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Listeria monocytogenes-Infected Human Peripheral Blood Mononuclear Cells Produce IL-1β, Depending on Listeriolysin O and NLRP3

Karolin Meixenberger,* Florence Pache,* Julia Eitel,* Bernd Schmeck,* Stefan Hippenstiel,* Hortense Slevogt,* Philippe N'Guessan,* Martin Witzenrath,* Mihai G. Netea,[†] Trinad Chakraborty,[‡] Norbert Suttorp,* and Bastian Opitz*

Different NOD-like receptors, including NLRP1, NLRP3, and NLRC4, as well as the recently identified HIN-200 protein, AIM2, form multiprotein complexes called inflammasomes, which mediate caspase-1–dependent processing of pro-IL-1β. *Listeria monocytogenes* is an intracellular pathogen that is actively phagocytosed by monocytes/macrophages and subsequently escapes from the phagosome into the host cell cytosol, depending on its pore-forming toxin listeriolysin O (LLO). In this study, we demonstrate that human PBMCs produced mature IL-1β when infected with wild-type *L. monocytogenes* or when treated with purified LLO. *L. monocytogenes* mutants lacking LLO or expressing a noncytolytic LLO as well as the avirulent *Listeria innocua* induced strongly impaired IL-1β production. RNA interference and inhibitor experiments in human PBMCs as well as experiments in Nlrp3 and Rip2 knockout bone marrow-derived macrophages demonstrated that the *Listeria*-induced IL-1β release was dependent on ASC, caspase-1, and NLRP3, whereas NOD2, Rip2, NLRP1, NLRP6, NLRP12, NLRC4, and AIM2 appeared to be dispensable. We found that *L. monocytogenes*-induced IL-1β production was largely dependent on phagosomal acidification and cathepsin B release, whereas purified LLO activated an IL-1β production independently of these mechanisms. Our results indicate that *L. monocytogenes*-infected human PBMCs produced IL-1β, largely depending on an LLO-mediated phagosomal rupture and cathepsin B release, which is sensed by Nlrp3. In addition, an LLO-dependent but cathepsin B-independent NLRP3 activation might contribute to some extent to the IL-1β production in *L. monocytogenes*-infected cells. *The Journal of Immunology*, 2010, 184: 922–930.

*L*isteria monocytogenes is an intracellular pathogen that causes severe diseases, particularly in immunocompromised individuals, pregnant women, and neonates. On infection of phagocytic cells, *L. monocytogenes* is actively internalized. The bacterium escapes from the phagosome into the host cell cytosol, depending on its pore-forming toxin listeriolysin O (LLO) encoded by *hly*, which is assisted by two phospholipases C encoded by *plcA* and *plcB* (1, 2). LLO is a member of a large family of cholesterol-dependent cytolysins that are secreted by numerous Gram-positive bacteria. However, *L. monocytogenes* is the only bacteria that secrets the toxin inside the host cell.

The innate immune system senses microbial components and endogenous danger-associated molecules by so-called pattern recognition receptors (PRRs), including the transmembrane TLRs as well as the cytosolic RIG-like receptors (RLRs) and NOD-like receptors (NLRs) (3–6). In humans, the NLR family consists of 22 members with only some of them functionally characterized (3, 4, 7, 8). Similar to the TLRs and RLRs, some NLRs such as NOD1/NLRC1 and NOD2/NLRC2 activate a NF-κB–dependent expression of proinflammatory genes. For example, the precursor pro-IL-1β is expressed after TLR, NOD1/NOD2, or RLR stimulation. A second signal provided by multiprotein complexes called inflammasomes is necessary for the release of the biologically active, mature IL-1β. Inflammasomes consist of one or two NLRs, including NLRP1, NLRP3, Naip5, or NLRC4, of caspase-1, which mediates cleavage of pro-IL-1β, and of the adaptor molecule ASC in most cases. The NLRP1 inflammasome is activated by *Bacillus anthracis* lethal toxin (9) or muramyl dipeptide (10, 11). The NLRP3 inflammasome mediates caspase-1 activation by a variety of molecules, including microbial RNA, gout-associated uric acid crystals, ATP, silica crystals, and aluminum salts (12–20). The NLRC4 inflammasome, which at least in some cases also includes Naip5/NAIP, responds to cytosolic flagellin but also to some nonflagellated bacteria (21–26). In addition to the NLRs, the HIN-200 family member AIM2 has recently been shown to mediate ASC-dependent caspase-1 activation after cytosolic DNA stimulation (27–30). Whereas AIM2 directly interacts with its ligand dsDNA in the cytosol, NLRP1 and NLRP3 possibly sense intermediate cellular signals induced by various stimuli instead of directly binding the molecules. Another subject matter of poor understanding is the function of the NLR members NLRP6 and NLRP12, which might also be capable of binding ASC and activating caspase-1 (31, 32).

L. monocytogenes can be recognized by TLR2 (33, 34), NOD1 (35–37), and NOD2 (38, 39), which leads to NF-κB–dependent proinflammatory gene expressions. Moreover, *L. monocytogenes* activates IFN-β responses are dependent on a yet-to-be-identified
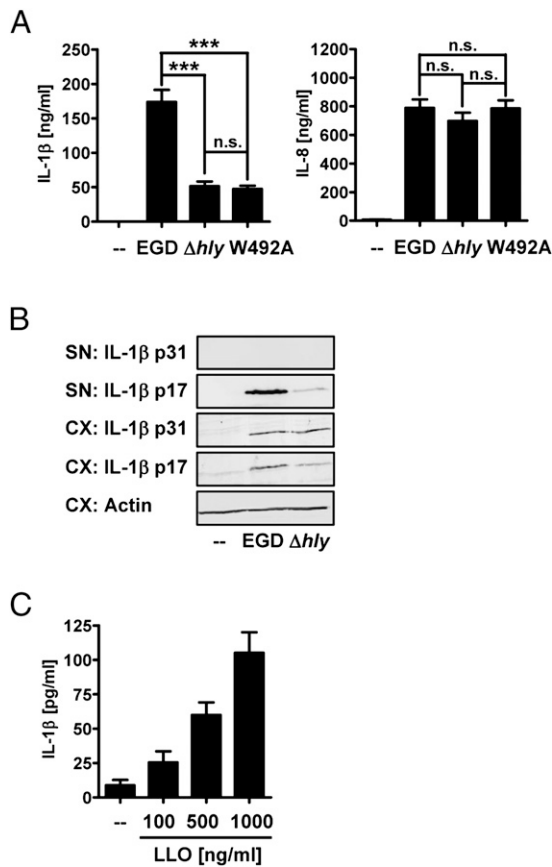
FIGURE 1. *L. monocytogenes* induced production of IL-1β depending on LLO. *A*, Human PBMCs were infected with *L. monocytogenes* EGD, Δ*hly*, or W492A. Production of IL-1β and IL-8 was determined by ELISA. *B*, Human PBMCs were infected with *L. monocytogenes* EGD or Δ*hly*. Amounts of IL-1β p17 and p31 in cell extract (CX) and supernatant (SN) were visualized by Western blot. *C*, Human PBMCs were treated with purified LLO. Production of IL-1β and IL-8 was assessed by ELISA. *A* and *C*, Results represent mean ± SEM of three independent experiments. Significant differences (***p < 0.001) were indicated. n.s., not significant differences (*p* > 0.05).

