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Cutting Edge: Cytosolic Bacterial DNA Activates the Inflammasome via Aim2

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Pathogens are detected by pattern recognition receptors that, upon activation, orchestrate an appropriate immune response. The TLRs and the nucleotide-binding oligomerization domain-like receptors (NLRs) are prototypic pattern recognition receptors that detect extracellular and cytosolic pathogens, respectively. Listeria monocytogenes has both extracellular and cytosolic NLR family members. These include two NLR members, NLRC4 and NLRP3, that, upon detection of cytosolic L. monocytogenes, induce the assembly of the inflammasome. Inflammasomes serve as platforms for the activation of the protease caspase 1, which mediates the processing and secretion of pro–IL-1β and pro–IL-18. We previously provided evidence that L. monocytogenes is also detected by a third inflammasome. We now use biochemical and genetic approaches to demonstrate that the third sensor senses bacterial DNA and identifies it as Aim2, a receptor that has previously been shown to detect viral DNA. The Journal of Immunology, 2010, 185: 000–000.

Macrophages have multiple classes of pattern recognition receptors that mediate a variety of immune responses. Activation of TLRs on the cell surface results in the induction and release of many cytokines, including TNF, IL-6, and IL-12 (1). TLRs also induce the production of pro–IL-1β and pro–IL-18. Secretion of these molecules only occurs after proteolytic processing by caspase 1, a component of the nucleotide-binding oligomerization domain-like receptor (NLR)-induced inflammasome (2, 3). NLRs are cytosolic pattern recognition receptors that contain a protein interaction domain (either a caspase recruitment domain [CARD] or Pyrin domain), a nucleotide binding site, and leucine-rich repeats. Upon activation, NLRs oligomerize and recruit procaspase 1 via the CARD domain, either directly through CARD–CARD interactions or indirectly through pyrin–pyrin interactions with the adaptor protein apoptosis speck protein containing a CARD (ASC), whose CARD domain then recruits procaspase 1. The assembled inflammasome forms a platform that mediates caspase 1 activation.

Listeria monocytogenes is a Gram-positive bacterium acquired through ingestion of contaminated food. It infects and replicates within a variety of cells types, including macrophages. After postphagocytosis, L. monocytogenes uses listeriolysin O (LLO) to degrade the vacuolar membrane and escape into cytosol (4). Infection propagates as L. monocytogenes uses actin-based motility to facilitate its entry into a neighboring cell (5). Cytosolic L. monocytogenes activates caspase-1 via NLRP3 and NLRC4 (6, 7). NLRP3 is a pyrin-containing NLR that requires the adaptor ASC. Although the specific agonist has not been defined, NLRP3 is known to be activated when L. monocytogenes escapes the vacuole (8). In contrast to NLRP3, NLRC4 is a CARD-containing protein that can interact with caspase-1 in an ASC-independent manner (9, 10). NLRC4 is activated by flagellin, a component of the bacterial flagellum (10).

We previously presented data that suggested the presence of a third cytosolic receptor for L. monocytogenes (6). This sensor was ASC-dependent, suggesting that it contains a pyrin domain (6). We demonstrate in this study that Aim2, a recently identified cytosolic DNA receptor (11–14), is the third sensor. Aim2 binds DNA (or its synthetic analog, polyA:T) directly through its HIN200 domain and ASC via its pyrin domain. This results in the formation of an atypical inflammasome because Aim2 lacks an oligomerization and a leucine-rich repeat domain and thus is not an NLR. We further demonstrate that Aim2 detects cytosolic L. monocytogenes DNA.

Materials and Methods

Bacterial and mammalian cell culture

Bone marrow-derived macrophages were cultured in 50% DMEM with 30% FBS, 20% L929 cell supernatant, penicillin, and streptomycin for 6 d before infection. Wild-type (WT) L. monocytogenes 10403s and LLO-deficient (ΔLLO) strain were gifts from Dan Portnoy (University of California, Oakland, CA).

