Caspase-8 and RIP kinases regulate bacteria-induced innate immune responses and cell death

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A number of pathogens cause host cell death upon infection, and *Yersinia pestis*, infamous for its role in large pandemics such as the "Black Death" in medieval Europe, induces considerable cytotoxicity. The rapid killing of macrophages induced by *Y. pestis*, dependent upon type III secretion system effector *Yersinia* outer protein J (YopJ), is minimally affected by the absence of caspase-1, caspase-11, Fas ligand, and TNF. Caspase-8 is known to mediate apoptotic death in response to infection with several viruses and to regulate programmed necrosis (necroptosis), but its role in bacterially induced cell death is poorly understood. Here we provide genetic evidence for a receptor-interacting protein (RIP) kinase–caspase-8-dependent macrophage apoptotic death pathway after infection with *Y. pestis*, influenced by Toll-like receptor 4-TIR-domain-containing adapter-inducing interferon-β (TLR4-TRIF). Interestingly, macrophages lacking either RIP1, or caspase-8 and RIP3, also had reduced infection-induced production of IL-1β, IL-18, TNF, and IL-6; impaired activation of the transcription factor NF-κB; and greatly compromised caspase-1 processing. Cleavage of the proform of caspase-1 is associated with triggering inflammasome activity, which leads to the maturation of IL-1β and IL-18, cytokines important to host responses against *Y. pestis* and many other infectious agents. Our results identify a RIP1–caspase-8/RIP3-dependent caspase-1 activation pathway after *Y. pestis* challenge. Mice defective in caspase-8 and RIP3 were also highly susceptible to infection and displayed reduced proinflammatory cytokines and myeloid cell death. We propose that caspase-8 and the RIP kinases are key regulators of macrophage cell death, NF-κB and inflammasome activation, and host resistance after *Y. pestis* infection.

The causative agent of plague, *Yersinia pestis* is well known to cause significant cell death upon infection (1–3). Like the activation of inflammatory pathways to produce cytokines, triggering cell death pathways is a common response of the mammalian immune system to infection. Death of immune cells can eliminate the replication niche of pathogens found within those cells, thus inhibiting the proliferation of the pathogens and exposing them to bactericidal mechanisms (4). Conversely, elimination of key immune cells can diminish the ability of those cells to respond to infection. Multiple host and microbial factors control cell death pathways (5). Caspase-8–dependent apoptosis, receptor interacting protein-1 (RIP1)- and RIP3-dependent necroptosis, and caspase-1/caspase-11–dependent pyroptosis constitute major modes of regulated cell death during infection (5, 6). Several viruses seem to induce caspase-8–dependent apoptosis (7). Caspase-8 has also been suggested to have additional functions, such as inhibiting necroptosis (7–9) and modulation of NF-κB activation in T and B cells (10). Signaling to the transcription factor NF-κB controls the transcription of cytokines such as IL-6, TNF, pro-IL-1β, and pro-IL-18, and stimulates cell survival. *Y. pestis* can induce cell death in macrophages and dendritic cells via the type III secretion system (T3SS) effector *Yersinia* outer protein J (YopJ; YopP in *Yersinia enterocolitica*), although it is unclear whether this is entirely by apoptosis (11, 12). All human-pathogenic *Yersiniae* (*Y. pestis*, *Yersinia pseudotuberculosis*, and *Y. enterocolitica*) harbor cytotoxic properties toward host cells, and YopJ production is associated with cell death in vivo and in vitro (13–16). YopJ-mediated inhibition of NF-κB by acetylation of Inhibitor of κB Kinase β (IKKβ), MAP kinase kinases, and TAK1 may modulate macrophage death via effects on inflammatory and prosurvival signals (2, 17–21). Inflammasome activation, culminating in the activation and processing of caspase-1, leads to the production of IL-18 and IL-1β, key inflammatory cytokines and antibacterial defenses, but can also be associated with caspase-1–dependent pyroptotic cell death (22). YopJ also participates in inflammasome activation (16, 23), leading to a host immune response. Thus, this single bacterial effector may induce both protective and harmful effects for the host. In the present study we investigated the mechanisms for *Y. pestis*-induced cell death, NF-κB activation, and triggering of inflammasome activation.

Results and Discussion

***Yersinia* Induces Cell Death via RIP1, Caspase-8, and RIP3.** Viable *Y. pestis* KIM5 can induce rapid cell death via YopJ (Fig. S1*A*). Rapid death in bone marrow-derived macrophages (BMDMs) is induced in a YopJ-dependent manner by *Y. pestis* or *Y. pseudotuberculosis* temperature-shifted from 26 °C to 37 °C (Fig. S1 *A*

Significance

Receptor-interacting protein-1 (RIP1) kinase and caspase-8 are important players in activation of apoptotic pathways. Here we show that RIP1, caspase-8, and RIP3 contribute to infection-induced macrophage cell death and also are required for activation of transcription factor NF-κB and caspase-1 upon infection with the bacterial pathogen *Yersinia pestis*, the causative agent of plague. Mice lacking caspase-8 and RIP3 are also very susceptible to bacterial infection. This suggests that RIP1, caspase-8, and RIP3 are key molecules with multiple roles in innate immunity during bacterial challenge.

