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Essential Roles of K63-Linked Polyubiquitin-Binding Proteins TAB2 and TAB3 in B Cell Activation via MAPKs

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Polyubiquitination of proteins plays a critical role in the activation of immune cells. K63-linked polyubiquitin-binding proteins TGF-β-activated kinase 1 (TAK1)–binding protein (TAB)2 and TAB3 are implicated in NF-κB signaling via TAK1 activation. However, TAB2 alone is dispensable for NF-κB activation in embryonic fibroblasts, and the functional roles of TAB2 and TAB3 in immune cells has yet to be clarified. In this study, we demonstrate that TAB2 and TAB3 are essential for B cell activation leading to Ag-specific Ab responses, as well as B-1 and marginal zone B cell development. TAB2 and TAB3 are critical for the activation of MAPKs, especially ERK, but not NF-κB, in response to TLR and CD40 stimulation in B cells. Surprisingly, TAB2 and TAB3 are dispensable for TAK1 activation in B cells, indicating that TAB2 and TAB3 activate MAPKs via a pathway independent of TAK1. In contrast to B cells, macrophages lacking TAB2 and TAB3 did not show any defects in the cytokine production and the signaling pathway in response to TLR stimulation. Furthermore, TAB2 and TAB3 were dispensable for TNF-induced cytokine production in embryonic fibroblasts. Thus, TAB2- and TAB3-mediated K63-linked polyubiquitin recognition controls B cell activation via MAPKs, but not the TAK1/NF-κB axis. The Journal of Immunology, 2013, 190: 000–000.

Pol yubiquitination chains are crucial for immune cell activation upon stimulation of TLRs, CD40, and Ag receptors. The synthesis of polyubiquitin chains linked by linear, K48, and K63 is critical for the activation of NF-κB and MAPKs (1–3). In unstimulated cells, NF-κB is sequestered in the cytoplasm by association with an inhibitory protein, IκBα. In response to stimuli, IκBα is rapidly phosphorylated by the inhibitor of κB kinase (IKK) complex comprised of IκKα, IκKβ, and NF-κB–specific modulator (NEMO; also known as IκKγ), triggering its degradation by the ubiquitin/proteasome pathway. This allows NF-κB to translocate to the nucleus and induce the expression of numerous proinflammatory genes (2). K48-linked polyubiquitination of IκBα leads to its degradation via the proteasome allowing nuclear translocation of NF-κB. Alternatively, conjugation of linear polyubiquitin chains to NEMO by a linear ubiquitin chain assembly complex (LUBAC), comprised of heme-oxidized IRP2 ubiquitin ligase 1 homolog (HOIL-1), HOIL-1–interacting protein, and shank-associated RH domain–interacting protein (SHARPIN), is responsible for activation of NF-κB (4, 5). Furthermore, K63-linked polyubiquitin chains are also reported to be essential for the activation of NF-κB and MAPKs (3, 6, 7). In response to TLR stimulation, TNFR-associated factor (TRAF)6 has been shown to catalyze K63-linked polyubiquitination by acting as an E3 ubiquitin ligase. An E2 ubiquitin ligase complex comprised of UBC13 and Uev1A generates K63-linked polyubiquitin chains, which are anchored to TRAF6 or act as an unanchored signaling mediator (8–10). Downstream of TRAF6 and UBC13, TGF-β–activated kinase 1 (TAK1) is known to be activated by these stimuli and to phosphorylate IKKβ and MAPK kinase 6 for activation (3). Although K63-linked polyubiquitination has been implicated in TNF signaling, mutation of ubiquitin K63 to R failed to impair IKK activation by TNF (11). Alternatively, K63-linked polyubiquitination was shown to be essential for IL-1β–induced IKK activation, suggesting that the contribution of K63-linked polyubiquitin functions in a stimulus-specific manner (11).

