TGF-β-induced IκB-ζ controls *Foxp3* gene expression

Takashi MaruYama [a, b, *]

[a] Laboratory of Cell Recognition and Response, Graduate School of Life Sciences, Tohoku University, Sendai, Miyagi 980-8578, Japan
[b] School of Medicine, Gifu University, Gifu 501-1194, Japan

ARTICLE INFO

ABSTRACT

Inhibitor of kappa B (IκB)-ζ, a member of the nuclear IκB family of proteins, is induced by the transforming growth factor (TGF)-β signaling pathway and plays a pivotal role in maintaining the balance of T helper (Th) cell subsets. IκB-ζ deficiency results in reduced percentages of Th17 cells and increased percentages of Th1 cells. In this study, the effects of IκB-ζ deficiency on T-cell subsets were examined further. The data showed that IκB-ζ-deficient T cells had a high capacity for generation of regulatory T cells (Tregs) when T cells were cultured under TGF-β stimulation in the presence of cytokine-neutralizing antibodies. Mechanistically, IκB-ζ itself negatively regulated activation of the *Foxp3* promoter in a nuclear factor of kappaB-dependent manner. Thus, this study showed that IκB-ζ controlled Treg differentiation.

1. Introduction

Inhibitor of kappaB (IκB)-ζ is a nuclear IκB family protein that is upregulated in macrophages in response to lipopolysaccharide (LPS) stimulation [1]. IκB-ζ is also upregulated in T cells in response to stimulation with transforming growth factor (TGF)-β and interleukin (IL)-6 stimulation and positively regulates *IL-17A* gene expression in cooperation with RAR-related orphan receptor (ROR) γt, a master regulator of Th17 cells [2]. Previous studies have shown that TGF-β stimulation alone was sufficient to induce IκB-ζ in T cells, thereby negatively regulating interferon (IFN)-γ production [3]. Thus, deficiency of IκB-ζ in T cells resulted in reduced capacity for generation of Th17 cells and increased capacity for generation of IFN-γ-producing helper T cells, called Th1 cells.

The balance among T helper cell differentiation determines many factors, including the balance among the expression of master regulators and cytokines. Foxp3 is a prominent master regulator of regulatory T cells (Tregs) and forms a complex with

RORγt, thereby preventing transcriptional activity and blocking the differentiation of Th17 cells [4]. On the other hand, RORγt can bind to the *Foxp3* promoter and negatively regulate Treg differentiation [5]. In addition, Gata3 (a master regulator of Th2 cells) can also bind to the *Foxp3* promoter and negatively regulate Treg differentiation [6]. Moreover, high levels of IFN-γ can inhibit Treg differentiation and promote Th1 cell differentiation [7], while high levels of IL-4 inhibit Treg differentiation and promote Th2 cell differentiation [8].

Previously, I have shown that IκB-ζ-deficient T cells have sufficient ability to induce Tregs in response to TGF-β *in vitro* [3]. However, IκB-ζ-deficient T cells produce large amounts of IFN-γ, even in the presence of TGF-β. Therefore, in this study, the role of IκB-ζ in maintaining the T cell balance was examined further. The data showed that production of high amounts of inflammatory cytokines from IκB-ζ-deficient T cells prevented the generation of Foxp3^{+} Tregs and that IκB-ζ itself could control *Foxp3* gene expression.

2. Materials and methods

2.1. Mice

C57BL/6 mice were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). *Nfkbiz*[flox/flox] [9] and Lck-Cre [10] mice were described previously. All mice were maintained under specific pathogen-free conditions in the animal facilities of Tohoku

University. All animal protocols were approved by the Institutional Animal Care and Use Committee.

2.2. Cells

EL4/LAF lymphoma cells [11] were kindly provided by Dr. Mathew C. Weber (Thomas Jefferson University) and were cultured in IMDM plus GlutaMax (Life Technologies Corp., Carlsbad, CA, USA) supplemented with 5% (v/v) heat-inactivated fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. $CD4^+CD62L^+$ T cells were prepared from spleens using a $CD4^+CD62L^+$ isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) unless otherwise described. $CD4^+CD62L^+$ T cells were cultured in RPMI1640 (Wako Pure Chemical Industries) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin.

2.3. Plasmids, antibodies, and cytokines

Expression vectors for FLAG-tagged mouse IκB-ζ and p65 (pcDNA3-FLAG-mIκB-ζ, -mp65) and plasmids for retroviral transduction (pMY-IRES-EGFP and pMY-FLAG-mIκB-ζ-IRES-EGFP) were described previously [9]. The pFoxp3-promoter vector was a kind gift from Dr. Akihiko Yoshimura (Keio University) [12]. pcDNA3 and phRL-TK were obtained from Life Technologies and Promega Corp. (Madison, WI, USA), respectively. APC-conjugated anti-IL-17A (TC11-18H10.1), Pacific Blue-conjugated anti-CD4 (GK1.5), and phycoerythrin (PE)-conjugated anti-Helios (22F6) antibodies were purchased from Biolegend, Inc. (San Diego, CA, USA). Anti−IFN−γ (XMG1.2) and anti-IL-4 (11B11) antibodies were from eBioscience, Inc. (San Diego, CA, USA). APC-conjugated anti-Foxp3 (FJK-16S) antibodies were from BD Biosciences (San Jose, CA, USA). Anti-IκB-ζ (C-15) antibodies and normal goal IgG were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Polyclonal anti-CD3 antibodies used for immunohistochemistry were purchased from Sigma Aldrich Co. LLC. (St. Louis, MO, USA). Recombinant human TGF-β1 was obtained from PeproTech, Inc. (Rocky Hill, NJ, USA).