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Conflict of interest statement: E.L. and J.D.G. have a patent application on the use of modified bacteria as used in vaccines. P.A.H., J.B., and P.J.G. are employees and shareholders of GlaxoSmithKline.

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and *B*), a condition that mimics the temperature change associated with infection via a fleabite. In addition to arming the T3SS, the temperature shift ensures the initial presence of some TLR4-stimulatory LPS (Fig. S1*C*) (24). Although caspase-1 is activated by *Y. pestis* (1, 25), macrophage death was independent of caspase-1/caspase-11, suggesting nonpyroptotic cytotoxicity (Fig. S2 *A* and *B*). Death was unaffected by Fas ligand (FasL) or TNF, indicating that those death receptor-mediated mechanisms are not involved; and independent of inflammasome-related NOD-like receptors (NLRs), the RNA-dependent protein kinase (PKR), the inflammasome adaptor apoptosis-associated speck-like protein containing a CARD (ASC), IL-1β, and IL-18 (Fig. S2 *C*–*G*) (1). Caspase-8 is a key enzyme in cell death induced by some viruses (26). Caspase-8 deficiency results in embryonic lethality, but mice deficient in both caspase-8 and RIP3 [RIP3$^{-/-}$ caspase-8$^{-/-}$ mice, double knockout (dKO)] are rescued. These data indicate a vital role for caspase-8 in suppressing necroptosis by targeting a component of the RIP3 pathway (8, 9). Macrophages from RIP3$^{-/-}$caspase-8$^{-/-}$ mice were remarkably resistant to cell death induced by *Y. pestis* and *Y. pseudotuberculosis*, but not by *Salmonella*, which induces pyroptotic death (4), or with the NLRP3 inflammasome-specific trigger nigericin (Fig. 1 *A* and *B*). YopJ-induced death is likely not necroptosis because RIP3-deficient cells are not protected (Fig. 1 *A* and *B*). Electron microscopy revealed that macrophages infected with *Y. pestis* displayed features consistent with apoptotic death, such as membrane blebbing and nuclear condensation and fragmentation. These effects were absent in visibly infected dKO cells (Fig. 1*C*). Moreover, infection of macrophages with *Y. pestis* led to DNA fragmentation patterns typically associated with apoptosis, and this was blocked by zVAD pan-caspase inhibition (Fig. S3*A*). Taken together, our data strongly suggest that *Yersinia* induces rapid macrophage death by apoptosis via caspase-8.

RIP3-mediated necroptotic death requires RIP1 (27–29), a serine/threonine kinase that can also contribute to NF-κB signaling (30) and apoptosis. RIP1$^{-/-}$ mice die shortly after birth (31), but

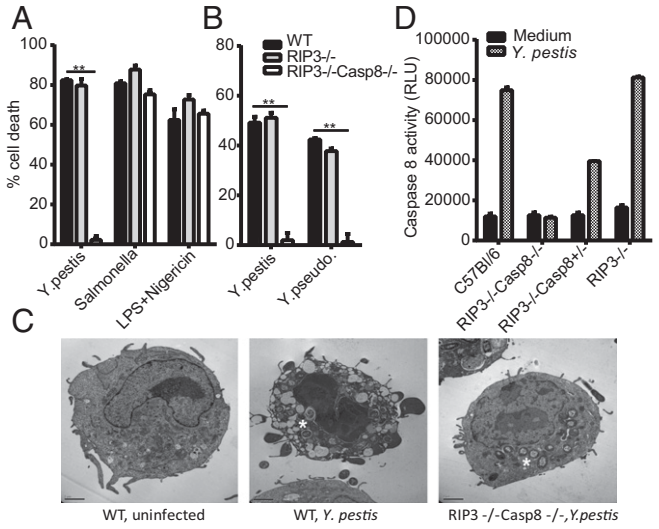

Fig. 1. Caspase-8–RIP3-deficient macrophages are protected against *Y. pestis* induced cytotoxicity. (*A* and *B*) Caspase-8$^{-/-}$ RIP3$^{-/-}$ (dKO), but not RIP3 KO BMDM, are protected from *Yersinia*-induced cytotoxicity measured by LDH release assay or (*C*) electron microscopy. (Scale bars, 2 μm.) Asterisks in *C* indicate bacteria. (*D*) Caspase-8 activity induced by *Y. pestis* infection (MOI 40, 2 h) in WT, RIP3$^{-/-}$ and dKO BMDMs. BMDM were infected with 10–40 MOI of *Yersinia*e or 1.5 MOI of *Salmonella typhimurium* for 4 h (*A* and *B*) or 2 h (*C* and *D*), and gentamycin was added after 1 h. Figures are representative for three to eight experiments performed. Bars indicate mean plus SD. $^{**}P < 0.01$ (two-tailed *t* test).

