NAIP proteins are required for cytosolic detection of specific bacterial ligands in vivo

Isabella Rauch,1,2 Jeannette L. Tenthorey,1 Randlea D. Nichols,1 Khatoun Al Moussawi,1,2 James J. Kang,1 Chulho Kang,3 Barbara I. Kazmierczak,4,5 and Russell E. Vance1,2,3

1Division of Immunology and Pathogenesis, 2Howard Hughes Medical Institute, and 3Cancer Research Laboratory, University of California, Berkeley, Berkeley, CA 94720

Abbreviations used: BMM, BM-derived macrophage; IRES, internal ribosome entry site; LDH, lactate dehydrogenase; NAIP, NLR family, apoptosis inhibitory protein; NLR, nucleotide-binding domain leucine-rich repeat-containing proteins; PA, protective antigen; pVCI, polyinosinic:polycytidylic acid; sgRNA, single guide RNA; T3SS, type III secretion system.

1To whom correspondence should be addressed. Email: rvance@berkeley.edu

Recent biochemical data implicating NAIP6 in flagellin detection. Our results provide genetic evidence that specific NAIPs (NLR family, apoptosis inhibitory proteins) are NLRs that appear to function as cytosolic immunoreceptors for specific bacterial proteins, including flagellin and the inner rod and needle proteins of bacterial type III secretion systems (T3SSs). Despite strong biochemical evidence implicating NAIPs in specific detection of bacterial ligands, genetic evidence has been lacking. Here we report the use of CRISPR/Cas9 to generate Naip1Δ/Δ and Naip2Δ/Δ mice, as well as Naip1Δ/ΔΔ mice lacking all functional Naip genes. By challenging Naip1Δ/Δ or Naip2Δ/Δ mice with specific bacterial ligands in vivo, we demonstrate that Naip1 is uniquely required to detect T3SS needle protein and Naip2 is uniquely required to detect T3SS inner rod protein, but neither Naip1 nor Naip2 is required for detection of flagellin. Previously generated Naip5Δ/Δ mice retain some residual responsiveness to flagellin in vivo, whereas Naip1Δ/ΔΔ mice fail to respond to cytosolic flagellin, consistent with previous biochemical data implicating NAIP6 in flagellin detection. Our results provide genetic evidence that specific NAIP proteins function to detect specific bacterial proteins in vivo.

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Several distinct inflammasomes have been described (von Moltke et al., 2013; Lamkanfi and Dixit, 2014), each responsive to distinct stimuli. NAIP (NLR family, apoptosis inhibitory protein)/NLRC4 (NLR family, caspase activation and recruitment domain [CARD] containing 4) inflammasomes are activated by the cytosolic presence of specific bacterial proteins. These include flagellin, as well as bacterial type III secretion system (T3SS) inner rod or needle proteins. C57BL/6 (B6) mice harbor four functional Naip genes (Naip1, 2, 5, and 6) that are arranged in a cluster on chromosome 13 (Fig. 1A). Interestingly, the Naip locus is variable between inbred mouse strains. For example, 129 mice additionally express Naip4 and Naip7. Biochemical experiments suggest that individual NAIPs detect specific ligands. For example, in B6 mice, NAIP1 interacts with T3SS needle proteins (Zhao et al., 2011; Rayamajhi et al., 2013; Yang et al., 2013), NAIP2 interacts with T3SS inner rod proteins (Kofoed and Vance, 2011; Zhao et al., 2011; Suzuki et al., 2014; Tenthorey et al., 2014), and NAIP5 and NAIP6 interact with flagellin (Kofoed and Vance, 2011; Zhao et al., 2011; Tenthorey et al., 2014). Human NAIP recognizes T3SS needle and flagellin (Zhao et al., 2011; Yang et al., 2013; Kortmann et al., 2015). Upon engagement of their cognate ligands, NAIPs associate with NLRC4 to form a high-molecular-weight ring-shaped oligomer (Hu et al., 2015; Zhang et al., 2015). The assembled NAIP/NLRC4 inflammasome then recruits and activates CASP1 via the CARD in NLRC4.

Although NAIPs have been extensively characterized biochemically, genetic evidence they are essential for specific cytosolic detection of bacterial ligands is lacking. Classic genetic studies established a key role for Naip5 in the innate response to Legionella pneumophila (Wright et al., 2003; Diez et al., 2003), a response later shown to require flagellin expression by L. pneumophila (Molofsky et al., 2006; Ren et al., 2006). The role of NAIP5 in detection of L. pneumo-
mophila was confirmed by the generation of Naip5−/− mice (Lightfield et al., 2008). However, Naip5−/− macrophages still undergo Nlr-4-dependent pyroptosis after transduction with flagellin (Lightfield et al., 2008) or infection with Salmonella enterica serovar Typhimurium (S. Typhimurium), presumably because of functional compensation by other Naip family members (Kofoed and Vance, 2011; Sellin et al., 2014). In addition, Naip5−/− mice show a partial inflammasome response to FlaTox, a toxin fusion that delivers flagellin systemically into the cytosol (von Moltke et al., 2012). To date, no other individual Naip gene has been disrupted by gene targeting, possibly because of the repetitiveness of the Naip locus. Mice lacking the entire Naip locus were recently reported (Sellin et al., 2014; Allam et al., 2015) and demonstrated the essentiality of Naip genes in the NLRC4 inflammasome response to S. Typhimurium. However, the requirement of specific Naip genes in the response to specific bacterial ligands remains unclear.

