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JunB and c-Rel cooperatively enhance Foxp3 expression during induced regulatory T cell differentiation

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ABSTRACT

The function and differentiation of induced regulatory T (iTreg) cells are tightly regulated by various signaling cascade. In this study, we have investigated the role of TCR signaling to induce *Foxp3* gene expression in cooperation with TGF-β/IL-2 stimulation. Activation of $CD4^+$ T cells by TCR signaling or TGF-β/IL-2 alone failed to enhance Foxp3 expression. Only when TCR stimulation is coupled together with TGF-β/IL-2, $CD4^+$ T cells expressed high levels of Foxp3 by maintaining open chromatin structure around its promoter region. Under this condition, stimulation-dependent recruitment of JunB together with c-Rel enhanced Foxp3 expression. Over expression of JunB and c-Rel significantly enhanced Foxp3 promoter activity while treatment of JunB siRNA or inhibition of TCR signaling by MAPK inhibitors significantly reduced Foxp3 expression. Collectively our results suggest that TCR signaling together with TGF-β/IL-2 stimulation cooperatively enhance *Foxp3* gene expression by maintaining accessible chromatin structure and by actively recruiting key transcription factors JunB and c-Rel.

1. Introduction

Maintenance of immune homeostasis is important to keep our body in healthy condition. Regulatory T (Treg) cells play pivotal role in regulating immune homeostasis by actively suppressing anti-self or innocuous antigens [1–3]. Natural Treg (nTreg) and inducible Treg (iTreg) are characterized by the presence of Forkhead box protein p3 (Foxp3) [1]. Foxp3 is a key transcription factor for the development and suppressive activity of Treg cells [4] and is necessary and sufficient for the development and function of nTreg cells [2,3,5–7]. Foxp3 in iTreg cells can be induced in peripheral $CD4^+$ T cells via specific antigenic and TGF-β/IL-2 stimulations [8,9]. Engagement of T cell receptor (TCR), costimulatory factor (CD28) and cytokine signaling in Foxp3 regulation are well documented [9]. The binding of TCR-mediated transcription factors such as Sp1, NFAT, and AP1 [10] and STAT5, CREB, ATF and Smad proteins induces Foxp3 transcription upon TGF-β treatment [9]. However, it is still unclear how TCR signaling induces *Foxp3* gene regulation under iTreg generation conditions. In addition, the exact role of TGF-β/IL-2 stimulation in iTreg generation is still unclear.

In this study, we have investigated and demonstrated the cooperative role of TCR and TGF-β/IL-2 signaling to induce *Foxp3* gene expression during iTreg differentiation. In addition, we tested the role of JunB as a latent transcription factor under the down-stream of TCR and TGF-β/IL-2 signaling. Our results demonstrate that TCR and TGF-β/IL-2 stimulation induce and maintain the accessibility of chromatin structure, and recruitment of JunB and c-Rel to the *Foxp3* promoter enhances Foxp3 expression during iTreg generation.

2. Materials and methods

2.1. Animals

$Foxp3^{gfp}$-reporter mice [5] and C57BL/6 mice were purchased from Jackson laboratory (Main, USA) and SLC (Hamamatsu, Japan), respectively. Mice were housed in specific pathogen-free barrier facilities and were used in accordance with protocols approved by the animal care and use committees of the Gwangju Institute of Science and Technology (GIST).

2.2. Antibodies, cytokines and siRNAs

The following antibodies were used: anti-RelA/p65, anti-c-Rel, anti-c-Jun, anti-JunB, anti-Foxp3, anti-β-actin, all from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-acetyl-H3K9 (AcH3K9), anti-acetyl-H4 (AcH4), anti-dimethyl-H3K4 (H3K4me2),

Abbreviations: Treg, regulatory T cells; TCR, T cell receptor; TGF-β, transforming growth factor; IL-2, interleukin-2; ChIP, chromatin immunoprecipitation.

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anti-trimethylated H3K27 (H3K27me3), and anti-IgG (Abcam, Cambridge, England). Goat anti-rabbit Cy5 and propidium iodide (PI) were purchased from Molecualr Probes (Eugene, USA). The following materials were purchased: micrococcal nuclease (MNase) from Worthington Chemical Company (Lakewood, NJ, USA), TGF-β from Peprotech (Rocky hill, NJ, USA), α-CD3 (145.2C11) from Pharmingen (San Diego, CA, USA) and control siRNA and JunB siRNA from Santa Cruz Biotechnology (Santa Cruz, CA, USA). rhIL-2 was provided by the National Cancer Institute, Preclinical Repository.

2.3. In vitro Treg cell differentiation and flow cytometry analysis

$CD4^+$ T cells were isolated from the spleen of 8–10 weeks old mice by magnetic beads (Miltenyi, L3T4). *In vitro* differentiation of iTreg cells was performed as previously described [11]. Foxp3 expression was analyzed by measuring GFP fluorescence by using quantitative flow cytometry. In inhibitor experiments, the cells were pretreated with the inhibitors BAY11–7085 (Calbiochem, Germany) or SP600135 (Calbiochem, Germany) for 6 h at the indicated concentration. SP600125 is a JNK inhibitor [12] and BAY11–7085 is a soluble inhibitor of IKK [13].

