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# *Leishmania major* promastigotes use phosphatidylserine for silencing of polymorphonuclear neutrophils

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# I Introduction

## 1. *Leishmania*

*Leishmania* are obligate intracellular protozoan parasites, named after W. B. Leishman, who developed a detection method for *Leishmania* in 1901. Altogether, 24 different *Leishmania* species are endemic in at least 88 countries (WHO, 2004). A spectrum of diseases is caused by *Leishmania*, which are subsumed under the term leishmaniasis. These range from the self-healing cutaneous leishmaniasis (CL, e.g. caused by *L. major*) to the potentially fatal visceral leishmaniasis (VL, e.g. caused by *L. donovani*) (Pearson and Sousa, 1996). More than 90% of cases are observed in underdeveloped countries. Leishmanial disease currently affects about 12 million people. About two million cases are reported annually and about 350 million people live under the risk of infection (WHO, 2004).

*Leishmania* transmission occurs via the bite of female sandflies (*Phlebotomus* spp., *Lutzomyia* spp., *Psachodopygus* spp.). These are tiny sand-colored blood-feeding flies that breed in forest areas, caves or the burrows of small rodents. The fly inoculates about 100 - 3000 promastigotes into the skin (Warburg and Schlein, 1986). Wild and domesticated animals as well as humans can act as a reservoir of infection. Within the mammalian host the motile promastigotes are ingested by resident or recruited cells of the monocyte/macrophage (MΦ) lineage (Ridley and Ridley, 1986). Intracellularly, the promastigotes develop into non-motile amastigotes. In MΦ, the best studied host cell for *Leishmania*, the parasites multiply until the cell eventually bursts resulting in infection of other phagocytic cells continuing the cycle (Solbach and Laskay, 2000) (fig.1).

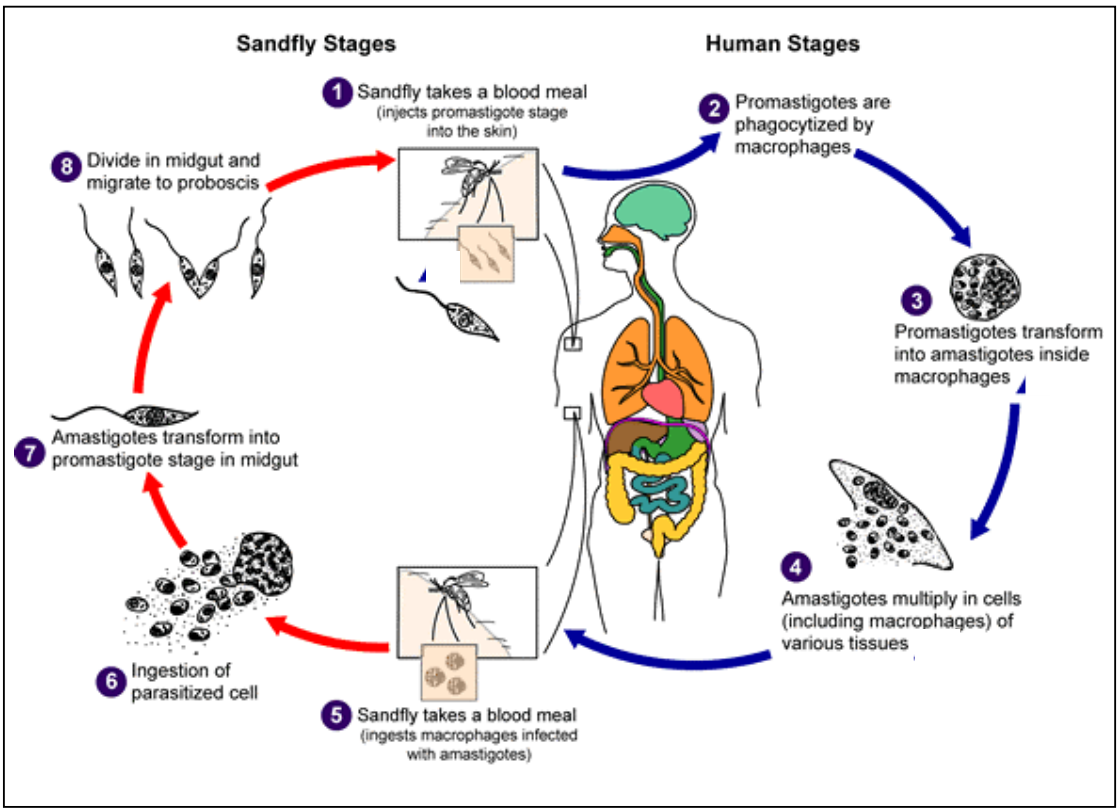


**Fig. 1: *Leishmania* life cycle (adapted from the Center for disease control, 2005)**

## 1.1 *Leishmania* promastigote development and infectivity

In *Leishmania* promastigote *in vitro* cultures, two distinct growth phases can be recognized, a logarithmic and a stationary phase. The logarithmic phase is marked by logarithmic growth during the first days of culture while the stationary phase is reached after seven to nine days of culture. *Leishmania* development from logarithmic to stationary growth phase is accompanied by a continuous increase of their infectivity thus stationary phase cultures are the infective ones. The development of this infective form is also termed metacyclogenesis (Giannini et al., 1974). This process is associated with the cell surface expression of a developmentally regulated glycolipid, the lipophosphoglycan (LPG) (McConville et al., 1992). During metacyclogenesis of so called procyclic (non-infective) parasites to metacyclic (infective) *Leishmania* LPG undergoes modifications where galactosyl side chains become capped with arabinosyl residues. Identical developmental changes of leishmanial LPG were also shown to occur in the sandfly vector (Sacks

and Perkins, 1984; Sacks et al., 1985). Within the sandfly, the LPG of procyclic parasites mediates attachment of the parasite to the midgut epithelium (Pimenta et al., 1992). During metacyclogenesis, the LPG of metacyclic parasites looses its binding capacity so that promastigotes are released for transmission by a blood meal (Sacks et al., 1990; McConville et al., 1995). Metacyclic parasites can be purified from stationary phase cultures by their failure to agglutinate with peanut lectin agglutinin (PNA). PNA can only bind to uncapped galactosyl chains present in LPG of procyclic parasites. Subsequently, non-agglutinated promastigotes are termed metacyclic, while agglutinated parasites are termed procyclic (Sacks et al., 1985).

### 1.2 *Leishmania* and the immune response

Immediately after infection the site of infection is infiltrated by polymorphonuclear neutrophilic granulocytes (PMN) followed by a wave of MΦ two days after infection (Laskay et al., 1997). *Leishmania* secrete a chemotactic factor, the so called *Leishmania* chemotactic factor (LCF), which facilitates PMN recruitment (van Zandbergen et al., 2002). Early after infection, extracellular parasites are exposed to serum components leading to the killing of most parasites by complement mediated lysis (Sacks and Perkins, 1984). Remaining parasites are phagocytosed by the accumulated phagocytes. *In vitro* experiments demonstrated that parasite surface molecules interact with phagocytic receptors on MΦ, such as the mannose-fucose receptor and the fibronectin receptor (Bogdan and Roellinghoff, 1998). Under physiological conditions the complement receptors (CR) 1 (CD35) and CR3 (CD11b/CD18), which bind complement components attached to lipophosphoglycan (LPG), are important for parasite internalisation (Rosenthal et al., 1996). Intracellularly, diverse anti-microbial effector mechanisms including the oxidative burst are responsible to combat *Leishmania*. Moreover, effective protection is correlated with the release of pro-inflammatory cytokines, predominantly INF-γ and IL-12, acting as potent activators of MΦ. In this context, the parasite has developed various mechanisms to modulate the immune defence thus achieving a silencing of their host cell. *Leishmania* can inhibit complement mediated lysis (Hermoso et al., 1991), but also facilitate their uptake via CR3 (Brittingham et al., 1995). An impairment of the oxidative burst is achieved by the parasite membrane component

gp63 (leishmanolysin) (Sorensen et al., 1994) and by LPG that is attributed to inhibit the protein kinase C (PKC) activity (Olivier et al., 1992; Moore et al., 1993; Spaeth et al., 2003). Moreover, there is evidence that *Leishmania* can prevent the induction of inflammatory mediators such as IL-12 and TNF-α (Carrera et al, 1996; Serinkan et al., 2005).

Concerning the adaptive immune response against *Leishmania*, resistance is conferred by a Th1-type immune response, whereas non-healing lesions are associated with a sustained Th2 response (Reiner and Locksley, 1995; Sacks and Noben-Trauth, 2002). Controversially, Anderson and colleagues recently showed that a *L. major* strain from a patient with non-healing lesions also produced non-healing lesions with ulcerations and high parasite burden in resistant C57BL/6 mice. These mice developed a strong, polarized and sustained Th1 response, as evidenced by high levels of INF-γ and the absence of counteracting IL-4 and IL-13. Hence, the Th1/Th2 concept failed to adequately explain the mechanisms underlying this non-healing form of leishmaniasis (Anderson et al., 2005).

Even though MΦ are supposed to be the favoured host cells for *Leishmania*, our research group clearly demonstrated that the parasite uses PMN as a host cell in the early phase of infection (Laufs et al., 2002). The exact interactions between *L. major* and PMN resulting in a productive infection are still unknown.

## 2. Polymorphonuclear neutrophilic granulocytes

Polymorphonuclear neutrophilic granulocytes (PMN) are leukocytes representing the first line of defence of the innate immune system against invading microbes (Witko-Sarsat et al, 2000). PMN are formed in the bone marrow from precursor stem cells. Before they enter the blood stream PMN pass six morphologically different stages (myeloblast, promyeloblast, myelocyte, metamyelocyte, non-segmented neutrophil, segmented polymorphonuclear neutrophlic granulocyte). PMN are permanently produced (up to $10^{11}$ per day in human adults, increasing up to $10^{12}$ during infection), but they are short living cells with a half-life of 6 to 10 hours before they undergo spontaneous apoptosis. In the blood stream, they account for 50% to 70% of the peripheral blood leukocytes. Circulating PMN bear chemokine receptors, which

enable them to migrate toward the site of infection. Among others the chemokine interleukin-8 (IL-8) serves as a potent chemoattractant for PMN (Premack and Schall, 1996). Upon PMN activation by chemoattractans their recruitment to the site of infection occurs in three steps: a rolling step, a tethering step and a subsequent diapedesis step. Reaching the inflamed tissue PMN can finally interact with infectious agents (Vaporciyan et al., 1993; Witko-Sarsat et al, 2000).

PMN are professional phagocytes that can phagocytose invading microorganisms including bacteria, fungi and protozoa. Microbial recognition occurs via common pathogen-associated molecular pattern (PAMP) and pathogen recognition receptors (PRR) present on phagocytes. Microbial phagocytosis by PMN can take place via two functional receptor classes. First, the Fc-receptors: FcγRI (CD64), FcγRII (CD32), FcγRIII (CD16) (Witko-Sarsat et al., 2000) and FcαR (CD89) (van Spriel et al., 1999). These receptors mediate phagocytosis if the microbe is opsonized with antibodies. The second uptake pathway is mediated through the complement receptors (CR)1 (CD35) and CR3 (CD11b/CD18) if the microbe is opsonized with complement components, such as C3b, which function as an adaptor between the microbe and the complement receptors (Sutterwala et al., 1996; Rosenthal et al., 1996). CR3 and FCγRII were shown to be functional for phagocytosis, whereas CR1 and FcγRIII mostly appear as co-receptors, which facilitate the function of CR3 and FCγRII (Witko-Sarsat et al., 2000).

After engulfment by PMN microbes are located in phagosomal compartments (Witko-Sarsat et al., 2000). Within minutes these so called phagosomes undergo remodelling and fusion with lysosomes, which contain anti-microbial proteins, peptides and enzymes, such as lysosomal hydrolases, cathepsins and glucuronidases (Lang et al., 1994). In the formed phagolysosome also the oxidative burst takes place. This process consists of the NADPH dependent production of radical oxygen species including superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$), hypochlorous acid (HOCl) and chloramines (Witko-Sarsat et al., 2000). Additional enhancement of the microbicidal activity is achieved by the degranulation of endocytic granules into the phagolysosome and the extracellular medium (Elsbach, 1998). Altogether, these mechanisms can lead to efficient degradation of internalised microbes by PMN.

The fact that *L. major* survive in PMN (Laufs et al., 2002) suggests that the parasite is able to silence the anti-microbial PMN effector functions. The mechanisms of PMN silencing are still unclear.

## 3. Apoptosis

The process of apoptosis as the opposing force of mitosis and proliferation is absolutely essential for embryonic morphogenesis and for the daily control of tissue homeostasis in every adult multicellular organism (Strasser et al., 2000). During apoptosis typical morphological changes occur. The early sign of apoptosis is the externalisation of phosphatidylserine (PS) from the inner to the outer leaflet of the cell membrane (Martin et al., 1995). At the same time a cascade of proteolytic enzymes termed caspases are activated. This results in proteolytic breakdown of the cell content, loss of membrane potential and cell shrinkage (Kerr et al., 1972; Kluck et al., 1997). The DNA becomes fragmentated and consequently nuclear condensation occurs in the late stage of apoptosis (Wyllie, 1980).

Apoptosis can be initiated by two different mechanisms that usually operate together and amplify each other. One is triggered by stimulation of receptors on the cell surface, such as CD95, through binding of death activators, such as CD95-ligand (CD95L). These mechanisms are termed "extrinsic trigger". Intracellularly, the signalling is mediated through an adapter protein following activation of various caspases, in particular caspase-3, -6 and -8, finally leading to apoptosis. The second mechanism is generated by signals arising within the cell and is called "intrinsic trigger". In this case, numerous cell damage pathways, e.g. UV-irradiation, converge on mitochondria and induce the release of mitochondrial proteins, such as cytochrom-c, subsequently resulting in apoptosis (Yang et al., 1997; Ferri and Kroemer, 2001). In contrast to necrosis, the plasma membrane of the dying PMN remains fully intact throughout the entire apoptotic process. Thus, the release of the cell content leading to inflammation is prevented (Dong et al., 1997).

PMN enter spontaneous apoptosis. This process is accompanied by the loss of functional properties (Haslett, 1997). However, in the presence of growth factors or pro-inflammatory agents the life span of PMN is extended (Cox et al., 1992; Colotta

et al., 1992; Lee et al., 1993; Kettritz et al., 1998). Infection with *L. major* also extends the life span of PMN. Infected PMN become apoptotic about two days after infection (Laufs et al., 2002; Aga et al., 2002). During this extended life span infected PMN release chemoattractans, such as MIP-1β, which drive the recruitment of macrophages to the site of infection (van Zandbergen et al., 2004). Subsequent apoptosis of infected PMN is accompanied by the externalisation of PS to the outer leaflet of the membrane thus allowing a silent removal by macrophages (Laskay et al., 2003).

## 4. Silent phagocytosis of apoptotic cells

Silent phagocytosis is generally known from the uptake of apoptotic cells by macrophages (MΦ) (Voll et al., 1997). To achieve the removal of apoptotic cells the dying cell releases a chemotactic signal, a so called “find-me” signal, which induces attraction of phagocytic cells. A potential candidate mediating phagocyte attraction is the phospholipid lysophosphatidylcholine (LPC) (Lauber et al., 2003, 2004). Subsequently, interaction between the phagocyte and the apoptotic cell is mediated by a wide range of surface molecules on the apoptotic cell. These molecules are also termed “eat-me” signals. These signals can orchestrate with corresponding receptors on MΦ representing a complex communication platform. An overview about the so called “phagocytosis synapse” is given by fig. 2.

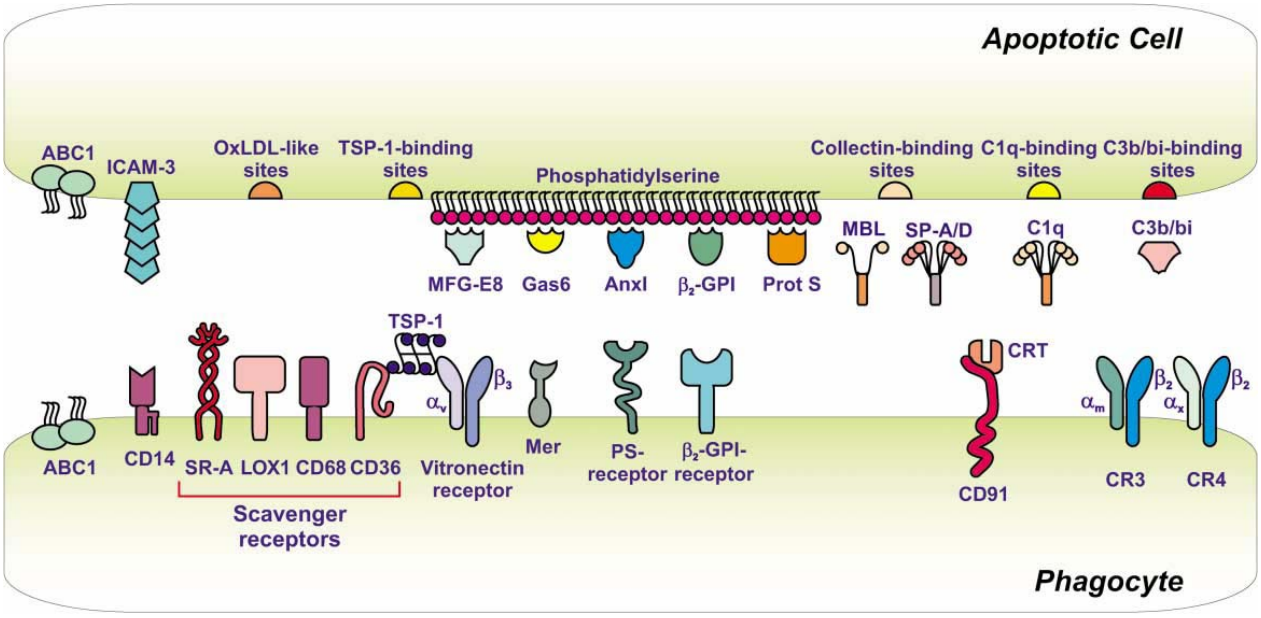


**Fig. 2: The phagocytosis synapse.** The apoptotic cell displays various eat-me signals that are recognized directly or indirectly via different bridging molecules by diverse phagocyte receptors. ABC1, ATP binding-cassette-transporter 1; AnxI, Annexin-I; ß2-GPI, ß2-glycoprotein-I; C1q, complement protein C1q; C3b/bi, complement protein C3b/bi; CD14, lipopolysaccharide receptor CD14; CD91, calreticulin/heat shock protein receptor; CR3, complement receptor 3; CR4, complement receptor 4; CRT, calreticulin; Gas6, growth arrest-specific 6; ICAM-3, intercellular adhesion molecule 3; LOX1, lectin-like oxidized low-density lipoprotein particle receptor 1; MBL, mannose binding lectin; Mer, receptor-tyrosin-kinase (expressed on monocytes and tissues of epithelial and reproductive origin); MFG-E8, milk-fat-globule-EGF-factor 8; OxLDL, oxidized low-density lipoprotein particle; Prot S, protein S; PS-receptor, phosphatidylserine receptor; SP-A/D, lung surfactant protein A or D; SR-A, class A macrophage scavenger receptor; TSP-1, thrombospondin-1 (adapted from Lauber et al., 2004).

Among these signals binding sites for collectins, the complement components C1q- and C3b/3bi-, trombospondin (TSP)-1, ATP-binding-cassette-transporter (ABC) 1, intracellular adhesion molecule (ICAM)-3 and also oxidized low-density lipoprotein particle (OxLDL)-like sites are involved in recognition. The mentioned find-me signal LPC also possess eat-me signalling properties due to its recognition by naturally occurring IgM antibodies (Kim et al., 2002). The most central eat-me signal known so far is the phospholipid phosphatidylserine (PS) (Fadok et al., 1992; Marguett et al., 1999; Callahan et al., 2000; Williamson et al., 2001).

The appropriate counterpart receptors for the recognition of eat-me signals include the scavanger receptor CD36 and the vitronectin receptor, which cooperatively bind

to TSP-1. Furthermore, complement receptors, additional scavanger receptors (CD68, LOX-1, SR-A), the heat shock protein receptor CD91 (Vandivier et al., 2002), CD14 (Devitt et al., 1998; Schlegel et al., 1999) and ABC1 also play a role in uptake. Nevertheless, the most important receptors for phagocytosis of apoptotic cells are receptors that interact either direct or indirect with PS.

Next to eat-me signals also “do not eat-me” signals exist. Brown and colleagues could demonstrate that expression of CD31 (adhesion receptor with signalling function) on both viable cells and phagocytes mediates active repulsion. During apoptosis the signalling of CD31 is disabled so that the apoptotic cell does not reject the phagocyte anymore (Brown et al., 2002).

The detailed signalling pathway resulting in cytosceletal rearrangements of the phagocyte, thus leading to the internalization of apoptotic cells, is largely unknown. In humans the transmembrane proteins CD91 and ABC1 are either directly or indirectly involved in corps recognition. Subsequent downstream signalling is mediated by the adaptor protein GULP, which interacts with CD91 (Moynault et al., 1998; Su et al., 2002). Moreover, a second signaling cassette was described consisting of so called CrkII, Dock180, Rac, and ELMO (Gumienny et al., 2001; Reddien and Horvitz, 2000; Wu and Horvitz, 1998; Zhou et al., 2001). However, it is still unclear which upstream receptor is involved in the activation of this signalling cassette. There is growing evidence that a PS binding receptor plays a key role (Hoffmann et al., 2001).

The conventional function of MΦ is clearance of apoptotic cells. Toward apoptotic cells MΦ do not respond with a full blown activation of their anti-microbial machinery. The uptake of apoptotic cells is characterized by an increased production of anti-inflammatory TGF-β, IL-10 and prostaglandine-2 and a decreased secretion of pro-inflammatory TNF-α, IL-1 and IL-12 by the phagocytosing MΦ (Savill et al., 1993; Voll et al., 1997; Fadok et al., 1998, 2000, 2001; Savill and Fadok, 2000; Huynh et al., 2002). Recently it was demonstrated in our laboratory that PMN are also capable to phagocytose apoptotic cells (Esmann et al., unpublished data). During infection PMN secrete TNF-α, which in turn enhances the expression of endothelial cell adhesion molecules and subsequently promotes PMN adherence to vascular endothelium. Additionally, TNF-α primes PMN for phagocytosis, degranulation and oxidative response (Witko-Sarsat et al, 2000). Moreover, PMN are able to produce TGF-β (Grotendorst et al., 1989). TGF-ß is synthesized in a latent form that must be

activated to be recognized by cell surface receptors and to trigger biological responses (Massagué et al., 1990). TGF-β acts in an immunosuppressive manner by down-regulating the production of pro-inflammatory mediators (Mac-Kay and Danielpour, 1991; Massagué et al. 1992). Whether the ingestion of apoptotic cells influences the capacity of PMN to release TNF-α or TGF-β1 is still not known.

The PS dependent up-regulation of TGF-β release and down-regulation of TNF-α secretion is a characteristic feature occuring during the silent uptake of apoptotic cells by MΦ (Fadok et al., 1998, 2000; McDonald et al., 1999, Serinkan et al., 2005). Hence, the recognition of PS by a PS binding receptor is the central point mediating immunosilencing properties.

## 5. Phosphatidylserine

### 5.1 Structure and distribution

Phosphatidylserine (PS), or 1, 2-diacyl-*sn*-glycero-3-phospho-L-serine, belongs to the family of phospholipids. Structurally, PS is characterized by anionic and ionizable groups, such as the phosphate moiety, the amino group and the carboxyl function, which presents a negative charge (fig. 3).

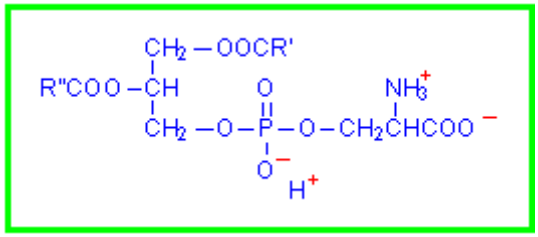


**Fig. 3: Structure of phosphatidylserine**

PS is an essential constituent of the lipid bilayer of virtually every cell membrane (in animals, plants and microorganisms) (Schlegel and Williamsen, 2001). Additionally, PS is also present in nuclear membranes, mitochondria and the endoplasmatic reticulum (ER). The ER is the organelle where PS is synthesized by a pair of enzymes, phosphatidylserine synthase (PSS) 1 (Voelker and Frazier, 1986; Kuge et al., 1997) and PSS 2 (Kuge et al., 1991, 1997). Both enzymes generate PS by

catalysing the exchange of serine for the head group of another phospholipid. PSS1 uses phosphatidylcholine (Voelker and Franzier, 1986; Kuge et al., 1986, 1991), whereas PSS2 uses phosphatidylethanolamine as a substrate (Kuge et al., 1997; Stone and Vance, 1999; Saito et al., 1998).

In living cells PS is mostly restricted to the inner leaflet of the plasma membrane so that its distribution across the bilayer is asymmetrical (Williamson and Schlegel, 1994). An enzyme activity, termed scramblase, acts to randomise the lipids between the two leaflets of the plasma membrane bringing PS to the surface. This process is ATP-independent and driven by the concentration gradient across the bilayer (Verhoven et al., 1992, 1995; Williamson et al., 1994; Smeets et al., 1994). The asymmetry is achieved by a P-type ATPase called the aminophospholipid translocase (APLT). This enzyme transports PS from the outer to the inner leaflet (Seigneuret and Devaux, 1984; Zachowski et al., 1987; Tang et al., 1996). During apoptosis, the translocase becomes inactive, whereas the scramblase keeps functional. Thus, PS appears and remains on the cell surface. Consequently, the asymmetrical PS distribution is lost and the so called membrane “flip-flop” occurs (Verhoven et al., 1995; Bratton et al., 1997). PS externalisation can be prevented by the broad spectrum caspase inhibitor zVAD-fmk, but also zVAD-fmk in-sensitive pathways do exist (Verhoven et al., 1999). Moreover, the ATP binding cassette transporter ABC1, which is present on both apoptotic cells and phagocytes, seems to be responsible for PS externalisation on apoptotic cells and on phagocytes (Hamon et al., 2000).

Externalized PS is negatively charged due to an oxidation process. Oxidated PS is recognized by different proteins, such as C-reactive protein (CRP) and annexin-V (AV) (Kagan et al., 2003). AV is a protein, which belongs to a highly conserved protein family present in plants, invertebrates and vertebrates (Crumpton and Dedman, 1990). Annexins all share a conserved core domain consisting of four or eight homologous repeats with phospholipid- and $Ca^{2+}$ binding sites. In this context, $Ca^{2+}$ binding is essential for efficient binding of AV to phospholipids (Raynal and Pollars, 1994). AV is involved in intra- and extracellular processes including blood coagulation, signal transduction, membrane trafficking, ion channel activity and anti-inflammatory processes (Raynal and Pollars, 1994; Liemann et al., 1995; van Heerde et al., 1995), but its exact biological function remains unknown. AV preferentially binds to PS (Andree et al., 1990; Gerke and Moss, 2002). In addition, a monoclonal

antibody to oxidized PS recently became available that can be used to detect PS on apoptotic cells (Biomol GmbH, Hamburg).

### 5.2 Recognition of phosphatidylserine

The first experimental evidence for the existence of a PS receptor came from Dr. Fadok and co-workers (Fadok et al., 2000). Their approach began with the production of monoclonal antibodies against “stimulated” MΦ. The recognition of apoptotic cells by these stimulated MΦ was inhibited by PS vesicles in a stereospecific fashion (Fadok et al., 1992). In contrast, the uptake of PS expressing target cells by “not stimulated” MΦ was insensitive to PS vesicles. One monoclonal antibody (mAb 217) was selected because it bound preferentially to stimulated MΦ, and this binding was inhibited by PS vesicles. The antigenic target of mAb 217 would thus appear to have the hallmarks of a PS receptor: it is on the cell surface, it recognizes PS and, as the authors showed, mAb 217 blocks the engulfment of apoptotic cells by MΦ (Fadok et al., 2000; Williamson and Schlegel, 2004). Moreover, the mAb 217 was shown to prevent PS mediated down-regulation of inflammatory responses. In western blot analysis, the mAb 217 revealed a protein of ~70 kDa and its deglycosylation resulted in a shift from ~ 70 to ~ 47 kDa (Fadok et al., 2000; Hoffmann et al., 2005). So far, these data seem to confirm that the appropriate gene found through mAb 217 is indeed responsible for the expression of the suggested PS receptor.

