Human NAIP and mouse NAIP1 recognize bacterial type III secretion needle protein for inflammasome activation

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Inflammasome mediated by central nucleotide-binding and oligomerization domain (NOD)-like receptor (NLR) protein is critical for defense against bacterial infection. Here we show that type III secretion system (T3SS) needle proteins from several bacterial pathogens, including *Salmonella typhimurium*, enterohemorrhagic *Escherichia coli*, *Shigella flexneri*, and *Burkholderia* spp., can induce robust inflammasome activation in both human monocyte-derived and mouse bone marrow macrophages. Needle protein activation of human NRL family CARD domain containing 4 (NLRC4) inflammasome requires the sole human neuronal apoptosis inhibitory protein (hNAIP). Among the seven mouse NAIPs, NAIP1 functions as the mouse counterpart of hNAIP. We found that NAIP1 recognition of T3SS needle proteins was more robust in mouse dendritic cells than in bone marrow macrophages. Needle proteins, as well as flagellin and rod proteins from five different bacteria, exhibited differential and cell type-dependent inflammasome-stimulating activity. Comprehensive profiling of the three types of NAIP ligands revealed that NAIP1 sensing of the needle protein dominated *S. flexneri*-induced inflammasome activation, particularly in dendritic cells. hNAIP/NAIP1 and NAIP2/5 formed a large oligomeric complex with NLRC4 in the presence of corresponding bacterial ligands, and could support reconstitution of the NLRC4 inflammasome in a ligand-specific manner.

NOD-like protein | innate immunity | caspase-1 | pyroptosis | pathogen-associated molecular pattern

Innate immunity in mammals relies on a group of germline-encoded pattern recognition receptors (PRRs) to sense conserved pathogen-associated molecular patterns (PAMPs) and defend against microbial infections (1). Cytosolic nucleotide-binding and oligomerization domain (NOD)-like receptor (NLR) proteins, characterized by an N-terminal caspase recruitment domain or a pyrin domain, a central NOD, and a C-terminal leucine-rich repeat domain, are a family of PRRs with increasingly appreciated function in innate immune defense (2, 3). The NLR family contains 23 members in humans and 34 members in mice, many of which are known or thought to form large oligomeric inflammasome complexes in response to particular stimulation. The inflammasome is present mostly in macrophage and dendritic cells, and functions as a signaling platform for caspase-1 autoprocessing and activation (4). Activated caspase-1 further cleaves IL-1β and IL-18 into mature forms, and also induces macrophage inflammatory death, or pyroptosis (5), both of which play important roles in restricting microbial infection (6).

The physiological function of most NLRs is not established, and only very few NLRs have defined ligands and stimulation signals. The NLRC4 inflammasome senses a wide spectrum of bacterial infections, including *Legionella pneumophila*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, enteropathogenic *Escherichia coli* (EPEC), and *Shigella flexneri*. NLRC4-dependent IL-1β production by intestinal phagocytes can discriminate pathogenic from commensal bacteria, contributing to immune defense against enteric bacterial infections (7). The NLRC4 inflammasome senses cytosolic flagellin as well as the rod component of bacterial type III secretion system (T3SS) (8–10). T3SS translocates effector proteins into host cells and is a general virulence mechanism for many Gram-negative pathogens (11).

How does NLRC4 sense the two different bacterial molecules? Recent identification of the NAIP family of inflammasome receptors provides significant insights into this question (12, 13). NAIPs are a family of NLRs with seven paralogs in mice (NAIP1–7) but only one family member in humans (hNAIP). NAIP5/6 and NAIP2 bind directly to bacterial flagellin and T3SS rod components, respectively (12, 13), mediating caspase-1 activation through direct interaction with NLRC4 (13). Intriguingly, the NLRC4 inflammasome in human U937 monocytes does not respond to flagellin and T3SS rod protein, but instead is activated by T3SS needle protein CprI in *Chromobacterium violaceum* infection (13).

Here we report that T3SS needle proteins can activate NLRC4 inflammasome in both human and mouse macrophages, and identify hNAIP and its mouse ortholog NAIP1 as responsible for recognizing cytosolic T3SS needle proteins. Recognition of the needle protein by hNAIP/NAIP1 stimulates formation of the large hetero-oligomeric hNAIP/NAIP1-NLRC4 inflammasome complex in 293T cell reconstitution. Further profiling of the inflammasome-stimulation activities of flagellin and T3SS rod and needle proteins from five bacterial pathogens reveals that each NAIP-bacterial ligand pair contributes differently to NLRC4-mediated innate immune detection of a particular bacterial infection. This extensive profiling also reveals a dominant role of NAIP1 recognition of T3SS needle protein in inflammasome detection of *S. flexneri* infection.

Significance

Our analyses uncover general immunogenic activity of bacterial T3SS needle protein and identify human NAIP and mouse NAIP1 as cytosolic innate immune sensors of bacterial T3SS needle protein. These results, together with our previous studies, establish a complete framework for understanding NLRC4-mediated detection of bacterial virulence products by the NAIP family inflammasome receptors. The inflammasome-stimulating activity of bacterial T3SS needle proteins and the difference between mouse and human NAIP inflammasome also may provide guidance in vaccine development.