fetal liver macrophages from RIP1$^{-/-}$ mice, in contrast to RIP3$^{-/-}$ macrophages, displayed a rescue from death induced by *Y. pestis* (Fig. 2*A*) and DNA laddering (Fig. S3*C*), suggesting that RIP1 activity contributes to apoptotic cell death upon infection, likely mediated by the induction of caspase-8 enzymatic activity and cleavage of procaspase-8 that precedes cell death (Figs. 1*D*, and 2 *A*, *B*, *E*, and *F* and Fig. S3 *D* and *E*). RIP1$^{-/-}$ macrophages were also protected from necroptotic cell death induced by heat-killed KIM5 plus zVAD or LPS plus zVAD (Fig. S3*F*). Potent and specific inhibitors of RIP3 (32) or RIP1 [GlaxoSmithKline (GSK): P.A.H., J.B., P.J.G.] kinase activity have recently been identified. The RIP1 inhibitor GSK'963, but not inactive enantiomer GSK'962, blocks *Y. pestis*-induced cell death (Fig. 2*C* and Fig. S3*B*) and caspase-8 activity (Fig. 2*D*). In addition to the genetic and pharmacological interactions between RIP1 and caspase-8, we found that RIP1 biochemically interacted with caspase-8 after *Y. pestis* challenge (Fig. 2*E*). Cell death, cleavage of procaspase-8, and enzymatic activity were partially reduced in the absence of TLR4 and TRIF, but not MyD88 (Fig. S4). Reduced death was also seen for bacteria grown at 37 °C and *Y. pestis*-EcLpxL, which constitutively generates a hexa-acylated LPS (Fig. S5 *A* and *B*). TLR4 signaling seems to enhance early caspase-8–mediated effects by *Y. pestis* YopJ, similar to those proposed for *Y. enterocolitica* YopP (33–35). Cell death induced by *Y. pestis* grown at 37 °C was inhibited by the presence of CaF1 capsule protein (Fig. S5 *C* and *D*), suggesting that the capsule prevented close contact between bacteria and host cells needed for T3SS effects.

The targeted deletion of caspase-8 in myeloid cells [conditional KO (cKO) caspase-8$^{fl/fl}$ LysM cre$^{+/+}$ generated by D.M.S.; Fig. S6 *A* and *B*] had little effect on *Y. pestis*-induced macrophage death (Fig. 2*C*). Although the generation of other mice with defects in caspase-8 in macrophages has been reported (36), our caspase-8 cKO BMDM appeared healthy in culture and did not display increased cell death in the presence or absence of infection (Fig. 2*C*). Blockade of RIP3 kinase activity with GSK'872 strongly reduced macrophage death in the absence of caspase-8, suggesting that deletion of caspase-8, or caspase inhibition by zVAD (Fig. 2*C* and Fig. S6*C*), may promote necroptosis by RIP3, presumably influenced by reduced *Y. pestis*-induced cleavage of RIP1 in the absence of caspase-8 (Fig. 2*F*).

Cleavage and activation of the downstream apoptotic executioner caspase-3 was also dependent upon YopJ and caspase-8–RIP3 (Fig. S6*D*). The caspase-8–RIP3 pathway also influenced death induced by *Y. enterocolitica* but not by *Salmonella* or *Pseudomonas*, which also harbor a T3SS (Fig. S6*E*). Thus, all human-pathogenic *Yersiniae*, but not all bacteria containing a T3SS, trigger cell death via the same pathway. Our results provide an explanation for how *Yersinia* induces macrophage cell death via caspase-8 and RIP kinases. In this model, caspase-8–dependent apoptosis represents the default, whereas caspase-8 absence may lead to RIP3-dependent necroptosis. RIP1 has a key upstream role for both modes of death, perhaps influenced by its ability to direct apoptosis under conditions of cIAP1 depletion (37) as seen with *Y. pestis* (Fig. S6*F*).

Effects on NF-κB Activity. Caspase-8 has also been suggested to regulate NF-κB activity (10, 38, 39). We found a reduction in TNF and IL-6 release, and pro-IL-1β expression, all controlled by NF-κB, in RIP1 KO, caspase-8 cKO, and RIP3$^{-/-}$caspase-8$^{-/-}$, but not in RIP3$^{-/-}$ macrophages upon infection or LPS treatment (Fig. 3 *A*–*F*). However, cytokine production by the TLR2 ligand Pam3Cys (Fig. 3 *A* and *B*) or Sendai virus (Fig. S7*A*) was largely preserved. The defect in cytokine release could be explained by a decreased NF-κB activation, as suggested by reduced IκBα degradation, IκBα phosphorylation, IKKα/β phosphorylation, and p65 nuclear translocation, particularly at later time points during *Y. pestis* or LPS challenge (Fig. 3 *G*–*J* and Fig. S7 *B* and *D*). Reduced signaling could also be observed in RIP1$^{-/-}$ macrophages (Fig. 3*E*

