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Listeria monocytogenes invades the cytoplasm of macrophages and induces the activation of caspase-1 and the subsequent maturation of IL-1β and IL-18. Although apoptosis-associated speck-like protein containing a caspase-activating and recruitment domain (ASC), an adaptor protein of nucleotide-binding oligomerization domain (Nod)-like receptors, has been shown to play an essential role in inducing this cellular response to L. monocytogenes, the mechanism has not been fully elucidated. In this study, we demonstrate the role of absent in melanoma 2 (AIM2), a recently described receptor of cytosolic DNA, in the activation of caspase-1 upon infection with L. monocytogenes. Secretion of IL-1β and IL-18 from Nod-like receptor family, pyrin domain containing 3 (NLRP3) and Nod-like receptor family, caspase-activating and recruitment domain containing 4 (NLRC4) knockout macrophages in response to L. monocytogenes was only slightly decreased compared with the levels secreted from wild-type macrophages, whereas secretion from ASC knockout macrophages was completely impaired, suggesting that receptors other than NLRP3 and NLRC4 also take part in inflammasome activation in an ASC-dependent manner. To identify such receptors, the abilities of several receptor candidates (NLRP2, NLRP6, NLRP12, and AIM2) to induce the secretion of IL-1β in response to L. monocytogenes were compared using the inflammasome system reconstructed in HEK293 cells. Among these receptor candidates, AIM2 conferred the highest responsiveness to L. monocytogenes-infected macrophages. These results suggest that AIM2, in cooperation with NLRP3 and NLRC4, plays an important role in the activation of caspase-1 during L. monocytogenes infection.

Listeria monocytogenes, a Gram-positive rod-shaped bacterium, often causes food-borne infections in immunocompromised hosts, including newborns and the elderly. As a facultative intracellular parasitic bacterium, L. monocytogenes invades a variety of host cells, such as hepatocytes, fibroblasts, and epithelial cells, and multiplies in the cytoplasm of these cells (1–4). Professional phagocytes, such as macrophages, are also permissive for intracellular growth of L. monocytogenes. After being engulfed by macrophages and trapped in the phagosome, L. monocytogenes survives by disrupting the phagosomal membrane by secreting a cytolsin, listeriolysin O (LLO), and other virulence factors, and then escapes into the cytoplasm. Indeed, L. monocytogenes strains lacking functional LLO are avirulent and are not able to grow in macrophages or escape from the phagosome (5–8).

In response to L. monocytogenes, innate immune cells, such as macrophages and dendritic cells, produce a wide variety of proinflammatory cytokines that are involved in host defense against this pathogen. Although the expression of many of these cytokines is induced due to recognition of bacterial cells or bacterial products by TLRs (9–13), some are induced independently of TLRs and instead are induced in response to sensing by intracellular surveillance mechanisms that are not yet fully understood. Upon infection of macrophages with L. monocytogenes, LLO-dependent bacterial entry into the cytoplasm but not TLR adaptors, MyD88 and Toll/IL-1R domain-containing adapter inducing IFN-β, is necessary for the induction of the expression of type I IFNs (14–17). Cytoplasmic entry of bacteria is also required for the induction of caspase-1 activation and subsequent maturation of IL-1β and IL-18 upon infection with L. monocytogenes (18–20). Besides the delivery of bacterial cells into the cytoplasm of macrophages, LLO has been shown to play a role in enhancing the activation of caspase-1, possibly by modulating some cellular signaling either directly or indirectly (21, 22).

Recent studies have revealed that nucleotide-binding oligomerization domain (Nod)-like receptors (NLRs), which are cytoplasmic proteins typically composed of C-terminal leucine-rich repeats, a central nucleotide-binding domain, and a variable N-terminal effector domain, such as a caspase-activating and recruitment domain (CARD), play central roles in caspase-1 activation in response to various exogenous and endogenous stimuli. These stimuli may include ATP, pore-forming toxins, uric acid crystals, alum, bacterial flagellins, and infection with microbial pathogens (23–29). NLR family, pyrin domain containing 3 (NLRP3) and NLR family, CARD domain containing 4 (NLRC4) are the most thoroughly investigated NLRs in this field, and a number of microbial pathogens have been reported to induce caspase-1 activation via one of these two recep-
In this study, we demonstrate that infection of macrophages with *L. monocytogenes* at a multiplicity of infection (MOI) of 1, without any prestimulation, is sufficient for the induction of a significant level of caspase-1 activation and this response is only partially dependent on NLRP3 and NLRC4. Furthermore, we demonstrate that apoptosis-associated speck-like protein containing a CARD (ASC), a key inflammasome component that bridges some NLRs and pro-caspase-1 (23), is essential in the induction of caspase-1 activation. In addition, our data suggest that absent in melanoma 2 (AIM2), a recently described intracellular DNA receptor (33–36), plays an important role in the activation of caspase-1 upon infection with *L. monocytogenes*.

**Materials and Methods**

**Reagents**

Ultra pure LPS was purchased from InvivoGen (Toulouse, France). Nigericin was purchased from Sigma-Aldrich (St. Louis, MO). Penicillin G and chloramphenicol were purchased from Nacalai Tesque (Kyoto, Japan). Ultra pure LPS was purchased from InvivoGen (Toulouse, France). Nigericin was purchased from Sigma-Aldrich (St. Louis, MO). Penicillin G and chloramphenicol were purchased from Nacalai Tesque (Kyoto, Japan).

