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Monocytes differentiate into macrophages, which deactivate invading pathogens. Macrophages can be resistant to cell death mechanisms in some situations, and the mechanisms involved are not clear. Here, using mouse immune cells, we investigated whether the differentiation of macrophages affects their susceptibility to cell death by the ripoptosome/necrosome pathways. We show that treatment of macrophages with a mimetic of second mitochondrial activator of caspases (SMAC) resulted in ripoptosome-driven cell death that specifically depended on tumor necrosis factor α (TNFα) expression and the receptor-interacting serine/threonine protein kinase 1 (RipK1)–RipK3–caspase-8 interaction in activated and cycling macrophages. Differentiation of macrophages increased the expression of pro-inflammatory cytokines but reduced RipK1-dependent cell death and the RipK3– caspase-8 interaction. The expression of the anti-apoptotic mediators, X-linked inhibitor of apoptosis protein (XIAP) and caspase-like apoptosis regulatory protein ($cFLIP_L$), also increased in differentiated macrophages, which inhibited caspase activation. The resistance to cell death was abrogated in XIAP-deficient macrophages. However, even in the presence of increased XIAP expression, inhibition of the mitogen-activated protein kinase (MAPK) p38 and MAPK-activated protein kinase 2 (MK2) made differentiated macrophages susceptible to cell death. These results suggest that the p38/MK2 pathway overrides apoptosis inhibition by XIAP and that acquisition of resistance to cell death by increased expression of XIAP and $cFLIP_L$ may allow inflammatory macrophages to participate in pathogen control for a longer duration.

Cell death plays an integral role in maintaining tissue homeostasis during normal development as well as during infection. Whereas apoptosis is vital for embryonic development and elimination of self-reactive immune cells (1), cell death induced by inflammasome and necrosome signaling is highly inflammatory (2, 3). There are various cell death platforms, each with different mechanisms and consequences for cell signaling (4–6). Proteins of the receptor-interacting protein kinase family, such as RipK1 and RipK3, play key roles in the assembly of the ripoptosome and necrosome signaling complexes (7, 8). The tumor necrosis factor (TNF)[2] receptor (TNF-R) engagement induces the formation of a cell survival complex that promotes activation of NF-κB and MAPKs (9–11). Cellular inhibitors of apoptosis proteins (cIAP1/2) maintain RipK1 in this complex, but the degradation of cIAPs by the second mitochondrial activator of caspases (SMAC) or other unknown mechanisms releases RipK1 from this complex and results in the interaction of RipK1 with FADD, RipK3, and caspase-8 to form the ripoptosome complex (12, 13). When caspase-8 activity is inhibited, RipK1, RipK3, caspase-8, and the pseudokinase MLKL instead form the necrosome (14–16). During necrosome signaling, RipK3 phosphorylates MLKL, which results in the formation of MLKL trimers that relocate to the cell membrane and cause cell rupture and release of intracellular DAMPs (17, 18).

Activation of cell death is particularly important in the immune system, which facilitates the control of pathogens (2, 19). Myeloid cells (neutrophils, monocytes, macrophages, and dendritic cells) provide the first line of defense against pathogens, and these cell types are highly vulnerable to cell death.

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This article contains Figs. S1–S4.

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[2] The abbreviations used are: TNF, tumor necrosis factor; TNF-R, TNF receptor; MAPK, mitogen-activated protein kinase; cIAP, cellular inhibitor of apoptosis protein; SMAC, second mitochondrial activator of caspases; $cFLIP_L$, caspase-like apoptosis regulatory protein; XIAP, X-linked inhibitor of apoptosis protein; M-CSF, macrophage colony–stimulating factor; LPS, lipopolysaccharide; BP, birinapant; Z, benzyloxycarbonyl; fmk, fluoromethyl ketone; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular signal–regulated kinase; JNK, c-Jun N-terminal kinase; PI, propidium iodide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Monocytes represent ~10% of the circulating leukocytes in human blood (20), which maintain homeostasis by replenishing the pool of tissue macrophages in steady state. Macrophages promote inflammatory responses (21) and also play an important role in resolving inflammation by remodeling and repairing tissues through efferocytosis (22). Different types of macrophages exist in different anatomical compartments (microglia, osteoclasts, Kupffer cells, alveolar macrophages, etc.) (22). Whereas most tissue macrophages are derived from blood circulating monocytes, embryonic precursors can also be the source of tissue macrophages, which undergo self-replication and are not dependent on replenishment by circulating monocytes (23, 24). Unlike monocytes, macrophages have a long life span, ranging from months to years (25).

Monocytes circulate in the blood for a few days before differentiating into macrophages or dendritic cells in the tissues in response to chemokines or cytokines (26). Upon differentiation, macrophages acquire resistance to cell death, but the mechanism has been unclear (27). We have evaluated the impact of macrophage differentiation on their susceptibility to cell death by the ripoptosome and necrosome pathway. Our results indicate that macrophage differentiation results in increased expression of endogenous inhibitors of cell death, $cFLIP_L$ and XIAP, which promotes resistance to cell death by the ripoptosome pathway.

Results

Differentiated macrophages are resistant to SMAC mimetic–induced cell death

In the course of our earlier work investigating the toxicity of SMAC mimetic compounds, we made an unexpected observation that macrophages showed different sensitivity, depending on their differentiation status. To further tease out the relationship of macrophage differentiation with cell death signaling, we purified monocytes from bone marrow ($CD11b^+Ly6C^+F4/80^-$) and differentiated them into macrophages for varying periods ($CD11b^+Ly\text{-}6C^-F4/80^+$) with M-CSF (Fig. 1*A*). The expression of CD11b increased upon differentiation with M-CSF (Fig. 1*B*), and there was a slight increase in the number of cells obtained with prolonged differentiation of cells (Fig. 1*C*). Cells that were harvested at day 12 of differentiation produced increased levels of pro-inflammatory cytokines following LPS stimulation, compared with cells harvested at day 5 of differentiation (Fig. 1*D*). Macrophages isolated at day 5 of differentiation showed slightly more proliferative potential compared with day 12 macrophages, as revealed by the expression of Ki67 (Fig. 1, *E* and *F*).

We evaluated the consequences of macrophage differentiation on their susceptibility to cell death by the SMAC mimetic, birinapant (BP). SMAC mimetics can induce cell death by the various RipK1-dependent pathways (12, 28). Macrophages harvested at later time intervals of differentiation progressively acquired resistance to BP-induced cell death (Fig. 1, *G–I*). Cell death was also inhibited by necrostatin-1 (*Nec-1*) (Fig. 1*J*), which inhibits the kinase activity of RipK1 (29). Inhibition of the kinase activity of RipK3, which also participates in ripoptosome signaling, by GSK872 did not inhibit cell death induced by BP (Fig. 1*J*). Similar results were obtained when cell death was induced by a different SMAC mimetic, BV6 (Fig. S1). These results suggest that macrophages isolated at day 12 of differentiation are resistant to ripoptosome-dependent cell death.

BP-induced cell death of early macrophages occurs through ripoptosome signaling

We have recently reported a knock-in mouse model where a conserved lysine in the kinase subdomain II of RipK1 was mutated to alanine (K45A), resulting in a kinase-inactive mutant of RipK1. The K45A mutation of RipK1 results in impairment in control of *S. enterica* Typhimurium infection *in vivo*, which correlated with decreased macrophage cell death, decreased reactive oxygen species production, and decreased activation of caspase-8 (30). We therefore evaluated the impact of the K45A mutation of RipK1 on BP-induced cell death. Our results indicate that Lys to Ala mutation at amino acid 45 of RipK1 abolished BP-induced cell death (Fig. 2*A*). Cell death was also dependent on RipK3 (Fig. 2*B*), and there was a modest impact of the adaptor protein *Trif*, but not *Myd88* or *Ifnar1* (Fig. 2, *C–E*). The Lys-45 activity of RipK1 was required for activation of caspase-8/9/3/7 following treatment with BP (Fig. 2, *F–H*). BP-induced cell death was highly dependent on *Tnfr1* and *Tnfα*, but not on *Tnfr2* signaling (Fig. 2, *I* and *J*). In the absence of *Tnfα*, phosphorylation of RipK1 (upper band) and MK2 was reduced (Fig. 2, *K* and *L*).

Impaired ripoptosome signaling in differentiated macrophages

Because expression of TNFα was required for BP-induced cell death (Fig. 2*J*), we measured the mRNA (Fig. 3*A*) and protein (Fig. 3*B*) levels of TNFα in macrophages following treatment with BP. Our results revealed similar levels of TNFα in macrophages isolated at day 5 or day 12 of differentiation. These results indicate that the impairment in day 12 macrophages to cell death induced by BP treatment is not related to TNFα expression. Macrophages isolated at day 12 of differentiation displayed poor phosphorylation of RipK1 following BP treatment in contrast to day 5 macrophages (Fig. 3, *C* and *D*). BP treatment did not induce any phosphorylation of RipK3 in day 5 or day 12 macrophages. Because ripoptosome-induced cell death is mediated by the activation of caspase-8, we evaluated the expression of caspase-8 in macrophages following treatment with BP. There was no difference in the basal level of caspase-8 in day 5 *versus* day 12 macrophages; however, active caspase-8 was detected only in BP-treated day 5 macrophages (Fig. 3*E*). Similar results were obtained when the activity of caspase-8 was measured by a colorimetric bioassay (Fig. 3*F*). Immunoprecipitation of caspase-8 revealed a RipK3–caspase-8 interaction in day 5 macrophages treated with BP, but not in day 12 macrophages (Fig. 3G). Taken together, these results indicate that continued differentiation of macrophages to day 12 results in impairment in ripoptosome signaling–induced cell death, without any impact on TNFα expression.

We evaluated whether concurrent engagement of TLR4 in day 12 macrophages would overcome their resistance to BP-induced cell death. Our results indicate that LPS + BP induces cell death in macrophages that operates through an atypical

