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Manipulation of Interleukin-1β and Interleukin-18 Production by *Yersinia pestis* Effectors YopJ and YopM and Redundant Impact on Virulence*

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Dmitry Ratner[‡], M. Pontus A. Orning[‡§], Kristian K. Starheim[‡§], Robyn Marty-Roix[‡], Megan K. Proulx[¶], Jon D. Goguen[¶], and Egil Lien[‡§1]

From the [‡]Program in Innate Immunity, Division of Infectious Diseases and Immunology, Department of Medicine, University of Massachusetts Medical School, Worcester, Massachusetts 01605, the [§]Centre of Molecular Inflammation Research, Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology, 7491 Trondheim, Norway, and the [¶]Department of Microbiology and Physiological Systems, University of Massachusetts, Worcester, Massachusetts 01655

Innate immunity plays a central role in resolving infections by pathogens. Host survival during plague, caused by the Gram-negative bacterium *Yersinia pestis*, is favored by a robust early innate immune response initiated by IL-1β and IL-18. These cytokines are produced by a two-step mechanism involving NF-κB-mediated pro-cytokine production and inflammasome-driven maturation into bioactive inflammatory mediators. Because of the anti-microbial effects induced by IL-1β/IL-18, it may be desirable for pathogens to manipulate their production. *Y. pestis* type III secretion system effectors YopJ and YopM can interfere with different parts of this process. Both effectors have been reported to influence inflammasome caspase-1 activity; YopJ promotes caspase-8-dependent cell death and caspase-1 cleavage, whereas YopM inhibits caspase-1 activity via an incompletely understood mechanism. However, neither effector appears essential for full virulence *in vivo*. Here we report that the sum of influences by YopJ and YopM on IL-1β/IL-18 release is suppressive. In the absence of YopM, YopJ minimally affects caspase-1 cleavage but suppresses IL-1β, IL-18, and other cytokines and chemokines. Importantly, we find that *Y. pestis* containing combined deletions of YopJ and YopM induces elevated levels of IL-1β/IL-18 *in vitro* and *in vivo* and is significantly attenuated in a mouse model of bubonic plague. The reduced virulence of the YopJ-YopM mutant is dependent on the presence of IL-1β, IL-18, and caspase-1. Thus, we conclude that *Y. pestis* YopJ and YopM can both exert a tight control of host IL-1β/IL-18 production to benefit the bacteria, resulting in a redundant impact on virulence.

Many pathogens rely upon a strong suppression or evasion of host immune responses to cause disease. *Yersinia pestis*, the etiologic agent of plague, achieves high virulence in part by actively suppressing the host immune system. A key component of this strategy is the type III secretion system (T3SS),[2] by which the bacterium delivers seven Yop (*Yersinia* outer protein) effectors into host immune cells. These Yops manipulate intracellular pathways to inhibit phagocytosis, motility, cytokine expression, and other vital immune processes (1). Two effectors, YopJ and YopM, have been extensively studied for over two decades, but key questions about their roles in disease remain unanswered.

In vitro studies have revealed YopJ to be an acetyl transferase targeting and inhibiting IKKβ (2), MAP kinase kinases (3, 4), and TAK1 (5, 6). YopJ has also been reported to behave like a deubiquitinase (7). YopJ robustly inhibits NF-κB-mediated transcription of pro-inflammatory cytokines and induces caspase-8/RIP1-mediated apoptosis, caspase-1 cleavage, and IL-1β release, effects that are dependent on its enzymatic activity (8–10). However, despite its powerful effects *in vitro*, YopJ appears perplexingly dispensable during *Y. pestis* infection *in vivo* (11, 12).

YopM has also been reported to limit the recruitment of monocytes, neutrophils, and NK cells to infected organs (13, 14). It was recently demonstrated that YopM inhibits caspase-1 (15, 16). Caspase-1 mediates maturation of IL-1β and IL-18, cytokines that promote a robust early immune response that enhances host resistance against *Y. pestis* infection (17, 18). Thus, YopM may promote virulence by inhibiting the processing of these cytokines. However, the impact of YopM on *Y. pestis* virulence appears minor (14, 19), and it is unclear whether IL-1β/IL-18 levels would increase *in vivo* during *Y. pestis* infection without YopM.

IL-1β and IL-18 are produced in a two-step process requiring an initial signal to trigger the transcription/translation of their pro-forms, and a second signal to trigger their processing by caspase-1 into their biologically active secreted forms (20). The first signal is NF-κB-dependent, typically downstream of stimulation via Toll-like receptors by microbially associated molecules such as LPS. The second signal is catalyzed by inflammasomes. A canonical inflammasome is formed when pathogen- or danger-associated molecular patterns are sensed

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[1] To whom correspondence should be addressed. E-mail: egil.lien@umassmed.edu.

[2] The abbreviations used are: T3SS, type III secretion system; NLR, NOD-like receptor; MOI, multiplicity of infection; BMDM, bone marrow-derived macrophage; BMDC, bone marrow-derived dendritic cell; DC, dendritic cell; TCR, T-cell receptor.

by a NOD-like receptor (NLR), such as NLRP3, NLRC4, or the non-NLR protein AIM2. This leads to recruitment and oligomerization of the adaptor protein ASC, which in turn recruits pro-caspase-1 dimers to be autoproteolyzed into the catalytically active p20 form. This process is normally accompanied by an inflammatory form of cell death called pyroptosis. Importantly, noncanonical inflammasomes involving caspase-8 (21, 22) or caspase-11 (23, 24) have also been described.

Because of the potential importance of IL-1β/IL-18 in fighting infection, the interaction of *Y. pestis* with host inflammasomes warrants close attention. Because YopJ and YopM appear to target the IL-1β/18 maturation pathway in distinct ways, we hypothesized that these two effectors may cooperate for optimal effects on the immune system. We set out to investigate whether such an interaction exists and what role this may play during infection. Surprisingly, we found that *Y. pestis* lacking both YopJ and YopM induced increased IL-1β/IL-18 compared with parental bacteria or strains lacking one of the effectors. We also found a strain lacking both effectors to be significantly attenuated in a bubonic plague model in an IL-18/IL-1β/caspase-1-dependent fashion, suggesting that tuning down inflammasome activity and IL-1β/IL-18 release are key features of *Y. pestis* pathogenesis.

Experimental Procedures

Bacterial Strains and Growth Conditions—The fully virulent KIM1001 strain of *Y. pestis*, the attenuated KIM5 (Δ*pgm*) BSL2 strain, and KIM5ΔYopJ were previously described (8, 18, 25). The ΔYopM and ΔYopM/J strains were generated both on the KIM5 and KIM1001 background as follows: an in-frame deletion removing amino acids 3–408 of 410 of the *yopM* gene was created via allelic exchange. PCR products made with primer sets yopM-A, yopM-B and yopM-C, yopM-D, respectfully, were used to make a fused product by overlap PCR using primers A and D (26). This product was cloned in the allelic exchange vector pRE107 (27) in *Escherichia coli* K12 strain β2155, a diamiopimelic acid auxotroph, and transferred to *Y. pestis* KIM1001 by conjugation. KIM1001 recombinants were selected on TB medium containing 100 μg/ml ampicillin but no diaminopimelic acid. Following counter selection with 5% sucrose, deletion mutants were identified by PCR. The same procedure was followed to construct an in-frame deletion mutant of *yopJ* and *yopE* in KIM1001 and KIM1001 Δ*yopM* using the respective gene specific A, B, C, and D primers shown in Table 1. Attenuated Δ*pgm* derivatives of each strain, bearing the designation KIM5 to indicate their altered chromosomal genotype, were derived from their respective KIM1001 parents by selection for loss of pigmentation on HIB Congo Red agar at 26 °C. KIM5 Δ*pla* was also generated as described (25). Loss of the pigmentation region (Δ*pgm*)/iron acquisition was confirmed by PCR with primers pgm-F, pgm-R; psn-F, psn-R; and hmsH-F, hmsH-R. Expression of YopM and YopJ was confirmed by RT-PCR.

