Foxp3 Inhibits RORγt-mediated IL-17A mRNA Transcription through Direct Interaction with RORγt*.†

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The cytokine, transforming growth factor-β1 (TGF-β1), converts naïve T cells into regulatory T cells that prevent autoimmunity. However, in the presence of interleukin (IL)-6, TGF-β1 has also been found to promote differentiation into IL-17-producing helper T (Th17) cells that are deeply involved in autoimmune and inflammation. However, it has not been clarified how TGF-β1 and IL-6 determine such a distinct fate. Here we found that a master regulator for Th17, retinoic acid-related orphan receptor γt (RORγt), was rapidly induced by TGF-β1 regardless of the presence of IL-6. IL-6 reduced Foxp3 expression, and overexpression of Foxp3 in a T cell line resulted in a strong reduction of IL-17A expression. We have characterized the IL-17A promoter and found that RORγt binding is sufficient for activation of the minimum promoter in the HEK 293T cells. RORγt-mediated IL-17A promoter activation was suppressed by forced expression of Foxp3. Foxp3 directly interacted with RORγt through exon 2 region of Foxp3. The exon 2 region and forkhead (FKH) domain of Foxp3 were necessary for the suppression of RORγt-mediated IL-17A promoter activation. We propose that induction of Foxp3 is the mechanism for the suppression of Th17 and polarization into inducible Treg.

T helper (Th)³ cells play a pivotal role in adaptive immunity. Upon stimulation by antigens, Th cells undergo distinct developmental pathways, attaining specialized properties and effector functions. CD4⁺ T cells are traditionally thought to differentiate into Th1 and Th2 cell subsets (1). Recently, a third subset of the polarized T cell subset Th17, characterized by the production of IL-17A, was identified and found to play an important role in autoimmune diseases, elimination of extracellular bacteria, and cancer (2–4). It has been shown that TGF-β1 plays a pivotal role in the induction of Th17 (5). Th17 differentiation of naïve T cells is initiated by IL-6 and TGF-β1 (6–8). In addition, IL-23 as well as IL-21 are thought to be a key cytokine for the maturation and/or maintenance of Th17 cells (9–11). However, TGF-β1 is a suppressor of Th1 and Th2 cell differentiation and drives the conversion of naïve T cells to Foxp3-positive Th cells with a regulatory phenotype, so-called inducible regulatory T cells (iTreg) (12). These observations suggest that the presence or absence of proinflammatory cytokines might lead to opposing immune consequences induced by TGF-β1.

RORγt has been shown to direct Th17 cell differentiation by inducing the IL-23 receptor (13). However, it has not been clarified whether RORγt is directly involved in IL-17A transcription. RORγt could be a STAT3 target since Th17 was not induced in STAT3-deficient T cells (14). STAT3 binding to the RORγt promoter may enhance its expression. However, it has been reported that RORγt can be induced in CD4⁺ T cells in response to TGF-β1 (15). Despite the existence of RORγt, naïve T cells activated by TGF-β1 alone differentiate into iTreg but not Th17. The mechanism in which Th17 development is suppressed by TGF-β1 alone has not been clarified.

CD4⁺CD25⁺Foxp3⁺ Tregs are crucial for the maintenance of immunological tolerance (16, 17). Treg produced in the thymus (naturally occurring Treg) constitutes 3–6% of CD4⁺ T cells (18). More recent studies have shown that Foxp3 is induced in CD4⁺Foxp3⁺ T cells after stimulation with TGF-β1 (19, 20). Foxp3 functions as a transcriptional repressor for various transcription factors such as nuclear factor of activated T cells (NFAT), NFκB, and Runx1 (21–23), which could be a part of the mechanisms for Foxp3-mediated suppression of immune responses.

In this study, using promoter analysis, we found that RORγt potently up-regulates IL-17A reporter activity without any other factors such as NFAT. We identified the two conserved

activated T cells; ZnF, zinc finger; Zip, leucine zipper; FKH, forkhead domain; Luc, luciferase.