A recent study demonstrated that the protein encoded by the cDNA of this PS receptor candidate is localized in the nucleus. This finding led to the suggestion, that this PS receptor candidate may serve a dual role both on the cell surface and in the nucleus (Cui et al., 2004). More insight was obtained by the use of knockout studies. A study using knockout mice for the assumed PS receptor encoding gene by Boese and colleagues showed perinatal lethality. Fetal liver-derived MΦ of PS receptor -/- mice were generated from embryos at E12.5 and E13.5. Using these cells the authors demonstrated that the suggested PS receptor gene is essential for the regulation of MΦ cytokine response, but the clearance of apoptotic cells was unaffected by the lack of this PS receptor (Boese et al., 2004). These data demonstrated that the gene detected by Dr. Fadok and colleages does not encode a

PS receptor. The gene rather appears to encode a nuclear protein that plays a role in development and differentiation (Boese et al., 2004).

Even though the PS recognizing receptor remains unidentified the important result of its interaction with PS is the creation of an anti-inflammatory milieu in MΦ. Whether PS exposure to PMN can influence their cytokine production is still unknown.

## 6. The apoptotic hallmark phosphatidylserine as immune evasion targets for *Leishmania*

Various single-celled eukaryotes express the apoptotic marker phosphatidylserine (PS) on the outer leaflet of their cell membranes. For parasites, the recognition of their externalised PS can be useful in different ways. For example, PS isolated from *Schistosoma mansoni* is recognized via toll like receptor (TLR)-2 on dendritic cells (DC). There, it polarizes the maturation of DC's resulting in a Th2 skewing by down-regulating the IL12p70 release. Additionally, PS recognition leads to the development of IL-10 producing regulatory T cells (van der Kleij et al., 2002). Interestingly, PS is also expressed by *Leishmania* spp, where it is suggested as an advantageous feature. PS of *L. amazonensis* amastigotes influences the surrounding cytokine milieu by the induction of TGF-β and IL-10 in MΦ. This process is accompanied by an inhibition of NO production in infected MΦ. Therefore, PS expression of *L. amazonensis* was termed "apoptotic mimicry" (Freitas Balanco et al., 2001). Other groups demonstrated that *Leishmania* have an apoptotic program. This parasite shows several apoptotic features including cytoplasmic DNA blebbing and vacuolization, chromatin condensation, cytochrom-c release and DNA fragmentation. Moreover, also a caspase-like activity achieved by paracaspases and metacaspases is identified (Lee et al., 2002; Debrabant et al., 2003; Nguewa et al., 2004). The PS exposure of *Leishmania*, which is dependent on an out-to-in and an in-to-out translocase activity, also indicates an ongoing apoptotic process (Tripathi and Gupta, 2003).

So far, the biological function of apoptosis in unicellular organisms is not yet fully understood. In this context, several hypotheses were postulated:

Apoptosis might have evolved together with the endosymbiontic incorporation of aerobic bacteria (the precursors of mitochondria) into ancestral unicellular eukaryotes (Kroemer et al., 1997). Hence, apoptosis could be a remnant process without any particular function. Moreover, a restriction of the individual parasite numbers might be meaningful to prevent prematurely death of the vector or the host cell or simply being a result of several factors such as cell differentiation, deprivation of nutrients or an increase of the cell density (DosReis and Barcinski, 2001). Others suggest apoptosis as a cell-sorting mechanism, e.g. for procyclic and metacyclic parasites so that those who fail to mature would become apoptotic (Nguewa et al., 2004). During infection the formation of a large vacuole with numerous *Leishmania* has been reported, which might be comparable with a pseudo-multicellular organism, which could favour altruistic mechanism and subsequently a survival of the fittest (Rittig and Bogdan, 2000; Debrabant et al., 2003). Furthermore, apoptosis could function as an advantageous feature, which might have developed during co-evolutionary adaptation for establishment and maintenance of the present day microorganism/host relationship by facilitating their survival (Nguewa et al., 2004; DosReis et al., 2001; Piacenza et al., 2001).

The apoptotic hallmark PS can be exploited by *Leishmania* amastigotes to gain survival advantages in MΦ. It remains unknown whether *Leishmania* promastigotes being the disease inducing form of this parasite can express PS on their membrane. In this case, PS on promastigotes could contribute to their “silent” entry into host phagocytes.

## 7. Aim of the study

Promastigotes are the disease inducing form of *L. major*. Polymorphonuclear neutrophilic granulocytes (PMN) make the first contact with this parasite. Since *L. major* promastigotes survive inside PMN, the role of phosphatidylserine (PS) for the silencing of PMN was investigated. In this thesis, the following questions were addressed :

Part I

1. Is the anti-inflammatory "eat-me" signal PS expressed on *L. major* promastigotes?
2. Are PS expressing *L. major* promastigotes apoptotic?
3. Does the presence of PS play a role for the silent phagocytosis of *L. major* by PMN?
4. Does the presence of PS influence the disease development *in vivo*?

Part II

1. Do PMN possess receptors for the "silent" phagocytosis of *L. major* promastigotes?

# II Material and Methods

## 1. Material

### 1.1 *L. major* parasites

The *L. major* (*Lm*) isolate MHOM/IL/81/FEBNI (Laskay et al., 1995) used for this study was originally isolated from skin biopsy of an Israeli patient and was a kind supply from Dr. Ebert (Bernhard-Nocht-Institute for Tropical Medicine, Hamburg). *Lm* promastigotes were cultured *in vitro* in biphasic Novy-Nicolle-McNeal (NNN) blood agar medium at 27°C in a humified atmosphere containing 5% $CO_2$ until they reach the stationary growth phase. Subsequently, parasites were passaged to a new agar plate. After every 12$^{th}$ passage a new *Lm* culture was obtained using freshly isolated *Lm* from popliteal lymph nodes of infected Balb/c mice (see 1.3).

### 1.2 Human leukocytes

Human polymorphonuclear neutrophilic granulocytes (PMN), macrophages (MΦ) and monocytes were obtained from fresh blood of voluntary donors. Subsequently, cells were purified or generated as described in methods (2.1.1. – 2.1.3).

### 1.3 Animals

Female Balb/c mice were purchased from Charles River Breeding (Sulzfeld, Germany) and maintained within the University of Lübeck animal care facility. Mice were six weeks old. Experiments were authorized by the Ministry of Social Affairs of Schleswig-Holstein.

### 1.4. Cell line

The cell line Mv1Lu (ICLC AL97002, mustela vision (mink), fetal lung fibroblast) was a kind supply by Dr. Dirk Haller (Technical University of Munich, Freising-Weihenstephan, Germany). Mv1Lu cells were cultured in 10 ml cell culture flasks with complete medium (see 1.5).

### 1.5. Media and buffers

| | |
|---|---|
| Annexin incubation buffer | 10 mM Hepes/NaOH pH 7.4 + 5 mM $CaCl_2$ in distilled $H_2O$ |
| Binding buffer | 20 mM Tris HCl + 0.5 M NaCl + 1 mM $CaCl_2$ + 2 mM EDTA, pH 7.4, in distilled $H_2O$ |
| Blocking buffer | PBS + 0.05% Tween-20 + 3% low-fat skimmed milk |
| Cacodylate buffer | 0.1 M cacodylate in PBS |
| Complete medium | RPMI 1640 medium + 10 mM Hepes + 2 mM L-glutamine + 100 U/ml penicillin + 100 µg/ml streptomycin + 10% heat inactivated FCS |
| Coupling buffer | PBS + 0.1 M $NaCO_3$ + 0.5 M NaCl, pH 8.3 |
| Dulbeccos Modified Eagle Medium (DMEM) | PAA Laboratories GmbH, Pasching, Austria |
| D-PBS (10x) | GIBCO Invitrogen Corporation, Karlsruhe |
| Elution buffer | 0.1 M glycine + 20 mM Tris HCl + 1 M NaCl + 1 mM $CaCl_2$ + 2 mM EDTA, pH 2.5, in distilled $H_2O$ |
| Erythrocyte lysis buffer | 155 mM $NH_4Cl$ + 10 mM $KHCO_3$ + 0.1 mM EDTA in distilled $H_2O$, pH 7.4 |
| FACS buffer | PBS + 1% human serum + 1% BSA + 0.01% $NaN_3$ |
| Inhibitor buffer | 1 mM PMSF + 10 µg/ml pepstatin-A + 2 mM levamisol + 0.5 mM benzamidin + 1 mM sodium orthovanadate + Complete Mini protease inhibitor (one tablet dissolved in 1 ml distilled $H_2O$ and used in a final dilution 1:5) |
| Lymphocytes Separation Medium | PAA Laboratories GmbH, Pasching, Austria |
| Lysis buffer (Mv1LU) | 50 mM β-glycerophosphate + 150 mM NaCl + 1.5 mM EGTA + 1 mM DTT + 0.1 mM sodium orthovanadate + 1 mM benzamidine + 10 µg/ml leupeptine + 1% triton X-100, pH 7.3 |
| MACS buffer | PBS + 0.5% bovine serum albumine (BSA) + 2 mM EDTA |
| Neutralisation buffer | 1 M Tris HCl, pH 9, in distilled $H_2O$ |

| | |
|---|---|
| Novy-Nicolle-McNeal (NNN) blood agar medium | 50 ml rabbit blood + 50 ml PBS + 2 ml penicillin/ streptomycin + 200 ml Brain Heart Infusion (BHI) medium (10.4 g in 200 ml distilled $H_2O$) |
| Phosphate buffer saline (PBS) | Pharmacy of the University of Lübeck |
| Roswell Park Memorial Institute (RPMI) 1640 medium | Sigma, Deisenhofen |
| Stripping buffer | 62.5 mM Tris-HCl pH 6.8 + 2% SDS + 100 mM β-mercaptoethanol |
| Washing buffer | PBS + 0.05% Tween-20 |

**5 x SDS-Page running buffer, pH 8.3 (1 litre)**

| | |
|---|---|
| Tris base | 15 g |
| Glycine | 72 g |
| SDS | 5 g |

Add distilled $H_2O$ to 1 litre (dilute to 1 x buffer before usage)

**Blot buffer (1 litre)**

| | |
|---|---|
| Tris base | 3.03 g |
| Glycine | 11.27 g |
| Distilled water | 800 ml |
| Methanol | 200 ml |

**4 x sample buffer**

| | |
|---|---|
| 0.5 M Tris-HCL, pH 6.8 | 1.0 ml |
| Glycerol | 1.6 ml |
| 10% SDS | 1.6 ml |
| 0.5% Bromphenol blue | 0.4 ml |
| Distilled water | 3.0 ml |
| (diluted to 1 x buffer before usage) | |
| ß-Mercaptoethanol (add before usage) | 1:20 |

## 1.6 Chemicals and other reagents

| | |
|---|---|
| Acetate | Merck, Darmstadt |
| Agarose | Sigma, Deisenhofen |
| Ammonium chloride ($NH_4Cl$) | Merck, Darmstadt |
| Anesthetic (Parke-Davis®) | Bayer Chemicals AG, Leverkusen |
| Annexin-V (recombinant) | Responsif GmbH, Erlangen |
| Annexin-V FITC | Roche Molecular Biologicals, Mannheim |
| Annexin-V Alexa 568 | Molecular Probes, Leiden, Netherlands |
| Araldite® | Fluka, Buchs, Switzerland |
| Benzamidine | Sigma, Deisenhofen |
| Biotin EZ-LinkTM Sulfo-NHS-LC | Pierce, Bonn |
| Bovine serum albumine (BSA) | Sigma , Deisenhofen |
| Bromphenol blue | Serva, Heidelberg |
| Cacodylate | Sigma, Deisenhofen |
| Calcium chloride dihydrate | Merck, Darmstadt |
| Chloroform | Roth, Karlsruhe |
| Complete Mini protease inhibitor | Roche Molecular Biologicals, Mannheim |
| Crystal violet staining solution | Sigma, Deisenhofen |
| Dihydrorhodamine 123 (DHR) | Medac, Hamburg |
| Dithiothreitol (DTT) | Sigma, Deisenhofen |
| Ethanol (96% pure) | Roth, Karlsruhe |
| Ethidium bromide | Sigma, Deisenhofen |
| Ethidium homodimer-1 | Molecular Probes, Leiden, Netherlands |
| Ethylene glycol-bis (ß-aminoethylether) -N,N,N',N'-tetra-acetic acid (EGTA) | Sigma, Deisenhofen |
| Ethylendiaminetetraacetic acid (EDTA) | Sigma, Deisenhofen |
| Fetal calf serum (FCS) | Sigma, Deisenhofen |
| D-Galactose | Serva, Heidelberg |
| L-Glutamin | Seromed-Biochrom, Berlin |
| β-Glucan | Serva, Heidelberg |
| Glutaraldehyde | Sigma, Deisenhofen |
| Glycerol | Sigma, Deisenhofen |
| β-Glycerophosphate | Sigma, Deisenhofen |

| | |
|---|---|
| Glycine | Serva, Heidelberg |
| Histopaque® 1077 | Sigma, Deisenhofen |
| Histopaque® 1119 | Sigma, Deisenhofen |
| IMAGEN® Mounting fluid | DAKO, Hamburg |
| Kaleidoscope prestained standard marker | Pierce, Bonn |
| N-2-Hydroxyethylpiperazine-N´-2-Ethansulfonic acid (HEPES) | Seromed-Biochrom, Berlin |
| Isopropanol | Roth, Karlsruhe |
| Lead citrate | Ultrostainer Carlsberg System, LKB, Bromma, Sweden |
| Levamisole | Sigma, Deisenhofen |
| Leupeptine | Calbiochem, Darmstadt |
| MACS CD14 MicroBeads | Miltenyi Biotec GmbH, Berg. Gladbach |
| May-Gruenwald-Giemsa staining solution | Sigma, Deisenhofen |
| β-Mercaptoethanol | Sigma, Deisenhofen |
| Methanol | J.T. Baker, Deventer, Netherlands |
| Milkpowder, low-fat | Töpfer GmbH, Dietmannsried |
| Mineral oil | Sigma, Deisenhofen |
| N, N, N´, N´-Tetramethylethylen-diamine (TEMED) | Sigma, Deisenhofen |
| Nonidet P-40 | Sigma, Deisenhofen |
| Osmium tetraoxyde ($OsO_4$) | Sigma, Deisenhofen |
| Paraformaldehyde | Sigma, Deisenhofen |
| Peanut lectin agglutinin (PNA) | Sigma-Aldrich Chemie GmbH, Munich |
| Penicillin/streptomycin | PAA Laboratories, Pasching, Austria |
| Percoll | Pharmacia, Uppsala, Sweden |
| Pepstatin-A | Sigma, Deisenhofen |
| Phenylmethylsulphonylfluoride (PMSF) | Sigma, Deisenhofen |
| p-Nitrophenylphosphate | Sigma, Deisenhofen |
| Propidium iodide solution | Sigma-Aldrich Chemie GmbH, Munich |
| Sepharose 4 Fast Flow CNBr-activated | Amersham Pharmacia Biotech, Freiburg |

| | |
|---|---|
| Serva-Blue G-250 | Serva Electrophoresis GmbH, Heidelberg |
| Sodium acetate ($NaCH_3CO_2$) | Roth, Karlsruhe |
| Sodium azide ($NaN_3$) | Merck, Darmstadt |
| Sodium chloride (NaCl) | Merck, Darmstadt |
| Sodium dodecyl sulfate (SDS) | Sigma-Aldrich Chemie GmbH, Munich |
| Sodium hydrogen carbonate ($NaCHO_3$) | Merck, Darmstadt |
| Sodium hydroxide pellets | Merck, Darmstadt |
| Sodium orthovanadate | Sigma, Deisenhofen |
| SuperSignal® West Dura Extended Duration Substrate | Pierce, Bonn |
| Rotiphorese® Acrylamide stock solution | Roth, Karlsruhe |
| SYTO-16 | Molecular Probes, Leiden, Netherlands |
| TGF-β1, human recombinant (ELISA) | Serva, Heidelberg |
| TGF-β1, human recombinant (Mv1LU stimulation) | Prepro Tech, Inc., Rocky Hill, NJ, USA |
| Tris base | Roth, Karlsruhe |
| Triton X-100 | Merck, Darmstadt |
| Trypan blue solution (0.4%) | Sigma, Deisenhofen |
| Tween 20 | Amersham Pharmacia Biotech, Freiburg |
| Uranyl acetate | Ultrostainer Carlsberg System, LKB, Bromma, Sweden |
| Viralex™ TRYPSIN/EDTA (1x) | PAA Laboratories GmbH, Pasching, Austria |

### 1.7 Laboratory supplies

| | |
|---|---|
| Cell culture flasks (5 ml,10 ml, 30 ml) | Nunc, Wiesbaden |
| Extra Thick Blot Paper Criterion ™ size | Bio-Rad Laboratories, Munich |
| Glas slides superfrost | Menzel, Braunschweig |
| Hamilton needles | Sigma, Deisenhofen |
| Hyperfilm ™ ECL | Amersham Biosciences, Buchinghamshire, England |
| MACS LS-Columns | Miltenyi Biotec GmbH, Berg. Gladbach |
| MACS Pre-Separation Filter (30 µm) | Miltenyi Biotec GmbH, Berg. Gladbach |

| | |
|---|---|
| MaxiSorb microtiter plates (12-, 48-, 96-well U-bottom) | Nunc, Wiesbaden |
| Microtestplates + lid (96 well, V-bottom) | Sarsted, Nümbrecht |
| Nitrocellulose (NC) membrane Optitran BA-S 83 reinforced NC | Schleicher & Schull, Dassel |
| Pipette tipps (1-10 µl, 10-100 µl, 100-1000 µl) | Greiner, Frickenhausen |
| Plastic tubes (15 ml, 50 ml) | Sarstedt, Nümbrecht |
| Reaction tubes (1.5 ml, 2 ml) | Sarstedt, Nümbrecht |
| Slide-A-Lyzer® Dialysis Cassettes (3 ml) | Pierce, Bonn |
| Tissue culture microtiter plates (96-well) | Nunc, Wiesbaden |
| Tissue culture plates (6-, 12-, 24-well) | Greiner, Frickenhausen |

**1.8 Instruments**

| | |
|---|---|
| Block thermostats: | |
| Unitek ™ block thermostat HB 130 | Peqlab, Erlangen |
| Block thermostat TCR 200 | Roth, Karlsruhe |
| Calipers | Kroepelin, Schlüchtern |
| Cell counting chambers | Neubauer, Marienfeld |
| Cell disruption vessel ($N_2$-bomb) | Parr instrument, Molin, Illinois, USA |
| $CO_2$-incubator IG 150 | Jouan, Unterhachingen |
| Centrifuges: | |
| Biofuge fresco | Heraeus, Hanau |
| Megafuge 2.0R | Heraeus, Hanau |
| Microfuge R | Beckmann, München |
| Cytocentrifuge Cytospin | Shandon, Frankfurt |
| Deep freezer, -20°C, -80°C | Liebherr, Ochsenhausen |
| Electron microscope EM 400 | Philips, Freiburg |
| FACS-Calibur® flow-cytometer | Becton, Dickinson, Heidelberg |
| Finn pipette | Labsystems, Helsinki, Finnland |
| Microscope Axiovert 25 fitted with a HRS 14 bits Aciocam | Carl Zeiss, Jena |
| Microwave oven | Bosch, Stuttgart |

| | |
|---|---|
| Midi MACS™ | Miltenyi Biotec GmbH, Berg. Gladbach |
| Mini-PROTEAN® 3 Cell | Bio-Rad Laboratories, Munich |
| Multichannel pipette | Eppendorf, Hamburg |
| pH-Meter Beckman 3500 digital | Beckman, Munich |
| Pipettes | Eppendorf, Hamburg |
| Precision Microplate Reader | Molecular Devices, Ismaning |
| Power supply P25 | Biometra, Göttingen |
| Shaker Vibrofix VF1 Electronic | Janke & Kunkel IKA® Labortechnik, Staufen |
| Sonificator Sonoplus HD 2070 | BANDELIN, Berlin |
| Transblot® Semidry Transfer Cell | Bio-Rad Laboratories, Munich |
| Transfer pipette | Brand, Wertheim |
| Ultracentrifuge L-70 | Beckman Coulter Inc., Fullerton, CA, USA |
| Ultracut-E | Reichert-Jung, Nußloch |
| UMAX Astra 6700 Scanner | UMAX Systems GmbH, Willich |
| UV-Stratalinker® UV Crosslinker | Stratagene, La Jolla, CA |
| Vacuum concentrator (UVS400A, SpeedVac® Plus SC110A) | Savant, New York, USA |
| Water bath | Bioblock scientific, Cedex, France |

### 1.9 Primary antibodies and dilutions

| | |
|---|---|
| Rabbit anti-human **ß-actin**, (pAb, IgG, 1:5000) | Sigma, Deisenhofen |
| Mouse anti-human **CD14** PE (mAb, IgG2a, Tük4, 1:100) | DAKO, Hamburg |
| Rat anti-human **CD11b** (mAb, IgG2b | BD Biosciences Pharmingen, Heidelberg |
| Rabbit anti-human **CR1** (mAb, IgG1, M1/70, 1:100) | BD Biosciences Pharmingen, Heidelberg |
| Mouse **IgG1 isotype** control (1:100) | DAKO, Hamburg |
| Mouse **IgM isotype** control (1:100) | BioCarta Inc. San Diego, CA, USA |

| | |
|---|---|
| Rabbit anti-human **Phospho Smad2** (pAb, IgG, Ser465/467, 1:750) | Cell Signaling, Beverly, MA, USA |
| Mouse anti-human **PS** (mAb, IgG, 1H6, 1:50) | Biomol GmbH, Hamburg |
| Hybridoma culture supernatant containing mouse anti-human **PSR** (mAb, IgM, 217, 1:50 (FACS), 1:3000 (western blot)) | Dr. Fadok, Denver, CO, USA |
| Hybridoma culture supernatant containing mouse anti-human **PSR** (mAb, IgG against PSR peptides encoded by exon 6 or 8 of PSR m-RNA, 1:50 (FACS) or 1:1000 (western blot)) | PD Dr. Dr. Herrmann and Dr. Voll, University of Erlangen-Nürnberg |
| Rabbit anti-human **PSR** (pAb, IgG, against PSR amino residues 363 to 381, cross reactivity to mouse PSR, 1:500) | Sigma, Deisenhofen |
| Chicken anti-human **TGF-β1** (mAb,IgG1,1:100) | R&D Systems, Wiesbaden |
| Mouse anti-human **TGF-β1, 2, 3** (mAb, IgG, 1:200) | R&D Systems, Wiesbaden |

**1.10 Secondary antibodies and dilutions**

| | |
|---|---|
| AP conjugated rabbit anti-chicken IgG, 1:10000 | Sigma, Munich |
| HRP conjugated goat anti-mouse IgG, 1:50000 | DAKO, Hamburg |
| HRP conjugated goat anti-mouse IgG, 1:2000 | Santa Cruz, Heidelberg |
| HRP conjugated goat anti-rabbit IgG, 1:5000 | Santa Cruz, Heidelberg |
| HRP conjugated goat anti-rat IgG, 1:50000 | DAKO, Hamburg |
| HRP conjugated streptavidine, 1:50000 | DAKO, Hamburg |
| HRPO conjugated goat anti-mouse IgM, 1:5000 | Biocarta, San Diego, USA |
| PE conjugated goat anti-mouse IgG, 1:100 | Biocarta, San Diego, USA |
| PE conjugated goat anti-mouse IgM, 1:100 | BD Biosciences Pharmingen, Heidelberg |

## 1.11 Ready-to-use Kits

| | |
|---|---|
| TUNEL assay, In Situ Cell Death Detection Kit OPTEIA ™ | Roch, Mannheim |
| ELISA kit for human TNF-α | BD Biosciences Pharmingen, |
| Annexin-V MicroBead Kit | Miltenyi Biotec GmbH, Berg. Gladbach |

## 1.12 Solutions for Leammli gel

| Chemicals: | Separating gel (20 ml): | | Stacking gel (10ml): |
|---|---|---|---|
| | **12%** | **7.5%** | **4%** |
| Acrylamide stock (30%) | 8.00 ml | 5.00 ml | 1.30 ml |
| 1.5 M Tris-HCl, pH 8.8 | 5.00 ml | 5.00 ml | - |
| 0.5 M Tris-HCl, pH 6.8 | - | - | 2.50 ml |
| 10% SDS | 0.20 ml | 0.20 ml | 0.10 ml |
| Distilled $H_2O$ | 6.69 ml | 9.69 ml | 6.04 ml |
| 10% APS | 0.10 ml | 0.10 ml | 0.05 ml |
| TEMED | 0.01 ml | 0.01 ml | 0.01 ml |

## 1.13 Software

| | |
|---|---|
| Adobe® Acrobat® 5.0 | Adobe Systems Inc., San Jose, CA, USA |
| Adobe® Photoshop® 6.0 | Adobe Systems Inc., San Jose, CA, USA |
| Axiovert 4.2 software (microscope) | Carl Zeiss, Jena |
| CellQuest Pro® software (FACS) | Becton, Dickinson, Heidelberg |
| Magellan ® (ELISA) | Tecan, Crailsheim |
| Microsoft ® EXEL, version 2000 | Microsoft Cooperation, Mountain View, CA, USA |
| Win MDI 2.8 | J. Trotter, The Scripps Research Institute, San Diego, California |

# 2. Methods

## 2.1 Cell isolation and culture methods

### 2.1.1 Isolation of human polymorphonuclear neutrophilic granulocytes

Heparinized blood collected by venipuncture from healthy adult volunteers or buffy coat (~ 50 ml, diluted 1:6 with sterile phosphate buffered saline (PBS)) was layered on a Histopaque® gradient consisting of Histopaque®1077 (top) and Histopaque® 1119 (bottom). Cells were centrifuged for 5 min at 300 x g followed by 20 min at 1045 x g without break. The plasma and the interphase were discharged. The polymorphonuclear neutrophilic granulocyte (PMN)-rich layer below the interphase was collected leaving the erythrocyte pellet on the bottom of the tube. The cells were washed twice in PBS and resuspended in complete medium (RPMI 1640 medium + 10 mM Hepes + 2 mM L-glutamine + 100 U/ml penicillin + 100 µg/ml streptomycin + 10% heat inactivated fetal calf serum (FCS)). Subsequently, the PMN-rich layer was fractionated on a discontinuous Percoll® gradient consisting of layers with densities of 1.105 g/ml (85%), 1.100 g/ml (80%), 1.093 g/ml (75%), 1.087 g/ml (70%) and 1.081 g/ml (65%). After centrifugation for 20 min at 1045 x g without break the interphase between the 80% and 85% Percoll® layers was collected, washed twice in PBS and resuspended in complete medium. All procedures were conducted at RT. The isolated cell suspension contained >99% granulocytes. More than 95% were PMN and 1 – 5% were eosinophils, as determined by May-Gruenwald-Giemsa staining (see in 2.4.1) of cytocentrifuged samples.

