Transcriptional and post-transcriptional regulation of \( \text{IκB-}\zeta \) upon engagement of the BCR, TLRs and FcγR

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Received 20 February 2013, accepted 3 April 2013

Abstract

\( \text{IκB-}\zeta \) is a nuclear IκB protein robustly induced in macrophages and fibroblasts upon TLR or IL-1R stimulation. IκB-\zeta associates with NF-κB in the cell nucleus and is essential for the induction of a subset of secondary response genes represented by IL-6. Here, we analyzed induction of IκB-\zeta in mouse B cells and found that IκB-\zeta is induced by BCR or TLR stimulation. Similar to TLR stimulation, BCR stimulation elicited NF-κB-mediated transcriptional activation and mRNA stabilization of IκB-\zeta via a cis-element in IκB-\zeta mRNA. Proteasome inhibitors inhibited transcriptional activation but not post-transcriptional activation, indicating independency of the two signals. Co-stimulation of the BCR and TLR9 or TLR7, but not TLR2/1, synergistically induced IκB-\zeta. Co-engagement of inhibitory Fcγ receptor suppressed BCR-mediated IκB-\zeta expression but not that induced by TLR stimulation alone or co-stimulation of TLR and the BCR. The PI3K inhibitor LY294002 inhibited BCR-mediated, but not TLR-mediated, induction of IκB-\zeta, consistent with the role of PI3K in BCR signaling and its suppression by FcγR. Analysis of IκB-\zeta-deficient B cells demonstrated that IκB-\zeta was essential upon stimulation of BCR or TLR for the expression of several genes including IL-10 and CTLA4. IκB-\zeta-deficient B cells exhibited impaired proliferation and enhanced up-regulation of CD86 following stimulation of TLR9, but not the BCR, indicating critical roles for IκB-\zeta in TLR signaling in B cells. Strict regulatory mechanisms for the induction of IκB-\zeta via multiple pathways and its essential function upon stimulation indicate that IκB-\zeta plays an important role in B cells.

Keywords: gene induction, NF-κB, nuclear IκB protein, mRNA stability

Introduction

In addition to the BCR, B cells express germ-line-encoded pathogen receptors, such as TLRs. Recognition of pathogen-derived molecules via TLRs in conjunction with the BCR induces proliferation, differentiation and production of cytokines and antibodies (1). Both BCR- and TLR-mediated responses induce activation of NF-κB, a nuclear factor that binds to the enhancer region of the immunoglobulin \( \kappa \) light chain (2), which acts as a key regulator of B-cell development, survival and functions (3). NF-κB activation by stimulation of TLRs and other innate pathogen receptors is essential for the induction of numerous inflammatory genes (4). NF-κB plays important roles not only in inflammation but also in various important biological processes such as embryonic development, cell differentiation and stress responses. Molecular studies of NF-κB activation have established a role for cytosolic IκB proteins such as IκB-α, -β or -ε, which associate with NF-κB. Phosphorylation-dependent degradation of cytosolic IκB proteins allows nuclear translocation of NF-κB, where it activates transcription. However, the precise mechanisms that ensure selective activation of appropriate genes in response to specific stimuli by this multifunctional transcription factor remain to be elucidated but may include cell-context-dependent chromatin structures, combinations of different NF-κB subunits or other transcription factors, and specific modifications of NF-κB. Recent studies indicated a critical role for NF-κB binding proteins or cofactors in specific gene induction (5).

\( \text{Nfkbi}z \) was originally identified as a gene induced in macrophages by LPS (6). Its product is a nuclear IκB protein, IκB-\zeta, harboring ankyrin repeats that associate with NF-κB. In contrast to cytosolic IκB proteins, IκB-\zeta is exclusively localized in the nucleus and does not affect nuclear translocation...
of NF-κB (6). IkB-ζ is induced upon receptor stimulation and modulates NF-κB activity in the nucleus, allowing chromatin remodeling essential for the induction of genes such as IL-6 in response to LPS (7–9).

In addition, IL-1β and TLR agonists other than LPS can induce IkB-ζ in macrophages or fibroblasts (6). Its induction requires NF-κB-mediated transcriptional activation (10) and post-transcriptional activation including specific mRNA stabilization (11), both of which are stimulated by MyD88-dependent signaling. A 165-nt element in the 3′-UTR of IkB-ζ mRNA acts as a cis-element that controls post-transcriptional regulation (12). Upon stimulation of TLR or IL-1R, IRAK1 together with TRAF6 activates the post-transcriptional mechanism (13). Interestingly, TNF-α, another potent proinflammatory cytokine, activates NF-κB-mediated transcriptional activation but does not elicit post-transcriptional activation, resulting in failure of IkB-ζ induction (11).

Recent studies have demonstrated that IkB-ζ functions in innate and adaptive immunities. For instance, IkB-ζ is critical for γδ T-cell differentiation and NK-cell activation (14, 15). In addition, we previously showed that IkB-ζ is induced in B cells upon BCR stimulation (16). Here, we show that either BCR or TLR stimulation of B cells elicits IkB-ζ induction via transcriptional and post-transcriptional activation. Interestingly, BCR-mediated signaling is inhibited by FcyR type IIb (FcyRIIB) co-engagement and co-stimulation of the BCR and TLR9 or TLR7 synergistically augments BCR- and TLR-mediated induction of IkB-ζ, induction. Fine-tuning of IkB-ζ induction via multiple regulatory mechanisms suggests its significance in B-cell functions.

