Cutting Edge: TLR13 Is a Receptor for Bacterial RNA

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Bacterial RNA (bRNA) can induce cytokine production in macrophages and dendritic cells (DCs) through a previously unidentified receptor. Gene expression analysis of murine DCs showed that bRNA induced gene regulation similar to that induced by stimulation of TLR7 with R848. Although TLR7 was dispensable for cytokine induction by bRNA, TLR-associated proteins MyD88 and UNC93B were required. TLR13 is an endosomal murine TLR that has been described to interact with UNC93B with, so far, no characterized ligand. Small interfering RNA against TLR13 reduced cytokine induction by bRNA in DCs. Moreover, Chinese hamster ovary cells transfected with TLR13, but not with TLR7 or 8, could activate NF-κB in response to bRNA or *Streptococcus pyogenes* in an RNA-specific manner. TLR7 antagonist IRS661 could, in addition, inhibit TLR13 signaling and reduced recognition of whole Gram-positive bacteria by DCs, also in the absence of TLR7. The results identify TLR13 as a receptor for bRNA.  

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Receptors of the innate immune system detect molecular patterns during the life cycles of infecting pathogens, depending on the localization and availability of these patterns. TLRs recognize a wide range of microbial components and are important for raising innate immune defenses against bacterial infections. Whereas LPS from Gram-negative bacteria commonly activates the innate immune system via TLR4 (1), it is less well understood how Gram-positive bacteria are recognized. Although TLR adaptor molecule MyD88 is important for activation of the innate immune system by *Staphylococcus aureus* (2) and strains of *Enterococcus* and *Lactobacillus* (3), activation by group A *Streptococcus* can occur independently of TLR2, 4, and 9 (4). Several studies have shown that bacterial RNA (bRNA) activates the innate immune system (5–8). RNA receptor TLR7, which recognizes single-stranded viral RNA (9), has been suggested to induce IFN-β (8) or IL-12 (3) production by myeloid dendritic cells (DCs) in response to bRNA. However, RNA of *Streptococcus pyogenes* induces IFN-β in myeloid DCs in an MyD88-dependent but TLR7-independent manner (6). In addition, RNA-dependent induction of TNF-α by group B *Streptococcus* in macrophages has been shown to be independent of nucleic acid-sensing TLR3, 7, and 9, although requiring MyD88 and UNC93B (10), needed for the function of endosomal TLRs (11). Thus, the receptor for bRNA, which may play an important role in recognizing Gram-positive bacteria, has not yet been identified.

We found that murine TLR13, an UNC93B-interacting orphan receptor (12, 13), could mediate the recognition of bRNA and whole Gram-positive bacteria when expressed in Chinese hamster ovary (CHO) cells. When TLR13 expression was targeted in DCs, using small interfering RNA (siRNA), cytokine induction by bRNA was reduced. Our results indicate that TLR13 is required and sufficient for the induction of cytokines in response to bRNA.

**Materials and Methods**

**Mice**

Wild-type (wt) C57BL/6 mice were purchased from Charles River Laboratories. MyD88−/− mice were provided by H. Wagner (Munich, Germany). TLR7−/− mice were provided by S. Bauer (Marburg, Germany). UNC93B mice harboring H412R missense mutation (3D) (11) were supplied by M. Freudenberg (Freiburg, Germany). Animal experiments were approved by local authorities.

**Cells**

Bone marrow-derived DCs were generated as previously described (14). GM-CSF was provided to the culture by adding supernatant of ×63-Ag8 cells, supplied by M. Lutz (Würzburg, Germany). DCs were harvested at day 7 and seeded in 24-well plates (8 × 10^5^ DCs per well). CHO cells (DSMZ, #ACC110) were grown in Ham’s F12 medium. Media were supplemented with 10% heat-inactivated FCS, penicillin G, and streptomycin sulfate (100 IU/ml) (Life Technologies).

**Bacteria and stimulations**

*Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6051), *Listeria monocytogenes* (ATCC 15313), *Enterococcus faecalis* (ATCC 29212), *Lactobacillus* sp. (patient isolate), and *Streptococcus pyogenes* (ATCC 12344) were grown to midlog phase. *S. pyogenes* was heat killed for 45 min at 70°C. RNA was purified from lysozyme-treated bacteria or eukaryotic cells (THP-1 or RAW 264.7), using TRIzol (Invitrogen) followed by High Pure RNA Isolation Kit (Roche) with DNase I digest. RNase A (Invitrogen) digestion of bRNA was performed at room temperature, bRNA (3 μg/ml from *L. monocytogenes* unless otherwise stated) was packaged with 2.5 μl DOTAP.
per microgram of RNA, as previously described (5). Ultrapure LPS from **Salmonella minnesota** (100 ng/ml) was provided by U. Seydel (Borstel, Germany). Other agents used were R848 (InvivoGen), polyinosinic–polycytidylic acid (pI:C, 50 μg/ml; Sigma-Aldrich), Pam3Csk4 (InvivoGen), and CpG ODN 1668 (MWG-Biotech). Phosphorothioate oligonucleotide inhibitor IRS661 (5′-TGCTTGCAAGCTTGCAAGCA-3′; Eurofins MWG Operon) was added 1 h before stimulation.

