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Differentiated Macrophages Acquire a Pro-Inflammatory and Cell Death-Resistant Phenotype Due to Increasing XIAP and P38-mediated inhibition of RipK1

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Running Title: Macrophage differentiation inhibits ripoptosome signaling.

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ABSTRACT

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. While 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 receptor (TNF-R) engagement induces the formation of a cell survival complex that promotes activation of NFκB and MAP kinases (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. Monocytes represent ~10% of the circulating leucocytes 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). While 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 mechanisms 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 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, in comparison to cells harvested at day 5 of differentiation (**Fig. 1 D**). Macrophages isolated at day 5 of differentiation showed slightly more proliferative potential in comparison to day 12 macrophages, as revealed by the expression of Ki67 (**Fig. 1 E, 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 that 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 *Salmonella typhimurium* infection *in vivo*, which correlated with decreased macrophage cell death, decreased ROS production, and decreased activation of caspase-8 (30). We therefore evaluated the impact of K45A mutation of RipK1 on BP-induced cell death. Our results indicate that K to A 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 K45 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, J**). In the absence of *Tnfα*, phosphorylation of RipK1 (upper band) and MK2 was reduced (**Fig. 2 K, L**).

Impaired ripoptosome signaling in differentiated macrophages

Since 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 BP-induced cell death 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, D**). BP treatment did not induce any phosphorylation of RipK3 in day 5 or day 12 macrophages. Since 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. 3 G**). 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 ripoptosome pathway that is less dependent on the kinase activity of RipK1, but more shifted towards 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 zVAD 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, 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+zVAD (**Fig. 4 C**), although the potency of this resistance was not as strong as was observed with BP alone. Day 12 macrophages displayed significant resistance to necroptosis induced by TNFα+zVAD (**Fig. 4 D**). Necroptosis was also induced in macrophages by treatment with LPS+zVAD, which was dependent on Ifnar1 and RipK3 (**Fig. 4 E, F**). Here again, day 12 macrophages displayed a small but significant resistance to cell death induced by LPS+zVAD (**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+zVAD resulted in phosphorylation of RipK1 and RipK3 in both day 5 and day 12 macrophages (**Fig. 4 I, 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 NFκB 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 NFκB showed similar results (**Fig. S3 A, 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 non-canonical NFκB pathway. Treatment with BP resulted in rapid degradation of cIAP1/2 in macrophages 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, 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, I**). XIAP did not appear to regulate cFLIP expression or RipK1 phosphorylation in day 12 macrophages; however, the phosphorylation of MK2 was enhanced in XIAP-deficient macrophages (**Fig. 5 J, K**). While 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+zVAD 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 p38MAPK 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 p38MAPK, was reduced in TNFα-deficient macrophages (**Fig. 2 K, L**), we evaluated the role of p38MAPK signaling in cell death resistance of day 12 macrophages. Our results indicate that prolonged macrophage differentiation results in reduced phosphorylation of p38MAPK and MK2 in response to BP treatment (**Fig. 6 A, S4 A, B**). Inhibiting p38MAPK abrogates their resistance to cell death in response to BP (**Fig. 6 B**), with a corresponding loss of MK2 phosphorylation (**Fig. 6 C, S4 C**) and an increase in caspase-8 activation (**Fig. S4 D, E**). There was no impact of inhibition of p38MAPK on the expression of XIAP or cFLIP (**Fig. 6 C, S4 F, G**). Day 12 macrophages displayed increased activation of JNK and ERK, which were elevated further by inhibition of p38MAPK (**Fig. 6 D, S4 H, I**). Unlike the impact of p38MAPK (**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 opposite results were obtained following BP treatment (**Fig. 6 I**). MK2 deficiency abrogated the resistance of day 12 macrophages to BP-induced cell death (**Fig. 6 J**), which correlated with increased activation of caspase-8 (**Fig. 6 L, S4 J**) and loss of RipK1 phosphorylation (**Fig. 6M, S4 K**). There was no impact of MK2 on the expression of XIAP (**Fig. 6 M**). Enhancement in cell death by inhibition of p38MAPK occurred through the ripoptosome pathway, as it was inhibited by Nec-1, not GSK872, and was still dependent on the K45 kinase region of RipK1 (**Fig. S4 L**). Taken together, these results indicate that activation of the p38MAPK-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 naïve 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 naïve 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 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 zVAD/GSK872 (**Fig 7D**). Based on these results, we propose that RipK1-mediated cell death pathways specifically target actively proliferating early macrophages, and selects 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 is 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 upregulation of endogenous inhibitors of cell death, which promote resistance against ripoptosome signaling.

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, while the expression of Ly6C, a monocyte marker (35) was diminished. CD11b is another marker of macrophage differentiation (36), which 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 naïve 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 is dependent 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 ripoptosome-induced cell death (41). Our results reveal a hierarchical role for various mediators in BP-induced cell death. TNFα and the K45 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. While 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. Since ripoptosome induced cell death is dependent 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. Since XIAP does not appear to impact necrosome signaling, it is conceivable that the marginal resistance of differentiated macropahges 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 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). While 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). While $cFLIP_L$ 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 case of ripoptosome signaling, $cFLIP_L$ has been shown to inhibit cell death, whereas the short isoform of cFLIP promotes ripoptosome signaling (40). We have failed to detect the expression of short isoform of cFLIP 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.