Recently, genome engineering of mice using the CRISPR/Cas9 endonuclease from Streptococcus pyogenes has been described (Wang et al., 2013). As Cas9 is targeted to genomic loci via a short ~20-nucleotide sequence present in an associated guide RNA, Cas9 is ideally suited for genomic engineering of highly repetitive loci such as the Naip gene cluster. Here we describe our use of CRISPR/Cas9 to generate mice specifically lacking Naip1, Naip2, or all functional Naips. By challenging these mice with specific bacterial ligands, we provide genetic proof for the specificity and essentiality of NAIP proteins for the detection of cytosolic flagellin, T3SS needle, and T3SS rod proteins in vivo.

**RESULTS AND DISCUSSION**

**Generation of Naip-deficient mice using CRISPR/Cas9**

We designed a single guide RNA (sgRNA) that targets Cas9 to a 20-nucleotide sequence in exon 6 (the fourth coding exon) of all four functional Naip genes (Naip1, 2, 5, and 6) in the

![Image of CRISPR/Cas9 targeting strategy](Figure 1. CRISPR/Cas9 targeting strategy. (A) Naip gene cluster in B6 mice. Guide RNA sequence for CRISPR/Cas9 targeting and protospacer-adjacent motif (PAM) are indicated. (B) The Naip1 locus in WT and Naip1−/− mice. (C) The Naip2 locus in WT and Naip2−/− mice. (D) The Naip1 and Naip5 loci in WT and Naip1-6ΔΔ mice (left) and the Naip2 locus in WT and Naip1-6ΔΔ (right).)
C57BL/6j genome (Fig. 1 A). This sequence is also present in Naip3. However, a cDNA for Naip3 has not been characterized (Grownrey and Dietrich, 2000), and thus, Naip3 is considered a pseudogene in the current mouse genome assembly (GRChm38; annotation release 105). The guide RNA sequence was not present in delta-Naip, which is truncated and believed to be nonfunctional. Cas9-targeted founder mice were screened for Naip mutations by PCR and sequencing of exon 6, and pure homozygous Naip mutant lines were then established.

The Naip1Δ line contains a 23-bp frameshift deletion in Naip1 (Fig. 1 B), whereas the Naip2Δ line contains a 4-bp frameshift deletion in Naip2 (Fig. 1 C). These deletions are predicted to result in Naip proteins truncated before the NBD, a domain essential for NAIP function (Kofoed and Vance, 2011). No other Naip mutations were detected in these mouse lines. We failed to obtain mouse lines with specific modifications in only Naip5 or Naip6. However, we did obtain mice lacking all functional Naip genes. In these Naip1−ΔΔΔ mice, Naip2 was inactivated by a 4-bp frameshift deletion, whereas the sequence between Naip5 exon 6 and Naip1 exon 6 (including the entire Naip6, Naip3, and delta-Naip genes) was deleted, leading to a nonfunctional fusion of Naip5 and Naip1 (Fig. 1 D). Thus, we used a single Cas9/sgRNA complex to generate three different Naip mutant mouse lines.

**Response of Naip-deficient macrophages to specific bacterial ligands**

To determine whether Naips are required for inflammasome responses to specific bacterial ligands, we used a previously described retroviral lethality assay (Lightfield et al., 2008). In this assay, BM-derived macrophages (BMMs) are transduced with a retroviral construct encoding a specific NAIP ligand (flagellin, rod, or needle) upstream of an internal ribosome entry site (IRES)–GFP reporter. As representative ligands, we used retroviruses expressing flagellin (FlaA) from *L. pneumophila*, the T3SS inner rod (PrgJ) from *S. Typhimurium*, and the T3SS needle (BTH_RS04290) from *Burkholderia thailandensis*. WT BMMs transduced with these constructs activate the NAIP/NLRC4 inflammasome and undergo pyroptosis, resulting in few GFP+ cells. As a control, transduced Nlrc4ΔΔΔ cells fail to undergo pyroptosis and thus express bacterial ligands and GFP. This assay permits analysis of responses to individual bacterial ligands independent of other ligands or confounding stimuli present during bacterial infections.