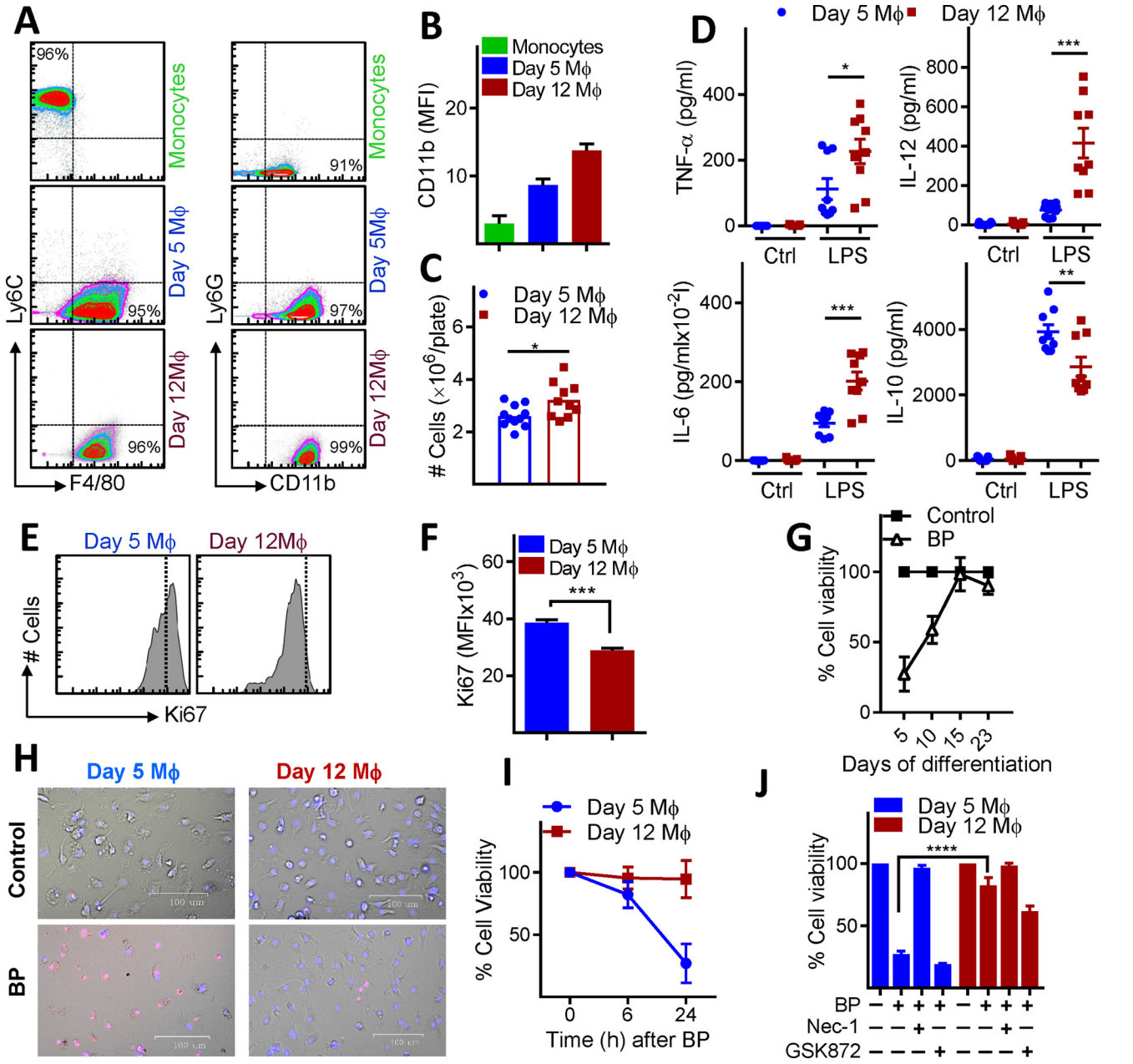

Figure 1. Differentiation of macrophages results in resistance to ripoptosome-induced cell death. Monocytes were isolated from the bone marrow of C57BL/6J (WT) mice and differentiated into macrophages with M-CSF, and the expression of Ly-6C, F4/80, and CD11b was evaluated by flow cytometry (*A* and *B*). Cell yield after 5 or 12 days of culture with M-CSF was enumerated (*C*). Day 5 or day 12 macrophages were stimulated with LPS (100 ng/ml), and the expression of various cytokines in the supernatants collected at 24 h was quantitated by ELISA (*D*). Intracellular staining for Ki67 was enumerated in cells collected at day 5 and day 12 post-treatment with M-CSF (*E* and *F*). Differentiating macrophages were harvested at various time intervals post-treatment with M-CSF and incubated with BP (10 μM) in 96-well plates, and cell viability was evaluated 24 h later by MTT uptake (*G*) or at 12 h by PI/Hoechst staining (*H*). Cell death was also evaluated at various time intervals post-BP treatment by MTT assay (*I*). Inhibitors against RipK1 (Nec-1, 10 μM) or RipK3 (GSK872, 10 μM) were added to determine the mechanism of BP-induced cell death (*J*). Each experiment was repeated three times with triplicate samples. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. *Error bars*, S.E.

ripoptosome pathway that is less dependent on the kinase activity of RipK1, but more shifted toward RipK3 dependence, and day 12 macrophages were still resistant to LPS + BP–induced cell death (Fig. S2, *A–I*).

Differentiated macrophages develop partial resistance to necrosome signaling

Considering the promiscuous interaction of RipK1 and RipK3 in the ripoptosome and necrosome signaling complexes, we sought to determine whether differentiation of macrophages influenced their susceptibility to cell death by the necrosome pathway. Inhibition of caspases with the pan-caspase inhibitor Z-VAD did not rescue BP-induced cell death; rather, the mechanism of cell death shifted to Ifnar1 and RipK3-dependent necroptosis, which was rescued by inhibitors against RipK1 and RipK3 (Fig. 4, *A* and *B*). Cell death was significantly dependent on Tnfr1 but not Tnfr2 signaling (Fig. 4*B*). Macrophages isolated at day 12 of differentiation displayed moderate but significant resistance to cell death induced by BP + Z-VAD (Fig. 4*C*), although the potency of this resistance was not as

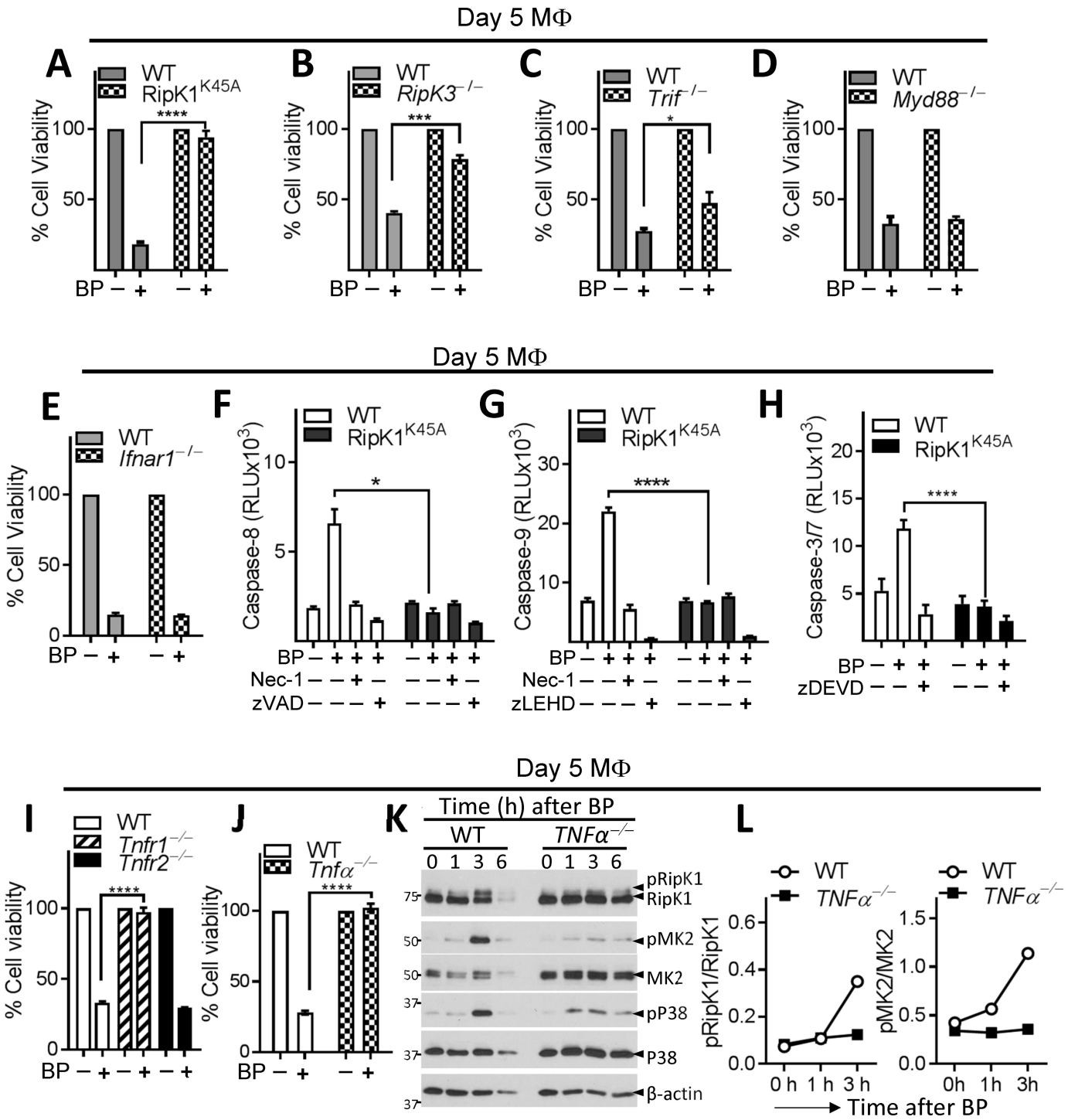

Figure 2. Early macrophages undergo cell death through a typical ripoptosome pathway. Bone marrow–derived macrophages were harvested at day 5 post-differentiation with M-CSF and treated with BP (10 μM) (*A–L*). Cell death was evaluated at 24 h post-treatment with BP by MTT uptake (*A–E*, *I*, and *J*). Macrophages were harvested at 4 h post-treatment with BP, and the activity of caspase-8/9/3/7 was evaluated by cleavage of specific substrates and corresponding chemiluminescence detection (*F–H*). Cell extracts were isolated at various time intervals after BP treatment of WT and TNF$\alpha^{-/-}$ macrophages, and the activation of RipK1, MK2, and p38 MAPK was evaluated by Western blotting (*K*). Densitometric analysis of Western blots was performed (*L*). Each experiment was repeated three times with triplicate samples. *, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$. *Error bars*, S.E.

strong as was observed with BP alone. Day 12 macrophages displayed significant resistance to necroptosis induced by TNFα + Z-VAD (Fig. 4*D*). Necroptosis was also induced in macrophages by treatment with LPS + Z-VAD, which depended on Ifnar1 and RipK3 (Fig. 4, *E* and *F*). Here again, day 12 macrophages displayed a small but significant resistance to cell death induced by LPS + Z-VAD (Fig. 4*G*). Differentiation of macrophages resulted in a progressive increase in resistance to necroptosis, although the potency of this resistance was not as great as that observed in response to ripoptosome signaling (Fig. 4*H*). Activation of necroptosis by LPS + Z-VAD resulted in phosphorylation of RipK1 and RipK3 in both day 5 and day 12

