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Activation of the NLRP3 Inflammasome by Group B Streptococci

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Group B *Streptococcus* (GBS) is a frequent agent of life-threatening sepsis and meningitis in neonates and adults with predisposing conditions. We tested the hypothesis that activation of the inflammasome, an inflammatory signaling complex, is involved in host defenses against this pathogen. We show in this study that murine bone marrow-derived dendritic cells responded to GBS by secreting IL-1 β and IL-18. IL-1 β release required both pro-IL-1 β transcription and caspase-1-dependent proteolytic cleavage of intracellular pro-IL-1 β . Dendritic cells lacking the TLR adaptor MyD88, but not those lacking TLR2, were unable to produce pro-IL-1 β mRNA in response to GBS. Pro-IL-1 β cleavage and secretion of the mature IL-1 β form depended on the NOD-like receptor family, pyrin domain containing 3 (NLRP3) sensor and the apoptosis-associated speck-like protein containing a caspase activation and recruitment domain adaptor. Moreover, activation of the NLRP3 inflammasome required GBS expression of β -hemolysin, an important virulence factor. We further found that mice lacking NLRP3, apoptosis-associated speck-like protein, or caspase-1 were considerably more susceptible to infection than wild-type mice. Our data link the production of a major virulence factor by GBS with the activation of a highly effective anti-GBS response triggered by the NLRP3 inflammasome. *The Journal of Immunology*, 2012, 188: 1953–1960.

The control of colonizing microorganisms and the elimination of those attempting invasion of normally sterile tissues are essential for the survival of multicellular organisms. For this reason, the innate immune system has evolved an arsenal of mechanisms to sense and destroy the microbial nonself (1). Such activities are based on the recognition of conserved microbial substructures (called pathogen-associated molecular patterns) by means of germline-encoded pattern recognition receptors (2). TLRs and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are the two major families of pattern recognition receptors involved in the host response to bacterial infection. Unlike membrane-bound TLRs, which sense

pathogen-associated molecular patterns on the cell surface or in endosomes, NLRs recognize microbial molecules in the host cytosol (3, 4). After microbial recognition, TLRs and NLRs induce the activation of distinctive host signaling pathways, which lead to innate and adaptive immune responses (1–3).

NLRs possess a characteristic domain architecture, consisting of an N-terminal pyrin (PYD) or caspase activation and recruitment domain (CARD), a central NOD (also known as NACHT), and C-terminal leucine-rich repeats. The CARD or PYD domains mediate homophilic protein–protein interactions with other CARD or PYD-containing proteins (3). At least two well-characterized NLRs, that is, nucleotide-binding oligomerization domain-containing 1 and 2 (NOD1 and NOD2), sense molecules produced during the synthesis and/or degradation of bacterial peptidoglycan, leading to the activation of transcription factor NF- κ B and MAPKs (4). Another group of NLRs participates in the formation of a large multiprotein complex called the inflammasome, whose assembly leads to the activation of caspase-1. Such activation involves cleavage of procaspase-1 into the active protease, which, in turn, is responsible for the maturation of the proinflammatory cytokines IL-1 β and IL-18 (5).

Many pathogens are controlled by caspase-1-mediated innate immune responses, which can be associated with the downstream effects of IL-1 β and IL-18 (5) or with caspase-1-induced cell death (6). The inflammasome includes at least one NLR and an adaptor protein called apoptosis-associated speck-like protein (ASC), which links the NLR to procaspase-1. Several NLR proteins can form inflammasomes, including NLR family pyrin domain-containing 3 (NLRP3; also known as cryopyrin or NALP3), NLR family CARD domain-containing 4 (also known as IPAF), and NLRP1 (also known as NALP1) (7). The inflammasomes are activated by different stimuli (4, 8). For example, mouse NLRP1b is involved in responses to the lethal toxin produced by *Bacillus anthracis* (9), whereas NLR family CARD domain-containing 4

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Abbreviations used in this article: ASC, apoptosis-associated speck-like protein; BMDC, bone marrow-derived dendritic cell; BMDM, bone marrow-derived macrophage; CARD, caspase activation and recruitment domain; GBS, group B *Streptococcus*; MOI, multiplicity of infection; NLR, nucleotide-binding oligomerization domain-like receptor; NLRP3, NLR family pyrin domain-containing 3; NOD, nucleotide-binding oligomerization domain; PYD, N-terminal pyrin; WT, wild-type.

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responds to cytosolic flagellin in cells infected with *Salmonella* (4, 10, 11), *Legionella* (12), and *Pseudomonas spp* (13). The NLRP3 inflammasome is activated by a large variety of stimuli, including microbial products [e.g., muramyl dipeptide, pore-forming toxins, and bacterial (5) and viral RNA (14)] and endogenous signals, such as urate crystals and ATP (15). A fourth inflammasome, the AIM2 inflammasome, was also recently identified. AIM2 is a member of the IFI202X/IFI16 (PYHIN) protein family, which can detect cytosolic DNA and activate caspase-1 (16–19).

Streptococcus agalactiae or group B *Streptococcus* (GBS) is a highly pathogenic Gram-positive bacterium that causes life-threatening infections in neonates, pregnant females, and elderly adults (20). GBS produces two membrane-damaging exotoxins, namely, β -hemolysin/cytolysin and CAMP factor. β -hemolysin is responsible for the characteristic zone of clearing (β -hemolysis) surrounding colonies grown on blood agar media. The innate immune response plays a major role in controlling in vivo growth of GBS. This bacterium is a potent inducer of TNF- α (21–23) and of IFN- β (24), both of which make a significant contribution to anti-GBS host defenses (25, 26). GBS can stimulate TNF- α release in two different ways, both of which absolutely require the TLR adaptor MyD88. In the first place, extracellularly released bacterial lipoproteins stimulate TLR2-TLR6 homodimers on macrophages by a mechanism that does not require cell to cell contact (27). Second, whole live or killed GBS can stimulate TNF- α production through activation of an as yet unidentified receptor(s) by a mechanism that requires phagocytosis and phagolysosomal processing. This second mechanism does not involve bacterial lipoproteins, peptidoglycan, or lipoteichoic acid, and, in murine macrophages, known TLRs such as TLR2, TLR4, TLR7, and TLR9 (27, 28). However, in conventional dendritic cells, TLR7 and TLR9 do recognize GBS nucleic acids in phagolysosomes after partial bacterial degradation (26), leading to IFN- β secretion (26). Because little is known of the ability of GBS to activate the inflammasome, we investigated in this study whether GBS can induce IL-1 β or IL-18 by caspase-1-dependent mechanisms and whether inflammasome activation plays a role in host defenses. We found that IL-1 β secretion in GBS-stimulated mouse dendritic cells is critically dependent on the NLRP3 inflammasome and on the production of β -hemolysin by GBS. Moreover, the NLRP3 inflammasome had a crucial role in in vivo anti-GBS defense.

