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Inflammasome Activation by *Campylobacter jejuni*

Lieneke I. Bouwman,* Marcel R. de Zoete,†,‡ Nancy M. C. Bleumink-Pluym,* Richard A. Flavell,†,‡ and Jos P. M. van Putten*


Inflammasomes are multiprotein complexes that form in the cytosol following sensing of intracellular threats like invading bacteria and viruses or cell damage. Inflammasome complexes generally consist of sensor proteins (members of the Nod-like receptor [NLR] or Pyrin and HIN200 domain [PYHIN] protein family) and effector procaspases (mainly caspase-1), which are bridged by the adaptor protein apoptosis-associated speck-like protein (ASC). After assembly, inflammasomes induce the activation of caspase-1 through autocleavage, which subsequently activates cytokines IL-1β and IL-18, and induce a form of cell death referred to as pyroptosis (1–5).

The best studied inflammasomes in relation to bacterial infection are the NLR family, CARD domain–containing 4 (NLR4) and the NLR family, pyrin domain–containing 3 (NLRP3) inflammasomes (6, 7). The NLRC4 inflammasome is formed after sensing cytosolic bacterial flagellin or components of the bacterial type III secretion system (T3SS) by distinct neuronal apoptosis inhibitory protein (NAIP) receptors in the cell (8–10). NLR3 inflammasome activation requires a two signal process. The first (priming) signal leads to the expression of NLRP3 and pro–IL-1β through activation of NF-κB via stimulation of TLRs, other pattern recognition receptors, or endogenous cytokines. Then, a second stimulus (e.g., pore-forming toxins, bacterial invasion, or uptake of large particulates) induces the formation of the NLRP3 inflammasome (4). Although NLRP3 “ligands” are highly diverse, they all seem to converge in the efflux of K+ from the cell, which is proposed to be the common trigger (11, 12).

The bacterial pathogen *Campylobacter jejuni* is the most common cause of bacterial foodborne disease worldwide. Symptomatic infection typically involves intestinal inflammation with abdominal pain, fever, and (bloody) diarrhea. In ~1% of the cases, serious complications may develop such as the acute autoimmune paralyzing neuropathy Guillain–Barre syndrome (13, 14). In contrast to most enteropathogens including *Salmonella*, *C. jejuni* lacks traditional virulence factors like T3SSs. The molecular cause of *C. jejuni* intestinal inflammation is still largely unknown. After ingestion, the bacteria travel deep down into the intestinal crypts of the colon where they colonize and replicate. At some point the epithelial barrier is breached, resulting in acute inflammation accompanied by strong neutrophil recruitment and activation of T- and B-cell responses (15, 16). Bacterial motility and chemotaxis are crucial for causing disease (17–20). Other proposed virulence traits include the polysaccharide capsule, secreted proteins (Cia proteins, HtrA protease), type 6 secretion (T6SS) effector molecules, apoptosis-inducing proteins (cytolysogenous toxin, FspA2), and bacterial adhesion and invasion promoting factors (FlaC, PEB1, JlpA, CapA, and CadF) (for review, see Refs. 21–23). The role of these factors in the development of human infection, however, remains to be demonstrated.

The induction of acute intestinal inflammation in response to *C. jejuni* infection suggests the activation of innate pattern recognition receptors (24, 25). Although *C. jejuni* flagellin and DNA escape TLR recognition, the bacterial LPS and lipoproteins potentially activate the TLR4 and TLR2 pathways (25). In addition, *C. jejuni* is internalized by monocytes and macrophages and activates NOD1 (24, 26–29). Cellular infection is accompanied by the secretion of several proinflammatory cytokines such as IL-6,
IL-8, TNF-α, and IL-1β (30–32). Considering the potential important role of IL-1β in the clinical manifestation of C. jejuni infection (33), we investigated in the current study the ability of C. jejuni to activate the inflammasome. Our results reveal that C. jejuni can induce inflammasome activation without cytotoxicity, which has thus far not been observed for other pathogens.

Materials and Methods

Cell culture and reagents

J774.A1 cells (ATCC TIB-67) were routinely cultured in DMEM plus 10% FCS at 37°C and 10% CO2. THP-1 null and THP-1 deNLRP3 (InvivoGen; thp-null, thp-dnlp) were grown according to the manufacturer’s protocol in RPMI 1640 medium plus 10% FCS in the presence of 200 μg/ml Hygogold (every other passage) at 37°C and 5% CO2. L929 cells were cultured in RPMI 1640 medium plus 10% FCS at 37°C and 5% CO2.

