Involvement of Absent in Melanoma 2 in Inflammasome Activation in Macrophages Infected with Listeria monocytogenes

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Involvement of Absent in Melanoma 2 in Inflammasome Activation in Macrophages Infected with *Listeria monocytogenes*

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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 *Listeria 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 *Listeria 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 *Listeria 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 NLPRP3 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 *Listeria monocytogenes* were compared using the inflammasome system reconstructed in HEK293 cells. Among these receptor candidates, AIM2 conferred the highest responsiveness to the bacterium on HEK293 cells. Knockdown of AIM2 significantly decreased the secretion of IL-1β and IL-18 from *Listeria monocytogenes*-infected macrophages. These results suggest that AIM2, in cooperation with NLPRP3 and NLRC4, plays an important role in the activation of caspase-1 during *Listeria monocytogenes* infection.

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In response to *Listeria 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 *Listeria 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 *Listeria 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 (NLPR3) 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-
tators (23). Two studies reported that *L. monocytogenes* is predominately recognized by NLRP3 (24, 30). However, another study reported that caspase-1 activation induced upon infection with *L. monocytogenes* is mediated by multiple receptors, including NLRP3, NLRC4, and other unidentified receptors (31). Moreover, Franchi et al. (32) showed that neither NLRP3 nor NLRC4 is necessary for the activation of caspase-1 in macrophages infected with *L. monocytogenes*. Therefore, the mechanism of caspase-1 activation upon infection with this pathogen remains controversial. Differences between the findings of these studies might result from differences in the experimental procedures, such as the bacterial strain used, the procedure for preparation of bacteria and macrophages, the infection dose, and pretreatment of macrophages with LPS (24, 30–32).

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 procaspase-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). Anti–IL-1β mAbs specific to IL-1β (Medical & Biological Laboratories, Nagoya, Japan) were used.

**Mice**

Female C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan). *L. monocytogenes* 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) (28), caspase-1 KO mice generated by Dr. Keisuke Kuida (Millennium Pharmaceuticals, Cambridge, MA), and IFN-α/β receptor 1 (IFNAR1) KO mice generated by Prof. Michel Aguet (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.

**Bacterial strains**

The parental wild-type (WT) *L. monocytogenes* strain used in this study was *L. monocytogenes* EGD (serovar 1/2a). Three isogenic mutants, Δ*hly*, Δ*hly:Δhly*, and Δ*hly:Δhly:*lmo*, 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). The two amplified fragments were ligated into the plasmid vector pH5-MCS, which carries a thermosensitive replication origin (21). The resulting plasmid was introduced into competent cells of WT *L. monocytogenes* EGD by electrotransformation. 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 at 30°C to select only those transformants with chromosomal integration of the plasmid. Co-integrants 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 (Δ*flaA*) 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% thioglycolate 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 eBioscience (San Diego, CA) and BD Biosciences (San Diego, CA), respectively. For the titration of IL-1β, a pair of biotin-labeled and unlabeled mAbs specific to IL-1β (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 (ToxaKaRa BiO, Shiga, Japan). The percentage of LDH release was calculated by using the following equation: percentage of LDH release = 100 × (experimental LDH release – spontaneous LDH release)/(maximal LDH release – spontaneous LDH release). 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
RLP3 KO mice were untreated (WT) or infected with WT or mutant L. monocytogenes at a MOI of 1. At the indicated time points, LDH release was determined. F, Adherent PECs from WT or NLRP3 KO mice were untreated (−) or pretreated (+) with 10 ng/ml LPS overnight. The cells were left uninfected (−) or infected with WT L. monocytogenes at a MOI of 1 or 50 for 2.5 h. G, Adherent PECs from WT mice were infected with WT or mutant L. monocytogenes strains at a MOI of 1 for 24.5 h. IL-1β (A, C, F, G) and IL-18 (B) 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. *p < 0.05.

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, NLRP6, 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. Results were analyzed with ABI PRISM 7000 (Applied Biosystems, Foster City, CA) using 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 NLRC4 in inflammasome activation during L. monocytogenes infection with WT or mutant 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 and 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 × 10⁶ 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 nonadherent cells were washed out with RPMI 1640 + FCS, and the transfected cells were used in experiments. The sense siRNA sequences were: AIM2-1, 5'-UUA UCU UCU GGA CUU UAA ACA ACA GGC C-3'; AIM2-2, 5'-UAA AGU CAU UGU CAC GCC GGG UGG C-3'. Negative control siRNA was purchased from Invitrogen.
Table I. Primers used for the construction of ΔflaA

