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TLR8 Senses Bacterial RNA in Human Monocytes and Plays a Nonredundant Role for Recognition of Streptococcus pyogenes

Tatjana Eigenbrod,* Karin Pelka, † Eicke Latz, † Bernd Kreikemeyer, ‡ and Alexander H. Dalpke* 

Microbial nucleic acids constitute an important group of pathogen-associated molecular patterns (PAMPs) that efficiently trigger innate immune activation. In mice, TLR13 has recently been identified to sense a highly conserved region within bacterial 23S rRNA. However, TLR13 is not expressed in humans, and the identity of its human homolog remains elusive. Moreover, the contribution of bacterial RNA to the induction of innate immune responses against entire bacteria is still insufficiently defined. In the current study, we show that human monocytes respond to bacterial RNA with secretion of IL-6, TNF, and IFN-β, which is critically dependent on lysosomal maturation. Using small interfering RNA and overexpression, we unambiguously identify TLR8 as receptor for bacterial RNA in primary human monocyte-derived macrophages. We further demonstrate that the sequence motif sensed by TLR8 is clearly distinct from that recognized by TLR13. Moreover, TLR8-dependent detection of bacterial RNA was critical for triggering monocyte activation in response to infection with Streptococcus pyogenes. Bacterial RNA within streptococci was also a dominant stimulus for murine immune cells, highlighting the physiological relevance of RNA sensing in defense of infections. The Journal of Immunology, 2015, 195: 1092–1099.

Rapid activation of the innate immune system upon pathogen encounter is initiated by the recognition of highly conserved pathogen-associated molecular patterns (PAMPs) through stimulation of a limited number of pattern recognition receptors. It has now been recognized that microbial nucleic acids, DNA as well as RNA, constitute an important group of PAMPs that, depending on their subcellular localization, are sensed by either endosomal or cytoplasmic receptors. Although the relevance of viral RNA and bacterial DNA recognition for initiation of innate immune responses has been intensively studied, bacterial RNA is a so far less well-characterized activator of innate immune responses and its physiological role in defense of infections is just beginning to be deciphered (1–6). Apart from inducing proinflammatory mediators such as TNF, IL-6, and type I IFN (7–9), bacterial RNA has previously been identified as an important trigger of Nlrp3 inflammasome activation, resulting in caspase-1–mediated cleavage of pro–IL-1β into its biologically active form (10–12). Moreover, bacterial RNA has been suggested to play an important role as ligand that indicates immediate danger to the host by functioning as so-called Vita-PAMP that allows the host to discriminate between live and dead bacteria (13).

Among the receptors that are recognizing bacterial RNA, the endosomal receptor TLR7 mediates sensing of bacterial RNA in both murine and human plasmacytoid dendritic cells (DCs), resulting in production of IFN-α (8, 14–16). By contrast, TLR13 has recently been identified as bacterial RNA receptor in myeloid dendritic cells (mDCs) and bone marrow–derived macrophages (BMDM) in mice (17, 18). TLR13 is unique among other receptors as it exclusively senses a specific nucleotide motif present in bacterial 23S rRNA. Of note, the same sequence is also the binding site for macrolide, lincosamide, and streptogramin antibiotics. N6-dimethylation within this sequence at position A2058 by erm methyltransferases or naturally occurring A2058G point mutations not only renders bacteria resistant to macrolide, lincosamide, and streptogramin antibiotics (19, 20), but also abolishes activation of TLR13, indicating a potential immune evasion mechanism (18). The concept that certain RNA modifications exert suppressive effects on immunostimulation has been acknowledged for a while. In this context, 2′-O-methylguanosine in Escherichia coli tRNA\textsuperscript{3\textprime}G at position 18 has been demonstrated to inhibit stimulation of TLR7 in human plasmacytoid DCs (15, 16). Importantly, TLR13 is not expressed in humans (18); yet, human monocytes respond to stimulation with bacterial RNA (8). Thus, the identity of the human TLR13 counterpart as well as the question whether N6-dimethylation at A2058 likewise impairs stimulation in the human system remains elusive. Moreover, most studies investigating immunostimulation by bacterial RNA were based on intracellular delivery of purified RNA into immune cells. Its physiological role for recognition of entire bacteria is ill defined to date.

