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TAK1-binding protein 2 (TAB2), but not TAB3, suppresses aberrant non- pyroptotic NLRP3 inflammasome activation of macrophages mediated by TNF-α/RIPK1/Caspase-8 axis

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Research Article

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Abstract

Background.

TAK1-binding protein 2 (TAB2) and TAB3 are two homologous adapter proteins that play overlapping yet indispensable roles in Toll-like receptor (TLR) signaling. It has also been suggested that TAB2 and TAB3 have their own unique functions. For example, TAB2, but not TAB3, is essential for preventing apoptosis induced by tumor necrosis factor (TNF)-α. However, the functional differences between TAB2 and TAB3 in the regulation of inflammatory responses remain unclear.

Methods.

Mouse bone marrow-derived macrophages (BMDMs) deficient in TAB2 and/or TAB3 were stimulated with TLR ligands. Cytokine production and the underlying mechanisms were analyzed using enzyme-linked immunosorbent assay, quantitative reverse transcription-polymerase chain reaction, and Western blot analysis.

Results.

TAB2-deficient (TAB2-KO) BMDMs, but not TAB3-deficient or wild-type BMDMs, secreted interleukin (IL)-1β upon stimulation with Pam3CSK4 alone via NLRP3 and caspase-1, without gasdermin D cleavage or pyroptosis. Pam3CSK4-induced IL-1β production in TAB2-KO BMDMs was significantly suppressed by an anti-TNF-α neutralizing antibody, a RIPK1 inhibitor, or TNFR1 or caspase-8 knockdown, indicating that autocrine TNF-α drives inflammasome activation through the TNFR1–RIPK1–caspase-8 axis.

Conclusion.

Our data suggest that TAB2 plays a unique and essential role as a gatekeeper suppressing aberrant NLRP3 inflammasome activation via the TNFR1–RIPK1–caspase-8 pathway triggered by autocrine TNF-α in macrophages.

Introduction

The innate immune system acts as a first line of defense against invading pathogens. It is also involved in the development of chronic diseases such as atherosclerosis, gout, or rheumatoid arthritis [1–3]. Macrophages recognize pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) through various pattern recognition receptors such as Toll-like receptors (TLRs)[4–7]. Upon binding to PAMPs or DAMPs, TLR signaling activates the nuclear factor (NF)-κB, mitogen-activated protein kinase (MAPK), and interferon regulatory factor (IRF) pathways, leading to the production of proinflammatory cytokines, chemokines, and type I interferons [4, 7]. Proinflammatory

cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β, orchestrate inflammation by regulating vascular permeability, recruiting immune cells to the site of inflammation, activating immune cells and regulating cell death in inflammatory tissues [8–10].

While TNF-α production is mainly regulated at the transcriptional and translational levels, IL-1β production is regulated by two steps [11]. The first step is called “priming”, which induces *Il1b* gene expression, leading to the accumulation of pro-IL-1β protein in the cytosol. The second step is called “activation”, which induces the processing of pro-IL-1β into mature IL-1β by caspase-1. Caspase-1 activity is controlled by a protein complex known as the inflammasome, which consists of NOD-like receptor (NLR) family proteins, the adaptor protein ASC, and pro-caspase-1. The NLRP3 inflammasome is the best-characterized and is known to be involved in both acute and chronic inflammation [12]. It is activated by various stimuli, including extracellular ATP, nigericin, monosodium urate crystals, and aluminum salts. These stimuli induce assembly of the NLRP3 inflammasome complex and activate pro-caspase-1. Active caspase-1 not only cleaves pro-IL-1β, but also cleaves gasdermin D (GSDMD), unleashing a pore-forming N-terminal domain (GSDMD-N) that induces lytic cell death known as pyroptosis [13].

Of note, TNF-α can also activate the NLRP3 inflammasome under certain conditions [14–18]. TNF-α is a pleiotropic cytokine that initiates complex signaling cascades through its receptor TNFR1, orchestrating cellular decisions between survival, death, and inflammation. Upon TNF-α binding to TNFR1, the receptor undergoes conformational changes that recruit adaptor proteins to form a membrane-proximal signaling platform termed Complex I. This complex consists of TNFR1-associated death domain protein (TRADD), RIPK1, TNF receptor-associated factor 2 (TRAF2), cellular inhibitor of apoptosis proteins 1 and 2 (cIAP1/2), and the linear ubiquitin chain assembly complex (LUBAC), and promotes cell survival [19, 20]. The post-translational modification of RIPK1 acts as a molecular switch towards cell death pathway. Under specific conditions, RIPK1 dissociates from the membrane-bound Complex I and translocates to the cytosol, where it nucleates the formation of death-inducing complexes collectively termed Complex II that recruit FADD and caspase-8. When caspase-8 is fully active, it executes apoptosis. But, when caspase-8 is inhibited or absent, RIPK1 engages RIPK3 to form the necrosome, leading to necroptosis [19]. Besides apoptosis and necroptosis, numerous studies have suggested the context-dependent regulatory role of RIPK1 and caspase-8 in pyroptosis via NLRP3 inflammasome [14, 15, 21–34]. However, the molecular mechanisms of TNF-α-mediated activation of NLRP3 inflammasome are not fully understood.

Transforming growth factor-β-activated kinase (TAK) 1 plays a pivotal role in the activation of NF-κB and MAPK in a wide variety of signaling pathways, including IL-1 receptor, TNFR, and TLR signaling [35]. TAK1 activity is regulated by three TAK1-binding proteins (TAB), namely TAB1, TAB2, and TAB3. TAB2 and TAB3 share similar structural features and function redundantly in TLR-mediated gene expression [36–39]. In addition to the NF-κB and MAPK activation, TAK1 functions as a critical gatekeeper that prevents RIPK1 kinase-dependent cell death and inflammation [16, 40, 41]. Inhibition or deficiency of TAK1 promotes TNF-α-dependent assembly of cytosolic RIPK1/FADD/caspase-8 complex (Complex IIb)

and induces apoptosis [40, 41]. Malireddi et al. demonstrated that TAK1-deficient BMDMs exhibit spontaneous activation of NLRP3 inflammasome via RIPK1 activated by autocrine TNF-α [16]. Recently, it was reported that TAB2, but not TAB3, is essential for preventing TNF-α-induced apoptosis via both TAK1-dependent and -independent mechanisms, suggesting the TAB2-specific function in regulating cell death [42, 43].