A type III secretion effector deficient strain (ΔT3SSe) was constructed by making sequential in-frame deletions, as described above, of *yopM* (amino acids 3–408 of 410), *yopE* (amino acids 40–197 of 220), *yopH* (amino acids of 3–467 of 469), *ypkA* (amino acids of 3–731 of 733), *yopJ* (amino acids 4–288 of 289), *yopK* (amino acids 4–181 of 183), and *yopT* (amino acids of 3–320 of 323) using the respective gene specific A, B, C, and D primers shown in Table 1. The deletions were made in *Y. pestis* KIM 1001, and a KIM5 derivative was generated as described above. This strain lacks Yops M/E/J/H/T/K and YpkA (also called YopO) but expresses Yops B/D and the machinery necessary to assemble a T3SS needle with a functional pore-forming translocon complex.

TABLE 1
Primers used for generation of bacterial strains

Primer name	Sequence 5′ to 3′
yopM-A	ATAGAGCTCTTCAAAAGGGGTACTGGATAC
yopM-B	GAACATATTGAATGCCTTTCT
yopM-C	AGAAAGGCATTCAATATGTTCGAGTAGTACGCAAGAGCGTTC
yopM-D	GGGTCTAGATTTACCAATTTTTTGATGGGG
yopJ-A	ATAGAGCTCCACTACTGATTCAACTTGGACG
yopJ-B	ACGGCAAATGCAGAGCAGTCCGATCATTTATTTATCCTTATTCA
yopJ-C	CTGCTCTGCATTTGCCGTTAATGTATTTTGGAAATCTTGCT
yopJ-D	GGGTCTAGACTGATGTCGTTTATTTCTGGGTAT
yopE-A	ATAGAGCTCAGCATTACACACTCCACAGTTGGGT
yopE-B	ACGCAGGCAGCAAATGAGATCAAA
yopE-C	CTCATTTGCTGCCTGCGTATATTGATCACTTGTTTG
yopE-D	ATATCTAGATATCCAGGCTGTTCAATGGTTGTCGAT
Pgm-F	CCGCAACAACATCATCCGTATTCA
Pgm-R	TTCGCTACCACTGAAATCCAAGAC
Psn-F	ATTGCTCCCCGCCATTGCTA
Psn-R	CATTGCTCTTACCCTGGTCGCCA
hmsH-F	CGTTTCAGTTGCCTGTGTGCTAAC
hmsH-R	CATCACTCGGTGTAGACATCGCT

All *Y. pestis* strains were grown using TB medium supplemented with 2.5 mM $CaCl_2$. KIM1001 and derivative strains were plated on agar incubated at 37 °C overnight and passed once before preparing inoculum for injection. Strains on the KIM5 background were plated overnight from frozen glycerol stocks and then grown at 26 °C in liquid broth overnight; on the day of infection, cultures were diluted 1:20 and grown for 2 h at 26 °C followed by a shift to 37 °C for 2 h. This transition is important to up-regulate expression of the T3SS while minimizing expression of F1 protein capsule (28), which interferes with cell-based assays. Bacteria were then washed three times in RPMI 1640 medium (prewarmed to 37 °C), quantified by A_{600}, and added to cells at a multiplicity of infection (MOI) of 10 bacteria per cell in a 10-μl volume within 1 h of preparation.

Cell Stimulations—Bone marrow-derived macrophages (BMDMs) were differentiated in RPMI 1640 medium supplemented with 10% FCS, 25 mM HEPES, 10 μg/ml ciprofloxacin, and 10% L929 conditioned medium containing M-CSF for 5 days. Bulk bone marrow-derived dendritic cells (BMDCs) were differentiated in R10 medium consisting of RPMI 1640 medium, 10% FCS, 20 mM HEPES, 2 mM L-glutamine, 50 μM β-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 ng/ml recombinant murine GM-CSF (Peprotech) for 9 days. BMDMs or BMDCs were harvested and seeded at a density of 100,000 cells/well in a 96-well plate format overnight and stimulated the following day. The cells were primed with 100 ng/ml LPS for 5 h (unless otherwise indicated) or allowed to rest in antibiotic-free RPMI 1640 medium with 10% FCS and 25 mM HEPES without antibiotic before addition of bacteria at an MOI of 10. End points were as follows: 2 h postinfection for RNA extraction, 3 h postinfection for caspase-8 enzymatic assays (Caspase-Glo 8 kit, Promega catalog no. G8200), 5 h

TABLE 2
Primers used for RT-PCR

Primer name	Sequence 5′ to 3′
GAPDH F	TGTGTCCGTCGTGGATCTGA
GAPDH R	CCTGCTTCACCACCTTCTTGA
Pro-IL-1β F	AGGCCACAGGTATTTTGTCG
Pro-IL-1β R	GCCCATCCTCTGTGACTCAT
Pro-IL-18 F	CAGGCTGTCTTTTGTCAACGA
Pro-IL-18 R	GACTCTTGCGTCAACTTCAAGG
IFN-β F	CTGTCTGCTGGTGGAGTTCA
IFN-β R	ATAAGCAGCTCCAGCTCCAA
IL-6 F	GAGCATTGGAAATTGGGGTA
IL-6 R	AACGATGATGCACTTGCAGA
YopM F	TTACCGCAGAGCCTGAAATC
YopM R	GCAACTCTGGCAATTCTTCC
YopJ F	TAGAAGTCATGCCCGCATTG
YopJ R	TGTCCTTATTGCCAGCATCG

postinfection for LDH assay (CytoTox 96, Promega), and 6 h for harvesting of supernatant and/or cells for analysis of cytokines by ELISA (R&D Systems) or caspase-1 activation by SDS-PAGE and Western blot (anti-caspase-1 catalog no. AG-20B-0042-C100, Adipogen; anti-IL-1β catalog no. DY401 840135, R&D Systems). For time points exceeding 3 h, 50 μg/ml gentamicin was added at 3 h postinfection Each graph represents results of two or more independent cell stimulations on separate days.

Cell Death Assays—The LDH assay was used to measure cell death at a fixed 5-h end point; for this assay, the RPMI 1640-based medium was replaced with X-vivo (Lonza) supplemented with 3.5% FCS and HEPES prior to infection. Additionally, a kinetic cell death assay using a DNA binding dye was performed as follows. The cells were incubated with 0.2 μM ethidium homodimer (EthD-1, Sigma catalog no. 46043) in the RPMI 1640 medium mentioned above 1 h before adding bacteria. Upon infection, the plate was placed in a Synergy H4 plate reader at 37 °C, and UV-induced fluorescence was measured every 10 min. Increased fluorescence correlates with DNA binding by EthD-1 upon entry through increasingly permeable cell membranes as cell death progresses.

RT-PCR—RNA was extracted using the Qiagen RNEasy kit, followed by RT-PCR with the SYBR Green DNA probe (Bio-Rad). $C(t)$ values were normalized to GAPDH internal controls, and the means were normalized to the unstimulated negative control group. For RT-PCR on RNA extracted from bacterial lysates, the results were normalized to *Y. pestis* 16S rRNA internal control. The primers used for RT-PCR are listed in Table 2.