The following reagents were used: gentamicin, kanamycin (kana), chloramphenicol (cat), Triton X-100, Tween 20, Tris, paraformaldehyde, PMA, TCA, and goat anti-rabbit IgG-HRP (A1941) (Sigma-Aldrich); FCS and Dulbecco’s PBS (PAA); DMEM, RPMI 1640 medium, Opti-MEM, penicillin, and streptomycin (Life Technologies); BCA Protein Assay Kit, Concentrators, 9K MWCO, and SuperSignal West Femto Chemiluminescent Substrate (Pierce); complete ULTRA Tablets EDTA-Free (EasyBlue) (LDH), and Cytotoxicity Detection Kit-LDHR (LDH) (Roche); WGA-Alexa Fluor 633, goat anti-rabbit-Alexa Fluor 488, primers, PFA DNA polymerase (Life Technologies); ATP, DNAse, 2′-deoxyxenosucilide 5′-triposphates, BamHI, KpnI, SacI, SacII, Phusion DNA Polymerase, GeneJET Gel Extraction Kit, CloneJET PCR Cloning Kit, and Rapid DNA ligation kit (Thermo); Mouse IL-1β ELISA Ready-SET-Go and Human IL-1β ELISA Ready-SET-Go (E Bioscience); Brilliant III Ultra-Fast Sybr Green qRT-PCR Kit (Agilent); reporter lysis buffer and Luciferase Assay Agent, pGEM-T easy (Promega); Saponin agar plates, Mueller Hinton plates, heart infusion (HI) plates, Luria–Bertani (LB) plates, LB broth, and HI broth (Biotrad); Campylobacter selective supplement and charcoal celofoperazone desoxycholate agar (SR0155) (Oxoid); RNA Bee (Bio-connect); Flusavore (Calbiochem); Qxax II gel extraction kit (Genom Tech); TAM fluorescence labeled inhibitor of caspase-1 (FLICA) Caspase-1 Assay Kit (Immunochemistry); and rabbit anti-caspase-1 (ab17820), rabbit anti-TMS1 (ASC) (ab64808), and rabbit anti-IL-1β (ab9722) (Abcam).

Cultivation of primary mouse macrophages

Bone marrow cells were isolated as described previously (34). Upon thawing, cells were collected (5 min, 485 × g, 20°C), resuspended in 10 ml bone marrow–derived macrophage (BMM) medium (RPMI 1640 medium plus 10% FCS and 30% L929 conditioned medium) with penicillin (100 IU) and streptomycin (100 μg/ml) and allowed to differentiate for 6 d in BMM at 37°C and 5% CO2. After 3 d, an additional 10 ml BMM medium was added to the cells. After differentiation cells were collected, counted, and seeded into a 96-well (1 × 104 cells/well) or 24-well (2.5 × 105 cells/well) plate in BMM medium, and used the next day. L929 conditioned medium was collected from L929 cells grown in 40 ml medium for 10 d in a T75 flask, filter sterilized (0.22-μm pore size), and stored at −20°C until use.

Bacterial culture

All C. jejuni strains (Supplemental Table I) were routinely grown under microaerophilic conditions at 37°C on saponin agar plates containing 4% lysed horse blood or in 5 ml HI broth at 160 rpm for 16 h. Kana (50 μg/ml) or cat (20 μg/ml) was added to the medium when appropriate. All C. jejuni strains had similar growth rates. Escherichia coli DH5a (Netherlands Culture Collection of Bacteria) was grown on LB agar plates or in 5 ml LB broth at 37°C in air.

Construction of C. jejuni luciferase reporter strains and fluorescent strains

The pMA5-metK-luc plasmid was introduced into several C. jejuni strains (81116, 108cheY:cat, 108cheY:cat, and 108etl:cat::Tn) via conjugation (35). Strain 108cheY:cat became either GFP or mCherry positive by introducing plasmid pMA1 containing GFP or mCherry via conjugation. In short, a 16-h culture of E. coli S17.1 containing the pMA5-metK-luc, pMA1-GFP, or pMA1-mCherry plasmid was diluted to an OD560 of 0.05 in 5 ml LB medium. Analogous 16-h cultures of the C. jejuni strains were diluted to an OD560 of 0.5 in 5 ml HI broth. When the E. coli culture reached an OD560 of 0.4, 1 ml C. jejuni culture was collected by centrifugation (10 min, 5000 × g) and suspended in 1 ml E. coli culture. The C. jejuni and E. coli mix was resuspended on a Mueller Hinton plate and incubated for 5 h (37°C) under microaerophilic conditions. Then, bacteria were collected in 1 ml HI, pelleted (10 min, 5000 × g), suspended in 100 μl HI, and plated on saponin agar plates containing 4% lysed horse blood, charcoal celluloperazone desoxycholate agar, Campylobacter selective supplement, 50 μg/ml kana, and when required, 20 μg/ml cat. Single antibiotic resistant colonies were collected after 48 h of incubation. The pMA1-mCherry plasmid was introduced in E. coli via chemical transformation.

Infection assay

J774.A1 macrophages were seeded into 24- or 96-well plates in DMEM plus 10% FCS, were seeded as described above. The next day, cells were primed by the addition of C. jejuni 108 lysate (equivalent of multiplicity of infection [m.o.i] 20) for 16 h when appropriate. THP-1 monocytes were co-cultured with C. jejuni (m.o.i. 10) for 16 h in RPMI 1640 medium plus 10% FCS and 50 μg/ml kana. Then, THP-1 monocytes were washed and stimulated with 2.5 or 5 mM ATP. After 20 min, the ATP was removed, and fresh medium was given. Extracellular Salmonella and E. coli were removed after 2 h by replacing the medium with fresh medium containing 50 μg/ml gentamicin. The experiment was stopped, and samples were collected after 12 h of incubation unless indicated otherwise.

