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FADD and Caspase-8 Mediate Priming and Activation of the Canonical and Noncanonical Nlrp3 Inflammasomes

Prajwal Gurung,* Paras K. Anand,* R. K. Subbarao Malireddi,* Lieselotte Vande Walle,[†,‡] Nina Van Opdenbosch,[†,‡] Christopher P. Dillon,* Ricardo Weinlich,* Douglas R. Green,* Mohamed Lamkanfi,[†,‡] and Thirumala-Devi Kanneganti*

The Nlrp3 inflammasome is critical for host immunity, but the mechanisms controlling its activation are enigmatic. In this study, we show that loss of FADD or caspase-8 in a RIP3-deficient background, but not RIP3 deficiency alone, hampered transcriptional priming and posttranslational activation of the canonical and noncanonical Nlrp3 inflammasome. Deletion of caspase-8 in the presence or absence of RIP3 inhibited caspase-1 and caspase-11 activation by Nlrp3 stimuli but not the Nlrc4 inflammasome. In addition, FADD deletion prevented caspase-8 maturation, positioning FADD upstream of caspase-8. Consequently, FADD- and caspase-8–deficient mice had impaired IL-1β production when challenged with LPS or infected with the enteropathogen *Citrobacter rodentium*. Thus, our results reveal FADD and caspase-8 as apical mediators of canonical and noncanonical Nlrp3 inflammasome priming and activation. *The Journal of Immunology*, 2014, 192: 1835–1846.

The Nlrp3 inflammasome responds to a variety of inflammatory triggers, including danger signals (e.g., ATP), microbial toxins (e.g., nigericin), and crystalline substances (1). This inflammasome is also critical for activation of the inflammatory cysteine protease caspase-1 in macrophages infected with enteric pathogens, such as *Vibrio cholerae*, *Escherichia coli*, and *Citrobacter rodentium* (2), and for mounting immune and host responses against *C. rodentium* in vivo (3). Enteropathogen-induced caspase-1 activation and secretion of the inflammasome-dependent cytokines IL-1β and IL-18 are referred to as the "noncanonical" Nlrp3 inflammasome pathway because it also requires caspase-11 for caspase-1 activation (2). Notably, noncanonical Nlrp3 activation in macrophages infected with *Salmonella typhimurium* grown to stationary phase, *E. coli*, or *C. rodentium* recently was shown to require TLR4- and MyD88-mediated Nlrp3 upregulation (4, 5), as well as TLR4/TRIF-mediated induction of caspase-11 expression (5–7). In contrast, caspase-11 is dispensable for canonical Nlrp3 inflammasome activation by danger signals, microbial toxins, and crystalline substances (2).

Engagement of death receptors, such as CD95, TRAIL receptor, and TNFR1, results in recruitment of caspase-8 and its adaptor protein FADD to initiate an apoptosis-inducing caspase cascade (8, 9). Notably, mice deficient for FADD or caspase-8 are embryonic lethal (10–12), and this lethality is rescued by deleting the necrosis-regulating kinase RIP3 (13, 14). These observations suggest that FADD/caspase-8–mediated apoptotic caspase activation and RIP1/RIP3-mediated necroptosis signaling are interconnected at the level of the death-inducing signaling complex. Recent work highlighted a previously unexpected role for caspase-8 in inducing inflammatory responses by promoting IL-1β production under conditions in which canonical inflammasome signaling is prevented (e.g. in caspase-1/11–deficient macrophages) and in response to infectious agents and stimuli that do not engage canonical inflammasome signaling (15–18). In addition, caspase-8 was shown to promote apoptosis induction in response to canonical inflammasome stimuli when the induction of inflammasome-dependent pyroptosis was prevented (19). Together, these studies suggest diverse roles and interconnections between apoptotic and inflammatory signaling pathways. However, the roles of RIP3 and FADD/caspase-8 in regulating canonical and noncanonical Nlrp3 inflammasome signaling in response to stimuli established to trigger activation of the inflammatory caspases-1 and -11 have not been explored. In this study, we revealed caspase-8 and FADD as upstream regulators of Nlrp3 inflammasome signaling, with dual roles in transcriptional priming and posttranslational activation of the canonical and noncanonical Nlrp3 inflammasome pathways, thus shedding light on a new level of interconnection between apoptotic and inflammatory signaling pathways.

Materials and Methods

Mice

Rip3$^{-/-}$ (20), *Rip3*$^{-/-}$*Casp8*$^{-/-}$ (14), *Rip3*$^{-/-}$*Fadd*$^{-/-}$ (21), *Nlrp3*$^{-/-}$ (22), *Asc*$^{-/-}$ (23), *Casp1*$^{-/-}$*Casp11*$^{-/-}$, *Casp1*$^{-/-}$*Casp11*^{Tg} (2), *IFNAR2*$^{-/-}$ (24), *Tlr4*$^{-/-}$ (25), *Trif*$^{-/-}$ (26), *MyD88*$^{-/-}$ (27), *Nod2*$^{-/-}$

*Department of Immunology, St. Jude Children's Research Hospital, Memphis, TN 38105; [†]Department of Medical Protein Research, Flanders Institute for Biotechnology, Ghent, B-9000, Belgium; and [‡]Department of Biochemistry, Ghent University, Ghent, B-9000, Belgium

P.G., M.L., and T.-D.K. designed the study; P.G., R.K.S.M., P.K.A., L.V.W., and N.V.O. performed experiments; P.G., M.L., and T.-D.K. analyzed data and wrote the manuscript; C.P.D., R.W., and D.R.G. provided essential reagents and scientific insight; and T.-D.K. oversaw the project.

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Address correspondence and reprint requests to Dr. Thirumala-Devi Kanneganti or Dr. Mohamed Lamkanfi, Department of Immunology, St. Jude Children's Research Hospital, MS #351, 570, St. Jude Place, Suite E7004, Memphis TN 38105-2794 (T.-D.K.) or Department of Medical Protein Research, Flanders Institute for Biotechnology, Albert Baertsoenkaai 3, B-9000 Ghent, Belgium (M.L.). E-mail addresses: Thirumala-Devi.Kanneganti@StJude.org (T.-D.K.) or mohamed.lamkanfi@vib-ugent.be (M.L.)

The online version of this article contains supplemental material.

Abbreviations used in this article: BMDM, bone marrow–derived macrophage; MDP, muramyl dipeptide; moi, multiplicity of infection; WT, wild-type.

(28), *TNF-α*$^{-/-}$ (29), and *TNFR1*$^{-/-}$ (30) mice were described previously. Caspase-8^{flx} mice were bred with *LysM*^{Cre} (*B6.129P2-Lyz2*$^{tm1(cre)Ifo}$*/J*; The Jackson Laboratory) mice to generate conditional caspase-8–knockout mice. C57BL/6 wild-type (WT; The Jackson Laboratory), *Rip3*$^{-/-}$*Casp8*$^{-/-}$, and *Rip3*$^{-/-}$*Fadd*$^{-/-}$ mice and littermate controls were bred at St. Jude Children's Research Hospital. Animal studies were conducted under protocols approved by St. Jude Children's Research Hospital's and Ghent University's Committee on the Use and Care of Animals.

Macrophage differentiation and stimulation

Bone marrow–derived macrophages (BMDMs) were prepared as described previously (5). In brief, bone marrow cells were grown in L cell–conditioned IMDM supplemented with 10% FBS, 1% nonessential amino acid, and 1% penicillin-streptomycin for 5 d to differentiate into macrophages. On day 5, BMDMs were seeded in six-well cell culture plates and were stimulated the next day with LPS (20 ng/ml) for 4 h, the last 30 min of which was in the presence of 5 mm ATP or 20 μM nigericin. Where indicated, BMDMs were treated with the caspase-8 inhibitor Ac-IETD-fmk (Calbiochem; 20 μM) before or after LPS priming, respectively. In other experiments, BMDMs that were pretreated or not with LPS were infected with *C. rodentium* or *E. coli* at a multiplicity of infection (moi) 25 for 24 h. Two hours postinfection, gentamycin (100 μg/ml) was added to the culture medium.

In vitro transcription/translation

^{35}S-labeled procaspases were produced in vitro using the SP6 High-Yield Wheat Germ Protein Expression System (Promega) and incubated with 100 U recombinant mouse caspase-8 (Enzo Life Sciences) or 35 ng mouse caspase-3 (Flanders Institute for Biotechnology) before caspase processing was analyzed by autoradiography.

Western blotting

Samples for immunoblotting were prepared by combining cell lysates with culture supernatants. Samples were denatured in loading buffer containing SDS and 100 mM DTT and boiled for 5 min. SDS-PAGE–separated proteins were transferred to polyvinylidene difluoride membranes and immunoblotted with primary Abs against caspase-1 (Adipogen; AG-20B-0042), pro–caspase-8 (Enzo Life Sciences; 1G12), cleaved caspase-8 (Cell Signaling Technology; D5B2), caspase-11 (Novus Biologicals; 17D9), FADD (Millipore; 1F7), Nlrp3 (Adipogen; AG-20B-0014), IL-1β (R&D Systems), IL-18 (31), and GAPDH (Cell Signaling Technology; D16H11), followed by secondary anti-rabbit, anti-rat, anti-mouse, or anti-goat HRP Abs (Jackson ImmunoResearch Laboratories), as previously described (22).

Flow cytometry and phagocytosis assay

BMDMs were stained with CD11b, F4/80, and CD86 Abs (eBioscience) or were preincubated with GFP-expressing *C. rodentium*, FITC-labeled zymosan A, or OVA (Molecular Probes) for 3 h prior to analysis on an LSR II (BD Biosciences) using FlowJo software.

