Neutrophils express distinct RNA receptors in a non-canonical way

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Capsule

Background
RNAs modulate immune responses. Neutrophils represent the major fraction of immune cells, but receptors by which neutrophils sense RNA are poorly characterized.

Results
Neutrophils and dHL-60 cells express the RNA receptors RIG-I, MDA-5 and TLR8. RIG-I and MDA-5 are localized in secretory vesicles.

Conclusion
Neutrophils express a distinct pattern of RNA receptors.

Significance
RNA receptors on neutrophils could have implications for RNA-based therapeutics.

RNAs are capable of modulating immune responses by binding to specific receptors. Neutrophils represent the major fraction of circulating immune cells, but receptors and mechanisms by which neutrophils sense RNA are poorly defined. Here we analyzed the mRNA and protein expression pattern and the subcellular localization of the RNA receptors RIG-I, MDA-5 and toll-like receptors (TLRs) 3, 7 and 8 in primary

Introduction
Aside from their role in transcription, RNAs have been shown to demonstrate immunomodulatory potential1. Immunostimulatory RNAs (isRNAs) are known to generate a potent immune response and are currently under intense investigation for new antiviral and anticancer treatment strategies2-6.
Recent evidence suggests that leukocytes recognize isRNA through cytosolic receptors\(^6,8\). The relevance of cytosolic RNA receptors is highlighted by the fact that small interfering RNA (siRNA) engage similar pathways\(^3,4\). Retinoic acid inducible gene I (RIG-I, also known as DDX58) was identified as a key candidate receptor for cytoplasmic viral RNA detection\(^1,9\). Together with melanoma differentiation-associated gene 5 (MDA-5) and laboratory of genetics and physiology 2 (LGP2), RIG-I forms the family of RIG-like helicases (RLH) based on their high similarities among helicase domains\(^9\). Activation of these receptors by RNA triggers production of type 1 interferons (IFN)\(^1,10\). For instance, RLHs interact with dsRNAs through their helicase domain, and dsRNA stimulation induces their ATP catalytic activity. The N-terminal caspase recruitment domains (CARDs) are then responsible for activating downstream signaling pathways that mediate, among others, dsRNA-induced IFN production\(^2\). 5’-triposphorylated and uncapped viral RNAs are recognized by RIG-I\(^6,7\). MDA-5 is thought to respond to uncapped, 5’-unmodified dsRNA, such as polyinosinic:polycytidylic acid (poly I:C)\(^6\).

One promising therapeutic approach is to stimulate RNA receptors with modified siRNA to knock-down cancer-related target genes and simultaneously boost the innate immune system\(^4\). Poeck et al. showed simultaneous knockdown of Bcl-2 and RIG-I-dependent IFN induction to be superior to either therapy alone utilizing an in vivo melanoma mouse model\(^1\). Moreover, messenger RNAs (mRNAs) have recently gained further attention as potential tools for gene therapy\(^11\). However, evidence about possible side effects of such RNA-based therapies is still scarce. Stimulating off-target cells in the circulation, such as neutrophils (polymorphonuclear leukocytes, PMNs), may limit the therapeutic potential of RNA in vivo.

Neutrophils represent the major fraction of circulating immune cells and provide the first cellular line of antibacterial and antifungal host defense, whereas their potential contribution to viral infections is rather enigmatic. Neutrophils sense pathogens through recognition of pathogen-associated molecular patterns (PAMPs) by innate pattern recognition receptors (PRR), such as the prototypical family of Toll-like receptors (TLRs). Previous studies found that primary neutrophils expressed a broad range of bacterial recognition TLRs, but lack expression of TLR3 and TLR7\(^12,13\). Notably, TLR8 was found to be expressed and was functional in mediating neutrophil effector responses\(^13\). A further study showed that neutrophils express RNA receptors of the RLH family and are capable of responding to intracellularly delivered polyinosinic:polycytidylic acid (poly I:C), suggesting that neutrophils could recognize viral RNA through helicases and may play a role in antiviral immunity\(^21\). Aside from these studies, the expression, subcellular localization and functionality of RNA receptors in primary neutrophils and neutrophil-like immortalized differentiated HL-60 (dHL-60) cells are still poorly defined.

### Experimental Procedures

**Isolation and culture of human neutrophils.** Peripheral blood was obtained from healthy volunteers in accordance with the institutional review board and approved by the Ethical committee of the LMU University of Munich and the University of Tübingen. After ammonium chloride erythrocyte lysis, human neutrophils and peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll-Hypaque density gradient centrifugation of heparinized blood from healthy volunteers. Neutrophils were additionally positive-enriched using anti-CD16 microbeads (Magnetic Cell Sorting, MiltenyiBiotec). Evaluation of the neutrophil population with FACS showed purity of 85% (SEM 2.5%) for isolation with Ficoll-Hypaque density gradient centrifugation and 95% (SEM 2%) for additional cell sorting with CD16 microbeads. Cells were cultured in RPMI 1640 (Biochrom) supplemented with 10% FCS, 10mM HEPES (from Sigma-Aldrich), 1.5mM L-glutamine, 100U/ml penicillin, and 100 µg/ml streptomycin (all from PAA Laboratories) in 96-well round-bottom plates for stimulation experiments.