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Results

Inflammasome Activation by Multiple Bacterial T3SS Needle Proteins in Human Monocyte-Derived Macrophages. The needle protein, conserved among Gram-negative bacterial pathogens, is a most critical component for assembly of a functional T3SS (14). When purified recombinant needle proteins from *S. typhimurium* (PrgI), enterohemorrhagic *E. coli* (EHEC) (EprI), *Burkholderia thailandensis* (BsaL), and *S. flexneri* (MxiH), were each introduced into U937-derived macrophages through the anthrax protective antigen (PA)-lethal factor amino terminal domain (LFn)-mediated protein delivery system (13), robust caspase-1 activation occurred (Fig. 1*A* and *SI Appendix*, Fig. S1*A*). The carboxyl terminus of the needle protein adopts a similar helical hairpin structure as the inflammasome-activation regions in flagellin and T3SS rod protein (14, 15). Similar to that observed with flagellin (13), mutations of two conserved hydrophobic residues in needle-protein helical hairpin regions (V69A and I79A in CprI and V65A and I75A in PrgI, referred to as the 2A mutant hereinafter) largely abolished the inflammasome stimulation activity (Fig. 1*A* and *SI Appendix*, Fig. S1*A*).

Human THP-1 monocyte-derived macrophages also showed robust caspase-1 activation, mature IL-1β production, and extensive pyroptosis on cytoplasmic delivery of PrgI and CprI, but not their 2A mutants (Fig. 1 *B* and *C* and *SI Appendix*, Fig. S1*B*). TAK-242, a TLR4 inhibitor (16), was added to THP-1 cells to prevent interference from residual endotoxin contaminants. Similar inflammasome activation patterns occurred when another TLR pathway inhibitor, BAY-11–7082, was used (*SI Appendix*, Fig. S2 *A* and *B*). Also similar to U937 cells, THP-1 cells did not respond to purified *L. pneumophila* flagellin (FlaA) and *B. thailandensis* T3SS rod protein BsaK (Fig. 1 *B* and *C* and *SI Appendix*, Fig. S2 *A–D*). Other bacterial needle proteins, including EprI, MxiH, BsaL, and PscF from the *P. aeruginosa* PAO1 strain, but not EscF from EPEC, showed similar activity in inducing caspase-1 processing in THP-1 macrophages (*SI Appendix*, Fig. S2 *C* and *D*). Omission of PA from this assay completely abolished caspase-1 activation (*SI Appendix*, Fig. S2*D*), confirming the requirement for the needle protein to enter into macrophage cytosol. Thus, sensing of bacterial T3SS needle proteins for inflammasome activation is a general innate immune response in human monocyte-derived macrophages.

S. flexneri and *S. typhimurium* infections were then performed with U937 cells, which are less sensitive to LPS compared with THP-1 cells. Both bacterial infections induced robust caspase-1 activation, which was largely diminished by genetic ablation of MxiH and PrgI from *S. flexneri* and *S. typhimurium*, respectively (Fig. 1*D*). U937 cells are deficient in flagellin-stimulated inflammasome activation, and infection with WT *L. pneumophila* resulted in little caspase-1 activation (13, 17). *L. pneumophila* does not harbor a T3SS, but notably, ectopic expression of CprI, but not of CprI_2A, in either *L. pneumophila* WT or Δ*flaA* strain stimulated evident caspase-1 activation (Fig. 1*D*). These findings confirm the important role of T3SS needle proteins in stimulating inflammasome activation in human macrophages.

hNAIP Mediates T3SS Needle Protein-Induced Inflammasome Activation. To test whether hNAIP functions similarly to NAIP2/5 in mouse but recognizes T3SS needle proteins, *hNAIP* stable knockdown U937 cells were generated by lentivirus-mediated transduction. Among four different *hNAIP* shRNAs ($1^{\#}$–$4^{\#}$) assayed, $1^{\#}$ shRNA effectively reduced *hNAIP* messenger RNA level by more than 60%, whereas other three shRNAs showed little knockdown effect (Fig. 2*A*). Consistently, only U937 cells harboring $1^{\#}$ shRNA exhibited defective caspase-1 activation and reduced IL-1β maturation on delivery of PrgI or MxiH (Fig. 2 *B* and *C*). Such inhibitory effect was also observed with other bacterial needle proteins, including BsaL, CprI, and EprI (Fig. 2*D*). For all of the five needle proteins assayed, *hNAIP* knockdown reduced caspase-1 activation to a similar extent as shRNA knockdown of *NLRC4* (Fig. 2 *B–D*). Knockdown of *hNAIP* and *NLRC4* did not affect double-strand DNA-stimulated AIM2 inflammasome activation (*SI Appendix*, Fig. S3*A*). Thus, hNAIP is required for human macrophages to sense T3SS needle proteins and activate the NLRC4 inflammasome.