Confocal immunofluorescence microscopy

WT, *Casp1*$^{-/-}$, and *Rip3*$^{-/-}$*Casp8*$^{-/-}$ macrophages grown on coverslips were left untreated (control), stimulated with LPS+ATP, or infected with *C. rodentium.* Cells were fixed with 4% paraformaldehyde and stained with caspase-1 (Adipogen) or caspase-8 (Cell Signaling Technology) Abs. Nuclei were counterstained with DAPI. Cells were mounted on glass slides using ProLong Gold Antifade Reagent (Life Technologies), and micrographs were taken with a Nikon C1 confocal microscope using a 40× objective lens. The images were processed and analyzed with ImageJ software. The images were taken at Cell and Tissue Imaging Center Light Microscopy Facility at St. Jude Children's Research Hospital.

LPS-induced endotoxemia and in vivo C. rodentium *infection*

In some experiments, cohorts of WT, *Rip3*$^{-/-}$, *Rip3*$^{-/-}$*Casp8*$^{-/-}$, and *Rip3*$^{-/-}$*Fadd*$^{-/-}$ mice were injected i.p. with 35 mg/kg LPS (Sigma-Aldrich) for 5 h before serum was collected for cytokine analysis. In other experiments, groups of WT, *Rip3*$^{-/-}$, *Rip3*$^{-/-}$*Casp8*$^{-/-}$, and *Rip3*$^{-/-}$*Fadd*$^{-/-}$ mice were infected with *C. rodentium* (ATCC 51459; 1×10^{10} CFU) by oral gavage. Food and water intake were stopped 8 h prior to infection and allowed to resume 1 h after infection. To determine bacterial counts, serial dilutions of homogenized feces were plated on MacConkey agar plates and incubated at 37°C for 24 h. Stool consistency was scored (stool score) according to standard protocol, as described previously (3). Briefly, 1 = well-formed pellets, 2 = semi-formed pellets that do not adhere to the anus, 3 = soft stool that do not form pellets and adhere to the anus, 4 = liquid stool that adhere to the anus, and 5 = liquid stool with blood.

Cytokine analysis

Concentrations of cytokines and chemokines were determined by multiplex ELISA (Millipore) or classical ELISA for IL-1β (eBioscience) or IL-18 (MBL International).

Real-time PCR

Total RNA was extracted from cells stimulated with LPS or infected with *C. rodentium* using TRIzol reagent (Life Technologies), according to the manufacturer's instructions. RNA was quantified, and 1 μg total RNA was reverse transcribed to complementary DNA with poly(dT) primers using the First-Strand cDNA Synthesis Kit (Life Technologies). Transcript levels of *proIL-1β* and *Nlrp3* were quantified by quantitative RT-PCR on an ABI 7500 real-time PCR instrument with SYBR Green (Applied Biosystems). *Gapdh* expression was used for normalization, and results are presented as fold induction over levels in untreated control cells.

Statistics

GraphPad Prism 5.0 software was used for data analysis. Data are presented as mean ± SEM. Statistical significance was determined by the Student *t* test; $p < 0.05$ was considered statistically significant.

Results

FADD is critical for potent canonical and noncanonical Nlrp3 inflammasome activation

Conditional deletion of FADD in hematopoietic progenitor cells affects myeloid cell differentiation into BMDMs (32). However, simultaneous deletion of RIP3 and FADD did not cause global defects in macrophage differentiation, because *Rip3*$^{-/-}$*Fadd*$^{-/-}$ macrophages looked morphologically normal and expressed normal levels of the myeloid cell/macrophage surface markers CD11b, F4/80, and CD86 (Supplemental Fig. 1A–D). In addition, phagocytosis and pinocytosis of GFP-labeled *C. rodentium*, fluorescently labeled zymosan, and FITC-ovalbumin were not affected (Supplemental Fig. 1E–G), indicating that *Rip3*$^{-/-}$*Fadd*$^{-/-}$ macrophages were not functionally impaired. To understand the roles of RIP3 and FADD in canonical Nlrp3 inflammasome activation, LPS-primed WT, *Rip3*$^{-/-}$, and *Rip3*$^{-/-}$*Fadd*$^{-/-}$ macrophages were stimulated with ATP or nigericin, and caspase-1 processing was monitored. Under these conditions, ATP- and nigericin-induced caspase-1 activation was significantly blunted in LPS-primed macrophages that lacked FADD in a RIP3-deficient background but not in cells lacking RIP3 only (Fig. 1A). ATP-induced caspase-1 processing also was reduced significantly in Pam3CSK4-primed *Rip3*$^{-/-}$*Fadd*$^{-/-}$ macrophages (Fig. 1B), indicating that the requirement for FADD was not restricted to TLR4-stimulated cells. In addition to these canonical Nlrp3 stimuli, FADD deficiency hampered noncanonical Nlrp3 inflammasome activation in macrophages infected with the enteropathogens *C. rodentium* and *E. coli*; cells lacking RIP3 and FADD were defective in procaspase-1 maturation, whereas WT and *Rip3*$^{-/-}$ macrophages responded to these enteropathogens with robust activation of caspase-1 (Fig. 1C). Because caspase-11 is required for caspase-1 maturation in enteropathogen-infected macrophages (2), defective caspase-1 maturation in *Rip3*$^{-/-}$*Fadd*$^{-/-}$ macrophages may be consequent to their inability to activate caspase-11. Indeed, *C. rodentium*– and *E. coli*–infected *Rip3*$^{-/-}$*Fadd*$^{-/-}$ macrophages also were blunted in maturation of procaspase-11 into the large catalytic subunit (p30) (Fig. 1C). In addition, they failed to secrete significant amounts of IL-1β (Fig. 1D) or IL-18 (Fig. 1E) in the culture medium. The roles of RIP3 and FADD in enteropathogen-induced Nlrp3 inflammasome activation are specific, because RIP3 and FADD were dispensable for *S. typhimurium*–induced caspase-1 activation (Fig. 1F), which proceeds through the

Nlrc4 inflammasome (23). Together, these results suggest a specific role for FADD in potent activation of the canonical and noncanonical arms of the Nlrp3 inflammasome.

FADD mediates caspase-8 maturation with canonical and noncanonical Nlrp3 inflammasome stimuli

FADD modulates apoptosis and necroptosis signaling through its associated effector protease caspase-8 (21). Therefore, we analyzed the expression and activation status of caspase-8 under conditions well-known to elicit activation of the canonical and noncanonical Nlrp3 inflammasomes. WT, RIP3-deficient, and *Rip3*$^{-/-}$*Fadd*$^{-/-}$ cells expressed comparable levels of procaspase-8, but procaspase-8 processing into the large catalytic (p17) subunit was markedly reduced in LPS-primed *Rip3*$^{-/-}$*Fadd*$^{-/-}$ macrophages that were treated with the canonical Nlrp3 inflammasome stimuli ATP and nigericin (Fig. 2A). Notably, combined stimulation with LPS and ATP was needed to induce caspase-8 maturation, because WT, RIP3-deficient, and *Rip3*$^{-/-}$*Fadd*$^{-/-}$ macrophages that were treated with only LPS or ATP failed to process caspase-8 (Supplemental Fig. 2A). Silica-treated macrophages also required FADD for potent

