# An unconventional role of an ASB family protein in NF-κB activation and inflammatory response during microbial infection and colitis

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Edited by Sankar Ghosh, Columbia University Medical Center, New York, NY, and accepted by Editorial Board Member Tadatsugu Taniguchi December 4, 2020 (received for review July 24, 2020)

**Nuclear factor κB (NF-κB)–mediated signaling pathway plays a crucial role in the regulation of inflammatory process, innate and adaptive immune responses. The hyperactivation of inflammatory response causes host cell death, tissue damage, and autoinflammatory disorders, such as sepsis and inflammatory bowel disease. However, how these processes are precisely controlled is still poorly understood. In this study, we demonstrated that ankyrin repeat and suppressor of cytokine signaling box containing 1 (ASB1) is involved in the positive regulation of inflammatory responses by enhancing the stability of TAB2 and its downstream signaling pathways, including NF-κB and mitogen-activated protein kinase pathways. Mechanistically, unlike other members of the ASB family that induce ubiquitination-mediated degradation of their target proteins, ASB1 associates with TAB2 to inhibit K48-linked polyubiquitination and thereby promote the stability of TAB2 upon stimulation of cytokines and lipopolysaccharide (LPS), which indicates that ASB1 plays a noncanonical role to further stabilize the target protein rather than induce its degradation. The deficiency of *Asb1* protects mice from *Salmonella typhimurium*– or LPS-induced septic shock and increases the survival of mice. Moreover, *Asb1*-deficient mice exhibited less severe colitis and intestinal inflammation induced by dextran sodium sulfate. Given the crucial role of ASB proteins in inflammatory signaling pathways, our study offers insights into the immune regulation in pathogen infection and inflammatory disorders with therapeutic implications.**

ASB1 | inflammatory response | NF-κB | TAB2 | ubiquitination

Inflammatory response is triggered by infection or tissue damage involving the recognition of damage-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) (1, 2). These PRRs recognize various microbial components such as bacterial lipoproteins, viral and bacterial nucleic acids, carbohydrates, and endogenous proinflammatory molecules released from damaged cells (3, 4). The activation of PRRs induces robust and quick expression and secretion of proinflammatory cytokines, chemokines, and type I interferon, initiating the immune responses (5). The ligation of the receptor induces formation of the receptor complex through the recruitment of adaptor proteins and further elicits a cascade of signaling networks, leading to the transcription factors of nuclear factor κB (NF-κB) and AP-1 activation through upstream kinases including IκB kinase (IKK) complex and mitogen-activated protein kinase (MAPK) pathways (6). During the signal transduction, TNFR-associated factors (TRAFs) function as E3 ligases to catalyze autoubiquitination or K63-linked polyubiquitination of substrates, which further recruit kinase complex consisting of TAK1 and TAK1-binding proteins TAB1, TAB2, or TAB3 (7). TAB2 or its homolog TAB3 binds to K63-linked polyubiquitin chains conjugated to TRAF6 or RIP1 and helps activate TAK1, that phosphorylates and activates IKK and MAPK kinase (MKK) (8, 9). IKKβ in turn phosphorylates the inhibitor of NF-κB (IκBα), resulting in IκBα degradation through ubiquitin proteasome system (UPS). The released NF-κB forms dimers and translocates into the nucleus to turn on various inflammatory gene expression (10). After activation by MKK, MAPKs phosphorylate transcription factor AP-1, cytoskeletal proteins, and other signaling proteins to regulate gene transcription and inflammatory response (11, 12). The activation of inflammatory response drives NF-κB– and AP-1–mediated transcription and, in turn, their transcriptional activities to induce various inflammatory gene expression that further amplify inflammation. Notably, dysregulation of NF-κB activation has been linked with several chronic inflammatory, infectious, or autoimmune disorders and cancer (13). However, the underlying regulatory mechanism of NF-κB in these diseases remains largely unknown.

The family of ankyrin (ANK) repeat and suppressor of cytokine signaling box (SOCS) (ASB) proteins consists of 18 members that contain a variable number of N-terminal ANK repeats and a C-terminal SOCS box (14). The ASB family and other four protein families form the SOCS box superfamily. SOCS box–containing molecules commonly comprise two functional

## Significance

**Regulation of inflammatory response is central to the outcome of microbial infection and inflammatory diseases. We demonstrated that ASB1, a member of the family of the ankyrin repeat and suppressor of cytokine signaling box (ASB) proteins, plays a previously unknown function in TAB2/TAK1-mediated inflammatory responses. ASB1 stabilizes TAB2 and consequently promotes the activation of downstream signaling pathways including NF-κB and MAPK pathways. The deficiency of ASB1 leads to less severe colitis and intestinal inflammation and protects mice from bacteria or lipopolysaccharide-induced septic shock. Such a finding has important implications in the understanding of inflammatory diseases and development of intervention measures related to ASB family proteins.**

Author contributions: P.H. and D.G. designed research; P.H., P.J., K.Y., Z.L., Y. Lin, and W.Z. performed research; P.H., P.J., K.Y., Z.L., T.T., Y. Lin, F.X., Y.C., C.L., Y. Liu, and D.G. contributed new reagents/analytic tools; P.H., P.J., K.Y., Z.L., T.T., and C.L. analyzed data; and P.H., P.J., C.L., and D.G. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission. S.G. is a guest editor invited by the Editorial Board.



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This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2015416118/-/DCSupplemental.

Published January 11, 2021.

protein–protein interaction domains (i.e., a substrate-binding domain and an SOCS box domain), which can function as E3 ubiquitin ligases (15, 16). Although some ASB proteins were reported to be involved in receptor signaling regulation, little is known about the detailed functions of the ASB family. Notch-induced ASB2 promotes the ubiquitination of Notch to control cell proliferation and differentiation and Notch1-ASB2-NF-κB pathway modulate the apoptosis and proliferation of T cell acute lymphoblastic leukemia (T-ALL) cells (17, 18). ASB3 promotes UPS-dependent degradation of TNF receptor 2, and its mutations promote the growth and metastasis of colorectal cancer cells (19, 20). A recent study reported that knockdown of ASB3 led to enhanced mitochondrial apoptosis and autophagy to promote cell death in hepatocellular carcinoma (HCC) (21). ASB4 promoted vascular differentiation through the promoting inhibitor of DNA binding 2 (ID2) degradation in the placenta and loss of function of ASB4 hindered tumor cell migration and invasion in HCC (22, 23). ASB6 was reported to facilitate degradation of adapter proteins with a pleckstrin homology and SH2 domain (APS) to regulate an insulin-signaling pathway (24). Inflammatory cytokines TNFα and IL-1α can up-regulate ASB10 expression that may be involved in glaucoma pathogenesis (25). The methylation status of the ASB1 gene relates with left ventricular performance in ischemic cardiomyopathy, and the epigenetic modification of the ASB1 gene is involved in anxiety-related immune dysregulation (26, 27). Recently, the structure and dynamics of the ASB9-Cul-RING E3 ligase complex were reported (28). Most of these studies suggested that ASB proteins assemble with Elongin B/C, Cullin 5, and Rbx2 to form ECS E3 ubiquitin ligases, which target the substrates for degradation through UPS.

In this study, we performed unbiased genome-wide library screen using the CRISPR-Cas9 system and identified the functions of ASB1 in regulating TAB2/TAK1-dependent inflammatory responses. Our study revealed that ASB1 enhances NF-κB– and MAPK-dependent signaling activity upon treatment with TNFα, IL-1β, and lipopolysaccharide (LPS). We demonstrated that ASB1 deficiency attenuates *Salmonella typhimurium* infection–induced sepsis, LPS-induced endotoxic shock, and dextran sodium sulfate (DSS)-induced chronic enteritis. Intriguingly, unlike other members of the ASB family, ASB1 does not function as an E3 ligase to mediate K48-linked polyubiquitination and degradation of substrate proteins but rather promotes TAB2 stability by inhibiting K48-linked polyubiquitination, leading to enhancement of NF-κB and MAPK activation. Such a mechanism was previously unknown to other members of the ASB family and represents a regulatory model of inflammatory signaling pathways.

## Results

**The Identification and Validation of ASB1 as a Positive Regulator of NF-κB–Mediated Inflammatory Response.** To identify novel genes that regulate the production of type I interferon and inflammatory response induced by virus infection, we performed genome-wide screen of a pooled CRISPR-Cas9 library. A quantitative flow, cytometry-based analysis was established for the screen by using enhanced green fluorescent protein (EGFP) reporter driven by a human interferon beta (IFN-β) promoter in A549 cells. We introduced the GeCKO library into A549 reporter cells, stimulated cells with Sendai virus (SeV), and sorted cells gated on high or low EGFP expression. The screen precisely identified several known regulators such as RIG-I (29), PSMB11 (30), SPNS2 (31), IRF3/IRF7 (32), IFIH1 (33), LCK (34), TRIM33 (35), and ATG12 (36) of immune signaling or development of immune cells, indicating that the screen was effectively executed. Among the top ranked genes, there are several that were previously unknown to be involved in immune and inflammatory responses, including CCDC50, which is a novel autophagic substrate receptor involved in negative regulation of RIG-I–mediated interferon signaling published very recently by our group (37), and ASB1 which was previously regarded as a substrate-recognition component of an E3 ligase complex for ubiquitination and proteasomal degradation (38). As the function of ASB1 had not been characterized previously, we went further to validate the function of ASB1 in type I IFN–mediated antiviral innate immune and inflammatory responses. As shown in Fig. 1*A*, ectopic expression of ASB1 enhanced SeV-induced activation of the IFN-β promoter in an ASB1 dose-dependent manner. Transcription of the *IFNB1* gene requires recruitment of the transcription factors IRFs, AP-1, or NF-κB to the corresponding binding sites in the *IFNB1* promoter. We then tested which signaling pathway was affected by ASB1. The results showed that ASB1 could promote SeV-triggered activations of IFN-β and NF-κB reporters but did not affect SeV-induced activation of the PRDI-III promoter or IFNγ-triggered activation of the IRF1 reporter in HEK293 cells (Fig. 1*B*), indicating that ASB1 might participate in the regulation of NF-κB signaling but not IRF3-mediated transcription. To confirm these results, we used classic cytokine stimuli TNFα and IL-1β to activate the NF-κB pathway. In similar reporter assays, ASB1 enhanced TNFα- and IL-1β-stimulated NF-κB activation in dose-dependent manners in HEK293 cells (Fig. 1*C*). Similarly, ASB1 also potentiated TNFα- and IL-1β–triggered IL-6 and IL-8 promoter reporters (*SI Appendix*, Fig. S1 *A* and *B*). In addition, transient expression of ASB1 significantly increased the transcription of *IFNB1* and proinflammatory cytokine *TNFα* but had no effect on the transcription of *IRF1* after induction with SeV or IFNγ, suggesting that ASB1 enhances virus-triggered activation of type I IFN through NF-κB signaling (Fig. 1*D*). Consistently, we observed that ASB1 increased SeV-induced phosphorylation of IKKα/β and TAK1 in the NF-κB pathway but did not change the level of the phosphorylation of TBK1 and IRF3 in the IRFs' pathway (Fig. 1*E*). Moreover, ectopic expression of ASB1 strengthened TNFα- and IL-1β–induced expression and secretion of proinflammatory cytokines including TNFα, IL-6, and IL-8 (Fig. 1 *F* and *G*). TNFα- and IL-1β–induced phosphorylation of IKKα/β and IκBα, a hallmark of NF-κB activation, was also markedly increased in ASB1-overexpressing cells in comparison with control cells (Fig. 1*H* and *SI Appendix*, Fig. S1*C*). Taken together, we demonstrated that ASB1 enhances type I IFN–mediated antiviral responses by promoting NF-κB-mediated inflammatory responses.

