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Alexandra Lainé, Bruno Martin, Marine Luka, Lucile Mir, Cédric Auffray, Bruno Lucas, Georges Bismuth and Céline Charvet

*J Immunol* published online 13 July 2015
http://www.jimmunol.org/content/early/2015/07/11/jimmunol.1500849

Supplementary Material
http://www.jimmunol.org/content/suppl/2015/07/11/jimmunol.1500849.DCSupplemental

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Foxo1 Is a T Cell–Intrinsic Inhibitor of the RORγt-Th17 Program

Alexandra Lainé, Bruno Martin, Marine Luka, Lucile Mir, Cédric Affravay, Bruno Lucas, Georges Bismuth, and Céline Charvet

An uncontrolled exaggerated Th17 response can drive the onset of autoimmune and inflammatory diseases, such as multiple sclerosis, rheumatoid arthritis, and psoriasis (1). These cells have been defined as the third subtype of effector CD4+ T cells beside the well-established Th1/Th2 duo, as they have specific functions related to their capacity to produce several proinflammatory cytokines, such as IL-17A and IL-17F (2–4). Depending on the context, different CD4+ Th17 cells have been described. One proposed classification follows the recently recommended nomenclature established for CD4+ regulatory T (Treg) cells (5), another T cell subset with immunosuppressive functions and characterized by the transcription factor (TF) Foxp3 (6), with three distinct categories, as follows: 1) thymic Th17 (tTh17) cells, which are selected by transcription factor Foxo1 downstream of the salt-sensing kinase SGK1 in Th17 development (22). Yet, the molecular mechanisms of transcriptional regulation involved in this process are still poorly understood, whereas at the same time it is totally unclear whether a T cell–intrinsic mechanism is responsible or rather some indirect regulation of Th17 differentiation by the defective Treg cell levels resulting from Foxo1 deficiency (18–20).

More recently, another layer of complexity was added to this picture by the discovery that Foxo TFs may also control Th cell differentiation. Indeed, a specific conditional knockout (ko) of Foxo1, alone or in combination with Foxo3, in mouse T cells and Foxp3+ Treg cells, impairs the generation and the suppressive function of Th17 and induced Treg (iTreg) cells (18–20). Both TFs regulate the expression of Foxp3 through a consensus DNA binding site in the proximal region of its promoter (21) as well as other Treg-associated genes, such as CTLA-4 (18–20). However, much less is known about the role of Foxo1 in Th17 generation. Some negative regulation by Foxo1 of IL-17A expression was suggested from the observed phenotype of mice lacking Foxo1 (16) and also from recent studies exploring the involvement of Foxo1 downstream of the salt-sensing kinase SGK1 in Th17 development (22). Yet, the molecular mechanisms of transcriptional regulation involved in this process are still poorly understood, whereas at the same time it is totally unclear whether a T cell–intrinsic mechanism is responsible or rather some indirect regulation of Th17 differentiation by the defective Treg cell levels resulting from Foxo1 deficiency (18–20).

In this study, by combining both in vivo and in vitro approaches, we show that Foxo1 is a direct negative regulator of the RORγt-Th17 differentiation program. Mixed bone marrow chimeras with Foxo1-deficient T cells as well as analysis of Foxo1-deficient mice reveals that Foxo1 is self-sufficient to negatively regulate Th17 cell generation. Using biochemical approaches, we clearly show...
that this inhibition relies on the direct binding of Foxo1 to RORγt via its DNA binding domain (DBD), resulting in impaired RORγt function at the level of genes controlled by this TF and involved in the generation of Th17 cells. Together, these findings demonstrate the existence of a de facto and T cell–intrinsic relationship between Foxo1 and RORγt at work in T cells. This reinforces the belief that Foxo1 is a promising target for therapies aimed at shutting down the harmful Th17-related cytokine activities often increased in autoimmune and/or inflammatory diseases.

Materials and Methods

Cells and mice

The 293T cells were maintained in DMEM supplemented with 10% FCS, penicillin/streptomycin, glutamine and sodium pyruvate. Foxo1fluorescent (Foxo1Flu), Foxo1Tko (Foxo1Flu × CD4-Cre), and Foxo1−/− (Foxo1−/−) mice have been previously described (16, 18, 23, 24). Foxo3−/− (Foxo3−/− × CD4-Cre) were generated by crossing Foxo3−/− mice to CD4-Cre mice (provided by S. Amigorena, Institut Curie, Paris, France). C57BL/6 mice were obtained from Charles River Laboratories. C57BL/6 wild-type (WT) CD45.1 and CD3ε−/− mice were bred in our own animal facility. All mice were main- tained in a pathogen-free condition in strict accordance with the French Veterinary Department guidelines. In vivo studies and procedures were performed in accordance with the European and National Regulation of Vertebrate Animals (CEEA34BL.002.12).