Pathogenic bacteria and viruses have evolved a diverse arsenal of effector proteins that specifically target the TAK1/TAB complex to evade or dampen innate immune responses. For example, the *Yersinia* outer protein J (YopJ, also known as YopP in *Y. enterocolitica*) is a multifunctional acetyltransferase that directly binds TAB1 and inhibits TAK1 activation through multiple mechanisms. YopJ acetylates critical serine and threonine residues in the activation loops of TAK1 and upstream kinases, preventing their phosphorylation and activation [27, 44, 45]. Another example is a methyl transferase NleE secreted by enteropathogenic *Escherichia coli* (EPEC), which methylates a critical cysteine residue (Cys673 in human TAB2) within the NZF domains of TAB2 and TAB3. This methylation abolishes the ability of TAB2 and TAB3 to bind K63-linked polyubiquitin chains, thereby preventing TAK1 recruitment to signaling complexes [46, 47]. Furthermore, it has recently been reported that the nucleocapsid (N) protein of SARS-CoV-2 targets TAB2 and TAB3, hinders the formation of the TAK1–TAB2/3 complex, and compromises the pro-inflammatory response [48]. While these pathogens inhibit the TAK1/TAB pathway, their PAMPs continuously activate pattern-recognition receptors such as TLRs. The consequences of this sustained simultaneous activation and inhibition of the TAK1/TAB pathway on innate immune function remain unclear.

In this study, we investigated the effects of TAB2 and TAB3 deficiency on macrophage cellular responses during sustained TLR activation. We show that TAB2, but not TAB3, is essential for preventing aberrant activation of NLRP3 inflammasome induced by an autocrine TNF-α, which acts through the TNFR1–RIPK1–caspase-8 axis. Interestingly, TAB2-deficient BMDMs secreted IL-1β upon sustained TLR stimulation without causing the cleavage of GSDMD, indicating that the pyroptosis-independent mechanism is involved in IL-1β secretion in TAB2-deficient BMDMs.

Materials and Methods

Mice and preparation of bone marrow-derived macrophages

Table 2 $^{flox/flox}$ mice have been published and were kindly provided by Shizuo Akira (Osaka University) [49]. Table 3$^{-/-}$ mice were used as described previously [50]. *LysM-Cre* transgenic mice (stock#: 004781) were purchased from Jackson Laboratories. Mice were maintained under conventional conditions and used for experiments at 8–16 weeks of age. BMDMs were generated by culturing bone marrow cells isolated from femurs and tibias in a bone marrow macrophage (BMM) medium containing RPMI-1640 supplemented with 10% heat-inactivated FBS (HI-FBS) (Sigma-Aldrich, F7524), 30% L929 cell-

conditioned medium, and penicillin/streptomycin (Nacalai Tesque, 09367-34) for 5 days. All the animal experiments were approved by the Animal Experiment Committee of the University of the Ryukyus (Approval No. A2019027, A2021069).

Cell culture

RPMI-1640 and MEM Eagle's medium were purchased from Sigma-Aldrich (R8758 and M4655, respectively). L929 cell-conditioned medium was prepared by culturing L929 cells in MEM Eagle's medium supplemented with 10% HI-FBS, non-essential amino acids (Gibco, 11140-050), sodium pyruvate (Gibco, 11360-070), and penicillin/streptomycin (Nacalai Tesque, 09367-34). All cells were grown at 37˚C in a 5% CO_2 humidified incubator.

Reagents and Antibodies

Ultrapure LPS from *E. coli* O111:B4 strain (#tlrl-3pelps) and Pam3CSK4 (#tlrl-pms) were purchased from Invivogen. Staurosporine (#S1421) was purchased from Selleck. Specific monoclonal and polyclonal antibodies against the following antigens were used for Western blotting: IL-1β (#12507), cleaved-IL-1β (#63124), caspase-8 (#4790), cleaved-caspase-8 (#8592) (Cell Signaling Technology), caspase-1 (#179515), GSDMD (#209845), GSDME (#215191) (Abcam), and β-Actin (SIGMA, #A1978). The secondary antibodies used were horseradish peroxidase (HRP)-conjugated antibodies against mouse and rabbit IgG (#7076 and #7074, respectively) (Cell Signaling Technology).

Caspase-1 and caspase-8 Assay

Protease activity of caspase-1 and caspase-8 was determined using Caspase-Glo 1 Inflammasome Assay (Promega, #G9951) and Caspase-Glo 8 Assay Systems (Promega, #G8200), respectively. BMDMs were seeded into white 96-well plates at 1×10^5 cells/well in 100 µL of BMM medium. Stimuli were added at volumes of 100 µL, and cells were incubated for 8 h or 24 h. For caspase-1 assay, 100 µL of luminescent substrate solution containing MG-132 proteasome inhibitor was added to each well, mixed for 30 seconds, and incubated at room temperature for 1.5 hours to allow signal stabilization. For caspase-8 assay, 25 µL of Caspase-Glo 8 assay buffer containing MG-132 was immediately added to the cells, followed by incubation in the dark for 60 minutes. Luminescence was measured using a Varioskan LUX plate reader (ThermoFisher Scientific).

Western Blotting

BMDMs were washed once with ice-cold PBS and lysed in cell lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1% Triton X-100 supplemented with protease inhibitor (Roche, Cat #05892791001) and phosphatase inhibitor (Roche, Cat# 04906837001) cocktails. Cellular debris was

removed via centrifugation at 20,000×g for 10 min at 4°C. Whole cell extracts were resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (GE Healthcare Life Scientific, Cat #10600023). The membranes were incubated with various primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies against rabbit or mouse IgG. The bound antibodies were visualized using Chemi-Lumi One Ultra reagent (Nacalai Tesque), and the chemiluminescent signal was captured using an ImageQuant LAS 4000mini image analyzer (GE Healthcare Life Scientific).

Enzyme-linked immunosorbent assay (ELISA)

BMDMs (5×10^5 cells) were cultured in 24-well plates in RPMI-1640 medium containing 10% HI-FBS and penicillin/streptomycin. The cells were treated with LPS (100 ng/mL) or Pam3CSK4 (100 ng/mL) for the indicated periods. Culture supernatants were collected and analyzed using ELISA kits for mouse IL-1β (ThermoFisher, #88-7013A-88) and TNF-α (BioLegend, #430904).

