

Selective and direct activation of human neutrophils but not eosinophils by Toll-like receptor 8

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Background: Granulocytes represent the largest fraction of immune cells in peripheral blood and are directly exposed to circulating Toll-like receptor (TLR) ligands. Although highly relevant for TLR-based therapies, because of the technical challenge, activation of the granulocyte subsets of neutrophils and eosinophils by TLR ligands is less well studied than activation of other immune cell subsets.

Objective: The aim of this work was to study direct versus indirect neutrophil and eosinophil activation by TLR7 and TLR8 ligands.

Methods: We used a new whole-blood assay, single cell-based cytokine detection, and highly purified primary human neutrophils and eosinophils to separate direct and indirect effects on these blood cell subsets.

Results: We found indirect but not direct activation of neutrophils but not eosinophils in whole blood by using unmodified immunostimulatory RNA (isRNA; TLR7/8 ligand). In contrast, direct activation and stimulation of the respiratory burst and degranulation was seen with nuclease-stable isRNA and with the small-molecule TLR8 agonist 3M002 but not 3M001 (TLR7). Neutrophils expressed TLR8 but none of the other 2 RNA-detecting TLRs (TLR3 and TLR7).

Conclusions: Together, these results demonstrate that neutrophils are directly and fully activated through TLR8 but not TLR7. Furthermore, the results predict that the clinical utility of small-molecule TLR8 ligands or nuclease-stable RNA ligands for TLR8 might be limited because of neutrophil-mediated toxicity and that no such limitation applies for unmodified isRNA, which is known to induce desired T_H1 activities in other immune cell subsets. (J Allergy Clin Immunol 2009;123:1001-1011.)

Key words: Toll-like receptor, immunostimulatory RNA, neutrophil, granulocyte, immunotherapy, small molecule, 3M001, 3M002, 3M007, R-848

Neutrophils represent the largest fraction of immune cells in peripheral blood and are the first immune cells to arrive at the site of infection. They quickly initiate microbicidal functions, allowing the acquired immune system enough time to generate sterilizing immunity and memory.^{1,2} Neutrophils are activated by IL-8, IFN- γ , and heat shock proteins³⁻⁵ or on binding of antibody- or complement-opsonized particles.⁶ Cytotoxic substances released by activated neutrophils are damaging to adjacent healthy tissue.⁷ The lifespan of granulocytes is limited and tightly regulated at stages of proliferation, differentiation, and apoptosis.⁸⁻¹⁰

Although in neutrophils the function of Toll-like receptors (TLRs) detecting conserved bacterial molecular patterns, such as endotoxin (TLR4) is well understood,^{11,12} relatively little and controversial information is available for the role of TLRs detecting nucleic acid patterns (TLR3, TLR7, TLR8, and TLR9). TLR7 detects short single- and double-strand RNA,¹³⁻¹⁵ TLR8 detects single-strand RNA,¹⁵ and TLR9 detects unmethylated CpG motifs in DNA.¹⁶ Small-molecule ligands are 3M001 (TLR7), 3M002 (TLR8) and R-848 (TLR7 and TLR8).¹⁷⁻¹⁹ The TLR7 ligand imiquimod is an approved drug for the local treatment of genital warts and actinic keratosis, and other TLR7 and TLR8 ligands are considered drug candidates, including small-interfering RNA, for which TLR7 activation is an unwanted side effect.

The expression profile and function of these nucleic acid immunoreceptors are well characterized in human PBMCs,^{20,21} but despite the significance for toxicology, only few studies have addressed the function of these receptors in granulocytes, specifically neutrophils. Here we studied the interaction of RNA and small-molecule TLR7 and TLR8 ligands with neutrophils.

METHODS

Cell culture

For whole-blood assays, blood from healthy donors was anticoagulated with lepirudin (20 μ g/mL; Pharmion, Hamburg, Germany) and diluted with an equal volume of 0.9% NaCl solution (B. Braun, Melsungen, Germany). One milliliter of this blood cell suspension was used in 48-well flat-bottom plates. For purification of neutrophils, polymorphonuclear leukocytes (PMNs) were separated from lepirudin-treated blood of healthy volunteers. Cells were obtained by means of density gradient centrifugation with Ficoll-Hypaque (Biochrome, Berlin, Germany) and subsequent sedimentation of erythrocytes with 3% dextran T500 (Sigma-Aldrich, St Louis, Mo). Residual red blood cells were lysed with PharmLyse (BD Biosciences, Heidelberg, Germany). Neutrophils were further purified by means of magnetic cell sorting (MACS) with positive selection with anti-human CD16 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's protocol.

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Abbreviations used

CD62L: CD62 ligand
fMLP: Formyl-Met-Leu-Phe
FSC: Forward scatter
HBSS: Hank's balanced salt solution
isRNA: Immunostimulatory RNA
MACS: Magnetic cell sorting
ORN: Oligoribonucleotide
PMN: Polymorphonuclear leukocyte
PTO: Phosphorothioate
SSC: Side scatter
TLR: Toll-like receptor

Neutrophil purity was greater than 99%, and viability was greater than 95%, as determined by means of flow cytometry and trypan blue staining. Cells were cultured in RPMI 1640 (Invitrogen, Karlsruhe, Germany) supplemented with 2% human AB serum (Cambrex, East Rutherford, NJ), 100 U/mL penicillin, 100 µg/mL streptomycin (PAA Laboratories, Pasching, Austria), and 1.5 mmol/L L-glutamine (Cambrex) in 96-well round-bottom plates at a concentration of 4×10^5 neutrophils/0.2 mL per well unless stated otherwise. Cells were always incubated at 37°C in a 95% humidified 5% CO₂ atmosphere. All compounds used for cell culture were purchased and endotoxin tested. Supernatants of PBMCs were negative for TNF-α (below detection limit of the ELISA, 7.8 pg/mL), a highly sensitive measure of endotoxin contamination in PBMC cell cultures.