cytosolic PRR and also involve NOD2 and its downstream kinase Rip2 (40–43). In addition, *L. monocytogenes* was shown to activate caspase-1 via the inflammasome adaptor ASC (44), as well as Nlrp3 in mice (17, 45), although another publication demonstrated contradicting results regarding Nlrp3 (46). A recent study further indicated that the Nlrc4 inflammasome contributed to IL-1β production in *L. monocytogenes*-infected mouse cells (45).

In this study, we examined the mechanism of IL-1β production in *L. monocytogenes*-infected human PBMCs and mouse macrophages.

Materials and Methods

Bacterial strains

The *L. monocytogenes* serotype 1/2a strain EGD was purchased from the American Type Culture Collection, (Manassas, VA). The isogenic mutants EGD Δ*hly* (further referred to as Δ*hly*) and EGD *hly* W492A (further referred to as W492A) have been described previously (47, 48). Bacteria were grown to the logarithmic growth phase in brain heart infusion (BD Biosciences, San Jose, CA) at 37°C with continuous shaking at 200 rpm. Bacteria were subsequently washed with PBS (PAA) by two steps of centrifugation (4000 rpm, 5 min) and diluted in RPMI 1640 (Invitrogen, Carlsbad, CA) for infection.

Human cell culture

The human monocyte cell line THP-1 was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cells were cultured in RPMI 1640 containing 10% FCS (Life Technologies,
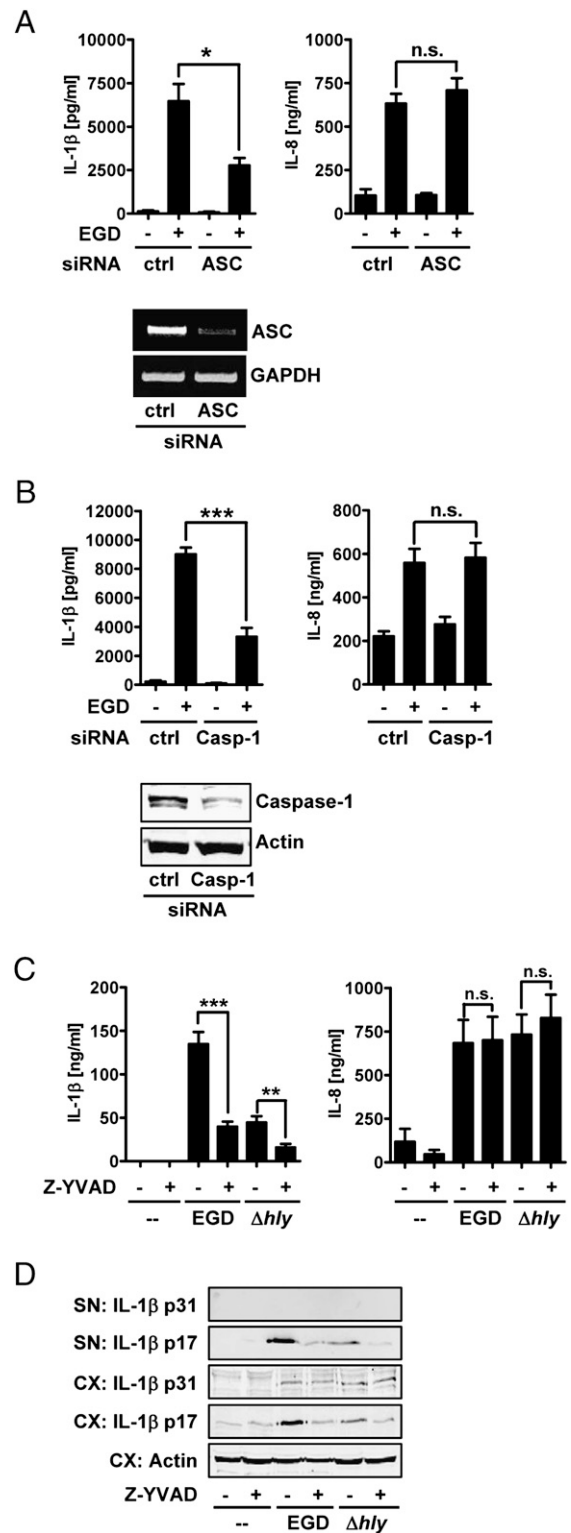
FIGURE 2. Role of caspase-1 and ASC in *L. monocytogenes*-induced IL-1β production. *A*, *B*, THP-1 cells were transfected with control siRNA or siRNA specific for ASC or caspase-1. After 72 h, cells were infected with *L. monocytogenes* EGD, and supernatants were subjected to IL-1β and IL-8 ELISA. Knockdown of ASC or caspase-1 was confirmed by RT-PCR or Western blot, respectively. *C*, *D*, Human PBMCs were infected with *L. monocytogenes* EGD or Δ*hly* in the presence or absence of 10 μM caspase-1 inhibitor Z-YVAD-FMK. Production of IL-1β and IL-8 was determined by ELISA and Western blot (IL-1β p17 and p31). Results obtained from ELISAs represent mean ± SEM of three independent experiments. Significant differences (***p < 0.001; **p > 0.01; *p < 0.05) were indicated. n.s., not significant differences (*p* > 0.05).

