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BRIEF DEFINITIVE REPORT

TAK1 restricts spontaneous NLRP3 activation and cell death to control myeloid proliferation

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The NOD-like receptor (NLR)–P3 inflammasome is a global sensor of infection and stress. Elevated NLRP3 activation levels are associated with human diseases, but the mechanisms controlling NLRP3 inflammasome activation are largely unknown. Here, we show that TGF-β activated kinase-1 (TAK1) is a central regulator of NLRP3 inflammasome activation and spontaneous cell death. Absence of TAK1 in macrophages induced spontaneous activation of the NLRP3 inflammasome without requiring toll-like receptor (TLR) priming and subsequent activating signals, suggesting a distinctive role for TAK1 in maintaining NLRP3 inflammasome homeostasis. Autocrine tumor necrosis factor (TNF) signaling in the absence of TAK1 induced spontaneous RIPK1-dependent NLRP3 inflammasome activation and cell death. We further showed that TAK1 suppressed homeostatic NF-κB and extracellular signal–related kinase (ERK) activation to limit spontaneous TNF production. Moreover, the spontaneous inflammation resulting from TAK1-deficient macrophages drives myeloid proliferation in mice, and was rescued by RIPK1 deficiency. Overall, these studies identify a critical role for TAK1 in maintaining NLRP3 inflammasome quiescence and preserving cellular homeostasis and survival.

Introduction

NOD-like receptor (NLR)–P3 inflammasome activation leads to the maturation of proinflammatory cytokines IL-1β and IL-18, and induction of pyroptotic cell death (Sharma and Kanneganti, 2016). Thus, NLRP3 is central in guarding the host against microbial infections, including bacterial, viral, fungal, and protozoan infections (Anand et al., 2011). Gain-of-function mutations in the NLRP3 gene are associated with inflammatory syndromes collectively known as cyropyrin-associated periodic syndromes (CAPS; http://fmf.igh.cnrs.fr/ISSAID/infevers/; Gurung and Kanneganti, 2016). Conventionally, activation of the NLRP3 inflammasome requires a priming signal and an activating signal. Previous studies demonstrated that the first priming signal—often provided by TLRs—serves to up-regulate NLRP3 and pro–IL-1β (Bauernfeind et al., 2009). Some of the proposed mechanisms for regulating NLRP3 inflammasome activation include potassium efflux, calcium mobilization, mitochondrial damage, and production of ROS (Sharma and Kanneganti, 2016). Molecularly, NEK7 (Schmid-Burgk et al., 2016), cardiolipin (Iyer et al., 2013), and caspase-8/FADD (Gurung et al., 2014) have been shown to directly regulate the NLRP3 inflammasome. Additional studies suggested that deubiquitination of NLRP3 by IRAK proteins is required to assemble the inflammasome complex after receiving the second activation signal (Juliana et al., 2012; Py et al., 2013). Herein, we sought to investigate the role of TAK1, a central signaling molecule, in regulating NLRP3 inflammasome activation and cell death.

Programmed cell death is central to homeostasis and orchestrates normal organismal growth and development. Failure to control cell death programs often results in devastating inflammatory pathologies and disease. TAK1 is a quintessential kinase that plays key roles in cellular homeostasis by positively regulating cell survival and proinflammatory signaling pathways (Yamaguchi et al., 1995; Wang et al., 2001; Ninomiya-Tsuji et al., 2003; Sato et al., 2005; Shim et al., 2005; Wan et al., 2006; Hayden and Ghosh, 2008; Zhang et al., 2017). Whereas inactivation of TAK1 induces apoptosis or necroptosis (Sanna et al., 2002; Mihaly et al., 2014; Guo et al., 2016), hyperactivation of TAK1 under conditions of its enforced expression or TAB2 deletion promotes necroptosis (Morioka et al., 2014). TAK1 is important for lysosomal rupture–induced inflammasome activation (Okada et al., 2014) and hypotonic stimulation (altering cellular volume–induced inflammasome activation; Compan et al., 2012). Currently, there is a tremendous interest in TAK1 inhibition as a therapeutic application for inflammatory disease

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management and cancer immunotherapy (Sakurai, 2012; Singh et al., 2012; Huang et al., 2015; Kilty and Jones, 2015; Guan et al., 2017). However, prolonged TAK1 inactivation also results in severe inflammation, bone disorders, and cancer development in mice and humans (Shim et al., 2005; Omori et al., 2006; Kajino-Sakamoto et al., 2008, 2010; Tang et al., 2008; Bettermann et al., 2010; Inokuchi et al., 2010; Lamothe et al., 2013; Le Goff et al., 2016; Wade et al., 2016). These findings are paradoxical because TAK1 is a well-accepted upstream kinase that drives inflammation through NF-κB and MAPK signaling cascades (Zhang et al., 2017). Furthermore, inactivation of NF-κB by deletion of IKKβ, NEMO/IKKγ, upstream TAK1-activating TAB proteins, or downstream antiapoptotic cIAP1/2 does not result in similar cell death phenotypes, and often requires priming to induce cell death in vitro (Shim et al., 2005; Vanlangenakker et al., 2011; Dondelinger et al., 2013; Mihaly et al., 2014). Moreover, repression of the deubiquitinase CYLD protects cells from RIPK1-mediated apoptosis in the absence of cIAP1/2 but not in TAK1-inactivated conditions (Dondelinger et al., 2013). Although TAK1 prosurvival function in different cell types is well established, there are conflicting studies regarding the mechanism and nature of cell death observed in TAK1 KO cells. In some studies, both RIPK1 and RIPK3 have been shown to promote necrotic and apoptotic cell death in TAK1-deficient cells (Vanlangenakker et al., 2011; Guo et al., 2016); however, other studies report that RIPK1, RIPK3 or both are dispensable for the cell death observed in TAK1 KO cells (Morioka et al., 2014; Dondelinger et al., 2015; Mihaly et al., 2017). Overall, the molecular mechanisms responsible for hyperactivation of the inflammatory immune response seen in conditions of TAK1 inactivation remain poorly understood. Herein, we sought to investigate the role of TAK1, a central signaling molecule, in regulating NLRP3 inflammasome activation and cell death.

Here, we show that TAK1 is a central regulator of NLRP3 inflammasome homeostasis. Absence of TAK1 in macrophages induced spontaneous activation of the NLRP3 inflammasome without requiring the priming and activating signals, suggesting a distinctive role for TAK1 in maintaining NLRP3 inflammasome homeostasis. Autocrine TNF signaling in the absence of TAK1 induced spontaneous RIPK1-dependent activation of the NLRP3 inflammasome and cell death. Our data further suggested that TAK1 suppresses homeostatic NF-κB and ERK activation to limit spontaneous TNF production. Moreover, the spontaneous inflammation resulting from TAK1-deficient macrophages drove myeloid proliferation in mice, which was rescued by RIPK1 deficiency. Overall, these studies identify a critical paradigm for the maintenance of inflammasome quiescence to preserve myeloid cell homeostasis.