### 2.1.2 Isolation and purification of human monocytes

Monocytes were isolated using two different methods. One method was conducted using magnetic cell sorting (MACS) and MACS CD14 MicroBeads. In short, the interphase (obtained after buffy coat separation as described in 2.1.1), which consists of peripheral blood mononuclear cells (PBMC) was collected. PBMC were washed once in PBS (1045 x g, 10 min, RT) and the pellet was resuspended in MACS buffer (PBS + 0.5% bovine serum albumine (BSA) + 2 mM EDTA) at a

concentration of 1 x $10^7$ cells per 80 µl. Subsequently, 20 µl MACS CD14 MicroBeads per 1 x $10^7$ cells were added, mixed and incubated for 15 min at 4°C. After incubation, cells were washed with MACS buffer and resuspended in MACS buffer (1 x $10^8$ cells per 500 µl). A MACS LS-column was placed in the magnetic field of a MACS separator, washed once with 3 ml of MACS buffer and the cell suspension was applied onto the column. CD14 negative cells passed through the column. After rinsing the column with 3 x 3 ml MACS buffer the column was removed from the separator. CD14 positive cells were flushed out using a plunger supplied with the column. Finally, the obtained CD14 positive monocytes were washed once in PBS and counted. Their purity was tested with a FACS staining using PE-labeled mAb to CD14.

A second method to obtain purified monocytes was conducted using a Percoll® gradient. Briefly, PMBC were harvested and washed once with PBS (1045 x g, 10 min, RT). The pellets were washed three times in PBS (140 x g, 10 min, RT) to remove thrombocytes. Cells were pooled, counted and centrifuged once more (1045 x g, 5 min, RT). The supernatants were discharged and the pellets were resuspended in 1 ml 100% Percoll® each. 2 ml-layers of 70%, 60% and 40% Percoll® were piled up and the gradient was centrifuged (1045 x g, 5 min, RT, without break). Monocytes were harvested (phase between 40% and 60% Percoll®), washed with PBS (1045 x g, 5 min, RT) and resuspended in complete medium.

### 2.1.3 Generation of macrophages

For macrophage (MΦ) generation 1.5 x $10^6$ Percoll® purified monocytes (see above) per ml complete medium were seeded in 12-well culture plates (1 ml cell suspension per well). Cells were allowed to adhere for 2 h in an incubator (37°C, 5% $CO_2$). Afterwards, non-adherent cells were removed. Adherent cells were washed twice with PBS (37°C) by shaking plates gently. Subsequently, complete medium supplemented with 10 ng/ml M-CSF was added. After at least four days of culturing, MΦ were harvested for further experiments.

## 2.2 SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli)

12% or 7.5% SDS-polyacrylamide gels were prepared according to a standard protocol (see materials) in small gel formats (8 cm x 10 cm x 1.5 mm). Either 30 µg of total protein or a total number of 1 x $10^6$ cells were diluted 1:4 with 4 x sample buffer (see materials) and placed in a boiling water bath for five min followed by a short spin (1045 x g, 2 min, RT). Samples were chilled on ice and loaded onto the gel. The electrophoresis was performed with constant 75 V for protein passage through the stacking gel and with constant 125 V for the separation gel.

## 2.3 Western blot analysis

Separated proteins were blotted onto a nitrocellulose membrane at 145 mA constant voltage for 60 minutes in a Transblot® Semidry Transfer Cell. The membrane was blocked with blocking buffer (PBS + 0.05% Tween 20 + 3% low-fat skimmed milk) for 2 h at RT, washed with washing buffer (PBS + 0.05% Tween 20) and subsequently exposed to a primary antibody (on, 4 °C) by gentle agitation on a shaker table. After extensive washing with washing buffer, the membranes were incubated with a HRP-, HRPO- or AP-coupled polyclonal secondary antibody on a shaker table (120 min, RT). The membrane was washed again and the protein bands were visualized using chemiluminescent western blotting detection system SuperSignal® West Dura Extended Duration Substrate. To control equal load of protein, the membrane was stripped with stripping buffer (62.5 mM Tris-HCl pH 6.8 + 2 % SDS + 100 mM β-mercaptoethanol) for half an hour at 56°C to release previously bound antibodies and additionally exposed to an anti-β-actin antibody. Afterwards, the membrane was washed, incubated with a 1:5000 dilution of polyclonal rabbit anti-human β-actin antibody for 120 min and subsequently proceeded as described above.

## 2.4 Staining methods

### 2.4.1 May-Gruenwald-Giemsa staining

1 x $10^5$ PMN or 5 x $10^5$ *Lm* were washed once in PBS and resuspended in 100 µl PBS. Cells were spun on slides in a cytocentrifuge at 54 x g for five min, RT. The slides were air dried and cells were fixed in methanol for five min. Afterwards, cell preparations were stained for 30 min in May-Gruenwald-Giemsa solution.

### 2.4.2. Live/dead staining

In order to discriminate microscopically viable from dead *Lm* promastigotes, live/dead staining was conducted. The live stain SYTO-16 (green) penetrates intact cell membranes and stains DNA, whereas the dead stain ethidium homodimer-1 (red) can only enter compromised membranes of dead cells. 5 x $10^5$ *Lm* were washed once in PBS and resuspended in 100 µl PBS supplemented with 5 µmol SYTO-16 solution and 5 µmol ethidium homodimer-1. Cells were incubated for 30 min at RT in the dark. Subsequently, cells were washed once in PBS. Then, cells were spun on glass slides and fixed with 1% paraformaldehyde-PBS. Cytocentrifuged slides were embedded in IMAGEN® mounting fluid and stored in darkness at 4°C. Viable and dead cells were determined using fluorescence microscopy.

## 2.5 Assessment of apoptosis

### 2.5.1 Phosphatidylserine detection with annexin-V

In order to stain PMN or *Lm* for phosphatidylserine (PS) fluorescent labeled annexin-V (AV) was used. Briefly, 1 x $10^6$ cells were washed once with annexin incubation buffer (10 mM Hepes/NaOH pH 7.4 + 5 mM $CaCl_2$). For fluorescence microscopy, cells were incubated with AV Alexa Fluor 568 conjugate (0.1 µg/100 µl) for 20 min on ice in the dark. Cells were washed and cytocentrifuged slides with 1 x $10^5$ PMN or 5 x $10^5$ *Lm* per slide were prepared. For FACS analysis, 0.5 x $10^6$ PMN or 1 x $10^6$ *Lm*

were incubated with AV-Fluos (0.1 µg/100 µl) in annexin incubation buffer for 20 min on ice in the dark in a 96-well V-bottom plate. As a negative control, cells were incubated in buffer alone. Cells were washed once and resuspended in 400 µl annexin incubation buffer. To quantify the ratio of PS expression, the cells were analysed by flow cytomerty using FACS Calibur® with CellQuest Pro® software. Similar incubation experiments were conducted in annexin incubation buffer without calcium supplementation.

### 2.5.2 Phosphatidylserine detection with anti- phosphatidylserine antibody

In addition to AV, also a mAb specific for PS was used. Briefly, 0.5 x $10^6$ apoptotic PMN (freshly isolated PMN were incubated for 18 h in complete medium to become apoptotic) or 1 x $10^6$ *Lm* were put into a 96 well-V-bottom plate and washed once in ice-cold FACS buffer (PBS + 1% human serum + 1% BSA + 0.01% sodium azide). Subsequently, cells were treated with either monoclonal mouse anti-human PS antibody (2 µg/100 µl), with the appropriate isotype control (2 µg/100 µl), or with FACS buffer alone for 20 min on ice. Cells were washed once with FACS buffer. Then, PE conjugated anti-mouse IgG antibody (1 µg/100 µl) was added. After 20 min of incubation on ice the stained cells were washed and fixed using 400 µl of 1% paraformaldehyde-PBS prior to FACS analysis.

### 2.5.3 TUNEL assay

The final stage of apoptosis was assessed using TUNEL technology (In Situ Cell Death Detection Kit, Roche, Mannheim), which detects apoptotic cell death by enzymatic labeling of DNA strand breaks with fluorescein-dUTP and TdT (Gorczyca et al., 1993). Briefly, 1 x $10^5$ cells were fixed in 4% paraformaldehyde-PBS (pH 7.4) for 60 min at RT, washed in PBS and resuspended in permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate in PBS) for 2 min on ice. Cells were washed and resuspended in 50 µl TUNEL reaction mixture or in 50 µl label solution alone as a negative control. The cell suspension was sealed and incubated for 60 min in a humidified atmosphere in the dark at 37°C followed by washing twice in PBS.

Subsequently, cells were resuspended with 400 µl PBS, and the green fluorescence of apoptotic nuclei was detected by FACS analysis and fluorescence microscopy.

### 2.5.4 Electron microscopy

In order to investigate apoptotic features on *Lm* morphologically, structural preservation electron microscopy was performed. *Lm* were fixed with 5% glutaraldehyde in 0.1 M cacodylate buffer for 1 h, treated with 1% osmium tetraoxyde for 2 h and dehydrated in graded ethanol series. The samples were embedded in Araldite® and ultra thin sections were cut on an Ultracut-E. The sections were contrasted with uranyl acetate and lead citrate. Examination was conducted with a Philips EM 400 electron microscope.

## 2.6 Coincubation of PMN with *L. major* promastigotes

In order to investigate the *in vitro* infection of PMN with *Lm*, 1 x $10^7$ PMN were coincubated with 5 x $10^7$ stationary phase *Lm* (*Lm* stat) in 1 ml complete medium. For infection, different *Lm* populations were used (*Lm* stat (70% PS+); *Lm* PS-, (7% PS+); *Lm* PS+, (95% PS+), see 2.7.2). Coincubation experiments were conducted for 3 h or 18 h at 37°C in a humidified atmosphere containing 5% $CO_2$. Afterwards, cells were centrifuged (1045 x g, 10 min, RT). The supernatants were taken and stored at –20°C for cytokine measurements. The PMN infection rate, defined as the number of PMN that have ingested one or more parasites, was determined using May Gruenwald Giemsa staining (see 2.4.1) or SYTO-16 green fluorescent live staining (see 2.4.2). The infection rate of PMN was assessed by microscopic evaluation of > 200 cells.

To investigate the cytokine release and the infection rate of PMN after blocking PS on stationary phase *Lm* promastigotes, parasites were pre-treated for half an hour on ice in annexin incubation buffer with annexin-V (AV) (2 µg/ml). Subsequently, similar coincubation experiments were conducted as described above. Additionally, the cytokine release and the infection rate were also investigated in MΦ using similar experimental setups as described for PMN (see above).

## 2.7 *L. major* purification methods

For the investigation of stationary phase and metacyclic *Lm* containing different ratios of PS expressing parasites, parasites were purified as described in the following methods (see fig. 4).

### 2.7.1 Peanut lectin agglutination assay

Agglutination with peanut lectin agglutinin (PNA) was used to purify metacyclic *Lm* promastigotes from *Lm* cultures. PNA agglutinates procyclic but not metacyclic promastigotes. A total number of 2 x $10^8$ *Lm* were incubated with 50 µg/ml PNA in complete medium for 30 minutes at RT. Agglutinated *Lm* were pelleted by centrifugation for 10 min at 200 x g at RT. Subsequently, the supernatant was centrifuged for 10 min at 1990 x g at RT to pellet the non-agglutinated *Lm.* These non-agglutinated parasites were metacyclic *Lm* (*Lm* met). To inactivate unbound PNA, *Lm* met were washed once in 10 ml DMEM medium supplemented with 20 mM galactose.

### 2.7.2 MACS separation

For separation of PS positive and PS negative *Lm* promastigotes, MACS separation with annexin-V (AV) MicroBead Kit was used. Briefly, 250 x $10^6$ stationary *Lm* (*Lm* stat) were incubated in binding buffer (provided by the kit) with 100 µl AV-beads for 15 minutes at 4°C. Then, *Lm* were washed and resuspended in binding buffer. A MACS LS-column was washed with 3 ml binding buffer and placed at in the Midi MACS. Subsequently, the *Lm* suspension was applied onto the column. The fluid passed through and the column was rinsed with 3 x 3 ml binding buffer. The obtained suspension contained the PS negative *Lm* fraction (*Lm* stat PS-). Subsequently, the column was removed from the Midi MACS and flushed out with 3 ml binding buffer resulting in the PS positive *Lm* fraction (*Lm* stat PS+). This method was similarly used for the separation of previously purified metacyclic *Lm* (*Lm* met) resulting in *Lm* met PS- and *Lm* met PS+ parasite samples. The purity of MACS separated *Lm* PS-

and *Lm* PS+ samples was proven using AV Fluos staining and FACS analysis as described above.

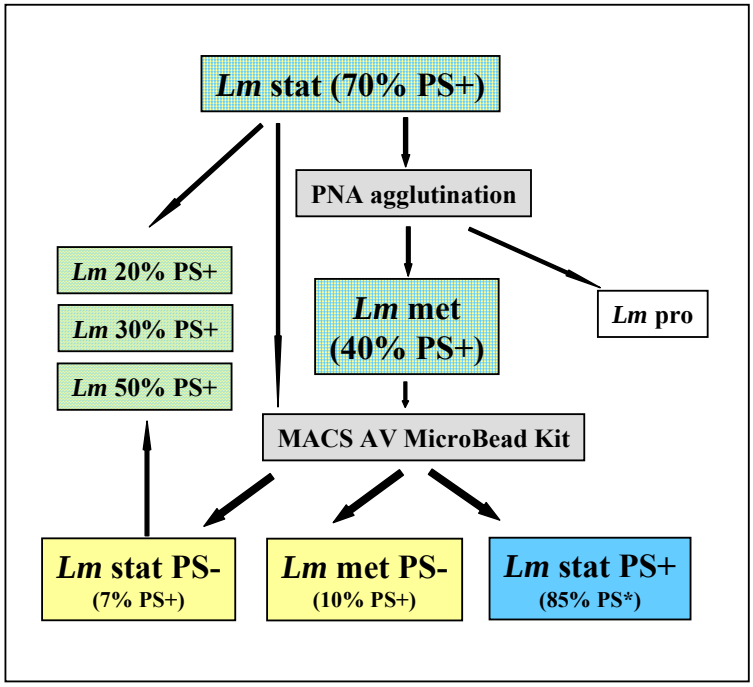


**Fig. 4: Schematic *Lm* separation using PNA and MACS**

## 2.8 End point titration method

End point titration is a method to determine parasite survival in time. This method was used to test the viability of MACS purified *Lm* PS+ and *Lm* PS- (see 2.7.2) or to quantify the number of viable parasites in infected PMN or in popliteal lymph nodes cells of infected mice (see 2.10.2.2). End point titration experiments were also used to investigate the presence of viable *L. major* in blood and intraperitoneal exudates of *Lm* infected mice.

To quantify the number of viable parasites inside PMN, 1 x $10^6$ PMN were coincubated for 3 h with a total number of 5 x $10^6$ *Lm* including a certain ratio of PS positive parasites. Cells were washed twice with PBS to remove extracellular *Lm* (140 x g, 10 min, RT). Subsequently, infected PMN were cultured for 18 h or 42 h in complete medium.

For end point titration either a total number of 1 x $10^5$ *Lm* or 5 x $10^3$ cells (PMN, blood- or intraperitoneal exsudate samples) were added in quadruplicate wells of 96-

well microtiter plates containing NNN blood agar medium (50 µl blood agar and 100 µl complete medium). End point titration with a dilution factor of 1.5 was carried out. Subsequently, the plates were incubated at 27°C in a 5% $CO_2$ humidified atmosphere for six days to allow the *Lm* to grow in number.

Calculation of the total number of viable parasites:
Dilution factor of the last well showing parasitic growth multiplied by the plating efficiency of 10 (see above).

Calculation of the total number of viable *Lm* per 1000 PMN:
A previously determined factor of 2500 was divided by the last well showing parasitic growth multiplied by the dilution factor. Subsequently, 1000 (PMN) was divided by the previously obtained value and multiplied by 100 (considering the plating efficiency of 10 (see above) and a previously determined factor of 10).

## 2.9 Assessment of PMN functions after contact with *L. major*

### 2.9.1 Detection of oxidative burst by PMN

In order to investigate the intracellular production of reactive oxygen radicals in PMN after coincubation with different *Lm* samples, the substrate dihydrorhodamine 123 (DHR) was used. In interaction with reactive oxygen species ($H_2O_2$ and HO) 123 DHR converts to a green fluorescent dye (Smith and Weidemann, 1993). A total number of 5 x $10^5$ PMN were centrifuged and resolved in 100 µl PBS + 1% FCS. Subsequently, 1 µM 123 DHR was added (5 min, 37°C). Afterwards, PMN were coincubated with *Lm* stat, *Lm* PS, *Lm* PS+ (see 2.7.2) in a ratio of 1:5, or PMN were stimulated with 10 ng/ml PMA (positive control) (15 min, RT). The fluorescence intensity of reactive oxygen radicals was determined using FACS analysis.

### 2.9.2 Detection of TNF-α in PMN supernatants by ELISA

The amount of TNF-α was measured in PMN/*Lm* coculture supernatants (see 2.6) using an enzyme-linked immunosorbent assay (ELISA) (OptEIA$^{TM}$ Set Human TNF-α, BD Bioscience, San Diego, USA). Similar TNF-α measurements were also performed with MΦ/*Lm* coculture supernatants (see 2.6).

### 2.9.3 Detection of TGF-β1 in PMN supernatants by ELISA

The presence of TGF-β1 in PMN/*Lm* coculture supernatants (see 2.6) was assessed using a TGF-β sandwich ELISA protocol according to Szymkowiak (Szymkowiak et al., 1995). Briefly, a 96-well microtiter plate was coated with a monoclonal mouse antibody against TGF-β1, 2, 3, blocked and washed. In order to activate latent TGF-β1, the samples were acidified to a pH of 2.0 (with 5 M HCl for 30 min, then neutralized with 5 M NaOH, 1 M Hepes (pH 7.4) and 200 mM EDTA). Immediately thereafter, the samples were applied to the plate for 1 h of incubation. TGF-β1 was detected using a chicken anti-human TGF-β1 antibody for 1 h of incubation. The plate was washed again and a polyclonal alkaline phosphatase (AP) conjugated rabbit anti-chicken IgG was added for 1 h of incubation. Subsequently, the substrate p-nitrophenylphosphate was used to visualize the presence of TGF-β1. Similar measurements were also performed using MΦ/*Lm* coculture supernatants (see 2.6).

### 2.9.4 Verification of TGF-β1 bioactivity using Smad2 phosphorylation

Because TGF-ß is synthesized in a latent form that must be activated to be recognized by cell surface receptors and to trigger biological responses (Massagué et al., 1990), the bioactivity of TGF-ß was investigated. Smad2 represents a downstream adapter protein within the TGF-β signaling pathway, which becomes phosphorylated after ligation of the appropriate receptor with only bioactive TGF-β (Derynck et al., 1998). In order to ensure that TGF-β1 released by PMN is bioactive, PMN/*Lm* coculture supernatants (see 2.6) were tested for Smad2 phosphorylation in TGF-β1 sensitive lung fibroblasts (cell line Mv1Lu, kindly supplied by Dr. Haller,

Technical University of Munich, Freising-Weihenstephan). Briefly, Mv1Lu cells were added to 24-well plates at a density of 5 x $10^5$ cells/well and allowed to adhere overnight. Adherent cells were washed twice using PBS. Cells were serum starved for 2 h before stimulation for 1 h with either recombinant TGF-ß1 (5 ng/ml) or with PMN/*Lm* coculture supernatants. Subsequently, Mv1Lu cells were washed twice in ice-cold PBS and lysed in 50 µl of 1 x sample buffer (30 min on ice). The resulting whole cell extract was heated at 100°C for five min and centrifuged for five min at 1045 x g. Western blotting was used to detect Smad2 phosphorylation using a rabbit anti-human Phospho Smad2 primary antibody and a HRP coupled polyclonal goat anti-rabbit secondary antibody.

## 2.10 *In vivo* experiments using a model for cutaneous *L. major* infection in Balb/c mice

### 2.10.1 Infection

To investigate the disease development in mice after infection with either viable PS negative (PS-) or viable plus apoptotic PS positive (PS+) *Lm* promastigotes, a cutaneous *Lm* infection model was used. Eight to ten weeks old female Balb/c mice were infected by s.c. injection of parasite samples (in 50 µl complete medium without FCS) into the left hind footpad. For infection, four different parasite samples were used: stationary phase *Lm* (*Lm* stat), *Lm* stat PS-, metacyclic *Lm* (*Lm* met), *Lm* met PS- (purified as described in 2.7.1 and 2.7.2). Because PS dependent MACS purification was not 100% efficient, *Lm* (stat/met) PS- samples did still contain a remaining amount of PS+ parasites. *Lm* samples were composed as demonstrated in table 1:

**Tab. 1: Composition of *L. major* samples**

| **1 x $10^6$ PS- *Lm* supplemented with PS+ *Lm*** | *Lm* stat PS- | *Lm* stat PS+ | *Lm* met PS- | *Lm* met PS+ |
|---|---|---|---|---|
| *Lm* stat | 1 x $10^6$ | 7 x $10^5$ | | |
| *Lm* stat PS- | 1 x $10^6$ | 7 x $10^4$ | | |
| *Lm* met | | | 1 x $10^6$ | 4 x $10^5$ |
| *Lm* met PS- | | | 1 x $10^6$ | 1 x $10^5$ |

| **1 x $10^6$ PS- *Lm* inclusive PS+ *Lm*** | *Lm* stat PS- | *Lm* stat PS+ | *Lm* met PS- | *Lm* met PS+ |
|---|---|---|---|---|
| *Lm* stat | 3 x $10^5$ | 7 x $10^5$ | | |
| *Lm* stat PS- | 9.3 x $10^5$ | 7 x $10^4$ | | |
| *Lm* met | | | 6 x $10^5$ | 4 x $10^5$ |
| *Lm* met PS- | | | 9 x $10^5$ | 1 x $10^5$ |

### 2.10.2 Measurements

#### 2.10.2.1 Footpad swelling

The footpad thickness of both the infected and the uninfected hind footpads were periodically measured with a calipers until the feet became necrotic and the mice were killed. The footpad swelling (in mm) was determined by calculating the difference between the footpad thickness of the infected and the uninfected foot.

#### 2.10.2.2 Parasite load in *L. major* infected Balb/c mice

The parasite load of differently infected mice (see above) was investigated using the popliteal lymph nodes after the mice were killed. Lymph nodes were homogenized by crushing them between two glass slides and additional rinsing the slides with 3 ml complete medium. To determine the parasite load, 50 µl of the popliteal lymph node homogenate were added to 50 µl complete medium in quadruplicate wells of a 96-

well NNN blood agar microtiter plate. Subsequently, end point titration was carried out as described in 2.8.

#### 2.10.2.3 Detection of *L. major* infected PMN or MΦ in peripheral blood and the peritoneum

In order to investigate whether *Lm* infected PMN or MΦ can be recruited into the peritoneum, an intraperitoneal phagocyte recruitment was induced by i.p. injection of 1 ml 1% glycogen in PBS per mouse. After 4 h, the mice were anesthesized with a lethal dose of Parke-Davis® anestheticum (0.4 ml of a mixture of 2 ml Ketanest, 0.5 ml Rompun, 10 ml NaCl (0,89%)). To obtain the recruited cells, the peritoneum was rinsed with 10 ml ice-cold PBS. Cells were washed with ice-cold complete medium (1045 x g, 10 min, 4°C), resuspended in complete medium and counted.

To investigate the presence of infected PMN or MΦ in the blood of infected mice, 0.5 - 1 ml blood per animal were taken by heart puncture. The blood was centrifuged (1045 x g, 10 min, 4°C) and the serum was stored at –20°C. The cell pellet was resuspended in 50 ml erythrocyte lysis buffer (155 mM $NH_4Cl$ + 10 mM $KHCO_3$ + 0.1 mM EDTA in destilled $H_2O$, pH 7.4) for 10 min, washed in PBS, resolved in complete medium and counted.

The presence of *Lm* in the obtained intraperitoneal and blood derived cells was investigated using May Gruenwald Giemsa staining (see 2.4.1) and end point titration (see 2.8).

### 2.11 Investigation of *L. major* binding PMN receptors

The presence of a specific PS receptor (PSR) on PMN was investigated with different methods. First, the existence of *Lm* binding molecules on PMN was investigated in coincubation experiments. Second, *Lm* binding PMN membrane fragments (PMN-MF) were isolated using precipitation and affinity chromatography. Finally, PSR membrane expression of PMN was assessed using different antibodies against the PSR with FACS- and western blot analysis.

### 2.11.1 Assessment of the PMN infection rate after coincubation with PMN membrane fragments pre-treated *L. major*

In order to investigate whether PMN possess *Lm* binding receptors on the membrane, coincubation experiments were performed. PMN were coincubated with stationary phase *Lm* either untreated, pre-treated with PMN membrane fragments (PMN-MF) (isolated as described below) (30 µg/1 x $10^6$ *Lm*) or pre-treated with BSA (30 µg/1 x $10^6$ *Lm*) for half an hour on ice. Subsequently, PMN were stained with May-Gruenwald-Giemsa solution and the infection rate was determined microscopically in > 200 PMN. Similar setups were used to investigate the infection rate of MΦ that were coincubated with *Lm* after pre-treatment with monocyte membrane fragments.

### 2.11.2 Biotinylation of PMN membranes and isolation of membrane fragments

Biotinylation and isolation of PMN membrane fragments (PMN-MF) was conducted with freshly purified PMN (at least a total amount of 1 x $10^8$ cells) that were washed three times in ice-cold PBS (pH 8) (1045 x g, 10 min, 4°C). For biotinylation, the cell pellet was resuspended in PBS (pH 8) supplemented with biotin (500 µg/ml/25 x $10^6$ PMN) and incubated overnight at 4°C. Subsequently, the biotinylation process was stopped using 2 mM $NH_4Cl$, which inactivates unbound biotin. Finally, biotinylated cells were washed three times with ice-cold PBS (pH 8) to remove unbound biotin.
With or without biotinylation, the cell pellet was resuspended in 1 ml protease inhibitor buffer (1 mM PMSF + 10 µg/ml pepstatin-A + 2 mM levamisol + 0.5 mM benzamidin + 1mM sodium orthovanadate + complete mini protease inhibitor (one tablet dissolved in 1 ml distilled $H_2O$ and used in a final dilution 1:5). The whole suspension was incubated for 30 min at 4°C and subsequently PBS was added to a final concentration of 25 x $10^6$ cells/ml.
Membrane fragments (MF) were obtained by sonification (15 min, 20 kHz, 21 w, RT). Disrupted cells were ultra-centrifuged (51000 x g, 1h, 4°C) and the pellet was resuspended in 1 ml protease inhibitor buffer (see above) with additional supplementation of 1% NP-40. After storage for 4 h at 37°C the cell suspension was ultra-centrifuged (105000 x g, 1h, 4°C). The MF containing supernatant was subsequently dialyzed against PBS to eliminate potentially toxic substances using

Slide-A-Lyzer® Dialysis Cassettes. The protein concentration of the dialysed MF solution was measured using Pierce method as suggested by the manufacturer. Finally, MF were stored at -80°C. Similar methods were also used to isolate MF from monocytes.

### 2.11.3 Precipitation of biotinylated PMN-MF with *L .major* and subsequent western blot analysis

In order to detect PMN membrane fragments (PMN-MF) that bind to *Lm*, a total number of 25 x $10^6$ stationary *Lm* promastigotes were incubated with 250 µg biotinylated PMN-MF in 100 µl PBS at 4°C overnight on a shaker. Then, *Lm* bound PMN-MF were precipitated by centrifugation for 20 min at 4°C at 4000 x g. The pellet was resuspended in 100 µl 1 x sample buffer and heated for 5 min. Afterwards, 25 µl of the solution were loaded per lane into a 12% denaturing polyacrylamide gel (see 2.2). Proteins were blotted (see 2.3) and visualized using a HRP conjugated streptavidine and chemiluminescent SuperSignal® West Dura Extended Duration Substrate.

### 2.11.4 Isolation of *L. major* membrane fragments

In order to isolate *Lm* membrane fragments (*Lm*-MF), from culture collected parasites were washed three times in ice-cold PBS (pH 8) (1200 x g, 5 min, 4°C). Then, *Lm* were resuspended in 5 ml of protease inhibitor buffer (see above). After incubation for 15 min, cells were disrupted using nitrogen cavitation. Briefly, 1 x $10^9$ *Lm* /ml were pressurized under N2-atmosphere (100 bar, 15 min, 4°C) with constant stirring in a nitrogen bomb. Cavitation of *Lm* was achieved by instant decompression of the cells into atmospheric pressure. Cavitates were spun twice at 1045 x g (10 min, 4°C) to pellet unbroken cells. *Lm*-MF were obtained using ultra-centrifugation as described in 2.11.2.

