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PATHOGEN-MEDIATED MODULATION OF SIGNAL TRANSDUCTION AND GENE EXPRESSION IN NEUTROPHIL GRANULOCYTES

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ABBREVIATIONS

Ab	Antibody
ACTB	Beta-actin
ANOVA	Analysis of variance
APC	Antigen-presenting cell
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BHI	Brain heart infusion
BLys	B-Lymphocyte stimulator
bp	Base pair
C	Cysteine
BSA	Bovine serum albumin
C3	Complement component 3
CD	Cluster of differentiation
cDNA	Complementary DNA
CTP	Cytosine triphosphate
DC	Dendritic cell
DCL	Diffuse cutaneous leishmaniasis
DC-SIGN	DC-specific ICAM-3-grabbing non-intregrin
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EBI3	Epstein-Barr virus-induced gene 3
ECL	Enhanced chemiluminescene
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycoltetraacetic acid
ELISA	Enzyme linked immuno sorbent assay
FACS	Fluorescence activated cell sorter
Fc	Fragment crystalline
FCS	Fetal calf serum
Gal_f	Galactofuranose
GIPL	Glycoinositolphospholipid

GM-CSF	Granulocyte-macrophage-colony-stimulating factor
GTP	Guanidine triphosphate
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
HRP	Horseradish peroxidise
ICAM	Intracellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRF	Interferon regulatory factor
JAK	Janus kinase
L.	*Leishmania*
LCL	localized cutaneous leishmaniasis
L. major, *L.m.*	*Leishmania major*
LPG	Lipophosphoglycan
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MCL	mucocutaneous leishmaniasis
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NC	Nitrocellulose
NET	Neutrophil extracellular trap
NK	Natural killer
OD	Optical density
P	Phosphate
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PIAS	Protein inhibitor of activated STAT

PIPES	1,4-Piperazinediethanesulfonic acid
PMA	Phorbol myristate acetate
PMN	Polymorphonuclear neutrophil granulocytes
PMSF	Phenylmethanesulfonyl fluoride
PS	Phosphatidylserine
PTP	Protein tyrosine phosphatase
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPA	RNase protection assay
RPE	R-Phycoerythrin
RPMI	Roswell Park Memorial Institute
SD	Standard deviation
SDS	Sodium dodecylsulfate
SOCS	Suppressor of cytokine signaling
STAT	Signal Transducer and Activator of Transcription
TBE	Tris borate-EDTA buffer solution
TBS	Tris buffered saline
TEMED	N, N, N´, N´-Tetramethylethylenediamine
TGF	Transforming growth factor
Th	T helper
TLR	Toll-like receptor
TMB	3,3´,5,5´-Tetramethylbenzidine
TNF	Tumor necrosis factor
Tris	Tris[Hydroxymethyl]aminomethane
UDP	Uridine diphosphate
UGM	UDP-galactopyranose mutase
UTP	Uridine triphosphate
UV-Vis	Ultra violet visible
VL	Visceral leishmaniasis
Y	Tyrosine

1 INTRODUCTION

The human immune system employs various mechanisms of defense in order to ensure protection from invading pathogens. Mechanical, chemical and biological surface barriers prevent microorganisms from intruding the human body. Epithelial cells of the skin form a physical border against pathogens while antimicrobial peptides and enzymes chemically protect mucosal surfaces. The commensal flora of the genitourinary and gastrointestinal tracts counteracts infection by modulating conditions in the environment and competing with pathogens for living space and nutrients. Yet, if these barriers have been surmounted, the human body elicits an immune response which prevents replication and spreading of pathogens. The immune system consists of two major subdivisions, the innate or non-specific immune system and the adaptive or specific immune system. Including defense mechanisms which, for the most part, are constitutively present and ready to be mobilized upon infection, the innate immune system provides an immediate response to microbial challenge. Cells of the innate immune system including macrophages, granulocytes (neutrophils, basophils and eosinophils), dendritic cells, mast cells, and natural killer cells directly eliminate pathogens or infected cells. Moreover, they function as important mediators in the activation of the adaptive immune system. If pathogens successfully evade the innate immune response, the adaptive immune system provides a further line of defense. In order to ameliorate recognition of a pathogen the immune system adapts its response during infection. After clearance of the pathogen, this improved response is preserved in the form of an immunological memory, enabling the adaptive immune system to respond more rapidly and efficiently to repeated attacks of the respective pathogen.

1.1 NEUTROPHIL GRANULOCYTES

Polymorphonuclear neutrophil granulocytes (PMN) were described by Paul Ehrlich (1) when fixation and staining techniques allowed visualization of the lobulated nucleus and the granules that have given these cells their name. Neutrophils belong to the

professional phagocytes in humans and play a major role in antimicrobial defense. They are the first cells to migrate from the blood into inflamed tissues, where they eliminate pathogens either within the cell following phagocytosis or outside the cell by release of toxic mediators. The latter function is associated with collateral tissue damage. Neutrophils amplify the inflammatory response by the release of cytokines (2, 3) and chemokines (4). They can therefore be considered as both inflammatory and immunoregulatory cells.

Neutrophils originate from hematopoietic stem cells in the bone marrow. These pluripotent self-renewing precursors give rise to the lymphoid and myeloid cell lineages, the latter of which represents progenitors of PMN. During their maturation which takes approximately 14 days, these cells undergo a variety of morphological changes which are referred to by the terms promyeloblast, myelocyte, and metamyelocytes. Granulocytes, including basophils, eosinophils and neutrophils emerge from metamyelocytes (5). Under normal conditions the bone marrow releases about 10^{11} PMN per day into the blood stream. Yet, in case of an infection, production of PMN may increase to up to 10^{12} per day (6). Within the blood stream, neutrophils form the most abundant population of leukocytes, representing more than 50 % of this cell type (5). Since neutrophils are constantly produced in large numbers, the same amount of cells needs to be eliminated within a defined time span in order to maintain homeostasis (7). PMN are inherently short-lived cells that undergo apoptosis within 6-8 hours. Safe turnover of these potentially harmful cells is achieved by apoptosis which is accompanied by common morphological features including condensation of the nucleus and intracellular organelles, aggregation and subsequent cleavage of chromatin, and formation of apoptotic bodies. Importantly, apoptotic changes involve downregulation of cellular functions such as phagocytosis, oxidative burst and degranulation which could otherwise damage surrounding vasculature or tissue (8). Apoptotic neutrophils display phosphatidylserine on their surface (9), which allows their recognition and subsequent uptake by macrophages (10-12). The duration of the neutrophil life span can be prolonged by signals from their microenvironment such as inflammatory mediators and infectious agents.

1.1.1 NEUTROPHILS IN INFLAMMATION

When pathogenic microorganisms infect the host, neutrophils migrate to the site of infection within 2-4 hours. Inflammatory signals originating from the site of infection mediate adherence of PMN to blood vessels at sites of tissue damage (13). Neutrophils then migrate through the endothelium (14). Tissue macrophages at the site of inflammation produce chemokines that induce the chemotaxis of leukocytes (15, 16). This accumulation of leukocytes forms the first step in immune surveillance and plays a key role in immune defense. Extravasation of leukocytes from the blood vessels into the inflamed tissue is a process occurring in four steps. During the first step, neutrophils reversibly bind to vascular endothelium which involves interactions between selectins induced on endothelium and their carbohydrate ligands on leukocytes (17). Since these interactions are rather weak, PMN roll along the blood vessels, partly tethering to the endothelium. In a second step, PMN firmly adhere to the vasculature. Tight binding depends on induction of ICAM1 on endothelium and leukocyte integrins LFA-1 (18) and Mac1 (19) on PMN. During the third step, which is referred to as diapedesis, PMN traverse the endothelial layer and basement membrane with the aid of metalloproteinases (20). Finally, during the fourth step, neutrophils migrate along a concentration gradient of chemokines, among which interleukin-8 (IL-8) is the most prominent one, toward the site of infection (15).

1.1.2 RECOGNITION OF PATHOGENS

At the site of infection, PMN identify microorganisms through a variety of receptors. Direct recognition occurs via Toll-like receptors (TLRs), detecting a broad range of molecular patterns (21, 22). These so-called pathogen-associated molecular patterns (PAMPs) occur in many pathogens. Furthermore, PMN express Fc receptors on their surface. Microorganisms which have been detected by Ig antibodies display the latter on their surface with the Fc region exposed to the exterior. Fc receptors of PMN recognize pathogens via the Fc region of bound antibody (23). Complement receptors detect the complement component C3b. Microorganisms which are coated with C3b can be identified by this receptor (24). A further means of pathogen detection is based on scavenger receptors which bind to a variety of polyanions on the surface of

microorgansims (25). Recognition of pathogens by TLRs, Fc receptors, complement receptors and scavenger receptors results in enhanced phagocytosis and activation of metabolic activity. Binding of infectious agents is furthermore associated with the release of proinflammatory cytokines such as interleukin-1 (IL-1), tumor necrosis factor (TNF) and interleukin-6 (IL-6).

1.1.3 KILLING OF PATHOGENS

After attachment of a pathogen, the neutrophil starts to extend pseudopods around the bacterium. These engulf the pathogen and finally enclose the infective agent within a phagosome. In the course of phagocytosis, neutrophil granules fuse with the phagosome and empty their digestive and antimicrobial contents. Several intracellular pathogens, however, prevent these fusion events, thus facilitating the pathogen`s survival within the cell (26-29). Phagocytes not only kill pathogens within phagolysosomes but can also secrete antimicrobial compounds. If neutrophils sense tissue damage and infection but fail to phagocytose a pathogen within a short time span, their antimicrobial products are released extracellularly. Moreover, PMN have been shown to extrude neutrophil extracellular traps (NETs), a web of fibers predominantly composed of chromatin and serine proteases that trap and kill pathogens (30). NETs not only display antimicrobial properties but are also thought to serve as a physical barrier that prevents the spread of pathogens.

Neutrophils basically contain two kinds of granules, the contents of which mediate the antimicrobial properties of these cells (31). Primary or azurophilic granules are predominantly abundant in newly formed PMN. They contain cationic proteins and defensins which kill pathogens, lysozyme that breaks down bacterial cell walls and proteolytic enzymes like elastase, proteinase 3 and cathepsin G. Characteristically, azurophilic granules store myeloperoxidase (32, 33) which catalyzes the generation of bactericidal hypochlorite. Specific or secondary granules occur in more mature PMN. Like azurophilic granules, they contain lysozyme. Particularly, lactoferrin, an iron-chelating protein, and B12-binding protein are stored in specific granules. They furthermore contain cytochrome b 245 (also named gp91 phox) (34, 35), which represents a major component of the NADPH oxidase involved in generation of toxic oxygen products (36). Killing of pathogens is thus on the one hand achieved by several

preformed compounds which mainly comprise enzymes. On the other hand these oxygen-independent antimicrobial functions of immune defense are complemented by oxygen-dependent mechanisms. During phagocytosis there is an increase in glucose and oxygen consumption which is referred to as respiratory burst. As a consequence of respiratory burst, a number of oxygen-containing compounds are produced. In this process NADPH oxidase catalyzes the formation of superoxide anions part of which are converted to hydrogen peroxide and singlet oxygen (37). Moreover, superoxide anion can react with hydrogen peroxide resulting in the formation of hydroxyl radicals and more singlet oxygen. Altogether, these reactions result in the production of toxic oxygen compounds such as superoxide anion (O_2), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hydroxyl radicals (OH). Myeloperoxidase is released into the phagosome as azurophilic granules fuse with the phagosome. This enzyme utilizes hydrogen peroxide and chloride ions to form hypochlorite, which represents a highly toxic substance that kills pathogens (38, 39).

1.1.4 REGULATION OF NEUTROPHIL FUNCTION

Neutrophil action is finely regulated by cytokines and chemokines. Proinflammatory cytokines, such as IL-1 and TNF amplify neutrophil functions, including their capacity of adhering to endothelial cells (40) and to produce reactive oxygen species (ROS) (41). Likewise, chemokines such as IL-8 form potent attractants favoring orientated migration toward the site of infection (16). Both, chemokines and cytokines may act as priming agents sensitizing neutrophils for further stimuli (42-44).

IFN-γ directs many antimicrobial functions in PMN (45, 46). Depending on environmental conditions and on further stimuli, it elicits a variety of responses including oxidative burst (47-50), differential gene expression (51-53) and antigen presentation (54, 55). IFN-γ signals via the Janus kinase signal transducer and activator of transcription (JAK-STAT) pathway (56, 57). Binding of IFN-γ results in assembly of the receptor α-chain (IFNGR1/CD119) and of the â-chain (IFNGR2) (58, 59). Subsequently Janus kinases which are constitutively associated with the receptor are activated by cross-phosphorylation (60, 61). Specific tyrosine residues on the receptor are then phosphorylated by (62), providing docking sites for STAT1 monomers that

exist as latent transcription factors in the cytosol. STAT1 monomers are phosphorylated by JAKs and dimerize (63, 64). STAT1 dimers then translocate to the nucleus, where they activate gene transcription (Fig. 1-1 A). Many IFN-γ-induced genes are synergistically activated by multiple transcription factors forming an assembly named enhancosome or successively exerting gene transcription. Interferon regulatory factor 1 (IRF-1) and PU.1, which binds to a purine-rich sequence called PU box, form an assembly enhancing transcription of a subset of IFN-γ-induced genes (65, 66) (Fig. 1-1 B).

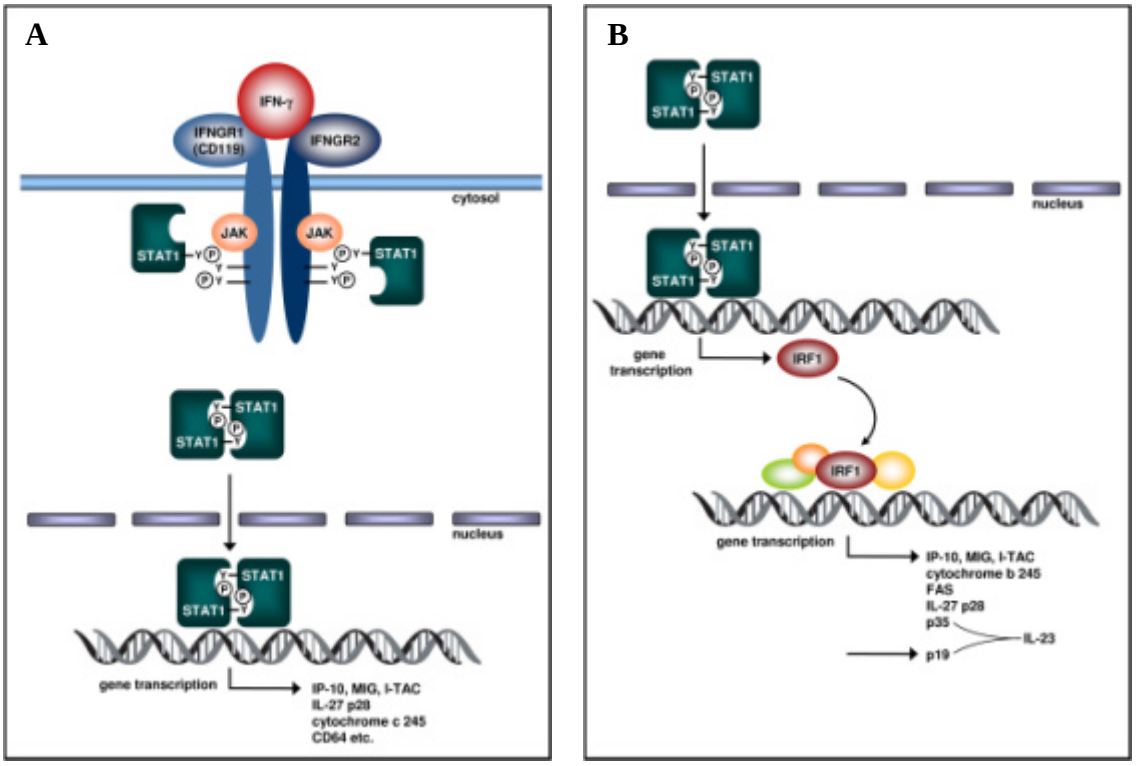


Fig. 1-1 A Induction of IFN-g signaling. After Binding of IFN-γ, receptor assembly occurs; receptor-associated JAKs are brought together, allowing cross-phophorylation (P). The activated JAKs tyrosine (Y)-phosphorylate and activate the IFN-ã receptor. STATs are recruited through specific interactions with phosphorylated tyrosine residues on the receptors and become phosphorylated by JAKs, which allows their dimerization and translocation to the nucleus. Here, STAT1 activates transcription of cytokine-responsive genes such as IP-10, MIG, I-TAC, IL-27p28, cytochrome b 245 and CD64.

Fig. 1-1 B Induction and activities of IRF-1. The IRF-1 gene is induced in response to IFN-γ by the transcription factor STAT1. IRF-1 binds to several other transcription factors which cooperatively activate a subset of IFN-γ-induced genes, among them IP-10, MIG, I-TAC, FAS, and IL-23 p23.

IFN-γ response is tightly regulated by several mechanisms. Suppressor of cytokine signaling 1 (SOCS1) can directly bind to JAKs, inhibiting their kinase activity while SOCS3 inhibits JAKs by binding to the IFN-γ receptor (67-69). Several different

protein inhibitors of activated STAT (PIAS) can inhibit JAKs and STATs (70, 71). Moreover, JAKs and STATs can be deactivated by protein tyrosine phosphatases (PTPs) and by proteasomal degradation (72-74) (Fig. 1-2).

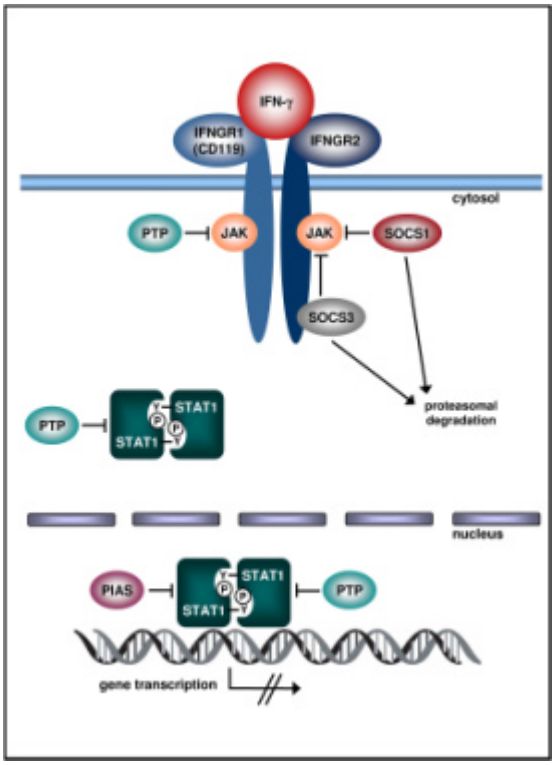


Fig. 1-2 Negative regulation of IFN-g signaling. The IFN-γ signaling is regulated at several levels. SOCS1 binds directly to tyrosine-phosphorylated JAKs while SOCS3 inhibits JAKs through binding of the receptor. Both promote ubiquitination and proteasomal degradation of their targets. JAKs can furthermore be negatively regulated by protein tyrosine phophatases. These can also negatively regulate STAT1. Transcriptional activity of STAT1 is moreover inhibited by PIAS proteins.

IFN-γ induces increased expression of the proinflammatory cytokines TNF and interleukin-1β (IL-1â) (75) as well as of the Th1 cell-attracting chemokines IFN-ã-induced protein of 10 kDa (IP-10), monokine induced by gamma-interferon (MIG) and interferon-inducible T cell alpha chemoattractant (I-TAC) (52, 76-78). Furthermore, expression of the Fcγ receptor I (FcγR1/CD64) is augmented by IFN-γ (79, 80), and the membranous subunit of the NADPH oxidase cytochrome b 245 is produced in higher amounts (53, 80, 81). Antigen presentation is enhanced in response to IFN-γ which is reflected by an increase in MHC class II molecules (54, 82), CD80, CD83 and CD86 (55, 83, 84) expression.

1.1.5 IMMUNOMODULATORY FUNCTIONS OF NEUTROPHILS

Neutrophils are not only a target but also a source of cytokines (2, 3). They represent key components of the inflammatory response that are able to exert immunomodulatory functions and to act as decision-shapers. Being a major source of cytokines and chemokines (4) at sites of infection, neutrophils contribute to recruitment, activation and programming of APCs. Neutrophils release chemotactic factors that attract monocytes and dendritic cells (85, 86). They furthermore direct macrophage differentiation (87). Proteolytic activation of prochemerin results in generation of chemerin which acts as an attractant for immature as well as for plasmacytoid DCs (88). TNF secreted by neutrophils activates macrophages and DCs and drives their differentiation (85, 87, 89). Activation of DCs is intensified by their contact to PMN, engaging neutrophil CD11b and DC-specific ICAM-3-grabbing non-integrin (DC-SIGN) (89). Neutrophil secretion of B-lymphocyte stimulator (BLys) promotes proliferation and maturation of B cells (90). Neutrophils have been shown to produce interleukin-12 (IL-12) (91). The latter plays a central role in promoting the differentiation of naï ve $CD4^+$ T cells into IFN-γ-producing Th1 effector cells, which are central for the development of a protective immunity to intracellular pathogens. By the release of IL-12, neutrophils have the potential of directing $CD4^+$ T cell differentiation toward a Th1 response (92, 93). Previous studies from our group aimed to clarify whether neutrophils express members of the IL-12 cytokine family (Fig. 1-3). RNase protection assays (RPA) revealed that neutrophils express IL-12 p40 and p19 which form the subunits of IL-23. Furthermore, the expression of the IL-27 subunits EBI3 and p28 was detected by RPA. Like IL-12, the cytokines IL-23 and IL-27 promote a Th1 response (94, 95). Yet, they exert distinct functions in Th1 development. While IL-27 is of major importance in the early stage of a Th1 response (96-98), IL-23 directs long term memory increasing proliferation of CD4+ memory T cells (97, 99). Moreover, IL-23 induces differentiation of Th17 cells which are a major source of IL-17 (100, 101). This cytokine, which has also been reported to be produced by neutrophils themselves, plays a crucial role in the induction of neutrophil-mediated inflammation and optimal Th1 response (102, 103). IL-17 is of importance in neutrophil homeostasis and recruitment (104, 105). The described

properties make IL-17 an efficient agent in the defense of intracellular pathogens (106-108).

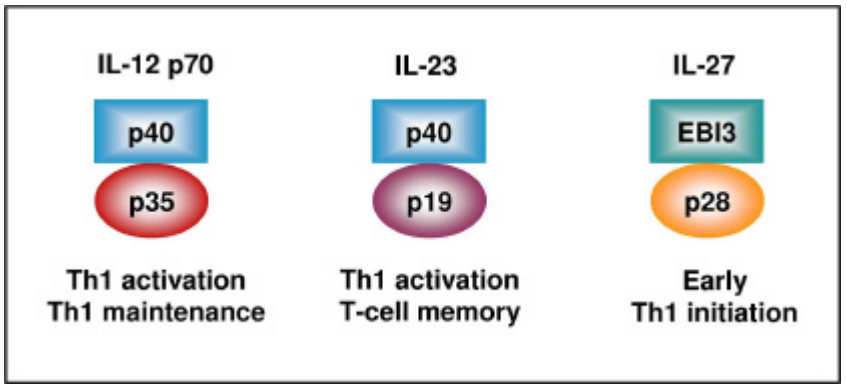


Fig. 1-3 The IL-12 cytokine family. Members of the IL-12 cytokine family promote differentiation of naï ve CD4+ T cells toward a Th1 response. IL-12 consists of a p40 and a p35 subunit and is crucial for Th1 activation and maintenance. IL-23 is composed of a p40 and a p19 subunit and is crucial for T-cell memory. IL-27 which consists of EBI3 and p28 plays a pivotal role in Th1 initiation and early Th1 response.

Neutrophils produce a great variety of chemokines (4). These form a group of structurally related cytokines that specifically recruit leukocytes subsets (109-111). Their primary sequence displays characteristic patterns of cysteine residues that form the basis for classification of these molecules into CXC and CC chemokines. The CXC family can be further subdivided due to the glutamate-leucin-arginine (ELR) motif that precedes the first two cysteines (109-111). ELR-CXC chemokines comprise among others IL-8 and growth-related gene product (GRO)-á and -â. Members of this group act as chemoattractants for neutrophils and display angiogenic properties (109-111). On the contrary, non-ELR chemokines recruit T lymphocytes and function as angiostatic agents (76, 77). Neutrophils have been shown to release MIG, IP-10 and I-TAC (52, 78), which primarily attract Th1 lymphocytes. By secreting various chemokines, PMN can successively recruit certain leukocytes subtypes to the site of inflammation, thus regulating local immune response. On a per-cell basis, neutrophils produce lower amounts of cytokines than mononuclear phagocytes. Since they outnumber the latter by far, they nevertheless, represent an important source of cytokines.