**Methods**

**Ethics statement**

Animal experiments were performed in compliance with the animal care and used guidelines of the Institutional Animal Care and Use Committee of Tohoku University. The animal protocol was approved by the same committee.

**Reagents**

Commercially available antibodies used in the study are shown in Supplementary Table S1, available at International Immunology Online. LPS from *Escherichia coli* O111:B4 was purchased from List Biological Laboratories Inc. (Campbell, CA, USA). A phosphorothioate-stabilized CpG oligodeoxynucleotide (ODN1826, 5′-TCCATGACGTTCCTGACGTT-3′) was synthesized by Sigma-Genosys Japan K.K. (Ishikari, Japan). S-[2,3-Bis(palmitoyloxy)-2(RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys₂-OH (Pam₃CSK₄) was synthesized by Peptide Institute, Inc. (Osaka, Japan). Imiquimod was purchased from Invivogen (San Diego, CA, USA). PMA and ionomycin were purchased from Sigma-Aldrich Japan K.K. (Tokyo, Japan). MG-132, lactacystin and LY294002 were purchased from Peptide Institute, Inc. (Osaka, Japan). Imiquimod was synthesized by Sigma-Genosys Japan K.K. (Ishikari, Japan). MG-132, lactacystin and LY294002 were purchased from Sigma-Aldrich Japan K.K. (Tokyo, Japan). MG-132, lactacystin and LY294002 were purchased from Sigma-Aldrich Japan K.K. (Tokyo, Japan).

**Plasmids**

Reporter plasmids for the IkB-ζ promoter, pNfkbiz(-1,725)-Luc, pNfkbiz(-786)-Luc, pNfkbiz(-786(x81M))-Luc, pNfkbiz(-786(x82M))-Luc, pNfkbiz(-786(x83M))-Luc, the 165-nt element-mediated post-transcriptional regulation (pGL4.12-SV40-[luc2CP]-Nfkbi-full, -Reverse, -After pA, -3′-UTR, and -165-nt) and the NF-κB reporter pELAM1-Luc were described previously (11, 12, 17). The Tet-off and pTRE2-Hyg-Luc were purchased from Clontech Laboratories, Inc. (Mountain View, CA, USA). pEF6/V5-His A was from Invitrogen Corp. (Carlsbad, CA, USA). The pEF-Tet-off plasmid was constructed by sub-cloning the tet repressor cDNA between the BamHI and EcoRI sites downstream of the elongation factor-1α promoter in pEF6/V5-His A. The pTRE2-Hyg-Luc-Nfkbi-3′-UTR plasmid was constructed by sub-cloning the 3′-UTR of IkB-ζ mRNA into the EcoRV site downstream of the luciferase open reading frame in pTRE2-Hyg-Luc.

**Cells**

The murine B lymphoma cell line A20 and the FcyRIIB-negative mutant cell line IIA1.6 derived from A20 (18) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate and 50 μM 2-mercaptoethanol at 37°C in 5% CO₂. The murine macrophage cell line RAW264.7 (ATCC® Number, TIB-71™) and a stable RAW264.7 line transfected with pELAM1-Luc (19) were maintained in DMEM with 10% heat-inactivated FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. Splenocytes from Balb/c mice were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-mercaptoethanol at 37°C in 5% CO₂. B cells were purified from the spleen of IkB-ζ-deficient mice (7) or wild-type littermate control mice by negative depletion of cells expressing CD43, CD4 or Ter-119 using a B-cell isolation kit (Miltenyi Biotech, Bergish Gladbach, Germany) according to the manufacturer’s protocol, resulting in purity > 95% of B220⁺ B cells. Mice were maintained under specific pathogen-free conditions in the animal facilities of Tohoku University.

**Northern blot analysis**

Total RNA was extracted using RNAiso plus (Takara Bio Inc., Otsu, Japan) and subjected to northern blot analysis with a [3P]-labeled probe for IkB-ζ, IkB-α or β-actin. Hybridized bands were visualized and quantified by phosphor imaging with FLA-3000 (FujiFilm Corp., Tokyo, Japan).

**Real-time RT-PCR**

Total RNA was prepared using RNAiso plus. mRNA levels were quantified by real-time RT-PCR with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and SYBR Premix EX Taq II (Takara Bio Inc.) using a LightCycler 3302 (Roche Diagnostics GmbH, by guest on 08 October 2017
**Immunoblotting**

Cells were lysed, subjected to 10% SDS-PAGE and analyzed by immunoblotting with anti-IκB-ζ or anti-β-actin antibodies and secondary antibodies conjugated with horseradish peroxidase. Bound antibodies were visualized by chemiluminescence following incubation with Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore).