In the current study, we demonstrate that TLR8 is the receptor for bacterial RNA in human monocytes. We further provide evidence that bacterial RNA is important for triggering monocyte activation in response to infection with group A streptococci.
**Materials and Methods**

**Reagents**

RPMI 1640 containing stable glutamine and DMEM was purchased from Biochrom (Berlin, Germany); FCS was obtained from Life Technologies (Darmstadt, Germany); Ficoll (1.078 g/ml) from Pan Biotech (Aidenbach, Germany); human M-CSF, GM-CSF, and IL-4 from R&D Systems (Abingdon, U.K.); Lipofectamine 2000 and TRIZol from Invitrogen Life Technologies (Darmstadt, Germany); Pam3CSK4, R848, Imiquimod, polyinosinic-polycytidylic acid [poly(lC)], and CpG 2216 from Invivogen (San Diego, CA); and N-[1,2-di-oioxy]propyl)-N,N,N-trimethylammonium methylsulfate (DOPAT) from Roth (Karlsruhe, Germany). Ultrapure LPS from Salmonella minnesota was provided by U. Seydel (Borstel, Germany). Primer sequences for quantitative real-time PCR were as follows: human GAPDH, forward, 5'-ACG GAT TTG GTC GTA TTG GGC-3'; reverse, 5'-ACG GAT TTG CAAT GGG CAC CAC CAC CTT TGG C-3'; and reverse, 5'-GAA CAG TTT TGC AAC ACC TTG TCT CTC C-3'; and reverse, 5'-GGA ATC CAA GCA AGT TGT AGC TC-3'. The sequence for the TLR3 stimulatory oligoribonucleotide (ORN) (18) was 5'-GCC CGU CUG UUG UGU GAC UC-3'. Primer sequences for quantitative real-time PCR were as follows: human GAPDH, forward, 5'-ACG GAT TTG GTC GTA TTG GGC-3'; reverse, 5'-ACG GAT TTG CAA GTG CCA TGG TG3-3'; and reverse, 5'-GCT TTG AGC CTA GAA GAC TTA CTA-3'; and human TLR4, forward, 5'-AGT ACC AAG AAC CAG TCT GTC TTC C-3'; and reverse, 5'-AGA CAT CCC CAT TGG TGG TGG C-3'; and human IFN-β1, forward, 5'-ATG ACC AAG AAC CAG TCT GTC TTC C-3'; and reverse, 5'-GCC CGU CUG UUG UGU GAC UC-3'. Mouse strains

**Isolation of human immune cells**

Human PBMCs were isolated from heparinized blood of healthy donors upon informed consent by standard Ficoll-Hypaque density gradient centrifugation. CD14-positive monocytes were isolated by positive selection using immunomagnetic cell separation (MACS-Microbeads; Miltenyi Biotec), according to the manufacturer’s instructions, in a semi-automated manner via AutoMACS device. The purity of CD14-positive cells exceeded 95%, as confirmed by FACS analysis. Human monocyte-derived macrophages and mDCs were generated by incubating CD14-positive monocytes with human RM-CSF (50 ng/ml) for 9 d or with human GM-CSF (10 ng/ml) plus IL-4 (20 ng/ml) for 6 d, respectively. For knockdown of human TLR8, monocyte-derived macrophages were transfected with 40 nM TLR8-specific small interfering RNA (siRNA; M-004715-01, SMARTpool; Dharmacon) with the transfection reagent DF4 (Dharmacon) on days 5 and 7 of differentiation. Cells were stimulated overnight on day 9. Nontargeting siRNA (D-001206-13; Dharmacon) served as control.

**Stimulation of PBMC**

All experiments were performed in RPMI 1640 supplemented with 10% FCS. For stimulation experiments, bacterial RNA (1 μg/ml, unless indicated otherwise) was encapsulated with DOPAT at a ratio of 3 μl DOPAT per 1 μg RNA, according to the manufacturer’s protocol. Unless otherwise indicated, bacterial RNA isolated from Streptococcus pneumoniae was used for experiments (transfection of highly purified bacterial RNA that is devoid of contaminating PAMPs induces comparable immune stimulation independently of the source from which the RNA was isolated (10).)

The absence of cell wall components within the RNA preparations in independent of the source from which the RNA was isolated (10, 12, 21). The presence of cell wall components within the RNA preparations that may trigger TLR2-dependent responses was confirmed using TLR2-deficient murine macrophages (data not shown). Stimulation was performed in 96-well flat-bottomed plates. For infection experiments, cells were infected overnight with different bacteria at the indicated multiplicity of infection (MOI), and penicillin/streptomycin was added after 90 min to kill extracellular bacteria. Where indicated, cells were stimulated with CpG2216 (1 μM), R848 (1 μg/ml), Imiquimod (1 μg/ml), Pam3CSK4 (1 μg/ml), LPS (50 ng/ml), or poly(lC) (10 μg/ml). For inhibitor experiments, bafilomycin A1 or cytochalasin D was added 1 h prior to cell stimulation. Levels of TNF and IL-6 were detected in cell-free supernatants by ELISA (BD Biosciences, Heidelberg, Germany). Secretion of IFN-β was assessed in HEK-Blue cells stably coexpressing human TLR8 together with a NF-κB-inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene (InvivoGen, Toulouse, France) were cultured in DMEM supplemented with 10% heat-inactivated FCS. Cells were stimulated with bacterial RNA or ssRNA40 (10 μg/ml) encapsulated with DOTAP at a ratio of 2 μl DOTAP per 1 μg RNA or with R848 (1 μg/ml) in HEK-Blue detection medium, according to the manufacturer’s instructions. HEK-Blue detection medium contains a specific SEAP substrate that produces a blue color upon hydrolysis by the secreted SEAP reporter protein. OD at 620 nm was measured after 18–22 h using a microplate reader. The same supernatants were analyzed for IL-8 levels by ELISA.