### TLR expression vectors

Murine TLR13 from (Mammalian Gene Collection; http://mgc.nci.nih.gov/) pCR-Blunt II-TOPO-TLR13 (I.M.A.G.E. IRAMp995J1823Q) was cloned into pcDNA3.1 (Invitrogen) using ApaI/XhoI. TLR8, from pCR4-TOPO-TLR8 (I.M.A.G.E. IRClp9951B0312D), was digested with SpeI/FspI and ligated into pcDNA3.1 cut with NheI/XbaI. TLR7 in pcDNA3 was a gift from T. Espevik (Trondheim, Norway).

### DNA transfection, selection, and luciferase reporter assay

CHO cells were cotransfected with vectors expressing TLR and NF-κB luciferase reporter pGL4.32[luc2P/NF-κB-RE/Hygro] (Promega), using Lipofectamine 2000 (Invitrogen) in 100-mm plates and incubated overnight. Bulk transfected cells were distributed in 24-well plates and stimulated upon seeding. Clones were generated by selection with G-418 sulfate (400 μg/ml; PAA Laboratories) and hygromycin B (100 μg/ml; InvivoGen) and distributed at 0.5 × 10⁵ per well in 48-well plates for stimulation.

### Quantitative RT-PCR

RNA was purified 6 h post treatment with the High Pure RNA Isolation Kit (Roche), including DNase I digestion. cDNA was generated using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and measured with Absolute SYBR Green Rox Mix Kit (ABgene House), using ABI PRISM 7700 (Applied Biosystems). Primer sequences are available on request.

### siRNA transfection

siRNA against TLR13 (target sequence: 5′-CACCTATGTTCTTGTAAGTTAA-3′) or Negative Control-N3 siRNA (Riboxx) was delivered with the Nucleofector Kit VPA-1011 (Lonza) or riboxx FECT Transfection Reagent (Riboxx) according to the manufacturer’s protocols. The riboxx FECT transfection was performed upon seeding 1 × 10⁵ DCs per 48 wells in 150 μl media. Each nucleofection was divided into 96 wells. Media were changed after 24 h, and the cells were stimulated 48 h post siRNA transfection.

### ELISA

Supernatants were analyzed using BD OptEIA Mouse ELISA Set IL-12p40 or IL-6 (BD Biosciences).

### FIGURE 1

**Induction of cytokines by bRNA requires MyD88 and UNC93B but is independent of TLR7.** Induction of cytokines in myeloid DCs from wt or knockout mice upon treatment with bRNA or RNase A-digested bRNA (bRNA RNase), pI:C, LPS, or R848 (1 μg/ml). (A) IL-12p40 mRNA after treatment of wt DCs with RNA from *L. monocytogenes* (L.m.), *B. subtilis* (B.s.), *E. faecalis* (E.f.), *S. aureus* (S.a.), or RNase A digests of respective RNA (RNA RNase) or eukaryotic RNA (n.t., Not tested). (B and C) Cytokine induction in DCs from wt or MyD88⁻/⁻ mice treated with bRNA or bRNA RNase; induction of (B) IL-12p40 mRNA and (C) IL-12p40 protein in supernatants at 17 h. (D) Fold induction of IFN-β mRNA over samples treated with bRNA RNase. (E) IL-12p40 mRNA in DCs from wt and TLR7⁻/⁻ mice. (F) IL-12p40 mRNA in DCs from wt and UNC93B-mutated 3D mice treated with bRNA, bRNA RNase, pI:C, LPS, R848, CpG DNA (1 μM), or Pam3Csk4 (1 μg/ml) (Pam3C). n = 3 mice from at least three independent experiments. Error bars show mean ± SD. **p ≤ 0.01, ***p ≤ 0.001.

