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TRAF-Interacting Protein with a Forkhead-Associated Domain B (TIFAB) Is a Negative Regulator of the TRAF6-Induced Cellular Functions

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Tumour necrosis factor receptor-associated factor (TRAF)-interacting protein with a forkhead-associated domain (TIFA) activates TRAF6 to induce NF-κB activation. TIFA-related protein, TIFAB, is highly expressed in the spleen and inhibits TIFA-mediated TRAF6 activation. However, little is known about cell types that express TIFAB and its function in those cells. Here, we show that TIFAB is mainly expressed in B cells rather than T cells in the spleen and that the expression level was much higher in dendritic cells (DCs) and macrophages than that in splenic lymphocytes. TIFAB expression was downregulated when B cells, DCs or macrophages were stimulated by TRAF6-mediated proliferative or maturation signals including those emanating from CD40, sIgM and TLRs. Furthermore, microinjection experiments using NIH3T3 cells revealed that TIFAB inhibited entry into the S phase of the cell cycle. Our results suggest that TIFAB could act as a negative regulator of the TRAF6-induced cellular function such as B cell proliferation and maturation of DCs and macrophages.

Key words: cell cycle, forkhead-associated domain, NF-κB, TIFA, TRAF.

Abbreviations: BrdU, bromodeoxyuridine; FHA, forkhead-associated; HPRT, hypoxanthine phosphoribosyltransferase; NF-κB, nuclear factor-κB; TIFA, TRAF-interacting protein with FHA domain; TRAF, tumour necrosis factor receptor-associated factor.

The tumour necrosis factor receptor (TNFR)-associated factor (TRAF) family of proteins are cytoplasmic proteins that mediate signalling emanating from members of the TNFR superfamily and the Toll/interleukin-1 receptor family (*1–5*). To date, seven members of the TRAF family have been described. TRAF6 plays essential roles in a variety of biological processes, including the differentiation of osteoclasts, the formation of lymph nodes, thymic microenvironment and skin appendices, and the development and maintenance of B cell follicles within the spleen during the late neonatal period (*2–9*). In these processes, TRAF6 activates transcription factors NF-κB (nuclear factor-κB) and AP-1 through IκB kinase (IKK) and mitogen-activated protein kinases (MAPKs) such as Jun N-terminal kinase (JNK), respectively. To elucidate the molecular mechanism by which TRAF6 mediates signal transduction, we screened for TRAF6-binding proteins and identified a protein designated TRAF-interacting protein with a forkhead-associated (FHA) domain (TIFA), which is also known as T2BP (*10*, *11*). FHA domains, which are predominantly found in eukaryotic nuclear proteins, are composed of 60- to 100-amino-acid residues (*12*). Some of the FHA domain-containing proteins bind directly to phosphoserine/phosphothreonine residues via the FHA domain in much the same way that SH2 domains interact with phosphotyrosine residues (*13*, *14*). Overexpression of TIFA activates NF-κB and JNK. Furthermore, introduction of a mutation into TIFA that abolishes the binding of TIFA to TRAF6 led to a loss of the ability of TIFA to activate NF-κB and JNK, indicating that the TRAF6 interaction is essential for TIFA activity. The binding of TIFA to TRAF6 promotes the oligomerization and Lys63-linked polyubiquitination of TRAF6, which leads to the activation of TAK1 and IKK (*15*). Interestingly, TIFA carrying mutations in the FHA domain that are known to abolish FHA domain binding to phosphopeptide (G50ES66A mutant) cannot activate NF-κB and JNK, suggesting that TIFA may be regulated by an unidentified phosphoprotein.

As another member of the TIFA family, TIFAB gene was identified by a homology search of the human and mouse genome databases. TIFAB shares a FHA domain that is similar to that of TIFA. Both TIFA and TIFAB mRNA are expressed at significant levels in the spleen.

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Although we have shown that TIFAB associates with TIFA but not with TRAF6 and blocks TIFA-mediated NF-κB activation (*16*), little is known about cell types that express TIFAB and its function in those cells.