Stimulation of cells

For all TLR ligands, dose response was performed to define optimal stimulating conditions in the studied cell type. Cells were stimulated with LPS from *Escherichia coli* (10 ng/mL; Invivogen, Toulouse, France), the synthetic TLR7 and TLR8 ligands 3M001 and 3M002, the combined synthetic TLR7/8 ligand 3M007 (R-848 analog; 10 µmol/L; all kindly provided by 3M Pharmaceuticals, St Paul, Minn), and the synthesized oligoribonucleotides (ORNs) polyA, polyU, and 9.2s,¹³ all purchased from Eurogentec (Cologne, Germany). All ORNs were 21mer, either with a phosphodiester or a phosphorothioate (PTO) backbone. PolyA and PBS (PAA Laboratories) were used as negative controls. All ligands were diluted in PBS to appropriate concentrations. ORNs (40 µmol/L) were complexed with the polycationic polypeptide poly-L-arginine (2 mg/mL; Sigma-Aldrich)¹³ at a ratio of 1:5 (poly-L-arginine/RNA; usually 0.9 µL:4.5 µL in 75 µL of PBS). In some experiments brefeldin A (4 µg/mL) or chloroquine (5 µg/mL, Sigma-Aldrich) were added to cultured cells 4 hours or 30 minutes before stimulation, respectively.

Detection of cytokines

Supernatants of cultured neutrophils were harvested 16 hours after stimulation. IL-1β, IL-6, TNF-α, IL-8, and macrophage inflammatory protein 1α levels were analyzed by using the Cytometric Bead Array or ELISA (BD Biosciences).

Quantification of enzyme release

MACS-purified neutrophils (see above) were adjusted to 1×10^7 /mL in Hank's balanced salt solution (HBSS, Invitrogen) supplemented with 5% FCS (Invitrogen), 0.5 mmol/L CaCl₂, and 1 mmol/L MgCl₂. Cells were treated with 5 µg/mL cytochalasin B (Sigma-Aldrich) for 5 minutes at 37°C, washed, and adjusted to 1×10^7 /mL in supplemented HBSS. Stimulation of neutrophils was performed in 96-well round-bottom plates with 1×10^6 neutrophils/0.2 mL per well. After 2 hours, cells were boosted with formyl-Met-Leu-Phe (fMLP; 1.34 µmol/L final concentration; Sigma-Aldrich) and further incubated for 2 hours. Activity of peroxidase in supernatants was determined according to the method of Menegazzi et al.²² As substrate, we used the 3,3', 5,5'-tetramethylbenzidine substrate reagent set

(BD Biosciences) that complies with the requirements of the assay. Enzyme activity was expressed as relative activity compared with that seen in untreated neutrophils and normalized to neutrophils pretreated with PBS and boosted with fMLP. Activity of β-glucuronidase was determined according to the method of Schroder et al.²³ Enzyme activity in supernatants of stimulated cells was expressed as relative activity compared with the maximum enzyme activity after total cell lysis in 0.2 mL of 0.4% (vol/vol) Triton X-100. Finally, the relative enzymatic activity was normalized to enzyme activity in the supernatants of neutrophils pretreated with PBS and boosted with fMLP.

Analysis of respiratory burst activity

The assay was performed according to the method of Rothe and Valet.²⁴ EDTA-free, MACS-purified neutrophils (see above) were adjusted to 1×10^7 /mL in HBSS supplemented with 5% FCS, 0.5 mmol/L CaCl₂, and 1 mmol/L MgCl₂. After adding carboxy-2', 7'-dichlorodihydrofluorescein diacetate (Invitrogen; 40 µmol/L final concentration), cells were incubated for 30 minutes at 37°C. Neutrophils (1×10^6)/0.2 mL per well were stimulated for 4 hours in 96-well round-bottom plates, with subsequent analysis of the mean fluorescence intensity on a FACSCanto flow cytometer (BD Biosciences).

Data are depicted as the fold induction of the respiratory burst compared with the PBS control.

Flow cytometry

After 4 hours of whole-blood stimulation (unless indicated otherwise) and red blood cell lysis with PharmLyse (BD Biosciences), the following mouse anti-human mAbs were used to stain neutrophils and eosinophils: anti-CD16 (fluorescein isothiocyanate), anti-CD11b (phycoerythrin), anti-CD62 ligand (CD62L; allophycocyanin), and anti-IL-8 (phycoerythrin). Doublets and dead cells (propidium iodide [Sigma Aldrich] positive) were excluded from flow cytometric analysis. Data were obtained on FACSCanto or LSR II flow cytometers (BD Biosciences) with FlowJo software (TreeStar, Inc, Ashland, Ore). For RNA isolation, cells were purified with density gradient centrifugation and dextran sedimentation, as described above. Cells were stained with anti-CD16 mAb, washed, and passed through a single-cell filter. CD16⁺/forward scatter (FSC)^{high}/side scatter (SSC)^{high} cells were sorted on a FACSDiva cell sorter (BD Biosciences) to a purity of greater than 99%, as determined by means of May-Grünwald-Giemsa staining and FACS analysis.

