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Gene Expression in the Gitr Locus Is Regulated by NF-κB and Foxp3 through an Enhancer

Yukiko Tone,*†,1 Yoko Kidani,†,1 Chihiro Ogawa,* Kouhei Yamamoto,* Masato Tsuda,* Christian Peter,‡ Herman Waldmann,‡ and Masahide Tone*†

Glucocorticoid-induced TNFR (Gitr) and Ox40, two members of the TNFR superfamily, play important roles in regulating activities of effector and regulatory T cells (Treg). Their gene expression is induced by T cell activation and further upregulated in Foxp3+ Treg. Although the role of Foxp3 as a transcriptional repressor in Treg is well established, the mechanisms underlying Foxp3-mediated transcriptional upregulation remain poorly understood. This transcription factor seems to upregulate expression not only of Gitr and Ox40, but also other genes, including Ctsa4, Il35, Cd25, all critical to Treg function. To investigate how Foxp3 achieves such upregulation, we analyzed its activity on Gitr and Ox40 genes located within a 15.1-kb region. We identified an enhancer located downstream of the Gitr gene, and both Gitr and Ox40 promoter activities were shown to be upregulated by the NF-κB–mediated enhancer activity. We also show, using the Gitr promoter, that the enhancer activity was further upregulated in conjunction with Foxp3. Foxp3 appears to stabilize NF-κB p50 binding by anchoring it to the enhancer, thereby enabling local accumulation of transcriptional complexes containing other members of the NF-κB and IκB families. These findings may explain how Foxp3 can activate expression of certain genes while suppressing others. 


Materials and Methods

Cell culture

EL4 subclones LAF and BO2 cells were cultured in IMDM with 1-glutamine and 25 mM HEPES (Cellgro) and 5% FBS. T cells were cultured in

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Abbreviations used in this article: Bcl-3, B cell lymphoma 3; ChIP, chromatin immunoprecipitation; Gitr, glucocorticoid-induced TNFR; HS, hypersensitive site; iTreg, induced regulatory T cell; NAL, NF-κB activation inhibitor; nTreg, natural regulatory T cell; poly(dI-dC), poly(deoxyinosinic-deoxycytidylic); Treg, regulatory T cell; WT, wild-type.
CD4+ T cells and T cells were isolated from mouse spleen using a CD4+ T cell isolation kit (Miltenyi Biotec) and a Pan T cell isolation kit (Miltenyi Biotec), respectively. CD4+CD25+ Treg cells were purified by a cell sorter using anti-CD4 (RM4-5, e Bioscience) and anti-CD25 (PC61.5, e Bioscience) from preisolated CD4+ T cells. Isolated CD4+CD25+ T cells were analyzed by an internal Foxp3 staining kit (e Bioscience), and Foxp3+ T cells were >95%. When required, T cells were stimulated with plate-coated anti-CD3 (KT3, 5 μg/ml) and 1 μg/ml anti-CD28. To generate Foxp3+ iTreg, cells were isolated from wild-type (WT) and Foxp3+GFP reporter mice (The Jackson Laboratory) and treated with TGF-β (5 ng/ml) (PeproTech), anti-CD28 (1 μg/ml), and anti-CD3 (plate-coated). To isolate Foxp3+ iTreg, the stimulated cells were sorted with GFP. Splenocytes and thymocytes were isolated from 129p50-deficient mice (The Jackson Laboratory) and WT mice.

The preparation of bone marrow–derived dendritic cells has been described previously (21). Briefly, bone marrow cells from BALB/c mice were cultured for 7 d with GM-CSF (5 ng/ml). When required, bone marrow–derived dendritic cells were stimulated with LPS (10 μg/ml) in PBS. Cells were harvested, and promoter or enhancer activity was analyzed by the luciferase assay (22).

RT-PCR
Total RNA was isolated from EL4 cells and T cells using an RNasey Mini Kit (Qiagen), and cDNA was prepared with an iScript cDNA synthesis kit containing random and oligo(dT) primer mixture (Bio-Rad). Quantitative RT-PCR was performed by using SsoFast EvaGreen Supermix (Bio-Rad). PCR primers used were as follows: Gitr, forward, 5′-GACCTCAGGC-CAAGATCTGC-3′, reverse, 5′-CTCTGACGTCGACAACCTC-3′; Ox40, forward, 5′-GTAAGCCAGCACCACCAAC-3′, reverse, 5′-GGCCAGACTGCTCTTGATTG-3′; Gapdh, forward, 5′-TGGTGAAGGGCTGTGTGAG-3′, reverse, 5′-TGGTGGCGTTGATTTGAG-3′; reverse, 5′-TGGGCCGGTGAAGCGTGCAG-3′; 18S rRNA, forward, 5′-CTTAAAGGCAACAGGTCGCG-3′, reverse, 5′-AATGCTAGCCAGCTAGTGA-3′; Gitr, Ox40, and Gapdh expression levels were normalized to 18S rRNA levels.

