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

Yukiko Tone,*† Yoko Kidani,*† 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 Clda4, 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 IkB families. These findings may explain how Foxp3 can activate expression of certain genes while suppressing others. The Journal of Immunology, 2014, 192: 3915–3924.

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Lucocorticoid-induced TNFR (Gitr; Tnfrsf18) and Ox40 (CD134, Tnfrsf4) are members of the TNFR superfamily (1) and are expressed on T cells. Expression of both receptors is upregulated by activation and, in particular, kept constitutively high on CD4+CD25+Foxp3+ regulatory T cells (Treg) (2–4), cells essential to the prevention of autoimmune disease and other forms of immunopathology (5–7). The development and function of Treg are regulated by the transcription factor Foxp3, which represses many genes by associating with other transcription factors, including NFAT (8), the p65 subunit of NF-κB (9), and Runx1 (10). However, a number of other genes such as Clda4, Il35, Cd25, Ox40, and Gitr are upregulated in Foxp3+ Treg (2, 11), with all of these gene products playing essential roles in Treg function (12). Signaling through Gitr and Ox40 has been shown to neutralize the suppressive effects of Treg in vitro (3, 13). Additionally, Ox40 signaling inhibits induced Treg (iTreg) development in periphery by blocking TGF-β/βcTCR signal–mediated induction of Foxp3 (14, 15). The Gitr and Ox40 genes are particularly interesting as models for Foxp3-mediated transcriptional upregulation, because they are located within a short 15.1-kb stretch of the mouse genome, suggesting control by a common regulatory region. We have previously shown a similar clustering of Ms4A gene family members, also upregulated by Foxp3 (16), where we postulated the existence of a comparable Foxp3-associated regulatory region at that gene locus. We selected the Gitr/Ox40 rather than the Ms4A gene locus for study because the latter is far larger, extending over 600-kb, and is therefore more challenging for analysis.

Not only are Gitr and Ox40 influential in Treg, but they also act as costimulatory receptors for effector T cells (Teff) (1, 17–19). Gitr and Ox40 are unique molecules playing seemingly different roles in Teff and Treg, with the distinct functions possibly determined by expression levels of these receptors on each T cell subset (low on Teff and high on Treg). In either case, the mechanisms underlying gene expression remain poorly understood, although we know that Ox40 expression is regulated by constitutively active transcription factors and upregulated by NF-κB in activated Teff (20). To define the molecular mechanisms responsible for upregulation of Gitr and Ox40 in more detail, in this study we sought clues within the Gitr/Ox40 gene locus in both Teff and Treg.

We identify an enhancer located downstream of the Gitr gene. Histone H4 molecules in this region are highly acetylated both in activated T cells and Foxp3+ Treg. We show that enhancer activity is regulated by NF-κB in activated Teff, and by NF-κB in conjunction with Foxp3 in Treg. We propose that Foxp3 stabilizes binding of the p50 subunit of NF-κB to the enhancer. Although p50 does not contain a transactivation domain, the p50/Foxp3 complex on the enhancer interacts with other members of the NF-κB and IkB family (e.g., p65, c-Rel, B cell lymphoma 3 [Bcl-3]) to supply transactivation domains to the complex. This may explain how Foxp3 can suppress expression of many genes while also upregulating 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
CD4+CD25+ Treg cells were purified by a cell sorter using anti-CD4 (RM4-5, ebioscience) and anti-CD25 (PC61.5, ebioscience) from preisolated CD4+ T cells, isolated CD4+CD25+ T cells were analyzed by an internal Foxp3 staining kit (ebioscience), and Foxp3+ T cells were >95%. When required, T cells were stimulated with plate-coated anti-CD3 (KT3, 5 μg/ml, eBioscience). To generate Foxp3+ T cells, Foxp3+ T cells were isolated from wild-type (WT) and Foxp3-3GP reporter mice (The Jackson Laboratory) and stimulated with TGF-β (5 ng/ml) (PeproTech), anti-CD28 (1 μg/ml), and anti-CD3 (plate-coated). To isolate Foxp3+ Treg, the stimulated cells were sorted with GFP. Splenocytes and thymocytes were isolated from Njkb p50-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). EL4 and CD4+ T cells were cultured with NF-κB activation inhibitor (NAI; EMD Biosciences) in DMSO. These cells were pretreated with NAI (1 μM) and stimulated with TGF-β (1 μg/ml) for 40 h, and NF-κB (Santa Cruz Biotechnology), anti-Sp3 (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.

