Published in final edited form as:
J Immunol. 2012 July 1; 189(1): 328–336. doi:10.4049/jimmunol.1103258.

Bacterial RNA mediates activation of caspase-1 and IL-1β release independently of TLR3, -7, -9 and Trif but dependent on Unc93B

Tatjana Eigenbrod[*,1], **Luigi Franchi**[†], **Raul Muñoz-Planillo**[†], **Carsten J. Kirschning**[‡], **Marina A. Freudenberg**[§], **Gabriel Núñez**[†], and **Alexander Dalpke**[*]

[*]Department of Infectious Diseases, Medical Microbiology and Hygiene, University of Heidelberg, 69120 Heidelberg, Germany

[†]Department of Pathology and Comprehensive Cancer Center, University of Michigan Medical School, Ann Arbor, Michigan 48109, USA

[‡]Institute of Medical Microbiology, University of Duisburg-Essen, 45147 Essen, Germany

[§]Max-Planck-Institute of Immunobiology and Epigenetics, 79108 Freiburg, Germany

Abstract

Recognition of foreign nucleic acids is important for the induction of an innate immune response against invading pathogens. Although the pathways involved in sensing bacterial DNA and viral RNA are now well established, only limited knowledge is available on mechanisms underlying recognition of bacterial RNA. It has been reported that intracellular delivery of *E. coli* RNA activates the Nlrp3 inflammasome but whether this is a general property of bacterial RNA remains unclear as well as the pathways involved in pro-IL-1β induction and caspase-1 activation by bacterial RNA. Here, we report that bacterial RNA from both gram-positive and gram-negative bacteria induces activation of caspase-1 and secretion of IL-1β by murine DC and BMDM. Stimulation was independent of the presence of 5′triphosphate termini and occurred with whole RNA preparations from bacteria but not from eukaryotes. Induction of pro-IL-1β as well as the priming for caspase-1 activation by bacterial RNA was dependent on Unc93B, an endoplasmic reticulum protein essential for delivery of TLRs to the endosome, whereas the established nucleic acid sensing endosomal TLRs 3, 7 and 9 were dispensable. In addition, caspase-1 activation and IL-1β production by transfected bacterial RNA were absent in MyD88 deficient cells but independent of Trif. Thus, our data indicate the presence of a yet unidentified intracellular nucleic acid receptor involved in bacterial RNA induced inflammasome activation and release of IL-1β.

Introduction

Infections with pathogenic microorganisms induce rapid activation of the innate immune system by activation of specialized pattern recognition receptors (PRRs) upon binding of pathogen-associated molecular patterns (PAMPs). Important groups of PRRs include toll-

[1]Address for correspondence: Tatjana Eigenbrod, Department of Infectious Diseases, Medical Microbiology and Hygiene, University of Heidelberg, INF 324, 69120 Heidelberg, Germany, Tel: +49-6221-5636771; tatjana.eigenbrod@med.uni-heidelberg.de.

like receptors (TLRs), NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs). The adapter molecule MyD88 is recruited to all activated TLRs except for TLR3 that mediates its signaling through Trif (1). As another exception, TLR4 triggers both MyD88- and TRIF-dependent signalling pathways. Recruitment of MyD88 and/or Trif triggers the activation of NF-κB and/or the IRF-pathway, resulting in the induction of genes encoding pro-inflammatory cytokines including IL-1β, IL-6, TNF and type I interferons, respectively (2). Microbial nucleic acids are an important group of immune stimulatory PAMPs that - depending on their subcellular localization - are being sensed by the endosomal TLRs 3, 7 and 9 or by cytosolic RLRs. Viral dsRNA and ssRNA have been identified as ligands for TLR3 and TLR7, whereas TLR9 senses unmethylated CpG motifs present in prokaryotic DNA (3–5). MDA-5 has been described to be activated by long stretches of viral dsRNA in the cytosol, whereas RIG-I initiates antiviral signaling in response to dsRNA or ssRNA with 5′triphosphate termini (6–8). Of note, it has been reported that transfected viral 5′triphosphate RNA can induce activation of caspase-1 and production of mature IL-1β in a RIG-I dependent but Nlrp3 independent manner although the differential roles of RIG-I and Nlrp3 for caspase-1 activation by different RNA viruses remain controversial (6, 9).

By contrast, it is largely unknown how bacterial RNA is sensed by the immune system although recent reports have pointed out the importance of bacterial RNA recognition for initiating an innate immune response against invading bacteria. Thus, delivery of bacterial RNA into immune cells using cationic lipid transfection reagents induced secretion of pro-inflammatory cytokines and recognition of bacterial RNA was important for the TNF response by several gram-positive bacteria (10–12). In addition, intracellular delivery of RNA isolated from *E. coli* has been described to activate caspase-1 via the Nlrp3 inflammasome and to induce secretion of active IL-1β (13, 14)

Production of active IL-1β is tightly regulated in a two step process: Usually mediated by PRR-induced NF-κB activation, IL-1β is initially synthesized as a 31-kDa inactive precursor molecule, pro-IL-1β, which in a second step is cleaved into the bioactive cytokine by caspase-1 (15). A critical step for activation of caspase-1 is the assembly of the so-called ‘inflammasome’, a multiprotein complex composed of procaspase-1, the adaptor molecule ASC and a sensor protein, for example Nlrp3, that mediates the autocatalytic self-cleavage of caspase-1 into the active enzyme (16). The activation of the Nlrp3 inflammasome requires two signals: Signal 1 provided by a microbial PAMP or an inflammatory cytokine functions as ‘priming’ signal, e.g. by up-regulation of Nlrp3 expression (17). In addition, a second signal is needed that can be delivered by ATP, pore-forming bacterial toxins or particulate substances (18–21). The exact mechanisms of inflammasome activation are not yet fully understood but the generation of reactive oxygen species (ROS), lysosomal damage and potassium efflux can contribute to it (22). It has not yet been investigated if activation of the Nlrp3 inflammasome by bacterial RNA is a unique feature of *E. coli* RNA or a general characteristic of bacterial RNA. In addition, pathways and mechanisms involved in both pro-IL-1β induction and caspase-1 activation by bacterial RNA have been poorly characterized. Here, we report that bacterial RNA from both gram-positive and gram-negative bacteria induced activation of caspase-1 and secretion of IL-1β a MyD88 dependent manner. However, the so far characterized nucleic acid sensing TLRs 3, 7 and 9 as well as

TLR adapter molecule Trif were dispensable. Yet, induction of pro-IL1β and Nlrp3 was dependent on Unc93B, thus indicating the existence of a novel, yet unknown nucleic acid detecting intracellular PRR for inflammasome activation and IL-1β release by bacterial RNA.

Materials and Methods

Reagents

RPMI 1640 containing stable glutamine and DMEM were purchased from Biochrom (Berlin, Germany), Lipofectamine 2000 and TRIzol were from Invitrogen Life Technologies (Darmstadt, Germany), FCS from Gibco (Darmstadt, Germany), Pam_3CSK_4, poly(I:C), R848 and nigericin from Invivogen (San Diego, CA, USA). Ultrapure LPS from *Salmonella minnesota* was provided by U. Seydel (Borstel, Germany). Silica, streptolysin O (SLO), dithiothreitol (DTT), N-acetyl-L-cystein (NAC), rotenone, 2′,7′-dichlorofluorescein diacetate (DCFDA), RNase A and Proteinase K were from Sigma (Munich, Germany), diphenylene iodonium (DPI) and bafilomycin A1 from Calbiochem (Merck, Darmstadt, Germany) and shrimp alkaline phosphatase from Fermentas (St. Leon-Roth, Germany). Primer for Nlrp3 (fw 5′-CCG AAG TGG GGT TCA GAT AA-3′, rev 5′-TTC AAT GCA CTG GAA TCT GC-3′) and pro-IL-1β (fw 5′-GGG CCT CAA AGG AAA GAA TC-3′, rev 5′-GGG GAA CTC TGC AGA CTC AA-3′) were custom synthesized by MWG-Biotech (Ebersberg, Germany).

Bacteria and mouse strains

The following microbial strains were used: *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Moraxella catarrhalis* (ATCC 9143), *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 121), *Listeria monocytogenes* (ATCC 15313), S*treptococcus pyogenes* (patient isolate), *Candida albicans* (ATCC 90028).

