Caspase-8 promotes c-Rel–dependent inflammatory cytokine expression and resistance against *Toxoplasma gondii*

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Caspase-8 is a key integrator of cell survival and cell death decisions during infection and inflammation. Following engagement of tumor necrosis factor superfamily receptors or certain Toll-like receptors (TLRs), caspase-8 initiates cell-extrinsic apoptosis while inhibiting RIPK3-dependent programmed necrosis. In addition, caspase-8 has an important, albeit less well understood, role in cell-intrinsic inflammatory gene expression. Macrophages lacking caspase-8 or the adaptor FADD have defective inflammatory cytokine expression and inflammasome priming in response to bacterial infection or TLR stimulation. How caspase-8 regulates cytokine gene expression, and whether caspase-8–mediated gene regulation has a physiological role during infection, remain poorly defined. Here we demonstrate that both caspase-8 enzymatic activity and scaffolding functions contribute to inflammatory cytokine gene expression. Caspase-8 enzymatic activity was necessary for maximal expression of *Il1b* and *Il12b*, but caspase-8 deficient cells exhibited a further decrease in expression of these genes. Furthermore, the ability of TLR stimuli to induce optimal IκB kinase phosphorylation and nuclear translocation of the nuclear factor kappa light chain enhancer of activated B cells family member c-Rel required caspase activity. Interestingly, overexpression of c-Rel was sufficient to restore expression of IL-12 and IL-1β in caspase-8–deficient cells. Moreover, *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ mice were unable to control infection by the intracellular parasite *Toxoplasma gondii*, which corresponded to defects in monocyte recruitment to the peritoneal cavity, and exogenous IL-12 restored monocyte recruitment and protection of caspase-8–deficient mice during acute toxoplasmosis. These findings provide insight into how caspase-8 controls inflammatory gene expression and identify a critical role for caspase-8 in host defense against eukaryotic pathogens.

caspase-8 | TLR signaling | c-Rel | IL-12 | *Toxoplasma*

Pathogen-associated molecular patterns, produced by both commensal and pathogenic microbes, trigger the activation of pattern recognition receptors (PRRs) located within the cytoplasm and at the host membrane (1, 2). Activation of a subset of PRRs known as Toll-like receptors (TLRs) initiates intracellular signaling cascades that lead to the nuclear translocation of transcription factors belonging to the nuclear factor kappa light chain enhancer of activated B cells (NF-κB) and AP-1 families, among others, which control the expression of inflammatory mediators (3). Conversely, pathogen-mediated or chemical blockade of these signaling pathways triggers cell death programs mediated by caspases (4–11).

Caspases are cysteine proteases that initiate various forms of programmed cell death (12). One member of this family implicated in both inflammatory (13, 14) and immunologically silent cell death is caspase-8. Caspase-8 is the initiator caspase that induces cell-extrinsic apoptosis in response to various death-inducing stimuli, such as Fas (15) and tumor necrosis factor (TNF) (16), but also has a prosurvival function in concert with its enzymatically inactive homolog cFLIP.

Under baseline conditions, caspase-8 is bound to cFLIP, forming a heterodimer that prevents the autoprocessing of caspase-8 required for its apoptotic function (17, 18). Up-regulation of cFLIP maintains survival of cells in response to TLR or TNF receptor family stimulation (18–22). Thus, during pharmacologic or pathogen-mediated blockade of gene transcription, lack of cFLIP up-regulation is thought to enable caspase-8 to form homodimers and undergo autoprocessing, thereby stabilizing its apoptotic conformation (23–26). Moreover, both prosurvival and proapoptotic conformations of caspase-8 negatively regulate

Significance

Caspase-8 is a critical regulator of apoptosis, programmed necrosis, and inflammatory gene expression. Caspase-8 is necessary for optimal transcription of inflammatory and antimicrobial host defense genes, including *Il12* and *Il1b*. While the mechanisms of caspase-8–mediated control of apoptosis and programmed necrosis are well described, how caspase-8 controls inflammatory gene expression is less clear. We show that caspase-8 is necessary for optimal nuclear translocation of the nuclear factor kappa light chain enhancer of activated B cells (NF-κB) family member c-Rel and, consequently, for recruitment of c-Rel to caspase-8–dependent gene promoters. Furthermore, caspase-8 deficiency leads to an inability to control acute *Toxoplasma gondii* infection. These findings provide insight into how caspase-8 contributes to inflammatory gene expression and host defense.

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programmed necrosis, in part through cleavage of the deubiquitinating enzyme CYLD (27–29).

In humans, caspase-8 deficiency is associated with defects in resistance against infection, suggesting that caspase-8–induced control of cell death contributes to antimicrobial defense (30). However, these patients also have defects in B, natural killer (NK), and T cell activation independent of cell death, raising the possibility that these patients' susceptibility to microbial infection may involve caspase-8–mediated control of immune cell activation. In murine models of caspase-8 deficiency, caspase-8 also plays a cell-intrinsic role in gene expression in response to gram-negative bacterial (31–35) and fungal infections (36), suggesting that cell-intrinsic control of gene expression by caspase-8 contributes to antimicrobial host defense independent of the role of caspase-8 in regulating cell death.

The mechanisms governing caspase-8–dependent control of gene expression in macrophages remain unclear. Initial observations of caspase-8 deficiencies in human patients suggested a role for caspase-8 in NF-κB signaling in response to B, NK, and T cell receptor (TCR) activation (37, 38). However, murine T cells lacking both caspase-8 and RIPK3 did not exhibit defects in TCR signaling or T cell activation, implying either that T cells deficient in caspase-8 alone undergo necroptosis upon TCR activation (39) or that human- and mouse-specific differences in these pathways impact the cellular response to activation. Mice lacking caspase-8 and RIPK3 have no discernable defects in T cell responses to mouse hepatitis virus infection or T cell proliferation (40). Caspase-8 scaffolding, but not catalytic activity, is important for TNF-related apoptosis-inducing ligand-induced inflammatory gene expression in HeLa cells (41). However, we previously observed that treatment of *Ripk3*$^{-/-}$ cells with pan-caspase inhibitors led to the inhibition of inflammatory cytokine production by macrophages, and that caspase-8 was required for maximal expression of *Il12b* and *Il1b* in response to multiple TLR ligands (31). To address these findings, and to mechanistically dissect the contribution of caspase-8 to macrophage inflammatory gene expression, we investigated the roles of caspase-8 scaffolding and enzymatic activities in the regulation of NF-κB family signaling.

We show that caspase-8 promotes inflammatory gene expression in macrophages through both enzymatic and nonenzymatic activities. Either caspase-8 deficiency or inhibition of caspase activity in *Ripk3*$^{-/-}$ bone marrow-derived macrophages (BMDMs) was associated with both reduced IκB kinase (IKK) phosphorylation and defective nuclear translocation of the NF-κB family member c-Rel, which has a particularly important role in regulating the expression of both *Il12* (39) and *Il1b*. Ectopic expression of c-Rel restored IL-12 and IL-1β expression in caspase-8–deficient cells, indicating that caspase-8 controls transcription of these key inflammatory cytokines in part via c-Rel. *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ mice succumbed to infection with the intracellular protozoan parasite *Toxoplasma gondii*, and this correlated with a failure to recruit monocytes to the peritoneum and with increased parasite burdens in the peritoneal cavity. Critically, both monocyte recruitment and control of *T. gondii* parasite burdens in *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ mice was restored by exogenous IL-12. Taken together, these findings demonstrate a previously undescribed role for caspase-8 in the control of c-Rel nuclear translocation and immune defense against a protozoan parasite.

