Built for the kill. Studies on the neutrophil NADPH oxidase
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Chapter 1

General Introduction
General introduction

Human neutrophils comprise 60 to 70 percent of the blood leukocytes and form a strong line of defense against invading microorganisms. Neutrophils, as professional phagocytes, have a highly specialized function: the killing of pathogens. To perform this killing, phagocytes have three characteristics that are unmatched by other cells of the immune system: their ability to migrate into an inflamed area, the amount of pathogens they can phagocytose and the array of antimicrobial products they can subject the pathogen to. These three features render the neutrophil a superb phagocyte and a very important factor in the clearance of infections. This introduction will provide an overview of the different processes occurring in the neutrophil that ultimately lead to killing of pathogens. Strong emphasis will be put on two aspects of neutrophil biology: the killing of microorganisms by reactive oxygen species (ROS) derived from the NADPH oxidase enzyme and the function of Toll-like receptors (TLRs), a family of receptors that is able to recognize specific bacterial components.

Migration from the blood stream to the site of infection.

Migration of neutrophils from the blood to the site of infection is a well orchestrated, multi-step process (Figure 1). In infected areas, low-molecular-weight factors are generated with the capacity to activate surrounding tissue cells, endothelial cells and leukocytes. Some of these factors are derived from microorganisms, others from tissue or immune cells. Activation of endothelial cells by TNF-α, IL-1β or lipopolysaccharide (LPS) leads to upregulation of adhesion proteins on these cells. The first step in the process of neutrophil extravasation is characterized by a weak interaction of the neutrophils with the activated vascular endothelium. This interaction of neutrophils with endothelium is mediated by adhesion molecules of the selectin family on the endothelium and their respective carbohydrate ligands on the neutrophils. Typically, because of the weak interaction, the neutrophils roll over the endothelium during this first step. The next step in neutrophil migration is mediated by signaling via sialyl Lewis X (on the neutrophils) after ligation with E-selectin (on the endothelial cells) as well as signaling mediated by cytokines produced and/or presented by the endothelium interacting with cytokine receptors on the neutrophils. These two signaling pathways lead to activation of β2 integrins, LFA-1 and Mac-1, on the surface of the neutrophils, and interaction with their ligands on endothelial cells, ICAM-1 and ICAM-2, establishing firm adhesion. Signaling by cell adhesion molecules
(CAMs) on the endothelial cells triggers the loss of endothelial cell-cell contact and allows the neutrophil to enter the next step of its migration, the crossing of the endothelial barrier. The actual transmigration across the endothelial cell layer is probably mediated by CD31 (PECAM-1), a member of the immunoglobulin gene superfamily. This protein is expressed on endothelial cells as well as on neutrophils and is capable of homotypic interactions, thereby providing the sites of attachment that are needed for the neutrophil to pull itself through the endothelial layer. When the neutrophil has passed the endothelium it ends up in the extracellular matrix (ECM), where it encounters signals that direct it towards the infectious site. These signals consist of chemotactic factors like chemokines (i.e. IL-8), cleavage products of the complement system (C5a) or bacterial products (the bacterial peptide fMLP). Presentation of these chemotactic factors by the ECM directs the migration of the neutrophils towards the site of infection and rimes the effector functions of the neutrophil for efficient killing of the invaded pathogens.