Cytologic analysis

Sorted cells were fixed to glass slides with a Shandon cytospin 4 centrifuge (Thermo scientific, Schwerte, Germany). Air-dried cells were stained with May-Grünwald-Giemsa solution according to standard protocols. Cells were analyzed with an Olympus (Hamburg, Germany) IX71 microscope equipped with a color-view camera and analySIS software (both from Soft Imaging Systems, Münster, Germany).

RNA isolation, cDNA synthesis, and quantitative real-time PCR

Sorted neutrophils were incubated with or without 500 U/mL recombinant IFN-β (Peprotech, Hamburg, Germany) for 3 hours at 37°C. Cells (1×10^7) were collected in 400 µL of lysis buffer from the MagnaPure mRNA Isolation Kit I (Roche, Mannheim, Germany) supplemented with 1% (wt/vol) dithiothreitol, and mRNA was isolated with the MagnaPure-LC device by using the mRNA-I standard protocol. The elution volume was set to 50 µL. An aliquot of 8.2 µL of RNA was reverse transcribed with AMV-RT and oligo-(dT) as a primer (First Strand cDNA Synthesis Kit, Roche) in a thermocycler according to the protocol provided. Parameter-specific primer sets optimized for the LightCycler (Roche) were developed and provided by SEARCH-LC GmbH, Heidelberg, Germany (www.search-lc.com). PCR was performed with the LightCycler FastStart

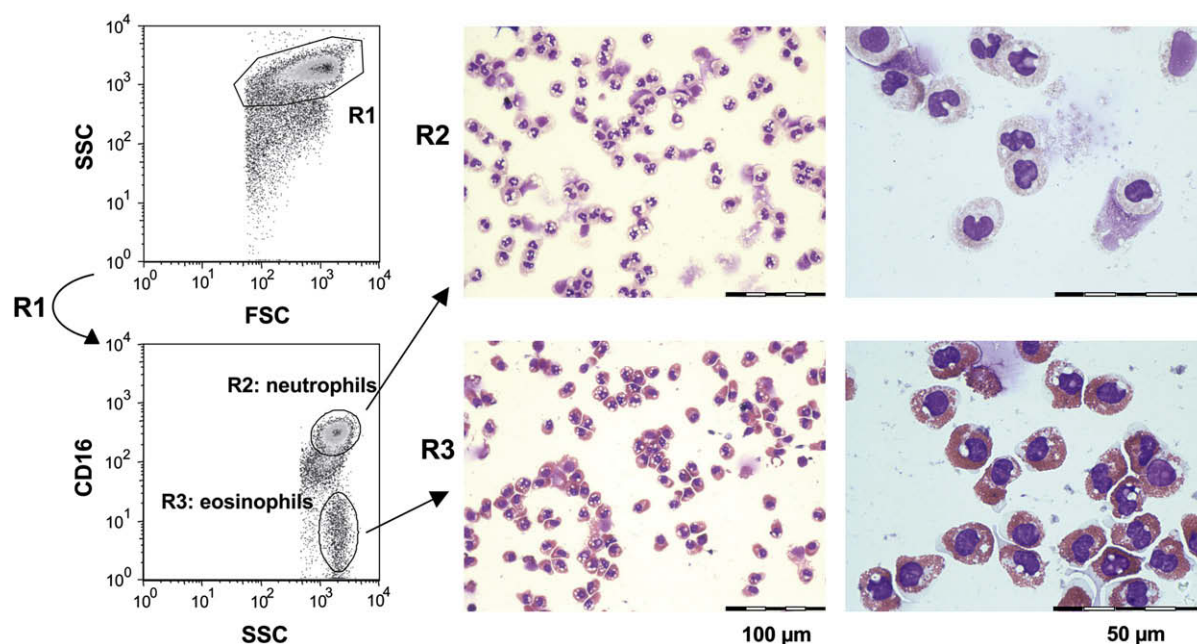


FIG 1. Identification of neutrophils and eosinophils in human whole blood. PMNs were stained with anti-human CD16 mAb. CD16⁺/FSC^{high}/SSC^{high} cells are neutrophils (R2), and CD16⁺/FSC^{high}/SSC^{high} cells are eosinophils (R3; left panels). Sorted cells were stained with May-Grünwald-Giemsa solution and analyzed microscopically (200-fold and 600-fold magnification; right panels, left to right). One of 2 independent experiments is shown.

