigote containing macrophages or released amastigotes are ingested. Inside the insect the *Leishmania* transform into motile, flagellated promastigotes and attach to the midgut of the phlebotomine sand fly.

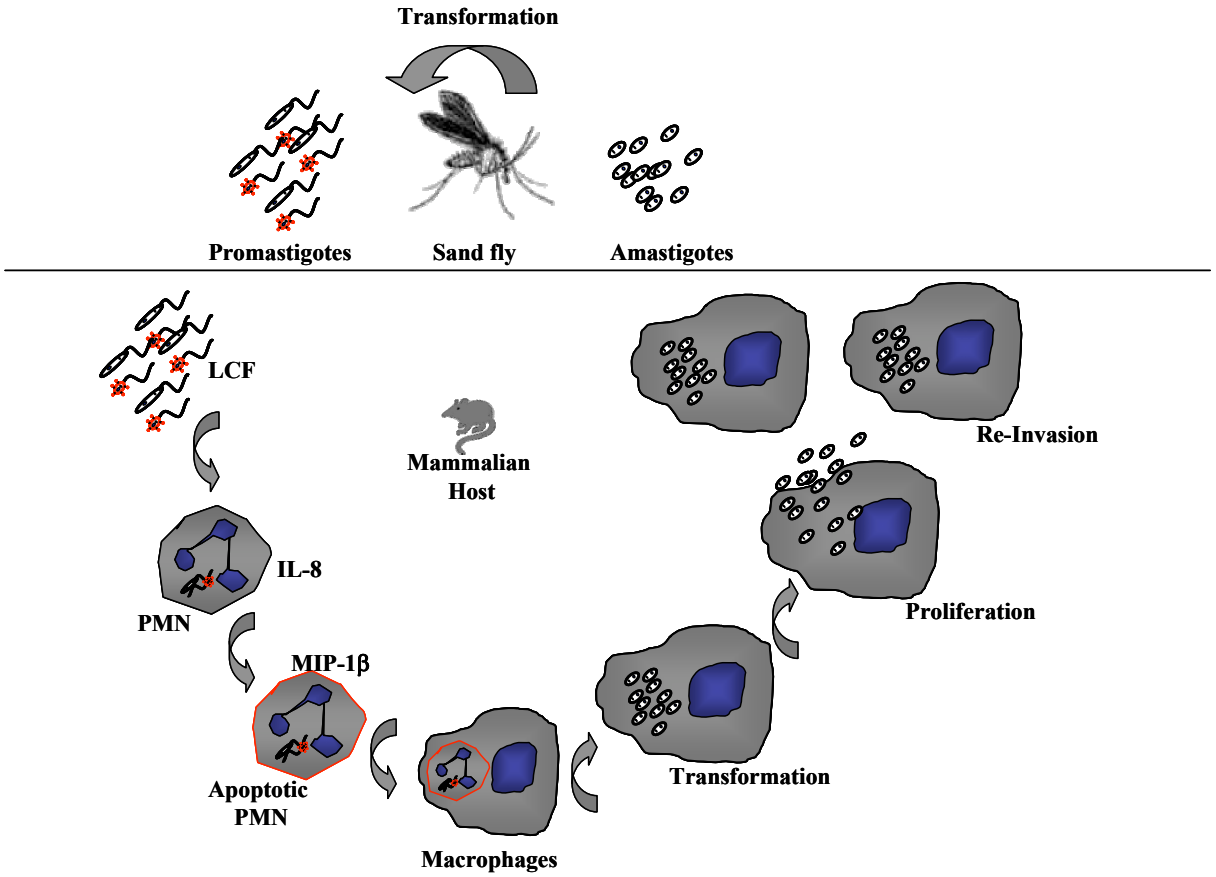

Fig. 1.1 Life cycle of *L. major* parasites

1.3 *Leishmania* biology

Leishmania genetics

Leishmania are asexual diploid protozoan parasites that feature some characteristics in gene regulation and expression. Unlike other eukaryotes *Leishmania* genes do not contain introns but are separated by short intergenic regions containing regulatory elements (Curotto de Lafaille et al., 1992; Stiles et al., 1999). Typical regulatory elements of eukaryotic promoters are missing (Borst, 1986; Kapler et al., 1990). The genes are transcribed in polycistronic manner that needs further processing by transsplicing and polyadenylation to generate monocistronic, mature mRNA. The mature mRNA are capped with a 39-nucleotide mini-exon (El-Sayed et al., 2005). Most of mitochondrial mRNA sequences are reprogrammed by RNA editing (Stuart et al., 2005). In recent years *L. major* genome analysis revealed that the parasites have a constitutively expressed genome. Comparing *L. major* procyclic and metacyclic promastigotes with amastigotes isolated from either infected mice or MΦ detected mere 0.2 – 5 % of total genes to be differentially expressed (Cohen-Freue et al., 2007; Leifso

et al., 2007). These gene expression analyses revealed that approximattly 90% of all gene are expressed continuously (Cohen-Freue et al., 2007). Moreover, only 113 genes were preferentially expressed in the amastigotes stage and 124 genes were specific for the promastigote stages (Cohen-Freue et al., 2007; Leifso et al., 2007). Furthermore, about half of the genes differentially expressed in the amastigote stage encoded hypothetical or novel proteins with no similarity to other known gene families (Leifso et al., 2007). Among those amastigote specific genes was the putative ABC transporter (LmjF11.0040). Specifically expressed genes for promastigote *L. major* included well documented genes as the recently described small hydrophilic endoplasmic reticulum associated protein (SHERP) (Knuepfer et al., 2001). The functions of the differentially regulated genes are currently not known. The constitutively expressed genome of the parasite demonstrates that the adaptation to different habitats is not regulated entirely on mRNA levels but can also be achieved by post-transcriptional means.

Lipophosphoglycan

In order to survive the different habitats *Leishmania* parasites need highly adapted cell membranes. The predominant cell surface structure of *Leishmania* promastigotes is LPG. LPG causes the parasites to attach and detach from the midgut of the sand fly vector (Sacks et al., 1995). The stage specific variations of LPG not only cause the transport of virulent metacyclic promastigotes to the saliva gland of the sand fly; it also protects the parasites from lysis through complement by shedding the membrane attack complex from the surface (Puentes et al., 1988; Sacks et al., 1995). Inside MΦ LPG is believed to delay the fusion on endosomal compartments by inhibiting protein kinase C. This delay might aid promastigotes to transform into amastigotes, by giving them more time (Naderer et al., 2004). Specific knock out mutants lacking LPG showed no conversion of promastigotes into amastigotes (Späth et al., 2000) *in vitro*, as well as a delayed disease development, *in vivo* (Kleczka et al., 2007; Späth et al., 2000). However, these data also indicate that other factors contribute to the virulence of *Leishmania*, especially since *L. mexicana* metacyclic promastigotes do not express LPG (Ralton et al., 2003). Moreover, surface expression of LPG is reduced considerably on amastigotes (Pimenta et al., 1992) that survive and divide in the acid parasitophorous compartment of MΦ.

Proteophosphoglycans

PPG constitute a family of secreted and membrane bound proteins that are closely related to LPG (Ilg, 2000b; Ilg, 2000a). It could be demonstrated that the filamentous, secreted form of PPG generates a viscous mesh in which the parasites are embedded (Ilg et al., 1999). PPG participate in the inhibiting enzymatic digestion inside the sand fly vector (Lawyer et al., 1991) that has been suggested to be involved in metacyclogenesis (Warburg and Schlein, 1986; Warburg et al., 1986). A *L. donovani* mutant, lacking PPG, was rapidly killed in sand fly midgut (Descoteaux et al., 1995). Moreover, this mutant was a-virulent in mouse and MΦ infection models (Späth et al., 2003b). These results suggest PPG to have an important role in mammalian survival. In addition PPG contributes to attachment and invasion of MΦ as well as protection from complement lysis during initial infection (Guha-Niyogi et al., 2001; Späth et al., 2003b).

Programmed cell death of *Leishmania*

Programmed cell death or apoptosis is historically linked to the physiological function in metazoan organisms to secure homeostasis (Henson and Hume, 2006). Therefore, many scientists deny the existence of a form of programmed cell death in unicellular life forms. But in a number of different classes of protists markers of apoptosis have been found (Deponte, 2008).

Apoptotic cells are subjected to a strict course of events to take place. As an early sign phosphatidylserine (PS) is externalised from the inner to the outer leaflet of the cell membrane (Martin et al., 1995). This correlates with the release of lysophosphatidylcholin (LPC) in the surrounding tissues to rapidly attract phagocytes. Next proteolytic enzymes called caspases are activated resulting in breakdown of cell contents. The membrane potential is lost and cell shrinkage is observed (Kerr et al., 1972). Last the nucleus condensates and the DNA is nicked resulting in typical DNA fragmentation (Wyllie et al., 1980).

Recent findings demonstrated that *Leishmania* have a cell death program. The externalisation of PS could be demonstrated on different *Leishmania* species. Though *Leishmania* lack caspases, caspase-like activity was detected correlating with the loss of mitochondrial membrane potential. Moreover, cell shrinkage and DNA fragmentation was found (Arnoult et al., 2002b; Sen et al., 2004; Nguewa et al., 2005; Alzate et al., 2006). In summary, these data support the existence of a programmed cell death in *Leishmania* though the exact mechanism has not been detected so far. In the virulent inoculum of *Leishmania major* promastigotes a

population of parastites with fragmented DNA as well as PS externalisation was described (van Zandbergen et al., 2006).

A common method to detect early apoptotic cells is by staining PS on the outer cell membrane with fluorescently labelled Annexin A5 (AnxA5). PS has a natural Ca+ dependent affinity towards AnxA5. The fragmented, nicked DNA of late apoptotic cells can be visualised by using terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) staining or by gel-electrophoreses as a typical DNA ladder.

1.4 Phosphatidylserine

The phospholipids phosphatidylserine (PS) (1,2-diacyl-sn-glycero-3-phospho-L-serine) constitutes about 3 % of the lipids of a eukaryotic cell membrane. In the endoplasmatic reticulum (ER) PS is synthesized by two enzymes, the phosphatidylserine synthase I (PSS1) and 2 (PSS2) (Voelker and Frazier, 1986; Kuge and Nishijima, 1997). Both enzymes substitute the head group of either phosphatidylcholine or phosphatidylethanolamine with a serine to synthesise PS. This reaction is strictly dependent on calcium ions and requires no further source of energy. Database research at the GeneDB of the Sanger Institute revealed that *L. major* have one gene (LmjF14.1200) with high homology to PSS2.

The biological function of PS is based on its anionic charge to which cationic proteins can bind. This effect is believed to re-direct proteins from one target membrane to another. In addition PS is an essential co-factor for protein kinase C, a key enzyme for signal transduction. All isoforms of this enzyme are strictly dependent on PS for activity (Epand et al., 1998; Quest and Bell, 1994).

As mentioned above in healthy cells PS is located mainly in the inner leaflet of the plasma membrane, while upon apoptosis essentially all cells expose PS on the outer leaflet of the plasma membrane (Vance and Steenbergen, 2005). The exact mechanism of this as membrane flip-flop known phenomenon is not clear. A family of P-type ATPases termed translocases have been considered to be responsible for the ATP-dependent establishment of the asymmetrical membrane distribution of PS (Tang et al., 1996). In apoptotic cells these enzymes would then be inactivated and PS redistributed randomly across the cell membrane. A second set of enzymes known as scramblases have been suggested to transport PS across the membrane bi-directionally in an ATP-independent manner (Sahu et al., 2007). Though four genes have been identified in mammals the first knockout mice for *Scr1* and *Scr3* did not show a defect in PS externalisation. Therefore the true identity of the involved scamblase has not been identified until now.

Apoptosis is often correlated with the generation of ROS that oxidate the sn-2 glycerol to a sn-2 acyl group of PS. This enables the incorporation of terminal γ-hydroxy-α,β unasaturated acyl moieties that are recognised by MΦ as a prerequisite for engulfment of apoptotic cells (Kagan et al., 2002; Arroyo et al., 2002).

1.5 Silencing of host phagocytes

Lipid mediated silencing

As mentioned above the first cells to be infected are PMN. These phagocytes are primary effector cells of the antimicrobial defence. They are the first cells to migrate into inflamed tissues where they encounter the responsible pathogens. These professional phagocytes eliminate pathogens by phagocytosis and subsequent intracellular lysis. In addition, PMN also can release toxic mediators like reactive oxygen species (ROS) to kill pathogens that cannot be phagocytosed. These toxic mediators are associated with collateral tissue damage that attracts more phagocytes to the site of inflammation leading to an amplification loop. Therefore, in order to avoid inflammatory disorders, PMN effector functions have to be tightly regulated. The resolution of acute inflammation is an active process governed by specific lipid mediators and involves an organised sequence of events (Bannenberg et al., 2005; Serhan et al., 2003; Serhan and Chiang, 2004). One of these endogenously generated lipid mediators that functions as stop signal of inflammation, is Lipoxin A4 (LxA4). Its receptor, the Lipoxin A4 receptor (ALX) formerly known as formyl peptide receptor-like 1 (FPRL-1) (Badolato et al., 1994), binds a family of structurally unrelated peptides, proteins and lipid mediators (Chiang et al., 2006). Moreover, ALX is a pleiotropic receptor expressed on PMN and MΦ. Depending on the ligand ALX signalling results in either pro- or anti-inflammatory responses (El Kebir et al., 2008). Binding of the acute-phase protein serum amyloid A (SAA) results in pro-inflammatory activity of PMN. This activation leads to prolonged life span and diapedesis of PMN into the inflamed tissues (Su et al., 1999). Furthermore, the secretion of IL-8 is induced (He et al., 2003) which causes the attraction of additional phagocytes to the site of infection. Binding of LxA4 overrides the signaling of SAA. The PMN are silenced to the degree that cytokines and chemokine release is blocked. The cells stop transmigration and their ability to release ROS becomes inactivated. Moreover, the intrinsic apoptosis program is induced (El Kebir et al., 2008).

PS-mediated silencing

In addition to this lipid-mediated shutdown of PMN, the best characterised, nonphlogistic process is the uptake of dying, apoptotic cells. Dying cells are subjected to well-orchestrated temporal events securing a rapid clearing of the remnants, before they become immunogenic. Initially, the succumbing cell release so called "Find Me" signals that attract phagocytes to the site of the dying cells. As a potential candidate for these signals LPC was identified (Lauber et al., 2003). Lauber et al could demonstrate that LPC was released form apoptotic cells in a caspase 3 dependent manner and that it attracted THP-1 cells to the releasing cells. Once the phagocyte has reached the dying cells these need to be identified as such. This is achieved by the surface expression of "Eat-Me" signals. Whereas "Find-Me" signals are diffusible extracellular factors, "Eat-Me" signals are displayed on the surface of dying cells very early in the apoptotic process (Henson et al., 2001). The best-studied "Eat-Me" signal is the externalisation of phosphatidylserine (PS) from the inner to the outer leaflet of the cell membrane (Fadok et al., 1998). Upon recognition of the "Eat-Me" signals the phagocytes engulf the apoptotic cells and degrades them. Currently there are three new receptors, TIM-4 (Miyanishi et al., 2007), BAI1 (Park et al., 2007), and stabilin-2 (Park et al., 2008) discussed to be responsible for the recognition of PS on dying cells. The process is characterised by the release of anti-inflammatory cytokines such as TGF-β and IL-10, thereby silencing the immune response towards the uptake of remnants from apoptotic cells (Voll et al., 1997).

It could be demonstrated that the virulent inoculum of *L. major* promastigotes consists of two distinct populations. Moreover, these two populations could be separated into viable and dead parasites. It was shown that the dead parasites are AnxA5-binding, a common method to detect PS on apoptotic cells. In addition, PS was detected using an antibody directed against PS or another PS binding protein, namely Protein-S. It was shown that depletion of PS-positive, AnxA5-binding *L. major* promastigotes from the virulent inoculum resulted in an activation of host PMN *in vitro* and in a proper immune answer *in vivo* (van Zandbergen et al., 2006). Therefore, the presence of AnxA5-binding parasites in the inoculum silences the phagocytes, thus enabling the viable parasites to survive inside PMN.

As mentioned earlier, *Leishmania* infection prolongs the short lived PMN´s lifespan to more than two days (Aga et al., 2002). After 2 days MΦ, the final host cells of *Leishmania,* arrive at the site of infection. By then the infected PMN have become PS positive themselves, enabling a nonphlogistic phagocytosis by MΦ. The uptake of apoptotic cells by MΦ induces the release of LxA4, which is a chemoattractant for MΦ and itself induces nonphlogistic phagocytosis of apoptotic PMN through MΦ (Godson et al., 2000). This results in a silencing-feed-back-loop

to take up more PS-positive, and in the case of *Leishmania* infection, infected PMN. There, the MΦ do not activate their full microbicidal repertoire after the phagocytosis of infected apoptotic PMN. This causes the intracellular parasites to survive and develop into amastigotes and start to replicate.

How these amastigotes propagate inside the host is poorly defined as well as what type of MΦ is infected.

1.6 The role of MΦ

Monocytes are generated within hematopoetic tissues and are released in circulation after a series of different matuaration and differentiation steps (Imhof and urrand-Lions, 2004). During Monocyte development, myeloid progenitor cells sequentially give rise to monoblasts, pro-monocytes and finaly monocytes (Porcheray et al., 2005)). Mature monocytes circulate in the bloodstream for 2-3 days after release from bone marrow, before they are recruited to tissues, where they differentiate into MΦ (Porcheray et al., 2005).

MΦ are central to the innate immune response in phagocytosing and killing invading pathogens (Hoebe et al., 2004). Furthermore, MΦ are important professional antigen presenting cells that initiate and regulate an adapted immune response (Unanue and Askonas, 1968). Moreover, MΦ are central to homeostasis, clearing 2 $x10^{11}$ apoptotic cells each day (Mosser and Edwards, 2008).

MΦ themselves are a heterogenic cell population. It could be shown that *in vitro* blood derived monocytes can be polarised into two subtypes of MΦ termed type I and type II MΦ (Verreck et al., 2004). Incubation with granulocyte-macrophage colony stimulating factore (GM-CSF) polarised the blood monocytes into type I MΦ. These MΦ were shown to be less active in phagocytosis as compared to type II MΦ (Xu et al., 2006). Type I MΦ release the pro-inflammatory cytokines IL-12, IL-23 and TNF-α. They are efficient producers of effector molecules like reactive oxygen and nitrogen intermediates. Moreover they release IL-1β and IL-6 and participate as inducers of a Th-1 response (Mantovani et al., 2004). Therefore, type I MΦ are termed pro-inflammatory phagocytes.

The incubation of monocytes with macrophage colony stimulating factor (M-CSF) led to Type II MΦ. In contrast to type I MΦ type II MΦ express the surface marker CD163. Moreover, these phagocytes have a higher phagocytosis capacity than type I MΦ (Xu et al., 2006; Xu et al., 2007). Type II MΦ were shown to release anti-inflammatory cytokines IL-10 and TGF-β upon uptake of apoptotic cells (Ehrt et al., 2001). Type II MΦ down-regulate IL-

12 production, leading to a resolution of inflammation (Krutzik et al., 2005). Therefore, these phagocytes are termed anti-inflammatory phagocytes.

In experimental as well as in human Leishmaniasis it is not entirely clear which MΦ are the preferential host cells for *Leishmania* to proliferate and propagate. It has been shown that immunity is mediated by T lymphocytes. The control of the disease is interceded by a T-helper 1 (Th-1) response, whereas a T-helper 2 (Th-2) response leads to disease development (Sacks and Sher, 2002). In mouse infection models with *L. major* parasites this dichotomy is reflected in the resistant C57BL/6 mice and the susceptible Balb/c mice. C57BL/6 mice release Th-1 cytokines INF-γ and TNF-α activate macrophages to actively destroy the intracellular parasites by the production of nitric oxide (NO). Balb/c mice produce IL-4 triggering a Th-2 response that does not eliminate the intracellular parasites. More recently it was demonstrated that the presence of IL-10 is responsible for the disease development (Anderson et al., 2005). The source of this IL-10 was shown to be CD4+ CD25- Foxp3- T cell infiltrating the site of infection (Anderson et al., 2005)), leading to down regulation of INF-γ. This prevents effective parasite elimination (Anderson et al., 2005).

1.7 Uptake mechanisms of MΦ

Phagocytosis is the main mechanism to clear apoptotic cells and take up and digest pathogens by professional phagocytes like PMN, MΦ and dendritic cells (DC), (Aderem, 2003; Greenberg and Grinstein, 2002). It is a multi-step process that is initiated by recognition of a particle by a variety of phagocytic receptors. Among these receptors are the mannose receptor (CD206), as well as class A scavenger receptors (SR-A). These receptors recognise pathogen-associated molecular patterns (PAMPs) on the surface of pathogens. Therefore, these receptors are known as pattern recognition receptors (PRR).

CD206 recognises branched polysaccharides with terminal mannose, fucose or N-acetylglucosamin that are expressed on the surface of a variety of pathogens like mycobacteria (Astarie-Dequeker et al., 1999) and *Candida albicans* (Lee et al., 2003). The leishmanial surface structure LPG as well as PPG contain in their repeating unit mannan residues. Therefore it is suggested that *Leishmania* interact with CD206 (Turco and Descoteaux, 1992). It was observed that pre-incubation of Leishmania promastigotes with mannose receptor ligands resulted in a decrease of 50% infected cells in murine macrophages (Palatnik et al., 1989). Moreover, it was found that *L. donovani* infect human MΦ in a CD206 dependent manner (Wilson and Hardin, 1988). Interestingly, *in vivo* infection studies in

CD206 deficient mice showed no significant reduction in the infection rate (Akilov et al., 2007).

Class A Scavenger receptors (SR-A) are a set of pattern recognition receptors mainly expressed on monocytes and macrophages. These receptors have a broad spectrum of possible ligands and are responsible for the phagocytosis of unopsonized microorganisms, apoptotic bodies as well as environmental dusts by alveolar MΦ (Peiser et al., 2002; Palecanda and Kobzik, 2001). The exact pattern SR-A recognise has not been defined jet. Due to the observation that the ligand repertoire is very hetereogenic, SR-A has been described as "molecular flypaper" (Krieger and Herz, 1994).

Phagocytosis of opsonised pathogens is mediated by a different class of receptors. Among these, complement receptors (CR) play an important role. These receptors recognise complement components attached to the surface of pathogens.

The complement receptor 3 (CR3) recognises the inactivated form of C3 (iC3b) that remain on the surface of the pathogens. But in contrast to signalling via CR1, ligation of iC3b with CR3 is sufficient to induce subsequent phagocytosis. Its involvement in the recognition and subsequent uptake of *L. major* promastigotes on PMN has been demonstrated (Laufs et al., 2002).

The complement receptor 1 (CR1 or CD35) recognises the complement component C3b on opsonised pathogens. Though on itself it cannot promote phagocytosis, it can lead to the uptake of a pathogen in the presence of other immune mediators. It has been reported to be essential for the uptake of *Leishmania* promastigotes (Da Silva et al., 1988; Da Silva et al., 1989).

Once a MΦ has made contact to a ligand with one of its phagocytosis receptors, large F-actin-coated vacuoles are formed and extended, embracing the particle to be engulfed (Diakonova et al., 2002). These pseudopods fuse at their tips to form the phagosome, which is subsequently internalised.

In addition to phagocytosis, cells can take up particles via clathrin- or caveolae-mediated endocytosis.

The assembly of the specific coat protein, clathrin, on the cytosolic leaflet of the plasma membrane, forms clathrin-coated-pits (CCP). Upon contact with a specific receptor on the outer leaflet of the membrane, the pit starts to invaginate until the intrusion is then sealed and internalised to fuse with endosomes (Conner and Schmid, 2003; Brodsky et al., 2001). This mechanism is often referred to as receptor-mediated endocytosis.

Caveolae are flask-shaped, cholesterol-enriched lipid rafts, stabilised by the name giving protein caveolin 1 (Fra et al., 1995). Upon contact with jet unspecified receptors these invaginations are internalised, sealed and then transported into the cytoplasm (Palade , 1953; Yamada , 1955). Though information on exact receptors are missing, it has been shown that caveolae are rich in receptor- and non-receptor protein kinases and GPI-anchored proteins (Simons and Ikonen, 2000). Therefore, these sites are suggested as especially active in signal transduction (Schlegel and Lisanti, 2001). Though the exact mechanism how caveolae uptake is induced remains to be analysed, it is known that this pathway is regulated by and impacts on cell signalling (Di Guglielmo et al., 2003; Sigismund et al., 2005).

1.8 Aims of this study

Leishmania are digenic protozoan parasites that alternate between phlobotomine sand flies and mammalian hosts. Inside the mammalian host they are obligate intracellular parasites residing in professional phagocytes like PMN and MΦ. In this study I focused on stage specific interactions of *L. major* parasites with PMN and MΦ.

1. I want to generate a *L. major* mutant strain that cannot present PS as a potential virulence factor. Therefore, I will generate *Leishmania* that cannot express PS or endogenously express AnxA5, thereby potentially shielding PS. Moreover, I want to develop transgene *Leishmania* strains that stably express eGFP or dsRED enabling us to detect and follow the development of intracellular parasites.

2. *Leishmania* promastigotes release a chemoattractant (LCF) specific for the receptor ALX on PMN. I want to assess the role of ALX activation on the initial uptake and survival of *L. major* promastigotes by PMN. Furthermore, I want to prove the Trojan Horse function of *L. major* infected PMN, *in vivo.*

3. I want to analyse, if lesion-derived and MΦ-derived amastigotes show hallmarks of apoptosis. Moreover, I will address the question whether axenic *L. donovani* amastigotes can also become PS-positive and / or TUNEL-positive.

4. In order to analyse the propagation of *L. major* amastigotes I want to generate an axenic *L. major* amastigote culture system. Subsequentially characterise morphologically, biochemically, genetically an axenic culture and analyse its infectivity of such amastigotes *in vitro* and *in vivo*.

5. Since it is not clear in which type of MΦ amastigotes are present during disease development, I will investigate both type I and type II MΦ as *Leishmania* host cells. I will focus on both phagocytotic and endocytotic pathways, investigating the role of CR1, CR3, Lectins and SR-A as possible entry receptors.

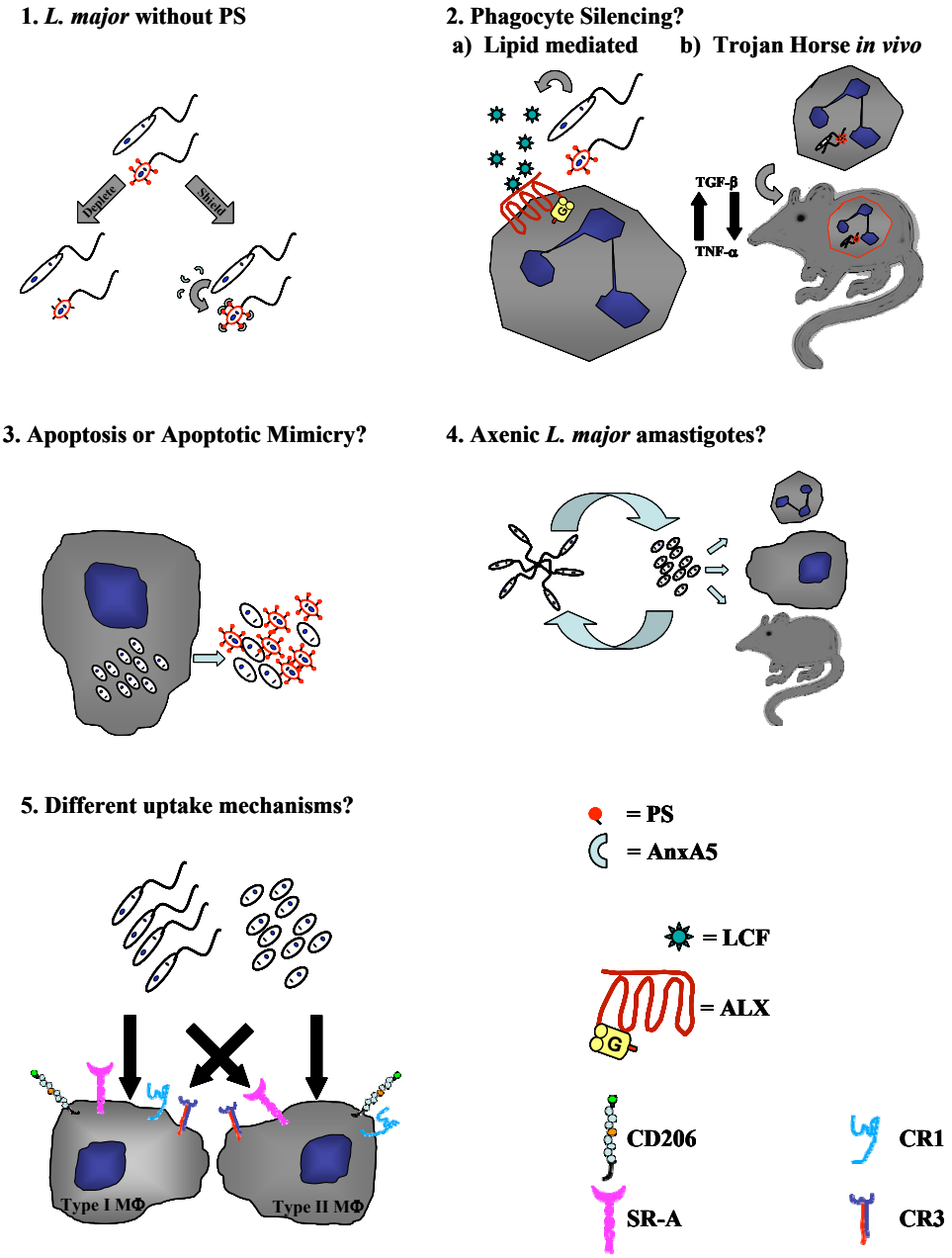

Figure 1.3 Schematic of the aims of this study (1) Generation of transgenic *Leishmania* that have no free PS by either targeted gene depletion or integration of endogenously expressing AnxA5, possibly shielding PS. (2) Analysing the influence of ALX activation on *L. major* promastigotes uptake by PMN. Transfer of the Trojan Horse model from *in vitro* to *in vivo* experiments. (3) Analysing the expression pattern externilised PS on MΦ-derived and lesion-derived L. major amastigotes. (4) Developing an axenic L. major amastigote culture enabling subsequent studies on propagation and infectivity. (5) Comparing infectivity of L. major promastigotes with amastigotes on pro-inflammatory type I MΦ and anti-inflammatory type II MΦ.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Eukaryotic cell lines

CHO-SCF	Recombinant murine SCF producing CHO cell line, a kind gift of Dr. Häcker
ER-HoxB8 SCF	Conditional HoxB8 murine PMN precurser cell line, a kind gift of Dr. Häcker
THP-1	Human acute monocytic leukaemia cell line (ATCC TIB-202™)

2.1.2 *Leishmania* strains

Leishmania donovani strain AG83 MHOM/IN1983/AG83	Originally obtained from an Indian Kala-azar patient.
Leishmania major isolate MHOM/IL/81/FEBNI	Originally isolated from a skin biopsy of an Israeli patient were kindly provided by Dr. F. Ebert (Bernhard-Nocht-Institute for Tropical Medicine, Hamburg)

2.1.3 Procaryotic cells

E. coli NovaBlue	*endA1 hsdR17* (rK12– mK12+) *supE44 thi-1 recA1 gyrA96 relA1 lac* F′[*proA+B+ lacIqZ*Δ*M15*::Tn*10*] (TetR) (Novagen)

2.1.4 Plasmids and cosmids

pGEM easy	PCR cloning vector	Promega, Mannheim, Germany,
pxG-Neo	*Leishmania* expression vector with neomycin resistance.	A kind gift of Dr. Beverley, St. Luis, USA
pcDNA3	Cloning vector	Invitrogen, Karlsruhe, Germany,
pBluescript II SK(+)	High copy number prokaryotic expression vector with ampicillin resistance	Stratagene
pssu-INT-eGFP	Cloning vector containing a gene	A kind gift of Dr.

	cassette for homologous recombination into the small subunit of rRNA locus of *Leishmania* Kinetoplast DNA bearing the EGFP gene.	Aebischer, Edingbourgh, UK
pssu-INT-dsRED	Cloning vector containing a gene cassette for homologous recombination into the small subunit of rRNA locus of *Leishmania* Kinetoplast DNA bearing the EGFP gene.	A kind gift of Dr. Aebischer, Edinburgh, UK
pQE60/AnxV	Plasmid containing the murine Anexin A5 gene inserted between the restriction sites of vector pQE60	A kind gift of Dr. Pöschl, Norwich, UK
pCR2.1hyg	Plasmid bearing the hygromycin phosphotransferase gene inserted between the BspHI and NheI restriction sites of vector pCR2.1 (Invitrogen, Karlsruhe, Germany)	A kind Gift of Dr. Wiese, BNI, Hamburg
pCR2.1phleo	Plasmid bearing the phleomycin binding protein gene inserted between the NcoI and AvrII restriction sites of vector pCR2.1	Gift of Dr. Wiese

2.1.5 Oligonucleotides:

2.1.5.1 Oligonucleotides used for qPCR:

ABC-fwd	5´-CGG GTT TGT CTT TCA GTC GT-3´
ABC-rws	5´-CAC CAG AGA GCA TTG ATG-3´
rRNA45-fwd	5´-CCT ACC ATG CCG TGT CCT TCT A-3´
rRNA45-rws	5´-AAC GAC CCC TGC AGC AAT AC-3´
SHERP-fwd	5´-GAC GCT CTG CCC TTC ACA TAC-3´
SHERP-rws	5´-TCT CTC AGC TCT CGG ATC TTG TC-3´

2.1.5.2 Oligonucleotides used for cloning strategy:

Hyg-fwd	5´-ATT AAT GAA AAA GCC TGA ACT-3´
Hyg-rws	5´-TCA GCA CTA GTC CTT TGC CCT C-3´
Lm-3´-UTR-fwd	5´-GAA TTC GGA CTA GTG GCG TGC CGT CCT-3´
Lm-3´-UTR-rws	5´-TGG TAC CCGC ATG CGA TGC AG-3´
Lm-5´-UTR-fwd	5´-CGA GTT CTA GAC AGC TCA CT-3´
Lm-5´-UTR-rws	5´-GAA TTC GCA TTA ATG TGC GTC AAG GAC GAA-3´
Lm-PSS-fwd	5´-ATG CGT CGA GGT CCG GAT G-3´
Lm-PSS-rws	5´-CTA GCG CCA AAA GAA GAT GTA CCG-3´
mAnxA5-fwd	5´-CCC GGG ATG GCT ACG AGA GGC ACT GT-3´
mAnxa5-rws	5´-GGA TCC TCA GTC ATC CTC GCC CCC GC-3´
Phleo-fwd	5´-CGC ACA CAT ATG GCC AAG TTG AC-3´
Phleo-rws	5´-TCA GCA CTA GTC CTG CTC G-3´