Materials and Methods

Mice

Gene-deleted mice were originally obtained from S. Akira (Osaka University, Osaka, Japan) (TLR2^{-/-}, TLR4^{-/-}, TLR9^{-/-}, MyD88^{-/-}, MAL^{-/-}, TRAM^{-/-}, and TRIF^{-/-}), as previously described (25). Caspase-1^{-/-} mice were obtained from A. Zychlinski (Max Planck Institute, Berlin, Germany), whereas ASC^{-/-}, NLRP3^{-/-}, and AIM2^{-/-} animals were obtained from V. Dixit (Genentech). All of the mice used were on a C57BL/6 background. C57BL/6 wild-type (WT) mice, used as controls, were purchased from Charles River Laboratories. The mice were housed and bred under pathogen-free conditions in the animal facilities of the Elie Metchnikoff Department, University of Messina.

Ethics statement

All studies were performed in strict accordance with the European Union guidelines for the use of laboratory animals (Directive 2010/63/EU). The protocols were approved by the Ethics Committee of the Metchnikoff Department of the University of Messina and by the relevant national authority (Istituto Superiore di Sanità).

Bacterial strains

GBS WT strain NEM316 serotype III and its isogenic β -hemolysin (Δ cylE), CAMP factor (Δ cfb), and double β -hemolysin/CAMP factor (Δ cylE Δ cfb)-deficient mutants, used for in vitro experiments, have been previously

described (24). GBS WT strain H36B serotype Ib was used for in vivo experiments. Bacteria were grown at 37°C in chemically defined medium (24) to late-log phase, washed twice in nonpyrogenic PBS (0.01 M phosphate, 0.15 M NaCl [pH 7.4]; Euroclone), and resuspended to the desired concentration in PBS. The endotoxin level of all the bacterial preparations was <0.06 EU/mg, as determined by *Limulus* amoebocyte lysate assay (PBI).

Bone marrow-derived cells

Bone marrow-derived cells were prepared by flushing femurs and tibiae with sterile RPMI 1640 (Euroclone) supplemented with 10% heat-inactivated FCS (Euroclone). The cells were collected by centrifugation and resuspended in hypotonic Tris-ammonium chloride buffer at 37°C for 5 min to lyse RBCs. After centrifugation, the cells were resuspended to a concentration of 4×10^5 cells/ml, seeded in 10-cm plates, and cultured for 7 d in medium consisting of RPMI 1640, supplemented with 10% FCS, in the presence of penicillin (50 UI/ml)/streptomycin (50 μ g/ml) (purchased from Euroclone). Medium was supplemented with 100 ng/ml M-CSF or 10 ng/ml GM-CSF (both from Peprotech) to obtain, respectively, bone marrow-derived macrophages (BMDMs) and conventional dendritic cells (bone marrow-derived dendritic cells [BMDCs]). On the third day, cultures were fed with 10 ml fresh cytokine-supplemented culture medium. Cells cultured in M-CSF were found to be, by flow cytometric analysis, >97% positive for CD11b, >87% positive for F4/80, and <4% positive for CD11c. Cells cultured in GM-CSF were found to be >86% positive for CD11c and CD11b and negative for B220. All Abs for flow cytometry analysis were purchased from Miltenyi Biotec. In some experiments, cell monolayers were treated with cytochalasin D (5 μ g/ml; Sigma-Aldrich) for 45 min before infection, or with bafilomycin A (1 μ M; Sigma-Aldrich) at 30 min postinfection. In preliminary experiments, cell viability, as determined by trypan blue exclusion, was not affected by any of the treatments described above.

Stimulation of BMDMs and BMDCs with bacteria

For stimulation, cells were washed in PBS, counted on a hemocytometer, and seeded in 96-well plates (5×10^5 /well for cytokine measurement by ELISA), in 24-well plates (1×10^6 /well for immunoblotting), or in 12-well plates (3×10^6 /well for real-time PCR measurements of cytokine mRNA). Cells were cultured in RPMI 1640 medium supplemented with 10% FCS for the indicated times. For immunoblot analysis, medium was replaced 24 h before the assay with RPMI 1640 medium (without phenol red; Euroclone) in the absence of FCS. Bacteria were added to the cell monolayers at the indicated multiplicities of infection (MOIs). All infections were carried out by centrifuging bacteria onto cell monolayers for 10 min at $400 \times g$, to facilitate bacterial adherence.

The number of viable bacteria used in each experiment was carefully determined by plate counting. After incubation at 37°C in 5% CO₂ for 25 min, the monolayers were incubated for various times in the presence of penicillin (250 UI/ml)/streptomycin (250 μ g/ml) to limit the growth of residual extracellular bacteria. Where indicated, cells were primed with *Escherichia coli* K12 ultrapure LPS (0.1 μ g/ml) or CL264 (5 μ g/ml) (both from InvivoGen) for 3 h before the addition of the inflammasome activator ATP (5 mM; Sigma-Aldrich) for 30 min.

Real-time PCR measurements of cytokine mRNA

For the quantification of IL-1 β and TNF- α mRNA, real-time quantitative RT-PCR assays were conducted, in duplicate, with an Applied Biosystems 7500 (Applied Biosystems), as described (29). Primers and TaqMan MGB probes for the above cytokines have been previously described (29) and were purchased from Applied Biosystems.