**Phagocytosis of pathogens by neutrophils**

In general, in the process of inflammation, plasma proteins are present at the site of infection. Among the plasma proteins are components of the complement system as well as immunoglobulins (Igs) and other proteins like mannose-binding lectin (MBL) and ficolin that are able to attach to the surface of microorganisms, a process called opsonization. When neutrophils have migrated into the site of inflammation and have come into contact with opsonized pathogens, these microbes are rapidly taken up. This uptake is mediated by two types of receptor expressed on the surface of neutrophils; the Fc receptors (FcR) and complement...
receptors CR1 and CR3. Complement receptor 3 (CR3) is a β2 integrin (αMβ2, CD11b/CD18, Mac1) with binding sites for adhesion proteins as well as for complement fragment iC3b sub. CR1 binds fragment C3b. The Fc receptors bind to the Fc region of immunoglobulins. After binding of the opsonized microbe to the neutrophil, actin polymerisation is induced, leading to protrusions of the neutrophil membrane around the microbe. To be able to engulf the microbe completely, extra membrane has to be recruited to the site of phagocytosis. This is achieved by the recruitment of granules, which by fusion with the plasma membrane provide the necessary extra membrane for the complete ingestion of the pathogen. Moreover, this fusion also initiates the release of the granule contents, i.e. microbicidal proteins, into the surroundings of the ingested microbes. After closure of the membrane around the microbe, the uptake has been completed and a phagosome containing the microbe has been formed. In the phagosome the killing of the microbe takes place. Phagocytosis, being a highly coordinated and complex process, requires extensive signal transduction. The ligation of FcRs as well as CRs by opsonized pathogens initiates signal transduction routes leading to the uptake of pathogens but also to the initiation of the killing process.

Killing of phagocytosed pathogens; role of the NADPH oxidase

The process of intracellular killing depends on the release of toxic proteins in the vicinity of the microorganisms (degranulation) and on the generation of reactive oxygen compounds, also close to the ingested microbes (figure 2). The latter process is mediated by an enzyme called leukocyte NADPH oxidase. The leukocyte NADPH oxidase consists of at least five components, two of which are located in membranes and the others in the cytosol (figure 2). The two membrane-bound components are called gp91phox (gp for glycoprotein and phox for phagocyte oxidase) and p22phox. The actual enzymatic unit is formed by gp91phox. This protein contains one FAD and two heme moieties, as well as a binding site for NADPH. Electrons donated by NADPH are transmitted through gp91phox via FAD and the hemes, and are finally transferred to molecular oxygen on the extracellular (or intraphagosomal) side of the membrane, thus generating superoxide (O2•−). This superoxide is the parent compound of other, more aggressive and longer-living oxygen compounds that the cell uses to kill microorganisms. The gp91phox protein is stabilized in the membrane by its association with p22phox; this gp91phox/p22phox complex is also called flavocytochrome b558.
Superoxide, and especially the other reactive oxygen compounds derived from it, are potentially also very harmful to the leukocyte itself. Therefore, the generation of these compounds must be strictly regulated in space and time. Neutrophils at rest do not produce these agents. Upon binding of microorganisms to cell surface receptors, the activity of the NADPH oxidase must be started. This is accomplished by an intracellular train of events that culminates in the movement of four cytosolic components ($p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and a small GTPase, Rac2) to the flavocytochrome in the membrane, complex formation that induces a conformational change in gp91$^{phox}$, and subsequent enhanced NADPH binding and enzymatic activity (figure 2)\textsuperscript{22}. In resting cells, the flavocytochrome is in the membrane of secretory vesicles and specific granules, and the other components are in the cytosol\textsuperscript{23,24}. Upon cell activation, the secretory vesicles and specific granules fuse with the phagosomal membrane that surrounds the ingested microorganism, thus localizing the flavocytochrome close to its target\textsuperscript{25}. At the same time, the cytosolic components move to the flavocytochrome and induce the enzymatic activity of the NADPH oxidase\textsuperscript{21}. In this way, the generation of reactive oxygen compounds is restricted to the site and to the moment that these compounds
are needed. It is not known yet in which way the enzymatic activity is turned off again.