2.1.5.3 Oligonucleotides used for homologous recombination strategy:

Lm-3´fwd	5´-CTC GAG GCA AGC TTG TGC GGA GGA AC-3
Lm-3´rws	5´-Ggt acc ctt agg agc gtt gat ggc gg-3
Lm-5´fwd	5´-TCT AGA GCT GCC GTT GAC CGT CTC CAA T-3
Lm-5´rws	5´-GGA TCC TCC GTT GTT GCC TGA GCC CA-3
Lm-HYG-fwd	5´-GCA CGC ATC ACG CAG GTT CGT CCT TGA CGC ACA CGC ATG AAA AAG CCT GAA CTC-3´
Lm-HYG-rws	5´-CCG TCG CCT TCT CTA CCT CTC GCT CTG TCA GCG CTA CTA TTC CTT TGC CCT CGG-3´
Lm-PHLEO-fwd	5´-GCA CGC ATC ACG CAG GTT CGT CCT TGA CGC ACA CGC ATG GCC AAG TTG ACC AG-3´
Lm-PHLEO-rws	5´-CCG TCG CCT TCT CTA CCT CTC GCT CTGG TCA GCG CTA AGT CCT GCT CCT CGG CCA-3´

2.1.6 Culture media and buffers

6 x DNA-Loading-Dye	4 M Urea
	50% (w/v) Saccharose
	0,1 M Na_2EDTA

	0,1 (w/v) Bromphenolblue
Alex-Amastigote-Medium	RPMI 1640 Medium
(AAM)	10% (v/v) FCS
	100 U / ml Penicillin
	100 µg / ml Streptomycin
	3 mM L-Glutamine
	pH 5.5, adjusted with 38 % (w/v) HCl
	sterile filtered
Complete-Medium	RPMI 1640 Medium
	10 % (v/v) FCS
	50 µM β-Mercaptoethanol
	2 mM L-Glutamine
	10 mM Hepes
	100 U / ml Penicillin
	100 µg / ml Streptomycin
Elektroporation-Buffer	20 mM Hepes
	137 mM NaCl
	5 mM KCl
	0.7 mM $NaHPO_4$
	6 mM Glucose
FACS Buffer	1x PBS
	1% (v/v) Normal Human Serum
	1 % (w/v) Bovine Serum Albumin
	0,01 % (w/v) Natrium-Azide
Hox-Basal-Medium	OptiMEM
	10 % (v/v) FCS
	100 U / mL Penicillin
	100 µg / ml Streptomycin
	30 µM β-Mercaptoethanol
Hox-Culture-Medium	Hox-Basal-Medium
	1:25 SCF
	1 mM β-Estradiol
Lm-FACS Buffer	1 x Ringer Solution
	1 % (v/v) Foetal Calf Serum

	1 % (w/v) Bovine Serum Albumin
Lm-Medium	RPMI 1640 Medium
	5 % (v/v) FCS
	50 µM β-Mercaptoethanol
	2 mM L-Glutamine
	10 mM Hepes
	100 U / ml Penicillin
	100 µg / ml Streptomycin
Lm-Suspension-Medium	Medium 199
	10 % (v/v) FCS
	40 mM Hepes
	5 ml 10 mM adenine, in 50 mM Hepes, pH 7.5
	1 ml 0.25% hemin, in 50 % triethanolamine
	0.5 ml 0.1% biotin, in 95% ethanol
	100 U / ml Penicillin
	100 µg / ml Streptomycin
MACS-Buffer	1 x PBS
	0.5 % BSA
	2 mM EDTA
Novy-Nicolle-McNeal	16.6 % (v/v) Defibrinated Rabbit Blood
blood agar medium	16.6 % (v/v) 1 x PBS
	66.2 % (v/v) Brain Heart Infusion Agar
	66.2 U / ml Penicillin
	66.2 µg / ml Streptomycin
PMN-Infect-Medium	RPMI 1640
	20 % (v/v) FCS
	50 µM β-Mercaptoethanol
	2 mM L-Glutamine
	10 mM Hepes
	100 U/mL Penicillin
	100 µg/mL Streptomycin
RF1	100 mM RbCl
	79 mM MnCl
	29.5 mM $C_2H_3KO_2$

	1 mM $CaCl_2$
	1.6 M $C_3H_8O_3$
	pH 5.8 in H_2O
RF2	4.8 mM MOPS
	50 mM RbCl
	3.75 mM $CaCl_2$
	0.8 M $C_3H_8O_3$
	in H_2O
Wash-Buffer	1 x PBS
	5 % Lm-Medium

2.1.7 Chemicals and Reagents

β-Estradiol	Sigma, Deisenhofen, Germany
β-Mercaptoethanol	Sigma, Deisenhofen, Germany
1 kb DNA-Ladder	Promega, Mannheim, Germany, Mannheim, Germany
Adenin	Sigma, Deisenhofen, Germany
Agarose, NEED Ultra-Qualtity	Carl Roth, Karlsruhe, Germany
Annexin-V-fitc	Responsif AG, Erlangen, Germany
Annexin-V-fluos	Roche Applied Science, Mannheim, Germany
Biotin	Sigma, Deisenhofen, Germany
Bovine Serum Albumin (BSA)	Sigma, Deisenhofen, Germany
Brain Heart Infusion Agar	Oxoid, Basingstoke, Hampshire, England
Bromphenol blue dye	Serva, Heidelberg, Germany
Calcium chloride	Sigma, Deisenhofen, Germany
Cytochalasin D	Sigma, Deisenhofen, Germany
Diff-QUIK®	Medion Diagnostics, Düdingen, Switzerland
Dimethyl Amiloride (DMA)	Sigma, Deisenhofen, Germany
EDTA	Sigma, Deisenhofen, Germany
Ethanol	Merck KgaA, Darmstadt, Germany
Ethidium Bromid	Sigma, Deisenhofen, Germany
Fillipin III	Sigma, Deisenhofen, Germany
Foetal Calf Serum (FCS)	Sigma, Deisenhofen, Germany

Glycerol	Sigma, Deisenhofen, Germany
HCl	Merck KgaA, Darmstadt, Germany
Hemin	Sigma, Deisenhofen, Germany
Hepes	Biochrom, Berlin, Germany,
Histopaque® 1119	Sigma, Deisenhofen, Germany
Human, recombinant Granolucyte Macrophage Colony Stimulating Factor (GM-CSF) (Leukine)	Bayer Healthcare Pharmaceuticals, Leverkusen, Germany
Human, recombinant Macrophage Colony Stimulating Factor (M-CSF)	PeproTech, Offenbach, Germany
Hygromycin B, solution	Invivogen, San Diego, CA, USA
Immersion Oil	Carl Zeiss, Jena, Germany
L-Glutamine	Biochrom, Berlin, Germany, Berlin, Germany
Lipoxin A 4, 15-epi	Calbiochem, Nottingham, UK
Lymphocyte Separation Medium 1077 (LSM 1077)	PAA, Pasching, Austria
Mannan	Sigma, Deisenhofen, Germany
Manganese chloride	Sigma, Deisenhofen, Germany
Medium 199	Sigma, Deisenhofen, Germany
Monodansylcadaverin (MDC)	Sigma, Deisenhofen, Germany
MOPS	Sigma, Deisenhofen, Germany
$NaHPO_4$	Sigma, Deisenhofen, Germany
Neomycin	Invivogen, San Diego, CA, USA
N-Formyl-L-Methionyl-L-Leucyl-L-Phenylalanine (fMLP)	Sigma, Deisenhofen, Germany
Opti-MEM	Invitrogen, Karlsruhe, Germany,
Paraformaldehyde (PFA)	Sigma, Deisenhofen, Germany
PBS, 1 x	PAA, Pasching, Austria
PBS, 10x	Biochrom, Berlin, Germany
Peanut Lectin	Sigma, Deisenhofen, Germany
Peanut Lectin Biotin	Sigma, Deisenhofen, Germany
Penicillin/Streptomcyin	Biochrom, Berlin, Germany
Percoll®	GE-Healthcare, Dornstadt, Germany

Phleomycin	Invivogen, San Diego, CA, USA
Phorbol 12-myristate 13-acetate (PMA)	Sigma, Deisenhofen, Germany
Phosphate Buffered Saline (PBS), 1x	PAA, Pasching, Austria
Polyinositylic acid (Poly I)	Sigma, Deisenhofen, Germany
Rabbit Blood, defibrinated	Elocin-Lab GmBH, Mülheim, Germany
Roswell Park Memorial Institute (RPMI) 1640 Medium	Sigma, Deisenhofen, Germany
Rubidium chloride	Sigma, Deisenhofen, Germany
Sodium Acetat	Sigma, Deisenhofen, Germany
Sodium Azide	Sigma, Deisenhofen, Germany
Sodium Cacodylate Trihydrosate	Fluka
Sodium Chloride	Sigma, Deisenhofen, Germany
Sodium Dodecyl Sulfate (SDS)	Sigma, Deisenhofen, Germany
Stem Cell Factor (SCF)	Cell culture supernatant of CHO-SCF Cells
Streptavidin-FITC	DakoCytomation, Hamburg, Germany
Triethanolamine	Merck, KGaA, Darmstadt, Germany
TRIS	Sigma, Deisenhofen, Germany
Urea	Sigma, Deisenhofen, Germany
Leishmania Chemotaktic Factor (LCF)	Supernatant of 10 days old L. major promastigote cultures, pelleted at 10000 x g for 30 minutes to remove residual parasites.

2.1.8 Antibodies

IgG1, X40	BD Pharmingen, Heidelberg, Germany
Isotype (APC), IgG2a, R35-95	Caltag Laboratories, Hamburg, Germany
Isotype (FITC), IgG2b, MPC 11	BD Pharmingen, Heidelberg, Germany
Isotype (PE), IgG2a, R35-95	BD Pharmingen, Heidelberg, Germany
Mouse anti-CD11b, IgG1, ICRF44	BD Pharmingen, Heidelberg, Germany
Mouse anti-CD14 (APC), IgG2a, TUK4	Caltag Laboratories, Hamburg, Germany
Mouse anti-CD163 (PE), IgG2, GHI/61	BD Pharmingen, Heidelberg, Germany
Mouse anti-CD206 (PE), IgG1κ, 19.2	BD Pharmingen, Heidelberg, Germany
Mouse anti-MHCII (FITC), IgG2b	Invitrogen, Karlsruhe, Germany
Mouse anti-Lm PPG/LPG, IgG1, WIC79.3	Kind Gift of Dr. Routier, Hannover, Germany

Rabbit anti-CR1, IgG	Kind Gift of Dr. Daha, Leiden, NL
Goat anti-mouse FAB2 (FITC)	DakaCytomation, Hamburg, Germany

2.1.9 Enzymes

FideliTaq™-DNA-polymerase	USB, Lonza, Wuppertal, Germany
lm-PromII reverse transcriptase	Promega, Mannheim, Germany
Shrimp Alkaline phosphatase	Promega, Mannheim, Germany
T4-DNA-Ligase	Promega, Mannheim, Germany

2.1.10 Restriction Enzymes

Ase I	Promega, Mannheim, Germany
Bam HI	Promega, Mannheim, Germany
EcoR I	Promega, Mannheim, Germany
Kpn I	Promega, Mannheim, Germany
Nde I	Promega, Mannheim, Germany
Nhe I	Promega, Mannheim, Germany
Pac I	New England Biolabs, Frankfurt am Main, Germany
Pme I	New England Biolabs, Frankfurt am Main, Germany
Sma I	Promega, Mannheim, Germany
Spe I	Promega, Mannheim, Germany
Xba I	Promega, Mannheim, Germany

2.1.11 Ready to use Kits

cDNA synthese Kit	Promega, Mannheim, Germany
In Situ Cell Death Detection Kit (TUNEL)	Roche Applied Science, Mannheim, Germany Applied Biosystems, Mannheim, Germany
LightCycler®FastStart Master Plus Syber Green I Kit	Roche Applied Science, Mannheim, Germany
Plasmid mini Purification Kit	Promega, Mannheim, Germany
Pure Yield Plasmid Maxi Kit	Promega, Mannheim, Germany
SVTotal RNA Isolation System	Promega, Mannheim, Germany

Wizard SV Gel & PCR Clean up System	Promega, Mannheim, Germany

2.1.12 Laboratory Supplies

Cell Culture Flask (25 cm^2; 75 cm^2)	BD labware Europe Le Pont de Claix, France
Cell Culture Plates (6 well; 24 well; 96 well)	BD labware Europe Le Pont de Claix, France
Centrifuge Tubes (15 mL; 50 mL)	BD labware Europe Le Pont de Claix, France
Cryo tubes Cryo.S	Greiner Bio-one, Frickenhausen, Germany
Electroparation cuvettes 1/2/4 mm	PeqLab, Erlangen, Germany
FACS-Tubes	Micronic, Lelystad, The Netherlands
FACS-Tubes	BD labware Europe Le Pont de Claix, France
LightCycler® Capillaries 20 µL	Roche Applied Science, Mannheim, Germany Diagnostics, Mannheim, Germany
Microtestplate, 96 well (V-Bottom)	Sarstedt, Nümbrecht, Germany
Multiplier-Tubes (0,65 mL), biopure	Sarstedt, Nümbrecht, Germany
Pipette filter tips (1-10 µL, 10-100µL, 50-200 µL, 100-1000 µL)	Sarstedt, Nümbrecht, Germany
Reaction Tubes (0,5 mL; 1,5 mL; 2,0 mL)	Eppendorf, Hamburg, Germany
Serological Pipettes, steril (2,5 mL; 5 mL; 10 mL; 25 mL)	Corning Inc., Corning, New York, USA
S-Monovette (9 mL), lithium heparin	Sarstedt, Nümbrecht, Germany
Transfer pipette (3,5 mL)	Sarstedt, Nümbrecht, Germany

2.1.13 Instruments

Balances	
Analytical balance AG204	Mettler Toledo, Giessen, Germany
Balance KERN470	Kern & Sohn GmbH, Balingen-Frommern, Germany
Cell Counting Chamber	Neubauer, improved, Marienfeld, Germany
Centrifuges	
Multifuge 3 SR	Heraeus, Thermo, Dreieich, Germany
Centrifuge 5471	Eppendorf, Hamburg, Germany
Variofuge 3.OR	Heraeus, Thermo, Dreieich, Germany
Cytocentrifuge Cytospin3	Shandon, Frankfurt, Germany
CO_2-Incubators	

Thermo Forma 3010	Thermo, Dreieich, Germany
Heraeus BBD 6220	Thermo, Dreieich, Germany
Heracell	Thermo, Dreieich, Germany
Electroporator EasyjecT®	EquiBio, PeqLab, Erlangen, Germany
Electroporator 2510	Eppendorf, Hamburg, Germany
Flow-cytometer FACS-Calibur II	Becton Dickinson, Heidelberg, Germany
Freezers	
-20°C	Bosch, Stuttgart, Germany
-80°C Herafreeze	Heraeus Sepatech GmbH, Osterode, Germany
Gel Documentation	Kappa opto-electronics GmbH, Gleichen, Germany
Gel Electrophoresis Chamber	Cti GmbH, Idstein, Germany
Laminar flow workbench,	
Laminar HBB 2448	Heraeus, Thermo, Dreieich, Germany
LightCycler®	Roche Applied Science, Mannheim, Germany Diagnostics, Mannheim
Magnetic stirrer MR3002	Heidolph, Leverkusen, Germany
Microscopes	
Axioscope	Carl Zeiss, Jena, Germany
Axiovert	Carl Zeiss, Jena, Germany
Microwave oven R-358	Sharp, Hamburg, Germany
PCR-Thermocycler TProfessional	Biometra, Göttingen, Germany
pH-Meter pH525	WTW, Wellheim, Germany
Photometers Ultraspec 3000	Pharmacia Biotech GmbH, Freiburg, Germany
Pipettes	Eppendorf, Hamburg, Germany
Power Supply ST606T	Gibco BRL
Shaker	VWR, Darmstadt, Germany
Water Bath	GFL, Burgwedel, Germany

2.1.14 Software

CellQuest® Pro	Becton Dickinson, Heidelberg, Germany
Excel for Mac 2004	Microsoft Co-operation, Mountain View, CA

	USA
LightCycler® Software Version 3.5	Roche Applied Science, Mannheim
WinMDI 2.8	Joseph Trotter, The Scripps Research Insitute, San Diego, CA, USA

2.2 Methods

2.2.1 Cell culture methods

All cell cultures were kept in humidified CO_2 incubators. The cells were passaged under steril conditions in endotoxin free environments.

2.2.1.1 Cultivation of *L. major* promastigotes

L. major promastigotes were cultured at 26°C, 5% CO_2 either in biphasic Novy-Nicolle-McNeal blood agar medium or in Lm-Suspension-Medium. *L. major* promastigote cultures were passaged in stationary growth phase. Up to ten serial passages were performed with each culture before it was discarded. To maintain a continuous pool of virulent parasites BALB/c mice were infected with 2 x 10^6 stationary-phase (state-phase) *L. major* promastigotes in the hind footpad. Subsequently, 2 weeks after infection parasites from popliteal lymph nodes were re-isolated and cultured on biphasic Novy-Nicolle-McNeal blood agar medium (NNN-Medium).

For Long time storage stationary-phase *L. major* were pelleted at 2400 x g at 4°C for 8 minutes. The pellet was resuspended in cold Lm-Medium supplemented with 20% FCS and 10% DMSO at a cell density of 1 x 10^8 *L. major* / ml. The cells were transferred into cryo – tubes and put into styropore boxes at -80°C overnight, thereafter, the vials could be stored in liquid nitrogen.

L. major were thawed in a 37°C water-bath, drop-wise added on top of Lm-Medium and centrifuged at 2400 x g for 8 minutes. The pellet was washed once more before the pellet was resuspended in 12 ml Lm-Medium. Of this suspension 100 µl / well was added onto NNN-Medium in 96 well plates. After 3 days the culture parasites were passaged for the first time.

2.2.1.2 Purification of metacyclic *L. major* promastigotes

Metacyclic *L. major* promastigotes were separated from stationary-phase cultures by Peanut lectin agglutination assay. Stationary-phase promastigotes were washed in DMEM twice and resuspended at a concentration of 2 x 10^8 *L. major* promastigotes / ml. 50 µg / ml Peanut lectin was added and incubated for 30 minutes at room temperature. Agglutinated procyclic promastigotes were pelleted by centrifugation for 10 minutes at 200 x g. The supernatant was transferred into a fresh tube and washed twice in DMEM supplemented with 20 mM D-galactose. The cells were pelleted at 2400 x g and counted.

2.2.1.3 Isolation of *L. major* amastigotes from lesions of infected mice

Infected Balb/c mice were sacrificed and the lesion was cut from the hind paw using sterile scalpels. The tissue was grinded in a tissue-grinder in RPMI-1640. The cell suspension was transferred into a 50 ml tube and the volume adjusted to 25 ml with Lm-medium and centrifuged for 10 minutes at 50 x g at 4°C. Supernatant was transferred into a new tube and successively filtered through a 30 µm mesh, a 20 µm mesh and finally through a 5 µm syringe filter. The cell suspension was washed 3-times at 1450 x g for 17 minutes at 4°C. Purity of cells were determined by analysing Diff QUIK® stained cytospin slides.

2.2.1.4 Isolation of *L. major* amastigotes from macrophages

The infected macrophages (MΦ) were washed twice with RPMI 1640 before they were incubated in RPMI 1640 supplemented with 0.01 % SDS for 30 seconds to 1 minute until the MΦ disintegrated. The incubation was stopped by adding AAM supplemented with 20 % FCS and the cells were harvested and centrifuged at 2400 x g for 8 min. The pellet was resuspendet and the cell suspension was centrifuged at 75 x g for 8 min. The supernatant was collected into a fresh tube and spun again at 75 x g for 8 min. This procedure was repeated twice. The remaining suspension was centrifuged at 2400 x g for 8 min and the *L. major* amastigotes were resuspended in AAM at a density of 20 x 10^6 *L. major* / ml. Purity of cells were determined by analysing Diff QUIK® stained cytospin slides.

2.2.1.5 axenic *L. donovani* amastigotes

Log phase (day 3-4 of culture) *L. donovani* promastigotes were pelleted at 2400 x g. The pettet was resuspended in Lm-Medium adjusted to ph 5.5 and centrifuged for 8 minutes at 2400 x g. The step was repeated once. The cells were adjusted to a density of 1 x 10^6 *L. donovani* / ml in Lm-Medium with ph 5.5. The culture was incubated for one week at 27°C. The culture was pelleted and resuspended in fresh Lm-Medium, pH 5.5. The cells were transferred into 6-well plates at a density of 5 x 10^6 / ml and incubated at 34°C.

2.2.1.6 Generation of *L. major* amastigotes *in vitro*

Log phase (day 3-4 of culture) *L. major* suspension cultures were pelleted at 2400 x g. The pellet was resuspended in 5 ml AAM and centrifuged for 8 min at 1450 x g. The step was repeated once. The cells were adjusted to a density of 20 x 10^6 *L. major* / ml in AAM. The culture was incubated for 10-14 days at 33°C. The cell culture was then harvested and centrifuged for 8 min at 2400 x g.

In order to separate the remaining promastigotes from the amastigotes a discontinuous Histopaque® 1119 density gradient was used. The pellet was resuspended in 50 % (v/v) (1.0595 g/ml) Histopaque® 1119 and layered on, from top to bottom, layers of 70 % (v/v) (1.0833 g/ml), 80 % (v/v) (1.0952 g/ml), 90 % (v/v) (1.1071 g/ml) and 100 % Histopaque® 1119 (1.119 g/ml). The gradient was centrifuged for 35 min at 2400 x g and the interphase between 90 and 100 % Histopaque® 1119 was collected, washed twice in AAM and resuspended in AAM at 20 x 10^6 Lm/ml. The purified *L. major* amastigotes were cultured in 25 cm^2 cell-culture flasks at 33°C until further use. Purity of cells were determined by analysing Diff QUIK® stained cytospin slides.

2.2.1.7 Transfection of *L. major*

2.2.1.7.1 Stable integration

Leishmania from suspension cultures were harvested in logarithmic-phase growth and washed twice in ice-cold Electroporation-Buffer at 1450 x g. The pellet was resuspended in ice-cold Electroporation-Buffer at a density of 2 x 10^8 *L. major* / ml. 100 µl of the cell-suspension was transferred in a cuvette with 2 mm gap and 1 µg DNA in H_2O was added. The mixture was incubated on ice for 1 minute, before the sample was electroporated with 2 consecutive pulses with a 5 seconds pause in between. The first pulse at 1500 V, 720 Ω and 25 µF was followed a second charge at 450 V, 720 Ω and 480 µF. The sample was kept on ice for 10 minutes before 4 ml Lm-Suspension-Medium was added and the cells were transferred to a 6-well plate and incubated overnight at 26°C.

2.2.1.7.2 Transient integration

Leishmania from suspension cultures were harvested in logarithmic-phase growth and washed twice in ice-cold Electroporation-Buffer. Cells were centrifugated at 1450 x g at 4°C. The pellet was resuspended in ice-cold Electroporation-Buffer at a density of 2 x 10^8 *L. major* / ml. 100 µl cell-suspension was transferred into a cuvette with 2 mm gap and 2 µg cosmid-DNA was added. The mixture was incubated on ice for on minute before the sample was subjected to 1 pulse at 450 V, 720 Ω and 480 µF. The sample was kept on ice for 10 minutes before four ml of Lm-Suspension-Medium was added. The cells were transferred to a 6-well plate and incubated overight at 26°C.

2.2.1.7.3 Selection of transfected *Leishmania*

After the overnight incubation the cell cultures were pelleted at 2400 x g and resuspended in suspension medium containing the appropriate antibiotics. The cell densities were adjusted to 4.8 *L. major* / ml and 100 µl / well of this cell suspension was transferred into a well in a 96-well plate. The cultures were incubated at 26°C, 5% CO_2 for up to 3 weeks. After 10 days of culture daily visual controls of the 96-well plates were performed to check for growth of monoclonal colonies.

2.2.1.8 Isolation of human PMN

Venous peripheral blood was collected from healthy adult volunteers using lithium-heparin S-monovetts. The heparinised blood was layered on a density gradient consisting of lymphocyte separation medium 1077 (LSM) on top of Histopaque 1119®. The gradient was centrifuged for 5 minutes at 300 x g followed by 25 minutes at 1024 x g. The plasma and the interphase between plasma and LSM, consisting mainly of PBMC, was discarded and the PMN rich Histopaque® 1119 phase was collected, leaving the erythrocyte pellet in the tube. The PMN were washed once in PBS, resuspended in PMN-Medium and layered on top of a Percoll® gradient consisting of layers with densities (from top to bottom) of 1.081 g/ml (65%), 1.087 g/ml (70%), 1.100 g/ml (80%) and 1.105 g/ml (85%). The gradient was centrifuged at 1024 x g for 25 minutes. The interphases between 70 and 80 % Percoll® was collected, washed once in Wash-Buffer and resuspended in PMN-Medium at a density of 10 x 10^6 PMN / ml. All procedures were performed at room temperature. Viability of the cells was determined by trypan blue exclusion and was usually above 97%. Purity of cells were determined by analysing Diff QUIK® stained cytospin slides.

2.2.1.9 Isolation of human PBMC

Monocytes were isolated from buffy coats from health adult blood donors. The buffy coats were diluted 1:8 with PBS and layered on top of lymphocyte separation medium 1077, centrifuged for 5 minutes at 300 x g followed by 25 minutes at 1024 x g. The plasma was discarded and the interphase consisting mainly of PBMC was collected. The cells were washed in Wash-Buffer once at 1024 x g and 3 times at 135 x g. Subsequently the pellet was resuspended in PMN-Medium at a density of 10 x 10^6 PBMC / ml.

2.2.1.10 Generation of blood derived MΦ

PBMC were cultured in 25 cm^2 cell-culture flasks and incubated for 90 minutes at 37°C. The supernatant was discarded and the adherent monocytes were washed twice with pre-warmed Wash-Buffer. The cells were covered with PMN-Medium supplemented with either 10 ng / ml GM-CSF to generate Type I MΦ or 10 ng / ml M-CSF to generate Type II MΦ and incubated for 5 days at 37°C.

2.2.1.11 Cultivation of HoxB8 PMN pre-cursor cells

HoxB8 cells were cultured in 6-well cell culture plates in OptiMEM supplemented with 10 % FCS, 30 µM β-Mercaptoethanol, Pen / Strep, (Basic Medium) 1 mM β-Estradiol and 1:25 SCF at 37°C. Every 48 h the HoxB8 PMN pre-cursor cells were passaged and cultured at a cell density of 2.5 x 10^5 cells / ml.

24 hours after passaging the pre-cursor cells could be differentiated into PMN by washing the cells twice with 1 x PBS supplemented with 10% (v/v) FCS and 2 % (v/v) SCF containing supernatant. Subsequently the pellet was resuspended in OptiMEM supplemented with 10 % FCS, 30 µM β-Mercaptoethanol, Pen / Strep and 4 % (v/v) SCF containing supernatant, without adding β-Estradiol, at a density of 1 x 10^5 cells / ml. The HoxB8 pre-cursor cells were brought out in 6-well plates and incubated for 96 h at 37°C.

For long time storage of HoxB8 cells in liquid nitrogen, HoxB8 cells were passaged a day before they were harvested, washed in ice cold Basic-Medium supplemented with 1 mM β-Estradiol and 1:25 SCF, 20 % FCS and 20 % DMSO. 5 x 106 HoxB8 cells were transferred into cryo vials and the same volume FCS was added. The cells were stored overnight at -70°C before the transfer into liquid nitrogen.

The HoxB8 cells were thawed in a 37°C waterbath, layered drop-wise on top of pure FCS, centrifuged once at 300 x g and the pellet resuspended in 3 ml Basic-Medium supplemented with 1 mM β-Estradiol and 1:25 SCF, transferred into 1 well of a 6 well cell culture plate and incubated overnight at 37°C in a humidified CO2 incubator. The next day 1 ml Basic-Medium supplemented with 1 mM β-Estradiol and 1:25 SCF was added to the culture. 48 hours after thawing the cells the cells were passaged the first time.

2.2.1.12 Cultivation of THP-1 cells

THP-1 cells were cultured in 75 cm^2 culture flasks at a density of 3 x 10^5 cell / ml in Complete-Medium. Cells were passaged every 72 h. In order to differentiate THP-1 cells into macrophages, cells were pelleted at 400 x g for 6 minutes and resuspended in Complete-

Medium supplemented with 10 ng / ml PMA and transferred into 6 well plates at a density of 5 x 105 THP-1 / well. The cells were incubated for 18 hours at 37C° in CO_2 incubators until further needed.

2.2.1.13 Cultivation of CHO-SCF cells

The adherent cells were cultured in Hox-Basic-Medium in 75 cm^2 culture flasks. To passage the CHO-SCF, the cells were washed twice with 1 x PBS and incubated with pre-warmed Trypsin / EDTA for 3 minutes at 37°C. After 3 minutes adding Hox-Basic-Medium stopped the reaction. The CHO-SCF cells were washed twice in Hox-Basic-Medium and a cell density of 2 x 10^5 / ml was adjusted and brought out in 75 cm^2 culture flasks.
48 hours after passaging CHO-SCF cells the supernatant was collected in 50 ml tubes and stored at -20°C until 500 ml SCF containing supernatant could be pooled, sterile filtered and stored at -20°C until use.

2.2.1.14 Incubation of PMN with *L. major*

Freshly isolated PMN were incubated with *L. major* at a ratio of 1:10 in Infection-Medium for 3 or 18 hours at 37°C in a humidified atmosphere in a CO_2 incubator. Non-phagocytosed parasites were removed by washing the cells four times at 135 x g in Wash-Buffer. Infection rates were assessed by counting parasitized cells in > 200 PMN in Diff-QUIK stained cytospin slides. Parasite burden was evaluated by counting parasites in 20 infected PMN.

2.2.1.15 Incubation of MΦ with *L. major*

After 5 days of incubation in the presence of either GM-CSF or M-CSF the MΦ were harvested, counted and 1 x 10^6 MΦ/ml were transferred into 24-well or 96-well cell culture plates. The cells were left to adhere for 90 minutes. The non-adherent cells were discarded and Complete-Medium with 10 x 10^6 stationary-phase *L. major* promastigotes or 3 days old *L. major* axenic amastigotes were added to the adherent cells. The plates were centrifuged at 135 x g for 3 minutes before they were incubated at 37°C in a humidified atmosphere in a CO_2 incubator. After 3 hours of incubation the extra-cellular parasites were removed by washing the adherent cells four times with pre-warmed Wash-Buffer. Infection rates were assessed by counting parasitized cells in > 200 MF in Diff-QUIK stained cytospin slides. Parasite burden was evaluated by counting parasites in 20 infected MF.

2.2.1.16 Blocking the uptake of *L. major* by MΦ

In order to block the uptake of the parasites the phagocytes were pre-incubated with 1 of the following pharmaceuticals. General uptake of particles was blocked by 15 minutes incubation of the phagocytes In Complete-Medium supplemented with 10 µM Cytochalasin D. Phagocytosis was blocked by treating the cells with 250 µM Dimethylamiloride (DMA) in Complete-Medium for 30 minutes. Caveolae-mediated endocytosis was blocked by a 30 minute incubation with 10 nM Fillipin III and Clatherin-mediated endocytosis was blocked by a 30 minute treatment with 250 µM Monodansylcadaverine (MDC) in Complete-Medium. The adherent cells were washed twice with pre-warmed Complete-Medium to remove the pharmaceuticals.

2.2.1.17 Blocking specific receptors of MΦ

Mannose Receptor (CD206) mediated uptake was blocked by pre-incubation of the phagocytes with 100 µg / ml Mannan for 15 minutes. By adding Polyinositolic acid (Poly-I) to the cells for 15 minutes Scavanger Receptor A (SR-A) was blocked. Complement Receptor I (CR-I) was blocked by treating the phagocytes with 10 µg / 1 x 10^6 / MΦ anti human CRI antibody for 20 minutes. Complement Receptor III (CR-III) was blocked with 1 µg / 1 x 10^6 / MΦ murine anti human CD11b antibody for 20 minutes. Before the addition of the *L. major* to the pre-treated macrophages washing the cells twice with fresh Complete-Medium eliminated the not bound blockers.

2.2.1.18 FACS analysis

2.2.1.18.1 FACS analysis of MΦ

FACS staining was performed in 96-well-v-bottom-plates in the dark on ice. MF were washed in FACS-Buffer once. 7.5 x 10^5 MΦ were resuspended in FACS-buffer and transferred into 1 well of the 96-well-v-bottom-plates. Cells were incubated with α-CD163-PE and α-MHC II-FITC or α-CD14-APC and α-CD206-PE in FACS-Buffer for 30 minutes. Cells were washed twice in FACS buffer, before being resuspended in FACS-Buffer supplemented with 1 % paraformaldehyd. PE-, FITC- or APC-conjugated matched mouse IgG1 and mouse IgG2 antibodies were used as isotype controls. Cells were analysed on a FACS Calibur® II flow cytometer using CellQuest® pro software.

2.2.1.18.2 FACS analysis of *Leishmania*

FACS staining of *Leishmania* were performed in 96-well-v-bottom plates in the dark on ice. 5 x 10^6 parasites were transferred into 1 well of, washed twice in Lm-FACS-Buffer.

In order to detect surface expression of PS the parasites were incubated in 100 µl Lm-FACS-Buffer supplemented with 0.1 µg Annexin A5 (AnxA5) –FITC or AnxA5-Fluos for 15 minutes and washed once.

LPG was detected by incubating *L. major* in 100 µl Lm-FACS-Buffer supplemented with 0.25 µg / ml PNL-biotin, or left untreated. Cells were incubated on ice for 15 minutes, washed once in Lm-FACS-Buffer and resuspended in 100 µl Lm-FACS-Buffer supplemented with streptavidin-FITC. Cells were incubated on ice in the dark for 30 minutes washed once.