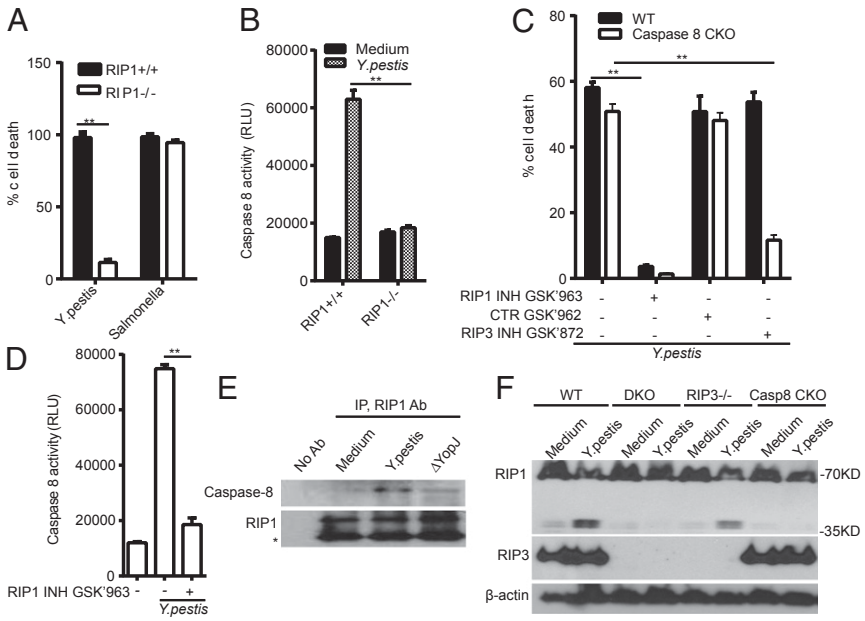

Fig. 2. RIP1 inhibition or deficiency protect macrophages from *Y. pestis*-induced cell death. (*A*) RIP1-deficient fetal liver macrophages are resistant to *Y. pestis*-induced killing (MOI 40, 4 h), detected by LDH release. (*B* and *D*) RIP1, but not RIP3, mediates caspase-8 enzymatic activity after infection of BMDM (*D*) or fetal liver macrophages (*B*) with *Y. pestis* for 2 h. (*C*) Caspase-8 conditional KO macrophages are protected from *Y. pestis*-induced death in the presence of RIP1 (GSK'963) or RIP3 (GSK'872) kinase inhibitors, but not by inactive compound GSK'962. (*D*) RIP1 kinase inhibitor GSK'963 inhibits caspase-8 enzyme activity after infection. (*E*) RIP1 forms a complex with caspase-8 upon infection (1 h), measured by co-IP. (*F*) RIP1 is cleaved after *Y. pestis* infection in a caspase-8 dependent fashion. Figures are representative for three to eight experiments performed. Bars indicate mean plus SD. $^{**}P < 0.01$ (two-tailed *t* test).

and Fig. S7*E*). How caspase-8 controls NF-κB activation is unclear and may not involve the enzymatic activity of caspase-8 (39) (Fig. S7*C*); However, TRIF-mediated pathways may be targeted because MyD88-dependent TLR2 signaling is not affected.

Subsequent experiments indicated that YopJ-dependent *Y. pestis*-induced IL-1β or IL-18 release was reduced in the absence of caspase-8 and showed further reduction by the absence or blockade of RIP3 or RIP1 kinase activity (Fig. 4 *A*–*E*).