Results and discussion

TAK1 deficiency in myeloid cells results in spontaneous inflammasome activation and secretion of IL-1β and IL-18

TAK1 deficiency is embryonically lethal in mice (Sato et al., 2005; Shim et al., 2005); thus, to study the function of TAK1, we generated mice lacking TAK1 specifically in the myeloid compartment (*Lyz2*$^{cre+}$ × *Tak1*$^{f/f}$ mice). We observed that TAK1-deficient bone marrow–derived macrophages (BMDMs) underwent spontaneous cell death (Fig. 1, A and B). This was expected, given the important role of TAK1 in the survival of different cell lineages including T cells, B cells, osteoclasts, and hematopoietic stem cells (Sato et al., 2005). Herein, we observed that TAK1-deficient macrophages also induced spontaneous caspase-1 activation in the absence of both priming and activating signals (Fig. 1 C). A two-hit (priming and activation) model is well established and accepted for optimal activation of the inflammasomes (Bauernfeind et al., 2009). However, in certain conditions where the inflammasome sensors have gain-of-function mutations (as observed with NLRP3 [CAPS; Hoffman et al., 2001a,b], NLRC4 [MAS, macrophage activation syndrome; Canna et al., 2014], and Pyrin [FMF, familial Mediterranean fever; French FMF Consortium, 1997; The International FMF Consortium, 1997]), only a priming or an activating signal is sufficient to assemble and activate the inflammasome. Given our data that caspase-1 activation in the TAK1-deficient BMDMs did not require any external stimuli (both priming and activation signals were not required), this demonstrates a previously unknown central regulatory role for TAK1 in maintaining inflammasome quiescence. In agreement with our observations in TAK1-deficient BMDMs, chemical inhibition of TAK1 kinase activity (5Z-7-oxozeaenol, herein referred to as TAK1 inhibitor or TAK1i) in WT macrophages also induced spontaneous cell death and subsequent caspase-1 activation (Fig. 1, D–F). One of the hallmarks of caspase-1 activation is the production of processed cytokines IL-1β and IL-18 (Gurung et al., 2015). Consistently, mature IL-1β and IL-18 were detected in the supernatants from cultured TAK1-deficient macrophages in a steady-state condition (Fig. 1, G and H). In addition, mRNA levels of pro–IL-1β were also up-regulated at steady-state in TAK1-deficient but not WT macrophages (Fig. S2 H), whereas mRNA levels of pro–IL-18, which is constitutively expressed in macrophages, were similarly expressed in both WT and TAK1-deficient macrophages (Fig. S2 I). These data suggested that TAK1 is a central homeostatic regulator of inflammasome activation in macrophages. More importantly, given that TAK1 deficiency promoted spontaneous IL-1β release, which requires a priming signal, our data suggested that TAK1 restricts spontaneous inflammatory signaling to promote cellular quiescence and homeostasis.

NLRP3 promotes spontaneous inflammasome activation observed in TAK1-deficient macrophages

We next asked if this spontaneous caspase-1 activation was dependent on ASC, a central adaptor molecule for inflammasome. We found that TAK1i-induced caspase-1 activation was dependent on ASC (Fig. 2 A). To identify the upstream inflammasome sensor, NLRC4-, AIM2-, and NLRP3-deficient cells were assessed for TAK1i-induced caspase-1 activation. Contrary to NLRC4 and AIM2, NLRP3 proved essential for TAK1i-induced inflammasome activation (Fig. 2, D, G, and J). Given the spontaneous activation of caspase-1 in TAK1-deficient macrophages (Fig. 1), we posited that the cells undergoing pyroptotic cell death could be rescued by the deficiency of NLRP3 inflammasome components. However, TAK1i-treatment induced robust cell death in ASC-deficient BMDMs similar to that observed in WT BMDMs (Fig. 2, B and C). To determine if the inflammasome sensors were involved in the induction of cell