1.2 INTRACELLULAR PATHOGENS

Phagocytes are generally supposed to kill ingested pathogens. However, several microorganisms manage to survive inside these cells. The bacterium *Anaplasma phagocytophilum* and the parasite *Leishmania major* represent two examples of microorganisms that survive inside PMN (112, 113).

1.2.1 *LEISHMANIA*

Protozoan parasites of the genus *Leishmania* are transmitted to mammalian hosts by the bite of phlebotomine sandflies (Fig. 1-4 A) of the genus *Phlebotomus* and *Lutzomyia* which occur throughout the world´s inter-tropical and temperate regions (114). The female insect infects itself while sucking blood from a vertebrate host of *Leishmania* in order to obtain the necessary proteins to develop its eggs. *Leishmania* parasites live and multiply inside macrophages of the vertebrate host as immobile, round amastigotes. These are ingested by the sandfly during a blood meal and thus get to the peritrophic membrane of the insect´s mid-gut where they transform to the mobile, elongate promastigote form. This developmental stage can be further subdivided. Procyclic stage parasites have a low virulence. They attach to the epithelial cells of the sandfly´s midgut and rapidly divide. Several days later the parasites differentiate into the virulent metacyclic stage and migrate to the foregut and esophagus of the sandfy where they are suspended in the saliva of the insect. During a further blood meal, the sandfly inoculates a new victim with the parasite, thus completing the life cycle of the parasite (115, 116).

Leihmaniasis is a group of diseases comprising a large spectrum of symptoms ranging from cutaneous over diffuse cutaneous and mucocutaneous to visceral forms. Several different *Leishmania* species account for various clinical manifestations of the disease. The localized cutaneous leishmaniasis (LCL), which is primarily caused by *Leishmania major* and *Leishmania tropica* (*L. major* and *L. tropica*) produces self-healing skin ulcers on exposed parts of the body. On the contrary, *Leishmania aethiopica* and *Leishmania mexicana amazonensis* (*L. aethiopica* and *L. mexicana amazonensis*) cause chronic diffuse cutaneous leihmaniasis (DCL). Progressive mucocutaneous leihmaniasis (MCL), the causative agent of which are *Leishmania braziliensis (L. braziliensis)* and

Leishmania mexicana pifanoi (L. mexicana pifanoi), can result in partial or total destruction of the mucous membranes. The most severe form of leishmaniasis, visceral leihmaniasis (VL) or kala azar, is caused by *Leishmania donovani, Leishmania infantum and Leishmania chagasi (L. donovani, L. infantum* and *L. chagasi)*. It affects the spleen, liver and bone marrow and is fatal if untreated.

In the mammalian host, *Leishmania* resides within macrophages (117, 118), dendritic cells (119, 120) and PMN (113, 121). Inside these phagocytes, the obligate intracellular parasite is protected from serum factors that promote its killing. In the presence of serum, *Leishmania* rapidly triggers the classical complement pathway resulting in opsonization by the complement component C3 and of complement-mediated lysis (122, 123). The parasite is furthermore detected by immunoglobulin G (IgG) that induces cell-mediated toxicity when binding to Fcã receptors of phagocytes with its Fc region (124). Moreover, recognition of *Leishmania* by TLRs has been reported to contribute to parasite clearance (125, 126). Thus, sequesteration inside phagocytes appears to protect the parasite from the detrimental effects of humoral immune response and its recognition by TLRs. Yet, phagocytes represent a potentially hostile environment requiring particular evasion strategies in order to escape their antimicrobial functions. Since macrophages are the main host cells for *Leishmania* replication, mechanisms of evasion from phagocyte antimicrobial effector functions have so far primarily been investigated in this cell. Mediating a large variety of antimicrobial effector functions, IFN-γ signaling has been the focus of many attempts to examine how *Leishmania* interacts with its host cell in order to facilitate intracellular survival. Nanadan et al. (127) showed that *L. donovani* attenuates IFN-γ-induced tyrosine phosphorylation of JAK1, JAK2 and STAT1 in mononuclear phagocytes, leading to inhibition of IFN γ signaling. Impaired signaling via STAT1 has furthermore been ascribed to enhanced proteasomal degradation of the transcription factor (128) and to increased expression of its dominant-negative variant STAT1-â (129). Moreover, negative regulation of the IFN-γ receptor has been demonstrated to contribute to inactivation of the cascade (129). The IFN-γ cascade employs a feedback loop limiting its action. SOCS3 forms part of these regulatory mechanisms. *L. donovani* exploits feedback inhibition by upregulating SOCS3 in order to ameliorate its survival conditions (130). Proteinphosphatases (PTP) such as Src homology-1 domain-

containing protein tyrosine phosphatase (SHP1) represent a further means of constitutive feedback inhibition for IFN-γ signaling. *L. donovani* alters signaling events to its advantage by triggering SHP-1-mediated JAK dephosphorylation (131). Thus, *Leishmania* inhibits IFN-γ signaling in macrophages by interference with various members of the cascade resulting in impaired antimicrobial effector functions. Among these, the generation of reactive oxygen species by NADPH oxidase and formation of NO by inducible nitric oxide synthase (iNOS) represent the most significant threats to the parasite (132, 133). *Leishmania*-infected macrophages are not capable of generating NO in response to IFN-γ (134). They furthermore loose the ability to form ROS (135). An effective immune response to *Leishmania* infection depends on cytokine production. *Leishmania* has been reported to prevent macrophage expression of pro-inflammatory cytokines such as IL-1 (136) and TNF (137). The parasite furthermore suppresses production of IL-12 (138, 139), which is essential for the development of host protective Th1 response. Processing and presentation of antigen is also targeted by *Leishmania*. *L. donovani* has been demonstrated to prevent antigen presentation by inhibiting the expression of MHC class II molecules on untreated and IFN-γ-stimulated macrophages (140).

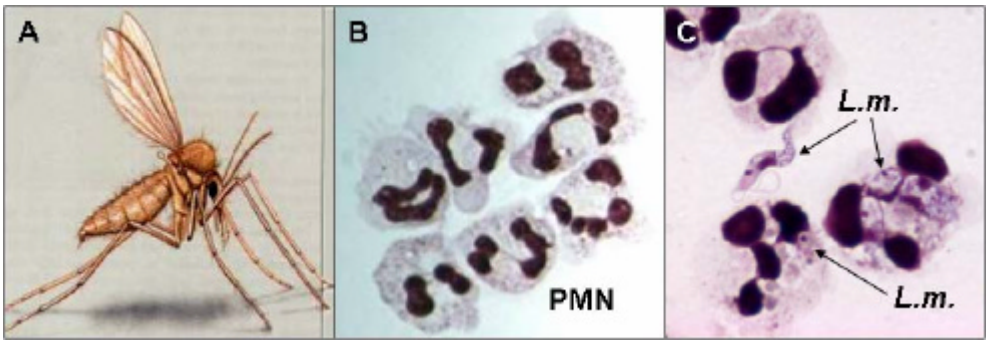


Fig. 1-4 A Phlebotomine sandfly. Infected phlebotomine sandflies inoculate *Leishmania* promastigotes into the skin of vertebrate hosts. (Source of picture: www.who.int/en/)
Fig. 1-4 B Neutrophil granulocytes. Giemsa staining of freshly isolated human neutrophil granulocytes. 1000x magnification of the original.
Fig. 1-4 C *L. major*-infected neutrophil granulocytes. Giemsa staining of *L. major* promastigotes and of *L. major*-infected neutrophils. 1000x magnification of the original.

Evasion of *Leishmania* from macrophage effector functions has been shown to depend on parasite interference with the host cell´s signaling machinery, resulting in impaired antimicrobial defense. Though leishmanial evasion strategies have been studied in much detail with regard to macrophages, the role of neutrophils (Fig. 1-4 B) in leishmania

infection has long been neglected. Laufs et al. could show that neutrophils phagocytose *L. major* (Fig. 1-4 C) and that the majority of the parasites survive intracellularly in the absence of opsonin (113).

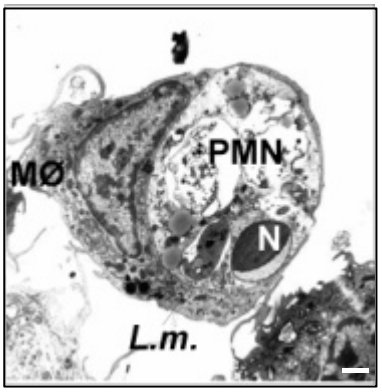


Fig. 1-5 Macrophages phagocytose *L. major*-infected apoptotic PMN. A transmission electron micrograph shows a completely engulfed apoptotic-infected PMN inside a macrophage (MØ) phagosome. The phagosomal membrane contains a complete apoptotic PMN (PMN) with condensed nucleus (N) and a structurally intact parasite (*L.m.*) (bar = 1 µm, magnification x6000). Photograph kindly provided by G. van Zandbergen (121).

Survival inside neutrophils, however, would not *per se* make sense since these cells have a short half-life of only a few hours. Yet, coincubation of PMN with *L. major* promastigotes delays neutrophil apoptosis by approximately 24 hours (141). Infected cells furthermore release MIP-1 â (macrophage inflammatory protein-1 â) which acts as a chemoattractant on macrophages (121). Van Zandbergen et al. demonstrated that macrophages readily phagocytose infected apoptotic PMN (Fig.1-5) and that parasites internalized by this indirect way survived and multiplied inside macrophages. Phagocytosis of apoptotic PMN induced secretion of the anti-inflammatory cytokine TGF-â (transforming growth factor-â) by macrophages (121). These results indicate that *Leishmania* can exploit neutrophils as a "Trojan horse" in order to gain silent entry to their final host cells and to remain unrecognized (142). Inside neutrophils, the parasites remain in the promastigote stage and do not multiply (121). PMN thus represent a transient host cell protecting *Leishmania* from the detrimental effects of serum factors until, approximately 24 hours later, macrophages infiltrate the site of infection. The neutrophil itself, however, is determined to kill invading pathogens and the parasite needs to circumvent antimicrobial functions of its host phagocyte. Van Zandbergen et al. could show that the virulent inoculum of *Leishmania* promastigotes contains a high ration of apoptotic parasites that are crucial for their disease-inducing ability (143).

Further data strongly suggest that similar to apoptotic cells, apoptotic *Leishmania* express phosphatidylserine on their surface, mediating "silent" phagocytosis (143). These results are in line with the finding that apoptotic parasites induce TGF-â release in human neutrophils (143). Moreover, IFN-γ-induced IP-10 production is decreased in the presence of *L. major* pointing to a possible effect of the parasite on IFN-ã signaling in PMN (144). In order to obtain a global picture of IFN-γ- and LPS-induced gene expression in infected neutophils, cDNA arrays were carried out in our group. Among a large amount of genes there was one group showing a particularly interesting expression pattern. Genes within this group are upregulated by LPS and IFN-γ. This upregulation, however, is prevented by *L. major* infection (145). These data indicate that *L. major* interferes with neutrophil expression of LPS- and IFN-γ-induced genes.

The parasite is furthermore protected by its surface molecules. These belong to the glycophosphadidylinositol family which comprises the most abundant molecule on *L. major* surface, Lipophosphoglycan (LPG) (146) as well as a heterogenous group of glycoinositolphospholipids (GIPLs) (147). LPG has been shown to be pivotal for *L. major* virulence. The LPG membrane anchor and GIPLs of *L. major* both contain a galactofuranose residue (Gal_f) (147, 148). The formation of this uncommon monosaccaride which is present on several pathogenic bacteria, fungi and protozoan parasites (149), requires the action of UDP-galactopyranose mutase (UGM) (150-154). Targeted replacement of the UGM encoding *GLF* gene affects synthesis of LPG and GIPLs and attenuates virulence of *L. major* (155). Whether IFN-ã-induced functions are impaired by infection of neutrophils with these parasites that are referred to as $UGM^{-/-}$ *L. major* in this study, remains undiscovered, yet.

1.2.2 *ANAPLASMA PHAGOCYTOPHILUM*

Anaplasma phagocytophilum (*A. phagocytophilum*) is a tick-borne obligate intracellular Gram-negative bacterium that infects neutrophil granulocytes of mammals, including man (112). Inside host neutrophils, *A. phagocytophilum* survives in cytoplasmic vacuoles and inhibits their fusion with lysosomes (156). The bacteria replicate within the host cell vacuole forming a microcolony called a morula (157, 158). *A. phagocytophilum* furthermore escapes antimicrobial effector mechanisms of its host cell by inhibition of ROS production (159-161) and modulation of neutrophil

chemokine response (112, 162). The bacterium delays PMN apoptosis, thus expanding the life span of its host cell. Still, the defense mechanisms against *A. phagocytophilum* infection are poorly understood. However, previous data point to a crucial role of IFN-γ in defense to *A. phagocytophilum.*

Anaplasma infection in immunocompetent mice is usually mild and self limiting. Mice deficient of TLR2, TLR4, MyD88, TNF, iNOS and NADPH oxidase were also able to control the infection (163). Yet, increased bacterial burden was observed in IFN-γ deficient mice (164, 165) and IFN-γ-receptor deficient mice had a prolonged bacteriemia (164). IFN-γ which is prominently produced during murine *A. phagocytophilum* infection was demonstrated to control pathogen burden in the early course of infection (164-166) but was dispensable for the eradication of persistent infection (167).

In a murine model of infection with the closely related *Ehrlichia spp*., the *Ixodes ovatus ehrlichia* (IOE) causing monocytic ehrlichiosis, adoptive transfer experiments demonstrated that $CD4^+$ T cell-dependent production of IFN-γ was required for protective immunity during low dose challenge infection. Importantly, production of IFN-γ by transferred wild-type $CD4^+$ T cells was sufficient to complement the susceptibility of IFN-γ deficient C57BL/6 mice (168).

Although previous findings suggest that IFN-γ-induced mechanisms might be of importance to activate antibacterial mechanisms in *A. phagocytophilum* infected HL-60 cells (169), it is not clear whether IFN-γ-mediated functions have any relation with the ability of PMN to control *Anaplasma* infection. Previous data from our group demonstrate a direct effect of IFN-γ on *A. phagocytophilum*-infected cells *in vitro*. Exposure to IFN-γ led to a marked decrease in bacterial load in HL-60 cells as well as in primary human neutrophils. An increased capacity to mount an oxidative burst was observed in *A. phagocytophilum* infected neutrophils after IFN-γ treatment, albeit to a lesser extent than in uninfected PMN. There is increasing evidence that IFN-γ is of central importance to *A. phagocytophilum* as it represents a potent modulator of neutrophil functions.

1.3 AIMS OF THE STUDY

Neutrophils are of major importance in the defense against pathogens since they are the first cells arriving at the site of infection. Displaying a large variety of antimicrobial and immunomodulatory functions, they can efficiently counteract infection. Pathogens can interfere with the effector and regulatory action of neutrophils in order to evade immune defense. This study investigated how the intracellular pathogens *L. major* and *A. phagocytophilum* affect defense mechanisms of their host cell, the neutrophil.

(I) Previous studies have revealed that *L. major* can survive inside neutrophils and alter the gene expression of its host cell. Microarray data indicated that the parasite downregulates the expression of LPS- and IFN-induced genes. The underlying mechanisms of parasite interference with its host gene expression remain unclear, so far. Since *Leishmania spp*. has been reported to impair IFN-γ signaling in macrophages, I aimed to investigate whether *L. major* modulates the respective signal cascade in PMN (Fig. 1-6). Referring to preliminary microarray data from our group, I furthermore intended to analyze LPS- and IFN-γ-induced gene expression in the context of *L. major* infection by means of real-time PCR in detail. Moreover, expression was to be examined on protein level. The eventual role of parasite viability as well as of LPG and GIPLs on the regulation of IFN-ã-induced neutrophil functions was to be examined by the example of IP-10.

(II) The obligate intracellular bacterium *A. phagocytophilum* survives inside neutrophils. Previous data have demonstrated that IFN-γ is of high relevance in defense against this pathogen and that it partly restores oxidative burst in neutrophils. I addressed the question if IFN-γ elicits further defense mechanisms in neutrophils and whether *A. phagocytophilum* interferes with IFN-γ signaling (Fig. 1-6).

(III) Neutrophils have been reported to secrete a large variety of cytokines, including IL-12. The cytokines IL-12, interleukin-23 (IL-23) and interleukin-27 (IL-27), form the IL-12 family, the members of which have in

common that they drive T cell differentiation toward a Th1 response that is essential in the defense against intracellular pathogens. Preliminary RPA data from our group indicate that neutrophils express IL-23 and IL-27. In this study I aimed to investigate the expression of IL-23 and IL-27 in detail with regard to *L. major* infection. The production of the Th1-recruiting chemokines IP-10, MIG and I-TAC were furthermore to be investigated in the context of infection.

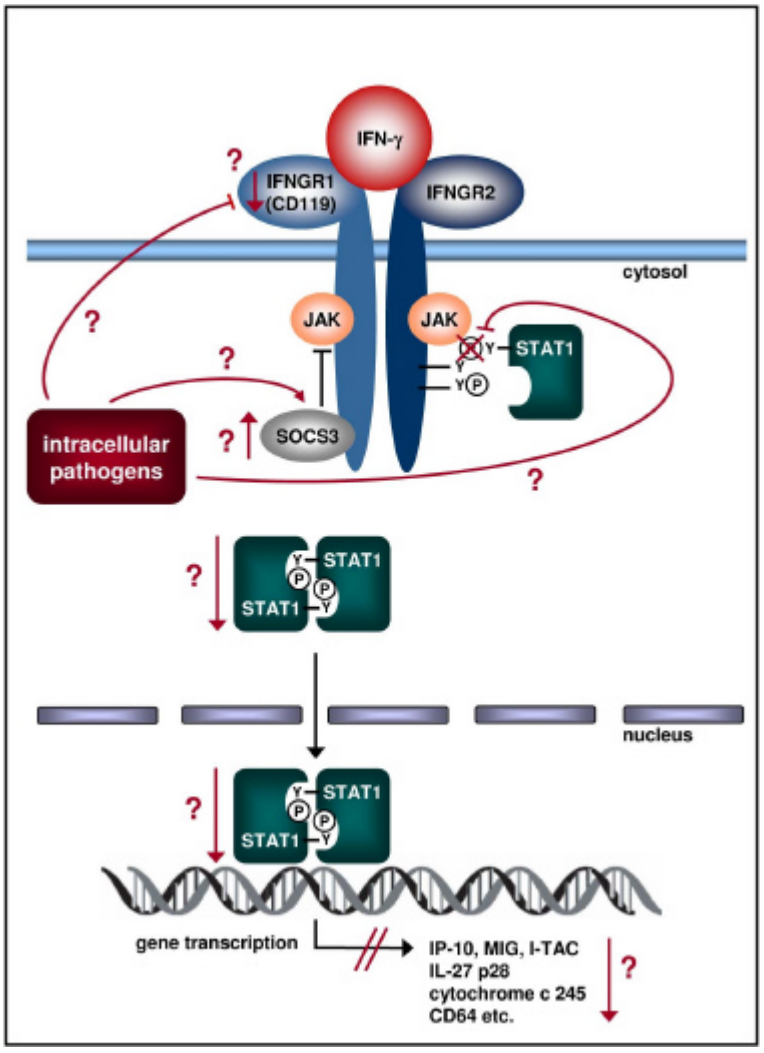


Fig. 1-6 Aims of the study I+II. Investigation of the IFN-ã signal cascade in the context of *Leishmania* and *A. phagocytophilum* infection. Regulation cascade members such as CD119, SOCS3 and pSTAT1 in the presence of these intracellular pathogens was to be analyzed in order to investigate, whether *Leishmania* and *A. phagocytophilum* evade antimicrobial functions of neutrophils by interfering with IFN-ã signaling. Various IFN-ã-induced genes were to be examined on gene and protein level.

2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 *LEISHMANIA* PARASITES

2.1.1.1 *LEISHMANIA MAJOR*

The *Leishmania major* isolate MHOM/IL/81/FEBNI used in this study was originally isolated from skin biopsy of an Israeli patient and was kindly provided by Dr. F. Ebert (Bernhard-Nocht-Institute for Tropical Medicine, Hamburg). In order to obtain a continuous pool of infectious parasites, *in vitro* cultures of promastigotes in the stationary phase were used to infect BALB/c mice. Amastigotes were then re-isolated from the spleen or footpad of the infected mice and cultured *in vitro* in biphasic Novy-Nicolle-McNeal blood agar complete medium at 26 °C in a humidified atmosphere containing 5 % CO_2. Stationary phase promastigotes were collected after 7 or 8 days in culture.

2.1.1.2 *LEISHMANIA DONOVANI*

L. dondovani strain AG83 (MHOM/IN/1983/AG83) was originally obtained from an Indian Kala-azar patient. Promastigotes were cultured *in vitro* in biphasic Novy-Nicolle-McNeal blood agar complete medium at 26 °C in a humidified atmosphere containing 5 % CO_2. Stationary phase promastigotes were collected after 7 or 8 days in culture.

2.1.1.3 UGM$^{-/-}$ *LEISHMANIA*

The UGM$^{-/-}$ *Leishmania major* from the isolate MHOM/SU/73/5ASKH used in this study, was kindly provided by Prof. Gerardy-Schahn (Medizinische Hochschule Hannover). Parasites were cultured *in vitro* in biphasic Novy-Nicolle-McNeal blood agar complete medium containing 50 µg/ml hygromycin and 5 µg/ml phleomycin at 26 °C in a humidified atmosphere containing 5 % CO_2. Stationary phase promastigotes were collected after 7 or 8 days in culture.

2.1.2 *ANAPLASMA PHAGOCYTOPHILUM*

The *A. phagocytophilum* MRK strain (formerly *Ehrlichia equi* MRK; 25) was cultured in HL-60 cells grown in RPMI 1640 medium containing 2 mM L-glutamine and 1 % FCS.