TAK1-binding protein (TAB)2 and TAB3 are homologous TAK1-interacting proteins comprised of an N-terminal CUE domain for ubiquitin binding, a coiled-coil domain, and a C-terminal Npl4 zinc finger (NZF) polyubiquitin-binding domain (12–14). The TAB2 NZF domain has been shown to interact with K63-type, but not linear, polyubiquitin chains (15–17). TAB2 and TAB3 interact with K63-linked polyubiquitin chains and activate TAK1-mediated NF-κB activation (18, 19). NEMO also has the potential to bind K63-linked polyubiquitin chains, but the affinity of NEMO for linear polyubiquitin chains seems to be even greater than for K63-linked ubiquitin chains (20, 21). It was shown that TAK1 and UBC13 are essential for the responses of macrophages and B cells to TLR and Ag receptor stimulation (22–24), leading to the activation of Ag-specific immune responses. Nevertheless, mouse embryonic
fibroblasts lacking TAB2 showed normal NF-κB activation in response to TNF and IL-1β (24, 25). The functional roles of TAB2 and TAB3 in immune cells are still unclear because of the lack of a TAB3-deficient mouse model and embryonic lethality of Tab2-deficient mice.

In this study, we generated conditional Tab2-deficient and Tab3-deficient mice to address the role of TAB2- and TAB3-mediated recognition of K63-linked polyubiquitin in B cells and macrophages, because TLRs, costimulatory molecules, and Ag receptor can activate these cell types. Interestingly, TAB2 and TAB3 are essential for the activation of MAPKs, but not NF-κB, in response to TLR and CD40 stimulation in B cells. Furthermore, TAB2 and TAB3 are essential for B cell activation in response to various stimuli and Ag-specific immune responses. Nevertheless, macrophages did not show a defect in the production of cytokines upon TLR stimulation even in the absence of both TAB2 and TAB3. These results demonstrate TAB2- and TAB3-mediated recognition of K63-linked polyubiquitin functions in a cell type- and stimulus-specific manner for activating MAPKs but not NF-κB.

Materials and Methods

Generation of Tab3-deficient mice

The targeting vector was constructed by replacing a 3.3-kb fragment containing almost all of exons 2 and 3 of the Tab3 gene with a neomycin-resistance gene cassette and inserting herpes simplex virus thymidine kinase driven by the PGK promoter into the genomic fragment for negative selection. After the targeting vector was transfected into embryonic stem cells, G418, and ganciclovir double-resistant colonies were selected and screened by PCR, and recombination was confirmed by Southern blot analysis. Homologous recombinants were microinjected into C57BL/6 female mice, and heterozygous F1 progenies were used for subsequent experiments. Mice were maintained in specific pathogen-free conditions and used at 8–10 wk age. All animal experiments were done with the approval of the Animal Research Committee of the Research Institute for Microbial Diseases, Osaka University, and of Kyoto University.

Mice and reagents

Tab2fl/fl mice were previously reported (25). LysM-Cre and CD19-Cre mice were as described (26, 27). LPS was purchased from Sigma-Aldrich. CpG DNA (oligonucleotide 1668) was synthesized by Invitrogen (28). Pam3CSK4 was purchased from InvivoGen. Agonistic anti-IgM and anti-CD40 were purchased from Jackson ImmunoResearch and BioLegend, respectively. α-Phosphatase was purchased from New England BioLabs. Abs specific for ERK (sc-94), IκBα (sc-371), and actin (sc-8432) were purchased from Santa Cruz Biotechnology. Newcastle disease virus (NDV) was previously described (29).

Purification of B cells

Resting B cells were isolated from splenocyte single-cell suspensions by positive selection with anti-B220 magnetic beads (Miltenyi Biotec) according to the manufacturer’s instructions.

Immunoblot analysis

Cells were lysed in a lysis buffer containing 1% Nonidet P-40, 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, and a protease inhibitor mixture (Roche). Lysates were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Bio-Rad). After membranes were blocked with Abs, proteins on membranes were visualized with an ECL system (PerkinElmer).

