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<td>Shao, Y; Kim, SY; Shin, D; Kim, MS; Suh, HW; Piao, ZH; Jeong, M; Lee, SH; Yoon, SR; Lim, BH; Kim, WH; Ahn, JK; Choi, I</td>
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TXNIP regulates germinal center generation by suppressing BCL-6 expression

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A B S T R A C T
The detailed mechanism driving the germinal center (GC) reaction to B cell lymphomagenesis has not been clarified. Thioredoxin interacting protein (TXNIP), also known as vitamin D3 up-regulated protein 1 which is an important tumor repressor, is involved in stress responses, redox regulation, and cellular proliferation. Here, we report that TXNIP has a potential role in the formation of GC in peripheral lymphoid organs where B lymphocytes divide rapidly. First, we compared changes in GC from wild type mice and Txnip−/−mice. After immunization, Txnip−/−mice exhibited higher expression level of BCL-6 and larger percentage of GC B cells with the reduction in antibody production and plasma cell numbers. In addition, Txnip−/−mice had a much larger population which expressed Ki-67, a marker of cell proliferation, in the red pulp border than WT spleens. Furthermore, the expression of BCL-6 was decreased in TXNIP overexpressing cells and elevated in TXNIP deficient cells. Taken together, we conclude that TXNIP may contribute to the formation of GCs after immunization. During this process, TXNIP suppresses BCL-6 expression.

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1. Introduction
Thioredoxin interacting protein (TXNIP; also known as VDUP1) was first identified in 1α,25-dihydroxvitamin D3-treated HL-60 cells, a human leukemia cell line [1]. TXNIP not only induces oxidative stress via interacting with thioredoxin [2–5], but also regulates cellular proliferation and the aging process. As a transcriptional repressor, TXNIP physically interacts with other corepressors including promyelocytic leukemia zinc finger protein, Fanconi anemia zinc finger protein and histone deacetylase 1 [6]. The expression of TXNIP is frequently lost in tumor cell lines and tissues, whereas the ectopic expression of TXNIP suppresses cellular proliferation along with cell-cycle arrest at the G1 phase by inhibiting JAB1 [6,7].

TXNIP plays an important role in the growth regulation of human T lymphocyte virus I (HTLV-I)-infected T cells. A significant lack of functional NK cells was found in Txnip−/−mice [8]. Dendritic cells (DCs) derived from Txnip−/−mice are defective in activating T cells [9]. TXNIP expression has been observed at different stages of B cell development and in many cell lines [10].

BCL-6 is a 95-kDa nuclear phosphoprotein that has a BTB/POZ zinc finger DNA binding motif. Although BCL-6 is transcribed in various cell types, its expression is mainly found in lymphocytes, and it was abundantly expressed in germinal center (GC) B cells and neoplastic B cells of GC origin [11–14]. BCL-6 deficient mice failed to form GCs during T cell-dependent immune responses and thus displayed a fatal inflammatory disease [15,16]. GCs are the sites of antigen-stimulated B cells proliferation and differentiation that is aided by follicular dendritic cells (FDCs) and follicular helper T cells (Tfh) [17–19]. GCs are crucial for the development of B cell responses including proliferation, apoptosis, somatic hypermutation (SHM), selection for high-affinity maturation, class-switch recombination (CSR), and differentiating into plasma cells or memory cells [18,20,21]. BCL-6 affects GC development via three mechanisms. First, BCL-6 induces the GC to undergo SHM and CSR by suppressing the activation of the apoptotic and cell-cycle arresting genes (p21, STAT2 and TP53) [22]. Second, BCL-6 represses genes expression (CD69, STAT1, and CD80) which are involved in

Abbreviations: CSR, class-switch recombination; DNP, 2,4-dinitrophenyl; FDC, follicular dendritic cell; GC, germinal center; KLH, keyhole limpet hemocyanin; MC, mantle zone; PNA, lectin peanut agglutinin; SHM, somatic hypermutation; TD, T cell-dependent; TI, T cell-independent.