Grand Island, NY) and seeded in appropriate culture dishes at a concentration of 10^6 cells/ml. THP-1 were adhered overnight with 100 ng/ml PMA (Sigma-Aldrich, St. Louis, MO), washed twice with PBS, and cultured 2 more d before infection. Human PBMCs were isolated from buffy coat preparations obtained from the German Red Cross Berlin (Berlin, Germany). The buffy coat was diluted with RPMI 1640 containing 5% FCS and 0.2 mM EDTA and centrifuged twice over Pancoll (PAN Biotech, Aidenbach, Germany) for 25 min at $800 \times g$. Cells were cultured in RPMI 1640 containing 10% FCS and seeded in appropriate culture dishes at a concentration of 5×10^6 cells/ml.

Generation of mouse bone marrow-derived macrophages

Wild-type C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA). Nlrp3 knockout (KO) mice were provided by Prof. J. Tschopp (University of Lausanne, Lausanne, Switzerland) (19). Rip2 KO bone marrow-derived macrophages (BMMs) were provided by Dr. L. Joosten (University of Nijmegen, Nijmegen, The Netherlands). BMMs were cultured in RPMI 1640 containing 30% L cell supernatant and 20% FBS and were replated 1 d prior to infection in RPMI 1640 containing 15% L cell supernatant and 10% FBS.

RNA interference

THP-1 cells were transfected using the Amaxa Nucleofector (Amaxa, Cologne, Germany) according to the manufacturer's protocol (Cell Line Nucleofector Kit V, Program T-08) with 2 μg/10^6 cells. Human PBMCs were transfected using the Amaxa Nucleofector according to the manufacturer's protocol (Human Monocyte Nucleofector Kit, Program Y-01) with 5 μg small interfering RNAs (siRNAs) per 10^7 cells (49). siRNAs were purchased from Ambion (Austin, TX) (Table II).

Bacterial infection

THP-1, human PBMCs, and mouse BMMs were infected overnight with *Listeria* spp., and supernatants were collected. When infecting THP-1, 1-h postinfection cells were washed twice with PBS and incubated with RPMI 1640 containing 50 μg/ml gentamicin (Invitrogen) for 1 additional h. Subsequently, cells were washed twice with PBS and incubated with RPMI 1640. In some experiments, human PBMCs and mouse BMMs were preincubated for 1 h with 10 μM CA-074 Me (Calbiochem, San Diego, CA), 10 μM Z-YVAD-FMK (Alexis Biochemicals, Lörrach, Germany), 100 nM bafilomycin A1 (Calbiochem), 130 mM KCl, or 130 mM NaCl, and incubation was continued during infection. The reagents used had no effect on bacterial growth or cell viability (data not shown).

LLO stimulation

Purified LLO isolated from a *L. innocua* strain engineered to overproduce listeriolysin (50) was added to human PBMCs or mouse BMMs, and supernatants were collected after overnight incubation. Mouse BMMs were primed with LPS (3 ng/ml) for 4 h before LLO treatment.

Western blot analysis

Cell-free supernatants were concentrated using Microcon Ultracel YM-3 Centrifugal Filter Devices (Millipore, Bedford, MA). Cell extracts or concentrated supernatants were separated by SDS-PAGE and blotted. Membranes were first exposed to Abs specific for IL-1β (no. 2022, Cell Signaling Technology, Beverly, MA), caspase-1 (no. 2225; Cell Signaling Technology) or actin (sc-1616; Santa Cruz Biotechnology, Santa Cruz, CA) and subsequently incubated with secondary Abs (Cy5.5-labeled anti-rat or IRDye 800-labeled anti-goat). Proteins were detected using the Odyssey Infrared Imaging System (LI-COR, Lincoln, NE).

ELISA

Concentrations of IL-1β, IL-8, IFN-β, murine (m)IL-1β, or mIFN-β in cell-free supernatants were quantified by commercially available sandwich ELISA kits (BD Biosciences; eBioscience, San Diego, CA; Fujirebio, Malvern, PA; and PBL Biomedical Laboratories, Piscataway, NJ).

Confocal laser scanning microscopy

Mouse BMMs were left untreated or were infected with *L. monocytogenes* EGD. After 2 h, extracellular bacteria were removed by washing with PBS, and cells were fixed with 3% paraformaldehyde (Sigma-Aldrich). Cells were permeabilized with 1% Triton X-100 (Sigma-Aldrich) and blocked with 5% goat serum (Invitrogen). Cathepsin B was stained by exposing cells to a specific Ab (ab58802; Abcam, Cambridge, UK), followed by incubation with AF546-conjugated anti-mouse IgG (Invitrogen). Host cell actin and actin assembly by cytosolic *Listeria* was stained with phalloidin AF488 (Invitrogen). Confocal laser scanning microscopy was conducted on a LSM5 Pascal (Zeiss).

RT-PCR Analysis

Total RNA from THP-1 or human PBMCs was isolated using RNeasy Kit (Qiagen) and reverse transcribed with either Moloney murine leukemia virus reverse transcriptase (Invitrogen) or High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The generated cDNA was amplified by semiquantitative PCR using REDTaq DNA Polymerase (Sigma-Aldrich) and specific primers (Table I) or by quantitative PCR using TaqMan Gene Expression Assays (Applied Biosystems) on the 7300 Real-Time PCR System (Applied Biosystems). The mRNA expression level of each target gene was normalized to the expression level of GAPDH.

Statistics

Results were statistically evaluated using Student's *t* test or one-way ANOVA with Newman–Keuls posttest. Values of $p < 0.001$ are indicated by three asterisks (***). Values of $p < 0.01$ are indicated by two asterisks (**). Values of $p < 0.05$ are indicated by one asterisk (*). Not significant differences ($p > 0.05$) are marked with n.s.