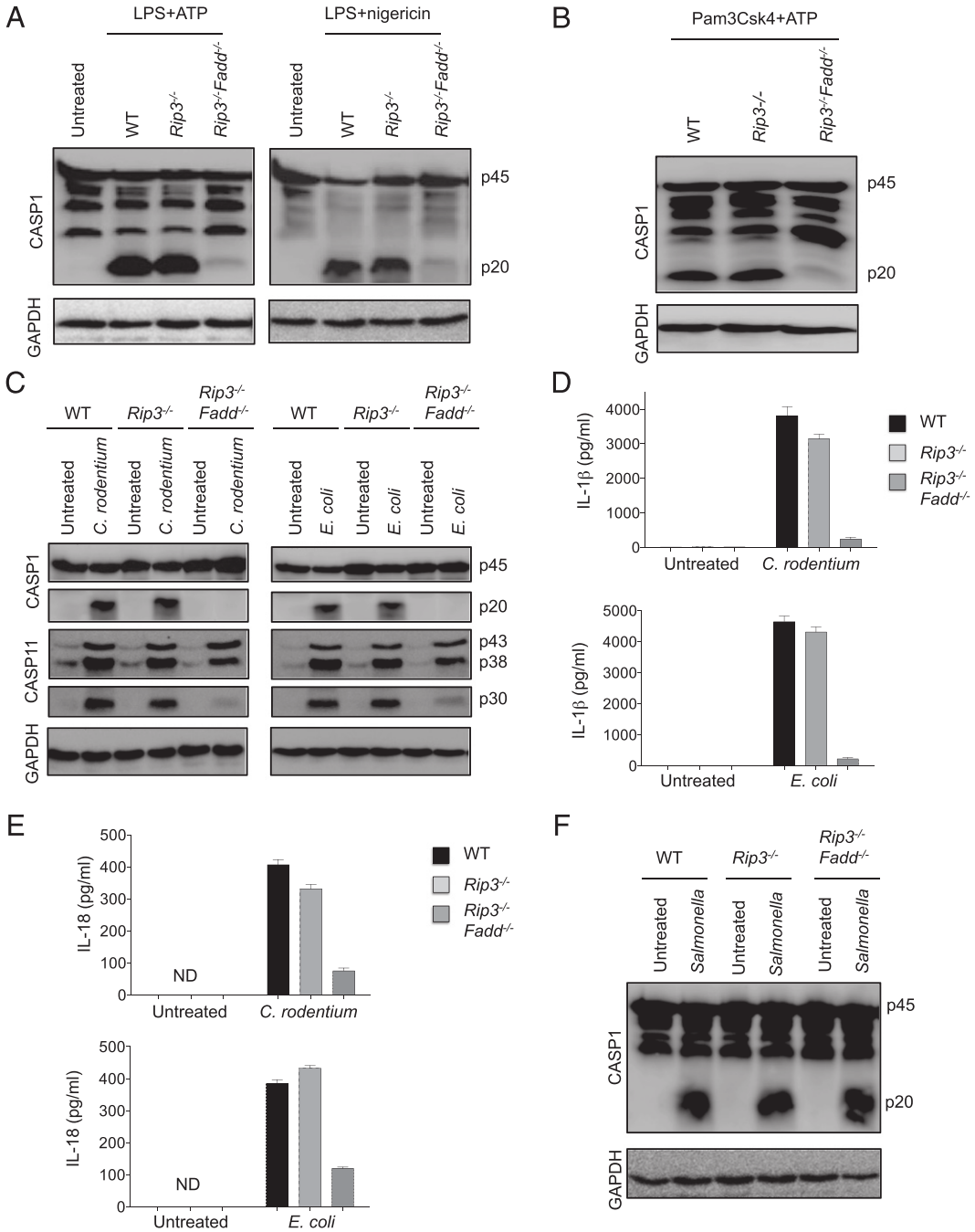

FIGURE 1. FADD is required for canonical and noncanonical Nlrp3 inflammasome activation. WT, *Rip3*$^{-/-}$, and *Rip3*$^{-/-}$*Fadd*$^{-/-}$ BMDMs were primed with 20 ng/ml LPS (**A**) or 2.5 μg/ml PAM3CSK4 (**B**) for 4 h, the last 30 min of which was in the presence 5 mM ATP or 20 μM nigericin. Lysates were immunoblotted for caspase-1. WT, *Rip3*$^{-/-}$, and *Rip3*$^{-/-}$*Fadd*$^{-/-}$ BMDMs were infected with *C. rodentium* or *E. coli* (moi 25) for 24 h before lysates were collected and immunoblotted for the indicated proteins (**C**), and culture supernatants were analyzed for secreted IL-1β (**D**) and IL-18 (**E**). (**F**) WT, *Rip3*$^{-/-}$, and *Rip3*$^{-/-}$*Fadd*$^{-/-}$ BMDMs were infected with *S. typhimurium* (moi 5) for 4 h before cell lysates were immunoblotted for caspase-1. ELISA data are shown as mean ± SEM, and all data are representative of at least three independent experiments.

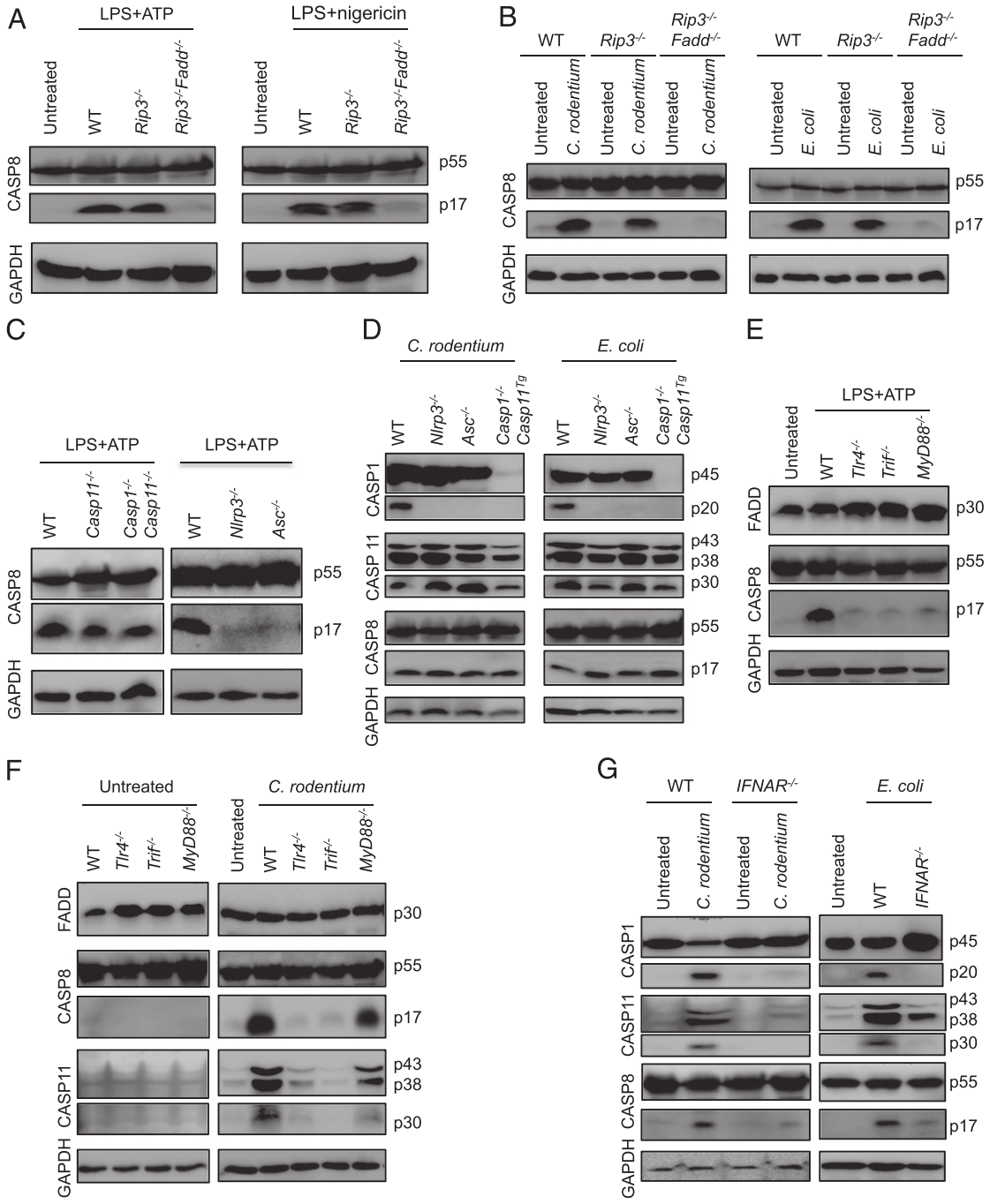

FIGURE 2. FADD is required for caspase-8 maturation in response to canonical and noncanonical Nlrp3 stimuli. (**A**) WT, *Rip3*$^{-/-}$, and *Rip3*$^{-/-}$ *Fadd*$^{-/-}$ BMDMs were stimulated with 20 ng/ml LPS for 4 h, the last 30 min of which was in the presence 5 mM ATP or 20 μM nigericin. Cell lysates were immunoblotted for the indicated proteins. (**B**) WT, *Rip3*$^{-/-}$, and *Rip3*$^{-/-}$*Fadd*$^{-/-}$ BMDMs were infected with *C. rodentium* or *E. coli* (moi 25) for 24 h before lysates were collected and immunoblotted for the indicated proteins. (**C**–**G**) WT, *Tlr4*$^{-/-}$, *Trif*$^{-/-}$, *MyD88*$^{-/-}$, *IFNAR*$^{-/-}$, *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, *Casp1*$^{-/-}$*Casp11*^{Tg}, *Casp11*$^{-/-}$, and *Casp1*$^{-/-}$*Casp11*$^{-/-}$ BMDMs were stimulated with LPS+ATP or infected with *C. rodentium* and *E. coli*, as above, before cell lysates were immunoblotted for the indicated proteins. All data are representative of at least three independent experiments.

induction of caspase-1 maturation and caspase-8 processing (Supplemental Fig. 2B). In addition, caspase-8 activation was reduced significantly in *C. rodentium*– and *E. coli*–infected *Rip3*$^{-/-}$*Fadd*$^{-/-}$ cells but not in *Rip3*$^{-/-}$ macrophages (Fig. 2B). Together, these results suggest that caspase-8 is processed under inflammasome-activating conditions. To further understand the relationship between FADD-induced caspase-8 maturation and Nlrp3 inflammasome activation, we monitored caspase-8 activation status in inflammasome-deficient macrophages. As reported for LPS+nigericin-treated macrophages (19), neither loss of caspase-11 nor combined loss of caspase-1 and -11 affected caspase-8 activation in LPS+ATP-stimulated cells, whereas loss of Nlrp3 or ASC inhibited LPS+ATP-induced caspase-8 processing (Fig. 2C). As reported (2), *C. rodentium*– and *E. coli*–induced caspase-1 processing was abolished in *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, and caspase-1/11–deficient macrophages in which caspase-11 expression was restored from a C57BL/6 bacterial artificial chromosome (*Casp1*$^{-/-}$*Casp11*^{Tg}) (Fig. 2D). Caspase-11 expression and maturation were not affected in these

cells. Notably, unlike during canonical Nlrp3 signaling, loss of Nlrp3, ASC, caspase-1, or caspase-11 or the combined loss of caspase-1 and caspase-11 did not affect enteropathogen-induced caspase-8 activation (Fig. 2D, Supplemental Fig. 2C), positioning FADD-dependent caspase-8 activation in enteropathogen-infected macrophages upstream of caspase-11 and the Nlrp3 inflammasome.

Previous studies (4, 5, 7) implicated TLR signaling in the canonical and noncanonical Nlrp3 inflammasome pathways. This prompted us to analyze whether TLR signaling modulated expression of FADD and/or caspase-8 activation in LPS+ATP-treated or enteropathogen-infected macrophages. FADD and procaspase-8 were constitutively expressed, and their expression levels did not change significantly in LPS+ATP-treated or *C. rodentium*–infected *Tlr4*$^{-/-}$, *Trif*$^{-/-}$, and *MyD88*$^{-/-}$ macrophages, respectively (Fig. 2E, 2F). However, both LPS+ATP- and *C. rodentium*–induced caspase-8 maturation were markedly reduced in TLR4- and TRIF-deficient macrophages (Fig. 2E, 2F). Moreover, caspase-8 processing was reduced in *MyD88*$^{-/-}$ macrophages (Fig. 2E and 2F), suggesting that, in addition to FADD, caspase-8 activation further required TLR-dependent signaling. Notably, *C. rodentium*– and *E. coli*–induced caspase-8 activation also was partially affected in *IFNAR1*$^{-/-}$ macrophages (Fig. 2G), suggesting that type I IFN signaling contributes to efficient caspase-8 activation in enteropathogen-infected macrophages. Together, these results implicate TLR and type I IFN signaling in caspase-8 activation by stimuli of the canonical and noncanonical Nlrp3 inflammasome, respectively. In addition, our observations that caspase-8 activation is defective in *Rip3*$^{-/-}$*Fadd*$^{-/-}$ cells and that Nlrp3 and ASC mediate caspase-8 maturation in response to canonical Nlrp3 stimuli suggest that caspase-8 may operate downstream of FADD in regulating Nlrp3 inflammasome responses.

