Built for the kill. Studies on the neutrophil NADPH oxidase
van Bruggen, R.

Citation for published version (APA):
van Bruggen, R. (2004). Built for the kill. Studies on the neutrophil NADPH oxidase

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 6

Activation of the neutrophil NADPH oxidase in response to Salmonella infection by intracellular Toll-like receptor 4 signalling.

Robin van Bruggen\textsuperscript{1,2}, Debby Zweers\textsuperscript{1}, Angela van Diepen\textsuperscript{3}, Hans Janssen\textsuperscript{4}, Jero Calafat\textsuperscript{4}, Niels Borregaard\textsuperscript{5}, Jaap T. van Dissel\textsuperscript{3}, Dirk Roos\textsuperscript{1}, and Taco W. Kuijpers\textsuperscript{2}.

\textsuperscript{1}Sanquin Research, and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.

\textsuperscript{2}Emma Children’s Hospital, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.

\textsuperscript{3}Department of Infectious Diseases, Leiden University Medical Center, Leiden, The Netherlands.

\textsuperscript{4}Division of Cell Biology, The Netherlands Cancer Institute, Amsterdam.

\textsuperscript{5}Department of Hematology, Rigshospitalet, University of Copenhagen, Denmark.
Abstract
Toll-like receptors (TLRs) form a family of transmembrane receptors that are essential for the recognition of pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS). The function of TLRs on the surface of immune cells has been widely investigated, and TLR signalling has been shown to lead to cytokine production and cell activation. A role for TLRs in phagosome sensing, i.e. binding of a pathogen’s PAMPs in the phagosome and subsequent signalling leading to killing of the phagocytosed pathogen, has been suggested, but until now there has been no proof that TLR signalling is indeed involved in this process. Here, we show with a new sensitive assay at the level of ingested microorganisms that LPS-mediated TLR4 signalling in phagocytes has a direct role in the activation of the NADPH oxidase. Infection of neutrophils with wild-type, LPS-expressing, *Salmonella* resulted in the generation of reactive oxygen species (ROS) in the *Salmonella*-containing vacuole, a phenomenon that was much less pronounced in an LPS mutant strain. Moreover, the generation of ROS in the wild-type *Salmonella*-infected neutrophils was largely inhibited by the action of a TLR4-specific cell-permeable peptide, but not by a TLR4 blocking antibody. This identifies intracellular TLR4 ligand binding and signalling as an essential factor in the sensing of phagosomes required for the killing of intracellular micro-organisms.

Introduction
The intracellular pathogen *Salmonella typhimurium* invades phagocytes, where it resides in a membrane-surrounded vacuole\(^1,2\). *S. typhimurium* is able to evade the host immune response by virtue of its pathogenicity islands, clusters of genes whose products induce the uptake of the bacterium by host cells and interfere with the killing of the pathogen\(^3\). A large portion of these genes exerts their effects by inhibiting or counteracting microbialicidal systems such as the NADPH oxidase\(^4\). Moreover, wild-type *S. typhimurium* restricts the activation of the NADPH oxidase after uptake, through the action of the *Salmonella* pathogenicity island (Spi)\(^2\,5\). This cluster of genes protects the intracellular bacterium against full activation of this microbialicidal system\(^4\,5\).

Resistance of *S. typhimurium* to host defense mechanisms increases as the LPS chain length increases, i.e., from avirulent strains containing a low number of sugars, so-called rough strains, to a high number of sugars found in smooth virulent...
bacteria. *Salmonella* strains of the rough chemotype are susceptible to complement-mediated lysis, either in the presence or absence of antibody\(^6\)\(^,\)\(^7\), and are non-invasive after oral challenge\(^8\)\(^,\)\(^9\). Intracellular killing by human neutrophils is enhanced by complement activity, and the survival of *Salmonella* spp. in the presence of serum and neutrophils decreases as the LPS chain length shortens\(^6\).