Table I.   *Primers*

| Primer	Forward	Reverse
AIM2	GCAGTGATGAAGACCATTCgTA	GCTGAGTTTGAAGCGTGTTGAT
ASC	ATGCGCTGGAGAACCTGA	AGGTAGGACTGGGACTCCCTTA
Casp-1	TGGGGTACAGCGTAGATGTGA	CGAACCTTGCGGAAAATTTC
GAPDH	CCACCCATGGCAAATTCCATGGCA	TCTAGACGGCAGGTCAGGTCCACC
IL-1β	TCCGACCACCACTACAGCAA	ATCTTTCAACACGCAGGCAA
IL-8	CTAGGACAGAGCCAGGAAGA	AACCCTCTGCACCCAGTTTTC
NAIP	ACATCGTCCAGTGCTTTTCC	ACAAAGTTCACCACGGCTCT
NOD2	AGCCATTGTCAGGAGGCTC	CGTCTCTGCTCCATCATAGG
NLRC3	TCACCTGCAGTGGAACTTCAT	TCACATTTCAACAGTGCACG
NLRC4	TCTGACTGACAGCTTGGGTAA	TGGGACCTCCTCCAAATGTT
NLRC5	TGGGAAGACACTCAGGCTAA	ATCATCGTCCTCACAGAGGTT
NLRP1	CGAGAACAGCTGGTCTTCTCCAGGGCTTCG	TCCCCCTTGGGAGTCCTCCTGAAAATGATC
NLRP2	CAGCTCAGCCAGGATGAGTT	AGCACCACCGTGTATGAGAA
NLRP3	AGCCACGCTAATGATCGACT	CAGGCTCAGAATGCTCATCA
NLRP6	TGCTGTACTGCCTGTACGAGA	TGTACCCTGACCGTCTGCA
NLRP8	AGCATGTGGCACAGGAAAT	ATATTGACACCGCTTCAGGCAA
NLRP12	CATGATGCTGCTTTGCGA	TCCATCCCAAATAACCAGAGG
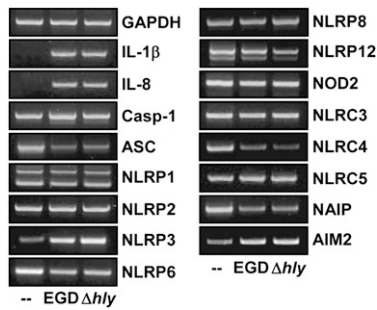
FIGURE 3. Expression of inflammasome components in human PBMCs. Human PBMCs were left untreated or were infected with *L. monocytogenes* EGD or Δ*hly* and lysed 8 h p.i. mRNA expression of selected genes was analyzed by RT-PCR.

Results

L. monocytogenes *and purified LLO-activated IL-1β production in human PBMCs*

First, we found that human PBMCs and THP-1 infected with wild-type *L. monocytogenes* (EGD) and *L. monocytogenes* mutants lacking both phospholipase C (Δ*plcA/B*) or lacking both internalins (Δ*inlA/B*) strongly activated the production of mature (p17) IL-1β (Fig. 1A, 1B, and data not shown). In contrast, *L. monocytogenes* lacking LLO (Δ*hly*) or expressing a non-pore-forming LLO (W492A) as well as *L. innocua* (INN) induced only a small IL-1β release (Fig. 1A, 1B, and data not shown). IL-8 production as well as IL-1β mRNA and pro-IL-1β protein expression were stimulated to a comparable degree by any of the tested bacterial strains (Figs. 1A, 1B, and 3, and data not shown). Moreover, we found that purified LLO isolated from a *L. innocua* strain engineered to overproduce listeriolysin activated a weak IL-1β release when added to the cells (Fig. 1C). Heat inactivation strongly impaired the capability of LLO to activate IL-1β release (data not shown). Overall, LLO was important for activating IL-1β production.

ASC and caspase-1 are required for L. monocytogenes-*induced IL-1β production*

Next, we tested the involvement of the common inflammasome components ASC and caspase-1 in the *L. monocytogenes*-induced IL-1β production. We found that ASC and caspase-1 siRNAs lowered the IL-1β production but not IL-8 secretion in *L. monocytogenes*-infected cells (Fig. 2A, 2B). Moreover, the caspase-1 inhibitor Z-YVAD-FMK also strongly reduced the release of the processed (p17) form of IL-1β but not of IL-8 or the pro-IL-1β expression in *L. monocytogenes*-infected cells (Fig. 2C, 2D). The caspase-1 inhibitor also further decreased the small IL-1β release induced by LLO-negative *L. monocytogenes* (Fig. 2C). This result suggests that two inflammasome activities contribute to the caspase-1–dependent IL-1β processing in *L. monocytogenes*-infected PBMCs: the one is LLO dependently activated; the other appears to be constitutively or LLO independently activated.

Expression of inflammasome components in L. monocytogenes-*infected human PBMCs*

Considering the hypothesis that *L. monocytogenes* activates one or more inflammasomes, we examined the expression of several inflammasome components in *L. monocytogenes*-infected cells by RT-PCR using specific primers (Table I). Besides ASC and caspase-1, NLRP1–3, NLRP6, NLRP8, NLRP12, NOD2, NLRC3–5, NAIP, and AIM2 were expressed in human PBMCs (Fig. 3). Upregulation of NLRP3 and AIM2 as well as IL-1β and IL-8 transcripts was observed in *L. monocytogenes*-infected PBMCs.

Role of different inflammasomes in L. monocytogenes-*activated IL-1β production*

Next, the involvement of different NLRs and of AIM2 in the *L. monocytogenes*-induced IL-1β production in PBMCs was examined by RNA interference experiments. Different siRNAs targeting NLRP1, NLRP3, NLRP6, NLRP12, NLRC4, AIM2, and NOD2 (Table II) attenuated expression of their corresponding transcripts (Fig. 4A–G). The NLRP3 knockdown, but not the knockdown of the other NLRs or AIM2, significantly inhibited the *L. monocytogenes*-activated IL-1β production (Fig. 4A–G). The IL-8 production was unaltered by all siRNAs tested (Fig. 4A–G). In addition, we tested involvement of Rip2, which mediates signaling downstream of NOD1/2. In accordance with the NOD2 siRNA experiments, *Listeria*-induced IL-1β production was not attenuated in Rip2$^{-/-}$ BMMs (Fig. 4H). These results indicate that the NLRP3 inflammasome mediates IL-1β production in *L. monocytogenes*-infected human PBMCs, whereas NLRP1, NLRP6, NLRP12, NLRC4, AIM2, NOD2, and Rip2 appear to be dispensable.