Results

Caspase-8 Catalytic Activity Regulates Activation of the IKK Complex. Caspase-8–deficient cells have defects in inflammatory gene expression, and this defect has been attributed to both a nonapoptotic function of caspase-8 catalytic activity and an activity-independent caspase-8 scaffolding function (31, 41). To resolve this apparent discrepancy, we analyzed the transcriptional induction of specific caspase-8–dependent genes (31) in *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ cells, which lack caspase-8 entirely, and in *Ripk3*$^{-/-}$ cells treated with the pan-caspase inhibitor zVAD-fmk, which blocks caspase activity without affecting caspase-8's scaffolding function (41). As expected, zVAD-fmk reduced the expression of *Il12b*, *Il1a*, *Il1b*, and *Il4i1* in response to lipopolysaccharide (LPS) stimulation; however, the impact of zVAD on gene expression was less than that seen in isogenic cells in which caspase-8 was entirely missing (Fig. 1*A*). These data imply that caspase-8 promotes the induction of TLR-dependent inflammatory gene expression via both enzymatic activity-dependent and -independent pathways. Importantly, the effect of z-VAD-fmk is independent of cell death, as *Ripk3*$^{-/-}$ cells are protected from cell death in response to zVAD treatment (42, 43).

As expected, zVAD reduced the secretion of IL-12p40, but again, this reduction was less than that occurring with complete loss of caspase-8 (Fig. 1*B*). In contrast, cells lacking caspase-8 and cells in which caspase activity was blocked with zVAD had equivalent levels of pro-IL-1β (Fig. 1*B*), suggesting that caspase-8 catalytic activity is critical for caspase-8–dependent expression of IL-1β in response to TLR stimulation.

Caspase-8 has been proposed to promote NF-κB signaling in both innate and adaptive immune cells (30, 37, 44), and caspase-8 promotes IKK phosphorylation in response to LPS (35) (Fig. 1*C*). Interestingly, we observed that *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ cells showed reduced IKK phosphorylation in response to both LPS and CpG (Fig. 1*D*), and that caspase activity was necessary for optimal IKK phosphorylation in response to both TLR stimuli (Fig. 1*E*). Intriguingly, loss of the adaptor protein FADD, which is required for the assembly of caspase-8–containing signaling complexes (33, 45, 46), also resulted in a defect in IKK phosphorylation and decreased inflammatory cytokine production in response to TLR stimulation (Fig. 1*F* and *SI Appendix*, Fig. S1). Importantly, the caspase-8–selective inhibitor IETD also significantly reduced levels of IL-12p40 secretion as well as *Il12b* expression (*SI Appendix*, Fig. S2 *A* and *B*). zVAD treatment abrogated caspase-8 activity in response to staurosporine treatment or TLR stimulation, and treatment with either a pan-caspase or caspase-8–selective inhibitor abrogated CYLD cleavage in response to LPS, which is mediated by the nonapoptotic activity of caspase-8 (28) (*SI Appendix*, Fig. S2 *C* and *D*). These data indicate that caspase inhibitors robustly block caspase activity in this system, suggesting that caspase activity contributes to inflammatory gene expression, at least in part, via regulation of IKK phosphorylation.

To further dissect the contributions of caspase-8 scaffolding or catalytic activities to inflammatory cytokine gene expression, we examined inflammatory cytokine production in immortalized *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ BMDMs (iBMDMs) reconstituted with Casp8^{WT} or Casp8^{D3A} mutant constructs in which all 3 aspartate residues in the interdomain linker between large and small catalytic subunits were mutated to alanines, and was previously shown to be deficient in IETDase activity (13). As expected, *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ iBMDMs produced minimal levels of TNF in response to TLR4 or TLR9 stimulation, and this was rescued by ectopic expression of wild-type (WT) caspase-8 (*SI Appendix*, Fig. S2 *E* and *F*). Intriguingly, the Casp8^{D3A} partially restored TNF expression, although not to the same extent as WT caspase-8 (*SI Appendix*, Fig. S2 *E* and *F*). Primary BMDMs from Casp8^{DA} mice, which have a single mutation at D387 that largely prevents autoprocessing and caspase-8–induced cell death (13, 25), showed WT levels of cytokine induction as well as IKK phosphorylation (*SI Appendix*, Fig. S2 *G* and *H*). However, in the absence of RIPK3, loss of caspase-8 autoprocessing led to intermediate effects on both transcription and IKK phosphorylation (*SI Appendix*, Fig. S2 *I–K*), suggesting that caspase-8 and RIPK3 function in concert to control optimal innate immune activation. Collectively, these data demonstrate a dual role for caspase-8 scaffolding and catalytic activities in regulating macrophage cytokine production in response to inflammatory stimuli.

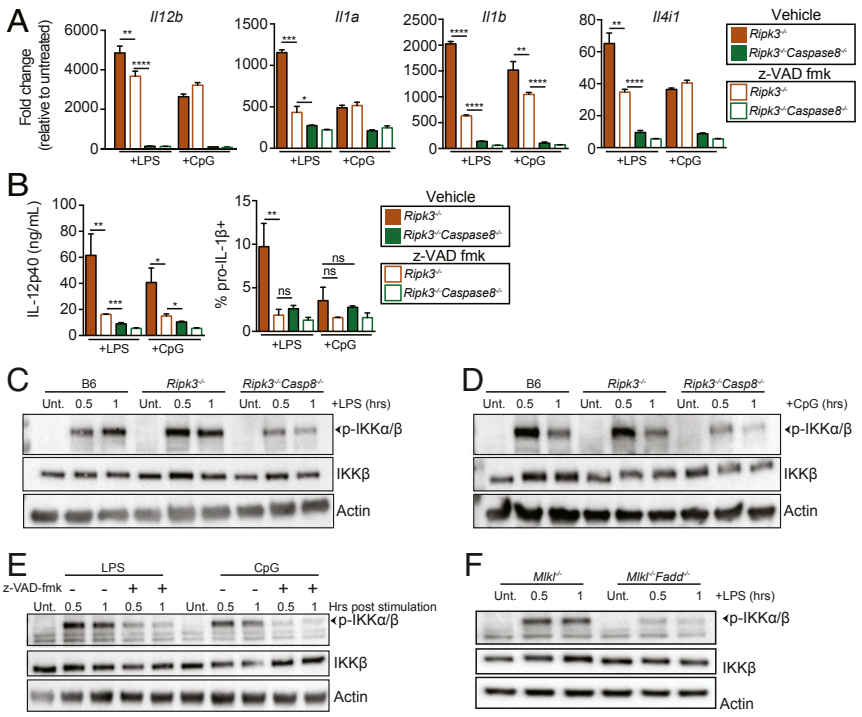


Fig. 1. Caspase-8 catalytic activity regulates activation of the IKK complex. (*A*) Fold change in mRNA expression of indicated genes in *Ripk3*$^{-/-}$ and *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ BMDMs pretreated with z-VAD-fmk or vehicle control for 1 h, followed by treatment with LPS or CpG as indicated in *Materials and Methods* for 2 h. (*B*) Levels of secreted IL-12p40 and frequency of intracellular pro-IL-1β expression by *Ripk3*$^{-/-}$ and *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ BMDMs pretreated with z-VAD-fmk or vehicle control for 1 h, followed by LPS or CpG treatment for 5 h. (*C* and *D*) Levels of phospho-IKKα/β and total IKKβ in B6, *Ripk3*$^{-/-}$, and *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ BMDMs following stimulation with LPS or CpG for indicated times, as determined by immunoblotting. (*E*) Levels of phospho-IKKα/β and total IKKβ in *Ripk3*$^{-/-}$ BMDMs pretreated with z-VAD-fmk or vehicle control for 1 h and stimulated with LPS or CpG. (*F*) Levels of phospho-IKKα/β and total IKKβ in *Mlkl*$^{-/-}$ and *Mlkl*$^{-/-}$*Fadd*$^{-/-}$ BMDMs stimulated with LPS for the indicated times. Error bars indicate ± SEM of triplicates. n.s., not significant; **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001, Student's 2-tailed unpaired *t* test. The data in *A*–*D* are representative of 3 independent experiments, and the data in *E* are representative of 2 independent experiments.

