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

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

*J Immunol* 2015; 195:1791-1803; Prepublished online 13 July 2015;
doi: 10.4049/jimmunol.1500849
http://www.jimmunol.org/content/195/4/1791

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

**References**
This article cites 56 articles, 16 of which you can access for free at:
http://www.jimmunol.org/content/195/4/1791.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2015 by The American Association of Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Foxo1 Is a T Cell–Intrinsic Inhibitor of the RORγt-Th17 Program

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

An uncontrolled exaggerated Th17 response can drive the onset of autoimmune and inflammatory diseases. In this study, we show that, in T cells, Foxo1 is a negative regulator of the Th17 program. Using mixed bone marrow chimeras and Foxo1-deficient mice, we demonstrate that this control is effective in vivo, as well as in vitro during differentiation assays of naive T cells with specific inhibitor of Foxo1 or inhibitors of the PI3K/Akt pathway acting upstream of Foxo1. Consistently, expressing this transcription factor in T cells strongly decreases Th17 generation in vitro as well as transcription of both IL-17A and IL-23R RORγt-target genes. Finally, at the molecular level, we demonstrate that Foxo1 forms a complex with RORγt via its DNA binding domain to inhibit RORγt activity. We conclude that Foxo1 is a direct antagonist of the RORγt-Th17 program acting in a T cell–intrinsic manner.

The Journal of Immunology, 2015, 195: 1791–1803.

D4+ Th17 cells are instrumental in cancer and autoimmune 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 (iTTh17) cells, which are selected by self-reactivity in the thymus (7); 2) peripheral Th17 (pTh17) cells, differentiated in vivo in response to a pathogen or an inflammatory stimulus in tissues; 3) in vitro induced Th17 (iTh17) cells, obtained after TCR stimulation of CD4 naïve T cells in the presence of a specific cytokine environment, usually TGF-β and IL-6, a condition that is supposed to mimic those found in vivo upon Ag recognition in the periphery, especially in an inflammatory context. However, in all cases, one hallmark of this Th17 differentiation program is the TF RORγt, which controls the production of the aforementioned Th17 cytokines (8, 9).

The TFs of the forkhead box O (FoxO) family (FoxO1, FoxO3, and FoxO4) are critical at the crossroad of different processes, such as quiescence, cell survival, and apoptosis and are considered as tumor suppressors in different cell systems (10). In T cells, Ag recognition activates the PI3K/Akt pathway, leading to the production of the second messenger phosphatidylinositol-3,4,5-trisphosphate, the activation of the serine/threonine kinases Akt and SGK1, and the phosphorylation of different substrates actively participating in the translation of these external stimuli into an effective T cell response (11, 12). Among these substrates, FoxO1 phosphorylation by Akt in the nucleus triggers its relocalization to the cytosol (13, 14), thereby shutting down its transcriptional activity. However, we and others highlighted new specific roles of FoxO1 in naïve T cells such as the regulation of CD62-L (L-selectin), CCR7, and IL-7Rα expression (15–17), giving this TF a determinant role in various major immune processes controlled by these receptors, that is, T cell homing, migration, and homeostasis.

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 thymic Treg 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. Foxo1wt (Foxo1), Foxo1Tko (Foxo1Δ5 × CD4-Cre), and Foxo1dKO (Foxo1Δ5) mice have been previously described (16, 18, 23, 24). Foxo3Tko (Foxo3f/f CD4-Cre), and Foxo3ctrl (Foxo3f/f) mice have been bred in our own animal facility. All mice were main-

