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Regulation of TLR10 Expression and Its Role in Chemotaxis of Human Neutrophils

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Keywords
Endotoxin · Chemotaxis · Immuno-gold electron microscopy · TLR-10

Abstract
Toll-like receptors are innate immune receptors that play a critical role in pathogen-associated molecular pattern recognition. TLR10 was recently identified and very limited data are available on its expression, mechanisms that regulate its expression, and its role in primary immune cells. To study the expression pattern of TLR10 in primary immune cells, we examined TLR10 protein expression in naive and *Escherichia coli* lipopolysaccharide (LPS)-activated human neutrophils. Human neutrophils challenged with LPS showed a decrease in total and surface TLR10 expression at 90 min. TLR10 in LPS-activated neutrophils colocalized with flotallin-1, a lipid raft marker, and EEA-1, an early endosomal marker, to suggest its endocytosis. There was increased colocalization of TLR10 with TLR4 at LPS 60 min followed by decrease at later LPS treatment times. Treatment with TLR4 neutralizing antibody decreased cytoplasmic localization of TLR10 in LPS-treated neutrophils. Reactive oxygen species (ROS) depletion and neutralization of p65 subunit of NF-κB in LPS-treated neutrophils decreased TLR10 expression. Live cell imaging of LPS-activated neutrophils showed TLR10 translocation in the leading edge and TLR10 knockdown in neutrophils reduced their fMLP-induced chemotaxis and the number of neutrophils with pseudopodia but without affecting the expression of key proteins of actin nucleation process, ARP-3 and Diap1. Taken together, our findings show that neutrophil activation alters TLR10 expression through ROS production and NF-κB regulation, and TLR10 knockdown reduced neutrophil chemotaxis.

Introduction

Toll-like receptors (TLRs) are innate immune receptors which were initially identified for their roles in embryonic development in *Drosophila* [1]. TLRs recognize ligands derived from various microorganisms including bacteria, viruses, protozoa, and fungi [2–11]. So far, 10 TLRs (1–10) have been reported in humans and 13 TLRs (1–9 and 11–13) in mouse. TLR10 was recently identified and is nonfunctional in mice due to retroviral insertions [12]. Co-immunoprecipitation studies show that activated TLR10 forms homodimers and makes heterodimers with TLR1/2 via extracellular domains [13]. The structural studies of the cytoplasmic domains of TLR10 re-

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vealed MyD88 as the probable adaptor molecule [14]. Currently, the information about TLR10 signaling pathway, ligand identity, and function is scarce. Hence, it remains as an "orphan receptor" of the innate immune system.

TLR10 protein is expressed in organs such as lymph nodes, spleen, thymus, lungs, and immune cells such as macrophages and neutrophils [15]. We reported TLR10 is widely expressed in lungs of pig, dog, cattle, and chicken and that its expression is altered in inflamed lungs [16]. The expression of TLR10 mRNA is found in early B-cell development and the translation commitment mainly observed during B-cell differentiation [17]. Recent studies indicate the role of TLR10 in Crohn's disease, an inflammatory bowel disease characterized by mucosal dysfunction [18]. TLR10 is identified as the key receptor in the innate immune response against *Listeria monocytogenes* through upregulation of chemokines CCL-20, CCL-1, and IL-8 through the nuclear translocation of NF-κB [19]. Kim and colleagues [20] showed the TLR10 activation in THP-1 cell line under hypoxic conditions and proposed intracellular reactive oxygen species (ROS) and NF-κB activation as downstream effects of the activation. But recently, Oosting and colleagues [21] reported that TLR10 is an anti-inflammatory receptor because its neutralization in peripheral blood mononuclear cells increased production of proinflammatory cytokines such as IL-1β. Polymorphisms of TLR1- TLR6- TLR10 super family have been reported in diseases such as sarcoidosis and prostate cancer [22, 23]. Taken together, although the few available data implicate TLR10 in inflammatory response, the regulation of TLR10 expression and immune functions remains poorly understood.

Neutrophils are central to the genesis of acute inflammation generated in response to bacteria or their products such as lipopolysaccharide (LPS) [24, 25]. LPS activates neutrophils upon binding to TLR4 expressed on their surface. Activated neutrophils in response to chemokines migrate into sites of inflammation, produce anti-microbial agents, undergo NETosis, and kill bacteria [26]. The anti-microbial agents such as ROS produced by activated neutrophils though necessary for host defense also cause significant damage to the tissues, which is credited with morbidity and mortality [27]. Neutrophils must extravasate from blood vessels to reach extravascular site of infection to tackle microbes [28]. The neutrophils move through a complex series of cellular and molecular events regulated by adhesive proteins, chemo-attractants, and cytoskeletal reorganization. Kubes and colleagues [29] have previously demonstrated the role of TLR4 in neutrophil chemotaxis in the lung. However, there are no data on the regulation of TLR10 expression and its role in neutrophil chemotaxis.

To gain an understanding of the regulation of TLR10 expression and to address its role in neutrophil chemotaxis, we performed a series of in vitro studies. The data show that TLR10 is expressed in human neutrophils and LPS treatment altered TLR10 expression in human neutrophils. TLR10 gene knockdown induced chemotactic arrest in HL-60-derived neutrophils, but it did not affect the expression of key actin nucleation proteins.

Materials and Methods

Reagents

E. coli LPS (L6529) was obtained from Sigma Chemicals (St. Louis, MO, USA). Anti-TLR10 polyclonal (ab53631), anti-beta actin (ab8226), anti-EEA1 (ab2900), anti-mouse IgG-FITC (ab6785), and anti-mouse IgG_1-FITC (ab106163) were from Abcam (Cambridge, MA, USA) and anti-flotillin-1 from Santa Cruz Biotechnology (sc74566; California, CA, USA). TLR4 neutralizing antibody (pab-hstlr4) and anti-Gr1 antibody (MAB1037-100) are commercially purchased from Invivogen USA and R&D systems (Minneapolis, USA). Peroxidase Substrate kit was obtained from Vector Laboratory Inc. (SK-4100; Burlinton, VT, USA) and protein assay kit obtained from Bio-Rad (5000201; Mississauga, ON, Canada). RPMI-1640 (30-2001) purchased from ATCC (Manassas, VA, USA), ROS detection kit was obtained from Invitrogen (I36007; Burlington, Canada), and all other chemicals from Sigma (St. Louis, MO, USA).

Isolation of Neutrophils from Human Blood

Blood was collected intravenously from healthy volunteers and carefully added to the density gradient of Histopaque 1,119, 1,083, and 1,077 as described previously [30]. Following centrifugation at 700 *g* for 20 min at room temperature, the layer between Histopaque 1,119 and 1,083 was aspirated and red blood cells were removed through hypotonic lysis. The cell pellet was suspended in RPMI-1640 with 10% FBS and viability checked by using trypan blue. Cells were stimulated with bacterial LPS (1 μg/mL) and harvested at 60-min, 90-min, and 120-min time interval. The purity of neutrophils was confirmed with light microscopic examination of cytospin preparations. Number of cells analyzed is identical for each experiment and counted 250–300 for any given quantification. Individual donor samples were used to run replicates and three different donors used for 3 independent replicates to minimize the biological variation.

Flow Cytometry for TLR10 Surface Expression in Neutrophils

FACSCalibur flow cytometer (BD Biosciences, Mississauga, ON, Canada) was used to perform the flow cytometry assays. LPS-treated cells were treated with Fc receptor blocker in order to reduce the background staining followed by the incubation with mouse antihuman TLR10 monoclonal antibody (0.35 μg/test; ab113446, Abcam) for 30 min at 4°C. PE-conjugated Gr-1 (0.30 μg/test; 108407, Biolegend) was used as the marker for preliminary

experiments to determine the neutrophil population and FITC-conjugated anti-mouse IgG_1as isotype control. FITC-labeled anti-mouse IgG antibody was used against TLR10. We analyzed 10,000 cells for quantification.

Confocal Microscopy for TLR10, EEA1, Flotilin-1, TLR4, ROS, and Actin

Confocal microscopy was performed using Leica TCS SP5 (Leica Microsystems, Wetzlar, Germany) with 63×/1.2 oil immersion objective for image acquisition. Isolated neutrophils were incubated at 37°C and 5% CO_2 in RPMI-1640 supplemented with 10% FBS and stimulated with *E. coli* LPS for 60 min, 90 min, and 120 min. Resting cells without LPS challenge in the same conditions were used as control for the experiment. Cells were fixed in 4% paraformaldehyde and permeabilized using 0.01% Triton X-100, blocking with 5% BSA followed by primary antibodies against TLR10 (1:250), early endosomal marker, EEA1 (1:200), flotilin-1 (1:150), Alexa 488 phalloidin (165 nM) according to the experiment and incubated 1 h at room temperature [31]. Fluorescent-labeled secondary antibodies incubation was done for 30 min at room temperature. DAPI was used to stain the nuclei. TLR4 neutralizing antibody (5 μg/mL) was co-incubated with isolated neutrophils prior to LPS treatment and ROS production was analyzed as the marker for the receptor neutralization. A targeted antagonist 4-N-[2-(4-phenoxyphenyl) ethyl]quinazoline-4,6-diamine (QNZ) (5 nM) (ab141588; Abcam, Cambridge, MA, USA) was used to inhibit NF-κB.

