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Cutting Edge: Mouse NAIP1 Detects the Type III Secretion System Needle Protein

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The NAIP/NLRC4 inflammasomes activate caspase-1 in response to bacterial type III secretion systems (T3SSs). Inadvertent injection of the T3SS rod protein and flagellin into the cytosol is detected through murine NAIP2 and NAIP5/6, respectively. In this study, we identify the agonist for the orphan murine NAIP1 receptor as the T3SS needle protein. NAIP1 is poorly expressed in resting mouse bone marrow–derived macrophages; however, priming with polynosinic-polycytidylic acid induces it and confers needle protein sensitivity. Further, overexpression of NAIP1 in immortalized bone marrow–derived macrophages by retroviral transduction enabled needle detection. In contrast, peritoneal cavity marrow–derived macrophages basally express NAIP1 and respond to needle protein robustly, independent of priming. Human macrophages are known to express only one NAIP gene, which detects the needle protein, but not rod or flagellin. Thus, murine NAIP1 is functionally analogous to human NAIP. The Journal of Immunology, 2013, 191: 000–000.

The detection of virulence factors is a key step in the early innate immune response to infection. Type III secretion systems (T3SSs) or type IV secretion systems (T4SSs) are commonly used bacterial virulence apparatus that inject effector proteins into the cytosol of target host cells, where they reprogram cellular functions to benefit the pathogen. Salmonella typhimurium is a flagellated bacterium that uses its SPI1 T3SS to induce macrophagocytosis and invasion in epithelial cells. Structurally, the T3SS apparatus is a hollow multiprotein complex composed of a basal body with an interior rod (PrgI in SPI1) and a hollow needle (PrgI in SPI1) that protrudes from the bacterial surface. During assembly, the secretion apparatus exports rod subunits into the interior of the basal body, where they polymerize. Subsequently, needle subunits are exported and polymerize to form a direct channel that links the bacteria to the host cell (1), enabling delivery of the effectors into the host cytosolic compartment.

There are many cytosolic-surveillance pathways, including the Nod-like receptors (NLRs). Several NLRs are inflammasomes that act as a platform to activate caspase-1, which carries out two major functions: processing of two inflammatory cytokines (pro–IL-1β and pro–IL-18) into their mature secreted forms and induction of a proinflammatory lytic cell death termed "pyroptosis." NLRC4 is an inflammasome that primarily responds to infection by bacteria that use T3SS and T4SS. NLRC4 was first shown to respond to flagellin in the host cell cytosol (2, 3), which is inadvertently injected by the T3SS (4). However, NLRC4 responded to naturally aflagellated Shigella flexneri (5), as well as Salmonella typhimurium and Pseudomonas aeruginosa flagellin mutants (6, 7). This flagellin-independent response was later attributed to detection of the T3SS rod protein (8), which presumably is also accidentally translocated into the host cytosol (9, 10). Interestingly, both flagellin and rod were detected through the same NLRC4 inflammasome (8). The detection of these two proteins through the same NLRC4 inflammasome was recently explained by the existence of distinct upstream NLRs in the NAIP family.

C57BL/6 mice have four functional NAIP transcripts (NAIP1, NAIP2, NAIP5, and NAIP6), whereas only one NAIP gene has been described in humans (11). In mice, NAIP2 detects rod proteins, whereas NAIP5 and NAIP6 detect flagellins, and NAIP1 remains an orphan receptor (12, 13). Although the single human NAIP is highly homologous to mouse NAIP5, it does not activate NLRC4 in the presence of flagellin. Instead, human NAIP detects the needle protein, whereas flagellin and rod are not detected (13).

In this study, we identify the T3SS needle protein as the agonist for the orphan mouse NAIP1, which is functionally homologous to human NAIP. This detection event was not observed previously because murine bone marrow–derived macrophages (BMMs) do not express sufficient levels of NAIP1 in the resting state.