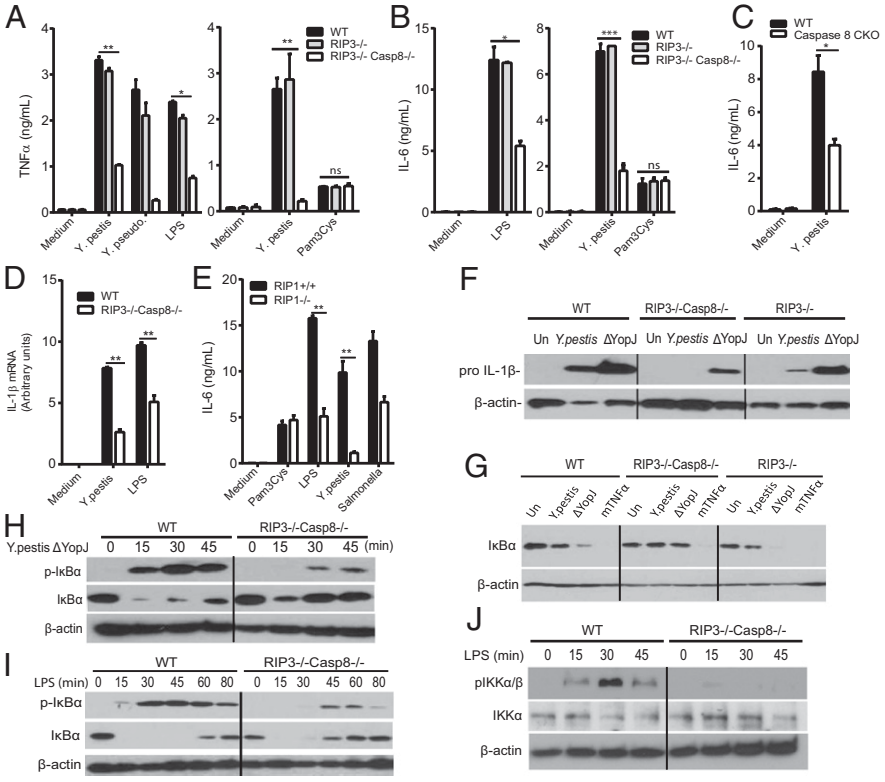

Fig. 3. Caspase-8 and RIP1 contribute to cytokine release and NF-κB activation. (*A*–*C*) WT or mutant BMDM were infected with *Y. pestis*, *Y. pseudotuberculosis* (MOI 10), or *Salmonella* (Sal, MOI 1.5) or treated with LPS (100 ng/mL) or Pam3Cys (500 ng/mL) for 6 h, and cytokine release was measured by ELISA. (*D*) BMDMs were infected with *Y. pestis* for 4 h, mRNA was isolated, and quantitative PCR for pro-IL-1β was performed. (*E*) WT or RIP1$^{-/-}$ fetal liver macrophages were stimulated with LPS (50 ng/mL), Pam3Cys (500 ng/mL), *Y. pestis* (MOI 10), or *Salmonella* (MOI 1.5) for 6 h. IL-6 release was measured by ELISA. (*F*) BMDMs were infected for 6 h and cell lysates probed for pro-IL-1β. (*G*–*J*) BMDMs were infected or treated with LPS, mouse TNF-α (10 min), and cell lysates were probed by immunoblot for the indicated proteins (IκBα, phospho-IκBα, phospho-IKKα/β, or β-actin). Figures are representative of two to five experiments performed. Bars indicate means plus SD. $^{**}P < 0.01$, $^{*}P < 0.05$ (two-tailed *t* test).

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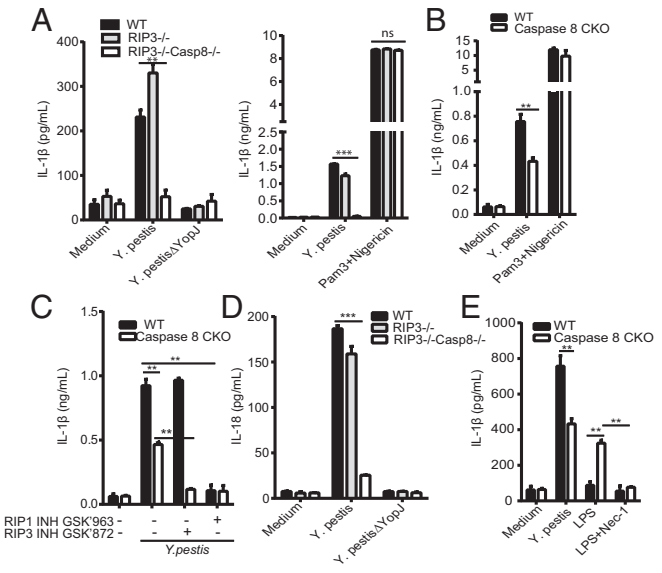

Fig. 4. *Y. pestis*-induced release of IL-1β and IL-18 is severely reduced in caspase-8/RIP3-deficient macrophages. (*A*–*E*) BMDMs were infected with *Y. pestis* or *Y. pestis* ΔYopJ for 6 h as indicated in Fig. 1, or stimulated with nigericin (10 μg/mL) for 1 h after priming with Pam3Cys (4 h, 500 ng/mL). IL-1β and IL-18 were analyzed by ELISA. (*C*) Some BMDMs were treated with RIP1 inhibitor GSK'963 (1 μM) or RIP3 inhibitor GSK'872 (10 μM) for 1 h before infection. (*E*) BMDMs were challenged with *Y. pestis* (MOI 10) for 6 h or LPS (50 ng/mL) for 10 h with or without Nec-1 pretreatment (20 μM). Figures are representative of three to five experiments. Bars indicate means plus SD. $^{**}P < 0.01$, $^{*}P < 0.05$ (two-tailed *t* test in *A*, *B*, and *D*, and two-way ANOVA with Tukey's posttest in *C* and *E*).

Cytokine release after stimulation with Pam3Cys and nigericin was unaffected (Fig. 4 *A* and *B*), implying that NLRP3 activation was not decreased. Although the absence of caspase-8 alone in macrophages decreased IL-1β release induced by infection, it increased IL-1β induced by LPS alone (Fig. 4*E*), as suggested for dendritic cells (40). Thus, more complex stimulations, as observed during infection, yield a different result than a purified ligand, possibly reflecting combined effects induced by both LPS and the *Yersinia* T3SS in the context of live bacteria.