2.4. Quantitative real-time PCR and immuoblotting

Quantitative real-time PCR (RT-PCR) was performed as previously described [14,15]. One microliter of cDNA was amplified by using the RT-PCR primer sets shown in Table 1. PCR conditions were 94 °C for 30 s, 62 °C for 20 s, and 72 °C for 30 s for a total of 35 cycles. In quantitative real-time PCR, the mRNA level of Foxp3 was determined by RT-PCR with SYBR green. Expression levels were normalized to β- actin levels in each sample. The immuoblotting procedure was performed as previously described [15].

2.5. Chromatin accessibility by real-time PCR (CHART-PCR)

Chromatin accessibility assays were performed as described previously [16]. Digested DNA samples were loaded and titrated on agarose gels (Supplementary Fig. 3A). DNA recovered from "Cut" (+MNase) and "Uncut" samples were used in qRT-PCR assays to measure the relative accessibility by using a series of primer pairs shown in Table 1. Data were normalized by the C_t value of each primer for genomic DNA. A comparative quantification method was used to determine the "Uncut"/"Cut" ratio for independent pairs of samples.

2.6. Chromatin immunoprecipitation (ChIP) assays

The ChIP assay was performed as described [14] with specific antibodies described above. As a loading control, PCR was done directly with input DNA purified from chromatin before immunoprecipitation. Quantification of the Foxp3 genome fragment following ChIP was performed by RT-PCR with primer pairs shown in Table 1. For normalization, 1% of 'input' DNA from each sample was analyzed in parallel and the amount of Foxp3 DNA in each sample was calculated. ChIPs with control antibody were used to ensure that C_t values from samples with specific antibodies resulted from specific immunoprecipitation.

2.7. Transfection, reporter assay and siRNA based knock-down assay

The Foxp3 minimal promoter (656 bp) for reporter assay was amplified using the primers listed in Table 1. Mutation of the κB or AP1 sites in the Foxp3 regulatory region was performed by using the mutated primers (Table 1). Luciferase activity was measured by the Dual Luciferase Assay System (Promega, Warrington, PA, USA). In the knock-down assay, $CD4^+$ T cells (1×10^6 cells/well; 24-well plate) were transfected with either 30 nM JunB siRNA or negative control (scrambled) by using the Nucleofection (Amaxa Biosystems, Basel, Switzerland).

2.8. Immunocytochemistry

Immunofluroscence assay was performed as described [14] with minor modifications. iTreg cells cultured for 2 days were fixed with 2% paraformaldehye for 20 min and washed twice with cold PBS. After permeablizaiton, the cells were incubated with anti-JunB at 1 μg/ml and PMA/ionomycin for 1 h. The cells were incubated with 2 μg/ml of Cy5-conjugated anti-rabbit-IgG (Molecular Probes) in PBS buffer for 1 h. After three washes in PBS, the cells were incubated in 10 μg/ml propidium iodide (Molecular Probes) and

Table 1
Primer sequences used for cloning, ChIP and DNA pull-down experiments.

Target genes	Sequences (5′–3′)	Experiments
FOXP3	F: GTTCATCGTGAGGATGGATGCATTAATATT	LUC constructs
	R: AATTGATATGGTCCCCATTGCTTGAACTA	
FOXP3 AP-1[a]	F: ATGCCTTTGTCAATTGACTTATTT	Mutagenesis
FOXP3 AP-2[a]	F: AATCATATTTCAAGATGACTTGTAAAG	Mutagenesis
FOXP3 k1[a]	F: CCTGGCTGCAATTCATTCACATGGCA	Mutagenesis
FOXP3 k2[a]	F: TGATTTGACTTATTAATTCTCAGT	Mutagenesis
FOXP3	F: GACTACTTCAAGTTCCACAACAT	qRT-PCR
	R: GGATGGTTTCTGAAGAAGGCAAACAT	
β-actin	F: CTGTGGCATCCATGAAACTACATTCAAT	qRT-PCR
	R: AGGAGGAGCAATGATCTTGATCTTCA	
FOXP3 (κ1)	F: GTTCATCGTGAGGATGGATGCATTAATATT	ChIP
	R: ACAGTTACTGAATGAGGAAAGAGAG	
FOXP3 (κ2 AP1–1)	F: GAGTAGGGCGAGGCCTCTGGGAA	ChIP CHART
	R: GCAGAGACACCATTCTGTGAGTGA	
FOXP3 (CNS2)	F: AGATGCAGACCCCGATATGAAAACATAA	ChIP
	R: ATACCGATTTCGGTGCAAATGGATGTTTT	
FOXP3 (AP1–2)	F: TTAAAAACTGTGGTTTCTCATGAG	ChIP
	R: TATGAGACTTAAACGGAAATTTTGAAAT	
Negative control (NC)	F: ATTATAGGATCCTGAAAAGCCAGTGCT	ChIP
	R: TCTTTCCCTGTCCAAGCCACGCAA	
FOXP3 AP1–1	F: GATGCCTTTGTGATTTGACTTATTT	DNA pull-down
IL4	F: GGTGTTTCATTTTCCAATTGGTCTGATT	DNA pull-down
Negative control (NC)	F: ATTATAGGATCCTGAAAAGCCAGTGCT	DNA pull-down

[a] Underline indicates the mutated sequence from original sequence.