**Mice**

Female C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan). NLRP3 knockout (KO) mice generated by Prof. Jürg Tschopp (University of Lausanne, Lausanne, Switzerland) (26), NLRC4 KO mice generated by Dr. Vishva Dixit (Genentech, South San Francisco, CA) (37), ASC KO mice generated by Prof. Shun-ichiro Taniguchi (Shinshu University, Nagano, Japan) (38), caspase-1 KO mice generated by Dr. Keisuke Kuida (Millenium Pharmaceuticals, Cambridge, MA), and IFN-α/β receptor 1 (IFNAR1) KO mice generated by Prof. Michel Aguett (Ecole Polytechnique Federale de Lausanne, Lausanne, Switzerland) were kindly provided as gifts. Mice were maintained in specific pathogen-free conditions and used at 7–9 wk of age. All of the experiments were performed in accordance with the Animal Ethics and Research Committee of Kyoto University Graduate School of Medicine, Kyoto, Japan.

**Bacterial strains**

The parental wild-type (WT) *L. monocytogenes* strain used in this study was *L. monocytogenes* EGD (serovar 1/2a). Three isogenic mutants, Δαhl, Δsyh, and Δαhl:Δsyh, were constructed from WT *L. monocytogenes* strain EGD using the homologous recombination method described in a previous study (21). An isogenic deletion mutant of the flaA gene in *L. monocytogenes* strain EGD was also constructed using the homologous recombination method. A 1875-bp fragment located upstream of the flaA gene was amplified from the chromosomal DNA of WT *L. monocytogenes* EGD by PCR with the P1 and P2 primer set (Table I). A 2012-bp fragment located downstream of the flaA gene was similarly amplified with the P3 and P4 primer set (Table I). The two amplified fragments were ligated into the plasmid vector pHS-MCS, which carries a thermostable replication origin (21). The resulting plasmid was introduced into competent cells of WT *L. monocytogenes* EGD by electroporation. Transformants were selected initially at 30°C on brain heart infusion (BHI) agar plates (Eiken Chemical, Tokyo, Japan) supplemented with erythromycin (5 μg/ml; Nacalai Tesque). Transformants were then grown on BHI agar plates with erythromycin (5 μg/ml) at 30°C to select only those transformants with chromosomal integration of the plasmid. Cointegrates were subsequently grown in BHI broth (Eiken Chemical) without antibiotic selection at 30°C to facilitate the isolation of derivatives in which integrated plasmids were resolved by a second recombination event. The in-frame deletion of the flaA gene was confirmed by PCR using the primer set P5 and P6 (Table I) and sequencing of the PCR product. The constructed mutant (ΔflaA) was not motile on low-agar BHI (0.375%) at 22°C (data not shown).

**Bacterial growth conditions**

For the preparation of bacterial stocks, all of the bacterial strains were grown overnight in BHI broth at 37°C with shaking. One volume of the overnight culture was added to 100 volumes of fresh BHI broth and cultured for a further 5 h. Bacterial cells were washed, suspended in PBS supplemented with 10% glycerol, and stored in aliquots at −80°C. Bacterial stocks were thawed and diluted in RPMI 1640 just before infection of macrophages.

**Cells**

Peritoneal exudate cells (PECs) of mice were obtained 3 d after i.p. injection of 2 ml 3% thiglycolate medium (Eiken Chemical). After being washed with RPMI 1640 media (Nacalai Tesque), PECs were suspended in RPMI 1640 supplemented with 10% FCS (+FCS) and incubated in 48-well microplates at a density of 2 × 10⁶ cells per well at 37°C in a humidified 5% CO₂ environment for 3 h. After incubation, the cells were washed with RPMI 1640 + FCS and adherent PECs were used for infection studies. Bone marrow cells were obtained from the tibiae of mice and cultured in RPMI 1640 + FCS containing gentamicin (10 μg/ml; Wako Pure Chemical Industries, Osaka, Japan) and recombinant mouse M-CSF (100 ng/ml; R&D Systems, Minneapolis, MN) for 5 d. Then, adherent bone marrow-derived macrophages (BMMs) were collected, suspended in fresh RPMI 1640 + FCS, and plated at 2 × 10⁶ cells per well in 48-well microplates. BMMs were used the following day for infection studies.

**Infection and stimulation of cells**

To infect macrophages, bacteria were added to the culture of adherent PECs or BMMs at the indicated MOI. After 30 min, gentamicin (final concentration of 5 μg/ml) was added to the culture to inhibit the growth of bacteria. However, in Fig. 1F, gentamicin was added to the macrophage culture 90 min after the addition of bacteria, and the cells were incubated for an additional 60 min, making a total infection time of 2.5 h. To stimulate cells with cytosolic DNA, Poly(dA):Poly(dT) (Sigma-Aldrich) or *Listeria* genomic DNA was introduced into adherent PECs or HEK293 cells using Lipofectamine LTX (Invitrogen, Carlsbad, CA).

**ELISA**

Levels of cytokines in culture supernatants were determined by two-site sandwich ELISA. Culture supernatants were stored at −80°C prior to cytokine measurement. The ELISA kits for IL-1α and IL-1β were purchased from e Bioscience (San Diego, CA) and BD Biosciences (San Diego, CA), respectively. For the titration of IL-18, a pair of biotin-labeled and unlabelled mAbs specific to IL-18 (Medical & Biological Laboratories, Nagoya, Japan) were used.