**Transfection**

A20 cells were transfected by electroporation with the indicated reporter plasmid and pEF6/V5-His A at a ratio of 19:1. One day after electroporation, cells were selected with 5 μg/ml blasticidin S for 10–11 days and used for stimulation. To obtain reporter stable cell lines, transfected cells were cloned by limiting dilution following selection. A20 Tet-off cells were obtained by transfecting A20 cells with pEF-Tet-off, followed by selection with 5 μg/ml blasticidin S and cloning. RAW264.7 cells were transfected with pTRE2-Hyg-Luc, selected with 200 μg/ml G418 and cloned. A20-Tet-off cells were transfected with pTRE2-Hyg-Luc, selected with 750 μg/ml hygromycin for 2 weeks and cloned. RAW264.7 cells were transfected with pGL4.12-SV40-[luc2CP]-NFkbiz-165nt and pcDNA3 at a ratio of 19:1 using HilyMax (Dojindo Laboratories, Kumamoto, Japan), selected with 1 mg/ml G418 and cloned.

**Luciferase assay**

Cells at 4 × 10^6 cells per milliliter were stimulated as indicated and lysed for the luciferase assay. Luciferase activity was measured by the Single-Luciferase Reporter Assay System according to the manufacturer’s instructions (Promega Corp., Madison, WI, USA).

**ELISA**

ELISA for IL-10 (PeproTech Inc., Rocky Hill, NJ, USA) and TNF-α (Invitrogen) was performed according to the manufacturer’s instructions. ELISA for IgM was carried out with the SBA Clonotyping System (SouthernBiotech Associates, Inc., Birmingham, AL, USA).

**Flow cytometry**

Cell-surface antigens were stained with the indicated antibodies in ice-cold PBS containing 2% FCS and anti-CD16/32 antibody (Fc block) in the dark at 4°C. The stained cells were washed and subjected to flow cytometric analysis with Gallios™ (Beckman Coulter, Inc., Brea, CA, USA). Data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

**Cell proliferation**

Cells were washed with PBS and incubated with 0.5 μM CFSE (eBioscience, San Diego, CA, USA) for 10 min in the dark. Then, cells were washed with RPMI containing 10% FCS, stimulated with 50 μg/ml mitomycin C for 48h and subjected to flow cytometric analysis.

**Statistical analysis**

Paired data were evaluated with Student’s t-test. A value of p < 0.05 was considered statistically significant.

**Results**

**IκB-ζ is induced by BCR and TLR stimulation of B cells**

To examine IκB-ζ induction in response to BCR engagement, mouse splenocytes were stimulated with F(ab)′, fragment of anti-immunoglobulin antibody. Northern blot analysis revealed that IκB-ζ mRNA was barely detectable in unstimulated splenocytes but was robustly induced in response to the TLR4 ligand LPS or PMA/ionomycin 1h after stimulation (Fig. 1A). Upon BCR stimulation with anti-IgM antibody, the induction of IκB-ζ mRNA was observed in primary splenic B cells. IκB-α, an NF-κB target gene, was also induced by anti-IgM antibody or LPS. As in splenic B cells, induction of IκB-ζ and IκB-α mRNA was observed in A20 B-cell lymphoma upon stimulation with anti-IgG F(ab)′, or with CpG ODN, a TLR9 ligand, indicating that IκB-ζ can be induced by either BCR or TLR stimulation (Fig. 1B and C).

In contrast to F(ab)′, stimulation, BCR engagement by intact IgG of anti-immunoglobulin elicited marginal IκB-α induction and no IκB-ζ induction in A20 cells (Fig. 1B and C). In a FcyRIIB-deficient cell line (IIA1.6), similar levels of IκB-ζ or IκB-α mRNA induction were observed by stimulation with the intact IgG of anti-immunoglobulin or F(ab)′ fragments (Fig. 1A). Thus, suppression was caused by co-cross-linking of FcyRIIB and the BCR via the Fc region of the antibody (20).

Induction of IκB-ζ protein was detected 1h after BCR stimulation with F(ab)′, and peaked at 2h (Fig. 1D). Co-engagement of FcyRIIB abolished this induction. Compared with BCR engagement, TLR9 stimulation by CpG ODN elicited greater induction at 2h after stimulation, which further increased at 4h.

**BCR stimulation elicits transcriptional and post-transcriptional activation of IκB-ζ**

Induction of IκB-ζ mRNA in LPS-stimulated macrophages requires NF-κB-mediated transcriptional activation of IκB-ζ (10, 11). We, therefore, analyzed activation of the IκB-ζ promoter by reporter analyses. Upon stimulation of A20 cells with F(ab)′, of anti-immunoglobulin antibody, a reporter harboring an IκB-ζ promoter fragment from −1725 to +115bp was significantly activated similar to the NF-κB reporter pELAM1-Luc (Fig. 2A). A further truncated IκB-ζ promoter from −786bp was also activated in response to BCR engagement (Fig. 2B). This promoter region contains three NF-κB binding sites, κB1, κB2 and κB3. Introduction of mutations at the κB2 site severely impaired activation in response to BCR engagement as in LPS-stimulated macrophages (11), indicating a critical role for NF-κB in the transcriptional activation. Mutations at the κB1 site moderately reduced activation, but mutations at the κB3 site, whose sequence is not conserved between mouse and human, did not affect activation.