100 μg/ml RNase (Sigma) in 2XSSC buffer for 30 min at 37 °C in the dark. After two washes in PBS, the cells were analyzed by using a FluoView microscope (Olympus, Hamburg, Germany).

2.9. DNA–protein pull-down assay

Nuclear lysate preparation and immunoblotting were performed as described [14,15] with minor modifications. Nuclear lysates were prepared from iTreg cells stimulated for 2 days with plate-bound α-CD3 plus TGF-β/IL-2. After pre-clearance with streptavidin-conjugated magnetic beads (Invitrogen, Oslo, Norway), nuclear lysates were incubated with biotinylated dsDNA probes (Table 1) followed by incubation with streptavidin-conjugated beads for 30 min each at 4 °C. The pelleted streptavidin-magnetic beads were washed and DNA–protein complexes were run on an SDS-12% polyacrylamide gel to do immunoblotting.

2.10. Statistical analysis

P-values of < 0.05 obtained with a two-tailed student's *t*-test were considered significant. Single asterisks (*) indicate $p < 0.05$, double asterisks (**) indicate $p < 0.005$.

3. Results

3.1. Coupling of TCR signaling with IL-2/TGF-β stimulation is required to induce stable Foxp3 expression

Although the importance of TCR stimulation on *Foxp3* gene expression has been reported [9], its exact role is still unclear. To test and confirm the requirement of TCR stimulation and TGF-β/IL-2 for Foxp3 expression, $CD4^+$ T cells were cultured under indicated stimulation conditions such as α-CD3 alone, TGF-β/IL-2 alone or α-CD3 together with TGF-β/IL-2. Foxp3 expression was analyzed by quantitative RT-PCR and flow cytometry during the 6 days culture period. TCR stimulation alone (α-CD3) or IL-2/TGF-β stimulation alone failed to enhance Foxp3 expression for 6 days culture periods (Fig. 1A and Fig. 1B) (Supplementary Fig. 1A–C). However, coupling of TCR stimulation together with TGF-β/IL-2 significantly induced Foxp3 expression (Fig. 1A and Fig. 1B) (Supplementary Fig. 1A–C). Under this condition, the population of $Foxp3^+$ T cells increased starting from 5.8% (day zero) to 62.2% (Day six) (Fig. 1B and Supplementary Fig. 1A). Interestingly, commitment of Foxp3 expression seems to be initiated within 2 days. Compared with cells stimulated with α-CD3 or TGF-β/IL-2 alone, a significant up-regulation of Foxp3 expression was observed under α-CD3/TGF-β/IL-2 within 2 days (Fig. 1A and Fig. 1B). Continuous TCR engagement in the presence of TGF-β/IL-2 further up-regulated the expression of Foxp3 mRNA, protein and $Foxp3^+$ Treg population over 6 days culture periods (Fig. 1 and Supplementary Fig. 1).

3.2. Coupling of TCR signaling with TGF-β/IL-2 stimulation induces a stable permissive chromatin structure at the Foxp3 promoter

To test whether commitment of *Foxp3* gene expression is also related with changes in the chromatin structure at the Foxp3 promoter locus (−391–−253) (Supplementary Fig. 1D), we performed chromatin accessibility by real-time PCR (CHART-PCR). $CD4^+$ T cells were left without stimulation (w/o) or stimulated with α-CD3 alone or α-CD3 together with TGF-β/IL-2 (α-CD3/TGF-β/IL-2) for indicated time periods (Fig. 1C). Purified nuclei from each treatment group were digested with MNase (Supplementary Fig. 3A) and then the amount of protected DNA was analyzed by RT-PCR with primer pairs specific for Foxp3 promoter locus (Table 1). $CD4^+$ T cells left without stimulation (w/o) have condensed chromatin structure (less accessible to MNase digestion). Interestingly, stimulation with α-CD3 alone increased chromatin accessibility within 12 h and then rapidly dropped to the basal level (Fig. 1C). This result is well correlated with Foxp3 expression profile since α-CD3 alone failed to enhance its expression (Fig. 1A). However, accessibility of chromatin structure at Foxp3 promoter was progressively enhanced in cells stimulated with α-CD3 together with TGF-β/IL-2 (Fig. 1C and Supplementary Fig. 3B). To further confirm whether stimulation-dependent changes in the chromatin structure around Foxp3 promoter locus are accompanied by epigenetic modification, we performed ChIP analysis with specific antibodies for modified histone molecules. In general, recruitment of acetylated H3 at lysine residue nine (AcH3K9), H4 (AcH4) or methylated H3 at lysine residue four (H3K4me2) are well correlated with actively transcribed region. Therefore, we examined whether TCR and TGF-β/IL-2 stimulation enhanced a recruitment of activation marker histones at the Foxp3 promoter during 48 h stimulation period. Similar to the chromatin accessibility results, coupling of TCR with TGF-β/IL-2 stimulation significantly increased the recruitment of AcH3K9, AcH4 and H3K4me2 at the Foxp3 promoter (Fig. 1D) and CNS2 region (Supplementary Fig. 3C). However, cells left without stimulation (w/o) or stimulated with only with α-CD3 alone showed much lower levels. Although stimulation with α-CD3 alone initially enhanced recruitment of active marker histone molecules, it failed to maintain the active statues of Foxp3 promoter (Fig. 1D). These results suggest that compared with cells stimulated TCR or TGF-β/IL-2 alone, enhanced Foxp3 expression by TCR with TGF-β/IL-2 stimulation is mediated by accessible chromatin architecture at the Foxp3 promoter.