Tlr2/3/7/9$^{-/-}$ *and Tlr2/3/4/7/9*$^{-/-}$ mice resulted from crossing-breeding of respective single knockout mice (23). *MyD88*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice have been described (14, 24). *Unc93B* mice harboring a H412R missense mutation (‘3d’) (25) and *lps2* mice harboring a mutation in the TRIF adaptor protein (26) were bred at the MPI, Freiburg, Germany. All knockout mice were backcrossed onto the C57BL/6 background for at least eight times. Wild-type (WT) C57BL/6 mice were maintained in the animal facility of the University of Heidelberg, Germany. All animal studies were approved by the local authorities.

Cell isolation and differentiation

Bone marrow GM-CSF-derived myeloid dendritic cells (DC) and bone marrow derived macrophages (BMDM) were prepared from 8–12 week-old mice as described previously (11). Briefly, for differentiation of DC, 8×10^6 bone marrow cells were seeded into 15 cm cell culture plates in differentiation medium (RPMI 1640 containing stable glutamine, supplemented with 10% FCS, 1% Penicillin/Streptomycin, 0.05 mM β-mercaptoethanol). Cell culture supernatant of LGM3 cells that produce GM-CSF was added as source of GM-CSF. Immature DC ($CD11c^+$, $B220^-$) were harvested at day 8. For generation of bone-marrow derived macrophages (BMDM), bone-marrow cells were seeded into 15 cm Petri

dishes in DMEM supplemented with 30% L929 supernatant, 10% FCS and 1% Penicillin/Streptomycin.

Cell stimulation and transfection

DC were stimulated in RPMI 1640 supplemented with 10% FCS and macrophages were stimulated in DMEM supplemented with 10% FCS. Unless specified otherwise, DC were stimulated overnight with 5 μg/ml bacterial RNA complexed with Lipofectamine 2000 at a ratio of 1 μl Lipofectamine 2000 per 1 μg of RNA. BMDM were transfected at a concentration of 10 μg/ml. For SLO experiment, cells were transfected for 4.5 h with bacterial RNA before replacing medium with PBS containing SLO (10μg/ml) and DTT (10 mM). After ca. 15–25 min, PBS was removed and fresh medium was added for another 3 h. As a positive control for caspase-1 activation, poly(dA:dT) complexed with Lipofectamine 2000 was transfected at a concentration of 1 μg/ml for 6 h. Alternatively, cells were incubated with the indicated TLR ligands for 1 h, followed by stimulation with silica for 5 h, or stimulated with LPS for 4 h followed by ATP (5 mM) for 45 min. For inhibitor experiments, DC were preincubated for 1 h with NAC or DPI at the indicated concentrations prior to transfection. Rotenone was added 3 h prior to collection of the cells.

Preparation of total bacterial RNA

Bacteria and yeast were grown in LB medium (*S. aureus, E. coli, P. aeruginosa, M. catarrhalis, C. albicans*) or BHI medium (*E. faecalis, L. monocytogenes, S. pyogenes*) and harvested within the mid log-phase growth. After a digestion step with lysozyme (1 h at 40 mg/ml for gram-positive and 5 mg/ml for gram-negative strains), bacterial RNA was isolated using TRIzol according to the manufacturer's protocol. The obtained RNA underwent a further purification step using the RNeasy mini kit (Qiagen, Hilden, Germany) including an on-column DNA digestion according to the manufacturer's protocol. Purity of the bacterial RNA preparations was validated by determining the 260/230 nm and 260/280 nm extinction ratio by NanoDrop (Thermo Scientific).

Isolation of mammalian RNA for quantitative real-time PCR

Total RNA from cells was isolated using the RNeasy mini kit including on-column DNA digestion (Qiagen, Hilden, Germany). RNA was transcribed into cDNA using the high capacity cDNA reverse transcription kit (Applied Biosystems, Woolston, UK) according to the manufacturer's instructions. Quantitative real-time PCR was performed with SYBRGreen (Applied Biosystems, Woolston, UK) using a standard protocol. Identity of amplicons was checked by melting curve analysis and no reverse transcriptase and no template controls were included. Analyses were performed in duplicates.

RNase digestion, RNA dephosphorylation, Proteinase K digestion and RNA fragmentation

Bacterial RNA was digested with RNase A at a concentration of 1 μg RNase A per 1 μg RNA for 60 min at 37°C, or with Proteinase K at a concentration of 500 μg/ml for 2 h at 37°C. Dephosphorylation of 5′-triphosphate ends was performed with shrimp alkaline phosphatase according to the manufacturer's protocol. Hydrolytic fragmentation of bacterial

RNA was performed at 50°C using RNA fragmentation buffer from Ambion (Austin, Texas, USA) according to the manufacturer's instructions.

Heat inactivation and RNase A digestion of bacteria

Bacteria were grown as described before, washed in PBS, and heat inactivated at 70°C for 45 min. Afterwards, an aliquot of these heat-killed bacteria was digested with RNase A at a concentration of 150 μg/ml for 60 min at 37°C.

Immunoblotting and cytokine measurements

For Western Blot analysis, complete cell culture supernatants were collected together with the cells in a lysis buffer containing 1% Nonidet-P40 supplemented with protease inhibitors leupeptin, aprotinin, pepstatin A at 1 μg/ml and PMSF at 1 mM. Proteins were then separated by 12% SDS-PAGE without prior precipitation and membranes were probed with a rabbit anti-mouse caspase-1 antibody provided by G. Núñez (University of Ann Arbor, Michigan, USA) (27). Alternatively, the same lysates were analyzed for pro-IL-1β expression using a goat anti-mouse IL-1β antibody (M-20, Santa Cruz, CA, USA).

Measurement of ROS production

DC were transfected with bacterial RNA (5 μg/ml) for 6 h in RPMI 1640 supplemented with 10% FCS. As positive control, cells were stimulated with LPS (100 ng/ml) for 4 h. 20 min prior to collection of the cells, 4 μM DCFDA was added. Cells were washed twice with PBS and subsequent FACS analysis was performed. Untreated cells not loaded with dye were used as a negative control to examine cellular autofluorescence.

Statistical analysis

Statistical significance between groups was determined by two tailed Student's *t* test. Differences were considered significant for $p < 0.05$ (*).

Results

RNA from both, gram-positive and gram-negative bacteria activates the Nlrp3 inflammasome in unprimed dendritic cells and macrophages

To investigate whether caspase-1 activation is a general characteristic of bacterial RNA, total RNA purified from a variety of clinically relevant bacterial strains was comparatively analyzed for its capacity to induce secretion of IL-1β by murine GM-CSF derived myeloid DC (DC). The panel tested included gram-positive and gram-negative, extracellular (*S. aureus*) and intracellular (*L. monocytogenes*) as well as spore-forming (*B. subtilis*) bacteria. As shown in Fig. 1A, transfected bacterial RNA from all tested strains induced IL-1β secretion in DC. By contrast, eukaryotic RNA derived from *C. albicans* and RAW264.7 macrophages failed to do so (Fig. 1A). IL-1β secretion by transfected bacterial RNA purified from gram-positive bacteria was completely abolished upon RNase A digestion, confirming that IL-1β release was indeed mediated by bacterial RNA and not by potential contaminants. However, residual activity was observed with bacterial RNA from gram-negative bacteria after RNase A digestion, indicating relevant contamination of those bacterial RNA

preparations with other PAMPs, most likely LPS that is found in the cell wall of gram-negative but not gram-positive bacteria. Therefore, only bacterial RNA purified from gram-positive bacteria was used for further experiments. Activation of caspase-1 by transfected bacterial RNA derived from different gram-positive strains was dependent on Nlrp3, as it has been described before for *E. coli* RNA (Fig. 1B). Bacterial RNA also induced IL-1β release in BMDM although side-by-side comparison demonstrated a higher sensitivity of DC towards stimulation with bacterial RNA (Fig. 1C). In line with a previous report (13), transfected bacterial RNA induced caspase-1 activation and release of IL-1β even in cells that received neither a separate priming nor an additional second signal (Figs. 1D, E). Although not required, additional stimulation of bacterial RNA transfected cells with the classical second signals ATP or pore-forming bacterial toxins nigericin and SLO enhanced and accelerated caspase-1 activation and release of IL-1β (Fig. 1D, E; note decrease in pro-IL-1β levels due to cleavage). As compared to overnight stimulation (Fig. 1A), IL-1β release induced by bacterial RNA alone was rather weak at early time-points after stimulation despite a sufficient up-regulation of pro-IL-1β (Figs. 1D, E).