### 2.11.5 Column affinity chromatography

As a second method to obtain PMN membrane fragments (PMN-MF) that bind to *Lm*, column affinity chromatography was used. In short, *Lm*-MF (~ 3000 µg protein) were coupled to CNBr-activated Sepharose 4 Fast Flow as specified by the manufacturer. The *Lm*-Sepharose coupling was stacked in a column, which was subsequently equilibrated with 200 ml binding buffer (20 mM Tris HCl + 0.5 M NaCl + 1 mM $CaCl_2$ + 2 mM EDTA, pH 7.4, in distilled $H_2O$). Then, PMN-MF (~ 3400 µg protein) were applied onto the column. Afterwards, the column was washed with 50 ml binding buffer and additionally eluted with 25 ml elution buffer (0.1 M glycine + 20 mM Tris HCl + 1 M NaCl + 1mM $CaCl_2$ + 2 mM EDTA, pH 2.5, in distilled $H_2O$). The eluted fractions were collected and neutralized with Tris-HCl (pH 8). The protein concentration of the collected fractions was determined using Pierce method as specified by the manufacturer.

### 2.11.6 Investigation of complement receptor 1 and 3 on PMN

Using 30 µg protein of either unbiotinylated PMN membrane fragments (PMN-MF) (see 2.11.2) or eluted fractions after column affinity chromatography (see 2.11.5), 7.5% SDS-PAGE (see 2.2) and western blot analysis (see 2.3) were performed. In order to detect CR1, a monoclonal rabbit anti-human CR1 primary antibody and a HRP conjugated goat anti-rabbit secondary antibody were used. For investigation of CR3 (CD11b/CD18), a rat anti-human CD11b primary antibody and a HRP conjugated goat anti-rat secondary antibody was used.

### 2.11.7 Investigation of the phosphatidylserine receptor expression on PMN

To investigate the phosphatidylserine receptor (PSR) expression on PMN using western blotting and FACS analysis, three different anti-PSR antibodies were used:

1.) Hybridoma culture supernatant containing monoclonal mouse anti-human PSR Ab (IgM, 217) (anti-PSR IgM) developed by Dr. Fadok and colleagues, Denver, CO, USA (Fadok et al., 2000)
2.) Hybridoma culture supernatant containing monoclonal mouse anti-human PSR Ab (IgG) (anti-PSR IgG) provided by PD Dr. Dr. Herrmann and Dr. Voll from the University of Erlangen-Nürnberg
3.) Commercial polyclonal rabbit anti-human PSR Ab (IgG) (Sigma, Deisenhofen)

To investigate the PSR expression on PMN using FACS analysis, 10 x $10^6$ PMN/ml were stimulated with 25 µg/ml β-glucan and 250 pg/ml TGF-β (37°C, on, in a humidified atmosphere containing 5% $CO_2$). To determine whether *Lm* promastigotes can induce PSR expression on PMN, PMN were coincubated for 5, 15, 30 and 60 min with stationary phase *Lm* (ratio 1:5) in complete medium, or PMN were incubated in complete medium alone. Subsequently, cells were washed with ice-cold PBS (1045 x g, 5 min, 4°C) and stored on ice. 5 x $10^5$ cells were put into a 96-well V-bottom microtiter plate and washed with FACS buffer. Cells were incubated with either anti-PSR IgM (2 µg/100 µl) or anti-PSR IgG (2 µg/100µl) or appropriate isotype controls in FACS buffer for 20 min on ice. After washing, the secondary antibodies PE conjugated goat anti-mouse IgM (1 µg/100 µl) or PE conjugated rabbit anti-mouse IgG (1 µg/100 µl) were added for additional incubation of 20 min on ice. Subsequently, cells were washed with FACS buffer and resuspended in 400 µl 1% paraformaldehyde-PBS and analysed with FACS-Calibur® flow-cytometer using CellQuest Pro® software. Similar experiments were performed to investigate the PSR expression on MΦ.

For western blot analysis, protein from 1 x $10^6$ freshly isolated PMN was separated using SDS-PAGE (see 2.2) and blotted (see 2.3). For PSR detection, the membrane was incubated either with primary anti-PSR IgM (1:3000), anti-PSR IgG (1:1000) or commercial anti-PSR IgG (1:500) at 4 °C overnight. After extensive washing, the membranes were incubated with HRP coupled secondary antibodies: polyclonal goat anti-mouse IgM (for detection of anti-PSR IgM), or polyclonal goat anti-mouse IgG (for detection of anti-PSR IgG), or polyclonal goat anti-rabbit IgG (for detection of commercial anti-PSR IgG).

## 2.12 Statistical analysis

Statistical calculations were performed using Microsoft Exel software (version 2000). Infection rates, cytokine contents, endpoint titration measurements and the footpad swelling of mice are presented as mean ± standard deviation (SD).

# III Results

## 1. Phosphatidylserine detection on *L. major* promastigotes

Data from Freitas Balanco and colleagues suggest that *Leishmania* amastigotes use phosphatidylserine (PS) in terms of an “apoptotic mimicry” to silence macrophages (Freitas Balanco et al., 2001). Other groups suggest an apoptosis-like system in *Leishmania* spp (Lee et al., 2002; Debrabant et al., 2003; Nguewa et al., 2004). Since promastigotes are the disease inducing form of the parasite, it was investigated whether *L. major* promastigotes can express PS on their membrane.

### 1.1 Phosphatidylserine is expressed on *L. major* promastigotes

The expression of PS on stationary phase *L. major* promastigotes was investigated using the PS binding protein annexin-V (AV) Alexa 568. PS was detected on *L. major*, as indicated by the red “patch-like” structures on the parasite (fig. 6). These patch-like structures were also observed to be present on the parasites flagellum (data not shown).

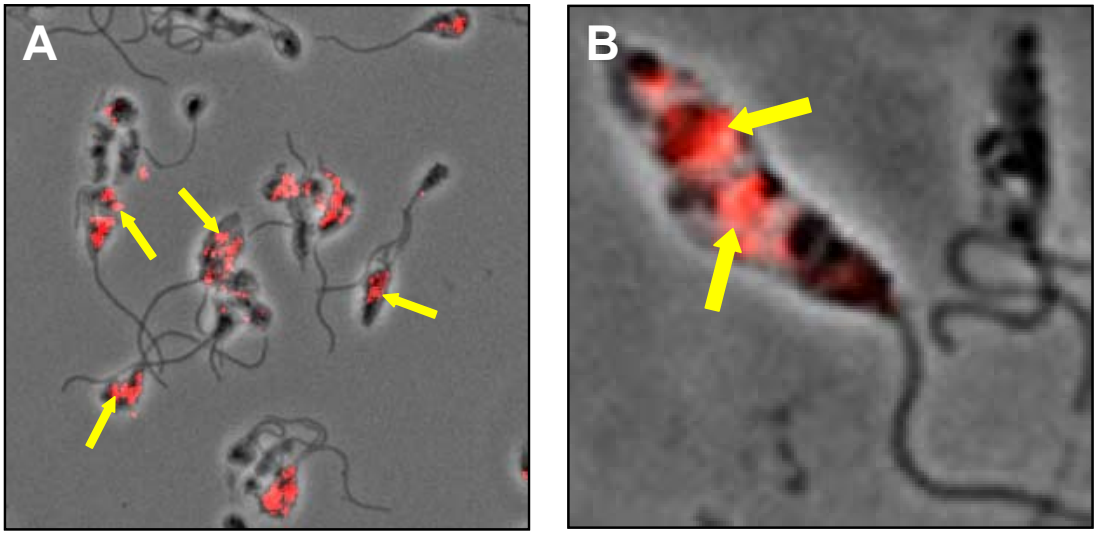


**Fig. 6: *L. major* promastigotes express phosphatidylserine.** Stationary phase *L. major* promastigotes were stained for PS with annexin-V (AV) Alexa 568 (red). AV stained parasites were analysed microscopically. The overlay of phase contrast micrographs and red fluorescent micrographs are depicted. The arrows indicate red “patch-like” structures on *L. major* promastigotes (magnification: x 630 (A), x 1000 (B)).

Since Raynal and Pollars described that the binding capacity of AV is calcium dependent (Raynal and Pollars, 1994), it was investigated whether AV binding to PS expressed on stationary phase *L. major* promastigotes is also calcium dependent. Therefore, *L. major* were stained with AV in the presence or absence of calcium. The data shown in fig. 7 demonstrate that in the presence of calcium 67.2% of stationary phase *L. major* promastigotes stained positive for PS. In the absence of calcium PS detection was abolished. Thus, AV binding to *L. major* promastigotes is calcium dependent.

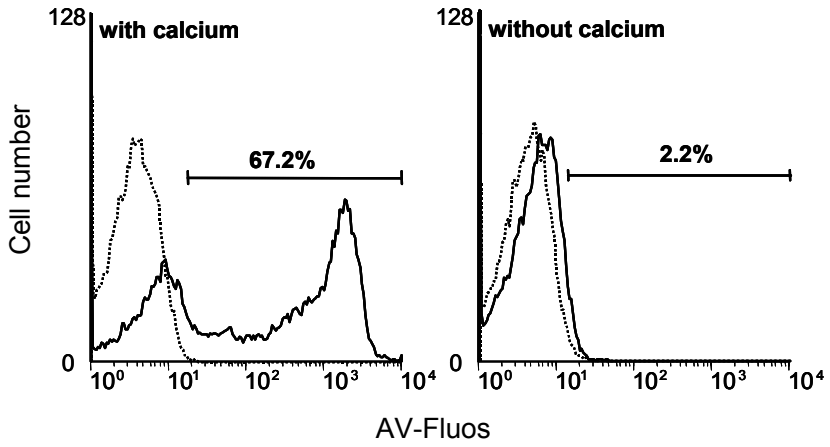


**Fig. 7: Annexin-V binding to phosphatidylserine on *L. major* promastigotes is calcium dependent.** AV Fluos (green) staining for PS was performed on stationary phase *L. major* promastigotes in the presence (left panel) and absence (right panel) of 5 mM $CaCl_2$. The ratio of AV stained parasites was assessed with FACS. The x-axis of the histograms shows the green fluorescence intensity. Dotted lines represent unstained parasite controls. Continuous lines represent AV stained *L. major* promastigotes. The ratio of AV Fluos stained parasites is shown. Representative histograms of three independent experiments are presented.

## 1.2 Anti-phosphatidylserine antibody also detects phosphatidylserine on the surface of *L. major* promastigotes

In addition to staining with AV, another possibility to assess PS expression is the use of a PS specific monoclonal antibody. As a positive control for PS expressing cells, apoptotic polymorphonuclear neutrophilic granulocytes (PMN) were used. Apoptotic PMN were stained with both AV Fluos and anti-PS antibody. As depicted in fig. 8, staining with AV Fluos revealed a ratio of 83.9% PS expressing apoptotic PMN. PS staining using a monoclonal anti-PS antibody demonstrated a ratio of 64.7% PS expressing apoptotic PMN. Similar staining experiments as for apoptotic PMN were performed with *L. major* promastigotes. As demonstrated in fig. 8, staining with AV Fluos detected a ratio of 46.3% PS expressing parasites. PS staining using the anti-PS antibody revealed a ratio of 35.7% PS expressing *L. major* promastigotes.

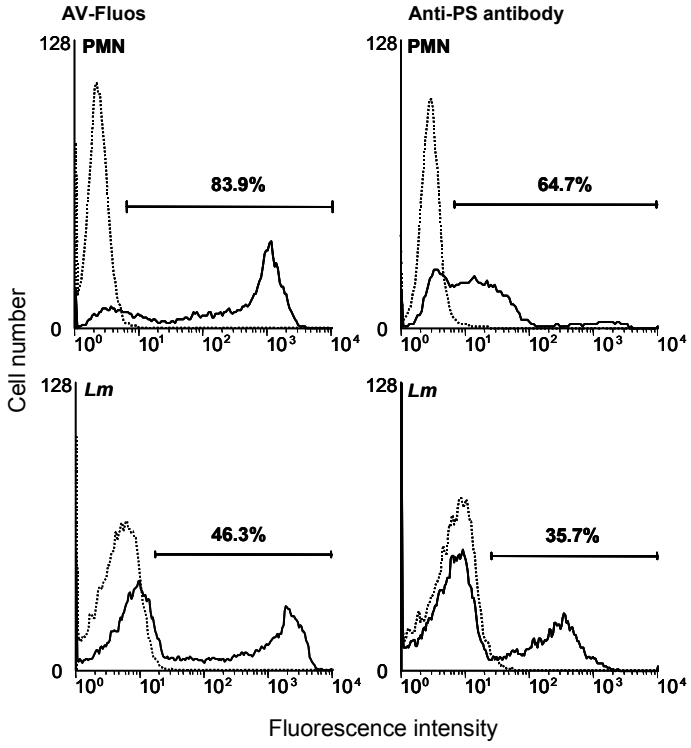


**Fig. 8: Detection of phosphatidylserine using a monoclonal anti-phosphatidylserine antibody.** For PS detection on apoptotic PMN (upper panels) and *L. major* promastigotes (day 5 of *in vitro* culture) (lower panels), FACS staining was performed using AV Fluos (left panels) and a monoclonal anti-PS antibody (right panels). Dotted lines represent the negative control (unstained cells or isotype control for anti-PS antibody). The anti-PS antibody was detected with a PE conjugated goat anti-mouse secondary antibody. Continuous lines represent stained cells. The ratio of positive stained cells is shown. Representative histograms of three independent experiments are presented.

## 1.3 The ratio of phosphatidylserine expressing *L. major* promastigotes increases during the growth period

In order to determine the growth curve of *L. major* promastigote during the period of culturing, the total number of parasites was determined every day. As depicted in fig. 9, the number of *L. major* promastigotes increases continuously form 1 x $10^6$ parasites per well at the beginning of the logarithmic phase (day 1) up to 25 x $10^6$ parasites per well in the stationary growth phase (day 9).

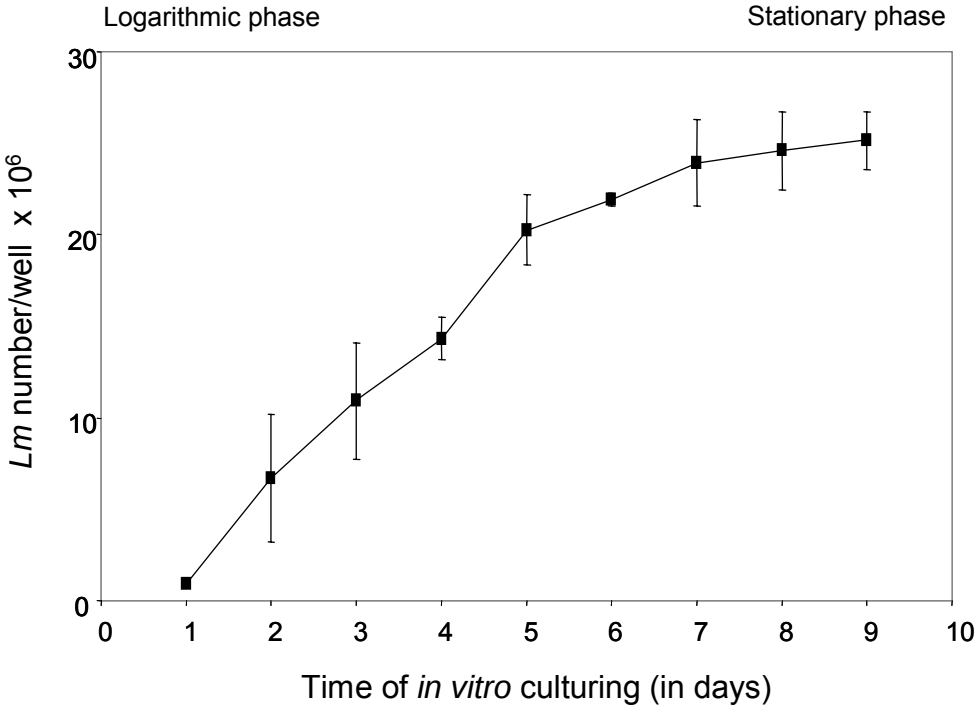


**Fig. 9: Growth curve of *L. major* promastigote cultures.** The total number of *L. major* promastigotes per well was determined microscopically during the culture period from day 1 to 9. The figure shows the mean ± SD of duplicate assays for every time point obtained from three independent experiments.

To analyse the PS expression on *L. major* promastigotes during the growth period, parasites were stained with AV at different days of culturing. AV binding was assessed using FACS analysis and fluorescence microscopy. The ratio of PS expressing *L. major* promastigotes increased from ~ 30% in the logarithmic growth phase (day 2) over ~ 50% at day 4 to 6 up to ~ 65% in stationary phase *L. major* cultures (day 7) (fig. 10).

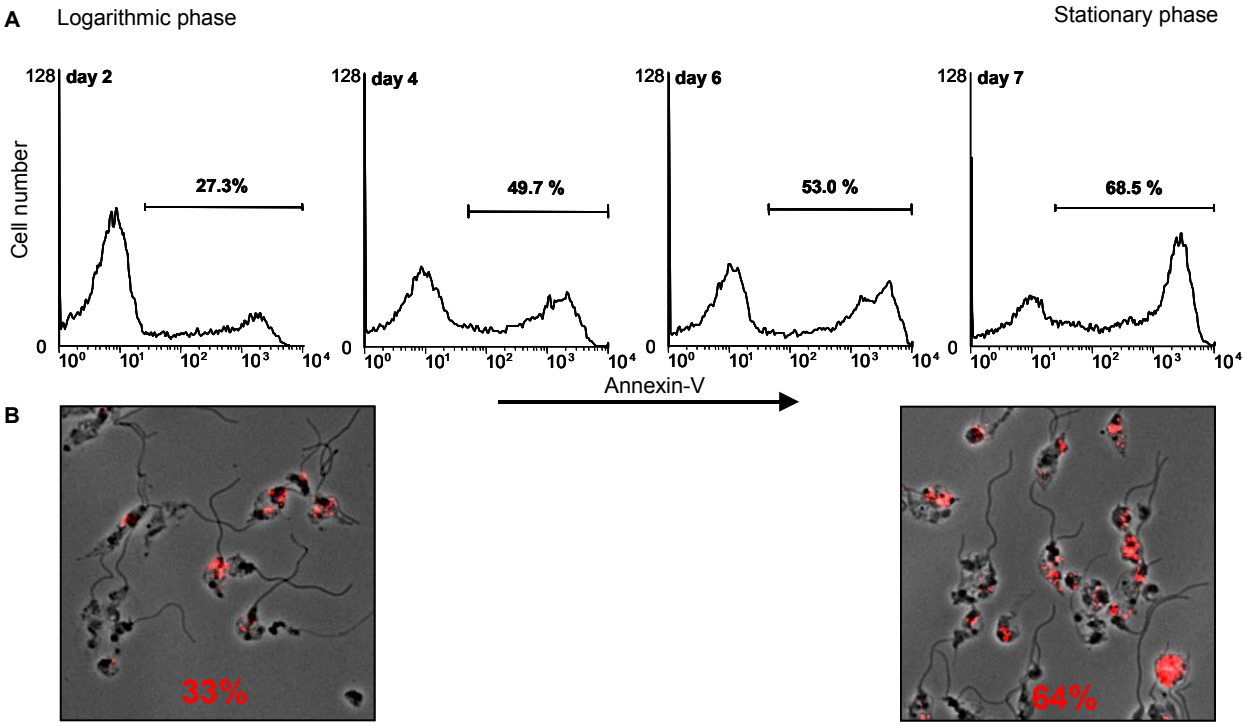


**Fig. 10: The ratio of phosphatidylserine expressing *L. major* promastigotes increases during the growth period.** *L. major* promastigotes were stained for PS with AV during the growth period from the logarithmic to the stationary growth phase (day 2, 4, 6 and 7). **A**, detection of PS expressing parasites using AV Fluos for FACS analysis. The x-axis shows the fluorescence intensity of stained *L. major* promastigotes. **B**, detection of PS expressing parasites using AV Alexa 568 (red) by microscopic evaluation of > 200 stained *L. major* promastigotes. The overlay of phase contrast micrographs and red fluorescent micrographs are presented. Magnification x 630. The ratio of PS expressing parasites is depicted. Representative data of three independent experiments are shown.

## 1.4 The ratio of phosphatidylserine expressing *L. major* promastigotes increases during metacyclogenesis

Metacyclogenesis was described as the development of infective *Leishmania* promastigotes occurring during the growth period from the logarithmic to the stationary phase (Giannini, 1974). According to the inability of infective promastigotes to agglutinate with peanut lectin agglutinin (PNA), non-agglutinated parasites were termed metacyclic (Sacks et al., 1985). In this study, infective *L. major* promastigotes are called metacyclic parasites. To quantify the ratio of metacyclic *L. major* promastigotes during metacyclogenesis, their number was assessed from day 4 to 7 of *L. major* cultures. As demonstrated in fig. 11, the ratio of metacyclic *L. major* increased continuously from 3.5% at day 4 up to 13.5% at day 7 of growth cultures.

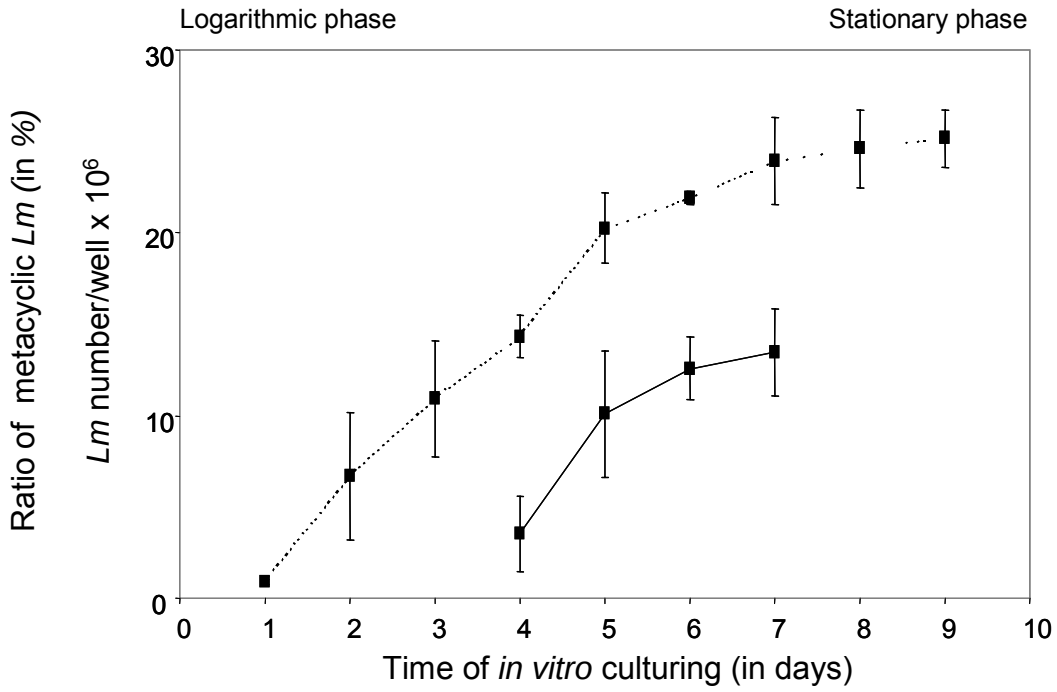


**Fig. 11: Ratio of metacyclic *L. major* promastigotes during metacyclogenesis.** Metacyclic *L. major* promastigotes were purified using peanut lectin agglutinin (PNA) at day 4 to 7 of promastigote cultures. The ratio of metacyclic parasites was determined microscopically. The y-axis represents the ratio of metacyclic *L. major* promastigotes (continuous line). The dotted line reminds of the growth curve of *L. major* promastigotes. The figure shows the mean ± SD of duplicate assays for every time point obtained from three independent experiments.

The finding that stationary phase *L. major* cultures contain high ratios of PS expressing promastigotes (see 1.3) suggests that PS might play a role for the infectivity of the parasite. Therefore, it was investigated whether the ratio of PS expressing metacyclic parasites increases during metacyclogenesis. Using PNA, metacyclic *L. major* were purified from *L. major* promastigote cultures, which were subsequently analysed for PS expression. As depicted in fig. 12, the ratio of PS expressing metacyclic *L. major* promastigotes continuously increased from 21.3% at day 4 up to 56.8% at day 7 of growth cultures.

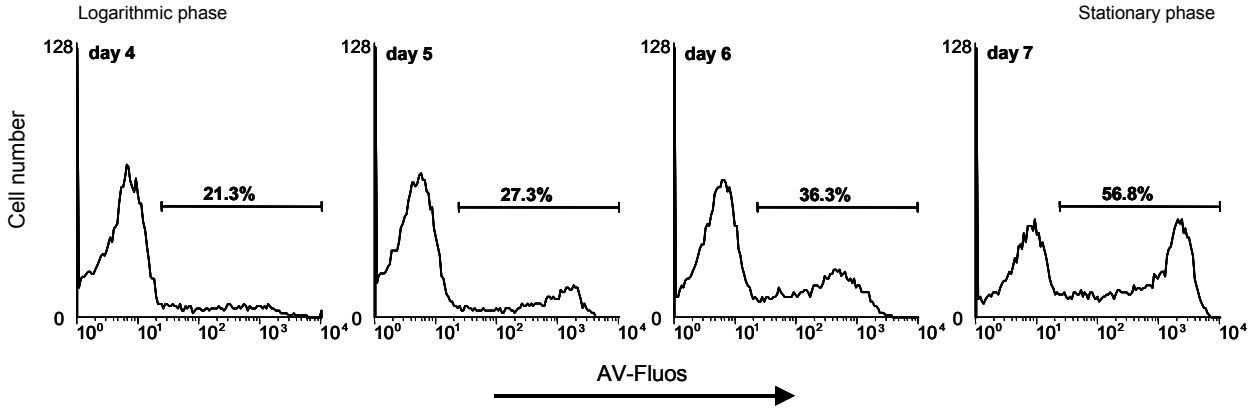


**Fig. 12: The ratio of phosphatidylserine expressing metacyclic *L. major* promastigotes increases during metacyclogenesis.** Metacyclic *L. major* promastigotes were purified using PNA. PS expression of metacyclic parasites was assessed using AV Fluos for FACS analysis. The x-axis of depicted histograms represents the fluorescence intensity of AV Fluos stained *L. major* promastigotes. The ratio of AV Fluos stained parasites is demonstrated. Representative histograms of three independent experiments are shown.

## 2. Detection of apoptosis in *L. major* promastigotes

The expression of PS on the outer leaflet of eukaryotic cell membranes is one of the characteristic features for the onset of apoptosis (Martin et al., 1995). The process of apoptosis is the transition from life to death. PS expression on *L. amazonensis* amastigotes was described as an active process of viable parasites, which was termed “apoptotic mimicry” (Freitas Balanco et al., 2001). In order to determine whether PS expressing *L. major* promastigotes are viable or apoptotic, first of all live/dead staining experiments were performed with stationary phase *L. major* promastigotes. It was found that 90% of the parasites were viable (data not shown). Moreover, a ratio of about 68% of stationary phase promastigotes was PS positive (see results, 1.3). These data led to the suggestion that viable *L. major* promastigotes might express PS in terms of an apoptotic mimicry. To prove this hypothesis, it was investigated whether *L. major* promastigotes enter apoptosis during the growth period using TUNEL assay. Additionally, the viability of PS expressing *L. major* promastigotes was investigated with end point titration method. The morphology of PS expressing parasites was observed using electron microscopy.

### 2.1 *L. major* promastigotes enter apoptosis during the growth period

The occurrence of late apoptotic DNA fragmentation in *L. major* promastigotes was investigated during the growth period from the logarithmic to the stationary phase using TUNEL assay. As demonstrated in fig. 13, the ratio of TUNEL positive *L. major* promastigotes continuously increased from 1.2% at day 2 up to 47.5% at day 7 of parasite growth cultures.