Construction of luciferase reporter plasmids and luciferase assay
A DNA fragment (1.22 kb) of the Gitr promoter and its deletion mutants were amplified by PCR, cloned into the pGL4 basic vector (Promega), and DNA sequences of the all inserted fragments were determined to remove deformed fragments generated by PCR errors. To construct enhancer luciferase reporter plasmids, pGrop Gitr promoter luciferase reporter plasmid was used. Potential enhancer DNA fragments (1.6-kb fragment [containing HS1 site], 1.15-kb fragment [containing HS2 site], and 0.28-kb fragment [containing HS3 site]) were PCR amplified and inserted into Sall site located downstream of the luciferase gene of the Ox40 promoter reporter plasmid (carrying 1.97-kb DNA fragment as Ox40 promoter) shown in a previous publication (20). The enhancer deletion mutant fragments were PCR amplified and integrated into the Sall site of the Gitr Pro8 reporter plasmid. The +39, +136, +183, and WT core enhancer fragments contain the same 3′ end (+286), and +71, +108, +193, and WT core enhancer fragments contain the same 5′ end (+1). These positions are shown in Supplemental Fig. 3A. Mutations were introduced in the enhancer sequence (Fig. 3A) were introduced into the enhancer fragment by PCR assemble procedure, and the mutated fragments were integrated into the Sall site in the Gitr Pro8 reporter promoter plasmid. DNA sequences of the all inserted fragments were determined to remove defective fragments generated by PCR errors.

For the luciferase assay, 5 × 104 EL4 LAf (Figs. 2, 3) and EL4 BO2 (Fig. 8) cells were transfected by Gene Pulser Xcell (Bio-Rad) with 5 μg luciferase reporter plasmids and 2 μg pRL-TK (Promega) as a internal control plasmid and cultured for 24 h in six-well plates. When required, cells were activated with plate-coated anti-CD3 (5 μg/ml in PBS). Cells were harvested, and promoter or enhancer activity was analyzed by the dual-Luciferase reporter assay system (Promega). Foxp3, NF-κB p50, p65, and c-Rel expression plasmids were transfected using pCMV-neo vector (expression is regulated by EF1α promoter). To remove the IκB homologous from p50, p50 cDNA encoding 1-423 aa was PCR amplified and cloned into pMFe-neo vector. For cotransfection experiments, 4 μg GITr promoter-enhancer plasmid was cotransfected with 4 μg each expression plasmid indicated in Fig. 8. Total DNA amount was adjusted with the empty pMFe-neo vector. EL4 BO2 subclone was used for this assay.

DNase I hypersensitive assay
Isolated nuclei were treated with DNase I (0–25 U/ml) at 25°C for 5 min in 60 mM KCl, 15 mM NaCl, 5 mM MgCl2, 0.1 mM EDTA, 15 mM Tris-HCl (pH 7.4) 0.5 mM DTT, 5% glycerol, and 10% sucrose, and DNAs were isolated and digested with KpnI, Sphi, EcoRI, XbaI, Xhol, or Sall. DNase I hypersensitive sites were analyzed by Southern blot hybridization, and probe positions for (KpnI digestion) are indicated in Supplemental Fig. 2.

EMSA
EMSA was performed with 32P-labeled probes and 2 μg nuclear extracts in 20 μl EMSA reaction buffer (2 μg poly(dI-dC)/poly(dI-dC) acid, 20 μM HEPS [pH 7.9], 1 μM MgCl2, 40 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 12% glycerol). Nuclear extracts were prepared from nonactivated or CD3-activated (for 1.5 and 24 h) EL4 cells as described previously (20). To perform competition assays, 100-fold excess of unlabeled competitor oligonucleotides was added to EMSA reaction mixture. To perform supershift assay, nuclear extracts in EMSA reaction buffer were incubated for 15 min with anti-Spi (Santa Cruz Biotechnology, PEP2), anti–Sp3 (Santa Cruz Biotechnology, D-20), and anti–NF-κB p50 (Santa Cruz Biotechnology, D-17), and anti–NF-κB p65 (Santa Cruz Biotechnology, C-20), and probes were added into the reaction mixture.

Chromatin immunoprecipitation
Chromatin immunoprecipitation (ChIP) assay was performed using Pan T cells, CD4+CD25+ T cells, TGF-β–induced iTreg (93% purity), and CD4+CD25+ Foxp3+ iTreg cells (purity >95%) as described previously (20). These cells were prepared (for 10 min at room temperature in 1% formaldehyde, 4.5 mM HEPS [pH 8.0], 9 mM NaCl, 0.09 mM EDTA, and 0.045 mM of ETA) and sonicated (Bioruptor) in lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris-HCl [pH 8.0]) with protease inhibitor (Sigma-Aldrich, P8340). Precleared lysates were incubated overnight at 4°C with polyclonal anti–acetyl histone H4 (Millipore), anti-NF-κB p50 (Abcam, anti–p105/p50/ChIP grade), or control rabbit IgG (Santa Cruz Biotechnology). DNA fragments were isolated from the immunoprecipitated chromatin and analyzed by real-time PCR with SsoFast EvaGreen Supermix (Bio-Rad). PCR primers for ChIP were used as follows: b1/ b2, forward, 5′-TATACCTGGAACCCACAGGTGCG-3′, reverse, 5′-TGTCGCTTCAAGCGAGTATA-3′; b3, forward, 5′-TGGATTCCAC TCACAGCCGCA-3′, reverse, 5′-GGGACACTGTCCTCAGTAC-3′.