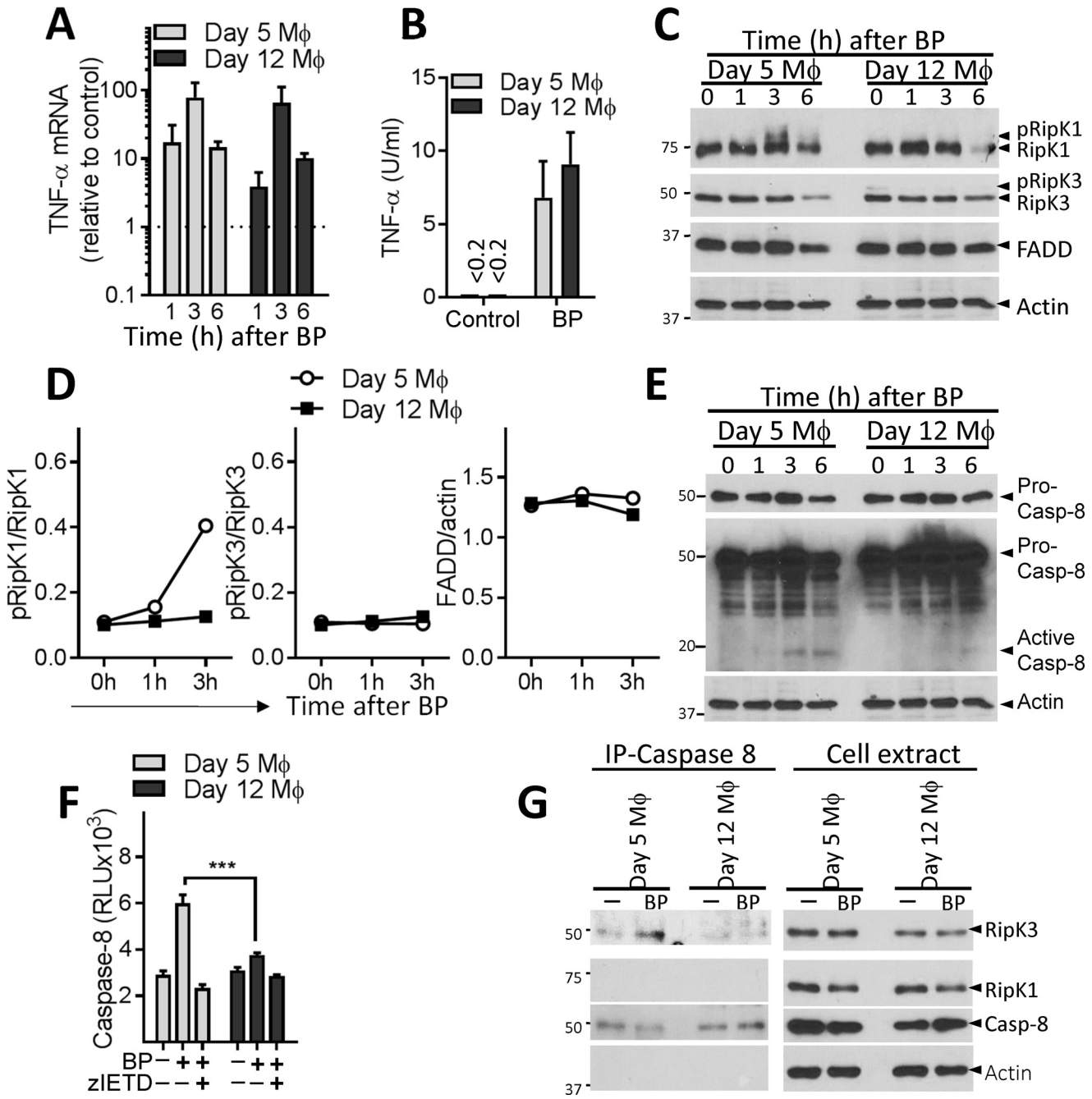

Figure 3. Macrophage differentiation results in impairment in RipK1 phosphorylation and caspase-8 activation. Macrophages from WT mice were harvested at day 5 or day 12 post-differentiation with M-CSF and treated with BP (10 μM) for various time intervals, and the expression of TNFα was evaluated at various time intervals by quantitative RT-PCR (*A*) or by the sensitive bioassay (*B*). Cell extracts were isolated, and Western blotting (*C*) and densitometric analysis (*D*) were performed. Activation of caspase-8 was determined by Western blotting of cell extracts at 4 h after BP treatment (*E*). Activity of caspase-8 was evaluated by cleavage of substrate and chemiluminescence detection (*F*). Cell extracts were collected from macrophages (control or BP-treated for 4 h), caspase-8 was immunoprecipitated, and the interacting proteins were revealed by Western blotting of immunoprecipitates (*G*). Western blotting was also performed in nonimmunoprecipitated cell extracts. Each experiment was repeated three times with triplicate samples. ***, $p < 0.001$. Western blotting of actin is reused in *C* and *D* and in Fig. 5*D*. *RLU*, relative light units. *Error bars*, S.E.

macrophages (Fig. 4, *I* and *J*). As expected, treatment with BP resulted in degradation of cIAP1/2 (Fig. 4*I*).

Increased expression of XIAP causes resistance of differentiated macrophages to cell death

Having observed increased cytokine expression by day 12 macrophages in response to LPS treatment (Fig. 1*D*), we considered the possibility that inhibition of NF-κB signaling might abrogate the resistance of day 12 macrophages to cell death by the ripoptosome pathway. Treating day 5 macrophages with low levels of IKK inhibitor did not significantly impact BP-induced cell death (Fig. 5*A*), whereas the resistance of day 12 macrophages to cell death was abrogated (Fig. 5*B*). Other inhibitors of NF-κB showed similar results (Fig. S3, *A* and *B*). Day 12 macrophages isolated from NIK-deficient mice were still resistant to BP-induced cell death (Fig. 5*C*), demonstrating no involvement of the noncanonical NF-κB pathway. Treatment with BP resulted in rapid degradation of cIAP1/2 in macro-

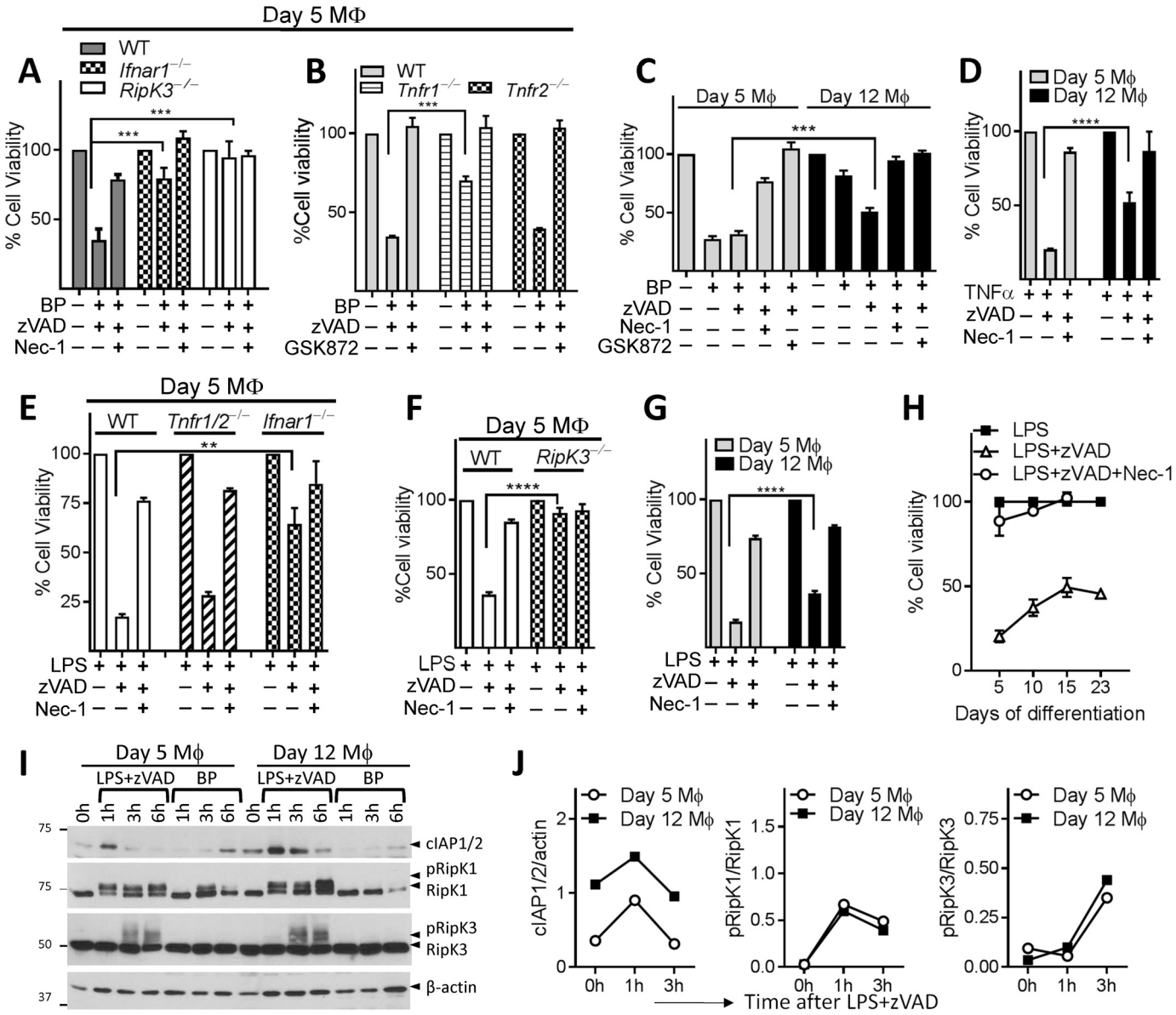

Figure 4. Macrophage differentiation results in a partial resistance to necrosome-induced cell death. Bone marrow–derived macrophages were treated with BP (10 μM) and Z-VAD (50 μM) in the absence or presence of Nec-1 (10 μM) or GSK872 (5 μM), and cell death was evaluated 24 h later by MTT assay (*A–C*). Macrophages were also treated with TNFα (50 ng/ml) (*D*) or LPS (1 ng/ml) (*E–H*) and Z-VAD (50 μM) in the absence or presence of Nec-1 (10 μM), and cell death was evaluated 24 h later by MTT assay. Western blot analysis was performed on cell extracts isolated at various time intervals after stimulation with LPS + Z-VAD or BP (*I*). Protein expression (*I*) was quantitated by densitometric analysis (*J*). Each experiment was repeated three times with triplicate samples. **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. *Error bars*, S.E.

phages as expected, but there was no difference in this degradation between day 5 and day 12 macrophages (Fig. 5*D*). Interestingly, the expression of the two endogenous inhibitors of the ripoptosome/necrosome pathways, XIAP and cFLIP$_L$, was elevated in day 12 macrophages following treatment with BP (Fig. 5, *D–F*) or LPS + BP (Fig. S3, *C–E*). The resistance of day 12 macrophages to BP-induced cell death was abrogated in XIAP-deficient macrophages (Fig. 5*G*), which correlated with increased TNFα expression and caspase-8 activation (Fig. 5, *H* and *I*). XIAP did not appear to regulate cFLIP$_L$ expression or RipK1 phosphorylation in day 12 macrophages; however, the phosphorylation of MK2 was enhanced in XIAP-deficient macrophages (Fig. 5, *J* and *K*). Although the deficiency of XIAP in day 12 macrophages resulted in cell death following BP treatment, the mechanism of cell death was still ripoptosome-dependent, as it was rescued by Nec-1, not GSK872 (Fig. 5*L*). XIAP had no impact on BP + Z-VAD–induced cell death by the necroptotic pathway (Fig. 5*M*). Taken together, these results confirm that differentiated macrophages express increased levels of XIAP, which promotes their resistance to cell death by the ripoptosome pathway. Resistance of day 12 macrophages to LPS + BP–induced cell death was also abrogated in XIAP-deficient macrophages (Fig. S3*F*).

Inhibition of p38 MAPK signaling pathway restores ripoptosome-induced cell death in differentiated macrophages

Having observed that TNF-R signaling was required for ripoptosome-induced cell death and that the phosphorylation of MK2, the downstream target of p38 MAPK, was reduced in