Infection of U937 macrophages with *S. flexneri* or *S. typhimurium* triggered robust caspase-1 activation in its needle protein-dependent manner (Figs. 1*D* and 2*E*). When infections were performed with *NLRC4* stable knockdown U937 cells, caspase-1 activation and IL-1β maturation were both diminished (Fig. 2*E*). Importantly, knockdown of *hNAIP* showed a similar inhibitory effect on *S. flexneri* and *S. typhimurium* infection-induced inflammasome activation (Fig. 2*E*). Moreover, caspase-1 activation by engineered *L. pneumophila* (Δ*flaA*) expressing a T3SS needle protein was also sensitive to *hNAIP* or *NLRC4* knockdown

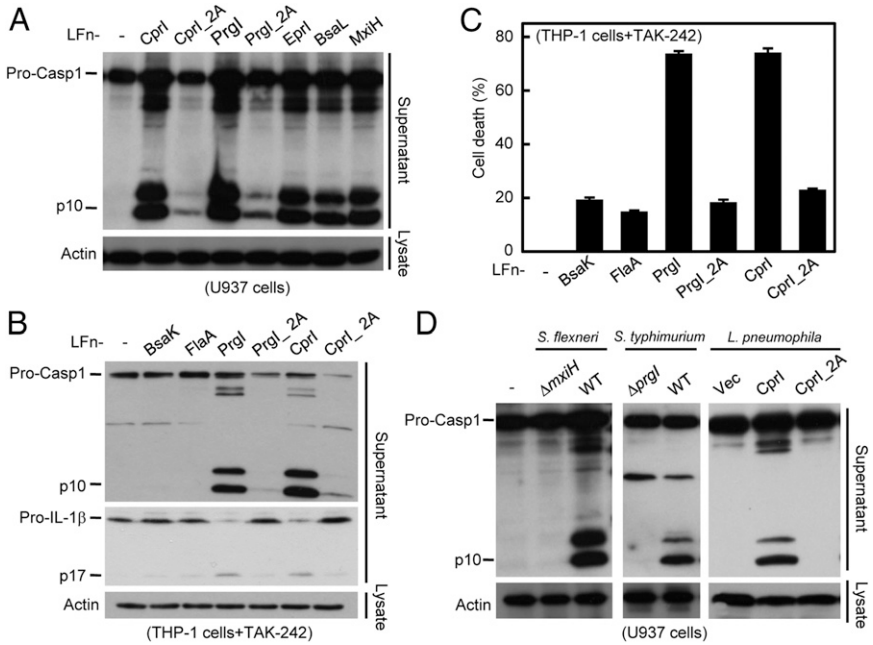

Fig. 1. Various T3SS needle proteins activate the inflammasome in human monocytes-derived macrophages. (*A–C*) Effects of cytoplasmic delivery of bacterial T3SS needle proteins on inflammasome activation in PMA-differentiated U937 (*A*) and THP-1 (*B* and *C*) cells. CprI, PrgI, EprI, BsaL, and MxiH are T3SS needle proteins from *C. violaceum*, *S. typhimurium*, EHEC, *B. thailandensis*, and *S. flexneri*, respectively. CprI_2A, CprI V69A/I79A; PrgI_2A, PrgI V65A/I75A; FlaA, *L. pneumophila* flagellin; BsaK, *B. thailandensis* T3SS rod protein. Shown in *A* and *B* are anti–caspase-1/IL-1β and anti-actin immunoblots of culture supernatants (*Upper*) and total cell lysates (*Lower*); p10 and p17 denote the mature forms of caspase-1 and IL-1β, respectively. Percentages of pyroptosis in *C* were determined by lactate dehydrogenase release as mean ± SD values (error bar) from three independent determinations. In *B* and *C*, 20 mM TAK-242 was used to block potential TLR ligand-induced NF-κB activation. (*D*) T3SS needle proteins activate the inflammasome in U937 cells during bacterial infections. WT or indicated needle protein-deficient strains of *S. flexneri* and *S. typhimurium*, as well as *L. pneumophila* Δ*flaA* expressing CprI, CprI_2A, or an empty vector (Vec) were used to infect PMA-differentiated U937 cells.

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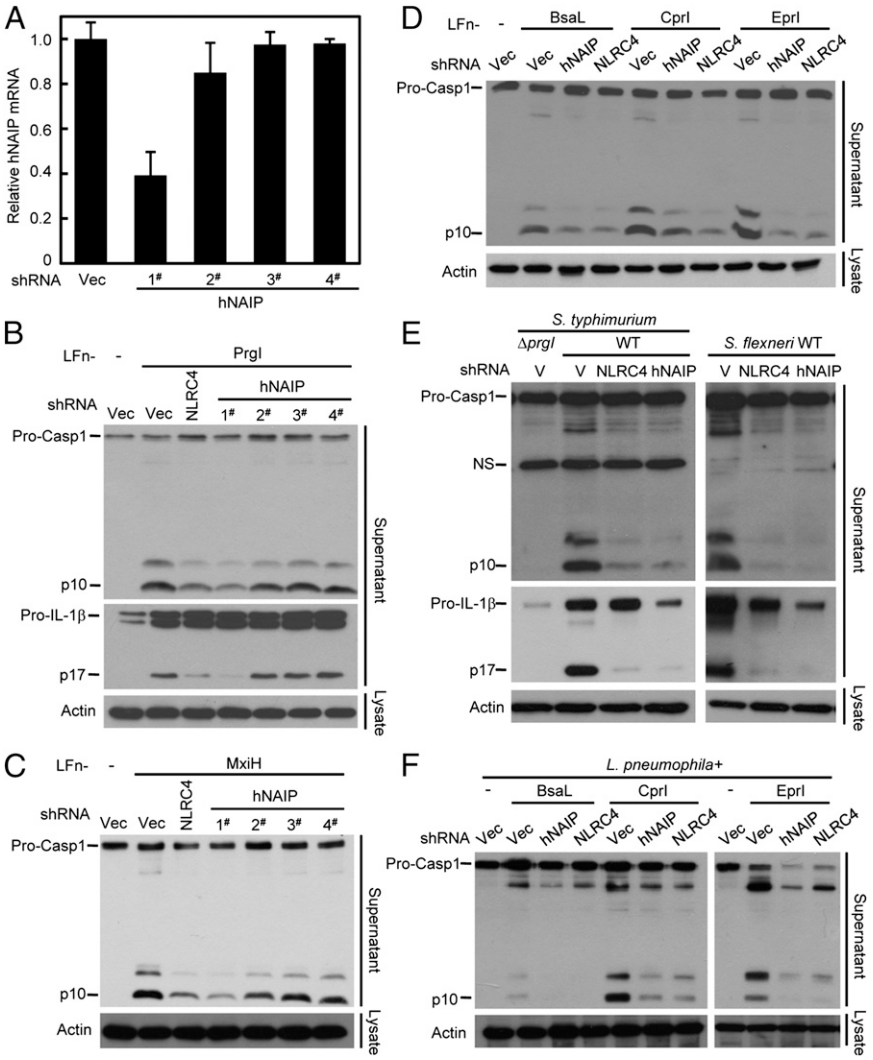