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premature B cell activation by T cells or other signals prior to GC formation [23]. Third, BCL-6 represses BLIMP1 to inhibit B cell differentiation [24].

Here, we present evidence that TXNIP regulates GC reactions by targeting BCL-6. TXNIP inhibited the expression of BCL-6, and TXNIP deficiency resulted in an increased immune response, especially in relation to GC reaction in mice, due to the increased expression of BCL-6.

2. Materials and methods

2.1. Mice and cell lines

Txnip−/− mice were generated as previously described [8]. All mice were maintained under specific pathogen-free conditions with standard temperature and lighting. Mice were given food and water ad libitum. All studies were approved by the institutional review board (KRRIBB Institutional Animal Care and Use Committee/KRRIBB-IACUC) and all procedures were performed in accordance with institutional guidelines for animal care. Human embryonic kidney-derived 293T cells were maintained in DMEM (Invitrogen) supplemented with 10% FBS (HyClone Laboratories) and antibiotics. The human Burkitt’s lymphoma cell lines (GC-derived B cell line) Raji and Ramos were maintained in RPMI (Invitrogen) supplemented with 10% FBS and antibiotics. All cell lines were obtained from the American Type Culture Collection.

2.2. Immunization

For induction of GC response, age-matched (6–8-week-old) WT and Txnip−/− male mice were immunized intraperitoneally with 100 μg KLH (Calbiochem, 100 μg/mice) precipitated in alum and analyzed as described [25]. Briefly, spleen was isolated from WT and Txnip−/− male mice at 10th day after immunization with KLH. For a kinetic analysis of GC B cells from WT and Txnip−/− spleen, GC B cells were examined by FACS at 0 day, 4 days, and 10 days after immunization. For induction of secondary response, age-matched (6–8-week-old) WT and Txnip−/− male mice were immunized intraperitoneally with DNP–Ficoll (25 μg/mice) in alum to elicit Tl immune response or DNP–KLH (100 μg/mice) in alum to elicit TD immune response.

2.3. Immunohistochemistry

The spleen and lymph nodes were fixed in 4% formaldehyde for 24 h, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. For immunostaining of tissue sections, the sections were stained with primary antibodies. The following primary antibodies were used for labeling mouse tissue sections: anti-PNA antibody (Vector Laboratories); anti-BCL-6 antibody (Santa Cruz Biotechnology); anti-CD3ε antibody and anti-F4/80 antibody (Abcam); anti-Mac-3 antibody, anti-CD11b antibody, anti-B220 antibody, anti-FDCM1 antibody (BD PharMingen), and anti-Ki-67 antibody (Dako). Negative controls were treated identically but the primary antibodies omitted.

2.4. Plasmid construction

To generate the luciferase constructs for the human BCL-6 promoter pBCL-6-Luc (p135–1288), a 1154 bp fragment spanning the 3' end of the human BCL-6 exon 1 and the 5’ region of intron 1 (nucleotides +135 to +1288) was isolated from human lung DNA and cloned into the KpnI/HindIII sites of the pGL3-Basic luciferase reporter plasmid (Promega). Additional deletion mutants (p265–, p346–, p436–, p696– and p703–1288) of the human BCL-6 promoter were constructed from pBCL-6-Luc (p135–1288) by PCR.

2.5. ELISA

Age-matched (6–8-week-old) WT mice and Txnip−/− mice were immunized intraperitoneally with DNP–Ficoll (25 μg/mice; Biosearch Technologies,) for TI immune response or DNP–KLH (100 μg/mice; Biosearch Technologies) for TD immune response. For primary response, sera were collected 10 days after primary immunization. For secondary response, these two groups of mice were boosted intraperitoneally with their original antigen 14 days after second immunization, and sera were collected 30 days later. Sera were then serially diluted, and added to 96-well ELISA plates precoated with 50 μl DNP–BSA (10 μg/ml; Calbiochem) per well. HRP-conjugated mouse IgM, IgA, and subclass IgG-specific antibodies (Pierce) were used to detect the different antibody isotypes and subclasses.