Abbreviations used in this paper: ASC, apoptotic speck protein containing a caspase recruitment domain; CARD, caspase recruitment domain; LLO, listeriolysin O; NLR, nucleotide-binding oligomerization domain-like receptor; shRNA, short hairpin RNA; WT, wild-type.

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Macrophage infection

Macrophages were stimulated with 50 ng/ml ultrapure LPS (List Biological Laboratories, Campbell, CA) for 3 h at 37°C to induce pro–IL-1β expression. *L. monocytogenes* were grown to midlog at ambient temperature. At time of infection, cells were spun at 1000 rpm (250 x g) for 5 min to increase contact between macrophages and *L. monocytogenes*. Macrophages were then incubated for 1 h before the addition of LPS and gentamicin to kill extracellular bacteria. Cells were then incubated for 3 h before supernatants were harvested.

**Bacterial lyse preparation**

WT *L. monocytogenes* was grown to stationary phase at 37°C with shaking. Bacteria were pelleted, washed once in PBS, and resuspended in 50 ml PBS. Bacteria were then passed twice through a French press at 1000 psi to lyse the bacteria. Lysates were centrifuged at 17,000 rpm to remove insoluble fraction.

**Microscopy**

Immortalized NLRP3/NLRC4DKO macrophages expressing ASC-V5 were treated with LPS. Log-phase *L. monocytogenes* was stained with Hoechst or DAPI stain at 50 μg/ml for 15 min at 37°C then washed five times in PBS. Macrophages were then infected with *L. monocytogenes*. After 4 h of infection, ASC was visualized with anti-V5. Listeria surface Ags were stained with anti-Listeria Ab (Virostat 4201, Virostat, Portland, ME).

**Retroviral transduction of short hairpin RNA**

Short hairpin RNA (shRNA) from Open Biosystems (Huntsville, AL; Aim2A: RMM1766-6343887; Aim2B: RMM1766-9344596; Aim2C: RMM1766-9353444; control TLR5: RMM1766-6343887; Aim2B: RMM1766-9344596; Aim2C: RMM1766-9353444; control TLR5: RMM1766-6343887; Aim2B: RMM1766-9344596; Aim2C: RMM1766-9353444) was cloned into the LMP retroviral vector (Emory University). Transduced cells were selected with puromycin.

**Results and Discussion**

Cytosolic *L. monocytogenes* activates IL-1β secretion independent of NLRP3 and NLRC4

We previously demonstrated that cytosolic *L. monocytogenes* induces inflammasome activation via three signaling pathways, including NLRP3, NLRC4, and a third unknown sensor pathway, with a combination of bacterial mutants and gene deletion macrophages (6). By using the allagulate ΔflaA mutant *L. monocytogenes* (to eliminate NLRC4 detection), we demonstrated that NLRP3−/− macrophages responded to *L. monocytogenes*, whereas ASC−/− macrophages did not. This indicated that the third sensor acts through ASC. To assess the relative contribution of each detector, we generated NLRP3/NLRC4DKO mice to specifically remove the two established sensors without altering the ASC adaptor protein or the infecting *L. monocytogenes*. In agreement with our previous data, NLRP3 mediated detection was predominant at 1 h postinfection (Fig. 1A), and the contribution of NLRC4 increased over time (Fig. 1B, 1C). By using NLRP3/NLRC4DKO cells, we determined that the third sensor induced IL-1β processing and secretion late, similar to NLRC4 (Fig. 1B–D). Further, we verified that this third sensor acts through ASC, as there was no processing or secretion of IL-1β in NLRC4/ASCDKO cells (Fig. 1). These results demonstrate that cytosolic *L. monocytogenes* is detected by a third sensor that signals through ASC and activates caspase 1.