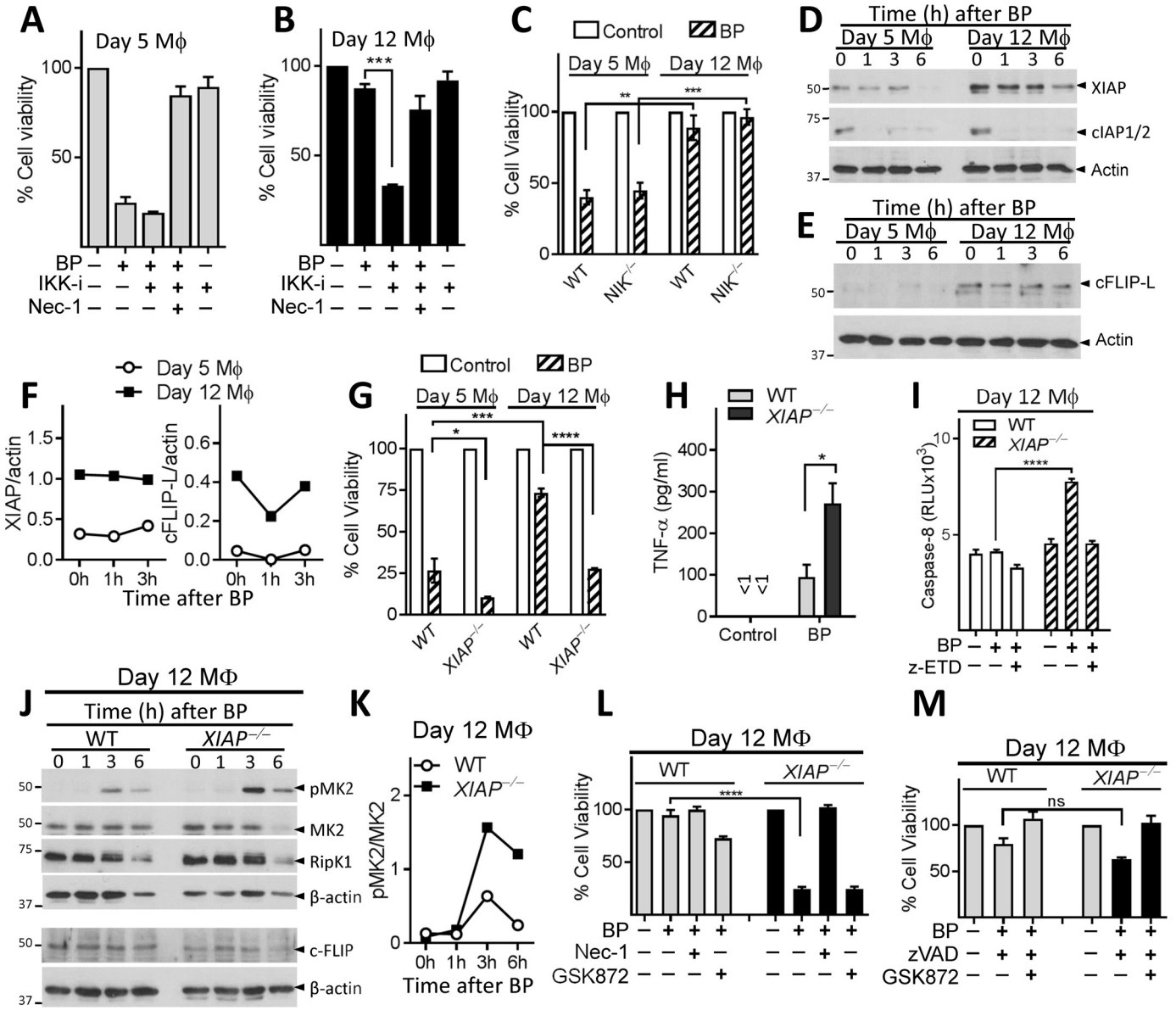

Figure 5. Macrophage differentiation results in increased expression of XIAP which promotes resistance of differentiated macrophages to ripoptosome-induced cell death. Bone marrow–derived macrophages were harvested from WT (*A–C*) or *NIK*$^{-/-}$ (*C*) mice at day 5 or day 12 post-differentiation and treated with BP (10 μM) in the presence or absence of NF-κB inhibitor (JSH-23, 1 μM) or Nec-1 (10 μM) (*A* and *B*). Cell death was evaluated at 24 h by MTT assay. Western blotting (*D* and *E*) and corresponding densitometric analysis (*F*) was performed on cell extracts collected from WT macrophages treated with BP for various time intervals. Bone marrow–derived macrophages were harvested from WT and *XIAP*$^{-/-}$ mice at day 5 and day 12 post-differentiation, and cell death was evaluated at 24 h after BP (10 μM) treatment (*G*). The level of TNFα secreted by WT and *XIAP*$^{-/-}$ macrophages was evaluated at 6 h post-BP treatment (*H*). Caspase-8 activity was evaluated at 4 h post-BP treatment by chemiluminescence assay (*I*). Western blotting (*J*) and corresponding densitometric analysis (*K*) were performed on cell extracts collected from WT and *XIAP*$^{-/-}$ macrophages treated with BP for various time intervals. WT and *XIAP*$^{-/-}$ macrophages (day 12 of differentiation) were treated with BP (*L*) or BP + Z-VAD (*M*) in the presence or absence of Nec-1 or GSK872, and cell death was evaluated 24 h later by MTT assay. Each experiment was repeated three times with triplicate samples. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; *ns*, not significant. Western blotting of actin shown in *D* is reused in Fig. 3 (*C* and *E*). *Error bars*, S.E.

TNFα-deficient macrophages (Fig. 2, *K* and *L*), we evaluated the role of p38 MAPK signaling in cell death resistance of day 12 macrophages. Our results indicate that prolonged macrophage differentiation results in reduced phosphorylation of p38 MAPK and MK2 in response to BP treatment (Fig. 6*A* and Fig. S4, *A* and *B*). Inhibiting p38 MAPK abrogates their resistance to cell death in response to BP (Fig. 6*B*), with a corresponding loss of MK2 phosphorylation (Fig. 6*C* and Fig. S4*C*) and an increase in caspase-8 activation (Fig. S4, *D* and *E*). There was no impact of inhibition of p38 MAPK on the expression of XIAP or $cFLIP_L$ (Fig. 6*C* and Fig. S4, *F* and *G*). Day 12 macrophages displayed increased activation of JNK and ERK, which were elevated further by inhibition of p38 MAPK (Fig. 6*D* and Fig. S4, *H* and *I*). Unlike the impact of p38 MAPK (Fig. 6*B*), inhibition of the MEK/ERK or JNK pathways did not have any significant impact on the resistance of day 12 macrophages to BP-induced cell death (Fig. 6, *E–G*). Expression of TNFα in response to LPS treatment was significantly reduced in MK2-deficient macrophages (Fig. 6*H*), whereas the opposite results were obtained following BP treatment (Fig. 6*I*). MK2 deficiency abrogated the

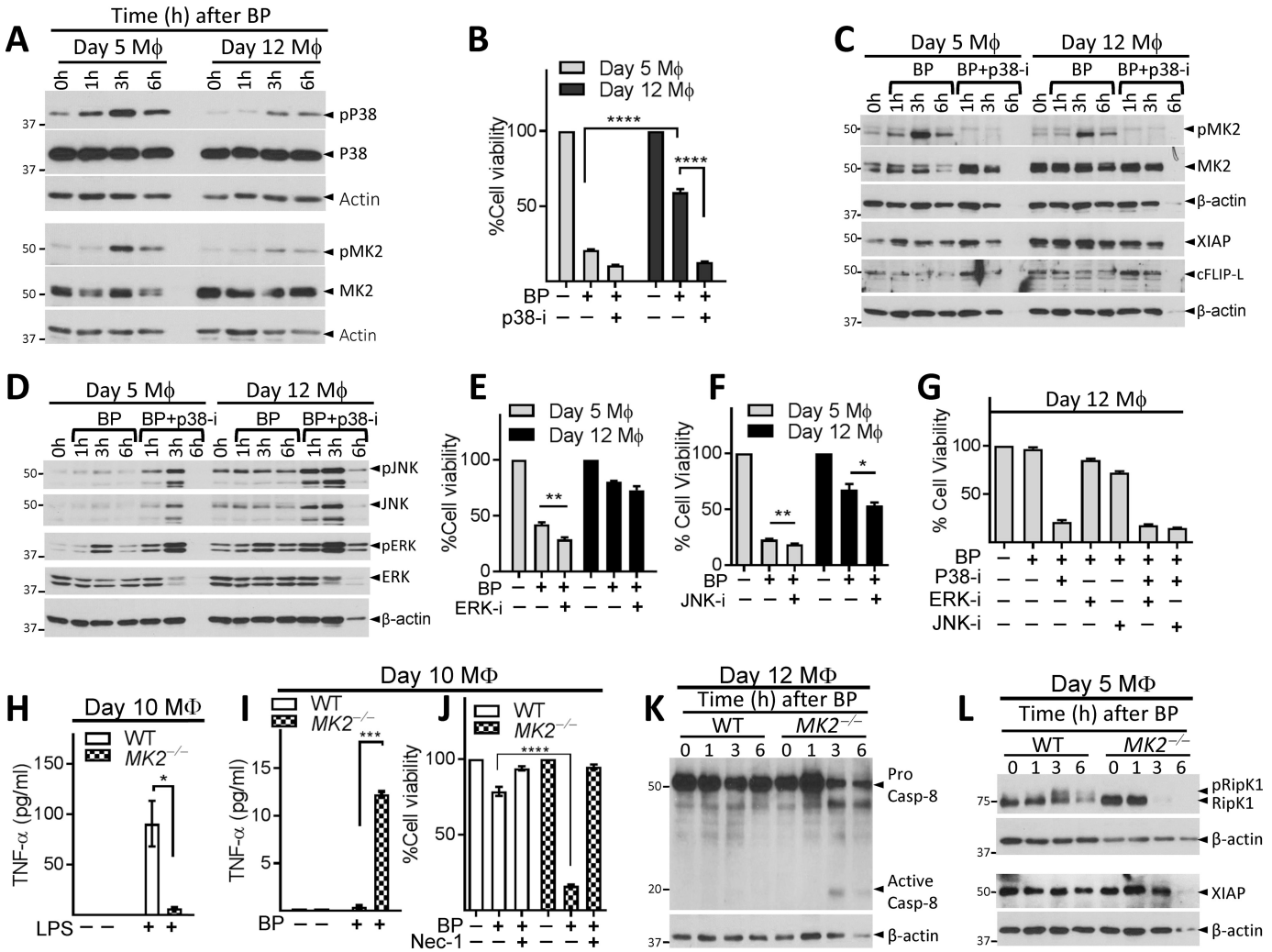

Figure 6. Inhibition of p38 MAPK pathway restores cell death of differentiated macrophages during ripoptosome signaling. Bone marrow–derived macrophages were harvested from WT mice at day 5 or day 12 post-differentiation and treated with BP (10 μM), and cell extracts were evaluated by Western blotting (*A*). Macrophages were treated with BP (10 μM) and/or p38 MAPK inhibitor (ralimetinib, 5 μM), and cell death was evaluated 24 h later by MTT assay (*B*). Cells were treated as described in *B*, and Western blot analysis was performed on cell extracts at various time intervals (*C* and *D*). Macrophages were also treated with BP and/or MEK/ERK inhibitor (PD0325901, 20 nM) or JNK inhibitor (SP600125, 20 μM), and cell death was evaluated 24 h later by MTT assay (*E–G*). TNFα secreted in the supernatants of WT and *MK2*$^{-/-}$ macrophages stimulated with LPS (1 ng/ml) (*H*) or BP (10 μM) (*I*) was evaluated at 6 h in the supernatants. WT and *MK2*$^{-/-}$ macrophages were treated with BP (10 μM), and cell death was evaluated 24 h later (*J*). Expression of caspase-8 (*K*), and RipK1 and XIAP (*L*) was evaluated by Western blotting of cell extracts at various time intervals after BP treatment. Each experiment was repeated three times with triplicate samples. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. *Error bars*, S.E.

resistance of day 12 macrophages to BP-induced cell death (Fig. 6*J*), which correlated with increased activation of caspase-8 (Fig. 6*K* and Fig. S4*J*) and loss of RipK1 phosphorylation (Fig. 6*L* and Fig. S4*K*). There was no impact of MK2 on the expression of XIAP (Fig. 6*L*). Enhancement in cell death by inhibition of p38 MAPK occurred through the ripoptosome pathway, as it was inhibited by Nec-1, not GSK872, and was still dependent on the Lys-45 kinase region of RipK1 (Fig. S4*L*). Taken together, these results indicate that activation of the p38 MAPK/MK2 pathway appears to be reduced in differentiated macrophages, yet inhibition of this pathway abrogates the resistance of differentiated macrophages to ripoptosome-induced cell death.

Activated and proliferating macrophages are eliminated by ripoptosome signaling

We also evaluated the impact of ripoptosome and necrosome signaling in splenic and peritoneal macrophages. Macrophages were purified from the peritoneum of naive mice or from mice that were injected with thioglycolate for 5 days. Treatment with BP resulted in modest cell death in peritoneal macrophages isolated from naive mice (Fig. 7*A*). In contrast, macrophages isolated from the peritoneum of thioglycolate injected mice were highly susceptible to BP-induced cell death (Fig. 7*B*), which was rescued only by inhibition of both apoptosis and necroptosis pathways. These results suggest that activation of resident macrophages enhances their susceptibility to BP-induced cell death and that the peritoneal macrophages are more flexible at switching between cell death pathways. Similarly, splenic macrophages were also susceptible to BP-induced cell death, which was rescued only after inhibition of both the apoptosis and necroptosis pathways (Fig. 7*C*). Staining of cells with the cell-cycling protein Ki67 revealed that only the cycling macrophages were susceptible to BP-induced cell death (Fig. 7*D*). Various phenotypic and functional subsets

