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

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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 induced 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

Abbreviations: IκB, inhibitor of kappaB; LPS, lipopolysaccharide; TGF, transforming growth factor; IL, interleukin; ROR, RAR-related orphan receptor; IFN, interferon; Treg, regulatory T cell; DMEM, Dulbecco's modified Eagle's medium; PE, phycoerythrin; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; NAB, neutralizing antibody; NF-κB, nuclear factor of kappaB.

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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 × *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′-AAGCGCCAGTTGTGTACAAATATC-3′ for the Foxp3 promoter; 5′-GTTTTGTGTTTTAAGTCTTTTGCACTTG-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 $Helios^-Foxp3^+$

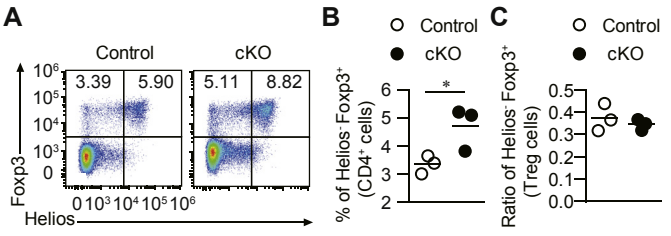

Fig. 1. Analysis of Helios expression in Tregs in T cell-specific IκB-ζ-deficient mice. (A) Flow cytometric analysis of CD4$^+$ lymphocytes cells in the spleens of 3-week-old *Nfkbiz*$^{f/f}$ (control) and *Nfkbiz*$^{f/f}$ Lck-Cre (cKO) mice. The data show a representative of three independent experiments. (B) Percentages of Helios$^-$Foxp3$^+$ in CD4$^+$ cells. (C) Relative percentages of Helios$^-$Foxp3$^+$ Tregs of total Foxp3$^+$ Tregs. The percentage of total Foxp3$^+$ Tregs was set to "1". The horizontal bars represent the mean. $^*p < 0.05$.

Tregs in Foxp3$^+$ Tregs were comparable between control and cKO mice (Fig. 1 C). Tregs from cKO mice would then also be expanded, depending on high levels of IL-2 secretion by CD4$^+$ T cells [3]; thus, IκB-ζ deficiency in T cells did not prevent peripheral-induction of Tregs *in vivo*.

A previous study showed that IκB-ζ-deficient T cells are generated in Foxp3$^+$ Tregs in response to TGF-β *in vitro* [2]. However, IκB-ζ-deficient T cells express high levels of IFN-γ, even in the presence of TGF-β [3]. In this study, IL-4 production from control and IκB-ζ-deficient T cells was significantly reduced in the presence of TGF-β, and IκB-ζ-deficient T cells showed high levels of IL-4 production (Fig. 2A). Because exogenous IFN-γ and IL-4 prevent Treg generation, I hypothesized that autocrine cytokine pathways in IκB-ζ-deficient T cells may prevent Foxp3$^+$ Treg differentiation. To test this hypothesis, purified naïve CD4$^+$ T cells cultured in the presence of TGF-β plus neutralizing antibodies (NABs: anti–IFN–γ and anti-IL-4). Interestingly, in the presence of TGF-β and NABs, IκB-ζ-deficient T cells showed greater induction of Foxp3$^+$ Tregs than that observed in control T cells (Fig. 2B). These results suggested that IκB-ζ-deficient T cells produced large amounts of IFN-γ and IL-4, even in the presence of TGF-β, thereby preventing Foxp3 differentiation.

3.2. IκB-ζ negatively regulated Foxp3 gene expression

Because IκB-ζ-deficient T cells had the ability to generate Foxp3$^+$ Tregs when the cytokine autocrine pathway was inhibited, I next asked whether IκB-ζ directly controlled *Foxp3* gene expression. To clarify the mechanisms involved in this process, mouse T cell lymphoma EL-4/LAF cells were used; these cells are known to upregulate *Foxp3* gene expression in response to TGF-β stimulation [11]. Moreover, IκB-ζ expression in T cells can be induced by TGF-β stimulation [3]. The data showed that IκB-ζ could be upregulated in response to TGF-β stimulation even in EL-4/LAF cells (Fig. 3A).

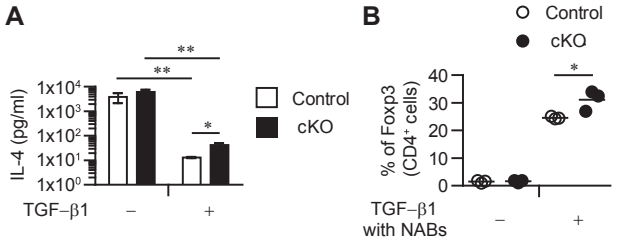

Fig. 2. IκB-ζ-deficient T cells cultured *in vitro*. (A) Enzyme-linked immunosorbent assay for IL-4 in the supernatants of naïve CD4$^+$ T cells cultured for 72 h. Numbers indicate the mean ± SD (n = 3). (B) Flow cytometric analysis of Foxp3 expression in CD4$^+$ T cells from *Nfkbiz*$^{f/f}$ (control) and *Nfkbiz*$^{f/f}$ Lck-Cre (cKO) mice cultured for 24 h in the presence or absence of TGF-β1 and neutralizing antibodies (NABs: anti–IFN–γ and anti-IL-4). Numbers indicate the percentage of Foxp3$^+$ Tregs. The horizontal bars represent the mean. $^*p < 0.05$, $^{**}p < 0.01$.