NFκB 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 NFκB signaling (12,54). In steady state, a cIAP1/2-TRAF2-TRAF3 complex regulates non-canonical NF-κB through constitutive K48-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 non-canonical 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 K63 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 NFκB 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 p38MAPK-MK2 pathway promotes the stabilization of TNFα expression (58). In contrast, during ripoptosome signaling in myeloid leukemia cells, p38MAPK 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 is dependent 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 p38MAPK, MK2, and RipK1. Rather, we observed that in day 12 macrophages, the phosphorylation of p38MAPK, 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 p38MAPK-MK2 pathway is reduced in differentiated macrophages during ripoptosome signaling, yet inhibition of the p38MAPK-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 K45A mutation of RipK1 reduces cell death, ROS production, and caspase-8 activation during infection of macrophages with *Salmonella typhimurium*, impairing *Salmonella typhimurium* control *in vivo* (30). RipK1 promotes capsase-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). Since 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 turnover more quickly to aid pathogen clearance and return to homeostasis. While 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 #0664), *TNF-R1,2*$^{-/-}$ (Stock #03243), *TNF-R1*$^{-/-}$ (Stock #03242), *TNF-R2*$^{-/-}$ (Stock #02620), *TNF*$\alpha^{-/-}$ (Stock #05540), *Trif-mutant* (Stock #05037), *Myd88*$^{-/-}$ (Stock #09088) and *NIK*$^{-/-}$ (Stock #025557) were obtained from Jackson Laboratory (USA). *RipK1*^{K45A} were obtained from Dr. Peter J. Gough (GSK, Philadelphia, USA), *RipK3*$^{-/-}$ were obtained from Dr. Visva Dixit (Genentech, USA), *MK2*$^{-/-}$ were obtained from Dr. Matthias Gaestel (Hanover Medical School, Germany), *XIAP*$^{-/-}$ were obtained from Dr. Robert Korneluk (Children's Hospital of Eastern Ontario, Canada) and *Ifnar1*$^{-/-}$ were obtained from Dr. Kaja Murali Krishna (Emory University, USA). Mice were maintained at the animal facility of the University of Ottawa, Faculty of Medicine. All procedures were approved by uOttawa Animal Care Committee.

Cell isolation, cell culture and viability measurements: Monocytes were purified from the bone marrow using beads obtained from Stem Cell 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 (Vancouver, Canada). Macrophages were plated in 96 well flat bottomed plate (Falcon, USA). Cells (7×10^4 per 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 hours, unless otherwise indicated, and cell viability was measured by various assays. Cell viability was measured by MTT uptake or by 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. (#33342) and used at a concentration of 2.5. Propidium iodide (PI) was obtained from BD Biosciences (550825) and used at a 1:10 dilution. Cell viability was measured by imaging cells under AxioObserver D1 microscope and the AxioVision Rel. 4.8 program was used to capture and analyze images.

In case of splenic macrophages, non-fractionated 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 determined by Flow cytometry.

Inhibitors and reagents: The SMAC mimetic, Birinapanat (BP, #S7015) was obtained from Selleckchem (#S7015) and used at a concentration of 10 µM. The SMAC mimetic BV6 was obtained from Genentech Inc. USA, and also used at a concentration of 10 µM. Ultrapure LPS from E.coli 055:B5 was obtained from Sigma Aldrich USA (#L4524) and used at a concentration of 1 ng/ml, if not indicated otherwise. Recombinant mouse TNFα was obtained from R&D systems USA (#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 USA (#S5567) and used at a concentration of 20 µM. Inhibitor of p38MAPK, Ralimetinib was obtained from Selleckchem (#S1494) and used at a concentration of 5 µM. RipK1 inhibitor, Nec-1, was obtained from Sigma-Aldrich, USA (#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 (zVAD-FMK, #A1902) and Caspase-8 inhibitor (zIETD-FMK, #B3232) were obtained from ApexBio USA (#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, USA and used at a concentration of 50 µM. The following inhibitors against NFκB 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 one hour. A PVDF membrane was soaked in 100% methanol and washed with the transfer buffer, then the transfer was run at 100V for an hour. Membrane was blocked in 5% skim milk in Tris Buffered Saline Solution (TBS – 0.5M Tris, 1.5 NaCl pH 7) with 0.5% Tween-20 (TBST) or 5% bovine serum albumin (BSA). The following primary antibodies were used for western blotting: mouse anti-RipK1 (BD Biosciences, #610459), rabbit anti-RipK3 (ProSci Inc, #2283), mouse anti-β-actin (BD Biosciences, #612656), rabbit anti-cIAP1/2 (Cyclex, #CY-P1040), rabbit anti-p38MAPK (Cell signaling, #8690), rabbit anti- phospho p38MAPK (Cell signaling, #4511), rat anti-caspase-8 (Enzo, #ADI-AAM-212-E), rabbit anti-XIAP (Cell signaling, #2042), rabbit anti-cFLIP (Santa-Cruz, #sc-8347), rabbit anti-MK2 (Cell signaling, #3007), rabbit anti-phospho MK2 (Cell signaling, #3042), rabbit anti-ERK1/2 (Cell signaling, #4695), rabbit anti-phospho ERK1/2 (Cell signaling, #4370), rabbit anti-JNK (Cell signaling, #9252), rabbit anti-phospho-JNK (Cell signaling, #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 #32106) or West Femto ECL substrate (Thermo Scientific #34095). Photosensitive film (Sigma-Aldrich, Carestream Health #785019) was used to examine protein expression. Results were quantified using densitometric analysis. The densitometric quantification of western blot signals was performed using ImageJ 1.48 software.

Flow cytometry: Cells were washed with staining buffer (1% Bovine serum albumin in PBS). FcBlock (anti-CD16/32) (BD Biosciences #553142) was added to the cells in the staining buffer and incubated for 10 minutes at 4^{o}C. Various flurophore-conjugated antibodies were added in the staining buffer. Cells were protected from the light and incubated with the antibodies for 30 minutes at 4^{o}C. After that, cells were washed with the staining buffer and re-suspended 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) and analyzed using Kaluza software (Beckman Coulter, version 1.2) or FlowJo V10 (FlowJo). The following antibodies were obtained from eBiosciences, USA: 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, Catalog #G8200), Caspase-Glo 9 (Promega, Catalog #G8210) and Caspase-Glo 3/7 (Promega, Catalog #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^{TM} F5 plate reader (Molecular Devices).

Immunoprecipitation: Cell lysates were immunoprecipitated with Dynabeads co-immunoprecipitation kit (Invitrogen, Life Technologies, Catalog #14321D, Burlington, Canada) with rat anti-caspase 8 (ENZO, Catolog. #1G12). The caspase 8 antibody was coupled with the dynabeads with incubation at 37^{o}C for overnight and then washing with various buffers. Next day, BMDMs were treated with Birinapant for 4 hours. Then, the cells were washed with ice-cold 1xPBS and lysed in IP lysis buffer. The lysates were sonicated for 10 seconds and then centrifuged at 5000xg for 5 minutes at 4°C to remove large debris and nuclei. 10µl of the supernatant was stored as input and the remaining clarified lysate was then incubated with antibody-coupled dynabeads for 16-24 hours at 4°C on a rocker platform. The unbound fractions were saved at -20°C and the bound proteins were eluted. 10µl of the 4X SDS-loading dye was added to the eluent and boiled for 5 mins. The final eluent was analyzed by western blotting.

