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The NLRP12 Inflammasome Recognizes *Yersinia pestis*

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SUMMARY

***Yersinia pestis*, the causative agent of plague, is able to suppress production of inflammatory cytokines IL-18 and IL-1β, which are generated through caspase-1-activating nucleotide-binding domain and leucine-rich repeat (NLR)-containing inflammasomes. Here, we sought to elucidate the role of NLRs and IL-18 during plague. Lack of IL-18 signaling led to increased susceptibility to *Y. pestis*, producing tetra-acylated lipid A, and an attenuated strain producing a *Y. pseudotuberculosis*-like hexa-acylated lipid A. We found that the NLRP12 inflammasome was an important regulator controlling IL-18 and IL-1β production after *Y. pestis* infection, and NLRP12-deficient mice were more susceptible to bacterial challenge. NLRP12 also directed interferon-γ production via induction of IL-18, but had minimal effect on signaling to the transcription factor NF-κB. These studies reveal a role for NLRP12 in host resistance against pathogens. Minimizing NLRP12 inflammasome activation may have been a central factor in evolution of the high virulence of *Y. pestis*.**

INTRODUCTION

Inflammasomes are multimolecular complexes consisting of inactive pro-caspase-1 and members of the nucleotide-binding domain-leucine-rich repeat (NLR) family of immune system proteins (Latz, 2010). The assembly of an inflammasome leads to proteolytic activation of caspase-1, which in turn cleaves pro-interleukin (IL)-1β and pro-IL-18 into mature forms (Latz, 2010). Active IL-1β and IL-18 are essential members of host defenses toward various pathogens and may also participate in sterile inflammatory processes. The NLR family has more than 20 members; however, many of these proteins have unknown functions (Martinon et al., 2009), and their relative roles in promoting resistance to infection are in many instances unclear. There is evidence supporting a function in bacterial recognition for several NLRs. These include NOD1/2 (recognizing peptidoglycan fragments) (Martinon et al., 2009), NLRP1 (sensing anthrax lethal toxin) (Averette et al., 2009), NLRP3 (activated by exposure to many pathogens, bacterial RNA, toxins, and crystal structures) (Davis et al., 2011; Duewell et al., 2010; Halle et al., 2008; Hornung et al., 2008; Kanneganti et al., 2006; Sander et al., 2011), NLRC4 (sensing of *Salmonella*, intracellular flagellin and bacterial type III secretion rod proteins) (Franchi et al., 2006; Miao et al., 2010), and Naip5 (promoting resistance to *Legionella*) (Kofoed and Vance, 2011; Molofsky et al., 2006; Ren et al., 2006). Recent results also suggested a role for NLRP6 in maintenance of bacterial homeostasis in the colon and for NLRP7 in the recognition of lipoproteins (Khare et al., 2012). NLRP12 (also called Nalp12, Monarch-1, and Pypaf-7) was the first NLR shown in biochemical assays to interact with the adaptor protein Asc to form an active IL-1β-maturing inflammasome (Wang et al., 2002). The role of NLRP12 in innate immunity has remained unclear. Both inflammatory and inhibitory functions have been suggested, as has a role in hypersensitivity (Allen et al., 2012; Arthur et al., 2010; Lich and Ting, 2007; Lich et al., 2007; Wang et al., 2002; Zaki et al., 2011). Interestingly, like for NLRP3, mutations in NLRP12 are linked to hereditary inflammatory disease (Jéru et al., 2008), and mutations may lead to increased Asc speckle formation and caspase-1 activity (Jéru et al., 2011b). It has been reported that patients carrying NLRP12 mutations associated with increased inflammasome activation have been successfully treated with anti-IL-1 therapy, similar to patients containing mutations in NLRP3 (Hawkins et al., 2003; Jéru et al., 2011a; Lachmann et al., 2009). No previous studies have addressed the role of NLRP12 in host resistance to infectious agents.

Evading innate immunity early in infection plays a key role in virulence of many microorganisms including the plague bacillus *Yersinia pestis* (Cornelis, 2000; Perry and Fetherston, 1997; Stenseth et al., 2008). This pathogen has several means of minimizing immune activation (Lathem et al., 2007; Monack et al., 1998; Mukherjee et al., 2006; Sodeinde et al., 1992; Zhou et al., 2005), with the effect that bacterial replication can proceed with minimal interference by the immune system. As a result,

plague is often characterized by very high bacterial numbers in patient sera and organs (Perry and Fetherston, 1997). Major factors neutralizing host defenses by active means include a complex type III secretion system (T3SS) (Cornelis, 2002; Perry and Fetherston, 1997), the plasminogen activator Pla (Lathem et al., 2007; Sodeinde et al., 1992), and a high-affinity iron acquisition system (Perry and Fetherston, 1997). The *Yersinia* T3SS delivers effector proteins, which disrupt signaling within the host cell to prevent phagocytosis, induce apoptosis, and evade the immune response (Cornelis, 2002). Many Gram-negative bacteria, including *Y. pseudotuberculosis*, a very close ancestor of *Y. pestis*, produce a hexa-acylated lipid A and LPS, which has the potential of strongly triggering innate immunity via Toll-like receptor 4 (TLR4)-MD-2 signaling (Munford, 2008; Raetz et al., 2007; Rebeil et al., 2004; Therisod et al., 2002). In contrast, *Y. pestis* generates a tetra-acylated lipid A-LPS that poorly induces TLR4-mediated cellular activation (Kawahara et al., 2002; Knirel et al., 2005; Montminy et al., 2006; Rebeil et al., 2006). We have reported that expression of *E. coli lpxL* in *Y. pestis*, which lacks a homolog of this gene, forces the biosynthesis of a hexa-acylated LPS (Montminy et al., 2006) and that this single modification dramatically reduces virulence in wild-type mice, but not in mice lacking a functional TLR4. This emphasizes that avoiding activation of innate immunity is important for *Y. pestis* virulence. It also provides a model in which survival is strongly dependent on innate immune defenses, presenting a unique opportunity for evaluating relative importance of innate immunity signals in protection against bacterial infection.

One implication of TLR4 engagement is the induction of the immature forms of the central proinflammatory cytokines IL-1β and IL-18. TLR4 signaling can also promote expression of inflammasome components such as *Nlrp3* (Bauernfeind et al., 2009). This establishes links between TLR4 activation and the inflammasome pathways. In this study, we have used wild-type *Y. pestis* and attenuated strains expressing a strong TLR4-activating hexa-acylated LPS as a model system to investigate the involvement of NLRP12 in pathogen recognition and IL-18 - IL-1β release.

Here, we show that NLRP12 is an inflammasome component that is central in the recognition of *Y. pestis* and that IL-18 signaling substantially contributes to resistance against bacteria. Compared to wild-type mice, NLRP12-deficient animals had higher mortality and increased bacterial loads after infection, correlated with lower amounts of IL-18, IL-1β, and IFN-γ. We propose a role for NLRP12 in the sensing of microbial pathogens.