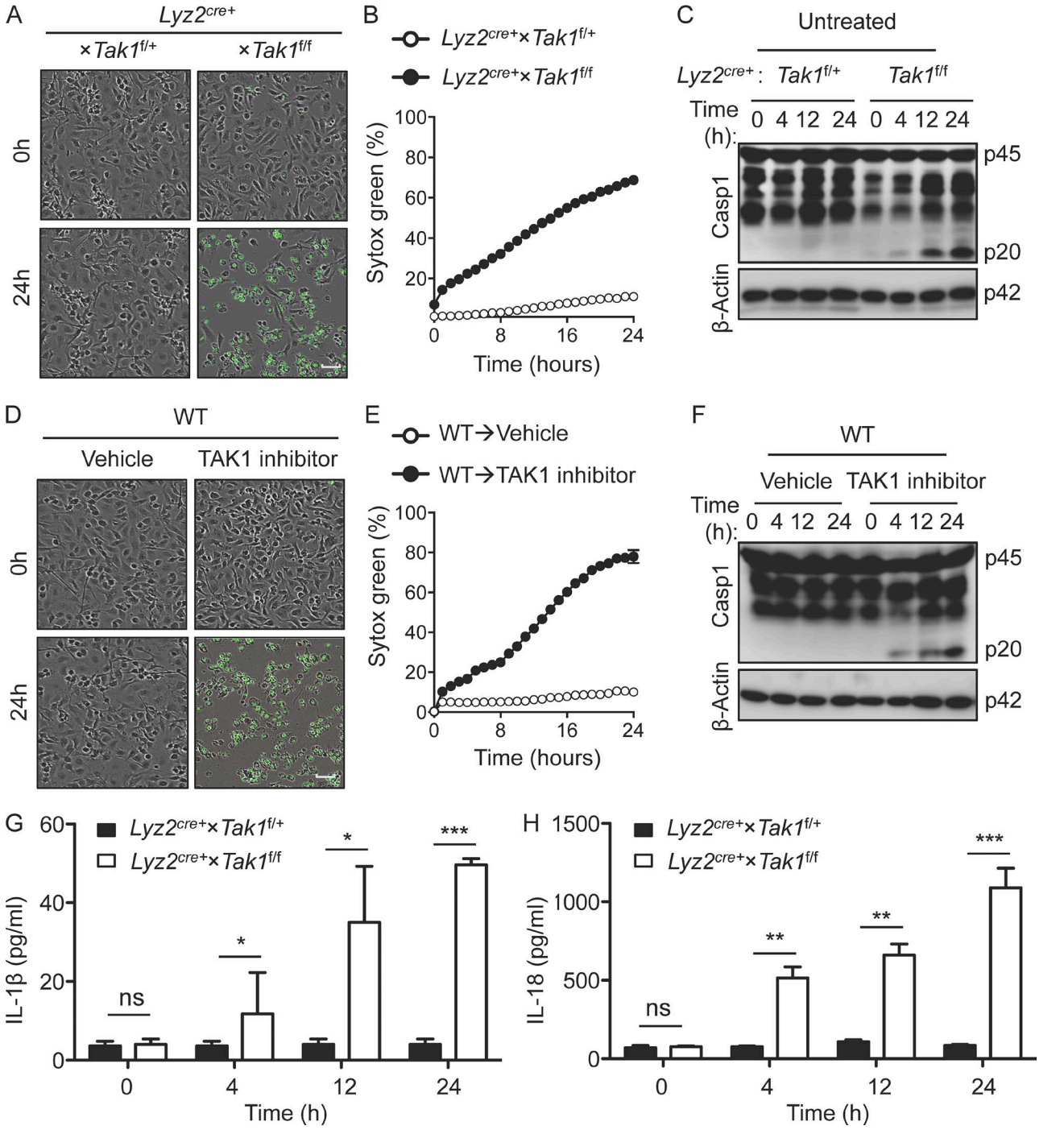

Figure 1. **TAK1 deficiency in myeloid cells results in spontaneous inflammasome activation and proinflammatory cytokine production. (A–C)** Cell death by Incucyte image analysis, (bar, 40 µm; A), time course quantification of dead cells (B), and immunoblot analysis of pro–caspase-1 (p45) and the active caspase-1 subunit p20 (p20; C) in unstimulated WT control (*Lyz2*$^{cre+}$ × *Tak1*$^{f/+}$) or TAK1-deficient BMDMs (*Lyz2*$^{cre+}$ × *Tak1*$^{f/f}$) assessed in culture at the indicated times after differentiation. **(D–F)** Cell death by Incucyte image analysis, (bar, 40 µm; D), time course quantification of dead cells (E), and caspase-1 activation (F) measured in BMDMs left unstimulated or treated with TAK1i for the indicated times in culture after differentiation. **(G and H)** Secretion of IL-1β (G) and secretion of IL-18 (H) in unstimulated *Lyz2*$^{cre+}$ × *Tak1*$^{f/f}$ (TAK1 KO) or WT BMDMs left untreated for the indicated times in culture. All data are presented as mean ± SEM (G and H). "p" in Western blots denotes protein molecular weight. $P < 0.05$ is considered statistically significant. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (two-tailed *t* test [G and H]). Data are representative of three independent experiments with $n = 2$ (A–F) and $n = 3$ in each repeat (G and H).

death, we treated NLRC4-, AIM2-, and NLRP3-deficient BMDMs with TAK1i. TAK1i-treatment induced similar cell death in both WT and the inflammasome sensor–deficient BMDMs (Fig. 2, E, F, H, I, K, and L).

To further establish the role of NLRP3 in TAK1-regulated spontaneous inflammasome activation, we generated BMDMs from mice that lacked both TAK1 and NLRP3 (*Lyz2*$^{cre+}$ × *Tak1*$^{f/f}$ × *Nlrp3*$^{-/-}$ mice). Similar to the results obtained with TAK1i treatment,