PPG was detected by incubating *L. major* in 100 µl Lm-FACS-Buffer supplemented with mouse-anti-*Leishmania*-antibody WIC79.3, or left untreated. Cells were incubated on ice for 15 minutes washed once and resuspended in 100 µl Lm-FACS-Buffer supplemented with anti-mouse IgG2-PE. Cells were incubated on ice in the dark for 30 minutes, washed once.

Next the pellets of the labelled parasites were resuspended in Lm-FACS-Buffer supplemented with 1 % (w/v) paraformaldehyde and analysed on a FACS Calibur® II flow cytometer using CellQuest® pro software.

In order to detect fragmentised DNA TUNEL staining was performed according to the manufactures protocol. In short, 5 x 10^5 *L. major* were fixed in 4% paraformaldehyd-PBS (pH 7.4) for 60 minutes at room temperature. Cells were washed once in PBS and resuspended in permeabilisation solution for 2 minutes on ice. TUNEL reaction mixture was prepared by adding 5 µl of enzyme to 45 µl of label solution. Cells were washed once in PBS and resuspended in 50 µl reaction mixture or in label solution al1 as negative control. The cells were incubated for 60 minutes in the dark at 37°C. Cells were washed twice in PBS and resupended in PBS 1 % (w/v) paraformaldehyde for us in FACS-analysis or fluorescence microscopy-analysis.

2.2.1.19 Purification of murine PMN

Murine PMN were isolated from whole blood or from air-pouch cell suspension using Anti-Ly-6G MicroBead Kit (Miltenyi Biotec) according to the manufactures instructions. Cell-suspensions were kept on ice at all times. Cells were pelleted at 300 x g for 10 minutes and cell concentrations of 5 x 10^8 cells / ml were adjusted in MACS-Buffer. 50 µl Anti-Ly-6G-Biotin / 1 x 10^8 cells were added and mixed. Cells were incubate at 4°C for 15 minutes. Cells were washed in 10 ml MACS-Buffer and pelleted for 10 minutes at 300 x g. The supernatant

was discarded. A LS-MACS Column was placed in the magnetic field of the MACS Separator and equilibrated by rinsing the Column with 3 ml MACS-Buffer. The cell suspension was applied onto the column and passed through the magnetic field by gravitational flow. The column was washed 3-times with MACS-Buffer. The collected flow-through harbours Ly-6G negative cells. The column was removed from the magnetic field and placed on top of a 15 ml tube. 5 ml MACS-Buffer was added to the column and passed through by gravitational flow. The elution step was repeated with additional 5 ml MACS-Buffer and the remaining cells were flushed out by firmly applying the supplied plunger. These collected cells are Ly-6G positive murine PMN. Cells were pelleted at 300 x g for 10 minutes and resuspended in RPMI-1640 for further use.

2.2.1.20 Cytocentrifuging cells

2 x 10^5 PMN, 1 x 10^5 MΦ or 2 x 10^6 *Leishmania* were washed once in Medium and resuspended in 100 µl 1 x PBS. Cells were spun on slides in a Shandon CytoCentrifuge at 54 x g for both PMN and MΦ or 500 x g for *Leishmania* for 5 or 10 minutes respectively. The slides were air-dried for further use.

2.2.1.21 Diff QUIK staining

Air-dried cytospins were incubated for 30 seconds in the fixation solution of a Diff QUIK® kit followed by 30 seconds incubation in Diff QUIK® staining solution I and 30 seconds incubation in staining solution II. Slides were rinsed in tap-water and used for microscopic analysis.

2.2.2 *In vivo* experiments

2.2.2.1 Air-pouch

8 weeks old female Balb/c (Charles River) mice were anaesthetised with ether before 3 ml of air was injected sub-dermal with a syringe between the shoulder blades. Subsequently, 1 x 10^8 *L. major* in 1 ml LCF containing supernatant were injected. 24 hours post infection the mice were sacrificed and the air-pouch was opened and rinsed thoroughly with 8 portions of 2 ml RPMI. The recovered cells were collected in 50 ml tubes and centrifuged at 400 x g. The pellet was resuspended in Complete-Medium and the cells were counted.

2.2.2.2 Infection of Mice

Either stationary-phase *L. major* promastigotes, 3 days old axenic *L. major* amastigotes or freshly isolated MF-derived *L. major* amastigotes were harvested and washed twice in RPMI 1640. Subsequently, 3 x 10^6 parasites were injected subcutaneously in the footpad of the mouse. Footpad swelling as a measurement of disease development was monitored weekly using a Vernier calliper.

2.2.3 Molecular biology methods

2.2.3.1 RNA isolation

Total RNA was isolated with *SV Total RNA Isolation System* from Promega according to the manufactures protocols. In short, 3, 18 or 42 h post infection 5 x 10^5 MΦ were washed twice with ice-cold PBS before they were lysed in 175 µl *RNA Lysis Buffer*. The lysate was transferred to a nuclease free conical minicentrifuge tube and 350 µl of *RNA Dilution Buffer* was added. The mixture was incubated for 3 minutes at 70°C in a heating block, followed by 10 minutes centrifugation at 12000 x g at room temperature. The cleared lysate was transferred into a fresh nuclease free conical microcentrifuge tube without disturbing the pellet and 200 µl pure ethanol was added. The mixture was transferred into a *Spin Basket* and centrifuged for 1 minute at 12000 x g. The flow through was discarded and the *Spin Basket* was washed once with 600 µl *RNA Wash Solution*. Onto the *Spin Basket*s membrane 50 µl *DNase I Incubation Mix* was added and incubated 15 minutes at room temperature. The incubation was stopped by adding 200 µl *DNase Stop Solution*. The *Spin Basket* was spun at 12000 x g for 1 minute before the membrane was washed twice with 600 and 250 µl *RNA Wash Solution*, respectively. A final centrifugation of 2 minutes was applied to dry the membrane before the RNA was eluted in 100 µl nuclease free water.

2.2.3.2 cDNA synthesis

cDNA synthesis was performed in thin walled nuclease free reaction tubes with the ImProm-II™ Reverse Transcriptase from Promega by the manufactures protocol. In short, 250 ng Total RNA was incubated together with 0,5 µg Random Primer in a final reaction volume of 5 µl at 70°C for 5 minutes followed by 5 minute incubation in ice-water. In the mean time the Reverse Tanscription Reaction Mix was prepared consisting of ImProm-II™ Reaction Buffer, 1.875 mM $MgCl_2$, 0.5 mM of each dNTP, 20 u Recombinant RNasin® Ribonuclease Inhibitor and 1 µl ImProm-IITM Reverse Transcriptase in nuclease free water in a final

volume of 15 µl. This Reaction Mix was added to the denatured RNA / Random Primer mix and incubated at 25°C for 5 minutes, followed by 1 hour at 42°C and a final incubation at 70°C for 15 minutes.

2.2.3.3 PCR

Polymerase chain reactions were performed in thin walled multiplier tubes under DNase I free conditions. Quantitative real-time PCR were performed in 20 µl LightCycler® Capillaries.

2.2.3.3.1 Colony PCR

In order to check for the presence of specific inserts in *Leishmania* transfectants or *E. coli* transformants, colony PCR was used. For the analysis *PRC Master Mix* (2 x) was used according to the manufactures protocol. In short, in a final volume of 25 µl the *PCR MasterMix* was supplemented with 200 nM forward primer, 200 nM reverse primer, 100 ng genomic DNA (*Leishmania* transfectants) or plasmid DNA (*E. coli* transformants). The reaction was heated to an initial denaturation at 94°C for 3 minutes followed by 35 cycles of 30 seconds denaturation at 94°C, 30 seconds annealing at 58°C and 30 to 60 seconds, depending on the analysed amplicon, elongation at 72°C, ending with a final extension of 5 minutes at 72°C, before the reactions was cooled down to 16°C. The sample was analysed on an agarose gel, stained with ethidiumbromid.

2.2.3.3.2 Amplification of DNA for cloning

To amplify DNA-fragments for cloning the proof reading enzyme *FideliTaq*™ was used according to the manufactures protocol. In short, in a final volume of 50 µl 500 ng DNA, 400 nM forward, 400 nM reverse Primer and *FideliTaq*™ *PCR Master Mix* was combined. The mixture was initially incubated for 120 seconds at 94°C followed by 35 cycles of 30 seconds denaturation at 94°C, 30 seconds annealing at 58°C and 150 seconds extension at 68°C finishing in a final extension at 68°C for 5 minutes before the sample was cooled down to 16°C. The sample was analysed on an ethidiumbormid stained agarose gel.

2.2.3.3.3 Quantitative real-time PCR

Differential gene expression was analysed by real-time PCR on a *LightCycler*® detection system using *LightCycler® FastStart DNA MasterPLUS SYBR* Green I kit according to the manufactures instructions. In short, each real-time PCR reaction consisted of a tenth volume of a cDNA synthesis, the *Master Mix* and 10 µM of forward and reverse primer in a final

reaction volume of 20 µl. The reaction was initiated by a 10 minutes denaturation at 95°C followed by 45 cycles with 10 seconds denaturation, 10 seconds annealing, 6 seconds extension at 72°C, 5 seconds melting of primer dimmers at 85°C, followed by the quantification of the fluorescence.

2.2.3.4 Agarose gel electrophoresis

DNA samples were diluted 1:6 with DNA-Loading Dye and separated on a horizontal agarose gel, stained with ethidiumbromide in TPE buffer. Electrophoresis was performed at 1 V / cm2 in TPE buffer. The DNA was visualized on a UV-table at 302 nm.

2.2.3.5 Gel-elution of DNA fragments

In order to purify DNA fragments of a specific length the DNA was separated on an agarose gel and the fragment of the desired size was excised. The gel slice was then melted and the DNA eluted with the Wizard® SV Gel and PCR Clean-Up System according to the manufactures instructions. In short, the gel slice was transferred into a 1,5 ml reaction tube, and dissolved in Membrane Binding Solution for ten minutes at 60°C. The solution was transferred onto a SV Minicolumn and incubated for 1 minute at room temperature before the column was centrifuged for 60 seconds at 16000 x g. The SV Minicolumn was washed twice with 700 and 500 µl Membrane Wash Solution before the DNA was eluted in 50 µl Nuclease Free Water.

2.2.3.6 Restriction Digestion

Restriction enzymes and buffer were used according to the manufactures instructions. In general 1 µg of DNA was incubated with 1 unit of enzyme overnight at the appropriate temperature.

2.2.3.7 Dephosphorylation of linearised DNA

In order to avoid self-ligation 1 µg linearized vector DNA was treated with 1 unit Shrimp Alkaline Phosphatase in 1 x reaction buffer for 15 minutes at 37°C. The enzyme was deactivated by heating the reaction to 65°C for 15 minutes before the DNA could be used in follow up experiments.

2.2.3.8 Ligation

50 ng of digested vector DNA and a 5 molar excess of insert DNA were incubated overnight at 4°C with 1 Weiss-U of T4-DNA-Ligase in a 20 µl reaction volume in the presence of 30 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 10 mM DTT and 1 mM ATP.

2.2.3.9 Plasmid isolation

2.2.3.9.1 Analytical plasmid isolation

For analytical plasmid isolation the *Wizard® Plus SV Minipreps DNA Purification System* was used according to the manufactures instructions. In short, 5 ml of selective LB-Medium were inoculated with a single cl1 from a selective LB-Agar-Plate and incubated at 37°C overnight in a shaking incubator at 150 rpm. 1.5 ml of the overnight culture were pelleted 5 minutes at 10000 x g. The pellet was resuspended in 250 µl *Cell Resuspension Solution* and mixed with 250 µl of *Cell Lysis Solution*. 10 µl *Alkaline Protease Solution* was added and incubated at room temperature for 5 minutes. The reaction was stopped by adding 350 µl Neutralizing Solution. The reaction tube was centrifuged 5 minutes at 10000 x g at room temperature and the cleared lysate was decanted onto a *Spin Column*. The DNA was bound to the silica membrane by centrifuging the sample for 1 minute at 10000 x g. The *Spin Column* was washed twice with *Wash Solution* with 750 and 250 µl respectively. The *Spin Column* was dried by centrifugation and the DNA was eluted in 100 µl nuclease free water.

2.2.3.9.2 Preparative plasmid isolation

Preparative plasmid isolations were carried out using PureYield™ Plasmid Maxiprep System according to the manufactures protocol. A 250 ml overnight culture of a single cl1 was pelleted at 5000 x g for 10 minutes at room temperature. The supernatant was discarded and the pellet resuspended in 12 mL Cell Resuspension Solution. The cells were lysed by adding 12 ml Cell Lysis Solution. After 3 minutes the reaction was stopped by adding 12 ml Neutralization Solution. The mixed lysate was centrifuged at 14000 x g for 20 minutes and the cleared solution was filtered with a PureYield™ Clearing Column, before the DNA was bound on a PureYieldTM Maxi Binding Column. By adding 5 ml Endotoxin Removal Wash possible endotoxins were removed. The column was washed once with 20 ml Column Wash, dried for 5 minutes until the DNA was eluted in 1,5 ml Nuclease Free Water by centrifugation. The DNA was quantificated and stored at 4°C until further need.

2.2.3.10 Transformation of bacteria

48 µl competent *E. coli* were thawed on ice for 5 minutes before 2 µl plasmid DNA or ligation mix were added. The mixture was incubated on ice for 20 minutes, followed by an 1 minute heat-shock at 42°C and a 2 minute cool down on ice. The mixture was resuspended in 500 µl pre-warmed LB-Medium and incubated 1 hour at 37°C and 150 rpm in a shaking incubator. A tenth of the reaction was then spread out on a selective LB-Agar-Plate and incubated overnight at 37°C.

2.2.3.11 Preparation of Chemically competent *E. coli*

A "starter colony" was grown in 5 ml LB medium at 37°C overnight. The next day 40 ml LB medium were inoculated with 400 µl of the overnight culture and incubated at 37°C in a shaking incubator until an OD600 of 0.4-0.5 was reached. The culture was centrifuged at 3000 x g for 10 minutes and the pellet resuspended in 23 ml RF1 and cooled on ice for 1 hour. The culture was centrifuged for 5 minutes at 3000 x g and the pellet resuspended in 3.2 ml RF2. The cells were incubated for 15 minutes on ice before they were divided into 100 µl aliquots that were shock-frozen in liquid nitrogen before they were stored until further use at -70°C.

3. Results

3.1 Generation of transgenic *L. major*

The virulent inoculum of *L. major* promastigotes contains two populations. One population consist of annexin A5 (AnxA5) -negative, viable *Leishmania*, the second population consist of AnxA5-binding and phosphatidylserine (PS) - positive, dead parasites. The PS-positive parasites have an immune-silencing effect on host phagocytes. This silencing enables the viable *Leishmania* to infect and survive inside the host's phagocytes. Having demonstrated that PS is a virulence factor for *L. major*, I wanted to develop a genetically altered organism that could not express PS by depleting its PSS gene, thereby potentially removing the parasites ability to generate PS.

3.1.1 Generation of a *L. major* PSS gene deletion mutant

Since *Leishmania* are diploid organisms, gene deletion needs two consecutive rounds of targeted gene replacement, to substitute each allele by a selection marker. Therefore, two constructs bearing different resistance markers had to be constructed. In order to knock-out the PSS gene.

3.1.1.1 Generation of the homologous gene cassette

In order to generate a gene cassette for homologous recombination the 500 bp upstream (5´-UTR) or downstream (3´-UTR) of the target gene *pss* were amplifyed by PCR. The used primers introduced restriction sites needed for subsequent cloning steps. At the 5´-UTR region *Xba* I, *Ase* I and *EcoR* I were inserted into the sequence. At the 3-UTR *EcoR* I, *Spe* I and *Kpn* I were introduced into the DNA fragment (Fig. 3.1.1 A). The restriction sites *Xba* I and *EcoR* I or *EcoR* I and *Kpn* I respectively were used to clone both fragments into the pcDNA-3 cloning vector. In a subsequent step both fragments were fused using the *EcoR* I site (Fig. 3.1.1 B). The cassette bearing 5´-UTR and 3´-UTR was then excised and sub-cloned into pGL3 cloning vector to avoid conflicting restriction sites in the pcDNA3 vector (Fig. 3.1.1 C). This plasmid was then linearised using *Ase* I and *Spe* I. By using these restriction sites the marker genes could be inserted in frame between the 5´-UTR and 3´-UTR securing a correct expression of the selection marker genes in *Leishmania* (Fig 3.1.1). The marker genes were generated by PCR from pCR2.1hyg and pCR2.1phleo (kind gifts from Dr. Martin Wiese, Edinburgh, UK) using primers that introduced *Nde* I at the 5´-end and *Nhe* I at the 3´-

end. These restriction sites are compatible to the enzymes mentioned above allowing an in frame cloning into a plasmid construct.

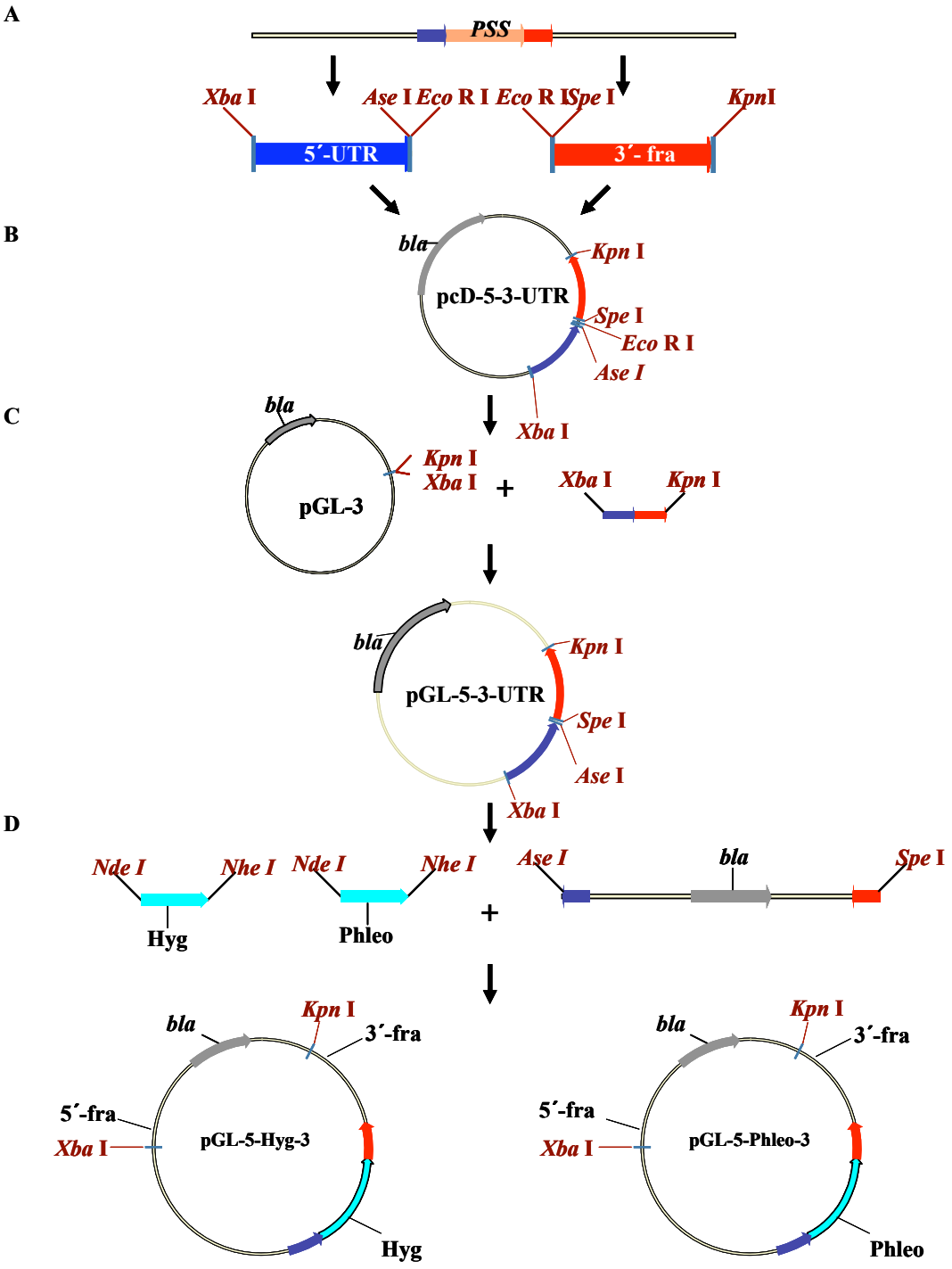

Figure 3.1.1 Scheme of the cloning strategy. A) The flanking regions of the target gene were PCR amplified, inserting restriction sites for *Xba* I, *Ase* I and *Eco* RI as well as *Eco* RI *Spe* I and *Kpn* I. B) The two regions were sub-cloned into pcDNA3 cloning vector using *Eco* RI to fuse the two fragments. C) The DNA fragment was excised with *Xba* I and *Kpn* I and sub-cloned into pGL-3. D) The plasmid DNA was linearised with *Ase* I and *Spe* I and fused with one of the selective marker genes coding for resistance against either phleomycin or hygromycin.

3.1.1.2 Targeted gene deletion of *L. major pss*

The hygromycin and phleomycin containing gene replacement cassettes were excised from the plasmids by *Xba* I / *Kpn* I digestion. The resulting fragments of 2 and 1.4 kb were separated on an agarose gel, excised and purified by gel extraction (Fig. 3.1.2). The purified DNA was quantified and 1 µg of DNA was electroporated into *L. major* promastigotes. The selection of the transfectants was assessed by growing the parasites on agar plates containing the appropriate selective drug, or by limiting dilution assays in liquid cultures in 96-well plates.

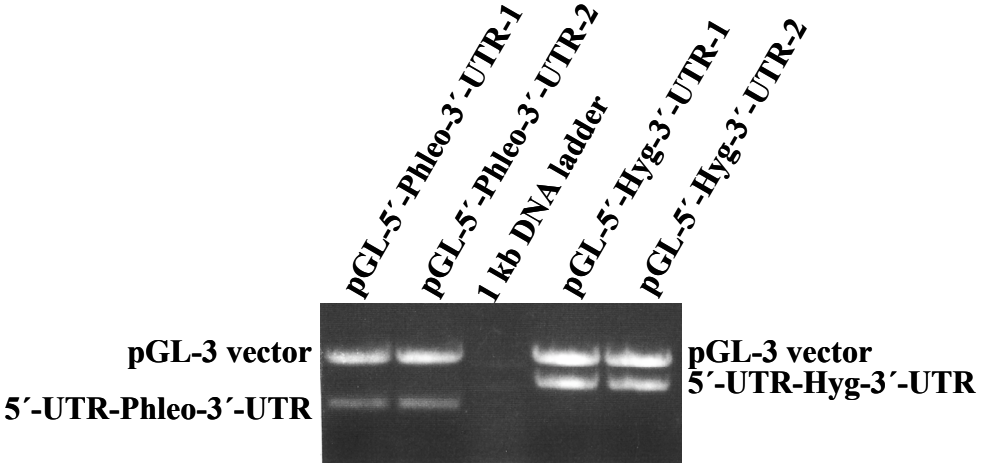

Figure 3.1.2 Excision of gene cassettes from plasmid DNA. Lane 1 and 2 pGL-5-UTR-Phleo-3´-UTR was digested with *Xba* I and *Kpn* I. Lane 3 1 kb DNA Ladder, lane 4 and 5 pGL-5´-UTR-Hyg-3´-UTR digested with *Xba* I and *Kpn* I. The depicted micrograph from 1 representative experiment of 5 independent experiments performed.

Different electroporation protocols (Tab. 3.1.1) that were published for genetic manipulations of eukaryotic cells were tested. It was tried to raise the electric charge (*Q*) of the electroporation by raising the initial voltage from 1500 to 2500 V (Tab. 3.1.1. Test 1-8), by raising the electric capacity from 25 to 480 and 500 µF (Tab. 3.1.1 Test 9-13) and by raising the gap width of the electroporation cuvette from 2 to 4 mm. Furthermore, it was tried to change the ratio of *L. major* to µg linearised DNA. Ratios ranged form 50 ng per 1 x 10^6 *L. major* to 200 ng per 1 x 10^6 *L. major*. Finally it was tried to use a two-pulse protocol (Tab. 3.1.1 Test 11-13) for the DNA to penetrate the plasma membrane as well as the membrane of the nucleus. The combination of two high charge pulses was tested in 2 and 4 mm cuvettes without revovering viable transfectants. Combining a low charge pulse with high charge pulse (Tab. 3.1.1 Test 13) was the last protocol applied for this knockout strategy. Despite, applying

these different electroporation conditions, no viable, heterozygote PSS-gene-knockout-transfectant could be recovered.

3.1.2 Shielding Leishmanial PS by endogenous expression of AnxA5

In addition to our knockout strategies for the PSS gene, I tried to shield PS in the *Leishmania* membrane by endogenous expression of PS-binding AnxA5. In order to achieve this, the complete coding region of the Annexin A5 gene (*anxa5*) was amplified from the pQE-musAnxV plasmid (a kind gift from Dr. E. Pöschl, Norwich, UK) inserting *Sma* I and *Bam* HI restriction sites 5´and 3´of the gene (Fig. 3.1.3 A). Using these restriction sites the gene was integrated into the *Leishmania* expression vector pXG-Neo (a kind gift from Dr. Beverley, St. Louise, USA) (Fig. 3.1.3 B), yielding the cosmid pXG-Neo_AnxA5 (Fig. 3.1.3 C).

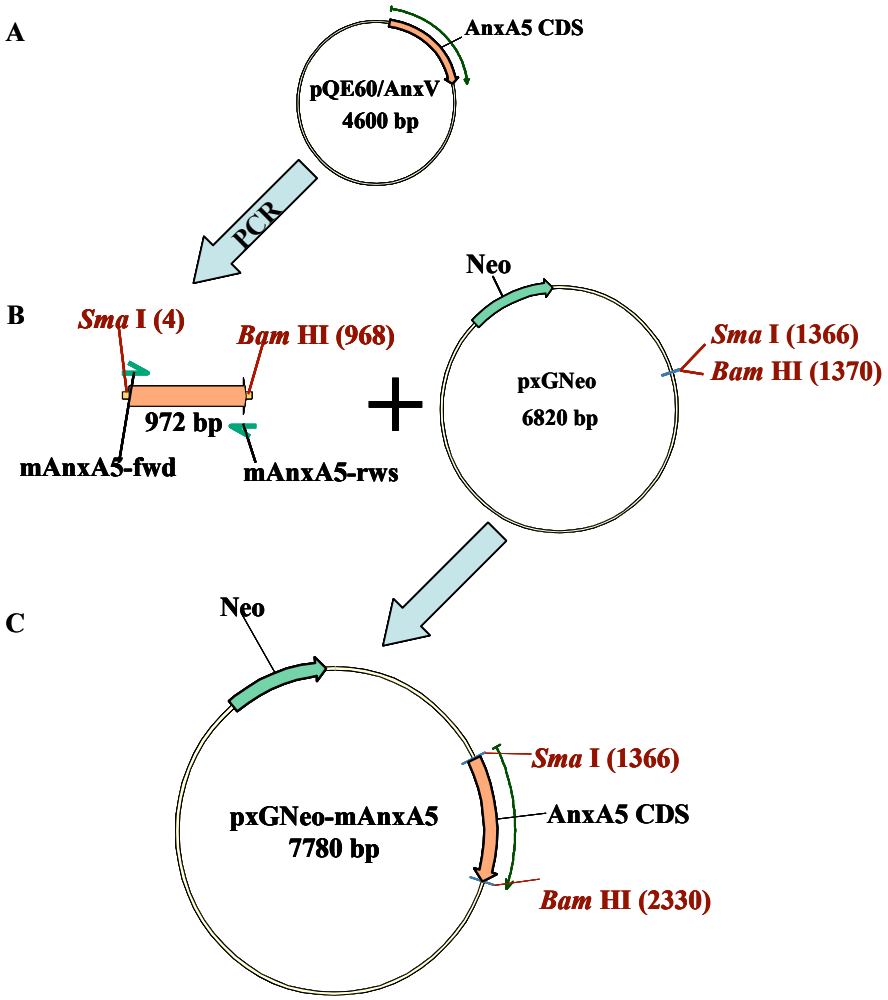

Figure 3.1.3 Scheme of the cloning strategy to shield PS. A) The murine AnxA 5 ORF was amplified by PCR from pQE60/AnxV plasmid inserting *Sma* I and *Bam* HI. B) pxGNeo was digested with *Sma* I and *Bam* HI and fused with murine AnxA 5 ORF. C) The endogenous expression vector pxGNeo-mAnxA5

L. major parasites were transfected with 2 µg of this cosmid using the single pule electroporation condition 10 (Tab. 3.1.1), targeting the DNA construct into the cytoplasm. The parasites were seeded out in 96-well plates at a concentration of 0.5 cells / well and cultured for 14 days at 27°C in selective medium containing 50 µg / ml Neomycin. Visible clones were expanded and cultured an additional week. DNA was isolated from all clones and checked for the presence of the Annexin A5 by PCR. From these clones the PCR was positive for clone 3 only (Fig. 3.1.4 A).

It was shown that at stationary phase growth (stationary-phase) of *L. major* about 50 % of the parasites become PS-positive and bind AnxA5 (van Zandbergen et al., 2006). Because the AnxA5-clone expresses endogenous AnxA5 we expected a decreased binding capacity of PS-positive, dead parasites towards exogenously added AnxA5, compared to wildtype parasites. Therefore, wildtype and AnxA5-clone 3 parasites were cultured in parallel for seven days. These cultures were stained with AnxA5-FITC and analysed by FACS. Though 40 % of wildtype parasites (Fig. 3.1.4 B, black line) were AnxA5-FITC positive in this experiment, only 10 % of the AnxA5-clone 3 parasites could be stained with AnxA5-FITC (Fig. 3.1.4 B, grey line). This is a decrease of the AnxA5 binding capacity by 80%.

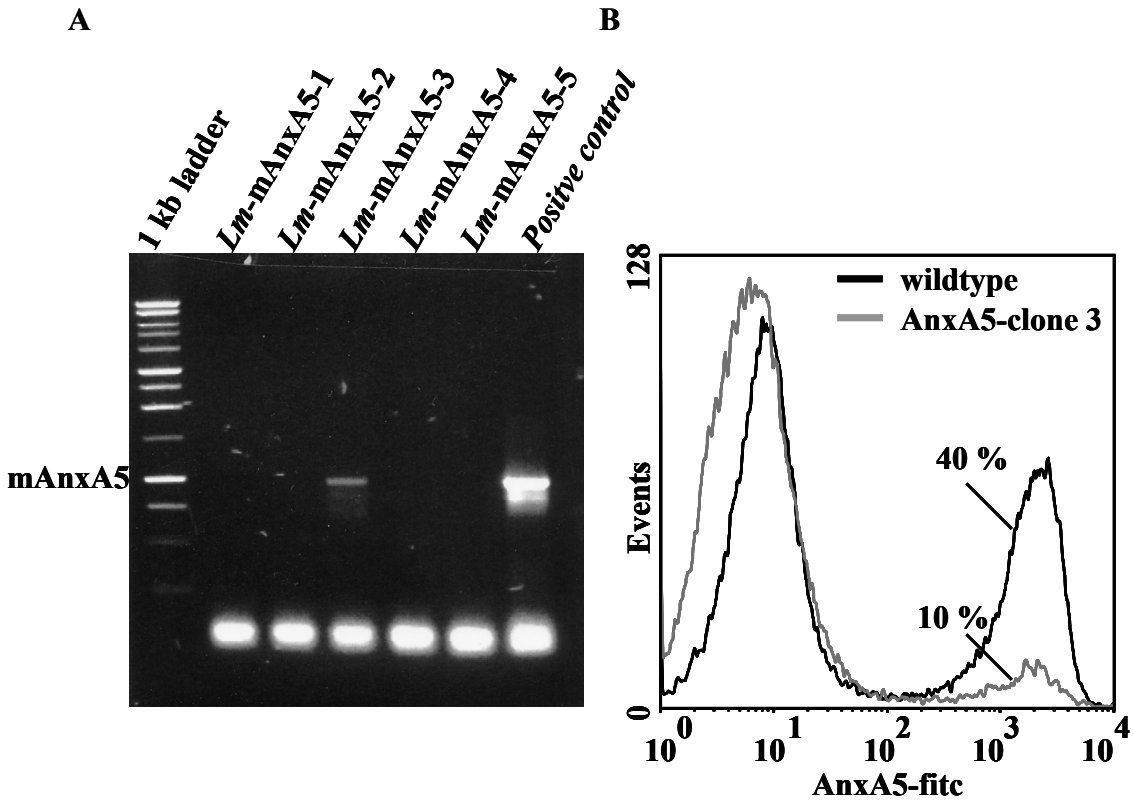

Figure 3.1.4 AnxA5 expressing transgenic *L. major* promastigotes. A) PCR screening of AnxA5 DNA in *L. major* promastigotes. Clone 3 is screened positive by PCR. **B)** Stationary-phase *L. major* promastigotes wild type (black line) and AnxA5-expressing transgenic *L. major* clone 3 (grey line) were stained with AnxA5-fitc.

3.1.3 Generation of eGFP and dsRED expressing *L. major*

In addition, I transfected *L. major* with the pSSU-int-eGFP and pSSU-int-dsRED constructs (a kind gift from Dr. Toni Aebischer, Edinbourgh, UK). These constructs code for a gene cassette for integration into a small genomic subunit of an rRNA locus. The transfectants stably express eGFP or dsRED respectively and allow a direct visual screening by FACS and fluorescent microscopy. (Misslitz et al., 2000; Paape et al., 2008). The gene cassette was excised from the plasmid by restriction with *Pac* I and *Pme* I according to the protocol of Dr. Aebischer. The DNA was mixed with 20 x 10^6 *L. major* promastigotes and electroporated with condition 13 (Tab. 3.1.1). Positive transfectants were selected for by limiting dilutions in 96-well plates in liquid medium containing 30 µg / ml hygromycin. Two weeks after electroporation colonies were visible and subjected to fluorescent microscopy analysis. Red and green fluorescent colonies could be recovered. These colonies were expanded and aliquots were stored in liquid nitrogen. By FACS analysis I found that most parasites of two day old cultures constitutively express either dsRED or eGFP (Fig. 3.1.5 A and C). At day seven of culture I found that about 50% of these cultures are not fluorescent any longer (Fig 3.1.5 B and D). Staining seven day old dsRED-expressing parasites with AnxA5-FITC demonstrated that the parasites were either dsRED-fluorescent or positive for AnxA5-FITC (Fig. 3.1.5 C). These parasites allowed a direct visual observation of the parasites inside the human MΦ (Fig. 3.1.5 D).