Bone marrow GM-CSF–derived myeloid DCs and BMDMs were prepared from 8- to 12-wk-old mice, as described previously (10). DCs and BMDMs were stimulated at a density of 2 × 10^5/well in antibiotic-free RPMI 1640 or DMEM, respectively, supplemented with 10% FCS. Cells were transfected overnight with bacterial RNA complexed with Lipofectamine 2000 at a ratio of 1 μl Lipofectamine 2000 per 1 μg RNA. Stimulation with R848 (1 μg/ml) or LPS (100 ng/ml) served as positive control.

**Cell lines**

HEK-Blue cells stably coexpressing human TLR8 together with a NF-κB–inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene (InvivoGen, Toulouse, France) were cultured in DMEM supplemented with 10% heat-inactivated FCS. Cells were stimulated with bacterial RNA or ssRNA40 (10 μg/ml) encapsulated with DOTAP at a ratio of 2 μl DOTAP per 1 μg RNA or with R848 (1 μg/ml) in HEK-Blue detection medium, according to the manufacturer’s instructions. HEK-Blue detection medium contains a specific SEAP substrate that produces a blue color upon hydrolysis by the secreted SEAP reporter protein. OD at 620 nm was measured after 18–22 h using a microplate reader. The same supernatants were analyzed for IL-8 levels by ELISA.

**Bacterial strains**

The following microbial strains were used: Staphylococcus aureus, erythromycin-sensitive (American Type Culture Collection [ATCC] 29253 or patient isolates, Department of Infectious Diseases, Heidelberg, Germany); S. aureus, erythromycin-resistant (patient isolates); Streptococcus pyogenes (ATCC 12344); S. pyogenes serotype M49 erythromycin-sensitive or carrying an ermB resistance gene in a pat18 vector (26) was provided by B. Kreikemeyer, University of Rostock; Streptococcus agalactiae (strain 15313); Streptococcus pneumoniae (patient isolates, provided by M. van der Linden, National Reference Centre for Streptococci, Aachen, Germany).

**Preparation of bacterial RNA**

The different bacterial strains were grown in Luria-Bertani medium or brain heart infusion broth (Merck, Darmstadt, Germany) and harvested within the mid log phase growth. Erythromycin-resistant strains were grown in the presence of 5 μg/ml erythromycin. After a digestion step with lysozyme (1 h at 40 mg/ml), total bacterial RNA was isolated using TRIzol reagent according to the manufacturer’s protocol. The obtained RNA underwent a further purification step using the RNAeasy mini kit (Qiagen, Hilden, Germany), including an on-column DNA digestion step. Purity of the RNA preparations was validated by determining the 260/230 nm and 260/280 nm extinction ratio by NanoDrop (Thermo Scientific).

**CFSE labeling of S. pyogenes**

Labeling of S. pyogenes with CFSE (100 μM) was performed by incubation for 20 min at room temperature. Bacteria were washed twice and used for PBMC infection. Cells were incubated at 37°C for 90 min, harvested, and washed twice (800 rpm, 5 min) to remove nonphagocytosed bacteria. After fixation in 4% paraformaldehyde, uptake of CFSE-labeled Green stained S. pyogenes was analyzed by flow cytometry. For analysis by fluorescent microscopy, the washing step of S. pyogenes-infected cells was omitted and cytospins were prepared.

**Intracellular cytokine measurement**

For intracellular cytokine staining, PBMCs were stimulated as indicated and brefeldin A (1 μg/ml; Merck, Darmstadt, Germany) was added 2 h later. After overnight incubation, cells were harvested, washed in FACs buffer (PBS plus 2% FCS), and stained with an anti–CD64-PE, anti–Slan-FITC Ab (both from Miltenyi Biotec) and anti–CD14-FITC or anti–CD16-Cy5PE Ab (BD Pharmingen) for 30 min at 4°C. After fixation in 4% paraformaldehyde/PBS, cells were washed three times, resuspended in
0.1% saponin in PBS/2% FCS, and incubated with an anti-IL-6-allophycocyanin Ab (eBioscience) for 20 min at 4°C. Cells were washed, resuspended in FACS buffer, and analyzed by flow cytometry on a FACS Canto flow cytometer (BD Biosciences, Heidelberg, Germany).