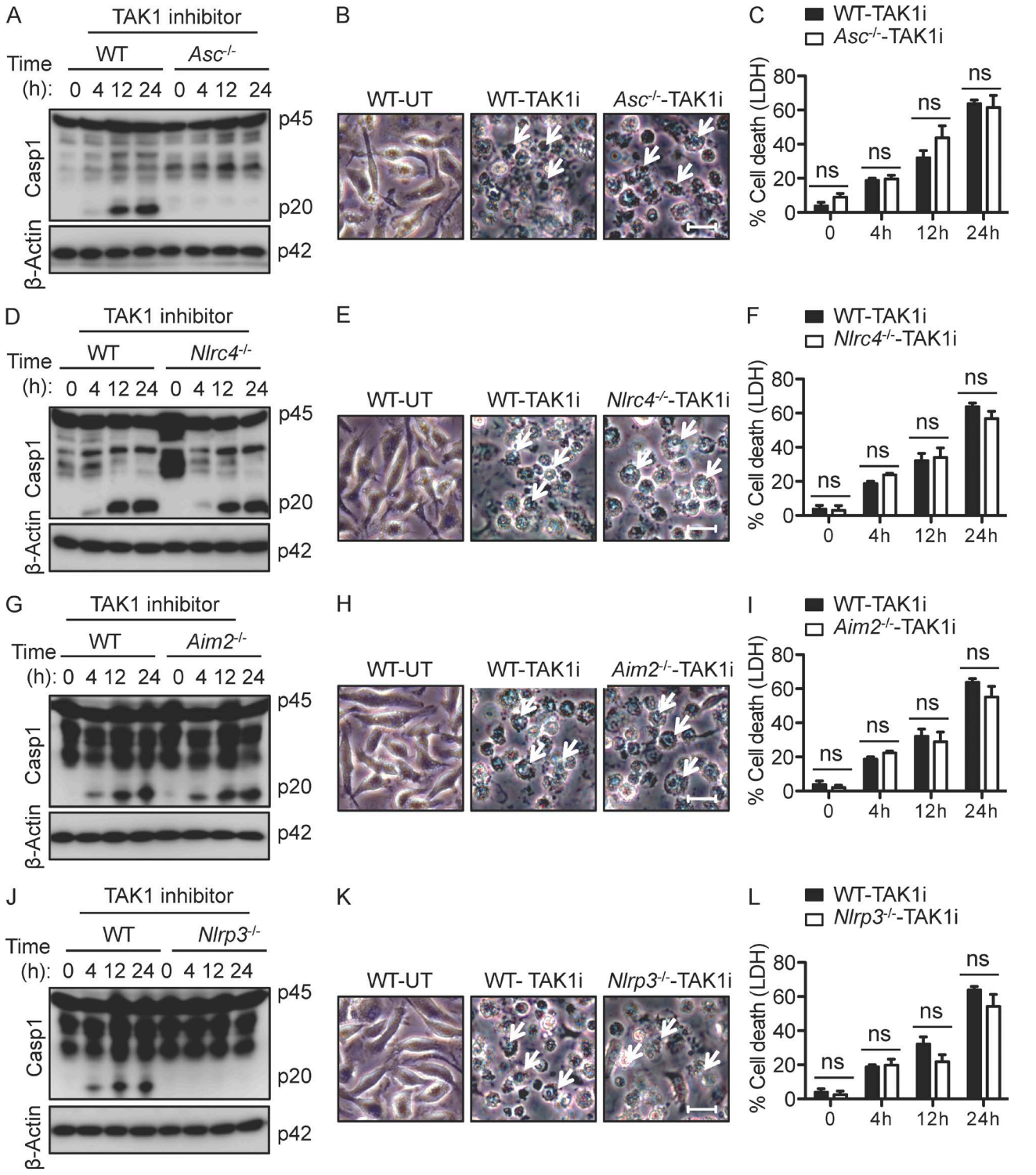

Figure 2. **NLRP3 promotes spontaneous inflammasome activation observed in TAK1-deficient BMDMs. (A–L)** WT or the indicated KO BMDMs were treated with TAK1i for the indicated times. Immunoblot analysis of pro–caspase-1 (p45) and the active caspase-1 subunit p20 (p20; A, D, G, and J), analysis of cell death by microscopy (bars, 20 µm; B, E, H, and K), or LDH secretion (C, F, I, and L) in TAK1i-treated BMDMs assessed at the indicated times after treatment in *Asc*$^{-/-}$ (A–C), *Nlrc4*$^{-/-}$ (D–F), *Aim2*$^{-/-}$ (G–I), and *Nlrp3*$^{-/-}$ (J–L). Arrows indicate dead cells (B, E, H, and K). Data are representative of three independent experiments with $n = 3$ (A–L). Error bars indicate SEM (C, F, I, and L). "p" in Western blots denotes protein molecular weight. $P < 0.05$ is considered statistically significant (two-tailed *t* test [C, F, I, and L]).

NLRP3 deficiency prevented spontaneous caspase-1 activation in TAK1-deficient BMDMs (Fig. 3 A). Consistently, treatment of TAK1-deficient BMDMs with MCC950 (a specific inhibitor of the NLRP3 inflammasome) prevented spontaneous caspase-1 activation (Fig. 3 C). Similar to the observation in TAK1i-treated WT and NLRP3-deficient cells (Fig. 2, K and L), genetic deficiency or

pharmacological inhibition of NLRP3 did not rescue cell death observed in TAK1-deficient BMDMs (Fig. 3, B and D).

These results showed that although NLRP3 and ASC deficiency reversed TAK1i-induced spontaneous caspase-1 activation, TAK1i-induced cell death could not be rescued. We have recently shown that IAV-induced cell death consists of all three forms of cell death that include apoptosis, pyroptosis, and necroptosis (Kuriakose et al., 2016). Given that the cells lacking NLRP3 and ASC (and thus pyroptosis) still underwent cell death, we hypothesized that TAK1 deficiency in BMDMs may induce all major forms of cell death that include apoptosis, pyroptosis, and necroptosis. Western blot data for caspase-3, caspase-7, and phospho-MLKL demonstrated that TAK1-deficient macrophages also exhibited the features of apoptotic and necroptotic cell death (Fig. S2, A–C). To this end, we used a combination of inhibitors that specifically block apoptosis, pyroptosis, and necroptosis to rescue spontaneous cell death observed in TAK1-deficient BMDMs. In accordance, we showed that inhibition of apoptosis, pyroptosis, or necroptosis individually was not sufficient to prevent cell death of TAK1-deficient BMDMs (Fig. S1, A–C). Also, the combined inhibition of apoptosis/pyroptosis, pyroptosis/necroptosis, and apoptosis/necroptosis did not completely rescue cell death in TAK1-deficient BMDMs (Fig. S1 D). However, when all cell death pathways were inhibited, TAK1-deficient cells were protected from cell death (Fig. S1 E), suggesting a redundant role for apoptosis, pyroptosis, and necroptosis in inducing cell death in TAK1-deficient BMDMs. Conversely, these data demonstrate that TAK1 plays an essential regulatory role in inhibiting cell death pathways and maintaining cellular homeostasis.

RIPK1 is upstream of spontaneous NLRP3 inflammasome activation and cell death in TAK1-deficient macrophages

Receptor interacting protein kinase (RIPK) 3 has been shown to be involved in regulating NLRP3 inflammasome activation under specific circumstances (Kang et al., 2013; Wang et al., 2014; Lawlor et al., 2015). Our results showed that TAK1i treatment of *Ripk3*$^{-/-}$ BMDMs results in normal NLRP3 inflammasome activation and cell death, similar to WT cells (Fig. 3, E and F). MLKL is a pseudokinase that upon activation intercalates in the plasma membrane to promote necroptosis (Wang et al., 2014). To test the role for MLKL, we treated WT or *Mlkl*$^{-/-}$ BMDMs with TAK1i. MLKL deficiency did not rescue TAK1i-induced caspase-1 activation or cell death (Fig. 3, I and J). To complement these studies, we treated TAK1-deficient BMDMs with RIPK3 or MLKL inhibitor (Fig. 3, G, H, K, and L), and our results showed that RIPK3 and MLKL are dispensable for spontaneous NLRP3 inflammasome activation. Concurrently, the cell death was also not rescued by RIPK3 or MLKL deficiency in TAK1-deficient BMDMs (Fig. 3, F, H, J, and L).

Next, we investigated whether RIPK1, an upstream kinase, was involved in spontaneous NLRP3 inflammasome activation and cell death induction. TAK1 inhibition of WT, but not *Ripk1*$^{-/-}$ macrophages (derived from fetal liver cells because the RIPK1 deficiency in mice causes day 1 postnatal lethality; Kelliher et al., 1998) induced spontaneous NLRP3 inflammasome activation and cell death (Fig. 3, M and N). Furthermore, TAK1-deficient BMDMs lacking RIPK1 kinase activity (*Lyz2*$^{cre+}$ × *Tak1*$^{f/f}$ × *Ripk1*^{K45A}) did not exhibit spontaneous caspase-1 cleavage or cell death (Fig. 3, O and P). Consistently, the levels of spontaneous IL-1β and IL-18 cytokines observed in *Lyz2*$^{cre+}$ × *Tak1*$^{f/f}$ macrophages were rescued in *Lyz2*$^{cre+}$ × *Tak1*$^{f/f}$ × *Ripk1*^{K45A} macrophages (Fig. S2, F and G). These results altogether suggest that TAK1 negatively regulates RIPK1 kinase activity independently of RIPK3 and MLKL to control spontaneous NLRP3 inflammasome activation and cell death.