RESULTS

IL-18 Signaling Is Essential for Resistance to Attenuated *Y. pestis*

We have found that all members of the genus *Yersinia* other than *Y. pestis*, and including the very closely related *Y. pseudotuberculosis*, contain the *lpxL* gene (S. Paquette et al., unpublished data). Absence of *lpxL* and the resulting production of a tetra-acylated LPS was proposed to be essential for *Y. pestis* virulence (Montminy et al., 2006). To study the evasion of TLR4 signaling in an evolutionary perspective, we cloned *lpxL* from the closely related *Y. pseudotuberculosis* and expressed it in *Y. pestis*, generating *Y. pestis*-pYtbLpxL, to determine its effects on virulence. *Y. pestis* grown at 37°C has a tetra-acylated lipid A (Figure S1A available online) (Montminy et al., 2006), whereas *Y. pseudotuberculosis* and *Y. pestis*-pYtbLpxL have a hexa-acylated lipid A (Figure S1B). Mice infected subcutaneously (s.c.) with 500 colony forming units (CFUs) of highly virulent *Y. pestis* KIM1001 rapidly succumb to infection (Figure 1A). All wild-type mice infected with KIM1001-pYtbLpxL expressing a hexa-acylated *Y. pseudotuberculosis*-like lipid A survived (Figure 1A), and the animals were protected toward challenge with virulent KIM1001 (Table S1). Survival of mice was strongly TLR4 dependent (Figure 1A). To determine the pathways responsible for in vivo clearance, we infected mice from several strains deficient in inflammatory cytokines or cytokine receptors s.c. with 500 CFUs of KIM1001-pYtbLpxL (Figure 1B). Interestingly, 100% of the animals lacking IL-18 and IL-18R died, as did the TLR4-deficient mice and 70% of the IL-1R1-deficient mice. Weaker effects were observed in animals lacking IFN-αβR, TNFR1, or IL-12p40 (Figure 1B). Resistance to infection in IL-1β- and IL-1R1-deficient animals was reduced to a similar degree, with ~30% of animals surviving (Figure 1C). However, IL-18 was critically important for resistance to infection in this model, given that IL-18 and IL-18R-deficient mice developed symptoms of bubonic plague and rapidly succumbed to disease when infected with KIM1001-pYtbLpxL (Figures 1B and 1D). Because inflammasomes are responsible for processing of IL-18 and IL-1β into mature forms, this result indicates that this infection model is well-suited for the study of inflammasome mechanisms and implications of IL-18 release. Mice deficient in MyD88, an adaptor molecule common to TLR, IL-1R, and IL-18R signaling pathways, were more susceptible to wild-type *Y. pestis* KIM1001 than wild-type C57Bl/6 mice (Figure S1C) and are also highly susceptible to strains expressing *lpxL* (Montminy et al., 2006). Intravenous (i.v.) infection causes systemic infection even when attenuated bacterial strains are used; hence the inflammatory capacity in tissues for various bacterial strains can better be compared with this route of delivery. We found elevated levels of spleen IL-1β and IL-18 after i.v. infection with *Y. pestis* and fully virulent KIM1001-induced lower cytokine levels as compared to KIM1001-pYtbLpxL producing the potent LPS (Figures 1E and 1F). A similar release pattern could also be seen in vitro with bone marrow-derived macrophages (BMDMs) (Figure 1G) after stimulation with KIM5 (a *pgm* mutant attenuated strain used for in vitro experiments) or KIM5-pYtbLpxL. Immunoblot analysis (Figure 1H) indicated that pro-IL-1β was indeed cleaved into mature IL-1β after infection with *Y. pestis* strains, a sign of inflammasome action. Infection with the *Y. pestis*-YtbLpxL strain markedly increased levels of pro- and cleaved IL-1β. These results indicate that minimizing inflammasome priming may have been an important implication of *lpxL* loss during evolution of *Y. pestis* from *Y. pseudotuberculosis*.

NLRP12 Is Involved in Recognition of *Y. pestis*

We next wanted to determine which NLRs were involved in resistance to *Y. pestis* strains and in IL-18 and IL-1β release. NLRP12 and NLRP3 have both been shown to interact with Asc in generating an IL-1β-processing inflamammasome

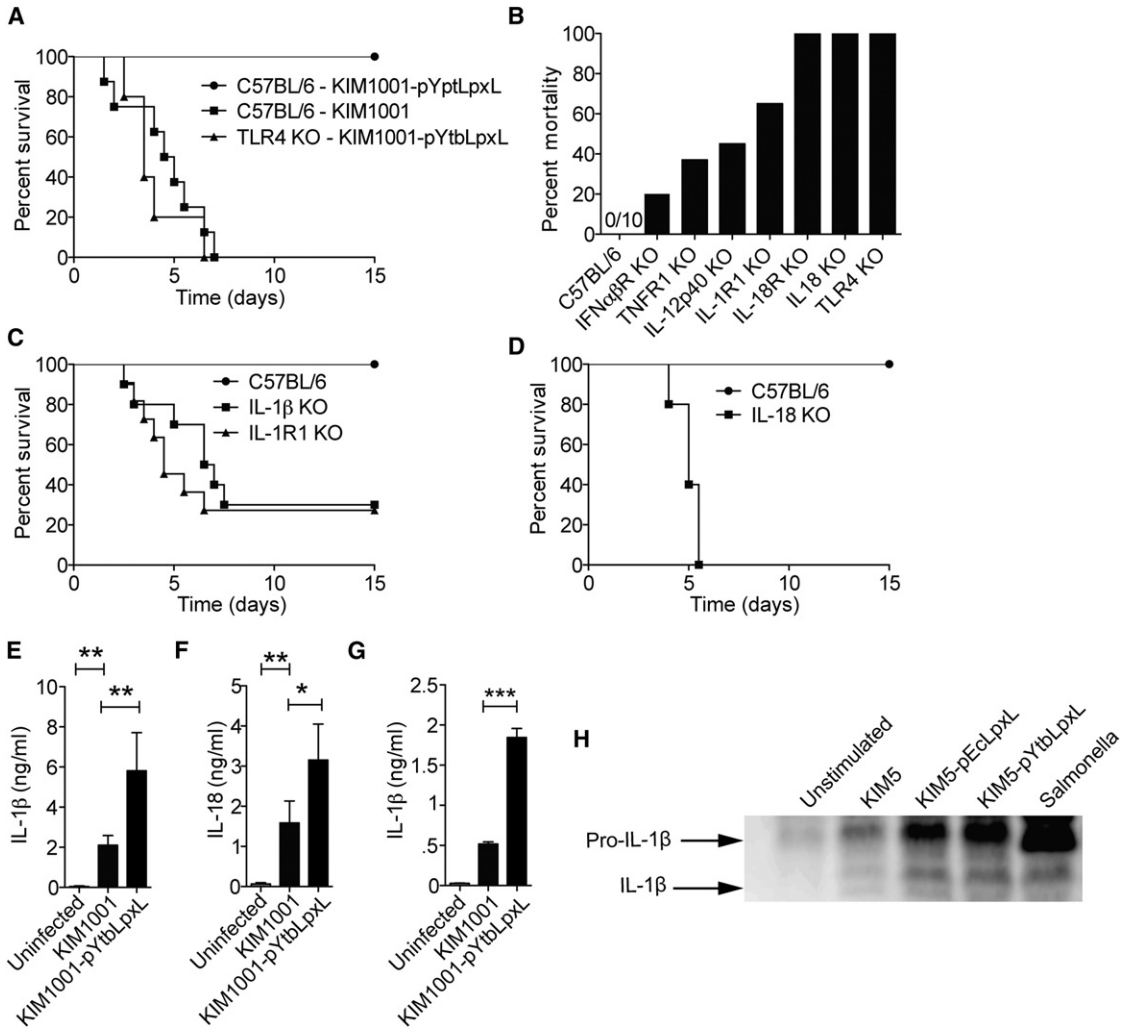

Figure 1. Infection of Mice with *Y. pestis*-pYtbLpxL Is Controlled by IL-18

(A) Survival of mice infected s.c. with 500 CFUs of *Yersinia pestis* KIM1001 (C57Bl/6: n = 8) or KIM1001-pYtbLpxL (C57Bl/6 and *Tlr4*$^{-/-}$ [TLR4 KO]: n = 10) bacteria.

(B) Mortality of animals infected s.c. with 500 CFUs of KIM1001-pYtbLpxL (n = 7 for *Ifnar*$^{-/-}$ [IFNαβR KO], 8 for *Il12b*$^{-/-}$ [IL-12p40 KO], 10 for C57Bl/6, *Il1r1*$^{-/-}$ [IL-1R1 KO], *Il18r1*$^{-/-}$ [IL-18R KO], *Il18*$^{-/-}$ [IL-18 KO], and TLR4 KO). Statistical differences in TLR4, IL-18, or IL-18R versus IL-1R and other strains: $p < 0.002$. Statistical differences in IL-12p40, IL-18, IL-18R, and TLR4 versus C57Bl/6: $p < 0.001$.