RNA interference (RNAi)

BMDMs were transfected with 20 nM Silencer Select Pre-designed siRNAs (ThermoFisher) targeting *Tnfrsf1a* (siRNA ID: s75276), *Tnfrsf1b* (siRNA ID: s75278), *Casp8* (siRNA ID: s63393), or a negative control siRNA (Silencer Negative Control No.1 siRNA, ThermoFisher #AM4611) using the Neon transfection system (ThermoFisher). Briefly, BMDMs were resuspended in Buffer R at 1×10^6 cells per 100 µL, combined with 20 nM siRNA, and electroporated using the following parameters: 1,900 V, 1 pulse, 20 ms pulse width. Following electroporation, cells were immediately transferred to antibiotic-free RPMI-1640 medium supplemented with 10% HI-FBS and incubated for 24 h. Transfected cells were then either harvested for assessment of target gene knockdown efficiency or stimulated with Pam3CSK4 for downstream analyses.

Quantitative reverse transcription-polymerase chain reaction (RT-qPCR)

Total RNA was extracted from the BMDMs using RNAiso Plus (TaKaRa, #9108) according to the manufacturer's instructions. One microgram of RNA was used for complementary DNA (cDNA) synthesis with the SuperScript VILO cDNA synthesis kit (Thermo Fisher, #11754050). Quantitative reverse transcription-polymerase chain reaction (RT-qPCR) was conducted using PowerUp SYBR Green Master Mix (Applied Biosystems, #A25741). The primer sequences used for SYBR Green-based qPCR were: *Il1b*: 5'-GCAACTGTTCCTGAACTCAACT-3' (forward) and 5'-ATCTTTTGGGGTCCGTCAACT-3' (reverse), *Nlrp3*: 5'-ATGGTATGCCAGGAGGACAG-3' (forward) and 5'-ATGCTCCTTGACCAGTTGGA-3' (reverse), *Casp8*: 5'-ATGGCTACGGTGAAGAACTGCG-3' (forward) and 5'-TAGTTCACGCCAGTCAGGATGC-3' (reverse), and *18S*: 5'-CGGACAGGATTGACAGATTG-3' (forward) and 5'-CAAATCGCTCCACCAACTAA-3' (reverse).

Statistical Analysis

One-way analysis of variance (ANOVA) with Tukey's multiple comparison test was performed to examine the differences among the mean values of the samples. Differences between groups were tested using two-way ANOVA with Tukey's multiple comparison test. GraphPad Prism software (GraphPad Software, San Diego, CA, USA) was used for statistical analyses. The *p* values are as follows: **p* < 0.05, ***p* < 0.01, ****p* < 0.001, and **** *p* < 0.0001.

Results

TAB2, but not TAB3 suppresses IL-1β production in BMDMs stimulated with TLR ligands

TAB2 and TAB3 play overlapping yet indispensable roles in the induction of immediate early response genes downstream of TLRs [36]. Some pathogens target and inhibit TAB2 and/or TAB3 to attenuate the TLR-mediated immune response. Although pathogen infection can trigger not only early-phase but also sustained activation of TLR signaling, the impact of TAB2 and/or TAB3 inhibition under conditions of sustained TLR stimulation remains unexplored. We therefore aimed to elucidate the effect of TAB2 and/or TAB3 deficiency in murine bone marrow-derived macrophages (BMDMs) upon sustained TLR stimulation. We prepared BMDMs from *Tab2*$^{flox/+}$ *Tab3*$^{+/-}$ *LysM-Cre/LysM-Cre* (Het), *Tab2*$^{flox/flox}$ *Tab3*$^{+/-}$ *LysM-Cre/LysM-Cre* (TAB2-KO), *Tab2*$^{flox/+}$ *Tab3*$^{-/-}$ *LysM-Cre/LysM-Cre* (TAB3-KO), and *Tab2*$^{flox/flox}$ *Tab3*$^{-/-}$ *LysM-Cre/LysM-Cre* (DKO) mice, and stimulated them with a TLR4 ligand LPS or a TLR2/1 ligand Pam3CSK4 for 24 hours. The BMDMs of Het control, TAB2-KO, or TAB3-KO produced comparable amounts of TNF-α, while BMDMs of DKO produced significantly less TNF-α upon stimulation with LPS or Pam3CSK4 (Fig. 1a). Interestingly, we observed IL-1β production from BMDMs of TAB2-KO and DKO (albeit in the DKO to a lesser extent than in TAB2-KO), but not from Het control or TAB3-KO BMDMs, when stimulated with LPS or Pam3CSK4 alone (Fig. 1b). We observed reduced cell viability only in DKO BMDMs upon stimulation with LPS or Pam3CSK4 (Fig. 1c). These results indicate that TAB2 and TAB3 play overlapping and indispensable roles in TNF-α production and cell survival at 24 hours post-TLR stimulation, whereas TAB2, but not TAB3, plays a unique and essential role in suppressing IL-1β production under the same conditions.

TAB2 deficiency causes aberrant inflammasome activation

As IL-1β maturation and secretion are regulated by the inflammasome, we next asked whether TAB2 deficiency might activate the inflammasome in BMDMs during sustained TLR stimulation. Western blot analysis of culture supernatants prepared from BMDMs stimulated with Pam3CSK4 showed elevated levels of mature IL-1β as well as the cleaved fragment of caspase-1 at 24 hours post stimulation in TAB2-KO compared to Het control (Fig. 2a). We also confirmed the elevated caspase-1 activity in TAB2-KO BMDMs upon Pam3CSK4 stimulation (Fig. 2b). The expression levels of pro-IL-1β in cell lysates were comparable between Het control and TAB2-KO BMDMs (Fig. 2a). Consistently, Pam3CSK4-induced expression of *Il1b* and *Nlrp3* mRNA was comparable between Het control and TAB2-KO BMDMs (Figs

S1a and S1b). These results suggest that TAB2 is not essential for the priming of macrophages to induce expression of *Il1b* mRNA and pro-IL-1β protein, but it plays a suppressive role in inflammasome activation under conditions in which macrophages are continuously stimulated with TLR ligand.