Pull-down assay
A 101-bp DNA fragment containing the b1 and b2 sites was amplified by PCR using biotinylated primers and purified using QIAEX II gel extraction system (Qiagen). Purified probes (500 ng) were mixed with nuclear extract (50 μl) in 400 μl binding buffer (60 μM KCl, 15 mM HEPS [pH 7.9], 4 mM Tris-HCl [pH 8.0], 1 mM EDTA, and 12% glycerol) containing 1.66 mM DTT, 0.06% BSA, and 20 μM poly(d-dC) acid at 4°C for 2 h. When required, 100 pmol Sp1 binding oligo from CD40 promoter (22) and 500 pmol indicated competitor oligonucleotides were added. Then, precleared streptavidin–agarose beads (Life Technologies) were mixed with the DNA-nuclear extract mixture for 2 h. The streptavidin–agarose beads were then washed five times with 1 ml binding buffer, and then 2× SDS sample buffer was added. The samples were analyzed by immunoblotting. PCR primers used were as follows: bK, forward, 5′-TATACCTGGAACCCACAGGTGCG-3′, reverse, 5′-TGTCGCTTCAAGCGAGTATA-3′; b3, forward, 5′-TGGATTCCAC TCACAGCCGCA-3′, reverse, 5′-GGGACACTGTCCTCAGTAC-3′.

Immunoprecipitation and immunoblotting
A nuclear extract was prepared from a p50 (1–423 aa)–Foxp3 (FLAG-tagged) transfectant. Nuclear extract (10 μg) was preincubated with protein G (Invitrogen) for 3 h in 5% glycerol, 12 mM HEPS (pH 7.9), 4 mM Tris (pH 8.0) 60 mM KCl, 0.1 mM EDTA, 1 mM DTT, poly(dI-dC)-poly(dI- dc) acid 20 μg, and 4 μl proteinase inhibitor (Sigma-Aldrich, P8340) with or without 92-bp DNA (16–108, Fig. 3). Anti-p50 (2 μg, Santa Cruz Biotechnology, C-19) or control goat IgG was added and incubated at 4°C overnight. Immunoprecipitated nuclear extracts were washed twice with 1 ml prewarmed binding buffer and analyzed by immunoblotting.

Immunoblotting was performed with anti-p50 (Santa Cruz Biotechnology, C-19), anti-p65 (Santa Cruz Biotechnology, C-20), anti-c–Rel

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CD3 response elements might be located near CD3-activated served in promoters and enhancers. Regulatory regions containing sites (HSs) were generated by disruption of nucleosome chromatin CD3 activation response elements in activated T cells. constitutively regulated through this region, suggesting that this gene Fig. 1). Gitr basal promoter activity seems, therefore, to be con- tivity was reduced to basal levels by deleting 39 bp from the 1.22-kb Gitr promoter. Regardless of activation, promoter ac- tivity was detected in both nonactivated and CD3-activated EL4 cells, but no CD3 activation response element was identified within the EL4 T cell line (which is known to retain many features of T cells), and we characterized the regulatory region in activated T cells. We then determined whether that regulatory region was functional in both TGF-β iTreg (23, 24) and nTreg and thereafter proceeded to investigate the molecular basis for the upregulation in Foxp3+ Treg. Because Foxp3 gene expression is maintained by transcription factors induced by TCR-CD3 signaling, these factors should translocate into the nuclei of Foxp3+ Treg. Knowing that Foxp3 can associate with these activated factors in Treg (8–10, 25), it was then conceivable that the Foxp3-responsive regulatory region coincides with those regions involved in CD3 activation. ox40 gene expression can be upregulated by CD3 activation in both T cells and EL4 cells (20), and as shown in Fig. 1A, Gitr gene expression is also upregulated in both cell types, suggesting that expression of these genes in primary T cells and the EL4 cell line is regulated by the same regulatory mechanisms. Regulation of Foxp3 gene expression in T cells and our EL4 subclone was also comparable (26). These data suggest that the EL4 T cell line can be used to probe Gitr and ox40 gene expression and that this information is relevant to primary Teff and iTreg.

Identification of an enhancer containing CD3 activation response elements

Using this scheme, we analyzed promoter activity in activated cells. We had previously identified an NF-κB binding site in the Ox40 promoter (20) that was involved in the response to CD3. We used the same approach in the present study to investigate the Gitr promoter by using a luciferase reporter assay with a series of deletion mutants (Pro1 to Pro8 in Fig. 1B). Gitr promoter ac- tivity was detected in both nonactivated and CD3-activated EL4 cells, but no CD3 activation response element was identified within the 1.22-kb Gitr promoter. Regardless of activation, promoter ac- tivity was reduced to basal levels by deleting 39 bp from −90 (Pro3) to −51 (Pro2). Binding of the transcription factor NFκB, which is known to be constitutively and ubiquitously expressed (27), to this region was detected using the EMSA (Supplemental Fig. 1). Gitr basal promoter activity seems, therefore, to be constitutively regulated through this region, suggesting that this gene expression is upregulated by other regulatory regions containing CD3 activation response elements in activated T cells.