Plasmids

MyC/His-tagged murine RORγt was inserted into EcoRI and EcoRV sites into pEFlb (Invitrogen) (mRORγt-MyC-His) under control of the EF1α promoter on a 2-kb NotI fragment (28). The IL-23R promoter-luciferase reporter has been provided by C. Dong (Institute for Immunology, Tsinghua University, Beijing, China) (28). The IL-23R promoter-luciferase reporter has been

provided by K. Sato (Faculty of Medicine, Saitama Medical University, Saitama, Japan) (29). The Renilla plasmid was from Promega (Madison, WI).

Retroviral infection

RORγtMyCIG (Addgene plasmid 24069) has been described (25), and FOXO1TM/pMIT was provided by D. Fruman (University of California, Irvine, Irvine, CA) (30). The mutant FOXO1TMΔDBD/pMIT has been generated using the primers (5′−3′) hFoxo1ΔDBD (forward), 5′-GGCCGCTCGCAGGCGGAGCAGCGATTTCTAGAGGCG-3′ and hFoxo1ΔDBD (reverse). 5′-CTCGGGTTTAACGAACTATCCTGAGGAGCC-3′ and the mutagenesis kit XL (Stratagene, Santa Clara, CA). Viruses were produced according to the protocol described, with some changes (31). Briefly, Platinum-E packaging cells (plast-E) (32) were seeded with 10 ml DMEM plus 10% FCS at 2.5 × 10⁵ cells/ml in a 10-cm dish. Twenty-four hours later, cells were transfected with the retroviral plasmid DNA (10 μg) using lipofectamine 2000 (Invitrogen, Grand Island, NY), according to the manufacturer. The next day, the medium was replaced by 6 ml IMDM plus 10% FCS. The retroviral supernatant was harvested 24 h later, filtered on a 45-μm filter, and frozen at −80°C. The medium was replaced with 6 ml IMDM plus 10% FCS for a second harvest. For retroviral infection, CD4 naïve T cells (CD44hiCD25− CD45.1−) were activated by coated anti-CD3 (CD3ε) at 1×10⁵ cells/ml in 24-well plates in the presence of IL-6 (10 ng/ml) plus TGF-β (20 ng/ml) in the case of the coinfection with RORγt and FOXO1TMΔDBD. Cells were then centrifugated (2000 rpm) for 1 h at 32°C and incubated 4 h at 37°C. The supernatant was then replaced by IMDM plus 10% FCS, IL-6 (10 ng/ml), and the pancaspase inhibitor QVD-OPh (20 μM) for 24 h. After the second harvest, viral supernatant was maintained for additional 3 d in culture. Infected cells were then activated with PMA plus ionomycin (0.5 μg/ml each) for 2 h plus Golgizip (1/1000); stained with anti–Thy-1.1-biotin (clone OX-7), followed by streptavidin-BV605 (both from BioLegend, San Diego, CA); fixed; permeabilized using the BD Cytofix/Cytoperm Plus Fixation/Permeabilization Kit (BD Pharmingen); stained with FITC anti-GFP (Thermofisher Scientific, Waltham, MA), allophycocyanin anti–IL-17A (BD Pharmingen); and analyzed by the FACS LSRII.

Real-time PCR

CD4 naïve T cells (CD44hiCD25− TCR−γδ NK1.1−) were activated in the presence of IL-6 (10 ng/ml) plus TGF-β (0.002 ng/ml) (Th17 conditions) with either the p108-PEK inhibitor (IC87114, 5 μM) or the Akt1a inhibitor (IC261, 1 μM) for 1 d. After the last infection (80 μg/ml), cells were activated with PMA plus ionomycin (0.5 μM) along with the IL-17A Prom plus CNS5-Luc (0.5 μg) (8); mIL17a (forward), 5′-GAGGTACATGCTGGCAGGCGGT-3′; and mL32 (reverse), 5′-TTGTGTCGCCCACCAAA-3′. At days 1 and 2, the medium was replaced by the retroviral supernatant supplemented with protamine sulfate (10 μg/ml) (or polybrene [5 μg/ml] in the case of the coinfection with RORγt and FOXO1TMΔDBD). Cells were then centrifugated (2000 rpm) for 1 h at 32°C and incubated 4 h at 37°C. The supernatant was then replaced by IMDM plus 10% FCS, IL-6 (10 ng/ml), and the pancaspase inhibitor QVD-OPh (20 μM) for 24 h. After the last harvest, cells were maintained for additional 3 d in culture. Infected cells were then activated with PMA plus ionomycin (0.5 μg/ml each) for 2 h plus Golgizip (1/1000); stained with anti–Thy-1.1-biotin (clone OX-7), followed by streptavidin-BV605 (both from BioLegend, San Diego, CA); fixed; permeabilized using the BD Cytofix/Cytoperm Plus Fixation/Permeabilization Kit (BD Pharmingen); stained with FITC anti-GFP (Thermofisher Scientific, Waltham, MA), allophyacocyanin anti–IL-17A (BD Pharmingen); and analyzed by the FACS LSRII.