Mice—All experiments involving mice were approved by the Institutional Animal Care and Use Committee. Mouse strains used in this study were described previously (17) and bred in-house. TCR$\beta^{-/-}\delta^{-/-}$ (TCRβδ dKO, lacking TCRαβ and TCRγδ) were from Jackson Laboratories and provided by Ray Welsh. BMDMs and BMDCs were differentiated from bone marrow harvested from the femurs of 6–20-week-old mice. Peritoneal macrophages were harvested by injecting mice intraperitoneally with 1 ml of thioglycolate and lavaging the peritoneal cavity 72 h later with RPMI 1640 medium. The cells were seeded at 500,000/well in 12-well format for subsequent stimulation.

To investigate a system where neutrophils are likely playing a major role in inflammatory signals, we conducted infections directly in the peritoneum of mice after stimulating heavy neutrophil recruitment with thioglycolate. The mice were injected intraperitoneally with 0.7 ml of 4% thioglycolate followed 3 h later by same side injection with 1×10^8 cfu of KIM5, KIM5ΔYopM, KIM5ΔYopJ, KIM5ΔYopM/J, or mock in 0.3 ml of PBS. The peritoneal cavity was lavaged with 3 ml of Hanks' buffered saline solution 6 h postinfection. The cells were centrifuged and analyzed by flow cytometry with anti-Ly6G) and anti-CD11b (Becton Dickinson), and the cleared lavage fluid was assayed for IL-1β by ELISA. This approach gave a similar cellular composition of 90–98% Ly6G/CD11b+ cells. Flow cytometry data were analyzed by FlowJo software.

Subcutaneous inoculations were conducted by injecting mice with 50–140 cfu in 50 μl of PBS in the nape of the neck behind the right shoulder blade; the mice were then either monitored for survival for up to 40 days or sacrificed for collection of organs at 90 h postinfection. Intravenous inoculations were conducted by injecting mice with 40–50 cfu in 200 μl of PBS via the tail vein and harvesting serum, spleen, and liver at 42 h postinfection. Upon dissection (subcutaneous infection), a single large lymph node on the side of infection was harvested from each mouse. Spleens and lymph nodes were collected in 0.7 ml of PBS and homogenized in the closed system Miltenyi gentleMacs homogenizer. 50 μl of homogenate was taken for serial dilutions and quantification of bacterial loads per organ. Excess spleen homogenate was treated immediately with protease and phosphatase inhibitor (Roche) and ciprofloxacin and analyzed for cytokines by ELISA. Blood was harvested by cheek bleed and processed for collection of serum as previously described (17). Livers were fixed in 10% formalin and stained by hematoxylin and eosin. Vaccination experiments were performed by infecting mice surviving infection with KIM1001ΔM/J on day 14 or 21 with virulent KIM1001 and monitoring survival for 25 days.

Statistical Analysis—*In vitro* assays were analyzed by two-way ANOVA followed by Bonferroni post-test. *In vivo* cytokines were tested for significance by one-way ANOVA followed by Bonferroni's multiple comparison. Nonparametric bacterial load data were analyzed by the Kruskal Wallis test followed by Dunn's post-test. Differences in survival were analyzed by Kaplan-Meyer analysis and log rank test. The values where $p < 0.05$ were considered significant.

Results

YopM and YopJ Differentially Influence Inflammasomes and Inflammatory Cytokines and Chemokines—Deletion of YopM results in increased IL-1β/IL-18 secretion and caspase-1 cleavage (Fig. 1, *A* and *B*) in BMDMs compared with the parental strain. By contrast, loss of YopJ alone results in reduced IL-1β/IL-18 secretion (Fig. 1*B*) and caspase-1 activation (Fig. 1*A*), despite an increase in pro-IL-1β and pro-caspase-1. Loss of both YopM and YopJ leads to a further increase in ASC/caspase-1-dependent IL-1β and IL-18 secretion but an insignificant increase in caspase-1 activation compared with loss of YopM alone (Fig. 1, *A*, *B*, and *D*). We confirmed Yop expression in the ΔYopJ and ΔYopM strains (Fig. 1*E*).

We found that YopE may play a part in triggering the IL-1β release that is inhibited by YopM (Fig. 1*C*). YopE is an effector that regulates GTPase activity in host cells (29). We are still uncertain of how YopE influences the signaling, but YopE may