### FIGURE 2

**bRNA and R848 induce similar gene regulation in DCs.** Microarray analysis of gene regulation in DCs treated with bRNA, RNAse A digest of bRNA (bRNA RNase), R848 (1 μg/ml), or mock (untreated); log2 of signal intensity values of (A) bRNA RNase versus bRNA, (B) bRNA RNase versus R848, (C) R848 versus bRNA, or (D) untreated versus bRNA RNase. Diagonal lines indicate 2-fold changes. n = 4 mice.
FACS

Brefeldin A (2 μg/ml; Sigma-Aldrich) was added at 5 h post stimulation. At 16 h, cells were fixed and stained using Cytofix/Cytoperm (BD Biosciences) with APC-conjugated anti-mouse IL-12 (p40/p70) Ab (BD #554480) and assayed using a FACS Canto (BD Biosciences).

Microarray

RNA was purified 6 h post stimulation, as described for quantitative RT-PCR, hybridized to Mouse Microarray Ref-8 v2.0 Expression BeadChips (Illumina) at the Genomics and Proteomics Core Facility at the Deutsches Krebsforschungszentrum (Heidelberg, Germany) and analyzed as described in the supplemental information online. The microarray data from this publication have been submitted to the ArrayExpress database, www.ebi.ac.uk/arrayexpress/ experiments/E-MTAB-1139, and assigned the identifier E-MTAB-1139.

Statistical analysis

Two-tailed unpaired Student t tests were used. Statistical significance indicated: *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001. Error bars show mean ± SD.

Results and Discussion

Cytokine induction by bRNA requires MyD88 and UNC93B but is independent of TLR7

RNA from Gram-positive bacteria, but not from eukaryotic cells, has been shown to induce cytokines in myeloid DCs when delivered to the endosome (5, 8, 10). We found that bRNA from a number of bacterial species induced IL-12p40.
mRNA in murine bone marrow-derived myeloid DCs (Fig. 1A). RNase A digestion of the bRNA significantly reduced IL-12p40 induction. Previous studies have been contradictory regarding the ability of bRNA to induce cytokines in the absence of MyD88. We showed that induction of IL-12p40 mRNA (Fig. 1B), secreted IL-12p (Fig. 1C), and IFN-β mRNA (Fig. 1D) by bRNA from Gram-positive bacteria required MyD88. TLR7 has been suggested to mediate IFN-β (8) or IL-12p40 (3) production in response to bRNA in myeloid DCs, whereas other studies have shown TLR7 to be nonessential for bRNA recognition (5, 6, 10). Using knockout cells, we found TLR7 to be dispensable for induction of IL-12p40 mRNA in response to bRNA (Fig. 1E).

UNC93B has previously been reported to be required for induction of TNF-α by bRNA in macrophages (10). We found that induction of IL-12p40 mRNA by bRNA was abolished in DCs from UNC93B defect 3D mice (11) (Fig. 1F).

bRNA induces signaling similar to that induced by TLR7 in myeloid DCs

To characterize gene regulation induced by bRNA in DCs, we performed a microarray analysis (Fig. 2, Supplemental Table I). Both bRNA and TLR7 ligand R848 induced more than 2-fold regulation of several hundred genes, compared with the RNase-digested control (Fig. 2A, 2B). However, gene regulation by bRNA and R848 showed a very high degree of similarity: Only seven genes were induced differently (Fig. 1C). Treatment with RNase A-digested bRNA and DOTAP did not induce any gene regulation, compared with untreated control, showing that the bRNA preparations did not contain any other components, in addition to RNA, capable of DC activation (Fig. 2D). The microarray result supported the hypothesis that bRNA induced signaling via an endosomal, MyD88-dependent TLR.

TLR13 activates NF-κB in response to bRNA and mediates recognition of Gram-positive bacteria

TLR13 is a murine orphan TLR expressed by myeloid DCs, B cells, and macrophages (13). To investigate whether TLR13 could mediate bRNA signaling, CHO cells were cotransfected with an NF-κB luciferase reporter and vectors expressing TLR13, 7, or 8, or empty vector, before treatment with bRNA (Fig. 3A). Treatment with bRNA induced NF-κB activation specifically in TLR13-transfected cells (Fig. 3A). A clone stably transfected with TLR13 and NF-κB luciferase reporter induced up to 6-fold NF-κB activation in a dose-dependent response to bRNA (Fig. 3B). CHO cells have a frameshift mutation rendering TLR2 defective, whereas they are capable of responding to LPS (15). Responses to bRNA were of the same magnitude as responses to stimulation by LPS. The TLR13-expressing CHO cell clone induced NF-κB luciferase reporter activity in response to both live and heat-killed S. pyogenes, but not in response to RNase A-treated bacteria (Fig. 3C), showing that TLR13 can contribute to recognition of group A streptococci. TLR7-inhibitory oligonucleotide IRS661 has previously been shown to inhibit bRNA signaling (3). We show that IRS661 inhibited TLR13-mediated activation of NF-κB luciferase reporter in response to bRNA, but not to LPS (Fig. 3D). We proceeded to investigate if TLR7/13 antagonist IRS661 could inhibit bRNA-induced signaling in DCs. Pretreatment with IRS661 completely inhibited IL-12p40 induction by bRNA in wt and TLR7−/− DCs (Fig 3E, 3F). The IL-12p40 production in response to several Gram-positive bacteria was significantly inhibited by IRS661 treatment of wt and TLR7−/− DCs, compared with induction by CpG via TLR9 or Pam3Csk4 via TLR2, which were not inhibited. Thus, inhibition of TLR13 reduced the innate immune induction by several Gram-positive bacteria independently of TLR7 (4, 6).