In the present study, we show that the level of TIFAB expression was higher in B cells than that in T cells in the spleen but that TIFAB mRNA is much abundant in DCs and macrophages compared to splenic lymphocytes. TIFAB expression was downregulated when B cells, DCs or macrophages were stimulated by TRAF6-mediated proliferative or maturation signals including those emanating from CD40, sIgM and TLRs. Furthermore, overexpression of TIFAB inhibited entry into the S phase of cell cycle. These results suggest that TIFAB may act as a negative regulator of the TRAF6-mediated cell proliferation and maturation.

MATERIALS AND METHODS

Reagents and Antibodies—Escherichia coli LPS (*E. coli* O111: B4) purchased from Sigma–Aldrich (St Louis, MO), an anti-mouse CD40 mAb (clone 1C10) and recombinant mouse CD40 ligand purchased from R&D Systems (Minneapolis, MN), an AffiniPure $F(ab')_2$ fragment goat anti-mouse IgM purchased from Jackson ImmunoResearch (West Grove, PA), R848 purchased from Pharma Tech (Shanghai, China), CpG-1668 (CpG-B) purchased from Hokkaido System Science (Sapporo, Japan), macrophage-activating lipopeptide 2 (MALP-2) purchased from EMC Microcollections (Tuebingen, Germany), the poly (I:C) purchased from GE Healthcare UK Ltd (Buckinghamshire, England) and flagellin purchased from Calbiochem (San Diego, CA) were used for cell stimulation. Anti-TIFAB antiserum generated by the injection of recombinant GST-TIFAB fusion proteins into rabbits, the anti-α-tubulin mouse mAb (DM1A) purchased from Calbiochem, the anti-rabbit IgG-horseradish peroxidase (HRP) and anti-mouse IgG-HRP secondary antibodies purchased from GE Healthcare were used for western blot analysis. The rabbit anti-c-myc (A-14) polyclonal antibody purchased from Santa Cruz Biotechnology (Santa Cruz, CA), the rabbit anti-β-galactosidase polyclonal antibody purchased from Cappel (Aurora, OH), the anti-BrdU (anti-bromodeoxyuridine) monoclonal antibody (BU6-4) purchased from Takara (Tokyo, Japan), and the Alexa Fluor®488 goat anti-rabbit IgG and the Alexa Fluor®568 goat anti-mouse IgG purchased from Invitrogen (Carlsbad, CA) were used for immunofluorescence staining.

Cell Culture—NIH3T3 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Mouse naïve B cells (>95% $B220^+$) and T cells (>80% $Thy1.1^+$) were isolated from the spleens of C57BL/6 mice (6 weeks of age) using the MACS B cell isolation kit and the Pan T cell isolation kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), respectively. To isolate the bone marrow-derived immature DCs and macrophages, bone marrow non-adherent cells from C57BL/6 mice (6–8 weeks of age) were cultured with Dulbecco's modified Eagle's medium (DMEM) containing 10 ng/ml GM-CSF and 10% FBS, and Minimum Essential Medium Eagle α modification (α-MEM) containing 10 ng/ml M-CSF and 10% FBS, respectively. Adherent cells obtained after 7 and 6 days of culture were used as DCs (>90% $CD11c^+$) and macrophages (>95% $Mac\text{-}1^+$, $F4/80^+$), respectively.