RIPK1 is a well-established regulator of TNF signaling. Thus, we hypothesized that TAK1 deficiency or inhibition may trigger spontaneous activation of the TNF signaling pathway. Indeed, we observed a significant amount of spontaneous TNF secretion in the culture by TAK1-deficient BMDMs (Fig. S2 D) and in the serum of *Lyz2*$^{cre+}$ × *Tak1*$^{f/f}$ mice (Fig. S2 E). To evaluate whether autocrine TNF was the upstream event that induced NLRP3 inflammasome activation and cell death in TAK1-deficient BMDMs, anti-TNF neutralizing antibody was used to block TNF signaling (Fig. S3, A–F). TNF neutralization rescued aberrant caspase-1 activation and cell death in both TAK1-deficient BMDMs and TAK1i-treated WT cells (Fig. S3, A–F). In addition, TNF neutralization also rescued the spontaneous production of IL-1β and IL-18 from TAK1-deficient macrophages (Fig. S2 G). To further examine the role of TNF signaling, we used TNF-deficient and TNFR-deficient BMDMs that were treated with TAK1i. Genetic deficiency of either TNF or TNFR rescued spontaneous caspase-1 activation and cell death responses in TAK1i-treated BMDMs (Fig. S3, G–L). However, TAK1 inhibition–induced caspase-1 activation from *Trif*$^{-/-}$ and *Ifnar1*$^{-/-}$ BMDMs was comparable to that observed in the WT BMDMs (Fig. S1 F). Altogether, these data demonstrated that the TNF signaling axes promote NLRP3 inflammasome activation and cell death in TAK1-deficient BMDMs.

TAK1 restricts RIPK1 kinase–dependent spontaneous NF-κB and ERK activation in macrophages and myeloid proliferation in mice

In addressing the mechanisms by which TAK1 promotes cellular quiescence, we posited that TAK1 deficiency activates inflammatory signaling pathways in the absence of exogenous stimuli, concurrent with our detection of spontaneous NLRP3 inflammasome activation and TNF production by TAK1-deficient BMDMs. In agreement with our hypothesis, we observed increased activation of ERK and NF-κB in TAK1-deficient BMDMs under homeostatic conditions (Fig. 4 A). Similarly, phospho-IKKα/β, upstream regulators of ERK and NF-κB, were also increased basally in TAK1-deficient BMDMs (Fig. 4 A). In concurrence with increased activation, basal protein expression of NLRP3 was also slightly increased in TAK1-deficient BMDMs (Fig. 4 A). These results were unexpected given the established role of TAK1 in promoting ERK and NF-κB activation. More importantly, this aberrant signaling in TAK1-deficient BMDMs was rescued when RIPK1 kinase activity was absent (Fig. 4 B). These data demonstrate that under homeostatic conditions, TAK1 restricts RIPK1-dependent spontaneous NF-κB and ERK activation.

Mice with myeloid specific deficiency of TAK1 develop progressive accumulation of neutrophils ultimately displaying signs of myeloid proliferation and death. Consistently, we also observed increased CD11b^{+} populations (myeloid cells) in the peripheral