Role of phagolysosomal acidification and of cathepsin B in L. monocytogenes- *and LLO-induced IL-1β production*

Recent studies demonstrated that lysosomal acidification, lysosomal damage, and release of phagosomal cathepsin B were involved in NLRP3 inflammasome activation by silica crystals, aluminum salts, and amyloid-β (13, 14). Considering LLO's role in mediating rupture of the phagosomal membrane during *L. monocytogenes* infection, we hypothesized a similar mode of NLRP3 activation in *L. monocytogenes*-infected cells. PBMCs were treated with bafilomycin A1 to block the vacuolar H$^+$ ATPase system required for acidification of phagolysosomes or with the cathepsin B inhibitor CA-074 Me (13, 14). We found that both inhibitors strongly impaired the *L. monocytogenes*-activated IL-1β production (Fig. 5A, 5C). Moreover, blockage of K$^+$ efflux by excess of KCl in the cell culture medium completely inhibited inflammasome activation by *L. monocytogenes* (Fig. 5E) as shown before for other NLRP3 activators (51). In contrast to the *Listeria*-induced IL-1β release, IL-1β production in cells treated with purified LLO was not significantly reduced by bafilomycin A1 or CA-074 Me but was inhibited by blockage of K$^+$ efflux (Fig. 5B,

Table II.   *siRNA*

| siRNA	Sense	Antisense
AIM2a	GCAACGUGCUGCACCAAAA	UUUUGGUGCAG CACGUUGC
AIM2b	GGAGAUAAGGUUCGACUUA	UAAGUCGAACC UUAUCUCC
ASC	GAUGCGGAAGCUCUUCAGU	ACUGAAGAGCU UCCGCAUC
Casp-1	GGUUCGAUUUUCAUUUGAG	CUCAAAUGAAA AUCGAACC
Control	UUCUCCGAACGUGUCACGU	ACGUGACACGU UCGGAGAA
NLRC4	GGUUCAAGCCAAAGUAUAA	UUAUACUUUGG CUUGAACC
NOD2	GGAAUUACCAGUCCCAUUG	CAAUGGGACUG GUAAUUCC
NLRP1	CGGUGACCGUUGAGAUUGA	UCAAUCUCAAC GGUCACCG
NLRP3	GGUGUUGGAAUUAGACAAC	GUUGUCUAAUU CCAACACC
NLRP6	CGUCAGUGUACCUGCUUUU	AAAAGCAGGUA CACUGACG
NLRP12a	GGAAAUGCACUGGAGGAUU	AAUCCUCCAGU GCAUUUCC
NLRP12b	GCAUAAUGAUCAGCCUCCU	AGGAGGCUGAU CAUUAUGC
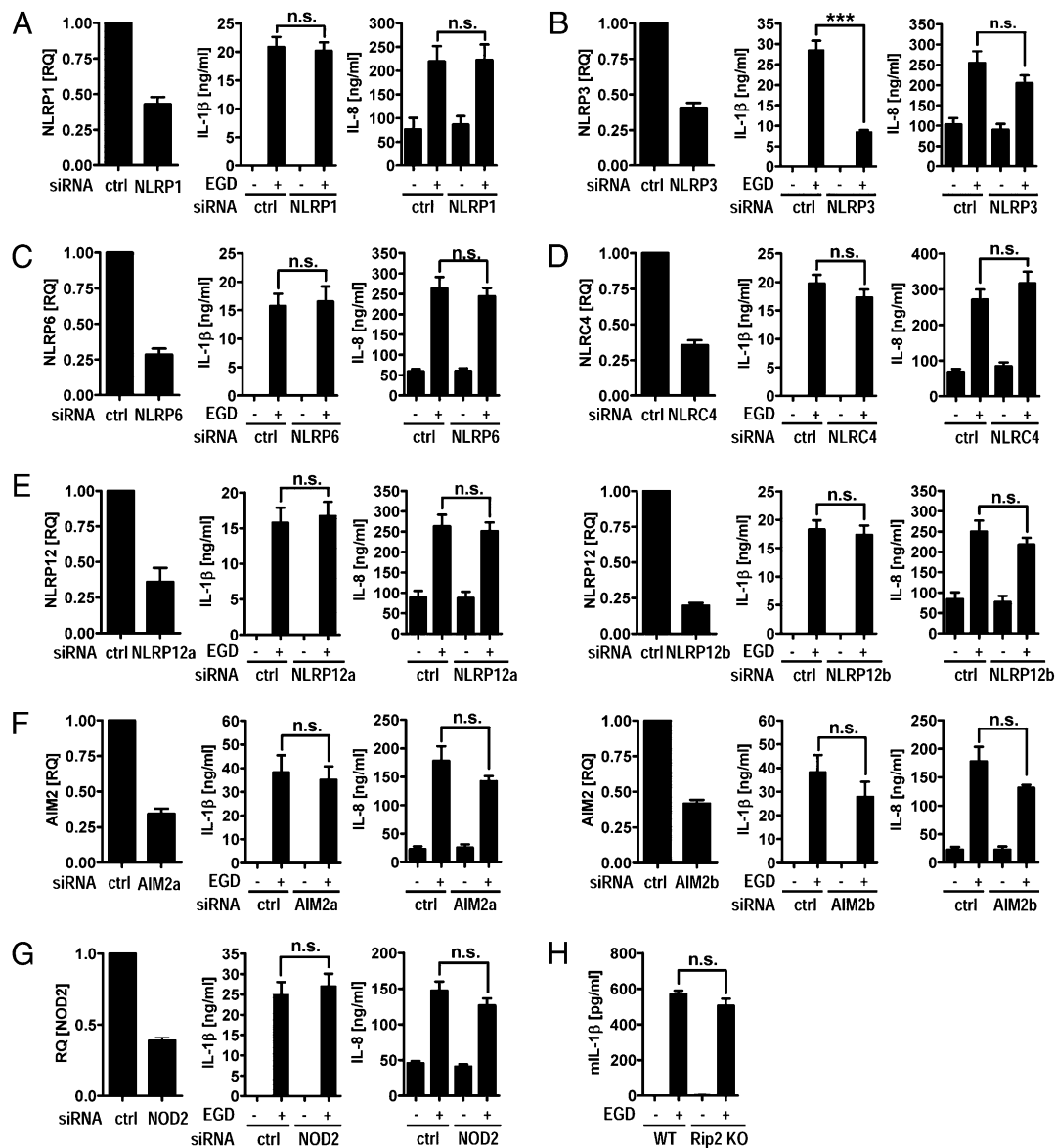
FIGURE 4. Role of NLRP1, NLRP3, NLRP6, NLRP12, NLRC4, AIM2, NOD2, and Rip2 in *L. monocytogenes*-induced IL-1β production. *A–G*, Human PBMCs were transfected with control siRNA or siRNA targeting specific genes as indicated. After 72 h, siRNA-mediated knock down of the specific genes was assessed by RT-PCR or cells were infected with *L. monocytogenes* EGD. Production of IL-1β and IL-8 was quantified by ELISA. *H*, Rip2$^{+/+}$ and Rip2$^{-/-}$ BMMs were infected with *L. monocytogenes* EGD, and production of mIL-1β was determined by ELISA. ELISA results represent mean ± SEM of three to five independent experiments. Significant differences (***$p < 0.001$) were indicated. n.s., not significant differences ($p > 0.05$).