**Western blot analysis**

PECs were cultured on six-well microplates at a density of 1 × 10⁶ cells per well in RPMI 1640 + FCS at 37°C for 3 h. The culture medium was replaced with Opti-MEM (Invitrogen), and adherent cells were infected with *L. monocytogenes* at a MOI of 1. Culture supernatants were collected 24.5 h postinfection, and the cells were lysed with RIPA buffer (Nacalai Tesque). The culture supernatants were concentrated 20-fold using 20% (w/v) trichloroacetic acid (Nacalai Tesque). The precipitates and cell lysates were subjected to SDS-PAGE and subsequently transferred to polyvinylidene difluoride membranes by electroblotting. The membranes were immunoblotted with anti–caspase-1 Ab (Santa Cruz Biotechnology, Santa Cruz, CA), anti–IL-1β Ab (R&D Systems), or anti–β-actin Ab (Sigma-Aldrich).

**Detection of lactate dehydrogenase release**

Lactate dehydrogenase (LDH) activity was measured using an LDH cytotoxicity detection kit (TaKaRa BIO, Shiga, Japan). The percentage of LDH released (maximal LDH release = 100 × (experimental LDH release – spontaneous LDH release)/ (maximal LDH release – spontaneous LDH release)) was determined. To determine the maximal LDH release, cells were treated with 1% Triton X-100.

**RT-PCR analysis**

Total cellular RNA of adherent PECs was extracted using the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany), according to the manufacturer’s instructions. The collected RNA (0.2 μg) was treated with...
RNase-free DNase (Promega, Madison, WI) to eliminate contaminating DNA before being subjected to reverse transcription using Superscript VILO (Invitrogen). Quantitative real-time RT-PCR was then performed on an ABI PRISM 7000 (Applied Biosystems, Foster City, CA) using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) with primers described in Supplemental Table I. Results were analyzed with ABI PRISM 7000 SDS software. Gene-specific transcript levels were normalized to the amount of β-actin mRNA. Extraction of total RNA from the spleen or ovary was carried out using a Sepasol-RNA I Super kit (Nacalai Tesque). The collected RNA was treated with RNase-free DNase and the cells were incubated for an additional 8 h. We then confirmed that L. monocytogenes was able to invade HEK293 cells and proliferate in the cytoplasm (data not shown).

**Reconstruction of the inflammasome system in HEK293 cells**

The full-length open reading frames of ASC, procaspase-1, pro-IL-1β, pro-IL-18, NLRP2, NLRP12, and AIM2 were amplified from cDNA preparations, which were obtained from the spleen or ovary of B6 mice by PCR using KOD Plus enzyme (TOYOBO, Osaka, Japan) and the primer sets indicated in Supplemental Table I. The amplified fragments were digested with restriction enzymes (Supplemental Table II) and inserted into the pFLAG-CMV2 vector (Sigma-Aldrich). The resulting plasmids were designated pFLAG-ASC, pFLAG-precaspase-1, pFLAG-pro-IL-1β, pFLAG-pro-IL-18, pFLAG-NLRP2, pFLAG-NLRP12, and pFLAG-AIM2, respectively. HEK293 cells obtained from the American Type Culture Collection (Rockville, MD) were maintained in DMEM supplemented with 10% FCS, 6 mM l-glutamine, 1 mM sodium pyruvate, and 5 μg/ml gentamicin. For experiments, HEK293 cells were plated in 24-well microplates at a density of 2 × 10^4 cells per well and incubated overnight. The cells were transfected with a total of 1 μg DNA per well using Transfectin Lipid Reagent (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer’s instructions. After 38.5 h, the culture medium was replaced with gentamicin-free medium, and the transfected cells were left uninfected or infected with 10^7 CFU of WT L. monocytogenes. Gentamicin (20 μg/ml) was added to the culture 1.5 h postinfection, and the cells were incubated for an additional 8 h. We then confirmed that L. monocytogenes was able to invade HEK293 cells and proliferate in the cytoplasm (data not shown).

**RNA interference**

PECs were transfected with Stealth small interfering RNA (siRNA) duplexes (Invitrogen) at a final concentration of 20 nM using siPORT Amine (Ambion, Austin, TX). Briefly, 0.6 μl siPORT was diluted in 20 μl Opti-MEM, incubated at room temperature for 10 min, and then combined with siRNA diluted in 20 μl Opti-MEM. The mixture was incubated at room temperature for 10 min, then transferred to a well in a 48-well microplate. PECs were suspended in Macrophage-SFM (Invitrogen) at a density of 1 × 10^5 cells per milliliter, and 0.2 ml of the suspension was plated in a well containing the siPORT–siRNA complex. The cells were incubated at 37˚C for 48 h. Then, the siPORT–siRNA complex and the transfected cells were used in experiments. The sense siRNA sequences were: AIM2-1, 5′-UAA UCU UCU GGA CUU UAA ACA GCC C-3′; AIM2-2, 5′-UAA AGU CAU UGU GAC CAC GGC GGG UGG C-3′. Negative control siRNA was purchased from Invitrogen.
Isolation of *L. monocytogenes* genomic DNA

An overnight culture of *L. monocytogenes* in BHI broth was centrifuged, and the pellet was suspended in an equal volume of 10 mM Tris buffer (pH 8) containing 50 mM EDTA, 20 mg/ml lysozyme, and 100 μg/ml RNase A and incubated at 37°C for 1 h. After being mixed with a 1:6 volume of 5 M NaCl and a 1:7.5 volume of 10% cetyltrimethylammonium bromide/0.7 M NaCl solution, the bacterial lysates were incubated at 65°C for 10 min. DNA was extracted from the mixture using chloroform/isoamyl alcohol and phenol/chloroform/isoamyl alcohol.