To evaluate TLR10 translocation in activated live neutrophils, isolated human neutrophils were stained with FITC-conjugated TLR10 antibody and washed gently to remove all unbounded dyes. Cells were resuspended in RPMI complete media supplemented with 20-mM HEPES and 250 μL of the suspension was added to the center of glass bottom culture dish (MatTek Corporation, 200 Homer Avenue, MA, USA) providing 37°C. Neutrophil activation was performed by adding LPS (1 μg/mL) to the cell suspension. Live cell activity was recorded for 120 min using time-lapse option. For quantification, we analyzed 250–300 cells.

Western Blotting for TLR10, Diap1, and ARP3

After activation with LPS, isolated primary human neutrophils and HL-60-derived neutrophils (control and TLR10 knockdown) were lysed in RIPA buffer (Sigma, St. Louis, MO, USA) with protease inhibitor cocktail. Immunoblots were prepared by anti-TLR10 antibody (1:600, 1 mg/mL; Abcam, Cambridge, MA, USA), anti-Diap1 antibody (1:500, Cell Signaling Tech, Danvers, MA, USA), anti-ARP3 antibody (1:400, Cell Signaling Tech, Danvers, MA, USA). Anti-β-actin (1:1,000, Abcam, Cambridge, MA, USA) was used as loading control. HRP-conjugated anti-goat or anti-rabbit (1:2000; DAKO, Burlington, VT, Canada) was used as the secondary antibody. Hybridization signals were detected with Amersham ECL Western blotting detection reagents (GE Healthcare, Mississauga, ON, Canada). Blots were digitally imaged and contrast adjustments were applied to all parts of the figure in an unbiased manner. Represented lanes indicate only parts of the blot for better visualization. Quantification of bands from immunoblots was performed with densitometry in Adobe Photoshop CS6 (San Jose, CA, USA).

Quantitative RT-PCR for TLR10

Total RNA was isolated from LPS-treated human neutrophils using Qiagen RNeasy Mini Kit followed by the treatment with RNase-free DNase (Qiagen, Ontario, Canada) according to the manufacturer's instructions. RNA was quantified by Nanodrop method and cDNA was prepared using Quantitect Reverse Transcription Kit (Qiagen, Ontario, Canada). Quantitative real time PCR was performed using Stratagene MX3005P PCR instrument and brilliant SYBR Green QPCR kit (Agilent Technologies, Santa Clara, CA, USA) was used for the reaction. TLR10 (FP: 3' ACTTTGCCCACCACAATCTC 5' and RP: 3' CCCAGAAAAGCCCACATTTA 5') and GAPDH (FP: 3' GAGTCAACGGATTTGGTCGT 5' and RP: 3' TTGATTTTGGAGGGATCTCG 5') primers were obtained from Invitrogen (Burlington, VT, Canada). ROX was used as reference dye for the PCR reaction. Specificity of the reaction was measured with nontemplate and no-reverse transcriptase controls and analysis of melting curves. GAPDH was used for normalization of the expression.

Immunoelectron Microscopy for TLR10 Expression in Neutrophils

Immunoelectron microscopy was performed on the neutrophils fixed in 2% paraformaldehyde and 0.5% glutaraldehyde in 0.1-M sodium cacodylate buffer (pH 7.2). Nonspecific binding was blocked by incubating with 1% BSA and 0.1% Tween 20 in 1X TBS (pH 7.9) for 30 min. The cells were incubated with primary goat antihuman polyclonal antibody (1:30) for 1 h at 25°C. Tissue sections were washed with TBS and incubated in 15-min gold particle-labeled secondary antibody for 1 hr [32].

Neutrophil Chemotaxis

The Boyden chamber assay was used for HL-60 chemotaxis experiments with fMLP as chemo-attractant at a concentration of 1 μM, whereas pipette tip diffusion technique with LPS (1 μg) was used for primary neutrophil live imaging experiments.

RNAi-Mediated TLR10 Silencing in HL-60 Cell Lines

HL-60 cell line was transfected with commercially available siRNA transfection reagent using liposomes (Santa Cruz Biotechnology, 10410 Dallas, TX, USA) after obtaining 80% confluence in culture as per manufacturer's instructions. In brief, 80 nM of siRNA for TLR10 (sc-40272; Santa Cruz Biotechnology) and missense oligonucleotide negative control (sc-36869; Santa Cruz Biotechnology) were incubated with transfection reagent for 45 min to form transfection reagent-siRNA complex and added to the cells washed with transfection medium. HL-60 cells were incubated for 8 h in the transfection mixture and were recovered by washing with RPMI-1640. Transfected cells were incubated in complete RPMI-1640 medium for 48 h followed by their differentiation into neutrophil lineage through incubation in 1.3% DMSO for 5 days. Visual confirmation of differentiation was done by light microscopy.

Statistical Analysis

One-way ANOVA was used for the comparison between the groups and p values with 0.05 or less than 0.05 were considered significant. Results are represented as mean ± SEM of three replicates. Graph Pad Software (Avenida de la Playa, CA, USA) was used for all the analysis.

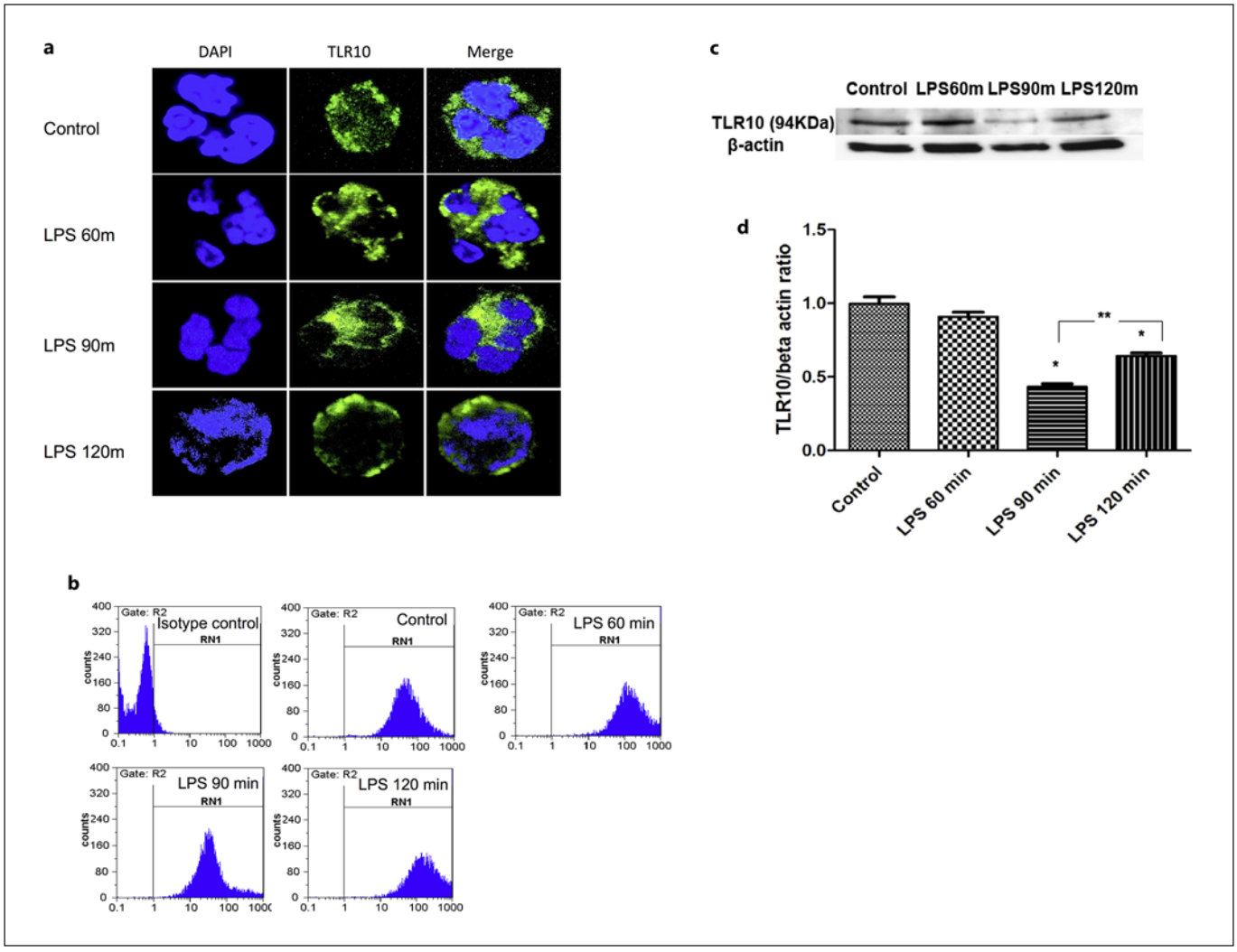

Fig. 1. LPS-mediated temporal expression changes of TLR10. **a** Time-dependent expression of TLR10 (in green) in human neutrophils (1×10^6). Nuclei stained in blue with DAPI. Neutrophils were treated with 1-μg/mL LPS for 60, 90, and 120 min ($n = 3$). Imaged the temporal expression changes and localization of TLR10 expression using confocal microscopy (magnification, 630; scale bar, 6 μm). **b** Neutrophils stimulated with LPS (1 μg/mL) for 60, 90, and 120 min and stained with antibodies against TLR10 and isotype-matching antibody for flow cytometry analysis. FITC-TLR10 fluorescent spectrum shift (qualitative) was used to analyze TLR10 surface expression changes. **c** Immunoblots lysates of neutrophils (2×10^6). Cells were stimulated with LPS (1 μg/mL) for 60 min, 90 min, and 120 min. Molecular weight is depicted on the left side of the blots. β-Actin showed in the lower panel referred as loading control. **d** Densitometry analysis showed the downregulation of TLR10 expression in neutrophils treated with 90 min and increased gene expression in LPS 120 min (*$p < 0.05$, compared with control; **$p < 0.05$, compared with LPS 60 min). One representative experiment of three in the above experiments is shown.