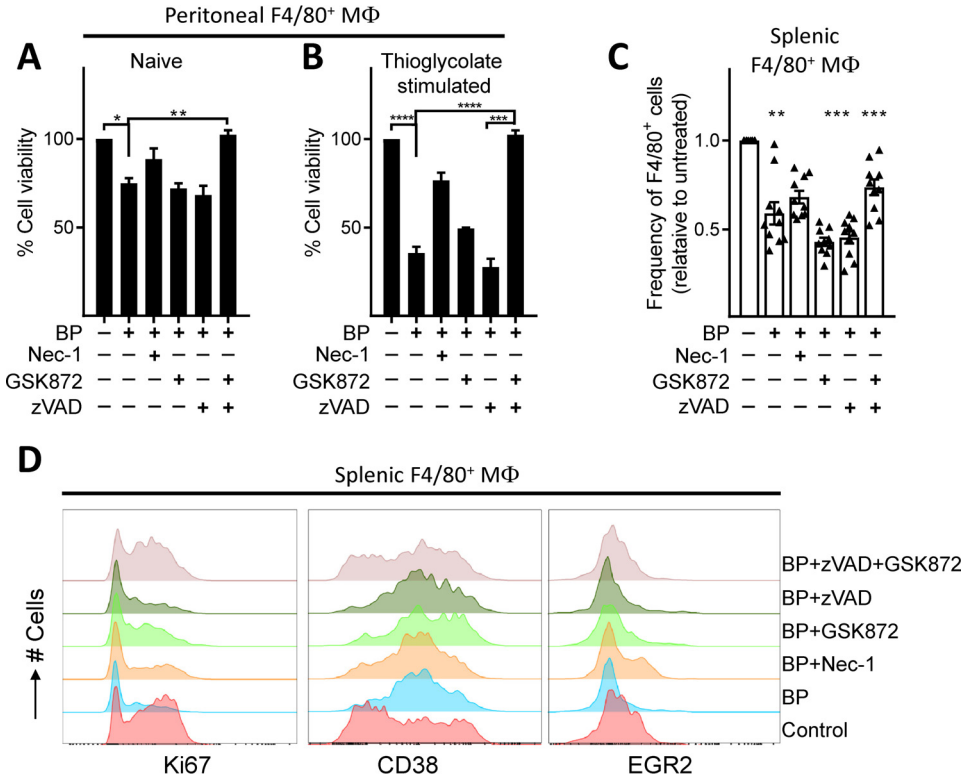

Figure 7. Activated and cycling macrophages are eliminated by ripoptosome signaling. Peritoneal macrophages from naive (*A*) or thioglycolate-injected (*B*) mice were treated with BP and/or Nec-1 (10 μM), GSK872 (5 μM), and Z-VAD (10 μM). Cell death was evaluated 24 h later by MTT assay. Spleen cells from naive mice (*C* and *D*) were incubated with BP (10 μM) and various inhibitors as mentioned above. After 24 h, cells were stained with antibodies against F4/80, CD3, CD19, and the zombie yellow viability dye, and samples were acquired using a high-throughput port by flow cytometry to ensure acquisition of 180 μl of each sample. The absolute number of acquired live cells or gated macrophages in 180 μl for each sample was determined (*C*). Cells were also stained for Ki67 (intracellular), CD38, and EGR2 using the labeled antibodies, and the impact of BP and various inhibitors on F4/80$^+$ macrophages was evaluated (*D*). Results are pooled from at least three experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. *Error bars*, S.E.

of macrophages have been identified that have been categorized as pro-inflammatory or anti-inflammatory (20, 26, 31), with CD38 being expressed on pro-inflammatory macrophages and EGR2 being expressed on anti-inflammatory macrophages (31). Thus, we investigated the expression of CD38 and EGR2 on BP-treated macrophages, where we observed a significant increase in CD38 and decrease in EGR2 expression, which could be partially blocked by co-treatment with Z-VAD/GSK872 (Fig. 7*D*). Based on these results, we propose that RipK1-mediated cell death pathways specifically target actively proliferating early macrophages, and select for survival of more differentiated and pro-inflammatory macrophage subsets.

Discussion

Monocytes migrate to the blood from the bone marrow on a continuous basis, and subsequently differentiate into macrophages to replenish the pool of tissue macrophages (20). Macrophages are the central cell type of the innate immune system that promote the innate immune response and mediate pathogen control (32–34). Considering that there are multiple types of macrophages (20, 26), the precise molecular steps and the relative timing that is required for monocytes to differentiate into macrophages are not clear. Unlike monocytes, macrophages have a long life span, ranging from months to years (25), and it is not clear how macrophages survive for prolonged periods in the face of ongoing pathogen insults and trauma. We have evaluated the impact of macrophage differentiation on their susceptibility to cell death by the ripoptosome and necrosome pathways. Our results reveal that differentiation of macrophages results in up-regulation of endogenous inhibitors of cell death, which promote resistance against ripoptosome signaling (Fig. 8).

Macrophages undergo dynamic changes in the expression of various cell surface molecules during differentiation (31). We observed that as monocytes differentiated into macrophages, expression of F4/80, a classic macrophage marker (21), was induced, whereas the expression of Ly6C, a monocyte marker (35), was diminished. CD11b is another marker of macrophage differentiation (36) that was increased during differentiation. The time required for macrophages to undergo differentiation is not clear, as macrophages have been isolated at various time intervals (37, 38). Interestingly, extending the differentiation of macrophages to 14 days resulted in enhanced control of mycobacteria (39), which suggests that macrophages continue to differentiate beyond day 7 of culture. Interestingly, our results indicate that peritoneal macrophages form naive mice are resistant to ripoptosome-induced cell death, akin to the 12-day differentiated macrophages.

Activation of the ripoptosome complex in fibroblasts results in cell death that depends on TNFα, RipK1, and RipK3 (12, 40). Nec-1 was identified as an inhibitor that binds to the kinase region of RipK1 and blocks its function (29) and inhibits ripop-

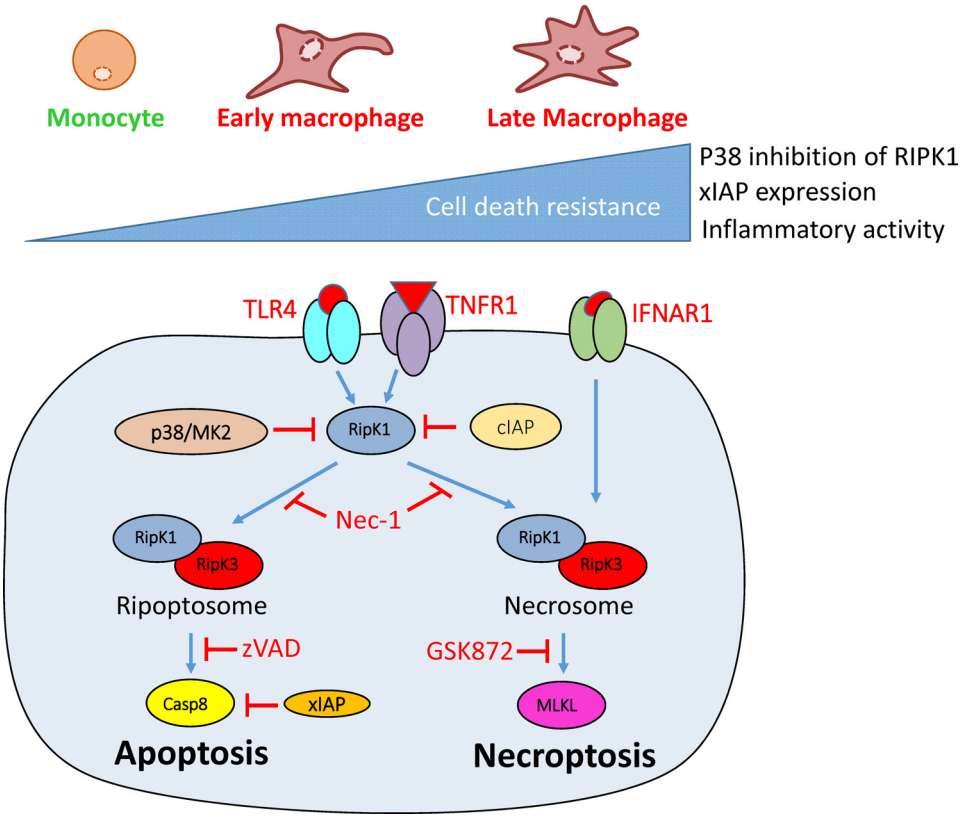

Figure 8. Differentiation of monocytes promotes resistance to ripoptosome signaling. As monocytes differentiate to macrophages, the expression of XIAP increases, which promotes resistance to cell death induced by ripoptosome signaling. In addition to XIAP, the p38 MAPK/MK2 pathway also promotes resistance to ripoptosome-induced cell death. Resistance to cell death in highly differentiated macrophages could promote their survival during infection and release of inflammatory cytokines for longer periods.

tosome-induced cell death (41). Our results reveal a hierarchical role for various mediators in BP-induced cell death. TNFα and the Lys-45 kinase activity of RipK1 had the highest impact on cell death, RipK3 had a moderate impact, and TRIF had a small impact. The specific mechanism through which RipK1 kinase promotes the formation of the ripoptosome is not very clear. Interaction of RipK1, RipK3, and caspase-8 appears to be required for ripoptotosome-induced cell death in fibroblasts, and our results indicate that this interaction is impaired in day 12 macrophages. Interestingly, we observed that the kinase activity of RipK1 promotes the activation of caspase-9 and caspase-3 in addition to the activation of caspase-8. Whereas activation of caspase-8 would result in downstream activation of caspase-3, the mechanism through which caspase-9 activation is influenced by RipK1 is not clear.

RipK3 participates in both ripoptosome and necrosome pathways; however, the impact of macrophage differentiation was noticeable mainly in ripoptosome signaling. Because ripoptosome-induced cell death depends on active caspase-8, inhibition of this caspase by increased expression of XIAP endows macrophages with a mechanism to resist cell death. On the other hand, necroptosis is induced only when caspases are inhibited (6); enhancing the expression of XIAP may not have much impact. Because XIAP does not appear to impact necrosome signaling, it is conceivable that the marginal resistance of differentiated macrophages to necrosome signaling may be related to increased cFLIP$_L$ expression.

IAPs act as endogenous inhibitors of cell death (42), and cancer cells often express high levels of IAPs (43–45). We have previously reported that SMAC mimetics induce rapid degradation of cIAP1/2 but not XIAP in macrophages (28). Our results indicate that the degradation of cIAP1/2 by SMAC mimetics was similar in day 5 and day 12 macrophages, ruling out the role of cIAP1/2 in the resistance of day 12 macrophages to cell death. On the other hand, the expression of XIAP was higher in day 12 macrophages, and XIAP inhibits caspase-3 and caspase-7 (46). XIAP has been previously reported to inhibit TNF and RipK3-dependent cell death and inflammasome signaling (47). Whereas various SMAC mimetics target IAPs for degradation, BP induces degradation of cIAP1/2, but not XIAP (48, 49). Lack of any impact of BP on XIAP levels therefore makes XIAP available to inhibit cell death in differentiated macrophages.

In addition to increased expression of XIAP, differentiated macrophages also expressed increased levels of cFLIP$_L$, which is a caspase-8 homologue that lacks key residues necessary for protease activity (50). Whereas cFLIP$_L$ functions solely as an inhibitor of caspase-8 through competition for binding sites, cFLIP$_L$ can act as an activator of caspase-8 by forming a heterodimeric complex with caspase-8, with substrate specificity that is similar to that of caspase-8 homodimers (51). In the case of ripoptosome signaling, cFLIP$_L$ has been shown to inhibit cell death, whereas the short isoform of cFLIP$_L$ promotes ripoptosome signaling (40). We have failed to detect the expression of

short isoform of $cFLIP_L$ in macrophages. In our experiments, if increased expression of $cFLIP_L$ were responsible for the resistance of differentiated macrophages to ripoptosome-induced cell death, then XIAP deficiency may not have had a major impact in abrogating this resistance.

NF-κB signaling promotes resistance against TNFα-induced cell death (52, 53), and our results indicate that NF-κB signaling is involved in promoting the resistance of day 12 macrophages to ripoptosome-induced cell death. Various studies have shown that cIAPs have divergent impact on NF-κB signaling (12, 54). In steady state, a cIAP1/2–TRAF2–TRAF3 complex regulates noncanonical NF-κB through constitutive Lys-48 –linked ubiquitination and proteasomal degradation of NIK, a member of the MAP3 kinase family (55). Degradation of cIAPs by SMAC mimetics results in stabilization of NIK and activation of the noncanonical NF-κB pathway (56). Our results with NIK-deficient macrophages did not reveal any impact of NIK on SMAC mimetic–induced cell death, suggesting that this pathway is not involved in the resistance of day 12 macrophages to cell death. It has also been shown that Lys-63–linked ubiquitination of RipK1 by cIAPs promotes activation of the canonical NF-κB pathway (12, 54), and degradation of cIAPs by SMAC mimetics may inhibit this pathway and favor cell death. In our experiments, the degradation of cIAPs by a SMAC mimetic was similar in day 5 and day 12 macrophages, which excludes the possibility that resistance of macrophages to cell death is due to the impact of cIAPs on NF-κB signaling. XIAP has also been shown to promote NF-κB signaling (57), and this may be another avenue through which increased expression of XIAP in day 12 macrophages promotes resistance to cell death.

