NLRP3 recruitment by NLRC4 during *Salmonella* infection

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NLRC4 and NLRP3, of the NOD-like receptor (NLR) family of intracellular proteins, are expressed in innate immune cells and are thought to nucleate distinct inflammasome complexes that promote caspase-1 activation, secretion of the proinflammatory cytokines IL-1β and IL-18, and a form of cell death termed pyroptosis. We show that NLRP3 associates with NLRC4 in macrophages infected with *Salmonella typhimurium* or transfected with flagellin. The significance of the interaction between the NLRC4 NACHT domain and NLRP3 was revealed when Nlrc4<sup>S533A/S533A</sup> bone marrow–derived macrophages (BMDMs) expressing phosphorylation site mutant NLRC4 S533A had only a mild defect in caspase-1 activation when compared with NLRC4-deficient BMDMs. NLRC4 S533A activated caspase-1 by recruiting NLRP3 and its adaptor protein ASC. Thus, Nlrc4<sup>S533A/S533A Nlrp3<sup>−/−</sup></sup> BMDMs more closely resembled Nlrc4<sup>−/−</sup> BMDMs in their response to *S. typhimurium* or flagellin. The interplay between NLRP3 and NLRC4 reveals an unexpected overlap between what had been considered distinct inflammasome scaffolds.

NLRC4 and NLRP3 each have a central nucleotide-binding oligomerization NACHT domain and carboxy terminus leucine-rich repeats (LRRs). NLRC4 has an amino terminus caspase-activation and recruitment domain (CARD), whereas NLRP3 has a pyrin domain (Lamkanfi and Dixit, 2009). Analyses of macrophages lacking NLRC4 or NLRP3 suggest that these proteins activate caspase-1 in response to distinct stimuli. For example, NLRC4 is essential for caspase-1 activation in macrophages infected with intracellular pathogens including *Salmonella typhimurium* (Mariathasan et al., 2004) and *Pseudomonas aeruginosa* (Sutterwala et al., 2007), whereas NLRP3 is required for caspase-1 activation by diverse agents including ATP, nigericin, and crystalline substances such as monosodium urate (Mariathasan et al., 2006; Martinon et al., 2006).

How NLRP3 senses cellular perturbations remains contentious, but mechanistic details of NLRP3 activation by pathogen–associated molecules are emerging. Bacterial flagellin or components of bacterial type 3 secretion systems, such as PrgJ or needle protein, bind to neuronal apoptosis inhibitory proteins (NAIPs), and this promotes interactions between the NAIPs and NLRC4 (Kofoed and Vance, 2011; Yang et al., 2013). The ligand-bound NAIPs between the NAIPs and NLRC4 (Kofoed and Vance, 2011; Yang et al., 2013) appear to drive conformational changes in NLRC4 that ex-
protein but, as expected, Ser533 phosphorylation was detected only in wild-type cells after infection with S. typhimurium, or after stimulation with LPS followed by flagellin transfection (Fig. 1 B). Interestingly, Nlrc4S533A/S533A BMDMs differed from Nlrc4−/− BMDMs in that they produced cleaved, active caspase-1 after infection, albeit ~1.5 h later than Nlrc4+/+ BMDMs (Fig. 1 C). Consistent with this finding, Nlrc4S533A/S533A BMDMs exhibited delayed caspase-1-dependent release of IL-1β and lactate dehydrogenase (LDH), the latter a measure of cell death (Fig. 1, D and E). Secretion of TNF after infection was not impaired by the NLRC4 S533A mutation, indicating a specific defect in caspase-1 activation (Fig. 1 F). In addition, only NLRC4-dependent caspase-1 activation was compromised because...
Nlrc4S533A/S533A BMDMs exhibited normal caspase-1 cleavage (Fig. 1G) and IL-1β secretion (Fig. 1H) in response to ATP or transfected double-stranded DNA (dsDNA), which stimulate NLRP3 and AIM2 inflammasome activity, respectively (Mariathasan et al., 2006; Jones et al., 2010).