The third sensor detects *L. monocytogenes* DNA in the cytosol. To identify the agonist that activates this third sensor, we transfected extracts from lysed *L. monocytogenes* into macrophages and examined the IL-1β response (Fig. 2). Macrophages secreted IL-1β in response to crude soluble extracts from *L. monocytogenes* after transfection into the cytosol. To further investigate the class of compound that was detected, we treated the extracts with either DNase or trypsin. Although protein digestion did not alter the IL-1β secretion observed, DNA digestion completely abrogated the response (Fig. 2A). We confirmed that DNA was the active component of the *L. monocytogenes* lysate by demonstrating that transfected genomic DNA purified from *L. monocytogenes* induced IL-1β secretion by WT and NLRP3/NLRC4DKO macrophages (Fig. 2B). The secretion of IL-1β occurred in a dose-dependent manner. In agreement with our infection data, this detection pathway requires ASC signaling as ASC/NLRC4DKO macrophages showed no IL-1β secretion (Fig. 2B). These data indicate that NLRP3/
NLRC4ΔKO macrophages can respond to L. monocytogenes DNA that accesses the cytosolic compartment of macrophages. L. monocytogenes DNA colocalizes with ASC specks

Upon NLR activation, components of the inflammasome, including ASC, are recruited into large complexes within cells termed specks (15). The vast majority of the soluble ASC within a macrophage is recruited into the speck, and this complex is the site of caspase 1 and IL-1β processing (15). Cytosolic polyA: T induces ASC speck formation (12), and we therefore examined whether L. monocytogenes DNA colocalizes with the ASC speck during infection of macrophages with the bacteria. NLRC4/NLRP3ΔKO macrophages expressing ASC-V5 were infected with DNA dye (DAPI or Hoechst) labeled L. monocytogenes. We observed that 48% of ASC specks colocalized with detectable L. monocytogenes DNA (Fig. 3A,3C). In contrast, ΔLLO mutants did not induce speck formation (Fig. 3A). As a control, specks induced by unstained L. monocytogenes were not fluorescent in the blue channel (Fig. 3B). To confirm that the DNA observed in the speck was released from bacteria, we visualized the L. monocytogenes cell wall in macrophages with ASC specks (Fig. 3C). DNA within intact bacteria was contained within the bacterial cell wall and did not colocalize with ASC (Fig. 3C, open arrow). DNA associated with lysed bacteria was proximal to but not contained by the bacterial cell wall and colocalized with ASC specks (Fig. 3C, closed arrow). As controls, we confirmed that DAPI staining does not alter bacterial fitness and that the ASC-V5 construct does not compromise inflammasome activation (Supplemental Fig. 1). These data suggest that a subset of L. monocytogenes lyases within the macrophage and that the ensuing release of DNA induces inflammasome activation.

Aim2 is the third sensor of cytosolic L. monocytogenes

Based upon recent reports that Aim2 is the sensor for viral DNA in the cytosol and that it signals through ASC (11–14), we hypothesized that Aim2 might also be detecting L. monocytogenes DNA during infection of macrophages. We functionally knocked down Aim2 signaling by stable expression of shRNA (Supplemental Fig. 2A,2B). Aim2 shRNA, but not an irrelevant shRNA, abrogated IL-1β secretion in NLRP3/NLRC4ΔKO macrophages infected with L. monocytogenes (Fig. 4A). In WT macrophages, the Aim2 deficiency was compensated by intact NLRP3 and NLRC4 signaling (Fig. 4B), consistent with our previous results (Fig. 1). As a control, Aim2 knockdown did not alter IL-1β secretion following infection with Salmonella typhimurium, which is detected by NLRC4 (Supplemental Fig. 2C). Thus, the Aim2 inflammasome detects L. monocytogenes during bacterial infection of macrophages.