RIP1, Caspase-8, and RIP3 Mediate Inflammasome Activation. Our previous data (Fig. 3) could partially explain reduced IL-1β release. However, caspase-8 or RIP1 inhibition minimized infection-induced caspase-1 cleavage (Fig. S7 *F* and *G*), indicating direct effects on inflammasome action. RIP3 has been involved in inflammasome activation under certain conditions with cIAP inhibiton (41). Infection-induced caspase-1 processing, only partially dependent upon NLRP12 (25), was not affected in RIP3$^{-/-}$ cells but was reduced in TLR4 or TRIF KO (Fig. S8 *A* and *B*) and caspase-8 cKO cells, and severely reduced in RIP1$^{-/-}$ or RIP3$^{-/-}$caspase-8$^{-/-}$ cells after *Y. pestis* infection (Fig. 5 *A*, *B*, and *D*). IL-1β processing was also affected (Fig. 5 *C* and *D*). Caspase-1 cleavage induced by *Salmonella* and Pam3Cys plus nigericin was not affected (Fig. 5 *A* and *B*), indicating that NLRC4- and NLRP3-mediated caspase-1 cleavage is not inherently reduced in RIP1$^{-/-}$ or dKO cells. Caspase-8 has been proposed to control IL-1β maturation and release in response to FasL stimulation or fungal and bacterial challenge (42, 43), perhaps by directly cleaving pro-IL-1β (44), and we cannot exclude this possibility. However, we propose that caspase-8 directs caspase-1 processing and activation, in a RIP3-enhanced manner, after *Y. pestis* challenge (Fig. 5 *B* and *D*), but caspase-1 does not control caspase-8 activation (Fig. S8*C*). Caspase-8 may be a critical component, but deletion or inhibition of RIP3 may block an alternative pathway in the absence of caspase-8, redundancy between caspase-8 and RIP3 may occur, or both molecules may be needed for stabilization of a signaling complex. The mechanism we describe seems independent of FasL, TNF, or type I IFN (Fig. S8 *D*–*F*) and may have some common features with responses induced by ER stress, certain chemotherapeutic drugs, or *Citrobacter* (45–47). The effect on caspase-1 cleavage may be mediated by the inflammasome adaptor ASC (Fig. 5*E*), because ASC can associate with caspase-8 after *Francisella* or *Salmonella* infection (38, 48), although the role of ASC may differ depending upon conditions and source of YopJ (16, 23, 25).

Role of Caspase-8 and RIP3 for in Vivo Resistance to Bacterial Infection. The in vivo relevance of our findings was emphasized by the fact that RIP3$^{-/-}$caspase-8$^{-/-}$ mice were more susceptible to s.c. infection with virulent *Y. pestis* KIM1001 (Fig. 6*A*). Because LD_{50} is very low for KIM1001 we used the attenuated strain KIM1001-EcLpxL, which constitutively generates a TLR4-activating hexa-acylated LPS (24), for survival analysis. dKO, or lethally irradiated WT mice with bone marrow transplant (BMT) from dKO, succumbed to s.c. infection with *Y. pestis*-EcLpxL (Fig. 6*B* and Fig. S9*A*). Resistance to *Y. pestis*-LpxL is heavily influenced by IL-18 and IL-1 (25). Moribund mice had large numbers of bacteria in their spleens compared with WT controls, suggesting that death occurred from uncontrolled systemic bacterial replication (Fig. 6*C*). This correlated with depressed IL-18, IL-1β, TNF, and IL-6 cytokine levels and reduced myeloid cell death (cells positive for live/dead stain and annexin V) in spleens (Fig. 6 *D*–*I* and Fig. S9 *B*–*G*) after i.v. infection. Reduced ability to suppress bacterial growth was also suggested by the presence of visible bacteria-containing pockets in inflammatory foci in the livers (Fig. 6*J*) of dKO BMT mice upon i.v. infection. Because irradiated mice that received RIP3$^{-/-}$caspase-8$^{-/-}$ BMT behaved similarly as dKO animals, we propose that protection toward infection is mediated by cells originating from the bone marrow, expressing caspase-8 and RIP3. Some questions still remain with respect to certain details of how caspase-8 and RIP3 are involved in caspase-1 processing, although it is possible that ASC has a central role. Our results provide a basis for increased understanding of how bacterial pathogens, via their T3SS, can interact with several aspects of host innate immunity via RIP kinases and caspase-8. The data also show how apoptosis, generally viewed as a "silent" cell death, can be accompanied by strong inflammatory reactions, via pathways with several common players. The host may have developed these pathways as an effective means of alerting cells to the infection. We propose that caspase-8 and RIP kinases are central regulators of cell death and innate immune responses to *Y. pestis*, and we establish a role for these components in antibacterial innate immune responses. Therapies that modulate the activity of these pathways may be useful in the treatment of bacterial infections.