2.1.3 CULTURE MEDIA AND BUFFER

Blocking solution (western blot)	TBS + 0.05 % Tween 20 + 5 % low-fat skimmed milk or 5 % BSA
Complete medium	Roswell Park Memorial Institute (RPMI) 1640 medium + 50 µM 2-mercaptoethanol + 2 mM L-glutamine + 10 mM HEPES + 100 U/ml penicillin + 100 µg/ml streptomycin + 10 % low endotoxin FCS
ELISA wash buffer	PBS + 0.05 % Tween 20
FACS buffer	PBS + 1 % normal human serum + 1 % BSA + 0,01 % Na-azide
Inhibitor cocktail	5 µg/ml leupeptin, 5 µg/ml pepstatin, 50 µM phenylarsin-oxide, 1 mM PMSF, 1 mM Na_3VO_4, 50 mM NaF, 1-5 mg/ml α_1-Antitrypsin
Lysis buffer for whole cell lysates	HEPES pH 7.9, 420 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 % NP-40, 1 mM DTT
Novy-Nicolle-McNeal blood agar medium	50 ml defibrinated rabbit blood + 50 ml PBS + 2 ml penicillin/ streptomycin + 200 ml Brain Heart Infusion (BHI) medium (10.4 g agar in 200 ml distilled water)
RNA loading dye	2.5 M Urea, 66 % formamide, 0.05 % xylene cyanole, 0.05 % bromphenol blue in TBS
Running buffer	0.125 M Tris pH 8.3, 0.96 M glycine, 0.5 % SDS
4x Sample buffer	1 M Tris pH 6.80, 2 % SDS, 720 mM 2-mercapto-ethanol + 30 % glycerol, 0.002 % bromphenol blue dye
Separating gel buffer	1.5 M Tris pH 8.8, 4 % SDS
Stacking gel buffer	0.5 M Tris-HCl pH 6.8, 4 %SDS
Stripping buffer	100 mM 2-mercapto-ethanol, 2 % SDS, 62.5 mM Tris pH 6.7
Transfer buffer	25 mM Tris pH 8.3, 192 mM glycine, 20 % methanol
TBS 10x	200 mM Tris base, 1.37 M NaCl

Seperating gel	**10 %**	**12.5 %**	**15 %**
Gel 30	10 ml	12.5 ml	15 ml
Separating gel buffer	7.5 ml	7.5 ml	7.5 ml
H_2O	12.4 ml	9.9 ml	7.4 ml
10 % APS	90 µl	90 µl	90 µl
TEMED	24 µl	24 µl	24 µl

Stacking gel 4 %	2 ml Gel 30, 3.75 ml stacking gel buffer, 9.2 ml H_2O, 60 µl APS, 12 µl TEMED
Acrylamide gel for RNA	21 g urea, 21.25 ml H_2O, 5 ml 10x TBE, 7 ml Gel 30, 40 µl TEMED, 10 µl APS

2.1.4 CHEMICALS AND OTHER LABORATORY REAGENTS

Agarose PeqGold Universal	Peqlab, Erlangen
Ammonium persulfate (10 %)	Sigma, Deisenhofen
α_1-Antitrypsin	Sigma, Deisenhofen
Aqua ad injectabilia	Delta Select, Pfullingen
Brain Heart Infusion (BHI)	Becton Dickinson, Heidelberg
Bovine serum albumin (BSA)	Sigma, Deisenhofen
Bromophenol blue dye	Serva, Heidelberg
Chloroform minimum 99 %	Sigma, Deisenhofen
Crystal violet	Sigma, Deisenhofen
Developer for Curix x-ray processing	Agfa, Mortsel, Belgium
Dithiotreitol	Sigma, Deisenhofen
DNA Molecular Weight marker	Peqlab, Erlangen
EDTA	Sigma, Deisenhofen
EGTA	Sigma, Deisenhofen
Ethanol absolute pro analysi	Merck, Darmstadt
Ethidum bromide	Roth, Karlsruhe
Fetal calf serum (FCS)	
(LPS content 0.523 ng/ml)	Sigma, Deisenhofen
Gel 30	Roth, Karlsruhe
Giemsa staining solution, modified	Sigma, Deisenhofen
L-Glutamine	Biochrom, Berlin

Glycerol	Sigma, Deisenhofen
Gycine	Sigma, Deisenhofen
Glycogen, RNA-grade	Fermentas, St. Leon-Rot
HEPES	Sigma, Deisenhofen
Histopaque® 1119	Sigma, Deisenhofen
Hygromycin, solution B	Merck, Darmstadt
Recombinant human IFN-γ	PeproTech, Offenbach
Immersion oil	Carl Zeiss, Jena
Immobilon Western Chemiluminescence HRP Substrate	Millipore, Billerica, MA, USA
Isopropanol for molecular biology	Sigma, Deisenhofen
Leupeptin	Sigma, Deisenhofen
Lipopolysaccharide *E. coli* 0111:B4	Sigma, Deisenhofen
6x Loading dye	Peqlab, Erlangen
Lymphocyte separation medium 1077	PAA, Pasching, Austria
2-Mercaptoethanol	Sigma, Deisenhofen
Methanol	J.T. Baker, Deventer, The Netherlands
Nonidet 40 (NP 40)	Serva, Heidelberg
low-fat skimmed milk (Sucofin)	TSI, Zeven
Paraformaldehyde	Sigma, Deisenhofen
PBS (1 ×) sterile solution	Pharmacy of University of Lübeck, Lübeck
PBS (10 ×) sterile solution	Gibco, Karlsruhe
PBS, Instamed, Dulbecco w/o Mg, Ca	Biochrom, Berlin
Penicillin/streptomycin	Biochrom, Berlin

Pepstatin-A	Sigma, Deisenhofen
Percoll®	Pharmacia, Uppsala, Sweden
peqGold RNApure™	Peqlab, Erlangen
Phenyarsineoxide	Sigma, Deisenhofen
Phleomycin	Invivogen, San Diego, CA, USA
PIPES	Sigma, Deisenhofen
PMSF	Sigma, Deisenhofen
Potassium chloride	Merck, Darmsatdt
Primer for real-time RT-PCR	TIB Molbiol, Berlin
Protein G Sepharose 4 fast flow	Amersham Bioscience, Heidelberg
Rabbit blood	Elocin-lab GmbH, Mülheim
Papid Fixer for x-ray processing	Agfa, Mortsel, Belgium
RNase away	VWR GmbH, Darmstadt
RPMI 1640 medium	Sigma, Deisenhofen
SDS	Roth, Karlsruhe
Sodium acetate	Sigma, Deisenhofen
Sodium azide	Merck, Darmstadt
Sodium chloride	Merck, Darmstadt
Sodium fluoride	Sigma, Deisenhofen
Sodium orthovanadate	Sigma, Deisenhofen
Sulfuric acid	Merck, Darmstadt
TBE (10 ×)	Amersham Bioscience, Heidelberg
TEMED	Roth, Karlsruhe
TMB Substrate Reagent Set	
BD OptEIA™	BD Biosciences Pharmingen, San Diego, CA, USA

10x TBE	USB, Cleveland, OH, USA
Tris-(hydroxymethyl)-aminomethane	Roth, Karlsruhe
Triton X-100	Merck, Darmstadt
Trypan blue solution 0.4 %	Sigma, Deisenhofen
Tween 20 for molecular biology	Sigma, Deisenhofen
Urea	Sigma, Deisenhofen

2.1.5 MONOCLONAL ANTI-HUMAN ANTIBODIES

Mouse anti-CD119 (RPE), IgG2b, clone GIR-94	BD Biosciences Pharmingen, San Diego, CA, USA
Mouse anti-CD119, IgG1, clone MMHGR-1	Calbiochem, San Diego, CA, USA
Mouse anti-STAT1	Cell Signaling, Danvers, MA, USA
Mouse anti CD95 (RPE), IgG1, clone DX2	Dako, Hamburg
Mouse anti-CD64, IgG1, clone 10.1	R&D Systems, Wiesbaden
Mouse IgG2b (PE)	BD Biosciences Pharmingen, San Diego, CA, USA
Mouse IgG1 (PE)	Dako, Hamburg

2.1.6 POLYCLONAL ANTI-HUMAN ANTIBODIES

Rabbit anti-phospho-STAT1	Cell Signaling, Danvers, MA, USA
Rabbit anti-beta-Actin	Cell Signaling, Danvers, MA, USA

2.1.7 SECONDARY ANTIBODIES AND DILUTIONS

Goat anti-mouse Ig (HRP), 1:1000	Santa Cruz, Santa Cruz, CA, USA
Goat anti-rabbit Ig (HRP), 1:1000	Santa Cruz, Santa Cruz, CA, USA

2.1.8 READY-TO-USE-KITS

DuoSet ELISA Development kits®	R&D Systems, Wiesbaden
human CXCL-9/MIG	
human CXCL-11/ITAC	
human CXCL-10/IP-10	
OptEIA™ h-TNF-α Set	BD Biosciences, Heidelberg
Human IL-23 ELISA Kit	eBioscience,
DNA-free® kit	Ambion (Huntingdon Cambridgeshire, GB)
Transcriptor First Strand cDNA Synthesis® kit	Roche Applied Science, Mannheim
LightCycler® FastStart DNA Master SYBR green I	Roche Applied Science, Mannheim

2.1.9 LABORATORY SUPPLIES

Cell culture flasks	Greiner bio-one, Frickenhausen
Cell culture plates (96, 24, 12, 6 well)	Greiner bio-one, Frickenhausen
Cell culture plates (24 well) nunclon™	Greiner bio-one, Frickenhausen
ELISA plate + lid Microlon, flat bottom	Greiner bio-one, Frickenhausen
Fine dosage syringe Omnifix® 1 ml	Braun, Melsungen
Gel-loader tips	Eppendorf, Hamburg
Hyperfilm™ ECL	Amersham Biosciences, Freiburg
LightCycler® Capillaries 20 µl	Roche Diagnostics, Mannheim
Microscope slides superfrost	Menzel, Braunschweig
Microtestplate + lid (96-well, V-bottom)	Sarstedt, Nümbrecht
Microtiter plates MaxiSorb™ (96-well)	Nunc, Wiesbaden
Millex-HA syringe driven filter unit	Millipore, Schwalbach
Nitrocellulose (NC) membrane	Biorad
Pipette 5, 10, 25 ml	Greiner bio-one Frickenhausen
Pipette filter tips	Nerbe plus, Winsen
Pipette tips (1-10 µl, 10-100 µl, 100-1000 µl)	Greiner bio-one, Frickenhausen
Plastic tubes (5 ml (PS) Falcon)	BD Biosciences, Heidelberg
Plastic tubes (15 ml (PS), 50 ml (PP))	Sarstedt, Nümbrecht
Reaction tubes (0.5, 1.5, 2 ml (PP))	Sarstedt, Nümbrecht
Reaction tubes (1.5; 2 ml (PP)) Biopure	Eppendorf, Hamburg
S-Monovette 9 ml, lithium-heparin	Sarstedt, Nümbrecht
Tissue culture plates (6, 12, 24, 48, 96-well, flat bottom)	Greiner bio-one, Frickenhausen
Transfer pipette 3.5 ml	Sarstedt, Nümbrecht

U-tubes for cytometry	Micronic, Lelystad, The Netherlands
Whatman Paper	Schleicher & Schuell, Dassel

2.1.10 INSTRUMENTS

Balances:	
Analytical balance BP61S	Sartorius, Göttingen
Balance	Sartorius, Göttingen
Block thermostats:	
Unitek™ block thermostat HB 130	Peqlab, Erlangen
Block thermostat TCR 200	Roth, Karlsruhe
Cell counting chambers	Neubauer, Marienfeld
Centrifuges:	
Biofuge fresco	Kendro (Heraeus), Langenselbold
Megafuge 2.0R	Kendro (Heraeus), Langenselbold
Multifuge 3 and SR	Kendro (Heraeus), Langenselbold
Microfuge R	Beckmann, Munich
Mikro 12-24	Hettich, Tuttlingen
Centrifuge 5417R	Eppendorf, Hamburg
Cytocentrifuge Cytospin3	Shandon, Frankfurt
CO_2- Incubator IG 150	Jouan, Unterhaching
Deep freezer, 20 °C, 70 °C	Liebherr, Ochsenhausen
Gel electrophoresis chamber:	
mini-Protean tetra™ system	Bio-Rad, Munich
Gel electrophoresis chamber	Biometra, Göttingen
Flow-cytometer FACS-Calibur®	Becton Dickinson, Heidelberg

Gel documentation	Vilber Lourmat, Marne La Vallée, France
Laminar flow workbench	Biohit, Cologne
LightCycler®	Roche Diagnostics, Mannheim
Magnetic stirrer Ikamag, Reo	IKA® Labortechnik, Staufen
Microscopes:	
Axiovert 25	Carl Zeiss, Jena
Axiostar plus	Carl Zeiss, Jena
Microwave oven	Severin, Sundern
Multichannel pipette	Eppendorf, Hamburg
PCR-Thermocycler UNO II	Biometra, Göttingen
pH-meter inolab	WTW GmbH, Weilheim
Pipettes	Eppendorf, Hamburg
Photometers:	
Tecan sunrise	Tecan, Crailsheim
ND-1000 UV-Vis Spectrometer	Nanodrop Technologies®, Wilmington, DE, USA
Power supply EPS 3500XL	Amersham Biosciences, Freiberg
Power supply P25	Biometra, Göttingen
Semi-dry protein transfer cell	Bio-Rad, Munich
Shaker Vibrofix VF1 Electronic	Janke & Kunkel IKA® Labortechnik, Staufen
Water bath	Köttermann, Uetze (Hänigsen)

2.1.11 SOFTWARE

Statistical analysis

GraphpadPrism®, Version 4.01	San Diego, CA,USA
JMP™, Version 5.1	SAS Institute, Cary, NC, USA

Instrument software

CellQuest® (cytometry)	Becton Dickinson, Heidelberg
Magellan® (ELISA)	Tecan, Crailsheim
LightCycler® Software Version 3.5	Roche Applied Science, Mannheim

2.2 METHODS

2.2.1 ISOLATION OF HUMAN PERIPHERAL BLOOD NEUTROPHIL GRANULOCYTES

(Approved by the ethics committee of the University of Lübeck on the 26.07.2005, reference number 05-124)

Venous peripheral blood was collected from healthy adult volunteers using lithium-heparin S-Monovettes. Alternatively, buffy coats provided by the Institute of Immunology and Transfusion Medicine, University of Lübeck were used. These contain the leukocyte-rich fraction obtained from peripheral blood after preparation of erythrocyte concentrates and plasma for transfusion purposes.

For isolation of granulocytes, heparinised blood or buffy coat diluted 1:5 with PBS was layered on a density gradient consisting of lymphocyte separation medium 1077 (upper layer) and Histopaque® 1119 (lower layer) and centrifuged for 5 minutes at 300 × g followed by 20 minutes at 800 × g. The plasma and the lymphocyte separation medium 1077 layer containing mainly lymphocytes and monocytes were discarded. The granulocyte-rich Histopaque® 1119 layer was collected leaving the erythrocyte pellet in the tube. Granulocytes were washed once in PBS and resuspended in complete medium. Further fractionation was achieved by discontinuous Percoll® gradient consisting of layers with densities of 1.105 g/ml (85 %), 1.100 g/ml (80 %), 1.087 g/ml (70 %), and 1.081 g/ml (65 %). After centrifugation for 25 minutes at 800 × g, the interface between the 80 and 70 % Percoll® layers was collected, washed once in PBS and resuspended in complete medium. All procedures were performed at room temperature. Cell viability was > 99 %, as determined by trypan blue exclusion. Cells were counted after staining with crystal violet solution.

2.2.2 PURTITY ANALYSIS OF CELL PREPARATIONS

The purity of isolated PMN was assessed by microscopic examination of Giemsa stained cytocentrifuge slides. The latter were prepared by spinning a suspension of 200,000 PMN per 100 µl PBS on slides in a cytocentrifuge at 400 × g for 5 minutes. Air

dried slides were fixed in methanol for 5 minutes and stained by Giemsa solution for 60 minutes. At least 200 cells were counted and determined as neutrophils, eosinophils, monocytes and lymphocytes by morphological analysis. Cell preparations contained more than 99 % granulocytes. The amount of eosinophil granulocytes varied between 0.5 to 15 %, depending on the donor.

2.2.3 INFFECTION OF PMN WITH *LEISHMANIA SPP.* OR *A. PHAGOCYTOPHILUM* AND COINCUBATION WITH OTHER STIMULI

PMN were cultured at a concentration of 5-10 $\times$ 10^6 cells per ml in complete medium. Cell preparations were incubated at 37 °C in a humidified atmosphere containing 5 % CO_2. The morphology of PMN in the cell culture was examined under an invert microscope.

2.2.3.1 INFECTION OF PMN WITH *LEISHMANIA SPP.*

Stationary phase promastigotes were collected from in vitro cultures in biphasic blood agar medium and washed with medium. After centrifugation at 2800 x g, *L. major* were taken up in complete medium and coincubated with PMN in a PMN: parasite ratio of 1:5. After time points indicated in the results the infection rate was assessed by Giemsa staining. For RNA isolation, circa 99 % of extracellular *Leishmania* were removed from PMN after coincubation by two washes with complete medium at 210 $\times$ g for 8 minutes.

2.2.3.2 TREATMENT OF PMN WITH ETHANOL-KILLED *L. MAJOR*

Stationary phase promastigotes were harvested from blood agar plates and washed with medium. After centrifugation at 2800 x g, *L. major* were taken up in PBS with 30 % of ethanol and left for 30 min at room temperature. Parasites were spun down at 2800 x g and the pellet was resuspended in complete medium. Mortification of the parasites was controlled under an invert microscope. All parasites were dead. Ethanol-killed *L. major* were counted. PMN were coincubated with killed parasites at a PMN:parasite ratio of 1:5.

2.2.3.3 TREATMENT OF PMN WITH *L. MAJOR* SUPERNATANTS

Parasites at a concentration of 25 $\times$ 10^6 per ml in complete medium were incubated over for 18 h at 27 °C. Samples were then centrifuged at 2800 x g. Supernatants were utilized

to resuspend freshly isolated PMN which were then incubated for 18 h. Furthermore, PMN were infected with *L. major* as described in 2.2.3.1 and incubated for 18 h at 37 °C. Samples were then spun down at 700 x g. Supernatants were collected and centrifuged again at 2800 x g. The obtained supernatants were used to resuspend freshly isolated PMN which were then incubated for 18 h.

2.2.3.4 INFECTION OF PMN WITH *A. PHAGOCYTOPHILUM*

20 x 10^6 cells of infected HL-60 cultures (>70 % infected cells as assessed by Romanowsky staining) were pelleted and resuspended in 2 ml of PBS. Subsequently, cell-free *A. phagocytophilum* was obtained as described (170). Briefly, infected HL-60 cells were passed through a 30 gauge needle 12 times followed by a centrifugation step at 750 x g for 10 min. Supernatant was collected and centrifuged at 2,500 x g for 15 min. Pellet containing cell-free *A. phagocytophilum* was resuspended in 1 ml of complete medium without antibiotics and added to PMN (final conc. 5 x 10^6) followed by an incubation for 5 h at 37 °C in humidified atmosphere containing 5 % CO_2. Subsequently, to remove non-ingested bacteria, PMN were washed 3 times with medium, at 256 x g for 10 min each. Immunohistochemical staining with an *A. phagocytophilum* polyclonal rabbit antibody was used to confirm infection in at least 90 % of the neutrophils. Washed infected PMN were cultured at a concentration of 5×10^6 cells per ml in complete medium at 37 °C in a humidified atmosphere containing 5 % CO_2 in tissue culture plates. In all above procedures involving *A. phagocytophilum*, culture medium without antibiotics was used.

2.2.3.5 STIMULATORY AGENTS

Stimulatory agents were used in the following concentrations: LPS 200 ng/ml and IFN-γ 200 U/ml.

2.2.4 EXTRACTION OF TOTAL RNA

Total RNA isolation was achieved utlizing RNApure™ reagent. After 5, 6 or 18h of incubation, 10 Mio PMN were lysed by 1 ml RNApure™. Further isolation procedure was carried out as recommended by the manufacturer. The air-dried pellet was resuspended in RNase-free sterile water. The RNA concentration and purity was determined by OD measurement of the RNA using Nanodrop-1000 UV-Vis

Spectrometer. Integrity of the RNA was tested on an 8 % acrylamide gel. RNA was added to the RNA loading buffer, heated at 95 °C for 5 minutes, cooled on ice and loaded on the gel which was run at 190 V for 45 minutes. Subsequently the gel was stained with 0.02 % ethidiumbromide in TBE. Remaining DNA was removed using DNA-free™ kit following the manufacturer's protocol.

2.2.5 REVERSE TRANSCRIPTION AND REAL-TIME PCR

Gene expression was investigated by real-time PCR. For this purpose 500 nanogram of total RNA were reverse-transcribed to single-stranded cDNA using Transcriptor First Strand cDNA Synthesis® Kit. The cDNA was stored at -20 °C until use. LightCycler® Detection System and LightCycler® FastStart DNA Master SYBR green I kit were used to carry out real-time PCR. The PCR protocol varied depending on Primers and length of the amplicon: Denaturation: 95 °C, 600 s; amplification (45 cycles): 95 °C, 10 s; 58-62 °C for 5 s, 72 °C for 5-9 s. Primers were used at HPLC-grade (Table 1).

For experiments involving *Leishmania spp*. a standard curve with five dilutions of a cDNA was carried out for each primer pair. One dilution of this cDNA was amplified within the same run as the samples of interest. Subsequently, the standard curve was imported by the software for determination of gene expression. Data were analyzed with the method of the second derivative maximum after an arithmetic baseline adjustment. The calculated imaginary concentrations were normalized to beta-actin. The data were shown as percental expression referring to beta-actin expression.

For the quantitative assessment gene expression in experiments involving *A. phagocytophilum*, expression of the gene of interest and host cell beta-actin gene expression was analyzed by relative quantification using of $2^{-\Delta\Delta C_T}$ method (171). The data are presented as fold change in gene expression normalized to the reference gene beta-actin and relative to untreated control. For the untreated control sample, $\Delta\Delta C_T$ equals zero and 2^0 equals one, so that the fold change in gene expression relative to the untreated control equals one by definition. For the treated samples, evaluation of $2^{\Delta\Delta C_T}$ indicates the fold change in expression relative to untreated control. The amount of target, normalized to an endogenous reference and relative to a calibrator is given by $2^{-\Delta\Delta C_T}$. Similarly, gene expression data of *A. phagocytophilum*-infected PMN are

presented as fold change in gene expression normalized to the reference gene beta-actin and relative to uninfected control. Different methods for quantification of gene expression in experiments involving *L. major* or *A. phagocytophilum* were used, because the respective experiments link to previous data. Both methods however yield equal results.

Table 1 Primers for real-time PCR

Gene Ref Seq number	Insert size	Primer sequence 5´to 3´
Beta-actin (house keeping) (NM_001101)	144 bp	Forward: CCT GGC ACC CAG CAC AAT Reverse: GGG CCG GAC TCG TCA TAC
Cytochrome b 245 (NM_000397)	117 bp	Forward: CAC AGG CCT GAA ACA AAA GA Reverse: GCT TCA GGT CCA CAG AGG AA
CD64 (NM_ 000566)	240 bp	Forward: CGC GTT CTA GGC ATA CAA G Reverse: GTA TCG CCG CTT CTC C
Fas (CD95) (NM_000043) + 7 further isoforms	218 bp	Forward: CTA GCC TGG TTT GGA G Reverse: GTA TGA CAA GAG CAA TTC C
Complement component C3 (NM_000064)	217 bp	Forward: GCC AAT GGT GTT GAC Reverse: GGT AGA ACC GGG TAC AG
EBI3 (NM_005755)	152 bp	Forward: AGC ACA TCA TCA AGC CCG AC Reverse: AGC TCC CTG ACG CTT GTA AC
IL-27 p28 (NM_145659)	132 bp	Forward: ATC TCA CCT GCC AGG AGT GAA Reverse: TGA AGC GTG GTG GAG ATG AAG
IRF-1 (NM_002198)	119 bp	Forward: CTT CCA CCT CTC ACC AAG AAC Reverse: CCA TCA GAG AAG GTA TCA GGG C
PU.1 (NM_001080547 + NM_003120)	103 bp	Forward: AAG ACC TGG TGC CCT ATG AC Reverse: TCC GAG TAA TGG TCG CTA TG
SOCS3 (NM_003955)	132 bp	Forward: GAA GAT CCC CCT GGT GTT GA Reverse: TTC CGA CAG AGA TGC TGA AGA

A melting curve analysis was performed to assure specificity of amplification. Additionally amplicons were loaded on a 2.5 % agarose gel to exclude artefacts due to unspecific primer binding.

Contribution of amplification from *L. major* or *A. phagocytophilum* genome was excluded by Blast search for the respective Primers

2.2.6 CELL LYSIS AND WESTERN BLOT ANALYSIS

After stimulation and / or infection, neutrophils (3 x 10^6/condition) were diluted 1:1 in ice-cold PBS containing 50 mM NaF and 1 mM Na_3VO_4. Cells were centrifuged twice at 500 x g for 5 min at 4 °C. Cells were then suspended in lysis buffer for whole cell lysates containing inhibitor cocktail (172). Following 15-min of incubation on ice, cell debris was spun down at 12,000 x g for 20 min at 4 °C. Supernatants were then boiled with 4 x sample buffer for 10 minutes at 95 °C. Lysates were stored at -80 °C.

For detection, lysates from 1 Mio PMN were electrophoresed on 7.5 % SDS-PAGE and subsequently transferred to nitrocellulose at 145 mA for 60 minutes in a Transblot® Semidry Transfer Cell. Membranes were first blocked for 1 h at room temperature containing 5 % BSA and then incubated overnight at 4 °C in the presence of the primary antibody at a dilution of 1/1000 in blocking buffer. After three washes with TBST membranes were probed with HRP-conjugated anti-mouse or anti-rabbit IgG at a dilution of 1/1000 in blocking buffer. The signal was revealed using the chemiluminescence system. To assure equal sample loading, membranes were stripped for 10 minutes at 50 °C and reprobed with anti beta-actin antibody. Detection was performed as described above.

For detection of tyrosine-phosphorylated STAT-1 (pSTAT1), lysates from 1 x 10^6 PMN were electrophoresed on 7.5 % SDS-PAGE and subsequently transferred to nitrocellulose at 145 mA for 60 minutes in a Transblot® Semidry Transfer Cell. Membranes were first blocked for 1 h at room temperature in TBST containing skim milk powder and incubated overnight at 4 °C in the presence of the Tyr701-phospho-specific anti-STAT1 rabbit polyclonal antibody diluted at 1/1000 in TBST containing 5 % BSA. After three washes with TBST, membranes were probed with HRP-conjugated goat anti-rabbit IgG antibody at a 1/1000 dilution in TBST containing 5 % BSA. The signal was revealed using the chemiluminescence system according to the manufacturer's instructions. To assure equal sample loading, membranes were stripped for 10 minutes at 50 °C and reprobed with rabbit polyclonal anti-beta-actin antibody or monoclonal anti-total STAT1 antibody and HRP-conjugated goat anti-rabbit IgG antibody.

2.2.7 FLOW CYTOMETRY

The cell surface expression of the IFN-γ receptor α-chain (CD119), the FcγRI (CD64) and FAS (CD95) was analyzed by flow cytometry. 5×10^5 PMN were resuspended in FACS-buffer in a V-bottom 96-well plate. After washing with FACS-buffer, PMN were stained with RPE-conjugated mAb to human CD119 and PE-conjugated mAb to CD64 or RPE-conjugated mAb to CD95 in FACS-buffer for 30 minutes on ice. Following two washes, cells were fixed with paraformaldehyde (1 % in PBS) and analyzed with a FACS Calibur® flow cytometer using CellQuest® software. PE-conjugated mouse IgG_1 and mouse IgG_{2b} antibodies were used as isotype controls.

