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Complement Receptor 3 and Toll-Like Receptor 4 Act Sequentially in Uptake and Intracellular Killing of Unopsonized *Salmonella enterica* Serovar Typhimurium by Human Neutrophils▽

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The uptake and subsequent killing of *Salmonella enterica* serovar Typhimurium by human neutrophils was studied. In particular, two pattern recognition receptors, complement receptor 3 (CR3) and Toll-like receptor 4 (TLR4), were found to be essential for the efficient uptake and activation, respectively, of the NADPH oxidase. The uptake of *Salmonella* was almost completely inhibited by various monoclonal antibodies against CR3, and neutrophils from a patient with leukocyte adhesion deficiency type 1, which lack CR3, showed almost no uptake of *Salmonella*. A lipopolysaccharide (LPS) mutant strain of *Salmonella* was used to show that the expression of full-length, wild-type, or so-called smooth LPS is important for the efficient killing of intracellular *Salmonella*. Infection with wild-type-LPS-expressing *Salmonella* resulted in the generation of reactive oxygen species (ROS) in TLR4-decorated, *Salmonella*-containing vacuoles, whereas ROS were not induced by an LPS mutant strain. In addition, the recognition of *Salmonella* by neutrophils, leading to ROS production, was shown to be intracellular, as determined by priming experiments with intact bacteria under conditions where the bacterium is not taken up. Finally, the generation of ROS in the wild-type-*Salmonella*-infected neutrophils was largely inhibited by the action of a TLR4-blocking, cell-permeable peptide, showing that signaling by this receptor from the *Salmonella*-containing vacuole is essential for the activation of the NADPH oxidase. In sum, our data identify the sequential recognition of unopsonized *Salmonella* strains by CR3 and TLR4 as essential events in the efficient uptake and killing of this intracellular pathogen.

The intracellular pathogen *Salmonella enterica* serovar Typhimurium invades phagocytes, where it resides in a membrane-surrounded vacuole (2, 27). *Salmonella* serovar Typhimurium is able to evade the host immune response by virtue of its pathogenicity islands, i.e., clusters of genes whose products induce the uptake of the bacterium by host cells and interfere with the killing of the pathogen (8). A large portion of these genes exert their effects by inhibiting or counteracting microbicidal systems, such as the NADPH oxidase (34). For instance, wild-type *Salmonella* serovar Typhimurium restricts the activation of the NADPH oxidase after uptake through the action of *Salmonella* pathogenicity island 2 (9, 34). This cluster of genes protects the intracellular bacterium against the full activation of this microbicidal system (9, 34).

The resistance of *Salmonella* serovar Typhimurium to host defense mechanisms increases as the lipopolysaccharide (LPS) chain length increases, i.e., from the lack of resistance of avirulent strains containing no or a very low number of sugars, so-called rough strains, to the high level of resistance of smooth, virulent bacteria containing a high number of sugars. *Salmonella* strains of the rough chemotype are susceptible to complement-mediated lysis, either in the presence or in the absence of antibody (23, 31), and are noninvasive after oral challenge (5, 25). 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 (31).

Neutrophils play an important role in the host defense against *Salmonella* (7). Neutrophils are equipped with various pattern recognition receptors, such as complement receptor 3 (CR3) (17), Dectin-1 (15), and several members of the Toll-like receptor (TLR) family (29). Together, these receptors enable the neutrophil to bind, phagocytose, and kill an array of pathogens without the need for the opsonization of these microorganisms. Two of these receptors, CR3 and TLR4, have been shown to interact with LPS (1, 35), one of the main components of the *Salmonella* outer membrane. Here, we investigated the role of CR3 and TLR4 in the killing of unopsonized *Salmonella* by neutrophils.

As a pattern recognition receptor, CR3 induces the uptake of a large variety of pathogens not covered by immunoglobulins or complement (14, 22). Furthermore, the spreading of *Salmonella* through the body in mice has been shown to be crucially dependent on the presence of CD18 (33). This phenomenon has been ascribed to the inability of CD18-deficient phagocytes to migrate through the different tissues, thereby limiting the spreading of the bacterium, since *Salmonella* species use phagocytes as a vector for their spreading. Since CR3 is important for the ingestion of different pathogens, we investi-

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gated the role of CR3 in the uptake of unopsonized *Salmonella*. Previously, different aspects of the opsonization by complement and the interaction with CR3 of opsonized *Salmonella* organisms have been investigated (11, 18). We found that, when *Salmonella* was incubated with neutrophils in the absence of serum, CR3 was the most essential component for its uptake.