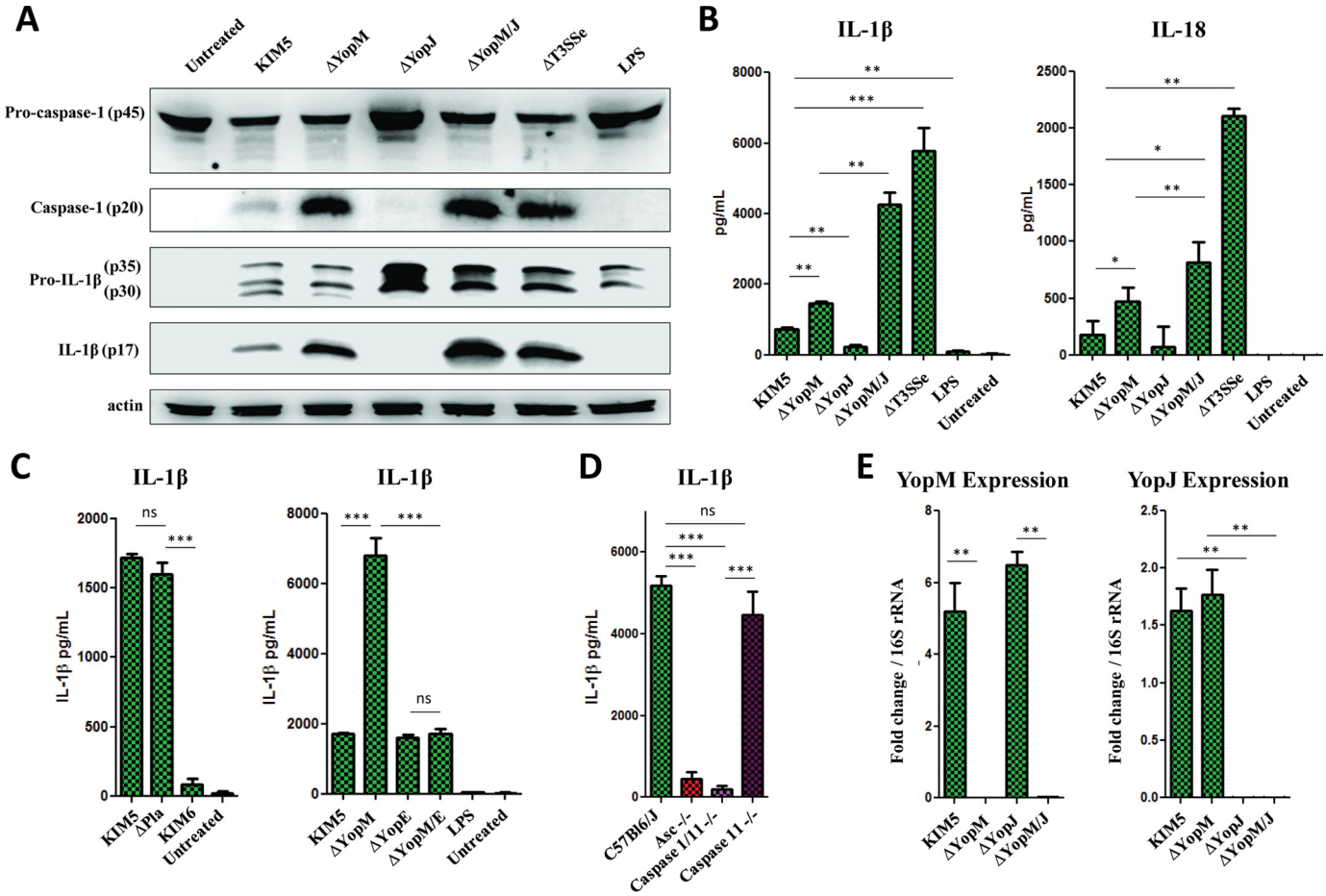

FIGURE 1. **YopM and YopJ have opposing effects on caspase-1 cleavage, but in sum reduce IL-1β and IL-18 levels during infection.** *A*, total protein from total BMDM samples (cell lysate and supernatant) at 6 h postinfection was separated by SDS-PAGE and analyzed by Western blot for IL-1β and caspase-1. *B* and *C*, BMDMs were infected with *Y. pestis* strains at MOI 10, and supernatants were harvested at 6 h postinfection for analysis of IL-1β or IL-18 for ELISA. *D*, supernatants from BMDMs from the indicated mouse genotypes were harvested 6 h postinfection with KIM5 ΔYopM/J (MOI 10) and assayed for IL-1β by ELISA. *E*, RNA was extracted from *Y. pestis* (grown at 37 °C to up-regulate T3SS expression), and amplified by RT-PCR using primers specific for YopM or YopJ. *C*(*t*) values for each primer pair were normalized to *Y. pestis* 16S rRNA internal control *C*(*t*) values. The figure is representative of three or more experiments. Shown are the means ± S.D. for triplicate wells. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *ns*, not significant.

impact bacterial effector translocation (30) or directly affect the host signaling pathways.

RT-PCR analysis demonstrated that YopJ, and not YopM, is primarily responsible for inhibiting the transcription of IL-1β/IL-18 precursors, as well as IL-6 and IFN-β (Fig. 2, *A* and *B*). We found that YopM confers protection from caspase-1-dependent cell death in the absence of YopJ but has only a weak cytoprotective effect when YopJ is present (Fig. 2, *C*, *D*, and *F*). We also confirmed that YopJ, but not YopM, drives the enzymatic activity of caspase-8 (Fig. 2*G*). Expression of TNFα, CXCL1, MCP1, CXCL5, and IL-10, which require no inflammasome processing, is also inhibited by YopJ but not YopM (Fig. 2*H*). Notably, the pCD1 plasmid containing the T3SS is essential for IL-1β induction, because the KIM6 strain lacking pCD1 failed to induce any IL-1β. (Figs. 1, *A*–*C*, and 2, *C*–*E*). The lack of another known virulence factor, the plasminogen activator protein (Pla) (25), had no effect on cell death or IL-1β release (Figs. 1*C* and 2*E*).

YopM and YopJ Have Redundant Impact on Virulence in Vivo—We found that in the mouse bubonic plague model, *Y. pestis* KIM1001 lacking both YopM and YopJ was significantly attenuated. In contrast, *Y. pestis* lacking either YopM or YopJ alone induced lethality comparable with the parent strain, with no significant differences from the fully virulent KIM1001 (Fig. 3*A*). In mice lacking caspase-1, IL-1β, or IL-18, the attenuation of the KIM1001ΔM/J strain was significantly reduced (Fig. 3*B*), suggesting that these components play central roles in the host defenses toward *Y. pestis* lacking both YopM and YopJ. We note that both WT and IL-18 KO mice die of low doses of the parental KIM1001 strain (17). On day 4 of subcutaneous infection, bacterial loads were significantly lower in mice infected with KIM1001ΔM/J both in the spleen and lymph nodes, but KIM1001ΔM and KIM1001ΔJ did not result in statistically different bacterial loads from the parental KIM1001 (Fig. 3*C*).

The increased activation of innate immunity as observed with the KIM1001ΔM/J may also impact adaptive immune responses. We found that wild-type mice that survive initial challenge with KIM1001ΔM/J were effectively protected from subsequent infection with fully virulent *Y. pestis* (Fig. 3*D*), indicating that strains containing deletions of YopM and YopJ, perhaps in combination with expression of LpxL (18), could be promising vaccine candidates. Adaptive immunity may also contribute to resistance of attenuated *Y. pestis* strains (18, 31),