Plasmids

Myeloma-tagged murine RORγt was inserted into EcoRI and EcoRV sites into pEhHis (Invitrogen) (mRORγt-Myc-his(EhFUS) upon PCR amplification of the template mRORγtpMIG (Addgene plasmid 24069) (25) using the primers (5′-3′) mRORγtipKozakEcoRI (forward), 5′-GAATTCACATGATGAGAAGGCGGCTTGCT TATCGGTTCCCTCCACAGGTGAGCTGACGAG-3′ and mRORγtEcoRV (reverse), 5′-ATGATGATATCCCTTGCACAGCGCCTCGGAGGATGATAAC-3′. The mutant RORγt AF2 was mutagenized using the primers (5′-3′) mRORγtAF2mut (forward), 5′-CCACTCGCGGGACAGCCGAGTAAATTTGCTAAGAGCCGA-3′ and mRORγtAF2mut (reverse), 5′-GATTCAACATCAGTGCTGGCGGCTTGC CCTTATAGAGTGGAGGGAAGGCGGCTTGGACCAC-3′. The 293T cells were seeded at 350,000 cells/ml in a 12-well plate. Twenty-four hours later, one-third of the cells was lysed and subjected to a nuclear fractionation, as described (14). Nuclear proteins were separated on a SDS-PAGE and analyzed by immunoblotting using antibodies against the FOXO1TM/pMIT was provided by D. Fruman (University of California, Irvine, Irvine, CA) (30). The mutant FOXO1TM/pMIT was provided by D. Fruman (University of California, Irvine, CA) (30). The IL-23R promoter-luciferase reporter has been provided by C. Dong (Institute for Immunology, Tsinghua University, Beijing, China) (32). The IL-23R promoter-luciferase reporter Xata has been provided by C. Dong (Institute for Immunology, Tsinghua University, Beijing, China) (28). The IL-23R promoter-luciferase reporter was generated by S. Amigorena, Institut Curie, Paris, France). C57BL/6 mice were obtained from Charles River Laboratories. C57BL/6 wild-type (WT) CD45.1 and CD45.2 mice were bred in our own animal facility. All mice were main-

Retroviral infection

RORγt/pMIG (Addgene plasmid 24069) has been described (25), and FOXO1TM/pMIT has been generated using the primers (5′-3′) huFOXO1ΔDBD (forward), 5′-GGCGCGCTCGGCGGGGACGCAAGCTATGGCGGCCAGGCACAGGAGCGCTGCC-3′ and huFOXO1ΔDBD (reverse), 5′-TGGCGTCTTACGAAAAATTCTACTGGGTGCCCGCCCGAGGCACG-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 (plat-E) (32) were seeded in 10 ml DMEM plus 10% FCS at 2.5×106 cells/ml in a 10-cm dish. At days 1 and 2, the medium was replaced by the retroviral supernatant supplemented with protamin sulfate (10 μg/ml) (or polybrene [5 μg/ml] in the case of the coinfection with RORγt and Foxo1TDMBD). 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 FCS, IL-6 (10 ng/ml), and the pancaspase inhibitor QVD-OPh (20 μM) for 24 h. After the first infection, cells were maintained for 3 d in culture. Infected cells were then activated with PMA plus ionomycin (0.5 μg/ml each) for 2 h plus Golgiplug (1/1000); stained with anti–Thy1.1-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 PE anti–IFN-γ (BD Pharmingen); and analyzed by the FACS LSRII.

Real-time PCR

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 (Myx 0.5 μg) along with either the Flg or Flag-Flag-tagged Foxo1/FOXO1wt, ΔDBD, FOXO1TM/pMIT, or FOXO1TM/pMIT expressing the template FOXO1TM/pMIT was provided by D. Fruman (University of California, Irvine, CA) (30). The IL-23R promoter-luciferase reporter has been provided by C. Dong (Institute for Immunology, Tsinghua University, Beijing, China) (32). The IL-23R promoter-luciferase reporter Xata has been provided by C. Dong (Institute for Immunology, Tsinghua University, Beijing, China) (28). The IL-23R promoter-luciferase reporter was generated by S. Amigorena, Institut Curie, Paris, France). C57BL/6 mice were obtained from Charles River Laboratories. C57BL/6 wild-type (WT) CD45.1 and CD45.2 mice were bred in our own animal facility. All mice were main-