B cell proliferation assay

Purified splenic B cells (1 × 10^6) were cultured in 96-well plates in RPMI 1640 medium supplemented with 10% FCS and 50 μM 2-ME for 48 h with various concentrations of various stimuli. Cells were pulsed with 1 μCi [3H]thymidine for the last 16 h and then [3H]uptake was measured with a beta-scintillation counter (Packard).

Cell cycle analysis

Cell cycles of B cells were analyzed with a BrdU flow kit (BD Biosciences) according to the manufacturer’s instructions. Cells (1 × 10^6) were cultured in RPMI 1640 medium supplemented with 10% FCS and 50 μM 2-ME with various stimuli for 24 h, pulsed with 10 μM BrdU for an additional 16 h, stained with FITC anti-BrdU and 7-aminomethylcoumarin D (7-AAD), and then analyzed by flow cytometry.

Cell viability assay

Purified splenic B cells (1 × 10^6) described above were stimulated with 1 μM CpG DNA, 10 μg/ml anti-IgM, or 1 μg/ml anti-CD40 for 12, 24, or 48 h. Cells were stained with FITC-conjugated annexin V and indocarbocyanine (BioVision) in annexin buffer (140 mM NaCl, 10 mM HEPES, and 2.5 mM CaCl2) for 10 min, and then cell viability was assessed with a FACSCanto II (Becton Dickinson).

Measurement of proinflammatory cytokine concentrations

B cells (1 × 10^5) or bone marrow (BM) macrophages (0.5 × 10^5) were stimulated with various stimuli (LPS, CpG DNA, or Pam3CSK4 for macrophages, CpG DNA for B cells) or infected with NDV for macrophages. The concentrations of IL-6, IL-12p40, and TNF in the culture supernatants were measured by ELISA according to the manufacturer’s instructions (R&D Systems).

EMSA

Purified splenic B cells (4 × 10^5) were treated with indicated stimuli. Nuclear extracts were purified from cells, incubated with a probe specific for the NF-κB DNA-binding site, separated by electrophoresis, and visualized by autoradiography as described (23).

In vivo immunization and ELISA

Mice were immunized i.p. with 50 μg nitrophenol–chicken γ-globulin (NP-CGG) (Biosearch Technologies) precipitated with Injext Alum (Pierce). Ag and isotype-specific Abs were measured by ELISA on plates coated with NP-BSA. Abs to mouse IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA were purchased from SouthernBiotech.

Preparation of thioglycolate-elicited macrophages and BM macrophages

Peritoneal exudate cells were isolated from the peritoneal cavities of mice 3 d after injection with 2 ml 4.0% thioglycolate medium (Sigma-Aldrich) as thioglycolate-elicited macrophages. BM cells were isolated from femurs and cultured in RPMI 1640 medium supplemented with 10% FCS, 50 μM 2-ME, and 20 ng/ml M-CSF (PeproTech) for 5 d. Cells were collected by incubation with 10 mM EDTA in PBS with gentle agitation as BM macrophages.

Preparation of mouse embryonic fibroblasts lacking Tab2 and Tab3

Mouse embryonic fibroblasts (MEFs) were prepared from E13.5 embryos and cultured in DMEM supplemented with 10% FCS and 50 μM 2-ME. MEFs were then introduced with a retrovirus expressing Cre. Two days after selection with puromycin (2 μg/ml), MEFs were subjected to the subsequent experiments.

Results

Generation of Tab3-deficient mice

To investigate the functional role of TAB3 in a variety of signaling pathways, we generated Tab3-deficient (Tab3−/−) mice by replacing exons 2 and 3 of mouse Tab3 with the neomycin resistant gene (Fig. 1A, 1B). Tab3−/− mice were born in Mendelian ratios, bred normally, and did not show any gross abnormality (data not shown). Abrogation of Tab3 mRNA expression was confirmed by PCR analysis (Fig. 1C). Furthermore, flow cytometric analysis revealed that the composition of T and B cells, dendritic cells, and macrophages was not altered between wild-type and Tab3−/− mice (data not shown).