Caspase-8 Regulates TLR-Induced Gene Expression Programs. In the setting of RIPK3 deficiency, caspase-8 has been proposed to mediate the expression of TLR4- or TLR3-induced genes, due to the link between TRIF and RIPK1/caspase-8 (13, 32, 33, 35). In previous studies, we had also observed a role for caspase-8 in regulating TLR2- and TLR9-dependent gene expression, which depends solely on MyD88 (31).

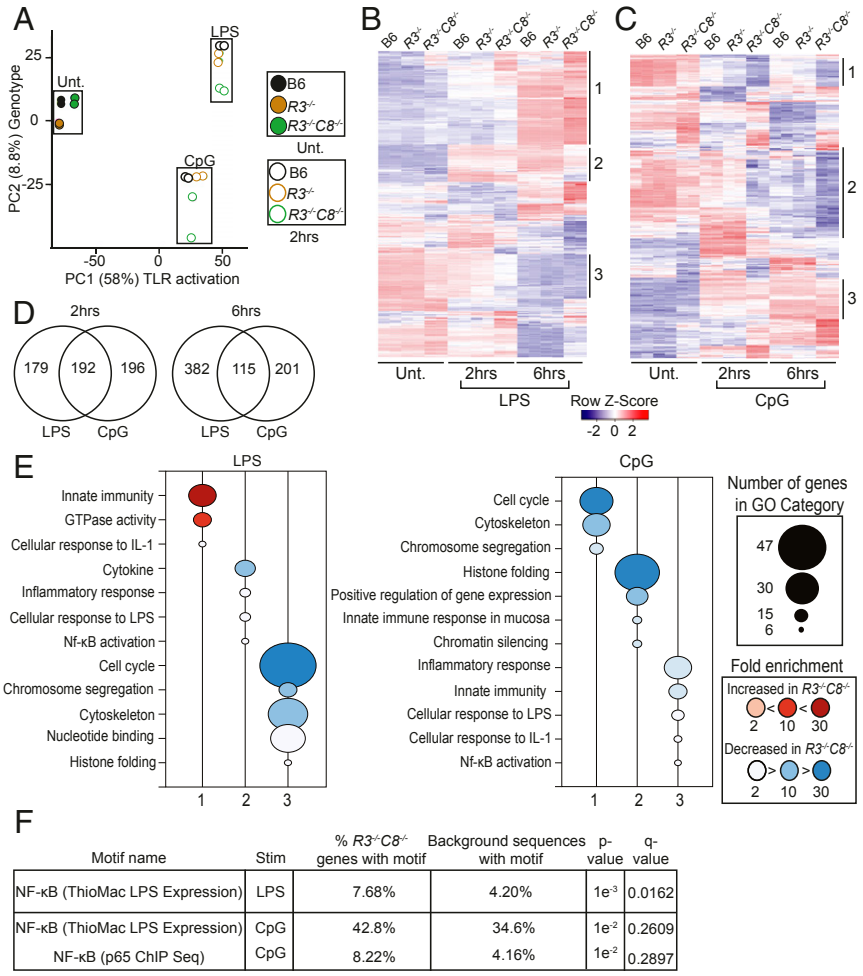
To gain further insight into caspase-8–dependent regulation of TLR-induced gene expression, we performed a global RNA-sequencing analysis of BMDMs stimulated with LPS or CpG for 2 h and 6 h (GEO accession no. GSE131171). Principal component analysis revealed that while the largest proportion (58%) of the variance in gene expression was driven by stimulation with either LPS or CpG, a substantial portion of the variance in gene expression (8.8%) was due to the presence or absence of caspase-8 (Fig. 2*A*). We considered genes caspase-8–dependent if they were differentially expressed in the absence of caspase-8 relative to both WT and *Ripk3*$^{-/-}$ BMDMs. We found that caspase-8 was required for WT expression levels of several hundred genes in response to both LPS (Fig. 2 *B* and *D*) and CpG (Fig. 2 *C* and *D*). Notably, 192 caspase-8–dependent genes at 2 h and 115 caspase-8–dependent genes at 6 h overlapped between LPS and CpG, representing approximately one-half of the respective LPS- or CpG-induced genes at 2 h and one-fourth to one-third of these genes at 6 h (Fig. 2*D*). This suggests a generally shared subset of genes regulated by caspase-8 during TLR engagement.

Hierarchical clustering of caspase-8–dependent genes and subsequent gene ontology enrichment analysis identified 3 clusters of genes significantly enriched for known biological processes or cellular components. Consistent with the role of caspase-8 in cytokine production (31) (Fig. 1), a subset of caspase-8–dependent genes induced in response to LPS or CpG stimulation were enriched for gene ontology terms broadly related to immune activation, including cytokine, inflammatory response, and cellular response to LPS (Fig. 2*E* and *SI Appendix*, Dataset S1).

While immune-related genes accounted for a significant fraction of caspase-8–regulated genes in both TLR4- and TLR9-stimulated datasets, we were also surprised to find a large number of genes enriched in gene ontology terms related to the cell cycle, chromosome segregation, and cytoskeleton (Fig. 2*E*). Intriguingly, recent studies revealed that the nonapoptotic activity of the caspase-8/RIPK1 complex regulates the function of Polo-like Kinase 1, and that loss of either caspase-8 or RIPK1 activity was associated with defects in chromosome segregation, mitotic spindle assembly, and genomic integrity in dividing cells (47). These data indicate that the nonapoptotic activity of caspase-8 has broader cellular functions beyond the control of inflammatory gene expression. Nevertheless, due to the enrichment of NF-κB–binding sites at the promoters of LPS- and CpG-induced caspase-8–dependent genes (Fig. 2*F*), together with the effect of caspase-8 on IKK activation (Fig. 1), we sought to define the contribution of caspase-8 to the regulation of NF-κB signaling.

Despite the requirement for caspase-8 in promoting IKK phosphorylation, the degradation of IκBα, a key regulator of NF-κB nuclear translocation, was unaffected in *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ cells, consistent with our previous observations (*SI Appendix*, Fig. S3*A*). However, both *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ and the *Mlkl*$^{-/-}$*Fadd*$^{-/-}$ BMDMs exhibited a small but consistently reproducible effect on degradation of another member of the IκB family, IκBε (*SI Appendix*, Fig. S3 *A*–*C*). In contrast, neither phosphorylation of IκBβ nor phosphorylation of p38 MAPK was affected by caspase-8 deficiency. These data indicate a selective effect of caspase-8 deficiency on IκBε.