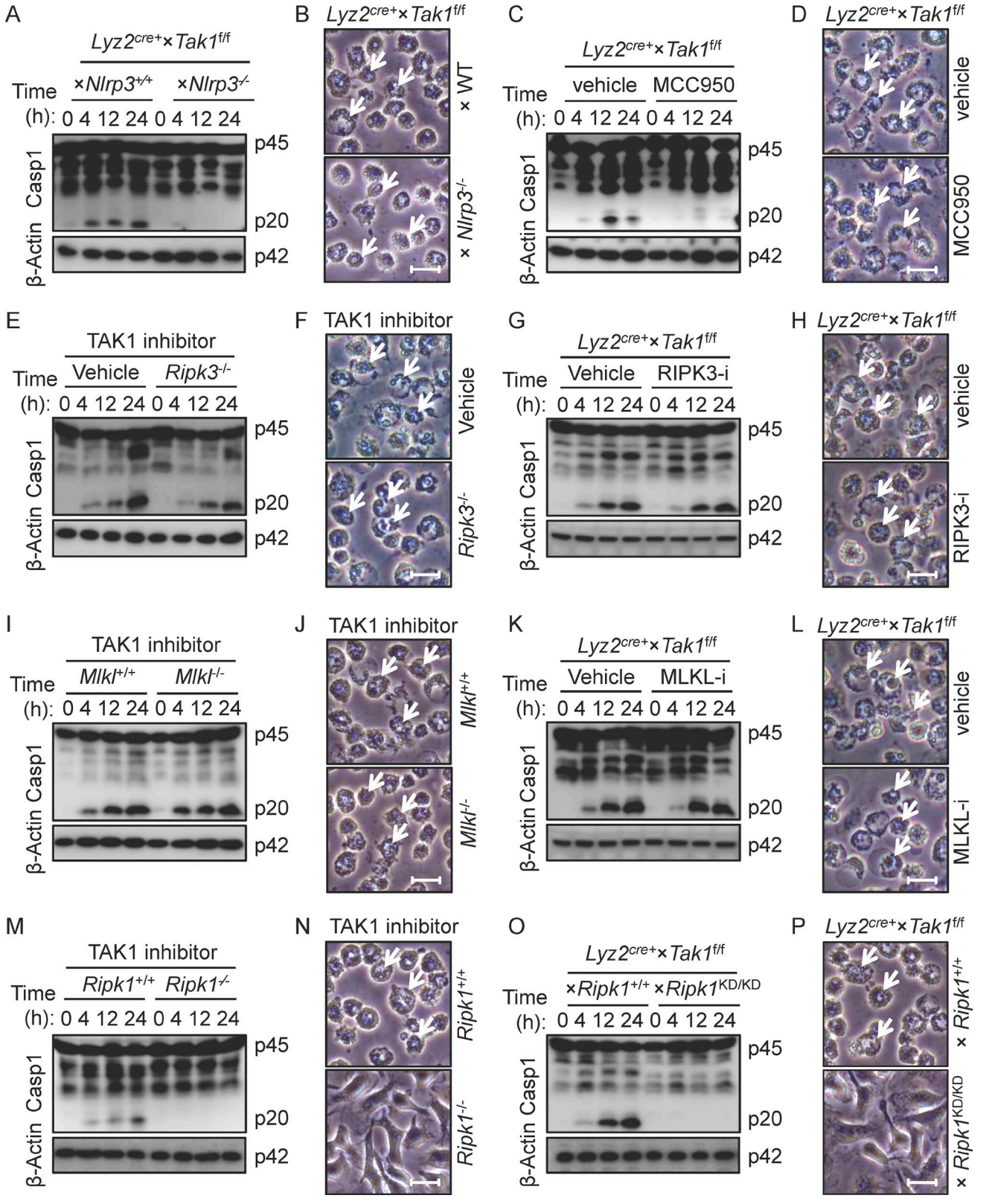

Figure 3. **RIPK1 promotes spontaneous NLRP3 inflammasome activation and cell death in TAK1-deficient BMDMs. (A and B)** Analysis of caspase-1 activation (A) and cell death by microscopy (B) in untreated *Lyz2*$^{cre+}$ × *Tak1*$^{f/f}$ (TAK1 KO) compared with *Lyz2*$^{cre+}$ × *Tak1*$^{f/f}$ × *Nlrp3*$^{-/-}$ (TAK1/NLRP3 DKO) assessed at the indicated times after differentiation of BMDMs in culture. **(C and D)** *Lyz2*$^{cre+}$ × *Tak1*$^{f/f}$ BMDMs were treated with vehicle or MCC950 (specific inhibitor of NLRP3) and probed for caspase-1 activation (C) and cell death (D). **(E and F)** Caspase-1 immunoblot (E) and cell death analysis (F) in TAK1i-treated WT and *Ripk3*$^{-/-}$ BMDMs at various time points indicated. **(G and H)** *Lyz2*$^{cre+}$ × *Tak1*$^{f/f}$ BMDMs were treated with vehicle or GSK'872 (RIPK3 inhibitor) and probed for caspase-1 activation (G) and cell death (H). **(I and J)** Caspase-1 immunoblot (I) and cell death analysis (J) in TAK1i-treated WT and *Mlkl*$^{-/-}$ BMDMs and assessed

blood (PBL) of *Lyz2*$^{cre+}$ × *Tak1*$^{f/f}$ mice (Fig. 4 C). A closer examination of CD11b$^+$ cells revealed that whereas neutrophil frequency was increased, monocyte frequency was decreased in *Lyz2*$^{cre+}$ × *Tak1*$^{f/f}$ mice when compared with littermate WT controls (Fig. 4, D and E). Importantly, the increased neutrophil and reduced monocyte populations in the PBL from *Lyz2*$^{cre+}$ × *Tak1*$^{f/f}$ mice were rescued in *Lyz2*$^{cre+}$ × *Tak1*$^{f/f}$ × *Ripk1*$^{KD/KD}$ mice (Fig. 4, F–H). To further corroborate these findings, we studied the TAK1i-induced acute neutrophilia and monocytopenia in mice (Fig. 4). TAK1i-treatment of WT mice significantly increased the frequency of CD11b$^+$ cells and neutrophils, whereas the frequency of the monocyte population was significantly reduced (Fig. 4, I–K), similar to the mice genetically lacking TAK1 in myeloid compartment (Fig. 4, C–H). Importantly, TAK1i-induced differences in neutrophil and monocyte populations were also dependent on RIPK1 kinase activity (Fig. 4, I–K). Collectively, these data demonstrate a critical role for RIPK1 kinase activity in regulating NLRP3 inflammasome activation and cell death to promote myeloid proliferation in the absence of TAK1 signaling.

The conventional role of TAK1 in propagating NF-κB and MAPK signaling events downstream of several PRR, growth, and cytokine receptors is well established (Ajibade et al., 2013; Zhang et al., 2017). Herein, we describe a previously uncharacterized, paradoxical role for TAK1 in regulating cellular quiescence and homeostasis by inhibiting spontaneous activation of IKKα/β. Early studies demonstrated that inhibition or deletion of IKKα/β activates NLRP3 inflammasome in the presence of priming signal alone (Greten et al., 2007; Zhong et al., 2016). Given these studies that show IKKβ deficiency or inhibition activated the NLRP3 inflammasome, which requires LPS priming, our study is fundamentally different because we demonstrate that TAK1 deficiency leads to enhanced basal activation of IKKα/β to promote TNF release and spontaneous inflammasome activation. This result is completely unexpected given the established role of TAK1 in promoting receptor-induced signaling events (Ajibade et al., 2013; Zhang et al., 2017). The absence of TAK1 in macrophages also induced spontaneous activation of the NLRP3 inflammasome without the requirement for exogenous priming and activation signals, which has not been reported before. Mechanistically, we have clearly demonstrated the role for TNF, TNFR, and RIPK1 in regulating spontaneous NLRP3 inflammasome activation and cell death. Physiologically, enhanced cell death and inflammation resulting from loss-of-function mutations of TAK1 drives myeloid proliferation in mice and humans (Ajibade et al., 2012; Lamothe et al., 2012). TAK1 loss-of-function mutations also cause death of a range of immune and nonimmune cells and disrupt tissue and bone homeostasis (Mihaly et al., 2014; Swarnkar et al., 2015; Le Goff et al., 2016; Wade et al., 2016). Our study identified several important effector molecules driving this cell death and inflammation downstream of TAK1-inactivation and hence potential therapeutic targets. Increased cell death of TAK1-deficeint resident macrophages has also been observed in in vivo mouse models with hematopoietic specific deletion of TAK1 (Sakamachi et al., 2017). Future studies will test whether similar pathways of cell death and inflammasome activation, as established in our study, are at work in these resident macrophages. These findings corroborate and provide a mechanistic explanation for the severe spontaneous inflammatory pathologies in TAK1 KO compared with the mice deficient for other NF-κB family members (Shim et al., 2005; Mihaly et al., 2014). More importantly, we have provided in vivo data targeting RIPK1 kinase activity to rescue the myeloid proliferation phenotype associated with TAK1 deficiency in mice. Our study uncovered previously unidentified functions of TAK1 with potential applications for therapeutically activating the innate immune system and managing myeloid proliferation in specific situations in which TAK1 functions are impaired.

Materials and methods

Mice

Ripk1^{K45A} (*Ripk1*$^{KD/KD}$; Berger et al., 2014), *Ripk3*$^{-/-}$ (Newton et al., 2004), *Nlrp3*$^{-/-}$ (Kanneganti et al., 2006), *Asc*$^{-/-}$ (Mariathasan et al., 2004), *Casp1*$^{-/-}$ × *Casp11*$^{-/-}$ (Kayagaki et al., 2011), *Tnf*$^{-/-}$ (Pasparakis et al., 1996), *Tnfr*$^{-/-}$ (Pfeffer et al., 1993), and *Mlkl*$^{-/-}$ (Murphy et al., 2013) were all described previously. *Tak1*$^{f/f}$ mice were bred with *Lyz2*$^{cre+}$ (*B6.129P2-Lyz2*$^{tm1(cre)Ifo}$*/J*; Jackson) mice to generate conditional Tak1 KO mice. C57BL/6 WT (Jackson) and littermate controls were bred at St. Jude Children's Research Hospital. Animal studies were conducted under protocols approved by St. Jude Children's Research Hospital on the Use and Care of Animals.