Test	V	µF	Ω	Gap in mm	Lm in 10^6	DNA in µg
1	1500	25	200	2	10	1
2	1500	25	200	4	10	1
3	2500	25	720	2	10	1
4	2500	25	720	2	20	1
5	2500	25	720	2	10	2
6	2500	25	720	4	10	1
7	2500	25	720	4	20	1
8	2500	25	720	4	10	2
9	450	480	720	2	10	1
10	450	480	720	2	10	2
11	450	480	720	2	10	1
				10 seconds pause		
	450	480	720			
12	450	480	720	4	10	1
				10 seconds pause		
	450	480	720			
13	1500	25	720	2	20	1
				5 seconds pause		
	450	480	720	2		

Table 3.1.1 Electroporation conditions tested to generate *L. major* transfectants.

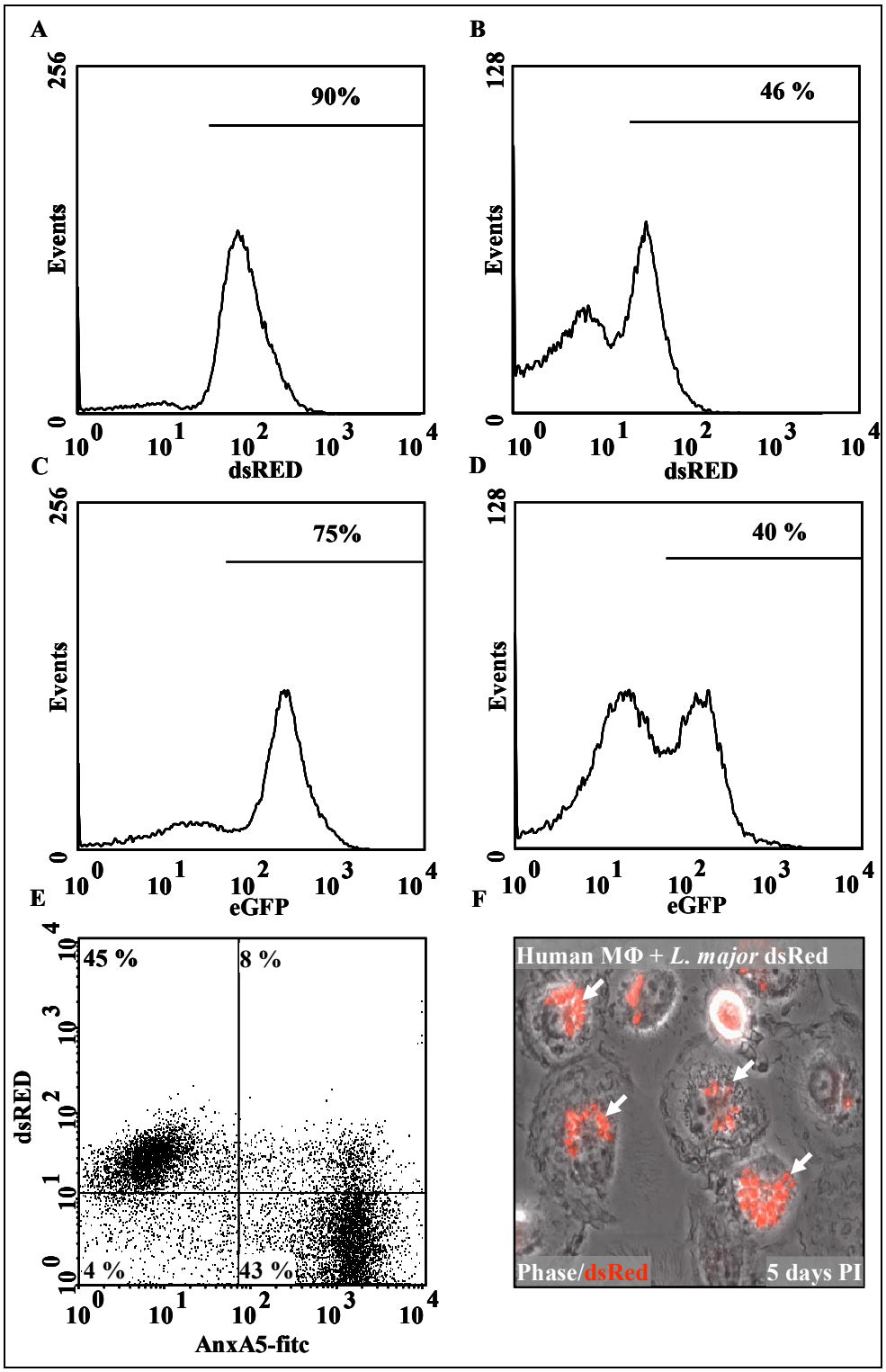

Figure 3.1.5 FACS analysis of stably transfected, transgenic *L. major*. 5 x 10^6 stably transfected *L. major* promastigotes were resuspended in PBS and analysed by FACS. Histograms of logarithmic-phase **(A)** and stationary-phase **(B)** eGFP expressing *L. major* promastigotes, or of stably transfected dsRED-expressing *L. major* promastigotes at logarithmic-phase **(C)** or stationary-phase **(D)**. Depicted are representative histograms of 1 out of 3 independent experiments performed. **E)** Stationary-phase dsRED expressing *L. major* promastigtotes were stained with AnxA5-FITC and analysed by FACS. Depicted is a representative dot-blot of 1 out of 3 independent experiments performed. **F)** Representative micrograph of human MΦ infected with dsRED expressing *L. major*.

Summary

The enzyme PSS, catalysing the synthesis of PS, was chosen as a target for gene replacement experiments in *Leishmania*, to generate PS negative parasites. Trying to knock out the gene was unsuccessful. An alternative approach to shield PS by endogenous expression of AnxA5 resulted in viable clones, expressing AnxA5 as detected by PCR. AnxA5 expressing parasites had a lower binding capacity for FITC labelled AnxA5 as compared to wild type parasites. Moreover, dsRED and eGFP expressing, fluorescent *L. major* promastigotes were generated. These parasites enable direct detection of *Leishmania*.

3.2 *L. major* – PMN interactions

PMN are the first cells to be infected by *L. major* promastigotes (Müller et al., 2001; Sunderkotter et al., 1993). It was shown that the parasites survive inside PMN, but do not replicate. Early PMN recruitment was explained by the release of a lipid, the Leishmania chemotactic factor (LCF) that specifically attracts PMN (van Zandbergen et al., 2002). Furthermore, it was demonstrated that LCF induces PMN migration through stimulation of the Lipoxin A4 (LxA4) receptor (ALX). ALX binds a variety of structurally unrelated peptides, proteins and lipid mediators. The signalling through this receptor results in either pro- or anti-inflammatory responses of the target cell, potentially silencing PMN effector functions.

3.2.1 The role of ALX on the silent uptake of *L. major*

First human PMN were incubated for 3 hours with *L. major* promastigotes. Not phagocytosed *L. major* were removed by washing. Cytospin slides were prepared 3 hours and 42 hours post incubation and stained by Diff QUIK®. Infection rates were assessed by counting parasitized cell in > 200 PMN. Infection rates were evaluated by counting intracellular *Leishmania* in 20 infected PMN (Fig. 3.2.1). I found that 3 hours post infection 35 ± 2 % (mean ± SEM) of all PMN were infected with *Leishmania*, while after 42 hours an infection rate of 22 ± 1 % PMN (Fig. 3.2.1 A) could be detected. A parasite burden of 1.9 ± 0.4 (mean + SEM) and 1.5 ± 0.3 intracellular *L. major* promastigotes 3 hours and 42 hours post infection was assessed, respectively (Fig. 3.2.1 B). To investigate the ALX dependent regulation of PMN effector functions in the context of *Leishmania* infection we infected human PMN with *L. major* promastigotes in the presence of the known ALX ligands LxA4 and fMLP and compared these with LCF containing supernatant or medium alone. Extracellular parasites were removed 3 hours after incubation and infection rates were assessed by counting parasites in Diff QUIK ® stained samples (Fig 3.2.2) as mentioned above. I found that incubating PMN with *L. major* promastigotes in the presence of LxA4 led to a significant increase in infection rates from 29 % ± 13 % to 34 % ± 13 % (Fig. 3.2.2. A and B). The stimulation of the PMN with LCF resulted in even higher and also significant increase to 42 % ± 14. (Fig. 3.2.2 C and D). Finally, I tested fMLP as a ligand for ALX on PMN. I detected the highest increase to 59 % ± 4 infected cells of all PMN (Fig. 3.2.2 E and F).

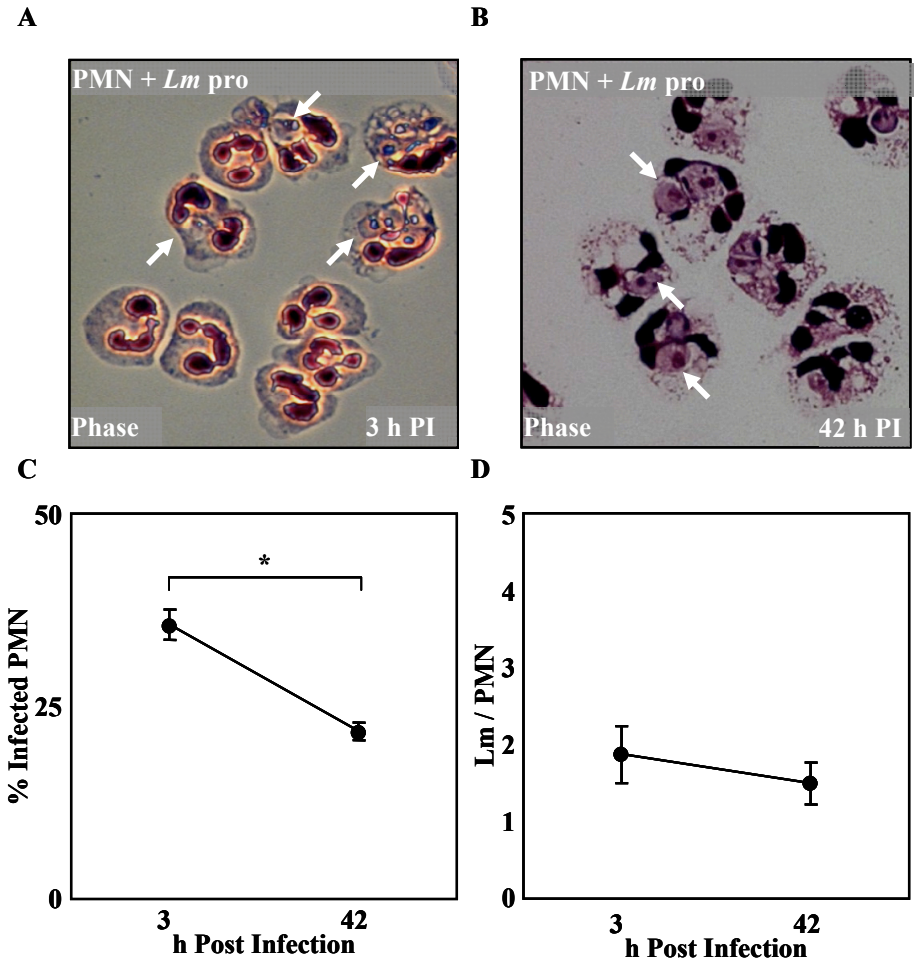

Figure 3.2.1 *L. major* promastigotes infect and survive inside human PMN. Human PMN were isolated from venous blood from healthy donors and incubated with stationary phase *L. major* promastigotes. Extracellular parasites were removed 3 hours post infection. Cytospin slides were prepared and Diff QUIK® stained. **A)** Representative micrograph of 1 experiments out of 5 independent experiments performed. Phase contrast micrograph of Diff QUIK stained, infected human PMN 3 hours post infection. White arrows indicate intracellular parasites. **B)** Micrograph of Diff QUIK stained human PMN infected with *L. major* promastigotes 42 hours post infection. White arrows indicate intracellular parasites. **C)** Depicted are the infection rates of human PMN in percent at 3 and 42 hours post infection. Values given are mean of 5 independent experiments +/- SEM. **D)** Parasite burden of infected human PMN 3 and 42 hours post infection. Values given are mean of 5 independent experiments +/- SEM. Statistical analyses were performed using a two-tailed, paired T-Test (* = $p<0.05$).

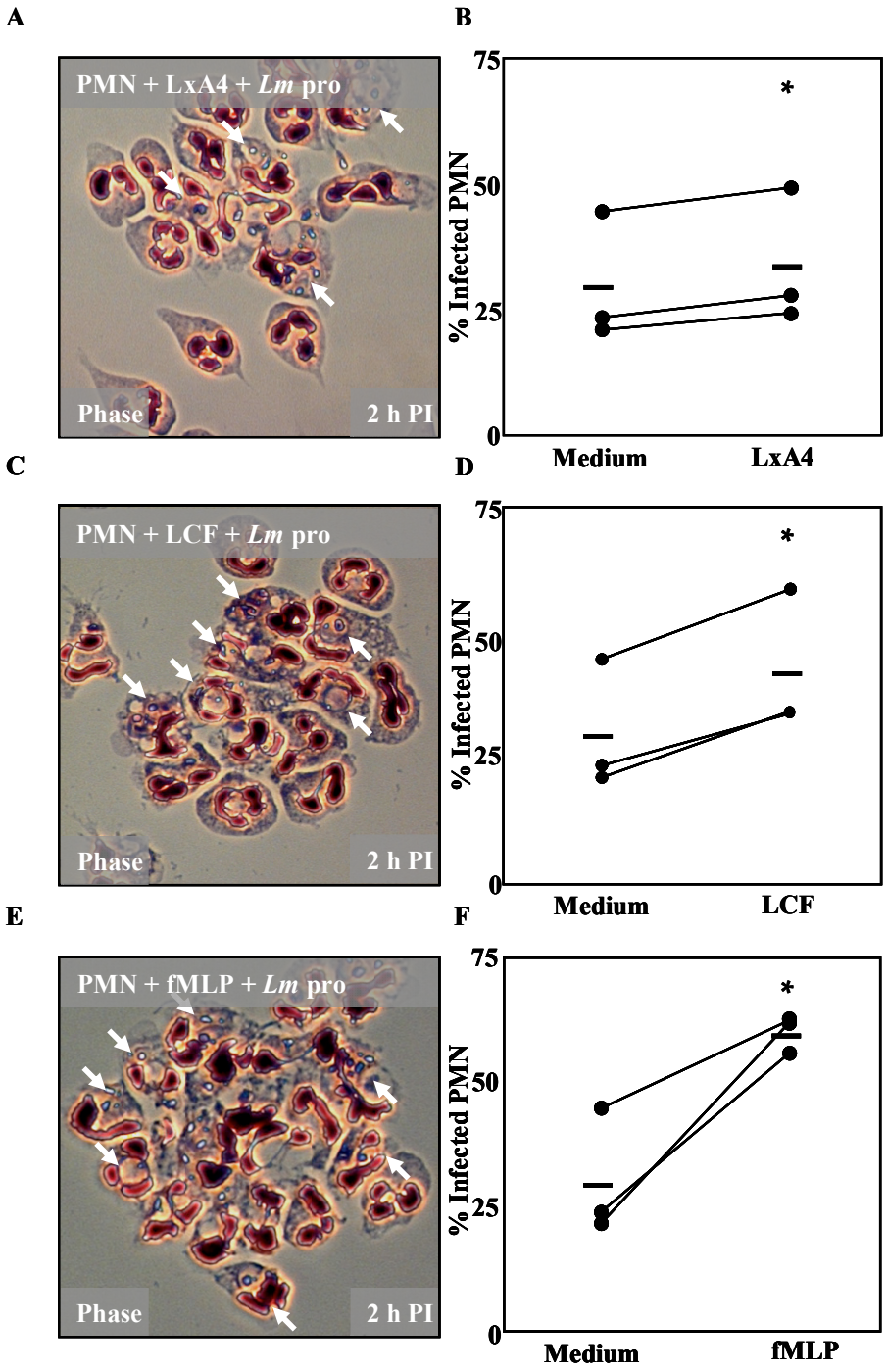

Figure 3.2.2 Stimulation of ALX on PMN increases *L. major* infection rates. PMN were pre-incubated for 5 minutes with medium alone or medium supplemented either with 25 µg/ml LxA4 (**A**, **B**), 1/2 volume LCF, prepared as described in Materials and Methods (**C**, **D**) or 1 µM fMLP (**E**, **F**). All samples were incubated with *L. major* promastigotes at a ratio of 1:10. After 2 hours extracellular parasites were removed. Cytospin slides were stained with Diff QUIK®. Infection rates were evaluated by counting parasitized cells of > 200 PMN. Depicted are representative micrographs of Diff QUIK® stained cytospin samples of 1 experiment of 3 experiments performed. Statistical analyses were performed using a two-tailed, paired T-Test (* = $p<0.05$).

3.2.2 PMN as vectors to induce disease development

It was shown that human PMN infected with *L. major* promastigotes serve as Trojan Horses to transmit the parasites silently into MΦ, *in vitro* (van Zandbergen et al., 2004). In order to analyse these *in vitro* findings *in vivo*, I tried to infect murine PMN. Subsequently, I wanted to inject $5x10^5$ of such infected PMN into the footpad of susceptible Balb/c mice. First PMN were isolated from blood or spleen form healthy mice using the Miltenyi Ly-6G MACS Kit. I obtained PMN with a purity of > 98 %, as assessed by evaluation of Diff QUIK® stained cytospin slides (Data not shown). Incubation of these PMN with *L. major* promastigotes did not result in infection of cells (data not shown).

From previous studies I knew that *L. major* injected into air-pouches resulted in the presence of *L. major* infected PMN. Therefore, I injected *L. major* promastigotes in air-pouches on Balb/c mice. Subsequently, I isolated cells from these air-pouches and used the Ly-6G MACS Kit to separate the PMN from the suspension. Initial experiments were performed to determine the optimal conditions to recover high amounts of infected PMN. At first 10×10^6 *L. major* in Xvivo15 were injected in the air-pouch. After 24 and 48 hours the mice were sacrificed and cells isolated from the air-pouch. Using the Ly-6G-MACS kit I purified the PMN and assessed the amount of recovered cells (Tab.3.2.1 Test 1 and 2). Since I did not obtain high numbers of PMN, I changed the experimental set-up by resuspending the parasites in RPMI-1640. 2×10^7 *L. major* were injected into the air-pouch and the mice sacrificed after 12 and 36 hours. The obtained cells were treated as mentioned before. Though the number of recovered cells in total was increased from 1.2×10^6 to 3.2×10^6 and 1×10^6 to 2.1×10^6 , the amount of Ly-6G positive cells remained low at 4×10^5 (Tab. 3.2.1 Test 3 and 4). Because I could show that the infection rates of human PMN increases in the presence of LCF (Fig. 3.2.2), I decided to inject *L. major* promastigotes that were resuspended in a LCF containing supernatant of *L. major* cultures. At first I injected 2×10^7 *Leishmania* and sacrificed the mice after 24 and 48 hours. Since I recovered only 1.7×10^6 and 2.5×10^5 cells in total, I did not proceed with MACS separation, and therewith the cell number of Ly-6G positive cells not determined (ND), but instead I evaluated the Diff QUIK® stained cytospin samples microscopically. 24 hours post infection 65 % of the cells isolated had PMN morphology (Data not shown) which was better than before (data not shown) but only about 6% of these cells were infected (Tab 3.2.1 Test 5 and 6). Injecting 1×10^8 and 2×10^8

Test	Medium	*L. major* injected	Time post infection	cells / mouse recovered	Ly-6G positive	Infection rate in %	Lm / cell
1	Xvivo15	1 x 10^6	24 hours	1.2 x 10^6	4.2 x 10^5	ND	ND
2	Xvivo15	1 x 10^6	48 hours	1 x 10^6	4 x 10^5	ND	ND
3	RPMI	2 x 10^7	12 hours	3.2 x 10^6	4 x 10^5	ND	ND
4	RPMI	2 x 10^7	36 hours	2.1 x 10^6	3.8 x 10^5	ND	ND
5	LCF	2 x 10^7	24 hours	1.7 x 10^6	ND	6 %	ND
6	LCF	2 x 10^7	48 hours	2.5 x 10^5	ND	ND	ND
7	LCF	1 x 10^8	12 hours	3.8 x 10^6	3 x 10^4	71 %	4
8	LCF	1 x 10^8	24 hours	3.6 x 10^6	ND	64	5.1
9	LCF	2 x 10^8	24 hours	5.7 x 10^6	4.6 x 10^5	ND	ND
10	LCF	5 x 10^7	24 hours	3.7 x 10^6	4.25 x 10^5	35 %	1.5

Table 3.2.1 Testing different conditions to recover infected PMN form air-pouch

Leishmania in combination with LCF containing supernatant in the air-pouch resulted in increased numbers of cells recovered in total, namely 3.8 and 5.7 x 10^6 cells in total, compared to 1.7 x 10^6 (Tab. 3.21. Test 7-8 compared to 5). An increase of Ly-6G positive cell could not be detected (Tab 3.2.1 Test 7-9). Finally, I injected 5 x 10^7 *L. major* in LCF into the air-pouches of Balb/c mice and waited for 24 hours, before the mice were sacrificed. The recovered cells, separated by MACS, were prepared for microscopy using cytospin-methods and Diff QUIK® staining. 35 % of the PMN were infected with *Leishmania* and had a mean parasite burden of 1.5 *Leishmania* per cell (Fig. 3.2.3 A). Furthermore, I found cells resembled PMN morphology in the Ly-6G negative fraction as well (Fig. 3.2.3 B).

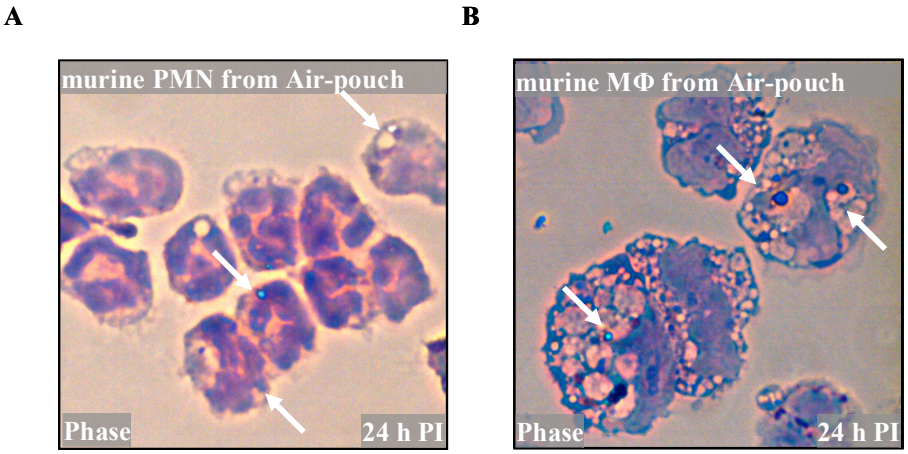

Figure 3.2.3 Infected murine cells isolated from air-pouch 24 hours post infection. A) MACS separated Ly-6G positive murine PMN. Black arrows indicate intracellular *Leishmania*. **B)** MACS separated Ly-6G negative murine phagocytes. Black arrows indicate intracellular *Leishmania*. Depicted are representative micrographs of Diff QUIK® stained cytospin samples.

Of these Ly-6G negative cells 25% were infected and had a mean parasite burden of 2 *Leishmania* per cell. Therefore, I used both fractions to infect Balb/c mice. The viability of the cells was assessed by trypan blue exclusion staining, as described in Materials and Methods. Using the obtained infection rates and parasite burdens, I calculated that the mean parasite burden in 1 x 10^5 Ly-6G positive and negative cells was 5.25 x 10^4 and 5 x 10^4 *L. major* respectively.

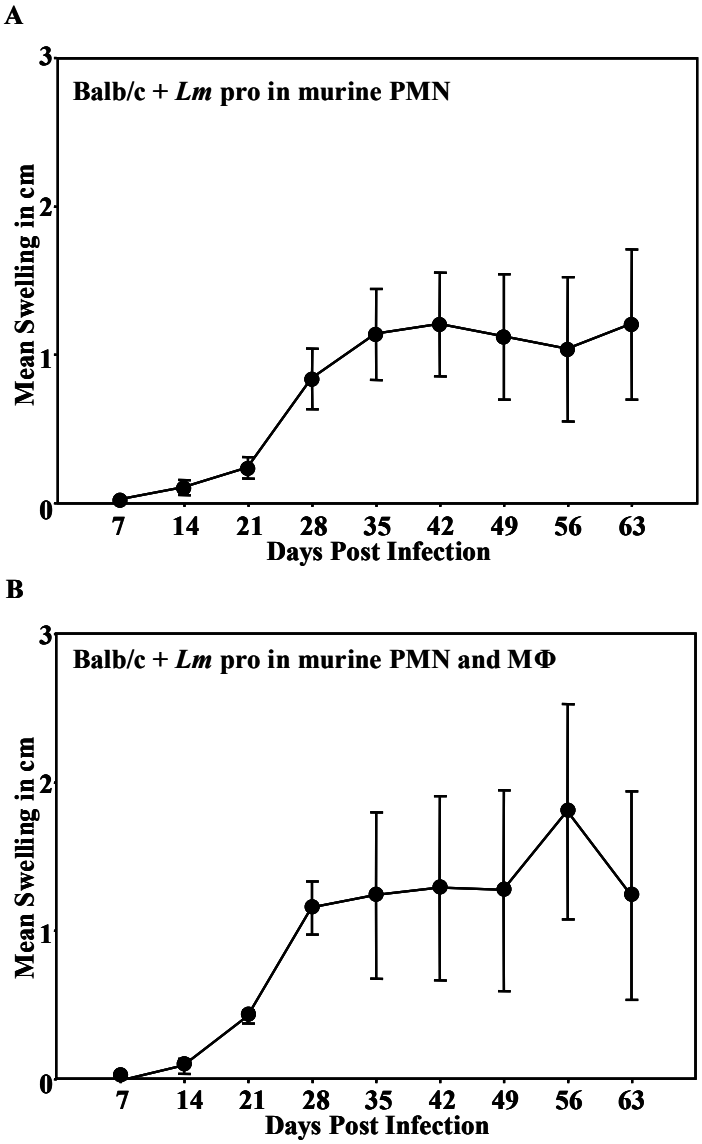

Figure 3.2.4 *Ex vivo* isolated infected murine PMN induce lesion formation in Balb/c mice. Murine PMN were isolated from air-pouches on Balb/c mice 24 hours post infection with *L. major* in LCF. The obtained cells were separated by MACS into Ly-6G positive and negative phagocytes. Cytospin slides were Diff QUIK® stained and infection rates in both fractions were determined. The cells were resuspended in RPMI and 9 x 10^4 cells were injected into the footpad of healthy female Balb/c mice. The disease development was monitored weekly, by measuring the increased lesion formation using a calliper.

Therefore, I adjusted both cell suspensions to the same amounts of *Leishmania* / ml. Next I injected 3.2 x 10^4 *L. major*, phagocytosed in either Ly-6G positive or negative cells, into the footpad of Balb/c mice. Disease development was monitored weekly, measuring increased lesion formation, using a calliper. I found that infected Ly-6G positive murine PMN (Fig. 3.2.4 A) as well as infected Ly-6G negative murine phagocytes (Fig. 3.2.4 B) induced footpad swelling.

3.2.3 Murine HoxB8 PMN as Trojan Horses for *L. major* parasites

The development of the HoxB8 PMN system (Wang et al., 2006) enabled us to work with murine PMN *in vitro*. I evaluated, if the HoxB8 PMN could be infected with *L. major* promastigotes and behaved similar as compared to human PMN. Therefore, I incubated the HoxB8 PMN with *L. major* promastigotes for 3 hours, removed the extracellular parasites and determined the infection rate 3 and 42 hours post infection (Fig. 3.2.5 A-D) as described above (3.2.1). I observed that after 3 and 42 hours post infection 46% ± 0.5 % and 35 % ± 0.3 % respectively of all HoxB8 PMN were infected (Fig. 3.2.5 C). Moreover, I found 3.1 ± 0.03 and 3.6 ± 0.4 intracellular parasites / HoxB8 PMN 3 and 42 hours post infection (Fig. 3.2.5 D). Similar to *L. major* promastigotes inside human PMN no significant increase of the intracellular parasites could be observed (Fig. 3.2.5 D). In parallel to 3.2.2 I analysed if *in vitro* infected HoxB8 PMN could induce disease development in susceptible Balb/c mice as compared to resistant C57BL76 mice. HoxB8 PMN were incubated with *L. major* promastigotes at a ratio of 1:10 for 3 hours at 37°C. The non-phagocytosed parasites were removed by washing and the infection rates as well as parasite burdens assessed by evaluating > 200 cells on Diff QUIK® stained cytospin slides (Fig. 3.2.6). I found that 44.4 % of the HoxB8 PMN were infected and had a mean parasite burden of 2.3 ± 0.9 *L. major* / cell. The viability of the cells was determined by trypan blue exclusion staining and was > 95%. The cells were resuspended at two different concentrations, the lower one contained 4 x 10^5 infected HoxB8 PMN and the higher contained 8 x 10^5 infected HoxB8 PMN. The infected cells were injected into the footpad and the disease development was monitored weekly by measuring increased lesion formation using a calliper. I found that infected HoxB8 PMN induced lesion formation in Balb/c mice. Moreover, I found that using *L. major* infected HoxB8 PMN induced a typical self-healing disease development in C57BL/6 mice.

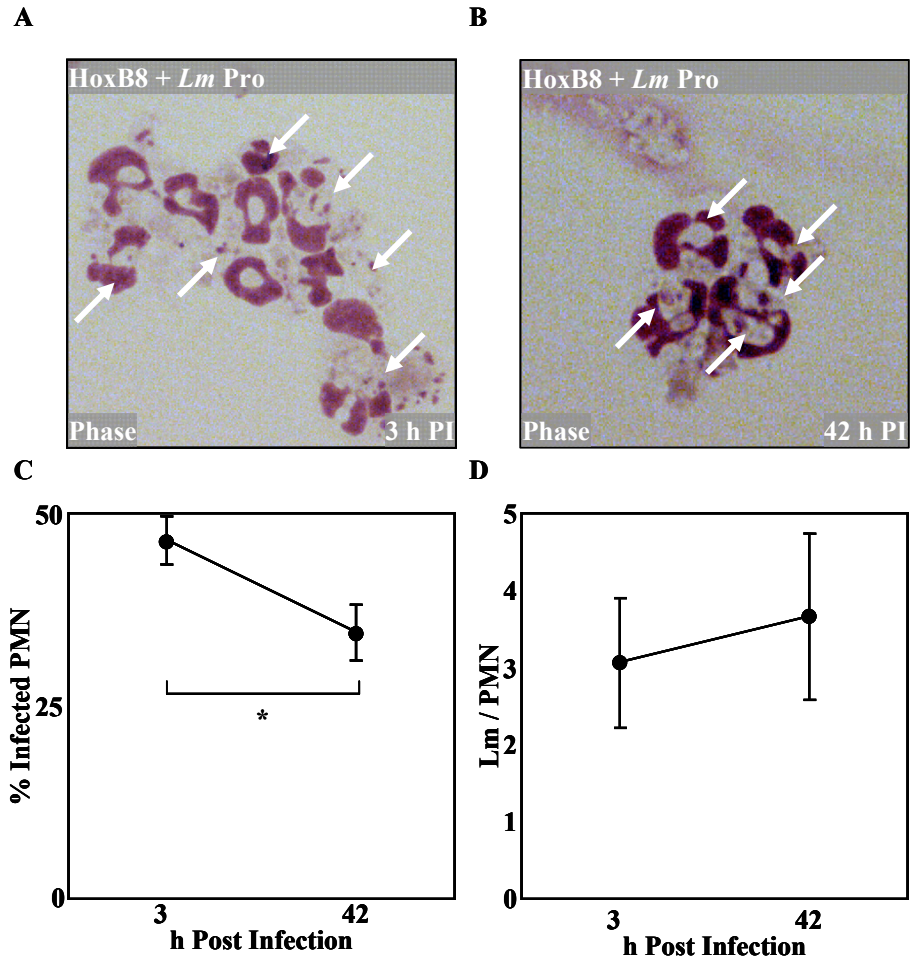

Figure 3.2.5 *L. major* promastigotes infect HoxB8 PMN
HoxB8 cells were differentiated 72 h into PMN before they were harvested and incubated for 3h with *L. major* promastigotes at a ratio of 1:10. Extracellular parasites were removed and samples taken 3 and 42 hours post infection, Diff QUIK® stained and infection rates as well as parasite burdens assessed. Values given are mean of 4 independent experiments ± SEM.**A)** Depicted is a representative micrograph of HoxB8 PMN 3 hours post infection. The arrows indicate intracellular parasites. **B)** Depicted is a representative micrograph of HoxB8 PMN 42 hours post infection. The arrows indicate intracellular parasites. **C)** Depicted is the mean percentage of infected PMN ± SEM in more than 200 cells 3 and 42 hours post infection. **D)** Depicted is the mean parasite burden ± SEM of 4 experiments of at least 20 infected PMN of each sample analysed 3 and 42 hours post infection.