Fig. 2. hNAIP and NLRC4 are required for T3SS needle protein activation of the inflammasome. (*A*) Quantitative real-time PCR (qRT-PCR) analysis of hNAIP transcripts in hNAIP stable knockdown U937 cells. Vec, a nontargeting shRNA. The transcript level of actin was used for normalization. Values are mean ± SD (error bar) from three independent determinations. (*B–D*) Effects of hNAIP and NLRC4 knockdown on T3SS needle protein-induced inflammasome activation in PMA-differentiated U937 cells. Purified PrgI, MxiH, BsaL, CprI, and EprI proteins were delivered into NLRC4 and hNAIP knockdown U937 cells using the LFn-PA system. Shown are anti–caspase-1/IL-1β and anti-actin immunoblots of culture supernatants (*Upper*) and total cell lysates (*Lower*). p10 and p17 denote the mature form of caspase-1 and IL-1β, respectively. (*E* and *F*) Effects of hNAIP and NLRC4 knockdown on bacterial infection-induced inflammasome activation in PMA-differentiated U937 cells. In *E*, *S. typhimurium* and *S. flexneri* were used for infection; in *F*, U937 cells were infected with *L. pneumophila* Δ*flaA* strain expressing indicated needle protein.

(Fig. 2*F*). *L. monocytogenes* activates mainly NLRP3 and AIM2 inflammasomes (18), which was not sensitive to shRNA knockdown of *hNAIP* and *NLRC4* (*SI Appendix*, Fig. S3*B*). These data highlight the important and specific role of hNAIP/NLRC4 in innate immune recognition of T3SS needle proteins in human cells.

T3SS Needle Proteins Activate the NLRC4 Inflammasome in Murine Bone Marrow Macrophages in an NAIP1-Dependent Manner. The NLRC4 inflammasome responds to bacterial T3SS rod component and flagellin in mouse but only detects the needle protein in human macrophages. Such selective response is intriguing from the evolutionary perspective. A possible explanation is that the NLRC4 pathway in mouse is indeed capable of detecting the T3SS needle protein, which is shielded by the activities of flagellin and rod protein in a standard bacterial infection assay. To this end, the LFn-PA protein delivery system was used to specifically examine the activity of T3SS needle proteins in mouse bone marrow-derived macrophages (BMDMs). Notably, several needle proteins, including CprI, BsaL, EprI, and MxiH, when transduced into C57BL/6 mouse-derived BMDMs, triggered robust caspase-1 activation, and these activations were completely abolished in *Nlrc4*$^{-/-}$ BMDMs (Fig. 3*A* and *SI Appendix*, Fig. S4*A*). When infected with *L. pneumophila* Δ*flaA* strain expressing MxiH or EprI, BMDM cells also showed evident caspase-1 activation (*SI Appendix*, Fig. S4*B*). Notably, CprI was less potent compared with MxiH, EprI, and BsaL in stimulating NLRC4-dependent caspase-1 activation (Fig. 3*A*). Such differential activity has been noted with bacterial flagellin and T3SS rod proteins (13). Thus, NLRC4-mediated innate immune detection of bacterial T3SS needle protein also occurs in mouse macrophages.

Among the seven mouse *Naip*s, *Naip1*, *Naip2*, *Naip5*, and *Naip6* are expressed in C57BL/6 mice-derived BMDMs (19). Knockdown of *Naip5* and *Naip2* did not affect MxiH-stimulated caspase-1 activation (*SI Appendix*, Fig. S5*A*). We then examined a possible role of NAIP1. Two independent siRNAs that specifically reduced *Naip1* expression for ∼70% were transfected into mouse BMDM cells (Fig. 3*B*). In contrast to *Nlrc4* knockdown that could attenuate flagellin, T3SS rod protein (BsaK), as well as needle proteins (MxiH and EprI), induced caspase-1 activation, knockdown of *Naip1* only inhibited MxiH/EprI-stimulated caspase-1 activation and IL-1β maturation (Fig. 3 *C* and *D* and *SI Appendix*, Fig. S5 *B* and *C*). Thus, NAIP1 is the mouse ortholog of hNAIP and mediates T3SS needle protein activation of mouse NLRC4 inflammasome.