(C and D) Survival of mice deficient in (C) IL-1β (*Il1b*$^{-/-}$, IL1β KO) and IL-1R and (D) IL-18 infected s.c. with 500 CFUs of KIM1001-pYtbLpxL (n = 10 of each genotype).

(E and F) Concentrations of spleen IL-1β and IL-18 from C57BL/6 mice infected i.v. with 500 CFU of KIM1001 or KIM1001-pYtbLpxL for 44 hr.

(G) IL-1β in supernatants from BMDM stimulated with 10 multiplicity of infection (m.o.i.) of KIM5 or KIM5-pYtbLpxL for 6 hr, 50 μg/ml of gentamicin was added to wells after 3 hr.p.i.; error bars represent the SD.

(H) Immunoblot of IL-1β in the combined lysates and supernatants of BMDMs stimulated with 10 m.o.i. of *Y. pestis* KIM5, KIM5-pEcLpxL, and KIM5-pYtbLpxL and 1 m.o.i. of *Salmonella typhimurium*. Both pro-IL-1β (upper band) and mature IL-1β (lower band) are shown. Shown is representative of three to five performed experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Also see Figure S1 and Table S1.

(Agostini et al., 2004; Manji et al., 2002; Wang et al., 2002), but little is known of the role of NLRP12 during infection. We infected both NLRP3-deficient and NLRP12-deficient mice (Figures 2A and 2B) s.c. with 500 CFUs of KIM1001-pYtbLpxL and found that only 20% of NLRP12-deficient mice survived the infection, whereas ~50% of mice lacking NLRP3 survived. This suggests that NLRP12 plays an important role in host defense against some bacterial pathogens. In contrast, NLRP12-deficient mice were resistant to infection with *Salmonella typhimurium*, whereas TLR4-deficient mice all succumbed to the infectious challenge (Figure 2C). This indicates that NLRP12 deficient animals are not universally more sensitive to infections. The function of NLRP12 is not well understood, but mRNA is detectable in several organs and immune cells (Figures S2A and S2B), including macrophages, although prolonged macrophage maturation led to a decrease in expression (Figure S2C). NLRP12-deficient mice (Figure S2D) had a normal composition of cell populations in spleen and bone marrow (Figure S2E).

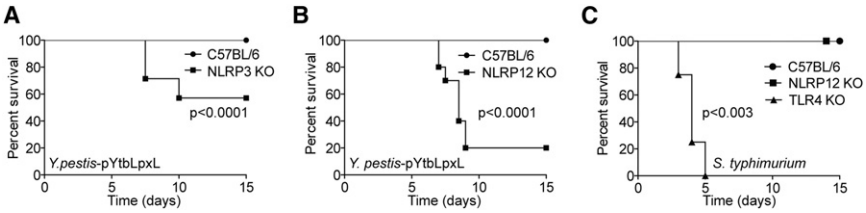

Figure 2. NLRP12 Is Involved in Host Resistance to Attenuated *Y. pestis*

(A and B) Survival of C57Bl/6 (circles), (A) *Nlrp3*$^{-/-}$ (NLRP3 KO, squares), and (B) *Nlrp12*$^{-/-}$ (NLRP12 KO, squares) mice infected s.c. with 500s CFU of KIM1001-pYtbLpxL.

(C) Survival of C57BL/6 (circles), NLRP12 KO (squares), or TLR4 KO (triangle) mice infected i.p. with 500 CFUs of *S. typhimurium*; $p < 0.003$ (NLRP12 KO or WT versus TLR4 KO). Also see Figure S2.

The possible involvement of NLRP12 in maturation of IL-1β and IL-18 led us to perform in vitro experiments with mouse cells to study inflammasome components that promote caspase-1 cleavage and IL-1β-IL-18 release after infection with *Y. pestis* and modified strains. Neutrophils express more *Nlrp12* than macrophages (Figure S2B), but the role of inflammasomes in pathogen-induced neutrophil release of IL-1β and IL-18 is not yet studied in detail for many microbes. We found that thioglycollate-elicited neutrophil-enriched peritoneal cells released IL-1β after *Y. pestis* infection (Figure 3A). When compared to cells from wild-type mice, the amounts of IL-1β, but not TNF (Figure S3A) released from the neutrophils lacking NLRP12, were markedly reduced after stimulation with *Y. pestis* strains. Moreover, infected neutrophils from the caspase-1-deficient mice lack IL-1β in the supernatant, suggesting that *Y. pestis*-induced neutrophil IL-1β release involves caspase-1 inflammasomes, although we cannot rule out a role for other neutrophil proteases (Netea et al., 2010). It is also unclear which role caspase-11 plays relative to caspase-1 in *Y. pestis*-induced inflammasome activation, given that the caspase-1-deficient mice utilized in this study contain the same truncated and apparently nonfunctional caspase-11 as previously published (Kayagaki et al., 2011). Macrophages deficient in NLRP12 or NLRP3 also had a reduced ability to release both IL-18 and IL-1β after infection with parental *Y. pestis* and *Y. pestis*-pYtbLpxL (Figures 3B and 3C). These observations are consistent with the survival data (Figure 2), which indicated that host recognition of *Y. pestis* involves NLRP12. Cells deficient in Asc and caspase-1 also had decreased IL-18 and IL-1β release (Figures 3B and 3C). Thus, NLRP12 signaling may occur parallel to or in cooperation with additional inflammasome components because NLRP12 deficiency did not completely block cytokine release. NLRP12 KO macrophages responded normally to alum, *S. typhimurium* (Figure 3C), nigericin, and poly(dA:dT) (Figures S3B and S3C), suggesting that NLRP12 may not participate in NLRP3, AIM2, or NLRC4 inflammasomes formed in response to those stimuli. None of the inflammasome proteins had an impact on TNF release (Figure 3D; Figure S3). Furthermore, NLRP12 deficiency had little impact on the expression of 31 selected macrophage genes, including *Il1b*, in the absence or presence of bacteria (Figure S3C). Many of those genes are controlled by NF-kB and/or MAP kinases. In a more detailed study, NF-kB signaling measured by IKK kinase assay and I-kB degradation was also largely preserved in NLRP12-deficient cells (Figures S3D and S3E). *Y. pestis* pregrown at 26°C naturally expresses a hexaacylated LPS (Montminy et al., 2006), and release of IL-1β in response to infection by 26°C grown bacteria was also influenced by NLRP12 (Figure S3B). Upon infection of wild-type and NLRP12 KO BMDMs with the human pathogens *Y. pseudotuberculosis* and *Y. enterocolitica*, ancestors of *Y. pestis* (Chain et al., 2004), we observed a reduction in secreted IL-1β from the cells lacking NLRP12 (Figure 3E), although TNF release was normal (Figure S3F). By using KIM6, a derivative of KIM5 that lacks the pCD1 virulence plasmid containing genes for the T3SS (Perry and Fetherston, 1997), we found that the secretion system was necessary for stimulating IL-1β release, even in the presence of a highly stimulatory LPS as found in KIM6-pYtbLpxL (Figure 3F). YopJ may participate in inflammasome activation (Zheng et al., 2011), and the deletion of YopJ or the T3SS translocon protein YopB reduced IL-1β release (Figure 3G). Experiments performed using a strain with the expression of *lpxL* on a YopJ mutant background suggested that YopJ is a key player controlling IL-1β release, even in the presence of a stimulatory LPS (Figure 3H), although other T3SS-dependent factors may also regulate IL-1β (Brodsky et al., 2010). The data suggest that the ligand(s) responsible for NLRP12 activation are dependent on the *Yersinia* T3SS. TLR4 plays a critical role in the IL-1β and IL-18 production after infection of the mouse macrophages (Figure 3I; Figure S3G), although the relative importance of mouse versus human TLR4-MD-2 in inducing *Y. pestis* responses may differ. Rodent cells have higher ability to recognize hypoacylated lipid A (Lien et al., 2000; Montminy et al., 2006). This might be influenced by a shallow positioning of the hypoacylated lipid A in mouse MD-2 compared to human MD-2, and the enabling of enhanced ionic interactions between hypoacylated lipid A and mouse TLR4, facilitating receptor cluster dimerization and signaling (Meng et al., 2010). Our results indicate a role for both TLR4 and NLRP12 in the proinflammatory macrophage response against *Y. pestis* strains.