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 (Myx 0.5 μg) along with either the Flg or Flag-Flag-tagged Foxo1/FOXO1wt, ΔDBD, FOXO1TM/pMIT, or FOXO1TM/pMIT expressing the template FOXO1TM/pMIT was provided by D. Fruman (University of California, Irvine, CA) (30). The IL-23R promoter-luciferase reporter has been provided by C. Dong (Institute for Immunology, Tsinghua University, Beijing, China) (32). The IL-23R promoter-luciferase reporter Xata has been provided by C. Dong (Institute for Immunology, Tsinghua University, Beijing, China) (28). The IL-23R promoter-luciferase reporter was generated by S. Amigorena, Institut Curie, Paris, France). C57BL/6 mice were obtained from Charles River Laboratories. C57BL/6 wild-type (WT) CD45.1 and CD45.2 mice were bred in our own animal facility. All mice were main-

Luminozones

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 (Myx 0.5 μg) along with either the Flg or Flag-Flag-tagged Foxo1/FOXO1wt, ΔDBD, FOXO1TM/pMIT, or FOXO1TM/pMIT expressing the template FOXO1TM/pMIT was provided by D. Fruman (University of California, Irvine, CA) (30). The IL-23R promoter-luciferase reporter has been provided by C. Dong (Institute for Immunology, Tsinghua University, Beijing, China) (32). The IL-23R promoter-luciferase reporter Xata has been provided by C. Dong (Institute for Immunology, Tsinghua University, Beijing, China) (28). The IL-23R promoter-luciferase reporter was generated by S. Amigorena, Institut Curie, Paris, France). C57BL/6 mice were obtained from Charles River Laboratories. C57BL/6 wild-type (WT) CD45.1 and CD45.2 mice were bred in our own animal facility. All mice were main-
T-cell-depleted Foxo1−/− CD45.2 bone marrow cells was then coinfected i.v. with 1 × 106 T-cell-depleted C57BL/6 CD45.1 bone marrow cells into lethally irradiated (950 rad) 6- to 8-wk-old C57BL/6 CD-1 mice. Analysis was performed 4 wk upon reconstitution, as indicated (n = 10 mice).

Ab and reagents

Anti–IFN-γ (clone XMG1.2), anti–γ–δ TCR (clone GL3), anti-CD25 (clone PC61), anti-CD4 (clone L3T4), anti–CD45R (clone 53-6.7), anti-CD69 (clone PK136), anti–TCR-β (clone H57.567), anti-CD44 (clone IM7), anti–IL-4 (neutralizing Ab; clone 11B11), FnBclCD16/32 (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; 3111) was from Thermoscientific. Anti–IL-6Rα (clone D7715A), anti–IL-17A (eBio17B7), and anti-Foxp3 (clone FJK-16B) Abs were from BioLegend. Anti-Myc (clone 9B11; 2276), anti–GFP-FITC (1-46326), and anti–IFN-γ (neutralizing Ab; clone R4-62A) Abs were from AbD Serotec. Murine rIL-6 (216-16) and human rTGF-β (100-21C) were from PeproTech. The p110α inhibitor IC87114 (528118), the Akti1/2 inhibitor IC87114 (528118), the Akti1/2 inhibitor (124017), and the Foxo1 inhibitor AS1842856 (344355) were from Calbiochem, and QVP-Oph (OPH001-01M) was from R&D Systems.

In vitro iTTh17 differentiation assay

293T cells were prepared as previously described (14). For immunoprecipitation of nuclear proteins, nuclear fraction were completed with a nucleus immunoprecipitation buffer (25 mM Tris-HCl [pH 7.6], 1 mM EDTA, 0.5% glycerol, 1% Nonidet P-40, 25 mM NaCl, and protease and phosphatase inhibitors; 150 mM final NaCl concentration). Anti-Foxo1 Ab was immunoprecipitated overnight at 4°C with protein A/G magnetic beads (Ademtech), followed by three washes with the BC100 buffer (36). Immune complexes were separated on SDS-PAGE, blotted, and revealed using anti-Myc, anti-Flag, or anti–phosphoT24-Foxo1 Abs.