IL-1β secretion by bacterial RNA requires intracellular delivery but is independent of RNA length and 5′-triphosphate termini

Next, the functional and structural requirement of bacterial RNA for inflammasome activation and IL-1β release were investigated. As demonstrated in Fig. 2A, intracellular delivery of bacterial RNA was essential for IL-1β secretion as stimulation with extracellular bacterial RNA showed no effect (Fig. 2A). It has recently been suggested that phagocytosis of *E. coli* RNA using heat-killed *E. coli* as a cargo resulted in strong release of IL-1β in murine BMDM (28). However, in the present study we did not observe additional IL-1β secretion when cells were stimulated with heat-inactivated *S. aureus* together with *S. aureus* RNA as compared to IL-1β release with heat-inactivated *S. aureus* alone (Fig. 2B). To investigate whether recognition of bacterial RNA was length-dependent, hydrolytic cleavage of RNA was performed. Fragmentation slightly increased IL-1β secretion although bacterial RNA in a size range between ca. 300 and 3000 bases had rather similar immunostimulatory capacity. It has been reported previously that viral 5′triphosphate RNA can activate caspase-1 upon recognition by the cytosolic helicase RIG-I (9). However, dephosphorylation of bacterial RNA using shrimp alkaline phosphatase did not alter IL-1β release (Fig. 2D) indicating that structures other than 5′triphosphate ends are crucial for inflammasome activation by bacterial RNA. Ribosomal 16S and 23S rRNA constitute approximately 80% of total bacterial RNA. As ribosomal RNA is tightly associated with ribonucleoproteins, it was investigated if potential RNA-associated proteins might be relevant for IL-1β secretion. As demonstrated in Fig. 2E, IL-1β release was not affected by treatment of bacterial RNA preparations with proteinase K, excluding a role for RNA/protein complexes.

Bacterial RNA mediated induction of pro-IL-1β and priming for Nlrp3 inflammasome activation are independent of TLR3, TLR7 and TLR9 but require Unc93B

To date, limited knowledge is available about the signaling pathways involved in innate immune sensing of bacterial RNA, especially regarding induction of pro-IL-1β and caspase-1 activation. To analyze these processes, the response of cells with a combined deficiency for the known nucleic acid sensing TLRs 3, 7 and 9 were analyzed for their

response to bacterial RNA. However, IL-1β release of DC and BMDM from TLR2/3/7/9 deficient mice transfected with bacterial RNA purified from all tested strains did not differ from WT cells (Figs. 3A, B). Of note, IL-1β secretion was unimpaired despite the deficiency for TLR2, a cell surface TLR involved especially in recognition of gram-positive bacterial cell wall components (29), further confirming the purity of the bacterial RNA preparations used. As all so far identified nucleic acid sensing TLRs are localized in the endosome, we next set up to investigate the role of Unc93B, an endoplasmic reticulum protein that is critical for delivery of nucleic acid sensing TLRs to the endolysosome (25, 30). IL-1β secretion was completely abolished in DC and BMDM harboring the missense mutation H412R in Unc93B (Figs. 3A, B). As this mutation abrogates signaling via all three endosomal TLRs - TLR3, 7 and 9 - it is also referred to as '3d'. Accordingly, TLR3, TLR7 and TLR9 ligands poly(I:C), R848 and CpG, respectively, failed to induce IL-1β secretion in Unc93B 3d cells but cells responded normally to the TLR2 ligand Pam_3CSK_4 and to the TLR4 ligand LPS (Fig. 3A). The lack of bacterial RNA induced IL-1β secretion observed in Unc93b mutated cells was a consequence of both insufficient up-regulation of pro-IL-1β and impaired cleavage of caspase-1 (Fig. 3C, D). Abolished caspase-1 activation correlated with defective priming, i.e. defective up-regulation of Nlrp3 which constitutes an important prerequisite for inflammasome assembly (17) (Fig. 3E). Next, we set up to investigate if apart from priming, Unc93b plays an additional direct role in caspase-1 activation by bacterial RNA. To this end, Unc93b mutated DC were first primed with Pam3CSK4 so circumvent deficient priming by bacterial RNA and were then either transfected with bacterial RNA or mock transfected. Under these conditions, IL-1β secretion did not differ between Unc93b mutated and WT cells (Fig. 3F), indicating that Unc93b is only mediating the priming signal but does not activate the inflammasome directly. As Unc93B mediates trafficking of TLRs to the endosome, we next pre-treated cells with bafilomycin A1, an inhibitor of endosomal acidification known to abrogate endosomal TLR signalling. As shown in Fig. 3G, bafilomycin A1 disrupted IL-1β secretion induced by bacterial RNA but not by LPS plus Silica.

Secretion of IL-1β upon bacterial RNA stimulation is dependent on MyD88 but independent of Trif

Next, the signaling pathways downstream of Unc93B were analyzed. To this end, DC and BMDM derived from mice deficient for MyD88, a key adaptor molecule for TLR signaling, were tested for their ability to respond to bacterial RNA. IL-1β secretion was completely abrogated in MyD88-deficient DC and BMDM (Fig. 4A). Similar to the data obtained in Unc93B mutated cells, both the induction of pro-IL-1β as well as the priming step for Nlrp3 inflammasome activation, i.e. up-regulation of Nlrp3 expression, were critically dependent on MyD88 (Figs. 4B, C). Accordingly, MyD88 deficient DC and BMDM failed to activate caspase-1 upon bacterial RNA transfection, whereas they responded normally to MyD88-independent stimulus poly(dA:dT) that mediates caspase-1 activation via the cytosolic receptor AIM2 (31–33) (Fig. 4C and data not shown). MyD88 is the adaptor molecule for all TLRs except for TLR3 which mediates its signal via Trif (1). In line with the previous data, Trif was dispensable for bacterial RNA-induced IL-1β release in DC and BMDM (Fig. 4D). Thus, it is clearly demonstrated that Unc93B and MyD88, but not the so far established nucleic acid sensing TLRs 3, 7 and 9 or the adaptor Trif were critical for the induction of

pro-IL-1β as well as for mediating the priming signal for inflammasome activation in murine DC and BMDM.

S. pyogenes induces IL-1β via recognition of its RNA in an Unc93B-dependent manner

Next, it was investigated if recognition of bacterial RNA was also relevant for infection with *S. pyogenes*, a bacterium that has previously been described to activate the Nlrp3 inflammasome and to up-regulate pro-IL-1β via a yet unidentified ligand (34). To this end, heat-inactivated *S. pyogenes* was depleted for its RNA by digestion with RNase A. Stimulation of DC with *S. pyogenes* induced expression of pro-IL-1β in a partly RNA-dependent manner, as the response was significantly diminished with RNase A treated *S. pyogenes* (Fig. 5A). As it was demonstrated before that release of IL-1β by transfected bacterial RNA was independent of TLR2, 3, 7 and 9 but required Unc93B (Fig. 3A), it was next tested if the same pathway was involved in *S. pyogenes* mediated IL-1β induction. Resembling the results obtained with transfected bacterial RNA, heat-inactivated *S. pyogenes* induced up-regulation of pro-IL-1β and Nlrp3 in WT and TLR2/3/7/9 deficient DC but not in Unc93B 3d mutated cells (Fig. 5B, C). The same pattern was observed for IL-1β secretion (Fig. 5D). The rather low capacity of heat-inactivated *S. pyogenes* to induce release of active IL-1β is most likely due to the lack of SLO production that has been shown to have a major impact on *S. pyogenes* mediated caspase-1 activation (34).