L. major and *A. phagocytophilum* infection leads to a shift of fluorescence to higher values of FL-2. This phenomenon is observed for unstained PMN (data not shown), isotype controls as well as for PMN stained with anti-CD119, anti-CD64 and anti-FAS Ab. FL-2 settings of the cytometer were adjusted for infected neutrophils so that unstained controls and isotype controls of infected and non-infected cells were congruent.

2.2.8 DETERMINATION OF CYTOKINES IN CELL CULTURE SUPERNATANTS

Cell free supernatants of PMN cultures from 5-10 $\times$ 10^6 cells/ml were collected after time points indicated in results and stored at –20 °C until cytokine or chemokine determination. MIG, IP-10 and I-TAC were measured by using the DuoSet ELISA Development kits® from R&D. The detection limits were 40 pg/ml (MIG), 15 pg/ml (IP-10) and 3 pg/ml (I-TAC). IL-23 was detected by Human IL-23 (p19/p40) ELISA Ready-SET-Go! Set from eBioscience which had a detection limit of 15 pg/ml The absorption was measured at 450 nm and wavelength correction was performed at 570 nm.

2.2.9 STATISTICAL ANALYSIS

Data from at least three independent experiments are presented as mean ± SD. Statistical evaluation of differences was determined with the Student's t test or two-way ANOVA and Bonferroni post-test. Results were considered statistically significant where $p < 0.05$.

3 RESULTS

3.1 INFECTION OF HUMAN NEUTROPHIL GRANULOCYTES WITH *LEIHMANIA SPP.*

The protozoan parasite *Leishmania* is ingested by phagocytes where it manages to survive. Hiding inside phagocytes allows *Leishmania* to escape from humoral immune response that would otherwise be directed against it. Yet, this necessitates inhibition of the phagocyte´s antimicrobicidal functions. A large number of immune mechanisms against intracellular pathogens are triggered by IFN-ã. Thus interference of *Leishmania* with the respective signaling cascade has been in the center of many attempts to elucidate how the parasite evades killing by its host cell. Previous studies have revealed that *L. major* and *L. donovani* attenuate STAT1-mediated signaling in macrophages (127-129, 173). However, if *Leishmania* infection not only impairs IFN-ã signaling in macrophages but also in neutrophils remains thus far unclear. Accounting for the largest leukocyte population and being the first cells to arrive at the site of an infection, neutrophils play a crucial role in host defense. I was thus interested in defining the mechanisms, enabling *Leishmania* to survive inside neutrophils. Previous microarray data from our group showed that LPS and IFN-ã upregulate a group of genes in neutrophils. This increased gene expression is prevented by *L. major* infection (145). In the present work, I intended to analyze several genes fom this group in more detail by real-time PCR and to accomplish investigation at the protein level.

3.1.1 *L. MAJOR* INFECTION DECREASES SURFACE EXPRESSION OF IFN-g RECEPTOR a -CHAIN (CD119)

The STAT1 signaling cascade is triggered by binding of IFN-ã to its receptor. Functional IFN-ã receptor is comprised of two ligand-binding IFN-ã receptor á-chains (CD119) which are associated with two signal-transducing IFN-ã receptor-â chains (58, 59). Flow cytometry analysis of CD119 cell surface

expression was performed in order to examine whether *L. major* interferes with the IFN-ã cascade at the receptor level.

PMN were infected with *L. major* for 6 h or left untreated. Subsequently, neutrophils were stained with anti CD119 mAb or with the respective isotype control antibody. FACS analysis (Fig. 3-1) showed that CD119 surface expression is diminished in infected PMN.

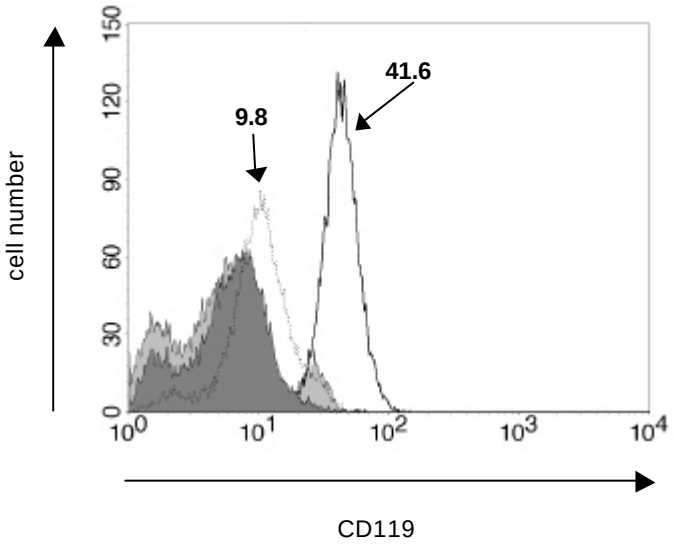


Fig. 3-1 *L. major* infection decreases surface expression of CD119. Freshly isolated human PMN were either left uninfected (solid line) or were infected with *L. major* (dotted line) at a ratio of 1:5. After 6 hours of incubation, neutrophils were stained with RPE-conjugated anti CD119 mAb (GIR94). The histograms and mean fluorescence intensities are from one representative experiments of three performed. The dark grey histogram shows the staining with isotype control antibody for infected PMN, the light grey histogram depicts the isotype control for uninfected PMN.

3.1.2 *L. MAJOR* INFECTION DOES NOT BLOCK STAT1 TYROSINE PHOSPHORYLATION

STAT1 signaling involves recruitment and subsequent phosphorylation of STAT1 monomers. Phosphorylated STAT1 monomers dimerize and translocate to the nucleus (62-64). As phosphorylation of the Tyrosine (701) site is a prerequisite for dimerization the impact of *L. major* infection on tyrosine phosphorylation was examined. PMN were either left untreated, infected with *L. major* or infected with *L. donovani*. After 6 h of

incubation, infected and control cells were stimulated with IFN-ã for 15 minutes.

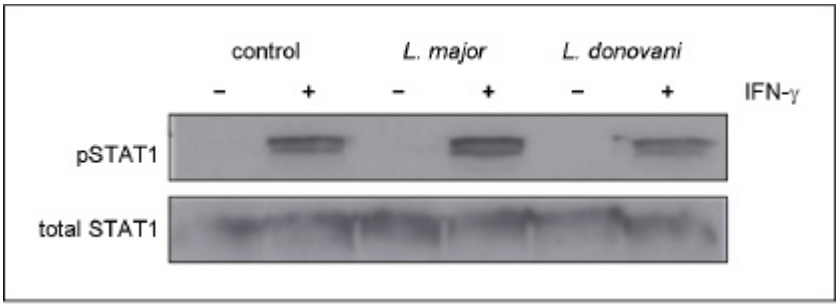


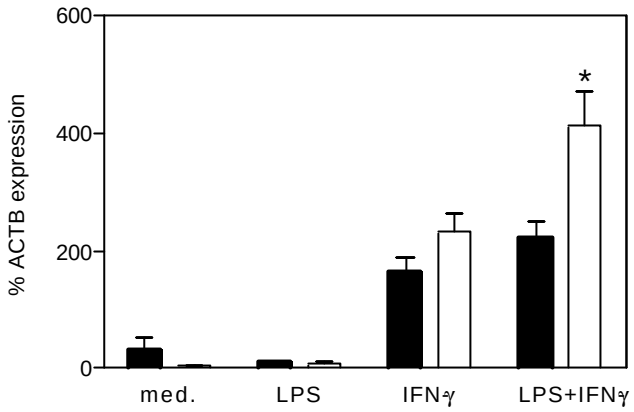
Fig. 3-2 *L. major* infection does not block STAT1 tyrosine phosphorylation. Freshly isolated human PMN were either left uninfected or were infected with *L. major* or *L. donovani* at a ratio of 1:5. Six hours later, the cells were stimulated with IFN-γ (200 U/ml) for 15 min. Whole cell lysates were then prepared, separated by SDS-PAGE and electroblotted. Blots were incubated with an Ab specific for phosphorylated STAT1 (Tyr701 STAT1) and bound Ab was visualized by enhanced chemiluminescence. To assure equal sample loading, membranes were stripped and reprobed with total STAT1 mAb. The results shown represent one of three experiments that yielded similar results.

Subsequently whole cell lysates were prepared, separated on SDS-PAGE and blotted onto nitrocellulose membrane for detection of pSTAT1 and total STAT1. Content of total STAT1 served as loading control. Western blot (Fig. 3-2) revealed that neither *L. major* nor *L. donovani* infection reduces tyrosine phosphorylation of STAT1 at this time point of infection.

3.1.3 *L. MAJOR* INFECTION INCREASES IRF-1 GENE EXPRESSION IN NEUTROPHILS

IRF-1 is intimately involved in mediating IFN-ã signaling. Basal IRF-1 expression has functions in constitutive gene expression (174), but STAT1 and nuclear factor (NF)-êB interaction with promoter elements dramatically increases IRF-1 transcription (175-177). IRF-1 drives inducible expression of many target genes through interaction with the IRF-E site. This sequence overlaps with the ISRE (IFN-stimulated response element) consensus site recognized by ISGF3 (interferon-stimulated transcription factor 3, gamma), which is induced by type-I IFN and to a lesser extent, type-II IFN (56). In this way, IRF-1 is able to directly induce a subset of the full spectrum of IFN-ã-

inducible genes. In order to investigate whether signaling events downstream of STAT1 were impaired by *L. major* infection, gene expression of IRF-1 was analyzed. PMN



were either left uninfected or were infected with *L. major*. Infected and control cells were stimulated with IFN-ã and/or LPS. After 6 h of incubation cells were lysed and processed for real-time PCR.

Fig. 3-3 *L. major* infection increases IRF-1 gene expression. Neutrophils were stimulated either with LPS or IFN-ã alone, or were concomitantly treated with LPS and IFN-ã. Control cells were incubated in medium (med.) without stimuli. Additionally, all samples were infected with *L. major* at ratio of 1:5 (white bars) or left uninfected (black bars). Neutrophils were lysed after 6h of incubation for isolation of total RNA. IRF-1 gene expression data obtained by real-time PCR are shown as the percentage expression relative to beta-actin. Values given are mean ±SD of three experiments. Significant increase (*) in gene expression after *L. major* infection is indicated (two-way ANOVA, Bonferroni post-test).

Quantitative PCR (Fig. 3-3) revealed that IRF-1 gene expression is induced by IFN-ã alone and to a higher extent by LPS and IFN-ã. IRF-1 expression is slightly increased in *L. major*-infected PMN stimulated with IFN-ã. *L. major* infection strongly increased IRF-1 gene expression in PMN simultaneously stimulated with LPS and IFN-ã.

3.1.4 *L. MAJOR* INFECTION DECREASES GENE EXPRESSION OF PU.1

PU.1 is a member of the Ets family of transcription factors which is selectively expressed in myeloid and lymphoid cells. Its function in cell differentiation and activation of basal transcription has been described long ago (178-180). PU.1 is upregulated during myeloid differentiation of multipotential human and murine cells and is expressed at highest levels in myeloid cells, most prominently in human neutrophils (178, 181). More recent studies also define a function of PU.1 in cytokine-

induced transcription activation and show its cooperation with STAT factors (65, 182). Functional cooperatively between PU.1 and STAT1 has been demonstrated to be necessary for IFN-γ-induced CD64 promoter activation (183, 184). Furthermore PU.1 is essential for IFN-γ-induced activation of the gp(91) phox gene in human neutrophils (185, 186). Since *L. major* infection did not alter STAT1 phosphorylation, another transcription factor involved in IFN-ã signaling may be modified by infection in a way that leads to altered expression of IFN-γ-induced genes. Quantitative PCR was performed in order to gain a first impression whether PU.1 may be affected by *L. major* infection.

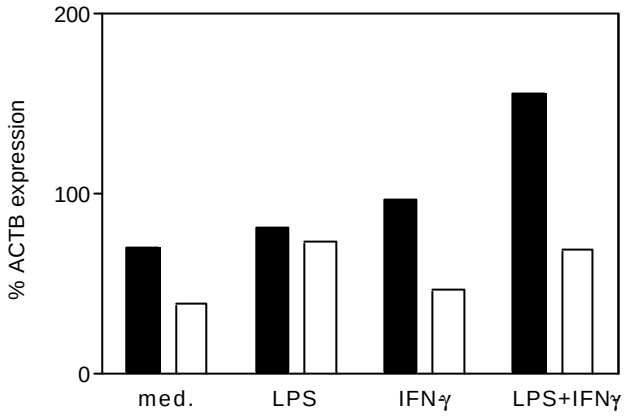


Fig. 3-4 *L. major* infection decreases PU.1 gene expression. Neutrophils were stimulated either with LPS or IFN-ã alone, or were concomitantly treated with LPS and IFN-ã. Control cells were incubated in medium (med.) without stimuli. Additionally, all samples were infected with *L. major* at ratio of 1:5 (white bars) or left uninfected (black bars). Neutrophils were lysed after 3h of incubation for isolation of total RNA. PU.1 gene expression data obtained by real-time PCR are shown as the percentage expression relative to beta-actin. The results show the means of two experiments.

Real-time PCR data (Fig. 3-4) revealed that infection diminished PU.1 expression in unstimulated PMN after 3h and 6h. LPS-treated neutrophils showed a similar PU.1 expression as untreated PMN. Almost no change was detected in comparison to infected cells. IFN-γ stimulation led to slightly increased expression levels. PU.1 expression was repressed in if IFN-γ-stimulated neutrophils were infected with *L. major*. Simultaneous stimulation of PMN with LPS and IFN-γ induced strong PU.1 expression which was decreased by *L. major* infection. Yet, no decrease of PU.1 expression was observed after 6h of infection (data not shown). Reduced levels of the transcription factor during

the early phase of infection may contribute to altered expression levels of LPS and IFN-γ-induced genes in presence of the pathogen.

3.1.5 *L. MAJOR* INFECTION INCREASES SOCS3 GENE EXPRESSION

Feedback inhibition is a mechanism commonly employed by cells to balance biological processes. Negative regulation of IFN-γ signaling is among others achieved by SOCS proteins (67-69).

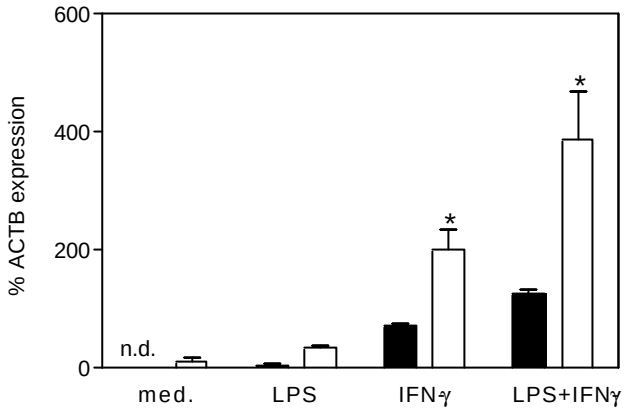


Fig. 3-5 *L. major* infection increases SOCS3 gene expression. Neutrophils were stimulated either with LPS or IFN-ã alone, or were concomitantly treated with LPS and IFN-ã. Control cells were incubated in medium (med.) without stimuli (n.d.: not detectable). Additionally, all samples were infected with *L. major* at ratio of 1:5 (white bars) or left uninfected (black bars). Neutrophils were lysed after 6h of incubation for isolation of total RNA. SOCS3 gene expression data obtained by real-time PCR are shown as the percentage expression relative to beta-actin. Values given are mean ±SD of three experiments. Significant increase (*) in gene expression after *L. major* infection is indicated (two-way ANOVA, Bonferroni post-test).

Analysis of SOCS3 gene expression (Fig. 3-5) was carried out to analyze whether *L. major* utilizes the feedback machinery of PMN to evade IFN-γ-induced immune response. In absence of stimuli no SOCS3 gene expression was detected in PMN. Infection of PMN with *L. major* did not induce SOCS3 expression, nor did stimulation with LPS alone. However, if PMN were treated with LPS and *L. major* simultaneously a slight SOCS3 expression could be detected. Substantial SOCS3 levels were detected after IFN-γ stimulation. These were further augmented by *L. major* infection. Stimulation with LPS and IFN-γ together led to even higher expression levels than IFN-γ alone. SOCS3 expression was strongly enhanced if PMN were additionally infected with *L. major*. Increase of SOCS3 expression after *L. major* infection suggests that the parasite misuses regulatory mechanisms of its host cell in order to downregulate antimicrobial effector mechanisms.

3.1.6 *L. MAJOR* INFECTION RESULTS IN DECREASED GENE EXPRESSION AND RELEASE OF CXC CHEMOKINES

IP-10, MIG and I-TAC belong to the group of CXC chemokines. The first two cysteine residues of CXC chemokines are separated by a single amino acid. IP-10, MIG and I-TAC form a subfamily of CXC chemokines that lack the ELR (Glu-Leu-Arg) motif in front of the first cysteine. Non-ELR-CXC chemokines recruit activated lymphocytes (187) and retard angiogenesis via their receptor CXCR3. CXCR3 is a G-protein-coupled receptor primarily expressed on activated T-cells. Among these, CXCR3 is higher expressed on the Th1 subset (188). Expression of non-ELR chemokines thus favours recruitment of Th1-cells. In addition to CXCR3, the non-ELR chemokines bind to CCR3, a CC chemokine receptor preferentially expressed on Th2 cells (189). However IP-10, MIG and I-TAC are antagonists for CCR3 (190). The opposing effects of CXCR3 and CCR3-bearing cells can enhance polarization of Th1 cell recruitment to sites of inflammation. IP-10, MIG and I-TAC expression is upregulated by IFN-ã. As a strong Th1-response promotes killing of intracellular pathogens suppression of non-ELR chemokines would be favourable for *Leishmania* survival. Gene expression and secretion of non-ELR CXC chemokines were examined in order to elucidate whether the parasite interferes with production of these immunomodulatory molecules.

3.1.6.1 *L. MAJOR* INFECTION DECREASES GENE EXPRESSION AND RELEASE OF IP-10

Real-time analysis of IP-10 (Fig. 3-6 A) revealed that after 6h of incubation no IP-10 expression was detectible in the absence of stimuli or if cells were treated with LPS. IP-10 expression was low if cells were stimulated with IFN-γ alone. Gene expression was high if PMN were stimulated with LPS and IFN-γ simultaneously. In presence of *L. major* IP-10 expression was decreased in comparison with expression in uninfected PMN. Secretion of IP-10 (Fig. 3-6 B) was induced by LPS alone, and to a higher extent by IFN-ã or LPS and IFN-γ. IP-10 release was prevented by *L. major* infection.

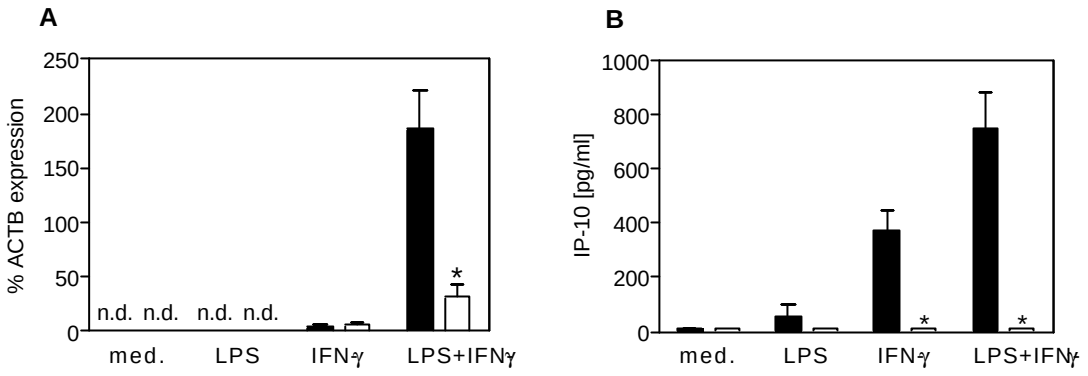


Fig. 3-6 A *L. major* infection decreases IP-10 gene expression (real-time PCR). Neutrophils were stimulated either with LPS or IFN-ã alone, or were concomitantly treated with LPS and IFN-ã. Control cells were incubated in medium (med.) without stimuli. Additionally, all samples were infected with *L. major* at ratio of 1:5 (white bars) or left uninfected (black bars). Neutrophils were lysed after 6h of incubation for isolation of total RNA. IP-10 gene expression data obtained by real-time PCR are shown as the percentage expression relative to beta-actin (n.d.: not detectable). Values given are mean ±SD of three experiments. Significant decrease (*) in gene expression after *L. major* infection is indicated (two-sided, paired t-test).

Fig. 3-6 B *L. major* infection decreases IP-10 release (ELISA). Neutrophils at a concentration of $5x10^6$/ml were stimulated and/or infected as described above and incubated for 18h. The IP-10 content of supernatants was quantified by ELISA. Values given are mean ±SD of three experiments. Significant decrease (*) in IP-10 release after *L. major* infection is indicated (two-way ANOVA, Bonferroni post-test).

3.1.6.2 *L. MAJOR* INFECTION DECREASES GENE EXPRESSION AND RELEASE OF MIG

Analysis of MIG gene expression (Fig 3-7 A) in neutrophils by quantitative PCR after 6h of incubation showed that neutrophils do not constitutively express MIG. Stimulation of neutrophils with either LPS or IFN-γ alone did not induce MIG expression. However, if neutrophils were treated concomitantly with LPS and IFN-γ substantial gene expression of MIG was observed. This expression was diminished by *L. major* infection. Neutrophils did not release MIG after 18h of incubation unless stimulated with either IFN-γ or with LPS and IFN-γ simultaneously. *L. major* infection inhibited MIG secretion in neutrophils treated with IFN-γ alone as well as in those stimulated with LPS and IFN-γ (Fig. 3-7 B).

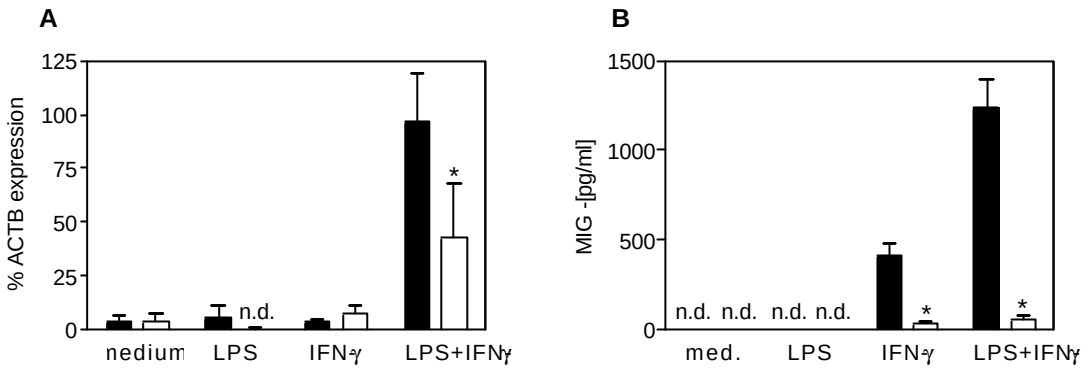


Fig. 3-7 A *L. major* infection decreases MIG gene expression (real-time PCR). Neutrophils were stimulated either with LPS or IFN-ã alone, or were concomitantly treated with LPS and IFN-ã. Control cells were incubated in medium (med.) without stimuli. Additionally, all samples were infected with *L. major* at ratio of 1:5 (white bars) or left uninfected (black bars). Neutrophils were lysed after 6h of incubation for isolation of total RNA. MIG gene expression data obtained by real-time PCR are shown as the percentage expression relative to beta-actin (n.d.: not detectable). Values given are mean ±SD of three experiments. Significant decrease (*) in gene expression after *L. major* infection is indicated (two-sided, paired t-test)

Fig. 3-7 B *L. major* infection decreases MIG release (ELISA). Neutrophils at a concentration of $5x10^6$/ml were stimulated and/or infected as described above and incubated for 18h. The MIG content of supernatants was quantified by ELISA (n.d.: not detectable). Values given are mean ±SD of three experiments. Significant decrease (*) in MIG release after *L. major* infection is indicated (two-way ANOVA, Bonferroni post-test).

3.1.6.3 *L. MAJOR* INFECTION DECREASES GENE EXPRESSION AND RELEASE OF I-TAC

Substantial gene expression levels of I-TAC (Fig. 3-8 A) were detected in PMN stimulated with IFN-γ and LPS. *L. major* infection abrogated I-TAC expression. Secretion of I-TAC Fig. 3-8 B) could solely be detected in supernatants from PMN treated with LPS and IFN-γ, but even in these supernatants only a low level of I-TAC could be measured. *L. major* infection completely abrogated I-TAC release.