<table>
<thead>
<tr>
<th>Primers</th>
<th>Primer Sequences</th>
<th>Restriction Enzymes</th>
</tr>
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<tbody>
<tr>
<td>P1</td>
<td>5′-TAG ATA TAA TGG TAG TAC CGG ATG TTG TCT ATT CAC CTG TCG-3′</td>
<td>KpnI</td>
</tr>
<tr>
<td>P2</td>
<td>5′-TAG ATA TAA TCC CGG GTP GTG TTT CCC TCC TAC ATT-3′</td>
<td>Xmal</td>
</tr>
<tr>
<td>P3</td>
<td>5′-TAG ATA TAA TCC CGG GTA ACA TAT CAA ATT CAT TTA-3′</td>
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</tr>
<tr>
<td>P4</td>
<td>5′-TAG ATA TAA TAA GCT GTA TCT GCG GTA CTG AAA CGG-3′</td>
<td>AatII</td>
</tr>
<tr>
<td>P5</td>
<td>5′-CAG AAG ATC TGG AAC AAT AGG GTG-3′</td>
<td></td>
</tr>
<tr>
<td>P6</td>
<td>5′-GAC GAG AGG GTA AGG GTA AAC AAT G-3′</td>
<td></td>
</tr>
</tbody>
</table>

Restriction enzyme sites are underlined.

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 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, B). By contrast, ASC KO macrophages did not secrete IL-1β or IL-18 postinfection with L. monocytogenes (Fig. 1A, B). 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 inflammasome activation in L. monocytogenes-infected macrophages, macrophages from WT mice were

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 RTPCR 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-protas-1 (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 1) or the LLO gene (*hly*). WT *L. monocytogenes* and Δ*flaA* similarly induced the secretion of IL-1β and IL-18, whereas Δ*hly* did not (Fig. 1G and data not shown), suggesting that sensing of flagellin by NLRC4 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 monocytic 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 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 monocytic 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

**FIGURE 3.** AIM2 is involved in the activation of caspase-1 upon infection of macrophages with *L. monocytogenes*. Adherent PECs were transfected with siRNAs (AIM2-1, AIM2-2, or control siRNA). A, Knockdown of AIM2 was verified by real-time RT-PCR. B, Knockdown of AIM2 resulted in a significant decrease in IL-18 secretion in response to 400 ng Poly(dA)·Poly(dT). C, Adherent PECs transfected with each siRNA were left uninfected or infected with WT *L. monocytogenes* at a MOI of 1 for 24.5 h. Culture supernatants and cell lysates were subjected to Western blot analysis as mentioned in Fig. 1. D–F, AIM2 knockdown macrophages were infected with WT *L. monocytogenes* at a MOI of 1 for 24.5 h. IL-1β (D), IL-18 (E), and IL-1α (F) in the culture supernatants were assessed by ELISA. G, AIM2 knockdown macrophages were stimulated with 10 ng/ml LPS overnight. Then, the cells were additionally stimulated with 20 μM nigericin for 3 h, and IL-1β in the culture supernatants was 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. *p < 0.05.
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 (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.

LISTERIA 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).
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-1β secretion induced by *L. monocytogenes* was significantly lower in IFNAR1 KO macrophages than that in WT macrophages (infection of untreated 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 attributable to the lower expression of AIM2, which is upregulated by the type I IFN 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 cytolysin, iva-nolysin O (ILO), instead of LLO, induced the activation of caspase-1 efficiently, even though these bacterial strains invaded and multiplied in the macrophage cytoplasm in a similar way. This suggested that the activation of caspase-1 is dependent not only on 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 pretreated with LPS and infected at a MOI of 50. In the current study, we performed a similar experiment, and a consistent result was obtained (Fig. 1F). It is noteworthy that IL-1β secretion in this context 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. Because 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
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 NLRC4 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 is essentially abrogated (Fig. 3B). In addition, because NLRP3 and NLRC4 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 LLO-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 invasive *L. monocytogenes*. We have recently found that, compared with the LLO-expressing strain, the LLO-expressing strain only moderately induced the expression of type I IFNs (data not shown), which transduce a signal essential for AIM2 upregulation upon *L. monocytogenes* infection through a common receptor composed of IFNAR1 (Fig. 6A). These data, together with the results of further analysis, will be presented in our future paper (Hara et al., manuscript in preparation).

AIM2 has also been shown to play a role in inflammasome activation during infection with DNA viruses (35, 55). It is therefore of interest whether AIM2 is also involved during infection with other pathogens, especially intracellular parasitic bacteria, such as *Shigella* and *Francisella*. A recent report has shown that activation of
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 (55, 57, 58). It has been suggested that each receptor contributes to the cellular response to a particular pathogen to varying degrees (55, 57, 58). It has been suggested that each receptor contributes to the cellular response to a particular pathogen to varying degrees (55, 57, 58).

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Disclosures

The authors have no financial conflicts of interest.

References