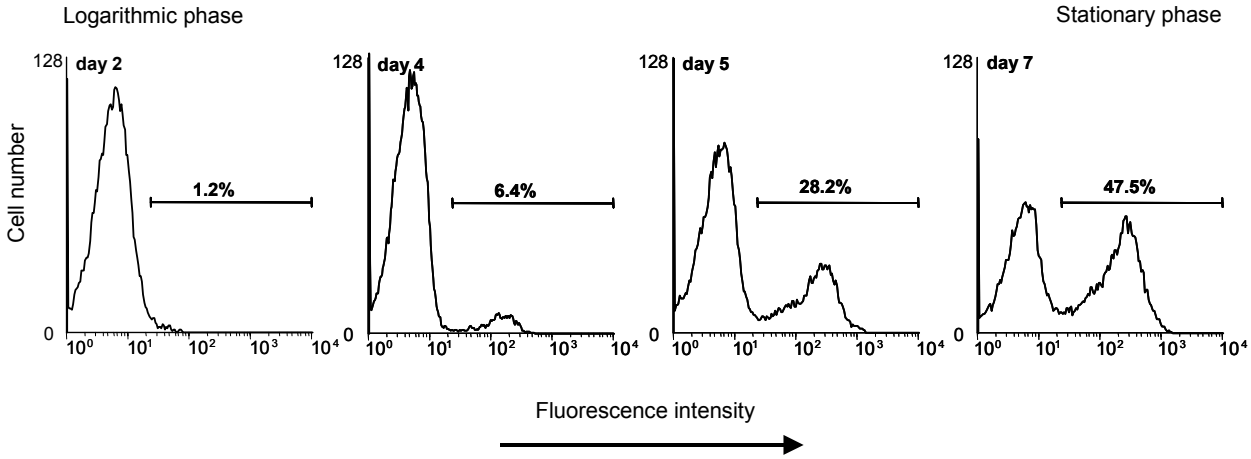


**Fig. 13: The ratio of apoptotic *L. major* promastigotes increases during the growth period.** The ratio of apoptotic *L. major* promastigotes from day 2, 4, 5 and 7 of growth cultures was assessed using TUNEL assay. TUNEL positivity was measured by FACS analysis. The x-axis shows the green fluorescence intensity of TUNEL positive parasites. The ratio of TUNEL positive parasites is depicted. Representative histogram profiles of three independent experiments are shown.

Taken together, the ratio of PS expressing *L. major* promastigotes and the ratio of TUNEL positive parasites increase almost parallel during the growth period from the logarithmic to the stationary phase (fig. 14).

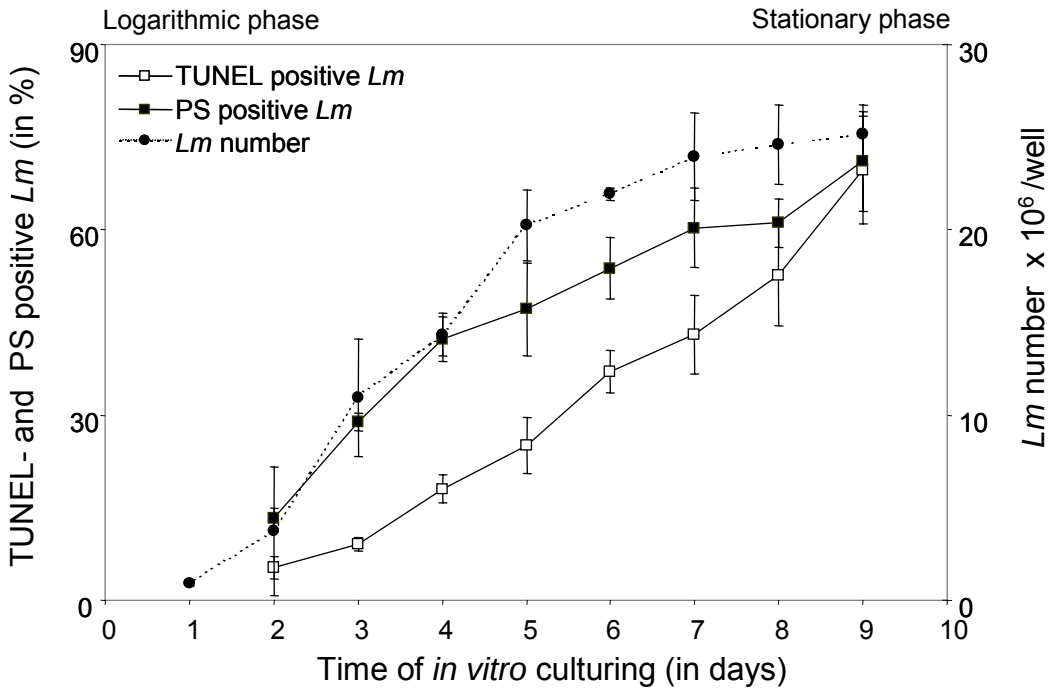


**Fig. 14: The ratio of TUNEL positivity, phosphatidylserine expression and *L. major* growth in number similarly increase during the culture period.** The number of *L. major* promastigotes was determined microscopically (closed circles). The ratio of PS expressing parasites was detected using AV Fluos (closed squares) and the ratio of apoptotic promastigotes was assessed by TUNEL staining (open squares) and analysed by FACS. The ratios of TUNEL positivity and PS expression are indicated at the left y-axis. The dotted line reminds of the growth curve of *L. major* promastigotes (right y-axis). The figure shows the mean ± SD of duplicate assays for every parameter and time point obtained from three independent experiments.

## 2.2 Phosphatidylserine expressing *L. major* promastigotes are not viable

The observation that the ratio of both the PS expressing *L. major* promastigotes and the TUNEL positive parasites similarly increase during the period of culturing led to the suggestion that PS expressing promastigotes enter apoptosis. In order to investigate the features of PS positive *L. major* (*Lm* PS+) and PS negative *L. major* (*Lm* PS-) in pure populations, the parasites were purified using magnetic cell sorting (MACS). To ensure that this method is suitable for the PS dependent purification of the parasites, purified populations of *Lm* PS+ and *Lm* PS- were investigated for their PS expression using AV Fluos. FACS analysis of AV stained *L. major* populations revealed a ratio of 85% PS expressing parasites in *Lm* PS+ samples and a ratio of

only 5% PS expressing promastigotes in *Lm* PS- samples (fig 15). Moreover, gated on *Lm* PS+ promastigotes in FACS analysis, these parasites displayed a lower forward-angle light scatter (FSC) as compared to *Lm* PS- parasites (data not shown).

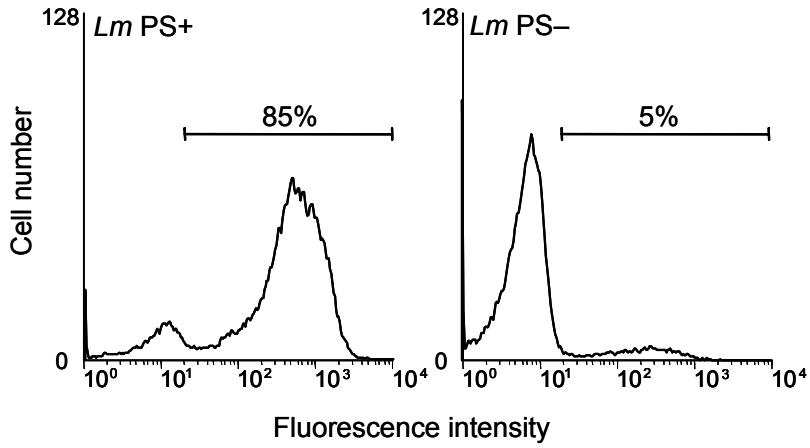


**Fig. 15: Phosphatidylserine expression of MACS separated *L. major* promastigotes.** Stationary phase *L. major* promastigotes were separated using MACS Annexin-V Microbead Kit and tested for PS expression with AV Fluos in FACS analysis. The x-axis of the depicted histograms shows the green fluorescence intensity of AV stained parasites. The ratios of AV positive staining purified PS positive *L. major* (*Lm* PS+) and PS negative *L. major* (*Lm* PS-) are indicated. Representative histograms of three independent experiments are shown.

The viability of PS expressing and PS depleted *L. major* promastigotes was investigated using end point titration with MACS purified *Lm* PS+ or *Lm* PS- populations. The total number of viable parasites was determined. As demonstrated in fig. 16, *Lm* PS- showed a total parasite number of 92000 ± 52300, whereas *Lm* PS+ only revealed a number of 102 ± 52 viable parasites.

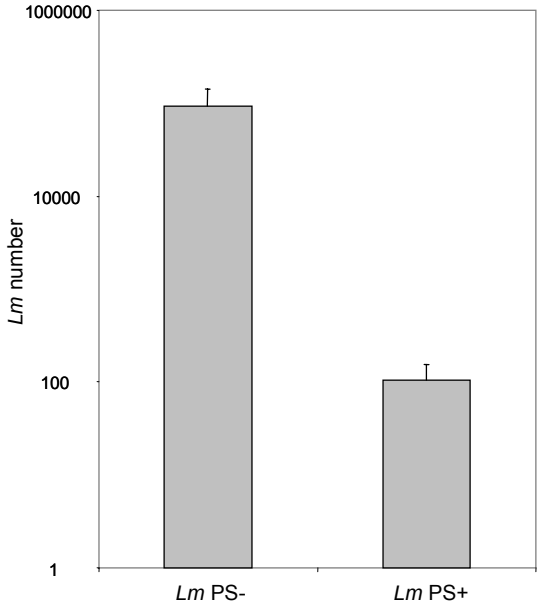


**Fig. 16: Viability of phosphatidylserine positive and phosphatidylserine negative *L. major* promastigotes.** Stationary phase *L. major* promastigotes were purified with MACS for PS expression. Purified *Lm* PS- and *Lm* PS+ were compared regarding their viability using end point titration. The bars represent the total number of viable parasites (see methods, 2.8). The mean ± SD of three independent experiments is shown (logarithmic scale).

Concerning the motility of *L. major* promastigotes, viable parasites are capable to swim in the surrounding medium by rapid movements of their flagellum. The microscopic investigation of MACS purified PS positive *L. major* promastigote populations revealed that PS positive parasites are incapable to move (data not shown).

## 2.3 Phosphatidylserine expressing *L. major* promastigotes show "apoptosis-like" morphology

To detect "apoptosis-like" features of PS expressing *L. major* promastigotes, a closer look was taken on the morphology of MACS purified PS negative and PS positive parasites using electron microscopy. The presented micrographs revealed that PS negative parasites have a granularity and a nuclear morphology typical for viable cells. They also showed fully intact tubular membrane structures. On the contrary, PS positive *L. major* revealed less granularity and the nuclei showed a rounded shape. Furthermore, the diameters of PS positive parasites appeared smaller in comparison to the PS negative parasites. The membrane of PS positive *L. major* showed a disturbed tubular organisation (fig. 17).

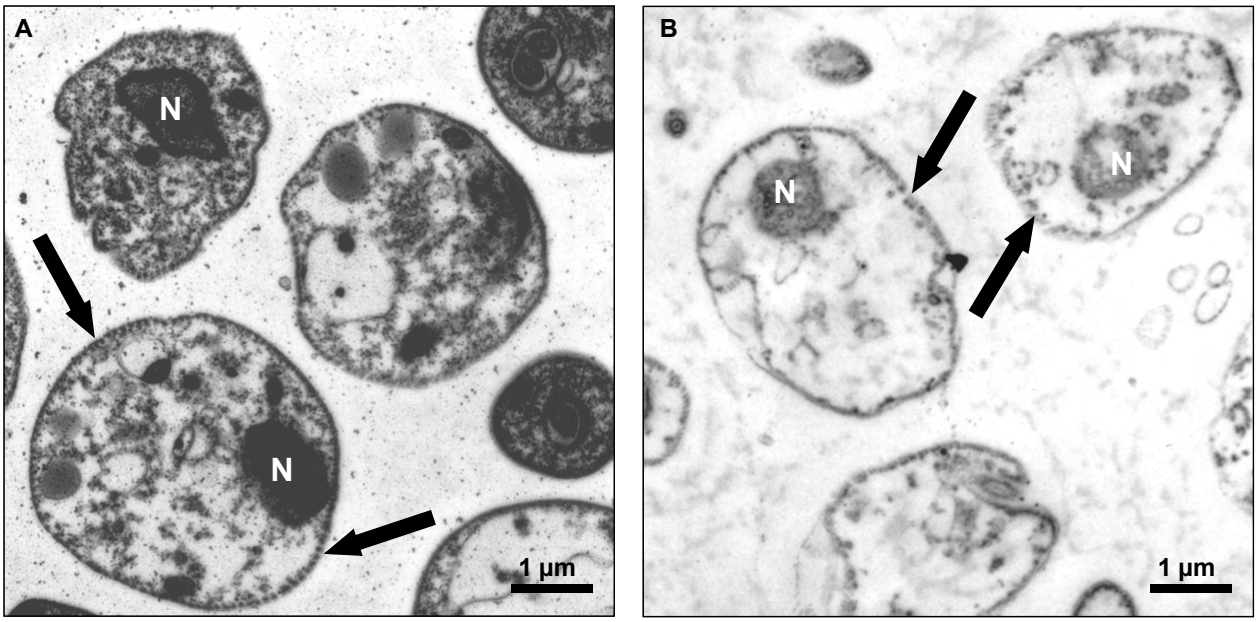


**Fig 17: Electron micrographs of phosphatidylserine negative and phosphatidylserine positive *L. major* promastigotes.** Cross-section electron micrographs of MACS purified PS negative *L. major* promastigotes (**A**) and PS positive parasites (**B**) are shown. The arrows indicate the tubular membrane structures. N = nucleus. Magnification x 7812. Electron microscopy was kindly supported by PD Dr. Klinger (Department of Anatomy, University of Luebeck).

## 3. Coincubation of phosphatidylserine expressing *L. major* promastigotes with PMN

Stationary phase *L. major* promastigotes were shown to contain high amounts of phosphatidylserine (PS) expressing parasites (see results, 1.3). Previous findings of different groups demonstrated that PS, either expressed on apoptotic cells or on *Leishmania amazonensis* amastigotes, possess immunosilencing capacities in macrophages (Fadok et al., 1998, 2001; Krahling et al., 1999; Callahan et al., 2000; Freire-de-Lima et al., 2000; Freitas Balanco et al., 2001). Therefore, it was investigated whether PS expression on *L. major* promastigotes plays a role in PMN silencing.

## 3.1 Phosphatidylserine expression on *L. major* promastigotes supports the infection rate of PMN

In order to investigate whether PS expression on *L. major* promastigotes has a function for the parasite uptake by PMN, coincubation experiments were performed. The “infection rate” as an objective parameter for these experiments is defined as the number of intracellularly present parasites. Since logarithmic phase *L. major* promastigotes were shown to contain lower amounts of PS expressing parasites as compared to stationary phase promastigotes, PMN were coincubated with parasites either in logarithmic or stationary growth phase. As demonstrated in fig. 18A, 41 ± 3% of PMN are infected after coincubation with logarithmic phase parasites. The infection rate was significantly higher (58 ± 7%) if PMN were coincubated with *L. major* promastigotes of the stationary growth phase. In addition, it was investigated whether the high amounts of PS expressing parasites available in stationary phase *L. major* cultures influences the infection rate. Therefore, PMN were coincubated with stationary phase *L. major* either untreated or pre-treated with annexin V (AV) to block PS. As demonstrated in fig. 18B, AV pre-treatment of the parasites significantly reduced the infection rate of PMN from 58 ± 9% down to 27 ± 12%.

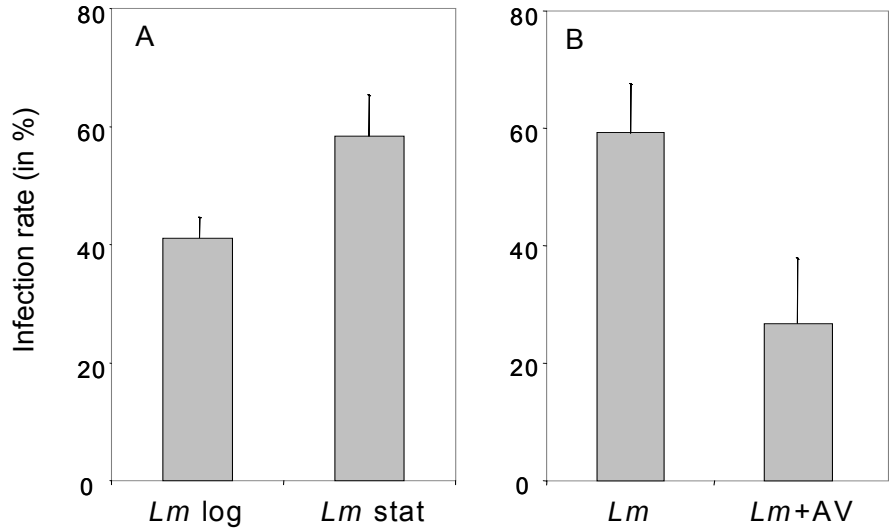


**Fig. 18: Phosphatidylserine expression on *L. major* promastigotes increases the infection rate of PMN.** Freshly isolated PMN were coincubated for 3 h with *L. major* promastigotes of logarithmic or stationary phase cultures (**A**). In addition, PMN were coincubated with stationary phase promastigotes either untreated or pre-treated with recombinant annexin-V (AV) (**B**). After the coincubation period, infected PMN were stained with May-Gruenwald-Giemsa solution. Cytocentrifuged preparations were analysed under the light microscope and the infection rate was determined in > 200 cells. The figures show the mean ± SD of duplicate assays obtained from three independent experiments.

## 3.2 Phosphatidylserine expression on *L. major* promastigotes does not affect the oxidative burst in PMN

PS expression on *Leishmania amazonensis* is involved in the impairment of the oxidative burst in MΦ (Freitas Balanco et al., 2001) but also in the parasite uptake by PMN (see results 3.1). These data raised the question, whether PS positive *L. major* promastigotes achieve silencing properties through prevention of the oxidative burst. In this context, PS negative *L. major* were suggested to induce the oxidative burst, whereas PS positive *L. major* were suggested to prevent it. To test this hypothesis, PMN were coincubated with either stationary phase promastigotes or with MACS purified PS negative or PS positive parasite populations. The production of reactive oxygen radicals was measured using dihydrorhodamine 1, 2, 3 (DHR). As shown in fig. 19, none of the *L. major* samples induced an oxidative burst in PMN.

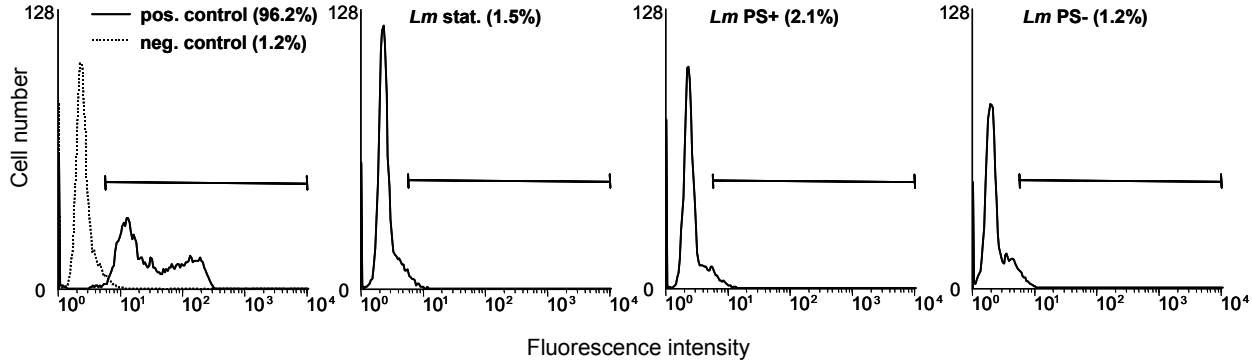


**Fig. 19: Phosphatidylserine expression on *L. major* promastigotes has no impact on the oxidative burst in PMN.** PMN were incubated for 15 min with stationary phase *L. major* promastigotes, or with MACS purified PS negative (*Lm* PS-) or PS positive (*Lm* PS+) promastigote populations. As a positive control, PMN were stimulated with 10 ng/ml PMA. The production of reactive oxygen radicals was assessed using dihydrorhodamine 1, 2, 3 (DHR) in FACS analysis. The x-axis shows the green fluorescence intensity of stained PMN corresponding to the production of reactive oxygen radicals. The ratios of positive stained PMN are depicted. Representative histogram profiles of three independent experiments are shown.

### 3.3. Phosphatidylserine expression of *L. major* promastigotes determines the amount of TGF-β1 and TNF-α release by PMN

Leishmanial PS does not affect the oxidative burst in PMN (see results, 3.2), but PS recognition by MΦ regulates the production of anti-inflammatory TGF-β1 and pro-inflammatory TNF-α (Savill et al., 1993; Voll et al., 1997; Fadok et al., 1998, 2001; Serinkan et al., 2005). According to these data, the silencing effect of *L. major* promastigotes in PMN was suggested to be achieved by the regulation of TGF-β1 and TNF-α. Therefore, it was investigated whether PS expression on *L. major* influences the cytokine production of PMN. Freshly isolated PMN were coincubated either with stationary phase *L. major* (70% PS+), MACS separated *L. major* samples (*Lm* PS- (7% PS+), *Lm* PS+ (95% PS+)), or PMN were incubated in medium alone. Additionally, PMN were also stimulated with stationary phase promastigotes after pre-treatment with annexin-V (AV). The supernatants of these cocultures were analysed for their content of TGF-β1 and TNF-α. The release of TGF-β1 by PMN increased correspondingly to the ratio of PS expressing *L. major* promastigotes (fig. 20A). The secretion of TNF-α showed an inverse correlation to the ratio of PS expressing *L. major* (fig. 20B). In comparison to the PMN coincubation with stationary promastigotes (70% PS+), a parasite pre-treatment with AV significantly reduced the release of TGF-β1 from 349 ± 46.8 pg/ml down to 174 ± 68.3 pg/ml (fig. 20A). In contrast, AV pre-treatment of the parasites increased the TNF-α release by PMN from 23 ± 7.5 pg/ml up to 32 ± 5.5 pg/ml (fig. 20B).

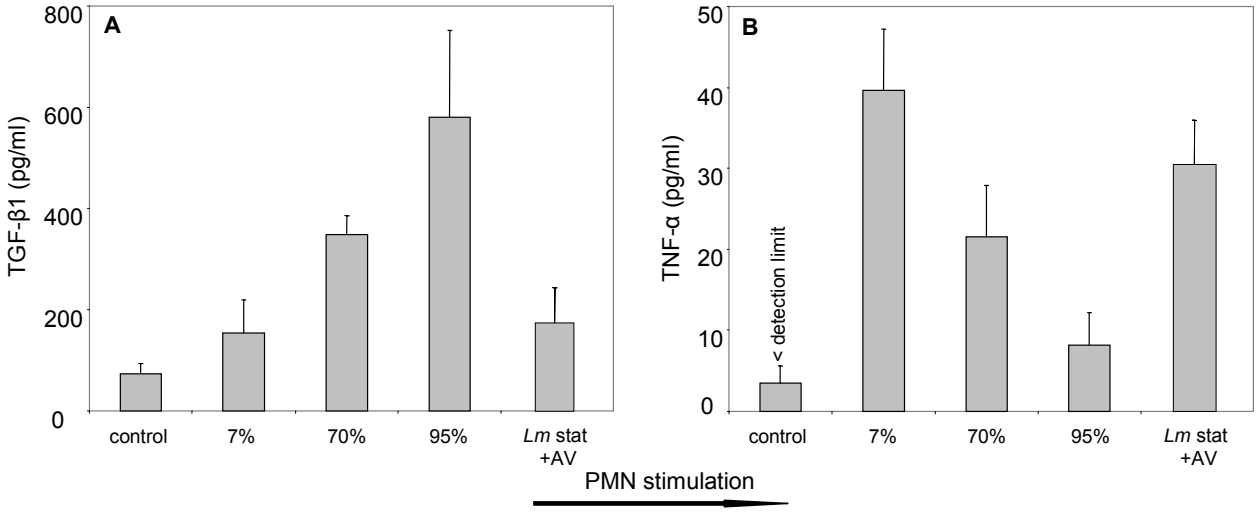


**Fig. 20: Phosphatidylserine expression on *L. major* promastigotes determines the amount of TGF-β1 and TNF-α release by PMN in coculture experiments.** PMN were coincubated for 18 h with stationary phase *L. major* promastigotes containing different ratios of PS positive parasites (7%, 70%, 95%), or with stationary phase *L. major* that were pre-treated for half an hour on ice with AV (*Lm* stat + AV). As a control, PMN were also incubated in medium alone. The cytokine content of the coculture supernatants was measured using ELISA. The figure shows the mean ± SD of duplicate assays for each condition obtained from three independent experiments.

### 3.4. PMN release bioactive TGF-β1 after stimulation with *L. major* promasigotes

TGF-ß1 is synthesized in a latent form that must be activated to be recognized by cell surface receptors and to trigger biological responses (Massagué et al., 1990). TGF-β receptor stimulation with bioactive TGF-β1 leads to the phosphorylation of the intracellular adapter protein Smad2 (Derynck et al, 1998). This fact allowed to analyse, whether TGF-β1 in coculture supernatants of PMN with *L. major* promastigote samples (see above) was bioactive. Western blot analysis was performed to detect Phospho Smad2 in TGF-β sensitive lung fibroblast cells (Mv1Lu) (Dr. Haller, Technical University of Munich, Freising-Weihenstephan). The Mv1Lu cells were stimulated with supernatants from PMN that were coincubated with stationary phase promastigotes or with MACS purified PS negative parasites. As a control, Mv1Lu cells were exposed to 1.5 ng TGF-β1 or cultured in medium alone. Smad2 phosphorylation did not occur in Mv1LU cells cultured in medium alone. The 58 kDa Smad2 posphorylation product was evident in Mv1Lu cells after stimulation

with TGF-β1 but also after exposure to both coculture supernatants. The Smad2 phosphorylation product showed an apparent stronger signal after Mv1Lu exposure to supernatant from PMN that were coincubated with stationary phase *L. major* promastigotes containing a ratio of 70% PS expressing parasites (fig. 21).

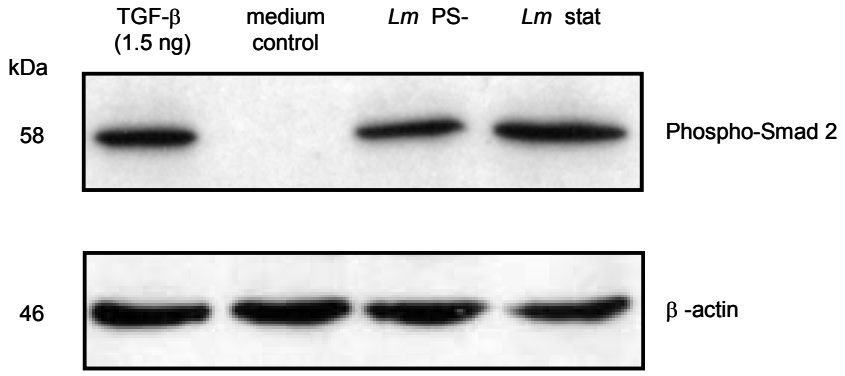


**Fig. 21: PMN release bioactive TGF-β1 after stimulation with *L. major* promastigotes.** Mv1LU cells were stimulated with supernatants deriving from PMN (10 x $10^6$ PMN/ml) that were previously coincubated for 18 h with stationary phase *L. major* promastigotes (*Lm* stat) or purified PS negative *L. major* (*Lm* PS-) samples. The lysat of 5 x $10^5$ stimulated Mv1Lu cells per line was separated in 12% denaturing SDS-PAGE. Phospho Smad2 was detected in western blot analysis using a polyclonal rabbit anti-human Phospho Smad2 antibody. Equal protein loading was determined by reprobing the membrane for β-actin using a polyclonal rabbit anti-human β-actin antibody. Representative blot from three independent experiments is shown.