Cytokine production: Cytokine expression by macrophages was measured in the supernatant at 24h 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 standard error of the mean. Unpaired two-tailed student's t-test or one-way ANOVA followed by a Bonferroni post-hoc test was used to determine statistical significance. All statistical analysis were performed using Graphpad Prism software.

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Author contributions: DR, AA, AW, KK, NA, ZA, ESA performed experiments and analyzed data. CT, MBM provided additional cell samples. RGK, MG, SM provided additional reagents. SS wrote the paper with SM.

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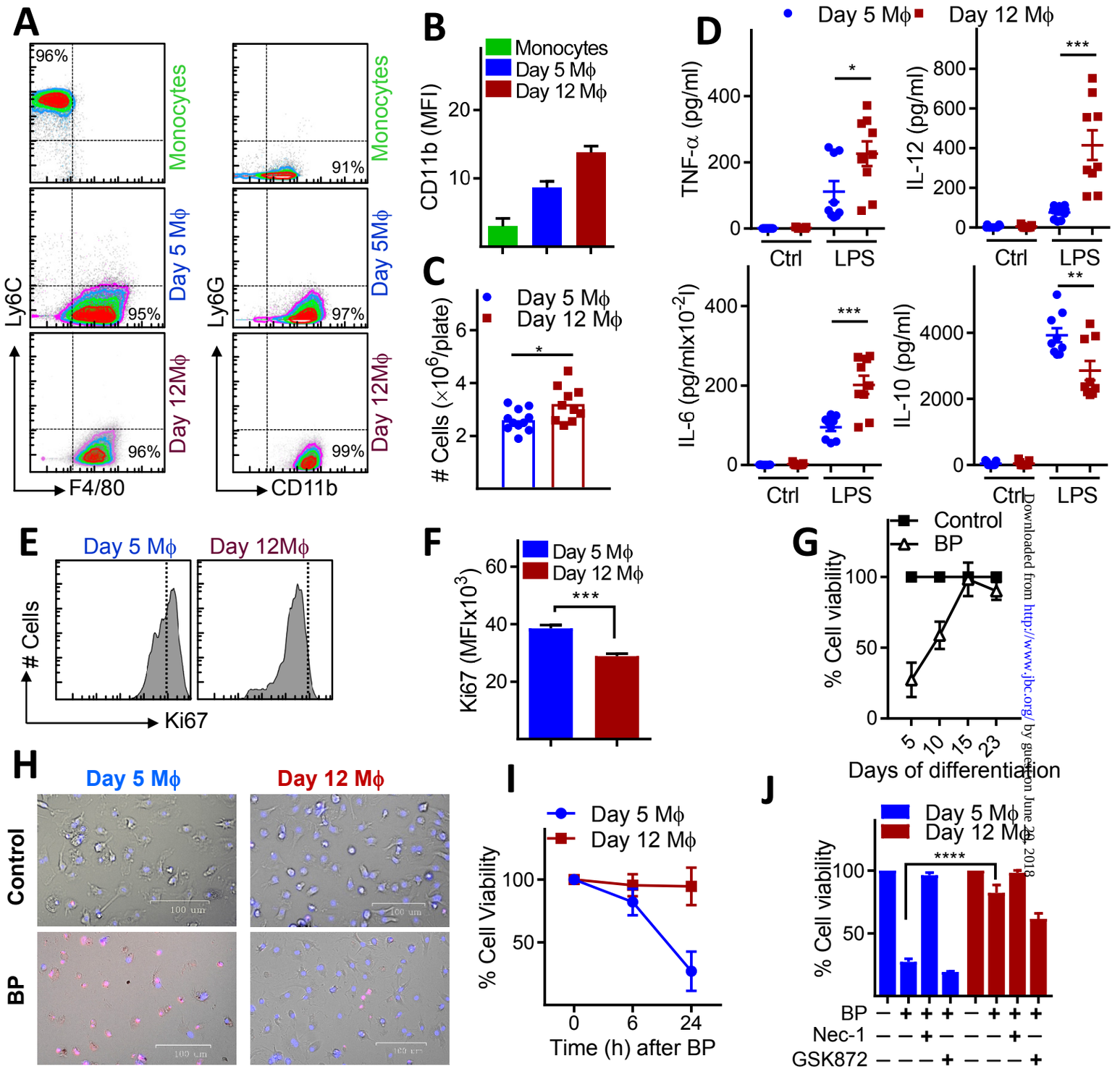

Figure 1: Differentiation of macrophages results in resistance to ripoptosome–induced cell death. Monocytes were isolated from the bone marrow of C57BL/6J (WT) mice and differentiated into macrophages with M-CSF, and the expression of Ly-6C, F4/80, and CD11b evaluated by flow cytometry (A, B). Cell yield 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 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, 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 evaluated 24h later by MTT uptake (G), or at 12 h by PI/Hoecst 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 thrice with triplicate samples. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