LPS is a ligand for Toll-like receptor (TLR) 4, a member of the well-conserved TLR family of pattern recognition receptors\(^10\)\(^,\)\(^11\). TLRs are able to recognize pathogen-associated molecular patterns (PAMPs), molecules that are exclusively found in pathogens and are often indispensable for pathogen viability in the host\(^10\)\(^,\)\(^11\). TLR signalling has been shown to lead to cytokine production and cell differentiation, ultimately influencing the outcome of the immune response\(^10\)\(^,\)\(^11\). TLR expression is found in cells from the immune system as well as in non-immune cells. Usually, signalling via TLRs is studied for cell-surface expressed TLRs, although signalling via intracellular TLRs has been suggested. Moreover, TLRs have been hypothesized to “sample” the contents of phagosomes for the presence of PAMPs\(^12\). Here, we investigated the role of LPS-mediated TLR4 signalling in the intracellular killing of wild-type and rough *Salmonella* strains in phagocytes. When infected in RAW 246.7 cells under serum-free conditions, LPS negative, rough, *Salmonella* bacteria were found to be less efficiently killed than the wild-type parent strain. This phenomenon was further explored in human neutrophils, where infection with the rough bacteria was shown to lead to lower levels of reactive oxygen species (ROS) generated by the NADPH oxidase as compared with the LPS-positive, wild-type, parent strain. To confirm the involvement of TLR4 in the activation of the NADPH oxidase upon infection with LPS positive wild-type *Salmonella*, TLR4 signalling was inhibited with a TLR4-specific, cell-permeable peptide. Indeed, this peptide prevented the activation of the NADPH oxidase. With a TLR4-blocking monoclonal antibody, the involvement of surface-expressed TLR4 in activating the NADPH oxidase in response to *Salmonella*-derived LPS in this system was ruled out. Together these data prove the intracellular ligand binding and signalling of TLR4 in response to *Salmonella*-derived LPS, which results in the activation of the NADPH oxidase. This is the first proof that TLR4 signals from intracellular compartments and directly activates microbicidal systems.
Methods.

Growth and labelling of bacterial strains

Single colonies of smooth parental *Salmonella enterica* serovar Typhimurium, and its rough Ra chemotype mutant were grown overnight in Luria-Bertani (LB) medium at 37°C while shaking (225 rpm). For infection of human neutrophils, overnight cultures of different *Salmonella* strains were diluted ten times in fresh LB medium. Bacteria were harvested in the log phase (OD=1 at 600 nm). Subsequently, bacteria were centrifuged and resuspended in phosphate-buffered saline (PBS). The bacteria were labelled with 1 μM dihydrorhodamine-1,2,3 (DHR) (Molecular Probes), for 10 min at room temperature in the dark, washed with PBS and resuspended in Hepes medium [132 mM NaCl, 6 mM KCl, 1 mM MgSO₄, 1.2 mM KH₂PO₄, 5.5 mM glucose, and 0.5% (wt/vol) human albumin (pH 7.4)].

NADPH oxidase-mediated fluorescence of intracellular bacteria

Neutrophils were purified from heparinized blood as described. 10⁶ neutrophils were incubated with 10⁷ bacteria of the different *Salmonella* strains at 37°C. At various time points, samples were taken and diluted twenty times in ice-cold PBS. After the last time point, all samples were centrifuged and resuspended in 100 μl of ice-cold PBS and analysed by flow cytometry in a Becton Dickinson FacsStar (Palo Alto, USA).

Electron microscopic analysis

Neutrophils were fixed for 24 h in 4% paraformaldehyde in 0.1 M PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM Mg Cl₂, pH 6.9) and then processed for ultrathin cryosectioning as previously described. For double immunolabelling the procedure described by Slot et al., was followed, with 10- and 15-nm protein-A conjugated colloidal gold probes (EM Lab., Utrecht University, The Netherlands). After immunolabelling, the cryosections were embedded in a mixture of methylcellulose and uranyl acetate and examined with a Philips CM 10 electron microscope (Eindhoven, The Netherlands). For the controls, the primary antibody was replaced by a non-relevant rabbit or mouse antibody.
Sub-cellular fractionation of human neutrophils

Subcellular fractions of neutrophils were obtained by nitrogen cavitation and centrifugation on a 3-layer Percoll gradient as described by Kjeldsen et al.\(^\text{16}\).  

Western Blot analysis of isolated neutrophil fractions

For immunodetection of TLR4, 20 μg of protein of isolated neutrophil fractions were subjected to SDS-PAGE and Western blotting as previously described\(^\text{17}\). Blots were incubated with polyclonal anti-TLR4 (clone sc-10741, Santa Cruz), secondary antibodies were swine-anti-rabbit horseradish peroxidase conjugated polyclonal antibodies (DAKO), and detection was performed with the enhanced chemiluminescence (ECL) kit (Amersham Pharmacia).