**Isolation of murine neutrophils.** To isolate murine neutrophils, bone marrow cells or splenic cells from healthy C57BL/6 mice were obtained and suspended in PBS and washed. Then, neutrophils were isolated as described above. Purity of neutrophil populations analyzed with FACS was 93% (SEM 3%). Animal studies were approved by the local regulatory agency (Regierung von Oberbayern, Munich, Germany).

**HL-60 cells.** HL-60 cells were passaged at three-day intervals to maintain exponential growth, and all experiments were performed with cells between passage 35 and 60. Differentiated
HL-60 (dHL-60) cells were obtained by treatment of 5x10^5 HL-60 cells/ml with 1.25% DMSO for 7 days as previously described. The obtained cells were neutrophilic based on microscopical and flow cytometrical criteria as described previously.

**Subcellular fractionation of neutrophils.**
Subcellular fractionation of neutrophils was performed by nitrogen cavitation and sedimentation of the postnuclear supernatant on a 4-layer Percoll density gradients, as described previously in detail by Clemmensen et al. In brief, neutrophils isolated from peripheral blood were resuspended in disruption buffer (100 mM KCl, 3 mM NaCl, 1 mM Na₂ATP 3.5 mM MgCl₂, 10 mM PIPES, pH 7.2) with a protease inhibitor cocktail added as described by the manufacturer (11836153001, Roche). Neutrophils were disrupted by nitrogen cavitation at 600 psi for 5 min and collected in 1.5 mM EGTA. Further, the cavitate was centrifuged at 400 g for 15 min to remove nuclei and unbroken cells and the supernatant was added to a Percoll solution with a density of 1.11 g/ml at a ratio of 1/1, resulting in a final density of 1.055. Next, nine milliliters were layered on top of a two-layer Percoll gradient (9 ml with density of 1.12 and 9 ml of density 1.09) in order to separate azurophil, specific, and gelatinase granules. Then, 9 ml of Percoll solution with density 1.03 was layered on top to create a flotation medium for separation of plasma membranes/cytosol and secretory vesicles. pH was adjusted to 7.0 by HCl. The four-layer gradient was centrifuged 20 000 g for 40 min., resulting in 5 major bands, the α-band enriched in primary/azurophil granules [marker: myeloperoxidase (MPO)], the β₁-band enriched in secondary/specific granules [marker: neutrophil gelatinase-associated lipocalin (NGAL)], the β₂-band enriched in tertiary/gelatinase granules [marker: gelatinase/MMP9], a γ₁-band enriched in secretory vesicles (marker: albumin), and the γ₂ band containing plasma membranes [marker: human leukocyte antigen (HLA)]. Samples were subjected to ELISA analysis or to SDS–PAGE and Western blot analysis. MPO, NGAL, MMP-9, HSA, and HLA were quantified in each fraction by ELISA and used as marker proteins for azurophil granule, specific granules, gelatinase granules, secretory vesicles, and plasma membrane, respectively. Where indicated, a three-layer percoll gradient was used.

**Western Blot.** Neutrophil fractions were separated on NuPAGE 4-12% Bis-Tris gels (Invitrogen), and immunoblotting was performed by standard procedures using XCell II blotting chambers (Invitrogen). After blocking, primary antibody against RIG-I or MDA-5 (both from Abcam) was incubated overnight. Blots were processed by using alkaline phosphatase conjugated secondary antibodies (Millipore) and BCIP / NBT solution (Millipore). Semiquantitative analysis was performed with the Quantity One software system (Bio-Rad).

**Stimulation of cells.** All TLR-ligands were titrated to define optimal stimulating conditions (activation of cells without affecting cell survival/apoptosis). Neutrophils were stimulated with LPS from Escherichia coli (Sigma-Aldrich), PMA (Sigma-Aldrich), 3M-003 (3M-Pharmaceutical) and poly(I:C) (Sigma-Aldrich). Poly(I:C) was subjected to 10 precipitations and washed in ethanol to remove endotoxin. Final concentrations of TLR-ligands used for stimulation are found throughout the text. For stimulation of TLR7 and TLR8, we used our previously described isRNA 9.2antisense (5'-UUGAAGGACAGGUAAAGCUdTdTT-3') synthesized by Eurogentec Germany GmbH. Upon stimulation, we complexed nucleic acid (200ng) using the poly-cationic polypeptide poly-L-arginine (P7762 by Sigma-Aldrich). As a control, we used non-stimulatory poly-A-RNA repeats, also purchased at Eurogentec Germany GmbH. Details on the synthesized stimulants are given in Table 2.