Statistical analysis

For comparisons between two groups, the Student's t test was used when the variances of the groups were judged to be equal by the F test. Multi-group comparisons of mean values were made according to ANOVA and the Fisher’s protected least significant difference post hoc test after the confirmation of homogeneity of variances among the groups using Bartlett’s test. Statistical significance was determined as p < 0.05.

Results

Involvement of NLRP3, NLRC4, and ASC in caspase-1 activation upon infection with *L. monocytogenes*

To clarify whether NLRP3 and NLRC4 are involved in the activation of caspase-1 upon infection with *L. monocytogenes*, adherent PECs from WT mice, NLRP3 KO mice, and NLRC4 KO mice were infected with the bacterium at a MOI of 1. The culture supernatants were collected 24.5 h postinfection, and the levels of IL-1β and IL-18 were determined. Although the secretion of these cytokines from NLRP3 KO macrophages and NLRC4 KO macrophages in response to *L. monocytogenes* infection seemed to be lower than that from WT macrophages, IL-1β and IL-18 were detected at a significant level even in the absence of NLRP3 or NLRC4 (Fig. 1A, 1B). By contrast, ASC KO macrophages did not secrete IL-1β or IL-18 postinfection with *L. monocytogenes* (Fig. 1A, 1B). Similar results were obtained when BMMs from each mouse strain were infected at a MOI of 1 or 2 (Fig. 1C and data not shown). To determine the level of activation of caspase-1 directly, the mature form of caspase-1 (p10 fragment) in the culture supernatants was detected by Western blotting using an anti-caspase-1 Ab. Caspase-1 p10, as well as mature IL-1β, were detected in the culture supernatants of macrophages from WT mice, NLRP3 KO mice, and NLRC4 KO mice infected with *L. monocytogenes*, although they were not detected in the culture supernatants of infected ASC KO macrophages (Fig. 1D). Moreover, LDH release induced upon infection with *L. monocytogenes* was significantly lower in caspase-1 KO macrophages and ASC KO macrophages but not NLRP3 KO macrophages than that in WT macrophages (Fig. 1E). These results indicated that ASC is indispensable for the activation of caspase-1 upon infection with *L. monocytogenes*, whereas NLRP3 and NLRC4 are only partly involved. It should be noted that, as shown in a previous report (24), NLRP3 was essential for the secretion of IL-1β and IL-18 when macrophages were pretreated with LPS and subsequently infected with *L. monocytogenes* at a MOI of 50 for 2.5 h (Fig. 1F and data not shown). This suggests that differences in the infection protocol might be the cause of the discrepancies in the significance of NLRP3 in the activation of caspase-1. To further examine the involvement of the recognition of flagellin by NLRC4 in inflammosome activation in *L. monocytogenes*-infected macrophages, macrophages from WT mice were

### Table I. Primers used for the construction of Δ*flaA*

<table>
<thead>
<tr>
<th>Primers</th>
<th>Primer Sequences</th>
<th>Restriction Enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>5'-TAG ATA TAA TGK TAC CGG ATT TGT ATT ATC CAC CTG TCG-3'</td>
<td>KpnI</td>
</tr>
<tr>
<td>P2</td>
<td>5'-TAG ATA TAA TCC CGG GTP GTG TGG TTT CCC TCC TAC ATT-3'</td>
<td>XmnI</td>
</tr>
<tr>
<td>P3</td>
<td>5'-TAG ATA TAA TCC CGG G7A ACA TAT CAA ATT CAT TTA-3'</td>
<td>XmnI</td>
</tr>
<tr>
<td>P4</td>
<td>5'-TAG ATA TAA TCC CGG G7A ACA TAT CAA ATT CAT TTA-3'</td>
<td>XmnI</td>
</tr>
<tr>
<td>P5</td>
<td>5'-GAC GAG AGG G7A AGG GTA AAA CCG-3'</td>
<td>XmnI</td>
</tr>
<tr>
<td>P6</td>
<td>5'-GAC GAG AGG G7A AGG GTA AAC AAT G-3'</td>
<td>XmnI</td>
</tr>
</tbody>
</table>

Restriction enzyme sites are underlined.