Materials and Methods

Tissue culture

BMMs were prepared from the femurs of C57BL/6 mice by culturing with L cell–conditioned supernatants. Human U937 monocytes, obtained from the...
American Type Culture Collection, were cultured in RPMI 1640 containing 10% FBS and treated with 50 ng/ml PMA for 48 h to induce differentiation on plastic plates. Differentiated cells were lifted with PBS containing 1 mM EDTA. Peritoneal lavage fluid from two or three mice was pooled for each experiment. Resident peritoneal cavity (PerC) macrophages were harvested from naive mice using ice-cold PBS containing 1 mM EDTA. Peritoneal lavage fluid from two or three mice was pooled for each experiment.

Cloning Salmonella PrgI and PrgJ into the pMXsIG retroviral system and the ensuing retroviral lethality screen were described elsewhere (8). Expression of bacterial proteins was assessed on day 2 posttransduction by flow cytometry. For Naip1 forced expression, Platinum-E cells (American Type Culture Collection) were transfected with MSCV2.2 retrovirus-based Naip1-IRES-GFP or empty vector (12). Retroviral supernatants were collected at 48 h, and spinfections were performed on Myc-immortalized BMMs (iBMMs). The GFP+ population was sorted using a Beckman Coulter Mo-Flo XDP and subcultured for further experiments.

Cells were stimulated for 3–4 h with 50 ng/ml LPS (List Biological), unless indicated otherwise. PrgI-6XHis and PrgJ-6XHis were purified using Talon beads (Clontech). Purified proteins were transfected into macrophages using the transfection reagent profect (P2), as described previously (8). Lactate dehydrogenase activity in the supernatant was determined with the CytoTox 96 transfection reagent (Promega), and IL-1β secretion from THP-1 and U937 cells, two differentiated human macrophage-like cell lines (Fig. 1A–D). However, there was no significant response to the PrgJ rod protein (Fig. 1D).

Interestingly, although we showed previously that BMMs cannot detect PrgI (2, 8), higher doses of cytosolic PrgI protein resulted in a weak response (Fig. 1E, 1F). To further confirm this finding, we used a retroviral lethality screen (8). BMMs from C57BL/6 or Nlrc4−/− mice were retrovirally transduced with GFP alone (empty vector), prgI-GFP, prgJΔLRRs-GFP, or prgJ-GFP. Survival of GFP+ cells was determined by flow cytometry 2 d later by flow cytometry. Some data in (G) and (H) (GFP alone and prgJ-GFP) were published previously (8); prgJΔLRRs-GFP and prgF-GFP transduction data from the same experiment are shown (H). Quantification of data in (G), Graphs show mean and SD of triplicate wells and are representative of at least three independent experiments.

Results and Discussion

We wanted to verify that human macrophages respond to cytosolic needle and not rod protein (13). Cytosolic delivery of purified PrgI needle protein induced robust pyroptosis and IL-1β release from THP-1 and U937 cells, two differentiated human macrophage-like cell lines (Fig. 1A–D). However, there was no significant response to the PrgJ rod protein (Fig. 1D).

Interestingly, although we showed previously that BMMs cannot detect PrgI (2, 8), higher doses of cytosolic PrgJ protein resulted in a weak response (Fig. 1E, 1F). To further confirm this finding, we used a retroviral lethality screen (8). BMMs from C57BL/6 or Nlrc4−/− mice were retrovirally transduced with GFP only (empty vector), prgI-GFP, prgJΔLRRs-GFP, or prgJ-IRES-GFP, and the surviving cells were analyzed by flow cytometry for GFP expression 2 d following transduction.

Real-time quantitative PCR analysis

Total RNA was isolated from indicated tissues, BMMs, or PerC macrophages using an RNeasy Mini Kit (QIAGEN), DNase treated (Promega RQ1), and reverse transcribed (Invitrogen), and quantitative TaqMan PCR was performed. The primers and probes used were described elsewhere (2). The amounts of mRNA analyzed were normalized to GAPDH or Rps17.