Recently, Reeves et al. provided evidence for a more sophisticated role of the NADPH oxidase in the killing of ingested microbes\textsuperscript{26}. These authors showed that the NADPH oxidase is also involved in the release of proteases from the matrix of the granules, resulting in the dispersion of these enzymes in the phagosome. Due to NADPH oxidase activity, large amounts of electrons are pumped into the phagosome, which changes the membrane potential over the phagosomal membrane. This membrane potential was believed to be totally compensated by the influx of $H^+$ into the phagosome. However, Reeves et al. showed that this potential is in part compensated by the influx of $K^+$. Inside the phagosome, the $K^+$ ions mediate the release of granular proteases from the strongly anionic sulphated proteoglycan matrix\textsuperscript{26}. Once released from the matrix, the proteases are able to degrade the ingested microbe, a crucial step in the process of killing. Thus, the NADPH oxidase is a crucial component for oxidative as well as proteolytic degradation of phagocytosed pathogens.

**Chronic Granulomatous Disease**

When the NADPH oxidase is not functional, the bacterial killing process is seriously impaired and a clinical syndrome develops that is characterized by life-threatening bacterial and fungal infections. This syndrome is called chronic granulomatous disease (CGD)\textsuperscript{27}. CGD is a rare congenital immunodeficiency seen in about one in 250,000 individuals. The patients suffer from severe recurrent infections, mostly pneumonia, lymphadenitis, cutaneous and lymphatic abscesses, osteomyelitis, and septicemia. These infections usually become apparent during the first year of life and are caused predominantly by *Staphylococcus aureus*, *Aspergillus* species, enteric gram-negative bacteria, *Serratia marcescens*, and *Burkholderia (Pseudomonas) cepacia*. In addition, CGD patients have diffuse granulomas (presumably caused by persistent microbes) that can become large enough to cause obstructive and painful symptoms in the esophagus, stomach, biliary system, ureters, or urinary bladder\textsuperscript{27}.

A deficiency of the leukocyte NADPH oxidase activity can be the result of any of a number of defects. In the first place, one of the structural components of the enzyme can be deficient. This is the case when mutations occur in gp91\textsuperscript{phox}, p22\textsuperscript{phox}, p47\textsuperscript{phox}, p67\textsuperscript{phox} or Rac2. The gene encoding gp91\textsuperscript{phox} is located on the X chromosome; therefore, mutations in this gene give rise to an X-linked form of
CGD (Table 1). The other four genes are autosomal. Mutations in the gene for p40 have not been described. Together, mutations in these five genes account for the overwhelming majority of CGD patients, with defects in gp91 found in about 70% of the cases and in p47 in about 25%. Defects in p22 and in p67 are very rare. Even rarer are mutations in Rac2. In that last case, the cellular abnormalities are not restricted to NADPH oxidase deficiency but also include defects in chemotaxis and degranulation.

This is explained by the fact that besides its role in NADPH oxidase activation, Rac2 is also involved in the regulation of a series of other cellular functions of neutrophils, like actin polymerization and chemotaxis in response to specific stimuli. Finally, defects in the process that provides the cell with substrate for the oxidase, i.e. NADPH, have also been described to lead to CGD. NADPH is generated in the hexose monophosphate pathway, in the successive glucose 6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) reactions. In some very rare instances, patients have been found with generalized G6PD deficiency. These patients present with CGD-like symptoms.

**Priming of the NADPH oxidase**

Several stimuli, particulate as well as soluble, are able to induce the production of superoxide by human neutrophils. In general, stimulation of neutrophils with soluble stimuli, such as the bacterial peptide fMLP, leads to secretion of ROS into the extracellular milieu. This production of ROS can be greatly enhanced by pretreatment with various priming agents such as platelet-activating factor (PAF), granulocyte/macrophage-colony stimulating factor (GM-CSF) and bacterial products such as LPS. The signal transduction responsible for the priming effects is complex and differs strongly between various stimuli, although the outcome of the priming is usually comparable for the different priming agents. In general, priming agents induce the fusion of granules with the plasma membrane, thereby providing extra flavocytochrome to participate in the production of superoxide. Moreover, some reports have suggested an increase in the concentration of the cytosolic proteins of the NADPH oxidase in the vicinity of the plasma membrane after priming with various priming agents.