Statistical analysis

Statistical significance between groups was determined by the two-tailed Student t test. Significance in TLR8 knockdown experiments was calculated using the linear mixed-effects model with Tukey’s rank test. Differences were considered significant for \( p < 0.05 \), \( **p < 0.01 \), and \( ***p < 0.001 \).

Results

\( N^6 \)-dimethylation at A2058 in 23S rRNA impairs activation of murine TLR13 but does not interfere with activation of human PBMCs

\( N^6 \)-dimethylation, the underlying cause of macrolide resistance induced by \( erm \) methylases within bacterial 23S rRNA at position A2058 (E. coli numbering), has previously been described to abolish activation of TLR13 in murine BMDMs and DCs (18). This raised the question whether the same pattern could also attenuate bacterial RNA-mediated activation of human PBMCs that are lacking TLR13. Confirming previous results, total RNA derived from erythromycin-resistant \( S. aureus \) clinical isolates failed to induce IL-6 production in murine BMDMs and DCs, whereas RNA derived from erythromycin-sensitive strains that lack 23S rRNA \( N^6 \)-dimethylation at A2058 strongly triggered cytokine secretion (Fig. 1A and data not shown). Furthermore, an \( erm \) methyltransferase expressing \( S. pyogenes \) strain also lacked the capacity to induce IL-6 (Fig. 1B). In a similar manner, total RNA derived from a \( S. pneumoniae \) clinical isolate bearing an A2058G point mutation failed to activate murine BMDMs (Fig. 1C). In line with previous results (18), a synthetic 19-mer oligoribonucleotide mirroring the TLR13 target sequence (23S rRNA ORN) (18) was sufficient to trigger IL-6 secretion in murine immune cells (Fig. 1D). In striking contrast, neither \( N^6 \)-dimethylated RNA nor RNA from \( S. pneumoniae \) with an A2058G mutation attenuated cytokine production in human PBMCs with respect to secretion of IL-6 (Fig. 1E–G). Moreover, the 23S rRNA ORN failed to activate human PBMC (Fig. 1G). Together, these data clearly demonstrate species-specific differences in recognition of bacterial RNA motifs in the murine versus the human system.

Monocytes are the main source of IL-6 production upon bacterial RNA stimulation

Although bacterial RNA has been described to trigger inflammatory cytokine production in human PBMCs, the relative contribution of different cell populations to date remained unclear. To address this question, PBMCs were stimulated overnight with bacterial RNA in the presence of brefeldin A and intracellular staining for IL-6 was performed. Analysis of size and granularity of IL-6-positive cells indicated that monocytes and DCs were the dominant source of this cytokine (Fig. 2A). To verify the presumed identity of IL-6-positive cells, a combined staining for the different cell populations was performed. This approach confirmed that CD64-positive monocytes represented the major origin of IL-6 upon stimulation with bacterial RNA as well as R848 (Fig. 2B, 2D). Both classical CD14\(^++\)CD16\(^-\) and intermediate CD14\(^+\)CD16\(^+\) monocytes could be activated by bacterial RNA, although intermediate monocytes constituted only a minor fraction of total monocytes (Supplemental Fig. 1). Bacterial RNA also triggered cytokine production in Slan-DCs (27, 28) and CD1c\(^-\) (BDCA-1\(^+\)) DCs, which constitute the majority of myeloid DC subsets within the blood (Fig. 2B, 2C). Yet, these cells contributed only slightly to overall cytokine production, owing to their low abundance and the lower IL-6 production at the single-cell level, as demonstrated by IL-6 mean fluorescence intensity (Fig. 2D, 2E). Only few IL-6-producing cells were identified among CD64\(^-/\)Slan\(^-\) double-negative cells. Although further identification of these cells was not performed, size and granularity indicated localization within the monocyte population.

Lysosomal acidification is essential for bacterial RNA recognition

As monocytes were identified as major source of proinflammatory cytokines upon bacterial stimulation, we next aimed to define the receptor involved in bacterial RNA sensing in this cell population.