Caspase-8 Promotes c-Rel Nuclear Translocation. Distinct IκB proteins preferentially regulate specific NF-κB family members (43). Notably, while IκBα preferentially binds to and inhibits nuclear translocation of p65, IκBε regulates c-Rel homodimers, as well as p50:c-Rel heterodimers (48–50). Because c-Rel is a critical regulator of macrophage *Il12b* expression (51, 52), and because *Il12b* was among the genes most strongly affected by the absence of caspase-8 in response to both LPS and CpG (31) (Fig. 1 and Dataset S1), we considered whether caspase-8–dependent regulation of gene expression could be due to an effect on c-Rel. Analysis of nuclear fractions from LPS- or CpG-simulated cells revealed a defect in c-Rel nuclear translocation following stimulation with either TLR ligand, whereas p65 nuclear translocation was unaffected, consistent with our previous findings (Fig. 3*A* and



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Motif name	Stim	% $R3^{-/-}C8^{-/-}$ genes with motif	Background sequences with motif	p-value	q-value
NF-κB (ThioMac LPS Expression)	LPS	7.68%	4.20%	$1e^{-3}$	0.0162
NF-κB (ThioMac LPS Expression)	CpG	42.8%	34.6%	$1e^{-2}$	0.2609
NF-κB (p65 ChIP Seq)	CpG	8.22%	4.16%	$1e^{-2}$	0.2897

Fig. 2. Caspase-8 regulates TLR-induced gene expression programs. (*A*) Principal component analysis of transcriptional profiling data from untreated and 2 h post–LPS- or CpG-treated B6, *Ripk3*$^{-/-}$, and *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ BMDMs. (*B* and *C*) Heat maps of global transcriptional response of B6, *Ripk3*$^{-/-}$, and *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ BMDMs after 2 or 6 h of stimulation with either LPS (*B*) or CpG (*C*). Gene clusters containing caspase-8–dependent genes following LPS or CpG stimulation are numbered and indicated in a sidebar to the right of each heat map. (*D*) Total number of caspase-8–dependent genes at 2 and 6 h after LPS or CpG stimulation. The Venn diagrams demonstrate the overlap of caspase-8–dependent genes in both the LPS- and CpG-treated BMDMs. (*E*) Bubble plot representation of gene ontology enrichment analyses on caspase-8–dependent gene clusters shown in *B* and *C*. Bubble color indicates the degree to which genes associated with each term were up-regulated (red) or down-regulated (blue). Bubble size represents the number of genes within a cluster associated with each term. (*F*) HOMER gene promoter analysis of caspase-8–dependent genes at 6 h after LPS or CpG stimulation.

SI Appendix, Fig. S4*A*). WT, *Ripk3*$^{-/-}$, and *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ BMDMs had similar basal and inducible levels of total c-Rel across all time points examined, indicating that the decrease in nuclear c-Rel levels was not due to differences in total c-Rel expression (Fig. 3*B*).

To further assess c-Rel nuclear translocation, and to quantify c-Rel translocation in individual cells, we analyzed nuclear translocation of c-Rel by confocal microscopy. Consistent with the defect in overall nuclear accumulation of c-Rel, we observed a significant defect in nuclear translocation of c-Rel in individual *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ BMDMs 2 h after stimulation with either LPS or CpG (Fig. 3 *C*–*E*), but no defect in translocation of p65 in response to LPS (*SI Appendix*, Fig. S4 *B* and *C*). Moreover, we observed a significant loss of c-Rel enrichment at the promoters of caspase-8–dependent genes in response to LPS in *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ BMDMs (Fig. 3*F*). The reduced accumulation of c-Rel at *Il12b*, *Il1b*, *Il6*, and *Tnf* promoters in *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ BMDMs was detectable as early as 30 min after treatment with LPS (Fig. 2*F*). In contrast to c-Rel, p65 recruitment to these promoters was unaffected in *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ BMDMs (*SI Appendix*, Fig. S4*D*), consistent with our previously published observations (31). These data demonstrate that caspase-8 selectively regulates nuclear translocation of c-Rel, but not p65, in response to LPS and CpG.

We next asked whether catalytic activity might contribute to caspase-8–dependent nuclear translocation of c-Rel following TLR stimulation. Intriguingly, *Ripk3*$^{-/-}$ BMDMs treated with z-VAD-fmk showed lower levels of nuclear c-Rel in response to both LPS and CpG at 2 h after stimulation (Fig. 4 *A* and *B*). Moreover, there remained a statistically significant difference between *Ripk3*$^{-/-}$ BMDMs treated with z-VAD and *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ BMDMs, suggesting that caspase-8 contributes to c-Rel nuclear translocation via both its enzymatic and nonenzymatic functions and consistent with our observation that caspase-8 controls cytokine gene expression via catalytic activity-dependent and -independent functions (Fig. 1).

c-Rel Promotes Expression of a Subset of Caspase-8–Dependent Genes. While most TLR-induced genes are regulated by p65, *Il12b* expression specifically requires c-Rel (52–54). Our observation that caspase-8 deficiency is associated with defects in c-Rel nuclear translocation and defective expression of a subset of inflammatory response genes led us to ask whether c-Rel might contribute to the expression of multiple caspase-8–dependent genes in response to TLR stimulation. Interestingly, *Rel*$^{-/-}$ BMDMs exhibited significant defects in transcriptional induction of *Il12b*, *Il6*, *Il1b*, *Il1a*, *Il4i1*, *Csf2*, and *Tnfsrf9* mRNA 2 h after stimulation with either LPS or CpG (Fig. 5*A*). The c-Rel–dependent defect in expression of *Il12b* and *Il1b* corresponded to a significant defect in both IL-12p40 and pro-IL-1β production in response to either LPS or CpG stimulation (Fig. 5 *B*–*D*). However, consistent with previous observations that c-Rel is not generally required for the expression of NF-κB–dependent genes (52, 55), there was no defect in TNF or IL-6 secretion by *Rel*$^{-/-}$ BMDMs (Fig. 5*D*), suggesting that compensatory mechanisms exist for the production of other cytokines in the absence of c-Rel. Overall, these data suggest that the defects in IL-12 and IL-1β expression observed in