**FIGURE 4.** siRNA targeting TLR13 reduces cytokine production in response to bRNA treatment. DCs were transfected with TLR13 or control siRNA, using riboxx FECT and treated with OptiMEM (Mock), S. aureus RNA (bRNA) (wedge represents 0.25 and 0.5 µg/ml), or CpG (100 nM). (A) Relative expression of TLR13 mRNA was assayed in mock-treated samples. The samples were analyzed for (B) IL-12p40 mRNA and (C) IL-6 mRNA. (D) Supernatants (6 h) were assayed for IL-6 by ELISA. (E) DCs were harvested at 16 h post treatment, stained with an anti–IL-12-APC Ab, and assayed by FACS. IL-12 is displayed as mean fluorescence intensity (MFI). DCs were transfected with TLR13 or control siRNA using nucleofection and treated with OptiMEM (Mock); S. aureus RNA (bRNA), 3 µg/ml; or CpG. The samples were analyzed for (F) IL-6 mRNA and (G) IL-12p40 mRNA. n = 5 mice. Error bars show mean ± SD. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
Decreasing TLR13 expression reduces induction of cytokines in response to bRNA

To determine whether TLR13 was required for bRNA signaling in DCs, TLR13 expression was targeted with siRNA. The TLR13 mRNA expression in cells treated with TLR13 siRNA was reduced to less than half of that in cells treated with a control siRNA (Fig. 4A). DCs transfected with siRNA against TLR13 produced significantly less IL-12p40 mRNA in response to bRNA than did DCs transfected with control siRNA, whereas IL-12p40 mRNA production in response to CpG did not differ (Fig. 4B). Production of IL-6 mRNA (Fig. 4C) and secreted IL-6 (Fig. 4D) was specifically reduced in TLR13 siRNA-transfected DCs in response to bRNA. Induction of secreted IL-6 could not be detected at 6 h (not shown). Instead, we stained the siRNA-transfected, bRNA-treated DCs for intracellular IL-12 production at 16 h and found that mean fluorescence intensity was significantly reduced in DCs treated with siRNA against TLR13. A similar reduction of the cytokine response to bRNA could be observed when the TLR13 siRNA was delivered by nucleoporation (Fig. 4F, 4G).

We show in this article for the first time, to our knowledge, that RNA from Gram-positive bacteria can activate previous orphan receptor TLR13 and that TLR13 activation contributes to the innate response to Gram-positive bacteria. Although TLR13 and TLR7 do not display a high degree of sequence similarity (16, 17), bRNA induces a gene expression profile similar to that induced by TLR7 ligand R848. Mice lacking MyD88 have been shown to be particularly sensitive to TLR7 and TLR9 signaling in DCs, TLR13 expression was targeted with siRNA. To determine whether TLR13 was required for bRNA signaling without involvement of TLR2, TLR4, and TLR9, we transfected DCs with siRNA against TLR13 produced significantly less IL-12p40 mRNA in response to bRNA than did DCs transfected with control siRNA (Fig. 4A). DCs transfected with siRNA against TLR13 (Fig. 4B) and secreted IL-6 (Fig. 4D) was specifically reduced in TLR13 siRNA-transfected DCs in response to bRNA. Induction of secreted IL-6 could not be detected at 6 h (not shown). Instead, we stained the siRNA-transfected, bRNA-treated DCs for intracellular IL-12 production at 16 h and found that mean fluorescence intensity was significantly reduced in DCs treated with siRNA against TLR13. A similar reduction of the cytokine response to bRNA could be observed when the TLR13 siRNA was delivered by nucleoporation (Fig. 4F, 4G).

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Disclosures

The authors have no financial conflicts of interest.