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**FIGURE 1.** \( N^6 \)-dimethylation of 23S rRNA at A2058 fails to activate murine TLR13 without affecting activation of human PBMCs. (A–C) Murine BMDMs (A and C) or DCs (B) were stimulated overnight with 3 \( \mu g/ml \) total bacterial RNA purified from (A) two different erythromycin-sensitive (ery-S) or erythromycin-resistant (ery-R) \( S. aureus \) clinical isolates, (B) \( S. pyogenes \) M49 WT or \( erm \)-methylase-expressing laboratory strains, (C) three different erythromycin-sensitive \( S. pneumoniae \) clinical isolates or a clinical isolate harboring a A2058G point mutation. (D) Murine BMDM were transfected with an ORN covering the 23S rRNA target sequence of TLR13 (23S oligo, \( c = 5 \mu g/ml \). (E–G)Human PBMCs were transfected as in (A)–(D) with different RNA preparations complexed with DOTAP at a concentration of 1 \( \mu g/ml \) bacterial RNA and 3 \( \mu g/ml \) 23S rRNA ORN. (A–G) Levels of murine or human IL-6 were measured in cell-free supernatants by ELISA. Values represent the mean of duplicate wells \( \pm \) SD. Results are representative of at least three independent experiments. \(* * * p < 0.001 \); n.d., not detected.
To test whether RNA sensing occurred in the cytosol (e.g., Nlrp3) or in the endolysosome compartment (nucleic acid recognizing TLRs), PBMCs were stimulated in the presence of bafilomycin A1, an inhibitor of endolysosomal acidification. Although cytokine responses toward LPS were unaffected, bafilomycin efficiently attenuated cytokine secretion upon stimulation with bacterial RNA or R848 in a dose-dependent manner, with similar efficiency for both stimuli (Fig. 3A). This observation strongly hinted toward an endosomal recognition pathway. To narrow down which receptor may be involved in bacterial RNA sensing, CD14-positive cells were sorted and subsequently stimulated with well-defined ligands known to activate specific nucleic acid–sensing TLRs. R848, a small molecule TLR7/TLR8 agonist, as well as bacterial RNA, was able to trigger IL-6 and TNF cytokine secretion in monocytes. By contrast, ligands specific for the other endosomal nucleic acid–sensing receptors TLR3 [poly(I:C)], TLR7 (imiquimod), and TLR9 (CpG) failed to do so (Fig. 3B), underpinning previous data that suggested TLR8, but not TLR3, 7, 9 expression in human monocytes (29). These data thus hinted toward TLR8 as the most likely candidate receptor. A similar stimulation pattern was observed in monocyte-derived mDCs (Fig. 3C).

Apart from NF-κB–dependent cytokines, nucleic acids classically trigger the release of type I IFN in target cells. Indeed, bacterial RNA and R848, but not TLR7 ligand imiquimod, strongly upregulated IFN-β expression in both CD14+ monocytes and mDCs (Fig. 3D).

TLR8 is the receptor for bacterial RNA in human monocytes
To investigate whether bacterial RNA is indeed a ligand for human TLR8, siRNA-mediated knockdown of TLR8 was performed in primary human monocyte-derived macrophages. Efficiency of TLR8 silencing was verified on the mRNA level, confirming a >90% decrease of TLR8, but not TLR4 transcript (Fig. 4A). Knockdown of TLR8 strongly attenuated bacterial RNA-induced

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**FIGURE 2.** Monocytes are the main source of IL-6 production upon bacterial RNA stimulation. (A) Human PBMCs were stimulated overnight with bacterial RNA (1 μg/ml) complexed with DOTAP or activated with R848 (1 μg/ml) or LPS (50 ng/ml). Intracellular staining for IL-6 was performed. Cells were analyzed by flow cytometry, and IL-6–positive (black dots) and IL-6–negative (gray dots) cells are shown for forward/side light scatter properties. (B–E) PBMCs were stimulated as in (A), and a combined staining for CD64 (PE), Slan (FITC), and IL-6 (allophycocyanin) was performed (B). CD64-positive, Slan-positive, and CD64/Slan–double-negative cells (B) or CD1c-positive cells (C) are shown for IL-6 production as dot blot from one representative donor (numbers within the FACS blot relate to the percentage of IL-6–positive cells within total PBMCs). (D) Pooled analysis of IL-6–positive cells within total PBMCs for the indicated populations from three to seven independent experiments (mean ± SEM). (E) Mean fluorescent intensity (MFI) (±SEM) of IL-6–positive cells from three to seven independent experiments.

**FIGURE 3.** Lysosomal acidification is essential for bacterial RNA recognition in CD14+ monocytes. (A) Human PBMCs were stimulated overnight, as indicated in the presence of different concentrations of bafilomycin. (B) Sorted CD14+ monocytes or (C) monocyte-derived mDCs were stimulated overnight with bacterial RNA (1 μg/ml for monocytes and 3 μg/ml for mDCs) or with the indicated TLR ligands. (A–C) Levels of IL-6 and TNF were analyzed in cell-free supernatants by ELISA. (D) Sorted CD14+ monocytes (left panel) or monocyte-derived mDCs (right panel) were stimulated as indicated, and relative expression of IFN-β was assessed by quantitative PCR after 5 h. The results represent mean data (±SEM) of 6–10 (A), 4 (B), or 3 (C and D) independent experiments with different donors. *p < 0.05, **p < 0.01.
production of IL-6, TNF, and IFN-β (Fig. 4B–D) as compared with a nontargeting control siRNA. This effect was specific as the response toward LPS was not affected (Fig. 4B, 4C). Activation of TLR8 by bacterial RNA was further confirmed in a complementary approach using HEK cells stably coexpressing human TLR8 together with a NF-κB–inducible SEAP reporter gene. Bacterial RNA induced both SEAP activity (Fig. 4E) as well as secretion of IL-8 (Fig. 4F), albeit to a lower extent than R848. In a similar manner, synthetic, short ssRNA40 also induced reporter gene activity as well as IL-8 secretion (Fig. 4E, 4F). Taken together, our data identify human TLR8 as receptor for bacterial RNA.