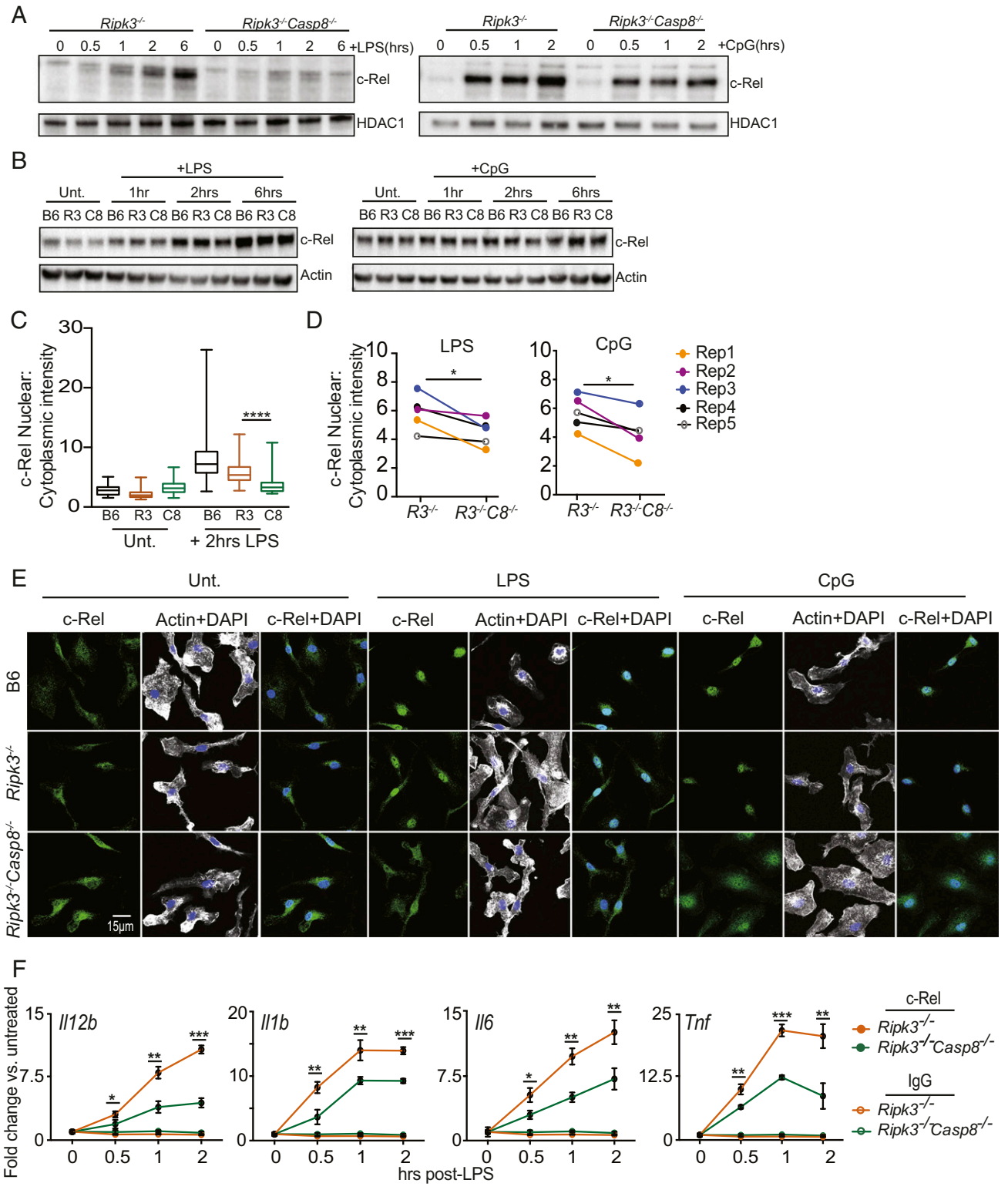

Fig. 3. Caspase-8 promotes c-Rel nuclear translocation. (*A*) Levels of c-Rel in nuclear fractions of *Ripk3*$^{-/-}$ and *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ BMDMs following LPS or CpG stimulation for the indicated times. (*B*) Total c-Rel expression in B6, *Ripk3*$^{-/-}$, and *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ BMDMs stimulated with LPS or CpG as indicated. (*C*) Quantification of the ratio of mean fluorescence intensity of nuclear:cytoplasmic c-Rel in *Ripk3*$^{-/-}$ and *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ BMDMs stimulated with LPS. The boxplots represent 60–90 individual cells per condition showing the median, upper and lower quartiles, and upper and lower extremes of the data. (*D*) Quantification of the ratio of mean fluorescence intensity of nuclear:cytoplasmic c-Rel in *Ripk3*$^{-/-}$ and *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ BMDMs stimulated with LPS or CpG. Each data point indicates the mean of an individual experiment. *$P < 0.05$, Student's 2-tailed paired *t* test. (*E*) Representative confocal images of experiment quantified in *C* and *D*. B6, *Ripk3*$^{-/-}$, and *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ BMDMs were stimulated with LPS or CpG for 2 h. c-Rel, actin, and DAPI are pseudocolored in green, white, and blue, respectively. (*F*) Fold-enrichment of c-Rel at indicated cytokine gene promoters in *Ripk3*$^{-/-}$ and *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ BMDMs following LPS treatment as determined by ChIP-qPCR at the indicated times poststimulation; n.s. not significant, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$, Student's 2-tailed unpaired *t* test. Error bars indicate ±SEM of triplicates. All data are representative of at least 3 independent experiments.

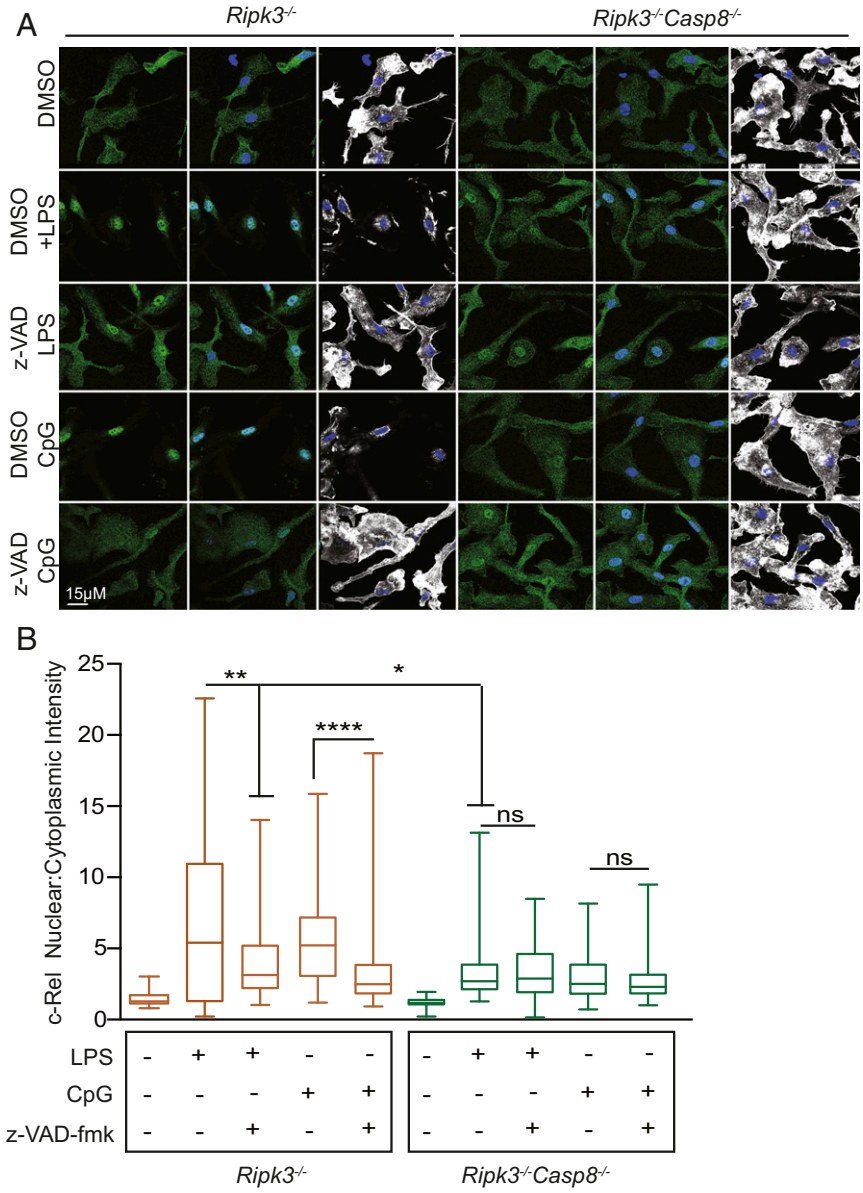

Fig. 4. Caspase activity contributes to c-Rel nuclear translocation in response to TLR signaling. (*A*) Representative confocal microscopy images of *Ripk3*$^{-/-}$ and *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ BMDMs pretreated with z-VAD-fmk or vehicle control for 1 h, followed by LPS or CpG as indicated for 2 h. c-Rel, actin, and DAPI are pseudocolored in green, white, and blue, respectively. (*B*) Quantification of the mean fluorescence intensity of nuclear:cytoplasmic c-Rel in *Ripk3*$^{-/-}$ and *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ BMDMs pretreated with z-VAD-fmk or vehicle control for 1 h, followed by LPS or CpG treatment for 2 h. Boxplots represent 60–90 individual cells per condition. The median, upper and lower quartiles, and upper and lower extremes of the data are shown. n.s., not significant; *$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$, Student's 2-tailed unpaired *t* test. All data are representative of at least 3 independent experiments.

caspase-8–deficient BMDMs are likely due to decreased levels of c-Rel translocation to the nucleus.

c-Rel Restores Expression of IL-12p40 to Caspase-8–Deficient BMDMs. Recent studies have identified cellular nucleic-acid binding protein (CNBP) as an important factor in regulating the nuclear translocation and nucleic acid-binding activity of c-Rel (56). However, caspase-8–deficient BMDMs exhibited similar or elevated levels of total and nuclear levels of CNBP compared with WT and *Ripk3*$^{-/-}$ BMDMs (*SI Appendix*, Fig. S5 *A* and *B*), indicating that caspase-8 regulates c-Rel nuclear translocation independent of CNBP.