NAIP1 in Dentritic Cells Mediates a Robust Response to T3SS Needle Proteins. On sensing of microbial products or infection, dendritic cells can mount robust innate immune defense responses, including NLRC4 inflammasome activation (20). Murine dendritic cells, such as primary bone marrow-derived dentritic cells (BMDCs) and immortalized DC2.4 cells, had higher levels of *Naip1* transcripts compared with BMDM cells (*SI Appendix*, Fig.

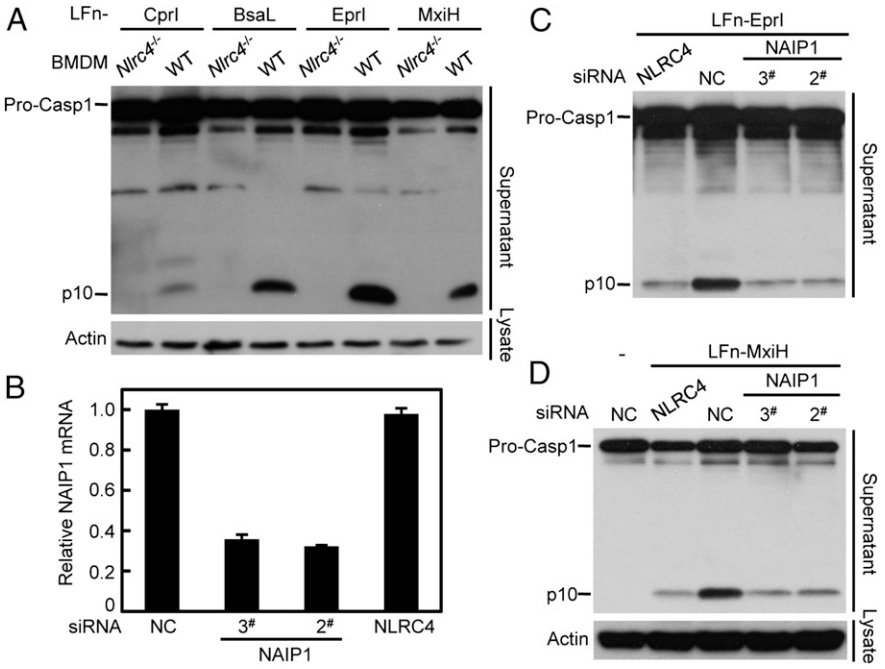

Fig. 3. Mouse NAIP1 mediates needle protein activation of the NLRC4 inflammasome in murine BMDM cells. (*A*) Purified CprI, BsaL, EprI, and MxiH proteins were delivered into BMDMs derived from WT (C57BL/6) or *Nlrc4*$^{-/-}$ mice. Shown are anti–caspase-1 and anti-actin immunoblots of culture supernatants (*Upper*) and total cell lysates (*Lower*). (*B*) qRT-PCR analysis of NAIP1 knockdown efficiency in mouse BMDMs. siRNA oligos targeting mouse NAIP1 (2# and 3#) or NLRC4 or a nontargeting siRNA (NC) were transfected into primary WT BMDM cells. The transcript level of actin was used for normalization. Values are mean ± SD (error bar) from three independent determinations. (*C* and *D*) Effects of NAIP1 and NLRC4 knockdown on needle protein-induced inflammasome activation in mouse BMDM cells. Purified EprI (*C*) or MxiH (*D*) proteins were transduced into mouse BMDM cells transfected with indicated siRNAs.

S6). Delivery of EprI and MxiH into DC2.4 cells resulted in a more robust caspase-1 activation than seen in BMDM cells, and this activation was highly sensitive to siRNA knockdown of *Nlrc4* (Fig. 4 *A* and *B*). When multiple independent *Naip1*-targeting siRNAs were transduced into DC2.4 cells (*SI Appendix*, Fig. S7*A*), caspase-1 activation induced by EprI and MxiH was drastically reduced (Fig. 4 *A* and *B*). In contrast, none of the four *Naip1*-targeting siRNAs affected flagellin-induced caspase-1 activation in DC2.4 cells, although it was sensitive to *Nlrc4* knockdown (Fig. 4*C*).

Differential Inflammasome-Stimulation Activity of Different Bacterial Flagellin and T3SS Rod and Needle Proteins. To obtain a comprehensive understanding of the inflammasome-stimulating activity