Tab3 alone is dispensable for B cell activation

We first examined the role of Tab3 in B cell activation. Proliferation of splenic B cells in response to stimulation with mitogens,
CpG DNA, anti-IgM, and anti-CD40 Ab was comparable between control Tab3+/− and Tab3+/+ B cells (Fig. 2A). Additionally, cell cycle profiles examined by staining with BrdU and 7-AAD revealed that cell cycle progression in response to mitogens was again comparable between Tab3+/− and Tab3+/+ B cells (Fig. 2B). When cultivated ex vivo without a mitogen, 50% of splenic B cells from Tab3+/− and Tab3+/+ mice underwent apoptosis within 12 h (Fig. 2C). Stimulation with CpG DNA and anti-CD40 Ab can prevent the apoptosis, and anti-IgM modestly inhibited the cell death both in Tab3+/− and in control Tab3+/+ B cells (Fig. 2C). Furthermore, production of IL-6 in response to CpG DNA stimulation was not impaired in Tab3+/− B cells (Fig. 2D). These results indicate that TAB3 alone is dispensable for the activation of B cells.

Critical role of TAB2 and TAB3 in B cell activation

B cell–specific deletion of Tab2 was achieved by crossing Tab2fl/fl mice (25) with mice expressing Cre under the CD19 promoter (CD19-Cre-Cre) (Fig. 1D). Immunoblot analysis showed that B cells from CD19-Cre−Tab2fl/fl and CD19-Cre−Tab2+/− mice had much less Tab2 protein than did control cells (Fig. 1E). As shown in Fig. 3A and 3B, B cells lacking Tab2 alone (CD19-Cre−Tab2fl/fl) proliferated and progressed in cell cycling normally in response to CpG DNA, anti-IgM, and anti-CD40 stimulation compared with CD19-Cre−Tab2+/− (control) B cells. In contrast, B cells lacking both Tab2 and TAB3 (CD19-Cre−Tab2fl/flTab3−/−; double KO [DKO]) showed impaired proliferation and incorporation of BrdU in response to the stimuli (Fig. 3A, 3B). We then examined whether TAB2 and TAB3 influence the survival of B cells. Whereas control and TAB2 KO B cells cultivated without mitogens underwent apoptosis in a similar time course, DKO B cells died more rapidly than did control and TAB2 KO B cells (Fig. 3C). The cell death was prevented comparably in response to stimulation with CpG DNA and anti-CD40 Ab and modestly inhibited in response to anti-IgM both in control and TAB2 KO B cells (Fig. 3C). In contrast, DKO cells underwent apoptosis more quickly than did control or TAB2 KO B cells in response to CpG DNA and anti-IgM, whereas the contribution of TAB2 and TAB3 in apoptosis was lower in response to anti-CD40 stimulation compared with other mitogens (Fig. 3C). Additionally, production of IL-6 in B cells in response to CpG DNA stimulation was impaired in the absence of both TAB2 and TAB3 (Fig. 3D). These data demonstrate that TAB2 and TAB3 are essential for B cell activation, and TAB2 and TAB3 function redundantly in B cells in response to BCR, CD40, and TLR9 stimulation.

TAB2 and TAB3 in the activation of signaling pathways in B cells

Next we examined the role of TAB2 and TAB3 in the signaling pathways triggered by TLR, BCR, and CD40 stimulation in B cells. Surprisingly, degradation of IκBα in response to CpG DNA was comparably induced in B cells from control, TAB2 KO, TAB3 KO, and DKO mice (Fig. 4A). Furthermore, anti-IgM and anti-CD40 stimulation degraded IκBα even in TAB2 and TAB3 DKO B cells (Fig. 4A). Consistently, an EMSA revealed that NF-κB–DNA binding activity was induced in response to CpG DNA, anti-IgM, and anti-CD40 stimulation even in the absence of both TAB2 and TAB3 (Fig. 4B). Thus, TAB2 and TAB3 are dispensable for the activation of NF-κB in response to various stimuli in B cells.