Real time RT-PCR

J774.A1 cells were seeded into a 24-well plate and stimulated the next day by the addition of 50 μg/ml lipopolysaccharide (LPS) of N. meningitidis, lysate of C. jejuni strain 108 (equivalent of m.o.i 20) or inoculated with viable C. jejuni 108 (m.o.i. 20). After 4 h of stimulation, RNA was isolated using RNA Bee, according to the manufacturer’s protocol. RNA was treated with 1 μg DNase/μg RNA for 30 min at 37°C. The DNase was inactivated by heating for 10 min at 65°C in the presence of 2.5 mM EDTA. mRNA levels were determined in the LightCycler 480 Real-Time PCR instrument using the Brilliant III Ultra-Fast SYBR Green qRT-PCR System (Agilent) according to the manufacturer’s protocol with the primers listed in Table I. The relative expression level was calculated as follows: 2^ΔΔCt. The primers and probes used are listed in Table I.
95°C. mRNA levels were calculated by subtracting the corresponding Ct values obtained for samples before (1) and after (2) treatment using the following formula: 1) \( \Delta Ct_{target} = Ct_{target \_pre \_treatment} - Ct_{Actin \_control} \) and 2) \( \Delta Ct \_target \_gene \_test = Ct_{target \_gene \_test} - Ct_{Actin \_pre \_treatment} \). The fold change in mRNA was determined by fold change = 2^{\Delta Ct_{target\_treatment} - \Delta Ct_{Actin\_treatment}} (36). Presented results are from three individual assays performed in duplicate.

ELISA

Cell culture supernatants were collected in a fresh plate, centrifuged (10 min, 485 x g) to remove dead cells, bacteria, and other debris, and stored at -80°C. For further analysis, the same assay was used. ELISA using the manufacturer’s protocol. Samples were diluted in assay diluent to stay within the range of the assay (8 - 1000 pg/ml for mice and 4 - 500 pg/ml for human). Plates were measured at 450 and 570 nm for wavelength correction on the FLUOstar Omega (BMG Labtech). Presented results are from three individual assays performed in duplicate.

Detection of caspase-1 and IL-1β cleavage

For caspase-1 cleavage J774.A1 macrophages were seeded into a 6-well in DMEM+10% FCS. The next day, the medium was replaced with Opti-MEM and primed by the addition of C. jejuni 108 lysate (equivalent of m.o.i. 20) for 16 h when appropriate. The macrophages were inoculated with C. jejuni or E. coli. After 2 h of infection, 250 μg/ml gentamicin was added to the E. coli–inoculated wells. After 6 h of infection, the supernatant was collected and frozen at -80°C for a minimum of 1 h with a protease inhibitor. For the detection of cleaved IL-1β in the supernatant, J774.A1 macrophages or THP-1 monocytes were seeded in a T25 flask and infected as described for the infection assay with one minor change. Before infection, the medium was replaced with Opti-MEM. Postinfection (12 h) the supernatant was collected and frozen at -80°C for a minimum of 1 h with a protease inhibitor. The thaw supernatant was concentrated via a concentrator (9K MWCO) (IL-1β detection) or TCA precipitation (caspase-1). TCA (40%) was added to the supernatant in a 1:1 volume and incubated for 30 min at 4°C. The precipitate was collected by centrifugation (10 min, 21,100 g), washed twice with ice cold acetone (10 min, 21,100 x g), dried (10 min at 50°C), and taken up in radioimmunoprecipitation assay buffer. Protein concentration was determined via BCA protein concentration kit, according to the manufacturer’s protocol. Protein (10 μg for caspase-1 detection and 50 μg for IL-1β detection) was loaded and run on a 12% SDS-PAGE gel. After transfer of the proteins via blotting, the polyvinylidene difluoride membrane was blocked in TBST plus 5% milk (1 h) and incubated (16 h) with rabbit anti-caspase-1 (ab17820; 1:1000) or rabbit anti-IL-1β (ab9722; 1:2500) in TBST plus 5% milk at 4°C. The next day, the membrane was washed three times with TBST plus 5% milk (10 min per wash), incubated (1 h) with goat anti-rabbit-HRP (ab9414; 1:10,000), and washed (10 min per wash) with TBST plus 5% milk, TBST, and TBS. HRP signal was detected using SuperSignal West Fermo Chemiluminescent Substrate on the ChemiDoc MP System (Bio-Rad). Data were analyzed using Image Lab software (Bio-Rad). The images have been cropped.

Luciferase reporter assay

Bacteria were cultured for 16 h under standard conditions with a start OD500 of 0.01 from an 8-h preculture. Infection assays were performed with C. jejuni strains containing pMA5-metK-luc as described above. After 6 h, the cells were washed twice, lysed with 1x reporter lysis buffer supplement, and centrifuged (10 min, 10,000 g). After washing, the pellets were resuspended in 100 μl 0.1 M Tris-HCl pH 8, and left at 4°C for 1 h. The luciferase activity was measured with the Dual-Luciferase Reporter Assay Kit (Promega, Madison, WI) according to the manufacturer’s protocol. Presented results are from three individual assays performed in duplicate.

Cytotoxicity assay

Prized J774.A1 macrophages were infected in 96-well plate as described above with some minor changes. The assay was performed in DMEM without FCS. After 12 h of infection, the total and secreted amount of LDH was determined using the Cytotoxicity Detection KitPLUS (LDH, according to the manufacturer’s protocol. Plates were measured at 492 and 690 nm for wavelength correction on the FLUOstar Omega (BMG Labtech). C. jejuni by itself had no effect on LDH and did not influence the assay. The percentage of cytotoxicity was calculated as the percentage of LDH release compared with the total LDH concentration (percentage of cytotoxicity = 100 × [LDH released/total LDH]). Presented results are from three individual assays performed in triplicate.