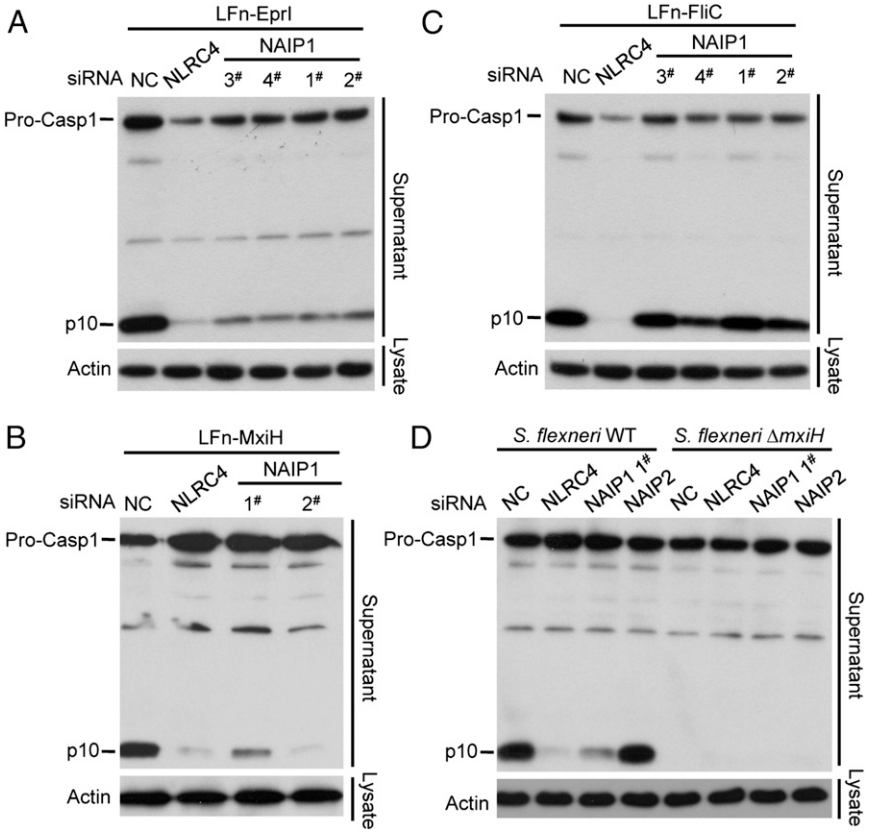

Fig. 4. The NAIP1/NLRC4 inflammasome in mouse dentritic cells mediates a robust response to cytoplasmic T3SS needle protein and *S. flexneri* infection. (*A*–*C*) Effects of NAIP1 and NLRC4 knockdown on T3SS needle protein-induced inflammasome activation in DC2.4 cells. Purified EprI, MxiH, and *P. aeruginosa* flagellin (FliC) were delivered into DC2.4 cells transfected with indicated siRNAs. Knockdown efficiency of the four independent NAIP1-targeting siRNAs (1#–4#) is shown in *SI Appendix*, Fig. S7*A*. Shown are anti–caspase-1 and anti-actin immunoblots of culture supernatants (*Upper*) and total cell lysates (*Lower*). (*D*) Effects of NAIP1, NAIP2, and NLRC4 knockdown on *S. flexneri*-induced caspase-1 activation. DC2.4 cells transfected with indicated siRNAs were infected with WT or the Δ*mxiH* strain of *S. flexneri*.