During TLR signaling, activation of the p38 MAPK/MK2 pathway promotes the stabilization of TNFα expression (58). In contrast, during ripoptosome signaling in myeloid leukemia cells, p38 MAPK has been shown to inhibit TNFα expression, and inhibition of MK2 results in enhanced cell death of leukemia cells (59). It was subsequently shown that MK2 mediates an inhibitory phosphorylation of RipK1, which restricts ripoptosome-induced cell death (60–62). Our results indicate that the phosphorylation of RipK1 in macrophages depends on MK2, and inhibition of MK2 results in augmentation of ripoptosome-induced cell death, particularly on day 12 macrophages. Paradoxically, day 12 macrophages did not display increased phosphorylation of p38 MAPK, MK2, and RipK1. Rather, we observed that in day 12 macrophages, the phosphorylation of p38 MAPK, MK2, and RipK1 was reduced in response to BP treatment, without any impairment in TNFα expression. Reduced phosphorylation of RipK1 in day 12 macrophages could be due to reduced MK2 phosphorylation in these cells. Our results indicate that although the activation of the p38 MAPK/MK2 pathway is reduced in differentiated macrophages during ripoptosome signaling, inhibition of the p38 MAPK/MK2 pathway abrogates their resistance to ripoptosome-induced cell death. The mechanism of this paradox remains unclear, but our observations should strengthen the view that the p38/MK2 pathway directly or indirectly inhibits RipK1-dependent cell death. It is important to note that XIAP-deficient macrophages displayed enhanced TNFα production and MK2 activation, which also supports the notion of complex cross-talk between p38/MK2 and XIAP.

We have recently reported that the K45A mutation of RipK1 reduces cell death, reactive oxygen species production, and caspase-8 activation during infection of macrophages with *S. enterica* Typhimurium, impairing *S. enterica* Typhimurium control *in vivo* (30). RipK1 promotes caspase-8 – dependent apoptosis or RipK3-dependent necroptosis in macrophages in response to *Yersinia* infection (63). These results reveal the key role of ripoptosome signaling in promoting bacterial control, and any impairment in this pathway may lead to compromised control of infection. On the other hand, in sterile inflammatory diseases, RipK1 might have detrimental effects. An inhibitor of RipK1, Nec-1, has been shown to have a protective role during ischemia-induced injury (64–68). Because RipK1 is the central component of the ripoptosome complex, there have been speculations that RipK1 might be playing a critical role in the pathogenesis of these diseases through ripoptosome, rather than necrosome, signaling (69). We have revealed here a mechanism whereby long-lived macrophages resist cell death due to increased expression of XIAP, which would allow them to continue expressing high levels of inflammatory cytokines and resist pathogen attack. On the other hand, newly differentiating/infiltrating macrophages may produce less inflammatory cytokines and turn over more quickly to aid pathogen clearance and return to homeostasis. Whereas XIAP-deficient mice do not show any obvious developmental abnormality (70), mutations in human XIAP result in immunodeficiency with aberrant activation of myeloid cells (71). These results further reinforce the role of ripoptosome signaling in human diseases.

Experimental procedures

Mice

C57BL/6J (stock no. 0664), *TNF-R1,2*$^{-/-}$ (stock no. 03243), *TNF-R1*$^{-/-}$ (stock no. 03242), *TNF-R2*$^{-/-}$ (stock no. 02620), *TNFα*$^{-/-}$ (stock no. 05540), *Trif-mutant* (stock no. 05037), *Myd88*$^{-/-}$ (stock no. 09088), and *NIK*$^{-/-}$ (stock no. 025557) were obtained from Jackson Laboratory. *RipK1*^{K45A} were obtained from Dr. Peter J. Gough (GSK, Philadelphia, PA), *RipK3*$^{-/-}$ were obtained from Dr. Visva Dixit (Genentech), *MK2*$^{-/-}$ were obtained from Dr. Matthias Gaestel (Hannover Medical School), *XIAP*$^{-/-}$ were obtained from Dr. Robert Korneluk (Children's Hospital of Eastern Ontario), and *Ifnar1*$^{-/-}$ were obtained from Dr. Kaja Murali Krishna (Emory University, Atlanta, GA). Mice were maintained at the animal facility of the University of Ottawa, Faculty of Medicine. All procedures were approved by the uOttawa Animal Care Committee.

Cell isolation, cell culture, and viability measurements

Monocytes were purified from the bone marrow using beads obtained from StemCell Technologies (Vancouver, Canada). Macrophages were generated from mouse bone marrow as per our previously published procedures (28). For *in vivo* macrophage activation, 1 ml of 3% thioglycolate solution was injected into the peritoneal cavity of mice, and cells were isolated at day 5. Peritoneal macrophages were purified by staining cells with anti-mouse F4/80-PE followed by capture with anti-PE beads from StemCell Technologies. Macrophages were plated in

96-well flat-bottomed plate (Falcon). Cells (7×10^4 cells/well) were seeded and incubated overnight. Different small-molecule inhibitors and agonists were added to cells. Following treatment with appropriate concentrations of inhibitors and agonists, cells were usually left for 24 h, unless otherwise indicated, and cell viability was measured by various assays. Cell viability was measured by MTT uptake or by propidium iodide (PI)/Hoechst staining (28, 72). MTT was obtained from Sigma-Aldrich (M5655). Absorbance of the dye was measured at 570 nm using a filterMax plate reader (Molecular Devices). Hoechst was obtained from Invitrogen Inc. (catalog no. 33342) and used at a concentration of 2.5. PI was obtained from BD Biosciences (catalog no. 550825) and used at a 1:10 dilution. Cell viability was measured by imaging cells under an AxioObserver D1 microscope, and the AxioVision release 4.8 program was used to capture and analyze images.

In the case of splenic macrophages, nonfractionated spleen cells were incubated overnight with various inhibitors and stimulants. Cells were then stained with anti-F4/80-PE-Cy7, anti-CD3-eFluor450, anti-CD19-eFluor450, and zombie yellow fixable viability dye. 180 μl of sample was acquired from each well by a high-throughput sample port, and the number of viable cells in gated macrophages was determined by flow cytometry.

Inhibitors and reagents

The SMAC mimetic BP (S7015) was obtained from Selleckchem (S7015) and used at a concentration of 10 μM. The SMAC mimetic BV6 was obtained from Genentech Inc. and also used at a concentration of 10 μM. Ultrapure LPS from *Escherichia coli* 055:B5 was obtained from Sigma-Aldrich (L4524) and used at a concentration of 1 ng/ml, if not indicated otherwise. Recombinant mouse TNFα was obtained from R&D Systems (410-MT) and used at a concentration of 50 ng/ml. The MEK/ERK inhibitor PD0325901 (S1036) was obtained from Selleckchem and used at a concentration of 20 nM. The JNK inhibitor SP600125 was obtained from Sigma-Aldrich (S5567) and used at a concentration of 20 μM. Inhibitor of p38 MAPK, ralimetinib, was obtained from Selleckchem (S1494) and used at a concentration of 5 μM. RipK1 inhibitor, Nec-1, was obtained from Sigma-Aldrich (catalog no. 9037) and used at a concentration of 10 μM. RipK3 inhibitor, GSK872, was obtained from Glixx (GLXC-03990) and used at a concentration of 5 μM. Pan-caspase inhibitor (Z-VAD-fmk, A1902) and caspase-8 inhibitor (Z-IETD-fmk, B3232) were obtained from ApexBio (A1902) and used at a concentration of 50 μM. Caspase-3 inhibitor (z-DEVD-FMK, FMK004) and caspase-9 inhibitor (zLEHD-FMK, FMK008) were obtained from R&D Systems and used at a concentration of 50 μM. The following inhibitors against NF-κB were used: JSH-23 (CAS749886-87-1, Calbiochem), Ly2409881 (S7697, Selleckchem), and TPCA-1 (S2824, Selleckchem). These were used at a concentration of 1–5 μM.

Western blotting

Macrophages were seeded in a 24-well plate at 3×10^5 cells/well and treated with inhibitors or agonists. At various time intervals, cells were washed with cold PBS and lysed in cold 1% SDS lysis buffer with 1% β-mercaptoethanol. Samples were boiled immediately and frozen. Lysates were run on a 1.5-mm 15-well 8, 10, 12, or 15% polyacrylamide gel, depending on the size of the proteins of interest. Gels were run at 130 V for 1 h. A polyvinylidene difluoride membrane was soaked in 100% methanol and washed with the transfer buffer, and then the transfer was run at 100 V for 1 h. Membrane was blocked in 5% skim milk in Tris-buffered saline solution (TBS: 0.5 M Tris, 1.5 NaCl, pH 7) with 0.5% Tween 20 (TBST) or 5% BSA. The following primary antibodies were used for Western blotting: mouse anti-RipK1 (BD Biosciences, catalog no. 610459), rabbit anti-RipK3 (ProSci Inc., catalog no. 2283), mouse anti-β-actin (BD Biosciences, catalog no. 612656), rabbit anti-cIAP1/2 (Cyclex, CY-P1040), rabbit anti-p38 MAPK (Cell Signaling, catalog no. 8690), rabbit anti-phospho-p38 MAPK (Cell Signaling, catalog no. 4511), rat anti-caspase-8 (Enzo, ADI-AAM-212-E), rabbit anti-XIAP (Cell Signaling, catalog no. 2042), rabbit anti-cFLIP$_L$ (Santa Cruz Biotechnology, Inc., sc-8347), rabbit anti-MK2 (Cell Signaling, catalog no. 3007), rabbit anti-phospho MK2 (Cell Signaling, catalog no. 3042), rabbit anti-ERK1/2 (Cell Signaling, catalog no. 4695), rabbit anti-phospho-ERK1/2 (Cell Signaling, catalog no. 4370), rabbit anti-JNK (Cell Signaling, catalog no. 9252), and rabbit anti-phospho-JNK (Cell Signaling, catalog no. 4668). Primary antibodies were diluted in appropriate blocking buffer (5% milk or BSA), added to the membrane in a sealed plastic pouch, and incubated overnight on the shaker at 4 °C. Membranes were then washed with TBST before adding secondary antibody diluted in the appropriate blocking buffer (5% milk or BSA). Membranes were washed and visualized using either luminol ECL substrate (Thermo Scientific, catalog no. 32106) or West Femto ECL substrate (Thermo Scientific, catalog no. 34095). Photosensitive film (Sigma-Aldrich, Carestream Health, catalog no. 785019) was used to examine protein expression. Results were quantified using densitometric analysis. The densitometric quantification of Western blotting signals was performed using ImageJ version 1.48 software.

Flow cytometry

Cells were washed with staining buffer (1% BSA in PBS). FcBlock (anti-CD16/32) (BD Biosciences, catalog no. 553142) was added to the cells in the staining buffer and incubated for 10 min at 4 °C. Various flurophore-conjugated antibodies were added in the staining buffer. Cells were protected from the light and incubated with the antibodies for 30 min at 4 °C. After that, cells were washed with the staining buffer and resuspended in flow fixative (PBS containing 1% paraformaldehyde and 0.02% sodium azide). Samples were then acquired on a CyAN-ADP analyzer (Beckman Coulter) or LSR-Fortessa or FACSCelesta (BD Biosciences) and analyzed using Kaluza software (Beckman Coulter, version 1.2) or FlowJo version 10 (FlowJo). The following antibodies were obtained from eBiosciences: anti-mouse Ly-6C-PE (12-5932-82), anti-mouse CD11b-Pe-Cy7 (2500112-81), anti-mouse Ly-6G-FITC (11-5931-82), and anti-mouse F4/80-APC-Cy7 (47-4801-80).