**Recognition of bacterial RNA is a physiological trigger of innate immune responses against streptococci in human monocytes**

Most previous studies investigating immune responses initiated by bacterial RNA were based on the transfection of bacterial RNA into immune cells. The relevance of RNA recognition for innate immune responses by whole bacteria is only poorly understood. To gain insights into the significance of bacterial RNA sensing for immune stimulation by entire bacteria, PBMCs were infected with a set of Gram-positive pathogens, including group A and group B streptococci (GBS), *S. aureus*, and *L. monocytogenes*. Inhibition of bacterial internalization using the phagocytosis inhibitor cytochalasin D strongly impaired IL-6 production in response to *S. pyogenes* and *S. agalactiae* in a dose-dependent manner (Fig. 5A, 5B). By contrast, cytochalasin D only partially suppressed cytokine production induced by *S. aureus* and *L. monocytogenes*, in line with the notion that TLR2 expressed on the cell surface has been implicated in sensing of both pathogens (30–35) (Fig. 5C). These results implicated a major contribution of intracellular recognition pathways for sensing of live streptococci. Further analysis revealed that inhibition of endosomal acidification by bafilomycin almost abolished IL-6 and IFN-β production (Fig. 5D, Supplemental Fig. 2A) in response to *S. pyogenes* in similar concentrations as observed before for inhibition of transfected bacterial RNA (Fig. 3A). PBMC activation by *S. agalactiae* (GBS) was also strongly attenuated, albeit to a lesser extent than for the *S. pyogenes* ATCC and the M49 strain (Fig. 5D). Within the PBMCs, CD14-positive monocytes constituted the major source of proinflammatory cytokines upon *S. pyogenes* infection (Supplemental Fig. 3A, 3B). Because our data suggested a predominant role of nucleic acid sensing in the endolysosome for innate immune sensing of streptococci especially of *S. pyogenes*, we next investigated the contribution of Unc93B1, an endoplasmic reticulum protein that is essential for delivery of all nucleic acid-sensing TLRs to the endolysosome (23). Unc93B1 knockout THP-1 cells transduced with Unc93B1 WT responded to *S. pyogenes* infection with TNF production, whereas THP-1 cells deficient in Unc93B1 or reconstituted with a H412R mutant protein failed to do so (Fig. 5E). The direct involvement of bacterial RNA-mediated TLR8 activation was confirmed by siRNA-mediated silencing of TLR8 in monocyte-derived macrophages. Cells with knockdown of TLR8 showed greatly suppressed IL-6, TNF, and IFN-β secretion when infected by whole *S. pyogenes* (Fig. 5F, 5G, Supplemental Fig. 2B). Together, these data identify TLR8-mediated recognition of bacterial RNA as a critical determinant for initiation of innate immune responses against *S. pyogenes* in human monocytes.

**Marine innate immune responses against *S. pyogenes* also depend on recognition of bacterial RNA**

Although we observed that the RNA sequence pattern recognized by human TLR8 clearly differs from that described for murine TLR13, we wanted to elucidate whether the concept that recognition of bacterial RNA is critical for immune cell activation by whole *S. pyogenes* bacteria is also operative in the murine system. To this end, BMDMs and DCs derived from TLR2/3/4/7/9–deficient mice (bearing a missense mutation in Unc93B (lacking responses to all endosomal TLRs, including TLR13) were tested for their response to live *S. pyogenes*. Strikingly, cytokine secretion triggered by *S. pyogenes* infection was almost completely abolished in macrophages or DCs lacking functional Unc93B, whereas TLR3, 7, and 9 were dispensable (Fig. 6A, 6B). These results indirectly suggested that recognition of bacterial RNA by TLR13 played a critical and nonredundant role in immune cell activation by live *S. pyogenes*. To further verify this assumption, the stimulatory capacity of an erythromycin-resistant *S. pyogenes* strain was investigated. Indeed, a *S. pyogenes* erm methyltransferase-expressing M49 strain carrying N6-dimethylation of 23S rRNA that lacks TLR13 stimulatory capacity (Fig. 1B) triggered only minor cytokine secretion in murine BMDM and DC as compared with the respective wild-type strain (Fig. 6C, 6D). Confirming specificity, *S. pyogenes* clinical isolates in which erythromycin resistance was mediated by efflux pumps showed no defects in cytokine production (data not shown). In conclusion, our data clearly demonstrate the pivotal role of bacterial RNA recognition
Discussion