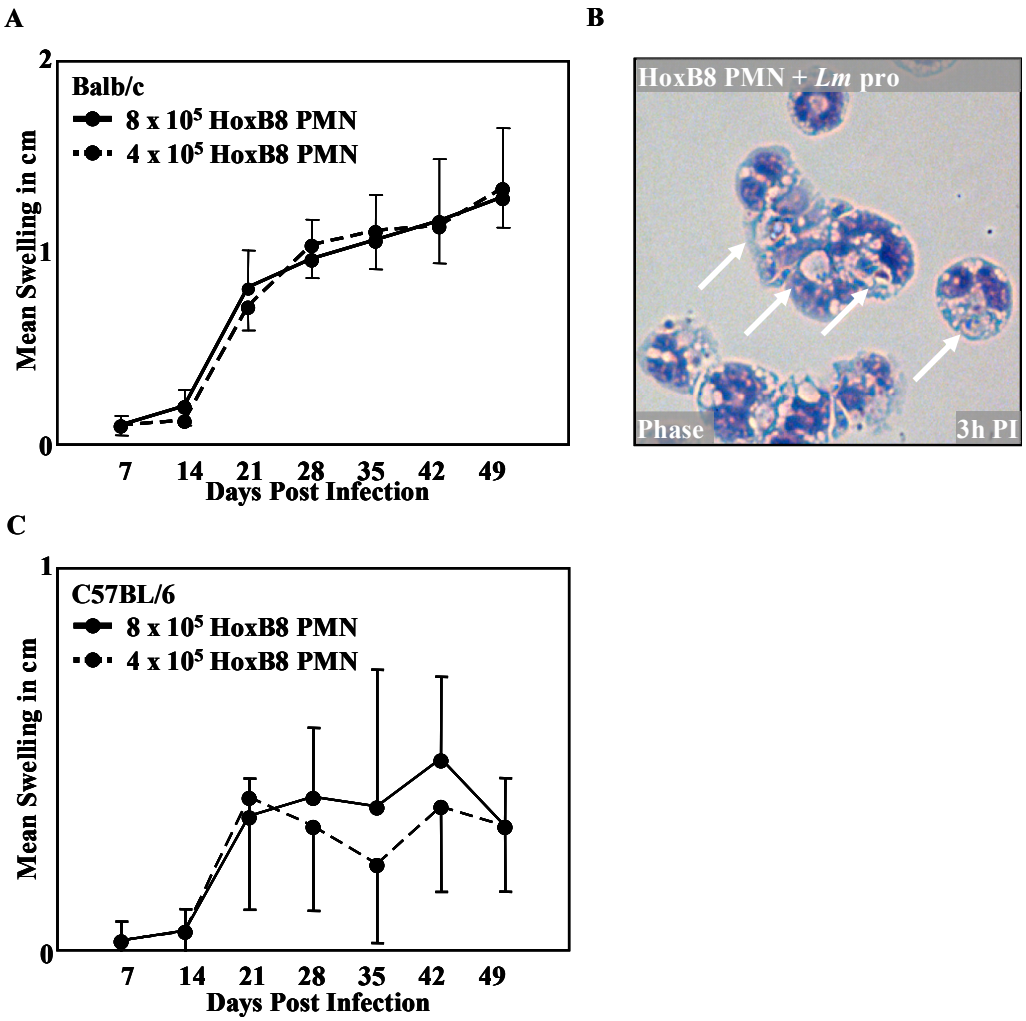

Figure 3.2.6 *In vitro* infected murine HoxB8 PMN induce lesion formation in Balb/c but not in C57BL/6 mice. HoxB8 cells were differentiated into PMN for 72 hours. The cells were harvested and incubated with *L. major* promastigotes for 3 hours. Extracellular parasites were removed and the infected HoxB8 PMN were injected into susceptible Balb/c mice (**A**) or resistant C57BL/6 mice (**B**). Disease development was monitored weekly by measuring the footpad swelling using a calliper. 2 different concentration of infected PMN were compared, dashed line 4 x 10^5 PMN, solid line 8 x 10^5 PMN. Depicted are the mean ± SEM increases of the footpad swelling of 1 experiment of two performed with 4 mice in each group. **(C)** Representative micrograph of Diff QUIK® stained cytospin sample of infected HoxB8 PMN injected into mice.

Summary *L. major*-PMN interactions:

Leishmania attract PMN via the release of the lipid chemoattractant LCF. The lipid stimulates the PMN in an ALX dependent fashion increasing phagocytosis of *L. major* promastigotes and subsequent survival. I could show that in the air-pouch experiment the injection of *L. major* promastigotes resuspended in LCF resulted increased numbers of PMN recovered and that these PMN had higher infection rates compared with air-pouch experiments performed with Leishmania resuspended in Xvivo15. Moreover, I demonstrated that infected murine

PMN can induce disease development in susceptible Balb/c mice. Furthermore I could show that the murine *in vitro* cultured HoxB8 PMN can be efficiently infected with *Leishmania*. Finally, I demonstrated that by injecting these infected cells in healthy Balb/c mice I could induce disease development

3.3 Apoptosis or apoptotic mimicry

Inside their final host cells *Leishmania* promastigotes transform into the amastigote stage and start to replicate. Until now little is known about the mechanisms how amastigotes propagate in mammalians enabling disease development. I could show that the virulent inoculum of *L. major* promastigotes contains two distinct populations, one viable and one AnxA5-binding, dead population (van Zandbergen et al., 2006).

3.3.1 Murine derived *L. major* amastigotes.

Since no *in vitro* culture system for *L. major* amastigotes existed, I first isolated lesion-derived amastigotes from infected Balb/c mice as described in Wanderley et al (Wanderley et al., 2006). I recovered 2 x 10^7 *L. major* amastigotes from the lesions from the infected Balb/c mice. The obtained amastigotes were analysed by electron microscopy (EM). Morphologically intact (Fig. 3.3.1 A black arrows) as well as not intact (Fig. 3.3.1 A white arrows) parasites were recovered. The intact amastigotes showed typical morphology of viable amastigotes, like elliptic shaped bodies and close proximity between nucleus and kinetoplast as well of lack of extracellular flagellum. Moreover, these intact amastigotes have tubular membrane structures (Fig. 3.3.1 B arrow 2) and an intact nucleus (Fig. 3.3.1 B arrow 3). Besides these intact cells I found amastigotes that lacked the typical intra-parasitic granular-like structures (Fig. 3.3.1 C arrow 4). These amastigotes lacked the tubular structures of the cell membrane (Fig. 3.3.1 C arrow 5) and had a nucleus with a condensed morphology (Fig. 3.3.1 C arrow 6). To analyse, if the morphological discrimination into intact and not intact amastigotes correlated with the late phase of cell-death, I performed TUNEL staining on these cells. I found TUNEL-negative (Fig. 3.3.1 D) as well as TUNEL-positive (white arrows) amastigotes. Infectivity analysis of these isolates could not be performed, since these murine-derived amastigotes transformed back into promastigotes within hours at room temperature (Data not shown).

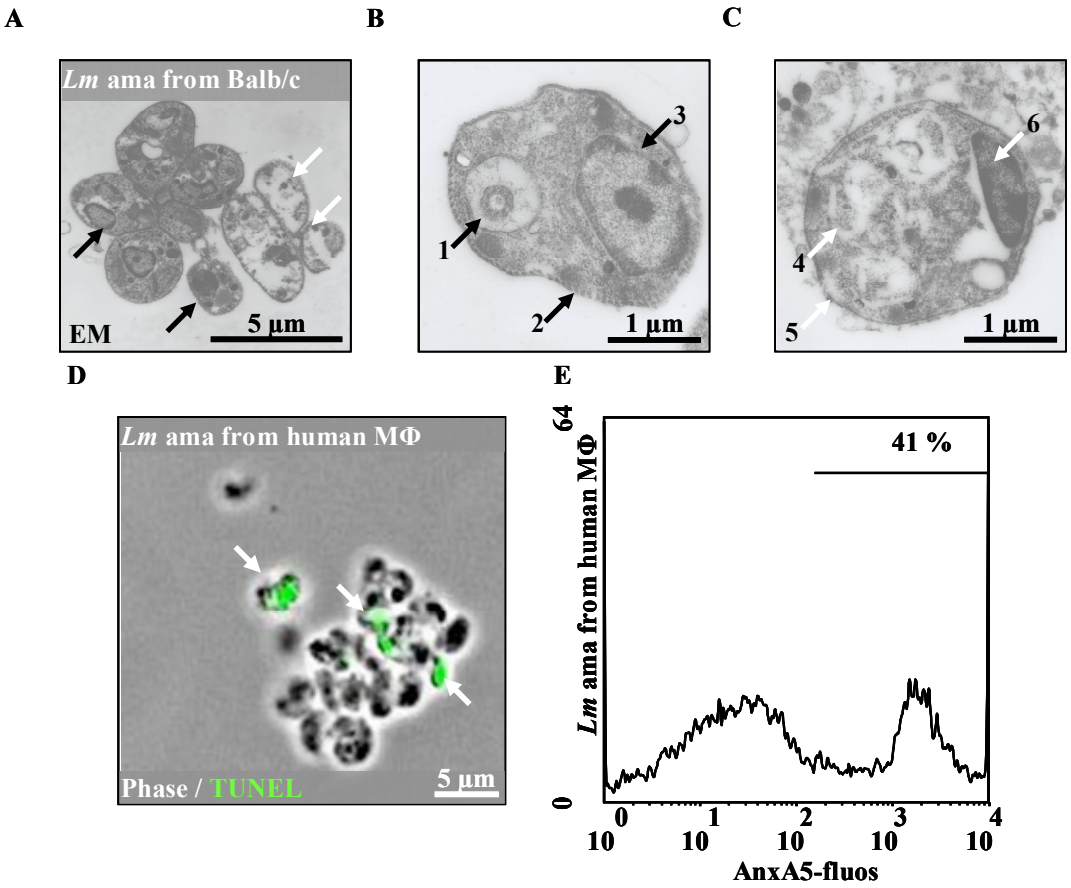

Figure 3.3.1 Electron microscopy of *L. major* amastigotes isolated from infected Balb/c. **A)** Depicted is a representative EM micrograph of *L. major* amastigotes isolated from infected Balb/c mice. Black arrows indicate intact cells. White arrows indicate not intact cells. **B)** Depicted is a representative EM micrograph of an intact *L. major* amastigote. Arrow 1 indicates the flagellum pocket. Arrow 2 highlights typical tubular structures beneath the *L. major* cell membrane. Arrow 3 points out intact nucleus. **C)** Depicted is a representative EM micrograph of a not intact *L. major* amastigote. Arrow 4 indicates the lack of intra-parasitic granular-like structures. Arrow 5 points at aberrant cell membrane structures. Arrow 6 indicates a condensed nucleus. **D)** Representative fluorescent micrograph of lesion derived *L. major* amastigotes from 1 out of 3 performed experiments. Balb/c mice were infected with *L. major* promastigotes. Two weeks after infection the mice were sacrificed and lesion-derived amastigotes isolated. The parasites were TUNEL stained and analysed by fluorescent microscopy. **E)** Representative FACS analysis of human MΦ-derived *L. major* amastigotes from 1 out of 3 performed experiments. Primary human MΦ were infected with *L. major* promastigotes and incubated for 5 days at 37°C. The developed amastigotes were isolated from the infected cells and labelled with AnxA5-fluos for FACS analysis.

3.3.2 Human macrophage derived *L. major* amastigotes.

To obtain amastigotes from human cells, I infected primary human macrophages (MΦ) with *L. major* promastigotes. 5 days post infection I isolated 1.2 x 10^7 *L. major* amastigotes from these cells. The parasites were stained with AnxA5-fluos and subjected to FACS analysis. I found AnxA5-negative (60 %) as well as AnxA5-binding (40 %) parasites (Fig. 3.3.1 E). I could not perform infectivity analysis of these isolates, because in culture these amastigotes

transformed into promastigotes-like parasites, within 1-2 hours at room temperature, as could be detected by a *de novo* grown flagellum and subsequent motility of the parasites (Data not shown).

3.3.3 *In vitro* generated axenic *L. donovani* amastigotes.

In order to analyse cell death of amastigotes in more detail, I analysed *L. donovani* next, because Debrabant *et al* developed a culture system for axenic amastigotes (Debrabant et al., 2004).

First, I investigated *L. donovani* promastigotes to see whether their virulent stationary-phase contained 2 populations as described for *L. major*. Staining two days old *L. donovani* promastigotes (logarithmic-phase) with AnxA5-fluos, I found only AnxA5-negative promastigotes (Fig. 3.3.2 A and B). Analysis of seven days old (stationary-phase) promastigotes revealed 57 % of the parasites to be AnxA5-negative and 43 % of the culture to be AnxA5-binding (Fig. 3.3.2 C and D). Last I stained stationary-phase *L. donovani* promastigotes by TUNEL methods and analysed these cells by fluorescent microscopy and FACS (Fig. 3.3.2 E and F). I found that 59 % of *L. donovani* promastigotes become TUNEL positive at stationary-phase growth.

Next, I generated *L. donovani* amastigotes *in vitro* by incubating the cells in acidic culture medium (pH5.5) for one week. Next I raised the culture temperature from 27°C to 34°C as well as 37°C (Debrabant et al., 2004). After 4 days of culture the parasites had an amastigote-like morphology at either of the two compared culture conditions (Data not shown). Two days old axenic *L. donovani* amastigotes were stained by AnxA5-fluos and analysed by fluorescent microscopy (Fig. 3.3.3 A) as well as FACS (Fig. 3.3.3 B). I found that at day two of culture all *L. donovani* amastigotes were AnxA5-negative. Fluorescent microscopy of AnxA5-fluos stained amastigotes at day 4 of axenic culture showed some AnxA5-positive parasites (Fig. 3.3.5 C). FACS analysis revealed that around 80 % of the parastites were AnxA5-binding (Fig. 3.3.5 D). Subsequently, I stained these parasites with TUNEL. Fluorescent microscopy analysis showed TUNEL positive (Fig. 3.3.3 E green) as well as negative parasites (Fig. 3.3.5 E). Quantification by FACS analysis showed around 50 % of the parasites to be TUNEL positive (Fig. 3.3.5. F).

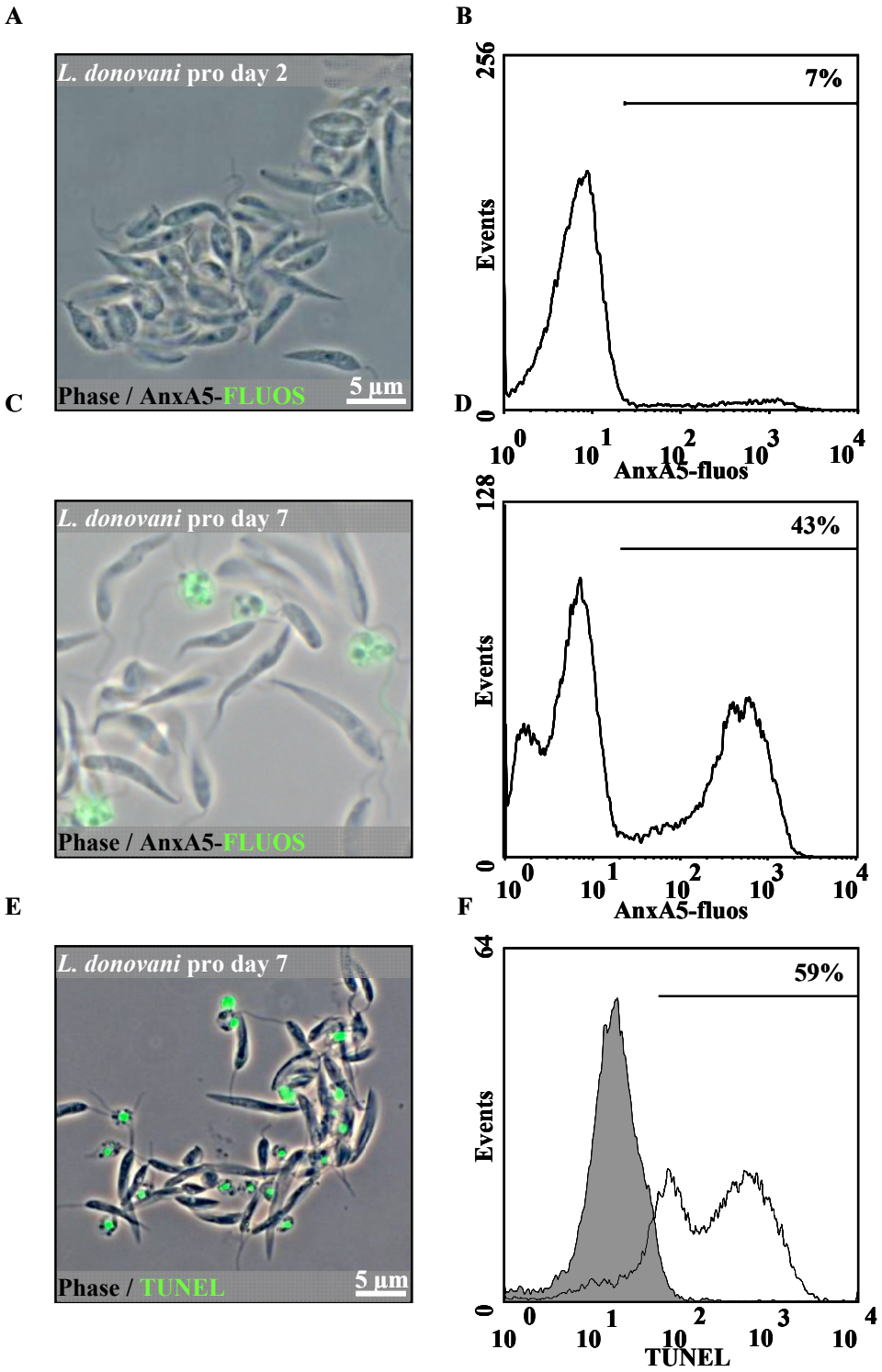

Figure 3.3.2 *L. donovani* promastigote cultures. Depicted are data from one representative experiment of 3 independent experiments performed. Logarithmic-phase *L. donovani* promastigotes cultures were subjected to AnxA5-FLUOS staining and analysed by fluorescent microscopy **A)** and by FACS analysis **B)**. Stationary-phase *L. donovani* promastigote were stained with AnxA5-FLUOS and analysed by fluorescent microscopy **C)** and FACS **D)**. TUNEL-stained stationary-phase *L. donovani* promastigotes analysed by fluorescent microscopy. **E)** and by FACS **F)**.

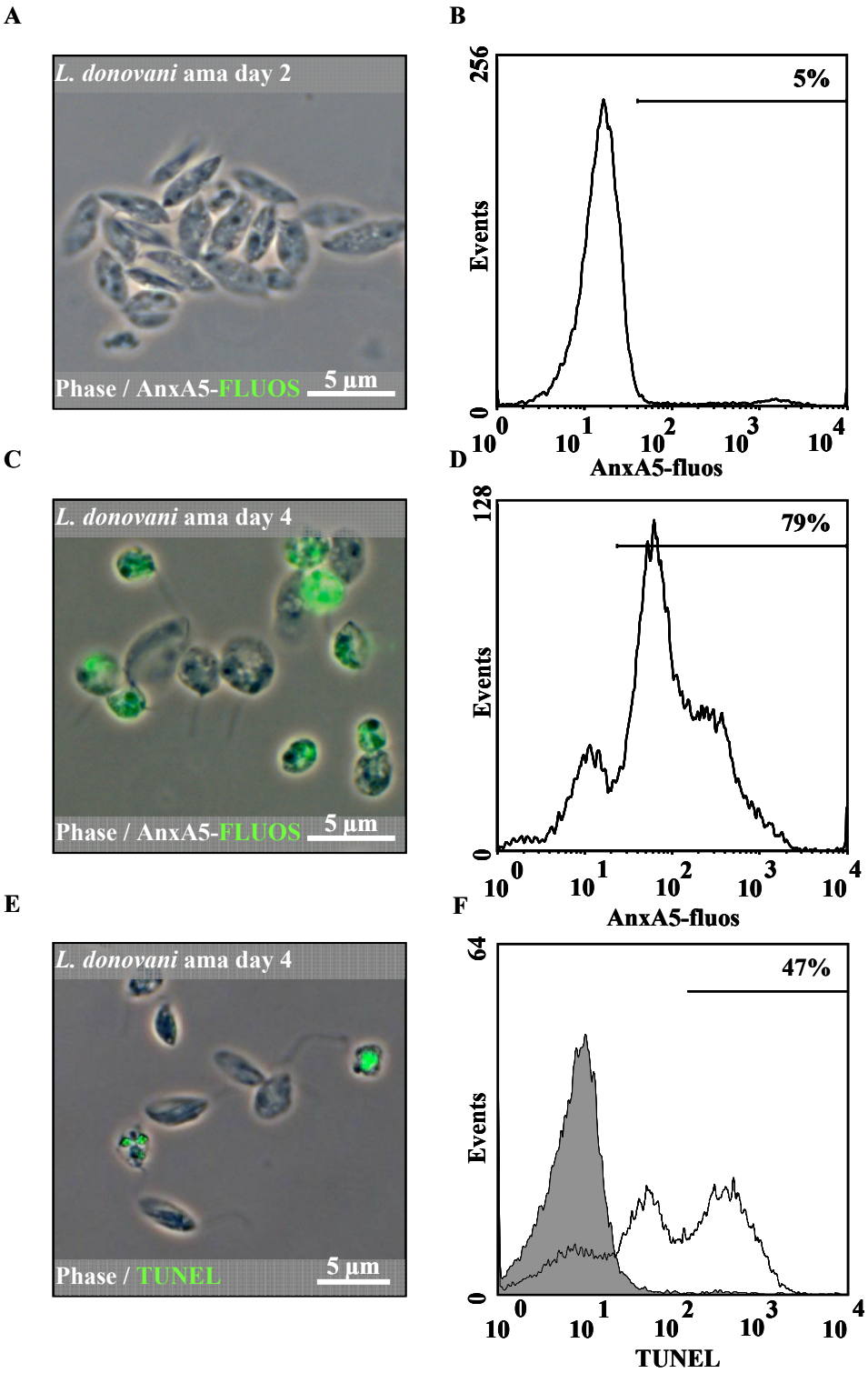

Figure 3.3.3 *L. donovani* axenic amastigote cultures. Depicted are data from one representative experiment of 3 independent experiments. **A)** Micrograph of AnxA5-fluos stained 2 days old *L. donovani* amastigotes **B)** FACS analysis of 2 days old *L. donovani* amastigotes stained with AnxA5-FLUOS. **C)** AnxA5-FLUOS labeled 4 day old *L. donovani* amastigote cultures were analysed by fluorescent microscopy as well as by **D)** FACS analysis **E)** Micorgraph of TUNEL stained 4 day old *L. donovani* amastigotes. **F)** FACS analysis of TUNEL stained *L. donovani* amastigotes.

Summary Cell death of *Leishmania* amastigotes:

I analysed lesion-derived *L. major* amastigotes from infected Balb/c mice. I isolated intact as well as not intact parasites. I could demonstrate that parts of the isolated Leishamania are AnxA5-binding and parts are not. I showed that some of the lesion derived amastigotes were TUNEl positive, while some were not. By isolating L. major amastigotes from human MΦ, I could confirm these results. Analysis of *in vitro* cultured *L. donovani* promastigotes revealed AnxA5- and TUNEL positive parasites in stationary-phase cultures similar to *L. major* promastigotes. In addition, axenic *L. donovani* amastigotes became in part AnxA5-positive and TUNEL-positve.

3.4 An axenic *L. major* amastigote *in vitro* cell culture system.

To study *L. major* amastigotes in more detail, I had to generate an axenic culture system. By culturing *L. major* promastigotes in AAM at 33°C for a prolonged time, I was able to generate a heterogeneous cell culture that contained around 30 % *L. major* promastigotes and 70 % of amastigote-like cells with an elliptic shaped body and no extracellular flagellum (data not shown). By density gradient centrifugation techniques, I was able to purify these elliptic cells. In contrast to murine-derived and human MΦ-derived *L. major* amastigotes, these parasites remained stable in their elliptic-like morphology. Moreover, I observed binary fission. Finally, on a temperature shift to 27°C induced a retransformation into promastigotes (data not shown). I analysed differential gene expression, morphological and biochemical features, enabling us to address these cells as axenic *L. major* amastigotes. Furthermore I analysed the virulence of these axenic *L. major* amastigotes in direct comparison with *L. major* promastigotes and MΦ-derived amastigotes.

3.4.1 Morphological characteristics of axenic *L. major* amastigotes.

Morphological differences between *Leishmania* promastigotes and amastigotes were analysed using different microscopic techniques. At first I investigated *Leishmania* promastigotes (Fig. 3.4.1). Light microscopy of Diff QUIK® stained samples of *in vitro* cultured *L. major* promastigotes showed typical micelles formation with the flagella pointing into the center of the micel (Fig. 3.4.1 A). Using SEM, revealed that promastigotes typically are 10 – 15 µm in length and 1.5 µm in width. They have a smooth surface with one apical flagellum (Fig. 3.4.1 B). The flagellum is anchored in the flagellum pocket as can be seen be EM (Fig. 3.4.1 C).

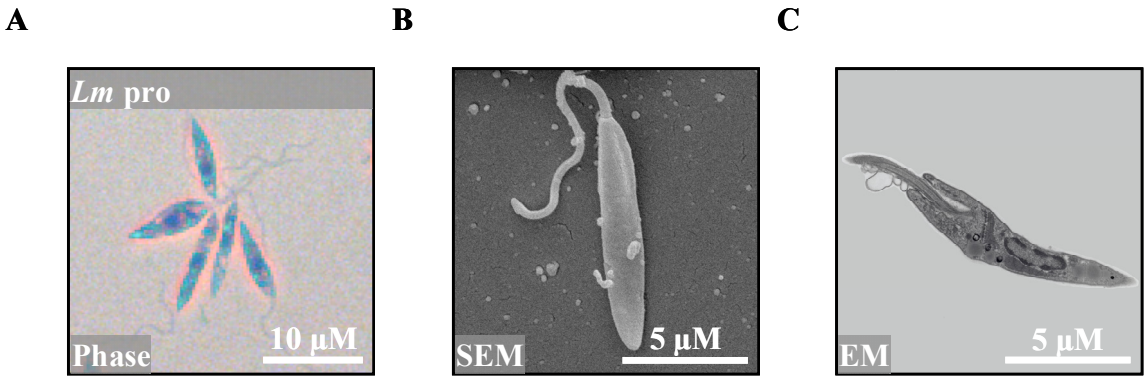

Figure 3.4.1 Morphological characteristics of *L. major* promastigotes. A) Representative phase contrast micrograph of stationary-phase Diff QUIK® stained *L. major* promastigotes. **B)** Scanning Electron Micrograph of stationary-phase *L. major* promastigotes. **C)** Transmission Electron Micrograph of stationary-phase *L. major* promastigote.

The prominent nucleus as well as the kinetoplast is visible in EM micrographs as well as in Diff QUIK® stained samples (Fig. 3.4.1 A and C). In comparison to these morphological characteristics of promastigotes, amastigotes from our axenic culture system have rounder, elliptic shaped bodies (Fig. 3.4.2 A – D). Small groups or clusters of parasites could be found in Diff QUIK® stained samples of axenic amastigote cultures, however no micelles were observed (Fig. 3.4.2. A). SEM and EM micrographs show that the *in vitro* cultured amastigotes lack the extracellular part of the flagellum (Fig. 3.4.2 B - D). Remnants of the flagellum could be found inside the flagellum pocket (Fig. 3.4.2 C). EM micrographs demonstrate that the *in vitro* generated amastigotes have typical tubular structured cell membranes and the kinetoplast is in closer proximity of the nucleus (Fig. 3.4.2 C).

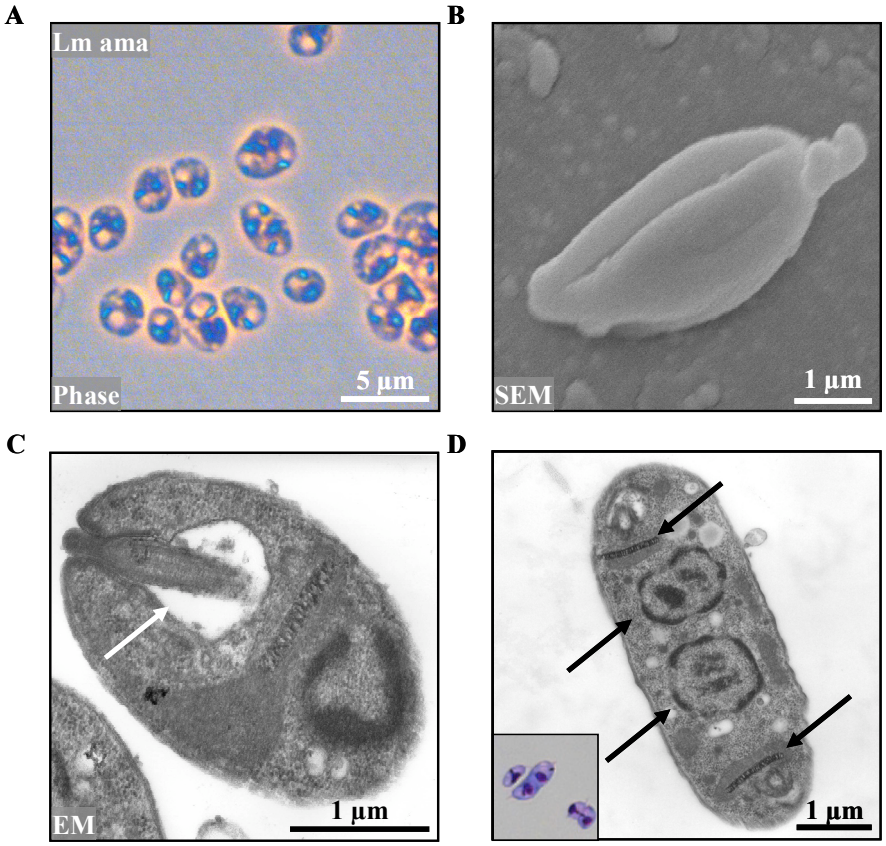

Figure 3.4.2 Morphological characteristics of *in vitro* generated *L. major* amastigotes. A) Representative phase contrast micrograph of Diff QUIK® stained *L. major* amastigotes. **B)** Scanning Electron Micrograph of *L. major* amastigotes. **C)** Electron Micrograph of *L. major* amastigotes. The Arrow indicates the flagellum pocket and the remains of the flagellum. **D)** Electron Micrograph of dividing *L. major* amastigotes. Arrows point out the two nucleae and the two kinetoplasts. Inlay: Diff QUIK® stained dividing *L. major* amastigotes typically found in 3 and 4 day old axenic cultures.

As compared to amastigotes isolated from Balb/c mice (Fig. 3.3.1) the axencic cultures have a more uniformal morphology, consisting mainly of intact amastigote like parasites (Fig. 3.4.2). Moreover, I found dividing cells in the axenic cultures (Fig. 3.4.2 D). In addition the axenic

amastigotes remained stable in amastigote form in the developed culture medium. In contrast, Balb/c or MΦ derived amastigotes readily transformed back into promastigotes (data not shown).

3.4.2 Molecular biological characterisation of axenic *L. major* amastigotes

In recent years *L. major* genome expression analysis revealed that the parasites have a constitutively expressed genome with as little as 3 % of the genes being differentially regulated (Cohen-Freue et al., 2007; Leifso et al., 2007). These analyses showed that the putative ABC-transporter (LmjF11.0040) is a reliable marker for *L. major* amastigotes. Furthermore, SHERP is expressed stronger on metacyclic *L. major* promastigotes than on amastigotes. Different gene expression analysis revealed that rRNA45 is a stable, endogenous reference gene to normalise expression analysis on. Using quantitative real-time PCR, I compared the mRNA expression of metacyclic *L. major* promastigotes, *in vitro* cultured axenic amastigotes and amastigotes isolated from human MΦ. Total RNA was isolated and quantified using photometric density analysis at 260 nm. According to these analyses, equal amounts of RNA were used in cDNA syntheses.

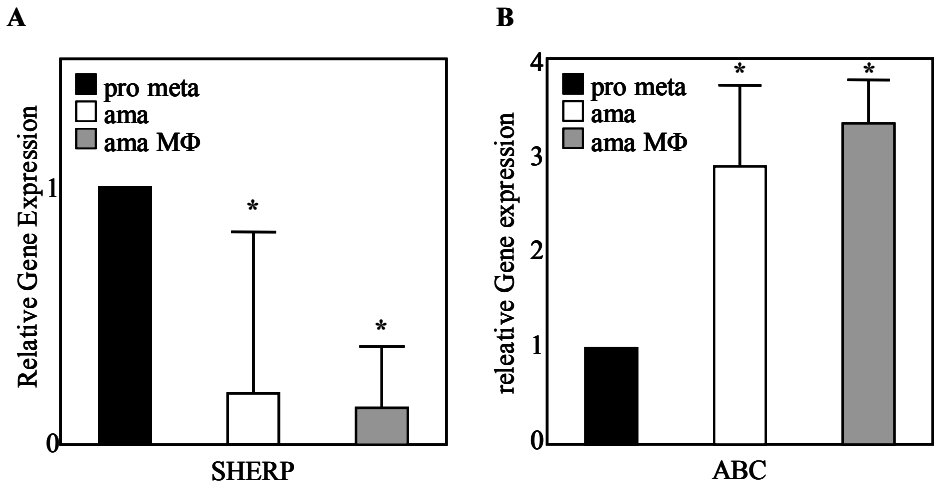

Figure 3.4.3 Stage specific Gene-expression *L. major*. Total RNA was isolated and cDNA generated from *L. major* amastigotes (white bars) or *L. major* amastigotes isolated from MΦ (grey bars) and compared with total RNA isolated from *L. major* metacyclic promastigotes (black bars). Depicted are fold mRNA change in relation to *L. major* metacyclic promastigotes. Values given are mean ± SD of 4 independent experiments. **A)** Differential Gene expression of the promastigote marker gene SHERP. **B)** Gene expression of the amastigotes marker gene ABC-Transporter. Significance was assessed by two-tailed, paired T-Test ($p < 0.05$)

The same volumes of cDNA were used in lightcycler ™ analysis. I found that metacyclic promastigotes express 6-times more SHERP than either axenic or MΦ-derived amastigotes

(Fig. 3.4.3 A). Moreover, I found that amastigotes, axenic as well as MΦ-derived, express 3 times more ABC-tansporter mRNA than metacyclic promastigotes (Fig. 3.4.3 B). In addition, axenic as well as MΦ-derived amastigotes have similar expression patterns for these markers (Fig. 3.4.3 A and B).

3.4.3 Growth curve analysis of *L. major*

The *in vitro* growth behaviour of axenic *L. major* amastigotes cultures was compared to the growth of *L. major* promastigotes on NNN-blood agar cultures (Fig 3.4.4 A and B). Both developmental stages grow with a similar sigmoid pattern, typical for microorganisms that are inoculated into fresh culture medium. An initial lagging phase is followed by an exponential increase (logarithmic-phase) of the *Leishmania* ending in the stationary phase (stationary-phase), were increase of parasites number has ceased. I found that to start an axenic culture, *L. major* amastigotes cultures were most stable at a cell density of about 2 x 10^7 cell / ml in the initial inoculum to grow as amastigotes.