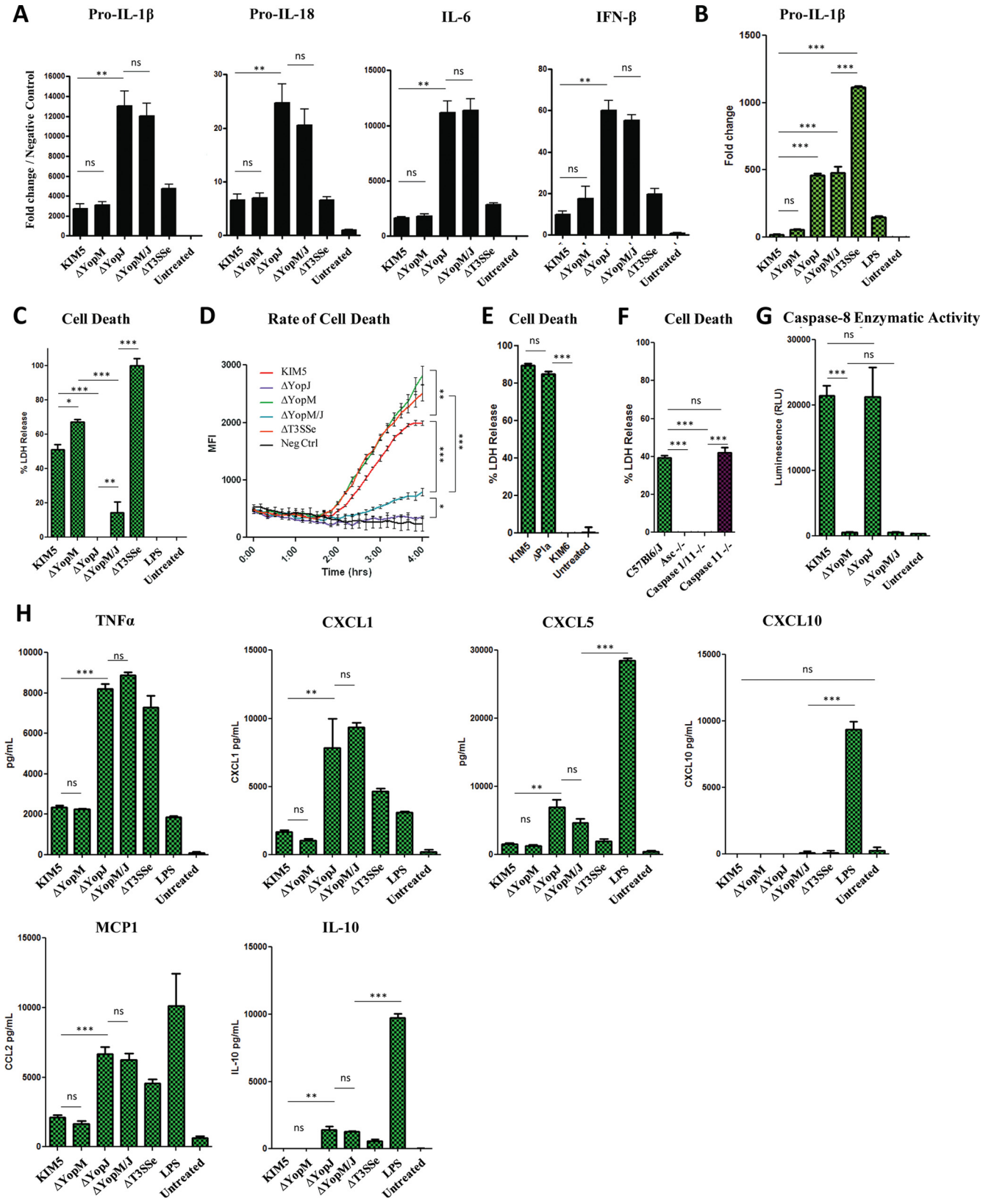

FIGURE 2. **YopJ, but not YopM, inhibits expression of pro-IL-1β and other cytokines and is a major driver of macrophage cell death.** *A* and *B*, RNA from peritoneal macrophages (*A*, unprimed) or BMDMs (*B*) were harvested at 2 h postinfection and amplified by RT-PCR using primers specific for pro-IL-1β, pro-IL-18, IL-6, or IFN-β. *C–E*, cell death in BMDMs was assayed either at 5 h postinfection by assaying LDH release (*C* and *E*) or continuously by measuring intracellular EtHD-1 entry (*D*). *F*, cell death was assayed at 5 h postinfection with KIM5 ΔYopM/J at MOI 10 by assaying LDH release. *G*, caspase-8 enzymatic activity in BMDMs was assayed at 3 h postinfection. *H*, unprimed BMDMs were infected with *Y. pestis* at MOI 10, and supernatants were harvested at 6 h postinfection for quantification of cytokines by ELISA. The figure is representative of two or more independent experiments. Shown are the means ± S.D. for triplicate wells. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *ns*, not significant.

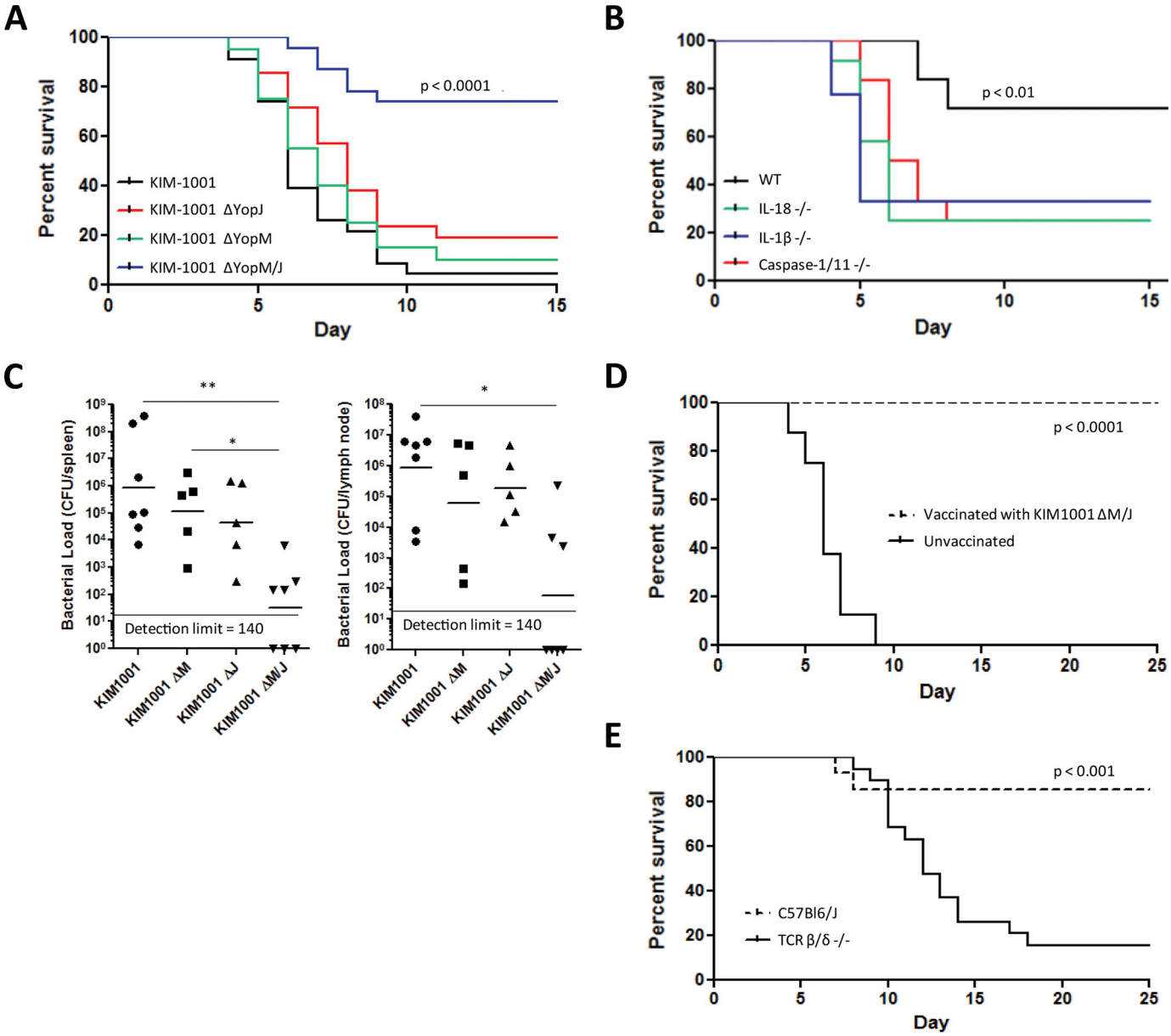

FIGURE 3. **Deletion of both YopM and YopJ attenuates *Y. pestis* in a manner dependent on caspase-1, IL-1β, and IL-18.** *A*, C57Bl6/J mice were injected subcutaneously with 50–140 cfu of KIM1001 ($n = 23$), KIM1001ΔM ($n = 21$), KIM1001ΔJ ($n = 20$), or KIM1001ΔM/J ($n = 23$) and monitored for survival. The results represent pooled data from two separate experiments. *B*, C57Bl6/J ($n = 9$), IL-18 KO ($n = 12$), IL-1β KO ($n = 9$), or caspase-1/11 KO ($n = 12$) mice were injected with 225 cfu of KIM1001ΔM/J subcutaneously and monitored for survival. The *p* values reflect comparisons between KIM1001ΔM/J and other bacterial strains (*A*) or between wild-type mice and other mouse strains (*B*). *C*, C57Bl6/J mice were injected subcutaneously with 50–140 cfu of KIM1001 ($n = 7$), KIM1001ΔM ($n = 5$), KIM1001ΔJ ($n = 5$), or KIM1001ΔM/J ($n = 7$); spleens and lymph nodes were harvested 90 h postinfection, and bacterial load was determined. Geometric mean values are shown. *D*, C57Bl6/J mice that survived infection with KIM1001ΔM/J ($n = 7$) or naïve controls ($n = 8$) were injected subcutaneously with 400 cfu of KIM1001 and monitored for survival for 25 days. The *p* value reflects the comparison of vaccinated *versus* naive animals. *E*, C57Bl6/J mice ($n = 14$) or TCR $\beta/\delta^{-/-}$ mice ($n = 20$) were injected subcutaneously with 120–180 cfu of KIM1001ΔM/J and monitored for survival for 25 days. The results represent pooled data from two separate experiments. The *p* value reflects comparison between wild-type and TCR $\beta/\delta^{-/-}$ mice.

and T cells appear to play a key role (32, 33). To explore this question further, we subjected mice lacking T cell receptors (TCR $\beta/\delta^{-/-}$ mice lack both $\alpha\beta$ and $\gamma\delta$ TCRs) (34) to the same subcutaneous challenge with KIM1001ΔM/J as in Fig. 3*B*. We found that the T cell receptors are very important for mice to survive the initial infection with KIM1001ΔM/J (Fig. 3*E*). Furthermore, the three TCR $\beta/\delta^{-/-}$ mice that survived the initial challenge were not protected from KIM1001; when infected with KIM1001 on day 21, all three died by day 30, whereas 100% (total 21 of 21) of the rechallenged wild-type controls survived beyond day 40. One limitation of these studies is that we cannot rule out a role of B-cells or antibodies; nevertheless, T cells are crucial for the resistance against bacterial challenge, suggesting participation of both innate and adaptive immune responses in optimal host responses to these bacterial strains.