Luciferase assays

The 293T cells were seeded at 350,000 cells/ml in a 12-well plate. Twenty-four hours later, cells were transfected in triplicates with RORγt−/− (MyC 0.5 μg) along with either Flag or Myc/Flag-tagged Foxo1TM, Foxo1TMΔDBD, or Foxo1TMΔTAD (0.01, 0.05, 0.1, and 0.5 μg) and the IL-17A Prom plus CNS-Luc (0.5 μg). IL-23 Prom-Luc (1 μg), or RORE-Luc reporter (0.05 μg) constructs and Renilla (0.1 μg) using lipofectamine 2000 (Invitrogen), according to the manufacturer. Twenty-four hours later, one-third of the cells was lysed and analyzed for firefly and Renilla measurement by a luminometer using the dual luciferase reporter assay kit (Promega), and the two-thirds left were subjected to a nuclear fractionation, as described (14). Nuclear proteins were prepared on a SDS-PAGE and analyzed by immunoblotting using indicated Abs.

Adaptive transfer of bone marrow cells

Bone marrow cells from C57BL/6 WT CD45.1 mice and from Foxo1TkoCD45.2 mice were incubated on ice for 20 min with anti-CD4 (GK1.5) and anti-CD8a (53-67.6) Abs, obtained from hybridoma supernatants, and then with magnetic beads coupled to anti-rat Ig (Dynal Biotech). A total of 4 × 10⁵
T cell–depleted Fxo1TKO CD4+CD8− bone marrow cells was then coinjected i.v. with 1 × 10^6 T cell–depleted C57BL/6 CD45.1 bone marrow cells into lethally irradiated (950 rad) 6- to 8-wk-old C57BL/6 CD5−/− mice. Analysis was performed 4 wk upon reconstitution, as indicated (n = 10 mice).

**Abs and reagents**

- Anti–IFN-γ (clone XMG1.2), anti–y8-TCR (clone GL3), anti–CD25 (clone PC61), anti–CD62L (clone L3T4), anti–CD8α (clone 53-6.7), anti–NK1.1 (clone PK136), anti–TCRβ (clone H57 567), anti–CD44 (clone IM7), anti–IL-4 (neutralizing Ab; clone 11B11), FcBlock CD16/32 (clone 2.4G2), anti–CD3 clone (clone 145-2C11), and anti–CD28 (clone 37.51) Abs were from BD Pharmingen. Goat anti–hamster IgG (H+L; 41–417) and human anti–TGF-β (100-21C) were from Serotec. Murine rIL-6 (216-16) and human rTGF-β (100-21C) were from PeproTech. Anti–IL-6Rα (clone D7715A), anti–IL-17A (eBio1B7), and anti–Foxp3 (clone FJK-16B) Abs were from eBioScience. Anti–Thy1.1 biotin (clone OX-7), anti–GFP–FITC (1-46326), and anti–IFN-γ (neutralizing Ab; clone R4-6A2) Abs were from BioLegend.
- Anti–MyC (clone 9B11; 2276), anti–PTEN (clone 4B10) Abs were from Millipore. Anti–Flag Ab (F1804 or F7450) were from Sigma-Aldrich, and anti–tubulin (clone C29H4, 2880), anti–Erk1/2 Ab (V114A) was from Promega. Anti–Myc Ab (clone 9B11; 2276), anti–GFP–FITC (1-46326), and anti–IFN-γ (neutralizing Ab; clone R4-6A2) Abs were from BioLegend.
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**Statistics**

Statistical significance was analyzed by a two-tailed Student t test or a one way ANOVA test, followed by Tukey’s multiple comparison tests. *p < 0.05, **p < 0.01, ***p < 0.001. Unless indicated otherwise, data represent the mean ± SD.

**Results**

Foxo1 deficiency in T cells leads to an increase of Th17 cells in vivo in a T cell–intrinsic manner

To directly question the role of Foxo1 protein in Th17 generation in vivo, we used mice with a T cell–specific deletion of Foxo1 (Fxo1TKO × CD4-Cre, designated in this work as Fxo1TKO) (16, 17) and littermate controls (Foxo1WT, designated in this work as Foxo1WT) (Supplemental Fig. 1A). We first analyzed the proportion of peripheral IL-17A− T cells (pTh17) in memory CD4 T cells (CD4+ TCRβ+ CD44−/low IL-17A+) in mLNs from Foxo1WT and Foxo1TKO mice (Fig. 1A). We also evaluated the tTh17 population in Foxo1TKO mice and found an increase of tTh17 of ~3-fold (Supplemental Fig. 1B, 1C). In contrast, similar experiments in Foxo3−/− mice showed no change in the number of pTh17 (Supplemental Fig. 1D) and tTh17 cells (data not shown).