Activation of the Nlrp3 inflammasome by bacterial RNA requires reactive oxygen species (ROS)

Although the exact mechanisms involved in Nlrp3 inflammasome activation by diverse stimuli remain elusive, a common role for reactive oxygen species (ROS) has been widely implicated (18, 35–38). Hence, it was investigated whether ROS might also play a role in bacterial RNA induced IL-1β secretion. Enhanced ROS generation was detected upon intracellular delivery of bacterial RNA as determined by FACS analysis of DCFDA loaded dendritic cells (Fig. 6A). ROS were induced to a similar extend as observed with LPS whereas transfection reagent alone showed only minor effects. Treatment of DC with N-acetyl-L-cystein (NAC), a well established and widely used ROS scavenger that reduces cellular levels of ROS, dose-dependently suppressed IL-1β release after transfection of bacterial RNA and impaired cleavage of caspase-1 (Figs. 6B, C). The major sources for cellular ROS that have been implicated in inflammasome activation are the NADPH oxidase and mitochondria (19, 36–38). As shown in Fig. 6D, the presence of NADPH oxidase inhibitor DPI diminished IL-1β release in response to bacterial RNA. In addition, treatment of DC with rotenone, a mitochondrial complex I inhibitor known to enhance mitochondrial ROS generation, caused a moderate but significant enhancement of IL-1β secretion upon transfection of bacterial RNA (Fig. 6E). Together, these data thus indicate that both NADPH oxidase and mitochondrial derived ROS formation contribute to inflammasome activation by bacterial RNA.

Discussion

Although the relevance of bacterial DNA and viral RNA recognition for inducing an innate immune response against a variety of pathogens has been extensively investigated, only few

studies have so far addressed the role of bacterial RNA. Some reports indicate that intracellular delivery of bacterial RNA in immune cells results in production of type I interferons and pro-inflammatory cytokines including TNF and IL-12 (10–12, 39). The significance of bacterial RNA recognition has been strengthened by the observation that DOTAP-packaged extracts from *S. pyogenes* failed to induce IFN-β in myeloid DC upon treatment with RNase A (40). Moreover, intracellular *E. coli* RNA mediates activation of the Nlrp3 inflammasome and release of IL-1β (13, 28). However, if this is unique for *E. coli* RNA or a general property of bacterial RNA has not been investigated and the signaling pathways involved remain poorly defined.

In the present study, we report that intracellular delivery of bacterial RNA derived from a variety of gram-positive and gram-negative bacteria induced Nlrp3-dependent activation of caspase-1 and secretion of IL-1β. By contrast, eukaryotic RNA from RAW 264.7 cells or *C. albicans* failed to induce caspase-1 activation. Lack of immunostimulatory capacity of eukaryotic RNA is in accordance with previous reports and attributable to a differential nucleotide modification profile of prokaryotic as compared to eukaryotic RNA (39, 41). Of note, IL-1β release by RNA derived from gram-positive bacteria was totally abolished upon treatment with RNase A but was independent of TLR2, a cell surface receptor involved in sensing cell wall components of gram-positive bacteria (29). Thus, a major strength of the current study is the use of bacterial RNA preparations devoid of relevant contaminating TLR ligands that could interfere with the immunostimulatory effect of the bacterial RNA itself. As indicated before, activation of the Nlrp3 inflammasome usually requires two consecutive signals (16). However, in line with a previous report (13), transfection of bacterial RNA was sufficient to drive secretion of processed IL-1β. Intracellular delivery of bacterial RNA was crucial for IL-1β release as extracellular stimulation had no effect. Although not required, activation of caspase-1 and secretion of mature IL-1β by transfected bacterial RNA were greatly enhanced and accelerated when cells received an additional secondary signal in form of ATP or pore-forming toxins nigericin and SLO. Thus, bacterial RNA can act in concert with danger signals and bacterial toxins that are released during infection to maximize inflammasome activation.

Not much is known about the structural requirements of bacterial RNA that are necessary for caspase-1 activation. It has been described that transfected viral 5′triphosphate RNA can induce inflammasome activation and release of IL-1β in murine DC and human PBMC (9). Although 5′triphosphate ends are also present in bacterial mRNA (42) and can thus be found in the total bacterial RNA preparations used in the current study, these structures were dispensable for inflammasome activation by bacterial RNA. This is in line with a previous report (28) and with the notion that inflammasome activation by transfected viral 5′-triphospate RNA required RIG-I but not Nlrp3, whereas Nlrp3 was critical for caspase-1 cleavage in response to bacterial RNA (9, 14). It is known that the length of viral dsRNA is critical for its differential recognition by either RIG-I or MDA5 (8). However, immunostimulatory capacity of bacterial RNA appeared not to be length-dependent and was rather similar in a size range of ca. 300–3000 bases.

The sensing of bacterial RNA is differentially regulated and cell-type specific. In this regard, it has been reported that transfected bacterial RNA triggered the release of IFN-α in human

PBMC and murine plasmacytoid DC in a TLR7 dependent manner (11). However, the role of TLR7 for induction of type-I interferon in murine myeloid DC is controversial: Mancuso *et al.* reported that group B streptococcus (GBS) induced IFN-β in mouse myeloid DC via recognition of its RNA by TLR7 (12). In contrast, Gratz *et al.* proposed that DOTAP-packaged lysates from *S. pyogenes* induced IFN-β in a RNA-dependent, but TLR7 independent manner (40). Similarly, production of TNF did not require TLR7 in human PBMC as well as in murine DC and BMDM (10, 11). The receptors participating in inflammasome activation by bacterial RNA in murine DC and BMDM still remain elusive. Here we demonstrate that both the induction of pro-IL-1β as well as the activation of caspase-1 by transfected bacterial RNA were independent of the established nucleic acid sensing TLRs 3, 7 and 9. Potential redundancies of the indicated receptors were excluded by using cells derived from mice lacking expression of all three TLRs. However, secretion of IL-1β was critically dependent on the TLR adapter molecule MyD88 and Unc93B, an endoplasmic reticulum protein that is required for trafficking of nucleic acid sensing TLRs to the endosome (25, 30). Lack of caspase-1 activation in Unc93B 3d mutant cells was due to interference with the priming step, i.e. impaired up-regulation of Nlrp3 which is required for Nlrp3 inflammasome activation (17). Of note, priming of Unc93b mutant cells with Pam3CSK4 prior to transfection of bacterial RNA restored IL-1β secretion, indicating that Unc93b does not play a direct role in caspase-1 activation. We further demonstrate that the same pathway was involved in both DC and BMDM and occurred for total RNA preparations from all tested bacterial strains.

It was recently proposed that intracellular delivery of extracellularly added *E. coli* RNA using heat-killed *E. coli* as a cargo for phagocytic uptake resulted in strong IL-1β release via the Nlrp3 inflammasome (28). Under these conditions, induction of pro-IL-1β was MyD88-dependent, whereas caspase-1 activation was regulated by Trif. However, we could not detect increased IL-1β secretion upon co-stimulation of cells with heat-inactivated *S. aureus* plus extracellular *S. aureus* RNA and IL-1β release upon transfection of bacterial RNA was independent of Trif in our study. The discrepancy between these two investigations might be due to the different bacterial strains used and/or potential contamination of *E. coli* RNA with other TLR-ligands that overlap with RNA mediated effects. However, the data of the current study are in line with a very recent report demonstrating that the TNF response of BMDM to RNA from *Streptococcus agalactiae* was independent of TLR7 and TLR9 but required Unc93B (10). As TLR8, another member of the endosomal TLR family that mediates recognition of GU-rich ssRNA in humans, is found to be non-functional in mice (4), these data suggest the presence of another intracellular nucleic acid receptor for sensing bacterial RNA. In which cellular compartment this recognition takes place is not entirely clear. As Unc93B delivers nucleic acid sensing TLRs to the endosome, it is possible that the unknown receptor shows the same localization. In addition, IL-1β secretion was abolished upon treatment with bafilomycin A1, an inhibitor of vacuolar H^+ATPase known to interfere with endosomal TLR signaling. However, lysosomal acidification might also be necessary for endosomal escape of bacterial RNA, which could then be sensed in the cytosol.