To identify the regulatory regions involved, DNase I hyper- sensitive assays were performed. Because DNase I hypersensitive sites (HSs) were generated by disruption of nucleosome chromatin structure by additional factor bindings, some of the HSs are ob- served in promoters and enhancers. Regulatory regions containing CD3 response elements might be located near CD3-activated T cell–specific HSs. We therefore compared positions of DNase I HSs in nonactivated and CD3-activated CD4+ T cells, EL4 cells, and bone marrow–derived dendritic cells (Fig. 1C, Supplemental Fig. 2). Many common HSs were detected in the Gitr/Ox40 gene locus (Fig. 1D). However, three additional sites were observed only in CD3-activated cells (Fig. 1C, 1D, Supplemental Fig. 2), which we term HS1, HS2, and HS3 (HS1 was detected only in CD3-activated CD4+ T cells).

We next examined enhancer activities in regions containing HS1, HS2, or HS3 using a luciferase reporter assay. As shown in Fig. 2A, DNA fragments containing these HSs were integrated downstream of the luciferase gene in the Gitr promoter reporter plasmid (Fig. 1B, Pro8). Promoter activity was enhanced with the fragment containing HS3 in both orientations (Fig. 2A, 2B) in CD3- activated cells but not in nonactivated cells. The enhancer activity was also analyzed using the Ox40 promoter (1.97 kb). Unlike the Gitr promoter, Ox40 promoter activity is itself increased 2-fold in CD3-activated cells (Fig. 2C, Pro) by NF-κB, as previously described (20), but this promoter activity was upregulated far more by this enhancer (Fig. 2C), suggesting strong enhancer activity. Enhancer activity was further analyzed by luciferase assays (Fig. 2A, 2D, 2E). Searching the transcription factor database, we found three potential NF-κB binding sequences in this enhancer region (referred to henceforth as κB1, κB2, and κB3) (Fig. 2D, 2E, gray boxes). Because the enhancer activity was only detected in CD3-activated cells but not resting T cells, we hypothesized that enhancer activity might be regulated by activated NF-κB through these sites. This possibility was investigated by using the 5′ (Fig. 2D) and 3′ (Fig. 2E) deletion mutants of the enhancer. Luciferase activity using the WT enhancer was reduced by dele- tions from +1 to +39 and from +39 to +136 (Fig. 2D). The deleted regions +1/+39 and +39/+136 contain κB1 and κB2 sites, respectively (Fig. 2D). Although the 5′ deletion mutant (+183) con- tained one potential NF-κB site (κB3), the enhancer activity was similar to that of the negative control (Fig. 2D, +183 and no en- hancer). To investigate whether the κB3 site is simply not func- tional, or whether the κB sites cooperate to regulate enhancer activity, we performed luciferase assays using the 3′ deletion mutants (Fig. 2E). Luciferase activity was reduced by the deletion containing the κB3 site (Fig. 2E, +193), suggesting that this 3′ re- gion containing κB3 cooperatively regulates enhancer activity as well. Such cooperative regulation was also deduced from the 3′ deletion mutant +71 containing κB1 but not κB2 and κB3 (Fig. 2E), as the enhancer activity of this deletion mutant was similar to the negative control. These results strongly suggest that the three NF-κB binding sequences, identified using the transcription factor database, are strong candidates as regulatory elements within this enhancer. We then analyzed histone H4 acetylation by ChiP as a marker for open chromatin in the κB1 plus κB2 (κB1 and κB2 cannot be an- alyzed separately by this assay because these two sites are too close to each other) and κB3 regions (Fig. 2F). H4 molecules in T cells became highly acetylated within 24 h of activation, suggesting that the chromatin of these regions opens up after CD3 activation. Histone H4 in these regions was also highly acetylated in nTreg but not in naive CD4+CD25+ T cells (Fig. 2G), suggesting that this regulatory region also functions in nTreg.
potential NF-κB binding sequences in the Gitr promoter/enhancer reporter plasmid (Fig. 3A, 3C). No factor binding to the mutated sequences could be detected by EMSA using a nuclear extract from CD3-activated cells (Fig. 3B). Mutation of any of the potential NF-κB binding sites reduced enhancer activity, and mutating all three sites eliminated it (Fig. 3C), suggesting that all three sites regulate enhancer activity. The kB1 site was identified as the strongest regulatory element in this enhancer because the enhancer activity was reduced by 20% by mutation of the kB1 sequence. However, similar reductions were observed with double mutations at the kB2 and kB3 sites. These results indicate that enhancer activity is cooperatively regulated by the transcription factors binding to kB1, kB2, and kB3 sites.

Transcription factor binding to each kB site was detected by a simple EMSA using a nuclear extract from CD3-activated cells (Fig. 3B). Although all probes encode potential NF-κB binding sequences, binding patterns were not exactly the same. We therefore investigated transcription factor binding in more detail (Fig. 4).