3.3. Stimulation dependent binding of Rel and Jun proteins to the Foxp3 promoter

To identify transcription factors involved in *Foxp3* gene expression we tested *in vivo* binding of AP1 (AP1–1 and AP1–2) and NF-κB (κ1 and κ1) (Supplementary Fig. 1D) at the Foxp3 regulatory locus by ChIP assay. $CD4^+$ T cells were left without stimulation (w/o) or stimulated for 24 h with α-CD3 plus TGF-β/IL-2. As predicted, AP1 family members, c-Jun and JunB, bound to the promoter regions, AP1–1 (−381) and AP1–2 (−247) sites (Fig. 2A) (Supplementary Fig. 1D). In addition, NF-κB family members, c-Rel and RelA, also bound to the regulatory regions such as κ1 locus (−494) and κ2 (−368) sites (Fig. 2B) (Supplementary Fig. 1D), which is well correlated with previous reported [17,18]. As a negative control (NC), the + 5011 locus (Supplementary Fig. 1D) was employed. To further confirm the physical binding of JunB to AP1–1 site, DNA–protein pull-down assay was performed. Biotinylated AP1–1 probe was incubated with nuclear extract prepared from $CD4^+$ T cells stimulated for 24 h with α-CD3 plus TGF-β/IL-2. In accordance with ChIP result, binding of JunB at the AP1 site was preferentially detected (Fig. 2C). Stimulation with α-CD3 plus TGF-β/IL-2 induced a nuclear translocation of JunB and co-localization of JunB signal is well matched with Foxp3 expression profile (Fig. 2D). In addition, compared with α-CD3 alone, α-CD3 plus TGF-β/IL-2 stimulation significantly enhanced JunB protein levels (Fig. 2E) and binding of JunB to the Foxp3 promoter during the culture periods (Supplementary Fig. 4). Collectively, these results suggest that upon stimulation with α-CD3 plus TGF-β/IL-2, Rel and Jun proteins bind to the Foxp3 promoter locus with permissive chromatin structure.

3.4. c-Rel and JunB cooperatively enhance Foxp3 promoter activity and modulate chromatin structure at the Foxp3 promoter

To further analyze the functional role of the JunB and c-Rel for foxp3 expression, we tested the effect of overexpression or