The present study further shows that recognition of its RNA was also important for IL-1β induction by heat-inactivated *S. pyogenes* and involved the same signalling pathways as

observed for transfected bacterial RNA. In line with these results, it was reported that induction of IFN-β by *S. pyogenes* in DC was probably mediated by its RNA and required MyD88 but not TLR3 and TLR7 (40). It was recently proposed that bacterial mRNA functions as so-called 'vita-PAMP' that is present in live bacteria but rapidly lost upon microbial cell death, thus enabling the immune system to distinguish between vital and dead bacteria (28). However, our data demonstrate that detection of foreign RNA was still relevant for cytokine induction by heat-inactivated *S. pyogenes*. Bacterial RNA was partially degraded after heat-inactivation (data not shown), but reduction of RNA size did not affect its immunostimulatory capacity (see Fig. 2C). The results of the present study are in line with a previous report demonstrating that several gram-positive heat-inactivated bacteria lost most of their TNF-inducing capacity when depleted of their RNA by RNase A digestion (10). Technical differences in inducing bacterial cell death may account for the divergent results observed in the different studies. In summary, our findings together with other reports strengthen the relevance of bacterial RNA recognition for the initiation of an innate immune response against invading pathogens and argue for the existence of another, yet unidentified intracellular nucleic acid receptor.

Acknowledgments

We thank A. Elfner, Å. Hidmark and H. Garbers for excellent technical support.

Abbreviations

BMDM	bone-marrow derived macrophages
DC	dendritic cell
MOI	multiplicity of infection
NAC	N-acetyl-L-cystein
o.n	over night
PRR	pattern recognition receptor
SLO	streptolysin O

References

1. Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, Takeuchi O, Sugiyama M, Okabe M, Takeda K, Akira S. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science. 2003; 301:640–643. [PubMed: 12855817]
2. Kawai T, Akira S. Toll-like receptor and RIG-I-like receptor signaling. Ann N Y Acad Sci. 2008; 1143:1–20. [PubMed: 19076341]
3. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature. 2001; 413:732–738. [PubMed: 11607032]
4. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K, Akira S. A Toll-like receptor recognizes bacterial DNA. Nature. 2000; 408:740–745. [PubMed: 11130078]
5. Lund JM, Alexopoulou L, Sato A, Karow M, Adams NC, Gale NW, Iwasaki A, Flavell RA. Recognition of single-stranded RNA viruses by Toll-like receptor 7. Proc Natl Acad Sci U S A. 2004; 101:5598–5603. [PubMed: 15034168]

6. Rajan JV, Rodriguez D, Miao EA, Aderem A. The NLRP3 inflammasome detects encephalomyocarditis virus and vesicular stomatitis virus infection. J Virol. 2011; 85:4167–4172. [PubMed: 21289120]
7. Hornung V, Ellegast J, Kim S, Brzozka K, Jung A, Kato H, Poeck H, Akira S, Conzelmann KK, Schlee M, Endres S, Hartmann G. 5′-Triphosphate RNA is the ligand for RIG-I. Science. 2006; 314:994–997. [PubMed: 17038590]
8. Kato H, Takeuchi O, Mikamo-Satoh E, Hirai R, Kawai T, Matsushita K, Hiiragi A, Dermody TS, Fujita T, Akira S. Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. J Exp Med. 2008; 205:1601–1610. [PubMed: 18591409]
9. Poeck H, Bscheider M, Gross O, Finger K, Roth S, Rebsamen M, Hannesschlager N, Schlee M, Rothenfusser S, Barchet W, Kato H, Akira S, Inoue S, Endres S, Peschel C, Hartmann G, Hornung V, Ruland J. Recognition of RNA virus by RIG-I results in activation of CARD9 and inflammasome signaling for interleukin 1 beta production. Nat Immunol. 11:63–69. [PubMed: 19915568]
10. Deshmukh SD, Kremer B, Freudenberg M, Bauer S, Golenbock DT, Henneke P. Macrophages recognize streptococci through bacterial single-stranded RNA. EMBO Rep. 2011; 12:71–76. [PubMed: 21164516]
11. Eberle F, Sirin M, Binder M, Dalpke AH. Bacterial RNA is recognized by different sets of immunoreceptors. Eur J Immunol. 2009; 39:2537–2547. [PubMed: 19662634]
12. Mancuso G, Gambuzza M, Midiri A, Biondo C, Papasergi S, Akira S, Teti G, Beninati C. Bacterial recognition by TLR7 in the lysosomes of conventional dendritic cells. Nat Immunol. 2009; 10:587–594. [PubMed: 19430477]
13. Kanneganti TD, Lamkanfi M, Kim YG, Chen G, Park JH, Franchi L, Vandenabeele P, Nunez G. Pannexin-1-mediated recognition of bacterial molecules activates the cryopyrin inflammasome independent of Toll-like receptor signaling. Immunity. 2007; 26:433–443. [PubMed: 17433728]
14. Kanneganti TD, Ozoren N, Body-Malapel M, Amer A, Park JH, Franchi L, Whitfield J, Barchet W, Colonna M, Vandenabeele P, Bertin J, Coyle A, Grant EP, Akira S, Nunez G. Bacterial RNA and small antiviral compounds activate caspase-1 through cryopyrin/Nalp3. Nature. 2006; 440:233–236. [PubMed: 16407888]
15. Thornberry NA, Bull HG, Calaycay JR, Chapman KT, Howard AD, Kostura MJ, Miller DK, Molineaux SM, Weidner JR, Aunins J, et al. A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. Nature. 1992; 356:768–774. [PubMed: 1574116]
16. Franchi L, Eigenbrod T, Munoz-Planillo R, Nunez G. The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. Nat Immunol. 2009; 10:241–247. [PubMed: 19221555]
17. Bauernfeind FG, Horvath G, Stutz A, Alnemri ES, MacDonald K, Speert D, Fernandes-Alnemri T, Wu J, Monks BG, Fitzgerald KA, Hornung V, Latz E. Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. J Immunol. 2009; 183:787–791. [PubMed: 19570822]
18. Cassel SL, Eisenbarth SC, Iyer SS, Sadler JJ, Colegio OR, Tephly LA, Carter AB, Rothman PB, Flavell RA, Sutterwala FS. The Nalp3 inflammasome is essential for the development of silicosis. Proc Natl Acad Sci U S A. 2008; 105:9035–9040. [PubMed: 18577586]
19. Dostert C, Petrilli V, Van Bruggen R, Steele C, Mossman BT, Tschopp J. Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. Science. 2008; 320:674–677. [PubMed: 18403674]
20. Halle A, Hornung V, Petzold GC, Stewart CR, Monks BG, Reinheckel T, Fitzgerald KA, Latz E, Moore KJ, Golenbock DT. The NALP3 inflammasome is involved in the innate immune response to amyloid-beta. Nat Immunol. 2008; 9:857–865. [PubMed: 18604209]
21. Mariathasan S, Weiss DS, Newton K, McBride J, O'Rourke K, Roose-Girma M, Lee WP, Weinrauch Y, Monack DM, Dixit VM. Cryopyrin activates the inflammasome in response to toxins and ATP. Nature. 2006; 440:228–232. [PubMed: 16407890]
22. Tschopp J, Schroder K. NLRP3 inflammasome activation: The convergence of multiple signalling pathways on ROS production? Nat Rev Immunol. 2010; 10:210–215. [PubMed: 20168318]