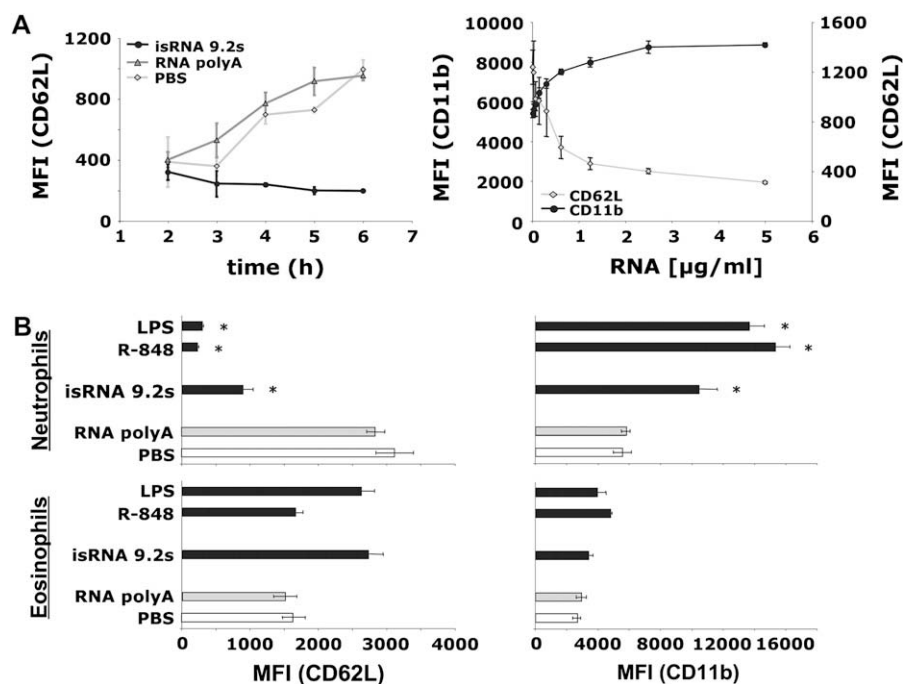


FIG 2. Activation of whole-blood neutrophils by isRNA. **A**, Whole blood stimulation with poly-L-arginine-complexed isRNA 9.2s or RNA polyA at 0.5 μ g/mL (kinetic; left panel) or at 5.0 to 0.02 μ g/mL (dose response; right panel). **B**, CD62L and CD11b expression on gated neutrophils and eosinophils. The mean $\pm$ SEM of 3 independent experiments is shown. * $P < .03$ versus PBS control. *MFI*, Mean fluorescence intensity.

DNA Sybr Green I kit (Roche). The calculated transcript numbers were normalized to the average expression of 2 housekeeping genes: cyclophilin B and β -actin. Data are presented as the input-adjusted transcript number per microliter of cDNA.

Statistical analysis

Data are depicted as the mean $\pm$ SEM. Statistical significance of differences was determined by using paired 1-tailed Student *t* tests with Microsoft Excel software.

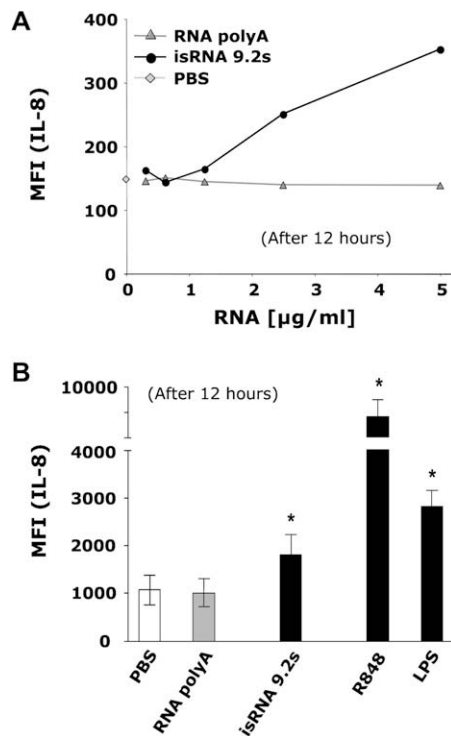


FIG 3. IL-8 induction in isRNA-stimulated neutrophils of whole blood. **A**, Analysis of intracellular IL-8 levels after 12 hours of whole-blood stimulation with poly-L-arginine complexed RNA polyA or isRNA 9.2s at 5.0 to 0.3125 µg/mL. One of 4 independent experiments is shown. **B**, Intracellular IL-8 expression on neutrophils on stimulation with poly-L-arginine-complexed RNA polyA, isRNA 9.2s (both 0.5 µg/mL), LPS, or R-848. The mean $\pm$ SEM of 3 independent experiments is shown. * $P < .03$ versus the PBS control.

RESULTS

Immunostimulatory RNA stimulates human neutrophils but not eosinophils in whole blood

Neutrophils isolated from whole blood undergo spontaneous apoptosis in cell culture. Therefore we established a whole-blood assay that allows analysis of neutrophil function in a close-to-natural environment. In preliminary studies we found that heparin, which is routinely used for anticoagulation in whole-blood assays, negatively interferes with the immunostimulatory activity of RNA oligonucleotides to be tested in neutrophils. The hirudin-like anticoagulant lepirudin showed no negative interaction with oligonucleotides and was used in all subsequent whole-blood analyses. In flow cytometry neutrophils are characterized as CD16⁺/SSC^{high}/FSC^{high} cells, whereas eosinophils are characterized as CD16⁺/SSC^{high}/FSC^{high} cells (Fig 1, left panels). CD16^{high} neutrophils were gated because senescent neutrophils or neutrophils undergoing apoptosis gradually downregulate CD16.^{25,26} Correct gating of neutrophils and eosinophils (>98% purity) was confirmed by means of fluorescence-activated cell sorting and subsequent May-Grünwald-Giemsa staining of sorted cells (Fig 1, right panels).