NLRP12 Is an Inflammasome Component

Upregulation of NLRP3 has been suggested to positively affect the activity of the NLRP3 inflammasome (Bauernfeind et al., 2009). We therefore studied expression of *Nlrp12* and *Nlrp3* (Figures 4A and 4B) after infection of macrophages with KIM5 or KIM5-pYtbLpxL. Expression of *Nlrp12* in BMDM was markedly increased after infection with *Y. pestis* strains and this may boost host responses to an infection. Treatment with LPS alone induced upregulation of *Nlrp12* gene expression (Figure S4A). Furthermore, *Y. pestis*-induced formation of cleaved and active caspase-1, as measured by an assay showing binding of active caspase-1 to a fluorescent substrate, was also impaired in NLRP12-deficient cells, providing evidence for NLRP12-dependent inflammasome function (Figures 4C and 4D). Caspase-1 cleavage measured by this assay is also

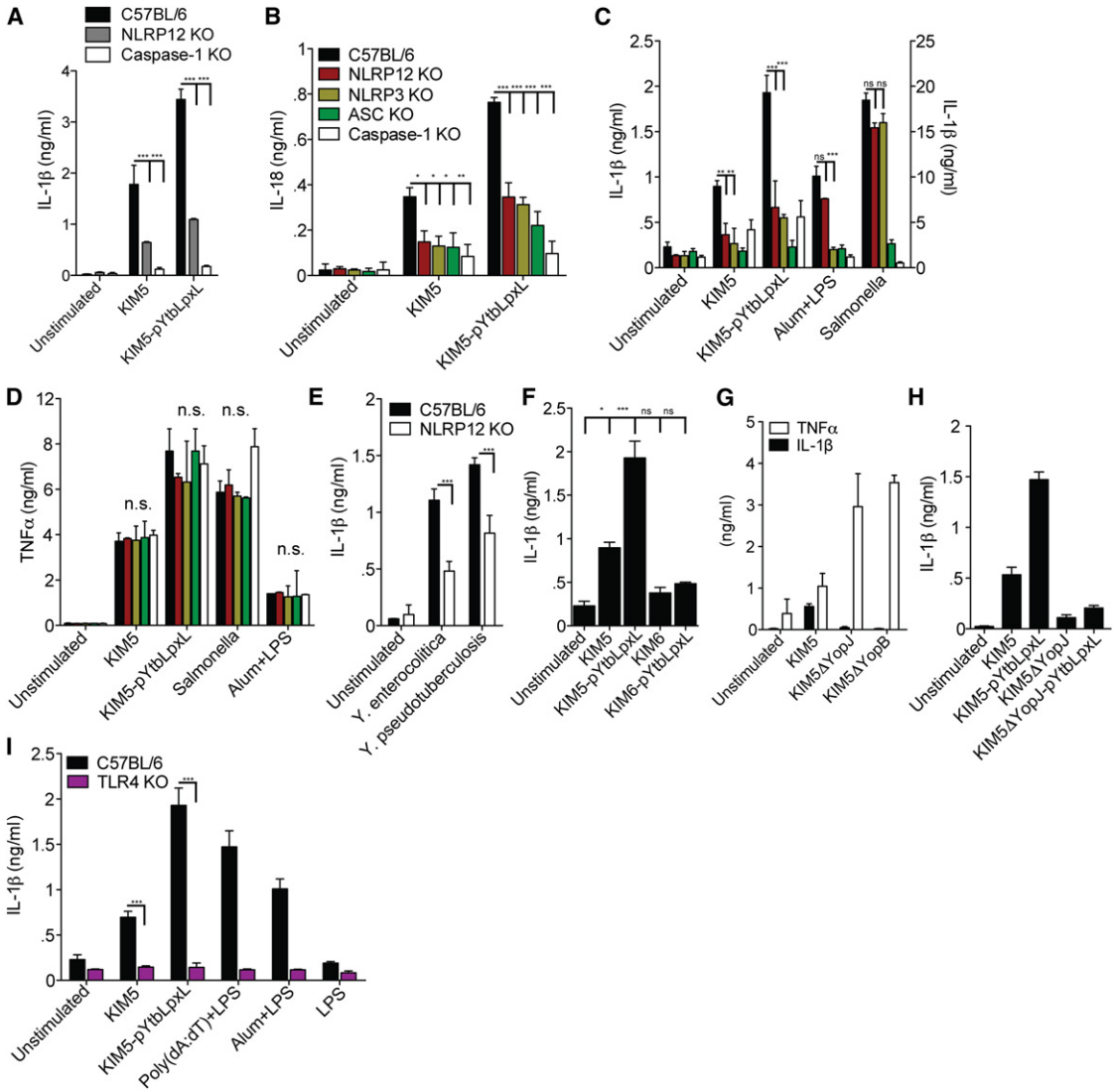

Figure 3. NLRP12 Mediates *Y. pestis*-Induced Release IL-1β and IL-18

(A) IL-1β released from neutrophil-enriched peritoneal cells from C57BL/6 (black bars), NLRP12 KO (gray bars), and *Casp1*$^{-/-}$ (Caspase-1 KO, white bars) mice. (B–I) IL-18 (B), IL-1β (C and E–I) and TNF (D and G) released from C57BL/6, NLRP12 KO, NLRP3 KO, ASC KO, and caspase-1 KO BMDM (B–D); C57BL/6 and NLRP12 KO BMDM (E); C57BL/6 BMDM (F and G); or C57BL/6 and TLR4 KO BMDMs (I). Infection with *Yersinia* strains occurred for 6 hr, with an addition of 50 μg/ml gentamicin to limit bacterial growth after 3 hr. *Yersinia* strains were added at 10 m.o.i., *S. typhimurium* at 1 m.o.i. Alum (130 μg/ml) stimulations (C and I) lasted 6 hr after priming for 3 hr with 10 ng/ml KIM5-YtbLpxL LPS. Shown are mean for triplicate cultures (with SD) in representative experiments out of three to ten performed. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Statistical comparisons are between wild-type cells and multiple mutant cells (A–E and I) or between unstimulated and multiple bacterial strains (F). Also see Figure S3.

decreased in spleen macrophages or neutrophils from NLRP12 KO mice 24 hr after infection with KIM1001 or KIM1001-pYtbLpxL (Figures 4E and 4F). *Il1b* gene expression was similar in infected wild-type cells and NLRP12 KO cells infected with *Y. pestis* (Figure 4G; Figure S3D). The macrophages infected in vitro showed a reduction in caspase-1 and IL-1β processing by immunoblotting (Figure 4H), also cells infected at a higher m.o.i. (Figure S4B). Thus, several lines of evidence support the hypothesis that NLRP12 is a component of inflammasomes formed after *Y. pestis* infection. Macrophage cell death induced by *Y. pestis* has been reported to be caspase-1 independent (Lilo et al., 2008). We confirmed those data (not shown), and in line with this observation, NLRP12-deficient cells did not show an altered cell death in response to *Y. pestis* infection (Figure S4C). Cell death may be induced by other mechanisms than pyroptosis in macrophages infected with *Y. pestis*.