Immunoprecipitation and immunoblotting

For immunoprecipitation experiments of proteins in the whole-cell lysates, 293T cells (4 × 105 in a 10-cm plate) were cotransfected with the indicated plasmids (10 μg each) using lipofectamine 2000 (Invitrogen). After a 24-h culture, cells were lysed and sonicated, and the tagged proteins were immunoprecipitated, as previously described (35, 36). Nuclear fractions of 293T cells were prepared as previously described (14). For immunoprecipitation of nuclear proteins, nuclear fraction were completed with a nucleus immunoprecipitation buffer (25 mM Tris-HCl [pH 7.6], 1 mM EDTA, 5% glycerol, 1% Nonidet P-40, 25 mM NaCl, and protease and phosphatase inhibitors; 150 mM final NaCl concentration). Anti-Foxo1 Ab was immunoprecipitated overnight at 4°C with protein A/G magnetic beads (Ademtech), followed by three washes with the BC100 buffer (36). Immune complexes were separated on SDS-PAGE, blotted, and revealed using anti-Myc, anti-Flag, or anti–phosphoT24-Foxo1 Abs.

Anti–IL-17A or anti-tubulin Abs were used as loading controls. For IL-6 signaling and anti-CD3 plus anti-CD28–induced proximal signaling events, CD4 naïve T cells were sorted and stimulated in serum-free medium with either IL-6 (5 ng/ml) or anti-CD3 plus anti-CD28 Abs (2 μg/ml each), followed by cross-linking with goat anti-hamster IgG (20 μg/ml) (Thermoscientific) for the indicated time. Cells were then resuspended in a lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA [pH 8.0], 5 mM Na3P, 1 mM Na2VO3, 20 mM NaF, 5 mM Na3VO4 [pH 7.6], 3 mM β-glycerophosphate, 10 mM NaF, 1% Triton X-100, and protease and phosphatase inhibitors [Roche]). The endonuclease Benzonase (Merck Millipore) and SDS (0.1%) were then added, and the samples were sonicated and centrifuged. For IL-6 signaling, phospho–Y705-STAT3 and STAT3 were detected by immunoblotting. Anti-phosphoS256 Foxo1, anti-Foxo1, anti-phosphotyrosine, anti–phospho-T202/Y204 Erk1/2, and anti–Erk1/2 were used for detection of TCR proximal signaling events.

Statistics

Statistical significance was analyzed by a two-tailed Student’s 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 (Foxo1flox × CD4-Cre, designated in this work as Foxo1Tko) (16, 17) and littermate controls (Foxo1f/f, designated in this work as Foxo1f/f) (Experimental Fig. 1A). We first analyzed the proportion of peripheral IL-17A+ T cells (pTh17) in memory CD4 T cells (CD4+ TCRβ+ CD44high CD69+) from mLNs and pLNs, essentially from WT origin. We consistently observed a higher proportion of effector memory cells expressing IL-17A in Foxo1Tko mice (Fig. 1A). We also evaluated the iTTh17 population in Foxo1Tko mice and found an increase of iTTh17 of ∼3-fold (Supplemental Fig. 1B, 1C). In contrast, similar experiments in Foxo3Tko mice showed no change in the number of pTh17 (Supplemental Fig. 1D) and iTTh17 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 CD45.2 mice with a mixture of bone marrows from WT (CD45.1) and Foxo1Tko (CD45.2) mice (Supplemental Fig. 1E). Foxo1 is deficient T cells. We purified CD4 naive T cells from LNs and Foxo1Tko (CD45.1) mice (Supplemental Fig. 1E). Foxo1 is essential for the maintenance of the expression of IL-7R on CD4 naïve T cells. As a result, CD4 T cell survival and proliferation are reduced in the absence of Foxo1 (Fig. 1F, 1G). 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 Foxo3− 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 protein in Th17 development by performing in vitro iTTh17 differentiation assays using Foxo1-deficient T cells. We purified CD4 naïve T cells from LNs 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 decreased with higher concentrations 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 iTreg 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 iTreg development.