Caspase-8/RIP3 deletion inhibits canonical Nlrp3 inflammasome activation

Having established a role for FADD in activation of caspase-8 and the Nlrp3 inflammasome, we next sought genetic confirmation of the potential role of caspase-8 in canonical and noncanonical Nlrp3 inflammasome activation. To this end, we differentiated BMDMs from caspase-8–deficient mice in a RIP3-deficient background (14), as well as from animals with a conditionally targeted deletion of caspase-8 in myeloid progenitors cells (*Casp8*$^{LysM\text{-}Cre}$) (33). As reported for an independently generated *Casp8*$^{LysM\text{-}Cre}$ mouse line (34), BMDM cultures of *Casp8*$^{LysM\text{-}Cre+}$ mice mainly contained cells in which deletion of the floxed *caspase-8* allele had failed to occur (Supplemental Fig. 2D, 2E). In contrast, caspase-8 was successfully deleted in *Rip3*$^{-/-}$*Casp8*$^{-/-}$ BMDMs (Supplemental Fig. 2D, 2E), suggesting that caspase-8–deficient myeloid progenitor cells might be sensitive to RIP3-mediated necroptosis. To characterize *Rip3*$^{-/-}$*Casp8*$^{-/-}$ BMDMs phenotypically, we confirmed that they appeared morphologically normal; that they expressed normal levels of the monocyte/macrophage markers CD11b, F4/80, and CD86 (Supplemental Fig. 1A–D); and that phagocytosis of pathogenic bacteria, fungal cell wall components, and antigenic peptides was not impaired (Supplemental Fig. 1E–G). In line with the hypothesized role for caspase-8 in mediating efficient canonical Nlrp3 inflammasome activation, ATP- and nigericin-induced caspase-1 maturation were affected significantly in LPS-primed *Rip3*$^{-/-}$*Casp8*$^{-/-}$ macrophages but not in macrophages lacking RIP3 alone (Fig. 3A). ATP-induced maturation of caspases-1 and -8 already was evident in WT and *Rip3*$^{-/-}$ macrophages 5 min after ATP exposure, and it increased further with similar kinetics in these cells (Fig. 3B). In addition to inducing rapid caspase-1 maturation, potent canonical Nlrp3 inflammasome stimulation induces rapid pyroptotic cell death, whereas induction of caspase-8–dependent apoptosis proceeds with slower kinetics and prevails in caspase-1–deficient macrophages (19, 35). Accordingly, we noted that both ATP-induced pyroptosis (Fig. 3C) and IL-1β secretion (Fig. 3D) followed suit and already were evident 10 min after ATP exposure. Consistent with an upstream requirement for FADD and caspase-8 in potent induction of caspase-1 activation (Figs. 1A, 3B), the time-dependent induction of pyroptosis and IL-1β secretion were specifically reduced in LPS+ATP-treated *Rip3*$^{-/-}$*Fadd*$^{-/-}$ and *Rip3*$^{-/-}$*Casp8*$^{-/-}$ macrophages (Fig. 3C, 3D). *Rip3*$^{-/-}$*Casp8*$^{-/-}$ macrophages were similarly defective in ATP-induced caspase-1 processing and IL-1β secretion after priming with the TLR2 ligand Pam3CSK4 (Fig. 3E, 3F), demonstrating that the role of caspase-8 in Nlrp3 inflammasome activation was not limited to LPS-primed macrophages. Both FADD and caspase-8 mediate cellular responses to TNF-α (36); however, their role in ATP-induced Nlrp3 inflammasome activation was independent of TNF-α signaling, because *TNF-α*$^{-/-}$ and *TNF-R1*$^{-/-}$ BMDMs responded to LPS+ATP stimulation with normal caspase-1 and caspase-8 activation, and they secreted normal levels of IL-1β (Supplemental Fig. 3A–C). Together, these results demonstrate that *Rip3*$^{-/-}$*Casp8*$^{-/-}$ macrophages are significantly impaired in canonical Nlrp3 inflammasome activation.

Enteropathogen-induced Nlrp3 activation is impaired in Rip3/caspase-8–*knockout macrophages*

To explore the role of caspase-8 in noncanonical Nlrp3 inflammasome signaling, macrophages were infected with the enteropathogens *C. rodentium* and *E. coli.* Notably, caspase-1 maturation was significantly impaired in *C. rodentium*– and *E. coli*–infected *Rip3*$^{-/-}$*Casp8*$^{-/-}$ macrophages (Fig. 4A, Supplemental Fig. 4A). In contrast, caspase-1 processing was normal in enteropathogen-infected macrophages lacking RIP3 alone (Fig. 4A). As was recently reported (37), caspase-1 maturation also was not affected in *S. typhimurium*–infected *Rip3*$^{-/-}$*Casp8*$^{-/-}$ cells (Fig. 4B), demonstrating specificity of these results. As with canonical Nlrp3 inflammasome stimuli, TNF-α signaling also was dispensable for enteropathogen-induced caspase-1 and caspase-8 activation in macrophages infected with *C. rodentium* (Supplemental Fig. 3D–F). Analysis of caspase-11 processing in *C. rodentium*– and *E. coli*–infected *Rip3*$^{-/-}$ and *Rip3*$^{-/-}$*Casp8*$^{-/-}$ macrophages further supported an upstream role for caspase-8 in mediating noncanonical Nlrp3 inflammasome activation, because caspase-11 maturation was specifically affected in *Rip3*$^{-/-}$*Casp8*$^{-/-}$ BMDMs (Fig. 4C). Concurrently, the time-dependent secretion of IL-1β (Fig. 4D) and the induction of IL-1α release and pyroptosis (Fig. 4E, 4F) were specifically reduced in *C. rodentium*–infected *Rip3*$^{-/-}$*Casp8*$^{-/-}$ macrophages but not in macrophages lacking only RIP3. Together, these results show that enteropathogen-induced activation of caspase-11 and the noncanonical Nlrp3 inflammasome are hampered severely in *Rip3*$^{-/-}$*Casp8*$^{-/-}$ macrophages.

Caspase-8 and FADD mediate transcriptional priming of the Nlrp3 inflammasome

Nlrp3 inflammasome activation is a highly regulated process at both the transcriptional and posttranslational levels. At the transcriptional level, Nlrp3 inflammasome–mediated caspase-1 activation and IL-1β secretion require TLR4/MyD88-mediated upregulation of *Nlrp3* and *proIL-1β* expression (4, 5). In addition, *C. rodentium*– and *E. coli*–induced Nlrp3 inflammasome activation requires TLR4/TRIF-mediated expression of caspase-11 (5–7). To further determine the level at which FADD and caspase-8