RNA Preparation, Reverse Transcriptase (RT) Reaction and PCR analysis—Total RNA was extracted from B and T cells (1×10^6 cells/1 ml culture), and the RT reaction was performed as described previously (*17*). The reaction mixtures were diluted fivefold with ddH_2O to give cDNA stocks that were stored at −20°C until PCR analysis. Primers used to amplify TIFAB were 5′-ATGGAGAGGC CCCTCACAGTCC-3′ and 5′-TCATTCATCTGTCTCCTG AGC-3′; those used to amplify HPRT were 5′-GTTGGAT ACAGGCCAGACTTTGTTG-3′ and 5′-GAAGGGTAGGCT GGCCTATAGGCT-3′. The PCR reactions contained 1× PCR buffer, 0.4 mM dNTPs, 2.5 ng forward and reverse primers, 0.005 U TaKaRa Ex Taq™ DNA polymerase (Takara) and 5 μl of the cDNA solution in a 10 μl volume. TIFAB and hypoxanthine phosphoribosyltransferase (HPRT) cDNAs were amplified for 30–35 and 29 cycles of 94°C (denaturation) for 1 min, 55°C and 60°C (annealing) for 1 min and 72°C (primer extension) for 1 min, respectively. The PCR products (5 μl) were analysed on 2% agarose gels in the presence of ethidium bromide. The expected fragments with the expected sizes were amplified for TIFAB (444 bp, 92–535) and HPRT (353 bp, 601–953), and the generated restriction endonuclease patterns were consistent with each of the respective genes.

Preparation of Cell Extracts, Immunoprecipitation and Western Blot Analysis—B cells (3×10^7 cells/30 ml culture) were either untreated or treated with LPS (100 ng/ml) for 24, 60 or 96 h. Cells were lysed in 3 ml of the TNE buffer (50 mM Tris–HCl, pH 8.0, 1% Nonidet P-40, 1 mM EDTA, 150 mM NaCl, 0.5 mM dithiothreitol) and centrifuged to remove cellular debris. Cell lysates were subjected to immunoprecipitation with 3 μg of the appropriate antibody and 60 μl of protein G-Sepharose (GE Healthcare). DCs and macrophages were unstimulated or stimulated with CD40 ligand or various TLR ligands, and then lysed. The resulting immunoprecipitates and the lysates were separated on 10% or 12.5% polyacrylamide/SDS gels, and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were immunoblotted with specific antibodies and visualized with appropriate HRP-conjugated protein A or secondary antibodies and the ECL western blotting system (GE Healthcare). The intensity of each band was analysed by the NIH ImageJ program.

Microinjection and Immunofluorescence Staining—NIH3T3 cells were cultured on coverslips (2×10^4 cells/coverslip) in DMEM containing 0.2% calf serum for 24 h, and then microinjected with pME-Myc-TIFAB, pME-Myc-TIFAB(G39ES54A), pME-Myc-TIFA and pME-LacZ (β-galactosidase) (200 μg/ml DNA). After 24 h of incubation under the same conditions, the medium was replaced with fresh DMEM containing 10% calf serum and 250 ng/ml BrdU (Sigma–Aldrich), and then the cells were incubated for another 18 h. The cells were fixed in 4% (w/v) paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 10 min at room

temperature and permeabilized with 0.2% (w/v) Triton X-100 in PBS for 10 min at room temperature. The permeabilized cells were treated with 2 N HCl for 10 min and 0.1 M borate buffer (pH8.5) for 5 min, and then with 2% (w/v) bovine serum albumin (BSA fraction V, Sigma–Aldrich) in PBS for 15 min. The myc-tag and β-galactosidase proteins were detected with specific antibodies and visualized with appropriate secondary antibodies.

RESULTS

TIFAB Is Expressed in Spleen B Cells during the Resting Stage and Downregulated by Growth-Stimulatory Signals—TIFAB mRNA is expressed at high levels in the spleen (*16*). To understand physiological roles of TIFAB, we determined the level of TIFAB mRNAs in splenic B and T cells. TIFAB mRNA was mainly expressed in the B cells but to a much lesser extent in the T cells (Fig. 1A). Since TIFAB inhibits TRAF6 activation, TIFAB mRNA expression was analysed after stimulation of TRAF6-mediated signals. We first determined the level of TIFAB mRNA in B cells that had been treated with LPS (TLR4 ligand), which induces TRAF6-dependent B cell proliferation (*3*). The expression level of TIFAB mRNA was significantly downregulated by 2 h of LPS treatment and the decreased expression level was sustained by LPS treatment for up to 96 h (Fig. 1B). Treatment of B cells with other growth-stimulating ligands, including anti-CD40, anti-IgM, R848 (TLR7 ligand) or CpG-B (TLR9 ligand), for 24 h resulted in the reduction of TIFAB mRNA expression (Fig. 1C). Furthermore, the expression level of TIFAB protein was also reduced in B cells in response to LPS stimulation (Fig. 1D). Compared with the B cell proliferation profile in response to LPS stimulation shown in Fig. 1E, TIFAB was expressed only in resting B cells and the expression level was reduced just before the initiation of proliferation. TRAF6 mediates signals emanating from CD40 and TLRs (*4*, *5*, *18*) and is thought to be involved in BCL10/MALT signalling known to connect T cell receptor (TCR) and B cell receptor (BCR) to NF-κB activation (*19*, *20*). Therefore, these findings implied that TIFAB acts as a negative regulator of TRAF6-dependent B cell proliferation.