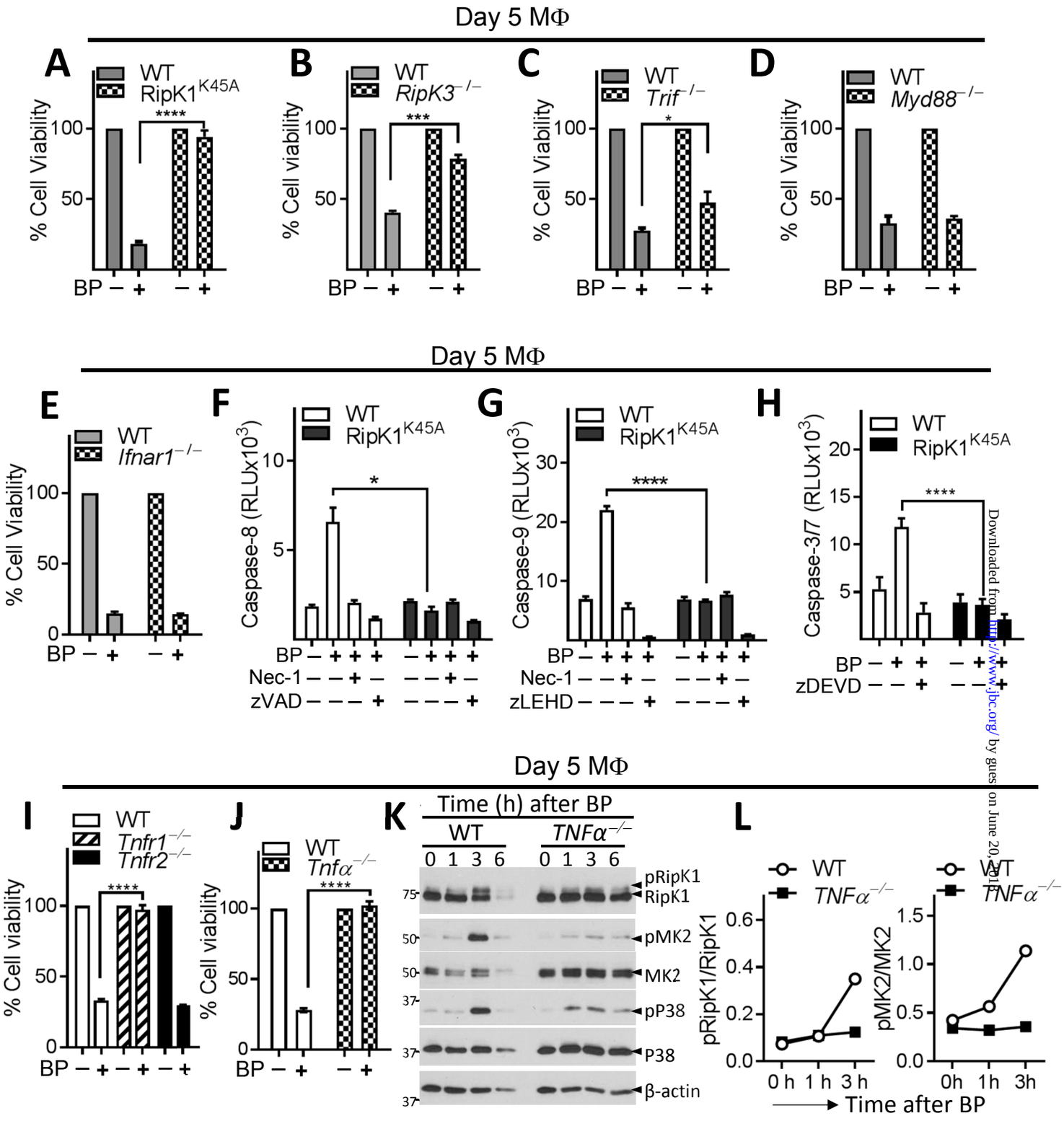

Figure 2: Early macrophages undergo cell death through a typical ripoptosome pathway. Bone marrow derived macrophages were harvested at day 5 post-differentiation with M-CSF and treated with BP (10 μM) (A-L). Cell death was evaluated at 24 h post-treatment with BP by MTT uptake (A-E, I, J). Macrophages 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 post BP-treatment of WT and TNFα$^{-/-}$ macrophages and the activation of RipK1, MK2, p38MAPK evaluated by western blotting (K). Densitometric analysis of western blots was performed (L). Each experiment was repeated thrice with triplicate samples. *P < 0.05, ***P < 0.001, ****P < 0.0001.