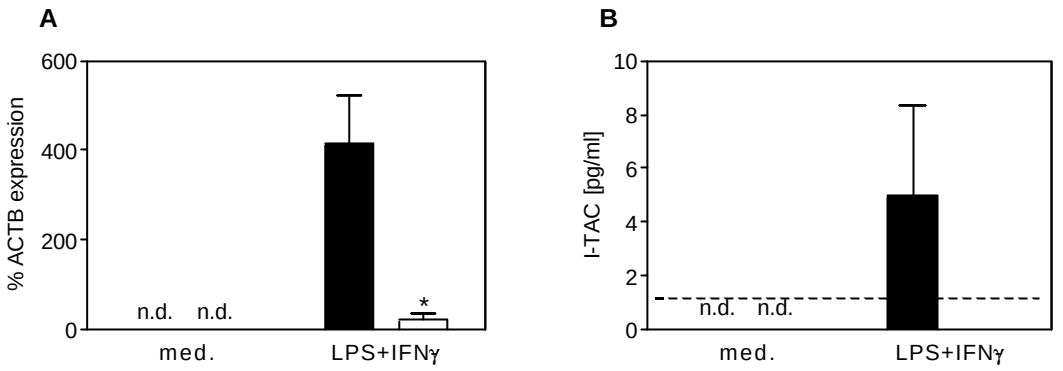


Fig. 3-8 A *L. major* infection decreases I-TAC gene expression (real-time PCR). Neutrophils were stimulated with LPS and IFN-ã. Control cells were incubated in medium (med.) without stimuli. Additionally, all samples were infected with *L. major* at ratio of 1:5 (white bars) or left uninfected (black bars). Neutrophils were lysed after 6h of incubation for isolation of total RNA. I-TAC gene expression data obtained by real-time PCR are shown as the percentage expression relative to beta-actin (n.d.: not detectable). Values given are mean ±SD of three experiments. Significant decrease (*) in gene expression after *L. major* infection is indicated (two-sided paired t-test).
Fig. 3.-8 B *L. major* infection decreases I-TAC release (ELISA). Neutrophils at a concentration of $10x10^6$/ml were stimulated and/or infected as described above and incubated for 18h. The I-TAC content of supernatants was quantified by ELISA (n.d.: not detectable). The dotted line indicates the detection limit of the assay. Values given are mean ±SD of three experiments. The detection limit is indicated as a dotted line.

3.1.6.4 DECREASE IN IP-10 RELEASE DOES NOT DEPEND ON LIPOPHOSPHOGLYCAN AND GIPLS

Downregulation of CD119 starts 30 minutes after infection (data not shown) when infection rate is still low. This suggests that the inhibitory effect L. major exerts on IFN-ã signaling may be mediated by contact of PMN to parasite surface. Liposphoglycan (LPG) is the most abundant surface component of *Leishmania* (146). Moreover, GIPLs form prominent molecules coating the parasite. These molecules are likely candidates for interaction with PMN. UGM is a key enzyme in biosynthesis of LPG and GIPLs. $UGM^{-/-}$ *L. major* that are deficient in LPG and GIPL synthesis (155) were used for infection of PMN in order to examine whether downregulation effects might be mediated by LPG or GIPLs. Secretion of the IFN-ã-induced protein of 10 kDa, IP-10, was chosen as read-out to gain insight into the regulative role of LPG and GIPLs with regard to PMN.

As in previous experiments, IP-10 secretion was induced either by IFN-γ alone or by LPS and IFN-γ. Release of IP-10 was completely abrogated by infection of PMN with wild type as well as with $UGM^{-/-}$ *L. major* indicating that the inhibition of IP-10 secretion is not dependent on LPG and GIPLs (Fig 3-9).

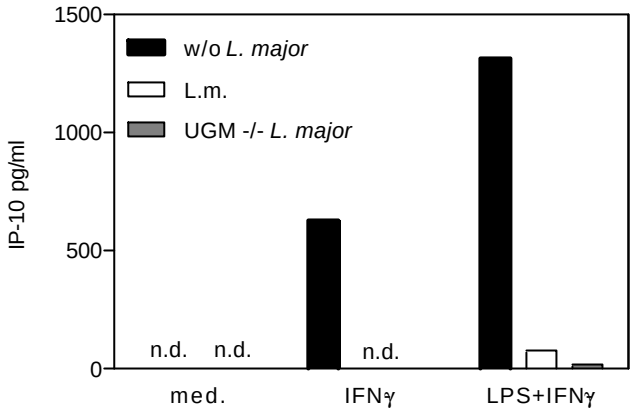


Fig. 3-9 Decrease in IP-10 release does not depend on Lipophosphoglycan. Neutrophils at a concentration of $10x10^6$/ml were stimulated with LPS and IFN-ã. Control cells were incubated in medium (med.) without stimuli. Additionally, all samples were infected with *L. major* or $UGM^{-/-}$ *L. major* at ratio of 1:5 or left uninfected. Neutrophils were incubated overnight. The IP-10 content of supernatants was quantified by ELISA (n.d.: not detectable). One representative experiment of two is shown.

3.1.6.5 DECREASE OF IP-10 SECRETION DOES NOT DEPEND ON VIABLE PARASITES, AND *L. MAJOR* SUPERNATANTS DO NOT DOWNREGULATE IP-10 RELEASE

As shown above *Leishmania* infection impairs IP-10 secretion. In order to get a first impression of whether this effect depends on viable parasites, ethanol-killed *L. major* were examined for their ability to decrease IP-10 production in PMN. In addition, supernatants from parasites incubated in medium overnight were examined for their ability to decrease IP-10 release in order to clarify if some agent secreted by *L. major* may interfere with IP-10 production. Moreover, supernatants from neutrophils co-incubated with *L. major* were prepared and and than added to freshly isolated neutrophils. The latter experiment was performed in order to examine whether either neutrophils or *Leishmania* secrete any agent in response to each other that may decrease IP-10 release. IFN-γ-induced IP-10 secretion (Fig. 3-10) was completely abrogated by viable as well as by ethanol-killed parasites. *L. major* supernatants, however, did not decrease IP-10 release in IFN-γ-stimulated cells. Thus downregulation of IP-10 secretion does not depend on viability of *L. major*. Moreover, decrease in IP-10 production is not mediated by any soluble factors released from the parasite or from PMN in response to the parasite.

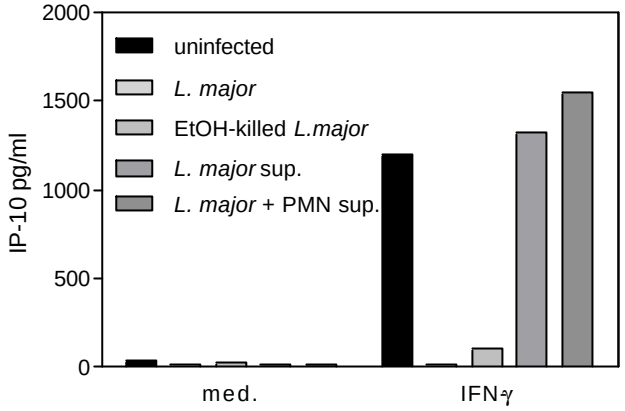


Fig. 3-10 Decrease in IP-10 release does not depend on viable parasites, and *L. major* supernatants do not downregulate IP-10 release. Neutrophils at a concentration of $10x10^6$/ml were stimulated either with LPS or IFN-ã alone, or were concomitantly treated with LPS and IFN-ã. Control cells were incubated in medium (med.) without stimuli. Additionally, samples were infected with *L. major* at a ratio of 1:5 or treated with the same amount of ethanol-killed *L. major*. Further samples were treated with supernatants prepared from *L. major* incubated overnight in medium or with supernatants from PMN co-incubated with *L. major* overnight. Samples were incubated for 18 h. The IP-10 content of neutrophil supernatants was quantified by ELISA. One representative experiment of two is shown.

3.1.7 NEUTROPHIL IL-27 GENE EXPRESSION AND IL-23 RELEASE ARE DECREASED BY *L. MAJOR*

IL-12 is of high importance for the defense of *Leishmania* as it promotes differentiation of naï ve T cells into IFNγ-secreting Th1 cells (98). Along with IL-23 and IL-27, IL-12 forms the IL-12 cytokine family. Each member of this group contributes to Th1 differentiation in a distinct and partly independent way.

IL-23 increases proliferation of $CD4^+$ memory T cells and production of IFN-γ (94) and shows a late-acting anti-leishmanicidal effect which is independent of IL-12 (191). IL-27 on the contrary directs early events leading to Th1 cell responses. The early regulation of Th1 differentiation is reflected by its importance during the initial phase of *Leishmania* infection (98, 192). We addressed the question if neutrophils produce IL-12 family cytokines, and how their expression may vary with respect to *Leishmania* infection. Neutrophils have been reported to secrete IL-12 (91) and IL-23 (193). However, whether PMN are able to produce IL-27 has not yet entirely been defined.

3.1.7.1 NEUTROPHILS EXPRESS IL-27 P28 AND EBI3 GENES, AND *L. MAJOR* INFECTION DECREASES GENE EXPRESSION OF BOTH SUBUNITS

Real-time PCR showed that neutrophils do not express p28 constitutively. LPS stimulation of PMN induced weak p28 gene expression. IFN-γ treatment led to slightly higher p28 expression than LPS. If neutrophils were stimulated with both, LPS and IFN-γ, substantial p28 gene expression could be detected. Gene expression of p28 was decreased if PMN were infected with *L. major* (Fig 3-11, p28).

Quantitative PCR revealed that neutrophils do not constitutively express EBI3 after 6h. Stimulation of neutrophils with IFN-ã alone did not result in EBI3 expression. Yet, EBI3 was expressed in response to LPS. Concomitant stimulation of neutrophils with IFN-γ and LPS led to expression of EBI3, too. Infection with *L. major* markedly inhibited EBI3 expression in neutrophils treated with either LPS alone or with LPS and IFN-γ (Fig 3-11, EBI3).

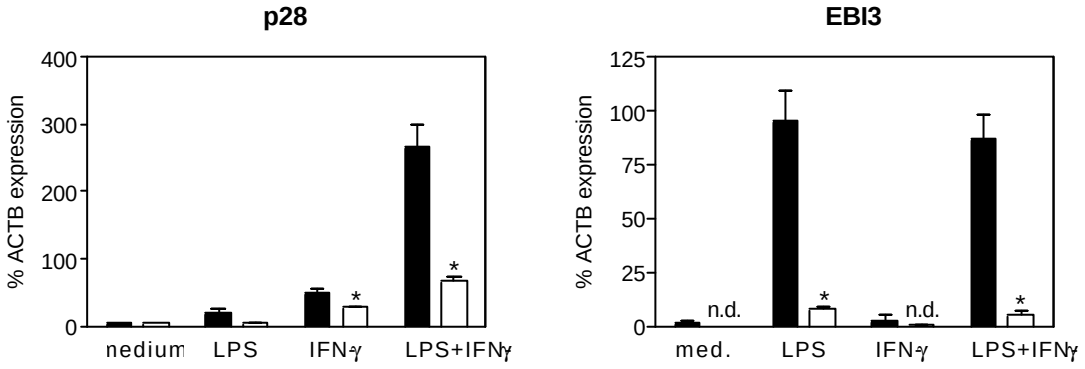


Fig. 3-11 Neutrophils express IL-27 p28 and EBI3 genes *L. major* infection decreases gene expression of both subunits. Neutrophils were stimulated either with LPS or IFN-ã alone, or were concomitantly treated with LPS and IFN-ã. Control cells were incubated in medium (med.) without stimuli. Additionally, all samples were infected with *L. major* at ratio of 1:5 (white bars) or left uninfected (black bars). Neutrophils were lysed after 6h of incubation for isolation of total RNA. EBI3 and p28 gene expression data obtained by real-time PCR are shown as the percentage expression relative to beta-actin (n.d.: not detectable). Values given are mean ±SD of three experiments. Significant decrease (*) in gene expression after *L. major* infection is indicated (two-way ANOVA, Bonferroni post-test).

3.1.7.2 NEUTROPHILS RELEASE IL-23 IN RESPONSE TO LPS AND IFN-g, AND *L. MAJOR* INFECTION DECREASES SECRETION OF IL-23

ELISA data (Fig. 3-12) showed that neutrophils did not release IL-23 after 18h in the absence of stimuli. Incubation of neutrophils with either LPS or IFN-γ alone did not result in IL-23 release. Marked release of IL-23 by PMN occurred solely if cells were stimulated with LPS and IFN-γ simultaneously. Infection by *L. major* led to decrease in IL-23 secretion.

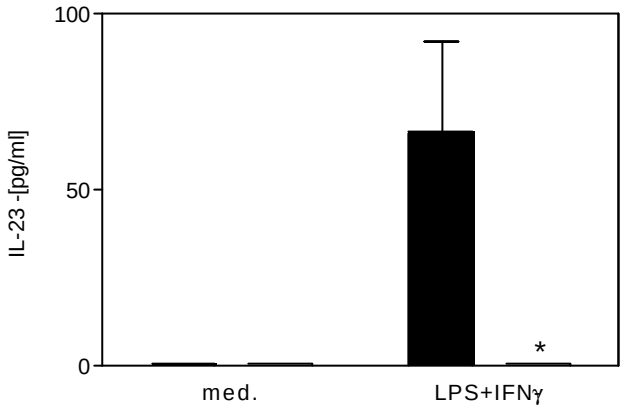


Fig. 3-12 Neutrophils release IL-23 in response to LPS and IFN-ã an *L. major* infection decreases secretion of IL-23. Neutrophils at a concentration of $10x10^6$/ml were either with LPS or IFN-ã alone, or were concomitantly treated with LPS and IFN-ã. Control cells were incubated in medium (med.) without stimuli. Additionally, all samples were infected with *L. major* at ratio of 1:5 (white bars) or left uninfected (black bars). After 18h of incubation, the IL-23 content of supernatants was quantified by ELISA. Values given are mean ±SD of three experiments. Significant decrease (*) in IL-23 release after *L. major* infection is indicated (two-sided paired t-test).

3.1.8 *L. MAJOR* INFECTION DECREASES GENE EXPRESSION OF TNF

TNF is a major proinflammatory cytokine. Its actions include the induction of phagocytic and cytotoxic activities of PMN and macrophages. Being a direct activator of oxidative burst, TNF plays an important role in host defense to *Leishmania*. We thus addressed the question whether Leishmania infection interferes with neutrophil expression of this cytokine.

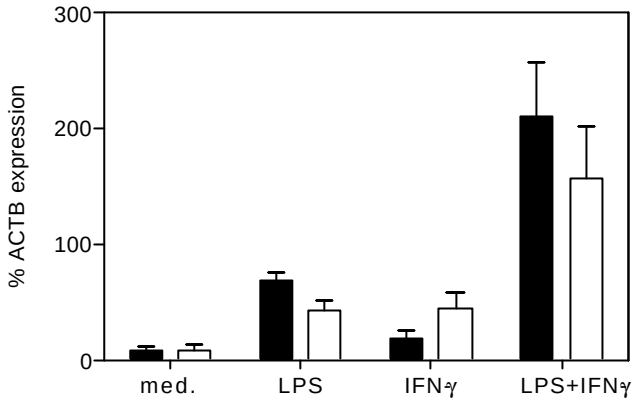


Fig. 3-13 ***L. major*** **infection decreases gene expression of TNF.** Neutrophils were stimulated either with LPS or IFN-ã alone, or were concomitantly treated with LPS and IFN-ã. Control cells were incubated in medium (med.) without stimuli. Additionally, all samples were infected with *L. major* at ratio of 1:5 (white bars) or left uninfected (black bars). Neutrophils were lysed after 6h of incubation for isolation of total RNA. TNF gene expression data obtained by real-time PCR are shown as the percentage expression relative to beta-actin. Values given are mean ±SD of three experiments.

Neutrophils express TNF in response to LPS, and to a minor degree, after stimulation with IFN-ã. Concomitant administration of LPS and IFN-ã induces a strong increase in TNF gene expression. Infection of neutrophils with *L. major* reduces gene expression levels in PMN that were at the same time stimulated with LPS or with LPS and IFN-ã. Yet, IFN-ã stimulated PMN showed an increased TNF expression when infected with *L. major* (Fig 3-13).

3.1.9 *L. MAJOR* INFECTION DECREASES CYTOCHROME B 245 GENE EXPRESSION

Forming a membranous subunit of the NADPH oxidase, cytochrome b 245 participates in oxidative burst of neutrophils (36). It has been shown that phagocytes decrease oxidative burst the presence of *Leishmania spp* (194), which may contribute to parasite survival. The underlying mechanism remains unclear. As production of hydrogen peroxide and superoxide anion is diminished in infected cells (194), a *L. major* mediated impairment of NADPH functions seems very likely.

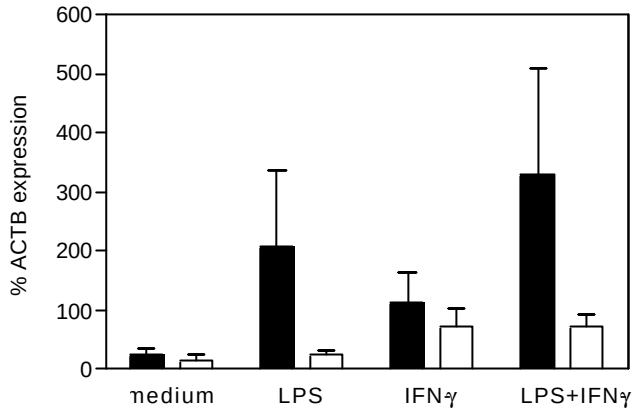


Fig. 3-14 *L. major* infection decreases cytochrome b 245 gene expression. Neutrophils were stimulated either with LPS or IFN-ã alone, or were concomitantly treated with LPS and IFN-ã. Control cells were incubated in medium (med.) without stimuli. Additionally, all samples were infected with *L. major* at ratio of 1:5 (white bars) or left uninfected (black bars). Neutrophils were lysed after 6h of incubation for isolation of total RNA. Cytochrome b 245 gene expression data obtained by real-time PCR are shown as the percentage expression relative to beta-actin. Values given are mean ±SD of three experiments.

Real-time PCR of cytochrome b 245 (Fig. 3-14) was carried out in order to elucidate if *L. major* interferes with gene expression of this NADPH oxidase subunit. Quantitative PCR revealed that expression of cytochrome b 245 is induced by LPS and to lesser extent by IFN-ã. Concomitant stimulation of PMN with LPS and IFN-ã led to higher expression levels than LPS or IFN-ã alone. *L. major* infection drastically decreased cytochrome b 245 expression. Reduced production of cytochrome b 245 and, consequentially, impaired NADPH oxidase activity may contribute to parasite survival inside PMN.

3.1.10 *L. MAJOR* INFECTION DECREASES GENE EXPRESSION OF COMPLEMENT COMPONENT C3

PMN rapidly phagocytose and kill *L. promastigtes* in the presence of fresh human serum (113) indicating a crucial role of serum factors in leishmanicidal defence mechanisms. *Leishmania* are known to trigger complement activation. Promastigote lysis has been shown to depend on C3 deposition on the parasite surface (122) thus playing a major role in killing of *Leishmania*. Most C3 is produced in the liver. However, with regard to the microenvironment of the inflammatory site, downregulation of neutrophil-derived C3 may be beneficial to parasite survival. I

addressed the issue, whether *L. major* interferes with gene expression of C3, in order to achieve better survival conditions.

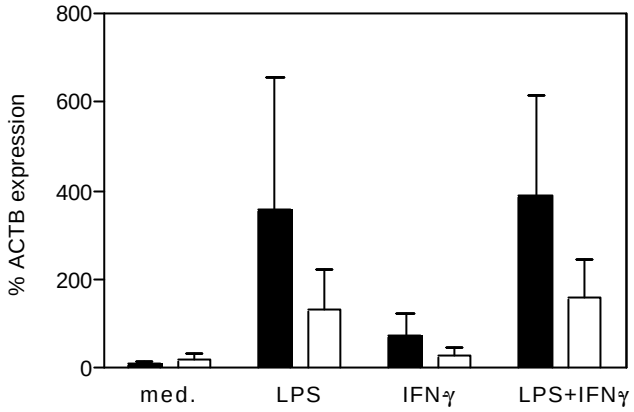


Fig. 3-15 *L. major* infection decreases gene expression of complement component C3. Neutrophils were stimulated either with LPS or IFN-ã alone, or were concomitantly treated with LPS and IFN-ã. Control cells were incubated in medium (med.) without stimuli. Additionally, all samples were infected with *L. major* at ratio of 1:5 (white bars) or left uninfected (black bars). Neutrophils were lysed after 6h of incubation for isolation of total RNA. C3 gene expression data obtained by real-time PCR are shown as the percentage expression relative to beta-actin. Values given are mean ±SD of three experiments.

Real-time PCR analysis (Fig. 3-15) showed that C3 expression is induced by IFN-ã alone as well as by LPS and IFN-ã. Slight C3 expression was measured if PMN were uniquely stimulated with LPS. *L. major* infection led to decrease in C3 gene expression, irrespectively of the applied stimulus. Downregulation of C3 might represent a means of escaping complement-mediated lysis.

3.1.11 *L. MAJOR* DECREASES CELL SURFACE EXPRESSION OF FC GAMMA RECEPTOR I (CD64)

Receptors of the Fc domain of immunoglobulin G (FcãR) constitute an interface between humoral and cellular immune responses. Mediating removal of antibody-coated infectious agents they provide an important means of host defense against invading pathogens. Antibody and FcãR interaction allows cells to identify organisms for phagocytosis and final degradation. As the mechanism of the pathogens entry into the phagocyte may determine whether the microorganism is killed or manages to survive, FcãR are of high interest with regard to interaction of *L. major* and PMN. FcãRI (CD64), the high affinity receptor of the Fc portion of IgG1 and IgG3, is induced on PMN following exposure to IFN-ã (195). I was interested in elucidating whether

L. major interferes with neutrophil CD64 expression in order to facilitate its intracellular survival.

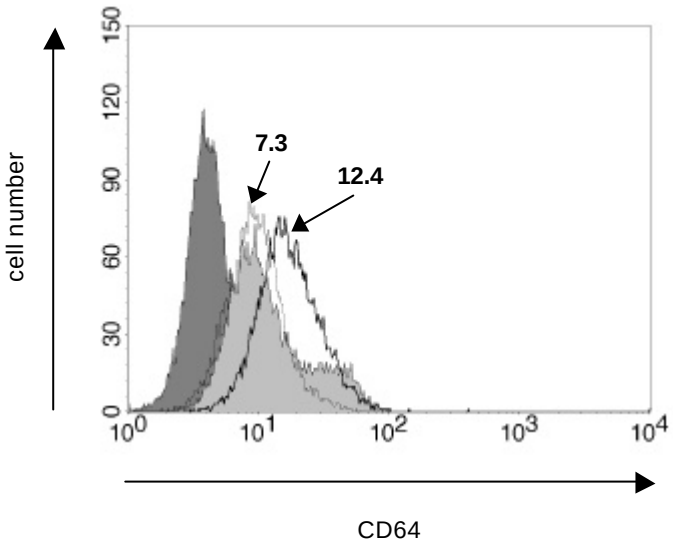


Fig. 3-16 *L. major* infection decreases surface expression of CD64. Neutrophils were either left untreated (light grey histogram), stimulated with IFN-ã (solid line), or concomitantly stimulated with IFN-ã and infected with *L. major* (dotted line). PMN were stained with RPE-conjugated anti CD64 mAb (clone 10.1) after 6h of incubation. The mean fluorescence intensities of CD64 staining are given for INF-ã-stimulated samples. The results are representative of three experiments. The dark grey histograms show the staining with isotype control antibody for untreated PMN.

Surface expression of CD64 was examined by flow cytometry (Fig. 3-16). FACS analysis asserted the induction of CD64 on PMN by IFN-ã. *L. major* infection decreased IFN-γ-induced CD64 surface expression indicating that the parasite may partially evade CD64-mediated phagocytosis.

3.1.12 *L. MAJOR* DECREASES GENE AND SURFACE EXPRESSION OF FAS (CD95)

Neutrophil granulocytes are inherently short-lived cells which have a half life of only about 6-10 h in the circulation. This process is characterized by typical phenomena comprising cell shrinkage, chromatin condensation, internucleosomal DNA fragmentation, membrane blebbing and finally, the decomposition into apoptotic bodies. Previous data from our group show that *L. major* infection delays intrinsic apoptosis in neutrophils allowing the pathogen to survive within these cells (141). The role of extrinsic, FAS-mediated apoptosis in *Leishmania* infection still remains controversial.

In order to investigate whether the parasite interferes with the extrinsic apoptosis pathway in PMN we examined FAS expression of neutrophils in the context of *L. major* infection.