Next, mutant-LPS, rough *Salmonella* bacteria were found to be less efficiently killed than the wild-type strain. Since LPS is also a well-known ligand for TLR4, a member of a family of receptors that has recently been shown to play an important role in the activation of the phagocyte NADPH oxidase (21), the role of TLR4 in NADPH oxidase activation was investigated. To confirm the involvement of TLR4 in the activation of the NADPH oxidase upon infection with wild-type *Salmonella* expressing full-length LPS, TLR4 signaling was inhibited with a TLR4-blocking, cell-permeable peptide. Furthermore, TLR4 was shown to signal from intracellular compartments under these conditions and did not recognize intact, unopsonized salmonellae in the extracellular milieu. In this study, we demonstrate that these two pattern recognition receptors, CR3 and TLR4, act sequentially in the uptake and killing of unopsonized *Salmonella* strains.

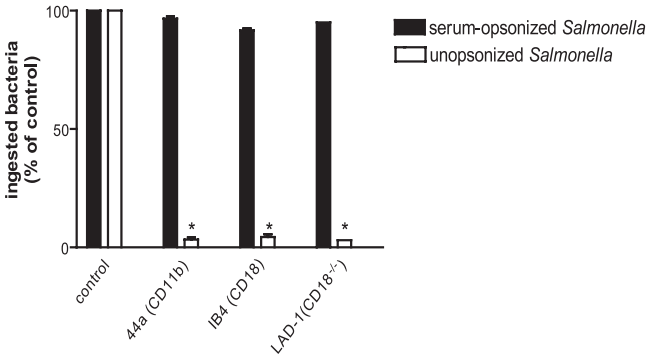

FIG. 1. The uptake of unopsonized *Salmonella* is dependent on CR3 expression. Numbers of ingested *Salmonella* bacteria, opsonized or unopsonized, by human neutrophils were determined for control neutrophils and for neutrophils pretreated with the MAb 44a or with the MAb IB4. Also, neutrophils from an LAD-1 patient were included in this experiment. Numbers of ingested bacteria are represented as percentages of the number of bacteria taken up by control neutrophils. Values are means from three experiments (± standard deviations) performed in duplicate. *, $P < 0.01$ (compared to values for the control; analysis by Student's *t* test).

MATERIALS AND METHODS

Growth and labeling of bacterial strains. Single colonies of smooth, parental *Salmonella enterica* serovar Typhimurium, strain 14208, and its rough Ra chemotype mutant, strain 14028r, were grown overnight in Luria-Bertani (LB) medium at 37°C with shaking (225 rpm). For infection of human neutrophils, overnight cultures of the *Salmonella* strains were diluted 10 times in fresh LB medium. Bacteria were harvested in the log phase (optical density at 600 nm of 1). Subsequently, bacteria were centrifuged and resuspended in phosphate-buffered saline (PBS). The bacteria were labeled with 1 μM dihydrorhodamine-1,2,3 (DHR) (Molecular Probes, Eugene, OR) 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 $MgSO_4$, 1.2 mM KH_2PO_4, 5.5 mM glucose, and 0.5% [wt/vol] human albumin [pH 7.4]).

Intracellular killing of *Salmonella*. The survival of *Salmonella* organisms in neutrophils was determined as follows. Neutrophils were incubated in a 96-well plate at a concentration of 2.5×10^5 cells/well with 5×10^6 bacteria in HEPES medium. Bacterial phagocytosis was allowed to proceed for 15 min, and then gentamicin (100 μg/ml) was added. To determine *Salmonella* survival in neutrophils, the cells were incubated for another 45 min in the presence of gentamicin. Neutrophils were then washed three times with PBS and lysed by resuspension in distilled water. The survival of intracellular bacteria was determined by the method described by Rada et al. (26). For inhibition of the NADPH oxidase, neutrophils were pretreated with the NADPH oxidase inhibitor diphenylene iodonium (DPI) (6) at a final concentration of 20 μM for 30 min.