Cytokine measurement

IL-1 β , IL-18, and TNF- α concentrations were measured in the supernatants of bone marrow-derived cultures using the mouse IL-1 β Quantikine Immunoassay and the mouse Duo-set TNF- α ELISA (both purchased from R&D Systems) or the mouse IL-18 ELISA kit (MBL). The lower detection limits of these assays were 16 pg/ml. Samples below the detection levels were assigned a theoretical value of one-half the detection limit (i.e., 8 pg/ml).

Immunoblotting

Supernatants were precipitated with 10% trichloroacetic acid (Sigma-Aldrich) and washed in cold acetone by centrifugation at $12,000 \times g$ for 10 min at 4°C. Next, pellets were dried and resuspended in sample buffer. For analysis of cell lysates, the plates were incubated on ice for 10 min,

and then the cells were detached with a scraper and transferred into 1.5-ml Eppendorf tubes. Cells were lysed with 300 μ l lysis buffer (50 mM Tris-HCl [pH 7.4], 100 mM NaCl, 1% Triton X-100, and 5% glycerol, all purchased from Sigma-Aldrich) supplemented with a mixture of protease inhibitors (Roche). The lysates were centrifuged at $12,000 \times g$ for 10 min at 4°C, to eliminate cellular debris, precipitated with 10% trichloroacetic acid, and processed, as described above. Proteins were separated on 15% polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad). Immunoblotting was performed using rabbit polyclonal Abs directed against the p-10 caspase-1 subunit (Santa Cruz Biotechnology) or using goat anti-IL-1 β (R&D Systems). All blots were developed with anti-rabbit or anti-goat IgG (both from Sigma-Aldrich).

Murine infection model

Adult 8-wk-old female mice were inoculated i.p. with 1×10^5 CFUs of the serotype Ib strain H36B. Bacteria were grown to the late-log phase in Todd-Hewitt broth and were diluted to the appropriate concentration in PBS before inoculation of animals. In each experiment, the number of injected bacteria was carefully determined by colony counts on blood agar plates (BD Biosciences). Each experiment included two groups of genetically defective mice and a WT control group. The groups did not differ in the age or the weight of the animals. Mice were observed twice daily for signs of disease and lethality. Mice with signs of irreversible disease (e.g., persistent hunching or piloerection, or lethargy) were euthanized. For quantification of bacterial burden, blood was collected and kidneys were weighed, homogenized, and diluted in 10-fold steps in PBS. Bacterial CFUs were determined by plating diluted homogenates in blood agar plates. All studies were performed in agreement with the European Union guidelines of animal care and were approved by the relevant national committees.

Data expression and statistical significance

Cytokine levels and log CFU were expressed as means \pm SD of the mean of several determinations, each conducted on a different animal in an independent experiment. Differences in cytokine levels and organ CFUs were assessed by one-way ANOVA and the Student-Keuls-Newman test. Survival data were analyzed with Kaplan-Meier survival plots, followed by the log-rank test (JMP Software; SAS Institute, Cary, NC) on an Apple Macintosh computer. When p values <0.05 were obtained, differences were considered statistically significant.

Results

GBS induces IL-1 β release in dendritic cells

We first investigated whether GBS induces IL-1 β secretion in BMDMs. GBS were added to macrophage monolayers at MOI ranging from 2.5 to 20 and incubated for 25 min. After this time, incubation was continued for 8 h in the presence of antibiotics to kill extracellular bacteria. Under these conditions, only a modest IL-1 β secretion was observed, despite the fact that cells readily responded to the positive control stimulus consisting of LPS followed by ATP (Fig. 1A). In contrast to IL-1 β , BMDMs potently produced TNF- α in response to GBS, as evidenced by high levels of this cytokine in culture supernatants (Fig. 1B). Because IL-1 β release requires the synthesis of pro-IL-1 β and its subsequent processing by proteolysis, lack of secretion of this cytokine could have resulted from an inability of GBS to induce pro-IL-1 β and/or to activate pro-IL-1 β cleavage. To discriminate between these possibilities, we looked at pro-IL-1 β production in GBS-stimulated BMDMs. Pro-IL-1 β was detectable by Western blot in cell lysates as early as 2 h postinfection and reached maximal levels at 8 h (Fig. 1C). In contrast, mature (p17) IL-1 β was barely detectable at 8 h postinfection. Pro-IL-1 β production in BMDMs was transcriptionally regulated, as specific mRNA was detected as early as 2 h and peaked at 4 h, after stimulation. Interestingly, GBS-induced pro-IL-1 β mRNA induction was robust, with values exceeding those observed after stimulation with optimal dosages of LPS (Fig. 1D). Moreover, the kinetics of pro-IL-1 β mRNA induction by GBS was delayed, relative to induction by LPS, suggesting the involvement of different mechanisms of response