Activation of the inflammasome is generally accompanied by the cleavage of gasdermin D (GSDMD). The cleaved p30 N-terminal fragment of GSDMD forms a pore on the plasma membrane, which leads to the secretion of IL-1β, and pyroptosis. Interestingly, we did not detect the cleaved fragment of GSDMD in Pam3CSK4-stimulated TAB2-KO BMDMs (Fig. 2a). Consistent with this observation, Pam3CSK4 stimulation did not induce plasma membrane damage in TAB2-KO BMDMs as assessed by flow cytometry using 7-AAD staining (Fig. 2c). These results also indicate that inflammasome activation in Pam3CSK4-stimulated TAB2-KO BMDMs is not caused by damage-associated molecules released from neighboring cells but rather by cell-intrinsic mechanisms. It has been reported that GSDME, another gasdermin family protein, permits IL-1β release in a sublytic phase that is independent of cell lysis [51]. However, we did not detect GSDME cleavage in Pam3CSK4-stimulated TAB2-KO BMDMs (Fig. S2). Therefore, it is likely that IL-1β is released through mechanisms independent of GSDMD- or GSDME-induced pore formation in TAB2-KO BMDMs upon sustained TLR stimulation.

We next examined the contribution of the inflammasome to the IL-1β secretion in TAB2-KO BMDMs. Pretreatment of TAB2-KO BMDMs with VX-765, a caspase-1 inhibitor, significantly decreased the IL-1β secretion upon sustained Pam3CSK4 stimulation (Fig. 3a). Similarly, pretreatment with MCC950, an NLRP3 inhibitor, almost completely suppressed the IL-1β secretion from Pam3CSK4-stimulated TAB2-KO BMDMs (Fig. 3b). Meanwhile, MCC950 and VX-765 did not reduce the cell viability or TNF-α secretion (Figs. 3c-3f). Therefore, it is suggested that the NLRP3 inflammasome is responsible for IL-1β secretion in TAB2-KO BMDMs upon sustained stimulation with TLR ligands.

TNF/TNFR1/RIPK1 axis induces IL-1β secretion in TLR-stimulated TAB2-KO macrophages

TAK1 deficiency results in spontaneous NLRP3 inflammasome activation mediated by autocrine TNF-α in myeloid cells [16]. We therefore examined the involvement of TNF-α in the IL-1β production from TAB2-KO BMDMs upon sustained TLR stimulation. Neutralization of BMDM-derived TNF-α by anti-TNF-α antibody significantly blocked the production of IL-1β without compromising cell viability, indicating that the autocrine or paracrine TNF-α is responsible for IL-1β secretion in TAB2-KO BMDMs stimulated with Pam3CSK4 (Figs. 4a and 4b). TNFR1 and TNFR2 play pivotal roles in mediating TNF-α signaling but exhibit distinct functions in regulating immune responses [19]. To clarify the involvement of TNFR1 or TNFR2 in the IL-1β production in TAB2-KO BMDMs, we knocked down TNFR1 or TNFR2 using their specific siRNAs. We found that the knockdown of TNFR1 expression markedly reduced the IL-1β secretion in TLR-stimulated TAB2-KO BMDMs, indicating the crucial role of TNFR1 in the activation of NLRP3 inflammasome in TLR-stimulated TAB2-KO BMDMs (Fig. 4c). In contrast, knockdown of TNFR2 resulted in a partial reduction of IL-1β secretion (Fig. 4c). Notably, the knockdown of TNFR1 or TNFR2 did not reduce cell viability or TNF-α production in TAB2-KO BMDMs stimulated with Pam3CSK4 (Figs.

4d and 4e), suggesting that the effect of TNFR1 knockdown on the reduction in IL-1β secretion was not due to the cell death or reduced TNF-α production, but due to the impaired TNF-signaling.

RIPK1 plays a pivotal role in TNF signaling downstream of TNFR1 and is involved in the aberrant activation of the NLRP3 inflammasome in TAK1-deficient BMDMs [16]. Thus, we hypothesized that RIPK1 may be involved in the IL-1β production from TAB2-KO BMDMs upon prolonged TLR stimulation. Necrostatin-1, a pharmacological inhibitor of RIPK1, completely suppressed the IL-1β production without affecting the TNF-α production and cell viability (Figs. 4f-4h). These results suggest that TNF-α/TNFR1/RIPK1 axis is responsible for the IL-1β production from TAB2-KO BMDMs upon prolonged TLR stimulation.

Caspase-8 is involved in IL-1β secretion in TLR-stimulated TAB2-KO macrophages

Since TNF signaling can activate the NLRP3 inflammasome via caspase-8 (reviewed by Pang and Vince [22]), we performed Western blotting to assess whether caspase-8 is activated in TAB2-KO BMDMs upon Pam3CSK4 stimulation. Cleaved caspase-8 was detected in TAB2-KO BMDMs at 8 hours after Pam3CSK4 stimulation, and it was diminished by 24 hours post-stimulation (Fig. 5a). We confirmed the protease activity of caspase-8 in TAB2-KO BMDMs which was increased at 8 hours after Pam3CSK4 stimulation and declined by 24 hours post-stimulation (Fig. 5b). To evaluate the contribution of caspase-8 in IL-1β production in Pam3CSK4-stimulated TAB2-KO BMDMs, we conducted RNAi knockdown of caspase-8 (Figs. S3). We found that caspase-8 knockdown markedly reduced the Pam3CSK4-induced IL-1β secretion without affecting TNF-α production in TAB2-KO BMDMs (Figs. 5c and 5d). Furthermore, Pam3CSK4-induced caspase-1 activation in TAB2-KO BMDMs was significantly decreased by caspase-8 knockdown (Fig. 5e), indicating that caspase-8 in part acts upstream of caspase-1. Taken together, these results suggest that TAB2 deficiency in macrophages causes aberrant caspase-8 activation, leading to the NLRP3 inflammasome activation during sustained TLR stimulation.

Discussion

In this study, we demonstrate that TAB2 suppresses NLRP3 inflammasome activation induced by autocrine or paracrine TNF-α in murine macrophages. Importantly, this function is unique to TAB2, as its homolog TAB3 does not exhibit such suppression of NLRP3 inflammasome. This finding suggests that while TAB2 and TAB3 exhibit functional redundancy in TLR signaling [36], there exists a clear functional divergence in the regulation of NLRP3 inflammasome. Our results provide new insight into the unique role of TAB2 as a gatekeeper of the NLRP3 inflammasome, thus preventing its aberrant activation.