2.4. In vitro cell culture

Naïve $CD4^+ CD62L^+$ T cells were cultured with plate-bound anti-CD3 (1 μg/mL) and soluble anti-CD28 (1 μg/mL) antibodies. TGF-β1 (2 ng/mL), anti−IFN−γ (10 μg/mL), and anti-IL-4 (10 μg/mL) were used as indicated [13]. For chromatin immunoprecipitation (ChIP) and western blotting, EL4/LAF cells were stimulated with plate-bound anti-CD3 (1 μg/mL) and soluble anti-CD28 (1 μg/mL) antibodies with or without TGF-β1 (2 ng/mL).

2.5. Retroviral transduction

Recombinant retroviruses were prepared by transfecting Plat-E packaging cells with the indicated plasmids using the calcium phosphate-DNA coprecipitation method [14]. Naive $CD4^+$ T cells (1×10^6 cells/mL) stimulated with plate-bound anti-CD3 (1 μg/mL) and soluble anti-CD28 (1 μg/mL) antibodies for 24 h were infected with fresh retroviral supernatant by centrifugation for 2 h at $780 \times g$ in the presence of 10 μg/mL polybrene (Sigma−Aldrich). Cells were further cultured for 2 days with or without TGF-β1 (2 ng/mL).

2.6. Flow cytometric analysis

Cell suspensions were prepared from thymus, spleen, or lymph nodes by sieving and gentle pipetting. Cell surface antigens were stained with the indicated antibodies in ice-cold phosphate-buffered saline containing 0.5% bovine serum albumin in the dark at 4 °C. Intranuclear staining of Foxp3 and Helios were performed with a Foxp3 staining buffer kit (eBioscience) according to the manufacturer's protocol. Stained cells were subjected to flow cytometric analysis with Gallios (Beckman Coulter, Inc., Brea, CA, USA). Data were analyzed with FlowJo software (Tree Star, Inc., Ashland, OR, USA).

2.7. Luciferase assay

HEK293 cells (1×10^5 cells) were transfected using the calcium phosphate-DNA coprecipitation method [14] with an individual reporter (0.45 μg), pcDNA3 (0.016−0.16 μg), pcDNA3-FLAG-mp65 (0.05 μg), and/or pcDNA3-FLAG-mIκB-ζ (0.1 μg), and pRL-TK-Luc (0.001 μg). Twenty-four hours after transfection, the medium was changed, and the cells were incubated for an additional 24 h. Luciferase activities were measured by the Dual-Luciferase Reporter Assay System according to the manufacturer's instructions (Promega).

2.8. ChIP assay

EL4/LAF cells were activated with plate-bound anti-CD3 and soluble anti-CD28 with or without TGF-β1 for 24 h. ChIP was performed by using anti-IκB-ζ antibodies and normal goat IgG according to our previously described methods [15]. Immunoprecipitated and input DNA were then analyzed by quantitative polymerase chain reaction (PCR) using SYBR Premix EX Taq (Takara Bio). The primer sequences were as follows: 5′-TTCCTCCCGCTCTCTGACTCT-3′ and 5′-AAGCGCCAGTTGTGTACAAA-TATC-3′ for the Foxp3 promoter; 5′-GTTTTGTGTTTTAAGTCTTTTG-CACTTG-3′ and 5′-CAGTAAATGGAAAAAATGAAGCCATA-3′ for the *Foxp3* CNS1; 5′-GTTGCCGATGAAGCCCAAT-3′ and 5′-ATCTGGGCCCTGTTGTCACA-3′ for the *Foxp3* CNS2.

2.9. Enzyme-linked immunosorbent assay (ELISA)

ELISA kits for IL-4 (eBioscience) were used to quantify respective cytokines in the culture supernatants according to the manufacturer's protocols.

2.10. Statistical analysis

Student's t-tests (two-tailed) were used to determine significant differences between two groups. Differences with p values of less than 0.05 were considered significant.

3. Results

3.1. IκB-ζ-deficient T cells produced large amounts of inflammatory cytokines, preventing Treg generation

T cell-specific IκB-ζ-deficient mice (*Nfkbiz*$^{f/f}$ Lck-cre; cKO) have been shown to have increased Foxp3$^+$ Tregs in the periphery [3]. Therefore, to further analyze this effect of IκB-ζ deficiency, the percentage of Helios$^-$Foxp3$^+$ Tregs (putative peripheral-inducing Tregs) [16] in the spleens from T cell-specific IκB-ζ-deficient mice (cKO) was examined. Interestingly, the data showed that these Tregs were present at higher percentages in cKO mice than in control mice (Fig. 1A and B). However, the ratios of Helios$^-$Foxp3$^+$