To test the hypothesis that defective expression of IL-12 in *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ cells is due to the effect of caspase-8 on c-Rel, we transfected *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ BMDMs with a c-Rel expression plasmid containing an IRES-GFP element (57). Approximately 30% of the total cell population was successfully transfected, as determined by the expression of GFP (*SI Appendix*, Fig. S5*C*). GFP expression remained robust up to 6 h posttransfection but then declined at 12 h and 24 h, commensurate with a decline in total actin (*SI Appendix*, Fig. S5*D*). These data indicate reduced viability of the cells at later times posttransfection, leading us to limit our analysis of the cells to no later than 6 h posttransfection. Electroporation of the c-Rel expression vector significantly increased the expression of both IL-12 and pro-IL-1β in the *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ GFP^{+} cells compared with GFP^{-} cells from the same transfection, as well as with GFP^{+} cells containing empty vector control (Fig. 6). Interestingly, c-Rel expression alone was sufficient to induce IL-12 production even in the absence of TLR activation,

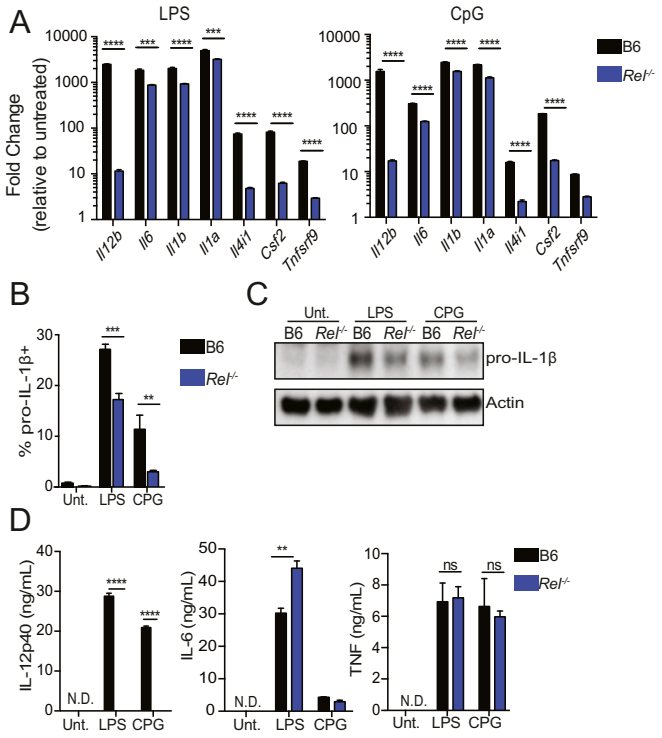

Fig. 5. c-Rel promotes expression of a subset of caspase-8–dependent genes. (*A*) Fold change in indicated inflammatory response genes in B6 and *Rel*$^{-/-}$ BMDMs at 2 h after stimulation with LPS or CpG, as indicated. (*B* and *C*) Flow cytometry quantification (*B*) and immunoblot analysis (*C*) of intracellular pro-IL-1β expression in B6 and *Rel*$^{-/-}$ BMDMs stimulated with LPS or CpG for 6 h. (*D*) ELISA measurement of secreted IL-12p40, IL-6, and TNF in B6 and *Rel*$^{-/-}$ BMDMs following stimulation with LPS or CpG for 6 h. n.s., not significant; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$, Student's 2-tailed unpaired *t* test. Error bars indicate ±SEM of triplicates. All data are representative of at least 3 independent experiments.

whereas pro-IL-1β was induced only in c-Rel transfected cells stimulated with LPS (Fig. 6).

While CpG stimulation increased the frequency of IL-1β–expressing cells, this increase was not statistically significant. This suggests that while c-Rel overexpression is sufficient to induce IL-12 in the absence of caspase-8, additional factors are required to enable optimal production of IL-1β in response to CpG. Notably, c-Rel transfection led to similar expression of IL-12 and IL-1β in both caspase-8–sufficient and caspase-8–deficient cells (*SI Appendix*, Fig. S6), indicating that ectopic c-Rel expression compensates for the lack of caspase-8 to enable IL-12 and IL-1β expression. Overall, these findings indicate that caspase-8 promotes the induction of IL-12 via its effect on c-Rel.

Caspase-8 Mediates Control of *T. Gondii* Infection via Innate Production of IL-12. *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ mice are highly susceptible to infection by the gram-negative bacterial pathogen *Yersinia* (13, 35), and human patients with homozygous inactivating *CASP8* mutations suffer from recurrent sinopulmonary and herpes simplex virus infections and poor responses to immunization (30). Given the broad role of caspase-8 in host defense, as well as previous observations that c-Rel and IL-12 promote control of the eukaryotic parasite *T. gondii* (58–62), we investigated whether caspase-8 might promote the control of this pathogen. Indeed, caspase-8 was required for survival of mice following i.p. infection with the Prugniard (Pru) strain of *T. gondii*, as 100% of *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ mice succumbed to *T. gondii* infection with kinetics similar to those of both *Il12b*$^{-/-}$ and *Rel*$^{-/-}$ mice, while WT and *Ripk3*$^{-/-}$ mice largely survived (Fig. 7*A* and *SI Appendix*, Fig. S7*A*).

Importantly, *Ripk3*$^{-/-}$*Casp*$^{DA/DA}$ mice exhibited intermediate survival between *Ripk3*$^{-/-}$ and *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ mice, indicating that autoprocessing of caspase-8 contributes to host defense against *T. gondii*, but that remaining scaffolding or catalytic activities are sufficient to partially restore antiparasitic immunity in this context (*SI Appendix*, Fig. S7*B*). Critically, a significantly higher frequency of peritoneal exudate cells (PECs) from *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ mice contained *T. gondii* parasites and harbored more parasites per cell than WT or *Ripk3*$^{-/-}$ mice, indicating a key role for caspase-8 in the control of *T. gondii* replication (Fig. 7 *B*–*D*). The function of caspase-8 in the control of *T. gondii* was mediated primarily by the hematopoietic compartment, as mice reconstituted with *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ bone marrow failed to control *T. gondii* infection, similar to *IL12b*$^{-/-}$ bone marrow chimeras (*SI Appendix*, Fig. S7 *C* and *D*). Furthermore, *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ mice showed significantly lower frequencies of IL-12–expressing Lin^{-}CD64^{-}CD11c^{+}MHCII^{+} dendritic cells (DCs) relative to control mice 5 d postinfection (dpi) (Fig. 7*E* and *SI Appendix*, Fig. S7 *E* and *F*).