To determine whether this mechanism was T cell intrinsic or promoted by a reduced Treg cell compartment, we next used a mixed bone marrow chimera strategy by reconstituting irradiated CD3ε− mice with a mixture of bone marrows from WT (CD45.1) and Foxo1TKO (CD45.2) mice (Supplemental Fig. 1E). Foxo1 is essential for the maintenance of the expression of IL-7R on CD4 naive T cells. As a result, CD4 T cell survival and proliferation are reduced in the absence of Foxo1 (16, 17). As previously described (18), we therefore used a 1:4 WT:Foxo1TKO ratio in the transfer experiments to recover a sufficient amount of Foxo1TKO CD4 T cells in the periphery. Four weeks later, although the 1:4 ratio was maintained in the thymus, we found an equivalent number of WT and Foxo1TKO total CD4+ cells in mLNs and pLNs (Fig. 1B). Most of these cells showed an effector memory phenotype (Supplemental Fig. 1F). Importantly, these chimeras expressed a high proportion of FoxP3+ Treg cells in mLNs and pLNs, essentially from WT origin (Supplemental Fig. 1G). Despite this, Foxo1-deficient memory CD4 cells were again clearly more prone to express IL-17A (10.9%) compared with the WT (2.7%) (Fig. 1C). These results indicate that Foxo1 negatively controls in vivo generation of Th17 cells via a cell-intrinsic mechanism.

**Modulation of Foxo1 protein level or Foxo1 activity leads to different outcomes in iTTh17 differentiation**

We next investigated the impact of Foxo1 in iTTh17 development by performing in vitro iTTh17 differentiation assays using Foxo1-deficient T cells. We purified CD4 naive T cells from mLNs and stimulated them with anti–CD3 and anti–CD28 Abs and IL-6-
combined with increasing TGF-β concentrations (37). In this assay, the percentage of iTh17 in the control condition reached a maximal peak at low TGF-β concentrations (0.031–0.25 ng/ml) when combined with IL-6, and gradually increased with higher doses of TGF-β (Fig. 2A). Unexpectedly, we found that Foxo1-deficient CD4 naive T cells differentiated less efficiently into iTh17 (Fig. 2A). Of note, Foxo3Tko and Foxo3ctrl CD4 naive T cells similarly differentiated into iTh17 in vitro (Supplemental Fig. 2A, 2B).

To explain these results and because the IL-6/STAT3 pathway is involved in the regulation of RORγt (8, 9), we hypothesized that the IL-6/IL-6R signaling pathway might be altered in the absence of Foxo1. To check this, we first measured the levels of IL-6Rα at the surface of CD4 naive Foxo1Tko cells. We found that its expression was significantly decreased by ∼30% (Fig. 2B, Supplemental Fig. 2C). We then sorted CD4 naive T cells from LNs of Foxo1ctrl and Foxo1Tko mice, stimulated them with IL-6 for the indicated period of time, and measured the status of STAT3 phosphorylation. In Foxo1ctrl cells, IL-6–induced STAT3 activation started at 5 min and gradually increased at 15 and 30 min (Fig. 2C). However, in the absence of Foxo1, its phosphorylation was strongly reduced (Fig. 2C). Of note, we could not find any difference in the phosphorylation events triggered by TCR/CD28 in the absence or presence of Foxo1, most likely excluding a signaling defect downstream of the TCR/CD28 costimulatory pathway (Supplemental Fig. 2D). These results suggest that the disturbed differentiation of iTh17 cells in the absence of Foxo1 is biased by an impaired IL-6/STAT3 signaling pathway.

To cope with this issue, we next tested how blocking directly Foxo1 activity with the recently described Foxo1 inhibitor (AS1842856) (38) could affect the T cell differentiation of normal naive CD4 T cells during differentiation assays in vitro. Inhibition of Foxo1 during iTh17 differentiation improved IL-17A expression (Fig. 2D). Strikingly, inhibition of Foxo1 by AS1842856 during iTreg differentiation with TGF-β dramatically increased IL-17A+ cells (Fig. 2E). These findings indicate that the direct and acute inhibition of Foxo1 strongly favors iTh17 development.

**Inhibition of the PI3K/Akt pathway decreases IL-17A and IL-17F expression and iTh17 differentiation triggered by RORγt at the transcriptional level**

To further consolidate this conclusion, we next sought to activate Foxo1 by inhibiting the PI3K/Akt pathway in vitro iTh17 differentiation assays. We used the p110δ-PI3K catalytic subunit inhibitor IC87114 and Akti1/2, an inhibitor of the two isoforms of Akt. Each inhibitor strongly decreased the emergence of iTh17 cells (Fig. 3A, 3B). We controlled in parallel that at these concentrations, the two inhibitors decreased Foxo1 S-256 phosphorylation induced upon CD3/CD28 stimulation (Fig. 3C), but had no effect on either IL-6–induced STAT3 Y705 phosphorylation (Fig. 3D) or RORγt expression (Supplemental Fig. 2E).

To complement these data, we directly tested the effect of the Akti1/2 inhibitor on RORγt–induced Th17 differentiation. For this purpose, purified CD4 naive T cells were activated for 24 h and transduced with either a retrovirus encoding RORγt-IRES-GFP or a control vector encoding GFP alone (pMIG). At day 2 postinfection, cells were treated with either the Akti1/2 inhibitor or DMSO as a control, and IL-17A expression was analyzed on GFP+–gated cells (Fig. 3E). RORγt overexpression strongly induced IL-17A (39.6%) compared with the pMIG control vector (8.44%). Inhibition of Akti1/2 strongly decreased both basal (4.29%) and RORγt–induced IL-17A (16%) levels (Fig. 3E).