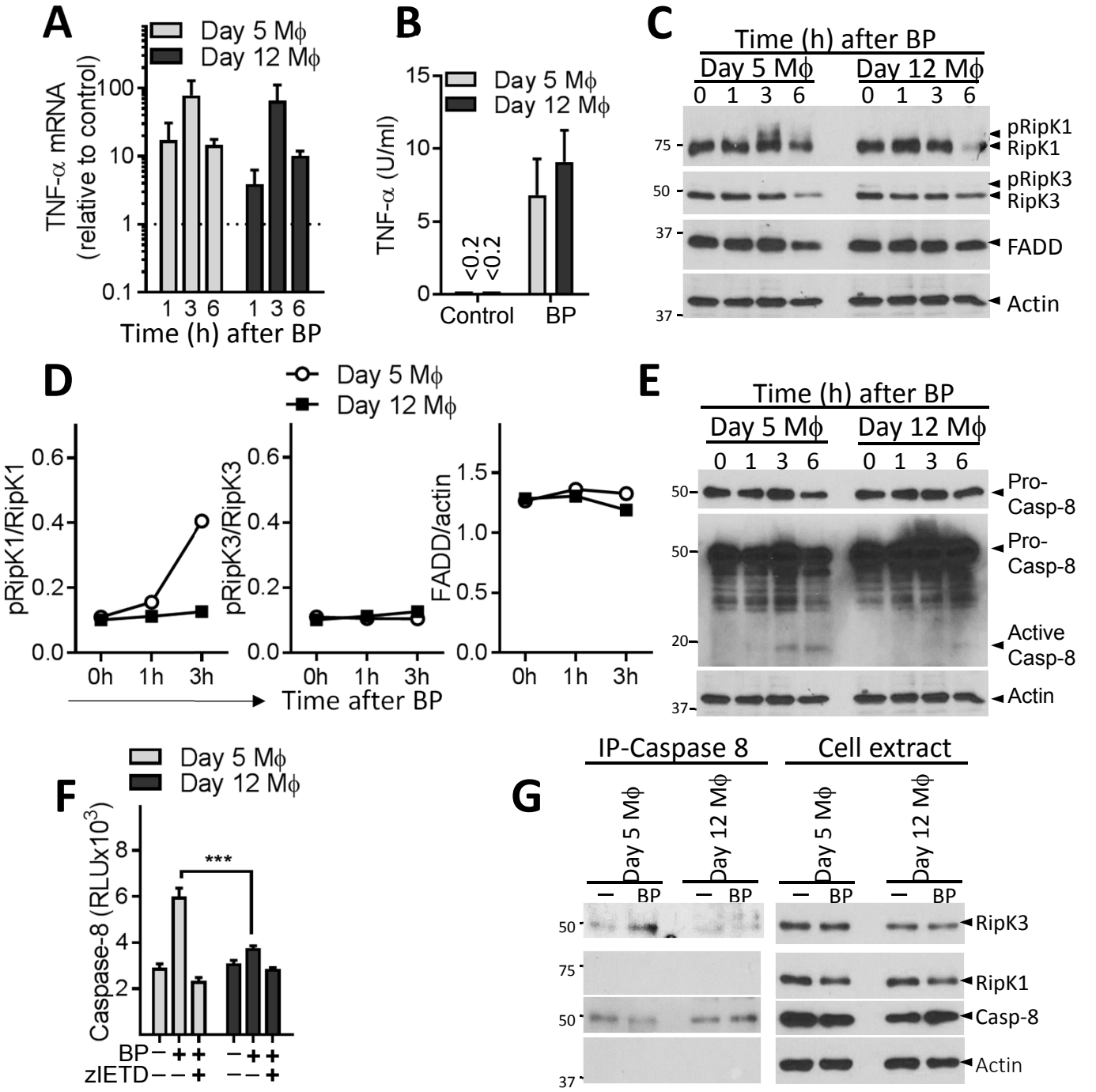

Figure 3: Macrophage differentiation results in impairment in RipK1 phosphorylation and caspase-8 activation. Macrophages from WT mice were harvested at day 5 or day 12 post-differentiation with M-CSF, treated with BP (10 μM) for various time intervals, and the expression of TNFα was evaluated at various time intervals by qRT-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 post 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 revealed by western blotting of immunoprecipitates (G). Western blotting was also performed in non-immunoprecipitated cell extracts. Each experiment was repeated thrice with triplicate samples. ***$P < 0.001$. Western blot of actin is reused in panels C, D and in Fig. 5 panel D.

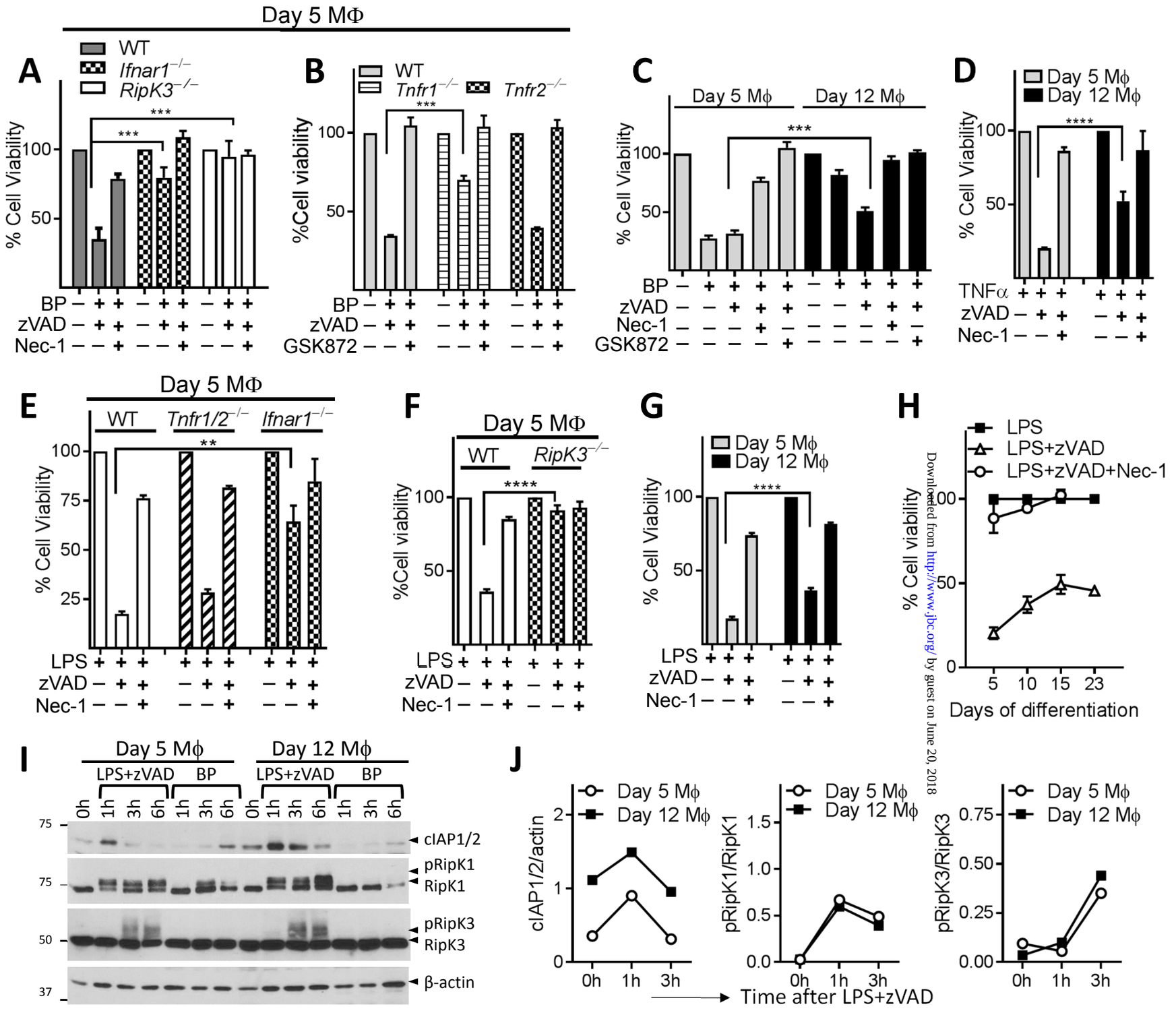

Figure 4: Macrophage differentiation results in a partial resistance to necrosome–induced cell death. Bone marrow derived macrophages were treated with BP (10 μM) and zVAD (50 μM) in the absence or presence of Nec-1 (10 μM) or GSK872 (5 μM) and cell death evaluated 24 h later by MTT assay (A-C). Macrophages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