First, we performed EMSA using nuclear extracts from nonactivated and CD3-activated cells (Fig. 4A). We detected both constitutive and induced binding to the kB1 and kB2 probes, but only induced binding to the kB3 probe (Fig. 4A). As shown in Supplemental Fig. 3, the constitutively active transcription factors binding to kB1 and kB2 probes were Sp1 and Sp3, which bind to sequences overlapping the NF-κB binding sites. Binding of the same induced factors to all probes was suggested by inhibition of binding to the 32P-labeled kB3 probe with excess of the “cold” kB1 and kB2 competitors (Fig. 4B). Because histone H4 acetylation in this enhancer region was induced by CD3 activation (Fig. 2F), binding of the induced transcription factors is likely to be the key to regulation. To identify binding of NF-κB to the kB1 and kB2 sites, excess cold competitor encoding an Sp1 binding sequence in the CD40 promoter (22) was added to block Sp1 and Sp3 binding to the labeled probe. We confirmed NF-κB p50 binding to these sites by supershift EMSA using anti–NF-κB p50. All complexes of transcription factors and the probes were supershifted with anti–NF-κB p50, and some of complexes were supershifted with anti–NF-κB p65, but unmoving complexes were still detected with this anti-p65 (Fig. 4C). This suggests that all complexes contain p50; in other words, all kB sites were bound by p50 homodimers, p50/p65 heterodimers, and/or p50 heterodimers with other transcription factors including other NF-κB members (as shown later, binding of c-Rel and Bcl-3 was detected by a DNA pull-down assay).

Any contribution of Sp1 and Sp3 to this enhancer activity is likely to be minimal, as they are present constitutively. However, it could be that they are bound in nonactivated cells but then replaced by NF-κB on activation, as described for Foxo1 and Foxp3 binding to Foxp3 gene (28).

NF-κB regulates gene expression in the Gitr/Ox40 locus

As shown above, enhancer activity is regulated by NF-κB. Because the EMSA result (Fig. 4C) suggests that NF-κB p50 is a main component of the binding factors (p50 homodimer and heterodimer with other transcription factors), we further analyzed p50 binding in T cells by ChIP. p50 binding to the kB1 plus kB2 and kB3 regions was increased in T cells 24 h after CD3 activation (Fig. 5A). This information, together with previous evidence that NF-κB p50 and p65 bind to the Ox40 promoter (20), indicates that gene expression in the Gitr/Ox40 locus is predominantly regulated by NF-κB p50. To test this, we analyzed Gitr and Ox40 expression in CD3-activated CD4+ T cells isolated from the spleens of mice lacking the Nf-κb p50 gene (Fig. 5B). Gitr and Ox40 expression was upregulated by CD3 activation on CD4+ T cells from both mice, but the Gitr high (36.8%) and Ox40 high (39.3%) cell populations from WT mice were less than half (Gitr high, 15.2%; Ox40 high, 18.7%) of values observed from p50-deficient mice. This result suggests that both Gitr and Ox40 expression are regulated by NF-κB p50. This conclusion was not matched by the observation
that a significant number of Gitr\textsuperscript{high} and Ox40\textsuperscript{high} cells could be detected in p50-deficient mice, despite NF-κB p50 preferentially binding to these NF-κB sites in the enhancer (Fig. 4C) and in the Ox40 promoter (20). We propose that the lack of NF-κB p50 is compensated by binding of a very similar family member, NF-κB p52, as previously suggested using NF-κB p50-, p52-, and p50/p52-deficient mice (29, 30). Unfortunately, the p50/p52 double-deficient mice would not be suitable to test this hypothesis, as these mice have a major problem in bone development and exhibit a profound immunodeficiency (29, 30). To overcome this problem, we analyzed Gitr and Ox40 expression by using NAI. Expression of Gitr and Ox40 RNAs was strongly inhibited by NAI in CD3-activated EL4 cells in a dose-dependent manner (Fig. 5C, 5D), in contrast to the housekeeping gene control (Gapdh) (Fig. 5E). Gitr and Ox40 cell surface expression on CD3-activated T cells was also inhibited by NAI (Fig. 5F). These results indeed suggest that Gitr/Ox40 expression depends on NF-κB.

NF-κB p50 regulates enhancer activity in Treg

Upregulation of Gitr and Ox40 expression in Foxp3\textsuperscript{+} Treg has been previously demonstrated (2–4). The enhancer we have identified in the present study might be involved in this process. To assess whether this enhancer functions in iTreg and nTreg, we examined NF-κB p50 binding to this region by ChIP assay in these cells. p50 binding to the enhancer (κB1 plus κB2 and κB3 regions) was detected in nTreg, but not in naive CD4\textsuperscript{+}CD25\textsuperscript{−} T cells (Fig. 6A). iTreg were generated using CD4\textsuperscript{+}CD25\textsuperscript{−} T cells from Foxp3-GFP reporter mice by stimulation with TGF-β plus anti-CD3 plus anti-CD28; 37% cells were Foxp3\textsuperscript{+} iTreg after 48 h, and these were isolated (93% purity) using cell sorting and GFP fluorescence (Fig. 6B). p50 was bound to the enhancer in these Foxp3\textsuperscript{+} iTreg, but not in CD4\textsuperscript{+}CD25\textsuperscript{−} T cells (Fig. 6B).