NLRP12 and IL-18 Mediate Host Resistance to *Y. pestis* Infection

As shown in Figures 1 and 2, NLRP12 knockout (KO) and IL-18 KO mice are more susceptible than wild-type mice to infection with *Y. pestis*-pYtbLpxL. To monitor changes in IL-18 and IL-1β in tissues during systemic disease, we subjected WT and NLRP12 mice to intravenous (i.v.) infection with fully virulent or

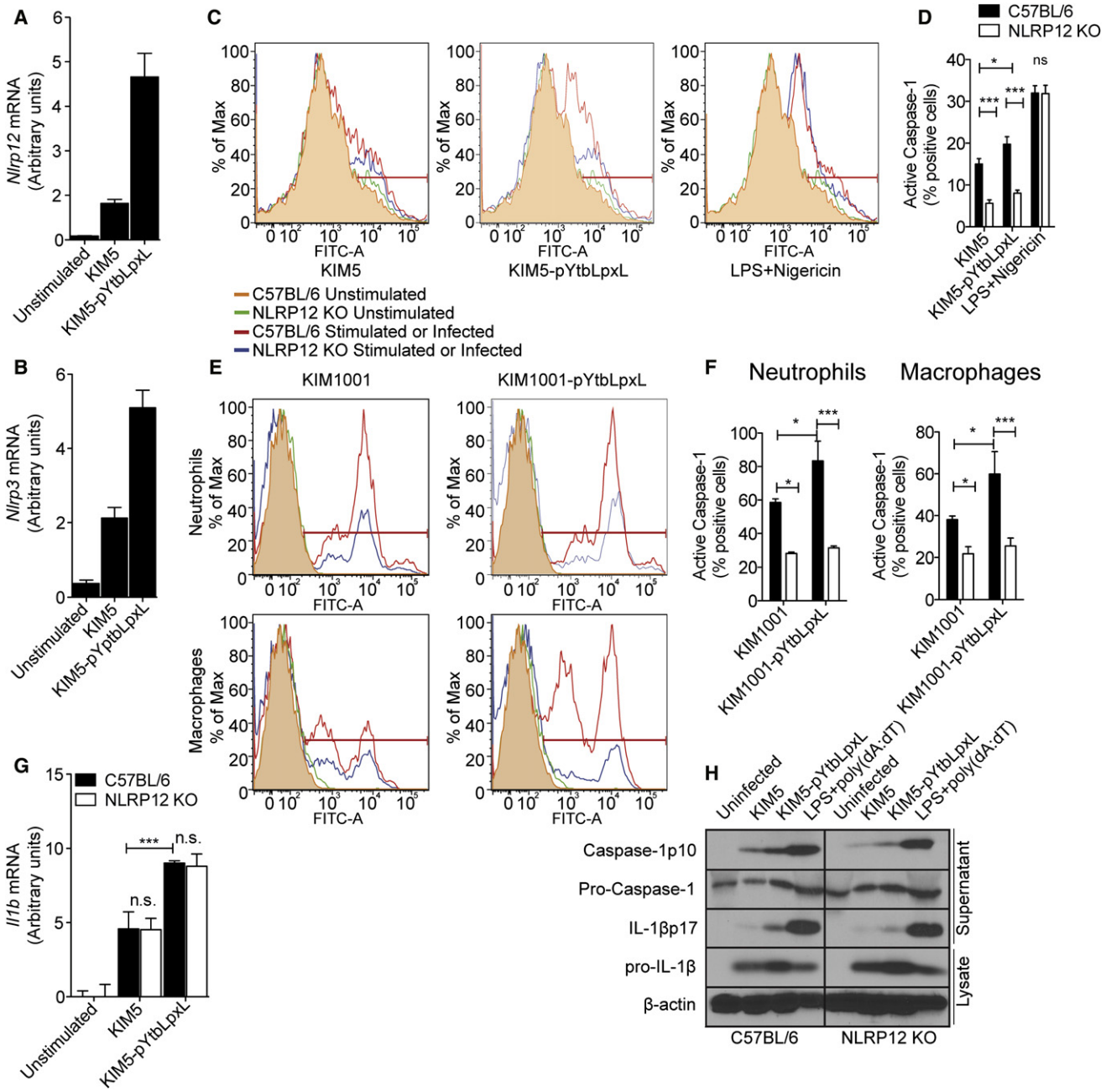

Figure 4. NLRP12 Is Necessary for Optimal Maturation of IL-1β and Caspase-1 after Infection with *Y. pestis*

(A, B, and G) Q-PCR of (A) *Nlrp12*, (B) *Nlrp3*, or (G) *Il1b* from BMDMs infected with 10 m.o.i. of *Y. pestis* KIM5 or KIM5-pYtbLpxL for (G) 4 hr or (A and B) 6 hr, with gentamicin addition after 3 hr. Error bars represent the SD.

(C–F) FACS histograms (C and E) showing active caspase-1 after FLICA reagent staining with corresponding (D and F) percent positive cells of (C and D) bone marrow cells after 6 hr of challenge with 10 m.o.i. of *Y. pestis* strains (gentamicin added after 3 hr) or (E and F) Ly6G- or F4/80-positive splenocytes from mice infected with 500 CFUs of KIM1001 or KIM1001-pYtbLpxL for 24 hr. Values from unstimulated cells are subtracted in (D). LPS primed cells treated with nigericin (10 μM) served as a control (C and D). Error bars in (D) and (F) represent the SD.

(H) Immunoblot for caspase-1 p10 or IL-1β p17 in supernatant or cell lysate from BMDMs exposed for 10 hr to poly(dA:dT) (LPS primed as in Figure 3), KIM5, or KIM5-pYtbLpxL. Shown is a representative of two (E–G) or three to five (A–D, H, and I) experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Also see Figure S4.

attenuated *Y. pestis* (KIM1001 or KIM1001-pYtbLpxL). At 44 hr after infection with KIM1001, IL-18 cytokine amounts were considerably lower in the NLRP12 KO mice, expressed as both cytokine normalized to the spleen bacterial load in each particular animal (Figure 5A) or simply as cytokine concentration in homogenate (Figure S5A). A decrease of IL-18 and IL-1β in the spleen (Figures 5B and 5C) and serum (Figures 5D and 5E) was also observed after KIM1001-pYtbLpxL infection in the