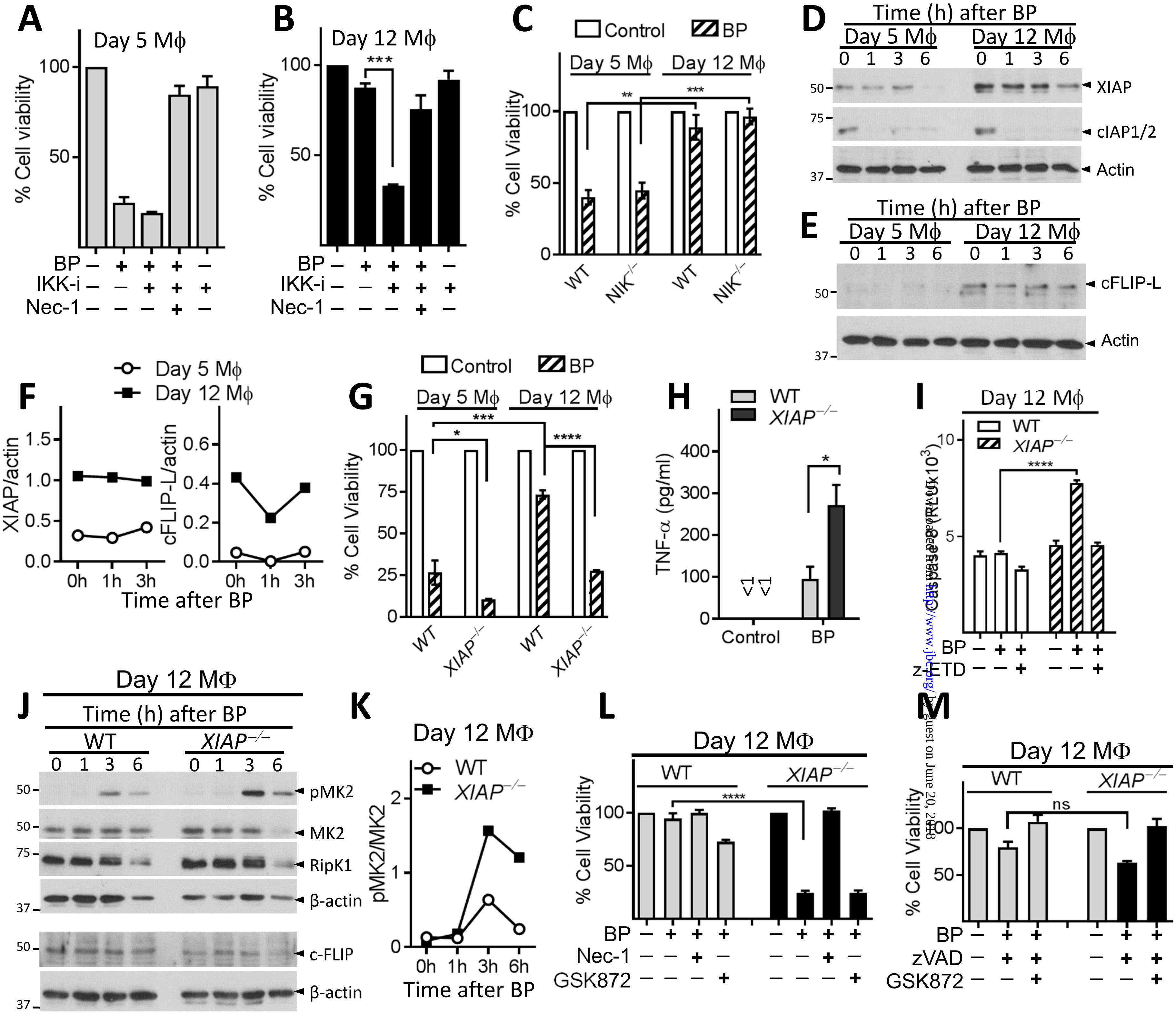

Figure 5: Macrophage differentiation results in increased expression of XIAP which promotes resistance of differentiated macrophages to ripoptosome–induced cell death. Bone marrow derived macrophages were harvested from WT (A, B, C) or *NIK*$^{-/-}$ (C) mice at day 5 or day 12 post-differentiation and treated with BP (10 µM) in the presence or absence of NF-kB inhibitor (JSH-23, 1 µM) or Nec-1 (10 µM) (A, B). Cell death was evaluated at 24 h by MTT assay. Western blot (D, 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 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 blot (J) and corresponding densitometric analysis (K) was 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+zVAD (M) in the presence or absence of Nec-1 or GSK872, and cell death evaluated 24 h later by MTT assay. Each experiment was repeated thrice with triplicate samples. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. Western blot of actin shown in panel D is reused in Fig. 3 panels C, E.