Cell-permeable peptides

The protein transduction domain of the HIV protein Tat\(^\text{18}\) was fused to amino acids 366-377 of TLR2 (YARAAARQARAGFKLCLHKRDFIPGKWI). As a control peptide, a peptide containing only the protein transduction domain was used (YARAAARRQARAG). Purified human neutrophils were pre-incubated with 200 μg/ml cell-permeable peptides for 1 min before priming or *Salmonella* infection.

Priming of ROS production of human neutrophils

Purified human neutrophils were primed at a concentration of 10^6 cells/ml in phosphate-buffered saline (PBS) containing 5.5 mM glucose and 0.5% (wt/vol) human albumin, with either 20 ng/ml LPS (Salmonella, Sigma), 10 μg/ml Pam3CysSK4 (EMC Microcollections, Tübingen, Germany) or 500 ng MALP-2 (EMC Microcollections) for 30 min at 37°C. Hydrogen-peroxide production of purified human neutrophils after fMLP (Sigma) activation was measured by the Amplex Red Assay (Molecular Probes) measured on a Perkin Elmer platereader.

Precipitation of cell-permeable peptides

Neutrophils (10^7/ml) were lysed in 100 mM TrisHCl (pH 7.4) containing 100 mM NaCl, 10% glycerol, 1% NP-40, 1 mM MgCl2 and protease inhibitors (Roche) for 15 min on ice. Lysates were centrifuged at 14,000g for 15 min at 4°C. Supernatants were incubated with 1 μg/ml of biotinylated peptides and 50 μl of streptavidin-agarose beads (Sigma) for 1 h at 4°C. Beads were then washed three times with lysis buffer. Proteins were extracted by boiling the beads in SDS sample buffer.
(125 mmol/L Tris, pH 6.8; 20% (w/v) SDS and 12.5% (v/v) β-mercaptoethanol) for 5 min at 95°C.

**Intracellular killing of Salmonella**

Persistence of *Salmonella* in RAW 264.7 macrophage-like cells was determined as follows: cells were allowed to adhere to plastic wells at a density of 1 x 10^5 cells/well during overnight incubation at 37°C in RPMI-medium containing 10% (v/v) inactivated fetal calf serum. Bacteria grown overnight in LB were added to the wells at a macrophage to bacteria ratio of 1:10, and centrifuged (10 min at 1200 rpm) onto the cells. Bacterial endocytosis was allowed to proceed for 10 min, and after three washes with PBS the cells were re-incubated at 37°C and 5% CO₂ in medium containing gentamicin. To determine persistence and/or replication in RAW 264.7 cells, gentamicin was added (100 μg/ml) for 10 min to kill remaining extracellular bacteria. After washing, the cells were again incubated in medium containing gentamicin (10 μg/ml) for determination of persistence after 3 and 24 hrs. The survival of intracellular bacteria against time was determined by plate counts following the removal of medium and hypotonic lysis of cells.

**Results and Discussion**

To assess the role of LPS for intracellular survival in the absence of complement we started to determine the intracellular survival of *S. typhimurium* 14028 strains with smooth (s) and rough (Ra) chemotype within macrophage-like cells after ingestion of non-opsonized bacteria. During the first hours after ingestion by macrophage-like RAW264.7 cells *in vitro*, the intracellular killing of the *Salmonella* 14028 strains was defective for LPS-deficient rough bacteria as indicated by higher intracellular outgrowth during the first 24 hours after uptake, as compared with its wild-type parental smooth strain (Figure 1). Thus, a *Salmonella* Ra strain that is killed extracellularly almost instantly in the presence of complement, is remarkably enough able to survive and replicate better intracellularly than its wild-type parental smooth strain. These differences in intracellular replication cannot be attributed to a differential susceptibility to antimicrobial peptides such as magainin 2 or defensins, because the length of the LPS sugar side chain does not determine bacterial susceptibility to these proteins^{19,20}. 
The NADPH oxidase of phagocytes—more so in neutrophils compared to macrophages—converts molecular oxygen to superoxide, the parent compound from which other, more aggressive reactive oxygen species (ROS), such as hydrogen peroxide and hypochlorous acid, are formed\textsuperscript{21}. Human neutrophils can be primed to secrete ROS by bacterial products such as LPS and proteoglycan (PGN), via TLRs expressed on the cell surface of the neutrophil\textsuperscript{22}. Triggered by the addition of the bacterial peptide fMLP, primed neutrophils show high NADPH oxidase activity, resulting in secretion of large amounts of ROS. *Salmonella*-derived LPS is also able to prime the secretion of ROS by human neutrophils in a TLR4-dependent fashion\textsuperscript{23}. Since *Salmonella* invasion of human phagocytes leads to the generation of ROS\textsuperscript{7}, we investigated the importance of LPS for the activation of the NADPH oxidase.