Caspase bioassays

The caspase-8, caspase-9, and caspase-3/7 activities were measured by using the caspase-Glo 8 (Promega, G8200), caspase-Glo 9 (Promega, G8210), and caspase-Glo 3/7 (Promega,

G8090) luminescent kits. The substrate was mixed with the buffer, and the mix was added to the cells in the 96-well plates. The luminescence generated in the wells was measured using a FilterMax™ F5 plate reader (Molecular Devices).

Immunoprecipitation

Cell lysates were immunoprecipitated with a Dynabeads co-immunoprecipitation kit (Invitrogen, Life Technologies, 14321D, Burlington, Canada) with rat anti-caspase-8 (ENZO, 1G12). The caspase-8 antibody was coupled with the Dynabeads with incubation at 37 °C overnight and then washing with various buffers. The next day, bone marrow–derived macrophages were treated with birinapant for 4 h. Then the cells were washed with ice-cold 1× PBS and lysed in immunoprecipitation lysis buffer. The lysates were sonicated for 10 s and then centrifuged at 5000 × *g* for 5 min at 4 °C to remove large debris and nuclei. 10 μl of the supernatant was stored as input, and the remaining clarified lysate was then incubated with antibody-coupled Dynabeads for 16–24 h at 4 °C on a rocker platform. The unbound fractions were saved at −20 °C, and the bound proteins were eluted. 10 μl of the 4× SDS-loading dye was added to the eluent and boiled for 5 min. The final eluent was analyzed by Western blotting.

Cytokine production

Cytokine expression by macrophages was measured in the supernatant at 24 h post-stimulation with LPS (100 ng/ml). In case of cells stimulated with BP, the level of TNFα was too low to be detected by ELISA. In this case, TNFα expression was measured by the WEHI-164 cell bioassay (73).

Statistics

All *error bars* show S.E. Unpaired two-tailed student's *t* test or one-way analysis of variance followed by a Bonferroni post hoc test was used to determine statistical significance. All statistical analyses were performed using GraphPad Prism software.

Author contributions—D. R., A. A., A. W., K. K., N. A., Z. A., and E. S. A. performed experiments and analyzed data. C. T. and M. B. M. provided additional cell samples. R. G. K., M. G., and S. M. provided additional reagents. S. S. wrote the paper with S. M.

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References

1. Elmore, S. (2007) Apoptosis: a review of programmed cell death. *Toxicol. Pathol.* **35,** 495–516 CrossRef Medline
2. Broz, P., and Monack, D. M. (2011) Molecular mechanisms of inflammasome activation during microbial infections. *Immunol. Rev.* **243,** 174–190 CrossRef Medline
3. Festjens, N., Vanden Berghe, T., Cornelis, S., and Vandenabeele, P. (2007) RIP1, a kinase on the crossroads of a cell's decision to live or die. *Cell Death Differ.* **14,** 400–410 CrossRef Medline
4. Schilling, R., Geserick, P., and Leverkus, M. (2014) Characterization of the ripoptosome and its components: implications for anti-inflammatory and cancer therapy. *Methods Enzymol.* **545,** 83–102 CrossRef Medline
5. Green, D. R. (2005) Apoptotic pathways: ten minutes to dead. *Cell* **121,** 671–674 CrossRef Medline
6. Pasparakis, M., and Vandenabeele, P. (2015) Necroptosis and its role in inflammation. *Nature* **517,** 311–320 CrossRef Medline
7. Vanden Berghe, T., Linkermann, A., Jouan-Lanhouet, S., Walczak, H., and Vandenabeele, P. (2014) Regulated necrosis: the expanding network of non-apoptotic cell death pathways. *Nat. Rev. Mol. Cell Biol.* **15,** 135–147 CrossRef Medline
8. Declercq, W., Vanden Berghe, T., and Vandenabeele, P. (2009) RIP kinases at the crossroads of cell death and survival. *Cell* **138,** 229–232 CrossRef Medline
9. Wang, C., Deng, L., Hong, M., Akkaraju, G. R., Inoue, J., and Chen, Z. J. (2001) TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature* **412,** 346–351 CrossRef Medline
10. Brenner, D., Blaser, H., and Mak, T. W. (2015) Regulation of tumour necrosis factor signalling: live or let die. *Nat. Rev. Immunol* **15,** 362–374 CrossRef Medline
11. Ea, C. K., Deng, L., Xia, Z. P., Pineda, G., and Chen, Z. J. (2006) Activation of IKK by TNFα requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO. *Mol. Cell* **22,** 245–257 CrossRef Medline
12. Tenev, T., Bianchi, K., Darding, M., Broemer, M., Langlais, C., Wallberg, F., Zachariou, A., Lopez, J., MacFarlane, M., Cain, K., and Meier, P. (2011) The ripoptosome, a signaling platform that assembles in response to genotoxic stress and loss of IAPs. *Mol. Cell* **43,** 432–448 CrossRef Medline
13. Vince, J. E., Wong, W. W.-L., Khan, N., Feltham, R., Chau, D., Ahmed, A. U., Benetatos, C. A., Chunduru, S. K., Condon, S. M., McKinlay, M., Brink, R., Leverkus, M., Tergaonkar, V., Schneider, P., Callus, B. A., *et al.* (2007) IAP antagonists target cIAP1 to induce TNFα-dependent apoptosis. *Cell* **131,** 682–693 CrossRef Medline
14. He, S., Wang, L., Miao, L., Wang, T., Du, F., Zhao, L., and Wang, X. (2009) Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-α. *Cell* **137,** 1100–1111 CrossRef Medline
15. Vanlangenakker, N., Vanden Berghe, T., Bogaert, P., Laukens, B., Zobel, K., Deshayes, K., Vucic, D., Fulda, S., Vandenabeele, P., and Bertrand, M. J. (2011) cIAP1 and TAK1 protect cells from TNF-induced necrosis by preventing RIP1/RIP3-dependent reactive oxygen species production. *Cell Death Differ.* **18,** 656–665 CrossRef Medline
16. Vandenabeele, P., Declercq, W., Van Herreweghe, F., and Vanden Berghe, T. (2010) The role of the kinases RIP1 and RIP3 in TNF-induced necrosis. *Sci. Signal.* **3,** re4 Medline
17. Robinson, N., McComb, S., Mulligan, R., Dudani, R., Krishnan, L., and Sad, S. (2012) Type I interferon induces necroptosis in macrophages during infection with *Salmonella enterica* serovar Typhimurium. *Nat. Immunol.* **13,** 954–962 CrossRef Medline
18. McComb, S., Cessford, E., Alturki, N. A., Joseph, J., Shutinoski, B., Startek, J. B., Gamero, A. M., Mossman, K. L., and Sad, S. (2014) Type-I interferon signaling through ISGF3 complex is required for sustained Rip3 activation and necroptosis in macrophages. *Proc. Natl. Acad. Sci. U.S.A.* **111,** E3206–E3213 CrossRef Medline
19. Miao, E. A., Leaf, I. A., Treuting, P. M., Mao, D. P., Dors, M., Sarkar, A., Warren, S. E., Wewers, M. D., and Aderem, A. (2010) Caspase-1-induced pyroptosis is an innate immune effector mechanism against intracellular bacteria. *Nat. Immunol.* **11,** 1136–1142 CrossRef Medline
20. Das, A., Sinha, M., Datta, S., Abas, M., Chaffee, S., Sen, C. K., and Roy, S. (2015) Monocyte and macrophage plasticity in tissue repair and regeneration. *Am. J. Pathol.* **185,** 2596–2606 CrossRef Medline
21. Murray, P. J., and Wynn, T. A. (2011) Protective and pathogenic functions of macrophage subsets. *Nat. Rev. Immunol.* **11,** 723–737 CrossRef Medline
22. Gordon, S., and Taylor, P. R. (2005) Monocyte and macrophage heterogeneity. *Nat. Rev. Immunol.* **5,** 953–964 CrossRef Medline
23. Schulz, C., Gomez Perdiguero, E., Chorro, L., Szabo-Rogers, H., Cagnard, N., Kierdorf, K., Prinz, M., Wu, B., Jacobsen, S. E., Pollard, J. W., Frampton, J., Liu, K. J., and Geissmann, F. (2012) A lineage of myeloid cells independent of Myb and hematopoietic stem cells. *Science* **336,** 86–90 CrossRef Medline
24. Yona, S., Kim, K. W., Wolf, Y., Mildner, A., Varol, D., Breker, M., Strauss-Ayali, D., Viukov, S., Guilliams, M., Misharin, A., Hume, D. A., Perlman, H., Malissen, B., Zelzer, E., and Jung, S. (2013) Fate mapping reveals origins