Construction of luciferase reporter plasmids and luciferase assay
A DNA fragment (1.22 kb) of the GIT 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 degraded fragments generated by PCR errors. To construct enhancer luciferase reporter plasmids, Pro8 GIT 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 pO4x0 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 in to the Sall site of the GIT promoter 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 in the enhancer sequence (Fig. 3A) were introduced into the enhancer fragment by PCR assemble procedure, and the mutated fragments were integrated in to the Sall site in the GIT promoter reporter 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 pGL3-TK (Promega) as an 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 protein G (Invitrogen) 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 pCMV-neo vector. For cotransfection experiments, 4 μg GIT promoter-enhancer plasmid was cotransfected with 4 μg each expression plasmid indicated in Fig. 8. Total DNA amount was adjusted with the empty pMFeo-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, XhoI, or SalI. 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 IP-labeled probes and 2 μg nuclear extracts in 20 μl EMSA reaction buffer (2 μg poly(dex20S)-poly(dex20S) acid, 20 mM HEPES [pH 7.9], 1 mM 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-Sp1 (Santa Cruz Biotechnology, PEP2), anti-Sp3 (Santa Cruz Biotechnology, D-20), 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 iTRG (93% purity), and CD4+CD25+Foxp3+ nTreg cells (purity >95%) as described previously (20). These cells were fixed (for 10 min at room temperature in 1% formaldehyde, 4.5 mM HEPES [pH 8.0], 9 mM NaCl, 0.09 mM EDTA, and 0.045 mM EGTA) and sonicated (Bioruptor) in lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris–HCl [pH 8.0]) with proteinase 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: Rbp1/TAR3, forward, 5′-TTACATGGAACCAACAGCAGTGG-3′, reverse, 5′-TGCCCCTGTTAATGCGGCTG-3′; Rbp1/TAR8, forward, 5′-GTGGATTT-3′, reverse, 5′-GGATTT-3′.

Pull-down assay
A 101-bp DNA fragment containing the Rbp1 and Rbp2 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 μg) in 400 μl binding buffer (60 μM KCl, 15 mM HEPES [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 μg poly(dI-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: Rbp1, forward, 5′-TTACATGGAGAACCAACAGCAGTGG-3′, reverse, 5′-TGCCCCTGTTAATGCGGCTG-3′; Rbp1, forward, 5′-TTACATGGAGAACCAACAGCAGTGG-3′; reverse, 5′-TGCCCCTGTTAATGCGGCTG-3′; C3, forward, 5′-TGACCCTCACGCGACTCATCCACC-3′, reverse, 5′-GGCCCATGTCCTCCATGAC-3′.

Immunoprecipitation and immunoblotting
A nuclear extract was prepared from a p50 (1–423 aa)-Foxp3 (FLAG)-tagged transfectant. Nuclear extract (10 μg) was preclotted with protein G (Invitrogen) for 3 h in 5% glycerol, 12 mM HEPES (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 0 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 and then preincubated with magnetic anti-GFP–conjugated beads (Amersham Pharmacia, Arlington, TX). Beads were washed with the 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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sensitive assays were performed. Because DNase I hypersensitive
CD3 activation response elements in activated T cells.