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¶ The abbreviations used are: Th, T helper; Th17, IL-17-producing helper T cell; TGF-β1, transforming growth factor-β1; CA-TAR, constitutively activated T cell reporter; iTreg, regulatory T cell; iTreg, inducible Treg; IL, interleukin; RORγt, retinoic acid-related orphan receptor γt; ROE, ROR-responsive element; Foxp3, forkhead box protein 3; RT-PCR, reverse transcription-PCR; ChIP, chromatin immunoprecipitation; Ab, antibody; GAPDH, glycerinaldehyde-3-phosphate dehydrogenase; WT, wild type; NFAT, nuclear factor of
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ROR-responsive elements (ROREs) on the IL-17A promoter, which are essential for RORγt-mediated IL-17A promoter activation. Interestingly, we found that IL-6 is not necessary for the induction of RORγt; TGF-β1 alone can up-regulate RORγt in primary naïve T cells as well as the EL4 T cell line. IL-6 rather suppressed Foxp3 expression in TGF-β1-treated naïve T cells. Thus, we hypothesized that Foxp3 negatively regulates RORγt expression in TGF-β1-induced IL-17A expression. We found that overexpression of Foxp3 reduced IL-17A expression in the EL4 cells and inhibited RORγt-mediated IL-17A promoter activity in the HEK 293T cells. This suppression was partly dependent on the physical interaction between RORγt and Foxp3. Our study provides a novel mechanism for the regulation of Th17 development by Foxp3.

**EXPERIMENTAL PROCEDURES**

Mice—C57BL/6 mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). Six- to twelve-week-old mice were used as experimental animals. All experiments were approved by the Animal Ethics Committee of Kyusyu University.

**Plasmid Constructions**—PCR was done to generate the IL-17A promoter plasmid by using mouse genomic DNA as a template. In the case of IL-17A promoter, an ~6-kb fragment corresponding to nucleotides from −6021 to +37 relative to the determined transcriptional starting site of IL-17A gene was subcloned into pGV-basic 2 vector (TOYOINKI). Series of deletion mutants were generated by restriction enzyme digests (−1812, −613, −301) or by PCR (−239, −153, −115, −94). The deletion mutants, −1812, −613, and −301, were constructed by the use of the restriction sites Bpu1102I, SplI, and PstI, respectively. The point mutations (TGCAAT or GTGGTA) in a putative RORE-1 (TGACCT) and RORE-2 (GTGGTC) were introduced by PCR. The point mutants were generated by using −239 plasmid as a template. The mouse RORγt cDNA was amplified by reverse transcription (RT)-PCR and was subcloned into pCMV7 vector for T7 tag. The Foxp3 mutants were subcloned into pCMV14 vector (Sigma) for FLAG tag. A constitutively activated TGF-β receptor I (CARTβRI) was provided from Dr. Joan Massagué and described previously (24). Transfection into HEK 293T cells and EL4 cells and luciferase assay were described previously (15, 17, 18).

**Naïve T Cell Preparation and Differentiation**—CD4+CD25− T cells were isolated from spleens and lymph nodes by negative selection using magnetic beads (Milteny BioTech) (typically >95% purity). For Th17 differentiation, 1 × 10⁶ CD4+CD25− T cells were cultured with 1 μg/ml plate-bound anti-CD3 antibody (Ab) (clone 145-2C11) and 0.5 μg/ml soluble anti-CD28 Ab (eBioscience) in the presence of 0.2 ng/ml recombinant murine IL-2 (Peprotech) and 10 μg/ml anti-interferon-γ Ab, 10 μg/ml anti-IL-4 Ab in the presence of 2 ng/ml recombinant human TGF-β1 (R&D Systems) and 20 ng/ml recombinant human IL-6 (R&D Systems). IL-6 was omitted for iTreg differentiation condition. Cell surface marker and intracellular cytokine staining were performed as described (25). Cell sorting was performed by fluorescence-activated cell sorter Aria (BD Biosciences) as described (26).