**FIGURE 2.** Screening of the receptors involved in caspase-1 activation during *L. monocytogenes* infection. A, Total RNA was isolated from the spleen or ovary, and the expression of each receptor was detected by RT-PCR using the primer sets indicated in Supplemental Table I. The amplification was performed for 38 cycles. B–D, HEK293 cells were cotransfected with empty vector (333.5 ng), pFLAG-pro-IL-1β (300 ng), pFLAG-pro-IL-18 (300 ng), pFLAG-pro-IL-18 (300 ng), pFLAG-pro-ASC (50 ng), or pFLAG-ASC (15 ng). The cells were also transfected with empty vector, pFLAG-AIM2, pFLAG-NLRP2, pFLAG-NLRP6, or pFLAG-NLRP12 (1.5 ng each). After 38.5 h, the transfected cells were left uninfected or infected with 10^7 CFU WT *L. monocytogenes* (B, C) or stimulated with 400 ng Poly(dA)·Poly(dT) (D). Gentamicin was added to the culture 1.5 h after the addition of bacteria, and the cells were incubated for an additional 8 h. IL-1β (B, D) and IL-18 (C) in the culture supernatants were assessed by ELISA. ELISA data are presented as the mean of triplicate assays and the SD. All of the experiments were repeated at least twice and similar results were obtained.
infected with WT *L. monocytogenes* or its isogenic mutant strains deficient in the flagellin gene (*flaA*) (Table I) or the LLO gene (*hly*). WT *L. monocytogenes* and ΔflaA similarly induced the secretion of IL-1β and IL-18, whereas Δhly did not (Fig. 1G and data not shown), suggesting that sensing of flagellin by NLRCA4 is not important for the activation of caspase-1 during infection with *L. monocytogenes* strain EGD.

**Screening of the receptors involved in caspase-1 activation in *L. monocytogenes* infection**

The data presented in Fig. 1A–D strongly suggested that, in addition to NLRP3 and NLRC4, some ASC-dependent receptors are involved in inducing caspase-1 activation during *L. monocytogenes* infection. To identify these receptors, we selected four receptor molecules (NLRP2, NLRP6, NLRP12, and AIM2) as candidates using the following criteria: 1) ability to interact with ASC via its pyrin domain (39–43); 2) ability to induce caspase-1 activation (40–43); and 3) expression in the spleen, a major target organ for *L. monocytogenes* in which innate and adaptive immune responses are actively induced upon infection in vivo (Fig. 2A). Although NLRP2 was not expressed in the spleen, we did not omit this NLR because previous reports have shown that NLRP2 is upregulated in the human mononcytic cell line THP-1 after stimulation with LPS (44) and mediates an innate immune response to *Fusobacterium nucleatum* in gingival epithelial cells (45). To test whether candidate receptors take part in the activation of caspase-1 in *L. monocytogenes*-infected cells, we reconstructed the inflammasome system in HEK293 cells. HEK293 cells were cotransfected with ASC, procaspase-1, pro-IL-1β, and pro-IL-18. The cells were also transfected with AIM2, NLRP2, NLRP6, NLRP12, or an empty vector. Transfected cells were then infected with *L. monocytogenes*, and levels of IL-1β and IL-18 in the culture supernatants were determined. Among these transfected cells, AIM2-transfected cells secreted the highest levels of IL-1β and IL-18 post-infection with *L. monocytogenes* (Fig. 2B, 2C). Although even HEK293 cells transfected with other receptors or the empty vector exhibited an increased secretion of IL-1β and IL-18 when infected with *L. monocytogenes* (versus uninfected control), the increase in the concentration was only ∼500 pg/ml. However, the increase in the concentration of IL-1β and IL-18 in the culture supernatants of AIM2-transfected cells infected with *L. monocytogenes* was >1500 pg/ml. These results suggested that AIM2 may play a role in sensing *L. monocytogenes*. We also observed that only AIM2-transfected cells were responsive to Poly(dA):Poly(dT), a canonical ligand for AIM2 (Fig. 2D) (33–36). It is important to note that we could not confirm whether NLRP2, NLRP6, and NLRP12 were functioning in the inflammasome system reconstructed in HEK293 cells, because ligands for these receptors (i.e., a positive control) are not known. However, we continued to investigate the function of AIM2 in macrophages.

**AIM2 plays an important role in inducing activation of caspase-1 in macrophages infected with *L. monocytogenes***

To examine whether AIM2 is involved in the induction of caspase-1 activation during infection of macrophages with *L. monocytogenes*, we performed a knockdown of AIM2 in macrophages using two siRNAs, AIM2-1 and AIM2-2, targeting independent sequences in the AIM2 transcript. The efficiency of the knockdown was verified
by real-time RT-PCR, and the expression of AIM2 was successfully inhibited by both siRNAs (Fig. 3A). These siRNAs did not inhibit the expression of ASC, procaspase-1, and pro-IL-18, as determined by real-time RT-PCR (data not shown). Moreover, knockdown of AIM2 significantly decreased the secretion of IL-18 from macrophages stimulated with Poly(dA)-Poly(dT) (Fig. 3B). Secretion of caspase-1 p10, IL-1β, and IL-18 from macrophages infected with L. monocytogenes into the culture supernatants was significantly decreased by knockdown of AIM2 (Fig. 3C–E), whereas secretion of IL-1α and TNF-α induced by L. monocytogenes genomic DNA was decreased by knockdown of AIM2 (Fig. 3F and data not shown) and secretion of IL-1β induced by LPS plus nigericin (Fig. 3G) were not affected. Moreover, knockdown of AIM2 did not affect intracellular growth of L. monocytogenes (Fig. 5A). Taken together, these findings suggested that AIM2 plays an important role in inducing activation of caspase-1 upon infection with L. monocytogenes.

