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TXNIP regulates germinal center generation by suppressing BCL-6 expression

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ABSTRACT

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*$^{-/-}$ spleens 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.

1. Introduction

Thioredoxin interacting protein (TXNIP; also known as VDUP1) was first identified in 1α,25-dihydroxyvitamin 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 B helper T cells (T_{FH}s) [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 (KRIBB Institutional Animal Care and Use Committee/KRIBB-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 TI 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–, 436–, 436–, 696– and 703–1288) 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 2×10^5 purified B cells were cultured in triplicate wells for the indicated times in B cell media (RPMI contains 100 U/ml penicillin, 100 mg/ml streptomycin, 50 mg/ml gentamycin, 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% CO_2 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-tetrazolium 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 94%. 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-CD11c-PE; anti-IgD-PE; anti-B220-PE-Cy7; anti-CD3ε-PE-Cy5; and anti-strep-avidin-FITC.