We also examined Gitr expression in CD4\textsuperscript{+}Foxp3\textsuperscript{+} Treg isolated from p50-deficient mice. Because Ox40 promoter activity is regulated by NF-κB, and NF-κB p50 binding to the promoter is detectable by ChIP assay (20), we investigated only Gitr expression to avoid any confusion. Splenocytes and thymocytes from these mice had a larger proportion of CD4\textsuperscript{+}Foxp3\textsuperscript{+} T cells with low Gitr expression than did those from control WT mice (Fig. 6C). This supports the argument that p50 is involved in upregulating Gitr expression in Treg. We speculate, therefore, that those Gitr\textsuperscript{high}CD4\textsuperscript{+} Foxp3\textsuperscript{+} T cells in p50-deficient mice are generated by com-

FIGURE 2. Identification of an enhancer in CD3-activated T cells and Treg. Assays were performed with nonactivated (Non) and CD3-activated (CD3) cells. (A) Structures of the promoter/enhancer reporter plasmids used in (B)–(E) are illustrated. DNA fragments containing HS1, HS2, or HS3 sites were inserted downstream of the luciferase gene in both orientations (AS, antisense; S, sense) and these plasmids were used in (B). The reporter plasmid containing the enhancer in sense orientation was used in (C)–(E). Luciferase assays were performed using EL4 cells under indicated conditions. (B) Luciferase reporter plasmids were constructed using the Gitr promoter as shown in (A). Luciferase activities were compared with that given by the negative control vector (Basic, no promoter and no enhancer). (C) The enhancer activity was detected with both the Ox40 and the Gitr promoter. The promoter in the Gitr promoter/enhancer with HS3 (sense) plasmid [shown in (A)] was swapped with the Ox40 promoter, and the luciferase assay was performed using the Ox40 and Gitr promoter (Pro) and the Ox40 and Gitr promoter/ enhancer (Pro plus HS3) reporters. (D and E) Luciferase assays were performed using the WT enhancer and 5′ deletion mutants (D) and 3′ deletion mutants (E). These deletion mutants are illustrated with the possible NF-κB sites indicated by gray boxes (κB1, κB2, and κB3). (F) Acetylation of histone H4 molecules in the chromatin region containing the κB1 plus κB2 and κB3 sites in CD3-activated T cells (T cells were activated by anti-CD3 for indicated times) was analyzed by ChIP using anti-acetyl-histone H4 (AcH4) or control IgG. (G) Acetylation of histone H4 was also analyzed using CD4\textsuperscript{+}CD25\textsuperscript{−} T cells (CD25\textsuperscript{−}) and nTreg as described in (F). Data are representative of more than three (B–G) independent experiments (error bars indicate the SD of triplicate samples).
pensatory NF-κB p52. Taken together, we conclude that Gitr expression is upregulated by the enhancer in conjunction with p50 molecules in both nTreg and iTreg.

Foxy3 binds both to p50 and to the enhancer DNA

Because the enhancer seems to be the key regulatory region in the locus, it is likely that Gitr and OX40 expression is further upregulated in Treg by Foxp3-mediated mechanisms operating through the enhancer. We noticed that within the enhancer the κB1 binding sequence is followed by a potential Foxp3 binding sequence, analogous to the NFAT-Foxp3 binding site in the IL-2 promoter (8) (Fig. 7A, Supplemental Fig. 3D). p50 binding to the κB2 site was weak, yet this site also possesses a similar arrangement analogous to NFAT/Foxp3 binding to the IL-2 promoter (but note to the κB probe was detected (Fig. 7C), and both binding to the enhancer probe was completely inhibited by the κB1 competitor (containing the κB1 binding site and the potential Foxp3 binding site, Fig. 7A; Fig. 7C, κB1), suggesting that p50 and Foxp3 bind to the competitor. Foxp3 binding was also inhibited by a Foxp3 competitor lacking the κB1 binding site but containing the potential Foxp3 binding site, Fig. 7A; Fig. 7C, Foxp3); this indicates that Foxp3 binds to the overlapping region containing the potential Foxp3 binding site (Fig. 7A). Because Foxp3 binds to both NFAT and the IL-2 promoter (Supplemental Fig. 3D), we examined whether Foxp3 binds directly to enhancer DNA and/or in association with p50. To examine this, p50 binding to the enhancer was inhibited in the presence of a competitor containing only a p50 binding sequence from the CD40 promoter (CD40–NF-κB competitor) (22) (Fig. 7A, 7D), but Foxp3 binding was minimally affected (Fig. 7D); this suggests that Foxp3 binds to DNA in the enhancer. However, p50 binding to the enhancer was not completely inhibited by a 500-fold excess of the CD40–NF-κB competitor (containing only NF-κB site) (Fig. 7D), but was completely inhibited by the κB1 competitor (containing NF-κB plus Foxp3 binding sites) (Fig. 7C). This suggests that a p50/CD40–NF-κB competitor complex binds to Foxp3 on the probe (i.e., a protein–protein interaction), as shown in Supplemental Fig. 3E. This possibility was supported by a coimmunoprecipitation experiment where FLAG-tagged Foxp3 was coprecipitated with anti-p50 without DNA (Fig. 7E). Foxp3 seems therefore to bind to both p50 and DNA in the enhancer (Fig. 8D).

Foxp3 upregulates gene expression in conjunction with p50

We confirmed that p50/Foxp3 binds to the enhancer in a manner analogous to NFAT/Foxp3 binding to the IL-2 promoter (but note

FIGURE 3. Three NF-κB binding sites in the enhancer core sequence. (A) DNA sequence of the enhancer core (286 bp) is shown. NF-κB binding sites (κB1, κB2, and κB3) are indicated in bold. Transcription factor binding to κB1, κB2, and κB3 probes (probe positions are underlined) with or without mutations (indicated by italic capital letters for the κB sites) were used for EMSA in (B) and Fig. 4, and enhancer activity with mutations of the κB sites were analyzed in (C). (B) Transcription factor binding to the WT and mutant κB probes [shown in (A)] were analyzed using the indicated probes and a nuclear extract from CD3-activated EL4 cells with anti-CD3 for 1.5 h. (C) Enhancer activity was analyzed using the enhancer fragment with or without mutations shown in (A). Luciferase assays were performed in nonactivated (Non) and CD3-activated (CD3) EL4 cells using the indicated plasmids, and activities were compared with the negative control plasmid (no promoter and no enhancer; Basic). Positions of the potential NF-κB binding sites (κB1, κB2, and κB3) are indicated by gray boxes. The mutated sites are indicated by ×. Data are representative of more than three (B, C) independent experiments (error bars indicate the SD of triplicate samples).