As previously reported, most WT BMMs transduced with T3SS needle protein undergo pyroptosis, despite low expression of Naip1 in unprimed BMMs (Rayamajhi et al., 2013), whereas Nlrc4−/− cells are protected (Fig. 2, A and B). BMMs from Naip1−/− and Naip1−ΔΔΔ mice were also protected from needle-induced pyroptosis, whereas Naip2−/− and Naip5−/− cells were susceptible (Fig. 2, A and B). Conversely, Naip2−/− BMMs were protected from rod-induced pyroptosis but were susceptible to needle-induced pyroptosis (Fig. 2, A and B). Both Naip1−/− and Naip2−/− were fully susceptible to flagellin-induced pyroptosis. Flagellin–expressing retrovirus was consistently less efficiently transduced into cells than needle- or rod-retrovirus, likely because of the larger size of the flagellin protein. Only Nlrc4−/− and Naip1−ΔΔΔ BMMs were protected from all ligands, with Naip1−ΔΔΔ mice recapitulating the full protection observed in Nlrc4−/− cells (Fig. 2, A and B). These results demonstrate that endogenous NAIP proteins are essential for NLRC4-dependent pyroptosis in response to bacterial rod, needle, and flagellin proteins and, in addition, demonstrate that NAIP1 and NAIP2 are specifically required for NLRC4 activation in response to T3SS needle and rod, respectively.

**Response of Naip-deficient macrophages to bacterial infection**

To examine the role of Naip genes in pathogen detection, we infected BMMs with three bacterial pathogens known to activate the NLRC4 inflammasome and assessed pyroptosis-dependent release of intracellular lactate dehydrogenase (LDH). First we infected BMMs with a WT *S. Typhimurium* strain (LT2) encoding stimulatory T3SS rod and needle proteins and two flagellin proteins, FliC and FljB. Consistent with a previous study using an independent Naip1−ΔΔΔ mouse line (Allam et al., 2015), BMMs from our Naip1−ΔΔΔ mice showed reduced levels of cell death, equivalent to Nlrc4−/− BMMs. In contrast, Naip1−/− and Naip2−/− macrophages released amounts of LDH comparable with WT. Naip5−/− cells showed a slight, but statistically significant, protection from *S. Typhimurium*–induced pyroptosis (Fig. 3 A). These results suggest there is redundancy among various NAIPs for detection of *S. Typhimurium*. To simplify the repertoire of NAIP-stimulatory ligands expressed by *S. Typhimurium*, we used a fliC/fljB mutant strain that lacks flagellin expression. This strain induces overall lower levels of pyroptosis, possibly because of its reduced motility or infectivity or because recognition of flagellin (via NAIP5/6) contributes more significantly to pyroptosis than recognition of rod or needle. Nevertheless, Naip2−/− BMMs showed significantly reduced cell death in response to the fliC/fljB mutant strain, as compared with infected WT BMMs (Fig. 3 A), whereas loss of the flagellin sensor Naip5 had no effect, as expected. IF BMMs are prestimulated with polyinosinic :polycytidylic acid (polyIC) to induce Naip1 expression (as previously reported [Rayamajhi et al., 2013]), Naip1−/− BMMs show a slight, but significant reduction in cell death as compared with WT BMMs. These data indicate that multiple NAIPs participate in NLRC4 activation by *S. Typhimurium*.

We next infected BMMs with *L. pneumophila*, a flagellated pathogen that encodes a type 4 secretion system but lacks a T3SS and thus inner rod and needle expression. As reported previously, Nlrc4−/− and Naip5−/− macrophages are protected from cell death upon *L. pneumophila* infection (Fig. 3 B; Lightfield et al., 2011). The lack of redundancy with NAIP6 in this experiment (as compared with results in Fig. 2) is most likely because lower levels of flagellin released during bacterial infection, as compared with retroviral trans-
duction, increase the sensitivity to loss of Naip5. Naip1-6Δ/Δ BMMs are also protected from L. pneumophila–induced pyroptosis, whereas Naip1−/− and Naip2−/− cells release equal amounts of LDH as WT. Upon infection with an aflagellate strain of L. pneumophila (ΔflaA), no Naip/Nlrc4-dependent cell death could be observed, confirming flagellin is the only NAIP ligand in this pathogen. Plating of infected cell lysates for CFU showed that the decreased cell death in S. Typhimurium– or L. pneumophila–infected Naip- or Nlrc4-deficient cells was not caused by decreased intracellular replication of pathogens (not depicted).

Pseudomonas aeruginosa, like S. Typhimurium, is a T3SS+ flagellated pathogen that was previously shown to activate NLRC4 (Sutterwala et al., 2007). ExoU is a T3SS-translocated phospholipase that kills cells independently of NLRC4. Upon infection of BMMs with an ExoU-deficient strain of P. aeruginosa, only Nlrc4−/− and Naip1-6Δ/Δ cells were significantly protected from cell death (Fig. 3 C). These results indicate that multiple NAIPs are involved in sensing P. aeruginosa T3SS components and flagellin, similar to what we observed with S. Typhimurium infection.