NLRP3 contributes to the NLRC4 inflammasome

The milder inflammasome activation defect of Nlrc4S533A/S533A BMDMs compared with Nlrc4−/− BMDMs prompted us to revisit our earlier experimental system of Nlrc4−/− immortalized macrophage progenitors reconstituted with NLRC4 S533A. Macrophages differentiated from these progenitors failed to activate caspase-1 after infection with S. typhimurium (Qu et al., 2012). These immortalized macrophages induced NLRP3 poorly in response to S. typhimurium when compared with primary BMDMs (Fig. 2A). Given that both NLRP3 and NLRC4 promoted caspase-1 activation after a long infection with stationary phase S. typhimurium expressing little SPI-1 (Broz et al., 2010a), we wondered if NLRP3 might have an unrecognized role during acute infection of primary BMDMs with SPI-1–expressing bacteria. To focus solely on NLRC4-dependent signaling events, we primed BMDMs with LPS to mimic infection-induced up-regulation of NLRP3 (Fig. 2B), and then transfected

Figure 2. NLRP3, but not caspase-11, contributes to NLRC4-dependent activation of caspase-1. (A and B) Western blots of immortalized macrophages (A) or BMDMs (A, B). Cells were primed for 4 h with LPS or Pam3CSK4, or infected for 2.5 h with S. typhimurium (MOI = 5). (C) Western blots of BMDMs and their culture medium. (D–F) Graphs show IL-1β (D), LDH (E) and TNF (F) released from BMDMs. (G) Western blots of NLRC4 immunoprecipitated with anti–FLAG antibody from BMDMs at 4 h after either infection (MOI = 10) or flagellin transfection. p–NLRC4, phosphorylated NLRC4. (H and I) Graphs show IL-1β (H) and LDH (I) released from BMDMs. Graphed data (mean ± SD) are pooled from three independent experiments for a total of three mice of each genotype. P-values were determined by the Student’s t test.* P < 0.05; ** P < 0.01.
the cells with flagellin from S. typhimurium to stimulate NLRC4 specifically (Franchi et al., 2006; Miao et al., 2006). Nlrc4S533A/S533A and Nlrp3−/− BMDMs processed caspase-1 and pro-IL-1β slower than Nlrc4+/+ BMDMs (Fig. 2 C) and, over the course of an experiment, this translated into reduced IL-1β (Fig. 2 D) and LDH release (Fig. 2 E). Nlrc4−/− BMDMs, as expected, failed to cleave caspase-1 and secrete IL-1β, whereas TNF secretion was equivalent between all the genotypes (Fig. 2 F). Phosphorylation of NLRC4 Ser533 was normal in Nlrp3−/− BMDMs (Fig. 2 G), indicating that NLRP3 contributed to NLRC4-dependent inflammasome signaling by some other mechanism.

LPS priming induces expression of caspase-11, as well as NLRP3 (Kayagaki et al., 2013). Unlike NLRP3, however, caspase-11 was dispensable for LPS/flagellin-induced secretion of IL-1β and LDH (Fig. 2, H and I). In control experiments, caspase-11 deficiency impaired IL-1β and LDH release in response to intracellular LPS as previously described (Kayagaki et al., 2013). These data indicate that NLRP3-dependent caspase-1 activation in BMDMs treated with LPS/flagellin was not merely a consequence of residual LPS from the priming step entering the cytoplasm during transfection and engaging caspase-11 (Kayagaki et al., 2011, 2013).

Given that NLRP3 is induced in BMDMs after infection with S. typhimurium or after treatment with TLR agonists (Fig. 2 B), we reasoned that NLRP3 probably contributed little to flagellin-induced inflammasome signaling in BMDMs that were not primed with LPS. Indeed, caspase-1 processing in Nlrc4+/+ BMDMs transfected with flagellin without priming was equivalent to that seen in similarly treated Nlrc4S533A/S533A and Nlrp3−/− BMDMs (Fig. 3 A). In addition, LDH release in the Nlrc4+/+, Nlrc4S533A/S533A, and Nlrp3−/− cultures was equivalent at 2 h after transfection with flagellin (Fig. 3 B). Collectively, our data suggest that up-regulation of NLRP3 after LPS stimulation of TLR4 allows NLRP3 to support NLRC4-dependent activation of caspase-1.