We then examined the role of TAB2 and TAB3 in the activation of MAPKs, including p38, JNK, and ERK in response to CpG DNA, anti-IgM, and anti-CD40 stimulation. Whereas TAB3 KO cells did not affect the MAPK activation, TAB2 deficiency partially impaired their phosphorylation in response to all tested stimuli (Fig. 4C). Furthermore, DKO B cells showed more profound defects in the activation of MAPKs than did TAB2 KO B cells (Fig. 4C). These results demonstrate that TAB2 and TAB3 control activation of MAPKs, but not NF-κB, in B cells in response to TLR, BCR, and anti-CD40 stimulation. Notably, the defect in the phosphorylation of ERK in DKO B cells was more severe than that of JNK or p38, suggesting that TAB2 and TAB3 might have a more important role in the activation of ERK than JNK or p38.

We next investigated whether TAB2 and TAB3 are required for the activation of Tak1. First, we found that the levels of Tak1 protein expression in B cells were comparable even in the absence of TAB2 and TAB3 (Fig. 4D). In response to anti-CD40 and CpG DNA stimulation, Tak1 migration slowly in a PAGE gel. The change in the mobility was cancelled by the treatment of cell lysates with α-phosphatase (Fig. 4E), indicating that the mobility change is due to the phosphorylation of Tak1 upon stimulation. Surprisingly, the appearance of slowly migrating Tak1 in response to CpG DNA and CD40 stimulation was still observed in the absence of TAB2 and TAB3 in B cells (Fig. 4F). These data demonstrate that Tak1 can be phosphorylated and activated even in the absence of both TAB2 and TAB3 in B cells.
Critical role of TAB2 and TAB3 in B cell development and immune responses

We next investigated the role of TAB2 and TAB3 in B cell activation in vivo. The B220^+CD5^+ B-1 B cell population was reduced in the peritoneal cavities of TAB2 KO mice, and DKO mice had a much lower frequency of B-1 B cells than did TAB2 KO mice (Fig. 5A). The reduction of the B-1 B cell population was due to the decrease in the B220^+CD5^+CD43^+ B-1a cell population (Fig. 5A). Moreover, we found that TAB2 KO and DKO mice had a lower frequency of B220^+CD21^hiCD23^lo marginal zone (MZ) B cells than did control mice (Fig. 5B). In contrast, the ratio of B cells to T cells in the spleen and the expression of IgM and IgD on mature splenic B cells were unaffected by the absence of TAB2 or both TAB2 and TAB3 (Fig. 5A). The reduction of the B-1 B cell population was due to the decrease in the B220^+CD5^+CD43^+ B-1a cell population (Fig. 5A). Moreover, we found that TAB2 KO and DKO mice had a lower frequency of B220^+CD21^hiCD23^lo marginal zone (MZ) B cells than did control mice (Fig. 5B).

Interestingly, the CD138^+CD19^dull plasma cell population was severely diminished in the absence of TAB2 or both TAB2 and TAB3 (Fig. 5B). Consistent with this notion, all serum Ig isotypes except IgA and IgM were lower in TAB2 KO and DKO mice than in control or TAB3 KO mice (Fig. 6A). To examine the role of TAB2 and TAB3 in Ag-specific Ab responses, we immunized mice lacking TAB2 and/or TAB3 in B cells with the T cell–dependent Ag NP-CGG. Although production of NP-specific IgM production was comparable, TAB2 KO and DKO mice showed significantly impaired production of NP-specific IgG1 (Fig. 6B). Thus, TAB2 and TAB3 are required for humoral immune responses in vivo by activating B cells.