TAK1 restricts spontaneous activation of the NLRP3 inflammasome and pyroptosis [16]. Since TAB2 functions as a binding partner of TAK1, it was conceivable that TAB2-mediated NLRP3 suppression might operate through a TAK1-dependent mechanism. However, we observed that TAB2-deficiency did not induce GSDMD/GSDME activation or cell death, indicating that TAB2-mediated NLRP3 regulation is TAK1-independent. This finding suggests that TAB2 has unique regulatory functions beyond serving merely as an adaptor molecule for TAK1. Recently, Delanghe et al. reported that TAB2 prevents TNF-α-

induced caspase-8 activation and protects mouse embryonic fibroblasts from apoptosis at the level of the TNFR1 complex II, independently of TAK1 [42]. Therefore, it is of great interest to determine whether similar mechanisms underlie the role of TAB2 in the suppression of TNF-α-induced caspase-8 and NLRP3 inflammasome activation in macrophages.

Table 2 and TAB3 are ubiquitously expressed, share high structural similarity, and have overlapping functions in the regulation of the NF-κB pathway through TAK1 activation in TLR, IL-1R, and TNFR signaling [37, 38, 52, 53]. Meanwhile, it has been shown that TAB2-deficiency, but not TAB3-deficiency, results in embryonic lethality due to the liver apoptosis, indicating that TAB2 has a non-redundant and essential role during development [49, 50]. It was recently demonstrated that TAB2, but not TAB3, suppresses TNF-α-induced apoptosis and necroptosis through TAK1-dependent or -independent mechanisms under different cellular context [42, 43]. However, the molecular basis underlying the functional divergence between TAB2 and TAB3 remains unclear. Further investigation is warranted to elucidate the distinct mechanisms by which TAB2 and TAB3 differentially regulate TNF-α-induced NLRP3 inflammasome activation and cell death.

A particularly noteworthy finding in this study is that IL-1β release induced in TAB2-deficient macrophages was not accompanied by GSDMD cleavage and pyroptosis. It is currently unclear how GSDMD cleavage is uncoupled from caspase-1 activation in TAB2-deficient macrophages. It was suggested that GSDMD, chaperoned by Cdc37/Hsp90, may be protected from cleavage by NLRP3/caspase-8 non-canonical inflammasome but still involved in IL-1β release via small extracellular vesicles in intestinal epithelial cells [54]. Further study is needed to determine whether a similar mechanism also exists in TAB2-deficient macrophages upon sustained TLR activation. Alternatively, Dupont et al. demonstrated that autophagy machinery mediates IL-1β secretion independently of cell death [55]. As TAB2 and TAB3 negatively regulate basal autophagy [56, 57], it may be possible that autophagy activation induced by TAB2-deficiency promotes IL-1β release. IL-1β can also be released through transmembrane emp24 domain-containing protein 10 (TMED10)-channeled unconventional protein secretion pathway or early endosome autoantigen 1 (EEA1)-mediated release pathway independently of GSDMD and pyroptosis [58, 59]. Furthermore, Pandori et al. showed that *Toxoplasma gondii* infection triggers robust IL-1β release from human monocytes without inducing GSDMD cleavage and pyroptosis, allowing sustained inflammatory signaling while preserving cellular antimicrobial functions [21, 60]. Thus, it is of considerable interest to determine whether TAB2 regulates IL-1β release under these conditions.

TAK1 complex plays a pivotal role in innate and adaptive immune responses. Some pathogens evade host immunity by targeting the TAK1 complex. For example, the effector molecule NleE of enteropathogenic *E. coli* (EPEC) is known to methylate cysteine residues of TAB2 and TAB3, thereby inhibiting ubiquitin chain recognition by TAB2 and TAB3 and suppressing NF-κB signal transduction [47]. Similarly, the 3C protease of enterovirus 71 (EV71) has also been reported to cleave TAB2 [61]. TAB2 targeting by these pathogens is a strategy to suppress host innate immune responses and establish infection. However, in light of our findings, suppression of TAB2 may also release the brake on NLRP3

inflammasome and induce IL-1β production. Even if pathogens temporarily suppress the NF-κB pathway using their effector proteins that target TAB2, the resulting activation of the NLRP3-IL-1β pathway ultimately triggers an inflammatory response, suggesting that TAB2 may function as a sensor to activate the host immune response, so-called “effector-triggered immunity” [62, 63]. In the future, it will be important to verify the role of TAB2 in IL-1β production and host defense during pathogen infection.

Declarations

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Conflict of interest

None of the authors has any financial interests related to this work.

Author contribution

Conceptualization, G.M. and G.T.; Methodology, O.T.; Investigation, T.A., N.A., G.M., G.T.; Writing-Original Draft, T.A. and G.T., Writing-Review & Editing, T.A., O.T., T.S., G.M., G.T.; Supervision, G.M. and G.T.

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Data Availability

The datasets supporting the conclusions of this article are included within this article and its supplementary information files.

Competing interests

The authors declare no competing interests.

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Figures

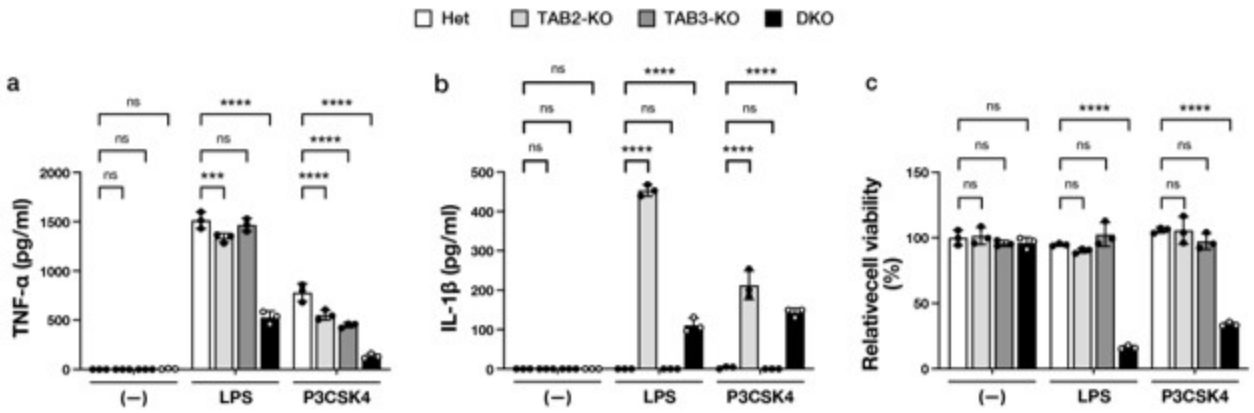


Figure 1

TAB2 deficiency promotes IL-1β production by BMDMs during prolonged TLR stimulation