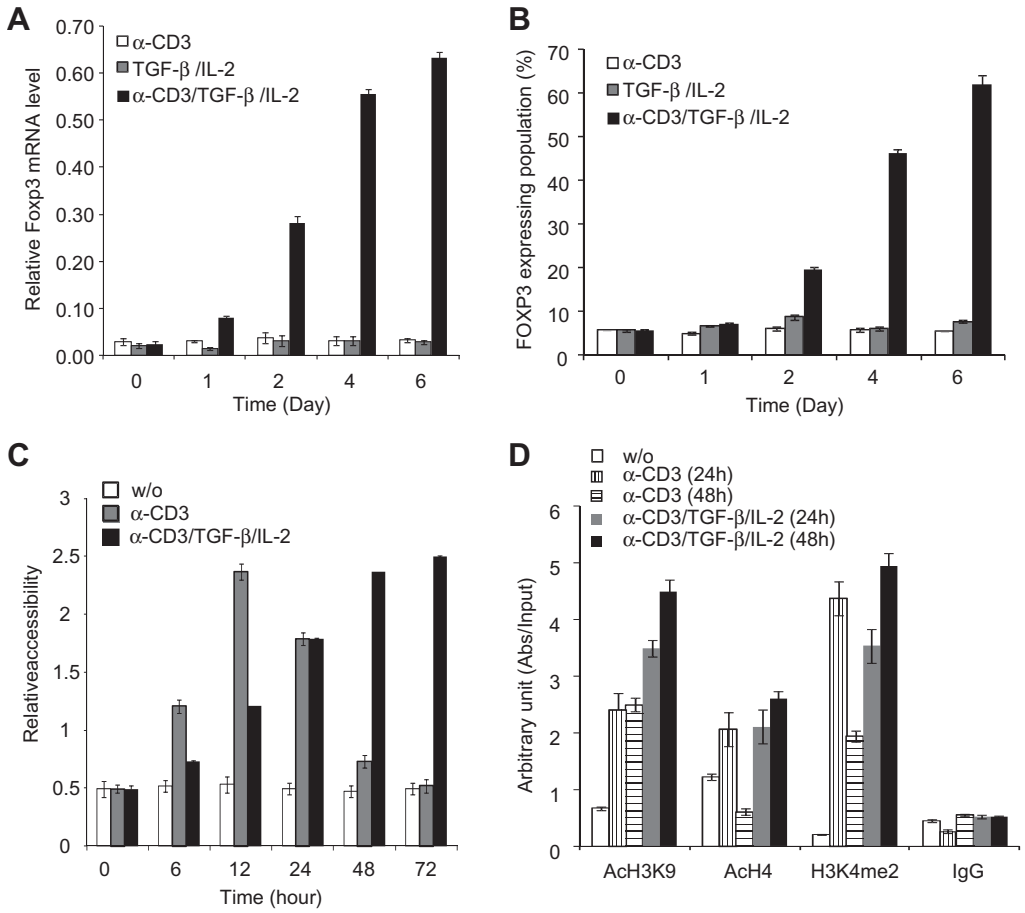

Fig. 1. Coupling of TCR signal with IL-2/TGF-β stimulation enhances Foxp3 expression by endorsing permissive chromatin structure. $CD4^+$ T cells were stimulated for indicated time periods and the levels of Foxp3 mRNA (A), Foxp3 expressing population (B) were analyzed. (C) Relative chromatin accessibility was determined with primer pairs specific for Foxp3 promoter locus (−487−−224) (Supplementary Fig. 1D). (D) Epigenetic modification around Foxp3 promoter locus were analyzed by ChIP analysis with indicated antibodies. Normal IgG was used a negative control for ChIP experiment. Results are shown for precipitated complexes and inputs. The means (+/−) SEM of three different samples are plotted. Data are representative of three independent experiments.

inhibition of JunB and c-Rel on Foxp3 promoter activity. First, we tested the cooperative effect of JunB and c-Rel on Foxp3 promoter activity. Jurkat $CD4^+$ T cells were co-transfected with Foxp3 promoter construct in the presence of JunB or c-Rel constructs and then Foxp3 promoter activity was measured. JunB or c-Rel alone failed to activate Foxp3 promoter (Fig. 3A). However, overexpression of JunB and c-Rel together significantly enhanced Foxp3 promoter activity (Fig. 3A). The cooperative activity was further enhanced upon stimulation (Fig. 3A). Addition of either NF-κB inhibitor (BAY11–7085; 5 μM) or the AP1 inhibitor (AP600125; 5 μM) significantly reduced Foxp3 promoter activity (Fig. 3B). Site-directed mutation of JunB or c-Rel binding site on Foxp3 promoter significantly reduced Foxp3 promoter activity (Fig. 3C). Next we tested the effect of inhibitor treatment on Foxp3 expression during iTreg differentiation. Purified $CD4^+$ T cells were stimulated with α-CD3 together with TGF-β/IL-2 in the presence or absence of indicated inhibitors. In accordance with Foxp3 promoter assay (Fig. 3B), treatment of NF-κB inhibitor (BAY11–7085) or AP1 inhibitor (SP600125) significantly reduced Foxp3 expression in mRNA (Fig. 4A) and protein (Fig. 4B) levels. Since the binding of c-Rel and JunB to the Foxp3 promoter is dependent on their accessibility at the Foxp3 promoter (Fig. 2A and B), we tested whether down-regulation of Foxp3 expression by the treatment of relative inhibitors is also mediated by modification of chromatin architecture around Foxp3 promoter (Fig. 4C). Indeed, treatment with NF-κB and AP1 inhibitor (BAY11–7085 + SP600125) significantly reduced chromatin accessibility around Foxp3 promoter locus (Fig. 4C). To further delineate the functional role of JunB, we tested the effect of knock-down of JunB expression. Primary $CD4^+$ T cells transfected with either mock siRNA (mock) or JunB siRNA (si-JunB) were stimulated with α-CD3 together with TGF-β/IL-2 for 48 h and then knock-down effect of JunB on Foxp3 expression was measured by RT-PCR. Compared with mock siRNA transfected cells, JunB siRNA (si-JunB) transfection significantly (about 60%) reduced Foxp3 expression levels (Fig. 4D). Treatment of JunB siRNA reduced endogenous JunB expression to 40% (Fig. 4D). As a control, transfection with mock or JunB siRNA did not alter control β-actin levels (Fig. 4D). Furthermore, knock-down of JunB significantly reduced chromatin accessibility (Fig. 4E). These results suggest that c-Rel and JunB cooperatively regulate Foxp3 expression by modulating the chromatin architecture of Foxp3 promoter region.