Inhibition of IL-1β and IL-18 Production Are Overlapping Functions of YopM and YopJ in Mice, but Other Effects Differ—During the subcutaneous infection described above (Fig. 3), it is likely that systemic IL-1β and IL-18 levels may correlate to bacterial loads, and thus this experimental setup may not be opti-

mal to study direct effects of YopM/J deletion on cytokine expression. For this reason, we injected the *Y. pestis* strains into mice intravenously and harvested tissue at 42 h to compare systemic IL-1β and IL-18 with smaller differences in host bacterial loads between the different strains (Fig. 4). We confirmed that bacterial loads in the spleen at this time were equivalent in all groups with no statistical differences, validating the comparison of cytokines between the groups (Fig. 4*A*). We found significantly elevated IL-1β and IL-18 in the serum of mice infected with KIM1001ΔM/J (Fig. 4, *B* and *C*). In contrast, however, we also detected a small but significant reduction in IL-18 when YopJ alone was deleted. Furthermore, although IL-1β and IL-18 expression in the KIM1001ΔM/J group was expressed in a caspase-1-dependent manner, KIM1001 induced low but significant levels of these cytokines independently of caspase-1, perhaps indicating a role of caspase-8 (8). The NF-κB-influenced chemokines CXCL1, CXCL10, CXCL5, and MCP1 were elevated in the absence of YopJ but not YopM (Fig. 4, *D–F* and *H*), similar trends as observed with macrophages *in vitro* (Fig. 2). Although the levels of CXCL5 were not significantly different in any strain, they trended higher in the absence of YopJ (Fig. 4*F*). IL-10 levels were not affected by either YopM or YopJ *in vivo* (Fig. 4*G*). On evaluation of liver histology, we noted that deletion of YopJ, but not YopM, appeared associated with the increased presence of both polymorphonuclear and mononuclear cells at foci of infection (Fig. 4*I*), possibly related to the regulation of chemokines (Figs. 2 and 4).

YopM and YopJ May Have Different Functions in Other Cell Types—In view of some of the overlapping effects of YopM and YopJ, we investigated how they affect IL-1β production in innate immune cells other than macrophages (35). We found that the relative roles of YopM and YopJ in IL-1β production *in vivo* in a neutrophil-enriched (90–98% Ly6G/CD11b+) peritoneal cell population (Fig. 5*A*) follow a pattern similar to macrophages *in vitro* (Fig. 1*B*). In BMDCs, however, IL-1β secretion is triggered by KIM5 and significantly reduced in the absence of YopJ. By contrast, IL-1β secretion is minimally altered by the absence of YopM unless YopJ is also absent (Fig. 5*B*). These results suggest that the bacterial regulation of IL-1β release in dendritic cells (DCs) is markedly different from macrophages and neutrophils.

Discussion

Our findings illustrate parallel strategies of a microbial pathogen to suppress generation of pro-inflammatory cytokines as a means of resisting host defense. Because IL-1β and IL-18 are central mediators of anti-microbial host defenses, it may be particularly desirable to block their production. These cytokines are produced in a multistep process, potentially as a failsafe mechanism because excess production may also contribute to shock (36). However, this complex regulation also opens opportunities for diverse microbial approaches to dampen their production and release. The *Y. pestis* effectors YopJ and YopM have apparent opposite effects on caspase-1 processing; however, in the absence of both effectors, it becomes apparent that the sum of the actions of YopJ and YopM suppresses IL-1β and IL-18 release more effectively than either YopM or YopJ alone. We summarize some of our major findings about the respective roles of YopM and YopJ in the regulation of IL-1β/IL-18 production in Fig. 6. In general, the dampening of caspase-1 processing mediated by YopM is dominant, and when YopM is deleted, YopJ appears to mainly suppress pro-IL-1β and not contribute to caspase-1 cleavage in a major way. Neither of these effectors appears to enhance disease progression when the other is present, and we propose that their actions promote virulence *in vivo* by a redundant net effect on IL-1β and IL-18. Nevertheless, their effects may vary by cell type and reflect the heterogeneity of the pathways regulating IL-1β/IL-18 production. Although their net effects on IL-1β/IL-18 overlap, their roles in regulating other aspects of the immune response are distinct and in some ways appear to oppose one another.

Previous studies have shown several immunosuppressive functions of YopJ (3, 5–7, 37), and it has been puzzling that this effector does not appear to be needed for *Y. pestis* virulence (11, 12) (Fig. 3*A*). In contrast, immune stimulation induced by this effector molecule, such as the activation of caspase-1 and IL-1β/IL-18 release, has also been reported (9, 10). One hypothesis is that the immunosuppressive and immune stimulatory actions of YopJ could balance each other, explaining why YopJ deletion has little effect on bacterial replication *in vivo*. Similarly, deletion of YopM has a modest effect on virulence via intradermal delivery of the CO92 strain (14), and no significant effect upon deletion in the KIM1001 strain upon subcutaneous delivery (Fig. 3*A*). Notably, the CO92 strain carries a variant of YopJ that is less catalytically active than the KIM1001 variant (10, 38, 39). It is possible that this variant of YopJ in CO92 partially unmasks the contribution of YopM to virulence. Another aspect worth considering is that reduced activity or secretion of YopJ/YopP may not necessarily benefit the host (40, 41). Nevertheless, our data suggest that YopJ and YopM, rather than being dispensable for plague virulence, are effective promoters of virulence capable of replacing each other's net effect *in vivo* by targeting different arms of the same immune defense mechanism.

The parental KIM5 strain normally triggers only low levels of IL-1β/IL-18 release in BMDMs compared with the ΔT3SSe strain that lacks translocated Yops; this reflects the efficient suppression of IL-1β/IL-18 by the cumulative action of these Yops. We and other groups have previously observed that YopJ triggers caspase-8 activation, which triggers some caspase-1 activation and IL-1β/IL-18 release (8, 9, 40); here, this is likely reflected in Figs. 1 (*A* and *B*) and 4 (*B* and *C*). Low levels of IL-1β/IL-18 observed in response to parental *Y. pestis in vivo* appear less influenced by caspase-1 and could be due to direct processing of the precursor forms by caspase-8 (42). Nevertheless, these IL-1β/IL-18 levels may not alter the outcome of infection (Figs. 3 and 4). It is possible that YopJ-induced activation is offset by the significant inhibition of pro-IL-1β and pro-IL-18 or other NF-κB-dependent factors (Figs. 2 and 4). This effect on signal 1 may explain why IL-1β/IL-18 levels remain relatively low in the absence of YopM *in vivo* (Fig. 4, *B* and *C*).