Both TAB2 and TAB3 are dispensable for responses to TLR, RIG-I–like receptor, and TNF in macrophages and MEFs

Macrophages sense the presence of pathogens by receptors such as TLRs and RIG-I–like receptors (RLRs). Whereas TLRs are responsible for recognizing various components from bacteria and viruses, RLRs recognize RNA virus infection in the cytoplasm (30). To address the role of TAB2 and TAB3 in macrophages, we crossed Tab2^−/− mice with LysM-Cre mice, which express Cre recombinase under the lysozyme M promoter. Southern blot analysis showed that Cre-mediated deletion produced a new 9.4-kb band corresponding to the mutated Tab2 allele in BM macrophages from the resultant LysM-Cre^+Tab2^−/− mice (Fig. 7A). Immunoblot analysis showed that LysM-Cre^+Tab2^−/− BM macrophages had much less TAB2 protein than did control cells (Fig. 7B). Furthermore, we generated MEFs lacking TAB2 and TAB3 by retrovirally expressing Cre in Tab2^−/− and control MEFs (Fig. 7C, 7D). When BM macrophages were stimulated with TLR ligands including Pam 2CSK4 (TLR2), LPS (TLR4), and CpG DNA (TLR9) or were infected with the RNA virus NDV (RIG-I), deficiency in TAB2 or TAB3 did not alter the production of TNF or IL-6 (Fig. 7E). Additionally, production of IFN-β was not impaired in the absence of TAB2 in macrophages (Fig. 7F). Furthermore, mice lacking both TAB2 and TAB3 in macrophages (LysM-Cre^+Tab2^−/−Tab3^−/−) produced IL-6, IL-12p40, and IFN-β in response to various stimuli comparable to control cells (Fig. 7E, 7F). These results indicate that TAB2 and TAB3 are dispensable for TLR-induced and RLR-induced cytokine production in BM macrophages. We found that cytokine production from peritoneal macrophages was not affected by the absence of TAB2 and TAB3 (data not shown). Furthermore, the production of IL-6 in response to TNF stimulation was comparable between control, TAB2 KO,
TAB3 KO, and DKO MEFs, indicating that TAB2 and TAB3 are dispensable for cellular responses to TNF stimulation. As shown in Fig. 7H, CpG DNA– and NDV-induced degradation of IκBα as well as the phosphorylation of the NF-κBp65 subunit were not impaired even in TAB2 and TAB3 DKO macrophages. Furthermore, phosphorylation of MAPKs, ERK, JNK, and p38 was comparably induced in control, TAB2 KO, TAB3 KO, and DKO macrophages (Fig. 7H). Collectively, both TAB2 and TAB3 are dispensable for NF-κB or MAPK signaling pathways as well as cytokine production in macrophages in response to TLR or RLR stimuli.

Discussion

In this study, we examined the role of TAB2 and TAB3 in immune cells by generating mice lacking both TAB2 and TAB3 in B cells and in macrophages. We found that TAB2 and TAB3 are critical for the development of B-1 and MZ B cells, as well as the activation of B cells in response to TLR ligands and BCR stimulation. Nevertheless, both TAB2 and TAB3 seem to be dispensable for the activation of NF-κB in B cells. Because MAPK activation was severely impaired in B cells lacking both TAB2 and TAB3, these K63-linked polyubiquitin-binding proteins are critical for the activation of signaling pathways leading to MAPKs for inducing B cell activation. It appears that TAB2 and TAB3 are more critically required for the activation of ERK than JNK and p38, although underlying mechanisms have yet to be clarified.

Mice lacking TAB2 and TAB3 in B cells show severe defects in the development of peripheral B-1 and splenic MZ B cells as well as defects in the activation of splenic B cells. A reduction in B-1 and MZ B cell populations was observed in mice lacking genes involved in the signaling pathway leading to NF-κB activation in B cells. For example, CARMA1, BCL10, and Malt1/paracaspase, proteins that act upstream of the IKK complex in Ag receptor signaling, are required for the development of B-1 and MZ B cells as well as the activation of NF-κB and MAPKs induced in response to BCR and CD40 stimulation (31, 32). Alternatively, lack of TAK1 in B cells affected B-1, but not MZ, B cell differentiation (22). These differences also support a model where TAB2- and TAB3-mediated K63-linked polyubiquitin recognition can function independent of TAK1 in vivo.