Naip1 is required for detection of T3SS needle protein in vivo

In the aforementioned infection experiments, it was not possible to address the role of Naips in response to individual bacterial ligands because cytosolic delivery of ligands requires a functional T3SS system, which itself requires simultaneous expression of both the T3SS inner rod and needle proteins. The retroviral lethality assay can be used to express individual bacterial proteins in BMM, but not to address the function of Naips in vivo. Thus, to address whether Naips are required for Nlrc4 activation in response to specific bacterial ligands in vivo, we used a previously described system in which bacterial ligands are fused to the N-terminal domain of anthrax lethal factor (LFn). The LFn domain acts as a signal sequence that targets proteins for cytosoli-
lic translocation via the co-administered protective antigen (PA) channel protein (Ballard et al., 1996; Kofoed and Vance, 2011; Zhao et al., 2011). Neither PA nor LFn fusion proteins used individually activate NLRC4, whereas the combination of PA and LFn-flagellin or LFn-rod protein are potent NLRC4 activators (Kofoed and Vance, 2011; Zhao et al., 2011; von Moltke et al., 2012). We previously found that recombinant PA or LFn fusion proteins purified from bacteria were unavoidably contaminated with LPS, which affects the in vivo inflammasome response (von Moltke et al., 2012). Therefore, to reduce the levels of bacterial contaminants as much as possible, we expressed and purified PA and LFn fusions from insect cells. Consistent with endotoxin contamination not playing a role, MyD88/Trif-deficient mice responded to NeedleT ox and RodT ox as robustly as WT mice (not depicted).

We first used the LFn-PA system to deliver the T3SS system needle protein from B. thailandensis into BMMs pretreated with polyIC to induce Naip1 expression. As expected, we found that WT, Naip2<sup>−/−</sup>, or Naip5<sup>−/−</sup> cells released LDH, whereas Nlrc4<sup>−/−</sup>, Naip1<sup>−/−</sup>, and Naip1-6<sup>−/−</sup> BMMs were fully protected from pyroptosis (Fig. 4 A). To determine which NAIPs are responsible for detection of T3SS needle protein in vivo, we treated mice with NeedleT ox intravenously. Resident peritoneal macrophages, a major contributor to FlaT ox-induced pathology (von Moltke et al., 2012), express robust levels of Naip1 and Naip2 (Rayamajhi et al., 2013). Furthermore, Naip1, 2, 5, and 6 are expressed at high levels in the spleen and small and large intestine (Rayamajhi et al., 2013; Sellin et al., 2014; Allam et al., 2015). Indeed, similar to treatment with FlaT ox (von Moltke et al., 2012), WT mice treated with NeedleT ox exhibited a drastic loss of body temperature (Fig. 4 B) accompanied by a highly significant increase in hematocrit (%RBC volume of blood; Fig. 4 C). Naip2<sup>−/−</sup> or Naip5<sup>−/−</sup> mice showed the same pathological response as WT animals, whereas Nlrc4<sup>−/−</sup>, Naip1<sup>−/−</sup>, and Naip1-6<sup>−/−</sup> mice were completely protected from NeedleT ox. These data show that systemic activation of the NAIP1/NLRC4 inflammasome produces rapid pathology and that NAIP1 is specifically required for detection of T3SS needle protein in vivo.

**Naip2 is required for detection of T3SS inner rod protein in vivo**

We fused LFn to the inner rod protein (PrgJ) from the S. Typhimurium SPI-1 T3SS and combined the resulting LFn-rod fusion protein with recombinant PA (both purified from insect cells) to generate “RodT ox.” Upon treatment of BMMs with RodT ox, WT, Naip1<sup>−/−</sup>, and Naip5<sup>−/−</sup> BMMs underwent pyroptosis, whereas Nlrc4<sup>−/−</sup>, Naip1<sup>−/−</sup>, and Naip2<sup>−/−</sup> BMMs were significantly protected from cell death (Fig. 4 D). Upon intravenous delivery of RodT ox, WT, Naip1<sup>−/−</sup>, and Naip5<sup>−/−</sup> animals exhibited a rapid hypothermic response and hematocrit increase, whereas Nlrc4<sup>−/−</sup>, Naip1-6<sup>−/−</sup>, and Naip2<sup>−/−</sup> animals were completely protected (Fig. 4, E and F). These results demonstrate that NAIP2 is required for NLRC4 inflammasome activation in response to T3SS rod protein in vivo.

**Naip1 and Naip2 are dispensable for detection of flagellin in vivo**

We expressed a fusion of LFn to L. pneumophila flagellin in insect cells and combined the fusion protein with PA to generate...
FlaTox. Upon treatment of BMM with FlaTox, we observed that WT, Naip2−/−, and Naip1−/− cells all underwent comparable levels of pyroptosis, whereas Naip5−/− macrophages were partially, but significantly, protected (Fig. 5 A). In contrast, Nlrc4−/− and Naip1-6Δ/Δ cells were fully protected from FlaTox. These results demonstrate that NAIP1 and NAIP2 are not required for NLRC4 activation in response to flagellin and suggest the involvement of an NAIP protein other than NAIP5 in the detection of flagellin. This NAIP gene is most likely to be NAIP6 based on previous biochemical experiments and its high degree of sequence identity to NAIP5 (Kofoed and Vance, 2011; Zhao et al., 2011; Tenthorey et al., 2014).