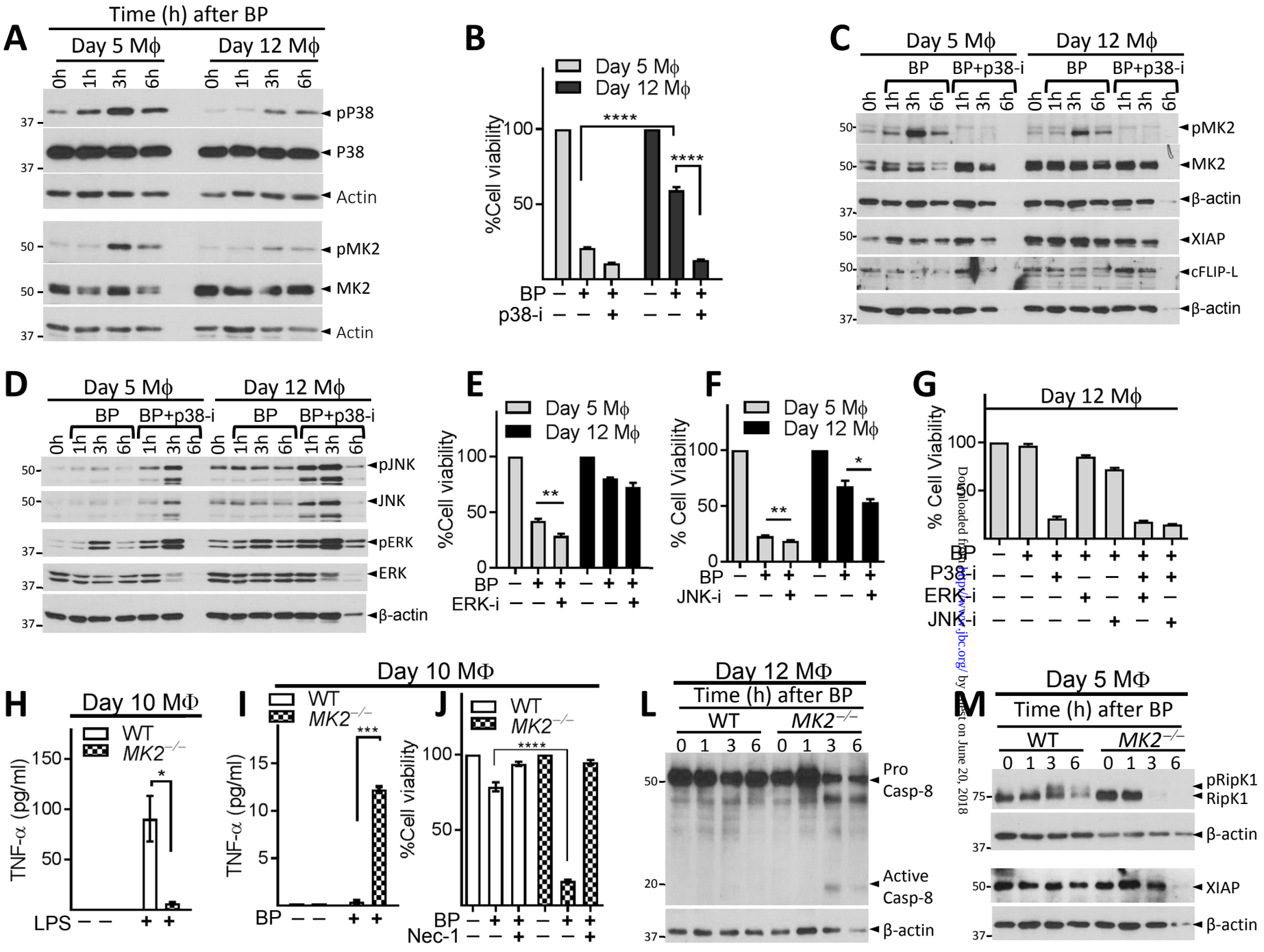

Figure 6: Inhibition of p38MAPK pathway restores cell death of differentiated macrophages during ripoptosome signaling. Bone marrow derived macrophages were harvested from WT mice at day 5 or day 12 post-differentiation and treated with BP (10 μM), and cell extracts evaluated by western blotting (A). Macrophages were treated with BP (10 μM) and/or p38MAPK inhibitor (Ralimetinib, 5 μM) and cell death evaluated 24 h later by MTT assay (B). Cells were treated as described in panel B and western blot analysis performed on cell extracts at various time intervals (C, D). Macrophages were also treated with BP and/or MEK/ERK inhibitor (PD0325901, 20 nM) or JNK inhibitor (SP600125, 20 μM), and cell death 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 evaluated 24 h later (J). Expression of caspase-8 (L), and RipK1 and XIAP (M) was evaluated by western blotting of cell extracts at various time intervals post BP treatment. Each experiment was repeated thrice with triplicate samples. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