The mechanisms involved in sensing of bacterial RNA in human monocytes—which we identify as leading source of NF-κB–dependent cytokines IL-6 and TNF—are poorly understood. Importantly, the expression profile of nucleic acid–sensing receptors in immune cells clearly differs between mice and humans. Although murine macrophages and myeloid DCs are equipped with TLR7, 9, and 13, human monocytes and macrophages have been reported to express TLR8, but not other endosomal TLRs (29). This is in line with our observation that only R848, a combined TLR7/8 agonist, but not poly(I:C) (TLR3), imiquimod (TLR7), or CpG (TLR9), triggered cytokine secretion in human monocytes. Extending current knowledge, we definitively identify TLR8 as receptor for bacterial RNA in primary human monocyte-derived macrophages by using siRNA-mediated silencing as well as TLR8 overexpression. Similarly to TLR7, TLR8 thus participates in sensing RNA of both viral and bacterial origin (2, 16). In contrast to the human system, TLR8 has been suggested to be nonfunctional in mice as it cannot be activated by ssRNA or small molecule agonists (36, 37). Unresponsiveness to these stimuli has been attributed to the absence of a 5-aa motif in the ectodomain of murine TLR8 that is required for ligand recognition (38). However, TLR8 deficiency is associated with the development of autoimmune diseases in mice, possibly by negatively controlling immunity (39, 40).

In murine macrophages and DCs, the endosomal receptor TLR13 has recently been identified as a sensor for bacterial RNA that specifically recognizes a conserved region within bacterial 23S rRNA. Recognition by TLR13 can be masked by N6-dimethylation at a critical adenosine residue (A2058, E. coli) within the target sequence (18). As this modification also renders bacteria resistant toward macrolide antibiotics (19, 20), it has been speculated that this resistance mechanism might additionally constitute an important immune escape mechanism. Broadening previous findings, we show in this study that a naturally occurring A2058G point mutation in S. pneumoniae 23S RNA abolishes TLR13 stimulatory capacity as efficiently as N6-dimethylation at this residue. Despite the striking effect of 23S rRNA A2058 modifications on TLR8 recognition, our data show in this study that a naturally occurring A2058G point mutation in S. pneumoniae 23S RNA abolishes TLR13 stimulatory capacity as efficiently as N6-dimethylation at this residue. Despite the striking effect of 23S rRNA A2058 modifications on TLR8 recognition, our data demonstrate that these alterations do not affect activation of human monocytes that sense bacterial RNA via TLR8. Our data thus unravel strictly species-specific differences in recognition of bacterial RNA motifs in the murine versus the human system. Given that antibiotic resistance is an emerging problem worldwide, the finding that macroline resistance does not cause immune silencing in human cells is of apparent importance.

Former studies investigating innate immune activation by bacterial RNA were mainly based on the intracellular delivery of purified RNA into immune cells (8, 10, 17, 18, 41). However, the relative contribution of RNA recognition for mounting immune responses against live pathogens is less well studied, especially in the human setting. First results indicated that RNA stimulation might be of importance for recognition of streptococci or for Borrelia burgdorferi (7, 42, 43), and certain polymorphisms in the TLR8 gene have been associated with susceptibility to tuberculosis (44). We show in this work that phagocytic uptake as well as endosomal acidification was essential for PBMC activation in response to infection with live S. pyogenes and S. agalactiae (GBS), indicating that endosomal recognition pathways play a predominant role in sensing of these pathogens. In accordance, UNC93B1 was required for TNF induction in THP-1

for mounting innate immune responses against S. pyogenes in both human and murine innate immune cells.

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FIGURE 5. Recognition of bacterial RNA is a physiological trigger of innate immune responses against streptococci in human monocytes. (A) Human PBMCs were infected with CFSE-labeled S. pyogenes (MOI 10) in the presence or absence of cytochalasin D (10 μM), and phagocytic uptake was analyzed by flow cytometry (upper panels, gated on the monocyte population) or by fluorescent microscopy (lower panels). (B–D) PBMCs were infected overnight with the indicated bacteria at different MOIs in the presence of cytochalasin D (B and C) or bafilomycin (D). IL-6 levels were analyzed in cell-free supernatants by ELISA. The results represent mean data (±SEM) of five (B and C) or six to nine (D) independent experiments. (E) Human UNC93B1-knockout THP-1 monocytes generated by CRISPR/Cas9-based gene editing were retrovirally transduced with UNC93B1-mCitrine WT or H412R or were