We next analyzed whether the regulation of IL-17A expression by the PI3K/Akt pathway occurred directly at the transcriptional level. For this aim, we performed an in vitro iTh17 differentiation assay in the presence of the PI3K/Akt inhibitors and analyzed IL-17A mRNA. A very high IL-17A mRNA expression (∼500-fold compared with control) was observed 48 h after stimulation. It was totally blocked by PI3K-p110δ (IC87114) or Akti1/2 (Akti1/2)
induced a decrease of RORγt (GFP+ Thy1.1high; 25%) (Fig. 4B). As a control, particularly pronounced in cells expressing the highest level of the two control viruses pMIG and pMIT (9.05%) (Fig. 4B).

We next examined the direct effect of Foxo1 on Th17 differentiation. We infected TGF-β/IL-6–induced IL-17f mRNA increase was strongly impaired by each inhibitor (Fig. 3G).

Taken together, these results show that inhibition of the PI3K/Akt pathway prevents iTTh17 development and production of Th17 cytokines induced at the transcriptional level by RORγt. **Foxo1 negatively regulates RORγt-induced Th17 differentiation**

We next examined the direct effect of Foxo1 on Th17 differentiation. For this aim, we used a constitutively active mutant of Foxo1 (Foxo1 triple mutant [TM]), mutated on the three residues phosphorylated by Akt (39), to see how it could impact Th17 development. We infected TGF-β/IL-6–induced iTTh17 cells with either FOXO1TM-IRES-Thy1.1 or a virus only encoding for Thy1.1 (pMIT) and analyzed the expression of IL-17A in the CD4+ Thy1.1+–gated cells. We found a ~2-fold decrease with FOXO1TM compared with the control virus (Fig. 4A). We extended these observations in coinfection experiments with combinations of the two previously used viruses and viruses encoding either Foxo1ctrl and Foxo1Tko mice were activated with anti-CD3 and anti-CD28 Abs in the presence or the absence of IL-6 (10 ng/ml) and increasing doses of TGF-β (0.015–8 ng/ml, as indicated) and analyzed on day 3 for IL-17A expression. The data shown are representative of five independent experiments. (B) IL-6/Stat3 phosphorylation on IL-6–stimulated CD4 naive T cells from Foxo1TM and Foxo1Tko mice. Quantification of four independent experiments. Data are representative of three independent experiments. (D and E) CD4 naive T cells were cultured in iTTh17 (D) or iTreg (E) conditions either in the presence of the Foxo1 inhibitor AS1842856 (25 nM) or absence (DMSO as a control) for 3 d. IL-17A expression on CD4+ cells is shown. Data are representative of two independent experiments. See also Supplemental Fig. 2. **p < 0.01.

Foxo1 inhibits IL-17A expression by dampening RORγt activity at the promoter level

We next examined the effect of Foxo1 on the activity of the IL-17A promoter triggered by RORγt. To this end, we used a luciferase reporter gene containing the IL-17A promoter (27). When coexpressed in 293T cells along with the IL-17A luciferase reporter, RORγt highly increased the activity of the IL-17A promoter (Fig. 4C). However, Foxo1TM strongly decreased this activity in a dose-dependent manner. Of note, Foxo1TM also strongly inhibited RORγt-induced IL-23R promoter activity (Supplemental Fig. 3B). To mediate this effect, Foxo1 could either directly bind on the IL-17A promoter and/or directly inhibit the transcriptional activity of RORγt. To test the first hypothesis, we analyzed the effect of a mutant of Foxo1TM, unable to bind DNA (Foxo1TM H212R) and transcriptionally inactive (Supplemental Fig. 3C). RORγt-induced IL-17A promoter activity was still reduced in the presence of Foxo1TM H212R, albeit less efficiently than with Foxo1TM (Fig. 4D). To test the second hypothesis, we used a luciferase reporter driven by a minimal promoter containing only three binding sites specific for ROR proteins (ROE-reporter) (28). We reasoned that, as this minimal promoter could not bind Foxo1, any inhibition would involve a suppressive activity of Foxo1 on RORγt. Expression of RORγt strongly increased ROE-reporter (~6-fold), compared with the control with empty vectors (Fig. 4E). However, addition of Foxo1TM strongly decreased RORγt activity in a dose-dependent manner. Again, the Foxo1TM H212R mutant inhibited RORγt activity, but slightly less than Foxo1TM (Fig. 4F). Altogether, these results indicate that Foxo1 directly inhibits RORγt transcriptional activity at the promoter level.
Foxo1 binds RORγt via its DBD