5*D*, 5*F*). Taken together, our results suggest that phagosomal rupture and release of proteolytic enzymes, including cathepsin B as well as K$^+$ efflux, are involved in IL-1β production in *L. monocytogenes*-infected human monocytes, whereas the LLO-stimulated IL-1β release is independent of cathepsin B.

Our results indicated so far that in *L. monocytogenes*-infected host cells, LLO mediates a cathepsin B release into the host cell cytosol, which is directly or indirectly sensed by NLRP3. To further test this hypothesis, we infected wild-type and Nlrp3 KO mouse BMMs, which were partially treated with the cathepsin B inhibitor, with wild-type *L. monocytogenes* or with *L. monocytogenes* lacking LLO. Our results showed that the *L. monocytogenes*-infected wild-type BMMs produced IL-1β, whereas there was almost no IL-1β production in the Nlrp3 KO macrophages (Fig. 6*A*). The amount of IL-1β released by Nlrp3 KO BMMs was comparable to the amount of IL-1β produced by wild-type BMMs infected

with *L. monocytogenes* lacking LLO (Fig. 6*A*). A parallel experiment in human PBMCs transfected with control or NLRP3 siRNA revealed a similar trend of results (data not shown). These experiments taken together with those shown in Fig. 5 strongly suggest that *L. monocytogenes* infection leads to a cathepsin B release from phagoendosomes to the cytosol, which triggers NLRP3 activation. This assumption is supported by confocal microscopy pictures showing a diminished punctual cathepsin B staining in BMMs infected with *L. monocytogenes* compared with noninfected cells (Fig. 6*B*). In addition, we found that IL-1β release stimulated by purified LLO was strongly diminished by NLRP3 siRNA in human PBMCs or in Nlrp3$^{-/-}$ BMMs (Fig. 6*C*, 6*D*), whereas cathepsin B was not required (Fig. 6*C*). Overall, our data suggest that the NLRP3 inflammasome is critical for IL-1β production in *L. monocytogenes*-infected host cells. NLRP3 appears to be activated by
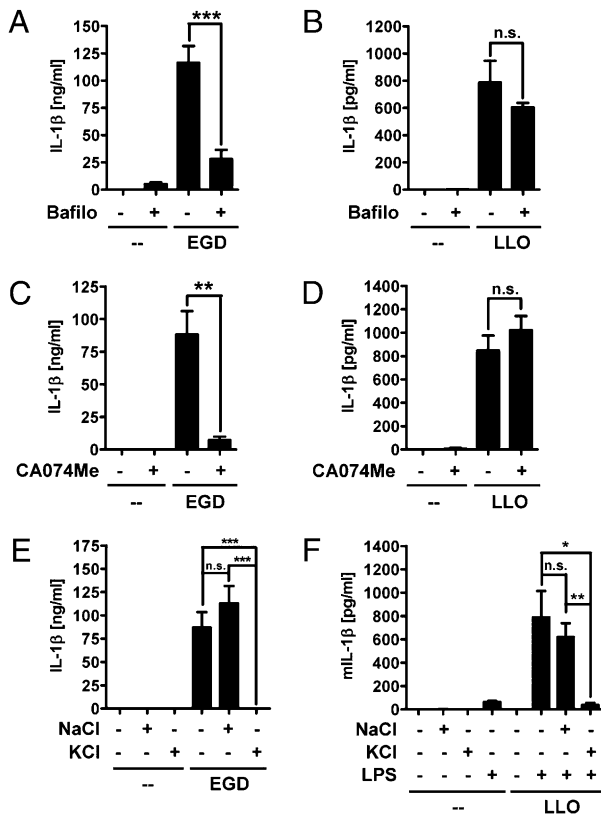
FIGURE 5. Role of phagolysosomal acidification, cathepsin B release, and K⁺ efflux in *L. monocytogenes*- and LLO-induced IL-1β production. *A* and *B*, Human PBMCs were infected with *L. monocytogenes* EGD (*A*) or were treated with purified LLO (*B*) in the presence or absence of 100 nM bafilomycin A1. *C* and *D*, Human PBMCs were infected with *L. monocytogenes* EGD (*C*) or were treated with purified LLO (*D*) in the presence or absence of 10 μM cathepsin B inhibitor CA-074 Me. *E*, Human PBMCs were infected with *L. monocytogenes* EGD in the presence or absence of 130 mM KCl and NaCl. *F*, Mouse BMMs were treated with LLO in the presence or absence of 130 mM KCl and NaCl. Production of IL-1β or mIL-1β was assessed by ELISA. Results represent mean ± SEM of three independent experiments. Significant differences (***$p < 0.001$; **$p < 0.01$) were indicated. n.s., not significant differences ($p > 0.05$).

an LLO-mediated phagosomal rupture and cathepsin B release and, to a small extent, by an additional mechanism activated by LLO which is independent of cathepsin B.