**ASB1 Deficiency Inhibits Viral Infection–Induced or Cytokine-Triggered NF-κB and MAPK Signaling.** To further explore the potential physiological functions of ASB1, we developed ASB1-deficient HEK293 and A549 cells by using the CRISPR-Cas9 system. ASB1-knockout (KO) HEK293 cells were generated by single cell cloning and confirmed by DNA sequencing and immunoblotting (Fig. 2*A* and *SI Appendix*, Fig. S2*A*). Consistent with the above results, KO of ASB1 attenuated SeV-triggered activation of the IFN-β and the NF-κB promoters in reporter assays (*SI Appendix*, Fig. S2*B*). ASB1 deficiency also significantly reduced the transcriptional levels of *IFNB1* and *IL8* induced by SeV (*SI Appendix*, Fig. S2*C*), which was consistent with reduced phosphorylation of IKKα/β and TAK1 in ASB1-deficient HEK293 cells (*SI Appendix*, Fig. S2*D*). In contrast, the level of phosphorylation of TBK1 and IRF3 was comparable between ASB1-wild-type (WT) and ASB1-KO cells (*SI Appendix*, Fig. S2*D*). Strikingly, ASB1 KO significantly lowered TNFα- and IL-1β-induced activation of the NF-κB promoter but not IFNγ-triggered activation of the IRF1 promoter (Fig. 2*B*). In line with this observation, the expression and secretion of NF-κB downstream genes *TNFα*, *IL6*, and *IL8* were reduced in ASB1-KO cells compared with those of ASB1-WT cells after TNFα or IL-1β stimulation (Fig. 2 *C* and *D*). Furthermore, ASB1-deficient cells exhibited a higher level of total IκBα and reduced levels of

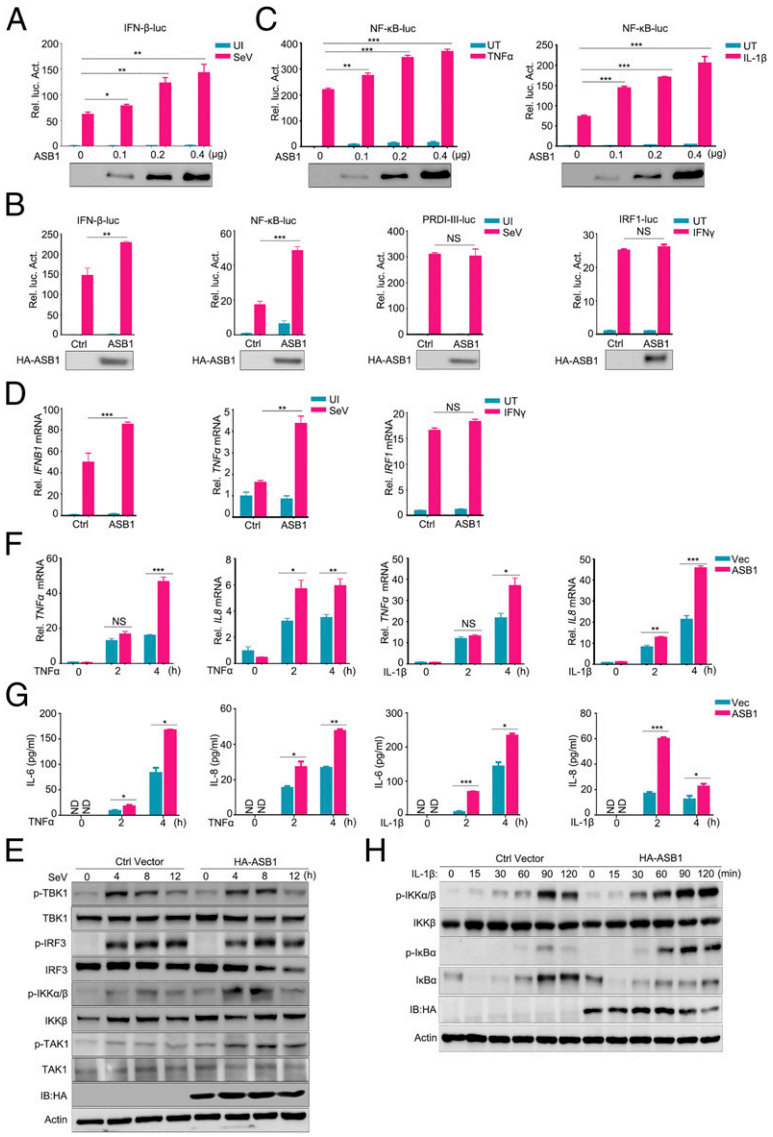


**Fig. 1.** The validations of ASB1 as a positive regulator of the NF-κB signaling pathway. (*A*) Dose-dependent effects of ASB1 on SeV-triggered IFNβ activation in HEK293 cells. HEK293 cells were transfected with the IFNβ-luc and RL-TK-luc as well as an increasing amount of HA-ASB1 plasmids for 24 h and then left infected or uninfected for another 8 h with SeV. The expression of ASB1 in HEK293 cells was examined by immunoblot analysis (*Lower*). (*B*) HEK293 cells were transfected with the IFNβ-luc, NF-κB-luc, PRDI-III-luc, or IRF1-luc plus RL-TK-luc as well as a control plasmid or plasmid encoding HA-ASB1 for 24 h and then infected with SeV for 8 h or stimulated with IFNγ for 10 h. The expression of ASB1 was examined by immunoblot analysis (*Lower*). (*C*) Dose-dependent effects of ASB1 on TNFα- and IL-1β–triggered NF-κB activation in HEK293 cells. The experiments were performed as in *A* except that the cells were treated with TNFα (10 ng/mL) and IL-1β (10 ng/mL) for 10 h. (*D*) qRT-PCR analysis of mRNA levels of *IFNB1*, *TNFα*, and *IRF1* in HEK293 cells transfected with control empty plasmids or plasmids encoding ASB1 for 24 h and then infected with SeV for 8 h or treated with IFNγ for 10 h. (*E*) Immunoblot analysis of HEK293 cells transfected with an empty vector (Ctrl) or HA-ASB1 for 24 h and then infected with SeV for indicated time points. (*F* and *G*) qRT-PCR analysis of mRNA levels of *TNFα* and *IL8* (*F*) and ELISA of production of IL-6 and IL-8 proteins (*G*) in HEK293 cells transfected with control empty plasmids or plasmids encoding ASB1 for 24 h and then stimulated with TNFα or IL-1β for indicated time points. (*H*) HEK293 cells were transfected with control empty plasmid or HA-ASB1 for 24 h, and then the cells were treated with IL-1β (10 ng/mL) for indicated time points. The whole-cell lysates were analyzed by immunoblotting with the indicated antibodies. mRNA levels are presented relative to those of untreated control cells; actin was used as a loading control. UT, untreated. ND, not detectable. NS, not significant; data are representative of three independent experiments and are shown as the mean with SEM (*A–D*, *F*, and *G*). See also *SI Appendix*, Fig. S1. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$; two-tailed unpaired Student's *t* test.

phosphorylated IκBα, p38, and ERK1/2, as well as JNK following TNFα or IL-1β stimulation, indicating that ASB1 was involved in positively regulating TNFα- or IL-1β–triggered NF-κB and MAPK activation and that ASB1 functioned upstream of MAPKs and IKKs (Fig. 2 *E* and *F*). To explore the potential roles of ASB1-mediated regulation in antiviral responses, we used vesicular stomatitis virus (VSV) to infect A549 cells with or without ASB1 knockdown with different multiplicities of infection and found that the replication of VSV-GFP was higher in ASB1 knockdown cells than that in their WT control cells, as determined by detection of messenger RNA (mRNA) levels of VSV and visualization by fluorescence microscopy (*SI Appendix*, Fig. S2 *E–G*). Consistently, KO of ASB1 significantly promoted virus replication as further determined by flow cytometry and plaque assays (*SI Appendix*, Fig. S2 *H* and *I*), suggesting that ASB1 inhibits viral infection and propagation. We then reconstituted ASB1 expression in ASB1-KO HEK293 cells with an ASB1-expressing plasmid which had a mutation in the PAM

motif and could resist Cas9 cleavage. Interestingly, the attenuation effect of ASB1 ablation on the activation of NF-κB was reversed (*SI Appendix*, Fig. S2*J*). Collectively, we proved that ASB1 specifically increased NF-κB and MAPK activation and proinflammatory cytokine production both in cellular antiviral responses and cytokine-triggered inflammatory responses.