**Generation of 3p-RNA.** For generation of in vitro transcribed double-stranded RNA the DNA templates of the sense and antisense strands were transcribed for six hours in separate reactions. An extra guanosine was added at the 5’ end to both the sense and the antisense strands in order to transcribe with T7 RNA polymerase. The reactions were then mixed and incubated overnight at 37°C to anneal the transcribed RNA strands. The DNA template was digested using DNase-I (Ambion) and subsequently RNAs were purified by phenol:chloroform extraction and alcohol precipitation. Excess salts and NTPs were removed by passing the RNAs through a Mini Quick SpinTM oligo Column (Roche). In order to test the immunstimulatory potential of 3p-RNA, T2-cells were incubated for 24 hours and MHC-I expression was compared to OH-siRNA with the same nucleotide sequence. RNA isolation, cDNA synthesis, and quantitative real-time PCR. For RNA preparation cells were washed with 0.9% sodium chloride, and cell pellets were lysed in 300 μl of...
lysis buffer from the MagnaPure LC mRNA isolation kit I supplemented with 1% DTT (Roche) and frozen at 80°C until further handling. Preparation of mRNA was performed with the MagnaPure-LC device using the mRNA-I standard protocol. An aliquot of 8.2 μl of RNA was reverse-transcribed using avian myeloblastosis virus-reverse transcriptase and oligo(dT) as primer (First Strand cDNA synthesis kit; Roche) according to the manufacturer’s protocol in a thermocycler. The reaction mix was diluted to a final volume of 0.5 ml and stored at 20°C until PCR analysis.

Parameter-specific primer sets optimized for the LightCycler (Roche) were developed and purchased from SEARCH-LC (Table 1). The PCR was performed with the LightCyclerFastStart DNA SYBR Green I kit (Roche) according to the protocol provided in the parameter-specific kits. The transcript numbers were normalized according to the expression of TBP (Tata-Box-binding protein) per microliter of cDNA.

Flow cytometry. Flow cytometric data were obtained on a FACSCalibur flow cytometer and analyzed with CellQuest software (BD Biosciences). Staining was performed following standard procedures. The mAbs used were anti-CD62L (FITC, BioLegend), anti-CD11b (APC, BioLegend), anti-RIG-I (Santa Cruz), anti-MDA-5 (Abcam), anti-TLR7 (FITC, Santa Cruz), anti-TLR8 (PE, Imgenex), Goat anti-Rabbit 2ndAb (FITC, Imgenex) and Donkey anti-Rat 2ndAb (FITC, Imgenex). Appropriate isotype controls were used for all applied detection antibodies. For intracellular cytokine detection cells were preincubated with Brefeldin A at 10 μg/ml. After surface staining, cells were fixed with PFA 2% and subsequently permeabilized with saponin 0.1% and then stained for intracellular proteins.

Confocal microscopy. For visualization of TLR8, MDA-5 and RIG-I, the samples were incubated with rabbit anti-human TLR8 (Imgenex, Clone: 44C143), MDA-5 (Abcam, polyclonal) or RIG-I antibodies (Santa Cruz, polyclonal). The primary antibodies were detected in CLSM by means of a secondary anti-rabbit Alexa Fluor 555 antibody (Invitrogen). DNA was stained with DAPI (Sigma-Aldrich) and concanavalin A Alexa 488 conjugate (Invitrogen) was used for detecting glycoconjugates in the cytoplasm. The specimens were analyzed with a CLSM (Olympus IX 51).

Immuno-Transmission electron microscopy (TEM). Isolated peripheral blood neutrophils were fixed, washed with PBS, permeabilized and blocked (10% normal goat serum, 10mM glycine, 0.2% Tween 20 in diluent containing 0.5% bovine serum albumin and 0.5% Triton X-100 in PBS). For visualisation of RIG-I, the grids were incubated with anti-RIG-I (Santa Cruz) and a gold-conjugated secondary antibody (ab27237, Abcam, Cambridge, UK, gold sphere diameter 5 nm). Finally, the grids were stained with 1% uranyl acetate (Sigma-Aldrich, Vienna, Austria). Negative controls were obtained by omitting the primary antibody.

RNA-FITC-labeling. RNA was labeled with a NHS-Fluorescein labeling kit (Thermo Scientific) according to manufacturer’s instructions. Briefly, 3p-RNA was incubated with the NHS-Fluorescein dye for two hours on ice. Excessive dye was removed by Zeba Desalt Spin Columns (Thermo Scientific) according to manufacturer’s instructions. Degree of Fluorescein labeling was determined by photometry.

ELISA. ELISA Kits by Invitrogen were used to quantify TNF-alpha levels in cell culture supernatants 40 minutes after stimulation. Protocols were performed according to the manufacturer’s recommendations.

Statistical analysis. Data are depicted as mean ± SEM. Statistical significance of differences was determined by paired two-tailed Student’s t test. In all tests, differences were considered significant at \( P<0.05 \).

Results

TLR8, MDA-5 and RIG-I are expressed by both primary neutrophils and immortalized neutrophil-like dHL-60 cells. We analyzed TLR3, TLR7, TLR8, MDA-5 and RIG-I expression at the mRNA and protein level in isolated primary neutrophils using RT-PCR, flow cytometry and confocal microscopy (Figure 1). Quantitative RT-PCR showed significant TLR8, MDA-5 and RIG-I expression levels with the highest mRNA expression for TLR8, while TLR3 or TLR7 expression was below the detection limit (Figure 1A, upper panel). In line with mRNA studies, we found a robust protein expression of TLR8, but could not detect any expression of TLR3 and TLR7 by human neutrophils at the protein level, (Figure 1A, lower panel). In contrast to the low/moderate mRNA expression levels, protein expression of MDA-5 and RIG-I in neutrophils were higher...
than TLR8 expression. Comparing intracellular/cytoplasmic and cell surface/membranous expression sites, MDA-5 and RIG-I protein expression was detectable both in cytoplasmic as well as in cell surface/membrane-associated compartments, whereas TLR8 seemed to be mainly restricted to the cytoplasm (Figure 1A, lower panel and Figure 1C, merged images). Confocal microscopy showed a more pronounced intracellular staining pattern for MDA-5 and RIG-I compared to flow cytometry (Figure 1A, lower panel and Figure 1C).