Propidium iodide uptake

Primed J774.A1 macrophages were infected in a 96-blackwell plate with a transparent bottom (see infection assay) with some minor changes. The assay was performed in Opti-MEM without phenol red. After 11 h of infection, propidium iodide (PI) (3 μM) was added to the wells, and incubated for 1 h after which the plate was measured. Plates were excited at 492 nm and measured at 610 nm using a fixed gain, with bottom optics (orbital average, 35 flashes) detected by the FLUOstar Omega (BMG Labtech). Fluorescence intensity (FI) was corrected for background fluorescence from an unstained non stimulated well. Presented results are from three individual assays performed in triplicate.

Confocal microscopy

J774.A1 cells or BMMs were grown on 12 mm circular glass slides, primed for 16 h with the indicated stimulus, and inoculated with fluorescent C. jejuni. THP-1 monocytes were PMA differentiated, as mentioned previously, on 12 mm circular glass slides and inoculated with fluorescent C. jejuni. For active caspase-1 detection cells were incubated (1 h, 37°C) with FAM-FLICA (0.5x) and washed twice (10 min, 37°C) before fixation. After incubation, cells were washed twice with DPBS and fixed with 4% of paraformaldehyde in 100 mM phosphate buffer (pH 7.4) (1 h, room temperature). The fixed cells were washed with DPBS and stained with WGA Alexa Fluor 633 (1:500 for 1 h in DPBS). When needed cells were permeabilized with 1% Triton X-100 plus 1% BSA in DPBS (30 min, 20°C). ASC was stained by incubation (16 h, 4°C) of the cells with anti-TMS1 plus 0.01% Triton X-100 plus 2% BSA in DPBS followed with the goat anti-rabbit-Alexa Fluor 488 (1:100) secondary Ab in DPBS plus 2% BSA (1 h, 20°C). After staining the slides were washed three times with DPBS, once with MilliQ, embedded in Fluorsave, and viewed in the Bio-Rad radiance2000 system or Leica SPE-II system. The slides used for C. jejuni uptake were viewed using fixed settings for section thickness (2 μm) and magnification. Per slide 4 random images were captured. Data were analyzed with ImageJ software.

Statistical analysis

Results were analyzed using GraphPad Prism 5 software. Where appropriate significance was calculated using a paired Student t test.

Results

C. jejuni infection primes the macrophages for inflammasome activation

As activation of the inflammasome may involve a two-step process (priming and activation), we first determined whether C. jejuni was capable of priming the cells for inflammasome activation (Fig. 1). The transcription levels of pro-IL-1β in J774.A1 macrophages were determined using real-time RT-PCR with the primers listed in Table I. Incubation of macrophages with C. jejuni strain 108 led to a 125-fold induction of pro-IL-1β mRNA at 4 h of infection (Fig. 1A). Lysed C. jejuni yielded an even stronger effect probably due to increased availability of TLR ligands after bacterial disintegration (25). The transcriptional levels of ASC, NLRP3, caspase-1, or caspase-11 were similar upon stimulation with E. coli or stimulated with ATP (known inflammasome activators). The amount of secreted IL-1β was reduced and subse-
primed macrophages with live C. jejuni resulted in a dose-dependent secretion of IL-1β (Fig. 2A) and was observed for several C. jejuni strains (Fig. 2B). Western blot analysis of the supernatant confirmed the presence of the active IL-1β (17 kDa) form upon infection with C. jejuni strain 108 (Fig. 2C). The C. jejuni lysate alone did not cause IL-1β secretion, suggesting the requirement for intact bacteria to activate inflammasomes.

To ascertain that the release of IL-1β from the macrophages was caused by activation of the inflammasome, we determined whether caspase-1 was cleaved and secreted into the supernatant upon infection (6 h) with E. coli or C. jejuni. The presence of the p20 cleavage fragment (containing the active domain) of caspase-1 in the supernatant was determined using Western blotting. Both E. coli and C. jejuni strain 108 induced caspase-1 cleavage, as evident from the increased appearance of the p20 protein band (Fig. 2D). For unknown reasons, the cleavage pattern for E. coli– and C. jejuni–infected macrophages were different. As expected, cleavage was most evident for primed macrophages. Inflammasome activation was also confirmed by the formation of the ASC speck (a large multiprotein complex is being formed containing ASC) upon infection with C. jejuni or E. coli (2 h) as determined by confocal microscopy (Fig. 2E). Clearly, these results demonstrate that C. jejuni is capable to activate the inflammasome.

**Cellular infection–dependent inflammasome activation by C. jejuni**

To learn more about the mechanism(s) of C. jejuni–induced inflammasome activation, a series of C. jejuni mutants with defects in putative virulence determinants was tested for their ability to induce IL-1β secretion (Fig. 3A–C). In these experiments an intermediate dose of C. jejuni (m.o.i. of 20) was used to infect the cells to avoid IL-1β secretion as result of bacterial depletion of media components or possible C. jejuni induced cell toxicity. Genetic manipulation resulted in successful inactivation of a vast number of putative virulence genes, albeit in different C. jejuni strains. Infection assays demonstrated that genetically defined mutants with defects in bacterial capsule assembly (kpsM::cat), LOS assembly (waaF::cat), bacterial motility (motAB::cat), production of flagellin and flagellin-like proteins (flaAB::cat; flaAB::cat + flaC::kan), cytolethal distending toxin (CDT) production (cdc::cat), or the type 6 secretion apparatus (hcp::kana + kpsM::cat) induced similar levels of IL-1β secretion as their corresponding parental strain (Fig. 3A–C). In contrast, mutants lacking the chemotaxis protein CheY or the energy taxis protein CetA elicited a strongly increased (cheY::cat) or reduced (cetA::cat) IL-1β secretion compared with the parent strain (Fig. 3A). Western blotting confirmed the presence of more cleaved IL-1β in the supernatant of cells infected with strain 108cheY::cat compared with the parent strain (108) (Fig. 2C). This suggests that bacterial taxis strongly influences the level of inflammasome activation.