In addition to transcriptional activation, post-transcriptional activation mediated via the 165-nt element in the 3′-UTR of IκB-ζ mRNA is critically involved in IκB-ζ induction in response to LPS or IL-1β (11, 12). Post-transcriptional activation was quantitatively analyzed using SV40 promoter-driven reporters that expressed a fusion mRNA for luciferase followed by IκB-ζ mRNA (Fig. 3) (12). Addition of the IκB-ζ mRNA sequence to luciferase mRNA resulted in suppressed expression of luciferase activity in unstimulated A20 cells (Fig. 3B, Full) but
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was stimulated upon BCR engagement with F(ab′)2 of anti-immunoglobulin (Fig. 3C). These effects were not observed when the IκB-ζ sequence was placed in the reverse orientation (Fig. 3A–C, Reverse) or when the IκB-ζ sequence was inserted downstream of the poly A signal so that the inserted sequence was not transcribed (Fig. 3A–C, After pA). These results indicate that the IκB-ζ mRNA sequence is acting as an orientation-specific cis-element in mRNA, rather than an enhancer element. The fusion mRNA harboring only 3′-UTR or the 165-nt element of IκB-ζ mRNA also exhibited similar expression (Fig. 3D and E), indicating that the 165-nt element in the 3′-UTR acts as a critical cis-element for the post-transcriptional regulation in BCR-mediated induction of IκB-ζ. A 168-nt fragment of chicken IκB-ζ mRNA, homologous to the mouse 165-nt element, also acted as a cis-element for post-transcriptional activation upon BCR stimulation of the chicken B-cell line DT40 (data not shown), indicating a conserved mechanism across the species.

Post-transcriptional regulation is mainly controlled by mRNA stability and translational efficiency. To evaluate mRNA stability of target mRNAs without possible cytotoxic effects of general transcriptional inhibitors such as actinomycin D, we established an A20 cell line constitutively expressing the tetracycline-controlled transcriptional transactivator (A20-Tet-off). A20-Tet-off cells were transfected with a plasmid containing the fusion luciferase mRNA with or without the 3′-UTR of IκB-ζ under the control of a tetracycline-responsive promoter. Following addition of doxycycline that specifically turned off transcription of target mRNAs, mRNA decay was quantitatively analyzed. Luciferase mRNA slowly decayed and its stability did not change upon BCR engagement with F(ab′)2 (Fig. 4A). The fusion luciferase mRNA harboring the 3′-UTR of IκB-ζ exhibited lower stability but was stabilized upon BCR stimulation to the level of the control luciferase mRNA without the element (Fig. 4B).

Transcriptional and post-transcriptional activation of IκB-ζ is independently regulated

We next examined whether transcriptional and post-transcriptional activation of IκB-ζ was coordinated upon stimulation. MG-132, a proteasome inhibitor, dose-dependently suppressed BCR-mediated activation of the ELAM1 reporter, indicating
inhibition of NF-κB (Fig. 5A). Similarly, transcriptional activation of the IκB-ζ promoter was also suppressed by the inhibitor. In contrast to transcriptional activation, post-transcriptional activation of IκB-ζ was not inhibited, but rather enhanced by MG-132 treatment. Similar results were obtained with another proteasome inhibitor, lactacystin. NF-κB activation by stimulation of TLR4 with LPS in macrophages was also inhibited by either some inhibitor, lactacystin. NF-κB, which is induced by TLR4 activation, was shown to be inhibited by lactacystin treatment. Similar results were obtained with another proteasome inhibitor, lactacystin (Fig. 5A).}

Co-engagement of FcγRIIB and BCR suppresses transcriptional and post-transcriptional activation of IκB-ζ

Because co-engagement of FcγRIIB with the BCR inhibited IκB-ζ mRNA induction as shown in Fig. 1, we analyzed the effect of co-engagement of FcγRIIB on the transcriptional and post-transcriptional activation of IκB-ζ. BCR-mediated activation of the ELAM1 promoter in F(ab′)2-stimulated cells was significantly inhibited upon co-engagement of FcγRIIB and the BCR by intact IgG of anti-immunoglobulin, demonstrating efficient NF-κB suppression (Fig. 6A). FcγRIIB co-engagement similarly inhibited activation of the IκB-ζ promoter as expected. Likewise, stabilization of mRNA harboring the 3’-UTR of IκB-ζ mRNA upon BCR stimulation was not observed using intact IgG, indicating suppression by the co-engagement of FcγRIIB and the BCR (Fig. 6B). Thus, stimulation with F(ab′)2 of anti-immunoglobulin, but not intact IgG, specifically up-regulated activity of the reporter harboring the 165-nucleotide element (Fig. 6C and D). Following pretreatment with the anti-FcγRIIB antibody 2.4G2, stimulation with intact IgG elicited post-transcriptional activation, further indicating that suppression was mediated by FcγRIIB.