By contrast, YopM appears not to have any effect on signal 1 (Fig. 2) but rather achieves IL-1β/IL-18 suppression by inhibiting caspase-1 activation (Fig. 1, *A* and *D*). Consistent with this role, YopM inhibits caspase-1-dependent cell death, although

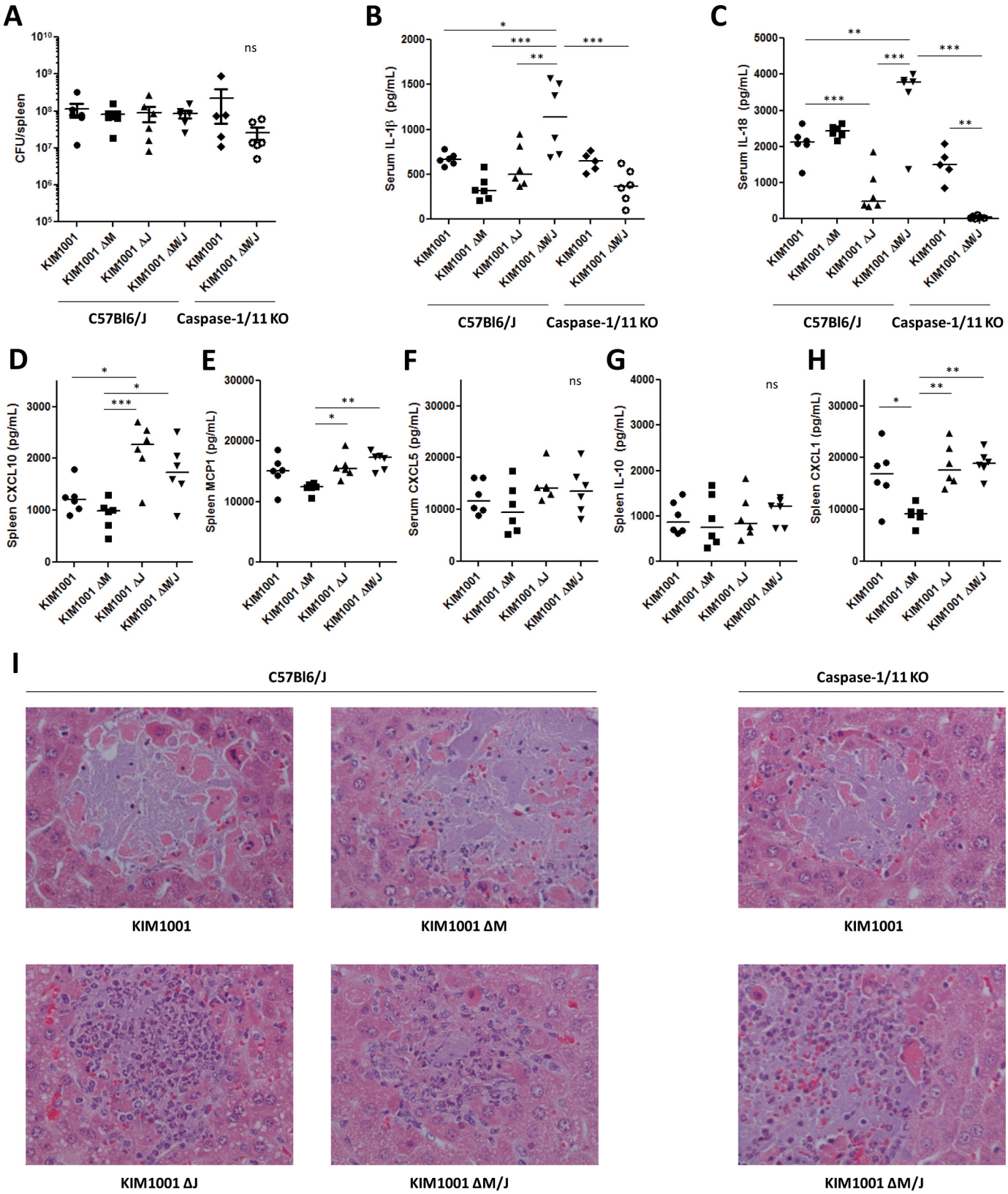

FIGURE 4. **YopM predominantly suppresses IL-1β and IL-18, whereas YopJ may limit cell recruitment in part by reducing chemokine expression during infection *in vivo*.** C57Bl6J or caspase-1/11 KO mice were injected intravenously with 40–50 cfu of KIM1001, KIM1001ΔM, KIM1001ΔJ, or KIM1001ΔM/J in 200 μl PBS ($n = 5$ caspase-1/11 KO mice infected with KIM1001ΔM/J, all other groups $n = 6$). Tissues were harvested at 42 h postinfection. *A*, bacterial load was determined from spleen homogenates. *B* and *C*, serum was used to quantify IL-1β (*B*) and IL-18 (*C*). *D–H*, spleen homogenates were used to quantify CXCL10, MCP1, CXCL5, IL-10, and CXCL1 by ELISA. Shown are median values. *I*, livers were stained with hematoxylin and eosin, and representative foci of infection were selected. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *ns*, not significant.

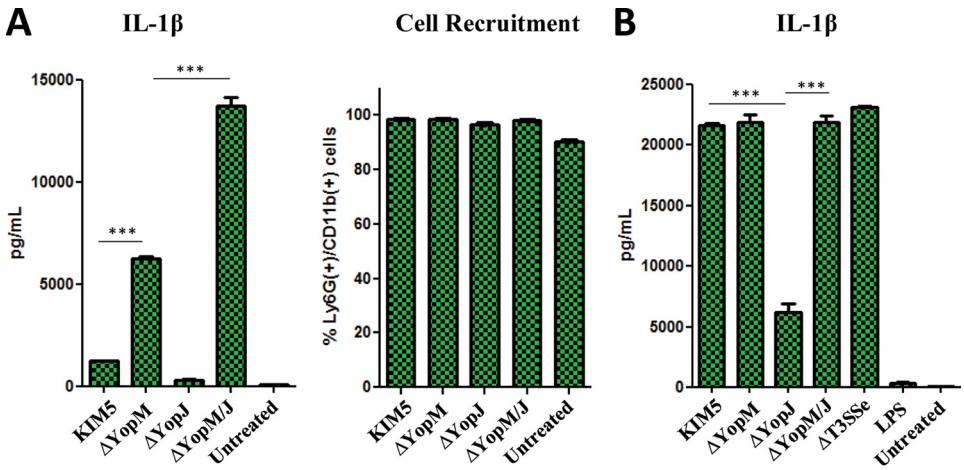

FIGURE 5. **YopJ and YopM may have different effects on IL-1β in dendritic cells compared with neutrophils and macrophages.** *A*, mice were injected intraperitoneally with 0.7 ml of 4% thioglycolate for 3 h to trigger heavy neutrophil influx, followed by injection with 1×10^8 cfu of the indicated bacterial strains in 0.3 ml of PBS for an additional 6 h. Peritoneal lavage fluid was assayed for IL-1β ELISA (*left panel*). The cells from peritoneal lavage were analyzed for cell surface markers by FACS to confirm comparable and robust influx of Ly6G/CD11b positive cells (*right panel*, % Ly6G+/CD11b+ of all conditions). *B*, BMDCs were infected with *Y. pestis* at MOI 10, and supernatants were harvested at 6 h postinfection and assayed for IL-1β by ELISA. Shown are means of triplicates, representative of two separate experiments.