The CheY- and CetA-defective bacterial phenotypes show, respectively, hyperinvasive and hypoinvasive behavior toward epithelial cells (18, 19, 37, 38). Therefore, we examined a possible correlation between the number of bacteria that infected the macrophages and IL-1β secretion for both taxis mutants and the parental strain 108 using a luciferase reporter assay (20). Macrophages were infected with different strains of C. jejuni—producing luciferase. The amount of luciferase produced correlates to bacterial luciferase activity. Ge-
Besides IL-1β secretion, a common downstream effect of inflammasome activation is cell death via pyroptosis. Indeed, incubation of primed J774.A1 macrophages with ATP, Salmonella, or E. coli (12 h) induced cytotoxicity as estimated from the release of LDH in the culture supernatants (Fig. 4A). In contrast, incubation of the cells with C. jejuni did not result in the release of detectable amounts of LDH despite activation of the inflammasome (Fig. 3A–C). Even the hyperinvasive cheY cat mutant did not cause any LDH release. Measurement of PI uptake in J774.A1 macrophages infected with C. jejuni (strain 108 and cheY:cat, 12 h) revealed a minor yet not significant increase (Figure 4B). In contrast, infection with Salmonella did increase PI uptake in these cells, whereas E. coli had a minimal effect under the conditions used. Taken together, these results suggest that C. jejuni activates the inflammasome without causing cell death.

To assess whether the lack of cytotoxicity may be related to rapid killing of the intracellular C. jejuni, we measured the intracellular survival in primed J774.A1 macrophages using the bacterial luciferase reporter assay (Fig. 4C). After 12 h of infection, very few viable intracellular bacteria were detected and none after 24 h. There was also no major difference in survival between strain 108cheY:cat and the hyperinvasive strain 108cheY:cat.

C. jejuni activates the NLRP3 inflammasome in primary mouse macrophages

To determine which type of inflammasome is activated by C. jejuni, we used BMMs derived from either C57BL/6 wild-type or knockout mice deficient in distinct inflammasome components. The wild-type BMMs showed the expected secretion of IL-1β after stimulation with ATP and the enhanced IL-1β secretion postinfection with Salmonella (Fig. 5A). Both ATP and Salmonella required priming of the primary macrophages to be effective. Infection of the wild-type macrophages with C. jejuni strain 108cheY:cat also elicited the release of IL-1β but without the need to previous prime the macrophages. In fact, in primed cells, C. jejuni did not induce IL-1β secretion (Fig. 5A).

The type of inflammasome that was activated by the various stimuli was determined using BMMs from caspase-1/11−/−, ASC−−, NLRP3−−, and NLRC4−− mice (Fig. 5B, 5C). As expected, ATP induced IL-1β secretion in wild-type and NLRC4−− BMM but not in BMMs deficient in NLRP3, ASC, and caspase1/11, which are all components of the NLRP3 inflammasome (Fig. 5B). Infection with Salmonella induced IL-1β secretion in both wild-type and NLRP3−− BMMs, whereas secretion was severely reduced in NLRC4−− and ASC−− macrophages, consistent with previous reports (Fig. 5B) (39). C. jejuni strain 108 and the cheY:cat mutant induced equal levels of IL-1β secretion in wild-type BMM and BMMs isolated from NLRC4−− and ASC−− mice (Fig. 5C). C. jejuni–induced IL-1β secretion was not detected postinfection of caspase-1/11−/− and ASC−− BMMs and severely reduced in NLRP3−− BMMs. This suggests that C. jejuni activates the NLRP3 inflammasome and that caspase-1/11 and ASC are critical for the activation.
To ensure that the lack of inflammasome activation in *C. jejuni*–infected NLRP3<sup>-/-</sup> BMMs was not caused by poor infection of these cells, we determined the number of intracellular bacteria. Wild-type and NLRP3<sup>-/-</sup> cells were incubated (1 h) with mCherry-producing *C. jejuni* strains 108 and 108<sup>cheY::cat</sup>, and bacteria were visualized by confocal microscopy (Fig. 5E, 5F). Both cell types contained equal numbers of *C. jejuni* strains 108 and 108<sup>cheY::cat</sup>. The <sup>cheY::cat</sup> mutant was more infective (4-fold) than the parent strain, as was already noted for the J774.A1 macrophages postinfection (12 h) with *C. jejuni* strain 108 (m.o.i. 20 or 200), 108<sup>cheY::cat</sup> (m.o.i. 20), *E. coli* (m.o.i. 20), or without bacteria (nonstimulated). Lanes were loaded with 50 µg protein. (D) Western blot probed for the cleaved caspase-1 p20 fragment in the supernatant of nonprimed or primed J774.A1 macrophages postinfection (6 h) with *C. jejuni* strain 108 (m.o.i. 200), *E. coli* (m.o.i. 20), or without bacteria (nonstimulated). Lanes were loaded with 10 µg protein. (E) Confocal microscopy showing ASC speck formation (<) in primed J774.A1 macrophages postinfection (2 h) with mCherry-fluorescent *C. jejuni* strain 108 (m.o.i. 40) or *E. coli* (m.o.i. 20) (red). ASC foci were stained with anti-ASC Ab in combination with goat-anti-rabbit-Alexa Fluor 488 (green). Cell surface was stained with WGA-Alexa Fluor 633 (blue). Scale bar, 10 µm.