**BCR and TLR9 or TLR7 synergistically activate IκB-ζ induction**

Because the BCR and TLRs independently elicited transcriptional and post-transcriptional activation of IκB-ζ, we examined IκB-ζ induction following co-stimulation of the two types of receptors. IκB-ζ mRNA was induced by BCR stimulation with the F(ab′)2 fragment of anti-immunoglobulin or by TLR9 stimulation with CpG ODN, and the induction level was not affected by co-stimulation of BCR and TLR9 in A20 cells (Fig. 7A). In contrast to IκB-α, induction of IκB-ζ mRNA was robustly enhanced by co-stimulation of the BCR and TLR9 (Fig. 7B). The synergistic induction of IκB-ζ mRNA was not observed with TLR2/1 stimulation with a lipopeptide (Pam3CSK4; Fig. 7C and D), indicating TLR specificity. Enhanced induction of IκB-ζ, but not IκB-α, by co-stimulation of BCR and TLR9 was also observed in purified splenic B cells (Fig. 7E and F).

Furthermore, we determined whether co-engagement of FcγRIIB inhibited TLR-mediated induction of IκB-ζ. Co-stimulation with the F(ab′)2 fragment and intact IgG of anti-immunoglobulin did not elicit either IκB-α or IκB-ζ mRNA, indicating suppression of BCR-mediated induction (Fig. 7A–F). When cells were co-stimulated with CpG ODN or Pam3CSK4 and intact IgG, neither IκB-α mRNA nor IκB-ζ mRNA induction was suppressed by intact IgG stimulation (Fig. 7A–F). Notably, CpG ODN and intact IgG co-stimulation elicited robust induction of IκB-ζ mRNA to the levels observed for synergistic induction by CpG ODN and the F(ab′)2 fragment. The synergistic induction of IκB-ζ mRNA in A20 cells by the BCR and TLR9 was reflected in the protein expression levels (Fig. 8A and B). The similar induction of IκB-ζ was observed in the FcγRIIB-deficient II.A1.6 cells (Fig. 8C and D). The synergistic induction of IκB-ζ mRNA in splenic B cells was weaker than that observed in A20 cells, but robust induction of IκB-ζ was elicited at the protein level by co-stimulation with CpG DNA and either the F(ab′)2 fragment or intact IgG of anti-immunoglobulin (Fig. 8E and F). TLR7 stimulation by imiquimod also resulted in the similar synergistic induction of IκB-ζ (Fig. 8G and H). Thus, the co-engagement of FcγRIIB specifically inhibits BCR-mediated induction but not TLR-mediated induction of IκB-ζ or the synergistic induction.

We next explored the relationship between BCR- and TLR-mediated induction of IκB-ζ and its suppression by FcγRIIB.
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After screening approximately 300 inhibitors, we focused on inhibitors of PI3K, which is critical in BCR-signaling (21) and is suppressed by FcγRIIB co-engagement. The PI3K inhibitor LY294002 dose-dependently inhibited BCR-mediated induction of IkB-ζ mRNA but not TLR-mediated induction (Fig. 7G and H).

IkB-ζ regulates gene expression in activated B cells

To evaluate the significance of IkB-ζ induction upon B-cell activation, we examined gene expression in B cells from IkB-ζ-deficient mice. Because different B-cell subsets exhibit distinct gene expression profiles (22), we analyzed compositions of B-cell subsets by measuring expression levels of several B-cell markers on splenocytes of IkB-ζ-deficient mice including B220, AA4.1, IgM, IgD, CD21 and CD23 (Supplementary Fig. S1, available at International Immunology Online). Equivalent numbers of B220+ cells were present in splenocytes obtained from wild-type and IkB-ζ-deficient mice (Supplementary Fig. S1A, available at International Immunology Online). Although CD21hiCD23lo marginal zone B cells were slightly increased and AA4.1+ transitional B cells were decreased in splenocytes of IkB-ζ-deficient mice, similar numbers of mature B cells (IgM+ IgD+) or follicular B cells (CD21loCD23hi) were present as the majority of wild-type and IkB-ζ-deficient splenic B cells and their compositions were not altered by purification of B cells. Furthermore, no significant difference was observed in expression levels of surface IgM, FcγRIIB and TLR9 between B220, AA4.1, IgM, IgD, CD21 and CD23 (Supplementary Fig. S1, available at International Immunology Online). Equivalent numbers of B220+ cells were present in splenocytes obtained from wild-type and IkB-ζ-deficient mice (Supplementary Fig. S1A, available at International Immunology Online). Although CD21hiCD23lo marginal zone B cells were slightly increased and AA4.1+ transitional B cells were decreased in splenocytes of IkB-ζ-deficient mice, similar numbers of mature B cells (IgM+ IgD+) or follicular B cells (CD21loCD23hi) were present as the majority of wild-type and IkB-ζ-deficient splenic B cells and their compositions were not altered by purification of B cells. Furthermore, no significant difference was observed in expression levels of surface IgM, FcγRIIB and TLR9 between...
BCR- and TLR-mediated induction of IκB-ζ in B cells

Fig 4. Stabilization of IκB-ζ mRNA upon stimulation of BCR. (A and B) A20 cells stably transfected with pEF-Tet-off and pTRE2-Luc (A) or pTRE2-Luc-Nfkbiz-3′-UTR (B) were treated with 2 μg/ml Dox together with or without 10 μg/ml F(ab′)2 fragment of antimouse IgG. Total RNA was extracted and mRNAs for luciferase and GAPDH were quantitated by real-time RT–PCR. Relative luciferase mRNA expression levels normalized to GAPDH are shown. Data represent the mean ± standard error of duplicate samples of one representative of three independent experiments.

