RNA and β-Hemolysin of Group B Streptococcus Induce Interleukin-1β (IL-1β) by Activating NLRP3 Inflammasomes in Mouse Macrophages

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Background: Group B Streptococcus (GBS) activates the NLRP3 inflammasome.

Results: GBS RNA escapes the phagolysosome and activates the NLRP3 inflammasome in a β-hemolysin-dependent fashion. RNA-NLRP3 interaction and activation are enhanced by lysosomal leakage.

Conclusion: RNA activates the NLRP3 inflammasome in synergy with phagolysosomal proteins.

Significance: The NLRP3 inflammasome responds to bacterial invasion via RNA recognition subsequent to phagolysosomal degradation.

The inflammatory cytokine IL-1β is critical for host responses against many human pathogens. Here, we define Group B Streptococcus (GBS)-mediated activation of the Nod-like receptor-P3 (NLRP3) inflammasome in macrophages. NLRP3 activation requires GBS expression of the cytolytic toxin, β-hemolysin, lysosomal acidification, and leakage. These processes allow the interaction of GBS RNA with cytosolic NLRP3. The present study supports a model in which GBS RNA, along with lysosomal components including cathepsins, leaks out of lysosomes and interacts with NLRP3 to induce IL-1β production.

The NLRP3 inflammasome responds to many human pathogens. Here, we define Group B Streptococcus (GBS)-mediated activation of the Nod-like receptor-P3 (NLRP3) inflammasome in macrophages. NLRP3 activation requires GBS expression of the cytolytic toxin, β-hemolysin, which is then secreted. Usually, NLRP3 activation is a two-step process. The first signal induces, via NF-kB activation, the expression of pro-IL-1β and NLRP3. The second signal is often generated by the stimulus itself, in the form of crystals, aggregated protein, ATP, or bacterial toxins (9–11).

Previously, we showed that GBS induces IFN-β production in a Toll-like receptor-independent fashion in macrophages by an unknown DNA sensor (12). We also showed that GBS induces IL-1β production in an NLRP3-dependent manner in dendritic cells (13). In this study, we demonstrate that GBS activates the NLRP3 inflammasome by a mechanism that requires hemolysin-mediated lysosomal leakage. We also show that the interaction of bacterial RNA with NLRP3 is greatly enhanced by phagolysosomal damage, suggesting a critical role for lysosomal components in promoting a receptor-ligand-like interaction (RNA-NLRP3) that results in IL-1β production.

EXPERIMENTAL PROCEDURES

Bacterial Strains

S. agalactiae strains NEM 316 (type III) were obtained from the blood of a neonate with sepsis as described (12). Strains NEM ΔcyIE and NEM Δcfb were NEM 316-derived mutants that lack CyIE, a putative n-acetyltransferase in ornithine rhamnolipid biosynthesis, or Cfb, which encodes the co-hemolysin (CAMP [Christie Atkins Munch-Petersen factor], (12). Two hyperhemolytic strains were produced: NEM 2802 by deleting

Group B Streptococcus (GBS, 2 Streptococcus agalactiae) is a commensal organism of the normal intestinal and female genital tracts, but is potently inflammatory once it becomes invasive (1). GBS is a leading cause of meningitis and sepsis in newborn, elderly, and immunocompromised individuals. Up to 50% of patients surviving meningitis suffer from neurological problems (2). A GBS virulence factor that may substantially contribute to the pathogenesis of meningitis and sepsis is the pore-forming toxin β-hemolysin, which is an ornithine rhamnolipid (3). Rhamnolipids, known as exotoxins in Pseudomonas spp., have pore-forming properties and may allow molecules such as nucleic acids to cross the phagosomal membrane.

Upon infection, the host innate immune system senses the pathogen and initiates a cascade of events resulting in the formation of cytokines, which recruit host immune cells and eliminate the pathogen. Microbial effectors are sensed by distinct classes of germline-encoded “pattern recognition receptors” such as Toll-like receptors (TLRs) (4), Nod-like receptors (NLRs) (5), cytoplasmic DNA sensors such as AIM2 (6, 7), and the DEcD/H box helicases DHX9 and DHX36 (8).

The NLRs and AIM2 are intracellular sensors that, upon stimulation, oligomerize with apoptosis-associated speck-like protein (ASC) and trigger the autocatalytic activation of caspase-1 (7). Caspase-1 cleaves pro-IL-1β to form mature IL-1β, which is then secreted. Usually, NLRP3 activation is a two-step process. The first signal induces, via NF-kB activation, the expression of pro-IL-1β and NLRP3. The second signal is often generated by the stimulus itself, in the form of crystals, aggregated protein, ATP, or bacterial toxins (9–11).