## 4. Coincubation of phosphatidylserine expressing *L. major* promastigotes with macrophages

In order to investigate the ability of PS expressing *L. major* promastigotes to silence the infection of macrophages (MΦ), MΦ were coincubated with stationary phase *L. major* promastigotes either untreated or pre-treated with annexin-V (AV). For coincubation experiments and cytokine measurements similar experimental setups were used as described before for PMN (see 3.1 and 3.3). Again, the term “infection rate” is defined as the number of intracellularly present parasites.

## 4.1 Phosphatidylserine expression on *L. major* promastigotes supports the infection rate of macrophages

As depicted in fig. 22, MΦ that were coincubated with stationary phase *L. major* promastigotes showed an infection rate of 69 ± 9.6%. Blocking of PS with AV resulted in a significant reduction of the infection rate down to 43 ± 10.7%.

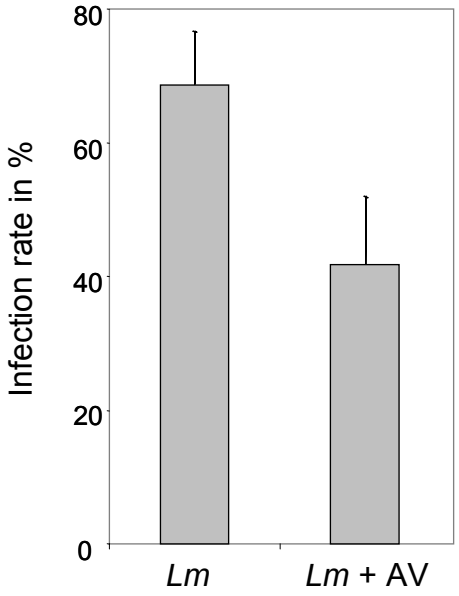


**Fig. 22: Blocking of phosphatidylserine on *L. major* promastigotes reduces the infection rate of macrophages.** MΦ were coincubated for 3 h with stationary phase *L. major* either untreated (*Lm*) or pre-treated with recombinant AV (*Lm* + AV). After the coincubation period, infected MΦ were stained with May-Gruenwald-Giemsa solution. Stained MΦ were observed under the light microscope and the infection rate of > 200 cells was determined. The infection rate of MΦ is indicated at the y-axis. The figure shows the mean ± SD of duplicate assays for each condition obtained from three independent experiments.

## 4.2 Annexin-V pre-treatment of stationary phase *L. major* promastigotes blocks TGF-β1 release and enhances TNF-α secretion by macrophages

Coincubation of MΦ with stationary phase *L. major* promastigotes induced a TGF-β1 release of 545 ± 53.4 pg/ml. Pre-treatment of the parastites with AV reduced the TGF-β1 amount in the supernatants down to 328 ± 89.5 pg/ml (fig. 23A).

The measurement of TNF-α revealed that stationary phase *L. major* promastigotes induced a TNF-α secretion of 55 ± 46.2 pg/ml, whereas the amount was increased up to 144 ± 34.4 pg/ml if PS was blocked on the parasites (fig. 23B).

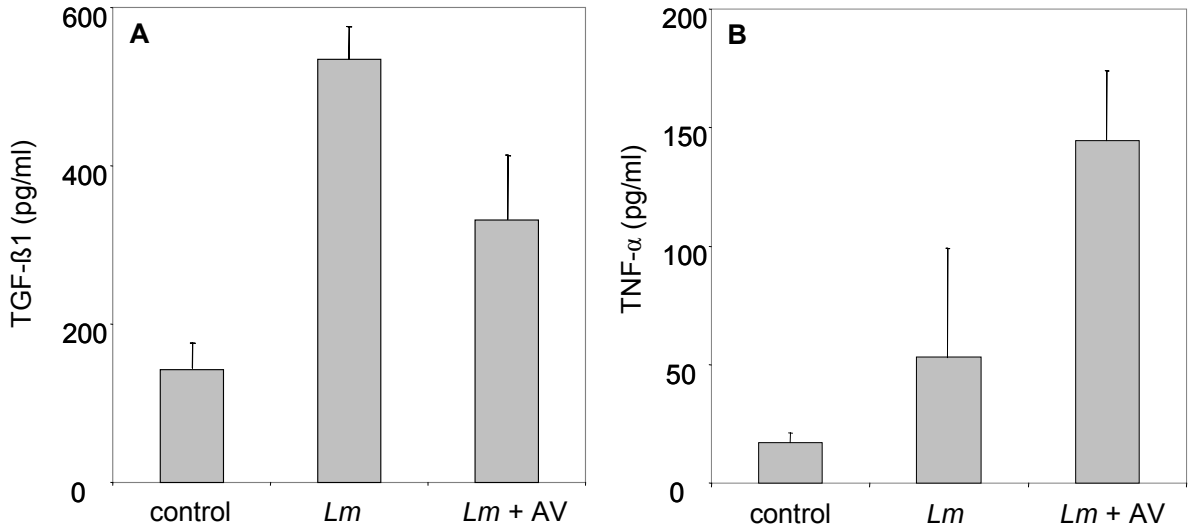


**Fig. 23: Phosphatidylserine blocking on stationary phase *L. major* promastigotes with annexin-V reduces TGF-β1 release and enhances TNF-α secretion by macrophages.** MΦ were coincubated for 18 h with stationary phase *L. major* promastigotes either untreated (*Lm*) or pre-treated for half an hour on ice with AV (*Lm* + AV), or cells were cultured in medium alone. The TGF-β1 content (**A**) and TNF-α content (**B**) of the coculture supernatants were measured using ELISA. The figures show the mean ± SD of duplicate assays obtained from three independent experiments.

## 5. Phosphatidylserine expressing apoptotic *L. major* promastigotes provide survival advantages for intracellular viable parasites in PMN

PS expressing apoptotic *L. major* promastigotes were shown to exert immunosilencing properties in PMN due to an up-regulation of TGF-β1 and a down-regulation of TNF-α (see results 3.3). These findings led to the suggestion that intracellularly viable parasites might profit from the anti-inflammatory milieu in terms of a facilitation of intracellular parasite survival. To test this hypothesis, PMN were coincubated with equal numbers of either stationary phase *L. major* or PS depleted stationary phase promastigotes. After 3 h, extracellular parasites were washed away and infected PMN were cultured for 18 h and 42 h. The infection rate of PMN is indicated in fig. 24. Over the time period from 3 h, 18 h to 42 h after infection, the infection rate remained almost constant in PMN infected with stationary phase *L.*

*major* (55 ± 3.9 (3 h) → 53 ± 6.3 (18 h) → 51 ± 4.2 (42 h) % of infection). In contrast, the infection rate was reduced during incubation period if PMN were coincubated with PS negative parasites (57 ± 7 (3 h) → 51 ± 6.4 (18 h) → 38 ± 7.5 (42 h) % of infection).

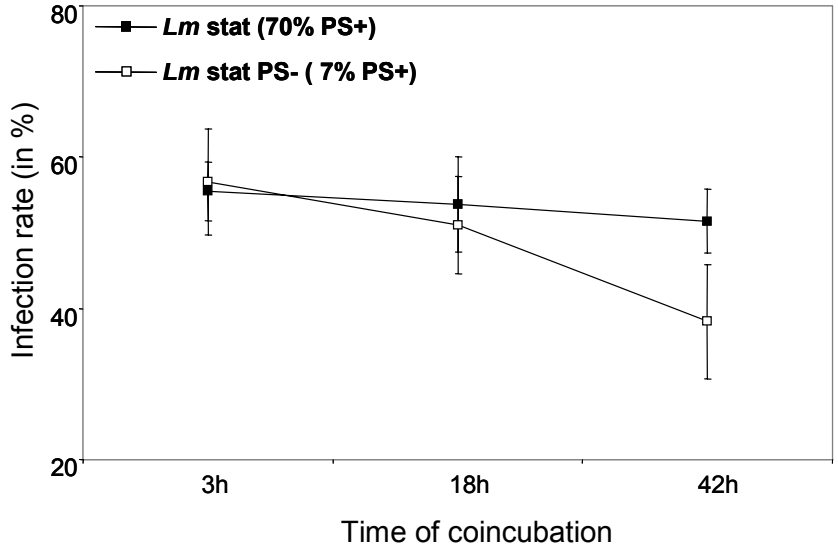


**Fig. 24: Phosphatidylserine expressing apoptotic *L. major* promastigotes maintain the infection rate of PMN.** PMN were coincubated with stationary phase *L. major* (*Lm* stat) (70% PS+) or PS negative stationary phase *L. major* (*Lm* stat PS-) (7% PS+). The infection rate was determined by microscopic evaluation of > 200 SYTO-16 stained cells on cytocentrifuged slides. The mean ± SD of the infection rate is shown for four independent experiments.

In order to quantify the number of intracellular surviving parasites, end point titration experiments were performed. If PMN were coincubated with stationary phase *L. major* promastigotes the number of parasites kept almost the same level over the time period from 3 h, 18 h to 42 h after infection (430 ± 73 (3 h) → 417 ± 79 (18 h) → 404 ± 39 (42 h) *Lm*/1000 PMN). In contrast, the parasite number significantly decreased over the time period if PMN were coincubated with PS negative parasite samples (409 ± 68 (3 h) → 342 ± 124 (18 h) → 257 ± 73 (42 h) *Lm*/1000 PMN) (fig. 25).

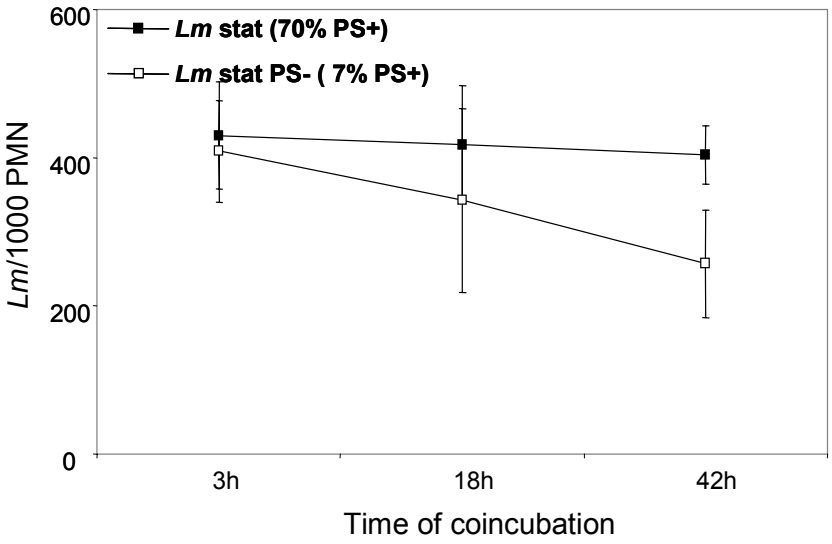


**Fig. 25: Phosphatidylserine expressing apoptotic *L. major* promastigotes provide survival advantages for intracellular viable parasites in PMN.** End point titration was performed with PMN that were previously coincubated with stationary phase *L. major* (*Lm* stat) (70% PS+) or with PS negative stationary phase *L. major* (*Lm* stat PS-) (7% PS+). The capability of *L. major* to survive inside PMN is represented by the relation of the viable parasite number per 1000 PMN (y-axis) over the time (x-axis). The mean ± SD is depicted for four times repeated assays obtained from four independent experiments.

To investigate how many PS expressing *L. major* promastigotes are needed for efficient maintenance of the infection rate and support of the parasite survival inside PMN over the time, parasite samples containing a ratio of 50%, 30% or 20% PS expressing *L. major* promastigotes were used for coincubation experiments. As depicted in fig. 26, a coincubation period from 18 h to 42 h led to a light reduction of the infection rate if PMN were coincubated with a parasite sample with a ratio of 50% PS expressing *L. major* (54% (18 h) → 51% (42 h)). In case of PMN infection with a parasite sample with a ratio of 30% PS expressing promastigotes the infection rate was reduced from 49% (18 h) to 43% (42 h). However, the strongest reduction of the infection rate was detected if PMN were coincubated with a parasite sample containing 20% PS expressing promastigotes (51% (18 h) → 35% (42 h)).

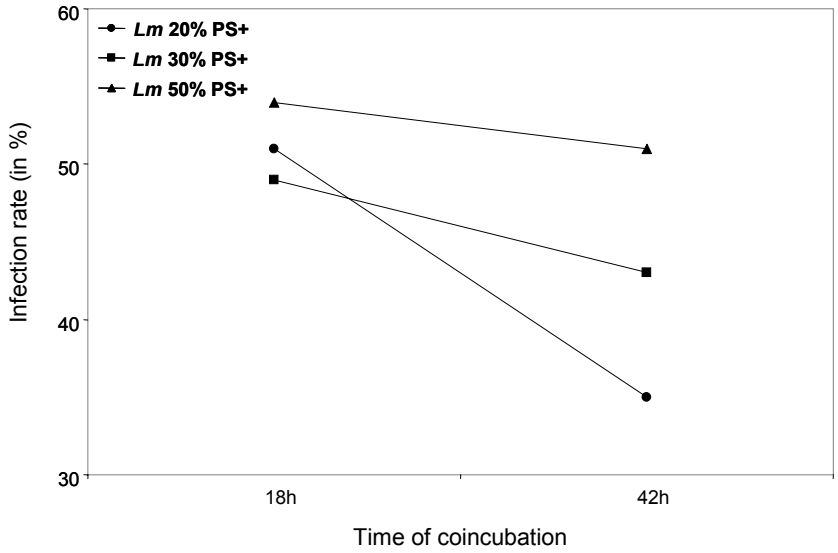


**Fig. 26: The maintenance of the infection rate of PMN with *L. major* over the time correlates with the ratio of phosphatidylserine expressing parasites.** PMN were coincubated with *L. major* samples containing a ratio of 20%, 30% or 50% PS expressing parasites. The infection rate was determined by microscopic evaluation of > 200 SYTO-16 stained cells on cytocentrifuged slides. The figure shows the infection rate (y-axis) during the time of coincubation (x-axis) for one out of four similar experiments.

Using end point titration experiments, the number of intracellularly surviving parasites was determined over a time period from 18 h to 42 h. As depicted in fig. 27, the number of viable parasites was maintained if PMN were infected with a parasite sample containing 50% PS expressing *L. major* (393 (18 h) → 387 (42 h) *Lm*/1000 PMN). PMN coincubation with a parasite sample containing a ratio of 30% PS expressing *L. major* showed a decrease of the parasite number from 392 *Lm*/1000 PMN (18 h) down to 330 *Lm*/1000 PMN (42 h). The strongest reduction of the viable parasite number was detected if PMN were infected with a parasite sample with a ratio of only 20% PS expressing *L. major* (392 (18 h) → 303 (42 h) *Lm*/1000 PMN).

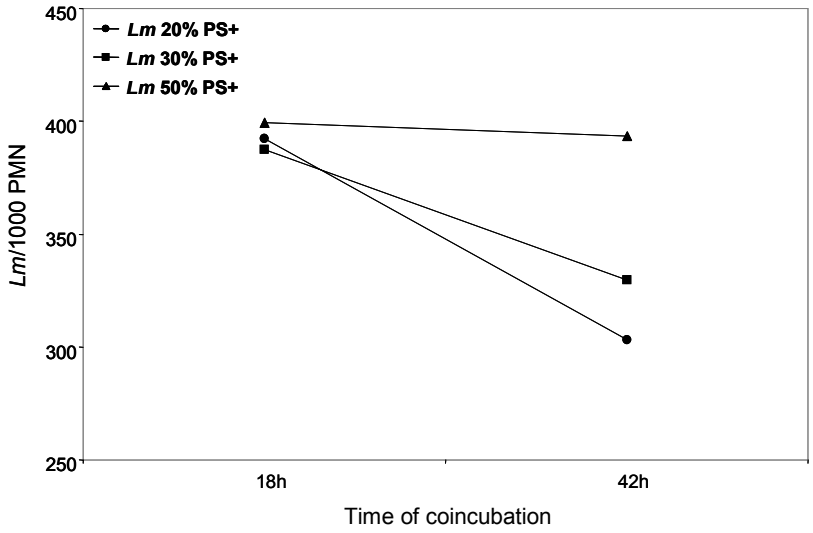


**Fig. 27: The maintenance of *L. major* survival inside PMN over the time correlates with the ratio of phosphatidylserine expressing parasites.** End point titration was performed with PMN that were coincubated with *L. major* samples with a ratio of 20%, 30% or 50% PS expressing parasites. The capability of *L. major* to survive inside PMN is represented by the number of parasite per 1000 PMN over the time. The figure depicts the data of one out of four similar experiments.

## 6. *In vivo* experiments

The *in vitro* studies described above demonstrated that PS expressing *L. major* support the intracellular parasite survival in PMN. This finding led to the hypothesis that PS expressing *L. major* might also promote disease development *in vivo*. In order to investigate the impact of PS on infection with *L. major* promastigotes, Balb/c mice were infected in the hind footpad with either stationary phase *L. major* (*Lm* stat) (70% PS+), MACS purified PS negative stationary phase *L. major* (*Lm* stat PS-) (7% PS+), PNA separated metacyclic *L. major* (*Lm* met) (40% PS+) or MACS purified PS negative metacyclic *L. major* (*Lm* met PS-) (10% PS+), as described in method 2.10.1. To assess the disease development, the footpad swelling was periodically determined until the feet became necrotic. Additionally, the parasite load of differentially infected mice was determined as a second parameter for disease development. The parasite number was measured in the popliteal lymph nodes after the mice were killed using end point titration experiments, as described in methods

2.10.2.2. In order to investigate whether infected PMN or macrophages (MΦ) are present in the blood of infected mice, and whether infected PMN or MΦ can be recruited into the peritoneum, blood samples and intraperitoneal exsudates of infected mice were microscopically investigated.

## 6.1 Phosphatidylserine expressing *L. major* support the disease development *in vivo*

Mouse infection experiments were carried out using 1 x $10^6$ viable PS negative *L. major* supplemented with apoptotic PS positive parasites (*Lm* stat or *Lm* met) or 1 x $10^6$ viable PS negative *L. major* (*Lm* stat PS- or *Lm* met PS-). The results demonstrated that the footpad swelling increased stronger if mice were infected with parasites containing a high ratio of PS expressing *L. major*. This is true for both stationary phase promastigotes (fig. 28) and purified metacyclic parasites (fig. 29).

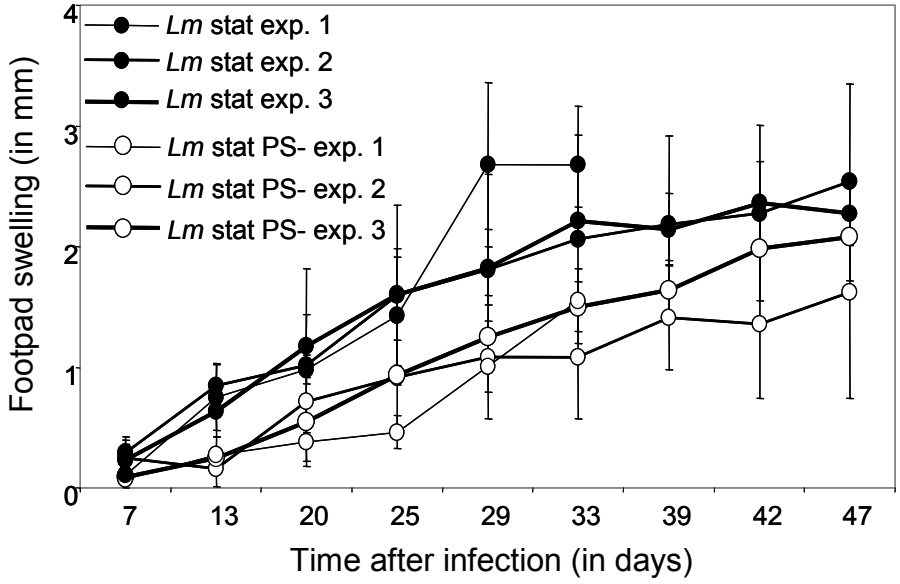


**Fig. 28: Disease development in mice after infection with either viable or viable + apoptotic stationary phase *L. major* promastigotes.** Balb/c mice were infected in the hind footpad with 1 x $10^6$ viable PS negative stationary promastigotes supplemented with 7 x $10^5$ apoptotic PS positive parasites (*Lm* stat) (closed circles) or with 1 x $10^6$ viable PS negative stationary promastigotes (*Lm* stat PS-) (open circles). Mice were observed over 47 days after infection. The footpad swelling in mm is shown as the mean ± SD (n = 4 for each condition in experiment (exp.) one and three, n = 3 in exp. two).

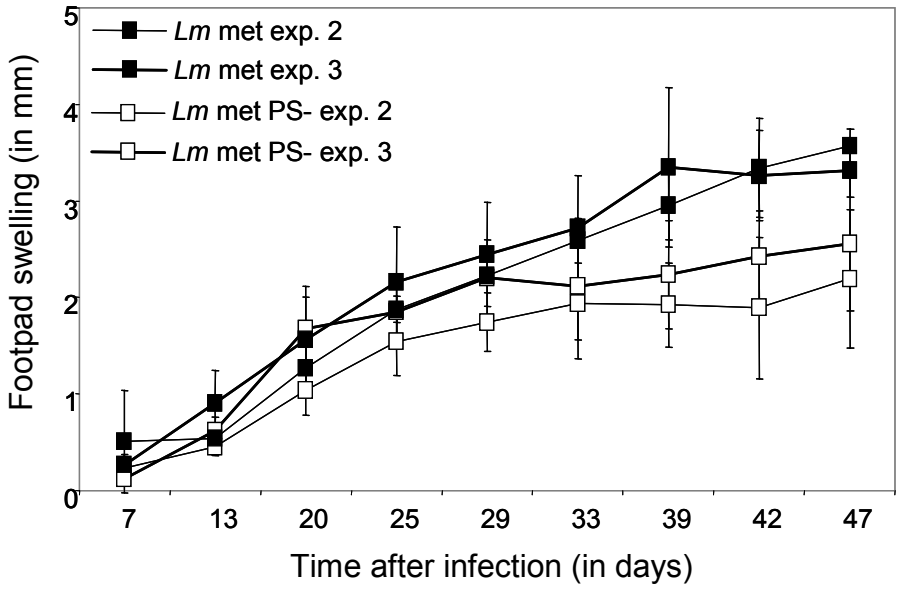


**Fig. 29: Disease development in mice after infection with either viable or viable + apoptotic metacyclic *L. major* promastigotes.** Balb/c mice were infected in the hind footpad with 1 x $10^6$ viable PS negative metacyclic promastigotes supplemented with 4 x $10^5$ apoptotic PS positive parasites (*Lm* met) (closed squares) or with 1 x $10^6$ viable PS negative metacyclic promastigotes (*Lm* met PS-) (open squares). Mice were observed over 47 days after infection. The footpad swelling in mm is depicted as the mean ± SD (n = 4 for each condition per experiment (exp.) except infection with *Lm* met in exp. 2, n = 3).

Additionally, a fourth experiment was performed using a total number of 1 x $10^6$ *L. major* including viable PS negative and apoptotic PS positive parasites (*Lm* met) or 1 x $10^6$ viable PS negative *L. major* (*Lm* met PS-). As demonstrated in fig. 30, infection with *Lm* met resulted in a significant increase of the footpad swelling up to 3.8 mm. In contrast, injection of *Lm* met PS- first led to a light increase of the footpad swelling till day 30 after infection. Afterwards, the footpad swelling remained at a low level and resulted in a final footpad swelling of only 0.4 mm.

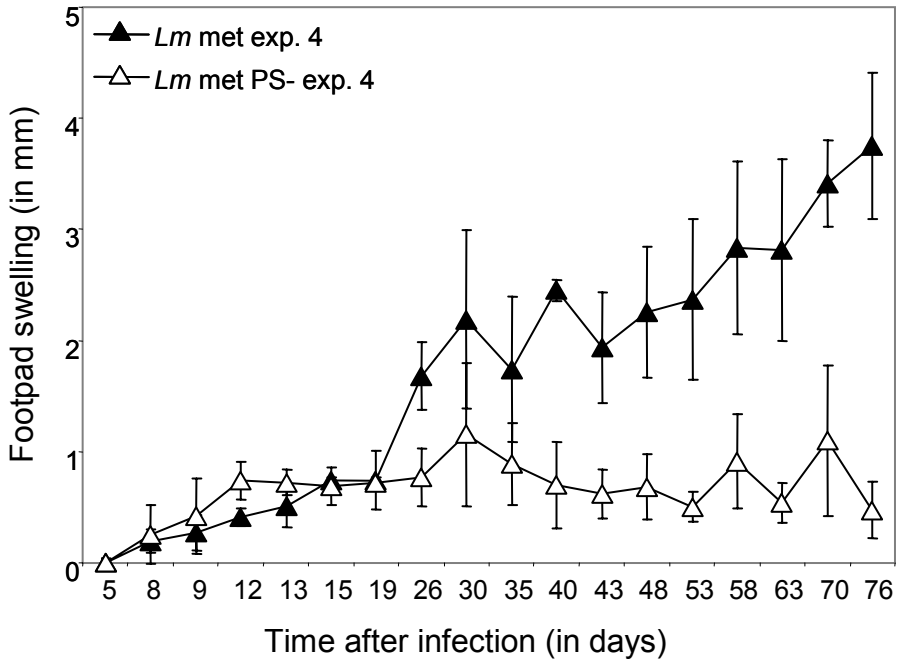


**Fig. 30: Disease development in mice after infection with either viable or viable + apoptotic metacyclic *L. major* promastigotes.** Balb/c mice were infected in the hind footpad with a total number of 1 x $10^6$ *L. major* including viable PS negative metacyclic promastigotes and apoptotic PS positive metacyclic parasites (*Lm* met) (closed triangle) or with 1 x $10^6$ viable PS negative metacyclic promastigotes (*Lm* met PS-) (open triangle). Mice were observed over 76 days after infection. The footpad swelling in mm is shown as the mean ± SD for one experiment ( for each condition n =4).

## 6.2 Parasite load in popliteal lymph nodes reflects the disease development in mice

The comparison of the parasite load in popliteal lymph nodes of infected mice showed remarkable differences if *L. major* samples used for infections contained PS expressing parasites or not. Infection with *Lm* stat resulted in a parasite load of 3.74 x $10^4$ parasites, whereas infection with *Lm* stat PS- showed a parasite load of only 2 x $10^3$ *L. major* promastigotes. Infection with *Lm* met revealed a parasite load of 7.79 x $10^4$ parasites. In contrast, infection with *Lm* met PS- showed a parasite load of about 2 x $10^3$ *L. major* promastigotes (fig. 31).

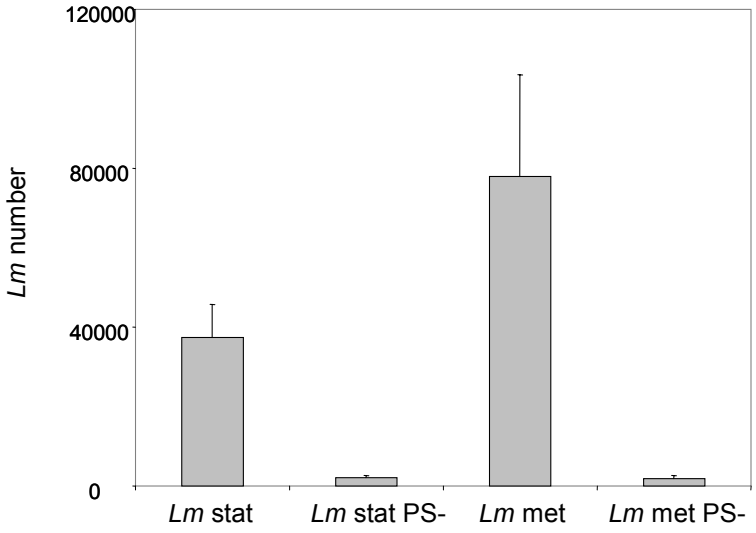


**Fig. 31: The presence of phosphatidylserine expressing *L. major* promastigotes increases the parasite load of popliteal lymph nodes in Balb/c mice.** Popliteal lymph nodes of mice that were infected for 47 days with *Lm* stat, *Lm* stat PS-, *Lm* met or *Lm* met PS- were homogenized in medium. To determine the parasite load (indicated as the number of viable parasites), end point titration experiments were performed using the homogenized lymph node cell suspension. The figure shows the mean ± SD of three independent experiments (n = 2).