Fig 5. Proteasome inhibitors inhibit NF-κB-mediated transcriptional activation but not post-transcriptional activation of IκB-ζ. A20 cells (A) or RAW264.7 cells (B and C) were transfected with pGL3-basic (None), pELAM1-Luc (ELAM1), pNlkbiz(-1,725)-Luc (IκB-ζ) or pGL4.12-SV40-[luc2CP]-Nlkbiz-165nt (165-nt) together with pEF6/V5-His A or pcDNA3 and selected with blasticidin or G418. Stably transfected clones were established with pELAM1-Luc and pGL4.12-SV40-[luc2CP]-Nlkbiz-165nt transfectants. Cells were pre-treated with the indicated concentrations of MG-132 or lactacystin for 30 min, stimulated with 10 μg/ml F(ab′)2 fragment of antimouse IgG for 3 h (A) or with 100 ng/ml LPS for 4 h (B) or 2 h (C), and luciferase activity was measured. Data represent the mean ± standard error of duplicate samples of one representative of three independent experiments.
of FcγRIIB, CD72 and CD22 was reduced in IκB-ζ-deficient B cells 4 h after TLR9 stimulation (Fig. 9A). Although induction of mRNAs for LIM domain containing protein Ajuba (Jub) (23) and the junction adhesion molecule 2 (Jam2) (24) was impaired in IκB-ζ-deficient B cells 24 h after BCR stimulation, induction of the C-type lectins Clec4d (25) and Clec4e (also known as Mincle) (26) was markedly up-regulated (Fig. 9B). In addition, induction of IL-10 mRNA was abolished in IκB-ζ-deficient B cells. On the other hand, significantly up-regulated TNF-α mRNA expression was observed at 24 h after TLR9 stimulation in B cells lacking IκB-ζ as in TLR-stimulated IκB-ζ-deficient macrophages (7, 8). We also found that mRNA for Ctl4 was synergically induced upon co-stimulation of the BCR and TLR9 in wild-type B cells but not in IκB-ζ-deficient B cells. Consistent with the mRNA expression levels, IL-10 production upon TLR9 stimulation was abolished in IκB-ζ-deficient B cells and TNF-α production was enhanced (Fig. 9C). TNF-α production upon BCR stimulation was unaffected in IκB-ζ-deficient cells. TNF-α expression was further enhanced by co-stimulation with CpG ODN and the F(ab′)2 of anti-immunoglobulin.

We further analyzed activation of IκB-ζ-deficient B cells. Proliferation in response to TLR9 stimulation, but not to BCR stimulation, was considerably impaired in IκB-ζ-deficient B cells (Fig. 10A). Whereas co-engagement of FcγRIIB inhibited BCR-mediated proliferation, FcγRIIB co-engagement did not elicit robust inhibition of proliferation by co-engagement of the BCR and TLR9 in our experimental conditions (Supplementary Fig. S4, available at International Immunology Online), contrasting with a previous report (27). Nevertheless, IκB-ζ-deficient B cells exhibited weaker proliferative responses upon co-stimulation of the three receptors. On the other hand, TLR9-mediated up-regulation of CD86 was enhanced in IκB-ζ-deficient B cells (Fig. 10B) whereas FcγRIIB up-regulation was inhibited (Fig. 10C). BCR-mediated proliferation or up-regulation of CD86 and FcγRIIB was not affected in IκB-ζ-deficient B cells. Although basal IgM production was higher in B cells of IκB-ζ-deficient mice as reported elsewhere (28), BCR stimulation abolished the IgM production as described previously (29). IgM production in response to CpG ODN was not impaired in IκB-ζ-deficient B cells. However, IgM secretion upon co-stimulation of TLR9, BCR and FcγRIIB was abolished in IκB-ζ-deficient B cells (Fig. 10D).

Discussion

IκB-ζ was originally identified in macrophages as a molecule induced by TLR stimulation but is also induced in B cells by BCR or TLR stimulation. The present study demonstrated that BCR engagement induces both NF-κB-mediated transcriptional activation and post-transcriptional activation via the
165-nt element in IκB-ζ mRNA, similar to that of TLR or IL-1β stimulation in macrophages or fibroblasts. mRNA stability analysis indicated that the 165-nt element acts as a cis-element to destabilize mRNA. Upon BCR stimulation, stability of mRNA with the 165-nt element was recovered to levels seen in the absence of the element, indicating relief from destabilization.

The proteasome inhibitors lactacystin and MG-132 inhibited transcriptional activation of the IκB-ζ promoter but not the post-transcriptional activation of IκB-ζ mRNA. Thus, the signaling pathway involved in post-transcriptional activation does not depend on transcriptional activation, which requires NF-κB activation. Rather, both inhibitors enhanced post-transcriptional activation, suggesting a negative effect by degradation of the signaling components.