Activation of neutrophils and eosinophils in whole blood was assessed based on the expression of CD62L and CD11b.^{11,27} We analyzed the effect of an established TLR7/8 RNA ligand (immunostimulatory RNA [isRNA] 9.2s¹³) and an inactive RNA control sequence (RNA polyA). RNA oligonucleotides were complexed with poly-L-arginine according to an established protocol.¹³

A kinetic analysis of the surface marker expression of neutrophils exposed to RNA (0.5 µg/mL) in whole blood revealed that neutrophils were activated by isRNA 9.2s but not by control RNA (downregulation of CD62L) starting at 2 hours (Fig 2, A, left panel). Activation of neutrophils was concentration dependent and reached a plateau at 5 µg/mL (Fig 2, A, right panel). Stimulation of neutrophils in whole blood with isRNA 9.2s was in the same range as with the small-molecule TLR7/8 ligand R848 or LPS, whereas eosinophils showed almost no response (Fig 2, B) except weak activation by R-848, which is consistent with the literature.²⁷ Next we examined the induction of IL-8 production in neutrophils in whole blood by means of intracellular cytokine staining. Accumulation of IL-8 in neutrophils at 12 hours was dose dependent (Fig 3). Unlike for the surface markers (CD62L and CD11b), stimulation of IL-8 production in neutrophils within whole blood on isRNA 9.2s was lower than with R848 or LPS (Fig 3). No increase of IL-8 levels was detectable in eosinophils (data not shown).

Neutrophils express functional TLR8 but not TLR7

Activation of neutrophils in whole blood could be a direct effect or indirectly mediated by other cell types. Direct activation requires the expression of the corresponding TLR in neutrophils. Highly purified neutrophils were isolated from peripheral blood of healthy donors by means of fluorescence-activated cell sorting of CD16⁺/FSC^{high}/SSC^{high} cells. Isolated neutrophils were incubated for 3 hours at 37°C in the presence or absence of IFN- β , and mRNA levels of TLR1 to TLR10 were analyzed with real-time PCR. Neutrophils were found to express considerable amounts of TLR8 but none of the other RNA-detecting receptors (TLR3 and TLR7, Fig 4). Similar results were obtained when neutrophils were preincubated with recombinant GM-CSF instead of IFN- β ($n = 3$, data not shown) or isolated by means of MACS. Exclusive activation of neutrophils through TLR8 but not TLR7 was confirmed by using selective small-molecule ligands for TLR7 (3M001) versus TLR8 (3M002). In the presence of brefeldin A (blockade of protein secretion), only the TLR8 ligand 3M002 but not the TLR7 ligand 3M001 was capable of activating neutrophils in whole blood (downregulation of CD62L and upregulation of CD11b; Fig 5, A). Furthermore, although the small-molecule TLR7/8 ligand R-848 also activated neutrophils in the presence of brefeldin A, unexpectedly, isRNA 9.2s did not. In the absence of brefeldin A (direct and indirect effects visible), all TLR7 and TLR8 ligands activated neutrophils (Fig 5, A). Direct activation of neutrophils by the small-molecule TLR8 ligand 3M002 and the TLR7/8 ligand R-848 but none of the other stimuli was confirmed by analyzing the induction of macrophage inflammatory protein 1 α in neutrophils purified from whole blood (Fig 5, B). Furthermore, stimulation of purified neutrophils through TLR8 (3M002) compared with control-induced IL-1 β (233 ± 59 vs 79 ± 5 pg/mL, $P < .03$, $n = 3$), IL-6 (28 ± 9 vs 5 ± 1.3 pg/mL, $P < .03$, $n = 3$), and IL-8 (25.4 ± 5.5 vs 1.3 ± 0.5 ng/mL, $P < .03$, $n = 3$) levels. In contrast, isRNA 9.2s did not induce significant amounts of either of these molecules (data not shown). These data indicate that neutrophils are directly and selectively activated through TLR8 but not TLR7 and that the G- and U-containing single-strand RNA isRNA 9.2s, although being a TLR8 ligand (unpublished results),¹⁵ does not directly activate neutrophils. The contribution of different immune cell subsets to indirect isRNA 9.2s-induced

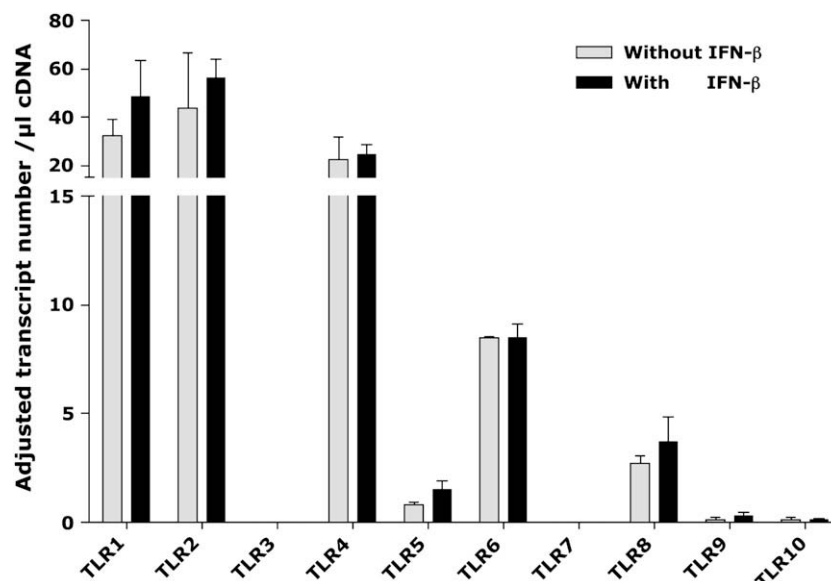


FIG 4. Quantitative analysis of mRNA expression of TLR1 to TLR10 in purified neutrophils. Neutrophils (purity >99%, viability >95%) were incubated with or without 500 U/mL recombinant human IFN- β for 3 hours. Expression of mRNA of TLR1 to TLR10 was determined by means of quantitative real-time PCR. The copy numbers are normalized to the housekeeping genes β -actin and cyclophilin B and are depicted as adjusted transcript number per microliter of cDNA ($\pm$ SEM of 3 independent donors).