Role of NLRP3, NOD2, and Rip2 in L. monocytogenes-*induced IFN-β production*

Recent data showed that production of type I IFNs (IFN-α/β) in *L. monocytogenes*-infected cells was largely dependent on LLO (42), similarly to the IL-1β production. Although NLRP3 mediates IL-1β production, the exact mechanism that mediates IFN-β production in *Listeria*-infected cells is unknown. NOD2 as well as its downstream kinase Rip2 have been indicated to contribute to IFN-β responses in some studies (40, 41). We therefore wanted to know whether NLRP3, NOD2, and Rip2 had a role in IFN-β responses in *L. monocytogenes*-infected human and murine cells. We found that PBMCs produced IFN-β after infection with *L. monocytogenes*, which was largely dependent on LLO (Fig. 7*A*). Knockdown of NOD2 or NLRP3 had little effect on the IFN-β production in PBMCs. Moreover, deficiency of Nlrp3 had no effect on the IFN-β production in *Listeria*-infected BMMs (Fig. 7*B*), whereas deficiency of the NOD1/2 signaling molecule Rip2 led to a somewhat reduced IFN-β response (Fig. 7*C*). These data
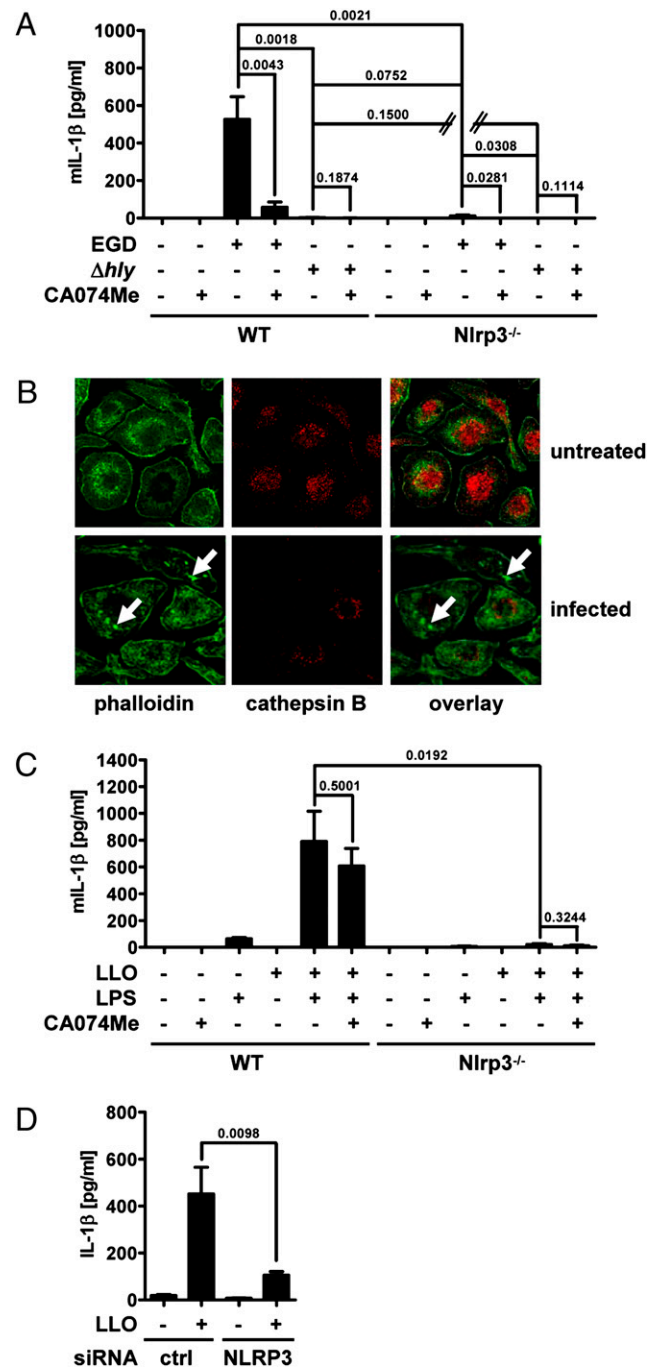
FIGURE 6. *L. monocytogenes*-infected host cells produced IL-1β depending on NLRP3, an LLO-mediated phagosomal rupture and cathepsin B release, whereas purified LLO activated an NLRP3-dependent IL-1β production independently of cathepsin B. *A*, Mouse BMMs obtained from wild-type or Nlrp3 KO mice were infected with *L. monocytogenes* EGD or Δ*hly* in the presence or absence of 10 μM cathepsin B inhibitor CA-074 Me. Production of mIL-1β was quantified by ELISA. *B*, Wild-type BMMs were left untreated or were infected with *L. monocytogenes* EGD for 2 h. Cathepsin B was visualized by confocal laser scanning microscopy using a specific Ab. Actin was stained with phalloidin AF488. Cytosolic *Listeria* were uncovered by actin assembly (see arrows). *C*, BMMs obtained from wild-type or Nlrp3 KO mice were primed with LPS and treated with LLO in the presence or absence of 10 μM cathepsin B inhibitor CA-074 Me. The production of mIL-1β was measured by ELISA. *D*, Human PBMCs were transfected with control siRNA or siRNA targeting NLRP3. After 72 h, cells were treated with LLO. Production of IL-1β was determined by ELISA. *A*, *C* and *D*, Results represent mean ± SEM of three to four independent experiments. The exact p values are indicated.
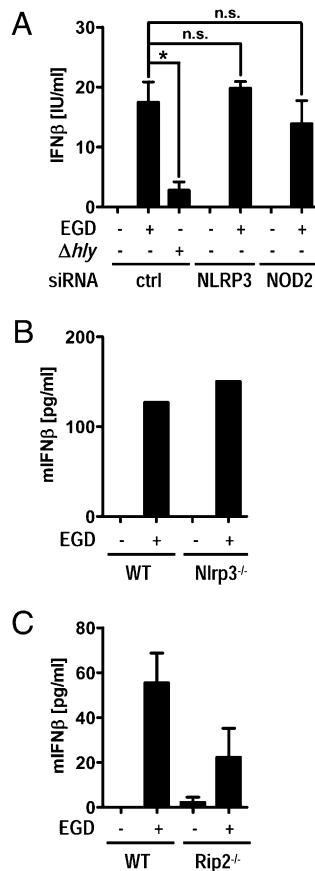
FIGURE 7. IFN-β production in *L. monocytogenes*-infected host cells. *A*, Human PBMCs were transfected with control siRNA or siRNA targeting NLRP3 or NOD2. After 72 h, cells were infected with *L. monocytogenes* EGD or Δ*hly*. Production of IFN-β was assessed by ELISA. *B* and *C*, Wild-type BMMs, Nlrp3$^{-/-}$ BMMs (*B*) or Rip2$^{-/-}$ BMMs (*C*) were infected with *L. monocytogenes* EGD. Production of mIFN-β was quantified by ELISA. *A* and *C*, Results represent mean ± SEM of three independent experiments. Significant differences (∗∗$p < 0.05$) were indicated. n.s., not significant differences ($p > 0.05$). *B*, Result shows one representative experiment in which supernatants of triplicates where pooled.

indicate that the pathways leading to IL-1β production and IFN-β release are clearly different, although both are triggered in an LLO-dependent manner.