Methods

Mice. RIP3 KO (49) and caspase-8$^{-/-}$ RIP3$^{-/-}$ (dKO) (9) have been reported. Caspase-8$^{fl/fl}$ LysM cre$^{+/+}$ cKO mice were generated by D.M.S. C57BL/6 mice were bred in house or from Jackson Laboratories. BMT was performed on lethally (900 rads) irradiated mice. Mice were infected s.c. or i.v. with 500 cfu of KIM1001-pEcLpxL and monitored for survival. Tissue for analysis was harvested at 42 h after infection, or at 68 h after s.c. infection with KIM1001 (300 cfu).

Bacterial Strains and Growth Conditions. *Y. pestis* KIM5 or KIM5ΔYopJ (24) (25) were grown in tryptose-beef extract broth with 2.5 mM $CaCl_2$ overnight with shaking at 26 °C. The next day the bacteria was diluted 1:8 in fresh media, cultured for 1 h at 26 °C, and shifted to 37 °C for 2 h or grown continuously at 37 °C when indicated. *Y. pseudotuberculosis* IP2666, *Y. enterocolitica* 8081, and *Salmonella enterica* serovar Typhimurium strain SL1344 were as reported (25) and grown at 37 °C. KIM5-EcLpxL and KIM1001-EcLpxL were as previously published (24).

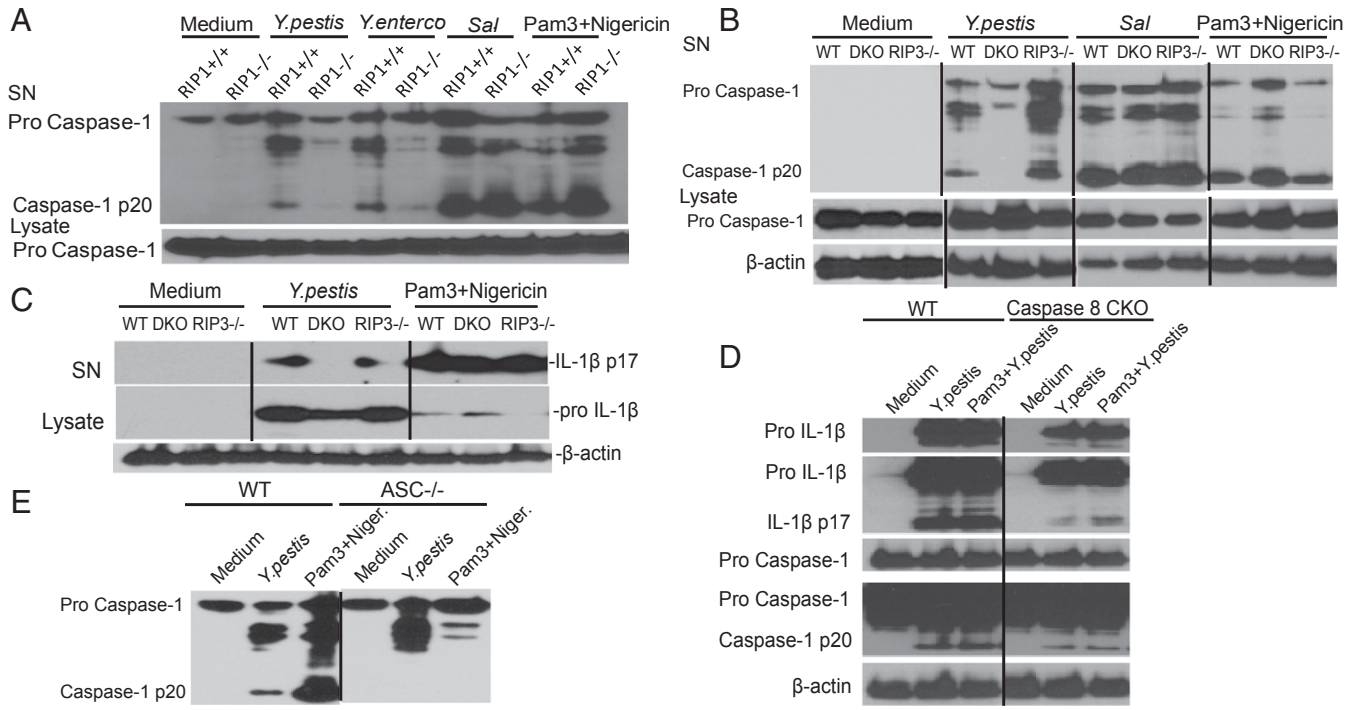

Fig. 5. RIP kinases and caspase-8 control caspase-1 cleavage induced by *Y. pestis*. (*A–E*) BMDM (WT, RIP3$^{-/-}$, RIP3$^{-/-}$caspase-8$^{-/-}$ dKO, caspase-8 cKO) or fetal liver macrophages (RIP1$^{+/+}$, RIP1$^{-/-}$) were infected with *Y. pestis*, *Y. enterocolitica*, or *Salmonella* (Sal) for 6 h or primed with Pam3Cys followed by nigericin for 1 h, and supernatants (SN) or lysates were analyzed for caspase-1 or IL-1β processing by immunoblots. Figures are representative of three to five experiments.