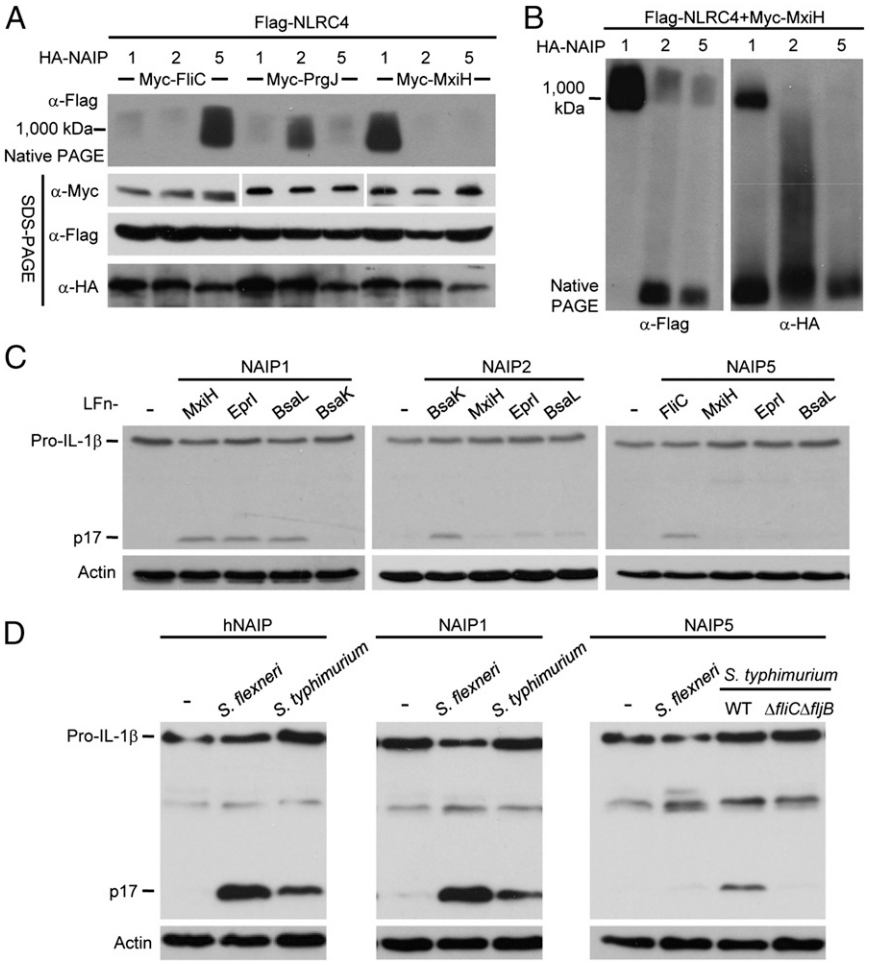

Fig. 5. Reconstitution of the NAIP1/hNAIP-NLRC4 inflammasome complex and its response to cytoplasmic needle proteins and bacterial infections. (*A* and *B*) NAIP1 recognition of T3SS needle proteins stimulates the oligomeric needle protein-NAIP1-NLRC4 inflammasome complex formation. The 293T cells were cotransfected with indicated combinations of Flag-NLRC4, HA-NAIPs (1, 2, or 5) and Myc-FliC, PrgJ, or MxiH. Anti-Flag and anti-HA immunoblotting of BN-PAGE gels were performed to examine the NAIP-NLRC4 inflammasome complex. Total cell lysates were also separated on the SDS/PAGE gels, followed by anti-Flag, HA, and Myc immunoblotting. (*C*) Ligand-specific reconstitution of the NAIP-NLRC4 inflammasome in 293T cells. The 293T cells transfected with NLRC4, procaspase-1, and pro-IL-1β together with an indicated NAIP expression plasmid were stimulated with indicated LFn-tagged NAIP ligands. Shown are immunoblotting analyses of IL-1β maturation; anti-actin blots serve as the loading control. (*D*) Responses of reconstituted NAIP1/hNAIP-NLRC4 inflammasome to bacterial infections. The 293T cells expressing indicated inflammasome components were infected with *S. flexneri* or *S. typhimurium* strains as indicated.

of bacterial flagellin and T3SS rod and needle proteins, we delivered equal molar amounts of the three bacterial products from five different pathogens (*C. violaceum*, *S. typhimurium*, *S. flexneri*, *B. thailandensis*, and EHEC) into primary BMDMs, BMDCs, and DC2.4 cells and compared inflammasome activation by quantifying pyroptotic cell death (*SI Appendix*, Fig. S8). This extensive profiling revealed several important findings. First, T3SS rod proteins, except for MxiI from *S. flexneri*, generally had a higher activity than the needle proteins and flagellin from the same bacterium, particularly in BMDM cells and BMDCs. Second, the same type of bacterial ligand from different organisms exhibited different activities, which was more evident for flagellin and the rod proteins. *S. typhimurium* flagellin and *S. flexneri* needle protein MxiH were the most active compared with their counterparts from other four bacteria. Third, compared with BMDM cells, BMDCs and DC2.4 cells exhibited a significantly decreased response to flagellin and rod proteins, but maintained a robust response to needle proteins. These findings suggest that the inflammasome-stimulation activity of a certain NAIP ligand is highly dependent on the bacterium that bears it, as well as on the cell type assayed; bacterial infection-induced NLRC4 inflammasome activation often results from a mixture of contributions from multiple NAIP receptors.

NAIP1 Recognition of T3SS Needle Protein Is Mainly Responsible for *S. flexneri*-Induced Inflammasome Activation. *S. flexneri* infection of mouse BMDM cells activates the NLRC4 inflammasome, but the underlying mechanism is not known (21, 22). *S. flexneri* is nonflagellar and does not express flagellin; *S. flexneri* T3SS rod protein MxiI was much less active than other bacterial rod proteins, whereas its needle protein MxiH was one of the most active needle proteins in mouse BMDM cells and BMDCs (*SI Appendix*, Fig. S8). Robust caspase-1 activation was observed in *S. flexneri*-infected DC2.4 cells, and this activation was drastically reduced by siRNA knockdown of *Nlrc4* expression (Fig. 4*D*). Importantly, deletion of *mxiH* from *S. flexneri* completely abolished infection-induced caspase-1 activation. Consistent with this observation, siRNA knockdown of *Naip1* also diminished *S. flexneri*-stimulated caspase-1 activation, whereas silencing of *Naip2* expression produced no such effects (Fig. 4*D* and *SI Appendix*, Fig. S7*B*). Thus, NAIP1 recognition of T3SS needle protein is the predominant innate immune response to *S. flexneri* infection, particularly in dentritic cells.

NAIP1/hNAIP Mediates Formation of a Needle Protein-NAIP1/HNAIP-NLRC4 Inflammasome Complex. We further tested whether NAIP1 and hNAIP can mediate formation of the needle protein-NAIP1/hNAIP-NLRC4 inflammasome complex. To this end, different NAIPs, together with NLRC4, were coexpressed with *S. typhimurium* FliC or PrgJ or *S. flexneri* MxiH in 293T cells, and inflammasome complex formation was examined by blue native (BN) polyacrylamide gel electrophoresis (PAGE). The presence of NAIP1, but not NAIP2/5, shifted the migration of NLRC4 to a high molecular weight position (>1,000 kDa) only when MxiH was expressed (Fig. 5*A*). This oligomeric NLRC4 complex also contained NAIP1 (Fig. 5*B*). Replacement of MxiH with FliC or PrgJ abolished NAIP1-NLRC4 inflammasome complex formation (Fig. 5*A*). Similar results were obtained when PrgI and hNAIP were assayed (SI Appendix, Fig. S9). Ser-533 in NLRC4 is phosphorylated by PKCδ on *S. typhimurium* infection, and this event is required for NLRC4 inflammasome activation (23). However, the foregoing reconstitution system appeared to have bypassed

the requirement of Ser-533 phosphorylation for NAIP-NLRC4 inflammasome complex formation (*SI Appendix*, Fig. S10).