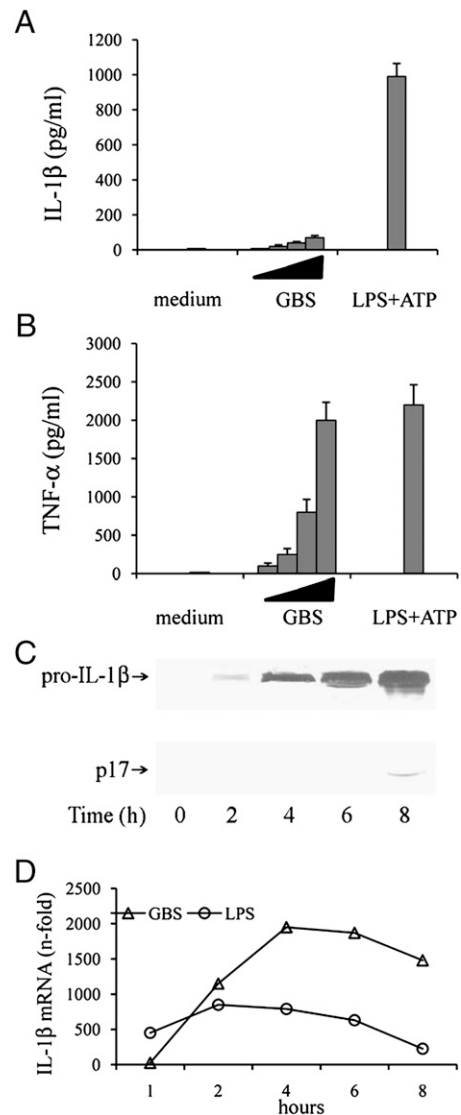


FIGURE 1. GBS induces the production and intracellular accumulation of high levels of pro-IL-1 β in BMDMs. BMDMs were stimulated with GBS at MOIs of 2, 5, 10, and 20 for 8 h. The positive control consisted of cells pretreated with LPS (0.1 μ g/ml) for 3 h and then pulsed with ATP (5 mM) for 30 min. IL-1 β (A) and TNF- α (B) release in culture supernatants were measured by ELISA. Data are expressed as mean \pm SD of three independent experiments. C, Lysates from BMDMs (1×10^6 /well) infected for the indicated times with GBS at a MOI of 10 were immunoblotted with anti-IL-1 β . Results are representative of two independent experiments. D, RT-PCR measurements of pro-IL-1 β mRNA expression in BMDMs at the indicated times after stimulation with GBS (MOI 10) or LPS (0.1 μ g/ml). Data are presented as fold increases in mRNA expression relative to uninfected cells, using a total of 3×10^6 BMDMs. Data are from one experiment representative of three independent ones.

to these two stimuli. Collectively, this first set of data indicated that GBS could readily induce, in BMDMs, the production and intracellular accumulation of high pro-IL-1 β levels. In contrast, its cleavage and the extracellular release of the mature IL-1 β form were relatively weak. Because dendritic cells, in addition to macrophages, have a central role in innate immunity responses, we also looked at the ability of BMDCs to produce IL-1 β in response to GBS. BMDCs released higher levels of IL-1 β in culture supernatants postinfection with GBS in a dose-dependent manner (Fig. 2A). The amounts of IL-1 β produced by GBS-stimulated BMDCs were ~ 10 -fold higher than those produced by similarly

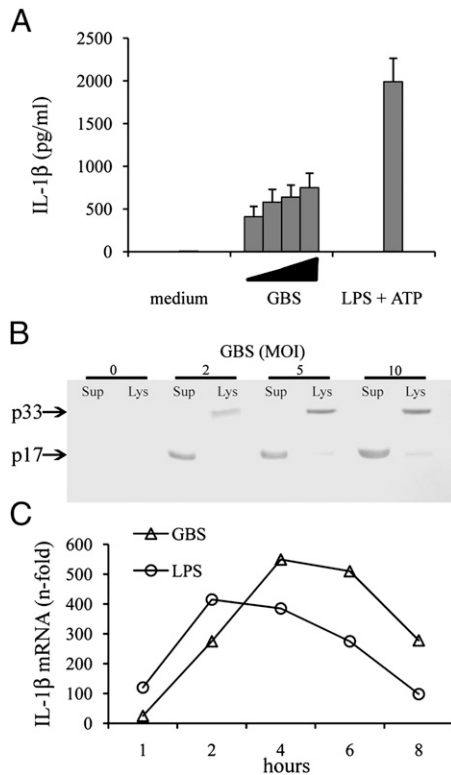


FIGURE 2. GBS induces the release of IL-1 β in BMDCs. *A*, BMDCs were stimulated with GBS at MOIs of 2, 5, 10, and 20 for 8 h. The positive control consisted of cells pretreated with LPS (0.1 μ g/ml) for 3 h and then pulsed with ATP (5 mM) for 30 min. IL-1 β release in culture supernatants was measured by ELISA. Data are expressed as means \pm SD of three independent experiments. *B*, Supernatants or lysates from BMDCs (1×10^6 /well) infected with GBS at the indicated MOIs were collected at 8 h postinfection, concentrated, and immunoblotted with anti-IL-1 β . Results are representative of two independent experiments. *C*, RT-PCR measurement of pro-IL-1 β mRNA expression in BMDCs at different times after stimulation with GBS (MOI 10) or LPS (0.1 μ g/ml). Data are presented as fold increases in mRNA expression relative to uninfected cells, using a total of 3×10^6 BMDCs. Data are from one experiment representative of three independent ones.

stimulated BMDMs (compare Fig. 1A with Fig. 2A). Moreover, BMDCs produced 2-fold higher IL-1 β levels, in comparison with BMDMs after LPS plus ATP stimulation. Western blot analysis of concentrated culture supernatants revealed that the mature, low molecular weight (p17) form entirely accounted for the immunoreactive IL-1 β released extracellularly after GBS stimulation (Fig. 2B). In contrast, cell lysates contained predominantly the high molecular weight, pro-IL-1 β form. Next, it was of interest to investigate whether increased IL-1 β release by BMDCs was the result of increased pro-IL-1 β mRNA induction. However, this did not seem to be the case because GBS-induced pro-IL-1 β mRNA elevations were actually lower in BMDCs relative to BMDMs (compare Fig. 1D with Fig. 2C). Collectively, these results indicate that GBS can induce pro-IL-1 β production in both BMDMs and BMDCs. However, release of mature IL-1 β occurs more efficiently in BMDCs than in BMDM, despite the ability of the latter to produce pro-IL-1 β in response to stimulation with GBS.

GBS-induced IL-1 β production absolutely requires MyD88 and is affected by phagocytosis

Previous studies have indicated that lipoproteins in staphylococcal culture supernatants can induce pro-IL-1 β synthesis (30) and that group A *Streptococcus* lipoproteins induce TNF- α secretion by

a TLR2- and MyD88-dependent mechanism (31). Therefore, it was of interest to investigate the role of TLR2 and MyD88 in IL-1 β induction by GBS. Fig. 3A shows that, in BMDCs, the release of either IL-1 β (Fig. 3A) or TNF- α (Fig. 3D) was entirely MyD88 dependent, but was not affected by the absence of other TLR