We further examined the expression level of TIFAB in each stage of B cell development. Notably, TIFAB mRNA level was much higher in pre-pro-B (Hardy's fraction-A)

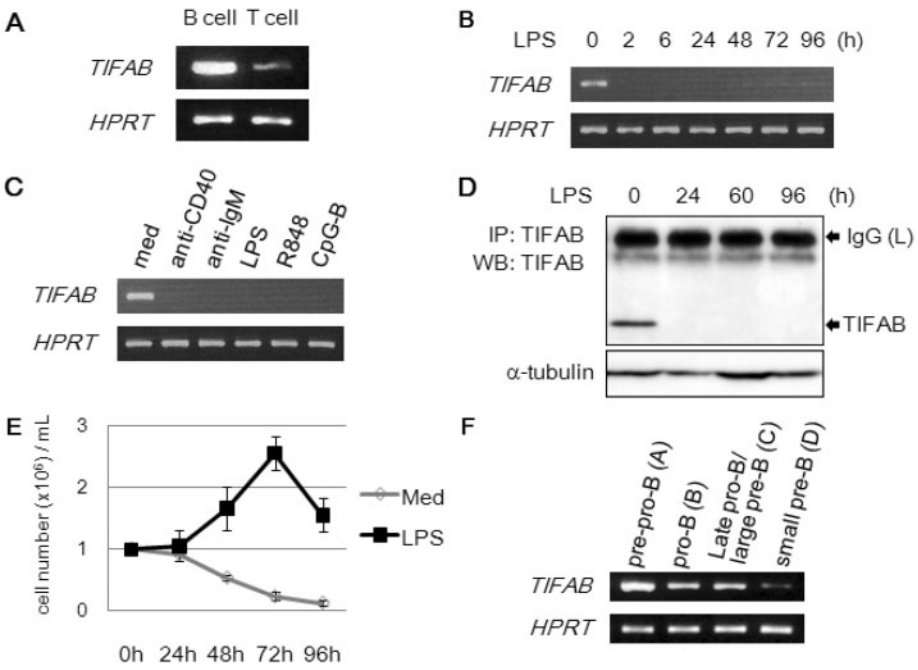

Fig. 1. **Rapid downregulation of TIFAB expression in splenic naïve B cells by growth stimulatory signals.** Cells were treated with or without LPS, R848, CpG-B, anti-CD40 or anti-IgM. After treatment, total RNA was isolated and both TIFAB and HPRT mRNA expression levels were determined by RT-PCR (A–C, F). (A) Naïve B cells and naïve T cells were isolated from mouse spleens. (B) Naïve B cells were stimulated with LPS (100 ng/ml) for 2, 6, 24, 48, 72 and 96 h. (C) Naïve B cells were stimulated with anti-CD40 (1 μg/ml), anti-IgM (10 μg/ml), LPS (100 ng/ml), R848 (1 μg/ml) or CpG-B (1 μM) for 24 h. (D) Naïve B cells were treated with LPS (100 ng/ml) for 24, 60 and 96 h and the cell lysates were subjected to immunoprecipitation with an anti-TIFAB antibody prior to subjecting the samples to western blot analysis. Aliquots of cell lysates used for immunoprecipitation were also separated by SDS–PAGE and the expression level of α-tubulin was visualized by western blotting with an anti-α-tubulin antibody. (E) Naïve B cells were treated with LPS (100 ng/ml) for 24, 48, 72 and 96 h. At the end of the incubation period, the number of alive cells was determined by staining the cell suspension with a 0.1% (w/v) solution of trypan blue and scoring stained (dead) and unstained (alive) cells on a haemocytometer chamber under light microscopy. Values are shown as the mean ± SD of five independent experiments. (F) Hardy's fraction-A: pre-pro-B cells ($B220^+/CD43^+/HSA^-/BP\text{-}1^-$); fraction-B: pro-B cells ($B220^+/CD43^+/HSA^+/BP\text{-}1^-$); fraction-C: late pro-B/large pre-B cells ($B220^+/CD43^+/HSA^+/BP\text{-}1^+$) and fraction-D: small pre-B cells ($B220^+/CD43^-$) were sorted from bone marrow cells.