**Figure 3. NLRP3 contributes less to NLRC4-dependent inflammasome activation in the absence of priming.** (A) Western blots of BMDMs and their culture medium. (B) Graph shows LDH release. (C–F) Western blots of BMDM lysates fractionated by size exclusion chromatography. In D and F, cells were infected for 3 h (MOI = 10). All cultures contained pan-caspase inhibitor Z-VAD-FMK to limit pyroptosis. One less fraction was collected in E and F than in C and D. Graphed data (mean ± SD) are pooled from three independent experiments for a total of three mice of each genotype.
uninfected cells (Fig. 3 E). Of note, however, pro–caspase-1 was absent from macromolecular complexes both before and after infection of Nlr4−/− BMDMs with S. typhimurium (Fig. 3, E and F). This last result was consistent with a role for NLR4 S533 phosphorylation in the optimal recruitment of pro–caspase-1 into the NLR4 inflammasome.
We explored the domains in NLRC4 required for binding to NLRP3 in a 293T overexpression system. The LRRs, CARD, and phosphorylation of S533 in NLRC4 were dispensable for the binding of NLRC4-Flag to NLRP3-eGFP (Fig. 5). The interaction was abolished, however, by loss of both the NACHT domain and LRRs in NLRC4. In light of these results, we hypothesized that caspase-1 activation in Nlrc4−/− BMDMs infected with S. typhimurium was delayed rather than abolished because the NLRC4 S533A mutant could recruit NLRP3 and ASC. Consistent with such a model, Nlrc4−/− BMDMs showed a marked reduction in NLRC4-dependent processing of caspase-1 and IL-1β after S. typhimurium infection (Fig. 6 A) or LPS/flagellin treatment (Fig. 6 B) when compared with Nlrc4−/− BMDMs, although we note that processing was not abolished completely. It is unclear if this residual processing of caspase-1 reflects the involvement of yet a third NLR family member or if the NLRC4 S533A mutant itself retains some limited capacity to recruit pro–caspase-1 directly via its CARD. In control experiments, Nlrc4−/− BMDMs processed caspase-1 and IL-1β normally after stimulation with LPS/dsDNA (Fig. 6 C). Nlrc4−/− BMDS also released less IL-1β and LDH than Nlrc4−/− BMDMs after infection with S. typhimurium (Fig. 6 D) or treatment with LPS/flagellin (Fig. 6 E). These findings indicate that NLRP3 is in large part responsible for NLRC4-dependent caspase-1 activation in Nlrc4−/− BMDMs.

A model arises wherein up-regulated NLRP3 is recruited to NLRC4 to amplify caspase-1 activation. We speculate that a NAIP—NLRC4 interaction permits NLRP3 recruitment and then a conformational change in NLRP3 permits its interaction with ASC. Whereas the CARD of NLRC4 can bind directly to the CARD of pro–caspase-1 (Poyet et al., 2001), the pyrin domain of NLRP3 presumably engages the pyrin domain of ASC, and then the CARD inASC binds to the CARD in pro–caspase-1. Our simplified model does not speak to the role that ASC appears to have in NLRC4 signaling that is independent of NLRP3. For example, NLRP3 is dispensable for the formation of an ASC focus that processes caspase-1, IL-1β, and IL-18 in BMDMs infected with S. typhimurium (Mariathasan et al., 2004, 2006; Broz et al., 2010a). In this complex, perhaps ASC is recruited via its CARD when NLRP3 is absent. Notably, NLRC4 deficiency has a much greater impact than ASC deficiency on caspase-1–dependent pyroptosis in BMDMs infected with S. typhimurium (Mariathasan et al., 2004). Some NLRC4 is proposed to activate caspase-1 in distinct complexes lacking ASC without causing caspase-1 autoproteolysis (Broz et al., 2010b).