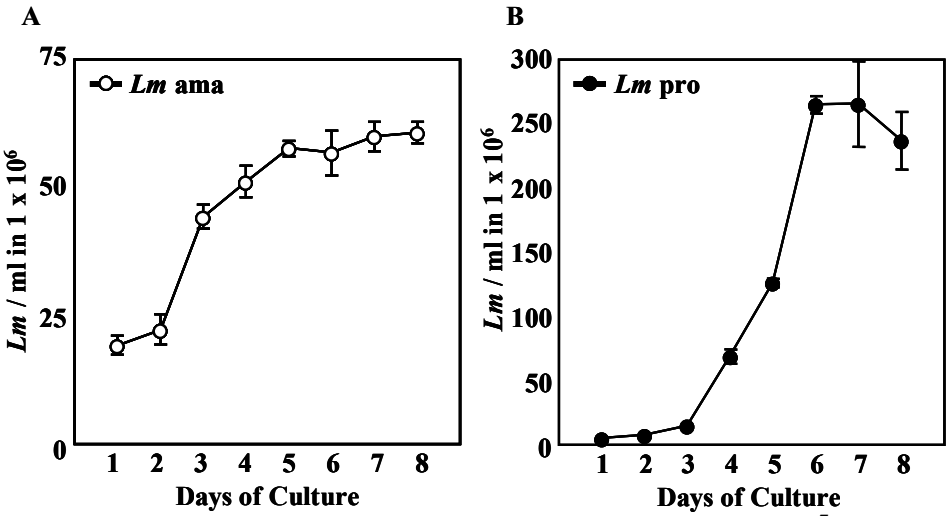

Figure 3.4.4 Growth curve analysis of *L. major* parasites. A) 2 x 10^7 Lm / ml *L. major* amastigotes were inoculated in 5 ml AAM. Amastigotes were counted daily as described (2.2.1.24). **B)** 1 x 10^6 Lm / ml *L. major* promastigotes were inoculated in NNN-semi-solid cultures. Promastigotes were counted daily as described (2.2.1.24). The values given are mean ± SEM of 4 independent experiments preformed.

Lower cell densities in some cases resulted in the re-transformation into promastigotes, whereas higher densities resulted in dead parasites within 3 to 4 days (data not shown). I observed the amastigote cultures to triple once during 8 days of culture namely from 2 x 10^7 / ml to 6 x 10^7 / ml, while promastigotes double 8 times from 1 x 10^6 to 3.5 x 10^8 *L. major* / ml during the same time period.

3.4.4 Biochemical characterisation of axenic *L. major* amastigotes

At first, I analysed surface expression of PS on the axenic *L. major* amastigotes using AnxA5-fitc. FACS analysis revealed only minor increase of PS-positive *L. major* amastigotes from 9 % ± 3.2 % (mean ± SD) at day 3 to 13 % ± 1.4 at day 7 cultures (Fig. 3.4.5 A and B). After 12 days of culture 32 % ± 3.2 % of *L. major* amastigotes were PS-positive (Fig. 3.4.5 C).

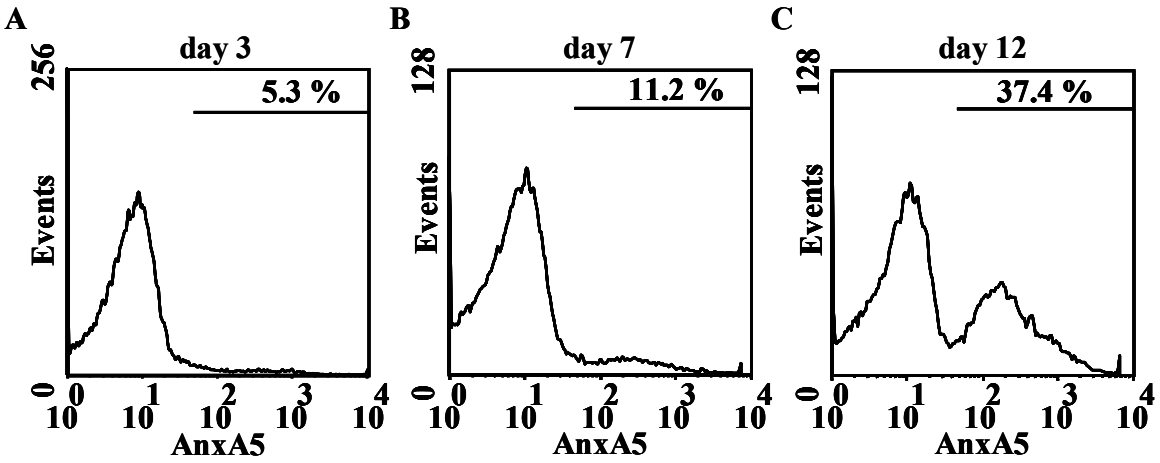

Figure 3.4.5 FACS analysis of axenic *L. major* amastigote cultures. A) 5 x 10^6 day 3 *L. major* amastigotes were stained with AnxA5-fitc. **B)** Day 7 *L. major* amastigotes were stained with AnxA5-fitc. **C)** Day 12 *L. major* amastigotes were stained with AnxA5-fitc. The graphs depicted show one representative experiment of four.

Next, I analysed the binding capacity for peanut lectin (PNL) on *L. major* promastigotes and compared it with amastigotes. I compared logarithmic-phase promastigotes and 3 days old amastigotes with each other as well as stationary-phase promastigotes with 6 days old amastigotes. I labelled *L. major* parasites with PNL-biotin and detected the biotin with Fitc-labelled Streptavidin. FACS analysis revealed that *in vitro* cultured amastigotes have a higher binding capacity of PNL with a geometric mean (GM) ranging from 100 – 1400 compared to promastigotes with a GM ranging from 57 – 600 (Fig. 3.4.6 A and B). Furthermore, I could detect that logarithmic-phase promastigotes (GM 100 – 600) as well as day 3 amastigotes (GM 300 – 1400) bound more PNL compared to stationary-phase promastigotes (GM 50 - 325) or day 6 amastigotes (GM 95 – 850), respectively (Fig. 3.4.6 A vs. B).

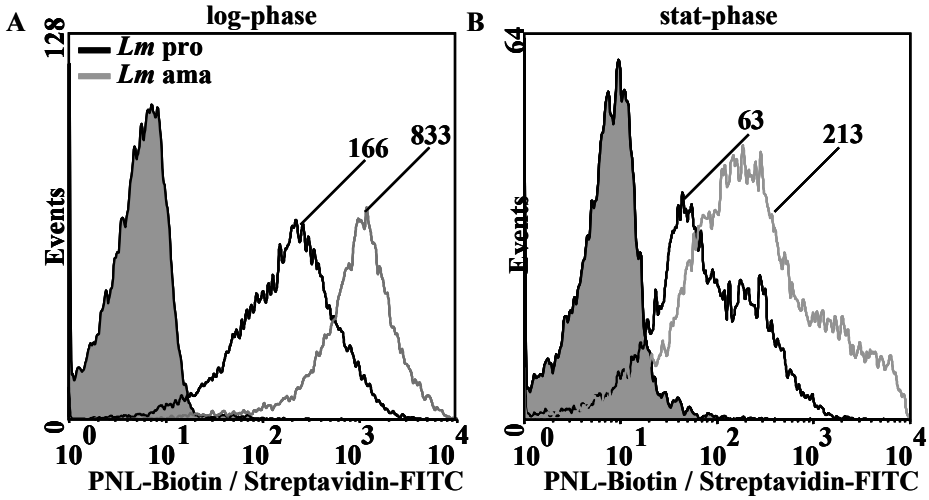

Figure 3.4.6 *L. major* amastigotes bind more Peanut Lectin than Promastigotes. *L major* parasites were incubated for 15 minutes with 0.2 µg / ml Peanut Lectin Biotin (PNL). The biotin was detected with FITC-labeled streptavidin. The cells were analysed by FACS analysis. (Isotype control: filled grey area, *L. major* promastigotes: black line, *L. major* amastigotes (grey line). Logarithmic-phase parasites (A) bind more PNL than stationary-phase parasites (B). Depicted is one representative experiment of three.

Finally, I analysed the surface expression of proteophosphoglycan (PPG) with the murine antibody WIC79.3. As mentioned above, I compared logarithmic-phase *L. major* promastigotes with day 3 amastigotes and stationary-phase promastigotes with day 6 amastigotes. *Leishmania* parasites were labelled with WIC79.3 or left untreated and subsequently stained with a PE-labelled anti-mouse antibody. FACS analysis showed that *L. major* amastigotes expressed more PPG (GM ranging from 120 – 2000) than promastigotes (GM 25 – 450) (Fig. 3.4.7 A and B). Furthermore, I could detect that the surface expression of PPG increases during maturation from logarithmic-phase (GM 35 – 20) or day 3 amastigotes (GM 120 -1300) to stationary-phase promastigotes (GM 70 -450) or day 6 amastigotes (GM 135 – 2000) (Fig. 3.4.7 A vs. B).

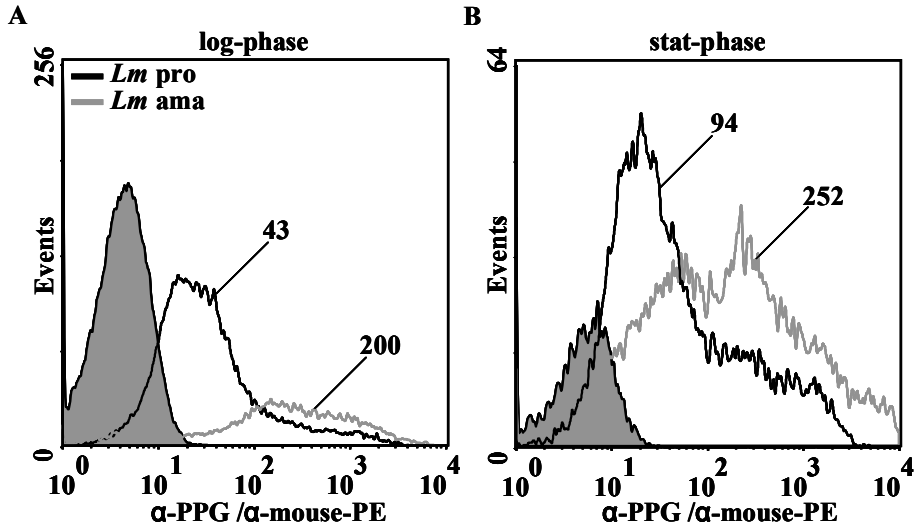

Figure 3.4.7 Proteophosphoglycan expression on *L. major* parasites. Stationary phase *L. major* promastigotes (black line) as well as *L. major* amastigotes (grey line) were stained with WIC79.3 (mouse α-Lm-PPG) or left untreated (grey filled). Bound WIC79.3 was detected with an antimouse-PE antibody. **(A)** PPG-expression of Logarithmic-phase promastigotes (black line) and day 3 amastigotes (grey line) were compared. **(B)**. PPG-expression on stationary-phase promastioges (black line) were compared with day 6 amastigotes. The depicted diagram shows one representative experiment of three.

3.4.5 Virulence of *in vitro* generated *L. major* amastigotes in *in vivo* mouse models.

In order to characterise the virulence of the *in vitro* generated amastigotes in an experimental *in vivo* model, I injected 3 x 10^6 *L. major* promastigotes, 3 x 10^6 axenic amastigotes or 3 x 10^6 MΦ derived amastigotes in the right footpad of Balb/c as well as C57BL/6 mice. The disease development was monitored weekly by measuring increasing lesion formation using a calliper. When inoculated with either *L. major* promastigotes, or axenic or MΦ-derived amastigotes, Balb/c mice presented lesions at the site of infection after 21 days of infection. In addition to infection with promastigotes, lesions developed similar when Balb/c mice were infected with amastigotes. I did not detect a significant difference between axenic and MΦ-derived amastigotes (Fig. 3.4.8 A).

Inoculation of C57BL/6 mice with promastigotes resulted in a self-healing phenotype. After 14 days of infection lesion formation could be detected. These lesions were not progressive and after 35 days of infection they started to heal again (Fig. 3.4.8. B, black dots). Axenic amastigotes induce a lesion formation after 21 days, but that subsided after 28 days (Fig. 3.4.8 B white dots). The MΦ-derived amastigotes induced a self-healing lesion after 35 days that healed 14 days later (Fig. 3.4.8 B grey dots).

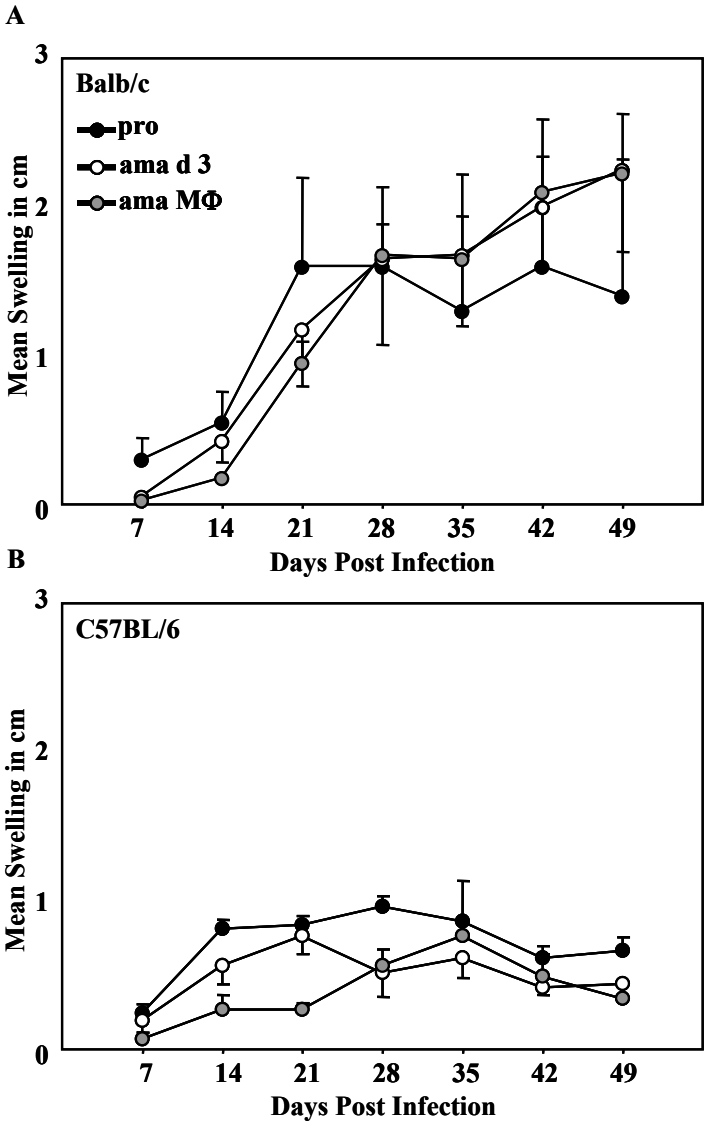

Figure 3.4.8 Virulence of axenic *L. major* amastigotes in mouse models. A) Female Balb/c mice were infected with 3 x 10^6 *L. major* promastigotes (black dots), *L. major* amastigotes isolated from macrophages (grey dots) or with *in vitro* generated *L. major* amastigotes (white dots). Values given are mean ± SEM from 1 out of 2 experiments with 4 mice. **B)** Femal C57BL/6 mice were infected with 3 x 10^6 *L. major* promastigotes (black dots), *L. major* amastigotes isolated from macrophages (grey dots) or with *in vitro* generated *L. major* amastigotes (white dots). Values given are mean ± SEM from 1 out of 2 experiments with 4 mice.

3.4.6 Infectivity of axenic *L. major* amastigote in human phagocytes

In order to analyse the virulence of the axenic amastigotes, I incubated different human phagocytes with the parasites at a ratio of 1:10. Extracellular parasites were removed three hours post infection, samples taken and the cells Diff QUIK® stained. Infection rates and parasite burdens were assessed by microscopically analysing the samples as described before (3.2.1).

Freshly, isolated PMN were incubated with either *L. major* promastigotes or axenic amastigotes. As described in 3.2.1 PMN readily phagocytosed *L. major* promastigotes (Fig.

3.2.1). I detected in mean 35 % ± 2 % infected PMN. Surprisingly, no infected cells could be detected when PMN were incubated with axenic amastigotes (Fig. 3.4.9).

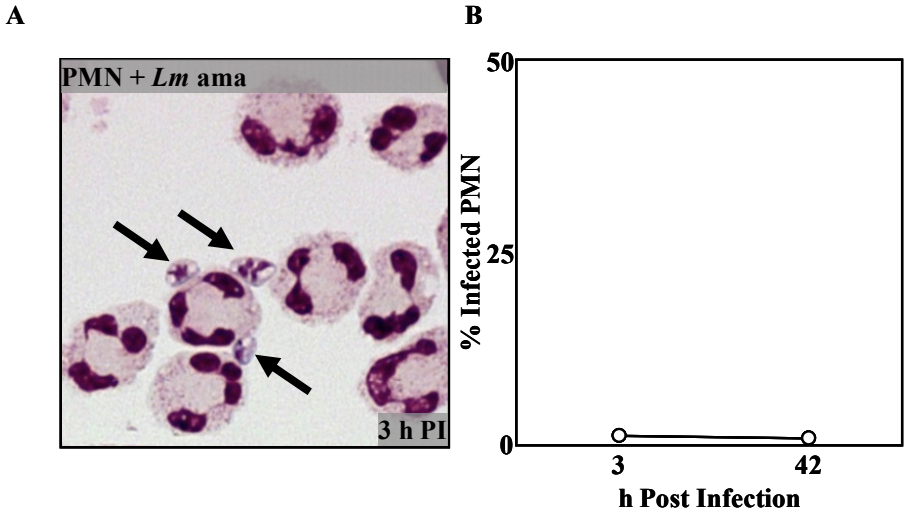

Figure 3.4.9 Human PMN do not take up *L. major* amastigotes Human PMN were incubated with *in vitro* generated *L. major* amastigotes at the ratio of 1:10. Samples were taken 3 hours and 42 hours post infection and Diff QUIK® stained. **A.)** Diff QUIK® stained cytospin of incubated PMN with *in vitro* generated *L. major* amastigotes. Arrows indicate extracellular *L. major* amastigotes. The micrograph is representative for 5 independent experiments. B.) Counting parasitized cells in > 200 human PMN infection rates were assessed 3 and 42 hours post infection. Values given are mean ± SEM of 5 independent experiments.

Since macrophages are the final host cells of *Leishmania* and contain the amastigote form in disease, I analysed the virulence for these cells, next. The PMA differentiated THP-1 cells were stimulated over night with 10 ng / ml PMA in 6-well plates. The cells were washed twice with pre-warmed medium before *L. major* parasites were added for three hours. Extracellular parasites were removed and Diff QUIK® stained samples prepared (Fig. 3.4.10 A - D). I found that 3 hours post infection 27 % ± 4.4 % of the THP-1 cells were infected with *L. major* promastigotes, in contrast to *L. major* amastigotes that infected 54 % ± 2 % of the THP-1 cells (Fig. 3.4.10 E). 66 hours post infection I detected similar infection rates, 27.8 % ± 5.8 % and 56 % ± 4 %, respectively (Fig. 3.4.10 E). Furthermore, I could observe that the parasite burden of THP-1 cell infected initially with *L. major* promastigotes increased from 2.8 ± 1.4 (mean + SD) to 3.4 ± 1.4. Cells that were infected with axenic amastigotes had an increase in intracellular parasites from13.5 ± 8.4 to 18.9 ± 15. (Fig. 3.4.10 F).

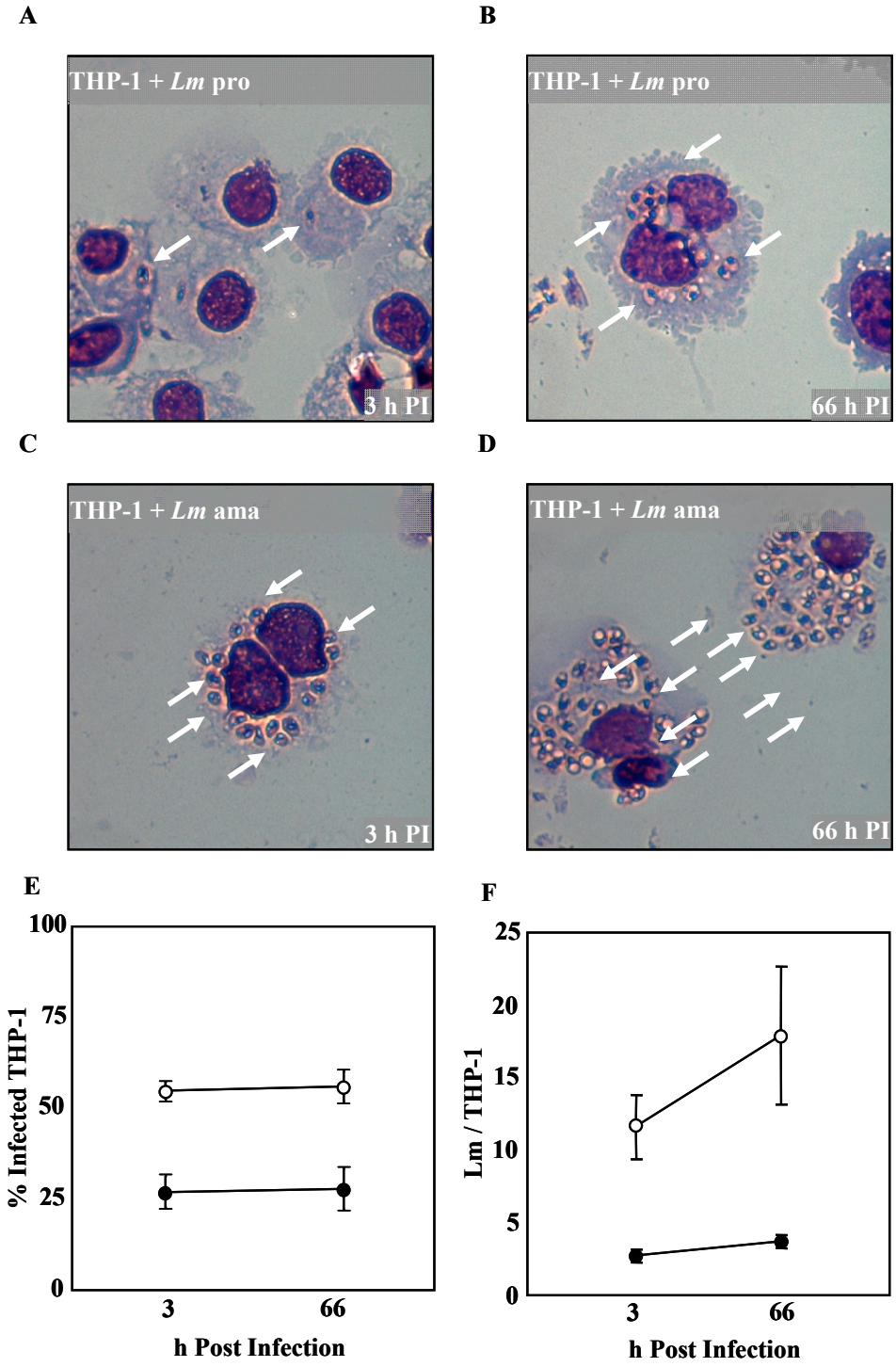

Figure 3.4.10 *L. major* amastigotes are highly virulent for human macrophages. Depicted are micrographs of Diff QUIK® stained THP-1 cells: 3 hours (**A** and **C**) post infection compared to 66 hours (**B** and **D**) post infection. The THP-1 cells were either incubated with *L. major* promastigotes (**A** and **B**) or amastigotes (**C** and **D**). **E)** Depicted are infection rates of THP-1 cells incubated with *L. major* promastioges (black dots) or amastigotes (white dots) 3 and 66 hours post infection. **F)** Parasite burdens of infected THP-1 cells 3 and 66 hours post infection. White dots THP-1 cells initially infected with amastigotes, black dots cells initially infected with promastigotes. Values given are mean ± SEM of 3 independent experiments. Statistical analysis were performed using a two-tailed, paired T-Test (* p=< 0.05).

3.4.6 Comparative analysis of different human primary macrophages

In cutane Leishmaniasis it is not known which type of MΦ plays a role in disease development or healing. Both pro-inflammatory type I MΦ as well as anti-inflammatory type II MΦ can be possible host cells. Therefore, I compared infectivity between both promastigotes and amastigotes on type I as well as on type II MΦ.

Initially, I characterised the two subtypes of MΦ by FACS analysis. At day 5 of culture in either M-CSF of GM-CSF containing growth medium cells were harvested and stained with CD163-PE antibody. I found that 95 % of M-CSF treated type II MΦ express the surface marker CD163, which is characteristic for type II MΦ, while this marker is absent on the GM-CSF derived type I MΦ (Fig. 3.4.11 A). Additionally, I found that type II MΦ express intermediate levels (GM 128) of MHCII (Fig. 3.4.11 B), low levels (GM32) of CD206 (Fig. 3.4.11 C) and high levels (GM 700) of CD14 (Fig. 3.4.11 D). In contrast, type I MΦ express intermediate levels (GM 80) MHCII (Fig. 3.4.12 B), CD206 (GM 53) (Fig. 3.4 11 C) and CD14 (GM 65) (Fig. 3.4.11 D).

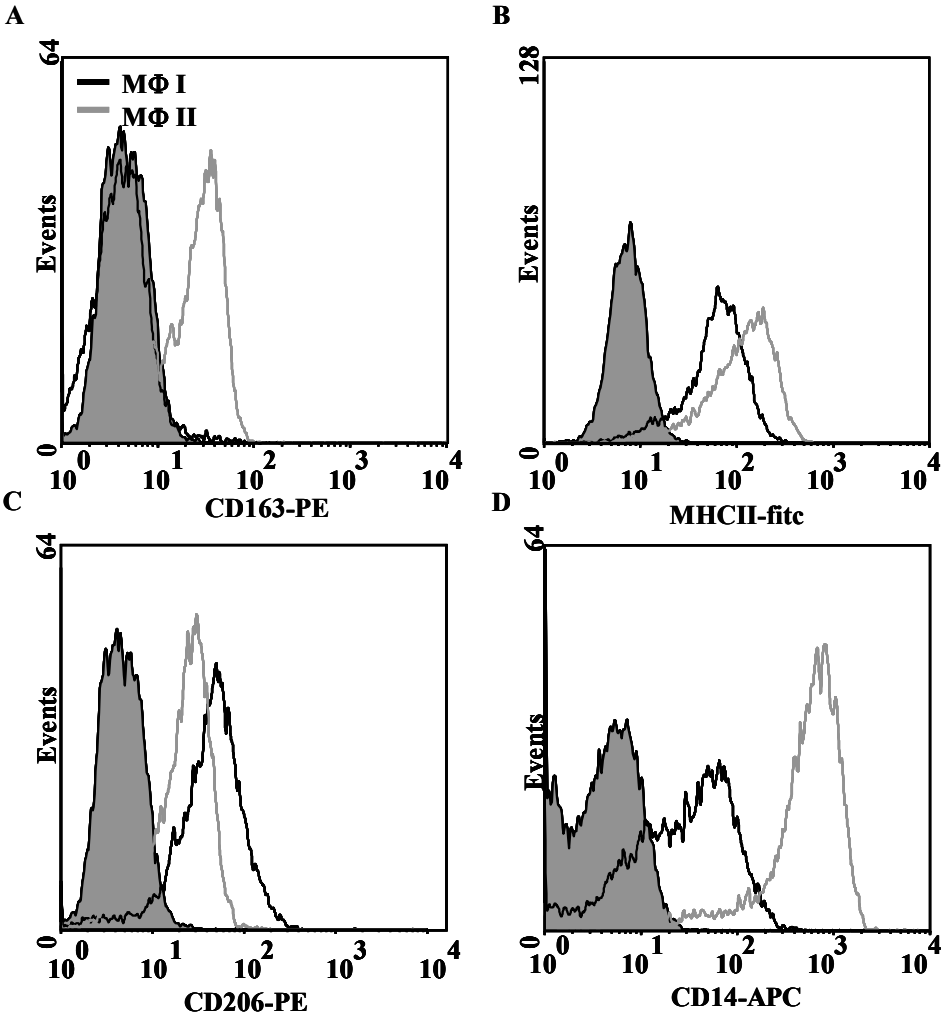

Figure 3.4.11 FACS characterization of type I and type II MΦ. Type I MΦ (black lines) and type II MΦ (grey lines) from the same donor were harvested and stained with anti CD163 (A), MHCII (B) CD206 (C), CD14 (D) or the respective isotype (grey filled) for 30 minutes. The cells were washed and analyzed by FACS. The diagram shows 1 representative experiment of 3 performed.

Comparative infection studies of type I and II MΦ from the same donor revealed that three hours post infection significantly more type II MΦ were infected compared to type I MΦ. Of all type I MΦ 38 % ± 3.5 % (mean ± SEM) were infected by promastigotes 3 hours after incubation compared to 46.7 % ± 2.4 % of all type II MΦ (Fig. 3.4.12 A). Moreover, 56 % ± 4.9 % of al type I MΦ were infected with axenic amastigotes, while 74.2 % ± 2.9 % of all type II MΦ were infected (Fig. 3.4.12 B). In addition I found that type I MΦ take up less *L. major* promastigotes compared to type II MΦ (2.7 ± 0.5 vs 3.2 ± 0.6) respectively (Fig. 3.4.12 C). Furthermore, type I MΦ took up less (7.5 ± 2.2) amastigotes compared with type II MΦ (13.5 ± 3).

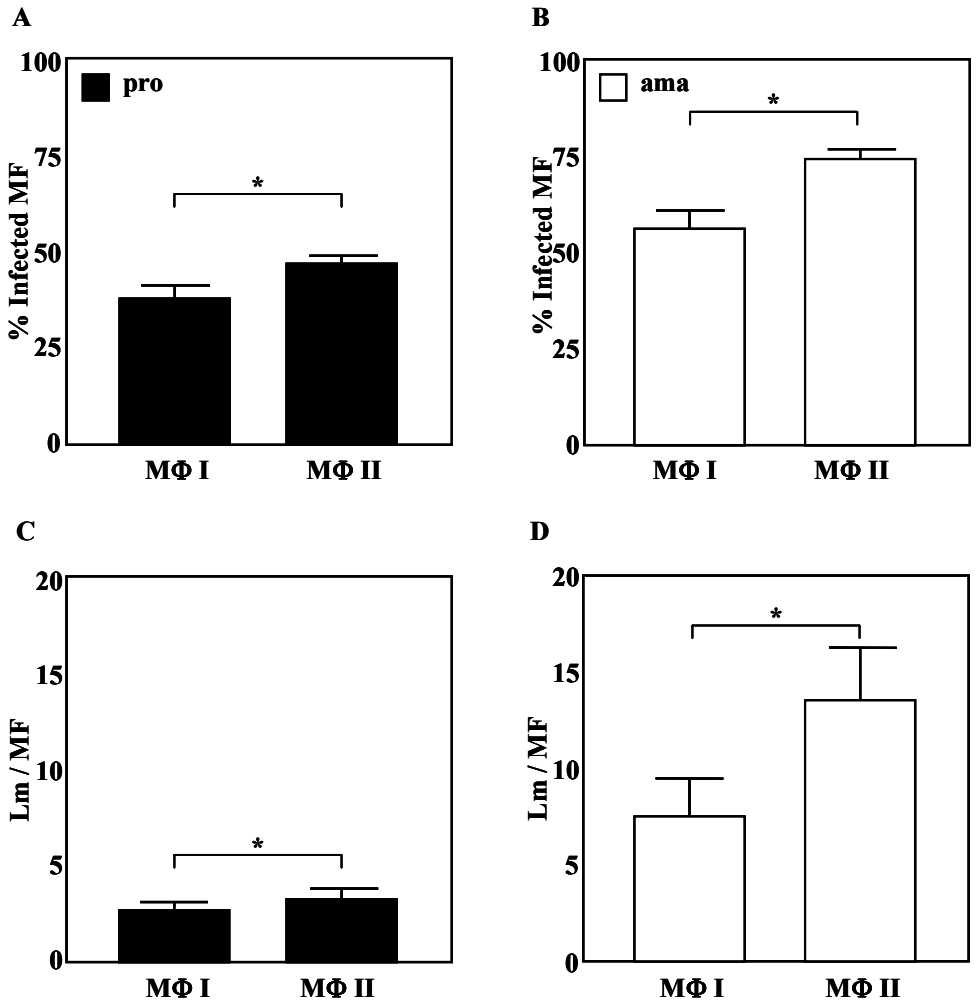

Figure 3.4.12 Comparison of type I and type II MΦ in *Leishmania* infection assays. Type I or type II MΦ were incubated with *L. major* promastigotes (A and C black bars) or *L. major* amastigotes (B and D white bars) at a ratio of 1:10 (MΦ : Lm). Extracellular parasites were removed 3 hours post infection and infection rates were determined by counting parasitized cells in > 200 phagocytes. Parasite burdens were assessed by counting parasites in 20 infected MΦ. The values depicted are mean + / - SEM of n = 11 independent experiments. Type two MΦ take up significantly more parasites in the same time frame as do type I MΦ. Statistical analyses were performed using a two-tailed, paired T-Test (* = $p < 0.0005$).

Summary Characteristics of *L. major* amastigotes

The *in vitro* generated axenic *L. major* amastigotes have a similar morphology to amastigotes isolated from infected MΦ. The established culture conditions lead to a sigmoid growth pattern similar to the growth curve of *L. major* promastigotes. I could show that the axenic amastigotes express the described amastigote marker the putative ABC transporter, while at the same time they decrease the expression for the metacyclic promastigote marker SHERP. Furthermore, these amastigotes have a higher binding capacity of PNL than promastigotes. I could show that the binding capacity for PNL decreases from 3 days to 6 days old amastigote

cultures. More PPG could be detected on the surface of axenic amastigotes compared to promastigotes. The axenic amastigote cultures have a disease inducing phenotype in Balb/c mice and induce self-healing lesions in C57BL/6 mice. Contrary to *L. major* promastigotes, amastigotes apparently are not virulent for human PMN, but highly infective for human macrophages. Finally, type II MΦ take up significantly more parasites, during 3 hours of incubation, as compared to type I MΦ.

3.5 On the uptake of *L. major* parasites

As it was demonstrated, *L. major* promastigotes are infective for PMN and MΦ, while axenic amastigotes apparently were not taken up by PMN. In addition I found that in *L. major* infection experiments type II MΦ take up more parasites.