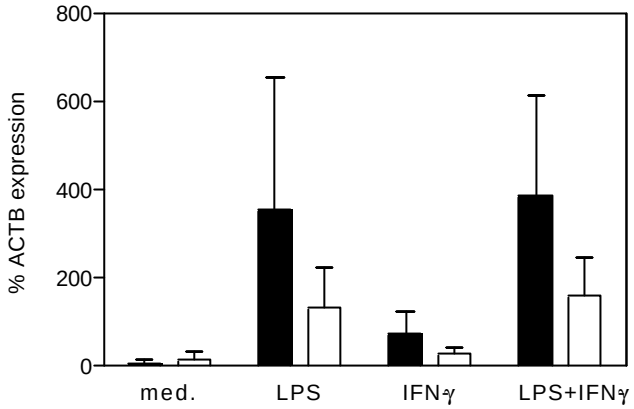


Fig. 3-17 *L. major* infection decreases gene expression of FAS (CD95). Neutrophils were stimulated either with LPS or IFN-ã alone, or were concomitantly treated with LPS and IFN-ã. Control cells were incubated in medium (med.) without stimuli. Additionally, all samples were infected with *L. major* at ratio of 1:5 (white bars) or left uninfected (black bars). Neutrophils were lysed after 6h of incubation for isolation of total RNA. FAS gene expression data obtained by real-time PCR are shown as the percentage expression relative to beta-actin. Values given are mean ±SD of three experiments.

Real-time PCR data (Fig. 3-17) indicate that after 6h, FAS is not expressed constitutively in PMN. Stimulation of neutrophils with IFN-ã induced weak FAS expression. Treatment with LPS increased FAS expression. PMN expressed similar amounts of FAS if stimulated with LPS and IFN-ã concomitantly. *L. major* infection inhibited induction of FAS gene expression in neutrophils stimulated with IFN-ã or LPS alone as well as in cells treated with LPS and IFN-γ simultaneously.

FAS surface expression (Fig. 3-18) was explored by flow cytometry. Exposure to IFN-ã induced a slight rise in FAS expression (data not shown). Constitutive FAS expression was decreased in infected PMN, independently of treatment with LPS or IFN-ã. Our findings showed that *L. major* suppresses IFN-ã-induced FAS expression in PMN. The parasite thus expands the life span of its host cell, employing not only intrinsic, but also extrinsic apoptotic pathways.

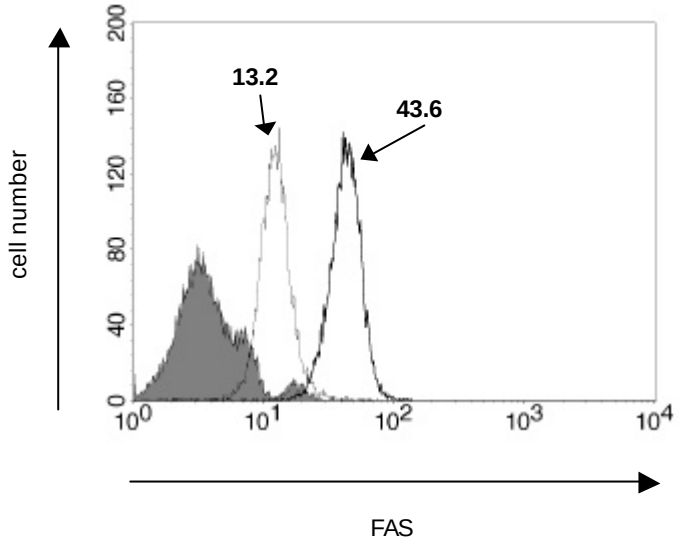


Fig. 3-18 ***L. major*** **infection decreases surface expression of FAS.** Infected (dotted line) and non-infected PMN (solid line) were stained with RPE-conjugated anti CD95 mAb (clone DX2) after 6h of infection. The mean fluorescence intensities of FAS staining are given. The results are representative of two experiments. The dark grey histograms show the staining with isotype control antibody for uninfected PMN.

Results I

Leishmania are ingested by neutrophil granulocytes and survive within these phagocytes. This study shows that the parasite employs a variety of mechanisms in order to inhibit IFN-ã- and LPS-induced antimicrobial functions of PMN. *L. major* impaired the host cell´s IFN-ã signaling cascade by downregulation of the IFN-ã receptor á-chain CD119 and by increasing expression of the inhibitor SOCS3. Moreover, the transcription factor PU.1 that is important for activation of several IFN-ã-induced genes was expressed in reduced amounts. On the contrary, phosphorylation of STAT1 remained unchanged after 6 hours of infection. Expression of IRF-1 which represents a transcription factor downstream of STAT1 was even slightly elevated.

Nevertheless, expression of IFN-ã-inducible genes and/or proteins analyzed in this study was decreased by *L. major* infection. The parasite furthermore diminished expression of genes and proteins induced either by LPS alone or by LPS and IFN-ã. Present data revealed that *L. major* reduced neutrophil gene expression and release of the CXC chemokines IP-10, MIG and I-TAC and of the cytokine IL-23. Gene expression of the IL-27 subunits p28 and EBI3, of TNF, cytochrome b 245, complement component C3 and of the death receptor FAS was diminished by *L. major* infection. In addition, surface expression of FAS and CD64 were decreased in presence of the parasite.

Infection of neutrophils with UGM$^{-/-}$ *L. major* that are deficient in surface LPG and GIPLs, revealed that these parasites are equally capable of diminishing IP-10 release as their wild type littermates. Moreover, neutrophils that were coincubated with ethanol-killed parasites secreted reduced amounts of IP-10. Yet, supernatants from *L. major* incubated either with neutrophils or alone overnight, did not alter IP-10 release of PMN. *L. major* impairs IFN-ã signaling, as well as IFN-ã- and LPS-induced antimicrobial functions of neutrophils. This is very likely to facilitate intracellular survival of the parasite.

3.2 INFECTION OF HUMAN NEUTROPHIL GRANULOCYTES WITH *A. PHAGOCYTOPHILUM*

Infection of HL-60 cells with *Anaplasma phagocytophilum*, the causative agent of human granulocytic ehrlichiosis, results in downregulation of the gp91 (phox) gene (159). As gp91 (phox) is induced by IFN-ã, the underlying mechanism of its reduced gene expression was initially thought to involve impairment of STAT1 signaling. Yet, Thomas et al. (169) could show, that rather expression of two transcription factors downstream of STAT1, notably of IRF-1 and PU.1, is diminished. HL-60 cells can be induced to differentiate into neutrophil granulocytes and are thus often used as a model for neutrophil functions. Their response to *A. phagocytophilum* infection, however, is eventually not a good model. So far, *A. phagocytophilum* is known to inhibit superoxide anion generation in human neutrophils (196) but it remains unclear whether repression of oxidative burst is based on similar mechanisms as in HL-60 cells. Data from our group show that IFN-γ partly restores neutrophil capacity to mount an oxidative burst. In order to examine whether further IFN-ã-induced effector functions are influenced by *A. phagocytophilum*, secretion of IP-10 and MIG was investigated. Subsequently, the IFN-ã signaling cascade was explored with the intention of elucidating possible mechanisms by which *A. phagocytophilum* may interfere with neutrophil gene expression.

3.2.1 *A. PHAGOCYTOPHILUM* INFECTION DECREASES SURFACE EXPRESSION OF THE IFN-g RECEPTOR a -CHAIN (CD119)

Binding of IFN-γ to its cell-surface receptor activates the STAT1 cell signaling pathway leading to expression of IFN-γ-induced genes (58, 59). The effect of *A. phagocytophilum* infection on surface expression of the IFN-γ receptor α-chain, CD119, was investigated. Flow cytometry (Fig. 3-19) showed the decreased surface expression of CD119 in PMN infected with *A. phagocytophilum* for 5h which can account for the inhibition of the IFN-γ-induced signaling cascade.

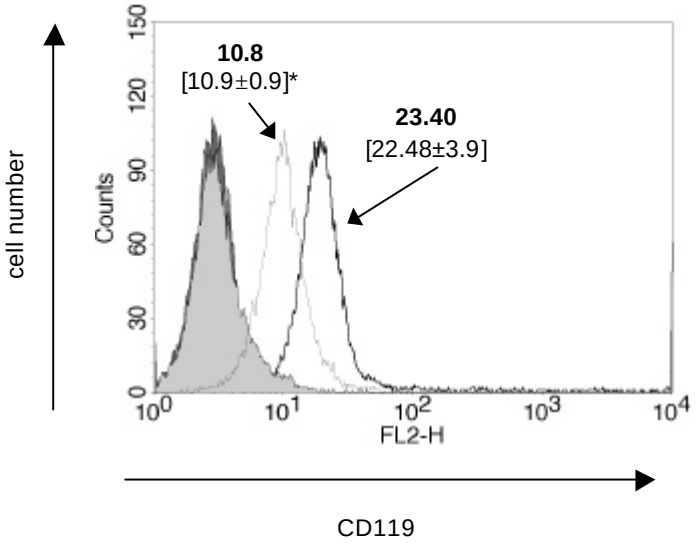


Fig. 3-19 *A. phagocytophilum* infection decreases surface expression of CD119. Freshly isolated human PMN were either left uninfected or were infected with *A. phagocytophilum.* Five hours later, the cells were washed in order to remove non-ingested bacteria. PMN were then incubated for further 5 hours. Infected (dotted line) and non-infected PMN (solid line) were stained with RPE-conjugated anti-CD119 mAb (GIR94). The histograms and mean fluorescence intensities are from one representative experiment. In brackets mean ± SD of the mean fluorescence intensities of three experiments are given. Significant downregulation (*) of the expression after *A. phagocytophilum* infection is indicated (two-sided, paired t-test). The dark grey histograms show the staining with isotype control antibody for infected PMN, the light grey histogram depicts the isotype control for uninfected PMN.

3.2.2 *A. PHAGOCYTOPHILUM* INFECTION BLOCKS STAT1 TYROSINE PHOSPHORYLATION

Stimulation of PMN with IFN-γ leads to phosphorylation and subsequent dimerization of STAT1 monomers (62-64). As phosphorylation of Tyrosine (701) is indispensable for function of the transcription factor, the effect of *A. phagocytophilum* infection on STAT1 tyrosine phosphorylation was investigated. After 5h of incubation *Anaplasma*-infected and control cells were incubated with IFN-γ for 15 min. Cells were then lysed and processed for antiphosphotyrosine western blot. Western blot analysis (Fig. 3-20) with Tyrosine (701)-phospho-specific STAT1 Ab, showed that phosphorylation of STAT1 was diminished in infected PMN. Infection thus reduced responsiveness to IFN-γ by decreased tyrosine phosphorylation.

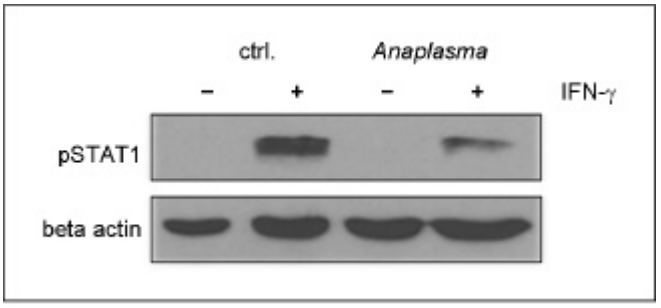


Fig. 3-20 *A. phagocytophilum* infection blocks STAT1 tyrosine phosphorylation. Freshly isolated human PMN were either left uninfected or were infected with *A. phagocytophilum.* Five hours later, the cells were washed in order to remove non-ingested bacteria. PMN were then either left untreated or stimulated with IFN-γ (200 U/ml) for 15 min. Whole cell lysates were prepared, separated by SDS-PAGE and electroblotted. Blots were incubated with an Ab specific for phosphorylated STAT1 (Tyr701 STAT1) and bound Ab was visualized by enhanced chemiluminescence. To assure equal sample loading, membranes were stripped and reprobed with anti-beta-actin mAb. The results shown represent one of three experiments that yielded similar results.

3.2.3 *A. PHAGOCYTOPHILUM* ALTERS GENE EXPRESSION OF IRF-1 AND PU.1

Along with several other transcription factors IRF-1 and PU.1 cooperate to increase several IFN-ã-induced genes (65, 185), in particular gp91 (phox) (186) which is part of the NADPH oxidase.

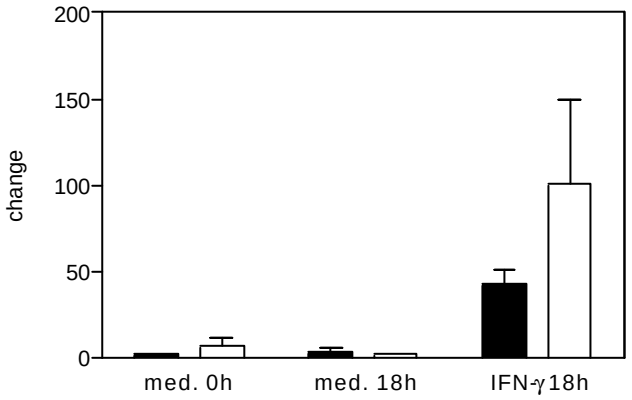


Fig. 3-21 *A. phagocytophilum* infection increases IRF-1 gene expression. Freshly isolated human PMN were either left uninfected (black bars) or were infected with *A. phagocytophilum* (white bars). Five hours later, the cells were washed in order to remove non-ingested bacteria. PMN were then lysed for isolation of total RNA (0h) or incubated for further 18 h. These samples were either left untreated or stimulated with IFN-γ (200 U/ml). Subsequently, neutrophils were lysed for isolation of total RNA (18h). Gene expression of IRF-1 was assessed by real-time RT-PCR. Using the $2^{-\Delta\Delta C}{}_{T}$ method (171), the data are presented as fold change in gene expression normalized to the reference gene beta-actin and relative to uninfected cells. Values given are mean ±SD of three experiments.

Expression of IRF-1 and PU.1 was examined in order to investigate whether transcription factors lying downstream of STAT1 are affected by *A. phagocytophilum* infection.

Real-time PCR analysis revealed that, irrespective of the incubation time, IRF-1 gene expression (Fig. 3-21) was very low in PMN in the absence of IFN-ã. *A. phagocytophilum* infection did not induce IRF-1 expression in these cells. Stimulation of neutrophils with IFN-ã, however, led to high gene expression of IRF-1 which was even augmented after infection with *A. phagocytophilum*.

A substantial expression of PU.1 (Fig 3-22) can be detected in untreated PMN. Expression of the transcription factor is strongly increased in infected cells 5h post infection, indicating that PMN mount an immune response against *A. phagocytophilum*. Yet, if infected PMN are incubated for further 18h, *A. phagocytophilum* infection doesn't lead to significant rise in PU.1 gene expression with regard to uninfected cells. PU.1 expression is slightly augmented upon IFN-ã treatment of PMN for 18h. Stimulation of infected PMN leads to a small increase in PU.1 expression with comparison to non-infected cells.

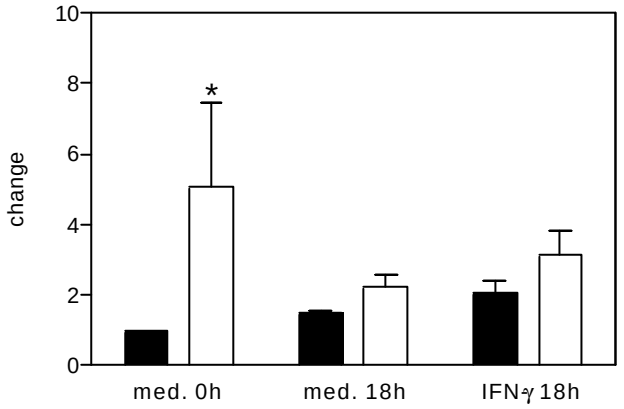


Fig. 3-22 *A. phagocytophilum* infection increases PU.1 gene expression. Freshly isolated human PMN were either left uninfected (black bars) or were infected with *A. phagocytophilum* (white bars). Five hours later, the cells were washed in order to remove non-ingested bacteria. PMN were then lysed for isolation of total RNA (0h) or incubated for further 18 h. These samples were either left untreated or stimulated with IFN-γ (200 U/ml). Subsequently, neutrophils were lysed for isolation of total RNA (18h). Gene expression of PU.1 was assessed by real-time RT-PCR. Using the $2^{\Delta\Delta C}{}_{T}$ method (171), the data are presented as fold change in gene expression normalized to the reference gene beta-actin and relative to uninfected cells. Values given are mean ±SD of three experiments. Significant increase (*) in gene expression after *A. phagocytophilum* infection is indicated (two-way ANOVA, Bonferroni post-test).

3.2.4 *A. PHAGOCYTOPHILUM* INCREASES SOCS3 GENE EXPRESSION

Negative regulation of IFN-γ signaling is among other things achieved by SOCS proteins (69). SOCS3 inhibits JAKs after binding to the IFN-γ receptor (197). Furthermore, SOCS interact with the cellular ubiquitination machinery and are thought to direct proteins, such as JAKs or receptors, for ubiquitin-mediated proteasomal degradation (198, 199). In order to investigate whether *A. phagocytophilum* infection affects negative regulation of IFN-γ signaling in PMN gene expression analysis of SOCS3 was carried out.

Real-time PCR (Fig. 3-23) showed a strong upregulation of SOCS3 in freshly infected cells. SOCS3 gene expression was lower, however, after 18h of infection. Non-infected PMN expressed SOCS3 after 18h of stimulation with IFN-ã. However, SOCS3 gene expression was even higher if PMN were additionally infected with *A. phagocytophilum*. Hence, *A. phagocytophilum* interferes with the IFN-ã cascade by increased expression of SOCS3.

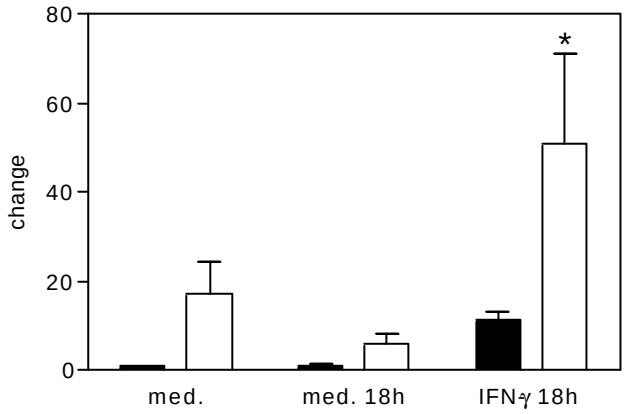


Fig. 3-23 *A. phagocytophilum* infection increases SOCS3 gene expression. Freshly isolated human PMN were either left uninfected (black bars) or were infected with *A. phagocytophilum* (white bars). Five hours later, the cells were washed in order to remove non-ingested bacteria. PMN were then lysed for isolation of total RNA (0h) or incubated for further 18 h. These samples were either left untreated or stimulated with IFN-γ (200 U/ml). Subsequently, neutrophils were lysed for isolation of total RNA (18h). Gene expression of SOCS3 was assessed by real-time RT-PCR. Using the $2^{-\Delta\Delta C}{}_{T}$ method (171), the data are presented as fold change in gene expression normalized to the reference gene beta-actin and relative to uninfected cells. Values given are mean ±SD of three experiments. Significant increase (*) in gene expression after *A. phagocytophilum* infection is indicated (two-way ANOVA, Bonferroni post-test).

3.2.5 *A. PHAGOCYTOPHILUM* INFECTION DECREASES SECRETION OF MIG AND IP-10

MIG and IP-10 are both IFN-ã-inducible chemokines. The capability of neutrophils to raise an IFN-ã-induced immunomodulatory response upon infection with *A. phagocytophilum* was investigated by measuring MIG and IP-10 secretion. Non-infected and *Anaplasma*-infected PMN were either left untreated or stimulated with LPS or IFN-ã for 18h at 37 °C. Culture supernatants were then assayed by ELISA for IP-10 and MIG (Fig. 3-24). Neutrophils secreted substantial levels of both chemokines when stimulated with LPS and IFN-γ for 18h. Infection of PMN with *A. phagocytophilum* decreased release of MIG and IP-10. Thus *A. phagocytophilum* infection decreases the release of two chemokines which depend on IFN-ã signaling.

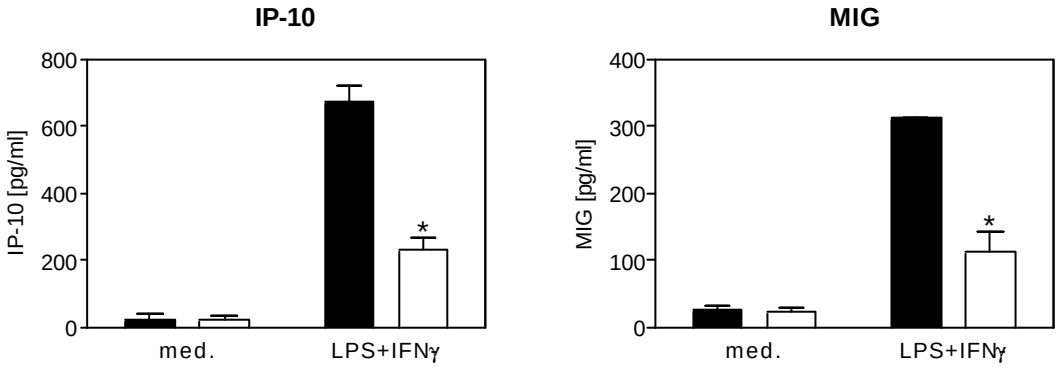


Fig. 3-24 *A. phagocytophilum* infection decreases release of IP-10 and MIG. Freshly isolated human PMN at a concentration of $5x10^6$/ml were either left uninfected (black bars) or were infected with *A. phagocytophilum* (white bars). Five hours later, the cells were washed in order to remove non-ingested bacteria. PMN were then either left untreated or stimulated with IFN-γ (200 U/ml) and LPS (200ng/ml) for additional 18h. IP-10 or MIG content of supernatants was quantified by ELISA. Values given are mean ±SD of three experiments. Significant decrease (*) in chemokine release after *A. phagocytophilum* infection is indicated (two-sided paired t-test).

3.2.6 *A. PHAGOCYTOPHILUM* INFECTION DECREASES CELL SURFACE EXPRESSION OF FAS

Neutrophils are short-lived cells which undergo apoptosis within 6-12 hours. However *A. phagocytophilum* prolongs the life span of its host cell by delaying spontaneous apoptosis (200). In order to investigate whether expression of death receptor FAS is influenced by *A. phagocytophilum* infection, FACS analysis was performed (Fig. 3-25).

Anaplasma-infected PMN at 18h post infection show reduced FAS surface expression compared to non-infected controls. These data confirm the findings of Ge et al. (201).

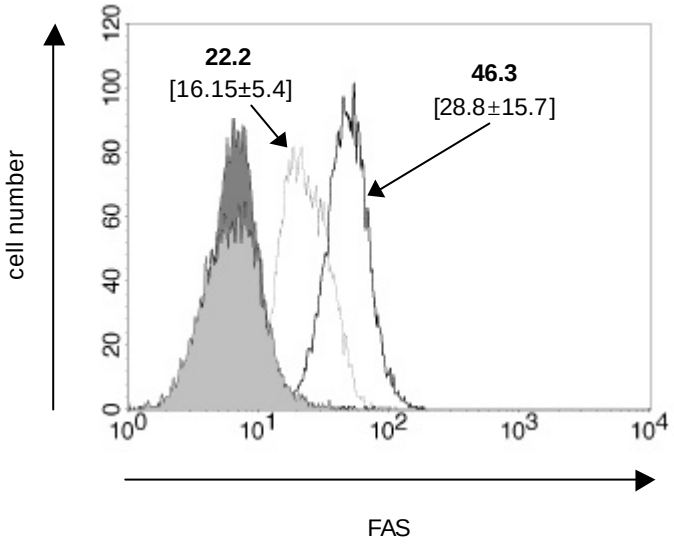


Fig. 3-25 *A. phagocytophilum* infection decreases surface expression of FAS. Freshly isolated human PMN were either left uninfected or were infected with *A. phagocytophilum.* Five hours later, the cells were washed in order to remove non-ingested bacteria. PMN were then incubated for further 18 hours. Infected (dotted line) and non-infected PMN (solid line) were stained with RPE-conjugated anti CD95 mAb (clone DX2). The histograms and mean fluorescence intensities are from one representative experiment. In brackets mean ±SD of the mean fluorescence intensities of three experiments are given. The dark grey histograms showed the staining with isotype control antibody for infected PMN, the light grey histogram depicts the isotype control for uninfected PMN.