Results

Bacterial LPS Alters the Expression of TLR10 in Human Neutrophils

First, we examined the effect of bacterial LPS on the expression of TLR10 in human neutrophils. As shown in Figure 1a, the LPS treatment resulted in changes in the localization of TLR10 in human neutrophils. Control neutrophils expressed TLR10 on their plasma membrane whereas at 60 min of LPS treatment, TLR10 was observed mainly in the cytoplasmic vesicles. However, TLR10 staining in the cytoplasm was diffused at 90 min of LPS treatment and reappeared on the membrane at 120 min. Flow cytometry confirmed reduction and reappearance of surface expression of TLR10 at 90 min and 120 min of LPS treatment, respectively (Fig. 1b). We examined

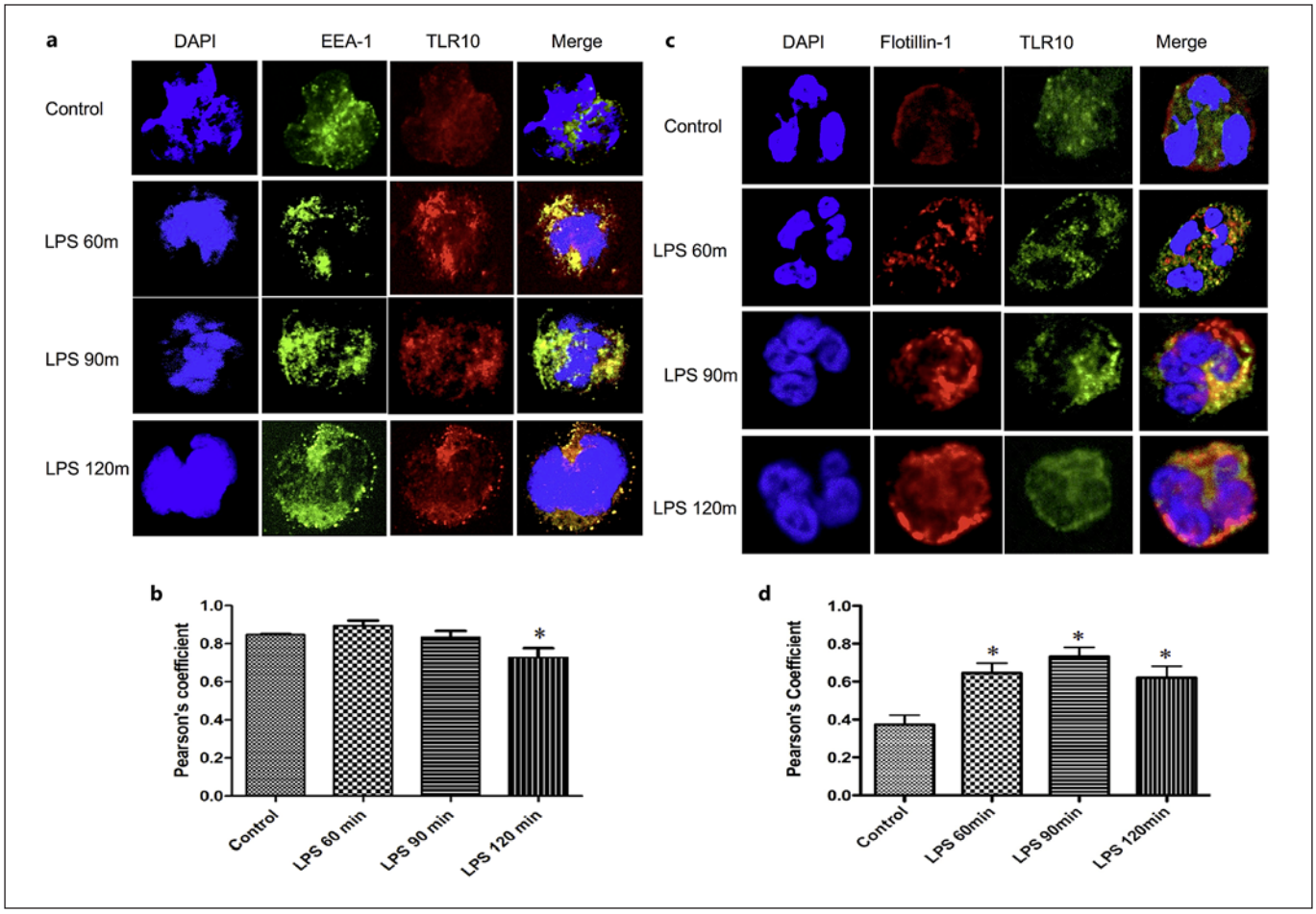

Fig. 2. Lipid raft-mediated endocytosis of TLR10. **a**, **b** Human neutrophils (1×10^6) adhered to FBS-coated coverslips were activated by LPS (1 μg/mL). Colocalization of TLR10 (red) and early endosomal antigen, EEA1 (green), are shown in merge panel. Treatment time points were 60 min, 90 min, and 120 min (magnification, 630; scale bar, 6 μm). Lower panel shows the graphical representation of quantification of colocalization in terms of Pearson's coefficient (*$p < 0.05$), analyzed by Imaris 7.4 (Bitplane Inc., Concord, MA, USA) using ImarisColoc module. **c**, **d** Colocalization of TLR10 (green) and flotillin-1, lipid raft marker (red) in LPS (1 μg/mL)-treated human neutrophils (1×10^6) adhered to FBS-coated coverslips. Intact plasma membrane in control cells and membrane rearrangement during 60–120 min was observed (magnification, 630; scale bar, 6 μm). Lower panel shows the graphical representation of quantification of degree of colocalization in terms of Pearson's coefficient (*$p < 0.05$), analyzed by Imaris 7.4 (Bitplane Inc., USA) using ImarisColoc module. Results in (**a–d**) show representative data of three independent experiments.

TLR10 expression in LPS-stimulated human neutrophils using Western blotting (Fig. 1c, d). The TLR10 expression was reduced at 90 min compared to control, 60 min and 120 min of the LPS treatment ($p < 0.05$). Lastly, we determined transcriptional level expression of TLR10 with quantitative RT-PCR and found an increase in mRNA level of TLR10 at 60 min of LPS treatment compared to the controls (online suppl. Fig. 1; see www.karger.com/doi/10.1159/000524461 for all online suppl. material).

LPS Induces Lipid Raft-Mediated Endocytosis of TLR10

We analyzed whether TLR10 is endocytosed in LPS-activated neutrophils. Quantitative colocalization of TLR10 with early endosomal antigen-1 (EEA-1), an endosomal marker protein, was performed at 60 min, 90 min, and 120 min of the LPS treatment. Figure 2a shows TLR10-EEA1 colocalization in the cytoplasm of neutrophils at 60-min and 90-min treatment. At 120 min of the LPS treatment, we observed more colocalization signal on the plasma membrane compared to 60 min and 90 min of

the treatment. The re-localization of TLR10 on plasma membrane at 120 min was consistent with the observation in Figure 1a. Quantification of colocalization in terms of Pearson's coefficient ($p < 0.05$) is shown in Figure 2b. The incubation of cells at low temperature impaired the cytoplasmic localization of TLR10 to suggest receptor-mediated internalization of TLR10 (online suppl Fig. 2).

To determine the role of lipid rafts in TLR10 endocytosis, we examined the colocalization of TLR10 with flotillin-1, a resident lipid raft protein. The data show significantly increased colocalization of TLR10 with flotillin-1 after the neutrophil activation by LPS. We used methyl-beta-cyclodextrin (β-MCD; 1 mM for 30 min) to disrupt the lipid raft and this led to the inhibition of TLR10 endocytosis (online suppl Fig. 3). Figure 2c shows plasma membrane staining of flotillin-1 in control neutrophils. Fluorescent intensity of colocalization signal of TLR10 and flotillin-1 was increased at LPS 60 min compared to the control neutrophils. Flotillin-1 staining on plasma membrane returned to control values at 120 min of LPS treatment. Figure 2d represents the quantification of colocalization in terms of Pearson's coefficient ($p < 0.05$).

The ultra-cellular localization of TLR10 was confirmed with immunoelectron microscopy of control neutrophils (Fig. 3a). Neutrophils treated with LPS for 60 min showed TLR10 aggregation on neutrophil pseudopods (Fig. 3b). TLR10 was detected on plasma membrane, cytoplasm, and nucleus of neutrophils at 120 min of LPS treatment (Fig. 3c).

TLR10 Colocalizes with TLR4 in LPS-Activated Human Neutrophils

Since bacterial LPS activates TLR4, we decided to examine the changes in TLR4 along with TLR10 expression on LPS challenge (Fig. 4a). At 60 min of the LPS treatment, cytoplasmic colocalization of TLR10 with TLR4 was significantly increased compared to control neutrophils as well as those at 90 min and 120 min of LPS treatment ($p < 0.05$; Fig. 4b).