Comparable with our in vitro results, Nlrc4−/− and Naip1-6Δ/Δ mice were fully protected from intravenous FlaTox treatment (Fig. 5, B–D). As previously observed, Naip5−/− mice showed a delayed loss of body temperature and a slight, but statistically significant increase in hematocrit compared with WT animals after 30 min (Fig. 5, B and C; von Moltke et al., 2012). At 90 min, when WT, Naip2−/−, and Naip1−/− animals had already succumbed to FlaTox, Naip5−/− exhibited a marked increase in hematocrit compared with Nlrc4−/− and Naip1-6Δ/Δ mice. Thus, NAIP5 and at least one other NAIP (likely NAIP6) are responsive to flagellin in vivo, whereas NAIP1 and NAIP2 are not required for NLRC4 activation in response to flagellin in vivo.

Collectively, our results provide unequivocal evidence for an essential role of NAIP proteins in NLRC4 inflammasome activation in response to specific bacterial ligands in vivo. In particular, we find that NAIP1 is required for T3SS needle detection, NAIP2 is required for T3SS inner rod detection, and NAIP5 participates in flagellin detection (most likely in conjunction with NAIP6). Our results provide genetic evidence that is entirely consistent with extensive prior biochemical and structural data. NLRC4 has previously been suggested also to require phosphorylation for its activation (Qu et al., 2012). Our data do not address this requirement, but do strongly imply that phosphorylation cannot circumvent the requirement for NAIPs in NLRC4 activation in response to bacterial ligands, consistent with previous conclusions (Matysiak et al., 2015). Lastly, our results illustrate the power of CRISPR/Cas9 to dissect genetically a highly repetitive gene cluster that had previously been technically difficult to target using conventional methodologies.

MATERIALS AND METHODS

Mouse experiments. All mice were specific pathogen free, maintained under a 12-h light–dark cycle (7 a.m. to 7 p.m.), and given a standard chow diet (Harlan irradiated laboratory animal diet) ad libitum. Naip5−/− mice on the B6 background...
ground were described previously (Lightfield et al., 2008). WT C57BL/6j mice were originally obtained from The Jackson Laboratory, and Nlrc4−/− animals were from V. Dixit (Genentech, South San Francisco, CA; Mariathasan et al., 2004). CRI SPR/Cas9 targeting was performed by pronuclear injection of Cas9 mRNA and sgRNA into fertilized zygotes from colony-born C57BL/6J mice, essentially as described previously (Wang et al., 2013). Founder mice were genotyped as described in Genotyping of Naip alleles, and founders carrying Naip mutations were bred one generation to C57BL/6J to separate modified Naip haplotypes. Homozygous lines were generated by interbreeding heterozygotes carrying matched Naip haplotypes. All animal experiments complied with the regulatory standards of, and were approved by, the University of California, Berkeley Institutional Animal Care and Use Committee.

Genotyping of Naip alleles. Exon 6 and the surrounding intronic regions were amplified by PCR using the following primers (all 5′ to 3′): Naip1 fwd, TCCGTGACCTGAGCA TCTA; and rev, CAAGTGCTTATAACTCCACCAT; Naip2 fwd, AAACGCATATAATCCTCCCAAAT; and rev, AGAGACAGAGACAGAGAC; Naip3 fwd, TGTTGGAA CTGCTATAATCCCT; and rev, AGACAGACAGACAGAC AGACA; Naip5 fwd, GCCCCGATCCCTCCTCCT GTGT; and rev, GAGGACACGGATTTAGTACAGT; and Naip6 fwd, TGAAGACAGGTACAGTGTTG; and rev, GTGGTGATGTTAGAAATGGG. All primers distinguish the specific Naip paralogues. Primers were used at 200 nM in a reaction with 2.5 mM MgCl2 and 75 µM dNTPs and 1 U Taq polymerase (Thermo Fisher Scientific) per reaction. Cleaned PCR products were diluted 1:16 and sequenced using Sanger sequencing (Berkeley DNA Sequencing facility).

Retroviral constructs and production. Genes encoding L. pneumophila flagellin (FlaA; accession YP_095369), S. Typhimurium T3SS inner rod protein (PrgJ, accession NP_461793), and B. thailandensis T3SS needle protein (BTH_RS04290; accession WP_009896110) were cloned into a replication-defective mouse stem cell retroviral construct (pMSCV2.2). Retroviral particles were generated with GP2-293 packaging cells and were used to transduce BM cells after 48 and 72 h of culture in macrophage colony-stimulating factor. Cells were analyzed 4 d after the first transduction.