and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity* **38,** 79–91 CrossRef Medline
25. Parihar, A., Eubank, T. D., and Doseff, A. I. (2010) Monocytes and macrophages regulate immunity through dynamic networks of survival and cell death. *J. Innate Immun.* **2,** 204–215 CrossRef Medline
26. Ginhoux, F., and Jung, S. (2014) Monocytes and macrophages: developmental pathways and tissue homeostasis. *Nat. Rev. Immunol.* **14,** 392–404 CrossRef Medline
27. Müller-Sienerth, N., Dietz, L., Holtz, P., Kapp, M., Grigoleit, G. U., Schmuck, C., Wajant, H., and Siegmund, D. (2011) SMAC mimetic BV6 induces cell death in monocytes and maturation of monocyte-derived dendritic cells. *PLoS One* **6,** e21556 CrossRef Medline
28. McComb, S., Cheung, H. H., Korneluk, R. G., Wang, S., Krishnan, L., and Sad, S. (2012) cIAP1 and cIAP2 limit macrophage necroptosis by inhibiting Rip1 and Rip3 activation. *Cell Death Differ.* **19,** 1791–1801 CrossRef Medline
29. Degterev, A., Hitomi, J., Germscheid, M., Ch'en, I. L., Korkina, O., Teng, X., Abbott, D., Cuny, G. D., Yuan, C., Wagner, G., Hedrick, S. M., Gerber, S. A., Lugovskoy, A., and Yuan, J. (2008) Identification of RIP1 kinase as a specific cellular target of necrostatins. *Nat. Chem. Biol.* **4,** 313–321 CrossRef Medline
30. Shutinoski, B., Alturki, N. A., Rijal, D., Bertin, J., Gough, P. J., Schlossmacher, M. G., and Sad, S. (2016) K45A mutation of RIPK1 results in poor necroptosis and cytokine signaling in macrophages, which impacts inflammatory responses *in vivo*. *Cell Death Differ.* **23,** 1628–1637 CrossRef Medline
31. Jablonski, K. A., Amici, S. A., Webb, L. M., Ruiz-Rosado Jde, D., Popovich, P. G., Partida-Sanchez, S., and Guerau-de-Arellano, M. (2015) Novel markers to delineate murine M1 and M2 macrophages. *PLoS One* **10,** e0145342 CrossRef Medline
32. Medzhitov, R. (2007) Recognition of microorganisms and activation of the immune response. *Nature* **449,** 819–826 CrossRef Medline
33. O'Brien, A. D., Metcalf, E. S., and Rosenstreich, D. L. (1982) Defect in macrophage effector function confers *Salmonella typhimurium* susceptibility on C3H/HeJ mice. *Cell. Immunol.* **67,** 325–333 CrossRef Medline
34. Tabas, I. (2007) Apoptosis and efferocytosis in mouse models of atherosclerosis. *Curr. Drug Targets* **8,** 1288–1296 CrossRef Medline
35. Ramachandran, P., Pellicoro, A., Vernon, M. A., Boulter, L., Aucott, R. L., Ali, A., Hartland, S. N., Snowdon, V. K., Cappon, A., Gordon-Walker, T. T., Williams, M. J., Dunbar, D. R., Manning, J. R., van Rooijen, N., Fallowfield, J. A., *et al.* (2012) Differential Ly-6C expression identifies the recruited macrophage phenotype, which orchestrates the regression of murine liver fibrosis. *Proc. Natl. Acad. Sci. U.S.A.* **109,** E3186–E3195 CrossRef Medline
36. Walker, E. B., Akporiaye, E. T., Warner, N. L., and Stewart, C. C. (1985) Characterization of subsets of bone marrow-derived macrophages by flow cytometry analysis. *J. Leukoc. Biol.* **37,** 121–136 CrossRef Medline
37. Chamberlain, L. M., Holt-Casper, D., Gonzalez-Juarrero, M., and Grainger, D. W. (2015) Extended culture of macrophages from different sources and maturation results in a common M2 phenotype. *J. Biomed. Mater. Res. A* **103,** 2864–2874 CrossRef Medline
38. Warren, M. K., and Vogel, S. N. (1985) Bone marrow-derived macrophages: development and regulation of differentiation markers by colony-stimulating factor and interferons. *J. Immunol.* **134,** 982–989 Medline
39. Vogt, G., and Nathan, C. (2011) *In vitro* differentiation of human macrophages with enhanced antimycobacterial activity. *J. Clin. Invest.* **121,** 3889–3901 CrossRef Medline
40. Feoktistova, M., Geserick, P., Kellert, B., Dimitrova, D. P., Langlais, C., Hupe, M., Cain, K., MacFarlane, M., Häcker, G., and Leverkus, M. (2011) cIAPs block ripoptosome formation, a RIP1/caspase-8 containing intracellular cell death complex differentially regulated by cFLIP isoforms. *Mol. Cell* **43,** 449–463 CrossRef Medline
41. Ofengeim, D., and Yuan, J. (2013) Regulation of RIP1 kinase signalling at the crossroads of inflammation and cell death. *Nat. Rev. Mol. Cell Biol.* **14,** 727–736 CrossRef Medline
42. Liston, P., Fong, W. G., and Korneluk, R. G. (2003) The inhibitors of apoptosis: there is more to life than Bcl2. *Oncogene* **22,** 8568–8580 CrossRef Medline
43. LaCasse, E. C., Mahoney, D. J., Cheung, H. H., Plenchette, S., Baird, S., and Korneluk, R. G. (2008) IAP-targeted therapies for cancer. *Oncogene* **27,** 6252–6275 CrossRef Medline
44. Tamm, I., Kornblau, S. M., Segall, H., Krajewski, S., Welsh, K., Kitada, S., Scudiero, D. A., Tudor, G., Qui, Y. H., Monks, A., Andreeff, M., and Reed, J. C. (2000) Expression and prognostic significance of IAP-family genes in human cancers and myeloid leukemias. *Clin. Cancer Res.* **6,** 1796–1803 Medline
45. Beug, S. T., Cheung, H. H., LaCasse, E. C., and Korneluk, R. G. (2012) Modulation of immune signalling by inhibitors of apoptosis. *Trends Immunol.* **33,** 535–545 CrossRef Medline
46. Roy, N., Deveraux, Q. L., Takahashi, R., Salvesen, G. S., and Reed, J. C. (1997) The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. *EMBO J.* **16,** 6914–6925 CrossRef Medline
47. Yabal, M., Müller, N., Adler, H., Knies, N., Gross, C. J., Damgaard, R. B., Kanegane, H., Ringelhan, M., Kaufmann, T., Heikenwälder, M., Strasser, A., Gross, O., Ruland, J., Peschel, C., Gyrd-Hansen, M., and Jost, P. J. (2014) XIAP restricts TNF- and RIP3-dependent cell death and inflammasome activation. *Cell Rep.* **7,** 1796–1808 CrossRef Medline
48. Krepler, C., Chunduru, S. K., Halloran, M. B., He, X., Xiao, M., Vultur, A., Villanueva, J., Mitsuuchi, Y., Neiman, E. M., Benetatos, C., Nathanson, K. L., Amaravadi, R. K., Pehamberger, H., McKinlay, M., and Herlyn, M. (2013) The novel SMAC mimetic birinapant exhibits potent activity against human melanoma cells. *Clin. Cancer Res.* **19,** 1784–1794 CrossRef Medline
49. Lawlor, K. E., Khan, N., Mildenhall, A., Gerlic, M., Croker, B. A., D'Cruz, A. A., Hall, C., Kaur Spall, S., Anderton, H., Masters, S. L., Rashidi, M., Wicks, I. P., Alexander, W. S., Mitsuuchi, Y., Benetatos, C. A., *et al.* (2015) RIPK3 promotes cell death and NLRP3 inflammasome activation in the absence of MLKL. *Nat. Commun.* **6,** 6282 CrossRef Medline
50. Wilson, N. S., Dixit, V., and Ashkenazi, A. (2009) Death receptor signal transducers: nodes of coordination in immune signaling networks. *Nat. Immunol.* **10,** 348–355 CrossRef Medline
51. Boatright, K. M., Deis, C., Denault, J. B., Sutherlin, D. P., and Salvesen, G. S. (2004) Activation of caspases-8 and -10 by FLIP(L). *Biochem. J.* **382,** 651–657 CrossRef Medline
52. Van Antwerp, D. J., Martin, S. J., Kafri, T., Green, D. R., and Verma, I. M. (1996) Suppression of TNF-α-induced apoptosis by NF-κB. *Science* **274,** 787–789 CrossRef Medline
53. Dondelinger, Y., Jouan-Lanhouet, S., Divert, T., Theatre, E., Bertin, J., Gough, P. J., Giansanti, P., Heck, A. J., Dejardin, E., Vandenabeele, P., and Bertrand, M. J. (2015) NF-κB-independent role of IKKα/IKKβ in preventing RIPK1 kinase-dependent apoptotic and necroptotic cell death during TNF signaling. *Mol. Cell* **60,** 63–76 CrossRef Medline
54. Gyrd-Hansen, M., and Meier, P. (2010) IAPs: from caspase inhibitors to modulators of NF-κB, inflammation and cancer. *Nat. Rev. Cancer* **10,** 561–574 CrossRef Medline
55. Fulda, S., and Vucic, D. (2012) Targeting IAP proteins for therapeutic intervention in cancer. *Nat. Rev. Drug Discov.* **11,** 109–124 CrossRef Medline
56. Zarnegar, B. J., Wang, Y., Mahoney, D. J., Dempsey, P. W., Cheung, H. H., He, J., Shiba, T., Yang, X., Yeh, W. C., Mak, T. W., Korneluk, R. G., and Cheng, G. (2008) Noncanonical NF-κB activation requires coordinated assembly of a regulatory complex of the adaptors cIAP1, cIAP2, TRAF2 and TRAF3 and the kinase NIK. *Nat. Immunol.* **9,** 1371–1378 CrossRef Medline
57. Hofer-Warbinek, R., Schmid, J. A., Stehlik, C., Binder, B. R., Lipp, J., and de Martin, R. (2000) Activation of NF-kappa B by XIAP, the X chromosome-linked inhibitor of apoptosis, in endothelial cells involves TAK1. *J. Biol. Chem.* **275,** 22064–22068 CrossRef Medline
58. Kotlyarov, A., Neininger, A., Schubert, C., Eckert, R., Birchmeier, C., Volk, H. D., and Gaestel, M. (1999) MAPKAP kinase 2 is essential for LPS-induced TNF-α biosynthesis. *Nat. Cell Biol.* **1,** 94–97 CrossRef Medline
59. Lalaoui, N., Hänggi, K., Brumatti, G., Chau, D., Nguyen, N. Y., Vasilikos, L., Spilgies, L. M., Heckmann, D. A., Ma, C., Ghisi, M., Salmon, J. M., Matthews, G. M., de Valle, E., Moujalled, D. M., Menon, M. B., *et al.* (2016) Targeting p38 or MK2 enhances the anti-leukemic activity of Smac-mimetics. *Cancer Cell* **29,** 145–158 CrossRef Medline

60. Jaco, I., Annibaldi, A., Lalaoui, N., Wilson, R., Tenev, T., Laurien, L., Kim, C., Jamal, K., Wicky John, S., Liccardi, G., Chau, D., Murphy, J. M., Brumatti, G., Feltham, R., Pasparakis, M., *et al.* (2017) MK2 phosphorylates RIPK1 to prevent TNF-induced cell death. *Mol. Cell* **66,** 698–710.e5 CrossRef Medline
61. Menon, M. B., Gropengiesser, J., Fischer, J., Novikova, L., Deuretzbacher, A., Lafera, J., Schimmeck, H., Czymmeck, N., Ronkina, N., Kotlyarov, A., Aepfelbacher, M., Gaestel, M., and Ruckdeschel, K. (2017) $p38^{MAPK}$/MK2-dependent phosphorylation controls cytotoxic RIPK1 signalling in inflammation and infection. *Nat. Cell Biol.* **19,** 1248–1259 CrossRef Medline
62. Dondelinger, Y., Delanghe, T., Rojas-Rivera, D., Priem, D., Delvaeye, T., Bruggeman, I., Van Herreweghe, F., Vandenabeele, P., and Bertrand, M. J. M. (2017) MK2 phosphorylation of RIPK1 regulates TNF-mediated cell death. *Nat. Cell Biol.* **19,** 1237–1247 CrossRef Medline
63. Weng, D., Marty-Roix, R., Ganesan, S., Proulx, M. K., Vladimer, G. I., Kaiser, W. J., Mocarski, E. S., Pouliot, K., Chan, F. K., Kelliher, M. A., Harris, P. A., Bertin, J., Gough, P. J., Shayakhmetov, D. M., Goguen, J. D., *et al.* (2014) Caspase-8 and RIP kinases regulate bacteria-induced innate immune responses and cell death. *Proc. Natl. Acad. Sci. U.S.A.* **111,** 7391–7396 CrossRef Medline
64. Degterev, A., Huang, Z., Boyce, M., Li, Y., Jagtap, P., Mizushima, N., Cuny, G. D., Mitchison, T. J., Moskowitz, M. A., and Yuan, J. (2005) Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. *Nat. Chem. Biol.* **1,** 112–119 CrossRef Medline
65. Chavez-Valdez, R., Martin, L. J., Flock, D. L., and Northington, F. J. (2012) Necrostatin-1 attenuates mitochondrial dysfunction in neurons and astrocytes following neonatal hypoxia-ischemia. *Neuroscience* **219,** 192–203 CrossRef Medline
66. Northington, F. J., Chavez-Valdez, R., Graham, E. M., Razdan, S., Gauda, E. B., and Martin, L. J. (2011) Necrostatin decreases oxidative damage, inflammation, and injury after neonatal HI. *J. Cereb. Blood Flow Metab.* **31,** 178–189 CrossRef Medline
67. Smith, C. C., Davidson, S. M., Lim, S. Y., Simpkin, J. C., Hothersall, J. S., and Yellon, D. M. (2007) Necrostatin: a potentially novel cardioprotective agent? *Cardiovasc. Drugs Ther.* **21,** 227–233 CrossRef Medline
68. Linkermann, A., Bräsen, J. H., Himmerkus, N., Liu, S., Huber, T. B., Kunzendorf, U., and Krautwald, S. (2012) Rip1 (receptor-interacting protein kinase 1) mediates necroptosis and contributes to renal ischemia/reperfusion injury. *Kidney Int.* **81,** 751–761 CrossRef Medline
69. Blander, J. M. (2014) A long-awaited merger of the pathways mediating host defence and programmed cell death. *Nat. Rev. Immunol.* **14,** 601–618 CrossRef Medline
70. Olayioye, M. A., Kaufmann, H., Pakusch, M., Vaux, D. L., Lindeman, G. J., and Visvader, J. E. (2005) XIAP-deficiency leads to delayed lobuloalveolar development in the mammary gland. *Cell Death Differ.* **12,** 87–90 CrossRef Medline
71. Marsh, R. A., Madden, L., Kitchen, B. J., Mody, R., McClimon, B., Jordan, M. B., Bleesing, J. J., Zhang, K., and Filipovich, A. H. (2010) XIAP deficiency: a unique primary immunodeficiency best classified as X-linked familial hemophagocytic lymphohistiocytosis and not as X-linked lymphoproliferative disease. *Blood* **116,** 1079–1082 CrossRef Medline
72. McComb, S., Shutinoski, B., Thurston, S., Cessford, E., Kumar, K., and Sad, S. (2014) Cathepsins limit macrophage necroptosis through cleavage of Rip1 kinase. *J. Immunol.* **192,** 5671–5678 CrossRef Medline
73. Sad, S., Marcotte, R., and Mosmann, T. R. (1995) Cytokine-induced differentiation of precursor mouse $CD8^+$ T cells into cytotoxic $CD8^+$ T cells secreting Th1 or Th2 cytokines. *Immunity* **2,** 271–279 CrossRef Medline

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