activation of neutrophils in whole blood was examined based on depletion of the corresponding cell subset (monocytes, B cells, plasmacytoid dendritic cell, and eosinophils) from PBMCs and cocubation of the remaining PBMCs with PMNs. We found considerably lower indirect activation of neutrophils in the absence of monocytes (1.6-fold increase of CD11b in the absence of monocytes vs 2.2-fold increase of CD11b in the presence of monocytes, $n = 2$), whereas B cells, plasmacytoid dendritic cells, and eosinophils did not contribute to indirect neutrophil activation (data not shown).

Because TLR7 expression of neutrophils is reported in the literature but not supported by our results, we suspected that the type of isolation protocol and the degree of purity of isolated neutrophils is critical for drawing the correct conclusions. Many publications use neutrophils recovered from density gradient centrifugation (termed PMNs) without additional purification. We analyzed the relevance of neutrophil purity for TLR7/8 stimulation experiments. PMNs were prepared and subdivided in a CD16⁻ and a CD16⁺ fraction. Identical numbers of PMNs, CD16⁻ cells, and CD16⁺ cells were stimulated with isRNA 9.2s (50 μ g/mL), control RNA polyA, and R-848. Supernatants were analyzed for IL-8 expression by using ELISA. Both total PMNs and CD16⁻ cells responded to R-848 (PMNs, 26.3 ± 0.5 ng/mL; CD16⁻ cells, 21.4 ± 0.1 ng/mL; $n = 2$) and isRNA 9.2s (PMNs, 9.4 ± 1.6 ng/mL; CD16⁻ cells, 14.8 ± 0.5 ng/mL; $n = 2$) stimulation, whereas purified CD16⁺ neutrophils only responded to R-848 stimulation (17.4 ± 1.3 ng/mL, $n = 2$), and IL-8 expression in CD16⁺ neutrophils on stimulation with isRNA 9.2s (also at a 10-fold higher concentration of RNA) was in the range of control RNA polyA (RNA polyA, 4.3 ± 0.4 ng/mL; isRNA 9.2s, 5.7 ± 0.2 ng/mL). These experiments confirm the need for high purification levels of neutrophils in experiments using TLR stimuli and explain discrepancies between studies using different isolation protocols.

TLR8 ligation induces degranulation and respiratory burst in neutrophils

To analyze the effect of TLR-mediated activation on neutrophil function, we performed respiratory burst and degranulation experiments. Purified neutrophils were stimulated with selective TLR7 (3M001) and TLR8 (3M002) agonists or with isRNA 9.2s. The bacterial peptide fMLP, which is known to induce respiratory burst and degranulation,²⁸⁻³⁰ was used as a positive control (Fig 6, A). The selective TLR8 agonist 3M002 but not the TLR7 agonist 3M001 or isRNA 9.2s induced significant respiratory burst activity in neutrophils. Significant degranulation was only seen for the small-molecule TLR8 ligand but not for the TLR7 ligands or isRNA 9.2s (Fig 6).

Nuclease-stable PTO-modified polyU RNA TLR8 ligand activates neutrophils

Neutrophils are expected to have specifically high nuclease activity in their phagocytic granules, which might be responsible for degradation of nucleic acid before recognition and signaling by TLRs. We compared a 21mer polyU RNA oligonucleotide with an unmodified phosphodiester backbone with a PTO-modified backbone (PTO increases the nuclease stability of RNA) for stimulation of isolated neutrophils. PTO-stabilized isRNA induced IL-8 in isolated neutrophils, whereas control RNAs were inactive (Fig 6, B). As expected for TLR8-mediated stimulation, preincubation with chloroquine, an inhibitor of endosomal acidification, abolished the observed effect.

DISCUSSION

Systemic activation of neutrophils in peripheral blood is expected to cause life-threatening toxicity and thus limits the clinical utility of compounds inducing such activation. Here we