Macrophage differentiation and stimulation

BMDMs were prepared as described previously (Gurung et al., 2012). In brief, bone marrow cells were grown in L cell–conditioned IMDM medium supplemented with 10% FBS, 1% nonessential amino acid, and 1% penicillin-streptomycin for 5 d to differentiate into macrophages. On day 5, BMDMs were counted, and 10^6 cells were seeded in 12-well cell culture plates in IMDM media containing 10% FBS, 1% nonessential amino acids, and 1% penicillin-streptomycin. For BMDMs generated from *Lyz2*$^{cre+}$ × *Tak1*$^{f/f}$ mice, as the precursor cells differentiate into macrophages, they will express Cre recombinase (under the control of myeloid-specific *Lyz2* gene) and delete the floxed *Tak1* gene, resulting in TAK1-deficient macrophages.

Where indicated, for pharmacological inhibition, BMDMs were pretreated with chemical inhibitors of apoptosis,

at the times indicated. **(K and L)** *Lyz2*$^{cre+}$ × *Tak1*$^{f/f}$ BMDMs were treated with vehicle or GW806742X (MLKL inhibitor) and probed for caspase-1 activation (K) and cell death (L). **(M and N)** Caspase-1 immunoblot (M) and cell death analysis (N) in WT and *Ripk1*$^{-/-}$ (generated from fetal liver cells) BMDMs treated with TAK1i and assessed at the indicated times. **(O and P)** Caspase-1 immunoblot (O) and cell death analysis (P) in *Lyz2*$^{cre+}$ × *Tak1*$^{f/f}$ and *Lyz2*$^{cre+}$ × *Tak1*$^{f/f}$ × *Ripk1*$^{KD/KD}$ BMDMs and assessed at the indicated times. Arrows indicate dead cells. Bars, 20 µm. "p" in Western blots denotes protein molecular weight. Data are representative of three independent experiments with $n = 3$ in each repeat (A–P).