Bacterial LPS-Induced TLR4 Activation Regulates TLR10 Expression and Localization Dynamics through ROS

Bacterial LPS activates TLR4 that leads to the nuclear translocation of NF-κB and production of ROS and proinflammatory cytokines [33]. Therefore, we assessed the role of LPS-induced TLR4 activation in TLR10 expression. TLR4 signaling pathway was inhibited with a TLR4

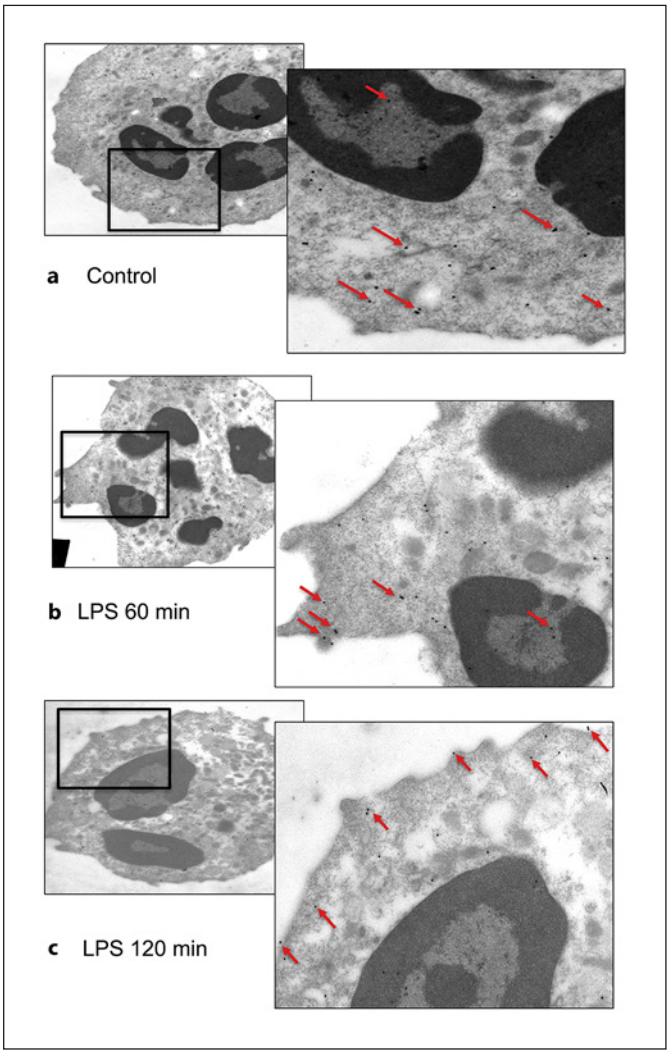

Fig. 3. Ultra-localization of TLR10 in human neutrophils. **a–c** Immuno-gold electron microscopy for TLR10 in human neutrophils shows the presence of TLR10 in nucleus, cytoplasm, as well as in the plasma membrane (red arrows). Note the TLR10 localization in pseudopodia of *E. coli* LPS (1 μg/mL)-activated neutrophils in panels (**b**, **c**). Magnification, 13,000.

Fig. 4. TLR10 colocalized with TLR4 on LPS challenge. **a** Isolated human neutrophils (1×10^6) adhered on FBS-coated coverslips were challenged with LPS (1 μg/mL) for 60 min, 90 min, and 120 min and examined by confocal microscopy for the colocalization of TLR10 (in green) and TLR4 (in red). Merged channel indicates the overlapping signals from TLR10 and TLR4 along with nuclear stain DAPI (magnification, 630; scale bar, 5 μm). **b** Quantification of colocalization in terms of Pearson's coefficient analyzed by Imaris 7.4 (Bitplane Inc.) using ImarisColoc module (*$p < 0.05$, compared with the control, and **$p < 0.05$, compared with LPS 60 min, and all data shown in terms of mean ± SEM). One representative of three in the above different experiments is shown.

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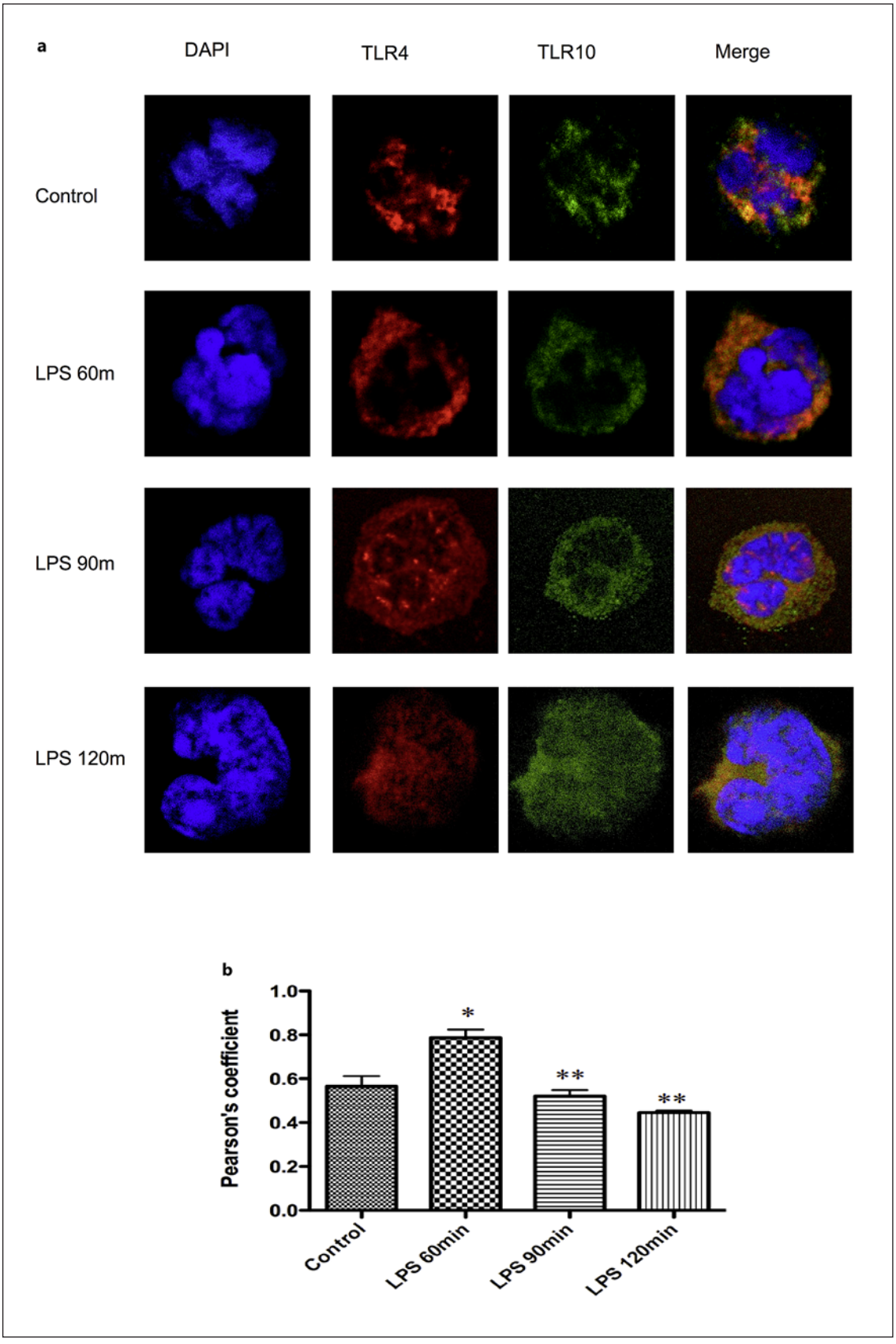
a
DAPI
TLR4
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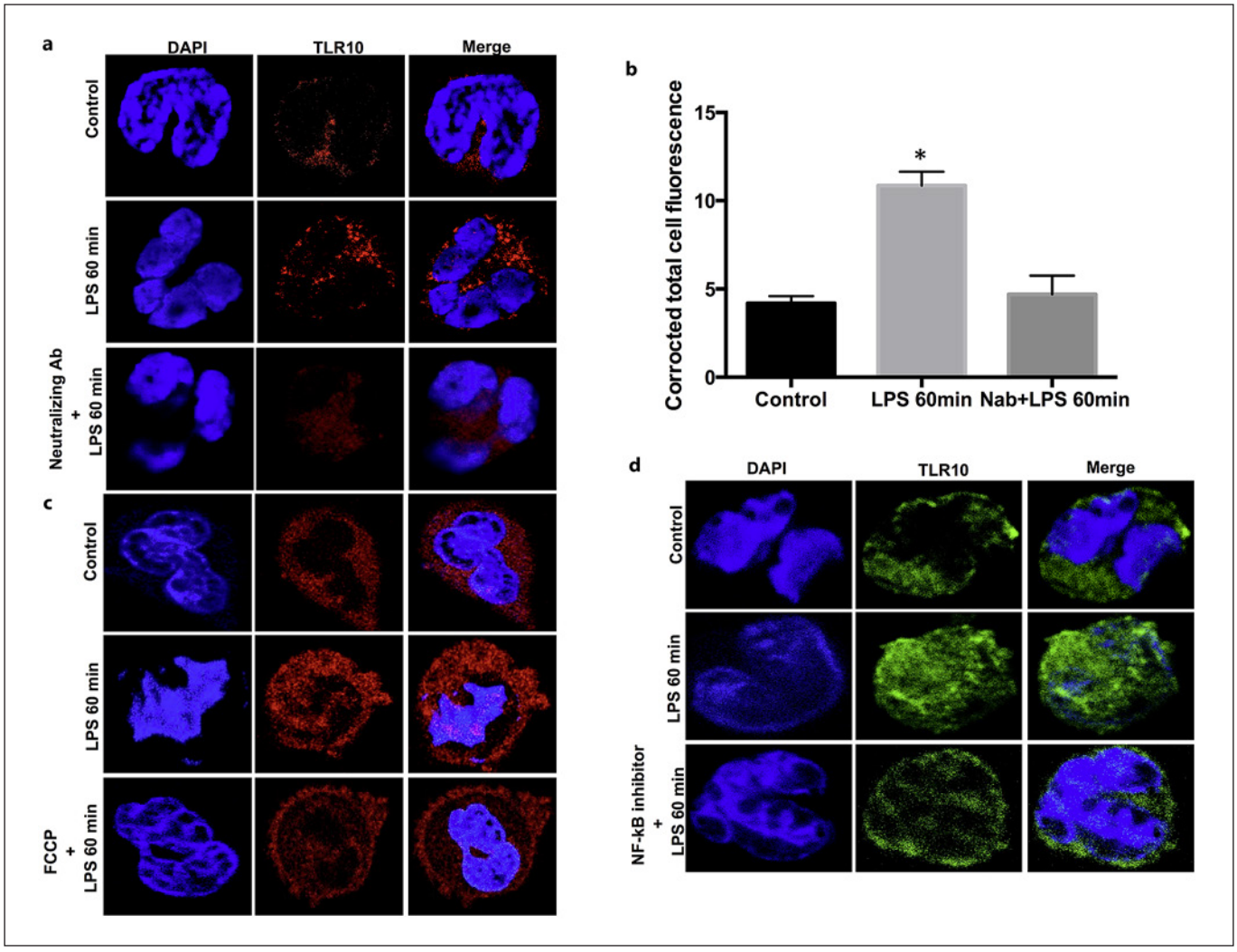