NADPH oxidase-mediated fluorescence of intracellular bacteria. Neutrophils were purified from heparinized blood as described previously, and experiments were performed with neutrophils from three healthy donors (28). Neutrophils (10^6) were incubated with 10^8 DHR-labeled bacteria of the different *Salmonella* strains at 37°C. At various time points, samples were taken and diluted 20 times in ice-cold PBS. After the last time point, all samples were centrifuged and resuspended in 100 μl of ice-cold PBS and analyzed by flow cytometry in a Becton Dickinson FACSStar (Palo Alto, CA).

Priming of ROS production of human neutrophils. Purified human neutrophils were primed at a concentration of 10^6 cells/ml in PBS containing 5.5 mM glucose and 0.5% (wt/vol) human albumin with either 20 ng/ml LPS, reextracted as previously described (16) (LPS from *Salmonella* serovar Typhimurium; Sigma, St. Louis, MO) in the presence of 1% (vol/vol) heat-inactivated human pool serum, 10 μg/ml Pam_3CysSK_4 (EMC Microcollections, Tübingen, Germany), or 500 ng/ml MALP-2 (EMC Microcollections) for 30 min at 37°C. Hydrogen peroxide production of purified human neutrophils after *N*-formyl-methionyl-leucyl-phenylalanine (fMLP; 10^{-6} M; Sigma) activation was measured by the Amplex Red assay (Molecular Probes) measured on a Perkin Elmer plate reader. For inhibition of *Salmonella* uptake by human neutrophils, these cells were preincubated with 10 μg/ml anti-CD18 (clone IB4; Ancell, Bayport, MN) for 15 min at room temperature.

CLS microscopy. Different *Salmonella* strains were labeled with Alexa 568 (Molecular Probes) according to the manufacturer's protocol. One million neutrophils were incubated with 10^8 labeled bacteria of the different *Salmonella* strains at 37°C. After 30 min, the cells were plated on glass coverslips coated with poly-L-lysine (100 μg/ml, 30 min, 37°C). The cells were allowed to adhere for 10 min and were then fixed with 3.7% (wt/vol) formaldehyde for 10 min at 4°C. The coverslips were then washed two times with PBS containing 0.2% (wt/vol) human serum albumin (HSA), and the cells were subsequently permeabilized with 0.1% (wt/vol) saponin. The cells were stained with a goat polyclonal antibody against TLR4 (clone sc-8694; Santa Cruz, Santa Cruz, CA) for 1 h in PBS-HSA. The coverslips were washed two times with PBS-HSA and subsequently incubated with Alexa 488-labeled rabbit anti-goat immunoglobulin G Fab_2 fragments (Molecular Probes) for 1 h. The coverslips were washed three times with PBS-HSA and were then analyzed with a confocal laser scanning (CLS) microscope (Zeiss, Göttingen, Germany).

Cell-permeable peptides. To construct the TLR4-inhibiting cell-permeable peptide, the protein transduction domain of the human immunodeficiency virus protein Tat (30) (YARAAARQARAG) was coupled to the following amino acids: FKLCLHKRDFIPGKWI. As a control peptide, a peptide containing only the protein transduction domain was used (YARAAARRQARAG). Purified human neutrophils were preincubated with 200 μg/ml cell-permeable peptides for 1 min before the priming or *Salmonella* infection.

RESULTS

The uptake of *Salmonella* serovar Typhimurium cells by human neutrophils is mediated by CR3. Since CR3 is an important pattern recognition receptor for different pathogens, we hypothesized that CR3 might also be very important in the uptake of unopsonized salmonellae. Two different monoclonal antibodies (MAbs) which block epitopes on CD11b (MAb 44A) or CD18 (MAb IB4) were used. The addition of these antibodies to neutrophils had dramatic effects on the rate of uptake of *Salmonella* cells by human neutrophils, reducing this process to 1% of untreated or control antibody-treated neutrophils (Fig. 1). In contrast, serum-opsonized bacteria were taken up by 44A- or IB4-treated neutrophils to the same extent as by control cells. To confirm this result, neutrophils from a patient with leukocyte adhesion deficiency type 1 (LAD-1),