To show that invaded *Salmonella* bacteria encounter ROS, wild-type and rough bacteria were labelled with dihydrorhodamine-1,2,3 (DHR), a dye that is converted to the fluorescent product rhodamine-1,2,3 in the presence of hydrogen peroxide and a peroxidase\textsuperscript{24}. After labelling, the bacteria were allowed to infect human neutrophils, and the fluorescence of both wild-type and rough *Salmonella typhimurium* strains inside the neutrophils was assayed at fixed times by flow cytometry (Figure 2a). Neutrophils infected by wild-type bacteria displayed a fluorescence signal that appeared after 30 min of infection and was maximal at 45 min. Although the fluorescence signal observed in neutrophils infected with the rough strain showed the same kinetics as the wild-type strain-infected cells, the observed fluorescence was much lower (Figure 2a,b). Infection rates of both strains were similar, as determined by counting the number of intracellular *Salmonella* bacteria after May-Grünwald-Giemsa staining (Figure 2c). The observed differences in fluorescence were not due to varying degrees of DHR labelling of the two strains, since phorbol myristate acetate (PMA) stimulation, resulting in vigorous NADPH oxidase activation and conversion of all DHR present into
rhodamine, showed equal fluorescence of wild-type and rough strain-infected neutrophils (Figure 2a). Together, these data show that at equal infection rates wild-type Salmonella encounters more ROS after infection of human neutrophils than does an LPS mutant strain, which is a strong indication that LPS-mediated signalling results in NADPH oxidase activation. Furthermore, wild-type Salmonella infection leads to activation of human neutrophils, as determined by expression of the cell-surface markers CD10, CD11b, CD14, and CD16 (Figure 2d). Clearly, infection of neutrophils with either wild-type or rough Salmonella bacteria leads to altered expression of activation markers, as compared to uninfected cells (PMN). However, wild-type Salmonella induced a more pronounced expression of CD11b and CD14, while infection with rough bacteria...
Chapter 6

led to marked shedding of CD16 and a slight increase in CD10 expression. Furthermore, infection with wild-type *Salmonella* induced clustering of the infected neutrophils (Figure 2e).

It was then investigated whether the differences in fluorescence between wild-type and rough bacteria were due to priming effects of LPS present on the wild-type bacteria via TLR4 expressed on the cell surface of the neutrophils. Human neutrophils express TLR4 at low but detectable levels on their cell surface (Figure 3a). TLR2 expression can also be detected in all donors tested, but the expression of TLR1 and 6 was variable between donors (Figure 3a). To inhibit TLR4 signalling, a TLR4-blocking monoclonal antibody was used. This antibody inhibits *Salmonella* LPS-mediated priming of ROS secretion by human neutrophils (Figure 3b). However, pre-incubation of neutrophils with this monoclonal antibody had no effect on the observed fluorescence after infection with wild-type *Salmonella* (Figure 2a). This suggests that signalling via surface-expressed TLR4 does not play a major role in the activation of the NADPH oxidase after *Salmonella* infection and that signalling, if mediated by TLR4, occurs largely via an intracellular pool of this receptor, where the antibody is not able to interfere with signalling.

Since it was unknown whether TLRs are localized intracellularly in human neutrophils, attempts were made to determine the subcellular localisation of TLR1, 2, 4 and 6 by electron microscopy. This technique showed that TLR1, 2 and 6 reside both in the azurophil and in the specific granules of neutrophils (Figure 3c,d,e,f). Due to poor antibody labelling with different antibodies, electron microscopic analysis of the localisation of TLR4 failed. Therefore, subcellular fractions of human neutrophils were prepared and analysed by Western blotting for TLR expression (Figure 3g). TLR4 was detected in all neutrophil granule fractions, proving the existence of intracellular pools of this receptor. Together with the finding that TLR4-blocking antibodies are not able to inhibit the observed NADPH oxidase activity in response to *Salmonella* infection, these data strongly suggest that signalling mediated by intracellular TLR4 receptors leads to activation of microbicidal systems in the phagosome.