Superoxide production after phagocytosis is an even more complicated process, especially when the pathogen or particle is opsonized with both IgG and complement. In this situation the NADPH oxidase is activated by different pathways, from both the FcRs as well as from the CRs, the latter being the more
potent in inducing NADPH oxidase activation (van Bruggen et al., unpublished results). This becomes apparent when both pathways are activated independently, by opsonizing particles with either immunoglobulins or complement, and allowing neutrophils to phagocytose them. Particles opsonized by immunoglobulins are rapidly taken up, but the extent of the respiratory burst is diminished in comparison to serum-opsonized particles (van Bruggen et al., unpublished results). In contrast, particles opsonized by complement are taken up at a slower pace but show superoxide production that is comparable to serum-opsonized particles (van Bruggen et al., unpublished results). The intimate association of NADPH oxidase activation with phagocytosis is underscored by the fact that, besides deletion of structural proteins of the NADPH oxidase, no protein has been found that can either be deleted or inhibited by pharmacological means to reduce the extent of NADPH oxidase activity while leaving phagocytosis fully intact. In other words, the signal transduction cascades leading to phagocytosis in neutrophils also lead to NADPH oxidase activation, no exceptions have been found yet. The probable cause for the close connection between phagocytosis and NADPH oxidase activation is their mutual dependence on rearrangements of the actin cytoskeleton. Both processes are dependent on actin polymerization. Moreover, Rac2, one of the structural components of the NADPH oxidase is also involved in organization of the actin cytoskeleton and plays a role in the actin rearrangements needed for phagocytosis. Besides the involvement of Rac2 in NADPH oxidase activity and the organization of the cytoskeleton, other components of the NADPH oxidase, p47phox and p67phox have also been shown to bind to proteins of the actin cytoskeleton, i.e. moesin and coronin, respectively.

Strategies to escape from killing by neutrophils

Pathogens have developed various strategies to circumvent or resist the killing process of neutrophils. Some pathogens interfere with their uptake by phagocytes, others have developed strategies to survive intracellularly. Microbial tactics for intracellular survival include lysis of the phagosomal membrane and escape to the cytoplasm, inhibition or delay in phagosome maturation and/or acidification and survival within the fused phagolysosome. One of the best studied intracellular pathogens is Salmonella typhimurium. This gram-negative bacterium is equipped with virulence factors that enable the uptake, survival and replication of this pathogen in phagocytes. First, the bacterium attaches to the phagocyte and injects virulence factors into the cytoplasm of the
host by a type-III secretion system. These virulence factors target the actin cytoskeleton of the host cell and promote the formation of membrane protrusions, leading to the uptake of the bacterium into a membrane-surrounded vacuole. In this vacuole Salmonella is protected from the microbicidal enzymes of the host cell by a second set of virulence factors. These virulence factors have various modes of action, such as the neutralization of ROS, the inhibition of superoxide generation by the NADPH oxidase and the prevention of fusion of the Salmonella-containing vacuole with lysosomes.

**Pattern recognition receptors in neutrophils**

Cells of the immune system are capable of discriminating invading microorganisms from host tissue. Unlike cells of the adaptive immune system, which recognise "microbial nonself" via an immense variety of receptors generated by the variable recombination of a set of germ line genes, cells of the innate immune system depend on the use of products of a limited number of germ line genes to discriminate "self" from "nonself". The proteins used by cells of the innate immune system to recognise "nonself" are collectively termed pattern recognition receptors (PRRs). PRRs are receptors with the ability to react with motifs specifically expressed by pathogens, so-called pathogen-associated molecular patterns (PAMPs). Examples of PAMPs are lipopolysaccharide (LPS), β-glucan and peptidoglycan (PGN). PRRs transmit signals that can lead to expression of inflammatory cytokines and chemokines and activation of microbicidal systems such as the production of ROS and release of antimicrobial peptides. Neutrophils express a number of PRRs (Table 1), which can influence the activation status, cytokine secretion and life span of these cells.