**Impaired Cellular Inflammatory Response in ASB1-Deficient Primary Immune Cells.** To further determine the biological functions of ASB1, we generated *Asb1* gene KO mice in a C57BL/6J background by using CRISPR-Cas9–based technology, and the detailed procedure is described in *SI Appendix*, *Materials and Methods*. In *Asb1*-KO mice, the entire fragment containing all exons of the ASB1 gene was removed (*SI Appendix*, Fig. S3*A*). The deletion of the *Asb1* gene was confirmed by the genotyping of genomic DNA from toes and tails of mice (*SI Appendix*, Fig. S3*B*). Although a previous study suggested that *Asb1* may be involved in spermatogenesis (39), the $Asb1^{-/-}$ mice were born in normal numbers, grew up in normal body sizes, and had no behavioral abnormalities compared with their WT littermates. We also verified the deletion of *Asb1* in primary immune cells derived from bone marrow and several organs including the liver, lung, and spleen (*SI Appendix*, Fig. S3 *C* and *D*). The cell number of the thymus and spleen and the percentages of lymphocytes of the spleen were comparative in $Asb1^{-/-}$ and $Asb1^{+/+}$ mice, which indicated that ASB1 did not influence the development of lymphocytes (*SI Appendix*, Fig. S3 *E*–*G*). To investigate whether CRISPR-Cas9 technology caused off-target events in the generation of the ASB1-KO mice, we predicted potential off-target sites for each guide RNA through CRISPOR software (crispor.tefor.net/). We next performed PCR amplification and Sanger sequencing of 16 top-ranked sites to determine whether there exists off-target mutations in the offspring mice (*SI Appendix*, Fig. S6). The results showed no mutations in these potential off-targeted sites, suggesting that the off-target mutations were negligible in our mouse model.

We then prepared bone marrow-derived macrophages (BMDMs) and dendritic cells (BMDCs) from $Asb1^{+/+}$ and $Asb1^{-/-}$ mice and stimulated them with TNFα and IL-1β. We observed that BMDMs and BMDCs from $Asb1^{-/-}$ mice showed significantly reduced mRNA expression of *Tnfα*, *Il6*, and *Cxcl1* compared with those of $Asb1^{+/+}$ counterparts (Fig. 3 *A* and *B* and *SI Appendix*, Fig. S4 *A* and *B*). Enzyme-linked immunosorbent assay (ELISA) also showed lower production and secretion of proinflammatory cytokines IL-6 and CXCL1 in $Asb1^{-/-}$ BMDMs and BMDCs after treatment with TNFα and IL-1β compared with those of immune cells from WT mice (Fig. 3*C* and *SI Appendix*, Fig. S4*C*). We also found that the phosphorylation of IKKα/β and IκBα was lower in $Asb1^{-/-}$ BMDMs than in $Asb1^{+/+}$ macrophages following TNFα and IL-1β stimulation (Fig. 3 *D* and *E*). Consistent with the phosphorylation data, IκBα was degraded less efficiently in *Asb1*-deficient BMDMs (Fig. 3 *D* and *E*). We also determined and confirmed this phenomenon in BMDCs prepared from $Asb1^{-/-}$ and $Asb1^{+/+}$ mice (*SI Appendix*, Fig. S4 *D* and *E*). Notably, the expression of *Asb1* was markedly higher than that in untreated cells, and the expression level changed in the time course of ligand stimulation in *Asb1*-WT cells (Fig. 3 *D* and *E* and *SI Appendix*, Fig. S4 *D* and *E*). Altogether, these findings indicate that ASB1 is involved in TNFα- and IL-1β–triggered NF-κB–mediated inflammation.

Next, we determined whether ASB1 is involved in LPS-induced activation of TLR4 signaling that belongs to the TLR/IL1-R superfamily. As is shown in Fig. 4 *A* and *B*, the mRNA expression of *Tnfα*, *Il6*, and *Cxcl1* in response to LPS stimulation was significantly reduced in *Asb1*-deficient BMDMs and BMDCs compared with their WT control cells. Furthermore, *Asb1* deficiency also reduced the protein production of IL-6 and CXCL1 induced by LPS in BMDMs and BMDCs isolated from $Asb1^{-/-}$ mice in comparison with that of $Asb1^{+/+}$ cells (Fig. 4*C*). Consistently, *Asb1* deficiency in BMDMs and BMDCs also obviously inhibited LPS-induced phosphorylation of IKKα/β, IκBα, ERK1/2, p38, and JNK, suggesting that ASB1 is involved in positively regulating TLR-induced NF-κB and MAPK activation (Fig. 4 *D* and *E*). Accordingly, the level of IκBα was increased in *Asb1*-deficient BMDMs and BMDCs. To investigate whether ASB1 is involved in noncanonical NF-κB signaling, we assessed CD40L-induced processing of p100 in BMDMs and BMDCs. No significant difference in the level of p100 and its cleavage product p52 was observed between *Asb1*-WT and *Asb1*-KO cells (*SI Appendix*, Fig. S4 *F* and *G*), indicating that ASB1 did not affect the alternative NF-κB pathway. Collectively, these results showed that deficiency of *Asb1* inhibited the activation of canonical NF-κB and MAPK signaling induced by TNFα, IL-1β, and LPS in mouse primary immune cells.

***Asb1*$^{-/-}$ Mice Were More Resistant to LPS- and *S. typhimurium*–Induced Septic Shock.** Considering the lethal effect caused by excessive inflammatory cytokine secretion, we examined whether loss of function of ASB1 protects mice from LPS- and *S. typhimurium*–induced septic shock. We first challenged $Asb1^{+/+}$ and $Asb1^{-/-}$ mice by injection intraperitoneally with LPS and found that $Asb1^{-/-}$ mice exhibited more resistance to LPS-triggered inflammatory death and increased survival rates compared with their $Asb1^{+/+}$ littermates (Fig. 5*A*). Compared with a 33.3% survival rate of $Asb1^{-/-}$ mice, $Asb1^{+/+}$ mice all died of excessive inflammatory response within 32 h post-LPS exposure. Moreover, less inflammatory cell infiltration and tissue damage was observed in the lung of $Asb1^{-/-}$ mice (Fig. 5*B*). Consistently, we detected reduced inflammatory gene expression of *Tnfα*, *Il6*, and *Cxcl1* in various organs including liver, lung, and spleen from $Asb1^{-/-}$ mice compared with $Asb1^{+/+}$ mice, indicating that *Asb1*-deficient mice exhibited a less robust inflammatory response (Fig. 5 *C*–*E*).

To further explore the role of Asb1 in pathogen-induced septic shock, we then challenged $Asb1^{+/+}$ and $Asb1^{-/-}$ mice with intraperitoneal administration of *S. typhimurium*. In agreement with the above results, the body weight of $Asb1^{-/-}$ mice decreased more slowly than that of WT mice within 48 h (Fig. 5*F*), and $Asb1^{-/-}$ mice showed a delayed death onset and increased survival rates in comparison with their WT counterparts (Fig. 5*G*). Similarly, $Asb1^{-/-}$ mice produced significantly lower level of inflammatory cytokines, including IL-6 and TNFα in response to *S. typhimurium* infection (Fig. 5*H*). Bacterial counts in the liver and spleen of *S. typhimurium*-infected $Asb1^{-/-}$ mice were higher than in those of WT mice at 30 h after infection (Fig. 5*I*), suggesting that ASB1-regulated inflammation limits systemic dissemination and growth of bacteria and that differential mortality of WT and $Asb1^{-/-}$ mice was due to the overactive inflammatory responses but not associated with bacterial burdens, which is consistent with previous studies showing that proinflammatory signaling is required for the host response to restrict bacterial systemic infection and that heightened inflammation would cause septic death (40–42). Taken together, these in vivo studies demonstrated that ASB1 deficiency protected mice from LPS- or bacteria-induced death, indicating that ASB1 may be involved in the positive regulation of endotoxin- and bacterium-induced septic shock.

**ASB1 Is Involved in DSS-Induced Chronic Inflammation in Mice.** Chronic inflammatory bowel diseases (IBDs) are classified into two types: ulcerative colitis and Crohn's disease, which are characterized by chronic inflammatory responses in the immune system of the gut (43). It is well documented that the excessive activation of NF-κB and its downstream proinflammatory cytokines causes increased severity of IBD (44, 45). Vice versa, inhibition of NF-κB can decrease inflammation and reduce the development of IBD (46). We then investigated the potential