Fig. 5. TLR4 neutralization, ROS depletion, and NF-κB inhibition reduced TLR10 expression. **a** Isolated human neutrophils (1×10^6) treated for TLR4 neutralization and activated using bacterial LPS (1 μg/mL). TLR10 (in red) was imaged using confocal microscopy (magnification, 630; scale bar, 5 μm). **b** Quantification of fluorescence in terms of corrected total-cell fluorescence analyzed by Image J v1.47 (nih.gov, Bethesda, MD, USA) using grey scale intensity analysis. (*$p < 0.05$, compared with the control and all data shown in terms of mean ± SEM). **c** Human neutrophils (1×10^6) pretreated with FCCP [34], 5 μg/mL, for 1 h at 37°C to deplete ROS production. FCCP pretreated cells were challenged with LPS (1 μg/mL) for 60 min and imaged for TLR10 (in red) using confocal microscopy (magnification, 630; scale bar, 5 μm). **d** Isolated human neutrophils (1×10^6) treated for NF-κB inhibitor and treated using bacterial LPS. TLR10 (in green) was imaged using confocal microscopy and merged image in the third column indicates the expression and cytoplasmic localization of TLR10 in control, LPS 60-min treated as well as NF-κB inhibitor pretreated cells challenged with bacterial LPS for 60 min (magnification, 630; scale bar, 4 μm).

neutralizing antibody (Polyclonal rat IgG, Cat# pab-hstlr4, Invivogen USA; 5 μg/mL) and production of ROS was monitored as marker for TLR4 neutralization. ROS production from TLR4-neutralized and LPS-treated neutrophils was significantly diminished when compared with LPS-treated cells without neutralizing antibody (online suppl Fig. 4). TLR10 expression was examined in untreated control cells, LPS-treated cells and the cells treated with LPS for 60 min, and TLR4 neutralizing antibody. TLR4 neutralization in LPS-treated neutrophils significantly reduced TLR10 expression compared to the controls (Fig. 5a, b).

Since ROS production is characterized as one of the effector events in LPS-treated neutrophils, we assessed

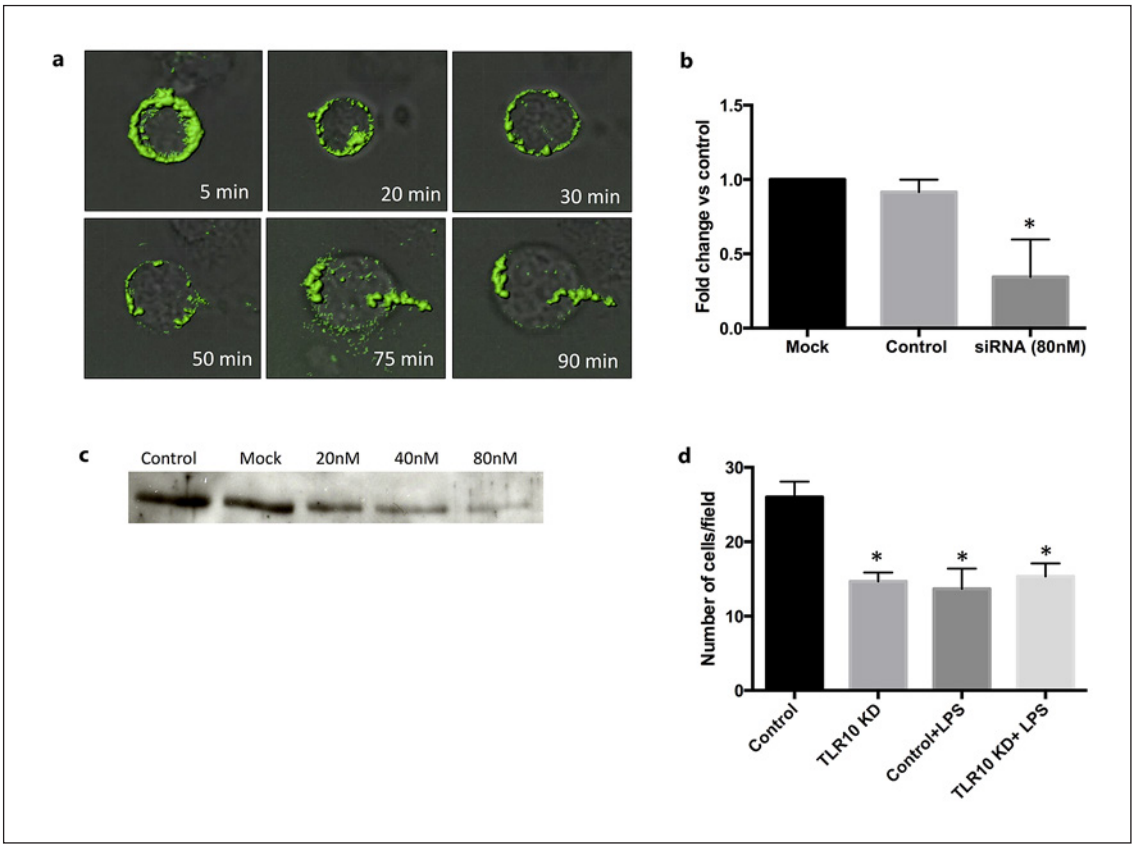

Fig. 6. Role of TLR10 in neutrophil chemotaxis. **a** Frames captured from live single-cell imaging of TLR10 (labeled in green) staining in human neutrophils. Neutrophils were activated with concentration gradient of LPS (1 μg/mL) attained by pipette tip diffusion and imaged for 100 min (magnification, 630; scale bar, 6 μm). **b** HL-60 cell line was transfected with 80 nM of TLR10 siRNA (sc-40272; Santa Cruz Biotechnology) using liposome-mediated transfection (Santa Cruz Biotechnology, 10410 Dallas, TX, USA) after obtaining 80% confluence in culture. HL-60 differentiation into neutrophils was achieved by the incubation with 1.3% DMSO for 5 days. Isolated mRNA used for quantitative real time PCR and calculated the fold change using ΔΔCt method. **c** Immunoblots lysates of HL60-differentiated neutrophils (2×10^6). Cells were treated with 20 nM, 40 nM, and 80 nM of TLR10 siRNA and total protein isolate was hybridized against anti-TLR10 antibody. Molecular weight is depicted on the left side of the blots. **d** Chemotaxis experiment was performed using Boyden chamber with control, TLR10 knockdown, LPS treated (60 min; 1 μg/mL), and TLR10 knockdown + LPS treated (60 min; 1 μg/mL). Migrated cells from at least 5 different fields were counted and tallied. One representative of two in the above experiments was shown.

the role of ROS in TLR10 expression. It has been demonstrated that LPS can rapidly induce ROS production through NADPH oxidase activation and lead to endothelial necrosis [35]. We used trifluorocarbonylcyanide phenylhydrazone (FCCP) as an uncoupler of oxidative phosphorylation and electron transport chain to prevent ROS induction in LPS-treated neutrophils. Neutrophils treated with FCCP (5 μg/mL) prior to incubation with LPS for 60 min showed decreased TLR10 expression and cytoplasmic localization when compared with LPS-treated as well as untreated controls (Fig. 5c). Furthermore, we analyzed the effect of nuclear translocation of p65 on TLR10 expression. Neutralization of p65 using an inhibitor peptide also reduced TLR10 expression (Fig. 5d). Taken together, these data show that ROS production and nuclear translocation of NF-κB after TLR4 activation by LPS regulate TLR10 expression.