Although both BCR and TLR stimulation induced IκB-ζ, sensitivity to PI3K inhibitors revealed distinct signaling pathways. PI3K is required for BCR-induced NF-κB activation but not that induced by TLRs (30). Activation of PI3K-Akt signaling causes phosphorylation of the RNA-binding protein KSRP, which stabilizes target mRNAs (31), and KSRP binds to various mRNAs for inflammatory proteins including IκB-ζ (32). Previously we
BCR- and TLR-mediated induction of \( \kappa B-\zeta \) in B cells demonstrated that TLR- or IL-1-induced activation of the post-transcriptional regulation of \( \kappa B-\zeta \) is mediated by IRAK1 and TRAF6 downstream of MyD88 (13). Although downstream mediators may be common to the activation pathways, the receptor-proximal events are different between the BCR and TLRs.

Increased mRNA stability and translational efficiency are two important mechanisms for post-transcriptional regulation. The degree of mRNA stabilization containing the 165-nt element was comparable with the up-regulation of reporter activity with the element. Therefore, mRNA stabilization accounts for most of the post-transcriptional activation of \( \kappa B-\zeta \) upon BCR stimulation, in contrast to IL-1 induction of \( \kappa B-\zeta \), which modulates mRNA stability and translational efficiency via the 165-nt element (11, 12, 33). \( \kappa B-\zeta \) protein expression by TLR9 stimulation was greater than that induced by BCR engagement.

Fig. 8. \( \kappa B-\zeta \) protein induction by engagement of BCR, TLR and FcγRIIB. A20 cells (A and B), IIA1.6 cells (C and D) or purified splenic B cells (E–H) were stimulated with 20 \( \mu \)g/ml F(ab')2 fragment, 40 \( \mu \)g/ml intact IgG of antimouse immunoglobulin, 1 \( \mu \)M CpG ODN (A–D), 30nM CpG ODN (E and F; +), 100nM CpG ODN (E and F; ++), or 0.3 \( \mu \)g/ml imiquimod (G and H; imiq, +) or 3 \( \mu \)g/ml imiquimod (G and H; imiq, ++) alone or in combination for 1h. The cells were lysed and analyzed by immunoblotting with anti-\( \kappa B-\zeta \) or anti-\( \beta \)-actin antibody (A, C, E and G). Data are a representative of three independent experiments. (B, D, F and H) Relative expression levels of \( \kappa B-\zeta \) protein normalized to \( \beta \)-actin were shown. Data represent the mean ± standard error of three independent experiments. *, \( P < 0.05 \); **, \( P < 0.01 \).
Although both elicited comparable levels of mRNA induction, consistent with the notion that translation of IkB-ζ mRNA was specifically up-regulated by TLR9 stimulation.

Although induction of IkB-ζ by BCR stimulation alone was weak, co-stimulation of the BCR and TLR9 synergistically elicited robust IkB-ζ induction in A20 cells and splenic B cells. Co-stimulation of the BCR and TLR9 can synergistically activate NF-κB and enhance proliferation and antibody production (34, 35). The synergy of IkB-ζ mRNA induction by TLR9 and BCR stimulation was less prominent in splenic B cells compared with that in A20 cells. The reason for this quantitative difference is currently unknown, but as A20 cells express surface IgG, the signaling pathways of naive B cells expressing surface IgM may be modulated following initial stimulation, leading to B-cell differentiation including immunoglobulin class switching. Nevertheless, remarkable synergistic induction of IkB-ζ protein was observed in both A20 and splenic B cells. This observation suggests functional significance of the synergistic induction, which is
BCR- and TLR-mediated induction of IκB-ζ in B cells

Fig. 10. Activation of IκB-ζ-deficient B cells. (A) Proliferation of wild-type (WT) and IκB-ζ-deficient (KO) splenic B cells. Purified splenic B cells were labeled with CFSE and stimulated with 20 μg/ml F(ab′)2 fragment of antimouse IgM, 40 μg/ml antimouse IgM or 100 nM CpG ODN alone or in combination for 48 h. The cells were then subjected to flow cytometric analysis. Percentages of proliferating B cells (indicated by horizontal bars) were assessed by CFSE dilution. (B and C) CD86 and FcγRII expression in WT and KO splenic B cells. Purified splenic B cells were stimulated as in (A) for 24 h. The cells were stained with anti-CD86 (B) or anti-FcγRII antibody (C) and subjected to flow cytometric analysis. Mean fluorescence intensities of anti-CD86 or anti-FcγRII staining are shown. Flow cytometric data are representative of three independent experiments. Quantitative data represent the mean ± standard error of three independent experiments. (D) IgM production of IκB-ζ-deficient B cells. Purified splenic B cells were stimulated as in (A) for 7 days. IgM concentrations in the culture supernatants were measured by ELISA. Data represent the mean ± standard error (n = 3). *, P < 0.05; **, P < 0.01.