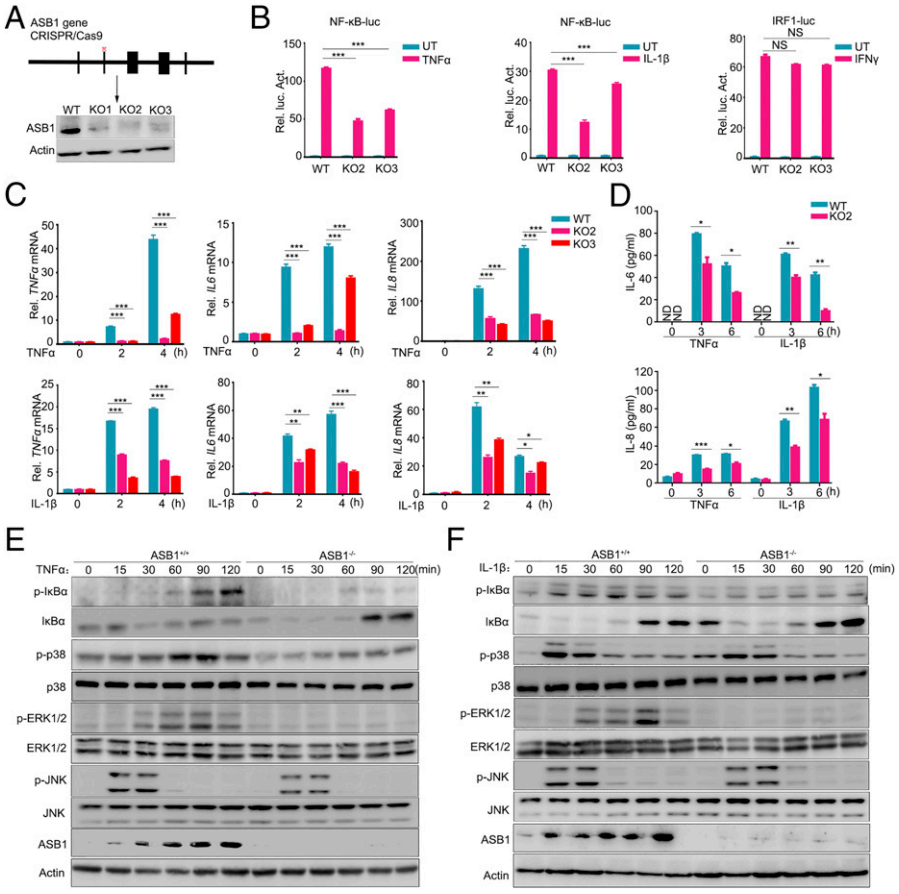


**Fig. 2.** Deficiency of ASB1 attenuated TNFα- and IL-1β–triggered signaling. (*A*) Schematic illustration of the target region of the ASB1 gene and immunoblot analysis of ASB1 expression in HEK293 cells. (*B*) WT or ASB1-KO HEK293 cells were transfected with NF-κB-luc or IRF1-luc and RL-TK-luc for 24 h, stimulated with TNFα, IL-1β, or IFNγ for another 10 h, respectively, and analyzed for promoter activities. (*C* and *D*) WT or ASB1-KO HEK293 cells were induced with TNFα or IL-1β for indicated time points and subjected to qPCR analysis for mRNA levels of TNFα, IL6, and IL8 (*C*) or ELISA analysis of secretion of IL-6 and IL-8 proteins (*D*). (*E* and *F*) WT or ASB1-KO HEK293 cells were induced with TNFα (*E*) or IL-1β (*F*) for indicated time points, and the cell lysates were harvested and analyzed by immunoblot; mRNA levels are presented relative to those of WT untreated cells. SSC, side scatter. Actin was used as a loading control; data are representative of three independent experiments and shown as the mean with SEM (*B–D*). See also *SI Appendix*, Fig. S2. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$; two-tailed unpaired Student's *t* test.

functions of ASB1 in the regulation of IBD in *Asb1*-deficient and WT mice. We used a DSS-induced colitis model, and the results showed that $Asb1^{-/-}$ mice exhibited less severe colitis associated with less decreased body weight and longer colon length compared with $Asb1^{+/+}$ control littermates after being orally fed with DSS (Fig. 6 *A–C*). In contrast to as high as a 50% lethal rate in $Asb1^{+/+}$ mice, $Asb1^{-/-}$ mice showed a 100% survival rate concomitant with less colon damage (Fig. 6 *D* and *E*). Histological analysis by immunohistochemistry was performed to assess intestinal damage, and $Asb1^{-/-}$ mice scored lower, thus suggesting that $Asb1^{-/-}$ mice had less severe colitis than $Asb1^{+/+}$ mice (Fig. 6*F*). To elucidate the molecular effect of Asb1 on colon tissue inflammation induced by DSS, colon tissue sections were stained and subjected for immunohistochemistry analysis of phospho-NF-κB p65 (p-p65) and proinflammatory cytokine IL-6. It showed that the level of p-p65 and IL-6 was significantly lower in $Asb1^{-/-}$ mice compared with that in $Asb1^{+/+}$ mice after DSS treatment (Fig. 6*G*). Consistently, the expression of proinflammatory cytokines, including *Tnfα*, *Il6*, and *Cxcl1*, was significantly lower in colons of $Asb1^{-/-}$ mice than in those of their WT littermates (Fig. 6*H*). Altogether, these results suggest that abruption of Asb1 ameliorates the DSS-induced colitis by suppressing proinflammatory signaling pathways.

**ASB1 Binds to TAB2 and TAB3 through Its ANK Repeat Domain.** We next determined the molecular mechanisms by which ASB1 enhances the activation of NF-κB pathway. We first detected the effects of ASB1 on the activity of NF-κB promoters induced by signal molecules in the NF-κB signaling pathway. The dual-luciferase reporter assay showed that ASB1 could further increase TRAF6-, TAB2-, and TAB3-triggered NF-κB promoter activity but had no effect on IKKβ-induced NF-κB promoter activity (Fig. 7*A*). Similarly, ASB1 deficiency substantially attenuated TRAF6-, TAB2-, and TAB3-induced NF-κB promoter activation, whereas IKKβ-induced NF-κB promoter activation was not affected by ASB1 KO (Fig. 7*B*). These data indicate that ASB1 functions upstream of IKK complex which is consistent with above observations (Fig. 2 *E* and *F*). Together with the finding that ASB1 could regulate both IL-1β– and TNFα-mediated NF-κB activation (Figs. 2 and 3), ASB1 could target most probably the TAB1/TAB2/TAB3 complex. To further interrogate whether ASB1 physically interacts with the TAB1/TAB2/TAB3 complex, we investigated their interaction and colocalization. Bidirectional coimmunoprecipitation showed that ASB1 could associate with TAB2 and TAB3 but not TAB1 (Fig. 7 *C* and *D*). Furthermore, in endogenous immunoprecipitation experiments, we observed that ASB1 had a strong association with TAB2 and TAB3 in TNFα- or IL-1β-induced cells,

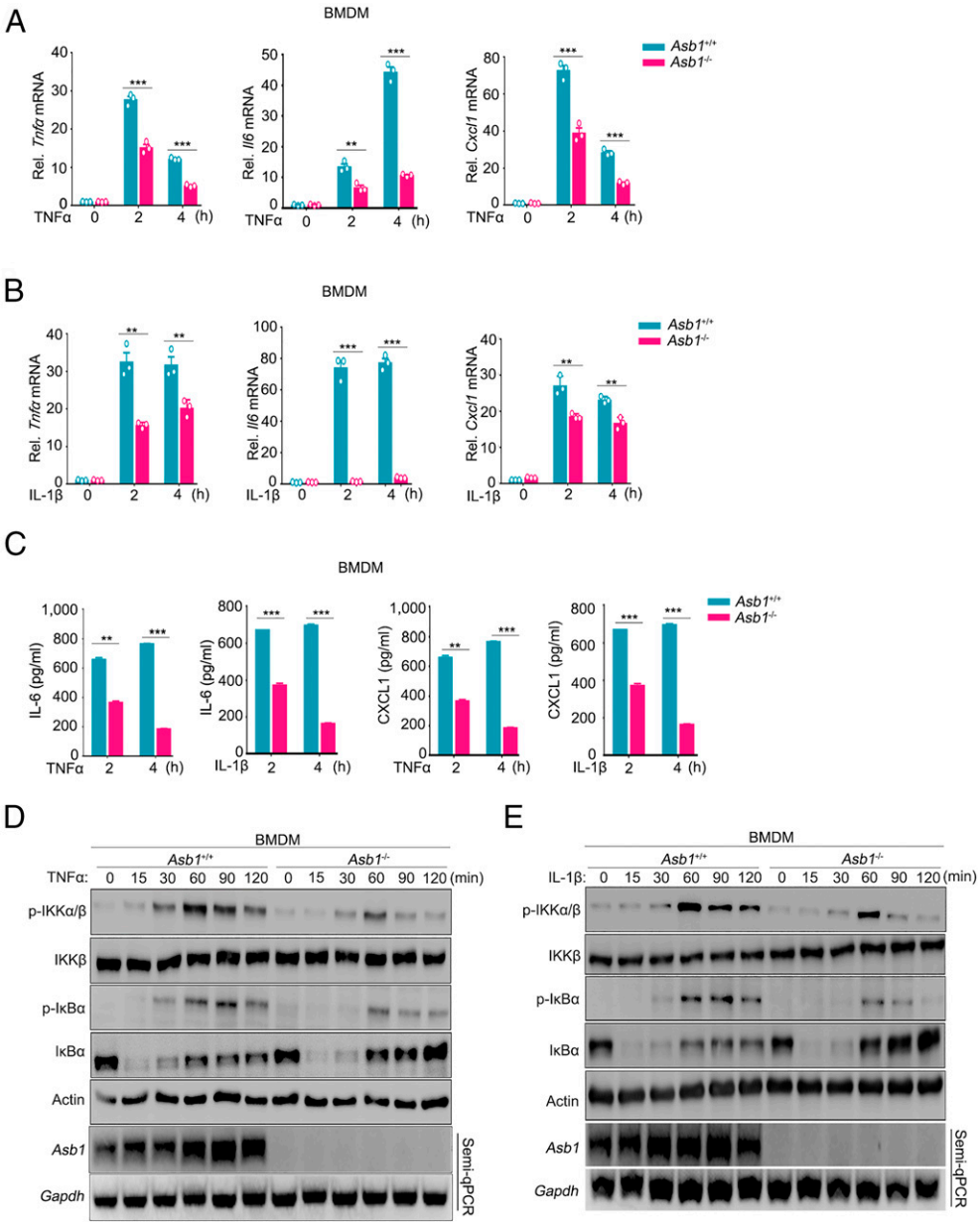


**Fig. 3.** Depletion of *Asb1* decreased TNFα- and IL-1β–induced inflammatory response in BMDMs. (*A* and *B*) qPCR analysis of mRNA levels of *Tnfα*, *Il6*, and *Cxcl1* in BMDMs isolated from $Asb1^{+/+}$ and $Asb1^{-/-}$ mice and stimulated with TNFα (*A*) or IL-1β (*B*) for indicated time points. (*C*) BMDMs from $Asb1^{+/+}$ and $Asb1^{-/-}$ mice were stimulated with TNFα or IL-1β for 2 or 4 h, and then the supernatant was prepared for ELISA. (*D* and *E*) BMDMs from $Asb1^{+/+}$ and $Asb1^{-/-}$ mice were treated with TNFα (*D*) or IL-1β (*E*) for indicated time points. The whole-cell lysates were analyzed by immunoblotting with the indicated antibodies; the expression of *Asb1* and *Gapdh* were determined by semiquantitative PCR (SemiqPCR). mRNA levels are presented relative to those of untreated WT cells. Data are representative of three individual experiments and shown as mean with SEM (*A*–*C*). See also *SI Appendix*, Figs. S3 and S4. $^{**}P < 0.01$ and $^{***}P < 0.001$; two-tailed unpaired Student's *t* test.