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Fig. 1). Gitr basal promoter activity seems, therefore, to be con-
activity was detected in both nonactivated and CD3-activated EL4
cells, but no CD3 activation response element was identified within
of deletion mutants (Pro1 to Pro8 in Fig. 1B). Gitr promoter ac-
used the same approach in the present study to investigate the
Ox40 promoter (20) that was involved in the response to CD3. We
had previously identified an NF-

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luciferase reporter assays were performed using mutations in the
regions +1/+39 and from +39 to +136 (Fig. 2D). The deleted
mutants (Fig. 2E). Luciferase activity was reduced by the deletion
fragment containing HS3 in both orientations (Fig. 2A, 2B) in CD3-
activated cells but not in nonactivated cells. The enhancer activity
also was 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-kB, 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-kB binding sequences in this enhancer
region (referred to henceforth as kB1, kB2, and kB3) (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-kB
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 kB1 and kB2 sites, respec-
tively (Fig. 2D). Although the 5‘ deletion mutant (+183) con-
tained one potential NF-kB site (kB3), the enhancer activity was
similar to that of the negative control (Fig. 2D, +183 and no en-
hancer). To investigate whether the kB3 site is simply not func-
tional, or whether the kB2 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 kB3 site (Fig. 2E, +193), suggesting that this 3‘ re-
gion containing kB3 cooperatively regulates enhancer activity as
well. Such cooperative regulation was also deduced from the 3‘
deletion mutant +71 containing kB1 but not kB2 and kB3 (Fig. 2E),
as the enhancer activity of this deletion mutant was similar to the
negative control. These results strongly suggest that the three NF-kB
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 kB1 plus kB2 (kB1 and kB2 cannot be an-
yalyzed separately by this assay because these two sites are too close
to each other) and kB3 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.

NF-kB regulates enhancer activity
As shown in Fig. 2, the 286-bp enhancer sequence (Fig. 3A) en-
codes three potential NF-kB binding sequences. Transcription factor
binding to these sites was analyzed by EMSA in Figs. 3B and 4. To
assess the contribution of these potential NF-kB sites and to inves-
tigate the existence of other regulatory elements in the enhancer,
luciferase reporter assays were performed using mutations in the

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-kB 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 NFI,
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 con-
istitutively 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
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tigate the existence of other regulatory elements in the enhancer,
luciferase reporter assays were performed using mutations in the
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 κB1 site was identified as the strongest regulatory element in this enhancer because the enhancer activity was reduced to half of the wild-type level (Fig. 3C). This result suggests that both Gitr and Ox40 expression are regulated by 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 κB1 plus κB2 and κB3 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 Gitrhigh (36.8%) and Ox40high (39.3%) cell populations from WT mice were less than half (Gitrhigh, 15.2%; Ox40high, 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$^{\text{high}}$ and Ox40$^{\text{high}}$ cells could be detected in p50-deficient mice, despite NF-kB p50 preferentially binding to these NF-kB sites in the enhancer (Fig. 4C) and in the Ox40 promoter (20). We propose that the lack of NF-kB p50 is compensated by binding of a very similar family member, NF-kB p52, as previously suggested using NF-kB 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-kB.

**NF-kB p50 regulates enhancer activity in Treg**

Upregulation of Gitr and Ox40 expression in Foxp3$^{\text{Treg}}$ 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-kB p50 binding to this region by ChIP assay in these cells. p50 binding to the enhancer (kB1 plus kB2 and kB3 regions) was detected in nTreg, but not in naive CD4$^{\text{+}}$CD25$^{\text{+}}$ T cells (Fig. 6A). iTreg were generated using CD4$^{\text{+}}$CD25$^{\text{+}}$ T cells from Foxp3-GFP reporter mice by stimulation with TGF-$\beta$ plus anti-CD3 plus anti-CD28; 37% cells were Foxp3$^{\text{Treg}}$ 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$^{\text{Treg}}$, but not in CD4$^{\text{+}}$CD25$^{\text{+}}$ T cells (Fig. 6B).