evident in IκB-ζ-dependent Ctf4 induction in splenic B cells. The quantitative difference at the mRNA and protein levels of the synergistic induction of IκB-ζ indicate that mRNA translation efficiency may be up-regulated by co-stimulation of the BCR and TLR9 in splenic B cells. Because synergistic induction of IκB-α mRNA was not prominent, post-transcriptional regulation of IκB-ζ is likely to be strongly activated by co-stimulation of the BCR and TLR9. Synergy was also observed by TLR7 stimulation but not by TLR2/1 stimulation, indicating the TLR specificity. As TLR7 and TLR9 are localized in the endosome, modulation of subcellular localization of TLRs by the BCR may underlie the specificity of the synergy (36).
In addition to NF-κB-mediated transcriptional activation, BCR-mediated post-transcriptional activation of IkB-ζ was suppressed by co-engagement of FcyRIIB. Co-igation of the BCR and FcyRIIB recruits the inositol phosphatase Src homology-2 domain-containing inositol polyphosphate 5'-phosphatase (SHIP) to immunoreceptor tyrosine-based inhibitory motif (ITIM) in the cytoplasmic region of FcyRIIB (20, 37). FcyRIIB signals suppressed NF-κB-mediated transcriptional activation and BCR-mediated mRNA stabilization of IkB-ζ. This finding is the first example of FcyRIIB-mediated suppression of a post-transcriptional mechanism.

Suppression by FcyRIIB was specific to BCR induction of IkB-ζ but not TLR. This is consistent with the SHIP-mediated suppressive effects of FcyRIIB observed for PI3K-dependent BCR activation but not for TLR activation, which does not require PI3K activity. Because FcyRIIB deficiency is associated with onset of systemic lupus erythematosus, FcyRIIB-mediated suppression of IkB-ζ expression may prevent the development of autoimmune diseases.

Synergistic induction by the BCR and TLR9 and suppression by FcyRIIB indicated that IkB-ζ induction is tightly regulated by interactions among these three receptors. Signals generated by microbial stimulation of TLRs and specific antigen recognition by the BCR upon infection are integrated and modulate B-cell functions (1). Therefore, the synergistic induction of IkB-ζ is noteworthy in both physiological and pathological contexts. Co-engagement of the BCR and TLR9 or TLR7 in response to chromatin-containing immune complexes is implicated in autoimmune B-cell activation in systemic autoimmune diseases (38, 39). Interestingly, suppression by FcyRIIB was not observed in the synergistic induction of IkB-ζ by BCR and TLR9 or TLR7, suggesting strongly that the effect of the BCR in the synergistic induction does not require PI3K-dependent transcriptional or post-transcriptional activation. This may be the molecular basis of inability of FcyRIIB to suppress detrimental activation by chromatin-containing immune complexes.

Our analysis on gene expression and activation of IkB-ζ-deficient B cells revealed that IkB-ζ plays essential roles in gene induction during B-cell activation. Because NF-κB activation does not appear to be impaired in IkB-ζ-deficient B cells, chromatin remodeling may be involved in IkB-ζ-mediated gene regulation as previously reported in macrophages (9, 40). IkB-ζ is required for TLR9-mediated, but not BCR-mediated, proliferation of B cells, consistent with our previous report (7). On the other hand, IkB-ζ-deficient B cells exhibited enhanced CD86 expression and reduced FcyRIIB in response to TLR9 stimulation. Upon BCR stimulation, up-regulation of CD86 and FcyRIIB expression was not affected in IkB-ζ-deficient B cells, indicating critical roles for IkB-ζ in TLR-mediated signaling. These observations highlight mechanistic differences between TLR- and BCR-mediated responses in B cells and suggest that another factor(s) is induced upon BCR stimulation, which compensates for the function of IkB-ζ.

Although further studies are required to elucidate the molecular mechanisms of IkB-ζ-mediated gene expression and its precise role in B cells, its role in the induction of cytokines such as IL-10 and TNF-α suggests it may be involved in the regulation of B-cell functions, such as regulatory B cells (22). Such studies using IkB-ζ-deficient mice are currently ongoing in our laboratory. Investigations on the role of IkB-ζ in B cells will provide new insights into the pathogenesis of autoimmune diseases and hopefully provide novel targets for the development of new diagnostics and therapeutics.

Supplementary data

Supplementary data are available at International Immunology Online.

Funding

Grant-in-Aid for Scientific Research on Innovative Areas [21117004] and Grant-in-Aid for Scientific Research on Priority Area [20060002, 20012005] from the Ministry of Education, Culture, Sports, Science and Technology of Japan to T.M.; Grant-in-Aid for Scientific Research [21390088, 24390076] from the Japan Society for the Promotion of Science to T.M.; Grant-in-Aid for JSPS Fellows [10J06513] to F.H.; and grants to T.M. from Takeda Science Foundation; the Naito Foundation; the Asahi Grass Foundation; Suzuken Memorial Foundation; the Novartis Foundation for the Promotion of Science; the Japan Foundation for Applied Enzymology.

Acknowledgements

We are grateful to Takashi Maruyama for discussion and to Tomoki Sasaki and Tomoyuki Kawasaki for technical assistance. We also thank the Screening Committee of Anticancer Drugs supported by a Grant-in-Aid for Scientific Research on Priority Area ‘Cancer’ from The Ministry of Education, Culture, Sports, Science and Technology, Japan, for providing the SCADS inhibitor kit.

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