**RT-PCR and Real-time PCR**—The cells were lysed in RNeasy (Takara) for RNA preparation. RT-PCR was performed with a standard procedure. The expression level of GAPDH was first evaluated as an internal control. The primer sequences were as follows: GAPDH, 5′-ACC ACA GTC CAT GCC ATC AC-3′ and 5′-TCCACCACCCTGTTGATGT-3′; TGF-β1, 5′-TAA TGG TGG ACC GCA ACA CAG C-3′ and 5′-GAC GGA ATA CAG GGC TTT CG-3′; Foxp3, 5′-CAC CCA GGA AAG ACA GCA ACC-3′ and 5′-GCA AGA GCT CTT GTG CAT TGA-3′; RORγt, 5′-ACC TCC ACT GCC AGC TGT GTG CTT GC-3′ and 5′-TCA TTT CTG CAC TTC TGC ATG TAG ACT GTC CC-3′; IL-17A, 5′-CAG CAG CGA TCA TCC CTC AAA G-3′ and 5′-CAG GAC CAG GAT CTC TTT CTG-3′. Real-time PCR was performed on cDNA samples using the SYBR Green system (Applied Biosystems). The relative quantification value is expressed as 2−ΔΔCt, where ΔCt is the difference between the mean Ct value of triplicates of the sample and of the endogenous GAPDH control.

**Chromatin Immunoprecipitation (ChIP) Assay**—The ChIP assay was performed using ChIP-IT express (Active Motif) according to the manufacturer’s instructions. The cell extracts were immunoprecipitated with polyclonal rabbit anti-T7 tag Ab (Medical & Biological Laboratories) or polyclonal rabbit antiserum specific for acetylated histone H4 Ab (catalog number 06-866, Upstate Biotechnology) and protein G magnetic beads for 4 h at 4°C. The DNA-histone complexes were incubated for 15 min at 94°C for reverse cross-linking and then treated with protease K. Purified DNA fragments were subjected to PCR. The PCR addressed for the IL-17A promoter region −52 to −246 and was performed using the following primers: 5′-GCA GCT CTG CTC AGC TTC TA-3′ and 5′-GGG CTT TTC TCC TTC TGT GG-3′. The PCR products were visualized using an ethidium bromide gel.

**Statistical Analyses**—The Student’s paired two-tailed t test was used. Values of p < 0.05 were considered significant. All error bars shown in the figures in this article are S.D.

**RESULTS**

**RORγt and Foxp3 Are Simultaneously Induced by TGF-β1 in Naïve T Cells**—As reported previously, Foxp3 was induced in naïve T cells in response to TGF-β1 (iTreg condition), whereas IL-17A was induced and Foxp3 was reduced in response to TGF-β1 plus IL-6 (Th17 condition) (Fig. 1A). However, the molecular basis in which IL-17A production is regulated has not been clarified. It has been proposed that TGF-β1 plus IL-6 induces RORγt, which in turn induces IL-17A transcription. However, several reports raised the possibility that TGF-β1 alone also induces the expression of both Foxp3 and RORγt in TCR-activated CD4+ T cells (15). Therefore, both Foxp3 and RORγt are expressed simultaneously in a single cell in the early phase of CD4+ T cell differentiation.

To analyze the relationship between Foxp3 and RORγt expression and IL-17A transcription, we investigated the time-dependent expression patterns under the iTreg or Th17 condition using RT-PCR (Fig. 1B). In agreement with previous studies (15), we found that TGF-β1 simultaneously induced the expression of RORγt and Foxp3 in CD4+CD25− T cells (Fig. 1B). IL-17A mRNA was induced under both Th17 and iTreg conditions; however, the Th17 condition induced much higher levels of IL-17A than the iTreg condition. Importantly, RORγt mRNA was induced within 24 h of stimulation regardless of the
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A

FIGURE 1. TGF-β1 induces Foxp3 and RORγt simultaneously. A, left, naive T cells were cultured with anti-TCR Ab and mlL-2 (Th0 condition) or anti-TCR Ab, mlL-2, and TGF-β1 in the presence (Th17 condition) or absence (iTreg condition) of IL-6 for 3 days. Foxp3 and IL-17A expressions were analyzed by flow cytometry. One representative experiment of three is shown. Right, the bar graph represents the average of CD4⁺CD25⁺Foxp3⁺ population out of three independent experiments. Means ± S.D. are shown. *, p < 0.05.