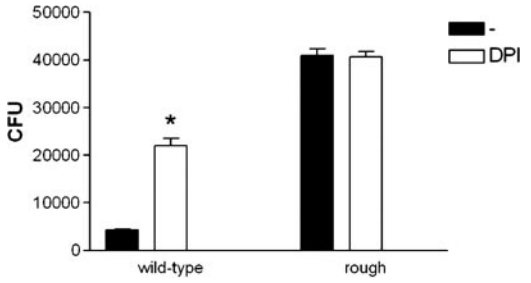

FIG. 2. Numbers of CFU of viable intracellular wild-type, smooth *Salmonella* serovar Typhimurium and its rough (Ra) chemotype within neutrophils 1 h after taking up nonopsonized bacteria in the absence of serum. To inhibit NADPH oxidase activity, neutrophils were pretreated with 20 μM DPI. Values are means from three experiments (± standard deviations) performed in duplicate. *, $P < 0.01$ (compared to values for untreated bacteria; analysis by Student's *t* test).

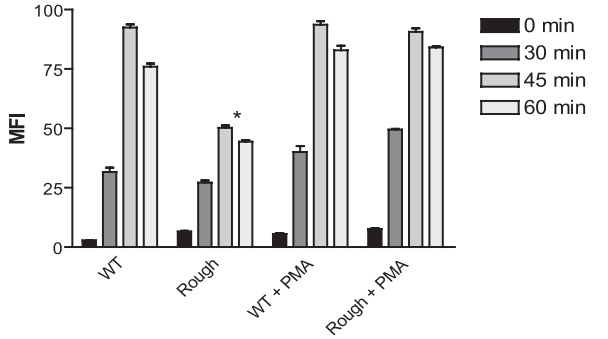

FIG. 3. Kinetics of hydrogen peroxide production by human neutrophils induced by infection with nonopsonized wild-type (WT) and rough *Salmonella* bacteria in the absence of serum. Values are means from three experiments (± standard deviations) performed in duplicate. *, $P < 0.01$ (compared to values for the wild type; analysis by Student's *t* test). MFI, mean fluorescence intensity.

which lack the expression of CR3 due to a mutation in CD18, were also exposed to *Salmonella* serovar Typhimurium. Indeed, bacterial uptake was absent in LAD-1 neutrophils (Fig. 1), proving that the expression of CR3 is crucial for the uptake of unopsonized *Salmonella* strains.

The expression of full-length LPS on *Salmonella* is essential for efficient killing by human neutrophils. Recently, the importance of TLR signaling for the efficient killing of *Salmonella* has been shown (21). To assess the role of LPS, the ligand for TLR4 and one of the main components of the *Salmonella* outer membrane, we determined the survival of smooth and rough *Salmonella* serovar Typhimurium 14028 strains (the LPS consists of the lipid A portion and the core region and lacks the O antigen) within neutrophils after ingestion of nonopsonized bacteria. During the first hour after ingestion by neutrophils in vitro, the intracellular killing of the *Salmonella* serovar Typhimurium strains was defective for the rough bacteria, as indicated by a higher intracellular outgrowth, compared to that of the wild-type parental smooth strain (Fig. 2). At an infection rate of 0.5 bacterium per cell, which was similar to the rates for wild-type and rough bacteria (infection rates were tested at several time points, but no differences were detected between wild-type and rough bacteria [data not shown]), the percentages of intracellular survival for the different strains were 3.4% for the wild-type strain and 32% for the rough strain. Thus, a *Salmonella* rough strain that is killed almost instantly in the presence of serum is better able to survive intracellularly than its wild-type parental smooth strain when serum is absent.

The expression of full-length LPS on *Salmonella* leads to intracellular activation of the NADPH oxidase. The NADPH oxidase of neutrophils 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 (12). To investigate the importance of the NADPH oxidase for the intracellular killing of *Salmonella*, neutrophils were pretreated with the NADPH oxidase inhibitor DPI before being infected with either wild-type or rough bacteria (Fig. 2). The intracellular survival of wild-type *Salmonella* serovar Typhimurium was greatly enhanced by DPI treatment; however, it was not increased to the level of rough bacteria, suggesting that other, nonoxidative microbicidal systems are also involved in the killing of wild-type *Salmonella*. DPI treatment did not affect the uptake or survival of rough bacteria, which is a strong indication that the neutrophil NADPH oxidase is not activated in response to infection with rough *Salmonella* strains.