L. monocytogenes genomic DNA can be a ligand for AIM2

AIM2 has recently been shown to recognize cytosolic DNA. Therefore, we proposed that DNA released from bacteria or organelles, such as the nucleus, damaged during the course of infection with L. monocytogenes could be responsible for the activation of caspase-1. We tested whether Listeria genomic DNA is able to induce the activation of caspase-1. Listeria genomic DNA was purified from bacterial culture, and part of the preparation was treated with DNase I (Fig. 4A). Untreated Listeria DNA clearly induced the secretion of IL-18 from macrophages, whereas the DNase I-treated sample did not, suggesting that Listeria genomic DNA is capable of inducing caspase-1 activation. Moreover, secretion of IL-18 induced by Listeria genomic DNA was decreased by knockdown of AIM2. These results indicated that Listeria genomic DNA is recognized by AIM2, thereby inducing the secretion of the caspase-1-dependent cytokine.

Dead bacteria in the cytoplasm induce the secretion of caspase-1-dependent cytokines in an AIM2-dependent manner

Even if it is assumed that DNA released from L. monocytogenes into the cytoplasm is responsible for the induction of caspase-1 activation upon infection of macrophages, the question regarding how DNA is released from the bacterium remains to be answered. After analyzing the kinetics of cytokine secretion and bacterial multiplication in host macrophages, we noticed a remarkable increase in secretion of IL-1β and IL-18 at >9.5 h postinfection at which stage the number of intracellular L. monocytogenes was reduced rather than increased (Fig. 5A, 5B, and data not shown). This result was consistent with our previous data that the activation of caspase-1 is detectable at >10 h postinfection with the LLO-expressing L. monocytogenes strain (22) and raised the possibility that DNA or other caspase-1-activating agents might be liberated from dead bacteria into the cytoplasm. To investigate whether L. monocytogenes killed in the cytoplasm were able to induce caspase-1 activation, a bactericidal antibiotic, penicillin G, was added to the culture 3.5 h postinfection. At that time, most of bacteria should have escaped into the cytoplasm (21). Penicillin G rapidly killed intracellular bacteria, as described in a previous report (46), and the number of intracellular L. monocytogenes at its peak was markedly fewer compared with the number of organisms in the absence of penicillin G (Fig. 5A). However, although the addition of penicillin G resulted in a decrease in the secretion of IL-1β and IL-18 in response to L. monocytogenes, the antibiotic did not severely impair secretion (Fig. 5C and data not shown). In contrast, chloramphenicol, a bacteriostatic antibiotic, almost completely impaired the secretion of IL-1β and IL-18 (Fig. 5C and data not shown). In our experiments, penicillin G never induced the secretion of IL-1β and IL-18 from uninfected macrophages (Fig. 5C) and never enhanced DNA-induced IL-18 secretion (data not shown). Accordingly, it is suggested that even a small number of L. monocytogenes can induce the activation of caspase-1 if artificially lysed in the cytoplasm. Moreover, secretion of IL-1β and IL-18 from infected macrophages occurred immediately after the addition of penicillin G (Fig. 5B and data not shown), and secretion was significantly decreased by knockdown of AIM2 (Fig. 5D and data not shown). Taken together, these findings indicate that L. monocytogenes is unable to proliferate and survive in macrophages for >12.5 h for unknown reasons and, at the stationary phase-like period, dead bacteria in the cytoplasm might liberate their contents, including DNA, which then induces the activation of caspase-1 via recognition by AIM2.

AIM2 expression is upregulated upon infection with L. monocytogenes

Because AIM2 is an IFN-inducible gene (47) and L. monocytogenes induces the expression of type I IFNs in host macrophages (15, 16), it could be expected that the expression of AIM2 might be upregulated upon infection with this pathogen. Indeed, the expression of AIM2 was significantly higher in infected macrophages compared with that in the uninfected control (Fig. 6A).
conceivable that the inferior ability of IFNAR1 KO macrophages to treated macrophages at a MOI of 1) (data not shown). Therefore, it is KO macrophages than that in WT macrophages (infection of un-
previous study, the LLO-expressing to play a role in enhancing the activation of caspase-1 (22). In our
between this study and our previous study in which LLO was shown
upregulated by the
utable to the lower expression of AIM2, which is upregulated by the

Furthermore, we found that the upregulation of AIM2 requires the type I IFN signal, because the expression of AIM2 in IFNAR1 KO macrophages was not altered by infection with L. monocytogenes (Fig. 6A). It has been reported that the type I IFN signal is required for the activation of caspase-1 during infection with L. monocytogenes (48). We also obtained a similar result that IL-18 secretion induced by L. monocytogenes was significantly lower in IFNAR1 KO macrophages than that in WT macrophages (infection of un-
treated macrophages at a MOI of 1) (data not shown). Therefore, it is conceivable that the inferior ability of IFNAR1 KO macrophages to activate caspase-1 in response to L. monocytogenes might be attrib-
utable to the lower expression of AIM2, which is upregulated by the type IIIFN signal. Finally, it is important to point out the relationship between this study and our previous study in which LLO was shown to play a role in enhancing the activation of caspase-1 (22). In our previous study, the LLO-expressing L. monocytogenes strain but not the L. monocytogenes strain expressing another cytolsin, ivanolsin O (ILO), instead of LLO, induced the activation of caspase-1 efficiently, even though these bacterial strains invaded and multi-
plied in the macrophage cytoplasm in a similar way. This suggested that the activation of caspase-1 is dependent on not only the entry of the bacterium into the cytoplasm but also on the distinct activity of LLO as a signaling ligand. To test whether LLO is involved in the upregulation of AIM2, in this study we analyzed the expression of AIM2 in macrophages infected with each L. monocytogenes strain. The LLO-expressing L. monocytogenes strain, but not the cytolsin-deficient strain or the ILO-expressing strain, significantly enhanced the expression of AIM2 (Fig. 6B), suggesting that some LLO-dependent signal might be necessary for maximum activation of caspase-1, at least in part, through the upregulation of AIM2.