B

To identify that Foxp3 negatively regulates RORγt-mediated IL-17A expression.

RORγt Up-regulates the IL-17A Transcription—To identify the regulatory mechanism of IL-17A transcription, we developed a simple reporter system with the use of the HEK 293T cell line. First, we cloned an ~6-kb DNA fragment upstream of the ATG initiation codon of the IL-17A gene using the mouse genomic data base. Next, this IL-17A promoter region was subcloned upstream of a luciferase reporter gene (termed mlL17p(−6021)-Luc) (Fig. 2A). This IL-17A promoter was activated by an anti-CD3 Ab in the EL4 cells (Fig. 2A). EL4 is a tumor cell line but maintains many T-cell properties and expresses RORγt constitutively (Fig. 3A). Then, IL-17A promoter activity was examined with or without an RORγt expression vector in the HEK 293T cells in which RORγt was not expressed. In the presence of RORγt, the promoter activity was significantly increased (Fig. 2A).

To define the RORγt binding sites within the IL-17A promoter, we generated a series of truncation mutants at the 5’ end of the promoter (Fig. 2B). These constructs were assayed for promoter activity in the HEK 293T cells co-transfected with or without RORγt. The truncations deleting the 5’ to ~153-bp did not markedly affect on the RORγt-mediated IL-17A promoter activity. However, a further 38-bp deletion (mlL17p(−115)-Luc) significantly reduced IL-17A promoter activity, and an additional 21-bp deletion (~mlL17p(−94)-Luc) almost completely abrogated the RORγt-mediated IL-17A promoter activity (Fig. 2B). This suggests that the region from −153 to −94 contains ROREs. RORγt has been shown to consist of the consensus core motif AGGGCA preceded by a 5-bp A/T-rich sequence (27). As shown in Fig. 2B, there are two potential ROREs (termed RORE-1 and RORE-2) within this region. These two ROREs and surrounding regions are highly conserved between rodent and human (Fig. 2C), suggesting an important, evolutionarily conserved function of this region.

RORγt Directly Binds to RORE1/2 of the IL-17A Promoter—To confirm the importance of these two ROREs, we introduced mutations into these two sites. As expected, mutations in one of the two ROREs severely impaired the RORγt-mediated IL-17A promoter activity, and mutations in both ROREs completely abrogated the RORγt-mediated promoter activity (Fig. 2D). These data indicate that these two ROREs are essential for RORγt-mediated IL-17A promoter activity.

To verify that RORγt actually binds to the ROREs within the IL-17A promoter, we examined the binding of RORγt to this region by ChIP analysis. HEK 293T cells were transfected with RORγt cDNA, and then nuclear extracts were immunoprecipitated with anti-RORγt Ab. DNA co-precipitated with RORγt was amplified by PCR with primers containing the region −153 to −94. As shown in Fig. 2E, RORγt was shown to bind to the IL-17A promoter area. In addition, histone H4 molecules were more highly acetylated in the presence of RORγt than in the absence of RORγt. Taken together, these data indicate that RORγt up-regulates the IL-17A expression by directly binding to the IL-17A promoter region containing RORE-1/2.

Forced Expression of Foxp3 Suppresses the IL-17A Expression in EL4 Cells—To investigate the effect of Foxp3 on IL-17A expression, we then examined the levels of IL-17A in EL4 cells. As shown in Fig. 3A, EL4 cells constitutively expressed RORγt, and IL-17A mRNA was induced by anti-CD3 Ab stimulation. Stable expression of Foxp3 consistently reduced the expression levels of IL-17A. Neither expression of RORγt nor expression of TGF-β1 was affected by Foxp3 overexpression. Thus, these data indicate that Foxp3 specifically inhibits IL-17A mRNA expression.