2.6. Luciferase reporter assay

Ramos cells were co-transfected with pBCL-6-Luc, its truncated reporter plasmid, or TXNIP expression plasmid by microelectroporator (Digital Bio Technology). A Renilla luciferase control vector (Promega) was used to monitor the transfection efficiency.

2.7. Western blot analysis

Whole cell lysates of 293T cells co-transfected with plasmid encoding HA-BCL-6 and HA-TXNIP were analyzed by western blot using anti-BCL-6 antibody (Santa Cruz Biotechnology), anti-TXNIP antibody (MBL), and anti-β-actin antibody (Sigma). For μM and μS blots, whole cell lysates from total splenocytes were subject to western blot with anti-IgM antibody (Sigma).

2.8. B cells proliferation assay

Splenocytes were harvested from WT and Txnip−/− mice. B cells were enriched by negative selection using a B Cell Isolation Kit (Miltenyi Biotec). A total of 2 × 10⁶ purified B cells were cultured in triplicate wells for the indicated times in B cell media (RPMI containing 100 U/ml penicillin, 100 mg/ml streptomycin, 50 mg/ml gentamicin, 2 mM l-glutamine, 1 mM sodium pyruvate, 50 mM 2- ME, and 20% FBS) and were stimulated with 10 μg/ml LPS (Sigma) at 37 °C in a 5% CO₂ incubator. The proliferation rate was then monitored with Cell Counting Kit-8 (CCK-8) by utilizing WST-8 (Dojindo) as described previously [26]. In principle, as cultured cell proliferates, there is an increase in activity of mitochondrial dehydrogenases, specially the succinate-tetraozolium reductase system. WST-8 is reduced by dehydrogenases in cells to give a yellow colored product (formazan). The amount of the formazan dye generated by the activity of dehydrogenases in cells is directly proportional to the number of living cells.

2.9. Flow cytometry and cell sorting

Purified B cells were collected by using a B Cell Isolation Kit (Miltenyi Biotec). GC B cells (B220⁺ PNA⁺) and none GC B cells (B220⁺ PNA⁻) were isolated from purified B cells using a FACSAria (BD Biosciences) with a purity of at least 98%. The following antibodies were used for labeling cells and were purchased from BD PharMingen or eBioscience except anti-PNA-FITC (Vector Laboratories); anti-CD4-FITC; anti-IgM-FITC; anti-IgG1-FITC; anti-CD38-FITC; anti-CD5-FITC; anti-CD11b-FITC; anti-CD8-PE; anti-F4/80-PE; anti-MHCII-PE; anti-CD138-PE; anti-strep-avidin-FITC.

Fig. 1. The effects of TXNIP on GC formation are dependent on BCL-6 expression. Immunohistochemical analysis of PNA (A) and BCL-6 (B) in spleen and lymph node sections from WT and Txnip−/− mice immunized with KLH. All immunohistochemical data through serial sectioning are representative of three WT and three Txnip−/− mice. (C) Quantitative analysis of PNA or BCL-6 staining from WT or Txnip−/− mice. Quantitative analysis of PNA or BCL-6 was based on the five randomly highest density areas from spleen and LN of WT or Txnip−/− mice. Data are shown as the mean ± SD, and the statistical significance was calculated from the Student’s t test between WT and Txnip−/− samples. (D) After WT and Txnip−/− mice were immunized with KLH, total RNA was extracted from sorted splenic GC B cells. The expression of Txnip and BCL-6 mRNA in GC B cells was examined by RT-PCR (top). The expression of Txnip and BCL-6 in nuclear extracts of splenic GC B cells was also examined by western blot (bottom). (E) Flow cytometric analysis of splenic B cells from WT and Txnip−/− mice immunized with KLH. Cells were labeled with anti-B220-PE-Cy7 and anti-PNA-FITC. The numbers indicate the percentage of B220+ PNA+ GC B cells. Data shown are representative dot plots of three independent experiments. Absolute numbers of GC B cells were determined before and after immunization. The results are presented as the mean ± SD of n = 3–5 mice per genotype. **P ≤ 0.01.
2.10. q RT-PCR analysis