Next we tested whether neutrophil stimulation by protein kinase C (PKC) activation changes the expression pattern / cellular distribution of the analyzed RNA receptors. Stimulation of neutrophils with the PKC activator phorbol 12-myristate 13-acetate (PMA, 40 ng/ml) led to an increase of intracellularly detectable MDA-5 receptor pools, whereas RIG-I surface expression levels decreased upon PMA stimulation (Figure 1A, lower panel), suggesting cell activation-induced redistribution/translocation mechanisms.

Due to the limited lifespan of primary neutrophils ex vivo/in vitro, differentiated HL-60 (dHL-60) cells, a leukemia cell line with characteristics and differentiation markers similar to human granulocytes, are frequently used to study neutrophil-like responses in cell culture conditions. Stimulation of HL-60 cells with DMSO results in differentiation towards mature granulocytic cells. We compared the expression of RNA receptors in undifferentiated and DMSO-differentiated HL-60 cells. Real-time PCR analysis showed that dHL-60 cells expressed a similar RNA receptor repertoire as primary human neutrophils did, with the exception of positive expression of TLR3 detectable in dHL-60 cells, which was completely absent in primary neutrophils (Figure 1C). Further studies showed that differentiation of HL-60 cells with DMSO decreased mRNA expression levels of TLR3 and TLR8, while expression levels of MDA-5 and RIG-I increased. Protein expression analysis by flow cytometry demonstrated TLR8, MDA-5 and RIG-I expression in HL-60 cells. Similar to human neutrophils, TLR3 and TLR7 were completely absent at the protein level. Intracellular flow cytometric stainings indicated that cytoplasmic TLR8, MDA-5 and RIG-I expression levels did not change upon DMSO differentiation. In contrast, surface MDA-5 and RIG-I expression levels were decreased or nearly lost, respectively, after DMSO differentiation.

These studies demonstrate that both primary human neutrophils and neutrophil-like dHL-60 cells express a distinct set of RNA receptors, namely TLR8, MDA-5 and RIG-I.

In depth-characterization and subcellular localization of RIG-I and MDA-5 in neutrophils. In contrast to the intracellular localization reported for other cell types, we initially observed that RIG-I and MDA-5 protein expression in human neutrophils seemed not to be restricted to intracellular sites, but were also detectable at the cell surface / membrane associated (Figures 1A, lower panel and Figure 1C, lower panel). Using flow cytometry, isotype-control-corrected mean fluorescence intensities of MDA-5 and RIG-I were equal or higher, respectively, on the cell surface compared to intracellular pools, independent of the blood donor or cell fixation/permeabilization protocols used (Figure 1A and data not shown). We used two different specific antibodies with distinct RIG-I binding sites in order to reduce the risk for unspecific binding. Both antibodies yielded similar staining results. Comparing human with murine neutrophils, we found a substantially higher RIG-I surface expression in human versus murine neutrophils with RIG-I being expressed mainly in the cytosol of murine neutrophils (Figure 2).

Since these observations were, however, mainly based on flow cytometric detection assays, we decided to study the subcellular localization of these RLH proteins in neutrophils in more depth comparing different protein detection and localization methods. For this purpose, previously established subcellular fractionation methods were performed. The subcellular components were stained for RIG-I or MDA-5 and were analyzed separately via western blotting (Figure 3A, B). As positive control, autologous PBMCs were utilized that are well known to express cytosolic RIG-I and MDA-5. These studies demonstrated that human neutrophils expressed both RIG-I and MDA-5 with a similar molecular weight as PBMCs did (Figure 3B). Remarkably, RIG-I and MDA-5 in neutrophils were detectable in fractions characteristic for secretory vesicles and plasma membranes, whereas the RLH proteins were absent in primary/azurophilic, secondary/specific or tertiary/gelatinase granule fractions. Besides secretory vesicles, the proteins were detectable in cytosolic fractions. In Immuno-TEM, RIG-I proteins were frequently associated with membranous / vesicle-like structures (Figure 3C, red arrows). When viewed
in combination, these studies provide evidence that, besides the well-known cytoplasmic expression sites reported for a variety of cell types, human neutrophils store the RLH RIG-I and MDA-5 in secretory vesicles. Since secretory vesicles communicate and fuse with the plasma membrane, we speculate that the vesicular localization of the RLHs in neutrophils could explain their appearance on the cell surface, an issue necessitating further investigation.