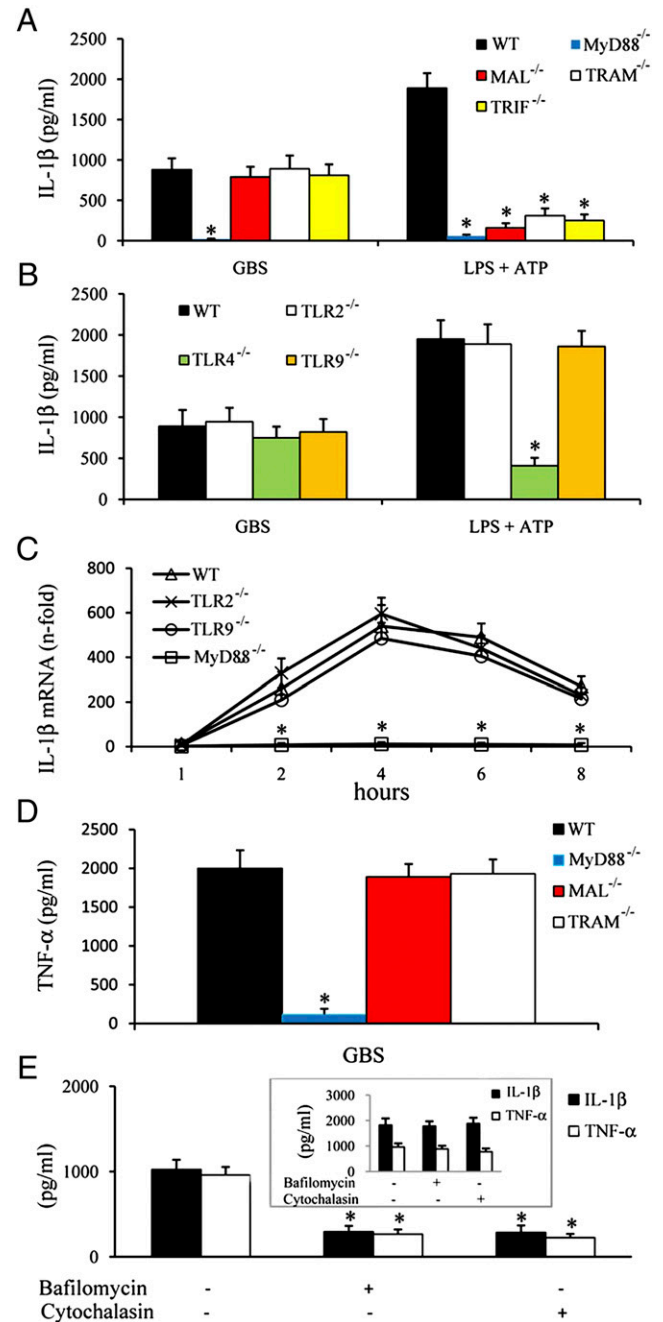


FIGURE 3. GBS-induced IL-1 β production is dependent on MyD88 and phagocytosis. IL-1 β (*A*, *B*) and TNF- α (*D*) protein secretion in culture supernatants of BMDCs lacking various TLR adaptors (*A*, *D*) or TLRs (*B*), after stimulation with GBS (MOI 10) or with LPS and ATP. *C*, RT-PCR analysis of the expression of pro-IL-1 β mRNA in BMDCs, lacking TLR-2, TLR-9, or MyD88, exposed for various times (*horizontal axis*) to GBS (MOI 10), presented as fold increase (*vertical axis*) in expression relative to uninfected cells. *E*, IL-1 β and TNF- α concentrations in supernatants of BMDCs treated with cytochalasin D (5 μ g/ml) or bafilomycin A (1 μ M) and stimulated with GBS (MOI 10) or LPS and ATP (*inset*). Data are expressed as means \pm SD of three independent observations, each conducted on a different animal. * $p < 0.05$ versus untreated cells by one-way ANOVA and the Student-Keuls-Newman test.

adaptors, such as MAL, TRIF, and TRAM. Moreover, IL-1 β release was not affected by lack of any of the TLRs tested, including TLR2, TLR4, and TLR9 (Fig. 3*B*). Lack of IL-1 β release in MyD88-deficient BMDC was accounted for by failure to produce pro-IL-1 β mRNA (Fig. 3*C*). Because our previous data indicated that TLR7 is involved in GBS-induced IFN- β secretion (26), we also tested whether TLR7 deficiency affected IL-1 β secretion. Supplemental Fig. 1 shows that this was not the case, as evidenced by similar IL-1 β secretion in TLR7-deficient and WT BMDCs. These data indicate that, similar to TNF- α (Fig. 3*D*), pro-IL-1 β is induced by a MyD88-dependent mechanism that, nevertheless, does not require any of the better-characterized TLRs, such as TLR2, TLR4, TLR7, and TLR9.

Because IFN- β production requires bacterial internalization and phagosomal maturation (26), in further experiments we tested whether the phagocytic ability of dendritic cells was required for GBS-induced IL-1 β release. Inhibiting actin polymerization, and thus phagocytosis, with cytochalasin D resulted in marked (70–

80%) inhibition of either IL-1 β or TNF- α release in BMDCs infected with GBS, suggesting that phagocytosis is needed to optimally activate the production of these cytokines (Fig. 3*E*). We also studied the effects of preventing phagosomal acidification with the V-ATPase inhibitor bafilomycin. Fig. 3*E* shows that bafilomycin treatment also inhibited GBS-induced secretion of IL-1 β , as well as pro-IL-1 β induction. Taken together, these data suggest that GBS-induced pro-IL-1 β production and the subsequent IL-1 β secretion require internalization and phagosomal acidification. This was in sharp contrast with LPS plus ATP-induced IL-1 β release that did not require phagocytosis or phagosomal maturation.

GBS-induced IL- β release requires caspase-1

IL-1 β release is known to depend on proteolytic cleavage of preformed pro-IL-1 β by cysteine-aspartic proteases, particularly caspase-1 (32). Therefore, in further experiments we investigated whether the GBS-induced IL-1 β release required caspase-1 using BMDCs from mice lacking this enzyme. Fig. 4*A* shows that IL-1 β release was largely caspase-1 dependent. Moreover, GBS infection resulted in cleavage of procaspase-1 into the active p-10 form (Fig. 4*C*). To determine the mechanisms by which GBS might activate caspase-1, we considered the possibility that GBS indirectly trigger inflammasomes by inducing the release of ATP or other activators from dying BMDCs. However, during the observation period, no cell toxicity or damage was observed, as evidenced by lactate dehydrogenase release or trypan blue exclusion (data not shown).