Total RNA was extracted from cells using TRizol (GibcoBRL). After reverse transcription, PCR reactions were performed with the following primers (5′ − 3′) were used for PCR:

<table>
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<th>Mouse genes</th>
<th>Forward primer (5′ − 3′)</th>
<th>Reverse primer (5′ − 3′)</th>
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<td>BCL-6</td>
<td>CTCGTGAATCGTGGGACTC</td>
<td>ACCAGGTTATTCACCTTG</td>
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<tr>
<td>Blimp1</td>
<td>TGGCATGTGGGACCTTG</td>
<td>TGGGACACCTCTTTGCTAG</td>
</tr>
<tr>
<td>TXNIP</td>
<td>TGCGAGCTGAGAGACCTC</td>
<td>TGCCCTAATACAGATGTCTTCATTTC</td>
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<tr>
<td>IgG1</td>
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<td>GATCCAGATTCAGGTCACC</td>
</tr>
<tr>
<td>IgG3</td>
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<tr>
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</tr>
<tr>
<td>β-Actin</td>
<td>AGCAGAGGGTGGGGTGG</td>
<td>TCATGAGGGTGGGGTCC</td>
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<tr>
<td>Blimp1</td>
<td>TGGCATGTGGGACCTTG</td>
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<tr>
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<tr>
<td>β-Actin</td>
<td>AGCAGAGGGTGGGGTGG</td>
<td>TCATGAGGGTGGGGTCC</td>
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3. Results

3.1. The effects of TXNIP on GC formation

To examine the impact of TXNIP on the maturation and activation of GC B cells, age-matched WT and Txnip−/− mice were immunized with TD antigens KLH. Immunohistochemical analysis for lectin peanut agglutinin (PNA) [27] demonstrated that the spleen and lymph nodes from Txnip−/− mice had a larger secondary follicle with GC structure than WT mice did (Fig. 1A). The expression of BCL-6, mainly found in GC B cells, was also elevated in the spleen and lymph nodes of Txnip−/− mice (Fig. 1B). There was no positive strain in negative controls (Fig. S1) and the difference of PNA and BCL-6 expression between WT and Txnip−/− mice was statistically significant (Fig. 1C). Txnip−/− GC B cells exhibited much stronger BCL-6 gene expression than WT GC B cells (Fig. 1D). In line with these observations, the percentage of GC B cells (B220+ PNA+ cells) was substantially higher in immunized Txnip−/− mice than that in immunized WT mice (Fig. 1E). During GCs formation, at the early stage (4 days after immunization), there is no difference between WT GC B cells and Txnip−/− GC B cells. However, at the mature stage (10 days after immunization), the population of GC B cells in Txnip−/− mice was much more than that in WT mice (Fig S2).

In addition, immunohistochemical analysis of spleen sections from Txnip−/− mice showed the normal proportions of Mac-3+ cells, T cells, but the increased proportions of CD11b+ cells and FDCs (FDCM1+)

(Fig. S3A). When we analyzed the population of T cells, macrophages, and DCs in spleen by flow cytometry, there were no significant differences except for macrophages between WT and Txnip−/− mice (Fig. S3B). We next examined B cell development in Txnip−/− mice. TXNIP did not affect B cell maturation (Fig. S4). Overall, these finding suggests that TXNIP is involved in GC formation.