## 6.3 *L. major* infected PMN and MΦ are not present in blood- or intraperitoneal exudate samples

In order to investigate whether *L. major* infected MΦ or PMN were present in the blood of infected mice, 47 days after infection blood samples were microscopically analysed. In these samples no infected cell at all was found (data not shown).

To look for infected MΦ or PMN in the peritoneum of infected mice, phagocytes were recruited into the peritoneum by i.p. injection of 1% glycogen in PBS. Microscopic investigation of the intraperitoneal exsudate did not reveal the presence of any infected cells (data not shown).

Using end point titration method (see methods, 2.8) the presence of living parasites was investigated in both blood- and intraperitoneal exsudate samples. These experiments demonstrated that no *L. major* promastigotes at all were present (data not shown).

# 7. Investigation of a phosphatidylserine receptor (PSR)

So far, this study demonstrated that phosphatidylserine (PS) expressing *L. major* promastigotes mediate a silencing of PMN and macrophages (MΦ). These data led to the assumption that a receptor for PS specific recognition is present on these cells, which can direct intracellular signalling toward the production of an anti-inflammatory cytokine milieu. Therefore, experiments were carried out with the aim to identify the PS receptor (PSR) on PMN.

## 7.1 PMN phagocytose *L. major* promastigotes

The process of *L. major* promastigote phagocytosis by PMN was investigated using light microscopy. *L. major* promastigotes made a first contact to PMN with their flagellum (data not shown). The subsequent engulfment of *L. major* by PMN is depicted in fig. 32.

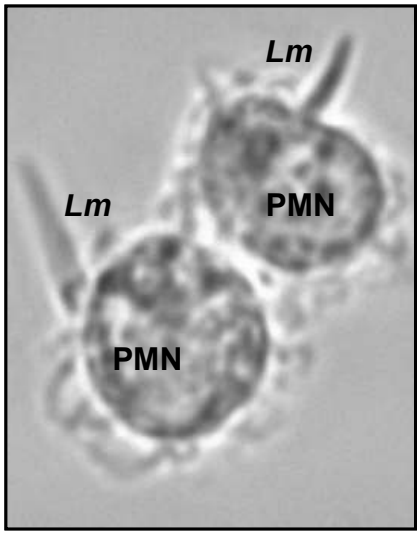


**Fig. 32: Phagocytosis of *L. major* promastigotes by PMN.** A coculture sample of PMN with *L. major* was continuously observed for 1 h using light microscopy. The picture indicates PMN engulfing *L. major* promastigotes (*Lm*). Magnification: 1 x 630.

## 7.2 PMN membrane fragments block the infection of PMN with *L. major* promastigotes

In order to prove that PMN possess *L. major* promastigote binding molecules, the capability of PMN membrane fragments (MF) to block *L. major* phagocytosis by PMN was investigated. Coincubation experiments were performed with PMN and stationary phase *L. major* either untreated or pre-treated with PMN-MF. As a negative control, PMN were coincubated with stationary phase *L. major* that were pre-treated with bovine serum albumin (BSA). Subsequently, the infection rate of PMN, as a parameter for intracellular present parasites, was assessed. PMN coincubation with untreated *L. major* samples resulted in an infection rate of 59 ± 5.7 %, whereas the coincubation of PMN with PMN-MF pre-treated parasites led to a significantly lower infection rate of only 4 ± 1.5%, whereas BSA pre-treatment of the parasites did not reveal any reduction of the infection rate. Additionally, the same experiment was repeated with MΦ and *L. major* that were pre-treated with monocyte-MF. The coincubation of MΦ with untreated *L. major* promastigotes showed an infection rate of 69 ± 4%, whereas the parasite pre-treatment again resulted in a strong reduction of the infection rate down to 13 ± 4.7% of infection (fig. 33).

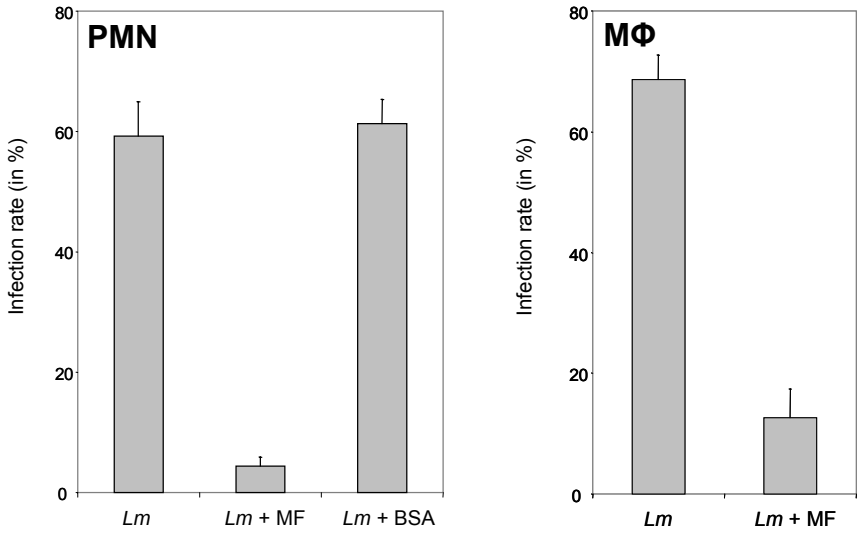


**Fig. 33: Pre-treatment of *L. major* promastigotes with membrane fragments.** Freshly isolated PMN (left panel), or MΦ (right panel), were coincubated for 3 h with untreated stationary *L. major* (*Lm*) or with stationary promastigotes that have been pre-treated with PMN-MF (*Lm* + MF). Additionally, PMN were coincubated with stationary phase *L. major* that were pre-incubated with BSA (*Lm* + BSA) as negative control. After coincubation, cells were stained with May-Gruenwald-Giemsa solution. The infection rate of > 200 cells was determined using light microscopy. The figures show the mean ± SD of duplicate assays for each condition obtained from three independent experiments.

## 7.3 Precipitation of *L. major* promastigote-binding PMN membrane fragments

Neither a PS receptor nor other receptors on PMN are known to play a role for *L. major* promastigote binding to PMN (except complement receptor (CR) 3). Therefore, a method was established to isolate proteins of the PMN membrane, which are capable to bind to the parasite. Biotinylated PMN membrane fragments (MF) were incubated with stationary phase *L. major* promastigotes and *L. major*-bound PMN-MF were precipitated. *L. major*-bound proteins were visualized using SDS-PAGE and streptavidine western blot analysis. PMN-MF showed many different proteins of various sizes. The *L. major*-bound PMN-MF revealed three distinct protein bands with a molecular weight of ~ 32, ~64 and ~ 84 kDa (fig. 34).

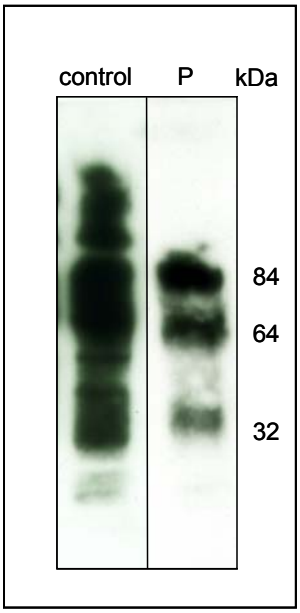


**Fig. 34: Precipitation of *L. major* binding PMN-MF.** Biotinylated PMN-MF were visualized using SDS-PAGE and streptavidine western blot analysis. The control lane shows biotinylated PMN-MF. The precipitate (P) indicates biotinylated *L. major*-bound PMN-MF. Representative blot from three independent experiments is shown.

## 7.4 Complement receptors CR1 and CR 3 are not present in *L. major* bound PMN membrane fragments

*L. major* binding receptors of PMN are CR1 (CD35) and CR3 (CD11b/CD18). To investigate the involvement of both receptors in *L. major* attachment to PMN, western blot experiments were performed using unbiotinylated PMN-MF precipitates. For visualisation, antibodies to CR1 or to CD11b were used. Neither CR1 nor CR3 was detectable in the precipitates (data not shown).

As a second method to isolate PMN surface molecules that can bind to *L. major* promastigotes column affinity chromatography experiments were performed. Therefore, *L. major* membrane fragments (MF) were coupled to CNBr activated sepharose. The coupled sample was put into a column and PMN-MF were applied, thus allowing PMN-MF to bind to *L. major*–MF. Subsequently, the bound PMN-MF were eluted. The obtained PMN-MF fractions were tested for the presence of CR1 and CR3 using western blot analysis, as described above. In the eluted PMN-MF neither CR1 nor CR3 could be detected (data not shown). These results are in line with the previous findings from the precipitation experiments.

### 7.5 Investigation of the PSR using anti-PSR antibodies

A PSR candidate was described by Dr. Fadok and colleagues and they generated a monoclonal mouse anti-human PSR (IgM) (217) (anti-PSR IgM) (Fadok et al., 2000). A second monoclonal mouse anti-PSR (IgG) (anti-PSR IgG) was provided by PD Dr. Dr. Herrmann and Dr. Voll. Additionally, a commercial polyclonal rabbit anti-human PSR (IgG) was available (Sigma). Using these three antibodies, the expression of PSR was investigated on PMN.

#### 7.5.1 PSR expression on PMN membrane is inducible with TGF-β and β-glucan

It has been published that the expression of the suggested PSR is inducible by stimulation of MΦ with TGF-β and β-glucan (Fadok et al, 1998). Therefore, it was investigated, whether this might also be true for the PSR expression on PMN. PMN were stimulated for 24 h with TGF-β and β-glucan and subsequently PSR detection was achieved using anti-PSR IgM. Evaluation with FACS analysis revealed that PMN cultured in medium for 24 h did not express the PSR. However, 15.9% of the PMN expressed the PSR after stimulation with TGF-β and β-glucan (fig. 35).

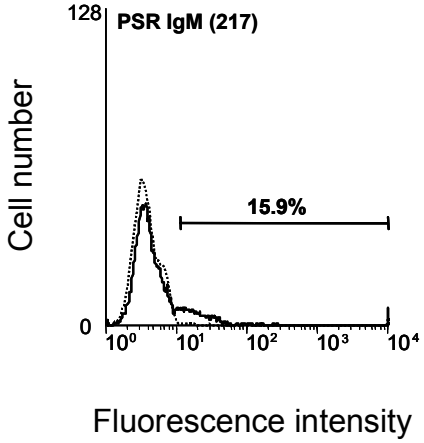


**Fig. 35: PSR on PMN is inducible with TGF-β and β-glucan.** PMN were stimulated for 24 h with TGF-β (256 pg/ml) and β-glucan (25 µg/ml) or cells were cultured in medium. FACS staining was conducted using anti-PSR IgM. For PSR detection by FACS analysis, a PE-labeled goat anti-mouse IgM secondary antibodies was used. As a negative control, an IgM isotype control was used. The dotted line represents cells in medium, the continuous line indicates stimulated cells. The x-axis shows the red fluorescence intensity. The ratio of positive stained cells is depicted. Representative histogram profile is shown of three independent experiments.

### 7.5.2 PSR expression on PMN membrane is inducible by *L. major* promastigotes

The inducibility of the PSR with TGF-β and β-glucan (see above) and the previous finding that *L. major* promastigotes express PS (see results, chapter 1) led to the suggestion that *L. major* might serve as a potent inducer of PSR expression on PMN. Therefore, PMN were stimulated with stationary phase *L. major* promastigotes for 15 min. Using anti-PSR IgM for FACS analysis, 33.5% of PMN revealed PSR expression. Apparently, PSR expression on PMN is inducible with *L. major*. Similar experiments were also performed with MΦ. Surprisingly, MΦ revealed only 13.5% PSR expression, which means 20% less than PMN (fig. 36).

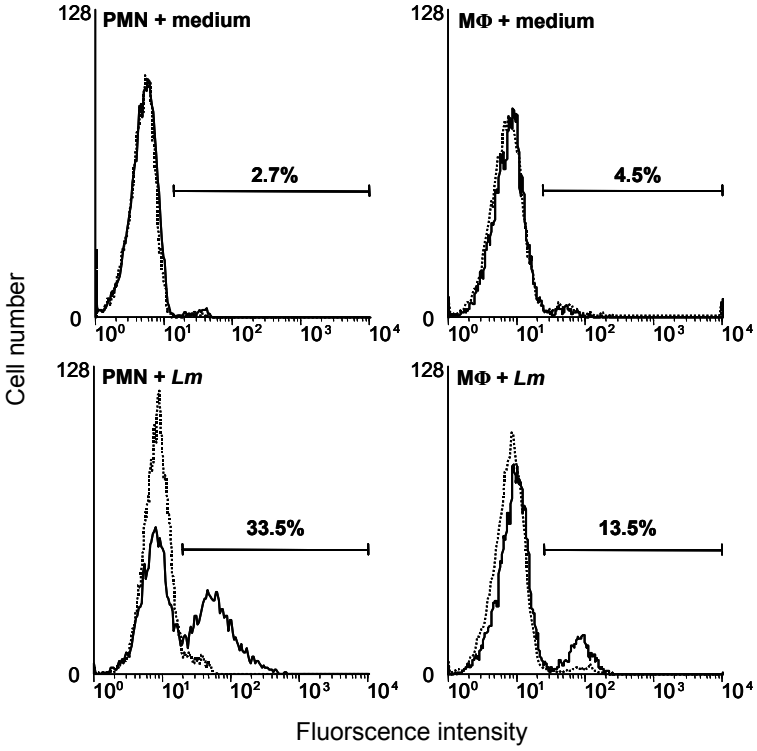


**Fig. 36: PSR expression on PMN and MΦ is inducible with *L. major* promastigotes.** PMN (left panels) or MΦ (right panels) were stimulated for 15 min with *L. major* promastigotes or cells were cultured in medium alone. FACS staining was conducted using unlabeled mouse anit-PSR IgM and a PE-labeled goat anti-mouse IgM secondary antibody. As a negative control, an IgM isotype control was used. The dotted lines represent isotype control, continuous lines indicate stained cells. The ratios of positive stained cells are depicted. Representative histogram profiles of three independent experiments are shown.

In order to confirm these findings, similar experiments were performed using anit-PSR IgG. However, this antibody did not reveal any PSR expression. Hence, the data obtained using anti-PSR IgM (see above) could not be confirmed (data not shown).

### 7.5.3 PSR expression on PMN in western blot analysis

The anti-PSR IgM was described to reveal a band of ~ 70 kDa in western blot analysis and a deglycosylation of the suggested PSR resulted in a shift from 70 to ~ 47 kDa in size ( Fadok et al., 2000; Hoffmann et al., 2005) In order to investigate the PSR expression of PMN, western blot experiments were conducted using PMN whole cell lysates. For PSR detection anti-PSR IgM was used. However, neither a ~ 70 kDa nor a ~ 47 kDa protein band was detectable (data not shown). Additionally, similar blotting experiments were conducted using anti-PSR IgG for PSR detection. The western blot analysis resulted in a ~ 47 kDa protein band. Similar western blot analysis was performed with MΦ whole cell lysates. Anti-PSR IgG also revealed a distinct protein band of ~ 47 kDa in size in MΦ (fig. 37).

Because of these contradictory results obtained by using the anti-PSR IgM and the anti-PSR IgG, similar western blot analysis was conducted using the commercially available anti-PSR antibody. These experiments revealed several protein bands of various sizes (data not shown).

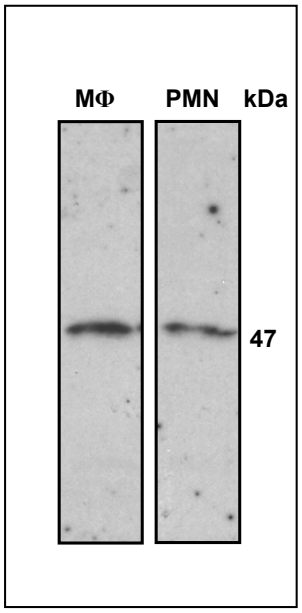


**Fig. 37: PSR expression in PMN and MΦ in western blot analysis.** Whole cell lysates from 1 x $10^6$ PMN or MΦ were separated in 12% denaturing SDS-PAGE and subsequently blotted. PSR was detected using anti-PSR IgG and HRP conjugated goat anti-mouse IgG secondary antibodies. Representative blots from three independent experiments are shown.

# IV Discussion

This study focussed on the identification of surface molecules on *L. major* promastigotes that are involved in the establishment of silent polymorphonuclear neutrophilic granulocytes (PMN) infection. In this context, the expression of the apoptotic “eat-me” signal phosphatidylserine (PS) on *L. major* and its influence on PMN infection was investigated. In addition, the membrane receptors on PMN that are involved in parasite uptake were studied.

It was demonstrated that *L. major* can express PS on their membrane. The ratio of PS expressing promastigotes increased during the culture period. In addition, stationary phase *L. major* promastigotes were demonstrated to enter apoptosis, as shown by TUNEL staining. TUNEL positivity increased parallel with the ratio of PS expressing parasites during the growth period from the logarithmic to the infective stationary phase. End point titration experiments demonstrated that PS expressing promastigotes do not grow in culture, thus PS expressing parasites are not viable. Moreover, electron microscopic analysis of PS expressing promastigotes revealed an “apoptosis-like” morphology, such as loss of granularity and cell shrinkage. Additionally, PS expressing *L. major* produced an anti-inflammatory milieu in PMN by an up-regulation of the TGF-β secretion and a down-regulation of the TNF-α release. The presence of PS expressing promastigotes promoted parasite survival inside PMN *in vitro*. Moreover, the presence of PS expressing parasites enhanced the disease development *in vivo*. In contrast, the lack of PS expressing promastigotes resulted in a reduced outcome or almost healed form of disease, which was dependent on the parasite number used for infection. The investigation of PMN receptors that can potentially interact with PS on *L. major* revealed three receptor candidates.

## 1. The role of phosphatidylserine in *L. major* infection

The data presented in this thesis demonstrate that PS is expressed on *L. major* promastigotes, which are the disease inducing form of this parasite. PS is detectable either with annexin-V (AV) in a calcium dependent manner, or using a mAb specific for PS. As shown in fig. 6, PS is arranged in “patches” on the membrane surface of *L.*

*major*. Additionally, these patches were also detectable on the flagellum. These observations led to the question of the possible role of such a patchy arrangement of PS. From the literature it is known that especially membrane standing molecules need to cluster to display biological functionality (Simson and Ikonen, 1997; Brown, 2002; Zhang et al., 2005). Therefore, it is conceivable that PS might appear randomly on the parasites membrane. In order to achieve biological functionality, this initial PS externalisation might be followed by a PS reorganisation leading to an accumulation of PS in clusters. Thus, the detected patches could represent such PS clusters suggesting an active molecular organisation.

Amastigotes of the *Leishmania* species *L. amazonensis* were suggested to express PS as an active form of “apoptotic mimicry” to gain survival advantages (Freitas Balanco et al., 2001). Therefore, PS expressing *L. major* promastigotes were first expected to be viable. However, PS is conventionally seen as the hallmark for the onset of apoptosis. The process of apoptosis represents the transition from life to death. During this process PS flips to the outer leaflet of the cell membrane (Martin et al., 1995), but the exact stage of PS expressing cells, whether they are still alive, apoptotic or already dead, is not known. Hence, the question was addressed whether PS expressing promastigotes are viable or apoptotic. The ratio of PS expressing *L. major* promastigotes increased during the period of culture. As indicated in fig. 13, the ratio of TUNEL positive parasites also increases during the period of culture and correlates with the increased ratio of PS expressing promastigotes (see fig. 14). End point titration experiments demonstrated that PS expressing *L. major* are not viable. However, Freitas Balanco and colleagues did not demonstrate whether PS expressing *L. amazonensis* amastigotes are viable or apoptotic (Freitas Balanco et al., 2001). Other groups suggested the presence of an apoptotic machinery in *Leishmania* (Lee et al., 2002; Debrabant et al., 2003; Nguewa et al., 2004). The time dependent increase of promastigotes expressing the apoptotic marker PS suggests a general ongoing aging process of the parasites ending in apoptosis. The finding that the ratio of PS expressing purified metacyclic promastigotes also increases during the period of culture supports this conclusion. Moreover, the increase of TUNEL positive promastigotes during the culture period indicates that apoptosis is emerging in *L. major* promastigotes. The expression of PS as a sign for the onset of apoptosis is followed by DNA fragmentation resulting in TUNEL positivity as a sign for a later

stage of apoptosis. Thus, not all PS expressing promastigotes are already TUNEL positive. However, the suggestion that both of these apoptotic signs occur in the same promastigotes was confirmed by the inability of purified PS expressing parasites to grow in culture, whereas PS negative promastigote samples did increase in cell number. According to these data, it was shown for the first time that PS expression on *L. major* promastigotes is not “apoptotic mimicry”. Instead, it is clearly demonstrated that PS expressing promastigotes are indeed apoptotic. Whether PS expressing amastigotes also undergo apoptosis is not known but would certainly be interesting to investigate.

Observations using electron microscopy revealed granular structures in PS negative *L. major* and their nuclear morphology showed the typical shape of viable cells. In contrast, PS expressing promastigotes have lost its granular appearance and revealed a rounded and smaller nuclear morphology as well as a smaller cell size. Moreover, the electron microscopic pictures indicated that the tubular membrane structure of PS expressing *L. major* promastigotes appears disorganized, whereas the membrane showed intact morphology (see fig. 17).

Taken all these findings into account they point to the direction of an apoptotic process in *L. major* promastigotes. In this context, apoptotic processes such as the loss of membrane potential as well as chromatin condensation and caspase-like activity have been reported to occur in different *Leishmania* species (Lee et al., 2002; Debrabant et al., 2003; Nguewa et al., 2004). So far, a disorganized tubular membrane structure was not described as a sign for apoptosis, but it is conceivable that an organized tubular structure is needed for membrane functionalities, such as the maintenance of the membrane potential. Thus, the observed disorganisation might represent a pro-apoptotic sign. However, a closer look using electron microscopy showed that the membrane of PS expressing promastigotes appeared to be still intact. This feature might explain why a ratio of over 90 percent of stationary phase promastigotes was alive in viability staining experiments. Furthermore, the previously mentioned chromatin condensation and caspase-like activity occurring in *Leishmania* serve as a possible explanation for the detected formative changes and degradations in PS expressing promastigotes. In FACS analysis, if gated on PS expressing promastigotes, these parasites displayed a lower forward-angle light scatter than PS negative promastigotes (data not shown). Thus, PS positive *L. major* are smaller than PS negative *L. major*. Since cells shrink during the process of

apoptosis, the shrinkage of *L. major* promastigotes might also represent a sign for apoptosis.

In addition, it was observed that the motility of purified PS expressing *L. major* is abolished. Viable *L. major* are mobile parasites capable of swimming in the surrounding fluid medium by a rapid movement of their flagellum. The mobility loss of PS expressing promastigotes in line with the TUNEL staining, end point titration experiments and electron microscopic investigation is concluded to be another hint for the conversion of PS expressing *L. major* into an apoptotic stage.

PS expression on apoptotic cells is the prerequisite for their uptake by macrophages (MΦ) (Fadok et al, 1992). Recent data of our group demonstrated that PMN are also able to phagocytose apoptotic cells (Esmann et al., unpublished data). Since PMN phagocytose apoptotic bodies and the uptake of apoptotic cells by MΦ exerts silencing properties in a PS dependent manner (Voll et al., 1997; Fadok et al., 1998, 2001; Huynh et al., 2002), the question was addressed, whether the engulfment of PS expressing apoptotic *L. major* promastigotes by PMN also results in silencing. The present data demonstrate for the first time that the infection rate of PMN, which is defined in this context as the number of intracellular present parasites, correlates with the ratio of PS expressing *L. major* promastigotes used for infection (see fig. 18). Moreover, the infection rate of PMN decreased if PS was specifically blocked using AV. These findings showed that PS expression on apoptotic *L. major* promastigotes has a functional role for parasite phagocytosis suggesting a direct involvement in PMN silencing.

*L. major* promastigotes need to enter PMN silently to ensure survival inside the host. Therefore, the next attempt of this thesis was to elucidate how the “silent entry” can be achieved. In this context, the leishmanial membrane constituents lipophosphoglycan (LPG) and gp63 (leishmanolysin) were described to impair the oxidative burst in MΦ. However, experiments using *L. major* lpg 1- mutants showed that these parasites still enter MΦ silently (Sorensen et al., 1994; Spaeth et al., 2003). Thus, LPG is obviously not of predominant importance to prevent host cell defence mechanisms such as the oxidative burst. The recognition of leishmanial PS was demonstrated to inhibit the NO production in MΦ (Freitas Balanco et al., 2001). Recent data of our group revealed that the uptake of *L. major* promastigotes does not

induce an oxidative burst in PMN by *L. major* (Laufs et al, 2002). According to these data, it was investigated, whether a silencing capacity of PS expressing *L. major* promastigotes might be responsible for the prevention of the oxidative burst. As depicted in fig. 19, PS expressing *L. major* are not involved in the lack of the oxidative burst, because neither PS positive nor PS negative *L. major* populations induced this defence mechanism in PMN. Hence, other membrane standing molecules on *L. major*, like gp63, as suggested in MΦ, might possibly play a role for the prevention of the oxidative burst in PMN. Consequently, leishmanial PS was suggested to exert other silencing functions.

The production of anti-inflammatory TGF-β correlates positively with the ratio of PS expression and controversially with the release of pro-inflammatory TNF-α during the removal of apoptotic cells by MΦ (Fadok et al., 1998, 2000; McDonald et al., 1999; Serinkan et al., 2005). As mentioned before, unpublished data of our group have shown that also PMN are able to phagocytose apoptotic cells. As PMN are the first professional phagocytes that appear in high numbers at the site of infection (Laskay et al., 1997), this study investigated the role of leishmanial PS for the establishment of immunosilencing properties in PMN. The present data demonstrate for the first time that increasing ratios of PS expressing promastigotes correlate with an up-regulation of TGF-β release by PMN. Additionally, the presence of PS expressing parasites is associated with a down-regulation of TNF-α secretion (see fig. 20). Thus, PS expressing *L. major* direct the surrounding cytokine milieu toward an anti-inflammatory situation. Although PMN produce lower amounts of TGF-β as compared to MΦ, PMN largely pre-dominate in cell number at the site of infection. Consequently, their lower production is still highly relevant. The functional relevance was further emphasized by the finding that TGF-β produced by *L. major* infected PMN results in the phosphorylation of the intracellular TGF-β receptor adaptor protein Smad2 in TGF-β sensitive Mv1Lu cells. This fact confirms that the released TGF-β is indeed bioactive. Possibly, secreted TGF-β acts via an autocrine loop on PMN themselves, because PMN possess a TGF-β receptor on their membrane (Brandes et al., 1991). Hence, *L. major* infected PMN could silence their own defence machinery that in turn might be responsible for intracellular parasite survival. Whether TGF-β might also affect *L. major* promastigotes, remains to be clarified.

In order to investigate whether the immunosilencing capacity of PS expressing *L. major* in PMN represents a more general principle, similar experiments were performed with MΦ. As demonstrated in fig. 22 and fig. 23, similar influences of leishmanial PS concerning the infection rate as well as the regulation of TGF-β and TNF-α production were found. However, it is questionable whether MΦ play a role for the immediate interaction with *L. major*, because PMN are the first cells at the site of infection. Additionally, it was demonstrated that *L. major* enter MΦ via infected PMN (Laskay et al., 2003). Consequently, PMN represent the most relevant phagocytes during the first hours of infection with *L. major*.