Finally, we investigated whether cell membrane-associated RIG-I binds to its ligand 3p-RNA and mediates downstream activation. We designed 3p-RNA containing a triphosphate chemical modification at the 5'-end of both strands as described previously. Confocal microscopy provided evidence that FITC-labeled 3p-RNA, at least partially, colocalized at the cell surface of neutrophils with RIG-I (Figure 4A). Therefore, we asked whether binding of 3p-RNA to membrane-associated RIG-I triggers neutrophil activation. We used LPS (ligand for TLR4), R848 (ligand for TLR7/TLR8), 3p-RNA, a double strand siRNA (with identical sequence but without the triphosphate modification of 3p-RNA), isRNA 9.2sense and lipofectamine or poly-L-arginine (RNA based ligand for TLR7/TLR8) for complexation and as controls (see also Table 2 for details). Neutrophil activation was analyzed by CD62L shedding (Figure 4B), CD11b upregulation (Figure 4B) and TNF-α production (Figure 4C). Both LPS and R848 dose-dependently decreased CD62L surface expression, triggered CD11b upregulation and elicited TNF-α production. While LPS activated neutrophils at a concentration of 0.1µg/ml, cell activation elicited by R848 required higher concentrations. Uncomplexed 3p-RNA or siRNA had no effects on neutrophil activation, while complexed RNAs has a slight, but non-significant, effect at higher concentrations on CD62L shedding (Figures 4B and data not shown). In particular, isRNA 9.2sense complexed with poly-L-arginine triggered CD62L shedding, but this effect was also observed for poly-L-arginine alone (Figure 4B, lower panel).

Discussion

Our studies demonstrate that both primary human neutrophils and immortalized dHL-60 cells express the RNA receptors TLR8, RIG-I and MDA-5 at the mRNA and protein level. These studies further demonstrate that both RLH RIG-I and MDA-5 are stored in secretory vesicles in neutrophils, suggesting that circulating granulocytes carry an intracellular reservoir of RNA receptors, reminiscent of other vesicle-stored receptors, such as CR1/CD35, Mac-1, CD13 and CD16. Further studies are required to understand the subcellular regulation and functional role of these receptors in the context of RNA recognition and their relevance for in vivo disease conditions.

Neutrophils are a substantial part of the innate immune system and recognize pathogens through PRRs, such as TLRs. These professional phagocytes are usually the first cell population to be involved in an immune response and subsequently modulate the response not only due to their potential to directly eliminate pathogens by phagocytosis, but also by the induction of modulatory chemokines / cytokines such as CXCL8 (IL-8) and others. Previous studies assessing TLRs in granulocytes, demonstrated that primary neutrophils express TLR7 and TLR8, respectively, and respond to TLR8 ligands functionally12,13. Beyond TLRs, Ekman and Cardell showed that neutrophils express functionally active non-TLR PRRs, particularly NOD-like receptors (NLRs)20. Despite these studies, our understanding of PRRs mediating RNA recognition in these immune cells is still limited. In this study, we comprehensively characterized TLR and non-TLR RNA receptors in primary neutrophils and immortalized neutrophilic-like cells at the mRNA and protein level. By using this approach we detected no protein expression of TLR3 and TLR7 in human neutrophils, regardless of their state of activation. TLR3 mRNA was expressed in untreated HL-60 cells but decreased upon DMSO differentiation. As for human neutrophils, TLR7 was not expressed in HL-60 cells. On the other hand, our studies provided evidence for a substantial expression of TLR8 in human neutrophils, which is in line with a previous study by Janke et al13. In HL-60 cells TLR8 mRNA expression decreased upon differentiation. We have currently no explanation for this phenomenon. When viewed in combination, our studies indicate that HL-60 cells express, with the exception of TLR3 mRNA, a similar RNA receptor expression pattern as primary human neutrophils do, and may therefore represent a useful tool in studying RNA receptor expression and regulation in an immortalized cell line.

Canonically, RIG-I is regarded as an intracellular RNA receptor, since cytosolic
expression has been reported for dendritic cells, monocytes, monocytes/PBMCs and other cell types \(^1,9,18,22\). In line with these previously reported cell types, we detected RIG-I protein expression in cytosolic neutrophil fractions at a similar molecular weight as in PBMCs that are well-known to express cytosolic RIG-I. Unexpectedly, we also observed that RIG-I was detectable at the cell surface of human, but not murine, neutrophils. We currently have no explanation for this interspecies discrepancy in RIG-I surface expression, but found this RIG-I staining pattern consistently in both murine bone-marrow- and peripheral blood-derived neutrophils independent of the isolation procedure. Since these initial surface expression findings were, however, mainly based on flow cytometric assays, that critically depend on antibody specificity and accessibility, we studied the expression and subcellular localization of the RLH proteins RIG-I and MDA-5 in greater detail using subcellular fractionation and immunoblotting. These studies demonstrated that both RIG-I and MDA-5 were localized in secretory vesicles, an easily mobilizable compartment, and in cytoplasmic fractions of human neutrophils. In both compartments, the RLH proteins were detected at a similar molecular weight as in PBMCs. Immuno-TEM analyses supported these findings by showing that RIG-I protein in neutrophils was, at least partially, associated to vesicle-like structures. Based on these studies, it is conceivable that RIG-I and MDA-5, in analogy to other secretory vesicle-associated receptors, such as complement receptors 1 (CD35) or (CD11b/CD18, Mac-1), shuttles between intracellular pools and the plasma membrane. The observed surface expression of RIG-I and MDA-5 by flow cytometry could be due to plasma membrane reorganization events after fusion with secretory vesicles, a process occurring during neutrophil activation or after cell isolation procedures. To define the precise subcellular localization and translocation/redistribution mechanisms of RLHs in neutrophil vesicles in greater detail, methods to separate plasma membrane vesicles from secretory vesicles such as free-flow electrophoresis followed by proteomic approaches will be required.