whereas there was a negligible interaction with TAB2 and TAB3 in cells in a resting state (Fig. 7*E*). Confocal microscopy also showed that ASB1 was diffused in resting cells and the treatment with TNFα and IL-1β caused clear colocalization between ASB1 and TAB2 or TAB3 (Fig. 7*F*). Since TAB2 and TAB3 are highly homologous and have redundant functions to bind to K63-linked polyubiquitin chains and act as adaptors to recruit TAK1 (47, 48), we next focused on the interaction between TAB2 and ASB1. ASB1 contains six ANK sequences and one SOCS box domain in the C-terminal extension. To define which domain of ASB1 was responsible for binding to TAB2, five truncations of ASB1 were generated (Fig. 7*G*). A domain-mapping experiment indicated that the ANK repeats of ASB1 (amino acids 36 to 265) were associated with TAB2 (Fig. 7*H*). We also constructed several TAB2 truncations containing various combinations of the TAB2 domains and performed coimmunoprecipitation. The result showed that the coiled-coil domain of TAB2 (amino acids 532 to 619) was responsible for its association with ASB1 (Fig. 7 *I* and *J*).

One previous study suggested that a certain degree of functional redundancy may exist among ASB family members and that ASB1 may play a similar function as ASB2 in promoting IκBα degradation in T-ALL cells (18). To investigate whether ASB1 and ASB2 have redundant functions, we studied the functions of ASB2 in the

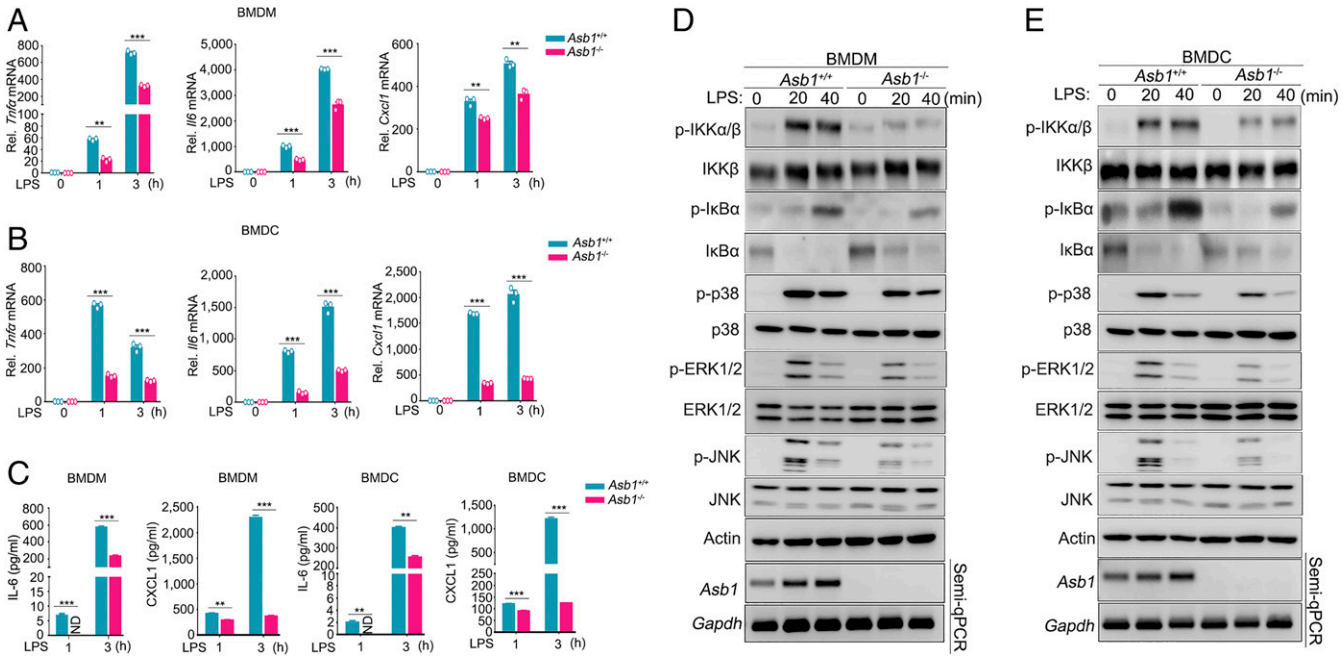


**Fig. 4.** KO of ASB1 specifically reduces LPS-induced inflammation. (*A* and *B*) qRT-PCR analysis of mRNA levels of *Tnfα*, *Il6*, and *Cxcl1* in BMDMs (*A*) and BMDCs (*B*) isolated from $Asb1^{+/+}$ and $Asb1^{-/-}$ mice and stimulated with LPS for indicated time points. (*C*) ELISAs of secretion of IL-6 and CXCL1 in $Asb1^{+/+}$ and $Asb1^{-/-}$ BMDMs and BMDCs treated with LPS for indicated time points. (*D* and *E*) Immunoblotting analysis of phospho-IKKα/β, IKBα, p38, ERK1/2, and JNK and total IKKα/β, IKBα, p38, ERK1/2, and JNK in $Asb1^{+/+}$ and $Asb1^{-/-}$ BMDMs (*D*) and BMDCs (*E*) treated with LPS for indicated time points. The expression of *Asb1* and *Gapdh* was determined by semiquantitative PCR (SemiqPCR); mRNA levels are presented relative to those of untreated WT cells. Data are representative of three individual experiments and shown as mean with SEM (*A*–*C*). $^{**}P < 0.01$ and $^{***}P < 0.001$; two-tailed unpaired Student's *t* test.

NF-κB signaling pathway. The results showed that ectopic expression of ASB2 could increase the activity of NF-κB slightly (*SI Appendix*, Fig. S5*A*). However, when we attempted to restore ASB1 with ASB1 and ASB2 in ASB1-KO cells, the TAB2-induced NF-κB activation was rescued by ASB1 but not by ASB2, suggesting that ASB1 and ASB2 function independently and that ASB2 cannot compensate for the functions of ASB1 (*SI Appendix*, Fig. S5*B*). Overall, these results demonstrate that ASB1 specifically targets and physically interacts with TAB2 and TAB3 through the ANK repeat domain of ASB1 and the coiled-coil domain of TAB2/TAB3.

**ASB1 Decreases K48-Linked Polyubiquitination and Enhances the Stability of TAB2.** Ubiquitination is critically important for the regulation of the NF-κB signaling pathway (49). As the proteins of ASB family are regarded to function as substrate-recognition components of E3 ligases and are involved in the UPS-dependent degradation of substrate proteins (16), we then tested whether ASB1 is involved in posttranslational modification and ubiquitination of TAB2/TAB3 in the NF-κB pathway. We first tested the stability of TAB2 in the presence of ASB1. Overexpression of ASB1 could increase the protein level of TAB2, and the cycloheximide (CHX) chase assay showed that ASB1 could also elongate the half-life of TAB2 (Fig. 8*A*). Consistently, ASB1 protected TAB2 from TNFα- and IL-1β–induced degradation, and deficiency of ASB1 increased the degradation of TAB2 in the presence of CHX (Fig. 8*B*). It was reported that the degradation of TAB2 induced by cytokines could be partially rescued by $NH_4Cl$ or MG132 (50). We found that ASB1 deficiency–mediated degradation of TAB2 was completely inhibited by MG132 but not $NH_4Cl$, indicating that ASB1 inhibits TAB2 degradation through suppression of UPS pathway (Fig. 8*C*). To further study how ASB1 regulates the stability of TAB2 and whether ASB1 is involved in modulating the ubiquitination state of TAB2, we performed ubiquitination assay of TAB2. The results showed that the polyubiquitination of TAB2 was robustly decreased in the presence of ASB1 (Fig. 8*D*). Then we explored which type of ubiquitin chains of TAB2 was suppressed by ASB1. We utilized hemagglutinin (HA)-tagged Ub-K48 and Ub-K63, in which all lysine residues were substituted by arginines except for the lysine at position 48 or position 63, respectively. We found that ASB1 decreased the polyubiquitination of TAB2 in the presence of Ub-K48 but not Ub-K63 (Fig. 8*E*). Furthermore, in vivo ubiquitination assays showed that KO of ASB1 increased K48-linked polyubiquitination of TAB2 but not K63-conjugated polyubiquitin chains (Fig. 8*F*).