Discussion

Inflammasomes have emerged as critical components of the innate immune system. They contribute to pathogen sensing and control posttranslational processing of important cytokines such as IL-1β and IL-18. Similarly to the TLRs, IL-1β and IL-18 activate via the IL-1 and IL-18 receptors a MyD88-dependent signaling and subsequent NF-κB– and MAPK-regulated gene expression. Accordingly, recent studies suggested that signals activated by LLO contribute to a TLR redundancy in *L. monocytogenes* innate immune recognition (52). In addition, IL-18 is crucial for inducing IFN-γ production, which in turn is essential for the innate intracellular defense against *L. monocytogenes* and for the T cell-mediated acquired immunity. Inflammasomes can therefore be seen as a major sentinel system of the innate defense that indirectly activate inflammatory gene expression as well as cell-autonomous resistance pathways in bacteria-infected host cells and contribute to the regulation of the adaptive immunity. In line with the assumption that inflammasomes are crucial for the host defense to *L. monocytogenes*, mice lacking caspase-1 or the IL-1β

receptor are significantly more susceptible to infection with *L. monocytogenes* (53, 54).

The present study examined the mechanism of inflammasome activation in *L. monocytogenes*-infected human PBMCs. We found that wild-type *L. monocytogenes*, but not *L. monocytogenes* lacking LLO or expressing a non-pore-forming LLO, stimulated a strong IL-1β production in human PBMCs. IL-1β secretion in human PBMCs and mouse BMMs was dependent on NLRP3, whereas AIM2 and all other NLRs examined, including NOD2 and its downstream kinase Rip2, were dispensable. In contrast, Rip2 signaling but not Nlrp3 were involved in signaling leading to IFN-β production in *L. monocytogenes*-infected mouse macrophages.

IL-1β secretion stimulated by *L. monocytogenes* was further largely dependent on phagosomal acidification and cathepsin B release. These results together with the recent observations on lysosomal damage as a trigger of the NLRP3 inflammasome (13, 14) point to a model in which inflammasome activation in *L. monocytogenes* infection is dependent on an LLO-mediated phagosomal disruption and cathepsin B release. A weak NLRP3-dependent IL-1β production, however, was also activated by purified LLO. The LLO-induced NLRP3 activation was independent of phagosomal acidification and cathepsin B. These results suggest that in addition to an LLO-mediated phagosomal disruption, the NLRP3 inflammasome is weekly activated by another mechanisms possibly involving LLO-mediated pore formation at the plasma membrane. Alternatively, LLO might be sensed in a more direct way within the host cell cytosol as recently suggested (55). Whether this "more direct NLRP3 activation" depends on a direct interaction of LLO and NLRP3, or more likely on still unidentified cytosolic mediator molecules upstream of NLRP3, is unknown. The hypothesis that LLO activates host cell responses through different pathways is supported by recently published results suggesting multiple mechanisms of LLO-stimulated, IL-18–dependent IFN-γ production (56).

Our results indicate that the *L. monocytogenes*-stimulated IL-1β production in human PBMCs does not require AIM2. AIM2 has recently been found to recognize bacterial DNA within the cytosol and evoke caspase-1 activation (27–30). Moreover, cytosolic detection of *L. monocytogenes* DNA is believed to be important for *Listeria*–host cell interactions (41, 57–59). In addition, a recent study indicated a role of type I IFNs in inflammasome activation by intracellular bacteria including *L. monocytogenes* (60), which might have fit to the observation that AIM2 is an IFN-inducible gene. Our data, however, do not argue for a major role of AIM2 in *L. monocytogenes*-induced inflammasome activation. Accordingly, pretreatment of PBMCs with IFN-β did not enhance the *L. monocytogenes*-stimulated IL-1β production in our experiments (data not shown).

Moreover, our examinations do not argue for a major role of NLRC4 in *L. monocytogenes*-activated IL-1β release. This is different to recent results, which described a contribution of flagellin sensing by the Nlrc4 inflammasome to the *L. monocytogenes*-stimulated IL-1β production in murine cells (45). The discrepancy is most likely caused by the fact that the *Listeria* strain used in our current study is known to express less flagellin at 37˚C as compared with the strain used previously (61).

Recent observations indicated a constitutive inflammasome activation in human PBMCs depending on a release of endogenous ATP (62, 63). In these studies, the LPS- or *Candida albicans*-stimulated IL-1β production in human PBMCs was solely dependent on the first signal provided by extracellular PRRs, including the TLRs. Pro-IL-1β expression therefore directly corresponded to the release of mature IL-1β. In our experiments, however, different *Listeria* were all capable of stimulating equal IL-1β mRNA and pro-IL-1β protein expression as well as IL-8

production, whereas only *Listeria* expressing pore-forming LLO also substantially activated release of mature IL-1β. Although the small caspase-1–dependent IL-1β production stimulated by the *L. monocytogenes* lacking LLO is possibly attributed to a constitutive inflammasome activity in PBMCs, our results clearly indicate an additional inducible inflammasome activation in *L. monocytogenes*-infected cells that was dependent on LLO.

Taken together, our results indicate that *L. monocytogenes*-infected human PBMCs produce IL-1β depending on NLRP3. NLRP3 appears to be activated by an LLO-mediated phagosomal rupture and cathepsin B release and—to a smaller extent—by an LLO-dependent but cathepsin B-independent mechanism.

Acknowledgments

Disclosures

The authors have no financial conflicts of interest.

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