23. Conrad ML, Ferstl R, Teich R, Brand S, Blumer N, Yildirim AO, Patrascan CC, Hanuszkiewicz A, Akira S, Wagner H, Holst O, von Mutius E, Pfefferle PI, Kirschning CJ, Garn H, Renz H. Maternal TLR signaling is required for prenatal asthma protection by the nonpathogenic microbe Acinetobacter lwoffii F78. J Exp Med. 2009; 206:2869–2877. [PubMed: 19995952]
24. Adachi O, Kawai T, Takeda K, Matsumoto M, Tsutsui H, Sakagami M, Nakanishi K, Akira S. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. Immunity. 1998; 9:143–150. [PubMed: 9697844]
25. Tabeta K, Hoebe K, Janssen EM, Du X, Georgel P, Crozat K, Mudd S, Mann N, Sovath S, Goode J, Shamel L, Herskovits AA, Portnoy DA, Cooke M, Tarantino LM, Wiltshire T, Steinberg BE, Grinstein S, Beutler B. The Unc93b1 mutation 3d disrupts exogenous antigen presentation and signaling via Toll-like receptors 3, 7 and 9. Nat Immunol. 2006; 7:156–164. [PubMed: 16415873]
26. Hoebe K, Du X, Georgel P, Janssen E, Tabeta K, Kim SO, Goode J, Lin P, Mann N, Mudd S, Crozat K, Sovath S, Han J, Beutler B. Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. Nature. 2003; 424:743–748. [PubMed: 12872135]
27. Kanneganti TD, Body-Malapel M, Amer A, Park JH, Whitfield J, Franchi L, Taraporewala ZF, Miller D, Patton JT, Inohara N, Nunez G. Critical role for Cryopyrin/Nalp3 in activation of caspase-1 in response to viral infection and double-stranded RNA. J Biol Chem. 2006; 281:36560–36568. [PubMed: 17008311]
28. Sander LE, Davis MJ, Boekschoten MV, Amsen D, Dascher CC, Ryffel B, Swanson JA, Muller M, Blander JM. Detection of prokaryotic mRNA signifies microbial viability and promotes immunity. Nature. 2011; 474:385–389. [PubMed: 21602824]
29. Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, Takeda K, Akira S. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity. 1999; 11:443–451. [PubMed: 10549626]
30. Kim YM, Brinkmann MM, Paquet ME, Ploegh HL. UNC93B1 delivers nucleotide-sensing toll-like receptors to endolysosomes. Nature. 2008; 452:234–238. [PubMed: 18305481]
31. Burckstummer T, Baumann C, Bluml S, Dixit E, Durnberger G, Jahn H, Planyavsky M, Bilban M, Colinge J, Bennett KL, Superti-Furga G. An orthogonal proteomic-genomic screen identifies AIM2 as a cytoplasmic DNA sensor for the inflammasome. Nat Immunol. 2009; 10:266–272. [PubMed: 19158679]
32. Fernandes-Alnemri T, Yu JW, Datta P, Wu J, Alnemri ES. AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. Nature. 2009; 458:509–513. [PubMed: 19158676]
33. Hornung V, Ablasser A, Charrel-Dennis M, Bauernfeind F, Horvath G, Caffrey DR, Latz E, Fitzgerald KA. AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. Nature. 2009; 458:514–518. [PubMed: 19158675]
34. Harder J, Franchi L, Munoz-Planillo R, Park JH, Reimer T, Nunez G. Activation of the Nlrp3 inflammasome by Streptococcus pyogenes requires streptolysin O and NF-kappa B activation but proceeds independently of TLR signaling and P2X7 receptor. J Immunol. 2009; 183:5823–5829. [PubMed: 19812205]
35. Allen IC, Scull MA, Moore CB, Holl EK, McElvania-TeKippe E, Taxman DJ, Guthrie EH, Pickles RJ, Ting JP. The NLRP3 inflammasome mediates in vivo innate immunity to influenza A virus through recognition of viral RNA. Immunity. 2009; 30:556–565. [PubMed: 19362020]
36. Nakahira K, Haspel JA, Rathinam VA, Lee SJ, Dolinay T, Lam HC, Englert JA, Rabinovitch M, Cernadas M, Kim HP, Fitzgerald KA, Ryter SW, Choi AM. Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. Nat Immunol. 12:222–230. [PubMed: 21151103]
37. Petrilli V, Papin S, Dostert C, Mayor A, Martinon F, Tschopp J. Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. Cell Death Differ. 2007; 14:1583–1589. [PubMed: 17599094]
38. Zhou R, Yazdi AS, Menu P, Tschopp J. A role for mitochondria in NLRP3 inflammasome activation. Nature. 469:221–225. [PubMed: 21124315]
39. Koski GK, Kariko K, Xu S, Weissman D, Cohen PA, Czerniecki BJ. Cutting edge: innate immune system discriminates between RNA containing bacterial versus eukaryotic structural features that

prime for high-level IL-12 secretion by dendritic cells. J Immunol. 2004; 172:3989–3993. [PubMed: 15034009]

40. Gratz N, Hartweger H, Matt U, Kratochvill F, Janos M, Sigel S, Drobits B, Li XD, Knapp S, Kovarik P. Type I Interferon Production Induced by Streptococcus pyogenes-Derived Nucleic Acids Is Required for Host Protection. PLoS Pathog. 7:e1001345. [PubMed: 21625574]
41. Kariko K, Buckstein M, Ni H, Weissman D. Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA. Immunity. 2005; 23:165–175. [PubMed: 16111635]
42. Bieger CD, Nierlich DP. Distribution of 5′-triphosphate termini on the mRNA of Escherichia coli. J Bacteriol. 1989; 171:141–147. [PubMed: 2464575]

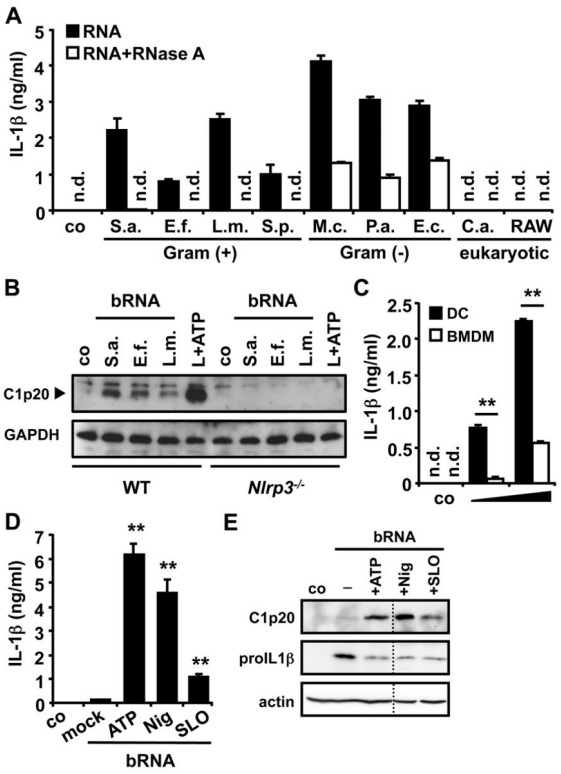

Figure 1. Transfected total RNA from gram-positive and gram-negative bacteria induces IL- 1β secretion in unprimed DC and BMDM

A, murine DC were transfected over night (o.n.) with total RNA purified from the indicated bacteria (5 μg/ml) or with eukaryotic RNA derived from *Candida albicans (C.a.)* or RAW264.7 cells. *E.c., Escherichia coli; P.a., Pseudomonas aeruginosa; M.c., Moraxella catarrhalis; S.a. Staphylococcus aureus; E.f., Enterococcus faecalis; L.m., Listeria monocytogenes; S.p., Streptococcus pyogenes. B*, DC from WT and Nlrp3 deficient mice were transfected o.n. with bacterial RNA from the indicated strains. Stimulation with LPS for 3 h followed by ATP (5 mM) for 30 min served as positive control. *C*, DC and BMDM were transfected o.n. with *S. aureus* RNA at 3 μg/ml or 9 μg/ml. *D*, BMDM and *E*, DC were transfected with *S. aureus* RNA at 10 μg/ml for 6.5 h before addition of ATP (5 mM) or nigericin (5 μg/ml) for 60 min. Alternatively, transfected cells were incubated with SLO as described in Materials and Methods. IL-1β levels were measured in cell-free supernatants by ELISA (*A*, *C–D*). Values represent the mean of duplicate wells ± SD. n.d. denotes not detected. (**) $p<0.01$. *B*, *E*, Extracts prepared from cells plus culture supernatants were immunoblotted with an antibody detecting active caspase-1 subunit p20 or with an antibody detecting pro-IL-1β. Results are representative of three (*A*, *C*) or at least two (*B*, *D–E*) independent experiments.