To further investigate the activation of the NADPH oxidase after ingestion of *Salmonella*, wild-type and rough bacteria were labeled with DHR, a dye that is converted to the fluorescent product rhodamine-1,2,3 in the presence of hydrogen peroxide and a peroxidase (32). Labeling of *Salmonella* serovar Typhimurium with DHR did not alter the uptake, viability, or killing of these bacteria (data not shown). After being labeled, the bacteria were allowed to be ingested by neutrophils under serum-free conditions, and the fluorescence of both wild-type and rough *Salmonella* serovar Typhimurium strains inside the neutrophils was assayed at fixed times by flow cytometry (Fig. 3). Neutrophils infected by wild-type bacteria displayed a fluorescence signal that appeared after 30 min of infection and was maximal at 45 min. With the rough-strain-infected cells, the observed fluorescence was much lower than that for the wild-type-infected cells, indicating that the ROS production by neutrophils infected with the rough strain is much lower than that for the wild-type strain (Fig. 3). The rates of uptake and the kinetics of both strains were similar, as determined by counting the number of intracellular *Salmonella* bacteria over time after May-Grünwald-Giemsa staining (approximately 2.4 bacteria per cell at 45 min under the conditions used [data not shown]). Moreover, the observed differences in fluorescence were not due to different degrees of DHR labeling of the two strains, since phorbol myristate acetate stimulation, resulting in vigorous NADPH oxidase activation and the conversion of all DHR present on the bacteria into rhodamine, produced equal levels of fluorescence of wild-type- and rough-strain-infected neutrophils (Fig. 3). Together, these data show that at equal rates of uptake, wild-type *Salmonella* encounters more ROS after the uptake of human neutrophils than does an LPS mutant strain, which is a strong indication that LPS-mediated signaling contributes to NADPH oxidase activation.

***Salmonella* LPS is detected intracellularly by human neutrophils.** It was then investigated whether the differences in fluorescence between wild-type and rough bacteria were due to the priming effects of the LPS present on the wild-type bacteria

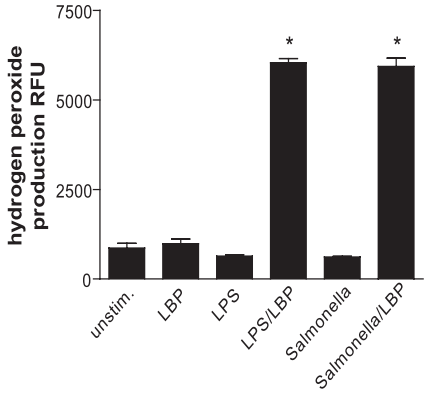

FIG. 4. Priming of human neutrophils by intact, unlabeled bacteria. Hydrogen peroxide production by neutrophils was induced by 1 μM fMLP stimulation after the neutrophils were primed with LPS or intact smooth *Salmonella* bacteria in the presence or absence of recombinant LBP. Bacterial uptake was prevented by the blockade of CR3 (CD11b/CD18) by MAb IB4 (CD18). Values are means from three experiments (± standard deviations) performed in duplicate. *, $P < 0.01$ (compared to values for unstimulated bacteria; analysis by Student's *t* test).

via TLR4 expressed on the cell surfaces of the neutrophils. Human neutrophils have been shown to express TLRs at their cell surfaces (13), and expression of TLRs 1, 2, 4, and 6 in flow cytometric analysis was also observed in this study (data not shown). Neutrophils can be primed to generate ROS by bacterial products such as LPS via TLRs expressed on their cell surfaces (20). Triggered by the addition of the bacterial peptide fMLP, primed neutrophils show high NADPH oxidase activity, resulting in the generation 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 (29).