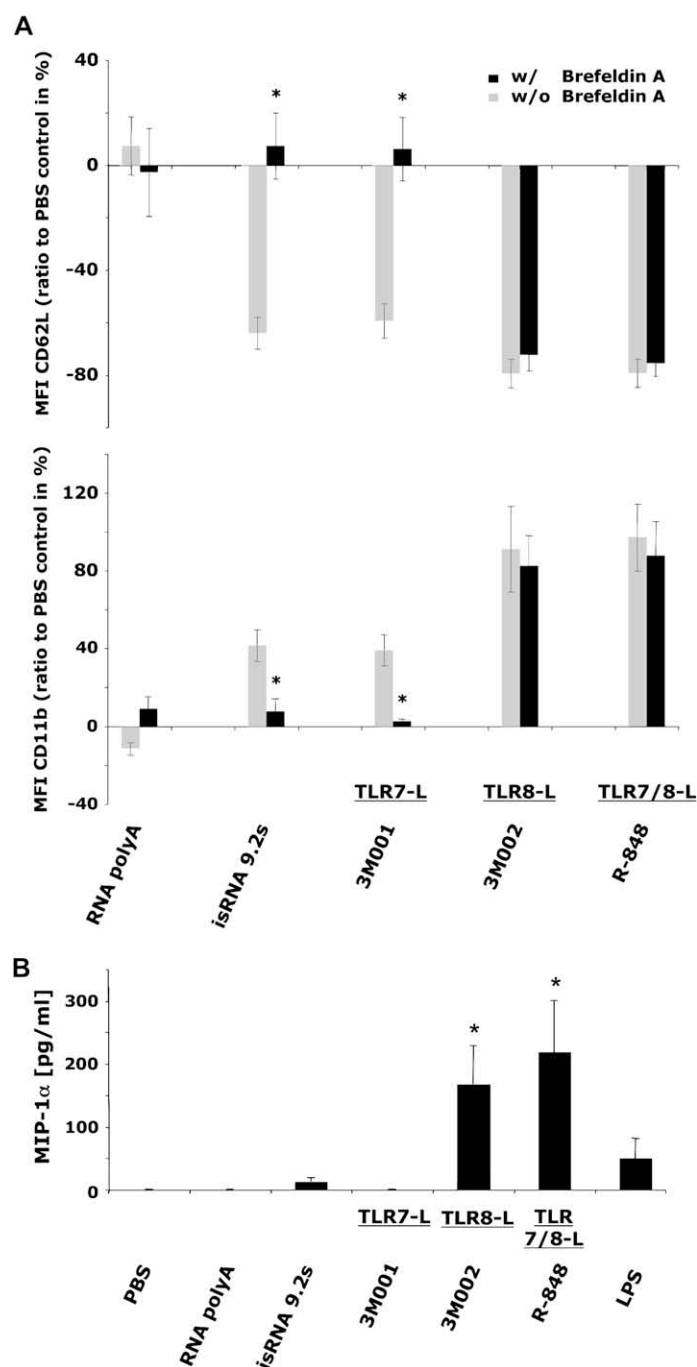


FIG 5. Selective direct activation of neutrophils by TLR8 but not TLR7. **A**, Whole blood was incubated with RNA polyA, isRNA 9.2s, (both 0.5 μ g/mL), 3M001, 3M002, R-848, and PBS. Data show the mean $\pm$ SEM fluorescence intensity of CD62L and CD11b on neutrophils as a ratio to PBS ($n = 3$). * $P < .03$, with brefeldin A versus without brefeldin A. *MFI*, Mean fluorescence intensity. **B**, Macrophage inflammatory protein (MIP) 1 α expression of 4×10^5 neutrophils stimulated with RNA polyA, isRNA 9.2s (both 5 μ g/mL), 3M001, 3M002, R-848, LPS, and PBS. The mean $\pm$ SEM is shown ($n = 3$). * $P < .03$ versus PBS.

found that human primary neutrophils with and without preincubation with IFN- β or GM-CSF express TLR8 but none of the other RNA-detecting TLRs (TLR3 or TLR7). Consistent with selective expression of TLR8, neutrophils are activated by a small-molecule ligand for TLR8 but not TLR7. Activation of neutrophils was reflected by downregulation of surface expression of CD62L, upregulation of CD11b, production of IL-8,

induction of respiratory burst, and release of preformed β -glucuronidase and peroxidase. The TLR8 RNA oligonucleotide ligand polyU (21mer) only activated neutrophils when the backbone was PTO modified (enhanced nuclease stability of the RNA). Unmodified ORNs showed no direct activation of neutrophils, even at high concentrations, despite their well-established TLR8 ligand activity.¹⁵

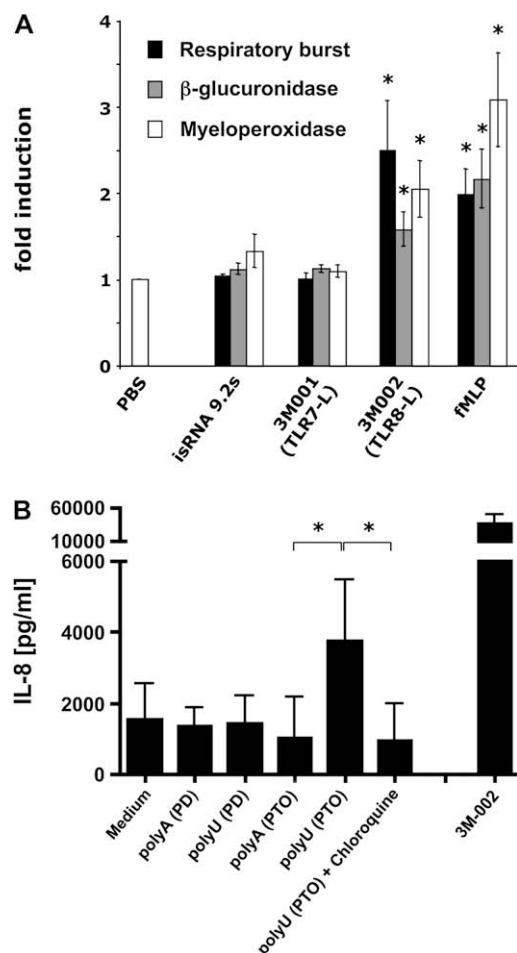


FIG 6. Activation of neutrophil function. **A**, Fold induction $\pm$ SEM of neutrophils stimulated with isRNA 9.2s (7 μ g/mL), 3M001, 3M002, or fMLP ($n = 4$; $*P < .05$ vs PBS) and of β -glucuronidase and myeloperoxidase activity ($n = 3$; $*P < .05$ vs PBS + fMLP boost). **B**, IL-8 expression of 4×10^5 neutrophils stimulated with 3M002, 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP)-complexed isRNA polyA, and polyU (7 μ g/mL) on a phosphodiester (PD) or PTO. The mean $\pm$ SEM is shown ($n = 3$). $*P < .05$.