We also examined Gitr expression in CD4$^{\text{+}}$Foxp3$^{\text{Treg}}$ isolated from p50-deficient mice. Because Ox40 promoter activity is regulated by NF-kB, and NF-kB 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$^{\text{+}}$Foxp3$^{\text{+}}$ 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$^{\text{high}}$CD4$^{\text{+}}$ Foxp3$^{\text{Treg}}$ T cells in p50-deficient mice are generated by com-
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.

**Foxp3 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 (Supplemental Fig. 3A). We therefore analyzed Foxp3 binding to the κB1 plus κB2 region using a DNA pull-down assay and nuclear extracts from CD4+CD25+ T cells stimulated for 72 h with TGF-β plus anti-CD3 plus anti-CD28. Foxp3 was coprecipitated with the biotinylated κB probe but not a control probe of the same length from the Gitr promoter (Fig. 7B). To confirm Foxp3 binding to the potential site (3′ side of the p50 binding site) (Fig. 7A), we performed a competition assay with κB1 and Foxp3 competitors (i.e., nonbiotinylated double-stranded oligonucleotides shown in Fig. 7A). Stimulation-specific NF-κB p50 and Foxp3 binding to the enhancer 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 coinmunoprecipitation 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. 7D).

**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

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**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 non-activated (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 X. Data are representative of more than three 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 ectopically expressed Foxp3 in CD3-activated T cells (activated by anti-CD3 for indicated times) were analyzed by ChIP using anti-p50 (p50) and control IgG. (B) Gitr and OX40 expression on CD4+ T cells from p50-deficient (p50 KO) and WT mice. Nonstimulated (Non) and CD3-activated (CD3) CD4+ T cells from indicated mice were analyzed by FACS using anti-Gitr and anti-Ox40. The percentages of CD4+Gitrhigh or CD4+Ox40high population are indicated. (C–E) EL4 cells were activated with anti-CD3 and cultured with or without NAI concentrations (nanomolars) are indicated. (C) Gitr, (D) OX40, or (E) Gapdh expression were analyzed by quantitative RT-PCR (expression levels normalized relative to 18S rRNA levels). (F) Gitr and OX40 expression in nonactivated (gray) and CD3-activated (solid line) T cells with or without NAI (100 nM) analyzed by FACS. Data are representative of more than three (A–F) independent experiments (error bars indicate the SD of triplicate samples).

**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 transcriptional 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 kB probe (Fig. 7C). We then asked what additional necessary part Foxp3 might play. We analyzed p50 binding to the kB 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 kB 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 transcriptional 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.](image)