BMDMs prepared from Het (*Tab2*$^{flox/+}$ *Tab3*$^{+/-}$ *LysM-Cre/LysM-Cre*, open bars), TAB2-KO (*Tab2*$^{flox/flox}$ *Tab3*$^{+/-}$ *LysM-Cre/LysM-Cre*, light grey bars), TAB3-KO (*Tab2*$^{flox/+}$ *Tab3*$^{-/-}$ *LysM-Cre/LysM-Cre*, dark grey bars), and DKO (*Tab2*$^{flox/flox}$ *Tab3*$^{-/-}$ *LysM-Cre/LysM-Cre*, black bar) mice were treated with LPS (100 ng/ml) or Pam3CSK4 (100 ng/ml) for 24 h or left untreated (–). Culture supernatants were analyzed by ELISA to measure the protein concentrations of TNF-α (**a**) and IL-1β (**b**), or subjected to cell viability analysis using Cell Count Reagent SF (**c**). Data were analyzed using two-way ANOVA followed by Tukey's multiple comparison tests. ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant. Data are shown as the mean ± SD of triplicate wells.

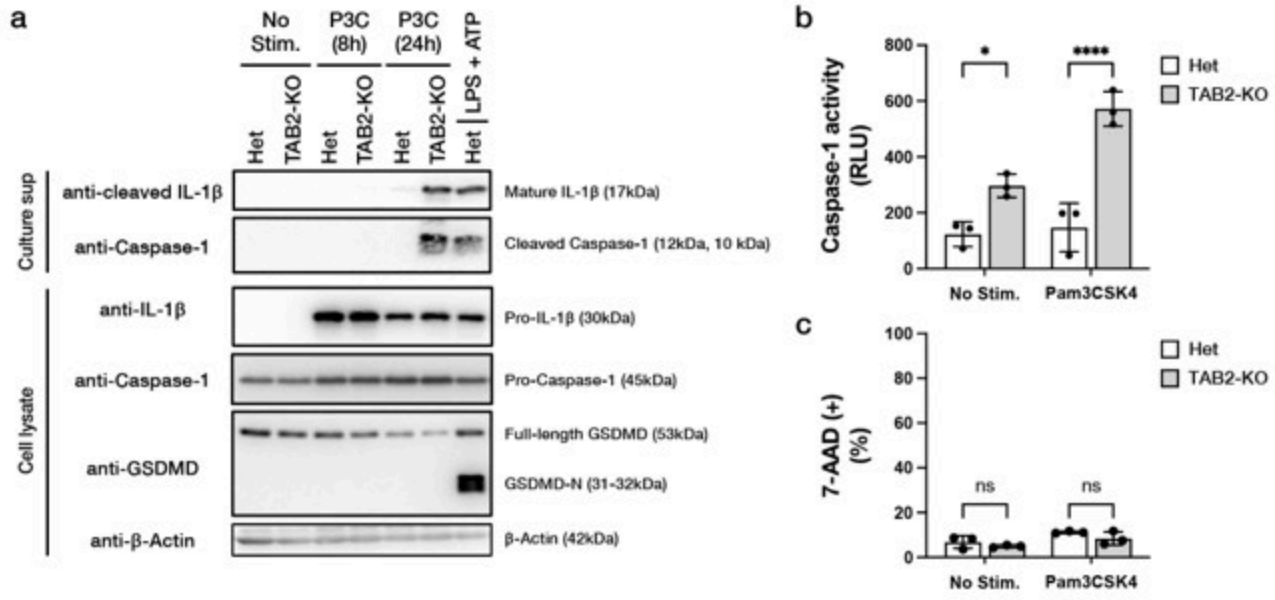


Figure 2

TAB2 deficiency causes caspase-1 activation in BMDMs during prolonged TLR stimulation

(a) BMDMs of the indicated genotypes were left unstimulated (No Stim.) or stimulated with Pam3CSK4 (100 ng/mL) for the indicated periods. Whole cell extracts and culture supernatant were subjected to SDS-PAGE followed by western blotting using the indicated antibodies to detect the corresponding proteins. β-Actin was used as a loading control. **(b)** BMDMs of the indicated genotypes were left unstimulated (No Stim.) or stimulated with Pam3CSK4 (100 ng/mL) for 24 h. Protease activity of caspase-1 was measured using a luminogenic substrate and expressed as relative light units (RLU). Data are shown as the mean ± SD of triplicate wells. **(c)** BMDMs of the indicated genotypes were left unstimulated (No Stim.) or stimulated with Pam3CSK4 (100 ng/mL) for 24 h. Cells were collected, stained with 7-AAD, and analyzed by flow cytometry. The percentage of 7-AAD-positive populations of each experimental group was shown. Data were analyzed using two-way ANOVA followed by Tukey's multiple comparison tests. *, $p < 0.05$; ****, $p < 0.0001$; ns, not significant. Data are shown as the mean ± SD of triplicate wells.