Y. pestis strain	Signal 1	Signal 2	Cell Death
WT (*Y. pestis*, YopJ, YopM)	+ TNFα + Pro-IL-1β + Chemokines	+ Cleaved Casp-1 + IL-1β + IL-18	++
ΔYopM (*Y. pestis*, YopJ)	+ TNFα + Pro-IL-1β + Chemokines	**++ Cleaved Casp-1** **++ IL-1β** **++ IL-18**	+++
ΔYopJ (*Y. pestis*, YopM)	**+++TNFα** **+++Pro-IL-1β** **+++Chemokines**	(-) Cleaved Casp-1 (-) IL-1β (-) IL-18	(-)
ΔYopM/J (*Y. pestis*)	**+++TNFα** **+++Pro-IL-1β** **+++Chemokines**	**+++ Cleaved Casp-1** **+++ IL-1β** **+++ IL-18**	+

FIGURE 6. **Summary of proposed YopM and YopJ effects on IL-1β/IL-18 production and cell death in host cells.** In the absence of YopJ and YopM, the cells respond to *Y. pestis* by expressing high levels of pro-IL-1β and pro-IL-18 and activating robust caspase-1 cleavage accompanied by pyroptotic cell death. YopM can inhibit IL-1β/IL-18 release and cell death by inhibiting inflammasome-mediated caspase-1 cleavage, but it has little effect on expression of pro-IL-1β/IL-18 or other cytokines not requiring inflammasome processing. In contrast, YopJ limits IL-1β/IL-18 production by suppressing expression of precursors and possibly by inducing rapid cell death, which may overshadow the cytoprotective effect of YopM. Additionally, YopJ alone triggers modest caspase-1 cleavage with some degree of IL-1β/IL-18 production, limited by the reduced levels of precursors, including pro-caspase-1 itself.

in the presence of YopJ, the cytoprotective role of YopM appears minor (Fig. 2, *C*, *D*, and *F*). It is unclear what the implications of this small effect would be *in vivo*. YopJ appears to be a major driver of cell death, with some inhibitory effects mediated by YopM. Very recent findings on cell death mediated by caspase-11 and caspase-1 via gasdermin D (43, 44) and by NEK7 via NLRP3/caspase-1 (45, 46) suggest that there are a number of additional players involved in regulation of inflammasome and pyroptosis pathways. Furthermore, other bacterial components, such as YopK (47), could differently impact the various death pathways. Several molecules, in addition to those studied here, are likely involved in regulating cytotoxicity induced by the different *Yersinia* strains. The identification of new cell death-mediating proteins could add to the understanding of why the various pathways induce different types and degrees of cell death.

Considering the seeming redundant effects of YopM and YopJ that we report here, it is notable that YopM and YopJ

sequences are well conserved in the *Y. pestis* genome and present in all virulent strains of *Y. pestis*, *Yersinia enterocolitica*, and *Yersinia pseudotuberculosis*; however, it is also worth considering that the CO92 strain, which contains a less catalytically active version of YopJ, may have been more recently evolved than the KIM1001 strain used in this study. Furthermore, *Y. pestis* infects a wide range of animals, with significant differences in immune systems and sensitivities to infection. Because a major strategy of *Y. pestis* is immune evasion to assist propagation rather than to kill the organism, it is possible that YopM and YopJ evolved to suppress the IL-1β/IL-18 producing pathway in alternative ways without a significant difference in host survival.

Our results underscore some important differences between YopM and YopJ, suggesting that although their effects on IL-1β/IL-18 and survival in mice overlap, their functions should not be viewed as redundant. There may be other factors important for the fitness of *Y. pestis* in its unique life cycle that make both effectors indispensable, independent of host survival. YopJ inhibits the expression of multiple cytokines and chemokines both *in vitro* (Fig. 2*H*) and *in vivo* (Fig. 4, *D–H*) in addition to modulating IL-1β/IL-18, whereas YopM appears to primarily inhibit IL-1β/IL-18 maturation by affecting caspase-1. One study shows that YopM has a minor influence on the expression of immunity-related genes *in vivo* (48), supporting the idea of a more targeted role compared with YopJ. Furthermore, although YopM and YopJ may have similar effects on IL-1β secretion in neutrophils and macrophages (Figs. 1 and 5*A*), their respective roles are less clear in DCs (Fig. 5*B*). It appears that the absence of YopM has little bearing on IL-1β secretion in DCs, although YopM may have an effect when YopJ is absent (Fig. 5*B*). In any case, we propose that regulation of IL-1β release by YopJ and YopM in DCs is markedly different from in macrophages and neutrophils. Evidence for a direct role of YopM in DCs is lacking, although YopM appears to limit recruitment of DCs, as well as other cell types (13, 49); however, these observations may be explained by the role YopM plays in suppressing IL-1β and IL-18 secretion. YopJ, in contrast, has been shown to inhibit signaling and cytokine production in DCs directly (50, 51). Importantly, YopJ prevents DCs from activating natural killer cells, T cell proliferation, and IFN-γ induction. Our findings support a critical role of T cells in antibacterial defenses during *Y. pestis* infection (Fig. 3*E*), and studies in *Y. enterocolitica* also argue for the importance of a DC-induced T cell response (52). The ability of YopJ to inhibit TNFα and IFN pathways likely contributes to a defective CD8+ T cell response in pneumonic plague (33), potentially promoting bacterial propagation independently of YopM in some circumstances. Taken together, we think this suggests that YopJ may be particularly important for regulating immune responses coordinated by DCs and T cells.

YopJ has been proposed as a key *Yersinia* effector inducing caspase-1 cleavage (9, 10). Our current results suggest that YopJ does not contribute to caspase-1 cleavage in a major way when YopM is absent; rather, other components of the *Y. pestis* T3SS trigger the majority of caspase-1 cleavage in the absence of YopM. Unexpectedly, we found that deletion of YopE seemingly neutralized the IL-1β induction blocked by YopM (Fig. 1*C*). Earlier studies in other cell systems suggested that YopE could inhibit caspase-1 activation (53) and signal 1 (54). Our findings warrant further studies, evaluating the contributions of YopE both on effector protein translocation (30) and on inflammasome signals 1 and 2.

Although it is unclear which mechanisms YopM uses for inhibition of inflammasome activation, direct interaction with caspase-1 and the scaffold protein Iqgap1 have been proposed (15, 16). Interactions between YopM and kinases such as RSK1/2 and PKN1/2 (55–57), known for binding to the effector, may also play a role in caspase-1 regulation. Regardless of the method for YopM-mediated inhibition, it is possible that the pathway blocked by YopM could be a dominant pathway in *Yersinia*-induced inflammasome activation. Multiple candidate pathways could potentially be blocked by YopM, because NLRP3, NLRC4, and NLRP12 have all been proposed as mediators of *Yersinia*-induced IL-1β/IL-18 production. We cannot exclude the possible involvement of additional pathways that sense type III secretion system molecules. We also cannot disregard the idea that bacterial molecules other than those belonging to the T3SS contribute to modulation of caspase-1 cleavage and IL-1β production *in vivo* (58). We add to a large body of evidence (Fig. 1) suggesting minimal macrophage IL-1β release in the absence of the pCD1 T3SS-containing plasmid or the YopB/YopD components of the pore-forming translocon (59). The role of the translocon is emphasized by data suggesting that increased translocation of YopB increases inflammasome activation (10, 60). YopK also participates in regulation of influx of other molecules into the host cell, and a net effect of YopK is inhibition of caspase-1 processing by the NLRP3 and NLRC4 inflammasomes (47, 60). The picture that emerges is complex, and this suggests that many T3SS-related molecules together regulate IL-1β/IL-18 release. Our experiments indicate that their relative roles may be dependent upon the presence or absence of other specific molecules.

The fact that so many bacterial components are involved in regulating IL-1β/IL-18 suggests that it is important for the bacterium to keep these cytokines in check. Indeed, our previous studies have indicated the power of IL-18 and IL-1β in inducing host antibacterial defenses toward *Y. pestis* strains (17). In that respect, *Y. pestis* serves as an interesting model system for investigating immune evasion by pathogens and microbial manipulation of host IL-1β and IL-18 release.

Author Contributions—D. R. performed experiments, with some help from M. P. A. O., K. K. S., and R. M.-R.; M. K. P. and J. D. G. generated bacterial mutants and established the EthD-1 cytotoxic assay. E. L. conceived the study and supervised all aspects of the work. D. R. and E. L. wrote the paper with input from all authors.

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