To ensure that the lack of inflammasome activation in *C. jejuni*–infected NLRP3<sup>-/-</sup> BMMs was not caused by poor infection of these cells, we determined the number of intracellular bacteria. Wild-type and NLRP3<sup>-/-</sup> cells were incubated (1 h) with mCherry-producing *C. jejuni* strains 108 and 108<sup>cheY::cat</sup>, and bacteria were visualized by confocal microscopy (Fig. 5E, 5F). Both cell types contained equal numbers of *C. jejuni* strains 108 and 108<sup>cheY::cat</sup>. The <sup>cheY::cat</sup> mutant was more infective (4-fold) than the parent strain, as was already noted for the J774.A1 macrophages postinfection (2 h) with mCherry-fluorescent *C. jejuni* strain 108 (m.o.i. 40) or *E. coli* (m.o.i. 20) (red). ASC foci were stained with anti-ASC Ab in combination with goat-anti-rabbit-Alexa Fluor 488 (green). Cell surface was stained with WGA-Alexa Fluor 633 (blue). Scale bar, 10 µm.

**FIGURE 2.** *C. jejuni* activates the inflammasome. (A and B) IL-1β secretion by primed J774.A1 macrophages infected (12 h) with different numbers of *C. jejuni*, *C. jejuni* lysate (~m.o.i. 20) (A) or with different *C. jejuni* strains (m.o.i. 200) (B). Infection with an m.o.i. higher than 20 (p < 0.05) or with different strains (p < 0.01) increased IL-1β secretion. Values are presented as the mean ± SEM of three independent experiments performed in duplicate. (C) Western blot probed for active IL-1β (17 kDa) (arrowhead) in the supernatant of primed J774.A1 macrophages postinfection (12 h) with *C. jejuni* strain 108 (m.o.i. 20 or 200), 108<sup>cheY::cat</sup> (m.o.i. 20), *E. coli* (m.o.i. 20), or without bacteria (nonstimulated). Lanes were loaded with 50 µg protein. (D) Western blot probed for the cleaved caspase-1 p20 fragment in the supernatant of nonprimed or primed J774.A1 macrophages postinfection (6 h) with *C. jejuni* strain 108 (m.o.i. 200), *E. coli* (m.o.i. 20), or without bacteria (nonstimulated). Lanes were loaded with 50 µg protein. (E) Confocal microscopy showing ASC speck formation (<) in primed J774.A1 macrophages postinfection (2 h) with mCherry-fluorescent *C. jejuni* strain 108 (m.o.i. 40) or *E. coli* (m.o.i. 20) (red). ASC foci were stained with anti-ASC Ab in combination with goat-anti-rabbit-Alexa Fluor 488 (green). Cell surface was stained with WGA-Alexa Fluor 633 (blue). Scale bar, 10 µm.
Luciferase assays on the infected THP-1 cells demonstrated higher bacterial values for the 108 \textit{cheY}::cat than for the parent strain (Fig. 6F), suggesting that also in human cells inflammasome activation varies with cellular infection levels. THP-1 defNLRP3 macrophages showed similar levels of luciferase activity as measured for the infected THP-1 cells excluding that the difference in IL-1β secretion in the deficient cells was caused by low infection rates. Finally, intracellular survival of \textit{C. jejuni} strain 108 (Fig. 6G) and 108 \textit{cheY}::cat (Fig. 6H) was followed by the luciferase reporter assay in differentiated THP-1 and THP-1 defNLRP3 cells. Intracellular levels of both strains severely reduced overtime and were almost absent after 24 h of infection. No difference in bacterial survival was observed between the THP-1 and the THP-1 defNLRP3 cells, indicating that the poor intracellular survival of \textit{C. jejuni} occurs independent of inflammasome activation. Overall, these results show that \textit{C. jejuni} also activates the human NLRP3 inflammasome and that activation varies with the amount of infection.

Discussion
In the current study, we provide evidence that the principal bacterial food-borne pathogen \textit{C. jejuni} induces the secretion of IL-1β via activation of the NLPR3 but not the NLRC4 inflammasome. The effect required the inflammasome components NLRP3, ASC and caspase-1/11 and was observed upon infection of both mouse and human macrophages. Inflammasome activation required viable

FIGURE 3. Cellular infection-induced activation of the inflammasome. (A–C) Induction of IL-1β secretion in primed J774.A1 macrophages (12 h) exposed to different \textit{C. jejuni} mutants and their respective parent strains 108, 81–176, and 81116 (m.o.i. 20). (D) Effect of \textit{C. jejuni} infection of primed J774.A1 macrophages (m.o.i. 20) on IL-1β secretion (C) (after 12 h) and bacterial viability (■) (after 6 h) as measured via the luciferase reporter assay (relative light unit [RLU]). The p values (A–D) for all mutant strains compared with the parent strain were not significantly different, except for the \textit{cheY}:cat and \textit{cetA}:cat (p < 0.05). (E) Intracellular bacterial viability of several \textit{C. jejuni} strains (m.o.i. 20) in primed J774.A1 macrophages (6 h) as measured via the luciferase reporter assay. Strain 81–176 was significantly (p < 0.01) more present intracellular than strain 108. Values are the mean ± SEM of three independent experiments performed in duplicate.