Previous studies proved that the activation of NF-κB was found as early as 2 min after stimulation and it can maintain for as long as 3 h, which required de novo synthesis of proteins and continual stimulation (51, 52). To explore the detailed molecular mechanisms by which ASB1 potentiates NF-κB activation, we performed a 3.5-h time course experiment. It showed that ASB1 was induced and reached its maximum level at 2 h after TNFα induction and did not change much at 3.5 h. In the meantime, the phosphorylation of IκBα increased at 1 h and decreased at 3.5 h, while the level of IκBα rapidly decreased. At 1 h, IκBα protein levels had a transient increase and returned to about maximum levels at 2 h and then slightly decreased at 3.5 h. ASB1-KO cells showed reduced phosphorylation and degradation of IκBα (*SI Appendix*, Fig. S5*C*). The interaction between ASB1 and TAB2 increased to maximum level at 2 h and started to decrease within 3.5 h (*SI Appendix*, Fig. S5*D*). The K48-linked polyubiquitination of TAB2 increased within 3.5 h, and ASB1-deficient cells exhibited increased level of K48-linked polyubiquitination of TAB2 (*SI Appendix*, Fig. S5*E*). Altogether, the 3.5-h experiment displayed two stages of NF-κB activation, and in the early stage, the effect of ASB1 is not obvious as its expression level is low. In the later stage, ASB1 displayed a more pronounced effect with higher expression and extends signaling activity, suggesting that the functions of ASB1 depended on transcription and protein synthesis.

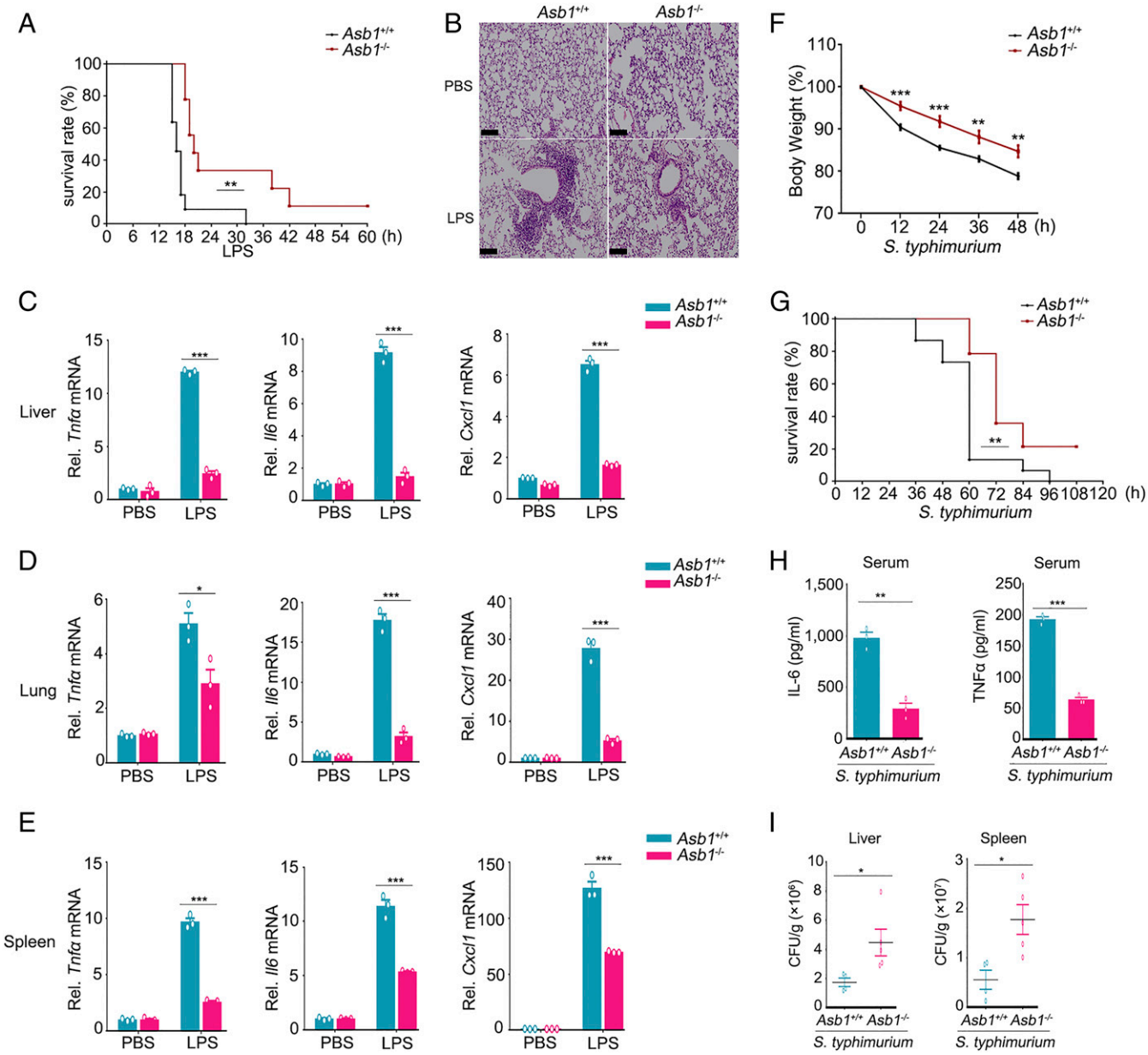


**Fig. 5.** ASB1 deficiency protects mice against LPS- and *S. typhimurium*–induced septic shock. (*A*) $Asb1^{+/+}$ and $Asb1^{-/-}$ mice were intraperitoneally injected with LPS (25 mg/kg), and the survival rates were recorded every 2 h ($Asb1^{+/+}$ mice, $n = 11$; $Asb1^{-/-}$ mice, $n = 9$). (*B*) Hematoxylin and eosin staining of lung tissues isolated from $Asb1^{+/+}$ and $Asb1^{-/-}$ mice 8 h postinjection with LPS (25 mg/kg) intraperitoneally. (Scale bars, 100 μm.) (*C–E*) qRT-PCR analysis of mRNA levels of *Tnfα*, *Il6*, and *Cxcl1* in liver (*C*), lung (*D*), and spleen (*E*) isolated from $Asb1^{+/+}$ and $Asb1^{-/-}$ mice treated as in *B*. (*F*) $Asb1^{+/+}$ and $Asb1^{-/-}$ mice were intraperitoneally infected with *S. typhimurium* ($10^5$ CFU/per mouse), and their body weights were recorded every 12 h ($Asb1^{+/+}$ mice, $n = 12$; $Asb1^{-/-}$ mice, $n = 12$). (*G*) $Asb1^{+/+}$ and $Asb1^{-/-}$ mice were treated as in *F*, and the survival rates were recorded every 12 h ($Asb1^{-/-}$ mice, $n = 14$; $Asb1^{+/+}$ mice, $n = 15$). (*H*) ELISAs of secretion of IL-6 and TNFα in serum from mice intraperitoneally infected with *S. typhimurium*. (*I*) $Asb1^{+/+}$ and $Asb1^{-/-}$ mice were intraperitoneally infected with *S. typhimurium*, and bacterial loads were detected in the liver and spleen 30 h after infection ($Asb1^{-/-}$ mice, $n = 5$; $Asb1^{+/+}$ mice, $n = 5$). PBS, phosphate-buffered saline; CFU, colony-forming units. mRNA levels are presented relative to those of untreated WT cells. Data are representative of three individual experiments and shown as mean with SEM (*C–E* and *H*). $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$; two-tailed unpaired Student's *t* test. Log-rank (Mantel–Cox) test was used for the analysis of mouse survival rates.

As TNFα not only can stimulate inflammation through the IKK/MKK but also can trigger apoptosis through caspase8 (53), we next determined whether ASB1 loss altered cell survival. ASB1-WT and ASB1-KO cells were untreated or treated with TNFα for 6 h, but both of the cells did not show much apoptosis and necroptosis (*SI Appendix*, Fig. S5*F*). Moreover, no significant difference was observed in TNFα-induced apoptosis/necroptosis between ASB1-WT and ASB1-KO cells, suggesting that the difference in inflammatory response was not due to altered cell survival. Together, these results indicate that ASB1 is involved in suppression of K48-linked ubiquitination and degradation of TAB2, unlike other members of the ASB family that function as core components of E3 ligases and promote the K48-linked ubiquitination and degradation of their respective substrates. Therefore, this study revealed a function of ASB family proteins in the promotion of the stability of the interacting partners.

## Discussion

It has been well documented that the NF-κB–dependent signaling pathway contributes to protective or pathological immune responses and inflammation. During signal transduction, TAB2/TAB3 functions as a part of TAK1 complex and binds to K63-polyubiquitin chains conjugated to TRAF6 or RIP1, which activates NF-κB and JNK/p38 signaling pathways in response to TNFR or TLR activation. However, the fine regulation of the immune response and inflammation in response to pathogens and inflammatory diseases is still poorly understood. In this study, we identified ASB1, a member of ASB family, as a positive regulator of inflammatory responses through interacting with TAB2/TAB3. The stability of TAB2/TAB3/TAK1 is crucial for TLR-induced TAK1 activation and activated TAK1 phosphorylates IKK and MKK that in turn phosphorylate IκBα and MAPK proteins, respectively. We revealed that ASB1 could promote the stability of TAB2/TAB3, which led to enhanced activation of NF-κB and MAPK pathways, thus promoting inflammatory responses. Therefore, we provide evidence that ASB1 is involved in the positive regulation of NF-κB– and MAPK-mediated inflammatory signaling pathways (Fig. 8*G*).

The ASB protein family belongs to the SOCS superfamily that also contains four other protein families, such as SPRY domain–containing SOCS box and WD40 repeat SOCS box. All the members contain a SOCS box motif that mediates the interaction with the Elongin B/C adaptor and Cul5, an adapter module in different E3 ubiquitin-protein ligase complexes targeting substrates for ubiquitination and degradation. SOCS superfamily proteins play intricate roles in regulating intracellular cytokine signaling and the development of immune system. As members of the SOCS box superfamily, ASB family proteins were reported to regulate cytokine signaling and several tumor-associated pathways by targeting the particular substrates for proteasomal degradation. Surprisingly, this study revealed that ASB1 behaves differently from other SOCS box–containing proteins. It does not promote the UPS-mediated degradation of its substrate but rather increases the stability of its substrate TAB2 by inhibiting K48-linked polyubiquitination. Therefore, we discovered a previously unknown function of ASB family proteins, which is different from that of other SOCS box members.