In further experiments, because caspase-1 activation is known to result in IL-18 release, in addition to maturation and release of IL-1 β , we measured IL-18 levels in BMDC supernatants after GBS infection. Indeed, GBS were able to induce caspase-1-dependent IL-18 release in BMDCs (Fig. 4*B*). Collectively, these results indicate that GBS-induced IL-1 β and IL-18 secretion occurs in a caspase-1-dependent manner.

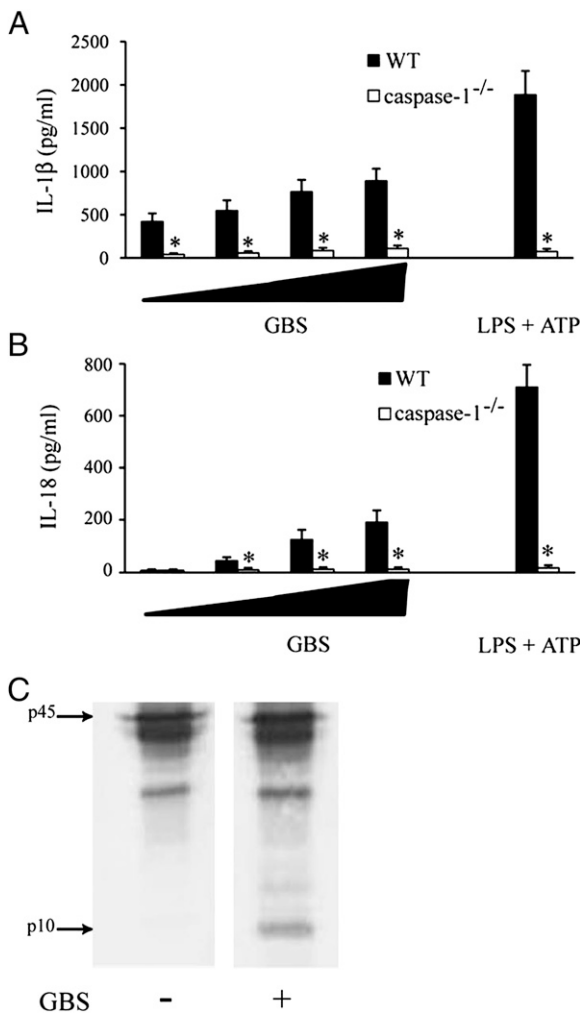


FIGURE 4. Caspase-1 is essential for IL-1 β secretion by BMDCs in response to GBS. IL-1 β (*A*) and IL-18 (*B*) protein secretion in culture supernatant of BMDCs from WT and caspase-1-deficient mice infected for 8 h with GBS at MOI of 2, 5, 10, and 20. The positive control consisted of cells pretreated with LPS (0.1 μ g/ml) for 3 h and then pulsed with ATP (5 mM) for 30 min. **p* < 0.05 versus untreated cells by one-way ANOVA and the Student–Keuls–Newman test. Data are expressed as mean \pm SD of three independent experiments. Western blot analysis (*C*) of BMDC lysates from WT mice infected with GBS (MOI 10) for 8 h, after treatment with anti-caspase-1. Results are representative of two independent experiments.

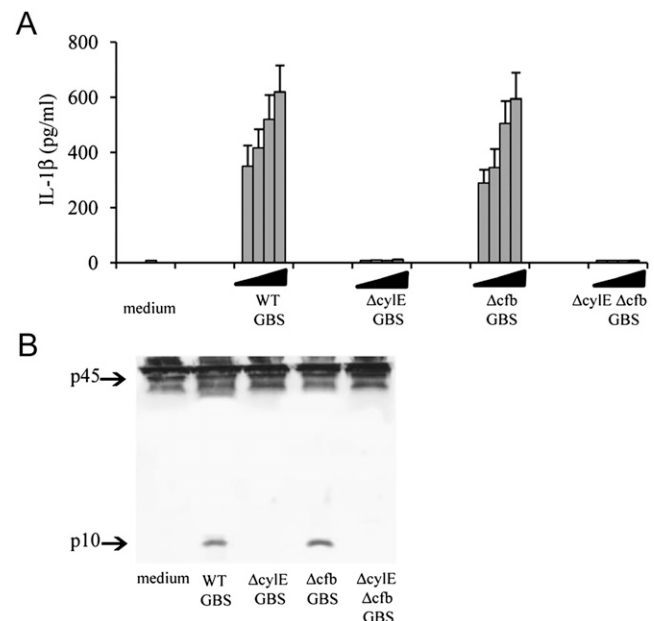


FIGURE 5. β -hemolysin is essential for GBS-stimulated IL-1 β release. IL-1 β release (*A*) and Western blot analysis (*B*) of BMDCs infected for 8 h with GBS (MOI 10). WT strain NEM316 serotype III or its isogenic mutants deficient in β -hemolysin (Δ cylE), in CAMP factor (Δ cfb), or in both (Δ cylE Δ cfb) were used. *A*, Data are expressed as means \pm SD of three independent observations, each conducted on a different animal. *B*, Results are representative of two independent experiments.

GBS-stimulated IL-1 β release is mediated by β -hemolysin

Previous studies have demonstrated that pore-forming toxins can activate the NALP3 inflammasome, resulting in the secretion of IL-1 β and IL-18 (33). Therefore, we sought to determine whether GBS-induced IL-1 β release was dependent on bacterial cytolysins. Specifically, we investigated whether IL-1 β release required β -hemolysin or CAMP factor, which are the known cytolysins of GBS. BMDCs were infected with GBS lacking β -hemolysin, CAMP factor, or both. Strains lacking β -hemolysin, but not CAMP factor, were unable to activate caspase-1 (Fig. 5B) and to secrete IL-1 β in BMDCs (Fig. 5A). These data indicate that GBS β -hemolysin, but not CAMP factor, is required for IL-1 β processing and secretion, and that, therefore, β -hemolysin has similar activities to those of other, genetically unrelated, pore-forming toxins of Gram-positive bacteria.