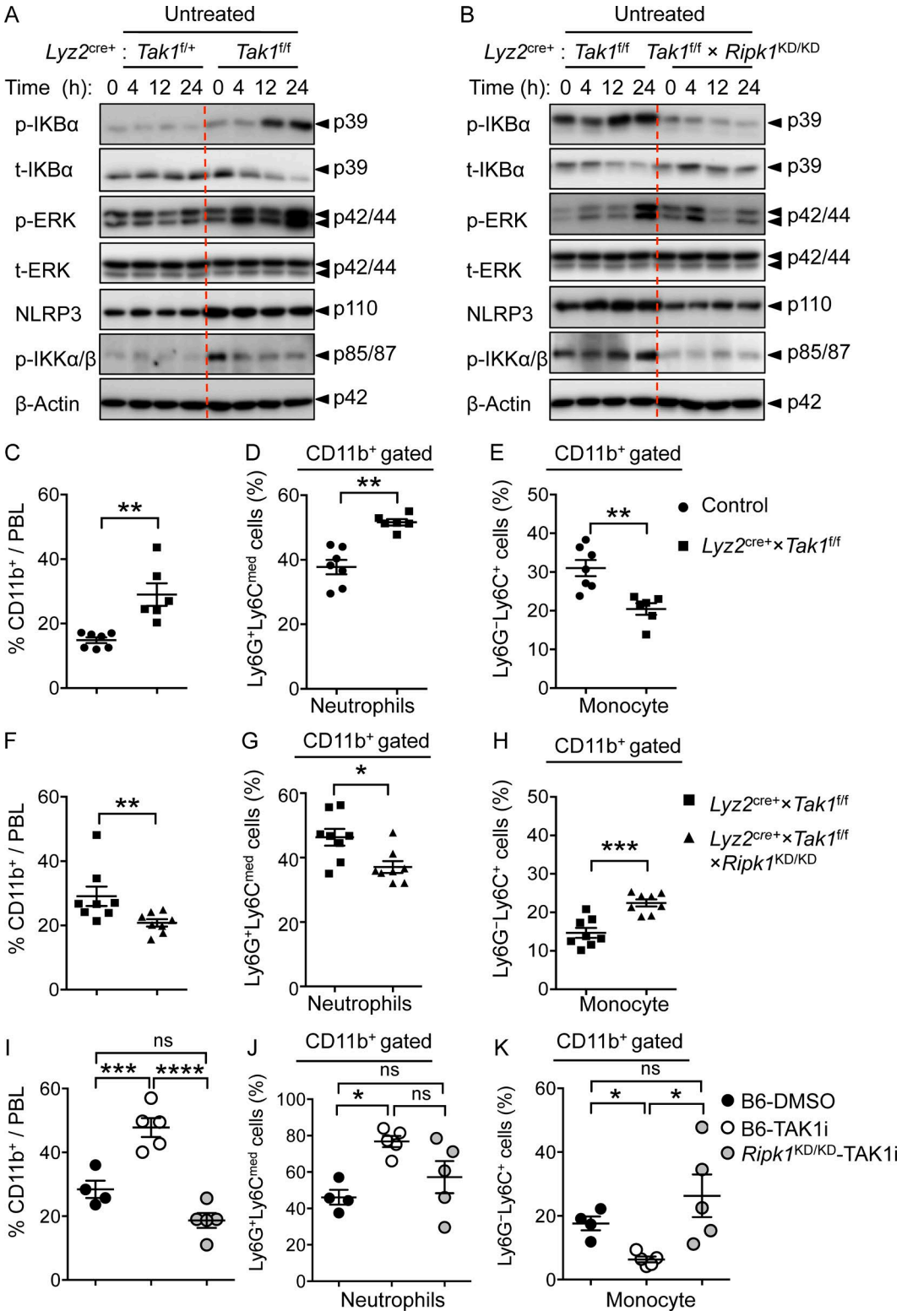

Figure 4. **RIPK1 kinase-dead mouse partially rescues the myeloid phenotype observed in TAK1-deficient mice in vivo. (A)** Immunoblot analysis of phospho-IκBα, phospho-ERK, NLRP3, phospho-IKKα/β, and β-actin (loading control) in untreated *Tak1*$^{f/+}$ × *Lyz2*$^{cre+}$ (HT ctrl) and *Tak1*$^{f/f}$ × *Lyz2*$^{cre+}$ (TAK1 KO) BMDMs assessed at the indicated times after differentiation in culture. **(B)** Immunoblot analysis as in A in untreated BMDMs from *Lyz2*$^{cre+}$ × *Tak1*$^{f/f}$ (TAK1 KO) and *Lyz2*$^{cre+}$ × *Tak1*$^{f/f}$ x *Ripk1*$^{KD/KD}$ (TAK1 KO with kinase-dead RIPK1) mutant mice. **(C–H)** Flow cytometry analysis of peripheral blood from control (n = 7), *Tak1*$^{f/f}$ × *Lyz2*$^{cre+}$ (n = 6, C–E; n = 8, F–H) and *Tak1*$^{f/f}$ × *Lyz2*$^{cre+}$ × *Ripk1*$^{KD/KD}$ (n = 8) mice. Littermate controls were used for the experiments, which included *Tak1*$^{f/f}$ and *Tak1*$^{f/+}$ × *Lyz2*$^{cre+}$ mice. **(C and F)** Cumulative dot plots of representing frequencies of CD11b$^+$ cells analyzed by flow cytometry from blood. **(D and G)** Cumulative dot plots representing frequency of neutrophil population in the CD11b$^+$-gated cells. **(E and H)** Cumulative dot plots representing frequency of monocyte population in the CD11b$^+$-gated cells. **(I–K)** Flow cytometry analysis of peripheral blood from control (n = 4) and TAK1i-treated WT (n = 5) and *Ripk1*$^{KD/KD}$ (n = 5)

pyroptosis, and necroptosis. In other experiments, BMDMs were treated with TAK1i 5Z-7-Oxozeaenol at 0.1 µM to study inflammasome activation and cell death.

Analysis of myeloid proliferation and TAK1i-induced PBL changes in vivo

All flow-cytometric analysis of in vivo myeloid phenotypes was conducted from *Lyz2*$^{cre+}$ × *Tak1*$^{f/f}$ (TAK1 KO) and *Lyz2*$^{cre+}$ × *Tak1*$^{f/f}$ × *Ripk1*$^{KD/KD}$ (TAK1 KO with kinase-dead RIPK1) mutant mice. For all in vivo TAK1i treatments, WT or genetically manipulated RIPK1 kinase–dead mice were i.p. injected with DMSO control or TAK1i at 50 mg/kg body weight. Blood samples were collected at 6 h after TAK1i treatment from mouse orbital sinus. PBLs were isolated using standard ACK RBC lysis protocol and stained for flow-cytometric analysis with the indicated antibodies.

Western blotting

Samples for immunoblotting were prepared by combining cell lysates with culture supernatants. Samples were denatured in loading buffer containing SDS and 100 mM DTT and boiled for 5 min. SDS-PAGE–separated proteins were transferred to PVDF membranes and immunoblotted with primary antibodies against caspase-1 (AG-20B-0042; Adipogen), Nlrp3 (AG-20B-0014; Adipogen), GAPDH (D16H11), and β-Actin (13E5; Cell Signaling Technology) followed by secondary anti–rabbit or anti–mouse HRP antibodies (Jackson ImmunoResearch Laboratories), as previously described (Kanneganti et al., 2006).

Lactate dehydrogenase assay

Secreted levels of lactate dehydrogenase from cell supernatants were determined using the CytoTox 96 Non-Radioactive Cytotoxicity Assay according to the manufacturer's instructions (G1780; Promega).

Flow cytometry

CD11b (M1/70), and Gr-1 (RB6-8C5) antibodies were purchased from eBioscience. LY6C (HK1.4), CD45.2 (104), and LY6G (1A8) were from BioLegend. Flow cytometry data were acquired on an upgraded eight-color FACScan and analyzed using FlowJo software (Tree Star).

Cytokine analysis

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

Microscope image acquisition

Light microscopy

Differentiated WT and mutant macrophages seeded in 12-well cell culture plates were either left untreated (control) or treated with TAK1i or different cell death inhibitors for the indicated times. Light microscopic images were obtained using an Olympus CKX41 microscope with a 40× objective lens. Digital image recording and image analysis were performed with the INFINITY ANALYZE Software (Lumenera Corp.). The images were processed and analyzed with ImageJ software.

Real-time cell death analysis

Real-time cell death assays were performed using a two-color IncuCyte Zoom in-incubator imaging system (Essen Biosciences). In brief, BMDMs were seeded in 24-well tissue culture vessels (250,000 cells/well) in the presence of 100 nM of the cell-impermeable DNA-binding fluorescent dye Sytox Green (S7020; Life Technologies), which rapidly enter dying cells on membrane permeabilization. Resulting images were analyzed using the software package supplied with the IncuCyte imager, which allows precise analysis of the number of Sytox Green–positive cells present in each image. Experiments were conducted using a minimum of three separate wells for each experimental condition and a minimum of four image fields per well. Dead cell events for each line of BMDMs were acquired via Sytox Green and plotted using GraphPad Prism software.

Statistical analysis

GraphPad Prism 5.0 software was used for data analysis. Data are shown as mean ± SEM. Statistical significance was determined by *t* tests (two-tailed) for two groups or one-way ANOVA (with Dunnett's or Tukey's multiple comparisons tests) for three or more groups.

Online supplemental material

Fig. S1 shows a combination of inhibitors that specifically block apoptosis, necroptosis, and pyroptosis rescue TAK1-deficient BMDMs from cell death. Fig. S2 shows TAK1 deficiency resulting in spontaneous TNF secretion in BMDMs. Fig. S3 shows the critical role of TNF signaling in spontaneous NLRP3 inflammasome activation in TAK1-deficient BMDMs.

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mice. Cumulative dot plots representing the frequencies of total CD11b$^+$ cells analyzed by flow cytometry from blood (I), and frequency of neutrophil (J) and monocyte (K) populations in the CD11b$^+$-gated cells. All data are presented as mean ± SEM (C–K), and each dot represents a single mouse. "p" in Western blots denotes protein molecular weight. Statistical significance between groups was determined by Mann-Whitney test, and P values less than 0.05 are considered statistically significant. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Data are representative of five (A and B) or two (C–H) independent experiments.

P. Gurung, J. Mavuluri, and T.K. Dasari performed experiments. R.K.S. Malireddi, P. Gurung, and T.-D. Kanneganti analyzed the data. R.K.S. Malireddi, P. Gurung, and T.-D. Kanneganti wrote the manuscript with input from the other authors. T.-D. Kanneganti oversaw the project.

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