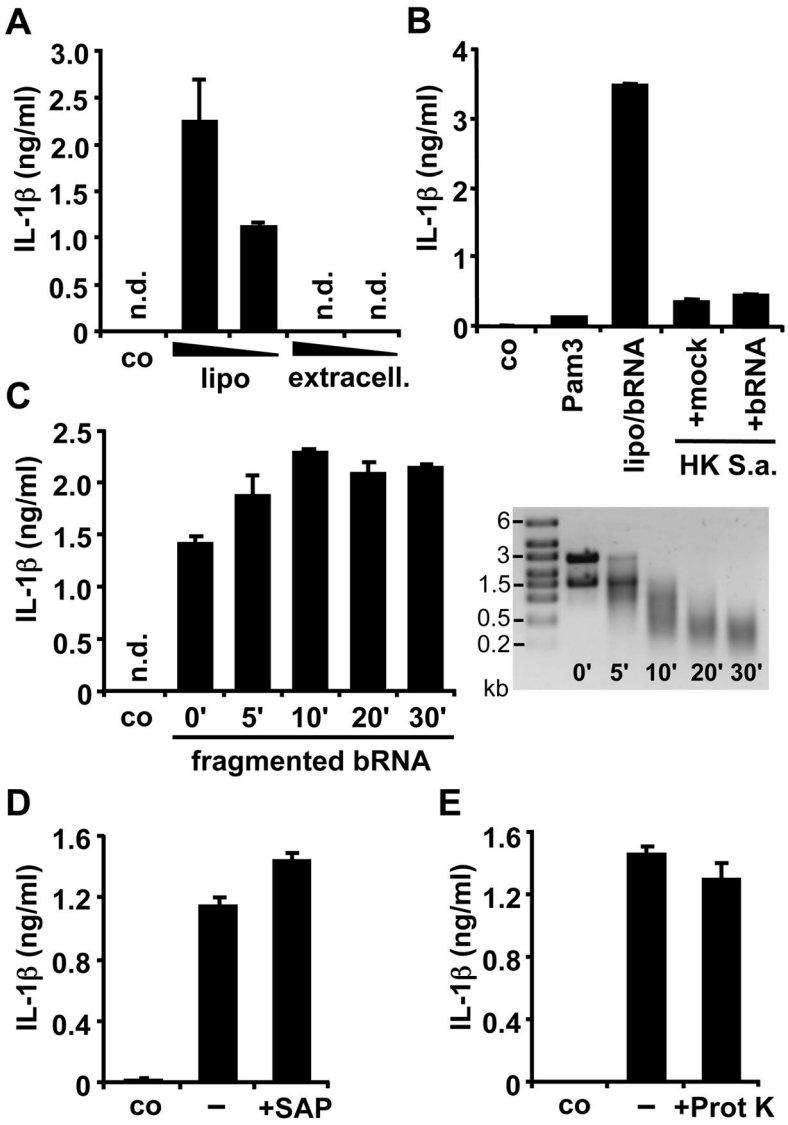

Figure 2. Bacterial RNA mediated release of IL-1β requires transfection but is independent of 5′triphosphate termini

A, DC were transfected over night (o.n.) or stimulated extracellularly with *S. aureus* RNA at 9 μg/ml or 3 μg/ml. *B*, DC were stimulated o.n. with heat-killed *S. aureus* (HK S.a.) at MOI 25 or with heat-killed *S. aureus* plus extracellular *S. aureus* RNA (10 μg/ml). Transfected *S. aureus* RNA (5 μg/ml) and Pam3CSK4 (1 μg/ml) served as controls. *C*, fragmentation of bacterial RNA was performed for the indicated times (min) as described in Material and Methods and transfected o.n. into DC. Fragmentation was visualized by agarose gel separation. *D*, bacterial RNA preparation was digested with proteinase K or *E*, shrimp alkaline phosphatase (SAP) prior to o.n. transfection into DC. IL-1β levels were measured in cell-free supernatants by ELISA. Values represent the mean of duplicate wells ± SD.

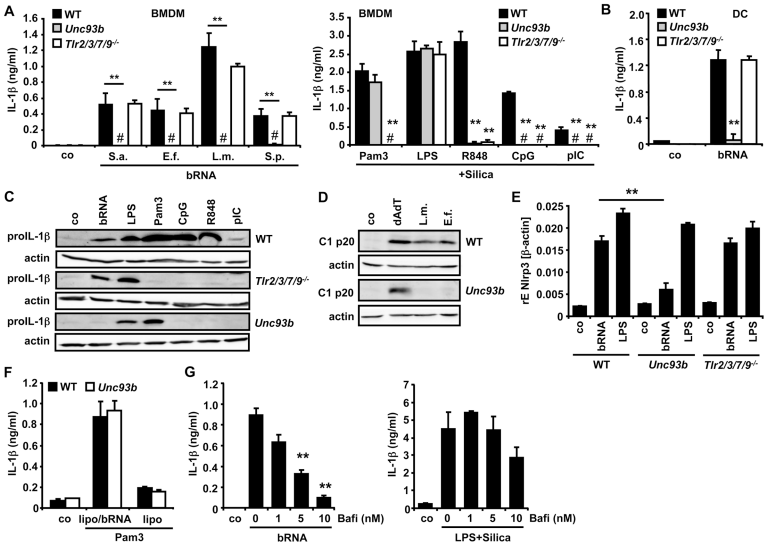

Figure 3. Unc93B, but not TLR 3, 7 and 9, are required for bacterial RNA induced IL-1β production and activation of caspase-1

A, BMDM and *B*, DC from WT and mutant mice with a combined deficiency for TLR2, 3, 7 and 9 or mice harboring the Unc93B 3d mutation were transfected o.n. with bacterial RNA purified from the indicated bacterial strains. Stimulation with Pam3CSK4 (1 μg/ml), LPS (100 ng/ml), poly(I:C) (10 μg/ml), R848 (1 μg/ml) or CpG (1 μM) plus Silica for 6 h served as control. Levels of IL-1β were measured in cell-free supernatants by ELISA. # denotes not detected. *C*, DC from WT and mutant mice were transfected for 6 h with bacterial RNA or stimulated with the indicated TLR-ligands and expression of pro-IL-1β was detected by WB. *D*, DC from WT and Unc93B mice were transfected o.n. with bacterial RNA from the indicated strains or with poly(dA:dT) for 6 h. Cleavage of caspase-1 was detected using an Ab recognizing caspase-1 subunit p20. *E*, Expression of Nlrp3 was detected by quantitative real-time PCR after transfection of *S. aureus* RNA for 6 h or stimulation with LPS for 2 h. *F*, DC were primed for 1 h with Pam3CSK4 (1 μg/ml) prior to transfection with bacterial RNA for 6 h. *G*, DC were transfected o.n. with bRNA or stimulated with LPS plus Silica for 6 h in the presence of bafilomycin A1. Levels of IL-1β were measured in cell-free supernatants by ELISA. Values represent the mean of duplicate wells ± SD. Results are representative of at least three independent experiments. (**) $p<0.01$.

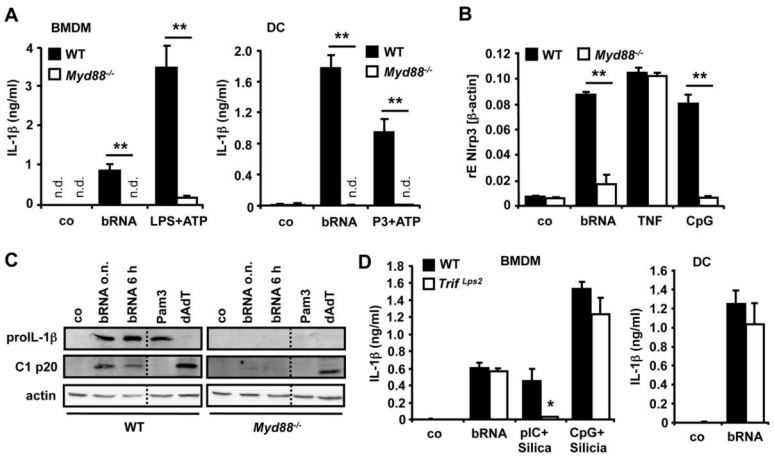

Figure 4. MyD88 is essential for induction of pro-IL-1β and activation of caspase-1 by bacterial RNA

A, BMDM and DC from wild-type (WT) or mutant mice deficient in MyD88 were transfected o.n. with *S. aureus* RNA and IL-1β was measured in cell-free supernatants by ELISA. Stimulation with LPS (100 ng/ml) or Pam3CSK4 (1 μg/ml) followed by ATP served as positive control. *B*, BMDM were transfected with bacterial RNA for 6 h and expression of Nlrp3 was detected by quantitative real-time PCR. TNF (100 ng/ml) and CpG (1 μM) were used as positive and negative controls, respectively. *C*, extracts were prepared from cells plus culture supernatants and immunoblotted with processed caspase-1 p20 Ab. Transfection with poly(dA:dT) (1 μg/ml) for 6 h served as positive control. *D*, BMDM and DC from wild-type (WT) or mutant mice harboring the Trif Lps2-mutation were transfected as in *A*. poly(I:C) (10 μg/ml) and CpG (1 μM) plus Silica for 6 h served as negative and positive controls, respectively. Values represent the mean of duplicate wells ± SD. (*) $p<0.05$, (**) $p<0.01$ between WT and mutant cells. Results are representative of three independent experiments.