To define the suppressive mechanism of IL-17A expression by Foxp3, we investigated whether Foxp3 expression affects the RORγt-mediated IL-17A transcription in the HEK 293T cells.
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As expected, Foxp3 strongly suppressed RORγt-mediated IL-17A promoter activity in a dose-dependent manner (Fig. 3B). However, Foxp3 did not affect CA-TBRI-mediated Smad transcriptional activity assayed with a Smad-binding element reporter (SBE) (Fig. 3C). Similarly, leukemia inhibitory factor-induced STAT3 transcriptional activity was not affected by Foxp3 (data not shown).

Then, we examined whether Foxp3 interferes with the association of RORγt with RORE-1/2 in the IL-17A promoter. Intriguingly, ChIP assay revealed that Foxp3 expression reduced RORγt binding as well as acetylation of histone H4 in the IL-17A promoter RORE-1/2 region (Fig. 3D). These data suggest that Foxp3 can specifically inhibit RORγt-mediated IL-17A transcription by reducing RORγt binding to the IL-17A promoter.

Foxp3 Suppresses RORγt Function by Physical Interaction—It has been shown that Foxp3 specifically represses the NFAT/activating protein-1 (AP-1)-driven transcription and forms a cooperative complex with NFAT on DNA (22). However, we could not observe any direct binding of Foxp3 to the IL-17A promoter (data not shown). Since Foxp3 inhibited the minimum IL-17A promoter (~153-Luc) (data not shown), which does not contain any Foxp3 binding elements, we hypothesized that Foxp3 inhibits the RORγt function by direct physical interaction with RORγt.

To test this hypothesis, we carried out immunoprecipitation and reporter assays using a series of Foxp3 deletion mutants (Fig. 4A). We detected strong interaction between wild-type Foxp3 and RORγt. The FOX3 protein encodes several functional domains, including a C2H2 zinc finger (ZnF), a leucine zipper (Zip), and a winged-helix/forkhead (FKH) domain. It has been shown that the N-terminal 188 amino acids and FKH domain are essential for the suppression activity of Foxp3. The Zip motif has been shown to be necessary for homodimerization (28); however, the role of ZnF has not been clarified. Interestingly, an N-terminal region corresponding to the exon 2 region of Foxp3 (Δ2) was necessary for interaction with RORγt. However, the other regions, namely the ZnF, Zip, and FKH domains, were unnecessary (Fig. 4A).

A reporter assay revealed that the exon 2 region as well as the FKH domain was necessary for the suppression of RORγt-mediated IL-17A promoter activation (Fig. 4B). This was confirmed in endogenous IL-17A induction in EL4 cells. EL4 cells were transfected with wild-type (WT) Foxp3 or mutants cDNAs linked to internal ribosomal entry site-enhanced green fluorescent protein. After sorting the green fluorescent protein-positive cells, IL-17A expression was measured by RT-PCR and real-time PCR. As shown in Fig. 4C, transient overexpression of WT Foxp3, but not Δ2 or ΔFKH, inhibited induction of IL-17A transcription (Fig. 4C). Taken together, these observations suggest that Foxp3 interacts with RORγt in the exon 2 region and that the FKH domain plays a critical role in the suppression of RORγt-mediated IL-17A transcription.

DISCUSSION

IL-17A is a signature cytokine of Th17, a new subset of helper T cells. It has been demonstrated that forced expression of RORγt is sufficient for the expression of IL-17A (Th17 differentiation) in T cells (13). Recently, RORA has also been shown
to induce IL-17A in T cells (29). However, the molecular basis for the activation of the IL-17A promoter by RORγt and its regulation by cytokines have not been clarified. This is the first report on characterization of the IL-17A promoter in vitro.