Effects of TLR7 and TLR8 ligands on granulocytes have recently been investigated by Janke et al\(^13\). TLR8 was found to be highly expressed by neutrophils, whereas it was absent in eosinophils. In the latter study, isolated human neutrophils were stimulated with isRNA or R848 and neutrophils, but not eosinophils, could be activated through the TLR8 downstream pathway. However, this effect was abolished when the RNA-backbone of isRNA consisted not of nuclease-stable phosphothioate but of regular phosphodiester bindings. Our findings support the data from Janke et al., since we were unable to observe neutrophil activation using unstable isRNA with a phosphodiester backbone compared to robust activation elicited by the TLR7/8-ligand R848. Taken together, these data highlight the notion that TLR8 ligands, including nuclease-stable RNA, could bear limitations for clinical applicability of RNA-based therapies due to potential off-target effects.

In a previous study by Hornung et al, 5’-triphosphate RNA was reported as a potent inducer of IFN-α in human monocytes and plasmacytoid dendritic cells (pDCs)\(^6\). The latter study used in vitro transcription to generate a dsRNA oligonucleotide with an overhang of one nucleotide at the 5’ position. The two single-stranded oligonucleotides and the double-stranded oligonucleotide induced comparable levels of IFN-α in monocytes, but not in pDCs. Cleavage of the 5’-overhang (including the 5’-triphosphate) of the dsRNA, or dephosphorylation of the 5’ end completely abrogated the IFN-α response. The authors also observed that plasmacytoid dendritic cells (pDCs) showed no decrease in IFN-α production when oligonucleotides were dephosphorylated. When viewed in combination, these results of Hornung et al. suggested that the 5’-triphosphate is at least one well-defined structural feature responsible for IFN-α-inducing activity of in vitro–transcribed RNA in monocytes, and that a 5’-triphosphate confers IFN-α–inducing activity to both single-stranded RNA (ssRNA) and dsRNA. In addition, they demonstrated a direct binding of RIG-I by 5’-triphosphate RNA in human embryonic kidney (HEK) 293 cells. In the light of these findings, the expression of RIG-I in human neutrophils and the functional response of RIG-I on human neutrophils was investigated in our study by treating isolated human neutrophils with 5’-triphosphate RNA, and monitoring RIG-I co-localization and neutrophil activation. We obtained evidence on co-localization of RIG-I and its labeled ligand 3p-RNA on the cell surface on neutrophils by confocal microscopy, thereby taking the possibility of early RNA degradation into account. However, the conclusions based on these findings are limited since they rely solely...
on confocal laser scanning microscopy, warranting future in-depth analyses to characterize the interaction of RIG-I and 3p-RNA in primary human neutrophils in detail. Despite the observed ligand-receptor colocalization at the cell surface, we were unable to detect specific activation of neutrophils by the RIG-I ligand 5′-triphosphate RNA under the experimental conditions used in this study. Of note, we did not study the effects of RIG-I ligands on gene expression, which might be more sensitive. A previous study nicely demonstrated that transfection with polyI:C, a synthetic mimetic of viral dsRNA activating TLR3, MDA-5 and/or RIG-I in a cell-type and length-specific manner, elicited an orchestrated immunoregulatory and antiviral gene expression program in primary neutrophils. While our study did not include polyI:C transfection or the analysis of antiviral gene expression patterns, both studies support the notion that RLH ligands, in particular polyI:C and 3p-RNA, are unable to elicit the secretion of pro-inflammatory cytokines despite increased mRNA transcripts, as found by Tamassia et al. The underlying reasons remain to be investigated and are discussed in the latter study in more detail.

Based on previous studies showing that neutrophils express non-canonical receptors at their cell surface, as demonstrated for the HIV co-receptor CCR5, and employ these receptors as ligand binding/scavenging instead of signaling receptors, we tempt to speculate that secretory vesicle-derived RIG-I in neutrophils could serve as RNA binding and inactivation receptor at sites of neutrophilic inflammation, a hypothesis that remains to be tested using in vitro and in vivo modeling models. Overall, the pathophysiological relevance of RNA receptors expressed by neutrophils in the modulation of immune responses remains a subject for future investigation.
REFERENCES