3.2. The effects of TXNIP on B cell proliferation

Ki-67, which is a marker of cell proliferation, is preferentially expressed during all active phases of the cell cycle (G1, S, G2, and M) of B cells. Ki-67 expression was detected in the spleen and lymph nodes of Txnip−/− mice (Fig. 2A). TUNEL analysis for detection of apoptosis in the spleen and lymph nodes of Txnip−/− mice was performed with anti-Ki-67 antibody and TUNEL staining (Fig. 2B). The proliferation of splenic B cells from WT and Txnip−/− mice was measured 10 days after immunization with PBS or KLH in alum using the CCK-8 Kit (Fig. 2C). The proliferation of splenic B cells from WT and Txnip−/− mice was measured 10 days after immunization with PBS or KLH in alum using the CCK-8 Kit. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
M phase), but not in resting cells (G0 phase) [28]. Txnip−/− spleens had a much larger population of Ki-67-positive cells in the red pulp border than WT spleens (Fig. 2A), indicating some Ki-67-positive cells in the red pulp proliferate actively on GC microenvironment. Meanwhile, TUNEL analysis showed that the number of apoptotic cells was similar in Txnip−/− and WT spleens (Fig. 2B). In CCK-8 assay, the proliferation of splenic B cells from Txnip−/− mice was also elevated after immunized with KLH (Fig. 2C). Together, the data indicate that the larger and more numerous GC in Txnip−/− spleen mainly due to the fast proliferation of activated B cells, not intrinsically apoptotic effect of B cells.

### 3.3. Role of TXNIP in plasma cell development

We further identified GC-derived plasma cells and memory B cells. Ten days after immunized with KLH, WT mice showed an increased percentage of CD138+ B220− early short-lived plasma cells (Fig. 3A). When mouse splenic B cells were stimulated with LPS, they proliferated and differentiated into Ig-secreting cells in vitro [29]. After KLH-immunized splenocytes were stimulated with LPS for 4 days, the number of CD138+ B220− plasma cells increased in WT cultures is much stronger than that in Txnip−/− cultures (Fig. 3A). The level of IgM protein in Txnip−/− splenocytes was analyzed by western blot. Txnip−/− splenocytes expressed less µM and less µS compared with WT splenocytes (Fig. 3B, left). When the expression of heavy chain C-region genes from WT and Txnip−/− spleen B cells was analyzed by RT-PCR, the production of class-switched germline IgG1 (γ1) and IgG3 (γ3) transcripts was reduced in Txnip−/− B cells (Fig. 3B, right). Furthermore, there were consistently less circulating DNP-specific IgM, IgG1, IgA, and κ light chain antibodies in the serum of Txnip−/− mice after immunization with DNP-KLH or DNP-Ficoll (Fig. 3C). IgG3 showed the biggest change between the response of mice immunized with KLH and those immunized with Ficoll. In addition, light chain isotypes were significantly reduced.

### 3.4. TXNIP suppresses BCL-6 expression

To clarify whether TXNIP regulates GC response via BCL-6, we next determined the effects of TXNIP on BCL-6 expression. The transfection of cells with a TXNIP expression plasmid resulted in the dose-dependent reduction in BCL-6 expression and a concurrent increase in BLIMP1 expression (Fig. 4A). Transfecting Raji cells with a TXNIP expression plasmid also resulted in a dose-dependent reduction in BCL-6 expression (Fig. 4B). Next, the effect of TXNIP on the activity of the human BCL-6 promoter was tested. It was found that TXNIP repressed BCL-6 promoter activity in a dose-dependent manner (Fig. 4C).

### 4. Discussion

TXNIP mRNA was induced in a cell density-dependent fashion concomitantly with the G0/G1 arrest [6]. The hyperproliferation of Txnip−/− GC B cells indicated that TXNIP is important in regulating GC B cell division. In addition, Txnip−/− GC B cells showed the increased BCL-6 gene expression. So far, the exact mechanisms that regulate BCL-6 expression during GC formation are not yet fully understood [14,30]. These reports made us investigate whether TXNIP could directly regulate BCL-6 expression and affect GC formation by modulating immune responses in GCs.