Discussion

In this study, we showed that AIM2 plays an important role in the activation of caspase-1 and the subsequent secretion of caspase-
1–dependent cytokines, IL-1β and IL-18, upon infection of macrophages with L. monocytogenes. However, our findings are conflicting with data presented in previous reports (24, 30). Our observations suggested that NLRP3 and NLRC4 are also involved in the secretion of IL-1β and IL-18 in response to L. monocytogenes, although these NLRs play a more minor role. Hence, we conclude that AIM2, in cooperation with NLRP3 and NLRC4, plays an important role in the activation of caspase-1 during L. monocytogenes infection.

In the first report to investigate the role of NLRs in L. monocytogenes infection, the NLRP3–ASC pathway was shown to be essential for the activation of caspase-1 in macrophages infected with L. monocytogenes (24). In that study, macrophages were pre-
treated with LPS and infected at a MOI of 50. In the current study, we performed a similar experiment, and a consistent result was ob-
tained (Fig. 1F). It is noteworthy that IL-1β secretion in this con-
text was more rapidly induced compared with induction upon infection of untreated macrophages with L. monocytogenes at a low MOI. Therefore, it appeared that the activation of caspase-1 in these two experiments was induced in a different manner. Be-
cause LPS facilitates the formation of the NLRP3–inflammasome by upregulating the expression of NLRP3 through the activation of NF-κB (49, 50), LPS pretreatment might enhance the contribution of NLRP3 to the induction of caspase-1 activation upon infection with L. monocytogenes. In another report by Warren et al. (31) the recognition of flagellin by NLRC4 was shown to be important for the activation of caspase-1 in macrophages infected with L. monocytogenes. It is known that the expression of flagellin is strictly

FIGURE 5. Relationship between intracellular growth of L. monocytogenes and the secretion of IL-1β from infected macrophages. A, B, and D. Adherent PECs were transfected with each siRNA (AIM2-1 or control sequence) and infected with WT L. monocytogenes at a MOI of 1. Penicillin G (100 U/ml) was added to some wells 3.5 h postinfection (indicated by arrows). At the indicated time points, the number of intracellular bacteria was determined by a CFU assay (A). Culture supernatants were also collected, and IL-1β in the culture supernatants was assessed by ELISA (B, D). C. Adherent PECs were left uninfected of infected with WT L. monocytogenes at a MOI of 1. Penicillin G (100 U/ml) or chloramphenicol (20 μg/ml) was added to some wells 3.5 h postinfection. Culture supernatants were collected 24.5 h postinfection, and IL-1β in the culture supernatants was assessed by ELISA. The multiplicity index is the CFU at the indicated times divided by the CFU at 1.5 h postinfection. CFU and ELISA data are presented as the mean of triplicate assays and the SD. All of the experiments were repeated at least twice and similar results were obtained. *p < 0.05.
inhibited at 37°C in *L. monocytogenes* strain EGD but not in the strain used in their study (10403s) (51, 52). Accordingly, differences in the regulation of flagellin expression among bacterial strains should be considered when discussing the role of NLRRC4 in inducing caspase-1 activation upon infection with *L. monocytogenes*. More recently, Meixenberger et al. (30) reported that NLRP3 is critical for IL-1β secretion from human PBMCs infected with *L. monocytogenes*, whereas AIM2 is not involved. One possible explanation for the discrepancy between our data and this previous report may be the differences between human PBMCs and primary mouse macrophages. For example, it has been reported that monocytes isolated from human PBMCs constitutively release ATP, which enables the cells to secrete bioactive IL-1β in response to LPS alone in a NLRP3-dependent manner (53). By contrast, LPS alone did not induce the secretion of IL-1β from adherent PECs obtained from WT mice (data not shown).

Our data clearly showed that AIM2 is involved in caspase-1 activation in response to *L. monocytogenes*. However, knockdown of AIM2 did not result in overall impairment of the secretion of caspase-1-dependent cytokines. One possible explanation for this is that AIM2 function in macrophages transfected with targeting siRNA might remain at a low level, because DNA-induced IL-18 secretion was also not completely abrogated (Fig. 3B). In addition, because NLRP3 and NLRRC4 appear to be partly involved in the secretion of IL-1β and IL-18 in response to *L. monocytogenes*, it is also possible that these NLRs partly compensate for the knockdown of AIM2. AIM2 KO mice were recently generated, and one group has shown that the activation of caspase-1 is partially induced in AIM2 KO macrophages infected with *L. monocytogenes*, suggesting a compensatory role for other receptors in the absence of AIM2 (54, 55). These mice would be useful in further clarifying the role of this DNA receptor during infection with *L. monocytogenes* in vivo.