GBS triggers caspase-1 activation via the NLRP3 inflammasome

To further investigate the mechanisms by which β -hemolysin mediates inflammasome activation, we examined IL-1 β release in NLRP3-deficient BMDCs during infection with GBS. To this end, we infected WT, NLRP3, and ASC and AIM2-deficient cells with GBS and then analyzed the secretion of IL-1 β . We observed that caspase-1 activation and IL-1 β secretion were dependent on NLRP3 and ASC, but not on AIM2, as revealed by almost complete abrogation of IL-1 β secretion in NLRP3- or ASC-deficient BMDCs (Fig. 6A). In contrast, NLRP3 and ASC were dispensable for the production of TNF- α in response to GBS (Fig. 6B). These

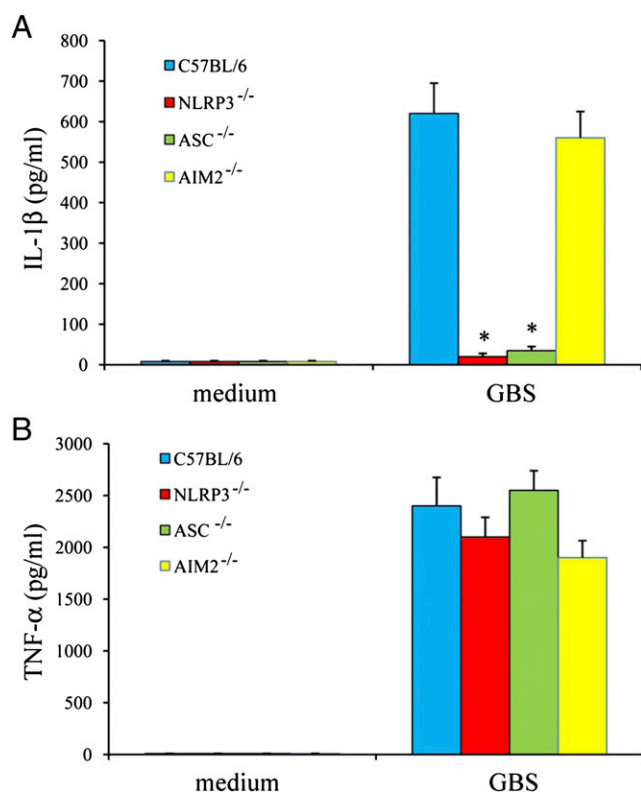


FIGURE 6. NLRP3 and ASC are essential for IL-1 β release from BMDCs infected with GBS. IL-1 β (top) and TNF- α (bottom) protein secretion in culture supernatants of BMDCs from WT and NLRP3-, ASC-, and AIM2-deficient mice infected with GBS for 8 h at a MOI of 10. Data are expressed as means \pm SD of three independent observations, each conducted on a different animal. * p < 0.05 versus cells from WT mice by one-way ANOVA and the Student–Keuls–Newman test.

results suggest that infection with GBS triggers caspase-1 via the NLRP3 inflammasome.

NLRP3 inflammasome is required for resistance against GBS

To further assess the role of the NLRP3 inflammasome during GBS infection, we investigated whether NLRP3 was critical for in vivo resistance against GBS. We infected i.p. WT and caspase-1-, NLRP3-, and ASC-deficient mice with a sublethal dose of GBS, consisting of 1×10^5 CFU/ml. We observed that all control mice survived the challenge, whereas the survival was significantly affected in caspase-1-, NLRP3-, and ASC-deficient mice (Fig. 7A). Increased lethality of NLRP3-, ASC-, and caspase-1-deficient mice was paralleled by increased bacterial burden in the blood and in kidneys (Fig. 7B). These data show that the NLRP3 inflammasome plays a crucial role in the control of in vivo GBS growth and in host resistance against these bacteria.

Discussion

Innate immunity plays a central role in the pathogenesis of GBS diseases. On the one hand, individuals at risk for contracting these infections lack protective Abs and rely exclusively on innate mechanisms to control GBS growth. On the other hand, massive release of proinflammatory mediators by innate immune cells causes the severe pathophysiological phenomena that are the hallmark of GBS-induced septic shock and lethality. The last 20 y have witnessed remarkable progress in the elucidation of the mechanisms underlying recognition of response to GBS. Infection with these bacteria is associated with the appearance, in a characteristic sequence, of some primary cytokines, such as TNF- α , IL-1, IL-12, and IL-18 (34–36). These few primary mediators are, in turn, responsible for the orchestration of a comprehensive antibacterial program involving a wide array of secondary and tertiary factors. Most studies have focused on the mechanisms whereby GBS in-

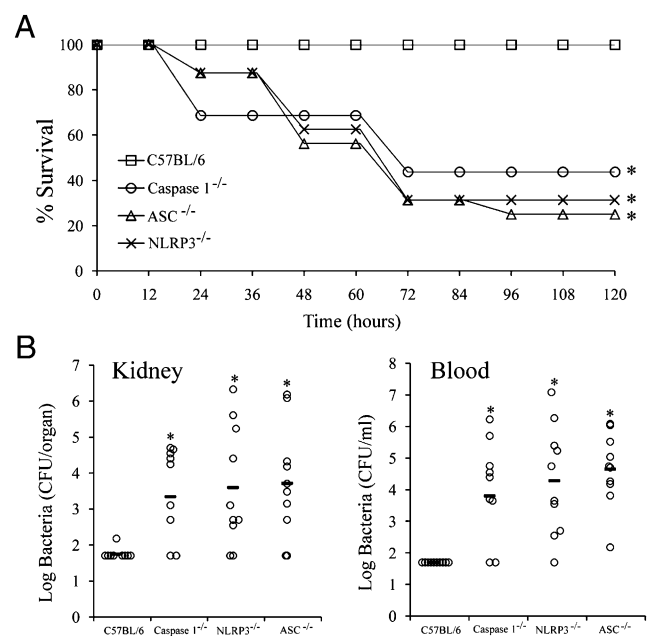


FIGURE 7. Mice lacking caspase-1, ASC, and NLRP3 are highly susceptible to GBS infection. *A*, Survival of WT C57BL/6, caspase-1-, ASC-, and NLRP3-deficient mice (16 per group) after i.p. challenge with 1×10^5 CFU GBS. * p < 0.05 versus WT mice by Kaplan–Meier survival plots. *B*, Bacterial numbers in the kidney and blood after challenge, as described above. Log CFU are expressed as means \pm SD of 10 determinations, each conducted on a different animal. * p < 0.05 versus WT mice by one-way ANOVA and the Student–Keuls–Newman test.