than that in pro-B (fraction-B) and late pro-B/large pre-B (fraction-C) cells and was severely suppressed in small pre-B cells (fraction-D) (Fig. 1F). These results suggest that TIFAB may play a crucial role in pre-pro-B cells, pro-B cells and late pro-B/large pre-B cells, but not in small pre-B cells.

TIFAB Is Expressed in Immature Macrophages and DCs and Is Downregulated by TRAF6-Dependent Signals—TLRs are expressed by not only B cells but also DCs and macrophages. Therefore, we investigated the expression level of TIFAB protein in bone marrow-derived macrophages and DCs. Interestingly, in immature macrophages and DCs, but not in B and T cells, TIFAB protein was detected by western blotting without concentration by immunoprecipitation (Fig. 2A), indicating that the TIFAB expression level was much higher in DCs and macrophages than that in B cells and T cells. To examine whether TIFAB expression is downregulated by TLR ligands in DCs and macrophages as well as B cells, we treated immature DCs and macrophages with CD40 ligand or TLR ligands such as MALP-2 (TLR2/6 ligand), poly (I:C) (TLR3 ligand), LPS, flagellin (TLR5 ligand), R848 or CpG-B. In contrast to the dramatic reduction of TIFAB expression in TLRs-stimulated B cells (Fig. 1B–D), the amount of TIFAB protein in DCs and macrophages was decreased gradually during first 24 h after these treatments except for the TLR3 ligand (Fig. 2B and C). These results may suggest the disparity between B lymphocytes and myeloid cells in the inhibitory fashion of TIFAB on the TRAF6-induced cellular functions such as B cell proliferation and the maturation, activation and development of DCs and macrophages.

TIFAB Blocks Cell-Cycle Progression from G_0/G_1 to the S Phase—To examine anti-proliferative function of TIFAB, we performed a microinjection experiment in NIH3T3 cells (Fig. 3). Injection of the TIFAB expression plasmid inhibited incorporation of BrdU into the cells, whereas microinjection of the β-galactosidase (LacZ) expression control plasmid scarcely inhibited BrdU incorporation (Fig. 3A, B and F). These data strongly suggest that TIFAB inhibits cell-cycle progression from G_0/G_1 to the S phase. Interestingly, inhibition of BrdU incorporation by the FHA domain mutant of TIFAB, TIFAB (G39ES54A), was weaker than that by wild-type TIFAB (Fig. 3C, D and F). Therefore, the unidentified protein that binds the FHA domain of TIFAB may modulate the growth suppressive function that is mediated by TIFAB. In addition, expression of TIFA blocked TIFAB-mediated G_0/G_1-arrest of cell cycle (Fig. 3E and F). Therefore, TIFA may act as an inhibitor of TIFAB-mediated cell-cycle arrest.