Previously, we showed that GBS induces IFN-β production in a Toll-like receptor-independent fashion in macrophages by an unknown DNA sensor (12). We also showed that GBS induces IL-1β production in an NLRP3-dependent manner in dendritic cells (13). In this study, we demonstrate that GBS activates the NLRP3 inflammasome by a mechanism that requires hemolysin-mediated lysosomal leakage. We also show that the interaction of bacterial RNA with NLRP3 is greatly enhanced by phagolysosomal damage, suggesting a critical role for lysosomal components in promoting a receptor-ligand-like interaction (RNA-NLRP3) that results in IL-1β production.

2 The abbreviations used are: GBS, Group B Streptococcus; NLR, Nod-like receptor; hNLRP3, human NLRP3; CAMP, Christie Atkins Munch-Petersen; BMDM, bone marrow-derived macrophages; BMDC, bone marrow-derived dendritic cells; MSU, monosodium urate; MOI, multiplicity of infection.
the transcriptional repressor CovR, and NEM 2424 by deleting the CovR binding site in the operon. The hemolysin-null strain NEM 2459 was made by deleting CyrE in NEM 2424. All strains were grown on 5% sheep blood agar plates.

**Macrophages**

Primary bone marrow-derived macrophages (BMDM) and dendritic cells (BMDC) were differentiated from mouse bone marrow as described (12, 13). The immortalized macrophage cell lines from wild type mice (WT; C57BL6) were generated as described previously (10).

**Stimulation of BMDM and BMDC**

GBS and *Salmonella* stimulations were carried out for 6 h. During stimulation, macrophages were maintained in antibiotic-free DMEM for the first 2 h, and then ciprofloxacin was added for the remaining time. For priming, LPS (100 ng/ml) was added 2 h prior to stimulation (unless otherwise noted).

**IL-1β Analysis**

Cell culture supernatants were analyzed for IL-1β by ELISA (R&D Systems). For Western blots, cell culture supernatants were precipitated as described (10). The protein pellet was suspended in Laemmli buffer and probed for IL-1β (R&D Systems). For pro-IL-1β levels, cells were lysed (in PBS, 200 mM NaCl, 1% Triton-X, protease inhibitor cocktail) and probed for IL-1β.

**hNLRP3-FLAG Expression and Purification**

*Expression*—The cDNA sequence for human NLRP3 was subcloned under the copper-inducible metallothionein promoter into the Ascl-NotI restriction sites of pSORTN1D5-FLAG, a derivative of the pRmHA3 *Drosophila* vector (14), which contains an extended polylinker harboring sequences for Ascl, NotI, and the FLAG tag to create a C-terminally FLAG-tagged protein. The plasmid was transfected into *D.mel-2* cells, stable cell lines were generated, and expression of hNLRP3-FLAG upon induction with 500 μM copper sulfate to the cells was confirmed by Western blot with the anti-FLAG M2 antibody (Sigma).

*Purification*—D.mel-2/hNLRP3-FLAG cells expanded in serum-free HyClone SFX (Thermo Scientific) insect medium were split 1:1 and grown overnight. 500 μM copper sulfate was added for 4 h, and cells were harvested, lysed, and treated with Benzonase® overnight to digest DNA and RNA. The resulting lysate was used for protein purification over an anti-FLAG M2 resin (Sigma) column.

**Detection of RNA Associated with NLRP3 by PCR**

Immortalized WT macrophages were primed with 100 ng of LPS/ml for 1.5 h. Lysosomal damage was then induced by infecting with NEM 316 GBS for 6 h (MOI 12:1) or treatment with silica and monosodium urate (MSU). In the case of silica and MSU, cells were transfected with GBS RNA 1 h later. Cells were subsequently incubated for 5 h. Culture supernatants were collected and analyzed for IL-1β. The remaining cells on the dishes were lysed. Lysates were centrifuged at 3000 rpm for 5 min and then incubated with 20 μl of anti-FLAG M2 beads overnight at 4 °C. The beads were washed three times in lysis buffer and heated to 65 °C with nuclease-free water for 20 min to elute RNA off the beads. RNA (1 μg) was reverse-transcribed and amplified by GBS-specific GAPDH primers (forward, 5′-ATCCACGCATACTGGTGA-3′; reverse, 5′-AACAGGAAACAGGTGGTGAC-3′).