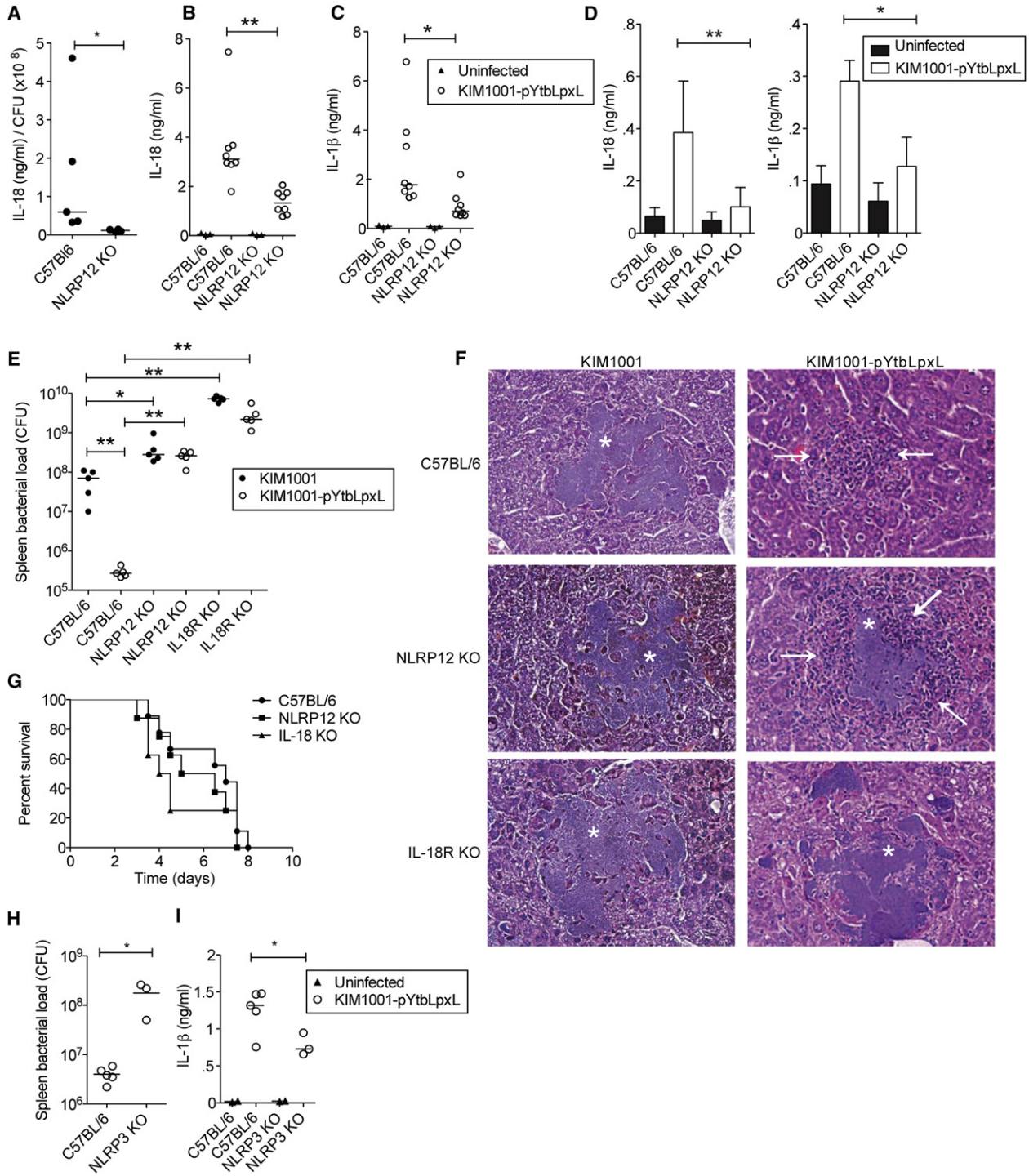

Figure 5. NLRP12 and IL-18 Control Infection with *Y. pestis* In Vivo

(A) IL-18 in serum (ng/ml) of C57Bl/6 or NLRP12 KO mice infected i.v. with 500 CFU *Y. pestis* KIM1001 for 44 hr, normalized by bacterial loads (for each animal); n = 5.

(B–D) IL-18 or IL-1β in (B and C) spleen or (D) serum of WT or NLRP12 KO mice infected i.v. with 500 CFU KIM1001-pYtbLpxL for 44 hr. In (B) and (C), uninfected mice: n = 3; infected animals: n = 8. In (D), uninfected mice: n = 4; infected mice: n = 5.

(E) Spleen CFUs of mice infected i.v. with either KIM1001 or KIM1001-pYtbLpxL (n = 5). Horizontal lines indicate mean values.

(F) Histology of fixed H&E stained liver sections from (top) WT, (middle) NLRP12 KO, or (bottom) IL-18R KO mice infected i.v. with (left) KIM1001 or (right) KIM1001-pYtbLpxL for 44 hr. Asterisks represent bacterial clusters; arrows represent foci of inflammatory cells, primarily neutrophils.

(G) Survival of C57Bl/6 (n = 10), IL-18 KO, and NLRP12 KO (n = 8) mice with 10 CFU s.c. of KIM1001.

(H and I) Spleen CFU (H) or spleen IL-1β (I) of C57Bl/6 and NLRP3 KO mice infected for 44 hr with 500 CFU i.v. of KIM1001-pYtbLpxL. Shown is a representative of three performed experiments. *$p < 0.05$; **$p < 0.001$. Also see Figure S5.

NLRP12 KO mice as compared to wild-type mice. Experiments with IL-1R KO, IL-1β KO, and IL-18R KO mice suggested that IL-18 signaling had the greatest impact on resistance to *Y. pestis*-pYtbLpxL, given that 100% of IL-18 and IL-18R KO animals died after infection (Figures 1B and 1D). IL-18R KO mice had reduced IL-18 and IL-1β in the spleens compared to WT mice after infection with KIM1001-pYtbLpxL (Figure S5B), suggesting a positive feedback loop via IL-18R for IL-1β and IL-18 production.

A reduction of several orders of magnitude in spleen bacterial load was seen when mice were infected i.v. with KIM1001-pYtbLpxL compared to wild-type KIM1001 (Figure 5E), indicating beneficial host responses induced by the presence of the hexa-acylated LPS. These differences in systemic bacterial load between the two bacterial strains were absent in mice lacking NLRP12 or IL-18R. NLRP12-deficient and IL-18R-deficient mice also had increased bacterial loads compared to wild-type mice when infected with the virulent *Y. pestis* KIM1001 (Figure 5E, $p = 0.01$, WT versus *Nlrp12*$^{-/-}$; $p < 0.001$, WT versus *Il18r1*$^{-/-}$). This is important in that it shows that NLRP12 and IL-18R participate in host resistance in vivo toward both virulent and attenuated strains of *Y. pestis*. Thus, it appears that *Y. pestis* has an inherent ability to activate NLRP12-dependent recognition and that the potent LPS found in strains expressing LpxL increases the formation of proforms and subsequently mature forms of inflammasome-controlled cytokines such as IL-1β (Figures 1H and 4C). Livers from animals infected with wild-type *Y. pestis* have large extracellular clusters of bacteria (Figure 5F, left panels, marked with an asterisk) and remarkably few signs of inflammation, probably reflecting active suppression of immunity combined with stealth via limited initiation of TLR4 signaling. Livers from animals infected with *Y. pestis*-pYtbLpxL display foci consisting of inflammatory cells (Figure 5F, upper right, indicated by arrows) and absence of visible bacterial masses, suggesting that recruitment of phagocytes limits bacterial growth (Montminy et al., 2006). Livers from NLRP12 KO mice infected with KIM1001-pYtbLpxL had recruitment of inflammatory cells (Figure 5F, arrows). Such masses of inflammatory cells typically contain large number of neutrophils and some mononuclear cells (Montminy et al., 2006), and a calculation of number of recruited cells showed no significant difference between infected wild-type versus NLRP12-deficient livers (Figure S5C). However, this cell recruitment did not correlate with suppression of bacterial growth, given that bacterial masses were visible (Figure 5F). These results suggest that NLRP12 may not play a major role in the attraction of phagocytes to infected sites in the liver, but is central to the effective antibacterial actions they perform. Few if any inflammatory cells were visible in livers of IL-18R-deficient mice (Figure 5F), indicating failures of both cell recruitment and antibacterial defenses.

Taken together, the results suggest that NLRP12 and IL-18 contribute to host resistance against *Y. pestis* and *Y. pestis*-pYtbLpxL. We also found that NLRP12 KO mice infected with KIM1001-pYtbLpxL had reduced amounts of TNF and the chemokine CXCL12 compared to C57Bl/6 mice (Figures S5D–S5F), possibly secondary effects of reduced IL-1β and IL-18 release, given that primary cells lacking NLRP12 did not display decreased TNF release in culture (Figure 2). In contrast, NLRP12-deficient mice injected with an alum-LPS mixture did not show decreased serum IL-1β, IL-18, TNF-α, and CXCL12 (Figure S5G). Furthermore, we found similar recruitment of neutrophils to the peritoneum of wild-type mice or NLRP12-deficient mice injected intraperitoneally (i.p.) with sterile thioglycollate (Figure S5H). Movement of neutrophils (Figure 5F; Figure S5C) and DCs (Figure S5I) during infection of NLRP12-deficient mice appears to be preserved. Differences in survival between NLRP12-deficient or IL-18-deficient mice and wild-type mice after s.c. infection with only 10 CFUs of fully virulent KIM1001 were not significant (Figure 5G). This result is of uncertain importance because the very low LD50 of *Y. pestis* by s.c. infection (less than 10 CFUs) makes it difficult to demonstrate reductions in host resistance impacting survival without the use of very large numbers of animals. Tissue bacterial loads (Figure 5E) appear to be more sensitive assays for analyzing host resistance to *Y. pestis.*

NLRP3 has also been proposed as an inflammasome component recognizing *Y. pestis* (Zheng et al., 2011) (Figures 2 and 3). NLRP3-deficient animals also were less resistant to infection by KIM1001-pYtbLpxL, in that they displayed increased bacterial loads in the spleen (Figure 5H) that correlated with reduced spleen cytokines (Figure 5I). In summation, NLRP12 and NLRP3 both contribute to the host resistance toward *Y. pestis* strains.