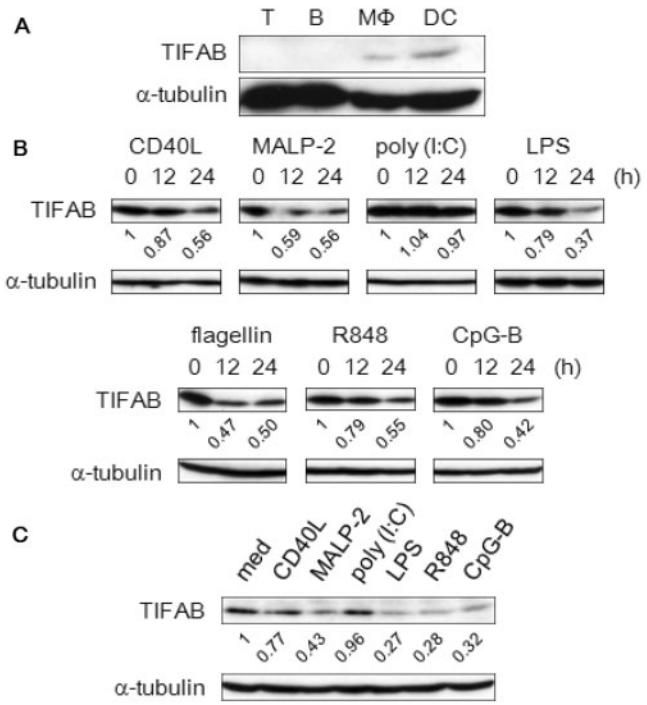

Fig. 2. **Downregulation of TIFAB expression in bone marrow-derived immature DCs and macrophages by CD40 and TLR signals.** (A) The cell lysates from naïve B cells (B; 2×10^6 cells/lane) and naïve T cells (T; 2×10^6 cells/lane) isolated from mouse spleens, and from bone marrow-derived immature macrophages (MΦ; 4×10^5 cells/lane) and immature DCs (DC; 4×10^5 cells/lane) were separated by SDS–PAGE and the expression levels of TIFAB and α-tubulin were visualized by western blotting with anti-TIFAB and anti-α-tubulin antibodies, respectively. (B) Bone marrow-derived immature DCs were stimulated with CD40 ligand (100 ng/ml), MALP-2 (50 ng/ml), poly (I:C) (10 μg/ml), LPS (100 ng/ml), flagellin (1 μg/ml), R848 (1 μg/ml) or CpG-B (1 μM) for 12 and 24 h. After treatment, the cell lysates were separated by SDS–PAGE and the expression levels of TIFAB and α-tubulin were visualized by western blotting with anti-TIFAB and anti-α-tubulin antibodies, respectively. Intensity of TIFAB band divided by that of α-tubulin at 0 h was set to 1 and the corresponding values after stimulation were shown. (C) Bone marrow-derived immature macrophages were stimulated with CD40 ligand (100 ng/ml), MALP-2 (50 ng/ml), poly (I:C) (10 μg/ml), LPS (100 ng/ml), R848 (1 μg/ml) or CpG-B (1 μM) for 24 h. After treatment, the cell lysates were separated by SDS–PAGE and the expression levels of TIFAB and α-tubulin were visualized by western blotting with anti-TIFAB and anti-α-tubulin antibodies, respectively. Intensity of basal TIFAB band divided by that of α-tubulin (med) was set to 1 and the corresponding values after stimulation were shown.

DISCUSSION

We have previously shown that TIFA promotes TRAF6-mediated NF-κB activation, whereas TIFAB associates with TIFA and blocks TIFA-mediated NF-κB activation. In the present study, we show that TIFAB is expressed in naïve B cells rather than in naïve T cells, and highly expressed in immature DCs and macrophages (Figs. 1A and 2A). Different expression patterns of TIFAB were also observed in each stage of B cell development. The TIFAB mRNA level was much higher in pre-pro-B (Hardy's fraction-A) than that in pro-B (fraction-B) and late pro-B/large pre-B (fraction-C) cells and was severely suppressed in small pre-B cells (fraction-D) (Fig. 1F). Tightly controlled p100 processing and RelB activation is essential for normal B lymphopoiesis and lymphoid/myeloid lineage decision in bone marrow (*21*). Since enhanced RelB activity blocks the transition from pre-pro-B to pro-B cells, TIFAB may suppress excessive RelB activation by downregulating TRAF6-mediated RelB induction as observed in the