FIGURE 4. NF-κB binding to the enhancer. Binding of transcription factors to the enhancer region was analyzed by EMSA performed using 32P-labeled indicated probes (Fig. 3A). (A) EMSA using nuclear extracts from nonstimulated (Non) and CD3-activated (CD3) (24 h activation) EL4 cells. (B) Competition EMSA using the κB3 probe with a nuclear extract prepared from CD3-activated (1.5 h) EL4 cells. Added cold competitors (100-fold excess) are indicated by +. (C) Supershift EMSA using a nuclear extract prepared from CD3-activated (1.5 h) EL4 cells, indicated probes, and anti–NF-κB p50 (anti-p50) and anti–NF-κB p65 (anti-p65). To block Sp1 and Sp3 binding to the κB1 and κB2 probes, cold Sp1 competitor (100-fold) was added to the reaction mixture. Added Abs are indicated by +. Data are representative of more than three independent experiments (A–C).
that Foxp3 represses IL-2 expression. We investigated the functional relevance of this binding using a luciferase reporter assay. Hori et al. (31) have demonstrated that Gitr expression is upregulated by coexpression of p50 and/or c-Rel (Fig. 8A) and p50 (Fig. 8B) in conjunction with Foxp3. It is well established that Foxp3 can bind to other transcription factors and inhibit activities of these factors (as shown in Fig. 8A, 8B, the activity of the p65 and c-Rel subunits of NF-κB was inhibited by coexpression of Foxp3). However, the enhancer activity was upregulated by coexpression of the p50 subunit of NF-κB with Foxp3. Foxp3 seems to stabilize binding of the NF-κB p50 to this enhancer. To explain how Foxp3 might stimulate p50 binding to the enhancer probe (Fig. 7C), suggesting that Foxp3 stabilizes p50 binding by itself binding to both p50 and DNA (Fig. 8D), thereby enabling accumulation of p50/p65 and p50/c-Rel heterodimer (Fig. 8E) and/or p50/p50 homodimer/ Blc-3 (Supplemental Fig. 3F) to the enhancer. To be able to understand the whole picture of the Foxp3-mediated transcriptional upregulation, further investigation is obviously required. However, it is clear that the activation function of the transactivation domains of p65 and c-Rel do not seem to be inhibited by such indirect binding of Foxp3 mediated via p50. This would be consistent with the enhanced luciferase activity seen when all components (Fig. 8A, p50, p65, and Foxp3; Fig. 8B, p50, c-Rel, and Foxp3) were coexpressed.

**Discussion**

We have identified a strong enhancer within the Gitr/Ox40 locus that is regulated by NF-κB and Foxp3. Gitr gene expression is upregulated by NF-κB through this enhancer in activated Teff, and further upregulated by NF-κB in conjunction with Foxp3 in Treg. It is well established that Foxp3 can bind to other transcription factors and inhibit activities of these factors (as shown in Fig. 8A, 8B, the activity of the p65 and c-Rel subunits of NF-κB was inhibited by coexpression of Foxp3). However, the enhancer activity was upregulated by coexpression of the p50 subunit of NF-κB with Foxp3. Foxp3 seems to stabilize binding of the NF-κB p50 to this enhancer. To explain how Foxp3 might stimulate p50-mediated transcription while lacking a transactivation domain (38), we suggest that p50 associates with other family members (e.g., through a p50/p65 heterodimer, p50/c-Rel heterodimer, or p50 homodimer/Bcl-3). In support of this, we detected p65, c-Rel, and Bcl-3 binding to the κB probe (Fig. 7C). We then asked what additional necessary part Foxp3 might play. We analyzed p50 binding to the κB probe with or without Foxp3. We considered using a nuclear extract from CD3-activated T cells (no Foxp3), but the amount of p50 in this nuclear extract is different from that in Foxp3+ T cells (stimulated with TGF-β). We therefore trapped Foxp3 protein in the same nuclear extract by adding a Foxp3 competitor (Fig. 7C) and performed a DNA pull-down assay using the κB probe. p50 binding to the enhancer probe was reduced as a result of inhibition of Foxp3 binding to the probe (Fig. 7C), suggesting that Foxp3 stabilizes p50 binding by itself binding to both p50 and DNA (Fig. 8D), thereby enabling accumulation of p50/p65 and p50/c-Rel heterodimer (Fig. 8E) and/or p50/p50 homodimer/ Bcl-3 (Supplemental Fig. 3F) to the enhancer. To be able to understand the whole picture of the Foxp3-mediated transcriptional upregulation, further investigation is obviously required. However, it is clear that the activation function of the transactivation domains of p65 and c-Rel do not seem to be inhibited by such indirect binding of Foxp3 mediated via p50. This would be consistent with the enhanced luciferase activity seen when all components (Fig. 8A, p50, p65, and Foxp3; Fig. 8B, p50, c-Rel, and Foxp3) were coexpressed.
regulated by negative feedback in activated T cells, we detected NF-κB p50, p65, and c-Rel in 72 h–stimulated T cells with TGF-β plus anti-CD3 plus anti-CD28. Nuclear translocation of these transcription factors sustained in Treg through TGF-β signaling and/or a Foxp3-mediated pathway. In CD3-activated T cells, nuclear p65 levels peak 2–6 h after activation (39, 40). Although we detected p65 binding to the κB probe using a nuclear extract from 72 h–stimulated T cells, p65 levels may have diminished from those found earlier after activation. c-Rel levels seem to remain high even after p65 decreased (40). The Foxp3 promoter activity is regulated by c-Rel (34), and nTreg development is also regulated by c-Rel through CNS3 in the Foxp3 gene (36). Taken together, p50/c-Rel heterodimers may be a key component in upregulating Gitr gene expression. Alternatively, transactivation domains might also be supplied by an IκB family member Bcl-3, which can bind to the p50 homodimer (41, 42) (Supplemental Fig. 3F). Gene expression upregulated by the p50 homodimer/Bcl-3 complex seems to depend on NF-κB sites (the p50 homo-