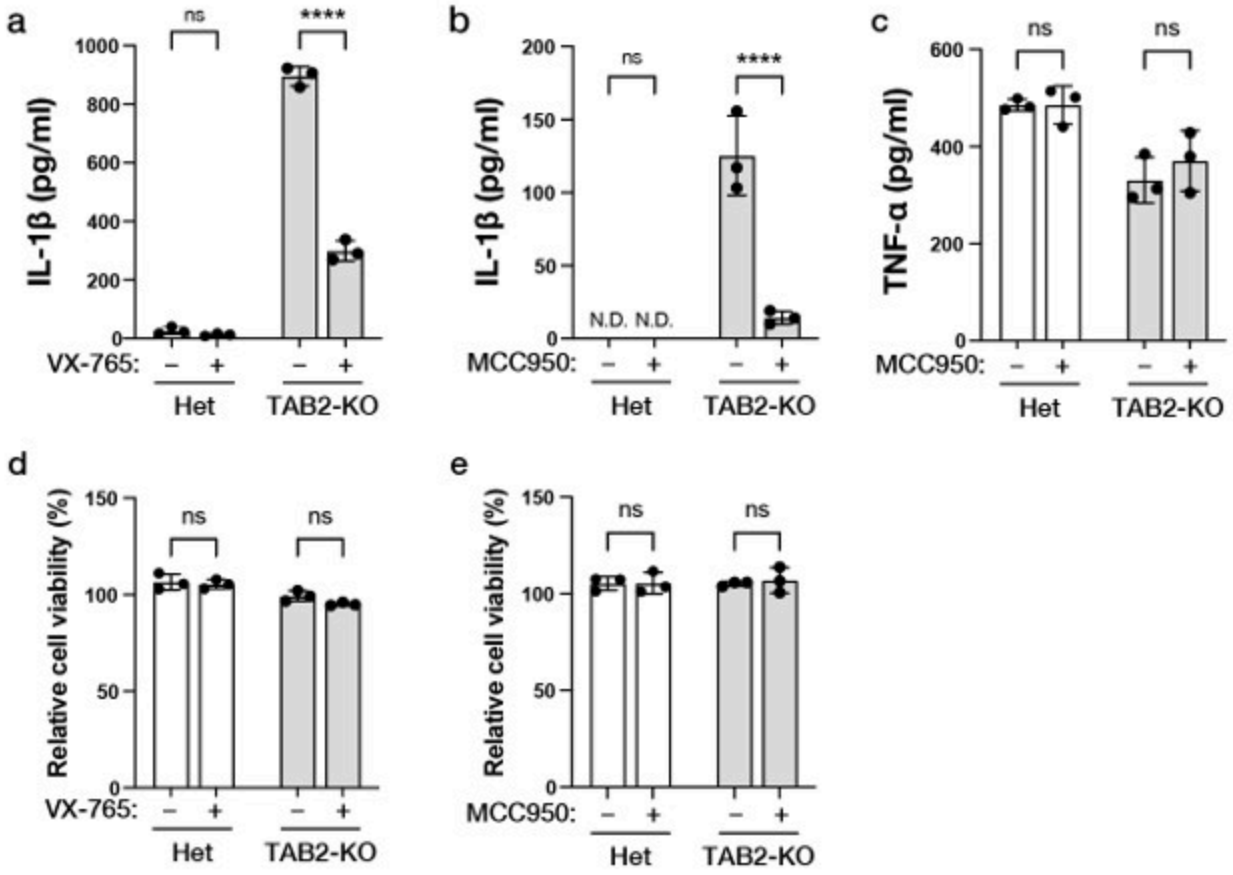


Figure 3

NLRP3 and caspase-1 are required for IL-1β production by TAB2-deficient BMDMs during prolonged TLR stimulation

(a) BMDMs of the indicated genotypes were left untreated (−) or pretreated (+) with VX-765 (10 μM) for 1 h, and stimulated with Pam3CSK4 (100 ng/mL) for 24 h. Culture supernatants were analyzed by ELISA to measure the protein concentrations of IL-1β. **(b, c)** BMDMs of the indicated genotypes were left untreated (−) or pretreated (+) with MCC950 (10 μM) for 1 h, and stimulated with Pam3CSK4 (100 ng/mL) for 24 h. Culture supernatants were analyzed by ELISA to measure the protein concentrations of IL-1β (**b**) or TNF-α (**c**). **(d, e)** Culture supernatants of A and B were subjected to cell viability analysis using Cell Count Reagent SF. Data were analyzed using two-way ANOVA followed by Tukey's multiple comparison tests. ****, $p < 0.0001$; ns, not significant. Data are shown as the mean ± SD of triplicate wells.

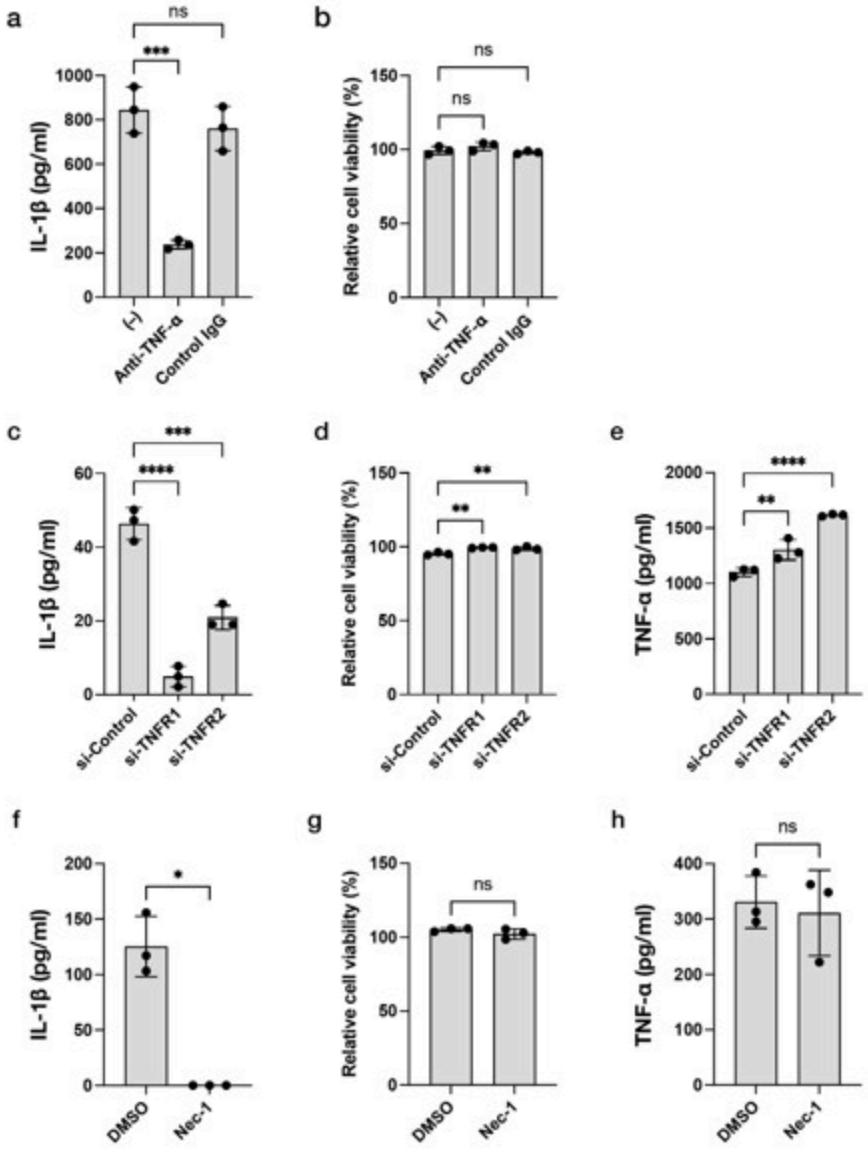


Figure 4

Autocrine TNF-α, TNFR1, and RIPK1 are required for IL-1β production by TAB2-deficient BMDMs during prolonged TLR stimulation