1. Hornung, V. et al. Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7. Nat. Med. 11, 263-270 (2005).
2. Castanotto, D. & Rossi, J.J. The promises and pitfalls of RNA-interference-based therapeutics. Nature 457, 426-433 (2009).
3. Poeck, H. et al. 5'-Triphosphate-siRNA: turning gene silencing and Rig-I activation against melanoma. Nat. Med. 14, 1256-1263 (2008).
4. Schlee, M., Hornung, V. & Hartmann, G. siRNA and isRNA: two edges of one sword. Mol. Ther. 14, 463-470 (2006).
5. Hornung, V., Barchet, W., Schlee, M. & Hartmann, G. RNA recognition via TLR7 and TLR8. HandbExpPharmacol 71, 86 (2008). doi:10.1007/978-3-540-72167-3_4
6. Hornung, V. et al. 5'-Triphosphate RNA is the ligand for RIG-I. Science 314, 994-997 (2006).
7. Kato, H. et al. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature 441, 101-105 (2006).
8. Kato, H. et al. Cell type-specific involvement of RIG-I in antiviral response. Immunity 23, 19-28 (2005).
9. Takeuchi, O. & Akira, S. MDA5/RIG-I and virus recognition. Curr. Opin. Immunol. 20, 17-22 (2008).
10. Berger, M. et al. TLR8-driven IL-12-dependent reciprocal and synergistic activation of NK cells and monocytes by immunostimulatory RNA. J. Immunother. 32, 262-271 (2009).
11. Kormann, M.S.D. et al. Expression of therapeutic proteins after delivery of chemically modified mRNA in mice. Nat. Biotechnol. 29, 154-157 (2011).
12. Hayashi, F., Means, T.K. & Luster, A.D. Toll-like receptors stimulate human neutrophil function. Blood 102, 2660-2669 (2003).
13. Janke, M. et al. Selective and direct activation of human neutrophils but not eosinophils by Toll-like receptor 8. J. Allergy Clin. Immunol. 123, 1026-1033 (2009).
14. Novak-Hofer, I., Bläuenstein, P. & Schubiger, P.A. Regulation of the cell surface expression of a nonspecific cross-reacting antigen variant during differentiation of HL-60 cells. Cancer Res. 50, 7437-7443 (1990).
15. Blair, O.C., Carbone, R. & Sartorelli, A.C. Differentiation of HL-60 promyelocytic leukemia cells: simultaneous determination of phagocytic activity and cell cycle distribution by flow cytometry. Cytometry 7, 171-177 (1986).
16. Clemmensen, S.N. et al. Alpha-1-antitrypsin is produced by human neutrophil granulocytes and their precursors and liberated during granule exocytosis. Eur. J. Haematol. 86, 517-530 (2011).
17. Udby, L. & Borregaard, N. Subcellular fractionation of human neutrophils and analysis of subcellular markers. Methods Mol. Biol. 412, 35-56 (2007).
18. Wörnle, M. et al. Novel role of toll-like receptor 3, RIG-I and MDA5 in poly(I:C) RNA-induced mesothelial inflammation. Mol. Cell. Biochem. 322, 193-206 (2009).
19. Jeong, E. & Lee, J.Y. Intrinsic and Extrinsic Regulation of Innate Immune Receptors. Yonsei Med. J 52, 379 (2011).
20. Ekman, A. & Cardell, L.O. The expression and function of Nod-like receptors in neutrophils. Immunology 130, 55-63 (2010).
21. Tamassia, N. et al. Activation of an immunoregulatory and antiviral gene expression program in poly(I:C)-transfected human neutrophils. J. Immunol. 181, 6563-6573 (2008).
22. Kawai, T. & Akira, S. Toll-like Receptors and Their Crosstalk with Other Innate Receptors in Infection and Immunity. Immunity 34, 637-650 (2011).
23. Ariel, A. et al. Apoptotic neutrophils and T cells sequester chemokines during immune response resolution through modulation of CCR5 expression. Nat. Immunol. 7, 1209-1216 (2006).
FOOTNOTES

Authorship. BM performed and designed research, analyzed data and wrote the paper, HC, BM, ML, LH performed research and analyzed data, MV designed and performed research, HA, MB and HL analyzed data and performed research, RN, WA and MK analyzed data, NO and vSD wrote the paper, KR designed research, HD designed research and wrote the paper.

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FIGURE LEGENDS

Fig.1. Expression of RNA receptors by neutrophils and HL-60 cells.
(A) Upper panel: Isolated neutrophils were analyzed by quantitative real-time RT-PCR for the expression profile of TLR3, TLR7, TLR8, RIG-I and MDA-5. TBP (Tata-Box-binding protein) was used as a reference for expression. The mean (±SEM) of 6 different healthy donors is shown. Lower panel: Intracellular (white bars) and cell surface (grey bars) expression of TLR3, TLR7, TLR8, MDA-5 and RIG-I in isolated human neutrophils analyzed by flow cytometry. Striped bars indicate experiments where isolated neutrophils were stimulated for 10 minutes with 40 ng/ml of PMA at 37°C. *p<0.05. The mean (±SEM) of 6 different healthy donors is shown.
(B) Upper panel: HL60 cells were either cultured untreated or treated with DMSO for 5 days and analyzed by quantitative real-time PCR for the expression profile of TLRs, RIG-I, and MDA5. TBP was used as a reference for expression. *p<0.05. Lower panel: Intracellular (white bars) and cell surface (grey bars) expression of TLR3, TLR7, TLR8, MDA-5 and RIG-I in HL-60 cells cultured untreated or treated with DMSO for 5 days analyzed by flow cytometry. Striped bars indicate experiments with DMSO treatment. *p<0.05.
(C) Subcellular localization for TLR8, MDA-5 and RIG-I (red), DAPI (DNA, blue) and cytosolic proteins (Concanavalin A, green) are shown using confocal microscopy of isolated neutrophils.

Fig. 2. Localization of RIG-I in human and murine neutrophils.
Cytosolic and membranous RIG-I expression in human and murine neutrophils. For both experiments, a representative histogram out of 6 independent experiments is shown. Intracellular flow cytometry was performed using cell permeabilization as described in detail in the methods section of the manuscript.