Therefore, priming experiments with intact, smooth *Salmonella* bacteria were undertaken to determine whether any TLR signaling from the cell surface occurred under the conditions of the infection experiments, i.e., without the addition of serum. The recognition of LPS by TLR4/CD14 depends on the presence of LPS-binding protein (LBP), which is usually provided by the addition of serum but was not present in our system. The uptake of *Salmonella* was prevented by blocking CR3 (CD11b/CD18) with the inhibitory CD18 MAb IB4 (the efficacy of the inhibition of bacterial uptake was tested in this assay and was found to be identical to the inhibition shown in Fig. 1 [data not shown]). Clearly, intact bacteria did not prime the respiratory burst induced by fMLP under the conditions used for the infection experiments (Fig. 4). However, when recombinant LBP was added to the system, neutrophils were primed to an extent similar to that with purified LPS (Fig. 4). These results strongly suggest that in the absence of serum, intact *Salmonella* bacteria do not trigger TLR signaling on the cell surfaces of human neutrophils.

The presence of TLR4 in *Salmonella*-containing vacuoles was then investigated by CLS microscopy. Vacuoles containing fluorescent wild-type and rough bacteria also stained brightly for TLR4 (Fig. 5). Since intact *Salmonella* serovar Typhimurium is unable to prime the secretion of hydrogen peroxide when its uptake is prevented, these data strongly suggest that signaling, if via TLR4, most likely occurs only intracellularly.

The activation of the NADPH oxidase by *Salmonella* LPS is mediated through intracellular TLR4 signaling. To confirm the involvement of intracellular TLR4 in mediating LPS-in-

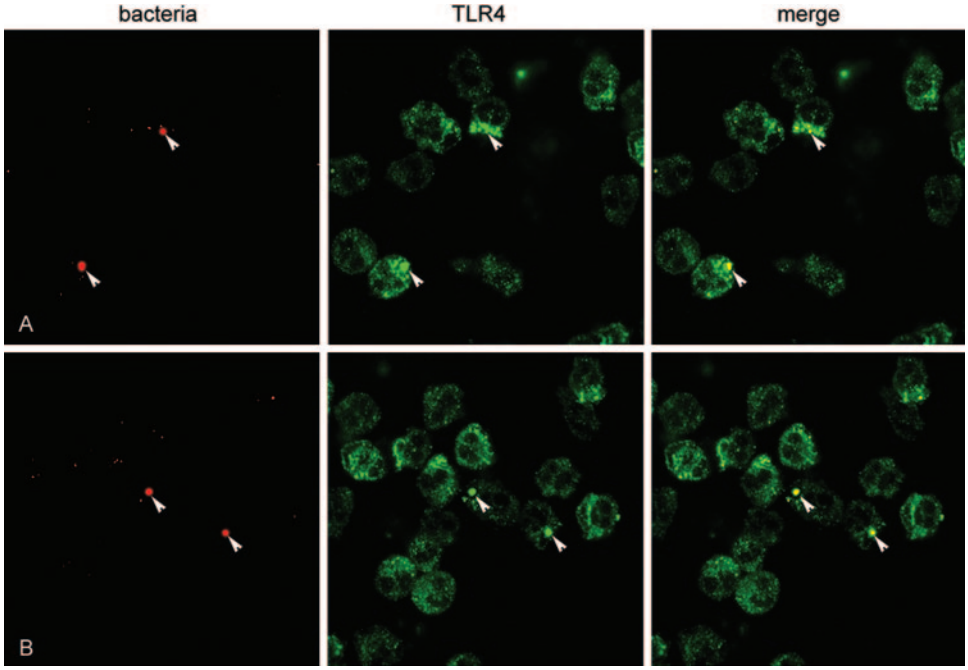

FIG. 5. Localization of TLR4 in wild-type- and rough-*Salmonella*-infected neutrophils. TLR4 localization in wild-type (A)- and rough (B)-*Salmonella*-infected neutrophils was examined by CLS microscopy 40 min after infection. Arrowheads indicate intracellular Alexa 568-stained *Salmonella* cells.