Toxins. Recombinant proteins (PA, LFn-flagellin, LFn-needle, and LFn-rod) were purified from insect cells. His6-LFn fused to the N terminus of the indicated gene (Ser-Thr-Arg linker) or His6-PA was cloned into a FastBac Dual (Invitrogen) using the SalI and NotI restriction sites. Recombinant bacmids were generated to infect Sf9 cells according to the manufacturer’s instruction (pFastBac Dual; Invitrogen). After 72 h of infection with P2 baculovirus, insect cells were lysed with buffered detergent (1% NP-40 in 20 mM Tris and 150 mM NaCl, pH 8.0) and then clarified by centrifugation at 50,000 g for 45 min at 4°C. Proteins were purified using Ni-NTA agarose (Qiagen), eluted with 500 mM imidazole in 20 mM Tris and 500 mM NaCl, pH 8.0, and then dialyzed into 20 mM Tris, pH 8.0, and subjected to anion exchange chromatography (Enrich Q column; Bio-Rad Laboratories).
using a gradient elution into high-salt buffer (20 mM Tris and 1 M NaCl, pH 8.0). Fractions containing protein were concentrated using Amicon-Ultra centrifugal filters (EMD Millipore) and quantified using a BCA kit (Thermo Fisher Scientific). Endotoxin contamination of the preparations (determined using a LAL chromogenic endotoxin quantitation kit; Thermo Fisher Scientific) was estimated to result in application of ~0.2 ng/mouse (Needle and RodTox) or 0.4 ng/mouse (FlaTox) of endotoxin, an amount below the threshold for systemic responses in mice (Copeland et al., 2005).

Toxin doses were 0.8 μg/g body weight of PA combined with 0.8 μg/g LFn-Fla, 80 ng/g LFn-needle, or 1 ng/g LFn-rod for intravenous (retroorbital) delivery or 4 μg/ml PA combined with 4 μg/ml LFn-flagellin, 200 ng/ml LFn-needle, or 10 ng/ml LFn-rod for in vivo delivery. Rectal temperature was determined using a MicroTherma 2T thermometer (BrainTree Scientific). Blood for hematocrit was collected by retroorbital bleed into StatSpin microhematocrit tubes (Thermo Fisher Scientific).

Macrophages. BM was harvested from mouse femurs, and cells were differentiated into macrophages by culture in RPMI supplemented with L929 cell supernatant and 10% fetal bovine serum (20% for P. aeruginosa experiments) in a humidified incubator (37°C, 5% CO₂). Cells were primed with 5 μg/ml polyIC for 24 h before S. Typhimurium infection or LFn-needle/PA treatment and with 50 ng LPS 3 h before P. aeruginosa infection.

Bacterial strains. L. pneumophila strains LP02 and LP02ΔflaA have been described previously (Ren et al., 2006). S. Typhimurium strain LT2 and the flagellin-deficient mutant were gifts from A. Van der Velden and M. Starnbach (Harvard Medical School, Boston, MA). S. Typhimurium was grown overnight in Luria-Bertani broth and was then reinoculated at a dilution of 1:40 and grown at 37°C to mid-exponential phase (3 h) to induce expression of the Salmonella pathogenicity island 1 T3SS. P. aeruginosa PA103ΔU fla⁺ (Jain and Kazmierczak, 2014), an isogenic strain of PA103ΔU, a T3SS-positive, naturally flagellate laboratory strain (Liu, 1966), was freshly streaked to VBMM agar from ~80°C glycerol stocks before each experiment. Single colonies were used to inoculate 3 ml Lysogeny broth (LB) overnight cultures (37°C, 225 rpm). Overnight cultures were diluted 1:100 into 3 ml LB, grown at 37°C with shaking to an OD₆₀₀ of ~0.2–0.3. Bacteria were washed in PBS.

Cytotoxicity assays. Cytotoxicity was measured by evaluation of the activity of LDH released from macrophages (Lightfield et al., 2008). Overnight cultures of L. pneumophila in stationary phase or SPI1⁺ S. Typhimurium were added at an MOI of 5 to a 96-well plate containing 5 × 10⁶ macrophages per well. P. aeruginosa was used at an MOI of 20 in 24-well plates at 2 × 10⁶ macrophages/well. Plates were spun for 10 min at 250 g for similar infectivity of motile and nonmotile strains. For S. Typhimurium, 20 μg/ml gentamicin was added 30 min after infection to kill extracellular bacteria. Culture supernatants were assayed for LDH activity after 2 (P. aeruginosa), 4 (L. pneumophila), or 6 h (S. Typhimurium). Note that LT2 is a highly passaged S. Typhimurium strain with low SPI1 expression, therefore necessitating analysis at a relatively late time point. Infection-specific lysis was calculated as the percentage of detergent-lysed macrophages.

ACKNOWLEDGMENTS
We acknowledge the assistance of the University of California, Berkeley Cancer Research Laboratory Flow Cytometry Laboratory, Eric Chen and Peter Dietzen for technical support, and the Vance and Barton Labs for discussions.

This work was supported by National Institutes of Health (NIH) grants to R.E. Vance (AI075039 and AI063302). R.E. Vance is a Burroughs Wellcome Fund Investigator in the Pathogenesis of Infectious Disease and an Investigator of the Howard Hughes Medical Institute. B.I. Kazmierczak is supported by an NIH grant (AI081825) and I. Rauch by the Austrian Science Fund (FWF; the Erwin Schrödinger Fellowship J3789-B22).

The authors declare no competing financial interests.