ASB1 contains six ankyrin repeats in its N-terminal region and one SOCS box domain at the C terminus (38). Ankyrin repeats are tandemly repeated modules of about 33 amino acids which mediate protein–protein interaction and are engaged in the recognition of the substrate of ASB-containing E3 ligases (54). In this study, we found that this ANK domain of ASB1 is involved in its association with TAB2/TAB3. Intriguingly, such association does not make TAB2/TAB3 as a substrate of the E3 ligase but inhibits the K48-linked ubiquitination of TAB2/3 (Fig. 8 *D–F*). However, how ASB1 inhibits the K48-conjugated polyubiquitination of TAB2/3 is still unknown. Two scenarios



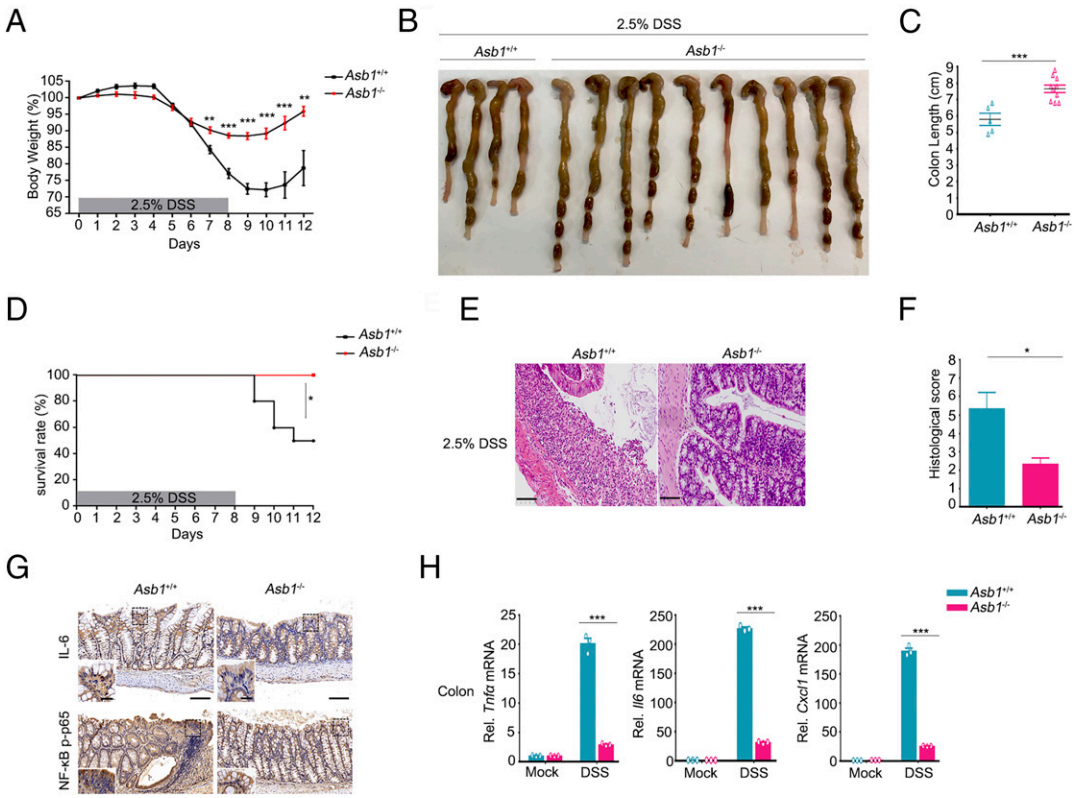


**Fig. 6.** ASB1 deficiency ameliorates colon inflammation in mice treated with DSS. (*A*) *Asb1*$^{-/-}$ and their WT littermates were fed with 2.5% DSS for a consecutive 8 d and then changed to regular animal facility water for another 4 d. Body weights were measured every day (*Asb1*$^{+/+}$ mice, $n = 10$; *Asb1*$^{-/-}$ mice, $n = 10$). (*B*) Representative images of colons of DSS-treated *Asb1*$^{+/+}$ and *Asb1*$^{-/-}$ mice (*Asb1*$^{+/+}$ mice, $n = 4$; *Asb1*$^{-/-}$ mice, $n = 10$). (*C*) The graph depicts the colon length of DSS-treated *Asb1*$^{+/+}$ and *Asb1*$^{-/-}$ mice (*Asb1*$^{+/+}$ mice, $n = 4$; *Asb1*$^{-/-}$ mice, $n = 10$). (*D*) Survival rates of *Asb1*$^{+/+}$ and *Asb1*$^{-/-}$ mice treated as in *A*, and the survival rates were recorded every day (*Asb1*$^{+/+}$ mice, $n = 10$; *Asb1*$^{-/-}$ mice, $n = 10$). (*E*) Hematoxylin and eosin–stained colon tissues isolated from *Asb1*$^{+/+}$ and *Asb1*$^{-/-}$ mice treated as in *A* on day 11. (Scale bars, 100 μm.) (*F*) Histological scoring of colon sections isolated from *Asb1*$^{+/+}$ and *Asb1*$^{-/-}$ mice treated as in *A* on day 11 (*Asb1*$^{+/+}$ mice, $n = 4$; *Asb1*$^{-/-}$ mice, $n = 4$). (*G*) Immunohistochemical staining for IL-6 and p-p65 in the colon tissues from *Asb1*$^{+/+}$ and *Asb1*$^{-/-}$ mice with colitis; nuclei were stained with 4′,6-diamidino-2-phenylindole, dilactate. (Scale bars, 100 μm; *Insets*, 25 μm.) (*H*) qRT-PCR analysis of *Tnfα*, *Il6*, and *Cxcl1* in colons of *Asb1*$^{+/+}$ and *Asb1*$^{-/-}$ mice treated with DSS on day 10. Data are shown as mean with SEM (*C*, *F*, and *H*). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$; two-tailed unpaired Student's *t* test. Log-rank (Mantel–Cox) test was used for the analysis of mouse survival rates.

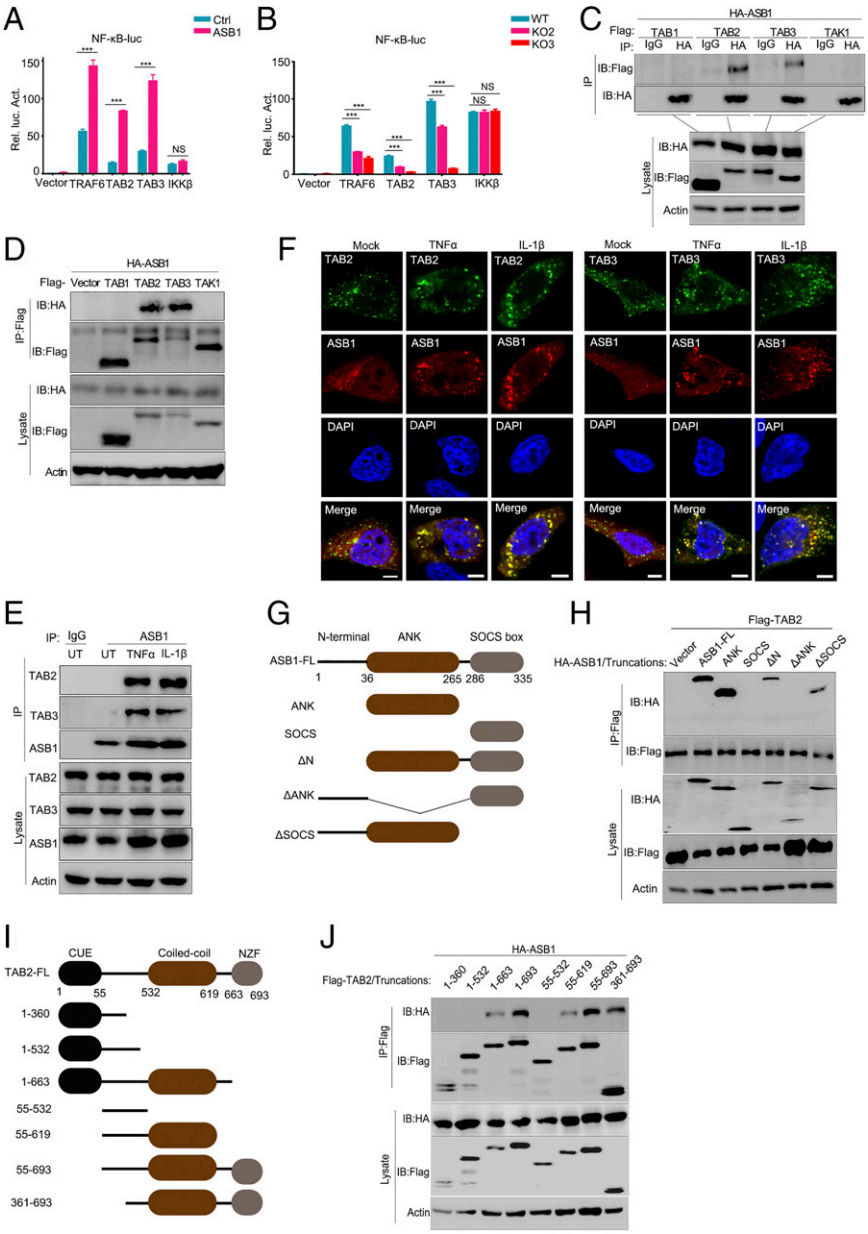


**Fig. 7.** ASB1 binds to TAB2/TAB3 but not TAB1. (*A*) Dual-luciferase activity of NF-κB-luc in HEK293 cells transfected with plasmids expressing TRAF6, TAB2, TAB3, or IKKβ along with ASB1 or empty control empty vector (Ctrl) for 24 h. (*B*) Dual-luciferase activity of NF-κB-luc in ASB1-WT and ASB1-KO HEK293 cells transfected with plasmids expressing TRAF6, TAB2, TAB3, or IKKβ for 24 h. (*C*) Immunoprecipitation (anti-HA-ASB1) and immunoblot analysis of the interaction between ASB1 and TAB1/TAB2/TAB3 proteins in cotransfected HEK293 cells. (*D*) Immunoprecipitation (anti-Flag) and immunoblot analysis of the interaction between TAB1/TAB2/TAB3 proteins and ASB1 in cotransfected HEK293 cells. (*E*) Immunoprecipitation (anti-ASB1) and immunoblot analysis of the endogenous interaction between ASB1 and TAB2/TAB3 in HEK293 cells stimulated with TNFα or IL-1β for 30 min. (*F*) Colocalization analysis of the association between TAB2/TAB3 and ASB1 in HeLa cells untreated or treated with TNFα or IL-1β for 30 min. (Scale bars, 5 μm.) (*G*) Schematic diagrams of human ASB1 and its truncated mutants. (*H*) Immunoprecipitation and immunoblot analysis of the interaction between TAB2 and ASB1 or its truncations in HEK293 cells cotransfected with Flag-TAB2 and HA-ASB1 or HA-tagged ASB1 truncations. (*I*) Schematic diagrams of human TAB2 and its truncated mutants. (*J*) Immunoprecipitation and immunoblot analysis of the interaction between ASB1 and TAB2 or truncations of TAB2 in HEK293 cells. Data are representative of two or three individual experiments and shown as mean with SEM (*A* and *B*). See also *SI Appendix*, Fig. S5. NS, not significant. ***$P < 0.001$; two-tailed unpaired Student's *t* test.