![Fig. 3. B cell differentiation and immune responses in Txnip−/− mice.](image-url)
First, we found that Txnip−/− mice have more GCs in spleen and LN sections after immunization with KLH. Txnip−/− spleens had more CD11b positive cells – macrophages (CD11b+/F4/80+) and FDCM1 positive cells (FDCs). A large population of macrophages that usually highly proliferate in red pulp are thought to be important to regulate antigen trafficking into the red and white pulp spaces [31]. Antigen trapped in the form of immune complexes is localized on FDCs resident within the follicle and, together with T follicular helper (Tfh) cells, drives rapid B cell proliferation to form GCs. Increased proportions of macrophages (CD11b positive cells) in spleen from Txnip−/− mice and a much larger population of Ki-67-positive cells in the red pulp border of Txnip−/− spleens suggest that macrophages act as Ki-67-positive cells proliferating massively in the red pulp to regulate GC microenvironment. Increased FDCs in Txnip−/− spleens could also explain the expansion of GC in Txnip−/− mice.

BCL-6 induces SHM in the dark zone of the GC. IRF4 controls plasma cell differentiation and CSR in the light zone of the GC. AID is critical in both processes, and XBP1 and BLIMP1 are involved in plasma cell differentiation [23]. After immunization, the expression level of BCL-6 in Txnip−/− splenic GC B cells was elevated, further indicating that TXNIP represses BCL-6 expression in the GC region. The reduction in BLIMP1 expression in Txnip−/− splenic GC B cells demonstrates the influence of TXNIP in plasma cell differentiation. Activated B cells undergo rounds of mutation and selection for higher affinity mutants in the GC, resulting in high-affinity antibody-secreting plasma cells and high-affinity memory B cells. Previous studies have suggested that the down-regulation of BCL-6 controls human plasma cell differentiation [32]. The reduction in the number of CD138+ B220− plasma cells in Txnip−/− mice suggests that TXNIP induces plasma cell differentiation. In Txnip−/− mice, the class-switch induction was partially impaired, resulting in lower level of DNP-specific IgM, IgG1, IgA, and α light chain antibodies in the serum (Fig. 3C). Therefore, the repression of BCL-6 by TXNIP would be beneficial for maintaining plasma cell differentiation.

To understand how TXNIP regulates BCL-6 expression during GC reaction in detail, more study about molecular mechanism of suppressing BCL-6 expression by TXNIP is required. The mouse BCL-6 promoter has previously been partially characterized and compared with human BCL-6 promoter [33]. A GC-rich 200-nt region upstream of the major transcription start site was shown to contain BCL-6 promoter activity in both human and mouse B cells. Specially, one Rel/NF-κB (from 172 to 178) and two STAT binding sites (from 216 to 239 and from 246 to 259) are within this region of both human and mouse BCL-6 promoter. Considering the Rel/NF-κB sites are functionally important in BCL-6 promoter assays, the relative importance of each of Rel/NF-κB in controlling BCL-6 expression in B cells in vivo needs to be determined.

JunD/AP-1 also is reported as crucial molecules inducing high BCL-6 expression in mouse GC B cells [33]. AP-1 DNA binding activity was inhibited in Txnip−/− fibroblast cells [7], suggesting that TXNIP may repress BCL-6 expression via interacting with AP-1 in GC B cells.

In conclusion, we found that TXNIP controls GC formation by inhibiting BCL-6 expression. Based on our current understanding of the role of BCL-6 in the programming of GCs and in the pathogenesis of B cell lymphomas, an incisive insight of the cross-talk between TXNIP and BCL-6 will contribute to overall understanding of the unique physiology of GC B cells and diffuse large B cell lymphogenesis.

Conflict of interest

The authors have declared that no conflict of interest exists.

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Appendix A. Supplementary data


References