FIGURE 4. Cell viability and intracellular survival after \textit{C. jejuni} induced inflammasome activation. (A–C) Cytotoxicity and bacterial survival in infected primed J774.A1 macrophages. (A) LDH release from primed cells at 12 h of incubation with ATP (5 mM), \textit{Salmonella} (m.o.i. 20), \textit{E. coli} (m.o.i. 20), or several \textit{C. jejuni} (m.o.i. 20) or mutants of strain 108. ATP (p < 0.001), \textit{Salmonella} (p < 0.01), and \textit{E. coli} (p < 0.01) caused significant cytotoxicity. None of the \textit{C. jejuni} strains induced cytotoxicity. (B) PI uptake by primed macrophages incubated (12 h) with the indicated strains. \textit{Salmonella} (p < 0.001) caused a significant increase in F.I. The increase F.I. induced by \textit{C. jejuni} \textit{cheY} mutant was not statistically significant. (C) Intracellular survival of \textit{C. jejuni} strain 108 and its \textit{cheY} derivative (m.o.i. 20) in primed J774.A1 macrophages as measured via the luciferase reporter assay. Luciferase activity (relative light unit [RLU]) after 6 h infection was set as 100% value. Values are the mean ± SEM of three independent experiments performed in triplicate.
bacteria and varied with the severity of the cellular infection. Strikingly, inflammasome activation by *C. jejuni* did not lead to cell death and occurred in primary mouse macrophages without the need of a priming signal.

The secretion of IL-1β and the structurally related IL-18 are important in the innate immunity and systemic response against bacterial infections (41). Many flagellated bacterial pathogens (*Legionella pneumophila*, *Pseudomonas aeruginosa*, *Salmonella Typhimurium*, *Shigella flexneri*, and enteropathogenic *E. coli*) activate the NLRC4 inflammasome (42, 43). Crucial for this activation is the translocation of flagellin or components of the T3SS into the cytosol, which are sensed by members of the NAIP family (8–10, 44–46). Our results with mouse NLRP3 failed to activate the NLRC4 inflammasome. This is consistent with the absence of a T3SS or T4SS in this pathogen. The apparent absence of translocation of flagellin or other NAIP ligands into the cytosol makes *C. jejuni* invisible for the NLRC4 inflammasome.

In this study, a large body of evidence indicates that *C. jejuni* activates the NLRP3 inflammasome. The *C. jejuni*-induced secretion of mature IL-1β in primed J774.A1 macrophages, the formation of an ASC speck, the generation of caspase-1 cleavage fragments, and the absence and severe reduction of IL-1β secretion in human and mouse NLRP3-negative cells respectively, all indicate NLRP3-dependent IL-1β secretion. Interestingly, some low residual IL-1β secretion remained in the NLRP3-deficient mouse macrophages, whereas this was not observed for the caspase-1/11 and ASC-deficient macrophages. This suggests a possible role for an additional inflammasome contributing to the response.

The NLRP3 inflammasome can be formed in response to a diverse array of agents (4, 7, 43) but, to our knowledge, for none of them binding of a specific ligand to NLRP3 has been demonstrated. The mechanism driving the activation of the NLRP3 by *C. jejuni* was investigated using different *C. jejuni* strains and series of genetically defined mutants. All of the tested strains induced IL-1β secretion, suggesting that inflammasome activation is a stable trait of the pathogen. This trait was preserved in mutants with defects in capsule formation, LOS biosynthesis, flagella synthesis and flagellin-like secretion, T6SS needle protein, CDT, and several assumed bacterial adhesion/invasion promoting factors. Inflammasome activation was affected after disruption of the cheY gene and, to a lesser extent, the cetA gene. Inflammasome activation was affected after disruption of the cheY gene and, to a lesser extent, the cetA gene.

**FIGURE 5.** *C. jejuni* activation of the NLRP3 inflammasome in primary mouse macrophages. (A) Secretion of IL-1β by non-primed () and primed (■) BMMs incubated (12 h) with *Salmonella* (m.o.i. 2), *C. jejuni* (m.o.i. 20), or ATP (2.5 mM). ATM (p < 0.05) and *Salmonella* (p < 0.001) significantly increased IL-1β secretion. *C. jejuni* significantly increased IL-1β in nonprimed cells only (p < 0.001). (B) Similar assay but with primed BMMs isolated from the indicated knockout mice and incubated with *Salmonella* (m.o.i. 2) or ATP (2.5 mM) (■). IL-1β secretion was significantly lower in caspase-1−/−/11−/− and NLR4−/− BMMs upon infection with *Salmonella* (p < 0.05). Upon ATM stimulation, IL-1β secretion was significantly reduced in caspase-1−/−/11−/−, ASC−/−, or NLRP3−/− BMMs (p < 0.05). (C) IL-1β secretion by nonprimed BMMs from several knockout mice infected (12 h) with *C. jejuni* strain 108 (○) or 108cheY::cat (■) (m.o.i. 20). Significant lower secretion was observed for caspase-1−/−/11−/− (p < 0.001), ASC−/− (p < 0.001), and NLRP3−/− (p < 0.01) BMMs upon stimulation with both *C. jejuni* strains. Strain cheY::cat induced more IL-1β secretion (p < 0.05) than the parent strain. (D) Secretion of IL-1β in nonprimed BMMs infected (12 h) with *C. jejuni* strain 108, 108cheY::cat, or 108cheY::cat+cheY (m.o.i. 20). Complementation of the defective cheY restored the high IL-1β secretion induced by strain 108cheY::cat (p < 0.01) to parental levels (p > 0.0.05). (E) Confocal microscopy on BMMs (wt and NLRP3−/−) infected (1 h) with mCherry fluorescent (red) *C. jejuni* 108 and 108cheY::cat (m.o.i 200); cells were counterstained with WGA-Alexa Fluor 633 (green). (F) Quantification of the number of intracellular bacteria in the wild-type (wt) (○) and NLRP3−/− (■) BMMs showed no significant difference in the number of intracellular bacteria between the wt and NLRP3−/− BMMs. Strain 108cheY::cat was more invasive (p < 0.001) than the parent strain in the BMMs. Values are the mean ± SEM of three independent experiments performed in duplicate.
likely cause a transmembrane ion flux (e.g., K⁺ efflux), which seems the common denominator of NLRP3 activation (12). At this time, it is tempting to speculate that C. jejuni–induced cell damage caused by the cellular infection contributes to the NLRP3 activation, although this was not evident from increased release of LDH from the cells (Fig. 4).