could be envisaged: first, it could recruit ubiquitin specific proteases (USPs) to deubiquitinate TAB2/3 in the manner of Fbw7, an SCF E3 ubiquitin-protein ligase complex that interacts with USP36 and USP28 to promote the deubiquitination and increase the stability of c-Myc (55, 56); and second, it may prevent the K48-linked ubiquitination by blocking the ubiquitination site of TAB2/TAB3 or by steric hindrance of other E3 ligases. Therefore, further studies of ASB1-mediated inflammation activation and the detailed molecular mechanisms are needed.

We further showed that the unexpected function of ASB1 has physiological and pathological relevance in animal disease models. The deficiency of ASB1 protects mice against *S. typhimurium*– and LPS-induced septic shock. We also explored the functions of ASB1 in intestinal homeostasis by studying its loss of function in a mouse model of inflammatory bowel disease, and the results showed that deficiency of ASB1 ameliorated DSS-induced colitis. These results indicated that ASB1 promotes inflammatory responses not only in primary immune cells but also in mouse

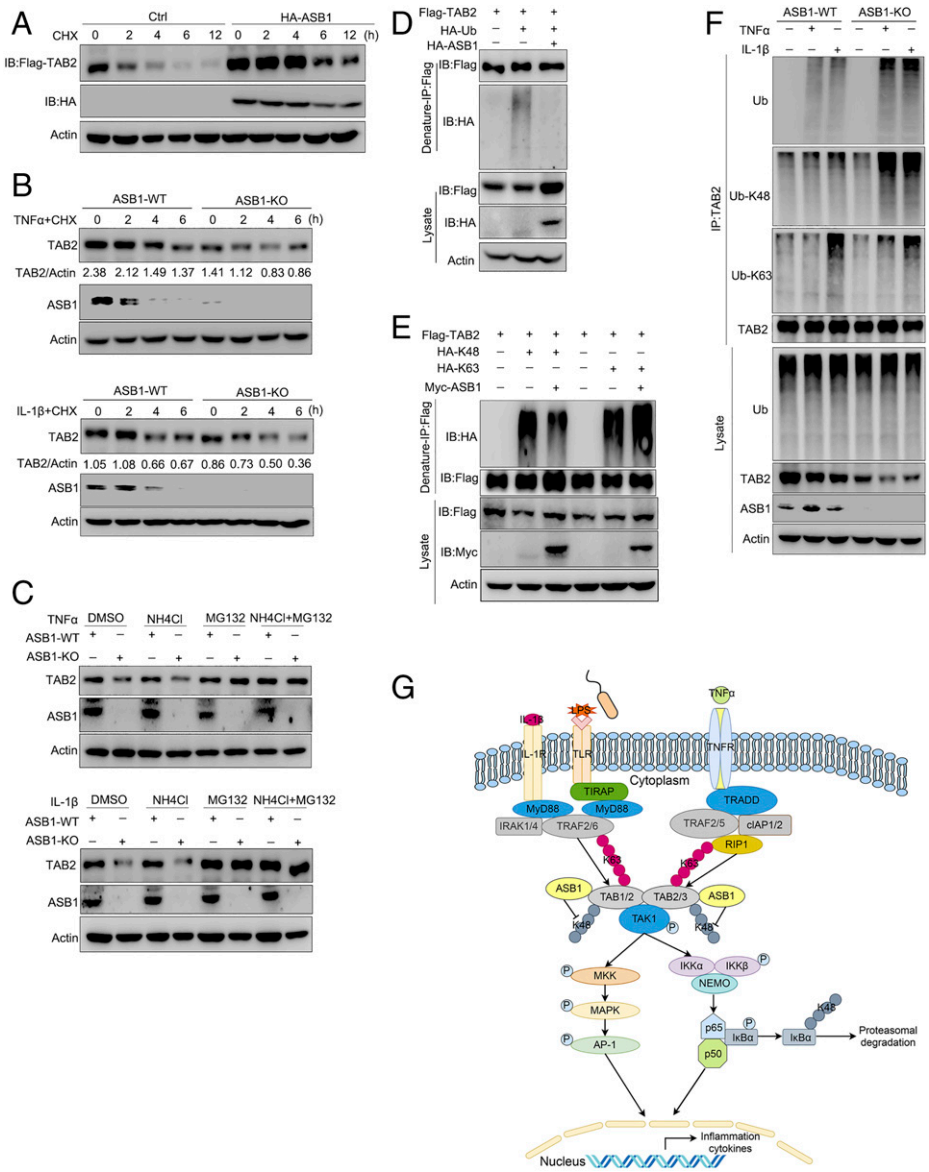


**Fig. 8.** ASB1 stabilized TAB2 through suppressing K48-linked polyubiquitination of TAB2. (*A*) Immunoblot analysis of HEK293 cells cotransfected with Flag-TAB2 and an empty plasmid or a plasmid encoding HA-ASB1 for 24 h and then left untreated or treated with CHX (100 μg/mL) for the indicated time points. (*B*) ASB1-WT and ASB1-KO HEK293 cells were treated with TNFα (*Top*) or IL-1β (*Bottom*) for 2 h and then treated with CHX for the indicated time points. The whole-cell lysate was analyzed by immunoblotting with the indicated antibodies. The expression levels of TAB2 and Actin were quantitated using ImageJ software. (*C*) ASB1-WT and ASB1-KO HEK293 cells were treated with DMSO, $NH_4Cl$, MG132, or $NH_4Cl$ plus MG132 for 4 h, and then the cells were treated with TNFα (*Top*) or IL-1β (*Bottom*) for another 2 h. The whole-cell lysate was analyzed by immunoblotting with the indicated antibodies. (*D*) Denature-IP (with anti-Flag) and immunoblot analysis of HEK293 cells transfected with the indicated plasmids for 24 h, lysed and immunoprecipitated with anti-Flag, and immunoblotted with anti–HA-Ub. (*E*) Denature-IP (with anti-Flag) and immunoblot analysis of HEK293 cells transfected with the indicated plasmids for 24 h and then lysed and immunoprecipitated with anti-Flag and immunoblotted with anti–HA-K48 or anti–HA-K63. (*F*) ASB1-WT and ASB1-KO HEK293 cells were treated with TNFα or IL-1β for 30 min. Cell lysates were subjected to denatured immunoprecipitation with TAB2 antibody, followed by immunoblotting with K48-Ub and K63-Ub antibodies. The cell lysates were immunoblotted with antibodies against ubiquitin, TAB2, ASB1, and actin; actin was used as a loading control. All experiments were repeated at least once. See also *SI Appendix*, Fig. S5. (*G*) Schematic illustration showing that ASB1 inhibits the K48-linked polyubiquitination and increased the stability of TAB2.

models. Given the importance of NF-κB– and AP-1–mediated transcription activities in regulating different aspects of immune functions, our findings provide insight into the regulatory mechanism of inflammatory signaling and have important physiological implications, which may contribute to establishing therapeutic strategies against microbial infection or inflammatory diseases.

## Materials and Methods

The *Asb1*-deficient mice were generated by GemPharmatech Co. Ltd (Nanjing, China) using CRISPR-Cas9–based technology and were maintained in a specific pathogen-free animal facility at Sun Yat-sen University. All animal experiments were supervised by the Institutional Animal Care and Use Committee of Sun Yat-sen University. The cell culture and preparation of BMDMs, BMDCs, and cell lines are described in *SI Appendix, Materials and Methods*. A detailed description of reagents, constructs, antibodies, transfection, LPS- and *S. typhimurium*–induced septic shock, DSS-induced colitis, hematoxylin and eosin staining and immunohistochemistry, immunoblotting, coimmunoprecipitation, qRT-PCR, ELISA, dual-luciferase reporter assay, plaque assay, fluorescent confocal microscopy, flow cytometry and data collection, and statistics used in this study are shown in *SI Appendix, Materials and Methods*.

**Data Availability.** Deep sequencing data have been deposited in Gene Expression Omnibus database (GSE143467). All other study data are included in the article and *SI Appendix*.

**ACKNOWLEDGMENTS.** The research is supported by the National Natural Science Foundation of China (Grant 81620108020 to D.G. and 81801574 to P.H.), Guangdong Province "Pearl River Talent Plan" Innovation and Entrepreneurship Team Project (2019ZT08Y464 to C.L.), and the China Postdoctoral Science Foundation (2018M633238 to P.H.). D.G. is also supported by Shenzhen Science and Technology Program (Grant KQTD20180411143323605), the Guangdong Zhujiang Leading Talents Programme, and the National Ten-Thousand Talents Programme. P.H. is supported by the Initiative Postdoc Supporting Programme (BX20180390).

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