The foregoing data suggest that caspase-8–deficient mice fail to control *T. gondii* replication due to an inability to robustly upregulate IL-12 expression. However, caspase-8 could also mediate other aspects of the immune defense against *T. gondii* independent of IL-12. To address this possibility, we asked whether exogenous IL-12 could restore survival in caspase-8–deficient mice, even in the absence of any other caspase-8–dependent factors. Critically, recombinant IL-12p70 fully rescued survival of caspase-8–deficient animals infected with *T. gondii* (Fig. 7*F*) and reduced parasite burdens in *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ and *Il12b*$^{-/-}$ mice to levels observed in control animals (Fig. 7 *G* and *H*). Furthermore, both *Il12b*$^{-/-}$ and *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ mice showed reduced frequency and numbers of Ly6C^{hi} monocytes recruited to the peritoneal

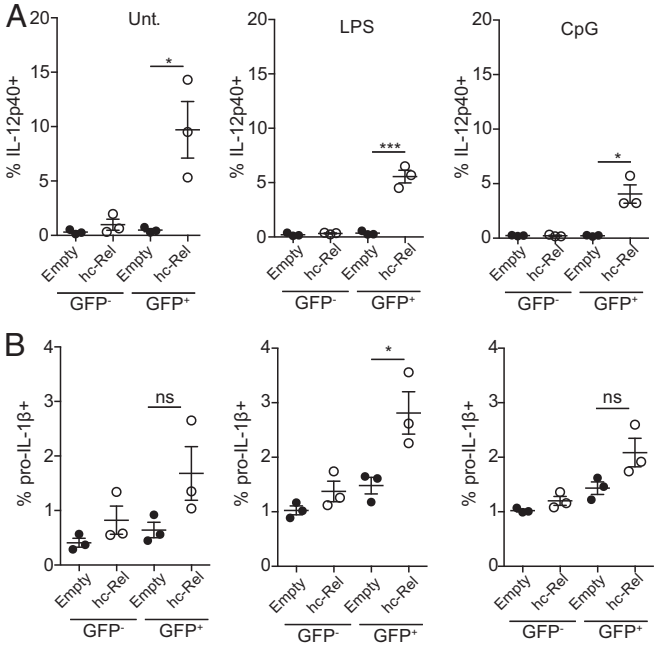

Fig. 6. c-Rel restores expression of IL-12p40 to caspase-8–deficient BMDMs. (*A* and *B*) Flow cytometry of intracellular IL-12p40 (*A*) and pro-IL-1β (*B*) in *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ BMDMs transfected with an IRES-GFP–expressing empty vector or hc-Rel-IRES-GFP–expressing plasmid and stimulated with LPS or CpG for 4 h. n.s., not significant; $^{*}P < 0.05$; $^{***}P < 0.001$, Student's 2-tailed unpaired *t* test. Error bars indicate ±SEM of technical replicates. All data are representative of at least 3 independent experiments.

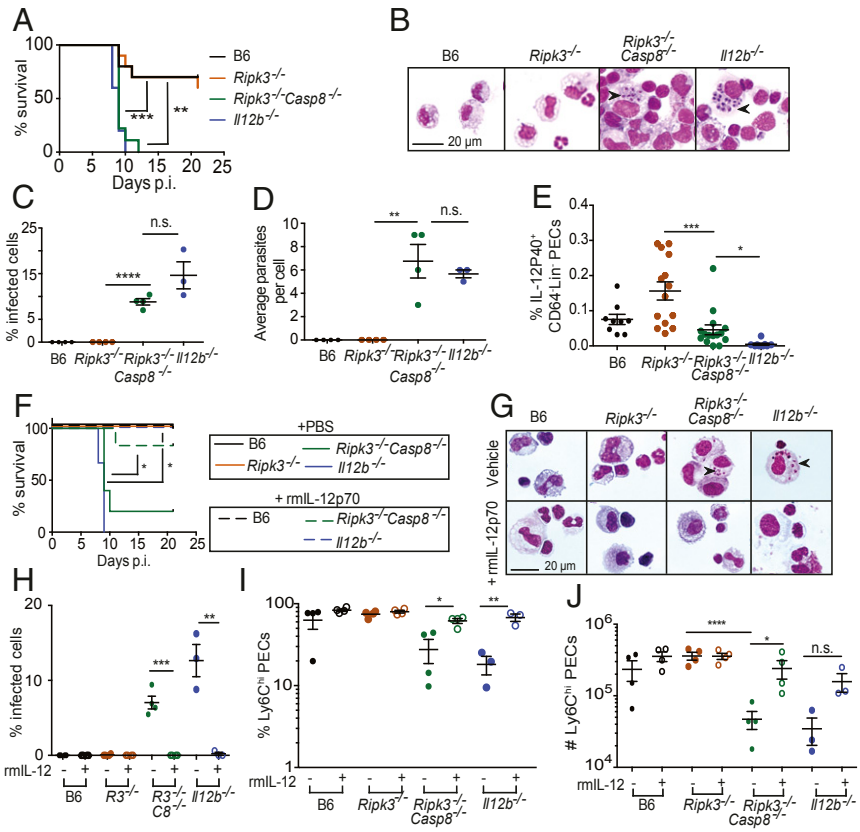

Fig. 7. Caspase-8 mediates control of *T. gondii* infection via innate production of IL-12. (*A*) Survival of B6, *Il12b*$^{-/-}$, *Ripk3*$^{-/-}$, and *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ mice (n = 5–10) infected i.p. with *T. gondii*. (*B*) Representative cytospin of infected cells in the PECs of *T. gondii*-infected B6, *Il12b*$^{-/-}$, *Ripk3*$^{-/-}$, and *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ mice at 5 dpi. (*C* and *D*) Quantification of infected cell frequency (*C*) and parasites per cell (*D*) in the PECs of *T. gondii*-infected B6, *Il12*$^{-/-/-}$, *Ripk3*$^{-/-}$, and *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ mice at 5 dpi. Each datapoint represents a single mouse. (*E*) Frequency of IL-12p40^{+} CD64^{-}Lin^{-} cells (Live^{+}Singlets^{+}CD19^{-}CD3^{-}B220^{-}NK1.1^{-}CD64^{-}MHCII^{+}CDllc^{+}) in the PECs of *T. gondii*-infected B6, *Il12b*$^{-/-}$, *Ripk3*$^{-/-}$, and *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ mice. (*F*) Survival curve of mice infected with *T. gondii* and injected i.p. with PBS or rmIL-12p70 (200 ng/mouse) for 7 dpi (n = 3–6). (*G*) Representative cytospins from PECs isolated at 5 dpi from *T. gondii*-infected B6, *Il12b*$^{-/-}$, *Ripk3*$^{-/-}$, and *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ mice injected with PBS or rmIL-12p70. (*H*) Quantification of infected cell frequencies in the PECs of PBS or rmIL-12p70–injected *T. gondii*-infected B6, *Il12b*$^{-/-}$, *Ripk3*$^{-/-}$, and *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ mice at 5 dpi. Each data point represents a mouse. (*I*) Frequency and (*J*) number of monocytes (Live^{+}Singlets^{+}CD19^{-}CD3^{-}B220^{-}NK1.1^{-} CD64^{+}MHCII^{+}LY6C^{hi} cells) in the PECs of PBS or rmIL-12p70-injected *T. gondii*-infected B6, *Il12b*$^{-/-}$, *Ripk3*$^{-/-}$, and *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ mice at 5 dpi. Each data point represents one mouse. For all infections, 10,000 Pru *T. gondii* parasites were injected i.p. In *A* and *F*, *P < 0.05, **P < 0.01; ***P < 0.001 Mantel–Cox test. In *C*–*E* and *H*–*J*, n.s., not significant; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001, Student's 2-tailed unpaired t test. Error bars indicate ±SEM. Data are representative of 3 (*A*–*F*) or 2 (*G*–*J*) independent experiments.

cavity following *T. gondii* infection, and this defect was rescued by exogenous IL-12 (Fig. 7 *I* and *J* and *SI Appendix*, Fig. S7*E*). Overall, these findings demonstrate that caspase-8 enables control of *T. gondii* infection via DC production of IL-12.