In our study separation of direct from indirect effects on neutrophils was achieved by means of (1) isolation of highly purified neutrophils with MACS and (2) comparison of neutrophil activation in whole blood in the presence or absence of brefeldin A (blocking protein secretion). Elimination of indirect effects turned out to be highly relevant for the correct interpretation of direct neutrophil activation. This is highlighted by the fact that in the presence of other immune cell subsets (whole blood and preparation of PMNs), indirect activation of neutrophils was also observed for a small-molecule TLR7 ligand and for unmodified RNA oligonucleotides (isRNA 9.2s and polyU), both of which are inactive on purified neutrophils. Of note, correct analysis of indirect activation of neutrophils in mixed cell populations, such as whole blood or PMNs, required single neutrophil-based analysis of surface marker expression and cytokine synthesis. Reciprocal regulation of surface expression of CD62L and CD11b ensured that general changes of surface molecule expression do not affect the analysis (general shedding of proteins from the surface or increased background staining). Monocytes were found to contribute to indirect neutrophil activation by isRNA 9.2s.

In the literature few studies examined the expression and function of TLR7 and TLR8 in human primary neutrophils. Hayashi et al³¹ reported mRNA expression of TLR7 and TLR8 in neutrophils (PMNs containing 95% neutrophils) and activation of PMNs by the TLR7/8 ligand R-848. The same TLR mRNA expression profile was reported by Nagase et al,²⁷ although TLR7 levels were very low (further purification of neutrophils from PMNs) and TLR7 function was not studied. Another group demonstrated expression of TLR8 protein on PMNs (containing neutrophils and eosinophils), as determined by means of intracellular flow cytometry, and coactivation (priming for fMLP-stimulated biosynthesis of 5-lipoxygenase) of PMNs by the TLR7/8 ligand R-848 but not by imiquimod (preferential TLR7 activation); TLR7 expression was not examined in this study.³² Francois et al³³ studied inhibition of neutrophil apoptosis by different TLR agonists in whole blood. In their study low levels of TLR7 expression (Western blotting) were found in HLA-DR⁺ cell-depleted PMNs, but similar to our results, a selective TLR7 ligand (loxoribine) showed no functional activity. In contrast, other TLR ligands, including R848 (TLR7/8 ligand), inhibited apoptosis of neutrophils, suggesting that the TLR8 activity of R848 is responsible for this effect. Of note, eosinophils contained in PMNs lack HLA-DR but express TLR7^{34,35} and thus might be responsible for the minimal TLR7 expression detected in their study. Considering the different isolation protocols (PMNs with different numbers of contaminating eosinophils and other TLR7 expressing cells), all these results do not contradict our data, which for the first time provide clear evidence for selective expression and function of TLR8 but not TLR7 in human neutrophils.

The issue of whether neutrophils directly or indirectly respond to different TLR ligands is highly relevant for the predicted toxicity of such ligands. The activity of certain TLR ligands to directly license neutrophils for respiratory burst and degranulation limits the clinical utility of such compounds for systemic treatment. This toxicity issue is obvious for LPS, which on the one hand has useful immunologic properties, such as the induction of IL-12 and IFN- β in immune cells of the myeloid lineage, but at the same time directly activates human neutrophil function, as reflected by markers of activation on the cell surface, cytokine production, and respiratory burst.^{11,12} This is in agreement with the expression of high levels of TLR4 in purified neutrophils and neutrophil activation by LPS seen in our study.

Similar considerations might apply for TLR8. Recently, severe adverse events, including fever, headache, shivering, and lymphopenia, were reported in a phase IIa clinical trial in which the TLR7/8 ligand resiquimod (R-848) was administered systemically for the treatment of patients with chronic hepatitis C.³⁶ Based on our study, the situation is different for RNA ligands. Despite the well-established TLR8 activity in immune cells of the myeloid lineage (IL-12 induction in monocytes³⁷), a polyU ORN with the natural phosphodiester backbone was not sufficient to directly activate human neutrophils. The lack of this activity might be explained by high nuclease activity in neutrophils because stabilization of the backbone against nucleases restored direct activation of neutrophils by the polyU ORN. Therefore the use of unmodified RNA avoids the unwanted direct activation of neutrophils while maintaining the wanted TLR8-mediated activation of immune cells of the myeloid lineage. Unmodified RNA with complexation has been successfully used in several animal models and thus might form the basis for the development of potent TLR8 ligands with no adverse events caused by neutrophil

activation. In contrast, PTO-modified RNA, for the same reason as small-molecule ligands of TLR8, might enhance activation of circulating neutrophils and eosinophils and thus requires careful consideration when systemic treatment is intended. However, it should be noted that because of the use of cytochalasin B for costimulation of neutrophils *in vitro*, the degranulation activity of different stimuli, including RNA, in the physiologic situation *in vivo* cannot be safely predicted.

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Clinical implications: Data question the clinical utility of small-molecule or nuclease-stable RNA TLR8 ligands because of direct neutrophil activation. There is no such activation by unmodified isRNA, which, based on T_H1 induction, is a candidate for allergy treatment.

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