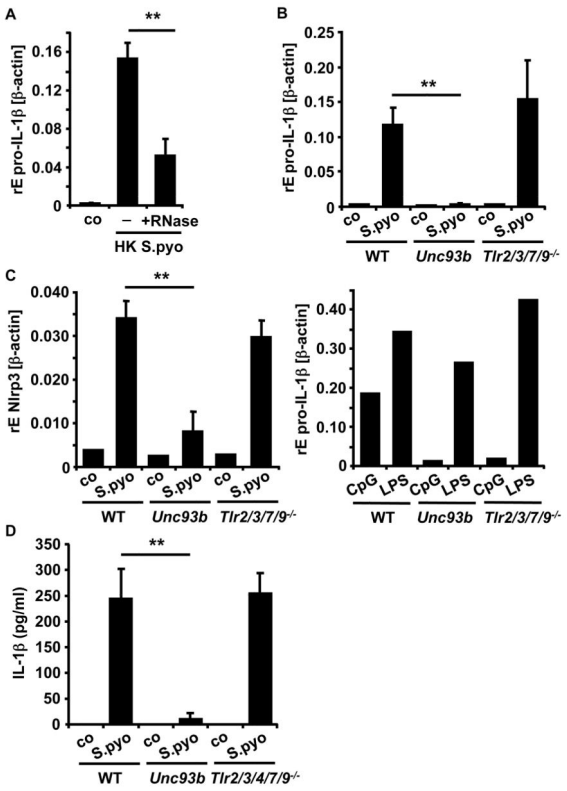

Figure 5. Heat-inactivated *S. pyogenes* induces IL-1β and Nlrp3 expression via recognition of its RNA in an Unc93B dependent manner

A, DC were stimulated with heat-inactivated *S. pyogenes* (MOI 30) for 6 h. Where indicated, heat-inactivated *S. pyogenes* was treated with RNase A as described in material and methods prior to addition to cells. Values represent pooled data from four independent experiments ± SEM. (**) $p<0.01$. *B* and *C*, DC from WT, *Tlr2/3/7/9*$^{-/-}$ and *Unc93B 3d* mice were stimulated with heat-inactivated *S. pyogenes* (MOI 15) for 6 h. Stimulation with LPS or CpG for 2 h served as control. Expression of pro-IL-1β and Nlrp3 was detected by real-time PCR. *D*, DC were stimulated o.n. with heat-inactivated *S. pyogenes* (MOI 50) and IL-1β release was measured in cell-free supernatants by ELISA. Values represent the mean of duplicate wells ± SD. (**) $p<0.01$ between WT and mutant cells. Results are representative of three (*B*–*C*) or at least two (*D*) independent experiments.

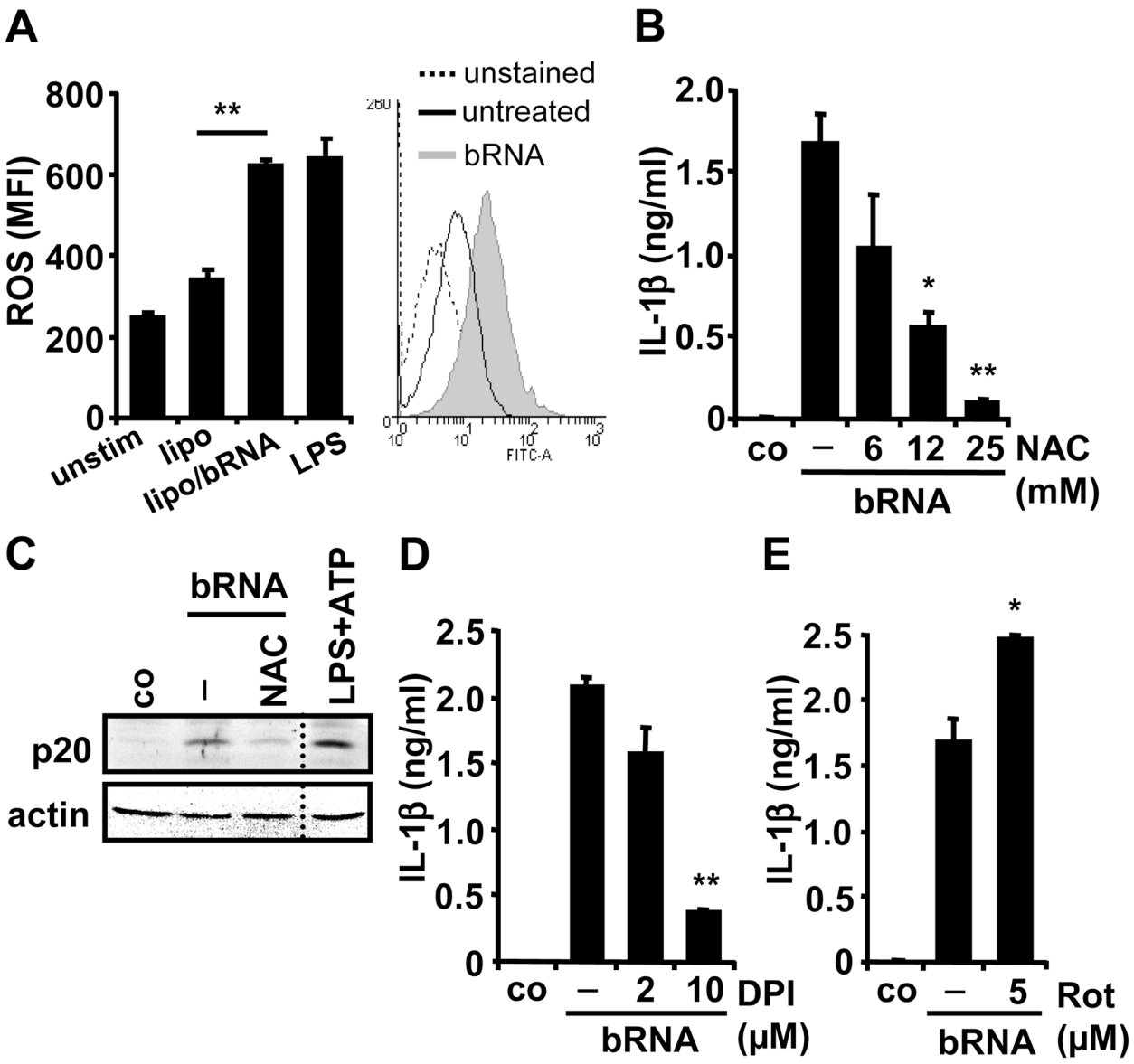

Figure 6. ROS formation is critical for IL-1β release by bacterial RNA

A, DC were transfected with bacterial RNA or mock transfected for 6 h or stimulated with LPS for 2 h. ROS formation was detected by FACS analysis using DCFDA labelling. Curves denote unstained (dotted lines), untreated DCFDA labelled (black line) and *S. aureus* RNA transfected DCFDA labelled (solid grey) cells. *B–E*, DC were transfected with bacterial RNA in the presence of the indicated inhibitors. IL-1β secretion was measured by ELISA in cell-free supernatants and caspase-1 cleavage was detected in extracts prepared from cells plus supernatants. Values represent the mean of duplicate wells ± SD. (**) $p<0.01$; (*) $p<0.05$. Results are representative of three independent experiments.