Submitted: 17 November 2015
Accepted: 16 March 2016

REFERENCES
Allam, R., M.H. Maillard, A. Tardivel, V. Chenupati, H. Bega, C.W. Yu, D. Velin, P. Schneider, and K.M. Maslowski. 2015. Epithelial NAIPs protect against colonic tumorigenesis. J. Exp. Med. 212:369–383. http://dx.doi.org/10.1084/jem.20140474
Ballard, J.D., R.J. Collier, and M.N. Starnbach. 1996. Anthrax toxin-mediated delivery of a cytotoxic T-cell epitope in vivo. Proc. Natl. Acad. Sci. USA. 93:12531–12534. http://dx.doi.org/10.1073/pnas.93.22.12531
Copeland, S., H.S. Warren, S.F. Lowry, S.E. Calvano, and D. Remick. Inflammation and the Host Response to Injury Investigators. 2005. Acute inflammatory response to endotoxin in mice and humans. Clin. Diag. Lab. Immunol. 12:60–67.
Duez, E., S.-H. Lee, S. Gauthier, Z. Yaraghi, M. Tremblay, S. Vidal, and P. Gros. 2003. Bin 1e is the gene within the Lgn1 locus associated with resistance to Legionella pneumophila. Nat. Genet. 33:55–60. http://dx.doi.org/10.1038/ng1065
Gronwey, J.D., and W.F. Dietrich. 2000. High-resolution genetic and physical map of the Lgn1 interval in C57BL/6J implicates Naip2 or Naip5 in Legasellna pneumophila pathogenesis. Genome Res. 10:1158–1171. http://dx.doi.org/10.1101/gr.10.8.1158
Hu, Z., Q. Zhou, C. Zhang, S. Fan, W. Cheng, Y. Zhao, F. Shao, H.-W. Wang, S.-F. Sui, and J. Chai. 2015. Structural and biochemical basis for induced self-propagation of NLR.C4. Science. 350:399–404. http://dx.doi.org/10.1126/science.aac5489
Jain, R., and B.I. Kazmierczak. 2014. A conservative amino acid mutation in NLRC4 self-propagation of NLRC4. Science. 345:399–404. http://dx.doi.org/10.1126/science.aac5489
Jain, R., and B.I. Kazmierczak. 2014. A conservative amino acid mutation in NLRC4. Science. 345:399–404. http://dx.doi.org/10.1126/science.aac5489
Kofoed, E.M., and R.E. Vance. 2011. Innate immune recognition of bacterial ligands by NAIPs determines inflammasome specificity. Nature. 477:592–595. http://dx.doi.org/10.1038/nature10394
Kortmann, J., S.W. Brubaker, and D.M. Monack. 2015. Cutting edge: Inflammasome activation in primary human macrophages is dependent on flagellin. J. Immunol. 195:815–819. http://dx.doi.org/10.4049/jimmunol.1403100
Lambkin, M., and V.M. Dixit. 2014. Mechanisms and functions of inflammasomes. Cell. 157:1013–1022. http://dx.doi.org/10.1016/j.cell.2014.04.007
Lightfield, K.L., J. Persson, S.W. Brubaker, C.E. Witte, J. von Molkke, E.A. Dunipace, T. Henry, Y.-H. Sun, D. Cado, W.F. Dietrich, et al. 2008. Critical function for Naip5 in inflammasome activation by a conserved carboxy-terminal domain of flagellin. Nat. Immunol. 9:1171–1178. http://dx.doi.org/10.1038/ni.1646

Lightfield, K.L., J. Persson, N.J. Trinidad, S.W. Brubaker, E.M. Kofoed, J.-D. Sauer, E.A. Dunipace, S.E. Warren, E.A. Miao, and R.E. Vance. 2011. Differential requirements for Naip5 in activation of the NLRc4 inflammasome. Infect. Immun. 79:1606–1614. http://dx.doi.org/10.1128/IAI.01187-10

Liu, P.V. 1966. The roles of various fractions of Pseudomonas aeruginosa in its pathogenesis. 3. Identity of the lethal toxins produced in vitro and in vivo. J. Infect. Dis. 116:481–489. http://dx.doi.org/10.1093/infdis/116.4.481

Marathi, S., K. Newton, D.M. Monack, D. Vucic, D.M. French, W.P. Lee, M. Roose-Girma, S. Erickson, and V.M. Dixit. 2004. Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. Nature. 430:213–218. http://dx.doi.org/10.1038/nature02664

Matusiak, M., N. Van Opherd, L. Vande Walle, J.-C.Sirard, T.-D. Kanneganti, and M. Lamkanfi. 2015. Flagellin-induced NLRc4 phosphorylation primes the inflammasome for activation by Naip5. PNAS. Natl. Acad. Sci. USA. 112:1541–1546. http://dx.doi.org/10.1073/pnas.1417945112

Molofsky, A.B., B.G. Byrne, N.N. Whitfield, C.A. Madigan, E.T. Fuse, K. Tateda, and M.S. Swanson. 2006. Cytosolic recognition of flagellin by mouse macrophages restricts Legionella pneumophila infection. J. Exp. Med. 203:1093–1104. http://dx.doi.org/10.1084/jem.20051659