Previous reports have shown that Foxp3 can bind RORγt via the LXXLL domain found in exon 2 of Foxp3 and antagonizes its function (25, 28, 44). Because Foxo1 decreased RORγt activity (Fig. 4), we explored the possibility that Foxo1 could act in a similar way as Foxp3 by binding RORγt. We coexpressed Flag-Foxo1WT or TM along with Myc-RORγt in 293T cells, and probed Flag-tagged Foxo1 immunoprecipitates for the presence of Myc-RORγt. As shown in Fig. 5A (left panel), both Foxo1WT and TM associated with RORγt. The Foxo1TM H212R mutant also bound RORγt (Fig. 5B, 5C, lane 8, Supplemental Fig. 3D), consistent with its inhibitory effect on RORγt function (Fig. 4). The binding of nuclear receptors to coactivators or corepressors involves an AF2 domain on the C-terminal tail of the receptors and a leucine-rich domain (LXXLL) on the copartner (45–47). We found that the TAD of Foxo1 contains a unique leucine-rich domain (LKELL) (Fig. 5B). We therefore mutated all the leucines into alanines (LKELL = >AKEAA), but the Foxo1TM AKEAA mutant was still able to bind RORγt/pMIG. Cells were then incubated with Akti1/2 (1 μM), and CD4+ GFP+ cells were analyzed for IFN-γ and IL-17A expression. Data are representative of three independent experiments. See also Supplemental Fig. 2.
A constitutive active Foxo1 represses RORγt-induced IL-17A expression by inhibiting RORγt function. (A) CD4 naïve T cells activated with CD3 plus CD28 mAbs in the presence of IL-6 (10 ng/ml) plus TGF-β (0.062 ng/ml) (iTh17 conditions) were transduced with either pMIT or FOXO1TM/pMIT. CD4+ Thy1.1+ cells were analyzed for IFN-γ and IL-17A expression. Data are representative of five independent experiments. (B) CD4 naïve T cells were coinfected with RORγt/pMIG and/or FOXO1TM/pMIT. GFP+ Thy1+ or GFP+ Thy1.1high cells were analyzed for IFN-γ and IL-17A expression. Data are representative of four independent experiments. (C) The 293T cells were transfected with the IL-17A promoter + CNS5-luciferase (Luc) reporter, Renilla, and the indicated plasmids. At day 1, the ratio firefly/Renilla was measured. Data are representative of three independent experiments. The graph shows means ± SD. Expression of the transfected proteins in the nucleus and the loading control Ku80 is shown. (D) The 293T cells were transfected with the IL-17A promoter + CNS5-Luc reporter, Renilla, and the indicated plasmids. At day 1, the ratio firefly/Renilla and the nuclear expression of the transfected proteins were analyzed as in (C). Data shown are representative of three independent experiments. The graph shows means ± SD. (E) The 293T cells were transfected the RORE-Luc reporter, Renilla, and the indicated plasmids. At day 1, cells were analyzed, as in (C). Data are representative of three independent experiments. The graph shows means ± SD. See also Supplemental Fig. 3. *p < 0.05, **p < 0.01, ***p < 0.001.
bind RORγt and found that, whereas Foxo1TMΔDBD had lost this capacity (Fig. 5G, left panel, Supplemental Fig. 3F). Taken altogether, these results indicate that the DBD of Foxo1 is required to form a complex with RORγt.

Binding of Foxo1 to RORγt is required to inhibit RORγt function

We then sought to determine the effect of FOXO1TM mutants on the activity of RORγt-induced target gene promoters, namely the IL-17A and IL-23R promoters. We found that the Foxo1TMΔDBD mutant was unable to repress RORγt-induced activity of both promoters (Fig. 6A, 6D). Surprisingly, the Foxo1TM mutant lacking the TAD domain was also unable to decrease RORγt-induced IL-17A promoter activity (Fig. 6B), whereas it was as efficient as Foxo1TM on the IL-23R promoter (Fig. 6D). We also tested the effect of these mutants using the previously used RORE minimal reporter gene. Consistently, the Foxo1TMΔDBD mutant was unable to repress RORγt function compared with Foxo1TM (Fig. 6E). Low expression levels of Foxo1TMΔTAD were unable to decrease RORγt function, but at higher level it repressed RORγt activity, albeit less efficiently compared with Foxo1TM (Fig. 6F).

To work in a more physiological context, we finally used the previously described infection system allowing the overexpression of FOXO1 and Thy1.1 molecules in CD4 naive T cells undergoing TGF-β/IL-6–induced Th17 differentiation. We found that, whereas FOXO1TM (Thy1.1+ cells) decreased the proportion of IL-17A+ cells compared with the control pMIT vector (∼2-fold; Fig. 7A, middle panels), the FOXO1TMΔDBD mutant, which does not bind RORγt, had no effect. No change in iTh17 differentiation was observed in noninfected cells (Thy1.1−) (Fig. 7A, lower panels). We then induced an iTh17 differentiation using the RORγt construct with the pMIG backbone, and confirmed that, particularly in highly infected cells (GFP+ Thy1.1high), RORγt-induced IL-17A expression (29.6%) was decreased in the presence of FOXO1TM (13.8%), but not with the FOXO1TM lacking the DBD (41.6%) (Fig. 7B, lower panels).

Together these results indicate that the DBD domain of Foxo1 is necessary to bind RORγt and decrease iTh17 differentiation triggered by either TGF-β/IL-6 or RORγt.