Results II

The Gram-negative bacterium *A. phagocytophilum* infects neutrophils and replicates within these cells. Survival in this hostile environment necessitates reduction of the phagocyte´s antimicrobial functions. This study revealed that *A. phagocytophilum* impairs IFN-ã signaling of the host cell by downregulating surface expression of the IFN-ã receptor á-chain CD119 in neutrophils. Moreover, phosphorylation of the transcription factor STAT1 was diminished in *Anaplasma*-infected PMN. Gene expression of SOCS3, an inhibitor of the IFN-ã cascade, was increased by infection. In contrast to these findings, IRF-1 and PU.1, two transcription factors that cooperate with STAT1 activating IFN-ã-induced genes, were expressed to an increased degree by infected PMN. Yet, LPS- and IFN-ã-induced production of MIG and IP-10 was diminished by the bacterium. *A. phagocytophilum* impaired IFN-ã signaling in neutrophils and reduced their release of CXC chemokines. Presumably, this facilitates evasion from antimicrobial functions of PMN.

4 DISCUSSION

4.1 MODULATION OF NEUTROPHIL FUNCTIONS BY *L. MAJOR*

Intracellular survival of *L. major* within the harsh environment of neutrophil granulocytes necessitates elaborate evasion strategies. The present study aimed to explore mechanisms by which the parasite escapes from killing by its host cell.

IFN-γ elicits a large variety of antimicrobial effector functions (46, 202), comprising oxidative burst (47, 48), and chemokine expression (4, 78) that contribute to parasite killing. Playing a pivotal role in host defence, IFN-γ signaling has been in the focus of many attempts to identify interactions between phagocytes and pathogens. Previous data indicate that macrophages become functionally deactivated during *Leihmania* infection (127, 129) and that such inhibitory effect alters transcription of IFN-γ-induced genes (203). So far, however, comparable studies have not considered *Leishmania*-infected neutrophils. Recent studies from our group aimed to investigate the effect of *L. major* on the transcriptional profile of PMN. Experiments employing cDNA arrays were carried out in order to gain a global picture of neutrophil gene expression in the context of *L. major* infection. These data revealed that a particular group of differentially expressed genes show upregulation by LPS and IFN-γ after 6h of incubation. The presence of *L. major*, however, prevents this increase in gene transcription (145). Linking to these results I examined the underlying mechanisms of this inhibitory effect *L. major* exerts on neutrophil gene expression. For this purpose, first the IFN-γ signal cascade was examined. Moreover, cDNA array data from selected genes were analysed in more detail by real-time PCR and additionally investigated on protein level.

4.1.1 *L. MAJOR* IMPAIRS IFN-g SIGNALING IN NEUTROPHILS

In the first instance, I examined whether neutrophil unresponsiveness to IFN-γ may involve deficiency in IFN-γ receptor expression. Various experiments revealed that after 6h of infection neutrophils show decreased surface expression of the α-subunit of the IFN-γ receptor which may account for reduced responsiveness to IFN-γ. In macrophages, CD119 is presented on the cell surface and stored in large intracellular pools. Binding of IFN-γ results in internalization of the receptor-ligand complex, which is then targeted to an acidified compartment. Whereas the ligand is translocated to lysosomes for degradation, the uncoupled IFN-γ receptor α-subunit is trafficked to an intracellular pool of CD119 and may be recycled for surface expression (204). Although the exact mechanisms of internalization and recycling haven't been explored in the same detail with regard to neutrophils there is evidence suggesting similar principles for PMN (59). The observed downregulation of CD119 on infected neutrophils may be a result of parasite interference with receptor recycling or a consequence of enhanced degradation. Microarray data have shown that CD119 is decreased after 6h of infection (145). Thus, diminished transcription of the IFNγ receptor α-chain may contribute to reduced surface expression as well. Disappearance of CD119 from the cell surface, however, starts after 30 minutes and reaches its maximum 2 hours after infection (data not shown) when infection rate is still low. This indicates that, at least in the early stage, downregulation of CD119 possibly depends on internalization. The further fate of the receptor remains yet to be discovered. The nearly immediate decrease in CD119 surface expression suggests that the effect may be mediated by leishmanial contact. Previous studies showed that despite its importance for infectivity, the most abundant surface molecule of *Leishmania*, LPG, does not alter IFN-γ receptor expression on U937 cells (173). Whether this holds true for neutrophils is yet to be determined. Furthermore the capability of *Leishmania* surface proteases to cleave neutrophil receptors should be investigated.

IFN-γ signaling is balanced by negative-feedback mechanisms, part of which is mediated by SOCS proteins (68). Among eight known SOCS proteins SOCS1 and SOCS3 appear to be the most efficient inhibitors of IFN-γ signaling (69). The gene

expression of SOCS3 was markedly enhanced in *L. major*-infected neutrophils. Here, I report for the first time the upregulation of SOCS3 in neutrophils by infection with an intracellular microorganism. In macrophages, infection with the intracellular pathogens *Leishmania donovani* (130) *Listeria monocytogenes* (205) and *Burkholderia pseudomallei* (206) were shown to induce SOCS3 gene expression. Since SOCS3 was demonstrated to partially reduce STAT1 phosphorylation and to interfere with IFN-ã signaling (69, 207, 208), these studies suggest a role for SOCS3 in pathogen-mediated interference with IFN-ã signaling in macrophages. Present data indicate enhanced SOCS3 gene expression and reduced IFN-ã -signaling in *L. major*-infected human neutrophils. The induction of SOCS3, therefore, appears to be a potent inhibitory mechanism by which intracellular microorganisms suppress cellular activation not only in macrophages but in neutrophils as well.

SOCS3 expression is among others induced by IFN-ã. This study however suggests that IFN-ã-induced gene expression is impaired by *L. major* infection which contradicts upregulation of SOCS3 in the presence of this parasite. SOCS3 is also induced by a variety of cytokines including IL-1â, IL-6, IL-12, IL-13 and TNF (209). Previous data from our group have shown that PMN secrete IL-1â in response to *L. major* infection. Moreover, cDNA arrays indicated that IL-13 expression is augmented in infected neutrophils (145). Yet, we could show that expression of TNF and members of the IL-12 cytokine family is decreased in the presence of *L. major*. An impact of cytokines released by neutrophils cannot be ruled out, so far. Further investigations should clarify, whether increase in SOCS3 is based on an autocrine effect. Baetz et al. have pointed out that SOCS3 can be induced by TLR agonists (210). Furthermore, de Veer et al. have shown that purified *Leishmania* LPG could activate innate immune signaling via TLR2 and that LPG is capable of inducing SOCS3 expression (126). These data indicate that upregulation of neutrophil SOCS3 in response to *L. major* infection may be based on interaction of leishmanial surface with TLRs. Yet, Bertholet et al. (130) have demonstrated that LPG does not induce SOCS3 expression in human macrophages. Whether neutrophil SOCS3 expression is triggered by LPG remains to be clarified.

SOCS3 has been suggested to promote ubiquitination and proteasomal degradation of target receptors (198, 199). This implies that upregulation of SOCS3 may contribute to decrease of CD119 expression in *L. major*-infected PMN. However, after 3h of

incubation infected neutrophils displayed only slightly higher SOCS3 expression than uninfected cells (data not shown), revealing the minor role of SOCS3 during the early phase of infection. At a later time point, however, SOCS3 may promote IFN-γ receptor degradation. *L. major* may profit from upregulation of SOCS3, as it inhibits IFN-γ-induced antimicrobial effector functions which would otherwise contribute to parasite killing. The exact mechanism by which *L. major* achieves SOCS3 induction in human neutrophils is yet to be discovered.

The transcription factor STAT1 exists in its latent form in neutrophil cytoplasm. Stimulation with IFN-γ results in phosphorylation, dimerization and subsequent nuclear translocation of STAT1. *Leishmania spp*. have been reported to block STAT1 phosphorylation (127, 129), resulting in impaired expression of IFN-γ-induced gene expression (203). The present study, however, shows that inhibition of IFN-γ-induced gene expression may be achieved in a different way in neutrophils. Western blot analysis revealed that, surprisingly, *L. major* infection does not diminish STAT1 tyrosine phosphorylation in PMN after 6h of infection. Since *L. donovani* has been shown to increase phosphorylation of STAT1 in macrophages, this species has been used in order to examine whether sustained phosphorylation of STAT1 I observed in neutrophils depends on *L. major*. Yet, *L. donovani* did not impair STAT1 phophorylation, either. These findings are in contradiction to downregulation of the IFN-γ receptor α-chain, as IFN-γ signaling is exclusively mediated by IFN-γ receptor comprising both receptor chains. Moreover, sustained pSTAT1 signal in infected PMN conflicts with increased SOCS3 expression since this inhibitory molecule blocks JAKs, thus reducing phosphorylation of STAT1.

The STAT1 phosphorylation I observed in spite of reduced surface expression of CD119 may indicate that IFN-γ signaling only requires very low receptor abundance in neutrophils. Apart from the IFN-γ receptor, many other receptors have been reported to mediate STAT1 activation, among them epidermal growth factor (EGF) (211), platelet-derived growth factor (PDGF) (212) and seven-transmembrane (7TM) receptors (213). Hence, activation of STAT1 by IFN-γ is possibly achieved via an alternative receptor which is yet to be determined.

Increase in SOCS3 gene expression due to *L. major* infection occurs 6h after infection while only a very slight increase is observed after 3h. This indicates that SOCS3 is

likely to exert its full inhibitory effect later than 6h after infection and that an incline in STAT1 phosphorylation probably occurs at a later time-point. Yet, increased IFN-γ-induced gene expression is observed after 6h of infection pointing to an inhibitory effect which is operative during earlier stages of infection. Analysis of nuclear extracts might show reduced amounts of pSTAT1 depending on *L. major* infection. Whole cell extracts could, irrespectively of stimulation or infection, contain a comparably large amount of pSTAT1 that causes a relatively high background. Moreover, *L. major* infection may result in impaired nuclear translocation of the transcription factor, which should be analysed by western blots of nuclear lysates. Complete transcriptional activation of STAT1 requires not only tyrosine but also serine phosphorylation (214, 215), the latter of which has not been investigated so far. Reduced serine phosphorylation may account for impaired IFN-γ-induced gene expression.

Impaired transcriptional activation of IFN-γ-induced genes despite sustained phosphorylation of STAT1 has been observed in *Mycobacterium tuberculosis*-infected macrophages (216, 217) as well as in *Anaplasma*-infeceted HL-60 cells (169). In these cases, inhibition of IFN-γ signal transduction has been shown to occur downstream of pSTAT1. Regulation of transcription is among others achieved by binding of co-activators to the carboxy-terminal transactivation domain of STATs. The inhibitory effect of *Mycobacterium tuberculosis* was attributed to a decreased IFN-γ-induced association of STAT1 with the transcriptional co-activators CREB binding protein and p300, which is required for full transcriptional response (217). STATs interact with a variety of other transcription factors. Cooperative binding of these is necessary for introduction of many genes. Repression of gp91phox gene in HL-60 cells has been demonstrated to be associated with reduced expression of IRF-1 and PU.1, two transcription factors that occur downstream of STAT1 (169). I addressed the question whether altered expression of IRF-1 or PU.1 might account for downregulation of IFN-γ induced genes in *L. major*-infected PMN. Real-time-PCR revealed that IRF-1 gene expression remained nearly unchanged in IFN-γ-stimulated, *L. major*-infected PMN 6h post-infection. Yet, PU.1 gene expression was diminished upon *L. major* infection in PMN stimulated with IFN-γ alone or LPS and IFN-γ. Whether decreased PU.1 expression accounts for diminished transcription of IFN-γ-induced genes requires further study. Quantitative PCR allowed quick analysis of IRF-1 and PU.1 expression.

Hence, this method was chosen in order to get a first impression with regard to the question, whether *L. major* might exert its influence on IFN-ã-induced gene expression via these transcription factors. However, this issue requires further investigation involving analysis of the presence of these transcription factors in neutrophil nuclei and their binding to specific consensus sequences.

The above described impairment of IFN-γ signaling is reflected by the gene expression pattern we previously observed using cDNA arrays. Microarrays clearly showed that expression of IFN-γ-induced genes is abrogated in the presence of *L. major*. Expression of selected genes was analysed in more detail by real-time PCR. Further analysis was accomplished on the protein level. The chosen genes/proteins are of importance for neutrophil immunomodulatory and phagocytic function.

4.1.2 *L. MAJOR* AFFECTS IMMUNOMODULATORY PROPERTIES OF NEUTROPHILS

Neutrophils have long been considered to be devoid of immunomodulatory functions. Yet, during the last years, evidence has accumulated that PMN are involved in recruitment of various immune cells (4, 85, 86), as well as in regulation of their functions (85, 89-91). In this study chemokines and cytokines that recruit or rather activate Th1 cells have been analysed in the context of a *L. major* infection. Moreover, the expression of the proinflammatory cytokine TNF has been examined.

The non-ELR CXC-chemokines MIG, IP-10 and I-TAC regulate cell traffic and tissue localization of effector cells. They attract Th1 cells, which produce large amounts of IFN-γ, hence amplifying parasite killing. The prominent role of non-ELR CXC chemokines is reflected by the two different dermal chemokine profiles which accompany disease progression of healing localized and progressive diffuse cutaneous leishmaniasis mediated by *L. mexicana*. While dermal lesions of LCL show high expression of MIG and IP-10, these chemokines occur in small amounts in DCL. Different chemokine profiles influence the composition of cells infiltrating the dermis. While dermal lesions in LCL display high amounts of Th1 cells, a dominance of Th2 cells is observed in DCL lesions (218). Hence, chemokine expression patterns and the consequential composition of local inflammatory infiltrate point to the possibility that chemokines shift the adaptive immunity to either Th1 or Th2 type response.

Clearly, suppressing non-ELR CXC-chemokine release in neutrophils would be beneficial for parasite survival. Our data reveal that *L. major* infection results in downregulation of MIG, IP-10 and I-TAC in PMN, which is likely to facilitate survival of the parasite.

The proinflammatory cytokine IL-12 induces production of IFN-γ by macrophages and NK cells thus favouring differentiation of naïve $CD4^+$ T cells into mature Th1 cells. These form a source of IFN-γ themselves and consequently amplify IFN-γ-induced antimicrobial effector mechanisms. IL-12 thus has a central role in the development of a protective innate and adaptive immune response to intracellular pathogens such as *L. major* (219-221). The recently discovered cytokines IL-23 and IL-27 are closely related to IL-12 with regard to their structure and display overlapping biological effects. Like IL-12, IL-23 and IL-27 can promote production of IFN-γ by human T cells (94, 95). Nevertheless, despite these similarities, IL-27 and IL-23 have unique functions which are only partly related to T cell polarity.

IL-27 predominantly directs early events that lead to the development of a Th1 response. This is reflected by the delay of a parasite-specific Th1 response in *L. major*-infected C57Bl/6 mice deficient in the IL-27 receptor chain WSX1 (98). Furthermore, EBI3-deficient mice of the same background show susceptibility to *L. major* infection and display impaired Th1 response in the early course of infection (192). At later time points, *L. major*-infected $WSX^{-/-}$ mice produce normal levels of IFN-γ, although IL-4 levels remain high, suggesting that overproduction of Th2 cytokines during the early phase is then counteracted by IFN-γ production and Th1 development (222). These reports point to a transient requirement of IL-27 in the development of immunity to *L. major* infection. In the present study, real-time PCR of EBI3 and IL-27 p28 showed that neutrophils express IL-27. EBI3 was mainly induced by LPS or by LPS and IFN-γ. IL-27 p28 was produced in response to LPS or IFN-γ in low amounts while concomitant stimulation with LPS and IFN-γ led to strong expression. Thus neutrophils are likely to contribute to the development of protective Th1 response in the early phase of infection. *L. major* inhibits EBI3 and IL-27 p28 expression pointing to a probable mechanism by which the parasite suppresses the Th1 response thus facilitating its survival. In contrast to the finding that IL-27 is important for Th1 differentiation there is evidence for a regulatory role of this cytokine (223). In the context of *Leishmania* infection

Rosas et al. have reported that WSX1-deficient mice controlled *Leishmania donovani* infection significantly faster than wild type mice but displayed severe liver pathology (224). Since IL-27 limits the inflammatory response by antagonizing the development of IL-17-producing T cells (225), reduced expression of IL-27 or its putative receptor may contribute to a strong inflammatory response, implying tissue damage. The impact of IL-27 on the inflammatory response and its role in defence against *Leishmania* infection are not yet fully understood. The activation status of cells and the initial cytokine milieu may determine whether IL-27 rather exerts a proinflammatory or regulatory role (226, 227). However, all data regarding *L. major* infection point to a high importance of IL-27 for the development of a Th1 response during the early stage of infection. Thus, downregulation of IL-27 gene expression by *L. major* most likely contributes to parasite survival.

While IL-27 is mainly important during the early phase of infection, IL-23 is of particular importance for long term memory functions during infection, as it increases the proliferation of $CD4^+$ memory T cells (94). Recent data suggest a late acting antileishmanicidal effect of IL-23 (191). Moreover, stimulation of activated memory T cells in the presence of IL-23 induces secretion of IL-17 by lymphocytes and neutrophils. IL-17, which promotes maturation and recruitment of neutrophils (104), is thought to work together with IL-23 in increasing resistance to intracellular pathogens (106, 228). Here, I showed that neutrophils secrete IL-23 in response to LPS and IFN-γ. Infection with *L. major*, however, led to a decrease in IL-23 release. These data suggest that the parasite may evade late-acting immune defence by downregulating IL-23 production. A decrease in IL-23 should imply reduced production of IL-17. The role of IL-17 in leishmaniasis has not been investigated, so far. Since neutrophils secrete IL-17 (102) and are at the same time target cells of IL-17, examination of its function in the context of leishmaniasis may provide novel insights into mechanisms of the disease.

Tumor necrosis factor represents a further cytokine involved in Th1 response and control of *Leishmania* infection (229). TNF stimulates macrophages to produce nitric oxide. In neutrophils, TNF augments phagocytic activity and increases oxidative burst and degranulation (230, 231). Treatment of resistant C57BL/6 mice with anti-TNF monoclonal antibodies after *L. major* infection results in increased parasite burden and size of the lesion (232). The course of infection in TNF-deficient mice, however, may

vary depending on the parasite strain (233). Present data clearly show that neutrophils express TNF in response to LPS and IFN-γ, thus amplifying the inflammatory response. Yet, in the presence of *L. major* TNF expression of neutrophils is impaired, involving a reduced inflammatory response. Since TNF induces oxidative burst, which constitutes an efficient defence mechanism against intracellular parasites, *L. major* may evade killing by inhibiting TNF expression.

Decreasing IL-27 and TNF gene expression, as well as IL-23 release, *L. major* modifies the immunomodulatory functions of its host cell. Infected neutrophils are impaired in their ability to mediate a Th1 response that would favour killing of intracellular pathogens.

4.1.3 *L. MAJOR* MODULATES MECHANISMS OF UPTAKE AND INTRACELLULAR CONDITIONS

Cytochrome b 245, the membraneous subunit of the NADPH oxidase, participates in oxidative burst of neutrophils (234). Here I show that *L. major* inhibits cytochrome b 245 gene expression in LPS- and/or IFN-γ-stimulated neutrophils. Downregulation of cytochrome b 245 is very likely to facilitate intracellular survival of *L. major*.

In the presence of fresh human serum *L. major* is rapidly phagocytosed and killed by neutrophils (113). A role of complement in early uptake of the parasite is suggested by the fact that heat inactivation of the serum leads to marked decrease in phagocytosis. In normal human serum, *Leishmania* triggers complement activation (122). Within seconds the complement component C3 is deposited on the promastigote surface promoting classical pathway-mediated lysis. These data indicate that successful infection *in vivo* is likely to be dependent on rapid uptake of the parasite in an environment of low C3 abundance. Since neutrophils are the first cells arriving at the site of infection PMN may shape the early microenvironment of the transmitted parasite. Here I show that *L. major* downregulates gene expression of C3 which may be beneficial for parasite survival.

In addition to C3, phagocytosis in neutrophils is mediated by FcãRI. Cross-linking of the IFN-γ-inducible receptor by antibody triggers cell-mediated cytotoxicity thus augmenting neutrophil effector responses (235, 236). Our data reveal that *L. major*

reduces IFN-γ-induced CD64 expression in neutrophils and point to a possible mechanism by which the parasite evades host defence.

Previous data from our group showed that *L. major* infection delays intrinsic apoptosis in neutrophils allowing the pathogen to survive within these cells (141). Present findings suggest that *L. major* suppresses FAS expression in PMN, thus expanding the life span of its host cell by delay of extrinsic apoptosis. Though data on the impact of FAS/FASL pathway for *L. major* survival *in vivo* remain controversial (237, 238), I assume that on a cellular level, downregulation of FAS may contribute to survival of the parasite since reduced levels of FAS involve delayed apoptosis of the host cell.

4.1.4 MODULATORY EFFECTS DO NOT DEPEND ON VIABLE *L. MAJOR*, AND LPG, GIPLS OR SECRETED MOLECULES ARE NOT INVOLVED

The molecule mediating downregulation of IFN-ã-induced genes and impairing the IFN-ã signal cascade has not yet been identified. Since decrease of CD119 surface expression starts 30 minutes after infection (data not shown), when only very few parasites have been ingested, reduced IFN-ã signaling is likely to be dependent on *leishmanial* contact. This would imply that dead *L. major* might be capable of impairing IFN-ã-induced gene expression. Indeed, ethanol-killed parasites were able to decrease IP-10 secretion to the same extent as viable parasites. These findings are in line with the results from Bertholet et al. who showed that in macrophages, expression of the inhibitory molecule SOCS3 is increased by viable as well as by dead parasites (130). Thus blocking of IFN-ã signaling and decreased production of IP-10 does not seem to depend on viability of the parasite.

Since LPG and GIPLs represent prominent molecules on *Leishmania* surface, a contact-dependent downregulation effect would likely depend on LPG and/or GIPLs. Consequently, UGM$^{-/-}$ *L. major* that lack a key enzyme of LPG and GIPL synthesis (155) should show a decreased ability to impair IFN-ã-induced functions of neutrophils. Yet, UGM$^{-/-}$ *L. major* decreased IP-10 release to the same extent as their wildtype littermates. Betholet et al. reported that purified LPG did not increase SOCS3 expression in macrophages (130). Together with our data, these results suggest that a contribution of LPG and/or GIPLs to the above described downregulation of IFN-ã-mediated functions is rather unlikely.

In order to investigate whether any soluble factor released by *L. major* mediated downregulation of IP-10, freshly isolated neutrophils were taken up in supernatants from parasites that were incubated in medium during the preceding night. Furthermore, supernatants from neutrophils co-incubated with *L. major* overnight were added to freshly isolated PMN the following day. These supernatants did not alter IP-10 release, either. Taken together, these findings indicate that decrease of IP-10 does not depend on any soluble factor released by *L. major* alone or in response to neutrophils, nor do PMN secrete any factor causing diminished IP-10 release. Since production of IP-10 is strictly dependent on functional IFN-ã signaling, the respective signal cascade is presumably not affected by *L. major* supernatants, either. The above discussed findings are, however to be considered as preliminary. Experiments carried out in order to determine potential molecules mediating downregulation effects should be reproduced using read-outs other than IP-10 secretion. Moreover, such investigation should include gene expression analysis and examination of the IFN-ã signal cascade. Further *Leishmania* surface molecules such as the metalloprotease glycoprotein 63 should be considered to possibly impair IFN-ã signaling. Since dead parasites decrease IP-10 release and no soluble factor seems to be involved, *leishmanial* surface molecules, eventually such displaying proteolytic activity, presumably mediate downregulation effects.

Conclusions I

This study shows that *L. major* infection of PMN results in decreased CD119 surface expression as well as in increased SOCS3 (Fig. 4-1A) and diminished PU.1 gene expression (Fig. 4-1B). Altered presence of these molecules is likely to contribute to reduced transcription of IFN-γ-induced genes. In the course of infection, different mechanisms seem to account for impairment of IFN-γ-mediated signal transduction. Presumably, *L. major* evades antimicrobial effector mechanisms by interfering with IFN-γ signaling. Impaired IFN-ã signaling resulted in decreased expression of IFN-ã-induced genes. Among these are the chemokines IP-10, MIG and I-TAC as well as the cytokines IL-23 and IL-27, all of which promote a protective Th1 response. Moreover, expression of the Proinflammatory cytokine TNF is decreased by *L. major* infection. These findings suggest that the parasite alters the immunomodulatory actions of neutrophils to its own advantage. *L. major* infection furthermore leads to decreased expression of CD64 and complement component C3, thus avoiding being phagocytosed by mechanisms that favour its killing. Decreasing gene expression of cytochrome b 245, *L. major* reduces one of the most powerful defense mechanisms neutrophils mount against invading pathogens. The precise molecular mechanism mediating impairment of IFN-ã-induced gene expression is not yet defined. Preliminary experiments using IP-10 as an indicator of intact or reduced IFN-ã-mediated neutrophil functions, however, suggests that downregulation might not depend on viable parasites. It furthermore does not seem to depend on LPG, GIPLs or on any soluble factor released either by *L. major* or by PMN, themselves.