**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.](image)

**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 for 72 h. The coprecipitated proteins were analyzed by immunoblotting with anti-p50 and anti-Foxp3. (C) DNA pull-down assays were performed using the κB probe and nuclear extracts prepared from nonstimulated (Non) and TGF-β plus anti-CD3 plus anti-CD28–stimulated (72 h) CD4⁺CD25⁺ T cells (left panel). Competition DNA pull-down assay was performed using the same probe and nuclear extract (from stimulated cells) and using nonbiotinylated κB1 or Foxp3 competitor [shown in (A), right panel]. 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).
Supplemental Fig. 1. Transcription factor NFI binding to the sequence between −90 and −51 (A) The deleted sequence from Pro3 to Pro2 are shown, and the position of the EMSA probes (P1, P2 and P3) are indicated by underlines. (B) EMSA was performed using $^{32}$P-labeled P1, P2 and P3 probes and a nuclear extract from EL4 cells. (C) EMSA was performed with P3 wild type and mutant probes. DNA sequences of the P3 and its mutant probes are shown under the EMSA result. The transcription factor binding sequences were determined by EMSA and shown under the probe sequence list. This sequence is similar to the consensus sequence of a transcription factor NFI (TTGGCNNNNNGCCAA). (D) Previously, we showed NFI binding to the Gitr ligand (GL) promoter [Tone et al. (2003) PNAS 100, 15059–15064]. EMSA was performed with the GL probe (−). The NFI binding to the GL probe was disappeared with 100-fold excess the GL and the P3 competitors, indicating NFI binding to the P3 sequence. NFI is indeed binding to the Gitr promoter region.
**Supplemental Fig. 2.** DNase I hypersensitive sites in the *Ox40/Gitr* gene locus. (A) Positions of *Ox40* and *Gitr* genes in this locus are illustrated. Exons are indicated by gray boxes. The activation specific DNase I hypersensitive (HS) sites HS1 (green arrow), HS2 (orange arrow) and HS3 (blue arrow) are indicated with common HS sites (black arrows with dotted lines). Positions of the probes used in (B) and (C) are indicated by red bars. (B) DNase I HS assay was performed using non-activated (Non) and anti-CD3 activated (CD3) EL4 cells. Isolated nuclei were partially digested with different concentrations of DNase I (DNase I Conc., indicated by yellow triangles). DNA isolated from these nuclei was digested with *Kpn* I and analyzed by Southern blot hybridization using the indicated probes. Positions of the probes are shown in (A). Activation specific and common bands generated with the HS sites are indicated by arrows with the same colors shown in (A). (C) DNase I HS assay was performed as described in (B). Non-stimulated (Non) and LPS-stimulated (LPS) bone marrow derived dendritic cells (bmDC), and Non-activated (Non) and anti-CD3 activated (CD3) CD4+ T cells were used. Activated T cell specific bands generated with the HS sites are indicated by arrows with the same colors shown in (A).
Supplemental Fig. 3. The enhancer core sequence and binding of transcription factors to κB1 and κB2 sites. (A) The identified three NF-κB binding sites (κB1, κB2, and κB3) are indicated by in Blue. Potential Foxp3 binding sites are indicated in Green. The positions of the 5’-ends of deletion mutants in Fig. 2D are indicated by arrows under the sequence, and the positions of the 3’-ends of deletion mutants in Fig. 2E are indicated by arrows upper the sequence. To analyze Sp1 and Sp3 binding sites, mutations are introduced into either the probe κB1 or κB2 (Sp1-M1 to Sp1-M4), and mutated sequences and the probe names are indicated. (B) EMSA was performed using the κB1 and κB2 probes and a nuclear extract from non-activated EL4 cells with indicated competitors or antibodies (anti-Sp1 and anti-Sp3). Addition of the competitor or the antibody are indicated by +. The results suggest that the same transcription factors were bound to both probes, and that constitutively expressed Sp1 and Sp3 also bound to the probes. Shifted bands with Sp1 and Sp3 are indicated. (C) EMSA was performed using indicated probes. Nuclear extract was prepared from non-activated EL4 cells. Mutated sequences in the κB1 and κB2 probes are shown in (A). Shifted bands with Sp1 and Sp3 are indicated. The Sp1 binding site is largely overlapping with the κB1 NF-κB binding site, and located at the 3’-side of the κB2 site. (D) NF-κB binding sequence (Blue) in the κB1 site and the potential Foxp3 binding sequence (Green) are compared with NFAT (Orange) and FOXP3 (Green) binding sequences in the IL-2 promoter suggested by Wu et al. (2006, Cell, 126, 375-387). (E) A model of binding of NF-κB p50 with the CD40 NF-κB competitor and Foxp3 to the enhancer. (F) A model of binding of NF-κB p50 homodimer, Bcl-3 and Foxp3 to the enhancer. (G) A Ox40 promoter/enhancer luciferase reporter plasmid was co-transfected with the NF-κB p50 expression plasmid with or without the Foxp3 expression plasmid. Luciferase activity generated with negative control plasmid Basic (no promoter and no enhancer) and co-transfection with empty vector (Vector) are also shown. Co-transfected expression plasmids are indicated under the graph. The structure of the reporter plasmid is illustrated. Ox40 promoter activity is regulated by Sp1/Sp3, YY1 and NF-κB (Y. Tone et al. 2007, J. Immunol. 179, 1760-1767), and the relative binding positions are indicated. Relative positions of NF-κB binding sites (κB1 to κB3) in the enhancer are also indicated by gray boxes.