4. Discussion

TCR signal plays pivotal role to initiate the chromatin remodeling and recruitment of transcription factors to induce Foxp3 gene expression. In this study, we have demonstrated that TCR or TGF-β/IL-2 stimulation alone failed to induce high level Foxp3 expres-

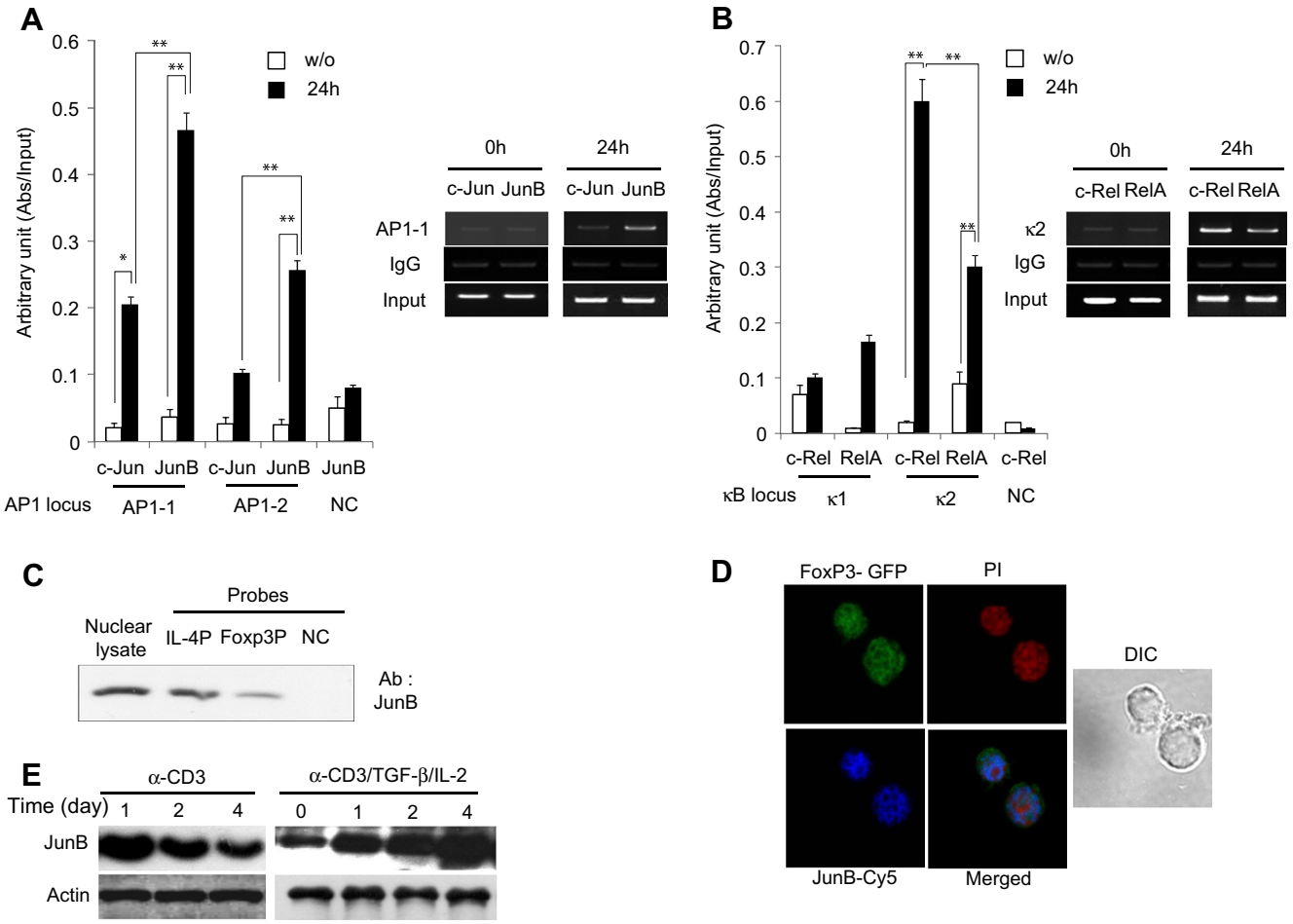

Fig. 2. Binding of NF-κB and AP1 to the Foxp3 promoter. $CD4^+$ T cells ($1X10^6$) were left without stimulation (w/o) or stimulated with α-CD3/TGF-β/IL-2 for 24 h. *In vivo* binding of AP1 (c-Jun and JunB) (A) or NF-κB (c-Rel and RelA) (B) to the Foxp3 promoter was tested by ChIP experiment. Nonspecific binding of each antibody was excluded by using negative control IgG. Arbitrary units were calculated and normalized with respect to the amplification of the 10% input of each sample. Error bars are computed as the standard error from three independent assay experiments. A negative control (NC) region corresponds at + 5011 locus (Supplementary Fig. 1D). (C) DNA–protein pull-down assay was performed. The IL-4 promoter (IL-4P) was used as a positive control for JunB binding. (D) Immunocytochemistry was performed by FluoView microscope; JunB (blue), Foxp3 (green), propidium iodide (PI, nuclear, red) and DIC (differential interference contrast). (E) The total cell lysate were harvested and JunB protein level was assayed by immunoblotting. β-actin was measured as a loading control. Data are representative of three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper)