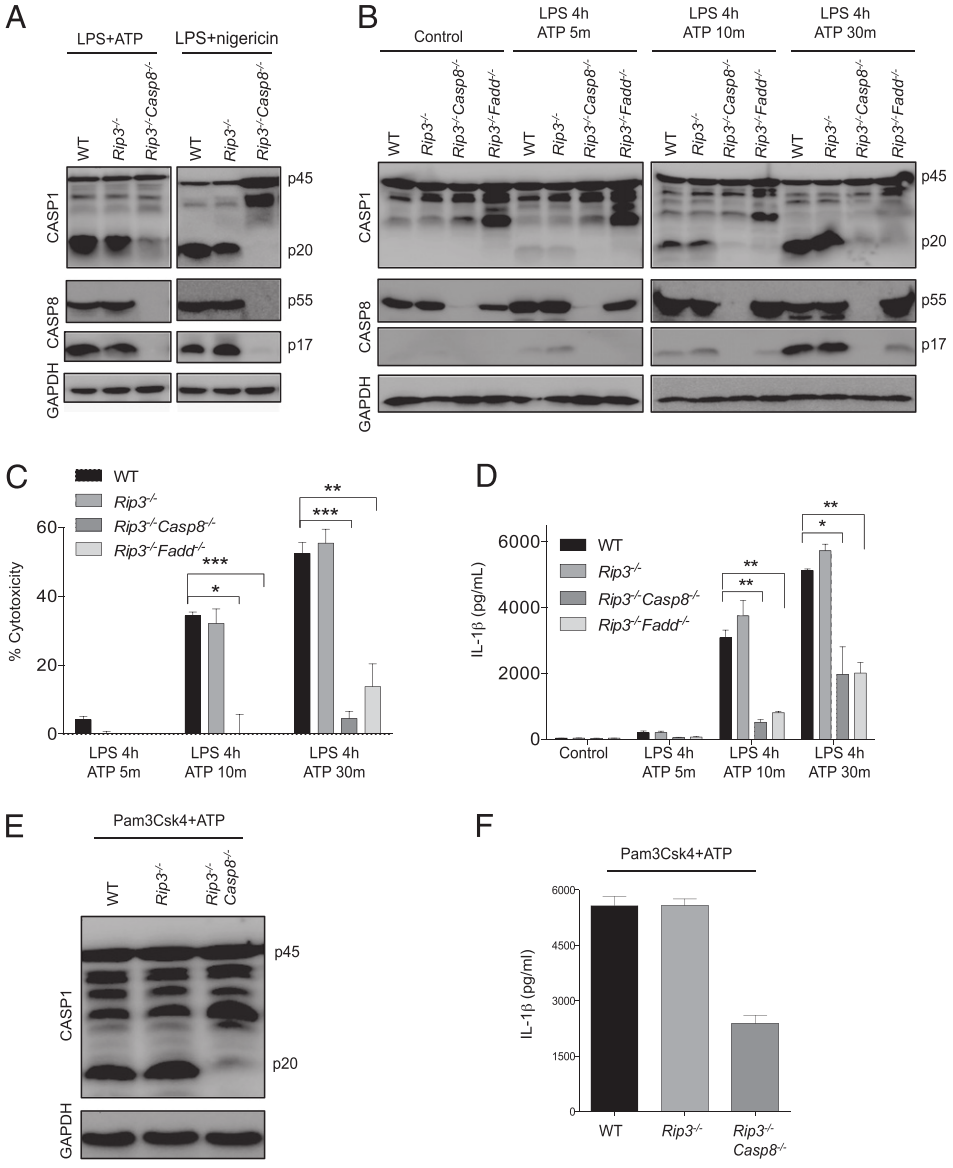

FIGURE 3. Caspase-8 is required for efficient activation of the canonical Nlrp3 inflammasome. (**A**) WT, *Rip3*$^{-/-}$, and *Rip3*$^{-/-}$*Casp8*$^{-/-}$ BMDMs were stimulated with 20 ng/ml LPS for 4 h, the last 30 min of which was in the presence 5 mM ATP or 20 μM nigericin. Lysates were immunoblotted for the indicated proteins. (**B**–**D**) WT, *Rip3*$^{-/-}$, *Rip3*$^{-/-}$*Casp8*$^{-/-}$, and *Rip3*$^{-/-}$*Fadd*$^{-/-}$ BMDMs were stimulated with LPS for 4 h, and 5 mM ATP was added for 5, 15, or 30 min. Lysates were immunoblotted for the indicated proteins (B), and culture medium was analyzed for LDH activity (C) and secreted IL-1β (D). (E and F) WT, *Rip3*$^{-/-}$, and *Rip3*$^{-/-}$*Casp8*$^{-/-}$ BMDMs were stimulated with PAM3CSK4 (2.5 μg/ml) for 4 h, the last 30 min of which was in the presence of 5 mM ATP. Lysates were immunoblotted for caspase-1 (**E**), and culture supernatants were analyzed for secreted IL-1β (**F**). ELISA data are mean ± SEM, and all data are representative of at least three independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

regulated Nlrp3 inflammasome activation, we analyzed Nlrp3 and proIL-1β expression levels in LPS-primed macrophages. Notably, LPS-induced upregulation of Nlrp3 mRNA (Fig. 5A) and protein expression (Fig. 5B) levels were reduced significantly in *Rip3*$^{-/-}$*Fadd*$^{-/-}$ macrophages. In agreement with FADD being responsible for these effects, loss of RIP3 alone did not significantly affect Nlrp3 expression (Fig. 5A, 5B). Concurrently, *Rip3*$^{-/-}$*Fadd*$^{-/-}$ macrophages were defective in LPS-induced proIL-1β mRNA (Fig. 5C), as well as in LPS-induced protein expression (Supplemental Fig. 2A) and in LPS+ATP- and LPS+silica-induced proIL-1β maturation (Fig. 5D, Supplemental Fig. 4B), suggesting that FADD regulated TLR4/NF-κB–dependent transcriptional priming of the Nlrp3 inflammasome. Indeed, LPS-induced phosphorylation of IκBα and ERK was specifically reduced in *Rip3*$^{-/-}$*Fadd*$^{-/-}$ macrophages but not in cells lacking RIP3 only (Fig. 5E). In agreement, LPS-induced secretion of the NF-κB–dependent cytokines IL-6 (Fig. 5F) and KC (Fig. 5G) was reduced significantly in the absence of FADD. In line with caspase-8 operating downstream of FADD, proIL-1β mRNA (Fig. 5H) and protein expression (Supplemental Fig. 2A) also were downregulated significantly in LPS-primed *Rip3*$^{-/-}$*Casp8*$^{-/-}$ macrophages. As a result, levels of both proIL-1β and mature IL-1β were significantly reduced in LPS+ATP- and LPS+silica-treated *Rip3*$^{-/-}$*Casp8*$^{-/-}$ macrophages (Fig. 5I, Supplemental Fig. 4B). To address whether FADD and caspase-8 were specifically involved in TLR-induced NF-κB activation, we stimulated cells with the NOD2 agonist muramyl dipeptide (MDP). As expected, NOD2-deficient macrophages failed to induce proIL-1β mRNA levels in response to MDP (Fig. 5J). Notably, *Rip3*$^{-/-}$*Fadd*$^{-/-}$ and *Rip3*$^{-/-}$*Casp8*$^{-/-}$ macrophages also were significantly affected in their ability to upregulate proIL-1β mRNA levels in response to MDP, albeit not to the extent of *Nod2*$^{-/-}$ macrophages (Fig. 5J). Together, these results demonstrate an accessory role for FADD and caspase-8 in NF-κB–dependent transcriptional upregulation of Nlrp3 and proIL-1β in cells primed with TLR and non-TLR agonists.

Role of caspase-8 in posttranslational activation of the Nlrp3 inflammasome

We next addressed whether caspase-8 also regulated Nlrp3 inflammasome activation at the posttranslational level. To formally explore this, WT macrophages were treated with the pharma-

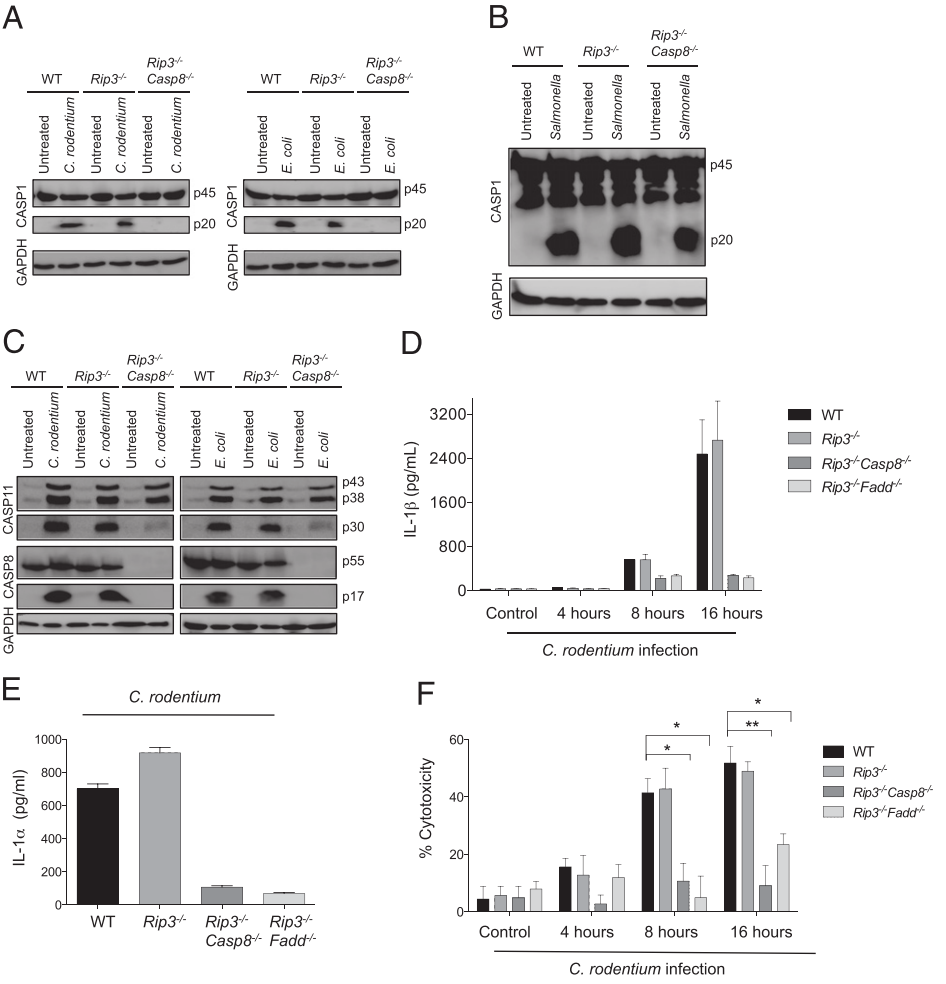

FIGURE 4. Caspase-8 mediates enteropathogen-induced Nlrp3 inflammasome activation. WT, *Rip3*$^{-/-}$, and *Rip3*$^{-/-}$*Casp8*$^{-/-}$ BMDMs were infected with *C. rodentium* or *E. coli* (moi 25) for 24 h (**A**, **C**) or with *S. typhimurium* (moi 5) for 4 h (**B**) before cell lysates were immunoblotted for the indicated proteins. WT, *Rip3*$^{-/-}$, *Rip3*$^{-/-}$*Casp8*$^{-/-}$, and *Rip3*$^{-/-}$*Fadd*$^{-/-}$ BMDMs were infected with *C. rodentium* (moi 25) for 4, 8, or 16 h before culture supernatants were analyzed for IL-1β (**D**), IL-1α (**E**), and LDH (**F**). Data are mean ± SEM, and all results are representative of at least three independent experiments. *$p < 0.05$, **$p < 0.01$.