TLR10 Knockdown Inhibits Neutrophil Chemotaxis

To examine the dynamic localization of TLR10 in live LPS-activated human neutrophils, we performed live single-cell imaging. The data show FITC-labeled TLR10 preferentially localized to the leading edge of the neutrophils (Fig. 6a; online suppl. Video 1). Because human neutrophils are short-lived cells in culture, we used HL-60 cell line to assess the role of TLR10 in neutrophil che-

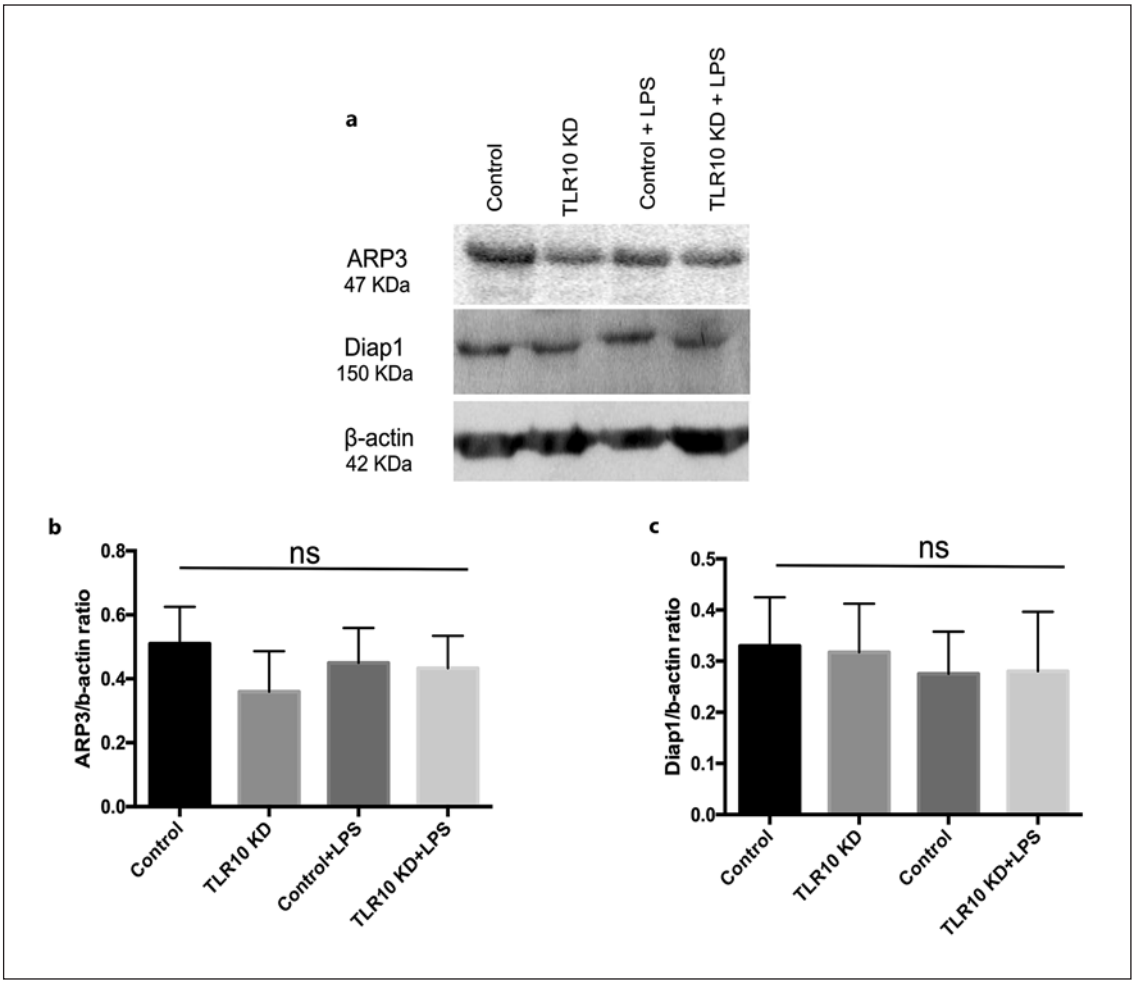

Fig. 7. TLR10 does not affect actin nucleation. **a** Immunoblots lysates of HL-60-derived neutrophils (2×10^6). TLR10 gene knockdown was performed as above and cells were stimulated with LPS (1 μg/mL) for 60 min. Actin nucleation proteins ARP3 (1:500) and Diap1 (1:400) were detected in the blot corresponding to the specific molecular weight. Molecular weight is depicted on the left side of the blots. β-Actin showed in the lower panel referred as loading control. **b**, **c** Densitometry analysis showed no significant change in actin nucleation proteins.

motaxis. The effective knockdown of TLR10 in HL-60 cells (Fig. 6b, c) led to significant reduction in their chemotaxis toward fMLP (50 nM) (Fig. 6d). To understand the underlying mechanism of regulation of chemotaxis by TLR10, we analyzed the changes in the expression of ARP3 and Diap1, which are key proteins involved in the process of actin nucleation. The Western blot data did not show differences in the expression of ARP3 and Diap1 (Fig. 7a–c). Confocal imaging on naive and TLR10 knockdown HL-60-derived neutrophils confirmed the inability of TLR10 knockdown neutrophils to form the pseudopodia against fMLP stimulus (Fig. 8a–e).

Discussion/Conclusion

Although TLR10 was characterized in early 2000 [15], there are very little data on signaling pathway, ligand, and role of TLR10 in innate immunity. Hence, it is known as an "orphan receptor" of innate immunity. Functional TLR10 is not present in mice due to retroviral insertions in the TLR10 encoding gene, thus precluding the use of mouse models to study the biology of TLR10. Neutrophils are the main effects of innate immune system and first responders against a bacterial infection [36]. Hence, in this study, we provide new data on the expression kinetics, and localization dynamics in

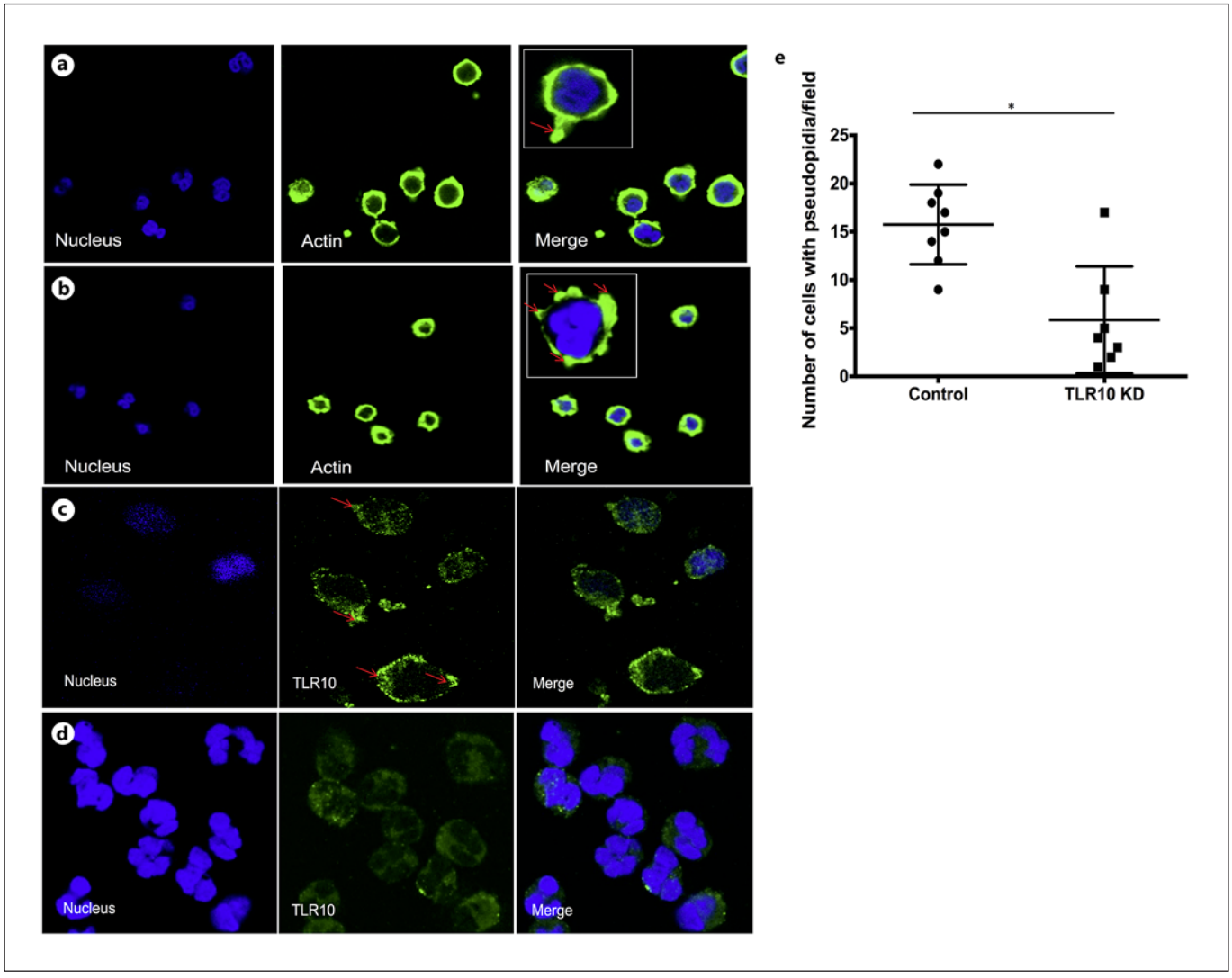

Fig. 8. TLR10 knockdown decreased formation of pseudopodia. Control (**a**) and TLR10-silenced (**b**) HL-60-derived neutrophils (1×10^6) were challenged with 1-μM fMLP for 1 min. F-actin (165 nM) stained in green and nuclei (blue) stained with DAPI. Arrows (red) indicate pseudopodia formation (scale bar, 12 μm). **c**, **d** Control and TLR10 knockdown HL-60-derived neutrophils (1×10^6) were challenged with 1-μM fMLP for 1 min. TLR10 stained in green and nuclei in blue stained with DAPI (scale bar, 10 μm). **e** Difference between the cells with pseudopodia in control and TLR10 knockdown groups. HL-60-derived neutrophils (1×10^6) were challenged with 1-μM fMLP for 1 min, imaged under confocal microscope and counted 8 different fields (double-blinded counting).

human neutrophils, and its role in neutrophil chemotaxis.