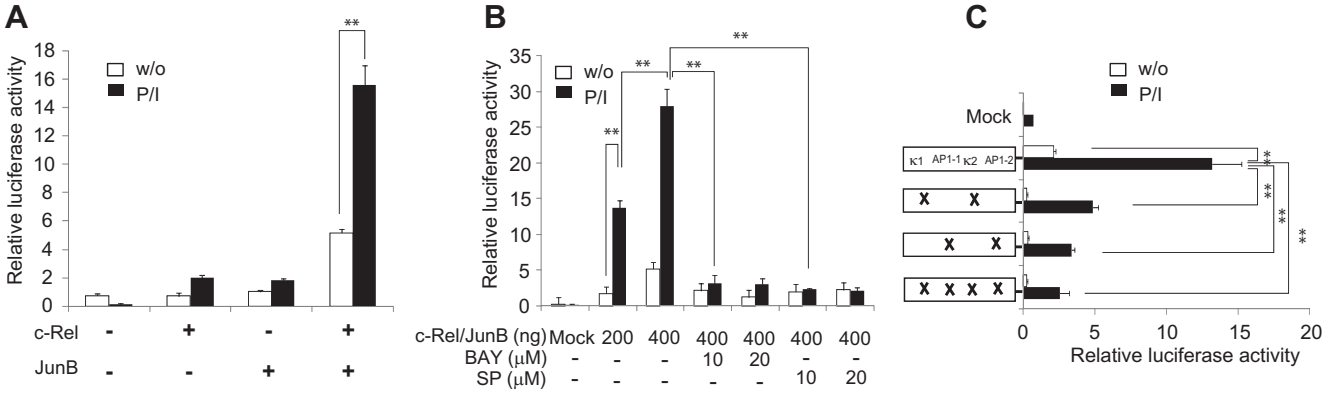

Fig. 3. c-Rel and JunB cooperatively enhance Foxp3 promoter activity. (A) Jurkat cells were co-transfected with luciferase construct driven by the Foxp3 promoter (−627/+29) together with the indicated plasmids and then left without stimulation (w/o) or stimulated with PMA/ionomycin (P/I) for 18 h. (B) Effect of NF-κB or AP1 inhibitors on Foxp3 promoter was measured. Jurkat cells transfected with c-RelA/JunB were stimulated in the presence or absence of indicated inhibitors (BAY11–7085 (BAY) or SP600125 (SP)). (C) Effect of mutation at the κB or AP1 binding site on Foxp3 promoter activity was measured. Relative luciferase activity was calculated by normalizing to Renilla luciferase activity. Error bars are computed from three independent experiments. Data are representative of three independent experiments.