cological caspase-8 inhibitor Ac-IETD-fmk prior to or after LPS priming. As expected, both proIL-1β expression and secretion of mature IL-1β (Fig. 6A, 6B) were significantly induced in macrophages treated with LPS and ATP in the absence of the caspase-8 inhibitor. In line with our genetic evidence that caspase-8 mediated LPS-induced production of proIL-1β (Fig. 5H, Supplemental Fig. 2A), macrophages incubated with the caspase-8 inhibitor prior to being exposed to LPS had reduced levels of proIL-1β expression relative to cells that were primed with LPS before incubation with the inhibitor (Fig. 6A). In agreement with caspase-8 regulating canonical Nlrp3 inflammasome activation posttranscriptionally, the caspase-8 inhibitor Ac-IETD-fmk markedly reduced ATP-induced proIL-1β maturation (Fig. 6A) and secretion of mature IL-1β (Fig. 6B) in these cells. Unlike proIL-1β, proIL-18 is constitutively expressed in macrophages, and its levels were not reduced in cells receiving Ac-IETD-fmk prior to LPS (Fig. 6A). Nevertheless, IL-18 secretion in the culture medium also was hampered severely in macrophages treated with Ac-IETD-fmk prior to ATP stimulation (Fig. 6C). In agreement with caspase-8 regulating Nlrp3 inflammasome activation at the posttranslational level, the caspase-8 inhibitor prevented caspase-1 and caspase-8 maturation, regardless of whether it was provided before or after LPS priming (Fig. 6D). In contrast, Ac-IETD-fmk failed to inhibit *S. typhimurium*–induced caspase-1 maturation (Fig. 6E), demonstrating that it did not target caspase-1 enzymatic activity directly. Our observation that Nlrp3 and ASC were required for LPS+ATP-induced caspase-8 maturation (Fig. 2C) suggests that caspase-8 interacts with inflammasome components. Thus, we hypothesized that caspase-8 may induce canonical Nlrp3 inflammasome activation by directly processing procaspase-1. Indeed, recombinant caspase-8 potently matured procaspase-1 into p30, p20, and p10 subunits associated with active caspase-1 (Fig. 6F). Such a role for caspase-8 appeared to be specific, because recombinant caspase-3 failed to cleave procaspase-1 under conditions that allowed it to efficiently process the caspase-3 and -7 zymogens (Supplemental Fig. 4C, 4D). To further address whether endogenous caspase-8 mediated caspase-1 activation by the canonical Nlrp3 inflammasome, lysates of untreated and LPS+ATP-stimulated WT, *Rip3*$^{-/-}$, and *Rip3*$^{-/-}$*Casp8*$^{-/-}$ macrophages were incubated with the caspase activity probe biotin-VAD-fmk, which covalently links to the catalytic cysteine of enzymatically active caspases. The amount of active caspase-1 recovered from streptavidin beads was analyzed by immunoblotting to determine the levels of active caspase-1 retrieved from LPS+ATP-stimulated macrophages of the different genotypes. As expected, active caspase-1 was not recovered from lysates of untreated BMDMs or from streptavidin beads loaded with lysates of LPS+ATP-treated WT macrophages in the absence of biotin-VAD-fmk (Fig. 6G), demonstrating specificity of the experimental setup. Notably, both the large catalytic subunit (p20; Fig. 6G, *lower panel*) and full-length caspase-1 (p45; Fig. 6G, *upper panel*) were pulled down efficiently from LPS+ATP-stimulated WT and *Rip3*$^{-/-}$ macrophage lysates but not from lysates of LPS+ATP-treated *Rip3*$^{-/-}$*Casp8*$^{-/-}$ BMDMs (Fig. 6G). The observation that caspase-8 expression was necessary to recover biotin-VAD–labeled unprocessed and processed caspase-1 from LPS+ATP-stimulated macrophages suggests that it may pro-

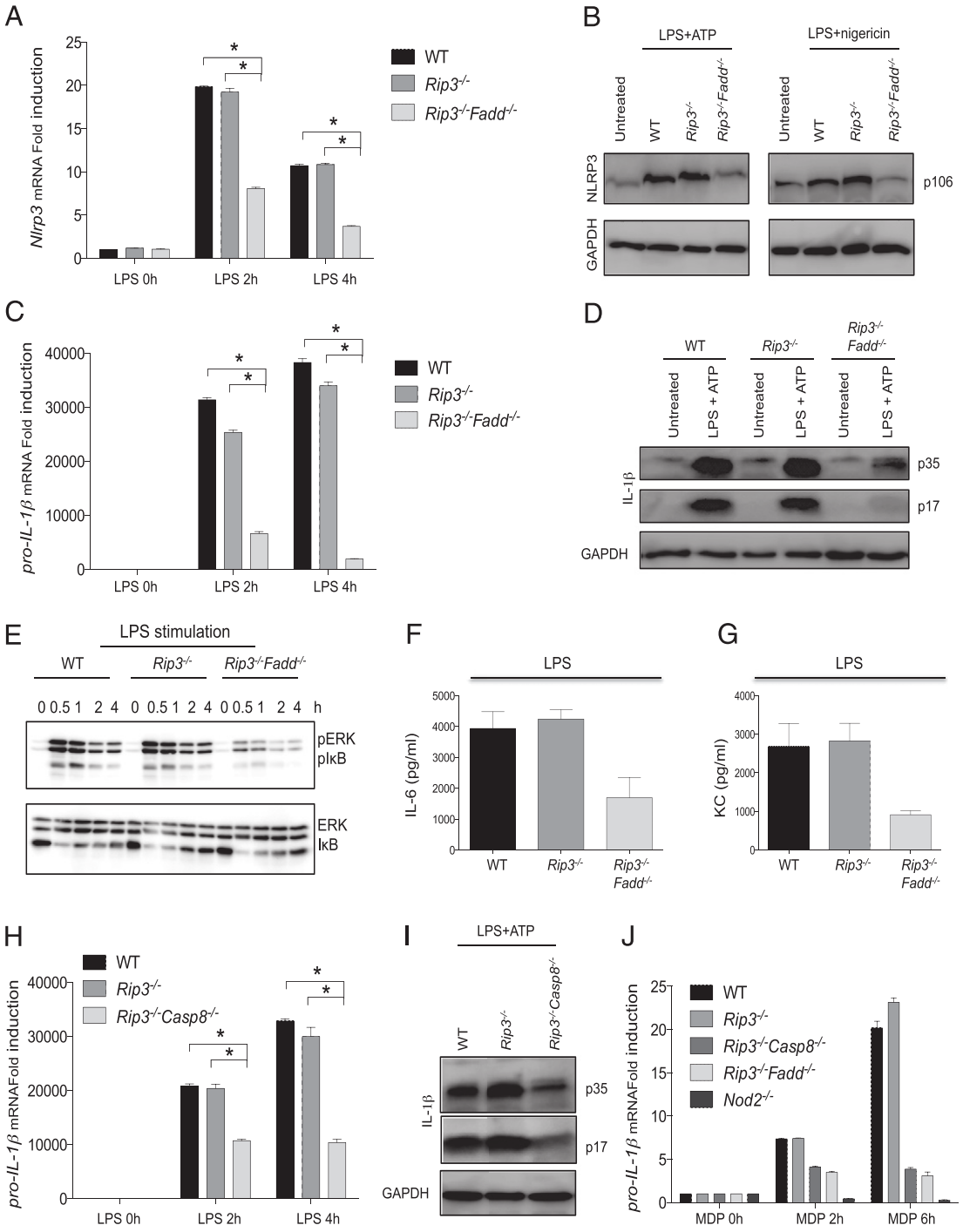

FIGURE 5. FADD and caspase-8 mediate transcriptional priming of the Nlrp3 inflammasome. WT, *Rip3*$^{-/-}$, and *Rip3*$^{-/-}$*Fadd*$^{-/-}$ BMDMs were left untreated or stimulated with 20 ng LPS for 2 or 4 h. Induction of *Nlrp3* (**A**) and *proIL-1β* (**C**) mRNA was determined as described in *Materials and Methods*. (B and D) WT, *Rip3*$^{-/-}$, and *Rip3*$^{-/-}$*Fadd*$^{-/-}$ BMDMs were stimulated with 20 ng LPS for 4 h, the last 30 min of which was in the presence of 5 mM ATP or 20 μM nigericin. Lysates were immunoblotted for Nlrp3 (**B**) and IL-1β (**D**). (**E**) WT, *Rip3*$^{-/-}$, and *Rip3*$^{-/-}$*Fadd*$^{-/-}$ BMDMs were stimulated with LPS for the indicated durations. Lysates were immunoblotted for total and phosphorylated IκBα and ERK. WT, *Rip3*$^{-/-}$, and *Rip3*$^{-/-}$*Fadd*$^{-/-}$ BMDMs were stimulated with 20 ng/ml LPS for 4 h, and the levels of secreted IL-6 (**F**) and KC (**G**) in culture supernatants were determined by multiplex ELISA. (H) WT, *Rip3*$^{-/-}$, and *Rip3*$^{-/-}$*Casp8*$^{-/-}$ BMDMs were stimulated with 20 ng LPS for 0, 2, or 4 h before proIL-1β mRNA levels were determined, as described in *Materials and Methods*. (I) WT, *Rip3*$^{-/-}$, and *Rip3*$^{-/-}$*Casp8*$^{-/-}$ BMDMs were stimulated with LPS+ATP, as described above, before combined cell lysates and culture supernatants were immunoblotted for IL-1β. (J) WT, *Rip3*$^{-/-}$, *Rip3*$^{-/-}$*Casp8*$^{-/-}$, and *Rip3*$^{-/-}$*Fadd*$^{-/-}$ BMDMs were stimulated with 10 μg MDP for the indicated durations before *proIL-1β* mRNA levels were determined, as described in *Materials and Methods*. ELISA data are shown as mean ± SEM, and all data are representative of three independent experiments. $^{*}p < 0.05$.

mote caspase-1 activation through both cleavage and proximity-induced autoactivation of procaspase-1 in the Nlrp3 inflammasome. In agreement, confocal immunofluorescence analysis showed significant colocalization of caspase-1 and caspase-8 in macrophages that were stimulated with LPS+ATP or infected with *C. rodentium* but not in untreated cells (Fig. 6H). This observation