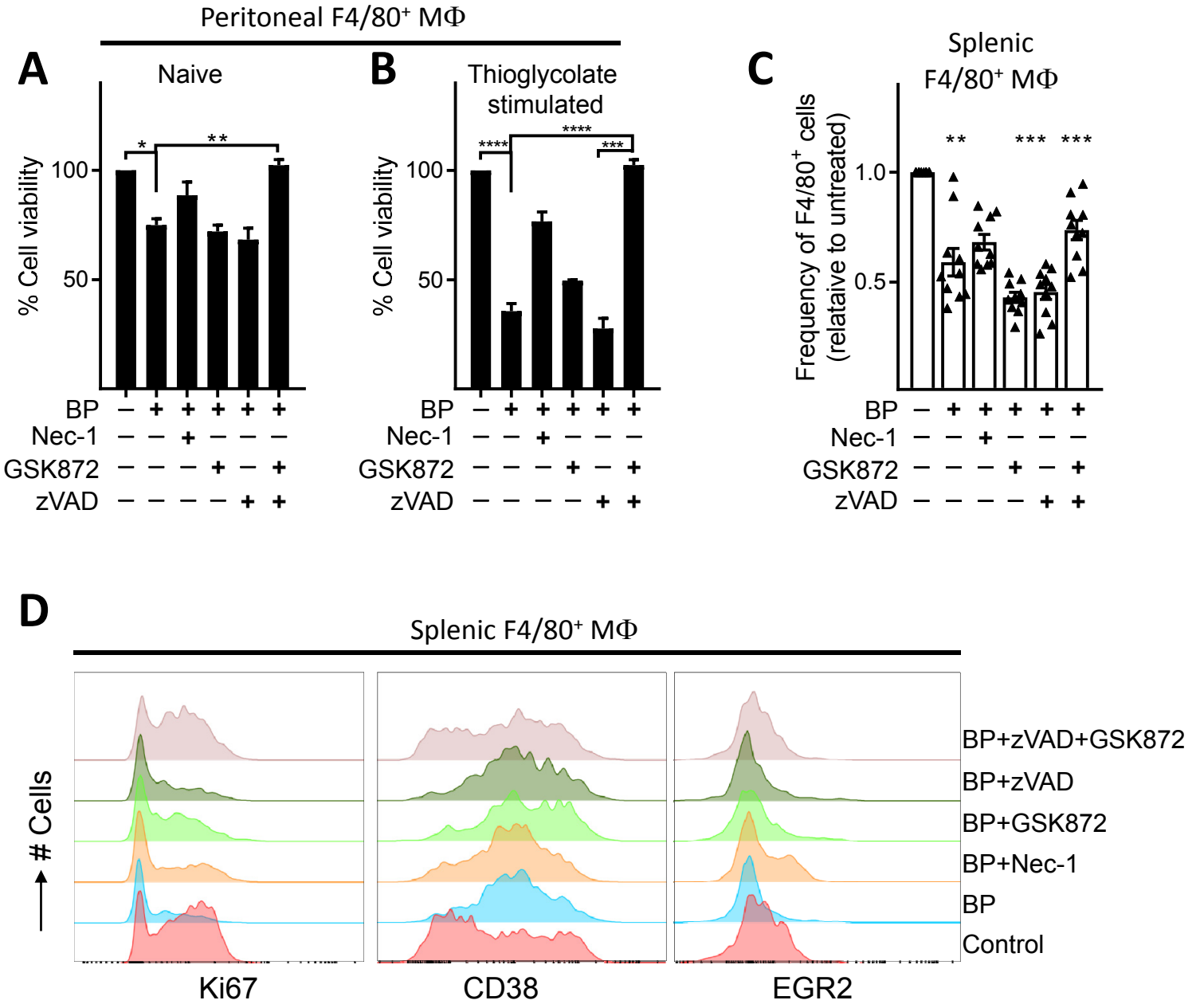

Figure 7: Activated and cycling macrophages are eliminated by ripoptosome signaling. Peritoneal macrophages from naïve (A) or thioglycolate injected (B) mice were treated with BP and/or Nec-1 (10 μM), GSK872 (5 μM), zVAD (10 μM). Cell death was evaluated 24 h later by MTT assay. Spleen cells from naïve mice (C, 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 acquired using a high-throughput port by Flow cytometry to ensure acquisition of 180 μl of each sample. 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 evaluated (D). Results are pooled from at least three experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

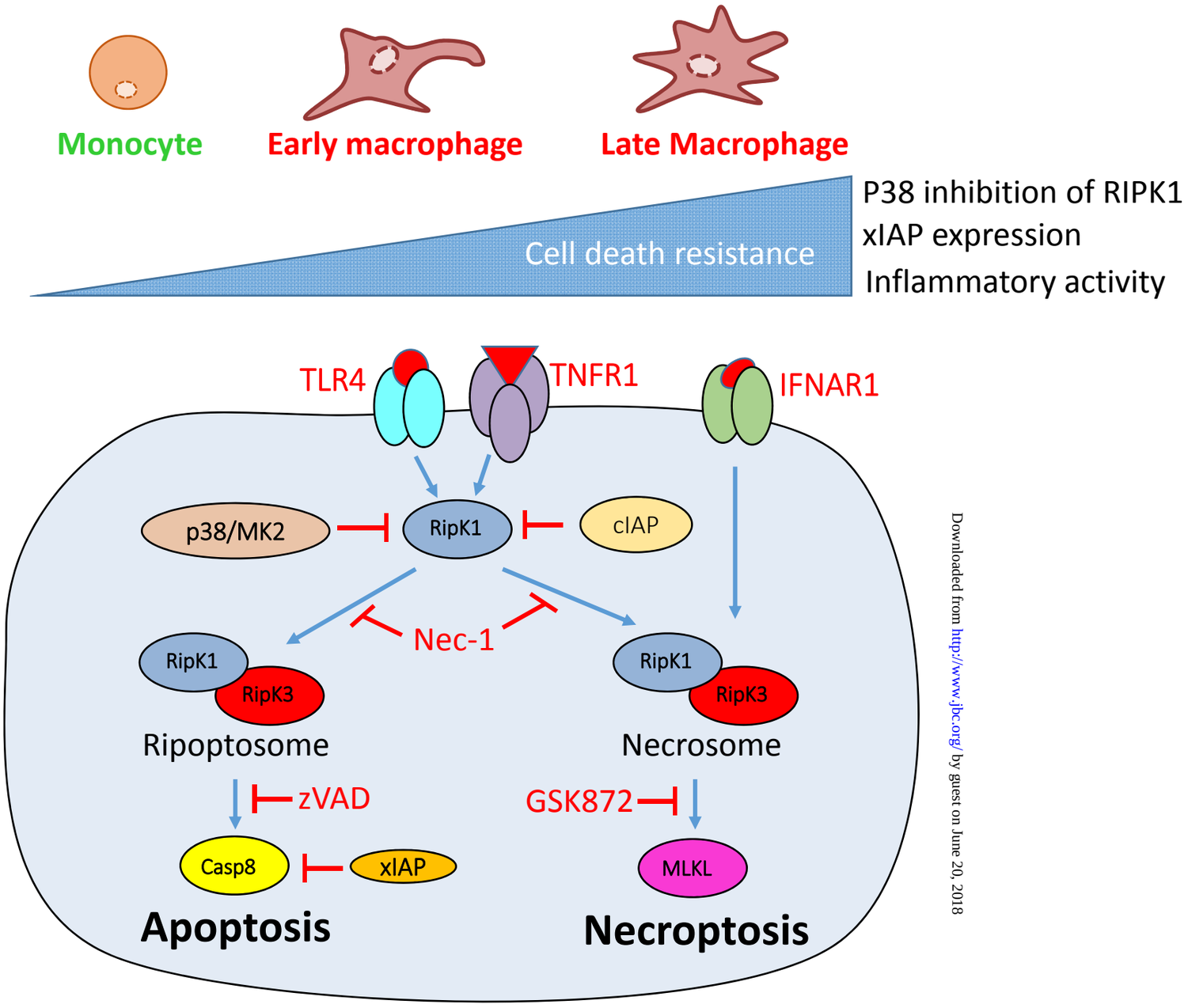

Figure 8: Differentiation of monocytes promotes resistance to Ripoptosome signaling. As monocytes differentiate to macrophages, the expression of XIAP increases which promotes resistance to cell death induced by Ripoptosome signaling. In addition to XIAP, the p38MAPK/MK2 pathway also promotes resistance 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.

Differentiated Macrophages Acquire a Pro-Inflammatory and Cell Death-Resistant Phenotype Due to Increasing XIAP and P38-mediated inhibition of RipK1

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