FIGURE 6. NF-κB p50 regulates Gitr expression in Treg. (A) p50 binding to the κB1 plus κB2 and κB3 regions in nTreg and CD4⁺CD25⁺ T cells were analyzed by ChIP using anti-p50 (p50) and control IgG. (B) CD4⁺CD25⁺ T cells from Foxp3-GFP reporter mice were cultured with TGF-β plus anti-CD3 plus anti-CD28 for 48 h, and Foxp3⁺ iTreg were isolated by sorting with GFP (93% were Foxp3⁺ T cells). p50 binding to the κB1 plus κB2 and κB3 regions in iTreg and CD4⁺CD25⁺ T cells was analyzed by ChIP using anti-p50 (p50) and control IgG. (C) Splenocytes and thymocytes were isolated from p50-deficient (p50 KO) and WT mice. These cells were stained with anti-CD4, anti-Foxp3, and anti-Gitr. Gitr expression in CD4⁺Foxp3⁺ population was analyzed. Data are representative of more than three (A–C) independent experiments (error bars indicate the SD of triplicate samples).

FIGURE 7. Binding of NF-κB p50 and Foxp3 to κB sites. (A) DNA sequences of the κB1, Foxp3, and CD40 NF-κB (from CD40 promoter) (CD40) competitors. NF-κB binding sequences are indicated by bold, and a potential Foxp3 binding sequence is indicated by bold italic. (B) DNA pull-down assays were performed using a biotinylated enhancer probe containing the κB1 plus κB2 sites (enhancer) or the same length of the Gitr promoter probe and a nuclear extract prepared from stimulated CD4⁺CD25⁺ T cells with TGF-β plus anti-CD3 plus anti-CD28. The coprecipitated proteins were analyzed by immunoblotting with anti-p50, anti-Foxp3, anti-p65, anti-c-Rel, and anti-Bcl-3. (C) DNA pull-down assays were performed with a biotinylated κB probe and the nonbiotinylated CD40 NF-κB competitor [shown in (A)]. Coprecipitated proteins were analyzed by immunoblotting with anti-p50, anti-Foxp3, anti-p65, anti-c-Rel, and anti-Bcl-3. (D) DNA pull-down assays were performed with a biotinylated κB probe and the nonbiotinylated CD40 NF-κB competitor [shown in (A)].Coprecipitated proteins were analyzed by immunoblotting with anti-p50 and anti-Foxp3. (E) p50 was precipitated by anti-p50 or control IgG (without DNA), and coprecipitated FLAG-tagged Foxp3 was detected using an anti-FLAG. Data are representative of more than three (B–E) independent experiments.
NF-κB p50 and Foxp3 cooperate to upregulate enhancer activity. (A and B) The Gitr promoter-enhancer luciferase reporter plasmid (Pro8 + Enhancer) was cotransfected with empty vector, p50, and/or p65 (A) or c-Rel (B) expression plasmids with or without Foxp3 expression plasmid. Cotransfected expression plasmids are indicated under the graph. Total amount of DNA for transfection was adjusted by addition of DNA from the empty vector. Luciferase activity with negative control plasmid (Basic; no promoter and no enhancer) and Pro8 (no enhancer) are also shown. *p < 0.05, **p < 0.005 (Student t test). (C) Gitr promoter-enhancer luciferase reporter plasmid (Pro8 + Enhancer) was cotransfected with 4 μg p50 expression plasmid (p50) and different amounts of Foxp3 expression plasmid (0–8 μg). DNA amounts of the expression plasmids are shown under the graph. The total amount of DNA was adjusted with addition of DNA from the empty vector. (D) Model of NF-κB p50 and Foxp3 binding to the κB1 site. (E) Model of NF-κB p50, Foxp3, and NF-κB p65 or c-Rel binding to the κB site. Data are representative of more than three (A–C) independent experiments (error bars indicate the SD of triplicate samples).
Disclosures

The authors have no financial conflicts of interest.

References