First, we examined TLR10 expression and its regulation in neutrophils. Because the identity of TLR10 ligand is still not known, we used LPS to activate neutrophils via TLR4 pathway to understand the effect of cell activation on TLR10 expression. TLR10 expression was reduced at 90 min of LPS treatment and recovered to control values by 120 min of the treatment. The new TLR10 gene transcription observed at 60 min probably restored the TLR10 protein expression at 120 min. Even more interesting is the rapid changes in plasma membrane expression of TLR10 through its endocytosis by activated neutrophils. Neutrophil activation led to changes in spatial and temporal expression of TLR10. Similar to TLR4 [37], TLR10 endocytosis is also dependent on integrity of lipid rafts because the endocytosis was inhibited following disruption of lipid rafts. While the flow cytometry data from large number of cells provide a global view of surface expression of TLR10, the IEM from only a few cells comple-

ments those data by providing fine localization of TLR10 in cytoplasmic vesicles. Taken together, the data show highly dynamic nature of TLR10 expression and trafficking in activated neutrophils.

Second, we analyzed the mechanisms of regulation of TLR10 expression in activated neutrophils. Neutrophils upon stimulation with LPS produce ROS and translocate NF-κB into their nuclei [38]. Earlier reports showed that ROS production is crucial in TLR4-dependent nuclear translocation of NF-κB [39]. ROS also contributes to LPS-TLR4 pathway-induced upregulation of TLR2 in lung endothelial cells [40, 41]. Kim et al. [20] reported the increase in TLR10 mRNA expression with an increase in intracellular ROS. Our data show that LPS-induced ROS production upregulates TLR10 expression in activated neutrophils because ROS depletion with FCCP decreased TLR10 expression. However, FCCP blocks only mitochondrial ROS and additional experiments with other ROS blockers such as Nox2 are needed in future experiments. Our data also show that LPS alters TLR10 expression through TLR4 pathway as the effects were neutralized with a TLR4 neutralizing antibody, and thus, showing potential cross talk between TLR4 and TLR10. Lastly, inhibition of nuclear translocation of NF-κB in neutrophils inhibited LPS-induced changes in TLR10 expression to underscore the role of new gene transcription in regulation of TLR10 expression. It is important to note lack of data showing any direct correlation between amount of mRNA and its translation into protein [42]. Taken together, these data show that TLR4 pathway-mediated production of ROS and nuclear translocation of NF-κB regulate the expression of TLR10 in activated neutrophils.

The potential interaction of TLR10 with TLR4 and the interference of TLR4 neutralizing antibody with access of TLR10 antibody to the receptor are not addressed by our experiments. The dimerization among various TLRs such as TLR1-TLR2 (Hasan et al. [13]) and TLR3-TLR10 [43] is well established. The endocytosis of TLR10 upon activation of TLR4 may suggest their dimerization either in control or activated state of neutrophils. We have now conducted some preliminary experiments to explore the interactions between TLR4 and TLR10. The data though inclusive at this point do lead us to conduct additional experiments in the near future. Furthermore, the effect of neutralization of TLR4 with the antibody resulting in reduced ROS production and NFκB suggests the cross talk between signals affecting the expression of TLR10. Lastly, while TLR10 mRNA was increased at 60 min of LPS treatment, the total protein showed a decrease at 90 min of LPS treatment. This discrepancy could be due to either degradation of mRNA in activated neutrophils or delayed translation of the mRNA into the protein.

There are very limited data on the role of TLR10 in neutrophil biology. We present the first evidence of role for TLR10 in neutrophil chemotaxis, one of the primal functions of neutrophils, using HL-60 cell lines. Even though TLRs are involved in PAMPs detection and regulation of proinflammatory cytokine secretion, which promotes leukocyte recruitment to the site of injury, many reports argue the role of TLR activation in inhibiting leukocyte chemotaxis [44–47]. However, there are contradictory data stating the modulatory effects of TLR activation on neutrophil chemotaxis. Fan and Malik [40] reported increased chemotaxis toward MIP-2 in LPS-challenged neutrophils. LPS-induced TLR4 activation downregulated G-protein-coupled receptor kinases (GRK) 2 and 5, which in turn desensitized chemokine receptors on neutrophils, and resulted in enhancing the chemotactic response [40, 48, 49]. Meanwhile, Alves-Filho et al. [46] showed LTA-induced TLR2 signaling activation upregulated GRK2 expression and inhibited neutrophil chemotaxis. Single-cell imaging of live neutrophils and immunoelectron microscopy showed aggregation of TLR10 on the leading edge of activated neutrophils. TLR10 knockdown with or without LPS treatment resulted in reduced chemotaxis of neutrophils toward fMLP, which are tyrosine phosphorylation and two distinct mitogen-activated protein-kinase [50]. LPS treatment alone also reduced neutrophil chemotaxis toward fMLP; there is a need for additional experiments to dissect the mechanism through which TLR10 influences chemotaxis. However, the quantification of plasma membrane pseudopods showed a reduction in their numbers in HL-60 cell line in which TLR10 was knocked down. Previous studies have shown that TLRs regulate actin polymerization, phagocytosis, and chemotaxis by modulating MAPK, Cdc42, and Rac1 pathways [46, 51, 52]. We further attempted to address the role of TLR10 in ARP3 and Diap1 proteins expression, which are involved in actin nucleation, one of the steps in pseudopod formation. However, the Western blot data showed no differences in the expression of these proteins in naive neutrophils or those subjected to TLR10 knockdown. Our data however do not address any changes in spatial localization of ARP3 and Diap1 proteins in the neutrophils. While there is reduction in membrane pseudopods following TLR10 knockdown in neutrophils, the role of TLR10 in neutrophil chemotaxis needs to be investigated further.

Genetic polymorphisms of TLR10 have been reported in a number of inflammatory diseases such as asthma, Crohn's disease, and prostate cancer [22, 53], indicating its functional role in immune system. However, the molecular mechanisms are still to be fully explored. Our data are the first to show detailed expression, dynamics, ultrastructural localization, and functional roles of TLR10 in bacterial LPS-induced innate immune response. It is plausible that TLR10 contributes a key regulatory role in innate immunity and these basic data open an intriguing area for future studies.

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Statement of Ethics

Study on human neutrophils from healthy volunteers was approved by Research Ethics Board of University of Saskatchewan (Bio#10-103), Saskatoon Canada. Licensed and trained personnel collected blood and got consent from all the volunteers from whom a written consent was obtained.

Conflict of Interest Statement

The authors declare they have no competing interests.

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Author Contributions

Y. Balachandran designed the study, performed the experiments, analyzed the data, and prepared the draft of the manuscript and approved it. S. Caldwell performed experiments, participated in the data, and approved the final draft. B. Singh secured the funding, mentored Y. Balachandran, analyzed the data, and prepared as well as approved the final draft of the manuscript. G.K.A. performed TLR4-TLR10 Co-IP experiments.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its online supplementary material. Further inquiries can be directed to the corresponding author.

References

1 Hashimoto C, Hudson KL, Anderson KV. The toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. Cell. 1988 Jan 29;52(2):269–79.
2 Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by toll-like receptor 3. Nature. 2001;413:732–8.
3 Hayashi F, Smith KD, Ozinsky D, Hawn TR, Yi EC, Goodlett DR, et al. The innate immune response to bacterial fagellin is mediated by toll-like receptor 5. Nature. 2001;410:1099–102.
4 Miettinen M, Sareneva T, Julkunen I, Matikainen S. IFNs activate toll-like receptor gene expression in viral infections. Genes Immun. 2001;2:349–55.
5 Takeuchi O, Sato S, Horiuchi T, Hoshino K, Takeda K, Dong Z, et al. Cutting edge: role of toll-like receptor 1 in mediating immune response to microbial lipoproteins. J Immunol. 2002;169:10–4.
6 Lund J, Sato A, Akira S, Medzhitov R, Iwasaki A. Toll-like receptor 9-mediated recognition of herpes simplex virus-2 by plasmacytoid dendritic cells. J Exp Med. 2003;198(3):513–20.
7 Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science. 2004 Mar 5;303(5663):1529–31.
8 Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, et al. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science. 2004 Mar 5;303(5663):1526–9.
9 Latz E, Visintin A, Espevik T, Golenbock DT. Mechanisms of TLR9 activation. J Endotoxin Res. 2004;10:406–12.
10 Takeda K, Akira S. TLR signaling pathways. Semin Immunol. 2004;16(1):3–9.
11 Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell. 2006 Feb 24;124(4):783–801.
12 Selvarajoo K, Govindaraj RG, Manavalan B, Lee G, Choi S. Molecular modeling-based evaluation of hTLR10 and identification of potential ligands in toll-like receptor signaling. PLoS ONE. 2010;5(9):e12713.
13 Hasan U, Chaffois C, Gaillard C, Saulnier V, Merck E, Tancredi S, et al. Human TLR10 is a functional receptor, expressed by B cells and plasmacytoid dendritic cells, which activates gene transcription through MyD88. J Immunol. 2005 Mar 1;174(5):2942–50.
14 Nyman T, Stenmark P, Flodin S, Johansson I, Hammarström M, Nordlund P. The crystal structure of the human toll-like receptor 10 cytoplasmic domain reveals a putative signaling dimer. J Biol Chem. 2008 May;283(18):11861–5.
15 Chuang T, Ulevitch RJ. Identification of hTLR10: a novel human toll-like receptor preferentially expressed in immune cells. Biochim Biophys Acta. 2001 Mar;1518(1–2):157–61.
16 Balachandran Y, Knaus S, Caldwell S, Singh B. Toll-like receptor 10 expression in chicken, cattle, pig, dog, and rat lungs. Vet Immunol Immunopathol. 2015 Dec 15;168(3–4):184–92.
17 Bourke E, Bosisio D, Golay J, Polentarutti N, Mantovani A. The toll-like receptor repertoire of human B lymphocytes: inducible and selective expression of TLR9 and TLR10 in normal and transformed cells. Blood. 2003 Aug 1;102(3):956–63.