To confirm the involvement of intracellular TLR4 in mediating LPS-induced NADPH oxidase activation, a cell-permeable peptide was used to specifically block TLR4-mediated signalling. This peptide was originally designed to inhibit TLR2 signalling and is composed of the protein transduction domain of the HIV protein TAT and part of the intracellular portion of TLR2, the BB-loop

106
Figure 3 Localisation of TLRs in human neutrophils. a, Flow cytometric analysis of surface expression of TLR1, 2, 4 and 6 on neutrophils of two different donors. White peaks represent fluorescence displayed by control IgG stained cells, gray peaks indicate fluorescence displayed by anti-TLR stained cells b, Inhibition of ROS production by a TLR4-specific MoAb. Hydrogen peroxide production by neutrophils was induced by fMLP stimulation after priming with different TLR ligands c,d,e,f, Immuno-electron microscopic analysis of TLR1, 2 and 6 (15 nm gold particles) localisation in human neutrophils. Lactoferrin (Lf) and myeloperoxidase (MPO) (10 nm gold particles) were taken as markers for specific granules and azurophil granules, respectively, arrows indicate TLR localisation. Numbers indicate the percentage of TLR-positive granules displaying colocalization of a TLR with the marker of these granules. For each experiment at least 200 positive granules were analyzed. g, Expression of TLR4 in different neutrophil fractions.

However, priming experiments with LPS and the TLR1/2 and TLR2/6 heterodimer ligands, Pam₃CysSK₄ and MALP-2, respectively, showed that the peptide is a potent inhibitor of TLR4 signalling and does not inhibit TLR1/2 or TLR2/6 signalling (Figure 4a). Furthermore, pull-down assays with a biotinylated form of
this peptide showed direct binding to TLR4 and not to TLR1, 2 or 6 (Figure 4b). The inability of the BB-loop peptide to bind to and inhibit signalling via TLR1/2 and TLR2/6 heterodimers can be explained by the recent observation that these heterodimers are co-translationally formed\textsuperscript{26}, which may lead to the masking of the intracellular target sequences within TLR1/2 and TLR2/6 heterodimers.

Pre-treatment of human neutrophils with the BB-loop peptide and subsequent infection with DHR-labelled wild-type \textit{Salmonella typhimurium} strongly diminished the observed fluorescence in comparison to untreated neutrophils (Figure 4c). A control peptide did not show this inhibitory effect, leaving fluorescence at the level of untreated cells. Neither peptide interfered with the uptake of the bacteria or diminished fluorescence by itself, as determined by May-Grünwald-Giemsa staining (not shown) and PMA stimulation, respectively (Figure 4c). Furthermore, inhibitors of p38MAPK and pERK –SB20358 and U0126 respectively– were effective in inhibiting bacterial fluorescence, suggesting

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4}
\caption{Inhibition of ROS production by a TLR4-specific, cell-permeable peptide. a, Hydrogen peroxide production by neutrophils upon fMLP stimulation after priming with different TLR ligands. b, Interaction of BB-loop peptide with TLR4 as determined by peptide pull-down. c, Effect of BB-loop peptide and inhibitors of MAPKs, SB20358 (10 μg/ml) and U0126 (10 μg/ml) on intracellular hydrogen peroxide production after \textit{Salmonella} infection.}
\end{figure}
that the signal transduction routes leading to activation of the NADPH oxidase by the intracellular bacteria are similar to those involved in priming of this enzyme complex by extracellular TLR4 stimuli (Figure 4c)\textsuperscript{27}.

In conclusion, these data demonstrate a crucial role for TLR4 in direct sensing of intracellular compartments and subsequent signalling, resulting in full activation of the NADPH oxidase system and subsequent killing of intracellular pathogens.

**Reference List**

Chapter 6


17. van Brugge R, Bautista JM, Petropoulou T, et al. Deletion of leucine 61 in glucose-6-phosphate dehydrogenase leads to chronic nonspherocytic anemia, granulocyte dysfunction, and increased susceptibility to infections. Blood. 2002;100:1026-1030.