(a, b) TAB2-KO BMDMs were left untreated (−) or pretreated with anti-TNF-α neutralizing antibody or isotype control IgG (5 μg/mL) for 1 h, and stimulated with Pam3CSK4 (100 ng/mL) for 24 h. Culture supernatants were analyzed by ELISA to measure the protein concentrations of IL-1β (**a**), or subjected to cell viability analysis using Cell Count Reagent SF (**b**). **(c-e)** TAB2-KO BMDMs were transfected with siRNAs against TNFR1 or TNFR2, or control siRNA, and incubated for 24 h. Cells were stimulated with Pam3CSK4 (100 ng/mL) for 24 h. Culture supernatants were analyzed by ELISA to measure the protein concentrations of IL-1β (**c**) or TNF-α (**e**), or subjected to cell viability analysis using Cell Count Reagent SF (**d**). **(f-h)** TAB2-KO BMDMs were left untreated (−) or pretreated (+) with Necrostatin-1 (Nec-1, 10 μM) for 1 h, and stimulated with Pam3CSK4 (100 ng/mL) for 24 h. Culture supernatants were analyzed by

ELISA to measure the protein concentrations of IL-1β (**f**) or TNF-α (**h**), or subjected to cell viability analysis using Cell Count Reagent SF (**g**). Data were analyzed using two-way ANOVA followed by Tukey's multiple comparison tests. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant. Data are shown as the mean ± SD of triplicate wells.

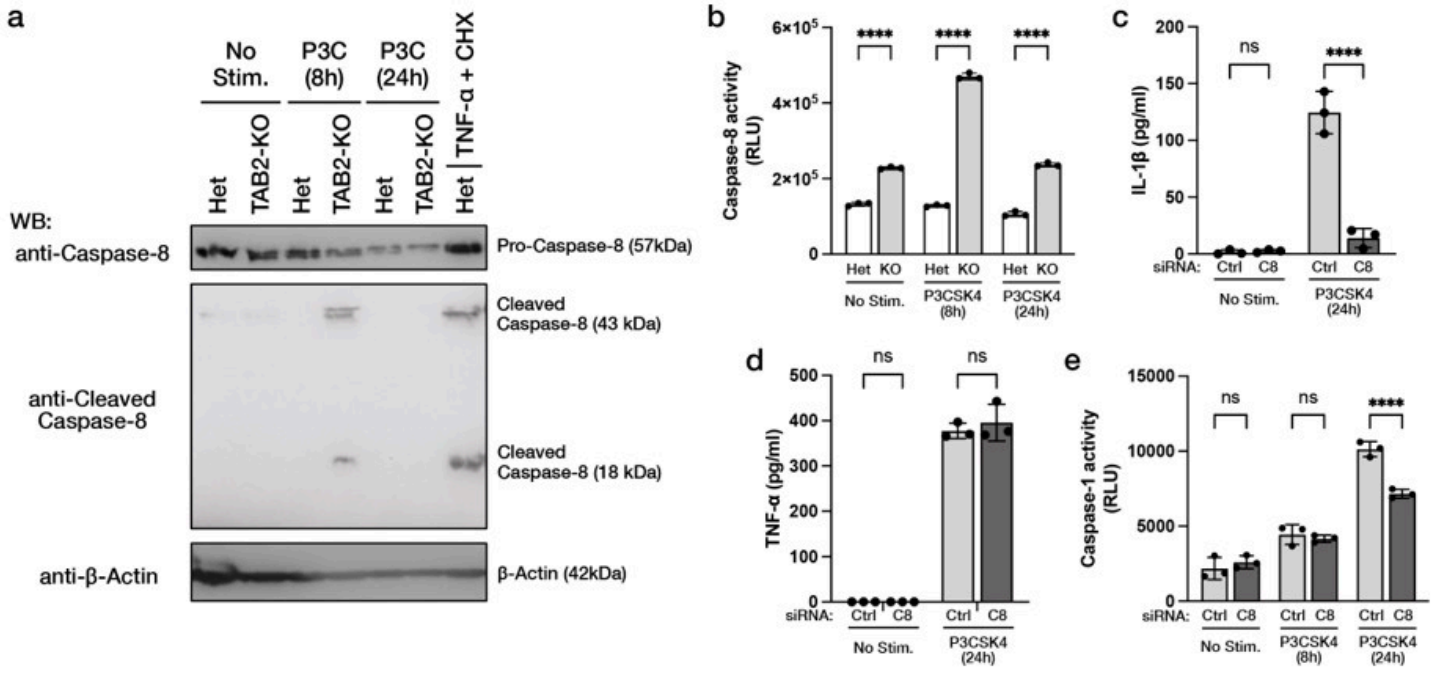


Figure 5

Caspase-8 is required for IL-1β production by TAB2-deficient BMDMs during prolonged TLR stimulation

(a) BMDMs of the indicated genotypes were left unstimulated (No Stim.) or stimulated with Pam3CSK4 (100 ng/mL) for the indicated periods. Whole cell extracts were subjected to SDS-PAGE followed by western blotting using the indicated antibodies to detect the corresponding proteins. β-Actin was used as a loading control. **(b)**BMDMs of the indicated genotypes were left unstimulated (No Stim.) or stimulated with Pam3CSK4 (100 ng/mL) for the indicated periods. Protease activity of caspase-8 was measured using a luminogenic substrate and expressed as relative light units (RLU). Data are shown as the mean ± SD of triplicate wells. **(c-e)** TAB2-KO BMDMs were transfected with siRNA against caspase-8 (C8), or control siRNA (Ctrl), and incubated for 24 h. Cells were stimulated with Pam3CSK4 (100 ng/mL) for 24 h. Culture supernatants were analyzed by ELISA to measure the protein concentrations of IL-1β (**c**) or TNF-α (**d**). Protease activity of caspase-1 was measured using a luminogenic substrate and expressed as relative light units (RLU) (**e**). Data were analyzed using two-way ANOVA followed by Tukey's multiple comparison tests. ****, $p < 0.0001$; ns, not significant. Data are shown as the mean ± SD of triplicate wells.

Supplementary Files

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