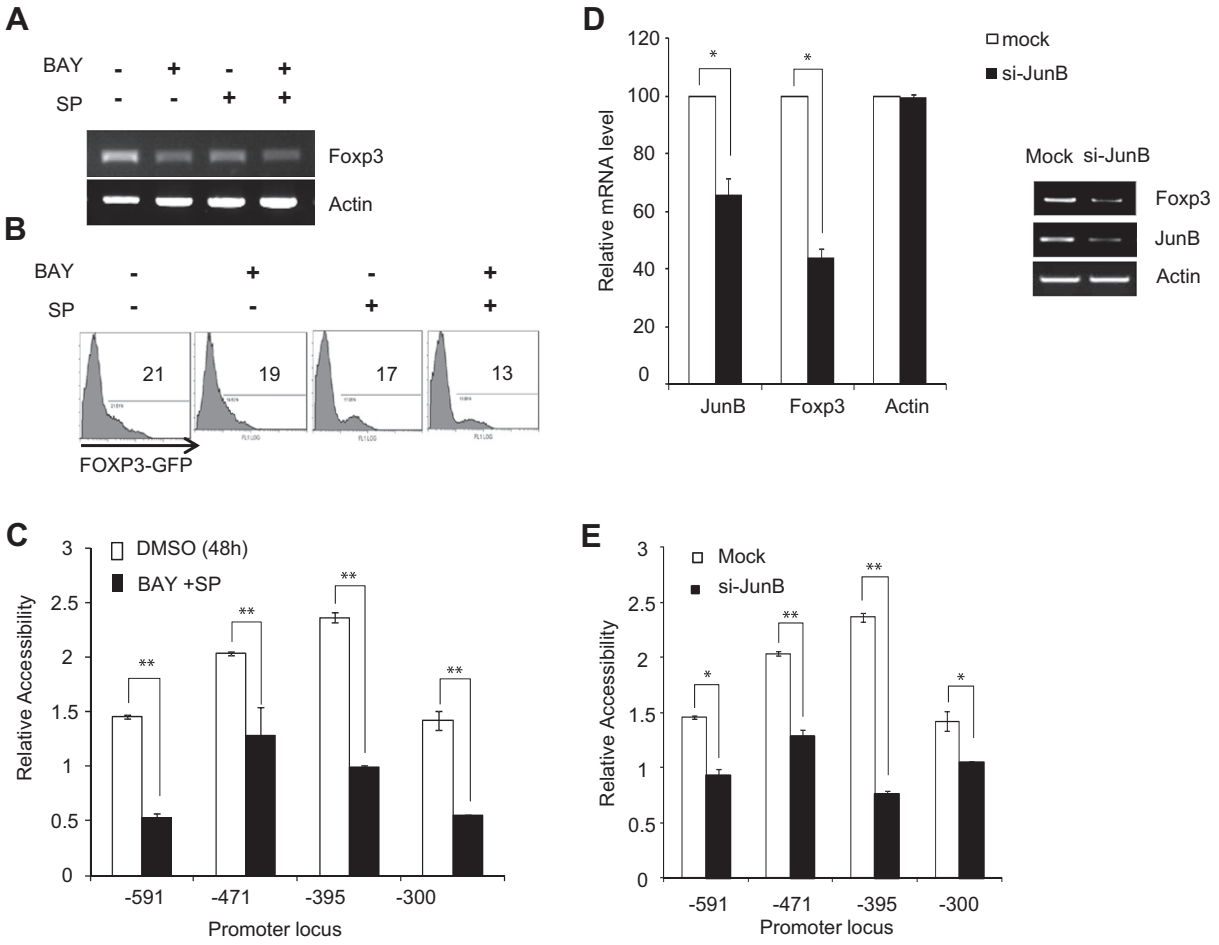

Fig. 4. Inhibition of c-Rel and JunB decreases Foxp3 expression and chromatin accessibility. $CD4^+$ T cells (1×10^6) were stimulated with α-CD3/TGF-β/IL-2 in the presence or absence of inhibitors BAY11–7085 (BAY) and SP600125 (SP). The effect of inhibitors for Foxp3 transcript level (A) or Foxp3 expressing population (B) was analyzed. (C) The effect of inhibitors on chromatin accessibility at the Foxp3 promoter was analyzed by CHART-PCR. (D) $CD4^+$ T cells transfected with either mock siRNA (mock) or JunB siRNA (si-JunB) were stimulated with α-CD3/TGF-β/IL-2 for 48 h and then knock-down effect of JunB on Foxp3 expression was measured by quantitative RT-PCR. (E) The effect of JunB siRNA on chromatin accessibility at the Foxp3 promoter was analyzed by CHART-PCR. Data are representative of three independent experiments. Error bars are computed as the standard error from three independent experiments.

sion, while coupling of TCR signaling with TGF-β/IL-2 stimulation significantly enhanced its expression by sustaining permissive chromatin structure at the Foxp3 promoter locus. Furthermore, recruitment of TCR-induced JunB and c-Rel to the accessible promoter induced a prolonged Foxp3 expression.

Previous studies have demonstrated the importance of TCR engagement [9] and the role of TGF-β/IL-2 as environmental cues for heterogeneous Foxp3-expressing population [19]. However, it is poorly understood how TCR activation integrates with other signaling pathways and transcription factors derived from TGF-β or/and IL-2 to regulate Foxp3 transcription. Although the chromatin state of Foxp3 promoter is accessible for binding of transcription factors [20], it failed to induce Foxp3 transcription under TCR stimulation alone (Fig. 1A). Our studies suggest that TCR stimulation induces a permissive chromatin structure while TGF-β/IL-2 signaling helps to maintain the chromatin accessibility (Fig. 1C), suggesting the necessity of both chromatin change and transcription factor binding for Foxp3 expression. The integration of these two signals may work through transcriptional complex formation with other basal or cell-type specific factors. Under TCR stimulation, addition of TGF-β/IL-2 at day zero induced maximal Foxp3 expression (Supplementary Fig. 2A). However, addition of TGF-β/IL-2 after day three failed to enhance Foxp3 expression (Supplementary Fig. 2A). In addition, TCR stimulation alone failed to enhance JunB expression while TCR and TGF-β/IL-2 stimulation induced a sequential JunB accumulation during the iTreg culture periods (Fig. 2E). TGF-β activates *JunB* gene expression *via* a non-canonical (Smad-independent) signaling pathway by recruiting and coordinating the various components of the transcriptional complex [21]. In this study, we have identified that JunB plays as a cooperative factor between TCR and TGF-β/IL-2 signals. JunB induces Foxp3 expression via a change in chromatin structure (Fig. 4E). Existence of JunB protein could be another key factor to induce Foxp3 gene expression. Previous reports also showed that TGF-β mediated Smad molecules also bind to the Foxp3 promoter at early time points upon simultaneous TCR and TGF-β/IL-2 stimulation [22]. However, these molecules are rapidly dissociated from the Foxp3 promoter during iTreg differentiation [18]. Prolonged Foxp3 expression by TGF-β/IL-2 requires secondary molecules induced by TCR signaling to replace the role of the primary occupied enhanceosome complex. We argued that the AP-1 family transcription factor JunB may act as a linker between the TCR and TGF-β/IL-2 signals.

Collectively, our results suggest that TCR engagement together with TGF-β/IL-2 signals plays pivotal role in Foxp3 expression by affecting chromatin architecture and recruitment of key transcription factors during iTreg differentiation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.02.126.

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