**In Vitro Binding of Purified NLRP3 and RNA**

Biotinylated GBS RNA (200 ng) was incubated with 700 ng of purified hNLRP3-FLAG for 4 h and then with 20 μl of 0.2% BSA-coated streptavidin magnetic beads overnight at 4 °C. The beads were washed and probed with anti-FLAG M2 antibody to detect hNLRP3-FLAG. Unbiotinylated GBS RNA (100 ng or 200 ng) was incubated with 350 or 750 ng of purified FLAG-tagged hNLRP3 for 4 h on ice and then with 20 μl of anti-FLAG M2 beads overnight at 4 °C. The beads were washed and heated to 65 °C with nuclease-free water for 20 min to elute off the RNA. RNA was reverse-transcribed, and PCR was carried out for GBS-specific GAPDH.
Bacterial RNA Extraction and Biotinylation

Overnight GBS cultures were centrifuged at 10,000 rpm for 10 min and washed with PBS. The cell pellet was vortexed on ice with glass beads and lysozyme (5 mg/ml). RNA was extracted with the RNA kit according to the manufacturer’s instructions (Zymo Research) and subsequently treated with DNase. RNA integrity was confirmed by measuring the A_{260/280} ratio (2.0–2.2). Isolated RNA was then biotinylated with the RNA 3’/H11032 end biotinylation kit (Pierce) as per the manufacturer’s protocol.

Biotinylated RNA-mediated NLRP3 Pulldown

Macrophages were primed with 100 ng of LPS/ml for 1.5 h. Lysosomal damage was then induced by infecting with NEM 316 GBS (MOI 12:1) for 1 h. Biotinylated RNA (20 ng/ml) isolated from NEM 316 and 2459 was then transfected and incubated for 5 h. Cells were lysed, and RNA was pulled down with streptavidin magnetic beads. The beads were further probed for NLRP3.

DNase/RNase Transfection into Macrophages

DNase/RNase transfection (10 or 100 ng/well) was performed with Pro-Ject protein transfection reagent (Pierce) for 3 h prior to LPS priming (100 ng/ml for 2 h) and then stimulated for 6 h with WT GBS (NEM 316), the hyperhemolytic strains NEM 2424 or 2802, or silica (500 μg/ml; positive control). Supernatants were measured by ELISA for released IL-1β.

RESULTS AND DISCUSSION

GBS Induces IL-1β Production via the NLRP3 Inflammasome in a β-Hemolysin-dependent Fashion—GBS produces two putative pore-forming toxins: ornithine rhamnolipid (β-hemolysin), a potent GBS virulence factor; and co-hemolysin CAMP (13). Our previous finding that GBS-induced IFN-β production requires β-hemolysin, but not CAMP (12), led us to analyze the IL-1β release by interacting with NLRP3.

FIGURE 2. GBS RNA induces IL-1β release by interacting with NLRP3. A, IL-1β production in WT BMDM transfected with DNase or RNase (10 or 100 ng), primed with LPS, and then stimulated for 5 h with either hyperhemolytic (NEM 2424 or 2802) GBS or NEM316 GBS at MOI 12:1, or with silica. In A and B, supernatant IL-1β was measured by ELISA. B, IL-1β production in WT BMDM primed with LPS for 1.5 h and then infected for 1 h with NEM316 or hemolysin-null (NEM2459) GBS at MOI 12:1. GBS RNA (20 ng/ml) isolated from NEM 316 and 2459 was then transfected and incubated for 5 h. Cells were lysed, and RNA was pulled down with streptavidin magnetic beads and incubated overnight at 4°C. Streptavidin beads were washed, boiled in Laemmli buffer, and analyzed by SDS-PAGE and Western blot for NLRP3 with the Cryo-2 antibody (Enzo Life Sciences). A fraction of the cleared supernatant was used to detect total NLRP3 content.
test whether β-hemolysin expression is required for IL-1β production. Neither a β-hemolysin-deficient GBS nor a β-hemolysin/CAMP double-deficient strain induced IL-1β production in BMDM. However, a β-hemolysin-competent, but CAMP-deficient strain, induced IL-1β at levels similar to NEM 316 GBS, indicating that β-hemolysin has a unique role in macrophage activation by GBS (supplemental Fig. 1A).