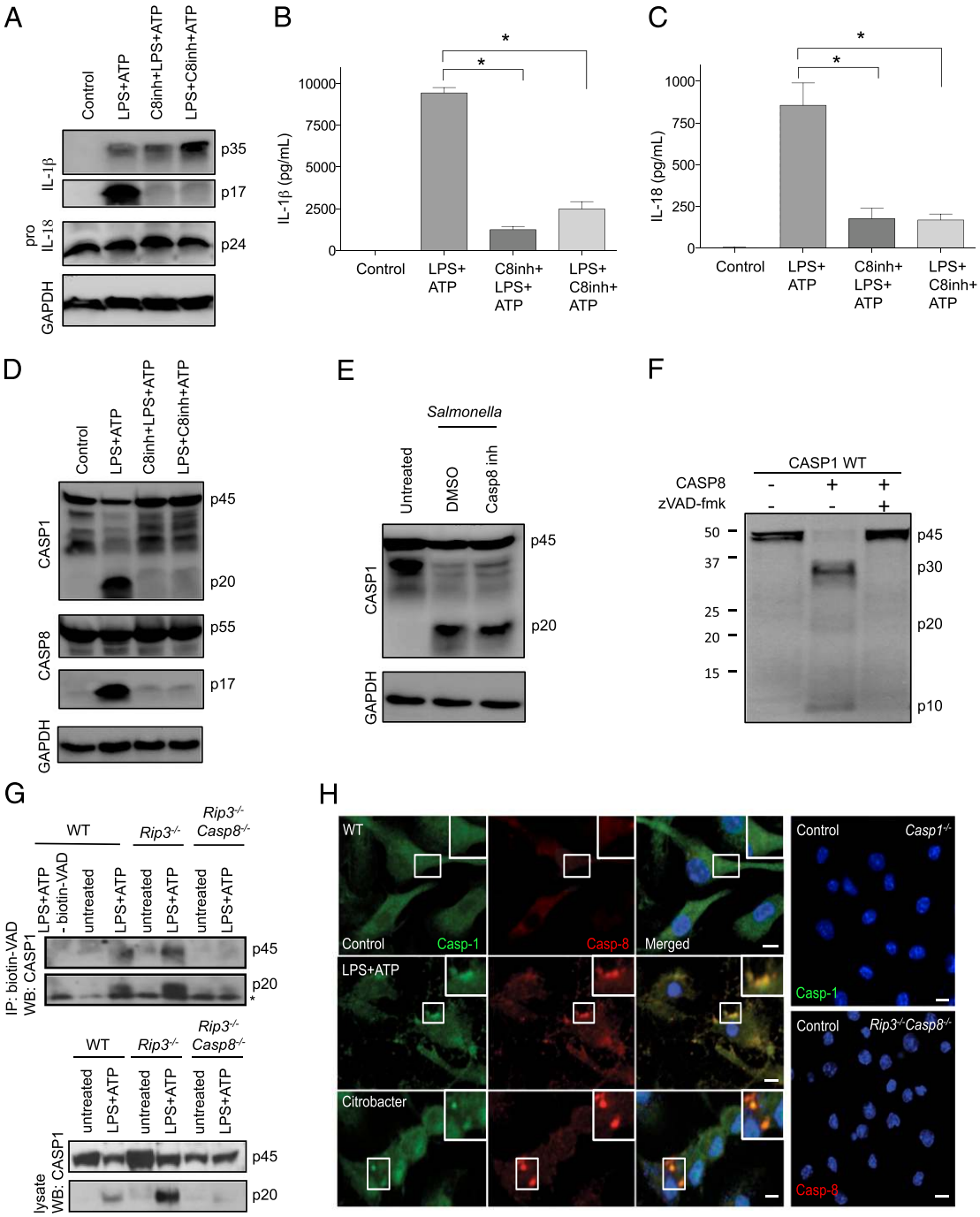

FIGURE 6. Caspase-8 is required for posttranslational activation of the Nlrp3 inflammasome. (**A**–**D**) WT BMDMs were stimulated with 20 ng LPS for 4 h, the last 30 min of which was in the presence of 5 mM ATP or 20 μM nigericin. In some setups, the caspase-8 inhibitor Ac-IETD-fmk (20μM) was added 10 min prior to LPS treatment, whereas in other setups it was added 10 min prior to ATP treatment. Lysates were immunoblotted for the indicated proteins (A, D), and cell supernatants were analyzed for secreted IL-1β (B) and IL-18 (C). (**E**) WT BMDMs were pretreated with vehicle control (DMSO) or 20 μM caspase-8 inhibitor Ac-IETD-fmk before being infected with *S. typhimurium* (moi 5) for 4 h. Lysates were immunoblotted for caspase-1. (**F**) ^{35}S-labeled procaspase-1 was produced in vitro and incubated with recombinant caspase-8 (100 U) at 37˚C for 1 h before caspase-1 processing was analyzed by autoradiography. In some setups, caspase-8 was incubated with 1 μM zVAD-fmk prior to coincubation with procaspase-1. (**G**) WT, *Rip3*$^{-/-}$, and *Rip3*$^{-/-}$ *Casp8*$^{-/-}$ BMDMs were stimulated with LPS and ATP as above, and cell lysates prepared by freeze-thawing in liquid N_2 were immunoblotted for caspase-1. Samples containing 1 mg protein were incubated in the presence or absence of 10 μM biotin-VAD-fmk (Enzo Life Sciences) for 45 min, followed by precipitation with streptavidin-agarose (Thermo Scientific) at 4˚C overnight before immunoblotting for caspase-1. Asterisk indicates nonspecific immunoreactive bands of the respective Abs. (H) BMDMs grown on coverslips were stimulated with LPS+ATP or infected with *C. rodentium*, as above. Cells were fixed and immunostained for caspase-1 (green) and caspase-8 (red). DAPI was used for nuclear staining. Scale bars, 10 μm. Data are representative of three independent experiments. *$p < 0.05$.

is in line with a recent report (19) demonstrating significant colocalization of caspase-8 and ASC in confocal micrographs of LPS+ nigericin-treated *Casp1*$^{-/-}$*Casp11*$^{-/-}$ macrophages. Together, our results suggest that FADD and caspase-8 interact with core components of the Nlrp3 inflammasome and promote activation of procaspase-1 in the complex.

In vivo role of FADD and caspase-8 in Nlrp3 inflammasome activation

To demonstrate the in vivo relevance of FADD and caspase-8 in Nlrp3 inflammasome signaling, cohorts of WT, *Rip3*$^{-/-}$, *Rip3*$^{-/-}$ *Casp8*$^{-/-}$, and *Rip3*$^{-/-}$*Fadd*$^{-/-}$ mice were challenged with a lethal dose of LPS. The LPS-induced endotoxemia model was selected because both caspase-1 (2) and caspase-11 (2, 38) are required for IL-1β production in LPS-challenged mice, whereas circulating IL-1α levels are regulated by caspase-11 independently of caspase-1 (2). Notably, circulating levels of both IL-1β (Fig. 7A) and IL-1α (Fig. 7B) were reduced significantly in LPS-challenged *Rip3*$^{-/-}$*Casp8*$^{-/-}$ and *Rip3*$^{-/-}$*Fadd*$^{-/-}$ mice relative to those of LPS-treated WT and *Rip3*$^{-/-}$ mice, thus extending the roles of FADD and caspase-8 in Nlrp3 inflammasome signaling to a relevant in vivo setting. The Nlrp3 inflammasome also plays a critical role in controlling *C. rodentium* replication in vivo (3). As in *Nlrp3*$^{-/-}$ and *Casp1*$^{-/-}$/*Casp11*$^{-/-}$ mice (3), bacterial burdens (Fig. 7C) and stool scores (Fig. 7D) were significantly elevated in *C. rodentium*–infected *Rip3*$^{-/-}$*Casp8*$^{-/-}$ and *Rip3*$^{-/-}$ *Fadd*$^{-/-}$ mice relative to those of infected WT and *Rip3*$^{-/-}$ mice.