FIGURE 1. Human macrophages detect cytosolic PrgI, whereas mouse macrophages show a weak response. Human monocytic cell lines U937 (A, B) and Thp1 (C, D) were differentiated into macrophages with PMA, primed with 50 ng/ml LPS, and transduced with purified PrgI or PrgJ. Two hours posttransfection, cytotoxicity (A, C) and IL-1β secretion (B, D) were determined by lactate dehydrogenase release and ELISA, respectively. BMMs primed with LPS (50 ng/ml) for 4 h were transduced with 125 ng/well PrgI or PrgJ for 1 h, and cytotoxicity (E) or IL-1β secretion (F) was determined. (G and H) Retroviral lethality screen was performed by transducing C57BL/6 and Nlrc4−/− BMMs with retroviruses expressing GFP alone (empty vector), prgI-GFP, prgJΔLRRs-GFP, or prgJ-GFP. Survival of GFP+ cells was determined by flow cytometry 2 d later by flow cytometry. Some data in (G) and (H) (GFP alone and prgJ-GFP) were published previously (8); prgJΔLRRs-GFP and prgJ-GFP transduction data from the same experiment are shown (H). Quantification of data in (G), Graphs show mean and SD of triplicate wells and are representative of at least three independent experiments.

FIGURE 2. Naip1 expression in BMMs, with or without stimulation with LPS or poly(I:C) from the innate immune database. (A) BMMs were left untreated or were primed with LPS or poly(I:C) for 23 h, and Real-time quantitative PCR was performed to assess Naip1 and Naip2 transcript levels relative to Rps17. (B) Cytotoxicity in response to cytosolic PrgI or PrgJ protein was determined at 2 h in unstimulated BMMs or BMMs primed with 6 µg/ml poly(I:C) for 2 d. Data are representative of two or three independent experiments.
Although GFP+ BMMs can be recovered from empty vector or prgJ/IRES-GFP transduction, all prgJ-GFP–transduced cells undergo pyroptotic cell death (Fig. 1G, 1H). Some prgJ-GFP BMMs could be recovered but at somewhat reduced percentages (Fig. 1G, 1H). Together, these findings suggest a weak needle detection by BMMs. This reduction was dependent on NLRC4 because Nlrc4−/− BMMs showed a higher recovery of prgJ-GFP–transduced cells (Fig. 1G, 1H), whereas empty vector was unaffected in the same experiment (47% in wild-type and 45% in Nlrc4−/−) (8). These findings further establish that BMMs are weakly responsive to cytosolic PrgJ in an NLRC4-dependent manner.

We hypothesized that PrgJ was detected through the orphan NAIP1 receptor. But why is this response so inefficient? Activation of some inflammasomes occurs in a two-step process. For example, NLRP3 requires a priming step achieved by a TLR agonist, and AIM2 inflammasome response is enhanced by type I IFN priming (14, 15). In contrast, NLRC4 inflammasome activation occurs without priming, because basal expression of all required components is sufficient to generate robust responses to flagellin and rod in vitro and in vivo. However, BMMs primed with LPS for 4 h show only a weak response to PrgJ delivered into the cytosol (Fig. 1E, 1F). We hypothesized that needle detection by BMMs could be augmented by different priming conditions. Therefore, we tested the TLR3 ligand, polyinosinic-polycytidylic acid [poly(I:C)], which is known to prime the noncanonical caspase-11 inflammasome pathway (16–18). Indeed, longer stimulation with LPS or poly(I:C) significantly enhanced Naip1 expression in BMMs (Fig. 2A). Consequently, BMMs primed with poly(I:C) showed increased pyroptosis in response to cytosolic PrgJ (Fig. 2B). LPS enhances the pool of pro–IL-1β while type I IFN induced by poly(I:C) treatment inhibits transcription of pro–IL-1β mRNA (19), so IL-1β secretion cannot be examined under these conditions. Therefore, although Naip2, Naip5, and Naip6 are basally expressed in BMMs, Naip1 induction requires additional priming.