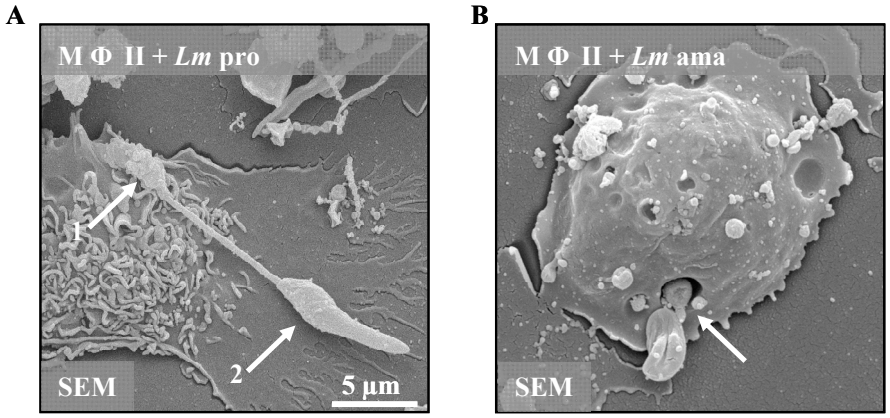

Figure 3.5.1 Scaning Electron Microscopy analysis of parasite uptake. Human primary Type II MΦ were incubated with *L. major* promastigotes (**A**) or amastigotes (**B**) for 15 minutes. Extracellular parasites were removed and the cells were fixed in Monti-fixation and analysed by SEM. **A**) *L. major* promastigote attached to MΦ. Arrow 1 indicates the flagellum tip attached to the MΦ. Arrow 2 points at the promastigotes body. **B**) *L. major* amastigotes are taken up by the phagocyte (white arrow).

It has been observed that the initial contact between *L. major* promastigotes and the phagocytes takes place via the flagellum tip and by an unknown mechanism (Fig. 3.5.1 A). Since amastigotes do not have an extracellular flagellum (Fig. 3.4.2) the first contact and subsequent recognition must be a different one. Interestingly, *L. major* amastigotes are not taken up by PMN. Therefore, I now focus on different mechanisms of cell-entry, such as phagocytosis and endocytosis. Moreover, I analyse a set of different receptors known to be involved in phagocytosic and endocytotic processes.

3.5.1 Do *L. major* invade MΦ or are they taken up by these host cells?

In order to analyse what mechanisms were responsible for the infection of human MΦ, I tried to discriminate, between an active seizure of the phagocytes, similar to *Toxoplasma gondii* (Baum et al., 2008) and a passive uptake of *Leishmania* by the cell. This was achieved by incubating MΦ with 10 µM Cytochalasin D for 15 minutes at 37°C. The cells were washed twice, before co-incubating them with *L. major* pro – or amastigotes for 3 hours. Extracellular

parasites were removed and infection rates determined by counting parasites in > 200 MΦ in Diff QUIK ® stained samples (Fig. 3.5.2 A-H).

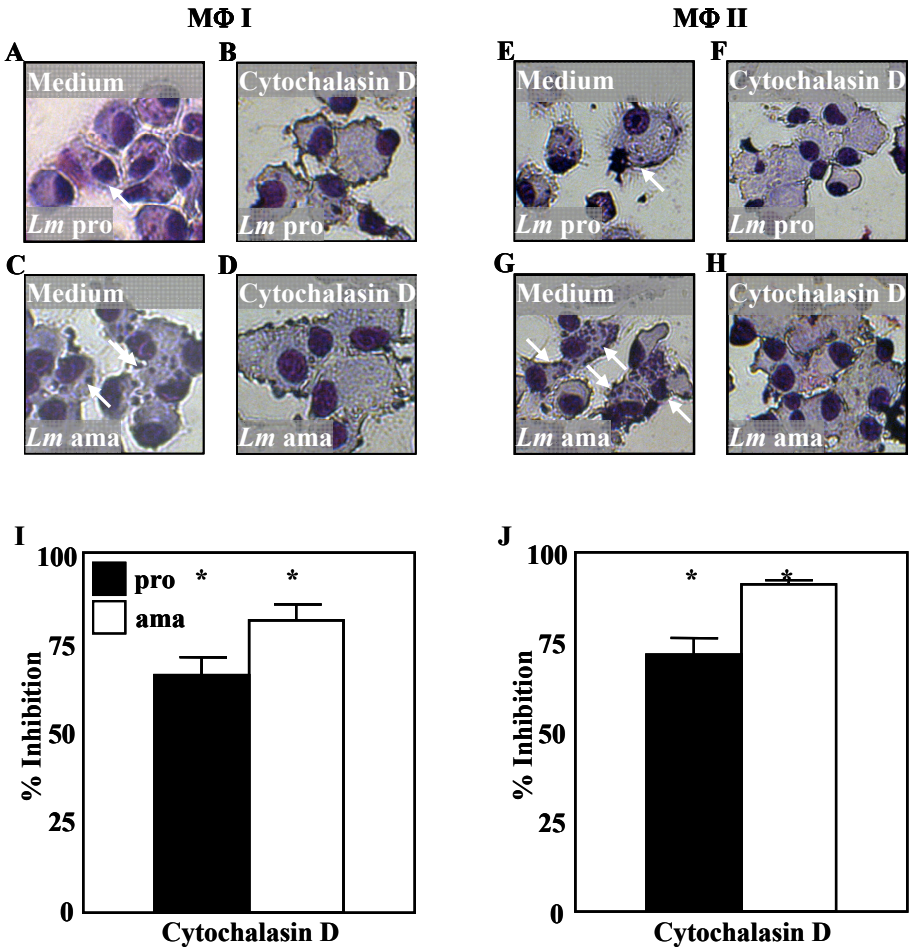

Figure 3.5.2 Cytochalasin D blocks infection with *L. major* parasites. Representative micrographs of Type I MΦ (**A – D**) or Type II MΦ (**E – H**) were incubated with 10 µM Cytochalasin D (**B, D, F, H**) or left untreated (**A, C, E, G**) for 15 minutes. The cells were washed twice in pre-warmed media before incubation with *L. major* promastigotes (**A, B, E, F**) or *L. major* amastigotes (**C, D, G, H**) Extracellular parasites were removed 3 hours post infection and samples Diff QUIK® stained. Infection rates were evaluated by counting more than 200 cells. **A)** Medium control of type I MΦ infected with *L. major* promastigotes, **B)** Cytochalasin D pre-treated type I MΦ incubated with *L. major* promastigotes. **C)** Medium control of MΦ I infected with *L. major* amastigotes. **D)** Cytochalsin D pre-incubated type I MΦ infected with *L. major* amastigotes. **E)** Medium control of type II MΦ infected with *L. major* promastigotes. **F)** Cytochalasin D pre-incubated type II MΦ incubated with *L. major* promastigotes. **G)** Medium control of type II MΦ infected with *L. major* amastigotes. **H)** Cytochalasin D pre-incubated type II MΦ incubated with *L. major* amastigotes. **I** and **J)** Depicted is the percentage of inhibition of *Leishmania* infection rate of type I MΦ (**I**) or type II MΦ (**J**) compared to medium controls. Values given are mean ± SEM from 3 independent experiments. Statistical analysis was performed by a two-tailed, paired T-Test ($p < 0.05$) as compared with controls.

During this period cells remained viable as was assessed by trypan blue exclusion staining (data not shown). The values depicted are mean ± SEM. Upon incubation of type I MΦ with

Cytochalasin D the infection rates for promastigotes and amastigotes were significantly reduced by 66 % ± 5.3 % and 81 % ± 6 % respectively (Fig. 3.4.2 I). The infection of type II MΦ was decreased by 71 % ± 4.7 % and 91 ± 0.7 % for promastigotes and amastigotes respectively (Fig. 3.5.2 J).

3.5.2 Phagocytosis versus endocytosis

In general phagocytes have three means by which they can take up particles, namely phagocytosis, clathrin-mediated endocytosis and caveolae-mediated endocytosis ((Ivanov, 2008).

Phagocytosis is blocked specifically, by inhibiting the sodium-proton exchange by incubating the target cells with dimethyl amiloride (DMA) (Ivanov, 2008; von Delwig et al., 2002). Clathrin-mediated endocytosis is inhibited by stabilizing clathrin-coated pits, when target cells are incubated with monodansylcadaverine (MDC) (Wang et al., 2003). Caveolae-mediated endocytosis is diminished by cross-linking cholesterol residues in the plasma membrane, incubating the target cells with the polyene antibiotic Filipin III (Kitajima et al., 1976).

3.5.2.1 Modulation of phagocytosis and endocytosis in human type I and type II MΦ

As mentioned earlier, type II MΦ take up more *L. major* parasites as compared to type I MΦ (Fig. 3.4.5). These observations in mind led us to investigate to what degree the two subsets of MΦ use one of the three pathways to take up *L. major* parasites.

The optimal concentrations of the pharmaceuticals were evaluated by pre-incubating PMA stimulated THP-1 cells before they were infected with Leishamnia parasites (data not shown). Transferring these concentrations onto primary type I and type II MΦ led to a loss of adherence of the MΦ. Therefore I continued with inhibiting the specific pathways by pre-treatment with either 250 µM DMA, 250 µM MDC or 10 nM Filipin III, concentrations at which the loss of attached cells was below 10% (data not shown).

Incubation with DMA significantly reduced the infection rates by 35 % ± 4.2 % in type I MΦ and 26 % ± 3.5 % in type II MΦ when incubated with *L. major* promastigotes, while no significant inhibition was observed on the infection by amastigotes. (Fig. 3.5.2 A and B).

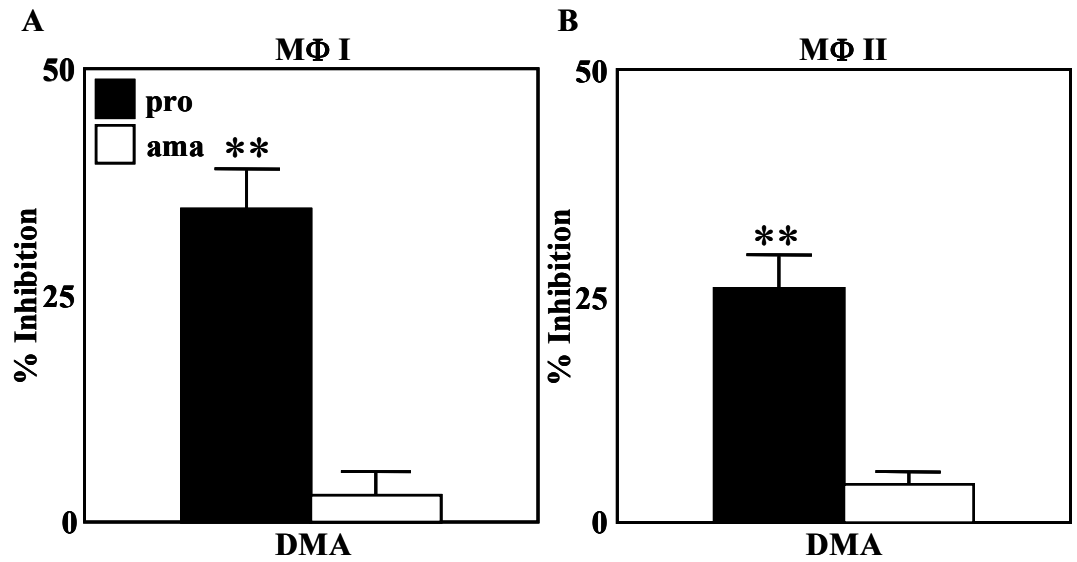

Figure 3.5.2 MΦ infections by promastigotes is blocked by DMA. Human primary type I macrophages (**A**) or type II MΦ (**B**) were incubated with 250 µM DMA for 30 minutes, washed twice with pre-warmed medium prior to incubation with *L. major* promastigotes (black bars) or *L. major* amastigotes (white bars). Extracellular parasites were removed 3 hours post infection, samples taken and Diff QUIK® stained. Infection rates were determined by counting > 200 cells per sample. Depicted is the relative decrease of the infection rate compared to medium controls. Values given are means ± SEM of 11 independent experiments. Statistical analysis was performed using a two-tailed, paired T-Test (* = $P < 0.0005$).

MDC pre-treatment reduced the endocytosis of *Leishmania* amastigotes by 35 % ± 6.2 % in type I MΦ and by 32 % ± 7.5 % in type II MΦ. At the same time phagocytosis of promastigotes was increased by 12.8 % ± 6.2 in type I MΦ and 9.2 % ± 7.5 % in type II MΦ (Fig. 3.5.3 A and B). The same pattern of inhibition was observed when caveolae-mediated endocytosis was blocked with Filipin III. Infection rates of *L. major* amastigotes were reduced by 36.5 % ± 3.4 % type I MΦ and 28.6 ± 3.6 % in type II MΦ (Fig. 3.5.4 A). Infection rates by *L. major* promastigotes were increased by 4.5% ± 3.9 % in type I MΦ and 7.9 % ± 4.3 % in type II MΦ. Combining the blockage of phagocytosis and either one of the endocytosis pathways did not show an additive effect (Fig. 3.5.5). The same was observed by combining MDC and Filipin III (data not shown).

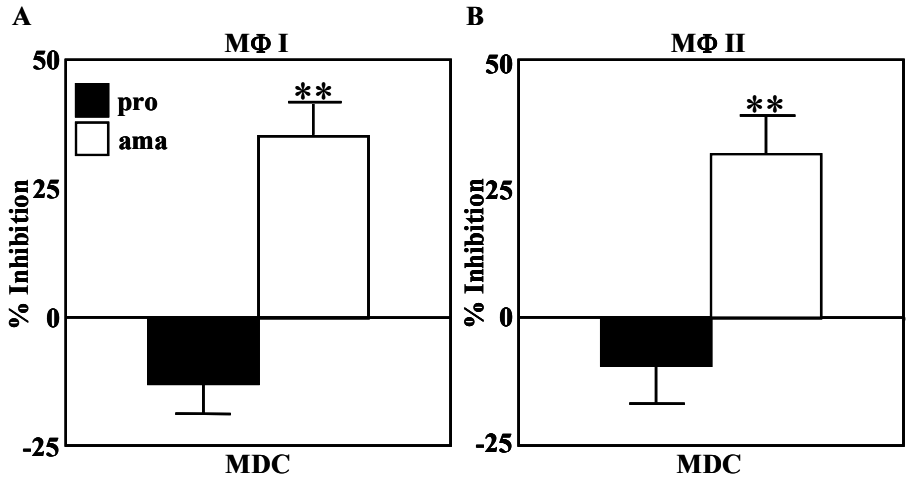

Figure 3.5.3 MΦ infection by amastigotes is blocked by MDC. Human primary type I macrophages (**A**) or type II MΦ (**B**) were incubated with 250 µM MDC for 30 minutes prior to incubation with *L. major* promastigotes (black bars) or *L. major* amastigotes (white bars). Extracellular parasites were removed 3 hours post infection, samples taken and Diff QUIK® stained. Infection rates were determined by counting more than 200 cells per sample. Depicted is the relative decrease of the infection rates compared to medium controls. Values given are means ± SEM of 11 independent experiments. Statistical analysis was performed using a two-tailed, paired T-Test (** = $P < 0.005$).

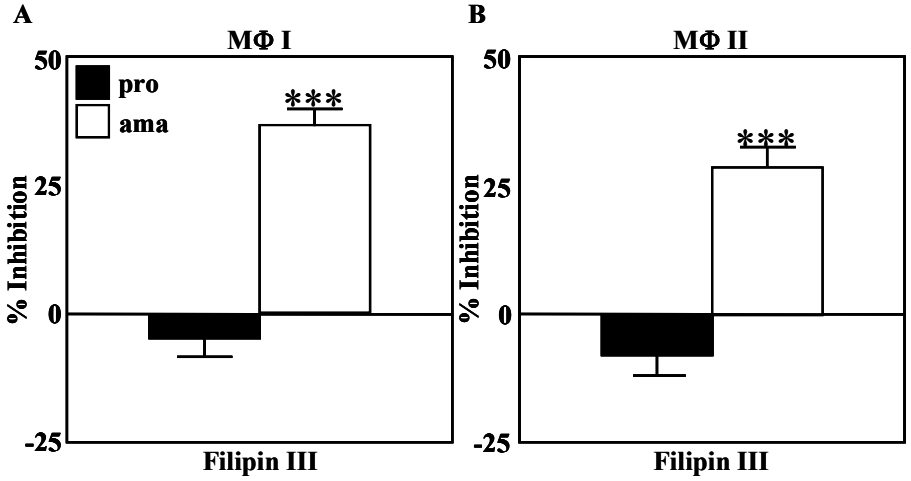

Figure 3.5.4 MΦ infection by amastigotes is blocked by Filipin III. Human primary type I MΦ (**A**) or type II MΦ (**B**) were incubated with 10 nM Filipin III for 30 minutes. The cells were washed twice with pre-warmed medium prior to incubation with *L. major* promastigotes (black bars) or *L. major* amastigotes (white bars). Extracellular parasites were removed 3 hours post infection, samples taken and Diff QUIK® stained. Infection rates were determined by counting more than 200 cells per sample. Depicted is the relative decrease of the infection rate compared to medium controls. Values given are means ± SEM of 11 independent experiments. Statistical analysis was performed using a two-tailed, paired T-Test (*** = $P < 0.000005$).

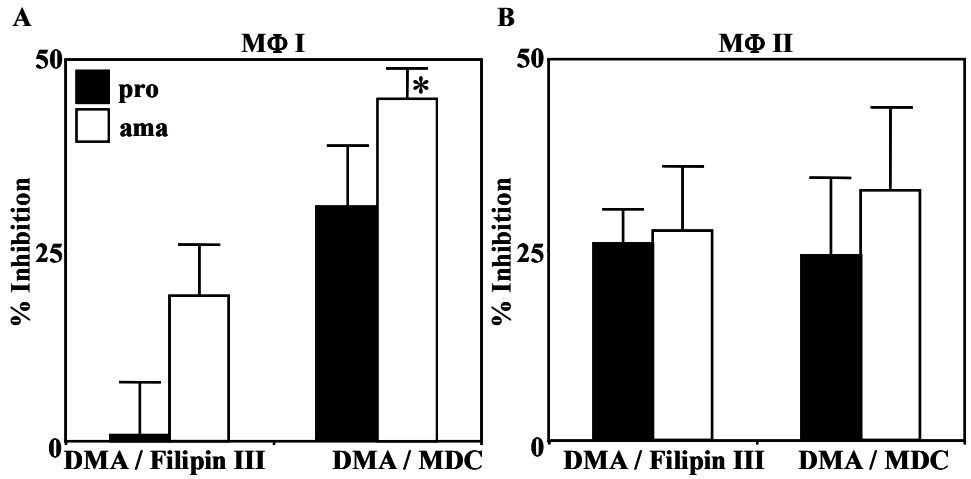

Figure 3.5.5 Combining inhibitors for phagocytosis with endocytosis inhibitors has no additive effect. Human primary type I MΦ or type II MΦ were incubated with the combination of 250 µM DMA and 10 nM Filipin III or 250 µM DMA and 250 µM MDC for 30 minutes prior to incubation with *L. major* promastigotes (black bars) or *L. major* amastigotes (white bars). Extracellular parasites were removed 3 hours post infection, samples taken and Diff QUIK® stained. Infection rates were determined by counting > 200 cells per sample. Depicted is the relative decrease of the infection rate compared to medium controls. Values given are means ± SEM of three independent experiments. Statistical analysis was performed using a two-tailed, paired T-Test (* = $P < 0.05$).

3.5.3 The role of complement receptor 1 (CR1, CD35) in *L. major* infection

Since both phagocytosis and endocytosis are mediated by specific receptors, I blocked a set of receptors known to be involved in either phagocytosis or endocytosis. First, I analysed the influence of CR1. I blocked the receptor on the surface of the target cells by incubation with 10 µg / 1 x 10^6 MΦ of specific rabbit anti human CR1 polyclonal antibody (A kind gift of Dr.M. Daha, Leiden, NL) or rabbit serum or left untreated for 20 minutes, according to the information supplied with the anti-body. The MΦ were treated as mentioned above and the infection rates evaluated 3 hours post incubation. Evaluation of the infection rates showed that treatment of type I MΦ with anti-CR1 antibody resulted in no significant reduction of promastigote infection. When incubated with *L. major* amastigotes, the infection rates in type I MΦ were significantly reduced (26.5 % ± 8%)(Fig. 3.5.6 A). Interestingly, the inhibition of CR1 on type II MΦ resulted in a significant reduction of *L. major* promastigote (38 % ± 8.8%) infection rates, but led no significant decrease of amastigote infection rates (Fig. 3.5.6 B)

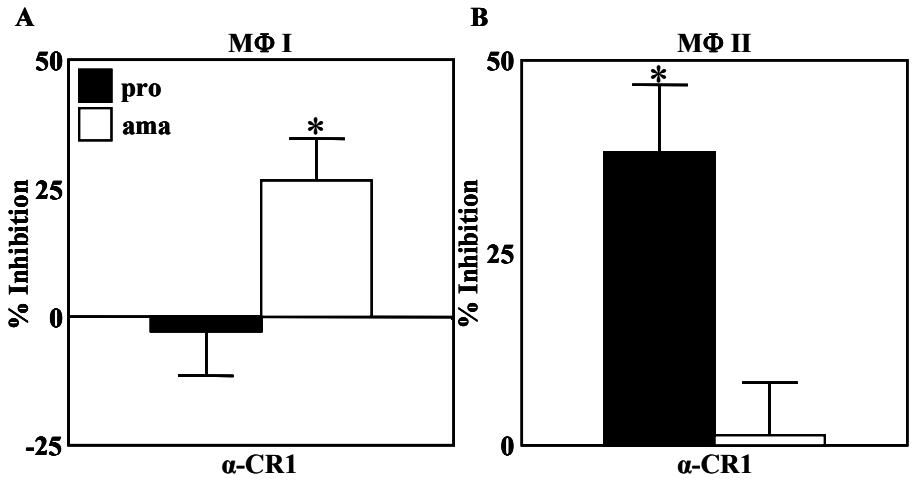

Figure 3.5.6 CR1 dependent infection of MΦ. Human primary type I MΦ (A) or type II MΦ (B) were incubated with 10 µg / 1 x 10^6 MΦ rabbit anti human polyclonal CR1 antibody, 10 µg/ 1 x 10^6 MΦ rabbit serum or medium for 20 minutes at 37°C. The cells were washed twice in pre-warmed medium before incubation with *L. major* promastigotes (black bars) or *L. major* amastigotes (white bars). Extracellular parasites were removed 3 hours post infection and samples Diff QUIK® stained. Infection rates were evaluated by counting >200 cells. Depicted are the mean percentages ± SEM of inhibition compared to medium controls of 5 independent experiments. Statistical analysis was performed by two-tailed, paired T-Test (* = $p < 0.05$). Isotype controls had no significant effect on the uptake of *L. major* parasites.

3.5.4 The role of compliment receptor 3 (CR3) in *L. major* infection

Next, I investigated the involvement of CR3 on the different subsets of MΦ by blocking the CD11b chain of the receptor with a monoclonal antibody (BD Pharmingen, Heidelberg, Germany). MΦ were incubated in the presence of 1 µg / 1 x 10^6 MΦ α-CD11b antibody, 1 µg / 1 x 10^6 MΦ of an matched isotype control monoclonal antibody or left untreated for 20 minutes at 37°C as described by Laufs et al (Laufs et al., 2002). MΦ were infected as mentioned above (3.5.2). In contrast to CR1, blockage of this receptor stimulated the phagocytosis of promastigotes (10% ± 5.7 %) by MΦ I, while the endocytosis of amastigotes was reduced (16.4 % ± 6 %) (Fig. 3.5.7 A). On type II MΦ the inhibition of CR3 had no influence on *L. major* promastigote infection rates, but reduced the infection of amastigotes by 13% ± 3.6 % (Fig. 3.5.7 B).

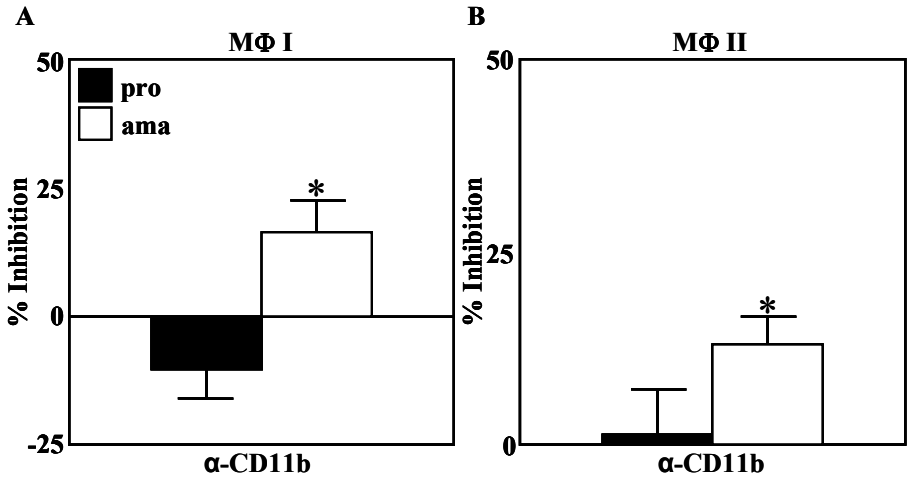

Figure 3.5.7 CR3 dependent infection of MΦ. Human primary type I (**A**) or type II (**B**) MΦ were incubated with 1 µg / 1 x 10^6 MΦ mouse anti human monoclonal anti CD11c antibody, isotype control or medium for 20 minutes at 37°C. The cells were washed twice in pre-warmed medium before incubation with *L. major* promastigotes (black bars) or *L. major* amastigotes (white bars). Extracellular parasites were removed 3 hours post infection and samples Diff QUIK® stained. Infection rates were evaluated by counting > 200 cells. Depicted are the mean percentages ± SEM of inhibition compared to medium controls of 5 independent experiments. Statistical analysis was performed by two-tailed, paired T-test (* $p < 0.05$). The isotype controls had no significant effect on the CR3 mediated uptake of *L. major* parasites.

3.5.6 The influence of mannan on *L. major* infection

Moreover, I analysed the influence of pre-incubation human MΦ with mannan on the up-take of *L. major* parasites. I incubated the target cells with 100 µg / ml mannan for 30 minutes or left the MΦ untreated, as was described by Xu et al (Xu et al., 2006) before proceeding as mentioned above (3.4.2). The infection rates of type I MΦ, incubated with *L. major* promastigotes, were reduced by 26 % ± 8.6 %. Moreover, infection rates were significantly reduced by 42 % ± 7.2 in type I MΦ, when incubated with amastigotes (Fig 3.5.8 A). In type II MΦ the infection with promastigotes was decreased by % 37% ± 7.4 %, while a significant reduction of amastigote uptake by 28 % ± 3.9 % in type II MΦ was detected (Fig. 3.5.8 B).

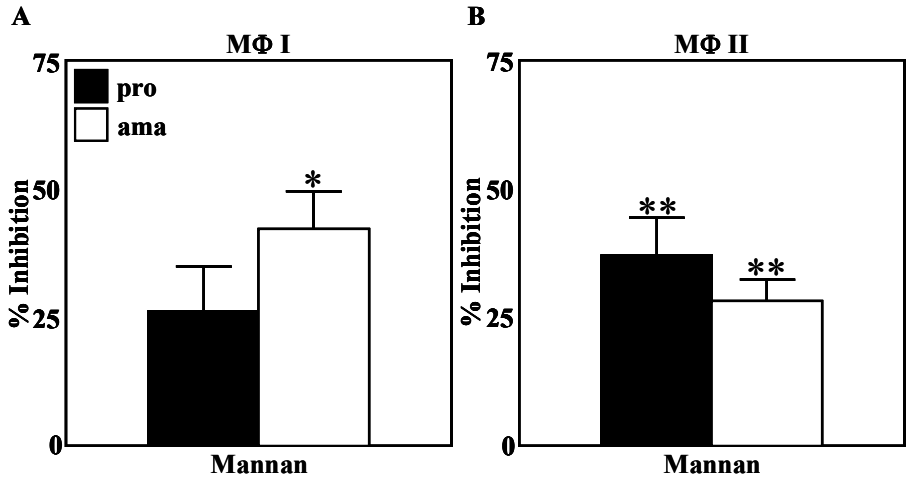

Figure 3.5.8 Mannan dependent inhibition of MΦ. Human primary type I (A) or type II (B) MΦ were incubated with 100 µg / ml mannan or medium for 30 minutes at 37°C. The cells were washed twice in pre-warmed medium before incubation with *L. major* promastigotes (black bars) or *L. major* amastigotes (white bars). Extracellular parasites were removed 3 hours post infection and samples Diff QUIK® stained. Infection rates were evaluated by counting >200 cells. Depicted are the mean percentages ± SEM of inhibition compared to medium controls of 5 independent experiments. Statistical analysis was performed by by two-tailed, paired T-Test (* $p < 0.05$, ** $p < 0.005$).

3.5.7 The role of class A scavenger receptors (SR-A)

Finally, I investigated the influence of scavenger receptor SR-A blockage on infection by *L. major*. Therefore, I used the non-selective, competitive SR-A inhibitor polyinosinic acid (Poly I). MΦ were pre-incubated with 50 µg / ml Poly I for 30 minutes at 37°C or left untreated, as was recommended by the distributor, before proceeding as mentioned above (3.5.2). Treatment with Poly I reduced the infection rates of type I MΦ incubated with *L. major* promastigotes by 36.6 % ± 4.5, while infections with amastigotes were reduced by 8 % ± 5.9 % (Fig. 3.5.9 A). Inhibition of SR-A on type II MΦ resulted in a reduction of infections with promastigotes by 20.1 % ± 6.1 % respectively (Fig. 3.5.9 B). Infection rates were reduced by 26.3 % ± 11.3 % on type II MΦ after Poly I pre-treatment, when incubated with amastigotes.

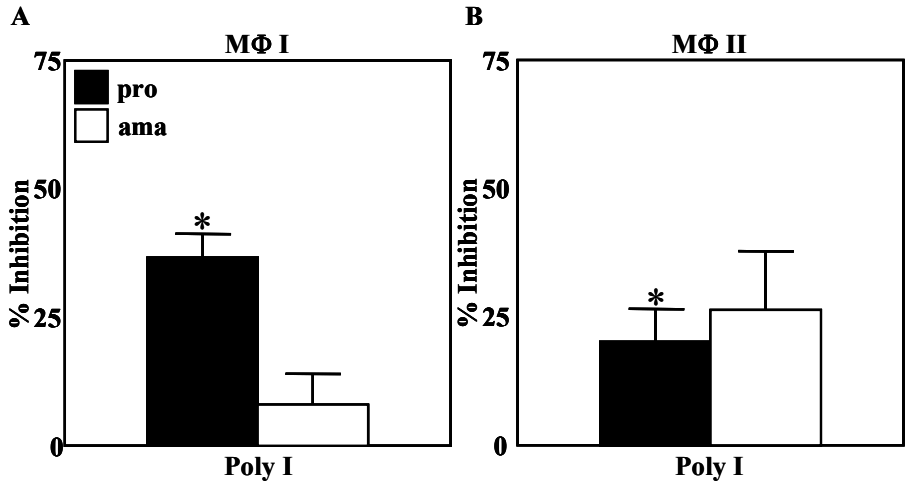

Figure 3.5.9 SR-A dependent infection of MΦ. Human primary type I (A) or type II MΦ were incubated with 50 µg / ml Polyinosinc acid (Poly I) or medium for 30 minutes at 37°C. The cells were washed twice in pre-warmed medium before incubation with *L. major* promastigotes (black bars) or *L. major* amastigotes (white bars). Extracellular parasites were removed 3 hours post infection and samples Diff QUIK® stained. Infection rates were evaluated by counting more than 200 cells. Depicted are the mean percentages ± SEM of inhibition compared to medium controls of 5 independent experiments. Statistical analysis was performed by two-tailed, paired T-Test (* = $p < 0.05$).

Summary Phagocytosis versus endocytosis:

The final host cells of *Leishmania major* parasites are the MΦ. These phagocytes take up the different stages of *L. major* parasites by different mechanisms. Whereas blocking of phagocytosis reduced *L. major* promastigote infections, amastigote infection rates were primarily decreased when endocytotic pathways were inhibited. I compared a set of receptors involved in phagocytosis and endocytosis. Results are summarised in Table 3.5.1

Receptor	MΦ I		MΦ II	
Leishmania stage	promastigotes	amastigotes	promastigotes	amastigotes
CR1	—	↓	↓	—
CR3	—	↓	—	↓
C-Type Lectins	↓	↓	↓	↓
SR-A	↓	—	↓	↓

Table 3.5.1 Role of distinct receptors on the infection with *L. major*.

4. DISCUSSION

When looking into infection experiments one has always to be aware of the system he or she works in. As Fang put it so correctly "Man is not a mouse" (Fang and Nathan, 2007) and type I MΦ are not type II MΦ as well as *L. major* promastigotes are not amastigotes. It is always difficult to transfer observations from on system to another and back again. Here I succeeded in transferring the Trojan horse model from *in vitro* infections in human MΦ to *in vivo* experiments in mice. I could show that *Leishmania* strains obviously have adopted different mechanisms to silence host phagocytes. Where *L. amazonensis* amastigotes, dividing in one giant parasitophorous vacuole, have adopted to mimic apoptosis (de Freitas Balanco et al., 2001) I could demonstrate here that *L. major* as well as *L donovani* amastigotes, residing in single vacuoles inside their host cells, contain virulent, viable as well as a-virulent, dead amastigotes. I could show that *L. major* promastigotes and amastigotes have different virulence for human PMN and MΦ. I demonstrated that in mice models, the, for human MΦ, highly virulent axenic amastigotes, do not induce disease development faster then stationary-phase promastigotes. I showed that promastigotes are mainly phagocytosed by MΦ while amastigote uptake involves endocytotic pathways. Moreover I could demonstrate that different MΦ subset use different receptors to recognise and engulf *L. major* pro- and amastigotes. Therefore, it is very important to clearly define the experimental system used to obtain the presented results. Using different subsets of MΦ might result in controversial experimental results.