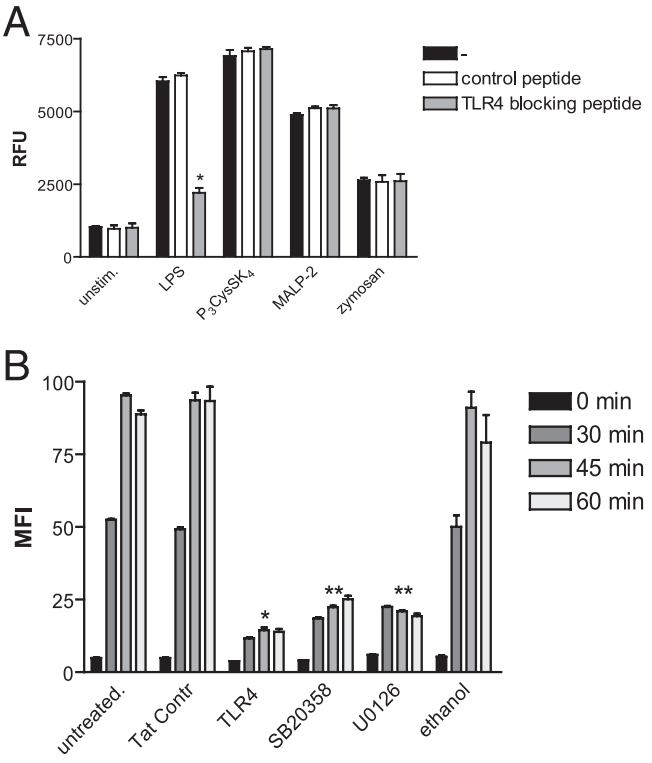

FIG. 6. Inhibition of ROS production by a TLR4-specific, cell-permeable peptide. (A) Hydrogen peroxide production by neutrophils upon 1 μM fMLP stimulation after they were primed with different TLR ligands (Pam_3CysSK_4 for TLR1/2, MALP-2 for TLR2/6, LPS for TLR4, and nonopsonized zymosan for TLR2). RFU, relative fluorescence intensity. (B) Effect of TLR4-blocking peptide (200 μg/ml) and inhibitors of p38MAPK (SB20358, 10 μg/ml), pERK (U0126, 10 μg/ml), and phospholipase D (ethanol, 1%) on intracellular hydrogen peroxide production after *Salmonella* infection. Values are means from three experiments (± standard deviations) performed in duplicate. *, $P < 0.01$ (compared to values for the control peptide); **, $P < 0.01$ (compared to values for untreated bacteria; analysis by Student's *t* test). Contr, control.

duced NADPH oxidase activation, a cell-permeable peptide was used to specifically block TLR4-mediated signaling. Priming experiments with LPS, the TLR1/2 and TLR2/6 heterodimer ligands Pam_3CysSK_4 and MALP-2, and the particulate TLR2 agonist zymosan showed that the peptide is a potent inhibitor of TLR4 signaling and does not inhibit TLR1/2 or TLR2/6 signaling (Fig. 6A). Furthermore, pull-down assays with a biotinylated form of this peptide showed direct binding to TLR4 and not to TLR1, TLR2, or TLR6 (data not shown).

Pretreatment of human neutrophils with the TLR4-blocking peptide and subsequent infection with DHR-labeled wild-type *Salmonella* serovar Typhimurium strongly diminished the observed ROS-dependent fluorescence in comparison to that in untreated neutrophils (Fig. 6B). A control peptide did not show this inhibitory effect, leaving fluorescence at the level in untreated cells. None of the peptides interfered with the overall uptake or the uptake kinetics of the bacteria or diminished fluorescence by themselves, as determined by May-Grünwald-Giemsa staining or phorbol myristate acetate stimulation, respectively (not shown). Furthermore, inhibitors of p38MAPK and pERK (SB20358 and U0126, respectively), but not an inhibitor of phospholipase D (ethanol), were effective in inhibiting bacterial fluorescence (Fig. 6B), suggesting that the signal transduction routes leading to activation of the NADPH oxidase by the intracellular bacteria are similar to those involved in the priming of this enzyme complex by extracellular TLR4 stimuli, such as LPS (4).