The TGF-β secretion by MΦ in response to leishmanial PS subsequently increases the susceptibility of MΦ to intracellular leishmanial growth (Freitas Balanco et al, 2001; Barral et al., 1993). This thesis demonstrated that PS expressing parasites also enhance the TGF-β release in PMN. In addition, the TNF-α production increases in PMN, if PS expressing parasites are depleted. TNF-α was demonstrated to enhance the anti-microbial host cell defence in MΦ, particularly against intracellular pathogens such as *Leishmania* (Beutler et al., 1993). Therefore, it was speculated that an enhanced TNF-α release might be responsible for parasite killing in PMN. To test this hypothesis, coincubation experiments of PMN with stationary *L. major*, either containing PS expressing parasites or PS depleted, were performed over a time period of 3 h, 18 h and 42 h. Subsequently, the intracellular parasite survival was investigated. Determination of the PMN infection rate, as a sign for intracellular present parasites, revealed that the infection rate is maintained over the observed time period in the presence of PS expressing promastigotes. The infection rate was decreased after 42 h of coculturing if PS was depleted. End point titration, as a sign for the presence of viable parasites, demonstrated that the presence of high amounts of PS expressing *L. major* supports the intracellular parasite survival. In contrast, the number of intracellular viable *L. major* was considerable reduced if PMN were infected with PS depleted parasite samples. These data clearly show that apoptotic PS expressing *L. major* support the intracellular survival of viable PS negative promastigotes in PMN. This survival advantage might be achieved by a PS dependent inhibition of the TNF-α secretion, as it was demonstrated that PS expressing parasites reduce the TNF-α secretion in PMN. The next attempt was to answer the question how many PS expressing promastigotes are needed to mediate

an efficient survival advantage for *L. major* in PMN. Therefore, coincubation experiments with PMN and *L. major* promastigotes containing a ratio of 20%, 30% or 50% PS expressing parasites were performed. Subsequently, the infection rate and the number of intracellularly surviving parasites was determined after 18 h and 42 h of coculturing. It was found that increasing ratios of PS expressing parasites contribute stepwise to the maintenance of the PMN infection rate and also to the survival of *L. major* promastigotes inside PMN. Thus, it is concluded that the higher is the ratio of apoptotic PS expressing promastigotes present in a *L. major* population, the more advantageous is the situation for viable PS negative parasites during the infection of PMN. This contribution of apoptotic *L. major* promastigotes to the biological fitness of viable parasites can be regarded as an altruistic mechanism, as suggested by Rittig and Bogdan (Rittig and Bogdan, 2000).

The fact that *L. major* gain survival advantages in PMN due to the presence of PS expressing promastigotes *in vitro* and the finding that PS expressing parasites down-regulate the release of TNF-α led to the suggestion that PS expressing *L. major* promastigotes might also support the disease development *in vivo*. In addition, this hypothesis was endorsed by the report that TNF is required early on to control *L. major* infection in mice (Murray et al., 2000; Wilhelm et al., 2001). Therefore, the regulation of TNF-α seems to be important for the final disease development. Using a cutaneous infection model in Balb/c mice, the disease development after injection of either *L. major* samples containing a high ratio of PS expressing promastigotes or PS depleted parasites populations was investigated. For the first three experiments, 1 x $10^6$ PS negative promastigotes or 1 x $10^6$ PS negative promastigotes supplemented with PS expressing *L. major* were injected. As depicted in fig. 28 and 29, the disease development is lower if mice were infected with PS depleted *L. major* samples. The difference between the infection with PS negative promastigotes or PS negative promastigotes supplemented with PS expressing *L. major* is especially notable if purified metacyclic parasites were used. The fourth experiment was performed using either 1 x $10^6$ PS negative promastigotes or a total amount of 1 x $10^6$ parasites including PS expressing *L. major* promastigotes. As demonstrated in fig. 30, the disease development is almost completely prevented if mice were infected with PS depleted *L. major* promastigotes. In contrast, parasite samples containing a high ratio of PS expressing promastigotes induce a distinct disease development.

Consequently, the presence of PS expressing parasites significantly enhances the disease development in Balb/c mice. Thus, PS expression on *L. major* promastigotes also has *in vivo* relevance. Moreover, the disease supporting effect of PS expressing promastigotes appears to be dose dependent. Possibly, this effect might even result in a stronger survival advantage for the parasite, if the total *L. major* number using for infection would be reduced. In this context, a reduction of the total parasite number would be closer to the natural *L. major* infection regarding the parasite transmission by the sandfly vector.

The relevance of PS expression on *L. major* promastigotes for disease development was emphasized by the finding that PS is also expressed on metacyclic promastigotes in the sandfly. In cooperation with Dr. Sacks and co-workers, the PS expression was investigated on metacyclic *L. major* promastigotes, which were freshly isolated from sandfly cultures. Using FACS analysis they demonstrated that metacyclic promastigotes contain a ratio of 43% PS expressing parasites (data not shown). Thus, the PS expression of *L. major* promastigotes is not limited to *in vitro* cultures but is also present under natural conditions.

The present data indicate that PS expressing *L. major* promastigotes are apoptotic. PS expression is present on both stationary phase and metacyclic *L. major* promastigotes in *in vitro* cultures but also on metacyclic parasites in the sandfly vector. Moreover, PS expression on *L. major* silence the immune response in PMN and MΦ. Importantly, PS expressing parasites reduce the disease development in *L. major* infected mice. Consequently, the infectivity of *L. major* promastigotes is clearly dependent on its expression of PS. Presumably, PS is more important for the establishment of *L. major* infection than LPG, because PS depleted parasites samples have lost their silencing capacity, whereas *L. major* lpg 1- mutants still silence the immune response of MΦ (Spaeth et al., 2003). Supplementation of non-infective procyclic promastigotes with PS expressing apoptotic parasites may offer a new model to investigate the contribution of PS expressing parasites to *L. major* infectivity. If procyclic promastigotes might subsequently show increased infectivity, an additional proof for a PS dependent infectivity would be given.

## 2. Investigation of the phosphatidylserine receptor on PMN

According to the data presented in the first part of this study, a PS dependent receptor might be responsible for the engulfment of apoptotic PS expressing parasites and thus for the silencing of *L. major* infection. On the other hand, phagocytosis of viable PS negative parasites is suggested to occur via receptor(s) other than PS receptor (PSR).

Coincubation experiments of PMN with *L. major* promastigotes revealed that the parasite uptake is almost completely abolished if the parasites were pre-treated with PMN membrane fragments. However, blocking of complement receptor (CR) 3 on PMN revealed a reduction of the infection rate less than 50% (Laufs et al., 2002). Therefore, it is assumed that more than only one receptor on PMN might be involved in the uptake of *L. major* promastigotes.
Precipitation of PMN membrane fragments with *L. major* promastigotes and subsequent western blot analysis revealed three distinct protein bands with a molecular weight of ~ 32, ~ 64 and ~ 84 kDa, respectively (see fig. 34). Complement receptor (CR) 3 and CR1 are known to be involved in *L. major* uptake by MΦ (Laufs et al., 2002). Using western blot analysis it was investigated whether these receptors were present in the precipitate or in PMN membrane fragment fractions derived from column affinity chromatography with *L. major* membrane fragments. In both samples neither CR1 nor CR3 were detected (data not shown). However, the three protein bands shown in the precipitate suggest that at least three different proteins are present on PMN that are involved in PMN binding to *L. major* promastigotes. So far, it is can not be distinguished whether these potential PMN receptors might be involved in PS dependent or –independent interaction with *L. major*.

Experiments using different anti-PSR antibodies showed contradictory results. According to a puplication by Dr. Fadok and colleagues, the stimulation of MΦ with TGF-β and β-glucan leads to expression of the suggested PS receptor (“PSR”) (Fadok et al., 2000). FACS analysis using a monoclonal mouse anti-human “PSR” IgM antibody (217) (anti-PSR IgM) (provided by Dr. Fadok) revealed that stimulation of PMN with TGF-β and β-glucan also induces “PSR” expression. Similar experiments with MΦ demonstrated next to a “PSR” expression also a positive signal

with an IgM isotype control (data not shown). Consequently, this finding led to the suggestion of an aspecific binding of the anti-PSR IgM. However, stimulation of both PMN and MΦ with stationary phase *L. major* promastigotes showed a normal surface expression of the “PSR”. In this case, the parasites themselves could possibly enhance an aspecific binding of the anti-PSR IgM through their own “stickiness”, which might also explain the higher “PSR” expression by stimulation with *L. major* as compared to stimulation with TGF-β and β-glucan. However, the anti-PSR IgM detected “PSR” expression on PMN could not be confirmed with a monoclonal mouse anti-human “PSR” IgG antibody (anti-PSR IgG) (provided by PD Dr. Dr. Herrmann and Dr. Voll).

In western blot experiments using PMN whole cell lysates, the situation was the other way around: The anti-PSR IgM did not detect any protein. In contrast, the anti-PSR IgG revealed a protein of the expected molecular weight of 47 kDa (fig. 36). Similar results were obtained using a whole cell lysate of MΦ.

In western blot analysis the three-dimensional protein structure is changed through linearisation. As a consequence, the "PSR” epitopes recognized by the anti-PSR IgM may be to far apart from each other for sufficient binding of the antibody. This might serve as a possible explanation for the lack of anti-PSR IgM binding to its ligand in western blot analysis. In contrast, the “PSR” epitopes recognized by the anti-PSR IgG might be localized inside the three-dimensional “PSR” protein structure present on intact PMN membranes. This epitope localisation might explain why the anti-PSR IgG can not detect its ligand using FACS analysis. However, Fadok and colleagues detected PS on MΦ using the anti-PSR IgM in western blotting. These conflicting results to the here presented findings might be caused by different western blot methods.

If the “PSR” detcetion using anti-PSR IgM in FACS analysis would be regarded as an aspecific binding, it is reasonable to suggest that the “PSR” is not present on the cell surface but intracellularly. This suggestion is in line with recent findings of other groups, who demonstrated that the protein encoded by the cDNA of this “PSR” is not localized on the cell membrane (Cui et al., 2003; Boese et al., 2004). A possible explanation for the misleading results achieved by the use of the “PSR” gene might be a cross reaction between the anti-PSR IgM and an epitope in phage display. A latest hint for this suggestion is a demonstrated weak cross reactivity of anti-PSR IgM

with a peptide within the protein encoded by the PSR gene (Williamson and Schlegel, 2004; Boese et al., 2004).

Taken together, the findings of this study and recent publications hint in the direction that it is more likely that the “PSR” is present exclusively intracellularly. However, the effects mediated by leishmanial PS on PMN corroborate the existence of a PSR. In case that PS might not directly interact with PSR, it is possible that PS recognition is mediated through bridging molecules. In this context, annexin-I, which is a releasable intracellular protein in PMN that mediates bivalent binding serves as a bridging molecule candidate (Francis et al., 1992). On the membrane surface annexin-I enhances phagocytosis of apoptotic cells in a PSR dependent manner (Arur et al., 2003; Fan et al., 2004; Vergnolle et al., 1995).

However, the PSR still remains to be clarified. The three protein bands obtained from the precipitate of PMN membrane fragments with *L. major* promastigotes possibly include a PS recognizing receptor of PMN. In order to identify a PS specific receptor, the precipitation method could be modified by using purified PS positive or PS negative parasites for precipitation experiments. If western blot analysis might detect a protein in the precipitate with PS positive *L. major*, which would not be present in the precipitate with PS negative parasites, this protein represents a new PSR candidate.

# VI Summary

Previous studies have shown that early after infection, polymorphonuclear neutrophil granulocytes (PMN) serve as host cells for the obligate intracellular parasite *Leishmania major* (*L. major*). This finding suggests that *L. major* succeed to enter PMN “silently”, without the activation of antimicrobial effector mechanisms of these cells. The present study aimed to investigate the surface molecules both of the parasites and of PMN that are involved in this silent entry process. A known silent way of phagocytosis is the ingestion of apoptotic cells by professional phagocytes. During this process, phosphatidylserine (PS) on the surface of apoptotic cells serves as “eat me” signal for the phagocytes. In addition, PS is thought to mediate the silencing of the pro-inflammatory response. Since PS was found to be present on the surface of *L. major*, it was investigated whether PS is responsible for the silent infection of human PMN with *L. major in vitro*. Labeling with both annexin-V and anti-PS antibody revealed that highly infectious stationary phase *L. major* cultures contain a high (>60%) ratio of PS positive parasites, whereas there are only a few PS positive parasites present in non-infectious logarithmic growth phase cultures. Importantly, TUNEL staining and viability tests revealed that the PS positive parasites are apoptotic, not capable of proliferation and destined to death. Electron microscopy also demonstrated that the PS positive *Leishmania* are morphologically not intact.

Infectious stationary phase *L. major* cultures contain both PS positive apoptotic and PS negative viable parasites. Interestingly, although the PS positive parasites are not viable, depletion of these parasites from stationary phase *Leishmania* cultures resulted in a decreased survival rate of the parasites in PMN, as compared to the survival rate of normal stationary phase cultures containing both PS positive and PS negative parasites. Coincubation of PMN with purified PS negative *L. major* resulted in the release of pro-inflammatory TNF-α, whereas the additional presence of PS positive parasites in stationary phase cultures shifted the cytokine response to an anti-inflammatory milieu, characterized by increased levels of TGF-ß release and down regulation of TNF-α production.

Based on these *in vitro* results I propose the model that the presence of an apoptotic population of the parasites is a prerequisite of infectivity of *L. major* promastigotes. Experimental infection of susceptible Balb/c mice proved this hypothesis *in vivo*. PS negative (i.e. viable) *L. major* did induce a significant lower disease development as

compared to parasite populations consisting of a mixture of PS negative and a high ratio of PS positive parasites. These data clearly proved the hypothesis that apoptotic parasites assist the viable parasites to establish infection.

Natural *L. major* infection occurs upon a bite of an infected sandfly. The finding that *L. major* isolated from infected sandflies also contain a high ratio of PS positive parasites suggest the importance of PS positive parasites regarding the natural *Leishmania* infection.

Pre-treatment of the parasites with the PS binding protein annexin-V blocked the uptake of *L. major* by PMN. This observation suggested that PMN express a PS receptor (PSR). However, in spite of repeated efforts to assess PSR expression on PMN, no conclusive results were obtained. However, precipitation experiments with biotinylated PMN membrane fragments revealed three until now unidentified PMN proteins that bind to *L. major*. Possibly, one of these three proteins represents a PS recognizing receptor and further studies are required to identify and characterize these molecules.

Taken together, the results of this thesis suggest that apoptotic *L. major* promastigotes, via the induction of anti-inflammatory and phagocyte deactivating cytokines, assist the viable parasites to survive inside phagocytes. Apoptotic parasites “silence” the immune response of PMN and enable the survival of non-apoptotic parasites to establish a productive infection.

# VII Zusammenfassung

Vorangegangene Studien haben gezeigt, dass polymorphonukleare neutrophile Granulozyten in der Frühphase der Infektion als geeignete Wirtszellen für den intrazellulären Erreger *Leishmania major* (*L. major*) dienen. Dieser Befund lässt vermuten, dass *L. major* auf „stille“ Art und Weise in PMN gelangt, ohne die antimikrobiellen Abwehrmechanismen dieser Zellen zu aktivieren. Die Zielsetzung der vorliegenden Arbeit war die Untersuchung von Oberflächenmolekülen des Parasiten als auch der PMN, die am Prozess des stillen Eintritts beteiligt sind. Ein bekannter Weg stiller Phagozytose ist die Aufnahme apoptotischer Zellen durch professionelle Phagozyten. Während dieses Prozesses dient Phosphatidylserin (PS) auf der Oberfläche apoptotischer Zellen als „friss mich“ Signal gegenüber des Phagozyten. Des weiteren wird angenommen, dass PS das Herunterregulieren der proinflammatorischen Antwort vermittelt. Nachdem herausgefunden war, dass PS auf der Oberfläche von *L. major* vorhanden ist, wurde untersucht, ob PS für die stille Infektion humaner PMN mit *L. major in vitro* verantwortlich ist. Die Markierung mit Annexin-V oder anti-PS Antikörpern zeigte, dass hoch infektiöse *L. major* Kulturen der stationären Phase eine hohen Anteil (>60%) PS positiver Parasiten beinhalten, wohingegen nur wenige PS positive Parasiten in Kulturen der nicht infektiösen logarithmischen Phase vorhanden sind. TUNEL Färbungen und Untersuchungen hinsichtlich der Lebensfähigkeit zeigten, dass PS positive *L. major* apoptotisch sind, unfähig zur Proliferation und somit im Absterben begriffen. Elektronenmikroskopische Untersuchungen zeigten zudem, dass PS positive Parasiten morphologisch nicht intakt sind.

Infektiöse *L. major* Kulturen stationärer Phase beinhalten sowohl PS positive apoptotische als auch PS negative lebendige Parasiten. Interessanterweise führte die Depletion PS positiver Parasiten, obgleich diese nicht lebendig sind, im Vergleich zur Überlebensrate normal stationärer Kulturen, welche PS positive und PS negative *L. major* beinhalten, zu einer Abnahme der Überlebensrate des Parasiten in PMN. Koinkubationen von PMN mit aufgereinigten PS negativen *L. major* führte zur Freisetzung von proinflammatorischem TNF-α, wohingegen die zusätzliche Anwesenheit PS positiver Parasiten in Kulturen der stationären Phase die Zytokinantwort hinsichtlich eines antiinflammatorischen Milieus veränderte,

charakterisiert durch eine gesteigerte Menge an freigesetztem TGF-ß und eine herunterregulierte TNF-α Production.

Basierend auf diesen *in vitro* Befunden stelle ich dir Hypothese auf, dass die Gegenwart apoptotischer Populationen eine Voraussetzung für die Infektiösität von *L. major* Promastigoten ist. Experimentelle Infektion empfindlicher Balb/c Mäuse hat diese Hypothese *in vivo* bestätigt. PS negative (d.h. lebendige) *L. major* induzierten eine deutlich geringere Krankheitsentwicklung im Vergleich zu Parasiten Populationen bestehend aus einer Mischung aus PS negativen und einem hohen Anteil PS positiver Parasiten. Diese Daten bestätigten die Hypothese, dass apoptotische Parasiten lebende Parasiten bei der Initiierung der Infektion unterstützen.

Die natürliche *L. major* Infektion erfolgt über den Stich einer infizierten Sandmücke. Der Befund, dass aus infizierten Sandmücken isolierte *L. major* ebenfalls einen hohen Anteil PS positiver Parasiten beinhalten, lässt die Wichtigkeit PS positiver *L. major* hinsichtlich der natürlichen Infektion vermuten.

Vorbehandlung der Parasiten mit PS bindendem Annexin-V blockierte die Aufnahme von *L. major* durch PMN. Diese Beobachtung legt nahe, dass PMN einen PS Rezeptor (PSR) exprimieren. Dieser PSR konnte auf PMN mittels der verfügbaren Antikörper allerdings nicht eindeutig nachgewiesen werden. Prezipitationsexperimente mit biotinylierten PMN Membranfragmenten zeigten jedoch drei bisher nicht identifizierte Proteine, die an *L. major* binden. Eines dieser drei Proteine könnte möglicherweise ein PSR sein. Diesbezüglich sind weiter Studien erforderlich, um diese Proteine zu identifizieren und zu charakterisieren.

Zusammengefasst legen die Ergebnisse dieser Dissertation nahe, dass apoptotische *L. major* Promastigoten mittels Induktion antiinflammatorischer und Phagozyten-deaktivierender Zytokine lebende Parasiten bezüglich ihrer Überlebensfähigkeit in PMN unterstützen. Apoptotische Parasiten regulieren die Immunantwort von PMN herunter und ermöglichen das Überleben nicht-apoptotischer Parasiten im Sinne der Initiierung einer erfolgreichen Infektion

# VIII Appendix

## 1. Abbreviations

| | |
|---|---|
| Ab | Antibody |
| ABC1 | ATP-binding-cassette-transporter 1 |
| AP | Alkaline phosphatase |
| APLT | Aminophospholipid translocase |
| AV | Annexin-V |
| BHI | Brain heart infusion |
| BSA | Bovine serum albumin |
| CD | Cluster of differentiation |
| c-DNA | Complementary desoxyribonucleic acid |
| CNBr | Cyanogen bromide |
| CR | Complement receptor |
| CRP | C-reactive protein |
| DC | Dendritic cell |
| DHR | Dihydrorhodamine |
| DMEM | Dulbecco's Modified Eagle Medium |
| DNA | Desoxyribonucleic acid |
| DTT | Dithiothreitol |
| ECL | Enhanced chemoluminescence |
| EDTA | Ethylendiaminetetraacetic acid |
| EGTA | Ethyleneglycoltetraacetic acide |
| ELISA | Enzyme-linked immunoabsorbent assay |
| ER | Endoplasmatic reticulum |
| Exp. | Experiment |
| FACS | Fluorescence-activated cell sorter |
| FADD | Fas-associated death domain |
| Fc | Fragment crystallisable (part of an immunoglobuline molecule) |
| FCS | Fetal calf serum |
| Fig. | Figure |
| FITC | Fluorescein isothiocyanate |
| FSC | Forward-angle light scatter |

| | |
|---|---|
| g | Gravitation |
| G-CSF | Granulocyte colony stimulating factor |
| h | Hour |
| HEPES | N-2-Hydroxyethylpiperazine-N'-2-Ethansulfonacid |
| HRP | Horseradish peroxidase |
| HRPO | Horseradish peroxidase |
| ICAM | Intracellular adhesion molecule |
| IFN | Interferon |
| Ig | Immunoglobulin |
| IL | Interleukin |
| i.p. | Intraperitoneal |
| kHz | Kilohertz |
| *L. major*, *Lm* | *Leishmania major* promastigotes |
| *Lm* met | Metacyclic *L. major* |
| *Lm* met PS- | PS negative metacyclic *L. major* |
| *Lm* met PS+ | PS positive metacyclic *L. major* |
| *Lm* PS- | PS negative *L. major* |
| *Lm* PS+ | PS positive *L. major* |
| *Lm* stat | Stationary phase *L. major* |
| *Lm* stat PS- | PS negative stationary phase *L. major* |
| *Lm* stat PS+ | PS positive stationary phase *L. major* |
| LPC | Lysophosphatidylcholine |
| LPG | Lipophosphoglycan |
| mAb | Monoclonal antibody |
| MACS | Magnetic cell sorting |
| M-CSF | Macrophage colony stimulating factor |
| met | Metacyclic |
| MF | Membrane fragments |
| MFR | Mannose-fucose receptor |
| min | Minute |
| MIP | Macrophage inflammatory protein |
| m-RNA | Messenger ribonucleic acid |
| Mv1Lu | Mustela vision (mink) lung fibroblast |
| МΦ | Macrophage |

| | |
|---|---|
| NC | Nitrocellulose |
| NNN | Novy-Nicolle-McNeal |
| NO | Nitrogen monooxyde |
| NP-40 | Nonidet-P40 |
| on | Over night |
| OxLDL | Oxidized low-density lipoprotein particle |
| pAb | Polyclonal antibody |
| PAGE | Polyacrylamide gel electrophoresis |
| PAMP | Pathogen-associated molecular pattern |
| PBMC | Peripheral blood mononuclear cell |
| PBS | Phosphate buffered saline |
| PE | Phycoerythrine |
| PECAM-1 | Platelet endothelial cell adhesion molecule-1 |
| PMA | Phorbol myristate acetate |
| PMN | Polymorphonuclear neutrophilic granulocyte |
| PMN-MF | Polymorphonuclear neutrophilic granulocyte membrane fragments |
| PMSF | Phenylmethanesulfonyl fluoride |
| PNA | Peanut lectin agglutinin |
| PRR | Pattern recognition receptor |
| PS | Phosphatidylserine |
| PS- | PS negative |
| PS+ | PS positive |
| PSR | Phosphatidylserine receptor |
| RPMI | Roswell Park Memorial Institute |
| RT | Room temperature |
| s.c. | Subcutaneous |
| SD | Standard deviation |
| SDS | Sodium dodecyl sulfate |
| stat | Stationary phase |
| TdT | Terminal deoxynucleotidyl transferase |
| TEMED | N, N, N', N'-tetramethylethylendiamin |
| TGF | Transforming growth factor |
| Th | T-helper |

| | |
|---|---|
| TLR | Toll like receptor |
| TNF | Tumor necrosis factor |
| Tris | Tris(hydroxymethyl)aminomethan |
| TSP | Thrombospondin |
| TUNEL | Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling |
| UTP | Uridine triphosphate |
| UV | Ultraviolett |
| W | Watt |
| WHO | World Health Organisation |
| WinMDI | Windows Multiple Document Interafce for Flow Cytometry |

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## 3. List of publications, meetings and awards

### Publications

- van Zandbergen, G., J. Gieffers, H. Kothe, J. Rupp, **A. Bollinger**, E. Aga, M. Klinger, H. Brade, K. Dalhoff, M. Maass, W. Solbach, and T. Laskay. 2004. *Chlamydia pneumoniae* multiply in neutrophil granulocytes and delay their spontaneous apoptosis. *J.Immunol.* 172:1768-1776.

- *Leishmania major* misuse the apoptotic "eat-me" signal phosphatidylserine for establishment of a productive infection.
  **Bollinger A**., M. Herrmann, R. Voll; A. Mueller, D. Haller, M. Klinger, D. Sacks, W. Solbach, T. Laskay, G. van Zandbergen; in preparation

### National and international meetings

- 27th Meeting of the North German Immunologists (26.11.2004), Borstel, Germany,
  Apoptotic *Leishmania major*promastigotes silence polymorphonuclear neutrophils. **Oral presentation**

- Joint Annual Meeting of the German and Dutch Societies of Immunology (20. – 23.10.2004) Maastricht, The Netherlands,
  Apoptotic *Leishmania major* promastigotes silence polymorphonuclear neutrophils. **Poster presentation**

- 8th Symposium "Infection und Immune Defense" (11. – 13.03.2004), Rothenfels, Germany, *Leishmania major* promastigotes use apoptotic mimicry for "silent" infection of phagocytes. **Oral presentation**

- 26th Meeting of the North German Immunologists (24.11.2003), Borstel, Germany,
  *Leishmania major* promastigotes use apoptotic mimicry for "silent" infection of phagocytes. **Oral presentation**

- 34th Annual meeting of the DGfI (24. – 27.09.2003), Berlin, Germany, *Leishmania major* promastigotes use phosphatidyl serine for “silent entry“ into polymorphonuclear leukocytes (PMN). **Oral presentation**

- 1st Spring Workshop of Immunology, DGfI (13. – 15.03.2003), Leipzig, Germany, *Leishmania major* promastigotes use phosphatidyl serine for “silent entry“ into polymorphonuclear leukocytes (PMN). **Oral presentation**

- 33th Annual meeting of the DGfI (25. – 28.09.2002), Marburg, Germany

**Award**

- Award for “The most Luminescent Presentation” (27th Meeting of the North German Immunologists (26.11.2004), Borstel, Germany)

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# X Curriculum vitae

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Born 13th January 1975 in Berlin

**Education:**

| | |
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| since 04/02 | **Doctoral candidate**<br>University of Luebeck<br>Institute of Medical Microbiology and Hygiene |
| 04/98 – 02/02 | **Studies in Biology** at the "Freie Universität" of Berlin, Germany, graduation: diploma in Biology |
| 04/97 – 12/97 | **Studies in Biology** at the University of Reykjavik, Iceland<br>Erasmus grant |
| 04/95 – 04/98 | **Studies in Biology** at the Gutenberg Universität of Mainz, Germany |
| 08/85 – 05/94 | **High School** in Wadern, Germany, graduation: Abitur |

**Further educations:**

| | |
|---|---|
| 04/02 – 09/05 | Advanced training (Graduiertenkolleg 288) |
| 28/02/02 – 04/03/05 | Advanced training “Spring school on Immunology”, Ettal, Germany |
| 04/98 – 02/02 | Employee of the Institute of Veterinary Medicine, Berlin, Germany |
| 01/94 – 04/95 | Practical training in the Maternité Lagune hospital of Coutonou, Benin, Africa |
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