Functional Reconstitution of Needle Protein Activation of the NLRC4 Inflammasome. To assay the functionality of the reconstituted NAIP1/hNAIP-NLRC4 complex, we transfected 293T cells with procaspase-1 and pro-IL-1β. In NAIP5- and NAIP2-expressing cells, intracellular delivery of FliC and BsaK, respectively, induced mature IL-1β production, whereas none of the three needle proteins (MxiH, EprI, and BsaL) demonstrated such activity (Fig. 5*C*). In NAIP1-expessing cells, delivery of any of the three needle proteins, but not of BsaK, induced mature IL-1β production (Fig. 5*C*). When the transfected 293T cells were subjected to *S. flexneri* infection, robust IL-1β maturation was observed with hNAIP/NAIP1, but not with NAIP5-reconstituted NLRC4 inflammasome (Fig. 5*D*). In contrast, NAIP1, NAIP5, and hNAIP were all capable of supporting IL-1β maturation after *S. typhimurium* infection. These data are consistent with the fact that *S. typhimurium* expresses an active flagellin, whereas *S. flexneri* does not. As expected, deletion of *fliC* abolished *S. typhimurium* activation of the reconstituted NAIP5-NLRC4 inflammasome complex (Fig. 5*D*). These results confirm that NAIP1 in mouse and hNAIP mediate recognition of bacterial T3SS needle proteins to stimulate NLRC4 inflammasome activation.

Discussion

Here we establish that T3SS needle proteins are a unique type of PAMPs recognized by cytosolic NAIP receptors in both human and mouse macrophages. Mice have multiple NAIPs that recognize flagellin (NAIP5/6) and T3SS rod proteins (NAIP2), as well as needle proteins (NAIP1). The presence of multiple NAIPs in mice, likely arising from gene duplications, maximizes mouse innate immune detection capacity in coping with bacterial infections. Human NLRC4 inflammasome recognizes only T3SS needle protein owing to the presence of a sole NAIP family member. T3SS and the flagella system are evolutionarily related, and many flagellated Gram-negative bacteria often contain a T3SS. The needle protein, most critical for T3SS assembly, is more prone to be exposed to host cytosol. These may explain why humans recognize only T3SS needle proteins. Compared with flagellin, T3SS is more directly associated with bacterial virulence. Recognition of the T3SS may help better distinguish pathogenic bacteria from those avirulent ones or commensals. Moreover, among the mouse NAIPs, NAIP1 is most closely related to hNAIP and has the shortest phylogenetic distance to hNAIP.

We demonstrate that each type of NAIP ligands have inflammasome-stimulation activities depending on the bacterial origin, as well as on the cell type examined. Each NAIP contributes differently to NLRC4 inflammasome-mediated innate immune detection of a particular bacterial infection. For example, the rather weak activity observed with flagellin and needle protein from EPEC agrees with the fact that knockdown of NAIP2 abolishes the large majority of EPEC-induced inflammasome activation in BMDMs (13). In contrast, NAIP1 recognition of the needle proteins dominates *S. flexneri*-stimulated inflammasome activation. Genetic analyses using *Nlrc4*-deficient mice have established a critical role of the NLRC4 inflammasome in innate immune defense against many types of bacterial infections in various animal and disease models. Identification and functional profiling of the three NAIPs described here provide a framework for further investigations of the contribution of each type of bacterial virulence factor to infection pathology and disease onset.

We report that inflammasome-stimulating activities of bacterial flagellin and TS33 rod and needle proteins emanate from their C-terminal helical hairpin regions and require a cluster of hydrophobic residues born there. We also demonstrate that NAIPs can support functional reconstitution of the NLRC4 inflammasome in 293T cells in a ligand-specific manner. A recent in vitro reconstitution and structural study showed that purified flagellin interacts directly with one NAIP5 molecule, which induces the recruitment and progressive incorporation of NLRC4 to form a hetero-oligomeric inflammasome (24). These findings should lead to future studies of the biochemical mechanisms of NAIP-NLRC4 inflammasome assembly and regulation.

Materials and Methods

cDNAs for mouse and human NAIPs, and plasmids for recombinant expression of needle proteins and PA, have been described previously (13). Purification of LFn-tagged flagellin, T3SS needle proteins, and rod proteins were also as described previously (13). Antibodies for caspase-1 and Myc epitope were obtained from Santa Cruz Biotechnology. Other antibodies used include IL-1β (3ZD; Biological Resources Branch, National Cancer Institute), HA epitope (Covance), Flag M2 (Sigma-Aldrich), and phospho-Ser533 NLRC4 antibody (monoclonal GEN-82 clone 3-3; Genetech). Cell culture products were from Invitrogen, and all other chemicals were Sigma-Aldrich products unless noted otherwise. Detailed information on mice, cell culture, inflammasome activation assays, RNAi knockdown, bacterial manipulation, and infection is presented in *SI Appendix, Materials and Methods*.

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