DISCUSSION

In the present study, the roles of CR3 and TLR4 in the uptake and subsequent killing of unopsonized *Salmonella* serovar Typhimurium by human neutrophils were investigated. First, the role of CR3 as a phagocytic receptor for *Salmonella* was determined. CR3 was found to be an indispensable factor for *Salmonella* uptake; blocking MAbs to both subunits of CR3, CD11b and CD18, inhibited almost completely the uptake of *Salmonella*. Moreover, neutrophils isolated from an LAD-1 patient that were deficient for CD18 were unable to ingest unopsonized *Salmonella* cells. To further dissect the killing process of *Salmonella* bacteria by neutrophils, killing by neutrophils of intracellular *Salmonella* bacteria that express a mutant form of LPS that consists of the lipid A portion and the core region and lacks the O antigen, a so-called rough strain, was compared to that of wild-type *Salmonella*. The rough strain was found to survive better when taken up by neutrophils.

The survival of the wild-type *Salmonella* cells was enhanced by inhibiting the activity of the NADPH oxidase. In contrast, the survival of the rough *Salmonella* cells was not affected when NADPH oxidase activity was inhibited. Taken together, these data suggest that NADPH oxidase is efficiently activated only by wild-type bacteria. Furthermore, the data imply that, aside from the NADPH oxidase, other antimicrobial systems are activated, since the survival of wild-type *Salmonella* was not increased to the level of rough *Salmonella* when NADPH oxidase activity was inhibited (Fig. 2).

By using DHR-labeled *Salmonella*, we found that, indeed, the NADPH oxidase is activated more potently by wild-type *Salmonella* than by rough *Salmonella*. Since TLR4 is able to recognize LPS, which can prime neutrophils to secrete ROS, we anticipated that differences in TLR4 signaling in infected neutrophils would lead to the observed differences in the levels of NADPH oxidase activity and killing of wild-type *Salmonella* and rough *Salmonella* strains. This possibility is supported by the fact that the efficient signaling of *Salmonella* LPS via human TLR4 critically depends on both the lipid A part and the sugar moieties of the LPS (24). This is in contrast to LPSs from most commonly used bacteria, such as *Escherichia coli*, of which the lipid A part alone is already sufficient to trigger TLR4 activation (24).

Moreover, extracellular intact bacteria, whose uptake was prevented by a CD18-blocking MAb, were not able to prime the activation of the NADPH oxidase in the absence of serum. Therefore, we conclude that the detection of LPS by TLR4 occurs intracellularly. In support of this notion, *Salmonella*-containing vacuoles, containing either wild-type or rough *Salmonella* bacteria, were found to colocalize with TLR4, which is consistent with previous reports (3). The identical localizations of TLR4 in wild-type- and rough-*Salmonella*-infected neutrophils strongly suggest that the routing of intracellular TLR4 to the *Salmonella*-containing vacuoles is the same under these

conditions and that correct intracellular localization of TLR4 does not require activation via this receptor. Unlike with TLR4's correct localization, the subsequent triggering and the killing efficiency of the neutrophil strongly depend on the subsequent recognition of wild-type *Salmonella* LPS as a proper ligand. It remains unknown whether the recognition of intracellular *Salmonella* depends on (i) the presence of LBP, which was not added in our system but may be provided by the neutrophil itself, and (ii) the surface expression of CD14. If LBP is not provided by the neutrophil, this means that LPS, once intracellular, is recognized in a manner different from that at the cell surface but still via TLR4. The latter possibility is consistent with the recent findings on the requirement of CD14 as the coreceptor for smooth LPS, whereas TLR4 is able to recognize rough LPS in a CD14-independent fashion (10, 19).

To inhibit the intracellular activation of TLR4, a cell-permeable inhibitory peptide that specifically blocks TLR4 was used. The cell-permeable peptide potently inhibited NADPH oxidase activation in response to infection with wild-type *Salmonella*. This proves that the efficient activation of the NADPH oxidase by *Salmonella* LPS requires TLR4 signaling and that this activation occurs from *Salmonella*-containing vacuoles within the neutrophil. These results are consistent with a recent report by Laroux et al. (21), who described the essential role of MyD88, one of the most important adapter molecules in TLR signaling, in NADPH oxidase activation after the uptake of gram-negative bacteria, including *Salmonella*.

Overall, our study shows that at least CR3 and TLR4 are required for the efficient uptake of unopsonized *Salmonella* and the subsequent activation of NADPH oxidase in response to this pathogen. At present, it is unknown whether TLR4 is recruited from intracellular stores or is internalized from the plasma membrane during the uptake of the bacterium.

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