Left: S. pyogenes

Right: S. agalactiae

FIGURE 5. Recognition of bacterial RNA is a physiological trigger of innate immune responses against streptococci in human monocytes. (A) Human PBMCs were infected with CFSE-labeled S. pyogenes (MOI 10) in the presence or absence of cytochalasin D (10 μM), and phagocytic uptake was analyzed by flow cytometry (upper panels, gated on the monocyte population) or by fluorescent microscopy (lower panels). (B–D) PBMCs were infected overnight with the indicated bacteria at different MOIs in the presence of cytochalasin D (B and C) or bafilomycin (D). IL-6 levels were analyzed in cell-free supernatants by ELISA. The results represent mean data (±SEM) of five (B and C) or six to nine (D) independent experiments. (E) Human UNC93B1-knockout THP-1 monocytes generated by CRISPR/Cas9-based gene editing were retrovirally transduced with UNC93B1-mCitrine WT or H412R or were left unconstituted (Unc93B1 none). Cells were infected overnight with S. pyogenes or simulated with rL-1β (10 ng/ml). Data represent the mean (±SEM) of three independent experiments. (F and G) siRNA-mediated knockdown of TLR8 was performed in human monocyte-derived macrophages. Cells were infected overnight with live S. pyogenes (ATCC strain) at three MOIs (1.5, 0.75, and 0.375). Levels of IL-6 and TNF were measured by ELISA. Each symbol pair represents one donor. *p < 0.05, **p < 0.01, ***p < 0.001. GBS, S. agalactiae; L.m., Listeria monocytogenes; S.a., Staphylococcus aureus; S. pyo, Streptococcus pyogenes.
monocytes. Further experiments in human monocyte-derived macrophages in which TLR8 was silenced by siRNA revealed that TLR8-mediated sensing of bacterial RNA was essential for cytokine induction by S. pyogenes. Thus, to our knowledge, we provide for the first time direct evidence that RNA represents the dominant immune stimulus of S. pyogenes in the human system and plays a largely nonredundant role in inducing innate immune responses. Residual activity observed in our experiments might be explained by incomplete silencing of TLR8 or may indicate the existence of additional, TLR8-independent recognition pathways. In a back-to-back manuscript with this study, Bergstrøm et al. (35) further demonstrate that TLR8 was also critical for IFN-β induction by S. aureus, whereas TLR2 and TLR8 mediated redundant effects on pro-inflammatory cytokines including TNF and IL-1β.

Although both the receptor for bacterial RNA and the sequence motif detected entirely differ between mice and humans, recognition of bacterial RNA was likewise critical for activation of murine macrophages and GM-CSF–derived DCs upon S. pyogenes infection. This supports the conclusion that detection mechanisms of S. pyogenes are evolutionarily conserved among different species.

Due to the clinical relevance of S. pyogenes infections, pathways involved in sensing of this pathogen had been subject of several investigations. Yet, the leading PAMP remained for a long time undefined. Initial studies described that production of NF-κB–dependent cytokines and IFN-β was dramatically impaired in macrophages and DCs derived from MyD88-deficient mice, but did not require signaling via TLR2, 4, and 9 (45, 46). This led to the hypothesis of either redundancy in TLR signaling or the existence of a yet undefined MyD88-dependent receptor (45, 46). Further analysis by Gratzi et al. (43) based on the transfection of RNase- or DNase-treated S. pyogenes lysates into immune cells indicated that IFN-β production by murine conventional DCs and macrophages was dependent on endosomal delivery of streptococcal nucleic acids. While this manuscript was in revision, Fieber et al. (47) published a study in which they provide evidence for a redundant involvement of TLR2 and TLR13 for TNF and IL-6 production by murine mDCs and BMDM upon S. pyogenes infection in vitro. By contrast, TLR2 and UNC93B1 played a nonredundant role in vivo in a s.c. S. pyogenes infection model. The discrepancy to the nearly complete RNA dependency in our in vitro experiments (Fig. 6) might be explained by the lower MOIs used in the present investigation (MOI 5 versus MOI 50).

Our data also suggest a leading role of nucleic-acid–sensing pathways for human PBMC activation upon infection with GBS. Similarly, heat-inactivated GBS have very recently been described to trigger activation of murine BMDMs and DCs in a RNA-dependent manner (48). However, TLR13-dependent recognition had a redundant role when live bacteria were used as stimulus, most likely due to functional compensation by other endosomal Unc93B1-dependent TLRs (48). Unc93B1-dependent but TLR2/7/9-independent sensing of live GBS had already been suggested by Deshmukh et al. (7). The authors proposed that sensing of bacterial ssRNA might even be relevant for all Gram-positive bacteria as treatment of heat-inactivated bacteria with RNase A reduced their TNF-inducing capacity. Yet, this is partially in conflict with the known TLR2 dependency of some of these bacteria, and recognition principles observed upon stimulation with dead bacteria cannot be transferred directly to live pathogens (13, 48). Thus, the participation of RNA recognition in innate immune responses against live bacteria in vitro and in vivo will need to be addressed in future investigations.

In summary, this work identifies human TLR8 as receptor for bacterial RNA and provides direct evidence for a nonredundant role of TLR8-dependent sensing of bacterial RNA in innate immunity.

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Disclosures
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