To show that Naip1 induction was sufficient among poly(I:C)-primed genes, we transduced iBMMs with retroviruses expressing GFP alone or Naip1-IRES-GFP. Untransduced iBMMs closely resemble primary BMMs and respond robustly to PrgJ but not to PrgI (Fig. 3A, 3B). As expected, Naip1 expression enabled detection of PrgJ, resulting in significantly enhanced pyroptosis and IL-1β secretion (Fig. 3C, 3D).

Naip1 is poorly expressed in BMMs as determined by quantitative PCR; however, it can be detected in some macrophage-rich tissues, including the spleen, small intestine, and colon (Fig. 4A, 4B). Interestingly, even under steady-state conditions, macrophages from the PerC of mice express substantial amounts of Naip1 (Fig. 4B). These findings are further corroborated in gene-expression profiling of immune cells by the ImmGen consortium (20). Therefore, we investigated whether PerC macrophages respond to PrgJ without poly(I:C) priming. Indeed, PrgJ induced robust IL-1β secretion from PerC macrophages compared with BMMs, and this was dependent upon NLRC4 (Fig. 4B). We also confirmed that the flagellar hook (FlgE), which is structurally analogous to the needle, is not detected by PerC macrophages (Fig. 4B). Therefore, primary macrophages that express NAIP1 in detectable amounts are fully capable of sensing the needle protein without poly(I:C) priming. The physiological relevance of priming of the Naip1 pathway in vivo in other macrophage populations remains to be investigated.

Together, our studies show that detection of the T3SS needle in the cytosol requires Naip1. These results underscore the...
importance of redundancy in the detection of T3SS. In mice, three distinct agonists, flagellin, rod, and needle, are targeted by four innate immune NAIP receptors, which converge upon a single NLRC4 inflammasome (Supplemental Fig. 1). Therefore, the ability to detect a variety of components of this important virulence structure ensures a thorough surveillance on the part of innate immune phagocytes.

Our findings reveal an unappreciated role for NAIP1 in the detection of T3SS needle and raise interesting questions about mouse versus human pathogen-recognition systems. Data published previously (13) and verified in this study indicate that humans seem to detect only the needle protein. Does the lack of rod and flagellin detection make humans more susceptible to Gram-negative pathogens that use T3SS? Future studies will examine the importance of flagellin, rod, and needle detection through NAIP5/6, NAIP2, and NAIP1, respectively.

Disclosures
The authors have no financial conflicts of interest.

References
Supplemental Figure 1.

A.

B.

MOUSE

HUMAN

Needle

Rod

Flagellin

NAIP5/6

NAIP2

MLP

T3SS apparatus

Periplasm

Inner Membrane (IM)

Outer Membrane (OM)

HOST cell cytosol

Plasma membrane

Vacuole

pro-IL1β

pro-IL-18

Caspase-1

Pyroptosis

Leucine-rich repeat (LRR)

CARD

BIR domain

NBD

Caspase 1

pro-IL1β

pro-IL-18

Caspase-1

Pyroptosis

hNAIP
Supplemental Figure 1. Models depicting the flagellar and the SPI1 secretion systems and activation of the NLRC4 inflammasome in mice and humans. (A) In the flagellar system, flagellin is transported through the hollow hook-basal body, and polymerizes to form filamentous flagella. In the T3SS SPI1 apparatus, rod protein lines the inside of the apparatus while needle protein polymerizes beyond the bacterial outer membrane and links with the pore complex inserted into the host cell membrane. b) Model shows the activation of NLRC4 inflammasome via distinct structural components of bacteria through NAIPs in mice and humans. In mice, NAIP2 and NAIP5/6 detect the cytosolic presence of rod and flagellins respectively. Here we describe that similar to accidental injection of rod and flagellins through the T3SS, needle protein monomers may also be exported into the host cytosol whereby they are detected by NAIP1. In humans, a single NAIP detect cytosolic needle protein. In both mice and humans, the NAIPs trigger NLRC4 oligomerization. NLRC4 then activates caspase-1, which processes IL-1β and IL-18 to their mature/secreted forms, as well as triggering pyroptotic cell death.