In contrast to TAK1, B cell–specific Ubc13-deficient mice also showed severe defects in the generation of B-1 and MZ B cells (23). Splenic B cell proliferation as well as cell survival in response to TLR, CD40, and BCR stimulation was severely impaired in the absence of UBC13. Nevertheless, UBC13 has been shown to be dispensable for the activation of NF-κB in B cells, whereas MAPKs were critically regulated by UBC13 (23). In this regard, the phenotypes of TAB2/TAB3 lacking B cells and Ubc13-deficient B cells resemble each other.

Surprisingly, TAB2 and TAB3 were dispensable for the phosphorylation of TAK1 in B cells. TAK1 regulates activation of not only MAPKs, but also NF-κB in B cells (22). Alternatively, UBC13-mediated generation of K63-linked polyubiquitin chains is critical for MAPKs, but not for NF-κB activation. These differences indicate that functions of TAB2/3 and UBC13 overlap significantly, although TAK1 and TAB2/3, at least in part, control distinct signaling molecules in B cells. Because UBC13 catalyzes polyubiquitination of NEMO and controls MAPK activation (23), TAB2 and TAB3 might activate MAPKs via K63-linked–ubiquitinated NEMO independent of TAK1 phosphorylation. Furthermore, it is also possible that TAB2 and TAB3 activate MAPKs independent of their recognition of the polyubiquitin chain. Further studies are required to uncover the precise mechanisms of MAPK activation.

TAB2 and TAB3 harbor polyubiquitin-binding domains CUE and NZF, and their binding to K63-linked polyubiquitin chains is critical for their function (17–19). Although it is still possible that
FIGURE 4. TAB2 and TAB3 are critical for the activation of MAPKs, but not NF-κB, in B cells. (A) Immunoblot analysis of the kinetics of IκBα degradation of whole-cell lysates of B cells from CD19-Cre"TAB2fl/fl Tab3"/" (control), CD19-Cre"TAB2fl/fl Tab3"/" (TAB2 KO), CD19-Cre"TAB2fl/fl Tab3"/" (TAB3 KO), and CD19Cre"TAB2fl/fl Tab3"/" (DKO) mice stimulated with 1 μM CpG DNA, 10 μg/ml anti-IgM, or 1 μg/ml anti-CD40 for indicated periods. IκBα levels relative to β-actin (%) compared with unstimulated samples of each genotype were quantified and are shown below the bands. (B) EMSA of NF-κB DNA-binding activity in nuclear extracts from purified splenic B cells in indicated genotypes stimulated with 1 μM CpG DNA, 10 μg/ml anti-IgM, or 1 μg/ml anti-CD40 for indicated periods. DNA-binding activities of NF-κB were quantified, and the relative increase to unstimulated samples of each genotype is shown below the bands. (C) Immunoblot analysis of MAPK activation of whole-cell lysates of B cells in indicated genotypes stimulated with 1 μM CpG DNA, 10 μg/ml anti-IgM, or 1 μg/ml anti-CD40 with Ab against p-p38, p-JNK, and p-ERK. Levels of p-MAPK relative to ERK (%) compared with the sample showing strongest signals were quantified and are shown below the bands. (D) Immunoblot analysis of whole-cell lysates of unstimulated B cells in indicated genotypes with Ab against TAK1. (E) (Figure legend continues)
K63-lined polyubiquitin-binding proteins other than TAB2 and TAB3 are responsible for the activation of NF-κB, no other protein has been found in the human or mouse genome that harbors a CUE domain and an NZF domain. Because cells lacking UBC13 were also shown to be dispensable for the activation of NF-κB in B cells, it is likely that the K63-linked polyubiquitin chain functions redundantly in activating the NF-κB signaling.