Discussion

During autoimmune inflammatory diseases, an accumulation of uncontrolled Th17 cells actively orchestrates the inflammation
FIGURE 6. The DBD domain of Foxo1 is required to inhibit RORγt function and RORγt-gene target-promoter activities. (A) The 293T cells were transfected with IL-17A promoter + CNS5-Luc reporter (0.5 μg) and Renilla along with the indicated plasmids. The graph shows the ratio luciferase/Renilla represented as means ± SD. The expression of the transfected proteins in the nucleus is shown. (B) The 293T cells were transfected with IL-17A promoter + CNS5-Luc reporter and Renilla along with the indicated plasmids. The graph shows the ratio luciferase/Renilla represented as means ± SD. The expression of the transfected proteins in the nucleus is shown. (C) The 293T cells were transfected with IL-23R Prom-Luc reporter (1 μg) and Renilla (0.1 μg). The graph shows the ratio luciferase/Renilla represented as means ± SD. The expression of the transfected proteins in the nucleus is shown. (D) The 293T cells were transfected with IL-23R Prom-Luc reporter and Renilla along with the indicated plasmids. The graph shows the ratio luciferase/Renilla represented as means ± SD. The expression of the transfected proteins in the nucleus is shown. (E) The 293T cells were transfected with RORE-Luc reporter (0.5 μg) and Renilla (0.1 μg) along with the indicated plasmids. The graph shows the ratio luciferase/Renilla represented as means ± SD. The expression of the transfected proteins in the nucleus is shown. (F) The 293T cells were transfected with RORE-Luc reporter and Renilla. The graph shows the ratio luciferase/Renilla represented as means ± SD. The expression of the transfected proteins in the nucleus is shown. For each panel, data are representative of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
process. Therefore, understanding how Th17 cells are physiologically generated is of major interest. In this study, we explored the contribution of the Foxo1 molecule, a master TF controlling T cell homeostasis, in the generation of Th17 cells. Our main conclusion is that Foxo1 is a direct negative regulator of Th17 program both in vivo and in vitro, acting via a T cell–intrinsic mechanism. Our work establishes the existence of a molecular complex between Foxo1 and the ROR\(\gamma\) TF and identifies the region of Foxo1 required for this interaction. It also demonstrates that this relationship between the two TF is critical for the generation of Th17 cells because it antagonizes ROR\(\gamma\) function and ROR\(\gamma\)-mediated transcription of several Th17 key genes, such as IL-17A and IL-23R, thereby preventing a proper Th17 differentiation program.  

Foxo1 binds ROR\(\gamma\) and antagonizes its function  

Foxo1 mainly mediates its transcriptional effects by directly binding to a consensus DNA-binding site in the promoter of target genes (39). However, it can also act in a DNA-binding independent manner by associating with other TFs, therefore increasing the panel of genes it regulates (48). In this study, we found that the nuclear constitutively active Foxo1TM was able to bind ROR\(\gamma\) and strongly decreased its activity and the induction of its target genes, such as IL-17A, II-17F, and IL-23R. Our data are in accordance with the recent finding that Foxo1 and ROR\(\gamma\) form an endogenous complex in primary Th17 cells (22). Interestingly, the mutant Foxo1TM H212R that lacked the capacity to bind to DNA and transactivate Foxo-responsive elements was still able to bind...
RORγt and inhibit RORγt function (Figs. 4, 5), suggesting that DNA binding of Foxo1 is not required for this interaction. It has been shown that Foxp3 interacts with RORγt via its LXXLL domain to inhibit RORγt function (25, 28, 44, 49). Such a motif on the C-terminal region of Foxo1 has also been described to be involved in the formation of the Foxo1-Sirt1 complex (50). However, we found that it is not necessary for the Foxo1/RORγt interaction; rather, we identified the DBD domain of Foxo1 as being critical. These findings indicate that, whereas both Foxo1 and Foxp3 can antagonize RORγt function, they probably bind different regions of the molecule, with the underlying hypothesis that a trimeric complex may be formed between these three TFs to regulate the Th17/Treg balance.

Interestingly, during the course of this study, we also found that a reciprocal antagonism between RORγt and Foxo1 takes place. Indeed, RORγt was also able to inhibit Foxo1 activity in vitro (Supplemental Fig. 4A). This may be related to the fact that the Foxo1/RORγt complex requires the DBD domain of Foxo1, thereby inhibiting its transcriptional activity. This observation suggests that if some active Foxo1 molecules remain during iTh17 differentiation, they would be inhibited by RORγt, which is highly expressed in this context.

**Foxo1 inhibits Th17 differentiation in a T cell–intrinsic manner**

We clearly observed an increased proportion of Th17 cells both in the thymus and the periphery in Foxo1lox/lox mice compared with WT animals. Consistent with the inhibitory binding of Foxo1 on RORγt, these results indicate that in vivo, the lack of Foxo1 most likely favors RORγt functioning, leading to an increase of IL-17A expression. A lack of functional pTreg in the absence of Foxo1 could explain this higher proportion of pTh17 (18, 19). However, using mixed bone marrow reconstitution, we clearly demonstrate that in the absence of Foxo1 a vast majority of CD4 cells showing an effector memory phenotype at the time of the analysis express IL-17A, indicating that these cells were more prone to differentiate into Th17 cells by a T cell–intrinsic mechanism, in a manner consistent with most of our in vitro experiments (luciferase assays and retroviral infections).