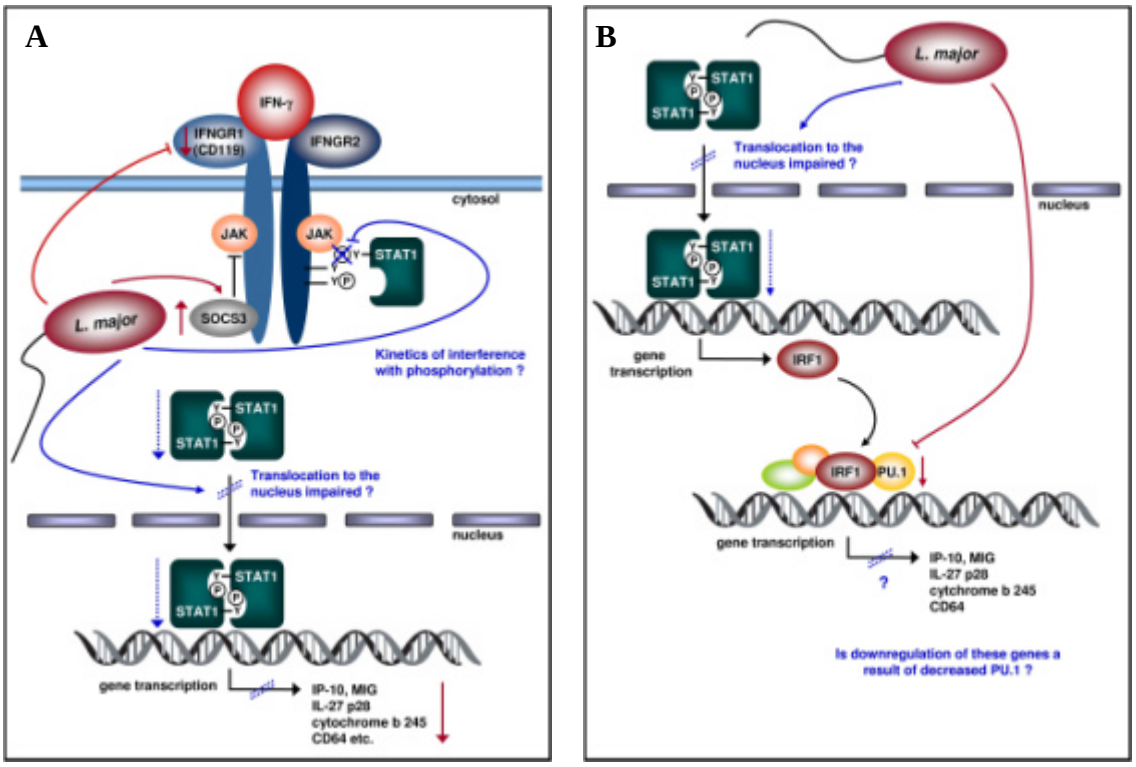


Fig. 4-1 A Interference of *L. major* with the JAK-STAT signaling. Neutrophil CD119 surface expression is diminished in the presence of *L. major.* Infection leads to increase in SOCS3 expression. Yet, phosphorylation of STAT1 seems to remain unchanged during infection. Possibly STAT1 phosphorylation is altered but the kinetics of parasite interference with phosphorylation are not fully understood. Moreover, translocation to the nucleus might be impaired. Expression of many IFN-γ-induced genes is decreased by *L. major* infection.

Fig. 4-1 B Interference of *L. major* with IRF-1 and PU.1 signaling. IRF-1 and PU.1 form an assembly that enhances transcription of various IFN-γ-induced genes. *L. major* infection leads to decreased expression of PU.1. which might contribute to diminished expression of these genes.

4.2 MODULATION OF NEUTROPHIL FUNCTIONS BY *A. PHAGOCYTOPHILUM*

In the present study I show that *A. phagocytophilum* inhibits IFN-ã signaling in neutrophils. Further data from our group, however, revealed that, in spite of compromised IFN-ã signaling, exposure to IFN-ã markedly reduced bacterial load in infected cells. Taken together, our investigations point to bi-directional interactions between IFN-ã and cells infected with *A. phagocytophilum*.

IFN-ã is known as an important mediator of cellular immune responses. In this regard, the most comprehensively investigated effect of IFN-ã is the activation of antimicrobial effector mechanisms in macrophages infected with intracellular pathogens. According to the Th1/Th2 paradigm, Th1-cell-derived IFN-ã is a major macrophage-activating mediator leading to control of intracellular pathogens such as *Mycobacteria, Toxoplasma* and *Leishmania* (48, 239-241). On the other hand, invasion strategies of intracellular pathogens comprise interference with IFN-ã signaling of the host cell leading to diminished effector mechanisms. *Mycobacteria and Leishmania* were reported to compromise IFN-ã signaling of their host cells (127, 129, 217). IFN-ã signaling thus mediates the host cells most powerful defense mechanisms but, it is at the same time a vulnerable point for pathogen interference.

The presented data show that *A. phagocytophilum* interferes with IFN-ã signaling in neutrophil granulocytes. Infected cells express the IFN-ã receptor-a chain CD119 at a lower level. Phosphorylation of STAT1 depends on proper assembly of IFN-ã receptor chains. The observed reduced STAT1 levels are therefore likely a result of diminished surface expression of the IFN-ã receptor á-chain CD119. Gene expression of SOCS3 was markedly enhanced in *A. phagocytophilum*-infected neutrophils. Since SOCS3 partially reduces STAT1 phosphorylation and thus interferes with IFN-ã signaling (69), this study suggests a regulatory role for SOCS3 in *A. phagocytophilum* interference with IFN-ã signaling.

In a previous study it has been shown that, in HL-60 cells, *A. phagocytophilum* infection inhibits expression of gp91 phox (159) which constitutes a major component of respiratory burst. Transcriptional activation of gp91 phox gene is basically induced by IFN-ã and mediated by the JAK-STAT signal cascade. Impaired gp91 phox gene

expression could, however, not be traced back on reduced levels of phosphorylated STAT1 (169). Decline of gp91 phox gene expression has rather been shown to occur downstream of pSTAT1 comprising reduced levels of the transcription factors IRF1 and PU.1 which bind to the gp91 phox promoter. Lower abundance of IRF-1 in HL-60 nuclei was partly shown to be a result of reduced binding of pSTAT1 to the STAT1 binding element of IRF-1. In contrast to these data, my findings show that, in neutrophils, IRF-1 and PU.1 expression is increased in infected cells. This study indicates that, in neutrophil granulocytes, *A. phagocytophilum* employs a different mechanism of interference with IFN-ã signaling. Phosphorylation of STAT1 is impaired in *A. phagocytophilum*-infected neutrophils. The promyelocytic cell line HL-60 serves as a valuable model to study *A. phagocytophilum* interaction with phagocytes. Yet, my results underline that mechanisms facilitating intracellular survival of *A. phagocytophilum* in its actual host cell, the neutrophil, may largely vary from the HL-60 model.

Decreased CD119 surface expression, upregulation of SOCS3 expression and the resulting decline in tyrosine phosphorylation may lead to compromised IFN-ã signaling in *A. phagocytophilum*-infected neutrophils. Consequently, IFN-ã-mediated antimicrobial effector mechanisms can be impaired. Present observations indicate that secretion of inflammatory chemokines MIG and IP-10 was markedly inhibited in infected neutrophils.

Since natural killer (NK) cell-derived IFN-ã was shown to be crucial for the control of intracellular pathogens such as *Leishmania* and *Toxoplasma* within the first days of infection (242, 243), NK cells are a possible source of IFN-ã which controls early *A. phagocytophilum* infection. Indeed, $NK1.1^+$ cells were recently shown to be involved in the early IFN-ã-mediated pathology of *A. phagocytophilum* infection in a murine model (244). After the first days of infection, other cell types such as Th1 cells are the likely origin of IFN-ã. Since IP-10 and MIG target activated T lymphocytes, especially Th1 cells, and NK cells, *A. phagocytophilum* infection may disturb the reciprocal interaction between IFN-ã -secreting cells and neutrophils releasing these chemokines. Decreased release of IP-10 and MIG by infected neutrophils can result in reduced recruitment of NK cells and Th1 cells leading to diminished antibacterial response.

Interactions between intracellular microorganisms and host macrophages have been thoroughly investigated in the last several decades. These studies indicate a delicate balance between antimicrobial effector functions of the host cell and escape mechanisms of the pathogens. Pathogens have evolved multiple strategies to avoid microbicidal actions of the host cells. They inhibit phagosome-lysosome fusion and respiratory burst, as well as the effect of activating cytokines. On the other hand, according to the classical view of a Th1-mediated cellular immune response, Th1 cell-derived cytokines such as IFN-ã can activate antimicrobial mechanisms of host macrophages and overcome the pathogen-mediated dysfunction leading to the killing of intracellular pathogens (245). Although such a balance between pathogen-mediated compromised IFN-ã -signaling and IFN-ã -mediated killing of the intracellular pathogen is well established in the context of macrophages, no such interplay has been shown so far regarding intracellular pathogens and neutrophil granulocytes.

Taken together, the present study and recent findings from our group indicate that, although *A. phagocytophilum* is able to impair effector functions of PMN by interference with its host cell´s signal transduction machinery, IFN-ã can still exert some antimicrobial effect on pathogens. Previous data showed that exposure of neutrophils to IFN-ã resulted in a marked reduction of bacterial load. This correlates with the ability of PMN to mount an oxidative burst in response to IFN-ã, if however, to lesser extent than in absence of *A. phagocytophilum*. These findings suggest that impaired IFN-ã signaling is still sufficient for mediating an immune response which leads, if not to complete bacterial clearance, so at least to reduction of bacterial load. Interactions between intracellular pathogens thus result in a delicate balance between antimicrobial effector mechanisms of the host cell and escape mechanisms of the pathogen.

Conclusions II

The Gram-negative bacterium *A. phagocytophilum* employs similar manipulation of IFN-ã signaling as the protozoan parasite in order to establish an infection. Like *L. major*, the bacterium decreases CD119 surface expression and increases SOCS3 gene expression. Besides this, STAT1 phosphorylation is decreased in *A. phagocytophilum*-infected neutrophils (Fig. 4-2). Decreasing IP-10 and MIG release, *A. phagocytophilum* presumably impairs the capacity of PMN to trigger T cell- mediated control of the pathogen. Although *A. phagocytophilum* manages to impair IFN-ã signaling, the remaining signal transduction can trigger oxidative burst in response to IFN-ã. Interference of *A. phagocytophilum* with IFN-ã signaling allows the bacterium to establish an infection which is counteracted by the remaining capacity of the neutrophil to respond to IFN-ã. Thus infection and clearance are a matter of balance between impairment and maintenance of IFN-ã signaling.

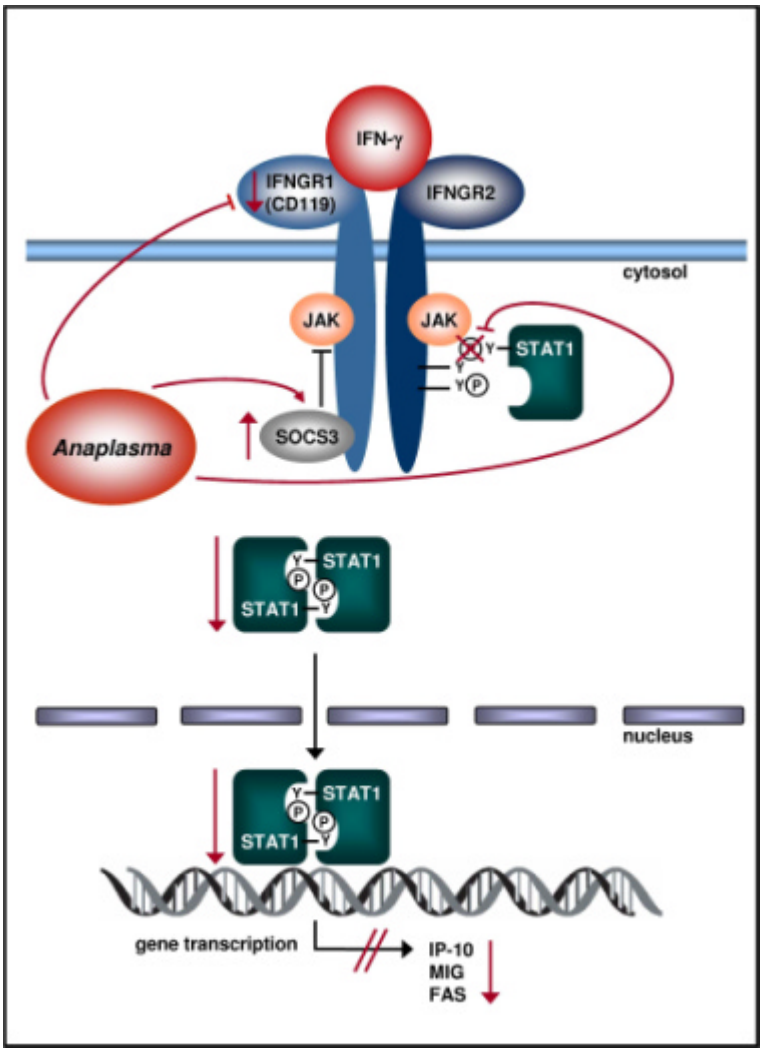


Fig. 4-2 Interference of *A. phagocytophilum* with the JAK-STAT signaling. Neutrophil CD119 surface expression is diminished in the presence of *A. phagocytophilum.* Phosphorylation of STAT1 is impaired in infected PMN. Since phosphorylation is a prerequisite for dimerization of the transcription factor, less functional STAT1 is formed. Furthermore, increased SOCS3 expression can contribute to inhibition of IFN-γ signaling in *A. phagocytophilum*-infected neutrophils. Impaired IFN-γ signaling results in diminished expression of IFN-γ-induced IP-10, MIG, and FAS.

5 SUMMARY

Polymorphonuclear neutrophil granulocytes form the first line of defense against invading pathogens. Following inflammatory signals, they migrate to the infected tissue, where they phagocytose and kill pathogenic microorganisms. Nevertheless, several microorganisms overcome neutrophil defense mechanisms and survive inside these phagocytes. The protozoan parasite *Leishmania major* and the Gram-negative bacterium *Anaplasma phagocytophilum* represent two microorganisms that infect neutrophils. The present study examined the molecular mechanisms by which *Leishmania major* and *Anaplasma phagocytophilum* achieve to survive in this harsh environment.

A large number of neutrophil defense mechanisms are triggered by IFN-ã. Therefore, the ability of both pathogens to compromise IFN-ã signaling was investigated. Present data show that *Leishmania major* infection decreases surface expression of the IFN-ã receptor á-chain CD119 and increases gene expression of the suppressor of cytokine signaling 3 (SOCS3). In spite of this, phosphorylation of the signal transducer and activator of transcription 1 (STAT1) remained unchanged in infected cells. Yet, gene expression of the transcription factor PU.1, which is thought to activate a variety of IFN-ã-induced genes was diminished by infection. These findings suggest that *Leishmania major* infection, at least partly, impairs IFN-ã signaling.

Analysis of IFN-ã-induced genes indicated that infected neutrophils express reduced amounts of several genes being pivotal to defense of the parasite. Releasing various cytokines and chemokines, neutrophils exert diverse immunomodulatory functions. These include recruitment and activation of specific immune cell subsets that mediate protection against intracellular pathogens. Infection led to decreased release and/or gene expression of several cytokines and chemokines promoting a protective Th1 response. Among these are the chemokines IFN-ã-induced protein of 10 kDa (IP-10), monokine induced by gamma-interferon (MIG) and interferon-inducible T cell alpha chemoattractant (I-TAC) that recruit Th1 cells. Ethanol-killed *L. major* and parasites deficient in production of the major leishmanial surface molecules lipophophoglycan (LPG) and glycoinositolphospholipids (GIPLs) were capable of impairing IP-10-release as efficiently as viable wildtype parasites. Supernatants from *Leishmania major*

cultures, however, did not reduce neutrophil IP-10 secretion. Two members of the IL-12 cytokine family, IL-27 and IL-23, which promote Th1 differentiation of naï ve CD4+ T cells, were shown to be produced in decreased amounts by infected cells. *Leishmania major* thus interferes with the immunomodulatory properties of neutrophils preventing theses cells from favouring a Th1 cell response.

Diminishing expression of the Fcã receptor I (CD64) and the complement component C3, *Leishmania major* avoids detrimental mechanisms of its uptake by neutrophils. The parasite, furthermore downregulated expression of cytochrome b 245, which forms the membranous subunit of the NADPH oxidase. The letter mediates generation of reactive oxygen species that represent a powerful means of killing invading pathogens.

Though phylogenetically dissimilar from *Leishmania major*, the bacterium *Anaplasma phagocytophilum* employed similar strategies in order to escape from neutrophil defense mechanisms. These comprise impairment of CD119 surface expression, increased gene expression of SOCS3 as well as diminished STAT1 phosphorylation. Reduced IFN-ã signaling is reflected by decreased secretion of IP-10 and MIG.

Taken together, this study shows that the intracellular pathogens *Leishmania major* and *Anaplasma phagocytophilum* impair neutrophil IFN-ã signaling and modulate antimicrobial functions of the phagocyte. This presumably facilitates survival of the pathogens within these cells.

6 ZUSAMMENFASSUNG

Polymorphkernige neutrophile Granulozyten repräsentieren die erste Verteidigungslinie des Immunsystems gegen eindringende Pathogene. Indem sie inflammatorischen Signalen folgen, migrieren Neutrophile in das infizierte Gewebe ein, wo sie Mikrooganismen phagozytieren und abtöten. Viele Erreger überwinden jedoch diese Abwehrmechanismen und überleben in Phagozyten. Der Parasit *Leishmania major*, welcher zu den Protozoen zählt, sowie das Gram-negative Bakterium *Anaplasma phagocytophilum* stellen zwei Pathogene dar, welche Neutrophile infizieren. In der vorliegenden Arbeit wurden die molekularen Mechanismen untersucht, welche es diesen Pathogenen erlauben, in einer derartig feindlichen Umgebung zu überleben.

Da eine IFN-ã eine Vielzahl von Abwehrmechanismen vermittelt, wurde hier die Fähigkeit beider Pathogene untersucht, die IFN-ã-Signaltransduktion zu beeinträchtigen. Die vorliedenden Daten zeigen, dass die Infektion von Neutrophilen mit *Leishmania major* zu einer verringerten Oberflächenexpression der IFN-ã-Rezeptor-á-Kette CD119 führt. Ferner wurde eine verstärkte Genexpression des *suppressor of cytokine signaling 3* (SOCS3) beobachtet. Die Phosphorylierung des *signal transducer and activator of transcription 1* (STAT1) hingegen war in infizierten Zellen unbeeinträchtigt. Der Transkripionsfaktor PU.1, welcher an der Aktivierung verschiedener IFN-ã-induzierte Gene beteiligt ist, wurde hingegen von infizierten Neutrophilen vermindert exprimiert. Diese Ergebnisse deuten darauf hin, dass *Leishmania major* die IFN-ã-Signaltransduktion zumindest teilweise beeinträchtigt.

Die Analyse IFN-ã-induzierter Gene zeigte, dass infizierte Neutrophile eine Vielzahl von Genen, welche eine zentrale Rolle in der Abwehr von Pathogenen spielen, in geringerem Maße exprimieren. Durch die Freisetzung verschiedener Chemokine und Zytokine können Neutrophile immunmodulatorische Funktionen ausüben, wie zum Beispiel die Rekrutierung und Aktivierung spezifischer Immunzellen, welche die Abwehr intrazellulärer Erreger vermitteln. Zytokine und Chemokine, die eine protektive Th1-Antwort begünstigen, wurden von infizierten Neutrophilen vermindert exprimiert. So wurde die Sekretion und Genexpression der Chemokine *IFN-ã-induced protein of 10 kDa* (IP-10), *monokine induced by IFN-ã* (MIG) und *interferon-inducible t cell alpha*

chemoattractant (I-TAC), welche Th1-Zellen rekrutieren durch *Leishmania major* herabgesetzt. Sowohl Ethanol-getötete *Leishmania major* als auch Lipophosphoglycan (LPG)- und Glycoinositolphospholipid (GIPL)-defiziente Parasiten waren in der Lage, die Freisetzung von IP-10 aus Neutrophilen im gleichen Maße wie lebendige Wildtypparasiten zu beeinträchtigen. Aus *Leishmania major*-Kulturen gewonnene Überstände verminderten die IP-10-Sekretion jedoch nicht. Es konnte gezeigt werden, dass zwei Mitglieder der IL-12-Zytokinfamilie, IL-27 und IL-23, welche die Th1-Differenzierung unterstützen, von infizierten Zellen in geringerem Umfang produziert werden. *Leishmania major* interferiert folglich mit den immunmodulatorischen Eigenschaften von Neutrophilen und verhindert, dass diese Zellen zur Ausbildung einer Th1-Antwort beitragen.

Indem *Leishmania major* die Expression des Fcã-Rezeptors (CD64) sowie der Komplementkomponente C3 herabsetzt, vermeidet der Parasit seine Phagozytose mittels dieser unzuträglichen Mechanismen. Ferner wird die Expression der membranständigen Untereinheit der NADPH-Oxidase, Cytochrom b 245, verringert. Dieses Enzym vermittelt die Generierung reaktiver Sauerstoffspezies, welche zur effektiven Abtötung von Pathogenen beitragen.

Obwohl dem Parasiten *Leishmania major* phylogenetisch sehr unähnlich, verwendet das Bakterium *Anaplasma phagocytophilum* ähnliche Stategien, um sich den Abwehrmechanismen Neutrophiler zu entziehen. Diese schließen eine beeinträchtigte CD119-Oberflächenexpression, die erhöhte Expression von SOCS3, sowie eine verminderte STAT1-Phosphorylierung ein. Die verringerte IFN-ã-Signaltransduktion spiegelt sich in der herabgesetzten Sekretion von IP-10 und MIG wieder.

Zusammenfassend konnte in dieser Studie gezeigt werden, dass die intrazellulären Pathogene *Leishmania major* und *Anaplasma phagocytophilum* die IFN-ã-Signaltransduktion in Neutrophilen beeinträchtigen und deren antimikrobielle Funktionen vermindern. Das Überleben dieser Pathogene wird hierdurch vermutlich erleichtert.

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8 LIST OF PUBLICATIONS, TALKS AND POSTERS

PUBLICATIONS

- **Human neutrophils infected with *Anaplasma phagocytophilum* are targets of antibacterial action of IFN-g despite compromised IFN-g signaling.**
  U. Bussmeyer, A. Sarkar, K. Broszat, T. Lüdemann, G. van Zandbergen, C. Bogdan, J. S. Dumler, F. D. von Loewenich, W. Solbach and T. Laskay
  Submitted

- **Infection with *Leihmania major* inhibits LPS and IFN-g-mediated activation of neutrophil granulocytes.**
  U. Bussmeyer, S. Lotz, R. Hoffmann, G. van Zandbergen, W. Solbach, T. Laskay
  In preparation

NATIONAL AND INTERNATIONAL MEETINGS

- **8th Symposium "Infection and Immune Defense", Burg Rothenfels, Germany, 2004.** Oral presentation.
- **36th Annual Meeting of the DgfI and SSI, Kiel, Germany, 2005.** Oral presentation.
- **2nd Joint Meeting of the German Society of Hygiene and Microbiology e.V. and of the Association for General and Applied Microbiology, Göttingen, Germany, 2005.** Oral presentation.
- **Signal Transduction Society (STS), 9th Joint Meeting, Weimar, Germany, 2005.** Poster presentation.
- **Spring School on Immunology, Ettal, Germany, 2006.** Poster presentation.
- **1st Joint Meeting of European National Socienties of Immunology (EFIS)/ 16th European Congress of Immunology (ECI), 2006, Paris, France.** Poster presentation.
- **35th Annual Meeting of the DgfI, Heidelberg, Germany, 2007.** Oral presentation. *In preparation*.

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10 CURRICULUM VITAE

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EDUCATION

04/2004 – 09/2007	PhD thesis in the group of Prof. Laskay, PhD, in Lübeck
10/1992 – 12/2003	University training in Pharmacy and Chemistry (Diploma) in Hamburg
08/1977 – 06/1991	Highschool: Gymnasium Schenefeld (Abitur)
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PROFESSIONAL EXPERIENCE

11/2001 – 12/2003	Student assistant in the animal facility at the Centre for Molecular Neurobiology, Medical School of the University of Hamburg
10/2000 – 10/2001	Technical assistant in the medical practice of Koppermann, MD and Hissnauer,MD
04/1999 – 10/2000	Intern and student assistant in the group of Prof. Schachner, PhD, at the Centre for Molecular Neurobiology, Medical School of the University of Hamburg
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02/1996 – 07/1996	Exchange student in the group of Prof. Dixneuf, PhD, Laboratoire de Chimie Coordination Organique, Université de Rennes, France
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SCHOLARSHIPS AND AWARDS

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