The *Foxp3* gene has several conserved noncoding sequences (i.e., the promoter, CNS1, and CNS2 regions), and these sequences play important roles in *Foxp3* gene expression [17]. To address the role of IκB-ζ in *Foxp3* gene expression, I performed ChIP assays and found that IκB-ζ could bind to the *Foxp3* promoter region in the presence of TGF-β (Fig. 3B).

IκB-ζ can also form a complex with nuclear factor of kappaB (NF-κB) and control NF-κB target gene expression [18]. In addition, the *Foxp3* promoter can be activated by NF-κB [19,20]. Therefore, I next confirmed that overexpression of the NF-κB p65 subunit could significantly activate *Foxp3* promoter activity, but that this function was negatively regulated by IκB-ζ (Fig. 4A). In addition, retroviral overexpression of IκB-ζ in T cells prevented Foxp3$^+$ Treg induction in response to TGF-β (Fig. 4B and C). Thus, these results suggested that IκB-ζ itself negatively regulated *Foxp3* gene expression.

4. Discussion

In this study, the role of IκB-ζ deficiency in maintenance of T-cell subsets was examined. The data showed that IκB-ζ had an important role in mediating the generation of Foxp3$^+$ Tregs, providing important insights into the immune cell balance and shifts in T-cell subsets.

Foxp3, a master regulator of Treg, is well known to control gene expression. TCR and TGF-β are two signaling molecules that play crucial roles in the regution of *Foxp3* gene expression [17]. The TGF-β signaling molecule 'Smad' first binds to the CNS1 region of the *Foxp3* gene [11, 21]. NF-κB enriched on the *Foxp3* promoter region then positively regulates *Foxp3* gene expression. In addition, the transcriptional factor 'TGF-β-inducible early gene 1' also plays an important role in the regulation of *Foxp3* gene expression by binding to the promoter region [22]. All these molecules positively regulate *Foxp3* gene expression. However, it is also important to prevent or suppress the expression of the negative regulators of *Foxp3* gene expression. Gata3, a Th2 master regulator, is known to bind to the *Foxp3* promoter region and negatively regulates *Foxp3* gene expression [6, 13]. It is also known that TGF-β inhibits Gata3 expression [23], thus promoting *Foxp3* gene expression. Although TGF-β plays crucial roles in the promotion of *Foxp3* gene expression [24], TGF-β-induced IκB-ζ negatively regulates *Foxp3* gene expression.

Exogenous inflammatory cytokines also affect *Foxp3* gene expression. Exogenous IFN-γ and IL-4 can inhibit Treg differentiation [7, 8]. In addition, the inhibition of IL-17 expression increases *Foxp3* gene expression [25]. Overexpression of IκB-ζ in T cells is hard to detect IL-17, even in the presence of TGF-β [2]. On the other

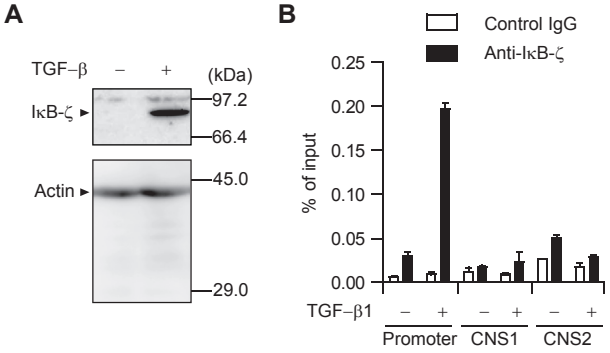

Fig. 3. IκB-ζ expression and enrichment on the *Foxp3* gene. (A) Expression of IκB-ζ in cultured EL4/LAF cells for 24 h (B) ChIP analysis of IκB-ζ binding to various positions in the *Foxp3* gene in EL4/LAF cells after TCR stimulation with/without TGF-β1 for 24 h (means + SDs, duplicate samples). Data are representative of two independent experiments (A, B).

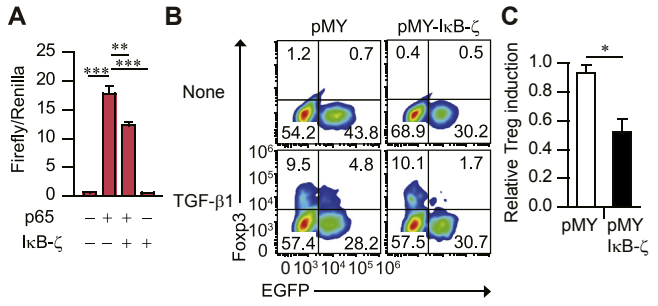

Fig. 4. IκB-ζ negatively regulated *Foxp3* gene expression. (A) Foxp3 reporter activity in HEK293 cells (mean + SD, triplicate samples). Data are representative of at least three experiments. (B) $CD4^+$ T cells were retrovirally transduced to express IκB-ζ and green fluorescence protein (GFP). $Foxp3^+$ Tregs were induced with 2 ng/mL of TGF-β1 for 48 h. (C) Relative Treg induction in GFP-positive populations. TGF-β1-induced Tregs in the GFP-negative population was set as "1" (mean + SE, n = 3). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

hand, overexpression of IκB-ζ in T cell strongly inhibits IFN-γ production [3]. Thus, overexpression of IκB-ζ in T cell prevents *Foxp3* gene expression, independent of cytokines autocrine pathways.

Taken together, the findings in this study indicated that IκB-ζ controlled cytokine production and NF-κB-dependent *Foxp3* gene expression, thus participating in the maintenance of the balance among Th cell subsets.

Conflict of interest

The authors have no conflict of interest.

Acknowledgments

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