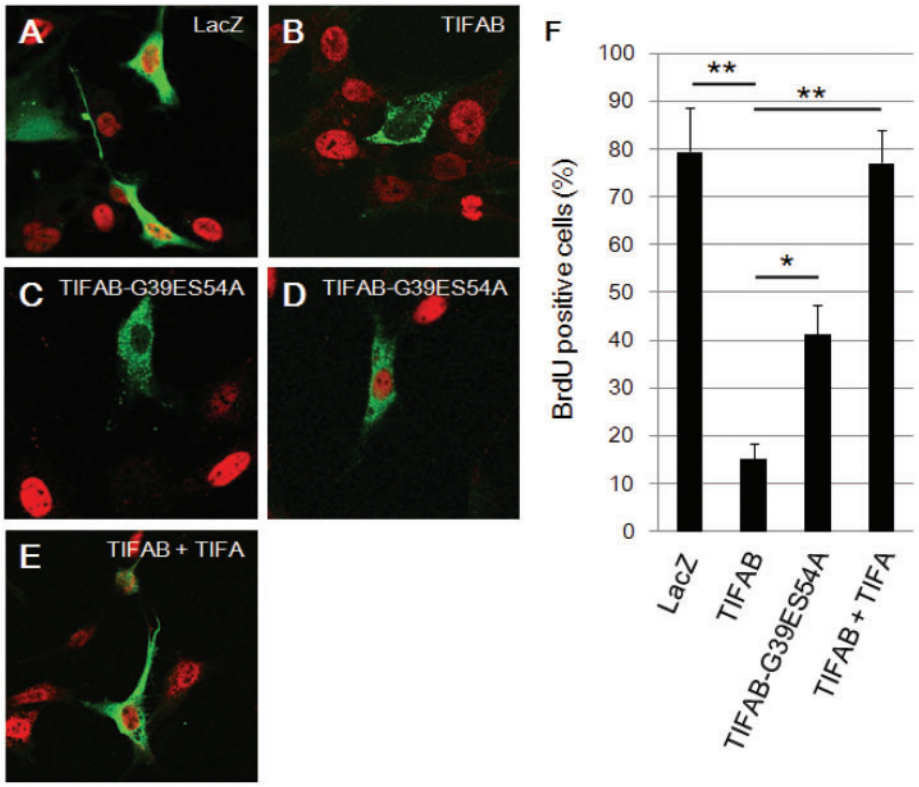

Fig. 3. **Inhibition of serum-induced G_0/G_1-S transition by exogenously expressed TIFAB.** Serum-starved NIH3T3 cells were microinjected with pME-LacZ (β-galactosidase) (A), pME-Myc-TIFAB (B), pME-Myc-TIFAB (G39ES54A) (C, D) or the combination of pME-Myc-TIFAB and pME-Myc-TIFA (E) and were re-fed with serum and then immunostained for Myc-tag (green), β-galactosidase (green) or BrdU (red). (F) The graph shows the percentage of BrdU incorporation into the NIH3T3 cells that expressed β-galactosidase, TIFAB, TIFAB (G39ES54A) or TIFAB in combination with TIFA. At least 70 cells were scored for each experimental condition. Values are shown as the mean ± SD of three independent experiments. Significant differences compared with TIFAB alone samples ($^{*}P < 0.05$, $^{**}P < 0.01$, Student's *t*-test; $n = 3$).

thymus (*8*). The large pre-B cells express pre-B cell receptor (pre-BCR) on the surface and are highly proliferative, whereas the small pre-B cells are derived from large pre-B cells that have downregulated pre-BCR and withdrawn from cell cycle (*22*, *23*). TIFAB may also act as a negative regulator of pre-BCR-mediated cell proliferation, and the expression of TIFAB may be properly downregulated because TIFAB is unnecessary in small pre-B cells that express neither pre-BCR nor BCR.