We then characterized the specific inflammasome involved in GBS-induced signaling. IL-1β production was completely abrogated in NLRP3−/− primary BMDM (Fig. 1A), but was unaltered in AIM2−/− primary BMDM (Fig. 1B), and primary BMDC from NLRP6−/− and NLRP12−/− mice (Fig. 1C). These findings suggest that GBS-induced IL-1β production depends primarily on NLRP3.

GBS RNA Induces IL-1β Release by Interacting with NLRP3—RNA has been shown to be a GBS effector that activates macrophages (15). To determine whether GBS RNA similarly activates the NLRP3 inflammasome, we transfected RNase or DNase into mouse macrophages and measured IL-1β release after GBS infection. Silica was used as a positive control. Transfection of RNase, but not DNase, diminished IL-1β induction upon infection with NEM 316 and hyperhemolytic GBS strains (Fig. 2A). The hyperhemolytic strains, which form larger hemolytic halos when compared with NEM 316 on sheep blood agar plates, produced more IL-1β as well (supplemental Fig. 1, B and C).

If RNase transfection diminishes GBS-induced IL-1β release, then exogenous RNA should increase it. Accordingly, we transfected RNA (20 ng/ml) isolated from NEM 316 or NEM 2459 GBS into BMDM that had been infected with NEM 316 1 h before and measured IL-1β release. RNA from both strains induced IL-1β (Fig. 2B). To determine whether RNA interacts with NLRP3, we transfected biotinylated RNA into GBS-infected macrophages. Cell lysates were precipitated with streptavidin magnetic beads and analyzed for NLRP3 by Western blot. More NLRP3 was pulled down after infection with NEM 316 than with β-hemolysin-deficient (NEM 2459) GBS, suggesting that lysosomal damage by β-hemolysin likely contributes to induction of the inflammasome by GBS RNA (Fig. 2C). This effect was not specific for GBS RNA because other bacterial RNAs also showed a similar result (data not shown). Endogenous NLRP3 was also found to interact with GBS RNA during live infection (supplemental Fig. 1D).

Given our observation that bacterially induced lysosomal damage is required for RNA interaction with NLRP3, we wanted to test whether crystal-induced lysosomal damage with silica or MSU also led to RNA-NLRP3 interaction. NLRP3-FLAG-overexpressing immortalized macrophages transfected with GBS RNA were subjected to anti-FLAG pulldowns. The beads were then analyzed for GBS GAPDH RNA by RT-PCR. We found NLRP3-associated GBS RNA only in the presence of lysosomal damage (Fig. 2D, top panel). Similarly, more IL-1β was released upon the addition of RNA after lysosomal damage (Fig. 2D, bottom panel). Importantly, the lysosomal acidifica-
tion inhibitor chloroquine abrogated GBS-induced IL-1β production (supplemental Fig. 1E).

Our observations strongly suggest an interaction between bacterial RNA and NLRP3. To explore whether these molecules interact in a cell-free system in vitro, we incubated semipurified hNLRP3-FLAG protein with unbiotinylated GBS RNA, immunoprecipitated the NLRP3 with anti-FLAG beads, subjected the beads to reverse transcription, and analyzed for GBS GAPDH by PCR. We found that RNA and NLRP3 interacted under these conditions (Fig. 3A). We also incubated semipurified hNLRP3-FLAG protein with biotinylated or unbiotinylated GBS RNA and subjected the mixtures to streptavidin bead pulldowns. We probed the beads for hNLRP3-FLAG and found substantial and subjected the mixtures to streptavidin bead pulldowns. We probed the beads for hNLRP3-FLAG and found substantial enrichment of NLRP3 in the presence of biotinylated RNA, further demonstrating that NLRP3 interacts with bacterial RNA (Fig. 3B).

These studies support a model for GBS-induced IL-1β production in mouse macrophages in which GBS-mediated activation of NLRP3 inflamasomes requires GBS uptake and phagolysosomal acidification and leakage (Fig. 3C). Our data suggest that: 1) IL1-β production in response to GBS infection is mediated by NLRP3 inflamasomes in the presence of the pore-forming toxin, β-hemolysin; 2) GBS RNA acts as a ligand for the NLRP3 inflammasome by interacting with NLRP3 (directly or via an evolutionarily conserved co-receptor (16, 17)); and 3) RNA interacts with NLRP3 only after lysosomal leakage, suggesting that lysosomal components are required for this interaction.

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