4.1 Generation of transgenic *Leishmania major* strains

Essential for the parasites survival inside the hostile environment of host phagocytes is the silencing of effector functions of PMN and MΦ alike. I could show that depletion of PS-positive *L. major* promastigotes from the virulent inoculum leads to clearing of the parasites *in vitro* and *in vivo* (van Zandbergen et al., 2006). Therefore I wanted to generate a *Leishmania major* strain that could not produce PS. Initial database researches in the GeneDB of the Sanger Institute showed one putative gene (LmjF14.1200) to have high ($E = 3.7 \times 10^{-47}$) homology to human PSS-2. Considering that *Leishmania* genes are transcribed in a polycistronic fashion (Stein et al., 1990; Stiles et al., 1999) and lack consensus elements characteristic for eukaryotic gene regulation, I generated a gene cassette suited for targeted gene deletion of PSS gene in *L. major*. After transfection of *L. major* promastigotes with linearised DNA of the gene cassette, I were not successful to recover viable parasites. Though

PS is a quantitatively minor phospholipid in eukaryotic cells comprising between 2 - 10% of total phospholipids (Vance and Steenbergen, 2005)), it is apparently essential for the generation of intact plasma membranes and viability. Others observed that depletion of predicted phosphatidylserine synthase (PPS1) in fission yeast, *Schizosaccharomyces pombe*, resulted in severe defects in cell growth and morphology. They could show that cytokinesis, actin cytoskeletal organisation and integrity was disrupted. Moreover stationary-phase survival or *pss1*Δ mutants perished (Matsuo et al., 2007). Interestingly in budding yeast, *Saccharomyces cerevisiae* deletion of PS synthase gene (*cho1*) resulted in growth defective phenotypes that could be rescued by the addition of either ethanolamine or choline. Moreover, in contrast to *S. pompe* deletion mutants, *S. cerevisiae cho1*Δ mutants had no obvious defects in cell morphology, cytokinesis or cell wall integrity (Letts et al., 1983). In addition, PS is a co-factor for PKC, an enzyme important in signal transduction, in eukaryotes (Epand et al., 1998). Though it has been reported that other anionic phospholipids can replace PS (Lee and Bell, 1992; Toker et al., 1994), it might very well be that in *L. major* even the loss of one allele of the PSS gene resulted in lethal phenotype. Others faced similar problems in generating a knockout strain of *Toxoplasma gondii*. They circumvented the problem by introducing an anhydrotetracycline (ATC)-responsive transactivator protein into toxoplasma and subsequently transfected with their target gene under the control of a conditional promoter (Mital et al., 2005). These mutants were than transfected with a knockout construct disrupting the endogenous target gene. Next ATC was added to the medium leading to inactivation of the introduced target gene. This resulted in a null-mutant of their chosen target gene (Mital et al., 2005; Meissner et al., 2005). In order to apply this strategy on *Leishmania*, one has to take into account that Toxoplasma are haploid organisms whereas *Leishmania* are diploid organisms. This necessitates two rounds of homologous gene replacement to eliminate endogenous gene transcription. At the same time, I did not succeed in generating a viable heterozygote $pss^{-/+}$ *L. major* clone, making it possible that a conditional knockout does not work for that PSS gene in *L. major*.

In addition, I transfected promastigotes with the *Leishmania* expression vector pxGNeo-mAnxA5. I could isolate mAnxA5 DNA from transfectants (Fig. 3.1.7). FACS analysis revealed that at stationary-phase 10 % of the culture could be stained by AnxA5-fitc. At the same time, 40 % of the respective wildtype culture was AnxA5-FITC positive (Fig. 3.1.7). This indicates that endogenous expression of mAnxA5 competitively binds PS on the surface of *L. major* promastigotes, thereby inhibiting binding of AnxA5-fitc. Further characterisation of theses clones need to establish if functional mAnxA5 protein is secreted into the cytoplasm

as well as into the cell culture supernatant. The growth pattern needs to be analysed to see whether these clones grow similar to wildtype parasites. Then infection assays would need to show to what degree shielding PS by endogenous expression of AnxA5 results in clearing of *L. major* parasites by PMN and MΦ. A drawback of this endogenous expression vector is that mAnxA5 is transiently integrated into *L. major*, not stably integrated in the genome. Others (Paape et al., 2008) and I observed that removing selective pressure resulted in the loss of the expression vector. Integration of the AnxA5 gene into the pssu-int vector constructed by Mislitz et al. could be used to generate a stably AnxA5-expressing transfectant with the drawback, that upon cell death in *Leishmania*, kinetoplast integrity is lost (Lee et al., 2002; Arnoult et al., 2002a) and transcription halted. Therefore, viable parasites would possibly be void of free PS but dead parasites still express PS on the outer surface of the cell membrane. To what extend the secreted amount of AnxA5 would suffice to shield externalised PS on dead *L. major* needs to be analysed.

Moreover, I succeeded in generating stably expressing eGFP and dsRED positive *Leishmania*. FACS analysis revealed that at logarithmic-phase all of these parasites express either eGFP or dsRED. At stationary-phase growth about 50% of the cultures lost their endogenous fluorescence. Staining these promastigotes with AnxA-FITC revealed that PS-positive parasites did not produce endogenous eGFP or dsRED anylonger. The pssu-int constructs used for stable gene integration targeted at the small ribosomal RNA subunit of kinetoplast DNA (Misslitz et al., 2000). Upon cell death kinetoplast membrane potential is lost and the kinetoplast disintegrates (Arnoult et al., 2002a) causing transcription and translation to halt. The remaining proteins might still be intact, since the half-life of eGFP and dsRED is between 2 and 5 hours. This might explain why FITC and dsRED positive parasites can be detected by FACS (Fig. 3.1.5 (E) upper right). As has been described these parasites enable direct analysis of intracellular promastigotes and amastigotes and assessment of infection rates (Paape et al., 2008; Misslitz et al., 2000). In addition these parasites will enable us to determine the ratio of viable and dead parasites during uptake and intracellular development. Comparing infection rates obtained by FACS analysis with data obtained by evaluating Diff QUIK® stained cytospin slides of the same samples, I found that FACS analysis resulted in lower infection rates as was counted in cytospins (Data not shown). Isolating the intracellular amastigotes from these samples revealed in subsequent FACS analysis that about 35 % of the cells were AnxA5 positive, dsRED negative (Data not shown). The discrepancy can be explained by the fact that Diff QUIK® stains all cells regardless if they are apoptotic or not.

4.2 The role of PMN in *Leishmania* infection

Previous studies demonstrated that *L. major* secrete a lipid chemotactic factor (LCF), that is a potent chemoattractant for PMN. It was shown that LCF signals through the Lipoxin A4 receptor ALX (van Zandbergen et al., 2002; Wenzel and van Zandbergen, 2009). It was demonstrated that stimulation of ALX resulted in accelerated phagocytosis of *L. major* promastigotes. Furthermore, an increase in parasite survival was detected. This indicates that stimulation of ALX resulted in a nonphlogistic uptake of *L. major* promastigotes. Signaling through ALX results in either pro- or anti-inflammatory reactions (El Kebir et al., 2008). It was shown that LxA4-ALX interactions on PMN resulted in inhibition of superoxide generation and IL-8 secretion, thereby silencing the PMN to the benefit of the intracellular parasites. Moreover, LxA4 is involved in the resolution of inflammation during chronic *Toxoplasma gondii* infection. *T. gondii* was shown to induce LxA4 production, which resulted in decreased IL-12 secretion of DC (Aliberti et al., 2002). Moreover, it was observed that *L. major* induced the production of LxA4 in PMN (van Zandbergen, unpublished). It was demonstrated that *L. major* infect and prolong the short lifespan of PMN (Aga et al., 2002) enabling infiltration of MΦ to the site of infection. Furthermore, LxA4 was shown to be a chemoattractant for MΦ and mediating nonphlogistic phagocytosis of apoptotic PMN (Godson et al., 2000). The rapid clearance of infected apoptotic PMN resulted in an anti-inflammatory environment beneficial for the intracellular survival of *Leishmania*.

Earlier findings showed that PMN can serve as Trojan Horses for *Leishmania* by transmitting intracellular virulent *L. major* to MΦ, *in vitro* (van Zandbergen et al., 2004). Only recently it was shown *in vivo* that PMN are the initial host cells for *L. major* promastigotes (Peters et al., 2008). Using *ex vivo* isolated, infected PMN as well as *in vitro* infected conditional ER HoxB8 PMN I succeeded inducing disease development in Balb/c but not in C57BL/6 mice. Though upon injection many *L. major* promastigotes are lysed by complement, transmission of PMN infected with *L. major* did not induce a faster lesion formation. ER HoxB8 are C57BL/6 derived PMN. It was shown that apoptotic PMN from C57BL/6 mice induce a pro-inflammatory response resulting in a potent host defence (Ribeiro-Gomes et al., 2004). Moreover it was shown that depletion of PMN in resistant C57BL/6 mice resulted initially in disease development that healed 35 days after inoculation (Lima et al., 1998) whereas depletion of PMN in Balb/c resulted in healing phenotype (Tacchini-Cottier et al., 2000). Therefore the PMN seem to be central to the response towards *Leishmania* infection (Charmoy et al., 2007). It was demonstrated that C57BL/6 PMN secrete the anti-

inflammatory cytokine IL-10 that could contribute to a suppression of inflammatory responses at the site of infection in C57BL/6 mice (Charmoy et al., 2007). Here I transmitted allogene PMN from C57BL/6 into Balb/c mice. This might explain why the lesion formation is not as strong as in mice infected with *L. major* promastigotes alone. The exact reasons explaining the less severe lesion formation observed needs to be analysed in future studies. For that reasons, the development of Balb/c derived ER HoxB8 PMN would be useful. Avoiding possible allogene reaction, the role of PMN in disease development could be analysed further. Moreover, the comparison of C57BL/6 and Balb/c PMN with each other in *L. major* infection studies could give an insight into the role PMN have in polarising the early immune response towards this intracellular parasite.

4.3 Cell death of *L. major* and *L. donovani*

The necessity of PS-positive, dead parasites in the virulent inoculum of *L. major* promastigotes has been demonstrated (van Zandbergen et al., 2006). Inside MΦ these promastigotes transform into amastigotes. As amastigotes, *Leishmania* start to replicate intracellular and subsequently propagates to surrounding phagocytes.

Here I demonstrated that *L. major* amastigotes isolated from lesions from infected mice or from infected human MΦ contain both viable and dead parasites. I could show morphologically that lesion derived amastigotes can be differentiated into intact and not intact parasites. The not intact parasites have characteristics of apoptotic cells like condensed nucleus (Fig. 3.2.1 C) and lack of typical intraparasitic granular-like structures. Moreover, I could show that part of the isolated amastigotes become TUNEL positive, meaning that these cells have fragmented DNA, which is a hallmark of late apoptosis. I could show that 40 % of all MΦ-derived amastigotes were PS-positive. In order to exclude that the observed hallmarks of apoptotic cells are artefacts of the isolation from tissue or cells, I analysed the occurrence of PS-externalisation and DNA fragmentation on *L. donovani* amastigote cultures. I found after 4 days of culture a disproportionate fraction (80 %) of *in vitro* generated *L. donovani* amastigotes to be PS-positive. TUNEL staining of these amastigotes revealed that 50% of the cultured parasites were dead. I think that this discrepancy could be the result of the presence of dead promastigotes in the samples. Parasite analysis revealed the presence flagella remnants (See 3.2.3 (C) and (E)). The use of PS as a silencing molecule of host MΦ by intracellular *L. amazonensis* amastigotes was discussed earlier (de Freitas Balanco et al., 2001). He and co-workers isolated lesion derived amastigotes and found 1 population of PS-positive parasites. De Freitas Balanco could show that blocking PS on the surface of

amastigotes with AnxA5 reduced the infectivity on MΦ and reduced the amount of TGF-β produced by the phagocytes. Moreover, Wanderley correlated infectivity and intracellular survival of *L. amazonensis* amastigotes with the amount of PS expressed on the surface of the cells (Wanderley et al., 2006). Apparently all intracellular *L. amazonensis* amastigotes became PS positive and remained virulent, meaning that at least part of the isolated amastigotes was still alive. A quantification of dead parasites by TUNEL staining was not performed in these studies. I could show that *L. donovani* as well as *L. major* promastigotes and amastigotes externalise PS in correlation with DNA fragmentation. Therefore, I found 2 distinct populations one PS-, TUNEL-positive, dead population and one PS-, TUNEL-negative, viable population. Therefore, I suggest that programmed cell death combined with subsequent PS externalisation are natural occurring steps of the survival strategy of these *Leishmania* species.

4.4 Characterisation of *L. major* amastigotes

For decades it has been tried to culture *L. major* amastigotes *in vitro*. Hitherto, all attempts have failed because relapsing *L. major* promastigotes contaminated the cultures at all times. Therefore, it was assumed that it would not be possible to culture *L. major* amastigotes *in vitro*. In the current study I succeeded to develope a culture system to generate *L. major* amastigotes. The chosen culture medium and culture conditions reflected the conditions amastigotes would encounter within the parasitophorous compartments in MΦ *in vitro* and *in vivo*. After incubation for prolonged time the *L. major* promastigotes adapted an amastigote-like phenotype. Fine-structure analysis by EM and SEM revealed that these parasites have the characteristics of lesion-derived amastigotes. In addition these parasites could be transformed back from the amastigote to the promastigote stage and into the amastigotes stage again. The change of phenotype from promastigote to axenic amastigote correlated with a change of gene-expression. Though gene expression analysis on stage specific genes showed that *L. major* mainly have a constitutive genome, 273 stage-specific genes could be identified (Cohen-Freue et al., 2007; Leifso et al., 2007). I found the expression of the metacyclic promastigote marker gene SHERP (Knuepfer et al., 2001) to be reduced in our axenic amastigotes and at the same time gene expression of the putative ABC transporter was increased. I found similar expression profiles from MΦ-derived *L. major* amastigotes. Global gene-expression analysis confirms our expression patterns (Cohen-Freue et al., 2007; Leifso et al., 2007). Therefore, I termed these elliptic cells to be the first axenic *L. major* amastigote culture.

Since the presence of PS-positive *L. major* promastigotes was shown to be important in establishing a successful infection I analysed the surface expression of PS on axenic amastigotes by AnxA5-staining. Suprisingly I could not detect increase in AnxA5-binding parasites in time of culture. Moreover, even in stationary-phase growth where cell division could not be observed any longer, I only detected low percentages of AnxA5-, PS-positive amastigotes. However, addition of apoptotic stimuli such as miltefosine, staurosporine and H2O2 readily induced a state of cell death and AnxA5-positive cells could be found. Therefore, I suggest that the rich culture medium causes the low amount of cell death observed. Looking into *L. major* amastigotes inside MΦ I could detect apoptotic cells (See section 3.2).

To further characterise the axenic amastigotes I analysed PNL binding capacities. I detected higher binding capacities for amastigotes compared to promastigotes. PNL is used to discriminate procyclic from metacyclic promastigotes (Sacks and Da Silva, 1987). It binds to terminal β-linked galactose residues of LPG branches on procyclic or logarithmic-phase promastigotes. These residues are substituted by arabinose on metacyclic promastigotes (McConville et al., 1992; Sacks and Perkins, 1984; Sacks et al., 1990). Since LPG is reported to be almost absent on *L. major* amastigotes (Späth et al., 2000; Späth et al., 2003a), it might be that terminal β-linked galactose residues on the remaining LPG molecules are accessible again and are responsible for the increased binding capacity compared to promastigotes. Moreover, I analysed the surface expression of PPG on *L. major* using the antibody WIC79.3. I found that amastigotes have higher binding capacity for this antibody compared to promastigotes. The specific antibody was used to detect secreted PPG in ELISA Assays (Ilg et al., 1996) and immunoblot analysis (Kavoosi et al., 2008). PPG represent a number of cell surface as well as secreted proteins, modulated by phosphoglycan side-chains similar to those found in LPG (Ilg, 2000b). It could be shown that PPG are protective against complement attack as well as proteases in the midgut of the sand fly (Descoteaux et al., 1995). It was demonstrated that *L. amazonensis* amastigotes secrete PPG modulating the formation of the parasitophorous vacuole (Peters et al., 1997)). Moreover it was shown that PPG are chemoattractant to MΦ. It is reasonable that in the absence of LPG the amastigotes express more PPG than promastigotes. But the antibody WIC79.3 recognises galactose substituted repeat units common to both PPG and LPG (Greenblatt et al., 1983). In an immunoblot one can discriminate PPG and LPG by the size of the two different phosphoglycans but our FACS based analyses could not discriminate different sized molecules on the surface of the

parasites. Therefore further biochemical analyses of the axenic amastigotes need to be done to find special characteristics of these parasite cultures.

With regard to infectivity I found that axenic amastigotes are as virulent as MΦ-derived amastigotes in Balb/c and C57BL/6 mice. Furthermore both types of amastigotes are not as infective as stationary-phase promastigotes in Balb/c mice. This might be explained by the fact that amastigotes are not virulent for PMN. Therefore, the amastigotes cannot use the PMN as a Trojan Horse to silently enter the MΦ and start to replicate in a silenced environment. Moreover, resident MΦ are described as pro-inflamamtory type I MΦ that are very capable in digesting intracellular parasites. This results probably in a significant decrease of amastigotes before they enter a phagocyte that enables them to replicate.

Interestingly primary human PMN could not be infected with *L. major* amastigotes. Previous experiments with *L. donovani* amastigotes revealed the same findings (Data not shown). An explanation might be that PMN were observed to internalise the *Leishmania* promastigotes via their flagellum that obviously is missing on amastigotes. A different explanation is that amastigotes are endocytosed while promastigotes are phagocytosed. Because endocytosis is often linked to nutrient uptake, it might just be that PMN are not capable for all endocytic pathways.

Interestingly I observed a much higher virulence for *L. major* amastigotes compared with promastigotes when I infected human MΦ. A higher infectivity of human MΦ could also be shown as a characteristic for axenic *L. donovani* amastigotes compared to promastigotes (Debrabant et al., 2004). Moreover, I found type I MΦ to be significantly less susceptible for *L. major* pro- and amastigote infection compared to type II MΦ. Type I MΦ have been described as classically activated or pro-inflammatory macrophages, while type II MΦ have been affiliated as regulatory or anti-inflammatory MΦ (Mosser and Edwards, 2008; Xu et al., 2006; Xu et al., 2007). It could be demonstrated that type II MΦ have higher phagocytic activity of apoptotic cells compared to type I MΦ (Xu et al., 2006). Moreover, these type II MΦ secrete IL-10 and TGF-β leading to a silencing of the immune system. It has been demonstrated that MΦ are polarised into type I MΦ in the presence of INF-γ and TNF *in vivo*. Type I MΦ secrete pro-inflammatory cytokines leading to control of acute infections (Shaughnessy and Swanson, 2007). It has been shown that bacteria actively interfere with the polarisation of MΦ. *Yersenia enterocolitica* stimulate a polarisation from type I into type II MΦ (Tumitan et al., 2007). Though many studies have focused on *Leishmania* interactions with MΦ little attention has been paid to the type of MΦ the parasites might infect. Resident tissue MΦ as well as blood monocyte derived MΦ will have different phenotypes and thereby

could be differently susceptible to *Leishmania* infection and intracellular replication. Since type II MΦ have higher capacities of phagocytosing apoptotic cells in a nonphlogistic manner that correlates with the production of IL-10 it is likely that these cells resemble the MΦ infiltrating a site of infection to cause resolution of infection (Mosser and Edwards, 2008). These specifically adapted cells cleare the apoptotic cells they encounter at the site of inflammation. Therefore, it is very likely that these phagocytes are those MΦ that take up infected PMN and are the final host cells of *Leishmania*.

4.5 On the uptake of *L. major* parasites

I analysed different mechanism that might explain the different virulence of *Leishmania* for PMN and MΦ. Blocking actin polymerisation in MΦ by Cytochalsin D revealed that neither *L. major* promastigotes nor amastigotes have mechanisms to actively penetrate the phagocytes plasma membrane, as is described for *Toxoplasma gondii* (Meissner et al., 2005). The differential blockage of phagocytosis, clathrin- and caveolae-mediated endocytosis showed that promastigotes and amastigotes are taken up by different mechanisms. Whereas *L. major* promastigotes are primarily phagocytosed, amastigotes are engulfed by endocytosis. As I could not detect significant differences between clathrin- and caveolae-mediated endocytosis, I must assume that either pathway is interchangeable for the uptake of *L. major* amastigotes. Incubation with both inhibitors for endocytosis showed no additive effect (Data not shown). The used pharmaceuticals to block either pathway are specific and should not cross-react with one another (Ivanov, 2008). The use of caveolae-mediated endocytosis, was described for a broad range of pathogens including *mycobacteria* (Gatfield and Pieters, 2000), simian Virus 40 (Anderson et al., 1996) and *plasmodium falciparum* (Lauer et al., 2000). A clear benefit for the caveolae-mediated endocytosis for these pathogens is that the caveosome not necessarily fuses with lysosomes. Therewith, these pathogens avoid killing inside the acidic compartments so phagolysosoms. In disease, *Leishmania* live and replicate in the acidic compartment of MΦ phagolysosomes (McConville et al., 2007). It has been demonstrated that promastigotes need low pH and high temperature to transform into the amastigote stage (Doyle et al., 1991). Therefore, it is not clear why amastigotes should enter the host MΦ by a mechanism that primarily avoids fusion with the lysosom. Since amastigotes do not need to transform they might not need the lysosom to replicate inside MΦ. Or maybe *L. major* amastigotes circumvent the need to silence the host phagocyte when taken up via endocytosis. But that has to be investigated in future studies. Another possibility could be that amastigotes use signalling properties of caveolea and clathrin coated pits to activate an

accelerated phagocytosis similar to the ALX-dependent uptake of *L. major* promastigotes by PMN (Wenzel and van Zandbergen, 2009). Endocytosis has been shown to be important on cell signalling (Seto et al., 2002). It has been found that caveolae are rich in receptor- and non-receptor protein kinases and GPI-anchored proteins (Simons and Toomre, 2000). Therefore it could be that *L. major* amastigotes can trigger one of these receptors through endoytotic pathways. If pharmaceuticals inhibit either caveolae- or clathrin-mediated endocytosis it might just be that not the invagination process is inhibited but the signalling process that starts the uptake of the parasite.

In the present study I analysed the role of CR1and CR3 as well as SR-A and CD206 on both type I and type II MΦ during binding and uptake of *L. major* pro- or amastigotes. Though the uptake of *Leishmania* by MΦ has been described in great detail, no study, to our knowledge, has compared type I and type II MΦ. During this study I focused on non-opsonised uptake of *L. major* pro- and amastigotes. I found that in the absence of serum pro-inflammatory type I MΦ use CR1 to bind *L. major* amastigotes but not promastigotes. Interestingly, I observed that anti-inflammatory type II MΦ use CR1 signalling to engulf promastigotes not amastigotes. I could show that both type I and type II MΦ use CR3 to take up *L. major* amastigotes but not promastigotes. Since complement proteins are serum components it is interesting that these receptors still play a role in the uptake of *Leishmania* in experiments without normal human serum. It was demonstrated that β-glucan sites of capsular polysaccharides mediate binding to CR3 by non-opsonised Mycobacterium tuberculosis (Cywes et al., 1997). Furthermore, it has been demonstrated that CR3 can bind *L. mexicana* LPG (Talamas-Rohana et al., 1990). Therefore, it might very well be that CR1 and CR3 recognise surface components of *L. major* pro- and amastigotes, since it was shown that in serum independent experiments C3 fragments do not mediate CR3 binding (Da Silva et al., 1989). Because, it has been demonstrated that CR1 alone could not promote receptor internalisation (Wright and Silverstein, 1983) CR1 can be seen as a co-receptor for uptake processes.

Therefore, it is likely that type I and type II MΦ use different receptors like PRR, to take up *L. major* promastigotes or amastigotes under non-opsonised conditions.

The pre-incubation of the MΦ by mannan revealed that mannan-binding receptors like the mannose receptor CD206 are involved in the uptake of *L. major* pro- and amastigotes. Furthermore I could not find a significant difference between type I and type II MΦ for these receptors. Recognising branched polysaccharides with terminal mannose, fucose or N-

acetylglucosamin, CD206 was suggested to bind to mannose residues of the repeating units of *Leishmania* LPG as well as PPG (Turco and Sacks, 1991; Turco, 1992). Infection studies in CD206 deficient mice showed no significant reduction in the infection rate (Akilov et al., 2007). *In vitro* studies showed that CD206 as well as CR3 needed to be in close local vicinity to take up *L. donovani* promastigotes (Blackwell et al., 1985). It is argued that both receptors act sequentially on attachment and subsequent uptake. Interestingly I could not detect a decrease of *L. major* promastigote uptake when CR3 was blocked but treatment with Mannan reduced the percentage infected MΦ significantly. Therefore, recognition and subsequent uptake of *Leishmania* by lectins overweigh the influence of CR3 under non-opsonised experimental set-ups.

Inhibiting SR-A on both subsets of MΦ revealed that type I MΦ apparently use SR-A to a greater extent to phagocytose promastigotes as compared to type II MΦ. These receptors have a broad spectrum of possible ligands and are responsible for the phagocytosis of unopsonised microorganisms, apoptotic bodies as well as environmental dusts by alveolar MΦ (Palecanda and Kobzik, 2001; Peiser et al., 2002). Since I used stationary-phase promastigotes, containing PS-positive cells it could be that blocking of SR-A leads to a decreased uptake of apoptotic *L. major* promastigotes. Since it could be demonstrated that type II MΦ are especially capable to phagocytose apoptotic cells, it is intriguing to speculate that these MΦ have additional or different receptors, like BAI 1, Tim-4 or stabilin-2 to recognise apoptotic *Leishmania* compensating the inhibition of SR-A. FACS analysis comparing surface expression of SR-A, BAI1, Tim-4 and stabilin-2 on type I and type II MΦ, should reveal which receptors are expressed by type I MΦ and type II MΦ. Future experiments using the dsRED or eGFP expressing *L. major* promastigotes will elucidate the degree of apoptotic cells taken up by type I and type II MΦ and if blocking of SR-A or CD206 results in a shift in the ratios of viable and dead parasites engulfed.

Since I could not inhibit uptake of neither pro- nor amastigotes completely, a redundancy for receptors capable in recognition and subsequent uptake of the parasites is most likely. This also would explain why a CD206 knockout mouse is not better protected against *Leishmania* infection. As I could demonstrate pro- and anti-inflammatory MΦ take up different numbers of parasites, it would be worth analysing by FACS different phagocytic and endocytic receptors. As this study demonstrated Leishamnia use different strategies to silent host phagocytes but as well they use stage specific different enty mechanisms. The axenic culture presented here will help to understand the propagation of L. major in host phagocytes better.

5. SUMMARY

Leishmania major (*L. major*) is an obligate intracellular parasite, which misuses the hosts apoptotic cell clearance system for disease development. The parasite enters polymorphonuclear neutrophil granulocytes (PMN) in its promastigote form. Hiding inside apoptotic PS-positive PMN, the parasite transfers into macrophages (MΦ), where it multiplies in its amastigote form. The presence of a dying and phosphatidylserine (PS)-positive population of promastigotes is responsible for the infectivity and survival of viable promastigotes inside PMN. Still relatively little is known about macrophage infection by the amastigote form of *L. major* propagating the disease.

In the present study a transgenic *L. major* strain, endogenously expressing the PS-binding protein AnxA5 was generated. Dying promastigotes of these AnxA5 transfectants contained significantly less free PS on their membrane. The data suggest that leishmania-produced AnxA5 can shield PS on dying *L.major* parasites.

In studies on the role of the lipoxin A4 receptor (ALX) it was demonstrated that the leishmania chemotactic factor (LCF) as well as other ligands use this receptor to increase infectivity of *L. major* promastigotes in PMN. Furthermore, injecting *L. major* infected murine PMN, into Balb/c mice induced disease development in the animals, corroborating the role of parasitized PMN as a Trojan horse for transfer of *Leishmania* to MΦ *in vivo*.

Focussing on *Leishmania* amastigotes it was shown that inside human MΦ *L. major* amastigotes consist of two populations, one viable and one dying PS-positive. This suggests that both amastigotes and promastigotes can use PS-externalisation to silence host phagocytes. A novel *in vitro* culture system for axenic *L. major* amastigotes was developed that made it possible to study stage specific interactions of both promastigotes and amastigotes with their host phagocytes. Axenic amastigotes were shown to be highly infective for MΦ, whereas PMN could not be infected with amastigotes. *L. major* promastigotes are taken up primarily by phagocytic mechanisms, whereas *L. major* amastigote uptake is associated with the endocytotic pathways. A comparative analysis of *L. major* infection using pro-inflammatory or anti-inflammatory MΦ revealed that anti-inflammatory MΦ take up significantly higher numbers of parasites. The different MΦ subsets use different receptors for a stage specific uptake of *leishmania*.

In conclusion, the results of the current study contribute to a more detailed understanding of stage specific interactions of *L. major* pro- and amastigotes with PMN and MΦ. The generated tools will be of help in the detailed analysis of PS as a virulence factor, as well as in future studies on the development of different T cell immune responses during amastigote propagation in infected host cells.

6. ZUSAMMENFASSUNG

Leishmania major (*L. major*) sind obligat intrazelluläre Parasiten, die auf elegante Art und Weise das System zum Entfernen von apoptotischen Zellen für die Krankheitsentwicklung missbrauchen. Als promastigote Form befällt der Parasit polymorphonukleäre Neutrophile Granulozyten (PMN). Der Parasit versteckt sich innerhalb Annexin A5 (AnxA5)- positiven PMN, um unerkannt in Makrophagen (MΦ) übertragen zu werden, in denen sich der Parasit in seiner amastigoten Form teilt. Es konnte gezeigt werden, dass die Gegenwart einer AnxA5-bindenden, promastigoten Population für das Überleben und die Infektiosität der lebenden Promastigoten innerhalb der PMN verantwortlich ist. Bis jetzt ist relative wenig bekannt über die Makrophageninfektion durch die amastigote Form der *L. major* Parasiten, die die Krankheit verbreiten.

In der vorliegenden Arbeit wurde ein transgener *L. major* Stamm, der endogen das PS-Bindende Protein Anxa5 expremiert, generiert. Sterbende Promastigote dieser AnxA5 Transfektanden enthielten erheblich weniger freies PS auf ihrer Membran. Diese Daten weisen darauf hin, dass das endogen produzierte AnxA5 das PS der sterbenden *L. major* abdeckt.

Studien über die Rolle des Lipoxin A4 Rezeptors (ALX) konnten zeigen, dass der Leishmanien Chemotaktische Faktor (LCF), sowie andere Liganden ALX benutzen, die Infektiösität der *L. major* Promastigote in PMN zu steigern. Des Weiteren, konnte durch die Injektion von infizierten murinen PMN eine Krankheitsentwicklung in Balb/c Mäusen induziert werden, wodurch die Rolle der parasitisierten PMN als Trojanisches Pferd zum Transfer von Leishmanien auf MΦ *in vivo* gezeigt wurde.

Im Hinblick auf die *Leishmania* Amastigoten, konnte gezeigt werden, dass *L. major* Amastigote innerhalb von human MΦ aus zwei Populationen, einer lebenden und einer sterbenden, PS-positiven, bestehen. Dieses deutet darauf hin, dass sowohl Amastigote als auch Promastigotes die Externalisierung von PS nutzen können, um die Wirtsphagozyten in ihrer Immunantwort zu drosseln. Ein neuartiges Kultur-System für axenische *L. major* Amastigote wurde entwickelt, welches es ermöglichte, stadien-spezifische Wechselwirkungen zwischen Parasit und Phagozyt zu untersuchen. Dabei zeigte sich, dass axenische Amastigote hoch infektiös sind für MΦ, nicht aber für PMN. Während *L. major* Promastigote hauptsächlich durch Phagozytose aufgenommen werden, ist die Aufnahme der Amastigoten mit Endozytose assoziiert. Eine vergleichende Analyse der *L. major* Infektionen in Pro- oder anti-inflammatorischen MΦ zeigte, dass anti-inflammatorische MΦ signifikant mehr Parasiten

aufnehmen. Außerdem konnte gezeigt werden das beide Typen von MΦ unterschiedliche Rezeptoren benutzen um Leishmanien aufzunehmen.

Zusammenfassend steuern die Ergebnisse der vorliegenden Arbeit dazu bei ein detaillierteres Verständnis der stadien-abhängigen Wechselwirkungen von *L. major* Pro- und Amastigote mit PMN und MΦ zu erlangen. Die entwickelten Werkzeuge werden in der detaillierten Analyse von PS als Virulenzfaktor helfen. Ebenso werden sie in zukünftigen Studien bei der Entwicklung unterschiedlicher T-Zell Immunantworten während der Amastigoten Verbreitung im Wirt hilfreich sein.

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8 LIST OF PUBLICATIONS, TALKS AND POSTERS

Publications

1. **Wenzel, A**., and van Zandbergen, G, 2009. Lipoxin A4 receptor dependent *Leishmania* infection. *Autoimmunity in press*.

2. van Zandbergen, G,., A. Bollinger, **A. Wenzel**, S. Kamhawi, R. Voll, M. Klinger, A. Müller, C. Hölscher, M. Herrmann, D. Sacks, W. Solbach, and T. Laskay. 2006. *Leishmania* disease development depends on the presence of apoptotic promastigotes in the virulent inoculum. *Proc. Natl. Acad. Sci. U. S. A.* 103:13837.

3. Kleczka, B., A. C. Lamerz, van Zandbergen, G,, **A. Wenzel**, R. Gerardy-Schahn, M. Wiese, and F. H. Routier. 2007. Targeted gene deletion of *Leishmania* major UDP-galactopyranose mutase leads to attenuated virulence. *J. Biol. Chem.* 282:10498.

4. Böttcher, K., **A. Wenzel**, and J. M. Warnecke. 2006. Investigation of the origin of extracellular RNA in human cell culture. *Ann. N. Y. Acad. Sci.* 1075:50-6.:50.

Manuscript in preparation:

A. Wenzel, E. Bank, C. Florian, T. Laskay, M. Klinger, N. Reiling, U. Ritter and G. van Zandbergen. Stage specific interactions of Leishmania parasites with human phagocytes.

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3rd Spring School on Immunology, Ettal, Germany February, 26th – March, 1st, 2007 Poster presentation: *Leishmania* disease development depends on the presence of apoptotic parasites

11th Symposium "Infection and Immune Defence" Burg Rothenfels, Germany, March 9th – 11th, 2007
Oral presentation: Do *Leishmania* spp. use apoptotic mimicry or altruistic behaviour for the establishment of a productive infection
37th Annual Meeting of the German Society for Immunology, Heidelberg, Germany, **September 5th – 8th, 2007**
Poster presentation: Cell death regulation controls *Leishmania* disease development

12th Symposium "Infection and Immune Defence" Burg Rothenfels, Germany, March 7th – 9th, 2008
Oral presentation: A Novel *Leishmania major* amastigote culture system

Joint Annual Meeting of Immunology of the Austrian and German Societies (ÖGAI, DGfI), Vienna, Austria September 3rd – 6th, 2008
Oral presentation: A Novel *Leishmania major* amastigote culture system

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10 CURRICULUM VITAE

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