**Toll-like receptors**

A recently identified family of PRRs is the family of Toll-like receptors (TLRs). TLRs are transmembrane proteins consisting of an extracellular leucine-rich repeat (LRR) for the recognition of PAMPs and a cytoplasmic tail that is responsible for the signal transduction after ligation of these receptors. Till now, at least nine members of the TLR family have been described in the human immune system. For most TLRs, a ligand in the form of one or more PAMPs has been identified (Table 2). TLRs are expressed on cells of the innate and the adaptive immune system, but expression of TLRs has also been described for cells...
Table 1. Pattern recognition receptors important for neutrophil function.

<table>
<thead>
<tr>
<th>PRR</th>
<th>Protein family</th>
<th>Ligand</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Secreted PRRs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MBL, Ficolin</td>
<td>C-type lectin</td>
<td>Terminal carbohydrate residues</td>
<td>Opsonization via the activation of the lectin complement pathway</td>
</tr>
<tr>
<td>CRP, SAP</td>
<td>Pentraxins</td>
<td>Phosphorylcholine on microbial membranes</td>
<td>Opsonization via the activation of the classical complement pathway</td>
</tr>
<tr>
<td>LBP</td>
<td>Lipid-transfer protein</td>
<td>LPS</td>
<td>LPS recognition</td>
</tr>
<tr>
<td><strong>Cell-surface PRRs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD14</td>
<td>Leucine-rich repeats</td>
<td>LPS, Peptidoglycan</td>
<td>Co-receptor for TLRs</td>
</tr>
<tr>
<td>CR3</td>
<td>Integrin</td>
<td>Zymosan</td>
<td>Phagocytosis, activation</td>
</tr>
<tr>
<td>Dectin-1</td>
<td>Lectin</td>
<td>β-glucans</td>
<td>Phagocytosis</td>
</tr>
<tr>
<td><strong>Toll-like receptors</strong></td>
<td>Leucine-rich repeats</td>
<td>various microbial products</td>
<td>Cell activation</td>
</tr>
</tbody>
</table>

MBL, mannan-binding lectin; CRP, C-reactive protein; SAP, serum amyloid protein; LBP, LPS-binding protein; LPS, lipopolysaccharide; TLR, Toll-like receptor; CR3, complement receptor 3.

that do not belong to the immune system. The cell-surface expression of TLRs enables cells to respond to PAMPs present in the extracellular milieu. Besides cell-surface expression, intracellular localization of TLRs on phagosomes in professional phagocytes has been demonstrated, leading to the hypothesis that TLRs are able to sample the content of the phagosome. Subsequently, after binding of a PAMP expressed by the ingested microbe to its specific TLR, signaling would occur. Although this is a very attractive hypothesis, there is as yet no evidence that binding of PAMPs is occurring in the phagosome.

TLRs share part of their signal transduction pathway with the Interleukin-1 receptor (IL-1R) family. Stimulation of both types of receptor ultimately leads to activation of the transcription factor NF-κB (Figure 3). The cytoplasmic tails of both TLRs and the IL-1R contain a conserved domain known as the Toll/IL-1 receptor (TIR) domain. This TIR domain is also present in the adaptor protein MyD88, and homotypic interaction between the TIR domain of the TLR/IL-1Rs with the TIR domain of MyD88 mediates the binding of this intracellular protein to ligand-activated receptors. After binding of MyD88 to TLR/IL-1Rs, IL-1R-associated protein kinase 1 (IRAK-1) is recruited by homotypic interaction...
between the death domain (DD) present in this kinase and in MyD88\textsuperscript{68,70,74}. This recruitment is mediated by a protein named Toll/IL-1R interacting protein (Tollip)\textsuperscript{75}. Tollip binds to IRAK-1 and is thought to bring IRAK-1 to the activated receptor complex and to allow binding of IRAK-1 to MyD88. IRAK-1 is then phosphorylated, detaches from MyD88 and subsequently binds to and activates a protein called TRAF-6 (TNF receptor-associated factor 6)\textsuperscript{76}. TRAF-6 triggers the activation of MKK6 and TAK1 which in turn activate JNK, p38 MAPK and NF-κB respectively\textsuperscript{77}. This cascade constitutes the basic signal transduction route after activation of TLR/IL-1Rs. This signal transduction route induces the expression of a set of genes that lead to inflammation like the induction of pro-inflammatory cytokines and the differentiation of various cell types into effector cells.