NLRP12 and IL-18 Signaling Induce IFN-γ that Limits Infection

IL-18 is a known inducer of IFN-γ (Okamura et al., 1995), a key protein in many host responses to pathogens. This suggests that signaling via NLRP12 and the IL-18R, resulting in the release of IFN-γ, could mediate resistance to *Y. pestis*-pYtbLpxL. Mice lacking both IFN-αβR and IFN-γR (dKO) were infected with KIM1001-pYtbLpxL s.c., and we found that all the dKO animals succumbed to the infection (Figure 6A). This phenomenon was largely attributed to IFN-γR signaling, given that only a few mice lacking IFN-αβR died upon infection, whereas almost all mice lacking IFN-γR succumbed (Figure 6B). No differences in IFN-γ concentrations were observed between spleens of uninfected WT, NLRP12-deficient, and IL18R-deficient mice (Figures 6C and 6D). However, the IFN-γ concentrations in spleens from KIM1001-pYtbLpxL-infected NLRP12-deficient mice compared to wild-type mice were drastically reduced (Figure 6C), as was also true for the mice lacking IL-18R (Figure 6D). Thus, we propose a cascade of signals from NLRP12 to IL-18 maturation that in turn mediates IFN-γ release after infection with *Y. pestis* strains.

DISCUSSION

We propose that recognition of *Y. pestis* expressing a stimulatory LPS by TLR4 leads to upregulation of NLRP12 and proinflammatory cytokines such as IL-18 and IL-1β. NLRP12 then recognizes a ligand produced upon *Y. pestis* infection and assembles into an inflammasome that processes IL-18 and IL-1β. Although the precise nature of the true NLRP12 ligand is unknown, and it may be a host or bacterial protein, the generation of the ligand appears to require the virulence-associated T3SS of Yersinia. Models for activation may include possibilities that cells sense membrane damage associated with the T3SS,

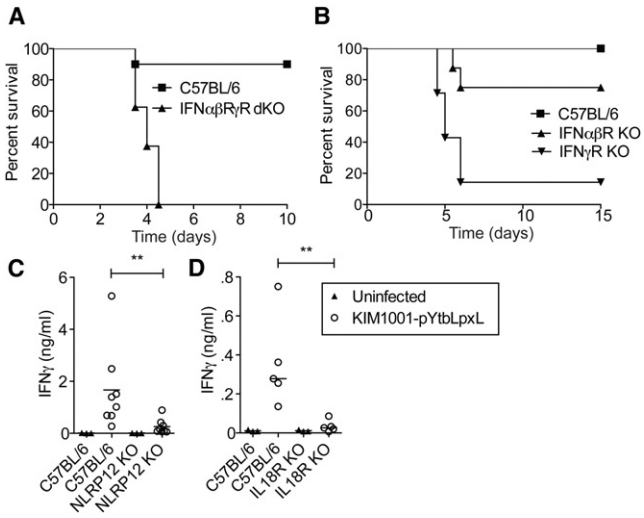

Figure 6. NLRP12 Induces IFN-γ via IL-18 Signaling

(A and B) Survival of mice: in (A), C57Bl/6: n = 10 and *Ifnar1*$^{-/-}$ × *Ifngr1*$^{-/-}$ (IFNαβR × IFN-γR DKO; triangles): n = 8, and in (B), C57Bl/6 (squares): n = 10, IFNαβR KO: n = 8, and IFN-γR KO: n = 7, infected s.c. with 500 CFUs of KIM1001-pYtbLpxL.

(C and D) IFN-γ in spleen homogenates from C57BL/6 and NLRP12 KO mice infected i.v. with 500 CFU of KIM1001-pYtbLpxL. Uninfected mice: n = 3; infected mice: n = 8 (C) and n = 5 (D). Samples were harvested 46 hr after infection. Horizontal lines indicate median values. Experiments shown are representative out of three performed. *p < 0.05; **p < 0.001.

secreted effectors or other molecules channeled by the T3SS, and modified host proteins. NLRP3 also contributes to IL-18-IL-1β release. IL-18 seems to be more critical than IL-1β and plays a key role in induction of IFN-γ.

We show that NLRP12 is an inflammasome component recognizing *Y. pestis* and contributes to in vivo resistance to infection with *Y. pestis* strains. To our knowledge, this is the first demonstration of a clear role for NLRP12 in resistance to infection. Our data suggest an inflammasome role for NLRP12 in pathogen recognition and that the NLRP12-IL-18-IFN-γ axis is effective in limiting infection with *Y. pestis*-pYtbLpxL. We also show that the expression of *Y. pseudotuberculosis* LpxL in *Y. pestis* increases TLR4-dependent release of IL-18 and IL-1β. This increase correlates with increased resistance to the modified pathogen. In fact, the results indicate that a major consequence of producing LPS with low TLR4-activating potential could be lack of priming necessary for effective synthesis of active IL-1β and IL-18. Therefore, *Y. pestis* is able to utilize inflammasome-activating components like the T3SS to neutralize the immune response without an effective activation of an inflammatory response. This phenomenon may have played a role in evolution of high virulence in *Y. pestis*.

These findings support the view that inflammasomes, the cellular protein complexes cleaving IL-18 and IL-1β into mature forms, are fundamental components of the host response to many pathogens. Indeed, several viral, bacterial and fungal microbes have strongly increased ability to induce disease in the absence of IL-1β, IL-18 and inflammasome components (Broz et al., 2010; Davis et al., 2011; Hise et al., 2009; Lamkanfi and Dixit, 2009; Rathinam et al., 2010). In spite of this, only a few mammalian NLRs out of a family of more than 20 members have currently been shown to directly participate in host defenses. Here, we show that NLRP12 participates in host responses to wild-type *Y. pestis* and modified *Y. pestis* strains expressing a potent LPS, although the factor(s) in *Y. pestis* responsible for directly activating the NLRP12 inflammasome are still unknown.

NLRP12 may also be involved in resisting infections caused by other human pathogens. It is unclear how NLRP12 may interact with other inflammasome components. NLRP12 deficiency did not cause a complete reduction in ability to release IL-18 and IL-1β after exposure to *Y. pestis* and *Y. pestis*-pYtbLpxL infection. Also, the increased mortality observed in NLRP12-deficient mice did not appear as great as observed in IL-18-deficient animals, and NLRP3 also plays a role in host defenses. Redundancy between NLRs may occur, and other NLRs may also participate in optimal responses to infection. This may support the idea that NLRs work together for optimal protection of the host (Broz et al., 2010). The generation of animals with combined deficiencies in NLRP12 and other NLRs may clarify how NLRP12 functions in cooperation with other signaling components. NF-kB signaling after bacterial challenge appeared normal in NLRP12-deficient cells.

IL-18, IL-1β, and IFN-γ are all cytokines active at the interface between innate and adaptive immunity. We have found that *Y. pestis* strains generating a hexa-acylated LPS could function as effective live vaccines (Montminy et al., 2006). It would be of interest to investigate the role of NLRP12 in promoting the development of adaptive immunity and protection after vaccination with both live and subunit + adjuvant vaccines.