In the current study, we hypothesized that *Listeria* DNA liberated from dead bacteria in the cytoplasm is recognized by AIM2. Indeed, *L. monocytogenes* in host macrophages exhibited a growth curve, which was very similar to that in broth media, and secretion of IL-1β and IL-18 became detectable from the stationary phase-like period (>9.5 h postinfection). We suppose that, in a macrophage compartmentalized by the plasma membrane, continuous proliferation of *L. monocytogenes* might cause the exhaustion of nutrients and subsequent bacterial death, possibly accompanied by the breakdown of the cell wall due to the action of autolysins and other hydrolytic enzymes. From our results, it appeared that lysis of *L. monocytogenes* in the host cell cytoplasm resulting in the liberation of the bacterial cell contents, including DNA, was able to induce the activation of caspase-1, because the addition of penicillin G at 3.5 h postinfection resulted in the rapid secretion of moderate levels of IL-1β in an AIM2-dependent manner (Fig. 5). In support of this idea, lysis of cytosolic *L. monocytogenes* by an expression-controlled autolysin or penicillin G has been implied to enhance the liberation of DNA from bacteria into the host cytoplasm (46, 56). However, in addition to dead bacteria, organelles of host macrophages, such as the nucleus, are also possible sources of DNA if they are damaged in the course of infection. Moreover, it is still possible that at the late stage of infection the number of intracellular *L. monocytogenes* decreased due to other reasons, such as host cell death or bactericidal host effector molecules. Furthermore, we did not show any evidence that DNA is actually responsible for the AIM2-dependent caspase-1 activation induced upon infection with *L. monocytogenes* because of the technical difficulties in proving this. Therefore, further research is required to fully elucidate the basis for AIM2–inflammasome activation upon infection of macrophages with *L. monocytogenes*.

In agreement with other reports (19, 20, 24, 30, 31), we showed that LLO was indispensable for the secretion of IL-1β from macrophages infected with *L. monocytogenes* (Fig. 1F), suggesting that bacterial entry into the cytosol is a key event in this context. However, in our study, we found that delivery of the bacterium into the cytosol alone was not sufficient for inducing inflammasome activation, because chloramphenicol, which was added to the macrophage culture after most bacteria had escaped into the cytosol, almost completely impaired the secretion of IL-1β in response to *L. monocytogenes* (Fig. 5C). Furthermore, we previously showed that not only the entry of bacteria into the cytoplasm but also the unique activity of LLO as a signaling ligand is necessary for the efficient activation of caspase-1 (22). In the current study, we showed that the expression of AIM2 was upregulated upon infection with the LLO-expressing strain but not the ILO-expressing strain (Fig. 6B). Therefore, the difference in expression of AIM2 may explain, at least in part, why LLO is required for efficient inflammasome activation upon infection with *L. monocytogenes*. We have recently found that, compared with the LLO-expressing strain, the ILO-expressing strain only moderately induced the expression of type I IFNs (data not shown), which transduce a signal that might cause the exhaustion of AIM2 (54, 55). These mice would be useful in further clarifying the role of this DNA receptor during infection with *L. monocytogenes* in vivo.

In the current study, we hypothesized that *Listeria* DNA liberated from dead bacteria in the cytoplasm is recognized by AIM2. Indeed, *L. monocytogenes* in host macrophages exhibited a growth curve, which was very similar to that in broth media, and secretion of IL-1β and IL-18 became detectable from the stationary phase-like period (>9.5 h postinfection). We suppose that, in a macrophage compartmentalized by the plasma membrane, continuous proliferation of *L. monocytogenes* might cause the exhaustion of nutrients and subsequent bacterial death, possibly accompanied by the breakdown of the cell wall due to the action of autolysins and other hydrolytic enzymes. From our results, it appeared that lysis of *L. monocytogenes* in the host cell cytoplasm resulting in the liberation of the bacterial cell contents, including DNA, was able to induce the activation of caspase-1, because the addition of penicillin G at 3.5 h postinfection resulted in the rapid secretion of moderate levels of IL-1β in an AIM2-dependent manner (Fig. 5). In support of this idea, lysis of cytosolic *L. monocytogenes* by an expression-controlled autolysin or penicillin G has been implied to enhance the liberation of DNA from bacteria into the host cytoplasm (46, 56). However, in addition to dead bacteria, organelles of host macrophages, such as the nucleus, are also possible sources of DNA if they are damaged in the course of infection. Moreover, it is still possible that at the late stage of infection the number of intracellular *L. monocytogenes* decreased due to other reasons, such as host cell death or bactericidal host effector molecules. Furthermore, we did not show any evidence that DNA is actually responsible for the AIM2-dependent caspase-1 activation induced upon infection with *L. monocytogenes* because of the technical difficulties in proving this. Therefore, further research is required to fully elucidate the basis for AIM2–inflammasome activation upon infection of macrophages with *L. monocytogenes*.
caspase-1 upon infection with Francisella depends on AIM2 (54). Thus, broad pathogens that deliver DNA into the host cytoplasm may likewise be sensed by AIM2.

In conclusion, in this study, we found that AIM2, in cooperation with NLRP3 and NLRC4, plays an important role in the activation of caspase-1 upon infection of macrophages with L. monocytogenes. This finding is consistent with reports published very recently, although there are some differences in the experimental conditions (55, 57, 58). It has been suggested that each receptor contributes to the cellular response to a particular pathogen to varying degrees depending on the infection conditions. It is therefore crucial that the in vitro infection conditions reflect, as closely as possible, the actual in vivo infection conditions when any host–pathogen interaction is examined.

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Disclosures

The authors have no financial conflicts of interest.

References

ROLE OF AIM2 IN LISTERIA INFECTION