One exception to this was our result showing that, in vitro, Foxo1-deficient naive T cells had a reduced capacity to differentiate into iTh17 cells. One explanation could be that Foxo1-deficient CD4 T cells are misdirected toward Th1 cells in these in vitro assays (18, 20). However, we could not restore a normal iTh17 differentiation when IFN-γ and IL-4 were neutralized (see Supplemental Fig. 4B, 4C). We also controlled that tyrosine phosphorylations triggered by TCR/CD28 were unaffected, suggesting no early activation defects downstream of these receptors (see Supplemental Fig. 2D); rather, in agreement with the critical role played by the IL-6/STAT3 pathway in RORγt expression (8, 9), we found a decrease in IL-6Ra expression and IL-6–induced STAT3 activation. The mechanism by which Foxo1 modulates the expression of IL-6Ra is not yet determined. However, using a ChIP-seq approach to determine Foxo1 binding site in Treg cells, Ouyang et al. (20) have identified a Foxo1 binding site located on the il6ra gene. Thus, as it had been shown previously for IL-7R (16, 17), Foxo1 may also regulate the expression of IL-6Ra at the transcriptional level (17, 18). Obviously, these findings question the role of IL-6 in Th17 differentiation in physiological conditions in vivo because we unambiguously found more Th17 cells in Foxo1lox/lox mice. However, it is still a matter of debate, as some authors claimed that the IL-6/STAT3 axis is important for Th17 generation in vivo (7), whereas others reported that it is dispensable (51). These observations therefore suggest that in Foxo1lox/lox cells differentiating into pTh17 cells in vivo, the lack of RORγt control by Foxo1 is probably more influential than the IL-6 signaling defect.

**Pharmacological modulation of Foxo1 activity controls Th17 differentiation**

Another set of experiments performed during this work using various pharmacological inhibition of the PI3K/Akt pathway also strongly argues for a negative role of Foxo1 on Th17 differentiation. Indeed, various drugs, leading to PI3K/Akt inhibition and Foxo1 activation, strongly decreased IL-17A–expressing CD4 T cells during in vitro differentiation of CD4 naïve T cells into Th17 cells. These results are consistent with previous studies having shown that the PI3K/Akt/mTORC1 axis is important for Th17 differentiation (52, 53). Our results also showed that this regulation of PI3K/Akt inhibitors on Th17 differentiation clearly occurs at the transcriptional level and that it can impact various Th17 genes simultaneously, as shown in this work for il-17a and il-17f genes (see Fig. 3F, 3G). A direct targeting of RORγt activity itself is most likely involved because our results also clearly show that inhibiting the PI3K/Akt pathway impairs Th17 development in activated CD4 + T cells directly transduced with RORγt.

One striking result observed during our study is that a pharmacological inhibition of Foxo1 itself in WT CD4 T cells could trigger IL-17A expression in the presence of TGF-β alone. This marked accumulation of iTTh17 cells suggests that, in this condition, RORγt could now freely exert its Th17 programming activity. As this Foxo1 inhibitor is assumed to bind the DBD domain of active Foxo1 (38), one can thus speculate that it prevents Foxo1 binding to RORγt, allowing RORγt to fully activate Th17 genes.

In recent years, the search for pharmacological inhibitors of RORγt has gained a lot of attention, as this TF represents a target of choice for the treatment of autoimmune inflammatory diseases (54, 55). In view of our results, Foxo1 can act as a repressor of RORγt, the master gene that unlocks the Th17 differentiation program. In human CCR6 + memory T cells, a similar mechanism might take place, as it has been recently reported that over-expression of FOXO1 decreases IL-17A (56). Activating Foxo1, with drugs acting either on pathways upstream this TF or directly controlling its activity, therefore offers a new means to control RORγt function and dampen Th17 responses. Hence, Foxo1 could be considered as a potent anti-inflammatory control switch directly acting on the Th17 program. As such, it may represent a new and promising target candidate molecule in the broad area of anti-inflammatory agents, including biological therapies and small drug molecules that block the inflammatory process and help fight autoimmune diseases.

**Acknowledgments**

We thank Stephen Hedrick for providing Foxo1lox/lox and Foxo3lox/lox mice and critical reading of the manuscript; Sebastian Amigorena for CD4-Cre mice; Dan Littman for mRORγt/pMIGR; Dominic Accili for mFoxo1wt/pCMV5 and David Fruman for FOXO1TM/pMIT constructs; Warren Strober for IL-17A promoter plus CNS5-luciferase; Chen Dong for RORE-luciferase and Kojiro Sato for IL-23R-luciferase reporter constructs; Stéphane Bécart for critical reading of the manuscript; Florence Lambolez for sharing protocols; Ulrich Maurer for helpful comments, critical reading of the manuscript, and sharing protocols; the cytometry facility (Cybio) for sorting CD4 naïve T cells; the animal facility of the Cochin Institute; and la Fondation pour la Recherche Médicale and the TOURRE Foundation for their support.

**Disclosures**

The authors have no financial conflicts of interest.
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