Qu, Y., S. Misaghi, A. Izrael-Tomasevic, K. Newton, L.L. Gilmour, M. Lamkanfi, S. Louie, N. Kayagaki, J. Liu, L. Kömüves, et al. 2012. Phosphorylation of NLRc4 is critical for inflammasome activation. Nature. 490:539–542. http://dx.doi.org/10.1038/nature11429

Rayamajhi, M., D.E. Zak, J. Chavarria-Smith, R.E. Vance, and E.A. Miao. 2013. Cutting edge: Mouse Naip1 detects the type III secretion system needle protein. J. Immunol. 191:3986–3989. http://dx.doi.org/10.4049/jimmunol.1301549

Ren, T., D.S. Zamboni, C.R. Roy, W.F. Dietrich, and R.E. Vance. 2006. Flagellin-deficient Legionella mutants evade caspase-1- and Naip5-mediated macrophage immunity. PLoS Pathog. 2:e18. http://dx.doi.org/10.1371/journal.ppat.0020018

Sellin, M.E., A.A. Müller, B. Felmy, T. Dolowischak, M. Diard, A. Tanivel, K.M. Masiowska, and W.-D. Harz. 2014. Epithelium-intrinsic Naip/NLRc4 inflammasome drives infected enterocyte expulsion to restrict Salmonella replication in the intestinal mucosa. Cell Host Microbe. 16:237–248. http://dx.doi.org/10.1016/j.chom.2014.07.001

Sutterwala, E.S., L.A. Mijares, L. Li, Y. Ogura, B.I. Kuzmierczak, and R.A. Flavell. 2007. Immune recognition of Pseudomonas aeruginosa mediated by the IPAf/NLRc4 inflammasome. J. Exp. Med. 204:3235–3245. http://dx.doi.org/10.1084/jem.20071239

Suzuki, S., L. Franchi, Y. He, R. Muñoz-Planillo, H. Mimuro, T. Suzuki, C. Sasakiwa, and G. Núñez. 2014. Shigella type III secretion protein MxiI is recognized by Naip2 to induce Nlr4 inflammasome activation independently of Pkcs5. PLoS Pathog. 10:e1003926. http://dx.doi.org/10.1371/journal.ppat.1003926

Tenthorey, J.L., E.M. Kofoed, M.D. Daugherty, H.S. Malik, and R.E. Vance. 2014. Molecular basis for specific recognition of bacterial ligands by NAIP/NLRc4 inflammasomes. Mol. Cell. 54:17–29. http://dx.doi.org/10.1016/j.molcel.2014.02.018

von Molkke, J., N.J. Trinidad, M. Moayeri, A.F. Kintzer, S.B. Wang, N. van Rooyen, C.R. Brown, B.A. Krantz, S.H. Leppla, K. Gronert, and R.E. Vance. 2012. Rapid induction of inflammatory lipid mediators by the inflammasome in vivo. Nature. 490:107–111. http://dx.doi.org/10.1038/nature11351

von Molkke, J., J.S. Ayres, E.M. Kofoed, J. Chavarria-Smith, and R.E. Vance. 2013. Recognition of bacteria by inflammasomes. Annu. Rev. Immunol. 31:73–106. http://dx.doi.org/10.1146/annurev-immunol-032711-135049

Wang, H., H. Yang, C.S. Shivalka, M.M. Dawlaty, A.W. Cheng, F. Zhang, and R. Jaenisch. 2013. One-step generation of mice carrying mutations in multiple genes by CRISPR-Cas-mediated genome engineering. Cell. 153:910–918. http://dx.doi.org/10.1016/j.cell.2013.04.025

Wright, E.K., S.A. Goodart, J.D. Growney, V. Hadinoto, M.G. Endrizzi, E.M. Kofoed, J. Chavarría-Smith, and R.E. Vance. 2013. Recognition of bacteria by inflammasomes. Annu. Rev. Immunol. 31:91–118. http://dx.doi.org/10.1146/annurev-immunol-032711-135049

Yang, J., J.Y. Zhao, J. Shi, and F. Shao. 2013. Human NAIP and mouse Naip1 recognize bacterial type III secretion needle protein for inflammasome activation. Proc. Natl. Acad. Sci. USA. 110:14408–14413. http://dx.doi.org/10.1073/pnas.1306376110

Zhang, L., S. Chen, J. Ruan, J.W., A.B. Tong, Q. Yin, Y. Li, L. David, A. Lu, W.L. Wang, et al. 2015. Cryo-EM structure of the activated Naip2-Nlrc4 inflammasome reveals nucleated polymerization. Science. 350:404–409. http://dx.doi.org/10.1126/science.aac5789

Zhou, Y., J. Yang, J. Shi, Y.-N. Gong, Q. Lu, H. Xu, L. Liu, and F. Shao. 2011. The NLRc4 inflammasome receptors for bacterial flagellin and type III secretion apparatus. Nature. 477:596–600. http://dx.doi.org/10.1038/nature09594