18 Abad C, González-Escribano MF, Diaz-Gallo LM, Lucena-Soto JM, Márquez JL, Leo E, et al. Association of toll-like receptor 10 and susceptibility to Crohn's disease independent of NOD2. Genes Immun. 2011;12(8):635–42.
19 Regan T, Nally K, Carmody R, Houston A, Shanahan F, Macsharry J, et al. Identification of TLR10 as a key mediator of the inflammatory response to Listeria monocytogenes in intestinal epithelial cells and macrophages. J Immunol. 2013 Dec 15;191(12):6084–92.
20 Kim D, Kim YJ, Koh HS, Jang TY, Park HE, Kim JY. Reactive oxygen species enhance TLR10 expression in the human monocytic cell line THP-1. Int J Mol Sci. 2010;11(10):3769–82.
21 Oosting M, Cheng SC, Bolscher JM, Vestering-Stenger R, Plantinga TS, Verschueren IC, et al. Human TLR10 is an anti-inflammatory pattern-recognition receptor. Proc Natl Acad Sci U S A. 2014 Oct 21;111(42):E4478–84.
22 Chen YC, Giovannucci E, Kraft P, Lazarus R, Hunter DJ. Association between toll-like receptor gene cluster (TLR6, TLR1, and TLR10) and prostate cancer. Cancer Epidemiol Biomarkers Prev. 2007 Oct;16(10):1982–9.
23 Veltkamp M, van Moorsel CH, Rijkers GT, Ruven HJ, Grutters JC. Genetic variation in the toll-like receptor gene cluster (TLR10-TLR1-TLR6) influences disease course in sarcoidosis. Tissue Antigens. 2012 Jan;79(1):25–32.
24 Standish AJ, Weiser JN. Human neutrophils kill Streptococcus pneumoniae via serine proteases. J Immunol. 2009 Aug 15;183(4):2602–9.
25 Kumar V, Sharma A. Neutrophils: cinderella of innate immune system. Int Immunopharmacol. 2010 Nov;10(11):1325–34.
26 Branzk N, Papayannopoulos V. Molecular mechanisms regulating NETosis in infection and disease. Semin Immunopathol. 2013 Jul;35(4):513–30.
27 Lee VM, Quinn PA, Jennings SC, Ng LL. Neutrophil activation and production of reactive oxygen species in pre-eclampsia. J Hypertens. 2003 Feb;21(2):395–402.
28 Mathias JR, Perrin BJ, Liu TX, Kanki J, Look AT, Huttenlocher A. Resolution of inflammation by retrograde chemotaxis of neutrophils in transgenic zebrafish. J Leukoc Biol. 2006 Dec;80(6):1281–8.
29 Andonegui G, Bonder CS, Green F, Mullaly SC, Zbytnuik L, Raharjo E, et al. Endothelium-derived toll-like receptor-4 is the key molecule in LPS-induced neutrophil sequestration into lungs. J Clin Invest. 2003 Apr;111(7):1011–20.
30 Oh H, Siano B, Diamond S. Neutrophil isolation protocol. J Vis Exp. 2008;(17):745.
31 Daniel L, Kai S. Lipid rafts as a membrane-organizing principle. Science. 2010;327:46–50.
32 Singh Suri S, Janardhan KS, Parbhakar O, Caldwell S, Appleyard G, Singh B. Expression of toll-like receptor 4 and 2 in horse lungs. Vet Res. 2006 Jul–Aug;37(4):541–51.
33 Yamada H, Arai T, Endo N, Yamashita K, Fukuda K, Sasada M, et al. LPS-induced ROS generation and changes in glutathione level and their relation to the maturation of human monocyte-derived dendritic cells. Life Sci. 2006 Jan 25;78(9):926–33.
34 Zablockaite D, Gendviliene V, Martisiene I, Jurevicius J. Effect of oxidative phosphorylation uncoupler FCCP and F1F0-ATPase inhibitor oligomycin on the electromechanical activity of human myocardium. Adv Med Sci. 2007;52:89–93.
35 Simon F, Fernández R. Early lipopolysaccharide-induced reactive oxygen species production evokes necrotic cell death in human umbilical vein endothelial cells. J Hypertens. 2009;27(6):1202–16.
36 Amulic B, Cazalet C, Hayes GL, Metzler KD, Zychlinsky A. Neutrophil function: from mechanisms to disease. Annu Rev Immunol. 2012;30:459–89.
37 Triantafilou M, Miyake K, Golenbock T, Triantafilou K. Mediators of innate immune recognition of bacteria concentrate in lipid rafts and facilitate lipopolysaccharide-induced cell activation. J Cell Sci. 2002;115:2603–11.
38 Neubauer O, Sabapathy S, Lazarus R, Jowett JB, Desbrow B, Peake JM, et al. Transcriptome analysis of neutrophils after endurance exercise reveals novel signaling mechanisms in the immune response to physiological stress. J Appl Physiol. 2013 Jun;114(12):1677–88.
39 Asehnoune K, Strassheim D, Mitra S, Kim JY, Abraham E. Involvement of reactive oxygen species in toll-like receptor 4-dependent activation of NF-kappa B. J Immunol. 2004;172:2522–9.
40 Fan J, Malik AB. Toll-like receptor-4 (TLR4) signaling augments chemokine-induced neutrophil migration by modulating cell surface expression of chemokine receptors. Nat Med. 2003;9(3):315–21.
41 Xiang M, Fan J, Fan J. Association of toll-like receptor signaling and reactive oxygen species: a potential therapeutic target for post-trauma acute lung injury. Mediators Inflamm. 2010;2010:916425.
42 Liu Y, Beyer A, Aebersold R. On the dependency of cellular protein levels on mRNA abundance. Cell. 2016 Apr 21;165(3):535–50.
43 Lee S, Yip T, Yan S, Jin D, Wei H, Guo R, et al. Recognition of Double-Stranded RNA and Regulation of Interferon Pathway by Toll-Like Receptor 10. Front Immunol. 2018;9:516.
44 Khan AI, Heit B, Andonegui G, Colarusso P, Kubes P. Lipopolysaccharide: a p38 MAPK-dependent disrupter of neutrophil chemotaxis. Microcirculation. 2005;12:421–32.
45 Sabroe I, Dower SK, Whyte MK. The role of toll-like receptors in the regulation of neutrophil migration, activation, and apoptosis. Clin Infect Dis. 2005;41 Suppl 7:S421–6.
46 Alves-Filho J, Freitas A, Souto F, Spiller F, Paula-Neto H, Silva J, et al. Regulation of chemokine receptor by toll-like receptor 2 is critical to neutrophil migration and resistance to polymicrobial sepsis. Proc Natl Acad Sci U S A. 2009;106:4018–23.
47 Yi L, Chandrasekaran P, Venkatesan S. TLR signaling paralyzes monocyte chemotaxis through synergized effects of p38 MAPK and global rap-1 activation. PLoS One. 2012;7(2):e30404.
48 Hayashi F, Means TK, Luster AD. Toll-like receptors stimulate human neutrophil function. Blood. 2003;102:2660–9.
49 Aomatsu K, Kato T, Fujita H, Hato F, Oshitani N, Kamata N, et al. Toll-like receptor agonists stimulate human neutrophil migration via activation of mitogen-activated protein kinases. Immunology. 2008 Feb;123(2):171–80.
50 Torres M, Hall FL, O'Neill K. Stimulation of human neutrophils with formyl-methionyl-leucyl-phenylalanine induces tyrosine phosphorylation and activation of two distinct mitogen-activated protein-kinases. J Immunol. 1993 Feb 15;150(4):1563–77.
51 Doyle SE, O'Connell RM, Miranda GA, Vaidya SA, Chow EK, Liu PT, et al. Toll-like receptors induce a phagocytic gene program through p38. J Exp Med. 2004 Jan 5;199(1):81–90.
52 Kong L, Ge BX. MyD88-independent activation of a novel actin-Cdc42/rac pathway is required for toll-like receptor-stimulated phagocytosis. Cell Res. 2008 Jul;18(7):745–55.
53 Lazarus R, Raby BA, Lange C, Silverman EK, Kwiatkowski DJ, Vercelli D, et al. TOLL-like receptor 10 genetic variation is associated with asthma in two independent samples. Am J Respir Crit Care Med. 2004 Sep 15;170(6):594–600.