Our results indicate that C. jejuni activates the NLRP3 inflammasome in murine J774.A1 macrophage cells, primary mouse BMMs, and human-differentiated THP-1 cells. A striking difference among these cell types, however, was the apparent lack of need to prime the primary cells to establish a strong IL-1β production upon C. jejuni infection. ATP only activated the inflammasome in primed BMMs, indicating that the cells were not already primed. Viable C. jejuni were most effective in inflammasome activation in nonprimed primary cells. In primed cells, IL-1β production was minimal probably because of the more efficient bacterial uptake and killing in activated macrophages.

Another unexpected finding was that C. jejuni activates the inflammasome without apparent cytotoxicity as evidenced by the absence of LDH release or an increased PI uptake by the infected cells. LDH release was noted postinfection with E. coli or Salmonella, indicating that the pyroptosis pathway is intact in these cells. Inflammasome activation by other bacterial species always seems to be followed by pyroptosis (2, 4, 6, 7, 42, 47). This has been linked with the presence of LPS in the cytosol (48, 49). It can be imagined that different bacterial metabolic demands, a low level of bacterial LPS in the cytosol, and/or the relative poor intracellular survival of C. jejuni prevent C. jejuni–induced cytotoxicity. Activation of the inflammasome was not required to kill the intracellular C. jejuni. Alternatively, the pathogen may have evolved a strategy to prevent bacteria-induced pyroptosis.

Our results that C. jejuni induces inflammasome activation in both murine and human cells without apparent cytotoxicity and in primary cells without a need of priming extends the known repertoire of inflammasome activation by bacterial pathogens. The data provide a molecular basis for the observed IL-1β secretion during C. jejuni infection and for key features in C. jejuni pathogenesis (22, 23, 31, 33, 50–52).

(○) and THP-1 defNLRP3 (●) cells infected with Salmonella (m.o.i. 2), E. coli (m.o.i. 20), or different C. jejuni strains (m.o.i. 20) (12 h). Salmonella (p < 0.01), E. coli (p < 0.01), and all C. jejuni strains (p < 0.05) induced IL-1β secretion upon infection. Strain cheY::cat induced more IL-1β secretion (p < 0.01) than the parent strain. C. jejuni strain 108 (m.o.i. 20)–infected E. coli (m.o.i. 20) (red). Cell surface was stained with WGA-Alexa Fluor 633 (blue). ASC foci (>10 μM) were stained with an anti-ASC Ab in combination with goat-anti-rabbit-Alexa Fluor 488 (green). Active caspase-1 (D) was detected with FLICA (green) at 1 h of infection. Scale bars, 10 μm. Western blot showing the presence of active (cleaved) IL-1β (17 kDa) in the supernatant of noninfected and C. jejuni strain 108 (m.o.i. 20)–infected (12 h) PMA-differentiated THP-1 cells. Lanes were loaded with 50 μg protein. Intracellular viability of C. jejuni mutants and parent strain in 6 h infected PMA-differentiated THP-1 (○) and THP-1 defNLRP3 (●) as measured with the bacterial luciferase reporter assay. No significant differences in RLU were measured between the THP-1 and THP-1 defNLRP3. Strain 108cheY::cat was more invasive (p < 0.001) than the parent strain. Intracellular survival (24 h) of C. jejuni strains 108G (G) or 108cheY::cat (H) in THP-1 (○) or THP-1 defNLRP3 (●) cells. Luciferase activity at 6 h of infection was set as 100%. The decrease in intracellular C. jejuni (108 and 108cheY::cat) at 12 h of infection was statistically significant (p < 0.05). Values are the mean ± SEM of three independent experiments performed in duplicate.
Acknowledgments
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Disclosures
The authors have no financial conflicts of interest.

References
**Table S1. Bacterial strain and plasmids used in this study**

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<td></td>
<td></td>
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<tr>
<td>81-176</td>
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<td>(2)</td>
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<tr>
<td>81116</td>
<td>Human enteritis</td>
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<td>(4)</td>
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<td>81116<em>waaF</em>::kana</td>
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**Other**

*E. coli* DH5α | Non-invasive *E. coli* | NCCB |

*E. coli* S17.1 | Used for conjugation | NCCB |

*Salmonella* Typhimurium SL1344 (SGSC # 438) | hisG46 strain | SSC |

*Salmonella* Typhimurium SL1344+pMW85 | GFP positive strain | R. van Aubel |

**Plasmids**

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<td>pMA1-mCherry</td>
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<td>pMW85</td>
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References