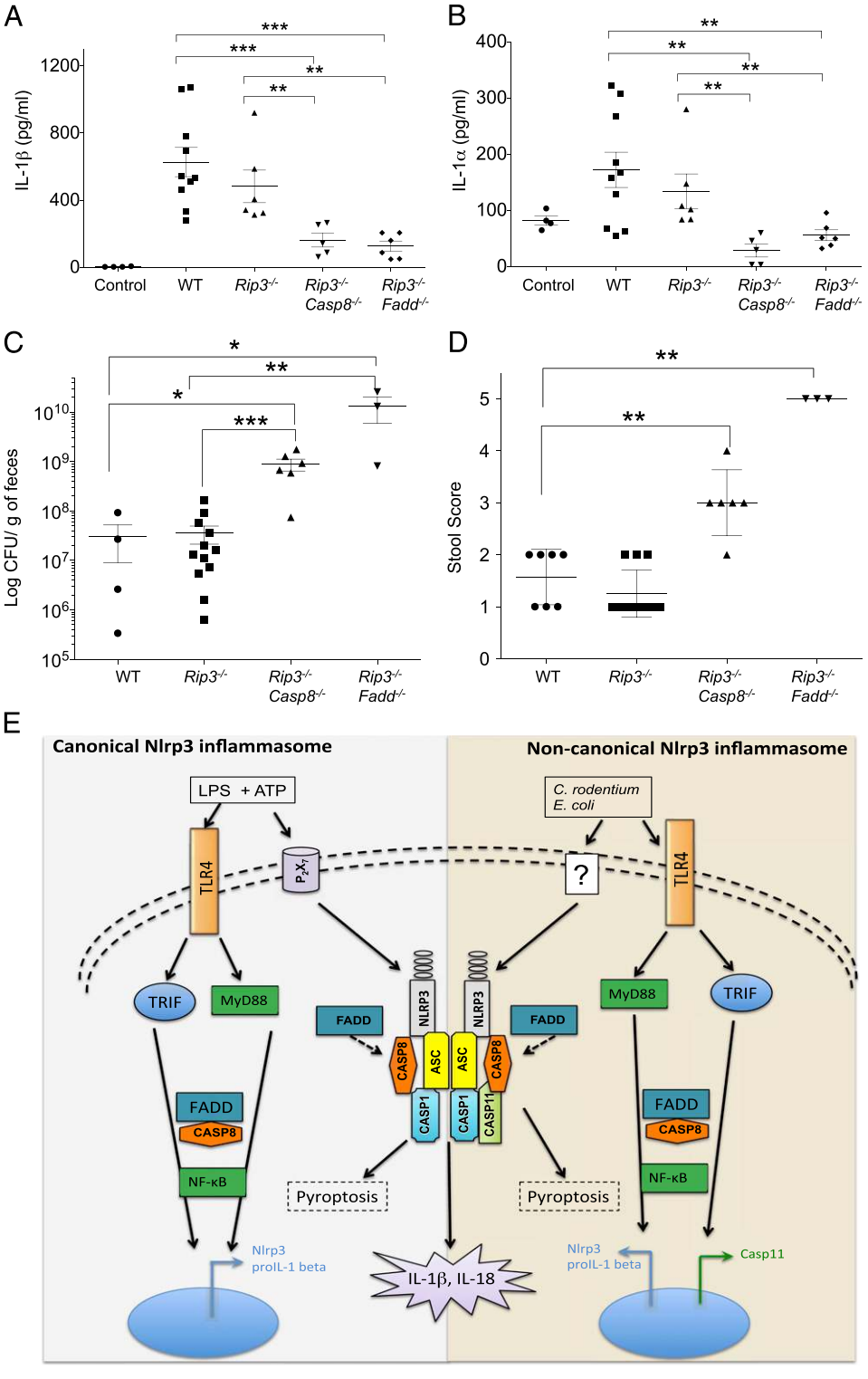

FIGURE 7. In vivo role of caspase-8 and FADD in inflammasome responses during LPS-induced endotoxemia and *C. rodentium* infection. Mice of the indicated genotypes were injected with 35 mg/kg LPS for 5 h before serum was collected and analyzed for IL-1β (**A**) and IL-1α (**B**). Mice of the indicated genotypes were infected orally with *C. rodentium*, and titers in feces (**C**) and stool consistency (**D**) were examined on day 15 postinfection. Each symbol represents a mouse. Data are mean ± SEM. (**E**) Schematic representation of the roles of FADD and caspase-8 in canonical and noncanonical Nlrp3 inflammasome signaling. LPS-induced TLR4 activation triggers MyD88-dependent upregulation of NLRP3 and proIL-1β levels, as well as TRIF-dependent procaspase-11 induction to prime the canonical and noncanonical Nlrp3 inflammasomes for activation by their respective ligands and bacterial pathogens. FADD and caspase-8 are required for efficient TLR4-induced transcriptional upregulation of NLRP3, procaspase-11, and proIL-1β (inflammasome priming). At the posttranslational level, FADD functions as a platform for procaspase-8 activation, and they both interact with core components of the Nlrp3 inflammasome to drive stimulus-dependent caspase-1/11 maturation. Inflammasome-activated caspase-1 processes and secretes IL-1β and IL-18. Independently of caspase-1, caspase-11 also mediates enteropathogen-induced pyroptosis. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Together, these results demonstrate that FADD and caspase-8 control pathological disease parameters in at least two in vivo mouse models of human disease that were previously attributed to Nlrp3 inflammasome activation.

Discussion

Caspase-8 was shown to directly mature proIL-1β in macrophages treated with TLR3 and TLR4 agonists in the absence of caspase-1 and Nlrp3 (17) and in response to extracellular fungi and mycobacteria (15). In addition, macrophages exposed to TLR ligands were shown to upregulate expression levels of Fas receptor, which allows significant secretion of mature IL-1β and IL-18 upon subsequent exposure to Fas ligand. Notably, Fas ligand–induced IL-1β and IL-18 secretion from LPS-primed macrophages and dendritic cells relied on caspase-8, whereas RIP3 and the inflammasome components ASC and caspases-1 and -11 were dispensable (16). A similar requirement for caspase-8, independently of ASC and caspase-1, recently was demonstrated for IL-1β secretion from BMDCs that were cotreated with LPS and proapoptotic chemotherapeutic agents, such as doxorubicin and Staurosporine (39). Moreover, when induction of more rapid caspase-1–mediated pyroptotic cell death is prevented, LPS-primed macrophages that are subsequently stimulated with nigericin (a canonical Nlrp3 inflammasome stimulus) or transfected with dsDNA (an activator of the AIM2 inflammasome) undergo caspase-8–mediated apoptosis (19, 40). Notably, caspase-8 physically interacted with the inflammasomes adaptor ASC under these conditions, and ASC, but not caspase-1, was required for caspase-8 maturation in response to LPS+nigericin and cytosolic DNA (19).

Our work clarifies the roles of FADD and caspase-8 in IL-1β production by the regular Nlrp3 inflammasome pathways (Fig. 7E). We showed that FADD and caspase-8 drive potent Nlrp3 inflammasome activation and IL-1β secretion at two regulatory checkpoints. At the transcriptional level, FADD and caspase-8 promoted NF-κB–dependent transcriptional upregulation of proIL-1β. Indeed, defective proIL-1β production also was observed recently to occur in *S. typhimurium*–infected *Rip3*$^{-/-}$*Casp8*$^{-/-}$ macrophages (37). This likely explains the significantly reduced secretion of mature IL-1β from these cells in the absence of defective caspase-1 maturation by the Nlrc4 inflammasome. In addition to proIL-1β, we noted that LPS-induced transcriptional induction of Nlrp3 expression levels, a necessity for Nlrp3 inflammasome priming, partially depended on FADD and caspase-8. A similar dependency on caspase-8 for transcriptional upregulation of proIL-1β and Nlrp3 was reported recently in LPS-primed BMDCs (39). The transcriptional role of caspase-8 in Nlrp3 inflammasome priming was evident at early time points, and it gained further significance when BMDCs were treated with LPS for extended durations (>8 h) (39). Notably, our observations further revealed that the transcriptional roles of FADD and caspase-8 were not confined to TLR4-induced signaling but extended to NF-κB and MAPK activation by the TLR2 ligand Pam3CSK4 and the NOD2 ligand MDP. Together, these results strongly implicate that FADD/caspase-8 play an accessory role in NF-κB and MAPK activation at the level of or downstream of the IKK complex, although further work is needed to reveal the precise mechanism by which they contribute to efficient NF-κB–mediated gene transcription.

At the posttranslational level, we observed that priming with LPS and exposure to canonical Nlrp3 inflammasome stimuli, such as ATP, nigericin, or silica, were both needed to induce efficient caspase-8 processing. Intriguingly, LPS+ATP-induced caspase-8 processing required the inflammasome components Nlrp3 and ASC, although caspase-1 was dispensable. This suggests that both caspase-1 and -8 are activated downstream of Nlrp3 and ASC in response to canonical stimuli of the Nlrp3 inflammasome. In agreement, a recent report showed that ASC mediates caspase-8 processing in LPS+nigericin-treated macrophages and physically associates with caspase-8 (19). A similar interaction between caspase-8 and ASC specks recently was reported to occur in macrophages that had been infected with *S. typhimurium* (37), a pathogen that may activate both the Nlrc4 and Nlrp3 inflammasomes (6, 41). Indeed, we observed that FADD and caspase-8 interacted with core components of the Nlrp3 inflammasome, and caspase-8 was required for caspase-1 activation, as well as for IL-1β and IL-18 secretion. Notably, $cFLIP_L$, an interaction partner of caspase-8, recently was demonstrated to interact with the Nlrp3 inflammasome, and hemizygous deletion of $cFLIP_L$ inhibited canonical Nlrp3 inflammasome activation, as well (42). Combined with the work presented in this article, it raises the possibility that cFLIP and caspase-8 form a proteolytically active complex that promotes Nlrp3 inflammasome activation, paralleling the cFLIP/caspase-8 complexes suggested to inhibit necroptosis (43) and to promote NF-κB signaling in activated T cells (44, 45). Together with the observation that TNF-α and its receptor were dispensable for Nlrp3 inflammasome activation, our data also suggest that FADD may serve as a platform for caspase-8 autoactivation in the inflammasome. In agreement, immunofluorescence micrographs showed caspase-1 and caspase-8 to colocalize in macrophages exposed to canonical and noncanonical activators of the Nlrp3 inflammasome. Notably, enteropathogens differed from canonical Nlrp3 inflammasome stimuli in that they activated caspase-8 independently of Nlrp3 and ASC; further work is needed to dissect the mechanism leading to caspase-8 processing in enteropathogen-infected macrophages. However, our observation that enteropathogen-induced caspase-11 maturation was significantly affected in FADD- and caspase-8–deficient cells suggests that these molecules might relay the signal for caspase-11 processing from a recently proposed intracellular LPS receptor of unknown identity (46). Taken together, our results support the hypothesis that FADD and caspase-8 contribute to both NF-κB–dependent priming and posttranslational activation of the Nlrp3 inflammasome. At first glance, this appears to contrast with a recent report (18) suggesting that caspase-8 negatively regulates spontaneous LPS-induced IL-1β secretion in dendritic cells, which was relayed by Rip3. However, the cited report focused on the mechanisms driving "spontaneous" LPS-induced inflammasome activation in caspase-8–deficient dendritic cells in the absence of inflammasome triggers, such as ATP and nigericin (18). Notably, unlike in dendritic cells with a conditional deletion of caspase-8, LPS stimulation alone fails to trigger inflammasome activation in WT and *Casp8*$^{-/-}$*Rip3*$^{-/-}$ macrophages and dendritic cells. Moreover, albeit higher than in WT controls, levels of spontaneously secreted IL-1β in LPS-stimulated *Casp8*$^{-/-}$ dendritic cells appeared significantly lower than those frequently noted with canonical triggers of the Nlrp3 inflammasome (18). Thus, it is likely that LPS-induced activation of Nlrp3 in the context of caspase-8 deficiency is mechanistically distinct from that leading to robust canonical and noncanonical Nlrp3 inflammasome activation in response to ATP and enteropathogens. Regardless, by identifying FADD and caspase-8 as upstream mediators of canonical and noncanonical Nlrp3 inflammasome priming and activation, our studies provide a framework for understanding how apoptotic and Nlrp3 inflammasome pathways interconnect.

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Disclosures

The authors have no financial conflicts of interest.

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