Multiple detection pathways that result in caspase-1 activation following L. monocytogenes infection indicate that the detection of pathogens in the cytosolic compartment is a critical component of immunosurveillance. NLRP3 is temporally activated first, likely detecting vacuolar rupture, followed

FIGURE 3. L. monocytogenes DNA colocalizes with ASC specks. NLRP3/NLRC4ΔKO macrophages expressing ASC-V5 were infected with L. monocytogenes. A, Hoechst-stained WT or ΔLLO L. monocytogenes DNA (blue) and ASC (green). B, DAPI-stained or unstained L. monocytogenes DNA (blue) and ASC (green). C, DAPI-stained L. monocytogenes DNA (blue), L. monocytogenes cell wall (red), and ASC (green). Enlarged merged images of L. monocytogenes DNA with cell wall from (1) an intact bacterium and (2) a lysed bacterium are shown without ASC green overlay. Corresponding phase images are shown. Scale bars, 15 μm. Original magnification ×60.

FIGURE 4. Aim2 responds to cytosolic bacteria. Control or Aim2 shRNA expressing NLRP3/NLRC4ΔKO (A) or WT macrophages (B) were generated by retroviral transduction and infected with L. monocytogenes at a multiplicity of infection of 10, and IL-1β secretion was determined after 4 h. C, Control or Aim2 shRNA expressing NLRP3/NLRC4ΔKO macrophages were infected with Francisella novicida for 8 h, and IL-1β secretion was determined. *p < 0.05.
by NLRC4 and Aim2 activation as flagellin monomers and bacterial DNA are released into the cytosol. For Aim2 detection, our data suggest that a small percentage of cytosolic bacteria lose cell wall integrity and release their DNA into the macrophage cytosol. Damage to the bacterial cell wall may be a result of vacuolar acidification and lysosomal fusion before vacuolar escape or may be due to as yet undefined antimicrobial mechanisms within the macrophage cytosol. Detection of cytosolic bacteria by Aim2 is likely a common mechanism to detect cytosolic bacteria; we demonstrate that Francisella novicida, a bacterial pathogen that also escapes the vacuole, is also detected by Listeria under review, other groups also reported that Aim2 detects Francisella (16–20). Previously, the scope of infections known to be detected by Aim2 was limited to viruses, and these results implicate Aim2 in the response to cytosolic bacterial pathogens.

Disclosures
The authors have no financial conflicts of interest.

References
Figure S1. DAPI staining and ASC-V5 expression do not compromise bacterial fitness or macrophage responses. A) Bacterial growth curve in BHI media for DAPI stained or unstained *L. monocytogenes*. B-D) WT macrophages were infected with DAPI stained or unstained *L. monocytogenes* at MOI 10 and B) IL-1β secretion by ELISA, C) cytotoxicity by LDH release, and D) bacterial replication by dilutional plating were determined. These results indicate that DAPI staining does not impact the fitness of *L. monocytogenes* in broth or in macrophages, nor does it impact the detection of *L. monocytogenes* by the inflammasome. E-F) Control or Aim2 shRNA expressing myc immortalized WT macrophages were infected with *L. monocytogenes* at MOI 10 for 8 hours or *S. typhimurium* at MOI 20 for one hour before E) IL-1β secretion and F) cytotoxicity were determined. Importantly, ASC-V5 expression did not prevent inflammasome activation. As expected, it slightly enhanced inflammasome signaling in response to both bacteria, but had minimal impact on cytotoxicity. (Pyroptosis is known to be ASC independent; unpublished data).

Figure S2. Functionality of Aim2 knockdown. We used three different shRNA to knockdown Aim2 in NLRP3/NLRC4DKO macrophages and found that, consistent with previous reports, one (Aim2C, in the text referred to as Aim2 shRNA) significantly reduced A) Aim2 mRNA levels within the cell measured by qPCR, and B) was associated with an 85% decrease in IL-1β secretion induced 4h after transfection of plasmid DNA with DOTAP into LPS primed macrophages. C) Aim2 shRNA did not alter the secretion of IL-1β in response to *S. typhimurium* via NLRC4 in WT macrophages. *p<0.05.