Interestingly, TIFA expression levels were also correlated with TIFAB expression levels in B cells, T cells, DCs and macrophages (data not shown), which is consistent with the notion that TIFAB regulates TIFA. Therefore, TIFAB expression may be appropriately downregulated in T cells because the inhibitory function of TIFAB is unnecessary in T cells that little express TIFA. Since B cells, DCs and macrophages, but not T cells, express CD40 and high levels of TLRs, these results suggest that TIFA and TIFAB may be required for CD40, TLRs or BCR signalling rather than TCR signalling.

Treatment of B cells with TLR ligands including LPS (TLR4), R848 (TLR7) and CpG-B (TLR9) also resulted in downregulation of TIFAB expression (Fig. 1C). TIFAB protein expression levels in DCs and macrophages were also downregulated by stimulation of various TLRs except for TLR3 (Fig. 2B and C). We have not tested TIFAB downregulation in B cells in response to TLR3 and TLR5 stimulations and that in macrophages in response to TLR5 stimulation. This is because naïve B cells barely express TLR3 and TLR5 and immature macrophages little express TLR5 (data not shown). TRAF6 is essential for the MyD88-dependent pathway, but not for the Toll/IL-1 receptor domain-containing adaptor-inducing IFN-β (TRIF)-dependent pathway in TLR signalling (*4*). TLR3 signalling is TRIF dependent, but not MyD88 dependent. Therefore, TRAF6 activation, which is initiated by various TLRs except TLR3, may induce suppression of TIFAB expression in naïve B cells, immature DCs and macrophages. Given that the TLRs-mediated TRAF6 activation induces B cell proliferation and the maturation, activation and development of DCs (*3*, *5*), we propose the hypothesis that TIFAB may act as a negative regulator of TRAF6 functions in B cells, DCs and macrophages.

Recently, the zinc finger protein ZCCHC11 was identified as a TLR signal regulator, which interacts with TIFA after LPS treatment and suppresses the TRAF6-dependent activation of NF-κB (*24*). TIFAB localizes to the cytoplasm (Fig. 3B), whereas ZCCHC11

predominantly localizes to the nucleus. ZCCHC11 translocates into the cytoplasm in response to LPS and binds to TIFA, whereas TIFAB binds to TIFA in the absence of stimulation (data not shown), but TIFAB is downregulated upon stimulation (Figs. 1B–D and 2B, C). Therefore, TIFAB may block accidental activation of TRAF6-mediated signalling in resting cells, while ZCCHC11 may act as a suppressor of TIFA in ongoing signalling.

Our results from microinjection experiments in NIH3T3 cells suggest that TIFAB strongly inhibits cell-cycle progression from G_0/G_1 to the S phase. Recent studies indicate that FHA-RING ligases play roles in negative regulation of the cell division cycle such as G_1- and G_2/M-arrest, apparently by coupling protein phosphorylation events to specific Lys48- or Lys63-linked ubiquitination of target proteins (*25*). Since TIFAB harbours the FHA domain and TRAF6 is a RING domain-containing ubiquitin ligase that synthesizes Lys63-linked polyubiquitination, TRAF6–TIFA–TIFAB complex in resting cells may have G_1-arresting function similar to FHA-RING ligases such as human RNF8 and yeast Chf1/Chf2 (*26*, *27*). Because growth rate of TRAF6-deficient mouse embryonic fibroblast (MEF) cells is significantly lower than that of wild-type MEF cells (data not shown), TRAF6 is likely to be involved in proliferation of NIH3T3 cells. Therefore, cell-cycle arrest induced by overexpression of TIFAB and its release by inactivation of FHA domain or coexpression of TIFA could be observed as long as proliferation of cells involves TRAF6 activation as in the case of B cell proliferation induced by TLR4 signalling.

In this study, we demonstrated that TIFAB expression was downregulated by TRAF6-mediated signalling in B cells, DCs and macrophages, and that overexpression of TIFAB inhibited G_0/G_1–S transition. Thus, we propose that TIFAB is a unique member of the FHA-containing proteins that functions as a negative regulator of the TRAF6-induced cellular functions such as B cell proliferation and the maturation, activation and development of DCs and macrophages.

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CONFLICT OF INTEREST

None declared.

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