The emerging role of inflammasomes as key players in host defenses during many infections makes them desirable targets for therapeutic intervention and drug development. We note that alum, one of the first components known to activate specific inflammasomes, already is in widespread use as one of the few vaccine adjuvants licensed for human use. However, a delicate balance between pathological effects and enhanced host defenses arising from inflammasome-stimulating treatments will be necessary. Mutations in NLRs are linked to inflammatory diseases (Hawkins et al., 2003; Jéru et al., 2011a), and anti-IL-1 treatment does in fact reduce symptoms in many such patients. More knowledge on the role of NLRs in inflammation and homeostasis is needed in order to fine-tune future NLR-based therapies.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions

Y. pestis KIM is originally a clinical isolate from a Kurdistan Iran man (Brubaker, 1970; Perry and Fetherston, 1997). *Y. pestis* strains KIM5, KIM5-pEcLpxL (containing *E. coli lpxL*, earlier called pLpxL) and KIM1001 were as reported (Montminy et al., 2006). *Y. pseudotuberculosis* IP2666 (containing a complementation of PhoP/PhoQ deficiency) and *Y. enterocolitica* 8081 were provided by J. Mecsas. Strains were grown in tryptose-beef extract (TB) broth with 2.5 mM $CaCl_2$ all by shaking at 37°C. *lpxL* of *Y. pseudotuberculosis* IP2666 including 480 basepairs upstream and 266 basepairs downstream from coding region was cloned with Pfu Ultra (Stratagene) and was ligated into the BamHI and SalI sites of pBR322, creating pSP::YtbLpxL (or "pYtbLpxL"). The resulting plasmid was electroproated into *Y. pestis* KIM5 (Goguen et al., 1984) or *Y. pestis* KIM1001 (Sodeinde et al., 1992), and bacteria were selected by growth on TB agar supplemented with 2.5 mM $CaCl_2$ in the presence of

100 μg/ml of ampicillin. All strains containing plasmids above remained tetracycline sensitive. KIM1001 (pPCP1+, pCD1+, and pMT1+) is highly virulent (Perry and Fetherston, 1997), whereas KIM5 bears the chromosomal deletion "Δ*pgm*," which substantially attenuates virulence. The *pgm* locus contains no genes thought to affect LPS biosynthesis. KIM6 is a KIM5 derivative lacking the T3SS-containing pCD1 virulence plasmid. KIM5-ΔYopB was provided by G. Plano (Torruellas et al., 2005). For the generation of KIM5-ΔYopJ, the following method was used: An in-frame deletion removing codons 4–287 was created via allelic exchange. PCR products made with primer sets A (5′-ATAGAGCTCCACTACTGATTCAACTTGGACG-3′), B (5′-5′-TCCGATCATT TATTTATCCTTATTCA-3′) and C (5′-TGAATAAGGATAAATAAATGATCGGAT AATGTATTTTGGAAATCTTGCT-3′), D (5′-GGGTCTAGACTGATGTCGTTTATT TCTGGGTAT-3′), respectfully, were used to make a fused product by overlap PCR using primers A and D (Horton et al., 1989). This product was cloned in the allelic exchange vector pRE107 (Edwards et al., 1998) in *E.coli* K12 strain B2155 and transferred to *Y. pestis* by conjugation; recombinants were selected on TB medium containing 100 μg/ml ampicillin but no diaminopimelic acid. After counter selection with 5% sucrose, deletion mutants were identified by PCR. For in vitro infections, bacteria were grown overnight at 37°C in TB broth with or without ampicillin, diluted 1:4 in fresh media, and cultured for three more hours at 37°C, then washed three times with PBS and resuspended in DMEM or RPMI. *S. enterica* serovar *typhimurium* strain SL1344 was provided by M. O'Riordan and strain M525P by C. Bryant.

Cell Stimulations

Mouse BMDMs were prepared by maturing fresh bone marrow cells for 5–7 days in the presence of M-CSF containing supernatant from L929 cells. Mouse neutrophils were enriched by injecting 1 ml of thioglycolate i.p., peritoneal cells (typically >80% Ly6G-positive cells; Nilsen et al., 2004) were harvested 4 hr later after flushing with RPMI. Mouse BMDMs were plated at 2×10^5 per well in 96-well plates for ELISA or 2×10^6 per well in 12-well plates for immunoblotting. Stimulation was for 6 hr and supernatants were collected for cytokine analysis. Three hours after bacterial infections, 50 μg/ml of gentamycin was added. Alum was from Pierce; nigericin and poly(dA:dT) was from Sigma. IL-1β p17 and Caspase-1 p10 immunoblots were conducted mainly as described (Hornung et al., 2008) with antibodies from Santa Cruz Biotechnology (caspase-1 p10) and R&D (IL-1 β). The antibody against β-actin was from Sigma. Q-PCR for *Nlrp12* and *Nlrp3* in resting or infected BMDMs or magnetic bead (StemCell Technologies)-isolated neutrophils was performed with the RNeasy Mini Kit (QIAGEN) and the iScript cDNA Synthesis Kit (BioRad). PCR was performed on transcribed cDNA or mouse tissue cDNA (Clontech) with primers for detection of mouse *Nlpr12* (5′-TGCAAGCTTCGAGTCCTGT-3′, 5′-CCTGG TCGGCTTCATTCTG-3′), *Nlrp3* (5′-AACCAATGCGAGATCCTGAC-3′, 5′-AT GCTGCTTCGACATCTCCT-3′), or *Il1b* (5′-GCCCATCCTCTGTGACTCAT-3′, 5′-AGGCCACAGGTATTTTGTCG-3′) with SYBR green (BioRad) in accordance with the manufacturer's instructions. ELISA kits for IL-1β, TNF-α, IL-8, CXCL12, IFN-γ (R&D), and IL-18 (MBL) were used for cytokine detection. Reagents for FACS detection of active and cleaved caspase-1 by FLICA-FITC substrate were from Immunochemistry Technologies.

Mice

All experiments involving animals were approved by the Institutional Animal Care and Use Committee. ASC (*Pycard*$^{-/-}$), NLRP3 (*Nlrp3*$^{-/-}$), and NLRP12-deficient (*Nlrp12*$^{-/-}$) mice were generated by Millennium Pharmaceuticals and were backcrossed 8–11 generations to C57BL/6 background. Mice deficient in TLR4 (*TLR4*$^{-/-}$) and MyD88 (*Myd88*$^{-/-}$) were from S. Akira, and mice lacking caspase-1 (*Casp1*$^{-/-}$) were from M. Starnbach. C57BL/6 mice and mice deficient in IL-1R1 (*Il1r1*$^{-/-}$), IL-18R (Il18r1$^{-/-}$), IL-18 (Il18$^{-/-}$), TNFR1 (*Tnfr1*$^{-/-}$), IL-12p40 (*Il12b*$^{-/-}$), and IFN-γR (*Ifngr1*$^{-/-}$) were all from Jackson Laboratories. J. Sprent (The Scripps Research Institute) provided the IFN-αβR1 (*Ifnar1*$^{-/-}$) and IFN-γR1 × IFN-αβR1 doubly deficient mice. IL-1β (*Il1b*$^{-/-}$)-deficient mice (Horai et al., 1998) were provided by Y. Iwakura. Wild-type (from Jackson Laboratories or bred at UMass) and knockout mice were infected s.c. in the nape of the neck with *Y. pestis* and their survival was monitored twice a day for 30 days. Mice were infected with 1000 CFUs of *S. typhimurium* M525P i.p. and survival was monitored as described above. For cytokine and CFU analysis, mice were infected either s.c. or i.v. and sacrificed at the indicated time points. Serum was generated by centrifugation in microtainer tubes (BD), and spleens were homogenized in 0.5 ml PBS with a closed system Miltenyi gentleMACS dissociator and c-tubes to preserve intact cells; subsequently cells/debris were removed by centrifugation. Samples for cytokine analysis were subjected to protease inhibitor (Roche) treatment. Cytokine amounts normalized by bacterial loads were calculated by dividing IL-18 concentrations (ng/ml) by the bacterial load (CFUs × 10^8) for each animal. Hematoxylin and eosin (H&E) staining and microscopy were performed as published (Montminy et al., 2006).

In Vivo Caspase-1 Cleavage Analysis

Mice were infected with 500 CFUs of *Y. pestis* i.p. After 24 hr, spleens were harvested and homogenized and cell suspensions were stained with caspase-1 FLICA reagent.

Statistical Analysis

In vitro cytokine release was analyzed by two-way ANOVA with a Bonferroni post-test. Differences in spleen and serum cytokine concentrations were analyzed by the unpaired t test. Differences in survival were studied with Kaplan-Meyer analysis and the logrank test. Differences in spleen CFUs or cytokine/CFU ratio values between genotypes of mice were evaluated with the Mann-Whitney test or in more complex comparisons involving multiple mouse genotypes, with a generalized linear regression model of cubic transformed log CFU values (95% confidence interval), to meet normality assumptions. Values of $p < 0.05$ were considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2012.07.006.

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