Besides the above-mentioned signal transduction route leading to NF-κB activation, other signal transducing proteins have been identified to bind to specific TLRs. One of the recently identified signal transduction proteins is Mal-TIRAP\textsuperscript{78-80}, a MyD88-like adaptor protein. This protein is involved in signal transduction induced by TLR1, 2, 4 and 6, but not in signal transduction mediated by TLR7 and 9. In fact, TLR1, 2, 4 and 6 signaling is impaired in TIRAP-deficient mice\textsuperscript{80}, a surprising finding, since the proteins that are needed for IL-1R activation were present and this pathway proved to be unaffected in TIRAP-deficient mice.

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**Table 2. Human Toll-like receptors and their ligands.**

<table>
<thead>
<tr>
<th>Toll-like receptor</th>
<th>Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1 (Dimer with TLR2)</td>
<td>Bacterial lipopeptide</td>
</tr>
<tr>
<td>TLR2</td>
<td>Peptidoglycan</td>
</tr>
<tr>
<td>TLR3</td>
<td>Double-stranded RNA</td>
</tr>
<tr>
<td>TLR4</td>
<td>LPS, heat-shock proteins, Taxol</td>
</tr>
<tr>
<td>TLR5</td>
<td>Flagellin</td>
</tr>
<tr>
<td>TLR6 (Dimer with TLR2)</td>
<td>Mycoplasmal lipopeptide</td>
</tr>
<tr>
<td>TLR7</td>
<td>Imidazoquinolines (anti-viral compounds)</td>
</tr>
<tr>
<td>TLR9</td>
<td>Bacterial DNA (CpG DNA)</td>
</tr>
</tbody>
</table>
Moreover, signaling of other TLRs like TLR 7 and 9 proved to be intact too, illustrating a clear difference in the signaling requirements between TLRs. Besides TIRAP, other proteins have been implicated to confer specificity to TLR signaling. Recently, a novel protein containing a TIR domain was identified, termed TIR domain-containing adapter inducing IFN-β (TRIF)\textsuperscript{81}. This protein is probably responsible for the MyD88-independent induction of NF-κB-, IFN-β- and IFN-γ-inducible genes observed after TLR3 and TLR4 activation. The phenotype of TRIF-deficient mice will shed more light on the exact role of this protein in TLR signaling. Finally, one report has identified the Rac-PI3K-Akt pathway as a downstream target of TLR2 signaling\textsuperscript{82}. Rac was shown to bind to TLR2 in a ligand-dependent manner, and NF-κB activation in response to TLR2 signaling proved to be dependent on the Rac-PI3K-Akt pathway. These differences in TLR signaling enable the immune system to react differently to various pathogens and thereby orchestrate the immune response specifically for efficient killing of a particular pathogen. TLRs are seen as very important links between innate and adaptive immunity, since the adaptive immune response is highly influenced by the cytokine profile of macrophages and the antigen-presenting capacity of dendritic cells, two features that are highly influenced by the outcome of TLR signaling in these cells.
To protect the host from the harmful effects of the immune response, like endotoxic shock, it seems logical that the actions of TLRs can be modulated. Evidence emerges that indeed TLR signaling is modulated by several different mechanisms. One mechanism that interferes with TLR signaling is the downregulation of TLRs on the cell surface after challenge with PAMPs. However, downregulation of TLRs appears to be limited and transient. Furthermore, although signaling via a specific TLR does not induce downregulation of other TLRs in the same cell, unresponsiveness to signaling by these other TLRs is induced. Recently, two proteins have been identified that are involved in inhibiting TLR signaling and have a role in the induction of tolerance for different PAMPs. One of these proteins is the suppressor of cytokine signaling-1 (SOCS1), which is rapidly induced upon TLR4 activation by LPS. The role of SOCS1 was shown in SOCS1-deficient mice, which display increased sensitivity to LPS-induced shock and increased activation of p38 MAPK and NF-κB activation after challenge with LPS. Moreover, constitutive expression of SOCS1 inhibited TLR4-mediated NF-κB activation. Similar to SOCS1, IRAK-M has been identified as an inducible negative regulator of TLR signaling. This kinase-dead member of the IRAK family dampens the immune response to different PAMPs and, like SOCS1, protects mice against LPS-induced shock. Both suppressors seem to be active at a different level of TLR signaling. IRAK-M is thought to inhibit the actions of IRAK-1, whereas SOCS1 is operating downstream of IRAK-1 and probably interferes with TRAF6 function.