IL-6 was necessary for the induction of IL-17A expression (Fig. 1A). It has been thought that IL-6 is necessary because it induces RORγt in collaboration with TGF-β1. However, we and others found that RORγt is induced by TGF-β1 alone. We noticed that reduced expression of Foxp3 was inversely correlated with IL-17A expression, whereas RORγt levels were not directly correlated with IL-17A expression. Therefore, we suspected that RORγt is essential for the induction of IL-17A, whereas Foxp3 may negatively regulate IL-17A expression in iTregs. This was confirmed by overexpression of Foxp3 in EL4 cells in which RORγt is constitutively expressed (Fig. 3A). Furthermore, we showed that Foxp3 overexpression suppressed RORγt-mediated IL-17A promoter activity (Fig. 3B).

Therefore, we propose that one of the functions of IL-6 on Th17 induction is the suppression of Foxp3 expression. So how does IL-6 suppress Foxp3 induction? We have reported that TGF-β can activate 10.2-kb Foxp3 promoter in EL4 cells and that IL-4/STAT6 inhibits this promoter activation (30). We showed that STAT6 directly bound to the Foxp3 promoter and suppressed the Foxp3 promoter activation. However, unfortunately, we could not see any effects of IL-6 or activated STAT3 on the Foxp3 promoter activation in EL4 cells as well as primary T cells. In addition, IL-6 as well as STAT3C (active form of STAT3) overexpression did not inhibit TGF-β-mediated Smad transcriptional activity. Therefore, unlike STAT6, we still could not understand how STAT3 inhibits Foxp3 induction. There is a possibility that the STAT3 binding site was not included in our 10.2-kb promoter. Alternatively, epigenetic alternations on the promoter region can be induced by STAT3. We also could not rule out the possibility that genes induced by STAT3 are involved in STAT3 repression. Further
study is necessary for clarifying the mechanism of Foxp3 repression by IL-6.

Although the molecular mechanism for the suppression of Foxp3 by IL-6/STAT3 remains to be clarified, we propose a novel mechanism for the regulation of IL-17A expression by IL-6. IL-6 suppresses Foxp3 expression, and thus, RORγt is able to activate the IL-17A promoter.

In this study, we established a simple reporter system that facilitates analysis of the regulatory mechanism of IL-17A transcription in the HEK 293T cells. This is the first demonstration that RORγt functions as a direct transcriptional activator of IL-17A. Since HEK 293T cells can be used for high throughput screening, this system may be useful for identifying genes that modify IL-17A promoter activity. Using these systems, we demonstrated that Foxp3 suppressed RORγt-mediated IL-17A promoter activation by a physical interaction. Recent reports have shown that Foxp3 interacted with NFAT and suppressed the effector functions of Th cells by inhibiting the activity of NFAT (22). Foxp3 has also been shown to inhibit NF-κB transcriptional activity (21). A similar mechanism may be present for the suppression of RORγt by Foxp3. Interestingly, the interaction between Foxp3 and NFAT is reported to be dependent on the FKH domain (22), whereas we found that association between Foxp3 and RORγt was dependent on the exon 2 region. However, the FKH domain was still required for the suppression of RORγt activity. Thus, Foxp3 inhibits RORγt by direct binding through the exon 2 region and inhibits transcriptional activity of RORγt through the FKH domain.

It has been reported that the FKH domain is essential for nuclear localization and repressor functions as well as DNA binding ability of Foxp3 (28, 31). In addition, a large cohort study revealed that mutations in patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome were clustered primarily within the FKH domain and the leucine zipper but were also present within the N-terminal portion of the protein. Lopes et al. (28) identified a novel function of the N-terminal region, which is required for FOXP3-mediated repression of transcription as well as repressor function. The N-terminal region may be important for interaction with transcription factors including RORγt. In contrast, we found that deletion of the zinc finger and leucine zipper motifs did not severely impair suppression of IL-17A transcription. Therefore, the mechanism of the transcriptional repressor function of Foxp3 may vary according to the transcription factors that interact with Foxp3.

In conclusion, we propose a novel mechanism for regulation of IL-17A expression by TGF-β1 and IL-6. TGF-β1 is important for the induction of RORγt, whereas IL-6 is necessary to suppress expression of the transcriptional repressor Foxp3. Foxp3 may also regulate Th1 and Th2 through a similar mechanism.

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