Fig. 3. Subcellular localization of RIG-I in human neutrophils.
(A) Resting neutrophils were gently disrupted using nitrogen cavitation method followed by 4-layer gradient subcellular fractionation. Distribution profiles of MPO (marker of azurophil granules), NGAL (marker of secondary/specific granules), MMP-9 (marker of tertiary granules), HSA (marker of secretory vesicles) and MICA/HLA (marker of plasma membranes), as measured by ELISA. y-Axis represents an arbitrary scale where the highest measured value of each protein is normalized to 1. (B) Western blot analysis of neutrophil subcellular fractions in autologous neutrophils (PMN) and PBMCs. Upper panel: four-layer fractionation and MDA-5 immunoblotting, middle panel: four-layer fractionation and RIG-I immunoblotting. SV: secretory vesicles, PM: plasma membranes, 3°: tertiary granules, 2°: tertiary granules, 1°: primary granules. Lower panel: three-layer fractionation and RIG-I immunoblotting. Cyt.: Cytosol.
(C) Immuno-transmission electron microscopy (TEM): RIG-I immuno-labeling with 5 nm gold particles. Arrows indicate RIG-I staining. The red arrows mark RIG-I associated to a vesicle-like structure.

Fig. 4. Effect of RNAs on neutrophils.
(A) Neutrophils were isolated and stained for colocalization of RIG-I (red), DAPI (DNA, blue) and FITC-labeled 3p-RNA (green).

(B) Isolated neutrophils were stimulated at the indicated concentrations with LPS, R848, complexed 3p-RNA (complexed with lipofectamine), non-complexed 3p-RNA (without lipofectamine), siRNA (complexed with lipofectamine), isRNA9.2s (complexed with poly-L-arginine), poly-A-RNA (complexed with poly-L-arginine), lipofectamine only or poly-L-arginine only at the indicated concentrations. Forty minutes after stimulation, CD62L shedding and CD11b upregulation were examined by flow cytometry. The mean (±SEM) of three donors is shown.

(C) Isolated neutrophils were stimulated for 40 minutes with the indicated reagents and supernatants were analyzed for TNF-α production using ELISA (values are shown in pg/ml). siRNA was complexed with poly-L-arginine, 3p-RNA was complexed with lipofectamine.
### Table 1.

| Gene | Sequence | Reverse |
|------|----------|---------|
| **Forward** | **Reverse** |
| TLR3  | TCCCAAGCCTTCAACGACTG | TGGGTGAAGGAGAGCTATCCACA |
| TLR7  | TTACCTGGATGGAAACCAGCTAC | TCAAGGCTGAGAAGCTGTAAGCTA |
| TLR8  | GAGAGCGGAGACAAAAACGTTC | TGTCGATGTGGCAATCC |
| Mda-5 | ATGTGGCAGCAAGAGCATCC | GGTAAGGCCCTGAGCTGGAGTT |
Table 2.

**Stimulants synthesized by Eurogentec:**

| Stimulant            | Target | Detail                                      |
|----------------------|--------|---------------------------------------------|
| Poly-A-Control       |        | Sequence: 5`-AAAAAAAAAAAAAAAAAAAAA-3`       |
| RNA9.2s-unmodified   | TLR7/8 | Sequence: 5`-AGCUUAACCUGUCCUUCA-3`          |

**Stimulants synthesized by Ambion:**

| Stimulant            | Target | Detail                                      |
|----------------------|--------|---------------------------------------------|
| siRNA(OHBcl2_2)      | RIG-I  | Sequence(Sense): 5`-GCAUGCGACCUCUUGAUUUGAUU-3`  |
|                      |        | Sequence(antisense): 5`-UCAAACAGAGGUGCGCAUGUUU-3` |
| siRNA-Mismatch (Control) |   | Sequence(Sense): 5`-UUCUCCGAAACGUUCGACGUUU-3`  |
|                      |        | Sequence(antisense): 5`-ACGUGACACGUUCGGAGAAUU-3`  |

**In house-synthesized stimulants:**

| Stimulant            | Target | Detail                                      |
|----------------------|--------|---------------------------------------------|
| 3p-RNA               | RIG-I  | Sequence(Sense): 5`-pppGCAUGCGACCUCUUGAUUUGAUU-3`  |
|                      |        | Sequence(antisense): 5`-pppUCAAACAGAGGUGCGCAUGUUU-3` |
| 3p-RNA (Control)     |        | Sequence(Sense): 5`-pppUUCUCCGAAACGUUCGACGUUU-3`  |
|                      |        | Sequence(antisense): 5`-pppACGUGACACGUUCGGAGAAUU-3`  |
FIGURES

A. Relative target gene expression

B. Relative target gene expression

C. 

Figure 1

- Intracellular
- Surface
- Intracellular PMA
- Surface PMA

- Intracellular DMSO
- Surface DMSO
Neutrophils express distinct RNA receptors in a non-canonical way
Michael Berger, Chin-Yuan Hsieh, Martina Bakele, Veronica Marcos, Nikolaus Rieber, Michael Kormann, Lauren Mays, Laura Hofer, Olaf Neth, Ljubomir Vitkov, Wolf Dietrich Krautgartner, Dietrich von Schweinitz, Roland Kappler, Andreas Hector, Alexander Weber and Dominik Hartl

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