Although the expression and functioning of TLRs on macrophages and dendritic cells has been broadly investigated, practically nothing is known about the function of these receptors on neutrophils. The presence of TLRs 1, 2, 4 and 6 mRNA has been identified by Muzio et al. and TLR2 and TLR4 are expressed on the cell surface of neutrophils. Expression of these receptors on the cell surface renders the neutrophil able to respond to specific bacterial products that bind to these receptors. Although there is only one report on the function of TLRs in neutrophils, a lot of data has been generated about the effects of LPS on neutrophils. LPS is able to prime the secretion of ROS by the neutrophil in response to stimulation with the bacterial peptide fMLP. This priming involves the upregulation of the amount of cytochrome b58 on the cell surface via granule exocytosis, is p38 MAPK and CD14 dependent and leads to translocation of NF-κB to the nucleus. Moreover, the upregulation of TLR2 by GM-CSF increases the neutrophil responses to the ligands of this receptor, such as
peptidoglycan (PGN)\textsuperscript{89}. Since neutrophils express a functional IL-1 signaling cascade\textsuperscript{90} and because activation of NF-\(\kappa\)B and p38 MAPK take place upon LPS stimulation\textsuperscript{90}, which are hallmarks of TLR signaling, it is believed that at least some of the TLR signal transduction cascades observed in other leukocytes are also functional in neutrophils.

**Aim of this thesis.**

Neutrophils are a very important factor in the clearance of microbial infections and are well equipped to perform their role of phagocytosis and subsequent killing. One of the most important enzymes in microbial killing is the NADPH oxidase. This enzyme is responsible for the generation of ROS but clearly is also important for the activation of intraphagosomal proteases and therefore has a general role in the killing process. Although much work has been performed to elucidate structural aspects, modes and consequences of activation and microbial strategies to counteract actions, of the NADPH oxidase, many questions remain to be solved. Several of these questions are addressed in this thesis.

In Chapter II, an attempt was made to identify some of the residues in gp91\textsuperscript{phox} that are involved in binding of FAD. In Chapter III, a new mutation in glucose-6-phosphate dehydrogenase is identified, which leads to reduced superoxide generation by the NADPH oxidase. Chapter IV shows the continuous translocation of the cytosolic NADPH oxidase components p67\textsuperscript{phox} and Rac2 to the phagosomal membrane and the importance of the actin cytoskeleton for correct localization of these cytosolic factors. In Chapter V the function of a protein expressed by *Salmonella typhimurium* in protecting this bacterium from hydrogen peroxide, derived from NADPH oxidase activity, is identified. Finally, the importance of TLR-mediated signaling in activation of the NADPH oxidase by microbial products is described in Chapter VI. The findings described in these five chapters are summarized and discussed in Chapter VII.
Chapter 1

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