Discussion

Caspase-8 plays an important role in cell-extrinsic apoptosis, necroptosis, and inflammatory gene expression (31, 35, 43, 44, 63, 64). Both caspase-8 activity and scaffolding functions have been implicated in caspase-8–mediated control of gene expression, raising fundamental questions about the mechanism by which caspase-8 performs this role (31, 41, 44, 65). Caspase-8 promotes phosphorylation of IKK (35), but whether this is mediated by caspase-8 activity is currently unknown. Intriguingly, blocking caspase activity in *Ripk3*$^{-/-}$ BMDMs decreased levels of phospho-IKK to a similar extent as deletion of caspase-8 or FADD in response to either LPS or CpG stimulation, indicating that caspase-8 enzymatic activity, most likely in an FADD-containing complex, regulates IKK phosphorylation downstream of multiple TLRs. Consistent with previous observations, caspase-8 regulated a specific subset of genes downstream of TLR stimulation. This suggests that if caspase-8 is present in a TLR-proximal signaling complex, it is not absolutely required for the assembly of this complex, but functions as a modifier or amplifier of its activity.

Our data suggest that caspase-8 regulates inflammatory cytokine gene expression via multiple pathways. Specifically, we can classify inflammatory response genes in accordance with their requirement for caspase-8, c-Rel, and caspase-8 enzymatic activity. In particular, we observed genes that are caspase-8–dependent and c-Rel–dependent, such as *Il12b*, *Il1b*, and *Il1a*, as well as genes that are caspase-8–dependent but c-Rel–independent, such as *Tnf* and *Il6*. Furthermore, within the caspase-8–dependent subset are genes that are caspase activity-dependent as well as activity-independent. Finally, the requirement for caspase-8 activity appears to be TLR-specific, as *Il12b* and *Il4i1* transcription were caspase activity-dependent downstream of TLR4 stimulation but activity-independent downstream of TLR9 stimulation. Thus, while c-Rel is critical for the control of some caspase-8–dependent genes, additional NF-κB–independent mechanisms and transcription factors likely contribute to caspase-8–dependent control of inflammatory gene expression.

Inducible control of macrophage inflammatory response genes involves the concerted activities of multiple transcription factors and coregulators, including multiple NF-κB family members, IRF and AP-1 transcription factors, and other DNA-binding

proteins, such as CNBP (56, 66–68). Individual genes likely have different levels of sensitivity to the loss of particular transcription factors. Indeed, *Il12b* has a requirement for c-Rel, whereas macrophage expression of cytokines such as TNF or IL-6 requires p65 but not c-Rel (52). Thus, the combined action of multiple transcription factors in the regulation of TLR-induced gene expression programs provides mechanisms of redundancy for the control of important subsets of genes, as well as multiple regulatory nodes that can be used to fine-tune the expression of specific gene subsets.

We observed no defects in the nuclear translocation of p65 or in its recruitment to the promoters of caspase-8–dependent genes, indicating that the role of caspase-8 in regulating nuclear translocation of NF-κB family members is limited to c-Rel. c-Rel and p65 may be differentially sensitive to levels of IKK phosphorylation; therefore, hypophosphorylation of IKK, as occurs in *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ cells, may be sufficient to fully activate p65 but not c-Rel.

Consistently while we observed no effect of caspase-8 deficiency on IκBα degradation, caspase-8–deficient cells showed reduced degradation of IκBε in response to TLR stimulation, suggesting that caspase-8 promotes degradation of a negative regulator of c-Rel. Indeed, our findings demonstrate a role for caspase-8 in c-Rel nuclear translocation and recruitment to the promoters of caspase-8–dependent genes. Interestingly, we found that c-Rel controls the expression of additional genes other than *Il12b*, most notably *Il1b*. c-Rel is both necessary and sufficient to promote the production of IL-12 and pro-IL-1β, implicating c-Rel as an important regulator of caspase-8–dependent inflammasome priming responses. These findings provide insight into the molecular mechanism of caspase-8–mediated control of gene expression.

How caspase-8 regulates IKK activation is currently unclear. Caspase-8 interacts with TRIF and RIPK1 in Ripoptosome complexes downstream of TLR4 and TLR3 (19, 69–71). Dynamic control of Ripoptosome function or components may provide a mechanism by which caspase-8 is recruited to signaling complexes that mediate inflammatory gene expression or cell death. Alternatively, in keeping with our observation that FADD also is necessary for caspase-8–dependent control of gene expression and IKK phosphorylation, FADD may be recruited to MyDDosome complexes via death domain interactions, thereby enabling caspase-8 to interact with the MyDDosome as well.

Finally, our work reveals a previously unknown requirement for caspase-8 in host defense against the intracellular protozoan parasite *T. gondii*. Control of *T. gondii* requires early IL-12 production by DCs to rapidly induce T cell and NK IFN-γ, which is critical for anti-*Toxoplasma* immunity (59, 72). Thus, *Il12b*$^{-/-}$ mice die rapidly with overwhelming systemic *T. gondii* burdens due to the failure to control intracellular parasite replication (62). We find that caspase-8 plays a key role in induction of IL-12 in vivo in response to *T. gondii* infection, which is likely responsible for the failure of caspase-8–deficient animals to control *T. gondii*. Intriguingly, this was correlated with a defect in the recruitment of Ly6C^{hi} monocytes, which play key roles in immunity against *T. gondii* infection (73, 74). Indeed, *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ mice succumb to *T. gondii* infection with kinetics statistically indistinguishable from those of *Il12b*$^{-/-}$ mice, and their survival, ability to control parasite burden, and monocyte recruitment can be fully rescued by exogenous IL-12.

Thus, while caspase-8 deficiency has pleiotropic effects on innate immune responses and regulation of cell death, our observations identify a previously unappreciated role for caspase-8 in resistance to infection by *T. gondii* via the control of IL-12 expression. These studies also implicate the nonapoptotic function of caspase-8 in controlling the nuclear importation of NF-κB family member c-Rel, providing insight into how regulation of specific transcription factors fine-tunes inflammatory gene expression to enable robust antimicrobial immune defense.

Materials and Methods

Mice. C57BL/6 mice were obtained from The Jackson Laboratory. The *Ripk3*$^{-/-}$ *Casp8*$^{-/-}$ mice and *Mlkl*$^{-/-}$*Fadd*$^{-/-}$ mice used in these studies have been described previously (43, 75) and were provided by Doug Green (St. Jude Children's Research Hospital). *Ripk3*$^{-/-}$ mice were provided by Vishva Dixit (Genentech). *Il12b*$^{-/-}$ mice have been described previously (76). *Rel*$^{-/-}$ mice were generated by Hsiou-Chi Liou as described previously (77). Animal experiments were performed using age- and sex-matched 8- to 12-wk-old mice.

Animal Infections. For *T. gondii* infections, mice were injected i.p. with 10^4 tachyzoites of the Pru strain as described previously (78). At 5 dpi, the mice were killed and tissues harvested. Parasite burdens were determined by cytospin analysis of PECs. rm-IL-12p70 (200 ng/mouse; Peprotech) or PBS was injected i.p. each day for the first 5 d after infection for parasite burden measurements and for the first 7 d for survival analysis. All animal experiments in this study were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania.

Cell Culture. BMDMs were grown as described previously (79) in a 37 °C, 5% CO_2 humidified incubator in DMEM supplemented with 10% FBS, Hepes, sodium pyruvate (complete DMEM) and 30% L929 supernatant for 7–9 d. Cells were replated onto 96-, 48-, 24-, 12-, or 6-well dishes in complete DMEM containing 10% L929 supernatant 16–20 h before treatment. After stimulation, cells were incubated at 37 °C. zVAD-fmk (100 μM; SM Biochemicals) was added at 1 h before treatment with LPS (*Escherichia coli* O55:B5 LPS, 100 ng/mL; Sigma-Aldrich) or CpG (1 μM; IDT).

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