In contrast to B cells, lack of TAB2 and TAB3 did not affect cytokine production or NF-κB activation in macrophages. In addition to K63-linked polyubiquitination, linear ubiquitination has been implicated in the NF-κB signaling pathway (4, 5). MEFs lacking HOIL-1, a component of LUBAC, showed impaired TNF-mediated NF-κB activation. In contrast, activation of JNK has been shown to be enhanced in the absence of HOIL-1, suggesting that NF-κB and MAPKs are differentially regulated by linear polyubiquitination (5). The cpdm mutation of the Sharpin gene (Sharpincpdm/cpdm), encoding another component of LUBAC, leads to the impairment in gene induction in response to TNF, CD40L, and IL-1β in MEFs and B cells. Sharpincpdm/cpdm mice showed impaired activation of macrophages in response to TLR stimulation (33–36). We also found that macrophages from Sharpincpdm/cpdm mice showed severe defects in the production of IL-6 and IL-12p40 in response to TLR stimulation (D. Ori, unpublished observations), indicating that linear ubiquitination plays a critical role in activating macrophages. In this regard, linear ubiquitination and K63-linked polyubiquitination function in part redundantly in controlling im-

Immunoblot analysis of whole-cell lysates of wild-type B cells stimulated (+) or unstimulated (−) with 1 μg/ml anti-CD40 and with or without α-phosphatase treatment. (F) Immunoblot analysis of whole-cell lysates of B cells stimulated with 1 μM CpG DNA or 1 μg/ml anti-CD40 with Ab against TAK1. β-actin and ERK serve as a loading control. Data are representative of at least two independent experiments.
FIGURE 7. TAB2 and TAB3 are dispensable for macrophage activation. (A) Southern blot analysis of genomic DNA from Tab2+/+, Tab2fl/+ and Tab2fl/fl mice expressing a Cre transgene under control of the LysM promoter (LysM-Cre+). (B) Immunoblot analysis of cell lysates of BM macrophages from LysM-Cre+Tab2fl/+ and LysM-Cre+Tab2fl/fl mice. Asterisk indicated a nonspecific band. (C) Southern blot analysis of genomic DNA from Tab2fl/+ and Tab2fl/fl MEFs infected with a retrovirus expressing Cre recombinase. (D) RT-PCR analysis of RNAs from Tab2fl/+ and Tab2fl/fl MEFs as described above, amplifying Tab2 gene. (E) Production of IFN-β in the culture supernatants of BM macrophages infected with NDV (MOI of 1) for 24 h was measured by ELISA. (F) MEFs from Tab2fl/+Tab3fl/+ (control), Tab2fl/flTab3fl/fl (TAB2 KO), Tab2fl/+Tab3fl/fl (TAB3 KO), and Tab2fl/flTab3fl/fl (DKO) mice expressing Cre recombinase were stimulated with TNF (0.1, 1, or 10 ng/ml) for 24 h. Production of IL-6 in the culture supernatants was measured by ELISA. (H) BM macrophages were stimulated with 1 μM CpG DNA (10 or 20 min) or infected with NDV (MOI of 10) (4 or 8 h). Levels of indicated phosphorylated proteins relative to ERK (%) compared with the sample showing strongest signals were quantified and are shown below the bands (second to fourth). ERK serves as a loading control. Data are representative of at least two independent experiments.
mune responses. Thus, it is possible that different polyubiquitination systems function in a cell type–specific fashion.

In summary, this study clearly demonstrates that TAB2 and TAB3 are essential for the activation of B cell immunity in a B cell–intrinsic manner. Nevertheless, NF-κB activation and TAK1 phosphorylation do not require TAB2- and TAB3-mediated K63-linked polyubiquitin recognition. Macrophages do not require TAB2 and TAB3 even in the production of proinflammatory cytokines. These data demonstrate that the functions of TAB2 and TAB3 are specific depending on the cell types and stimulus. The two different ubiquitination systems, linear and K63 type, regulate different functions, both at the molecular and at the cellular levels. TAB2 and TAB3 represent a potential therapeutic target, as their inhibition would suppress aberrant B cell activation, inducing production of autoantibodies without affecting innate immune cells such as macrophages.

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Disclosures
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References