duce TNF- α (34, 37) and type I IFNs (24, 26). Much less is known about the mechanisms underlying GBS-induced release of IL-18 and IL-1 β , two key mediators of antibacterial defenses (36, 38). Whereas a single signal (e.g., TLR activation) is generally sufficient for cytokine secretion, the release of IL-1 β is strictly controlled by a two-signal system. First, a transcriptional response must be activated, leading to pro-IL-1 β synthesis. Subsequently, a separate pathway initiates caspase-1-dependent maturation and secretion. We found in this study that GBS can readily activate both signals. Stimulation of pro-IL-1 β synthesis by GBS required an as yet unidentified MyD88-dependent receptor, internalization of whole bacteria, and phagosomal acidification. This mechanism is reminiscent of that recently described by Deshmukh et al. (39). These authors found that TNF- α release by macrophages stimulated with whole killed Gram-positive bacteria required ssRNA, MyD88, and the chaperone protein UNC93B, which mediates translocation of nucleic acid-sensing TLRs from the endoplasmic reticulum to endosomes. Moreover, the established MyD88-dependent endosomal nucleic acid sensors TLR7 and TLR9 were not involved. Clearly, further studies are needed to identify the recognition receptor responsible for pro-IL-1 β induction and TNF- α secretion after stimulation with whole Gram-positive bacteria. It was previously shown that extracellular release of lipoproteins by GBS results in TLR2/TLR6-dependent NF- κ B activation and TNF- α secretion. However, this mechanism was not involved in pro-IL-1 β induction, at least under the experimental conditions we used. In fact, TLR2 was not required for pro-IL-1 β transcription or IL-1 β release. Moreover, a Δ lgt mutant, which is unable to produce lipidated proteins (28), was as potent as WT GBS at inducing IL-1 β (G. Mancuso and G. Teti, unpublished observations).

In addition to the mechanisms underlying pro-IL-1 β production, the current study investigated the pathway involved in GBS-induced cleavage of pro-IL-1 β and release of the mature cytokine. Our data showed that each component of the NALP3 inflammasome, namely NLRP3, ASC, and caspase-1, was required for secretion of IL-1 β or IL-18. Moreover, activation of this pathway made a significant contribution to in vivo anti-GBS defenses, as evidenced by increased susceptibility to infection of mice lacking any of the NALP3 inflammasome components. Only few studies have to date examined the effects of NLRP3 on the outcome of infections caused by extracellular bacteria. NLRP3 was redundant for host defenses against *Streptococcus pyogenes* (31), but was essential for anti-pneumococcal (40, 41) or anti-*Klebsiella pneumoniae* defenses (42). Therefore, the effects of NLRP3 inflammasome activation on the outcome of infection by extracellular bacteria may be pathogen specific. This is not surprising, in view of the different mechanism used by each bacterial species to evade recognition by the innate immune system or promote pathogenesis. An additional finding of the current study was that NLRP3 activation entirely depended on β -hemolysin, the main cytotoxin of GBS, but not on CAMP factor, a sphingomyelinase that also displays hemolytic activity. Our observations that β -hemolysin triggers NLRP3 activation and IL-1 β release in the context of GBS infection may provide a mechanistic explanation for the previously described proinflammatory effects of the toxin (43, 44), including its ability to cause microabscesses in the liver (45). Our results add GBS hemolysin to the growing list of structurally unrelated bacterial toxins, including several cholesterol-dependent cytotoxins and staphylococcal hemolysins, that are able to activate the NLRP3 inflammasome. Collectively, available data link the production of a highly conserved virulence mechanism, such as cytotoxin production, with a specific, host-protective response, namely NLRP3-dependent IL-1 β and IL-18

release. Thus, whereas many bacterial pathogens have evolved the ability to produce cytotoxins to escape host defenses, the host has developed highly effective means to detect these dangerous weapons and fight back. Another important finding of the current study is the existence of a marked cell-type specificity in the ability to respond to bacterial infection with NLRP3 inflammasome activation. For example, IL-1 β response was modest in BMDMs under the in vitro conditions tested in this work, despite robust levels of intracellular pro-IL-1 β . In contrast, in BMDCs, stimulation with GBS resulted both in pro-IL-1 β production and IL-1 β maturation and release. Moreover, BMDCs produced twice as much IL-1 β after treatment with LPS followed by ATP. The reasons for this differential responsiveness are not presently clear and are the subject of intensive investigation. Most of the inflammasome studies conducted to date have dealt with macrophages, and only few (40, 41, 46) used dendritic cells. To our knowledge, ours is the first study to directly compare macrophages with dendritic cells for their ability to respond to NLRP3-activating stimuli. Our data on the relatively weak IL-1 β response of macrophages to GBS are in agreement with previous studies conducted with other extracellular Gram-positive organisms, such as *Staphylococcus aureus*, *S. pyogenes*, and *Streptococcus pneumoniae* (31, 40, 47). For example, macrophages only substantially produced IL-1 β in response to *S. aureus* when cells were pretreated with LPS (48) or stimulated with very high MOIs (30). In full accordance with these data, we found that GBS induces IL-1 β release in LPS-pretreated macrophages or if higher MOIs were employed (G. Mancuso and G. Teti, unpublished observations). Although *S. pyogenes* induced IL-1 β release in BMDMs in the absence of LPS pretreatment (31), it was necessary to incubate bacteria with host cells for a prolonged time (3.5 h) in the absence of antibiotics, a condition under which streptococci can easily reach 2-log higher counts. Collectively, our data argue that dendritic cells are a major source of IL-1 β and IL-18 release during infection with GBS. Further studies are clearly needed to verify this possibility. In conclusion, infection of murine dendritic cells with GBS triggered the activation of caspase-1, resulting in IL-1 β and IL-18 release. Moreover, the NLRP3 inflammasome made a significant contribution to host defenses. This study identifies the production of a critical virulence factor of GBS, namely β -hemolysin, as the main stimulus for triggering a highly effective host-protective response through activation of the NLRP3 inflammasome.

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Disclosures

The authors have no financial conflicts of interest.

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