**MATERIALS AND METHODS**

**Mice.** Nlrc4−/− mice (Genoway) and Nlrc4−/− mice (Taconic) were generated from C57BL/6 embryonic stem cells. For Nlrc4−/− mice, TaqMan primers 5′-CCT ACAAGGACTTTCGTCACCA-3′ and 5′-CATCTT...
GCTCAGTGGTATTTCACGA-3' amplified an 83-bp fragment that was detected by a wild-type (AGGCAGGA TCAA) or knock-in (AGGCAGGAAGCCA) probe. For Nlrc4<sup>S533A</sup>/S533A animals, primers 5'-ACGGGCTGGGAGGT GA-3' and 5'-CTTGTAGGTGTTGGAATCA-3' amplified a 219-bp wild-type fragment and a 285-bp knock-in fragment.
fragment. \textit{Nlrc4}\textsuperscript{flx/flx}, \textit{Casp1,11\textsuperscript{-/-}}, \textit{Asc\textsuperscript{-/-}}, and \textit{Nlpr3}\textsuperscript{-/-} mice were described previously (Mariathasan et al., 2004, 2006; Kayagaki et al., 2011; Qu et al., 2012). The Genentech animal care and use committee approved all mouse protocols.

**Reagents.** Antibodies recognized NLRP3 (Cryo-2; Adipogene), FLAG (M2; Sigma-Aldrich), ASC (8E4.1; Genentech), caspase-1 (sc-514; Santa Cruz Biotechnology, Inc.), IL-1β (GTX74034; GeneTex), phospho-NLRC4 S533 (GEN-82 clone 3–3; Genentech), and NLRC4 (hamster monoclonal 1D4 for immunoprecipitation and a rabbit polyclonal for blotting; Genentech). Other reagents included Z-VA-D-FMK (MBL), \textit{S. typhimurium} flagellin (InvivoGen), LPS from \textit{Escherichia Coli O111:B4} (Invivogen), Pam3CSK4 (Invivo-gen), and DOTAP transfection reagent (Roche). LDH release was measured with a Cytotoxicity Detection kit\textsuperscript{plus} (Roche). Secreted IL-1β and TNF were measured with Meso Scale Discovery mouse kits.

**Macrophage cultures.** Bone marrow cells were cultured for 5–7 d in DMEM containing 20% L cell-conditioned medium, 10% heat-inactivated FBS, 100 U/ml penicillin, 10 mM l-glutamine, and 100 µg/ml streptomycin. BMMCs were replated in 6- or 24-well plates for experiments. BMDMs were primed with 0.5 µg/ml E. coli LPS serotype O1101:B4 for 30 min, DOTAP-transfected flagellin for 4 h, or 1 µg/ml Lipofectamine 2000-transfected pcDNA3.1 (Invitrogen) for 4 h. \textit{S. typhimurium} was from D. Monack (Stanford University). Bacteria were grown in LB overnight at 37°C and subcultured for 3–4 h before macrophage infection.

**Plasmids.** Mouse NLRC4 (wild-type or S533A) with a C-terminal FLAG tag was cloned into retroviral vector pMXGFP (Cell Biolabs). NLRC4\textsuperscript{NACHT-LRR-FLAG} and NLRC4\textsuperscript{CARD-FLAG} were generated in pcDNA3.1(-) (Life Technologies). NLRC4\textsuperscript{ΔLRR-FLAG} and NLRP3\textsuperscript{-eGFP} were generated in pHUSH-GFP (Genentech).

**Immunoprecipitation.** BMDM soluble lysates were incubated with anti-FLAG agarose or anti-NLRP3 antibody overnight at 4°C. Anti-NLRC4 antibody was added for 2 h at 4°C. NLRP3 or NLRC4 complexes were captured with protein A/G beads. Beads were washed three times with 20 mM Tris pH 7.5, 2.42 mM MgCl\textsubscript{2}, 92.5 mM NaCl, 0.1% Triton X-100 supplemented with protease and phosphatase inhibitors.

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