Cell Stimulations. BMDMs were prepared by maturing bone marrow cells for 6–7 d in the presence of L929 supernatant containing M-CSF. Some experiments were performed with BMDM immortalized with J2 retrovirus (42), or J2 immortalized RIP1$^{+/+}$ and RIP1$^{-/-}$ fetal liver macrophages (31). Cells were plated overnight and infected with bacteria at multiplicities of infection (MOIs) of 10 or 40, or stimulated with LPS from *Y. pestis* 26 °C (24) or *Escherichia coli*, or Pam3Cys (Invivogen). Gentamycin was added 1–2 h after infection. Cell death was estimated at 4 h by measuring lactate dehydrogenase (LDH) release (Promega). In some experiments, cells were pretreated with 1 μM GSK'963 or GSK'962, or 3 μM GSK'872 [RIP1 and RIP3 inhibitors (32) and GSK: P.A.H., J.B., P.J.G.], 20 μM Nec-1 (Enzo), 20 μM zIETD, zYVAD, or zVAD (Promega) for 1 h before infection. Cytokines and caspase-1 cleavage were measured

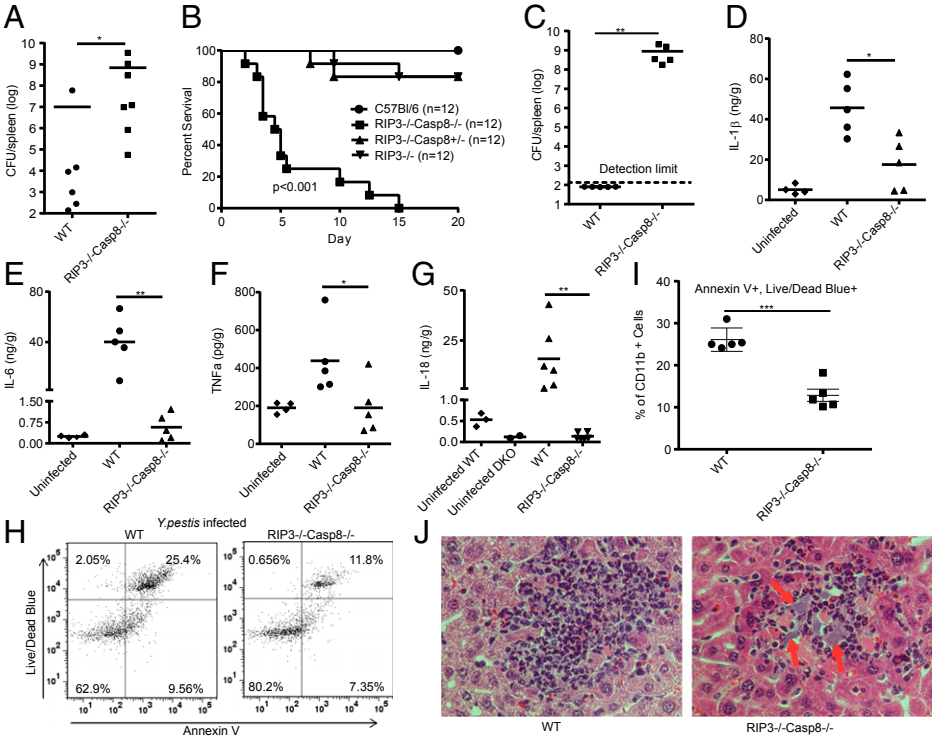

Fig. 6. Caspase-8 with RIP3 is critical for in vivo resistance to bacterial infection. RIP3$^{-/-}$ caspase-8$^{-/-}$ dKO or WT mice were infected s.c. with virulent *Y. pestis* KIM1001 (300 cfu) for 68 h and spleens analyzed for bacterial growth (*A*). Lethally irradiated mice, subjected to bone marrow transplantation (BMT) from the indicated genotypes (*B* and *C*), were infected s.c. with 500 cfu of *Y. pestis* KIM1001-EcLpxL and monitored for survival (*B*), $P < 0.001$ dKO vs. WT (log–rank test). Spleens from moribund dKO BMT mice and controls were analyzed for bacterial contents (*C*). (*D–J*) Mice from BMT as above (*D–F*, *H–J*) or regular dKO (*G*) were infected i.v. with KIM1001-EcLpxL (500 cfu) for 42 h. Spleens were homogenized and analyzed for cytokines by ELISA (as cytokine/g tissue) (*D–G*). (*H* and *I*) CD11b-positive myeloid cells in spleens were analyzed for cell death with live/dead blue and annexin V stain. (*J*) Liver sections were stained with hematoxylin and eosin and subjected to microscopy (400×). Foci containing inflammatory cells (mostly neutrophils) are shown, with visible pockets containing bacteria indicated by arrows. Shown is a representative experiment out of two to three performed. *$P < 0.05$, **$P < 0.01$ (Mann-Whitney *U* test).

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as previously indicated (25). Caspase-8 activity (Promega) was measured after 2 h.

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