Inhibition of the PI3K/Akt pathway decreases IL-17A and IL-17F expression and iTreg 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 iTreg 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 iTreg 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 vector encoding GFP alone (pMIG). We performed an in vitro RORγt 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).
inhibitors (Fig. 3F). Similarly, 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 RORγt or Foxo1 (Fig. 4B). As a control, particularly pronounced in cells expressing the highest levels of FOXO1 (GFP+ Thy1.1high, 25%) (Fig. 4B). As a control, particularly pronounced in cells expressing the highest levels of FOXO1 (GFP+ Thy1.1high, 25%) (Fig. 4B).

**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 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. 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, 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 AKEEA mutant was still able to bind RORγt (Fig. 5C, lane 6, Supplemental Fig. 3D). Consistent with this result, RORγt mutated on the AF2 domain (RORγt-AF2mut) was also still able to bind Foxo1TM (Fig. 5D, 5E, lane 6). This observation indicates that Foxo1 forms a complex with RORγt, which is not bringing into play the LXXLL domain involved in the Foxp3/RORγtc complex.

To gain further insights into this molecular interaction, we created two mutants of Foxo1TM in which either the DBD or the TAD was truncated (Fig. 5F). Both truncated mutants were transcriptionally inactive compared with Foxo1TM mutants (Supplemental Fig. 3E). A nuclear fractionation revealed that, whereas Foxo1TM was mainly expressed in the nucleus, both mutants could also be found both in the cytosol and the nucleus (Supplemental Fig. 3E). We tested the ability of both mutants to

**FIGURE 3.** Inhibition of the PI3K/Akt pathway strongly decreases IL-17A and IL-17F expression. (A) CD4 naive T cells (C57BL/6 WT 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) with the PI3Kδ inhibitor IC87114 (5 μM) or DMSO as a control. On day 3, CD4+ IL-17A+ cells were analyzed. Data are representative of three independent experiments. (B) CD4 naive T cells were stimulated as in (A) in the presence of the Akt inhibitor Akti1/2 (0.75 μM) or DMSO as a control. On day 3, CD4+ IL-17A+ cells were analyzed. Data are representative of three independent experiments. (C) Foxo1 S256 phosphorylation on CD4 naive T cells was incubated with either DMSO, IC87114 (5 μM), or Akti1/2 (0.75 μM) for 1 h prior to activation with CD3 plus CD28 Abs. Data are representative of two independent experiments. (D) STAT3 Y705 phosphorylation on CD4 naive T cells incubated with the inhibitors as in (C) prior to IL-6 stimulation. Data are representative of two independent experiments. (E) CD4 naive T cells were activated with CD3 plus CD28 Abs and transduced with either pMIG or 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. (F) and (G) CD4 naive T cells were cultured in either Th0 (no cytokines) or iTh17 (IL-6 [10 ng/ml] plus TGF-β [0.062 ng/ml]) conditions with DMSO, IC87114 (5 μM), or Akti1/2 (0.75 μM). On day 2, IL-17A (F) or IL-17F (G) expression was measured by real-time PCR and normalized to L32 mRNA expression. Error bars represent the SD of triplicate measurements. Data are representative of two independent experiments. See also Supplemental Fig. 2.
FIGURE 4. A constitutive active Foxo1 represses RORγt-induced IL-17A expression by inhibiting RORγt function. (A) CD4 naive 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 naive 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 shown are representative of three independent experiments. The graph shows means ± SD. (F) The 293T cells were transfected with 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, 6C). 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 TGFβ/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 FOXO1TM lacking the DBD (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, IL-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

**FIGURE 7.** The ROR\(_{\gamma}\)-interacting DBD domain of Foxo1 is required to repress IL-17A expression induced by TGF-β/IL-6 and ROR\(_{\gamma}\).

(A) CD4 naive T cells were activated with anti-CD3 plus anti-CD28 Abs (2 μg/ml each) in the presence of IL-6 (10 ng/ml) plus TGF-β (0.062 ng/ml) (Th17 conditions), transduced with either pMIT, FOXO1TM/pMIT, or FOXO1TM\(_{\Delta}\)DBD/pMIT, and cultured for additional 3 d. CD4\(^+\)Thy1.1\(^+\) or CD4\(^+\)Thy1.1\(^-\) cells were then analyzed for IFN-γ and IL-17A expression. Data are representative of three independent experiments. (B) CD4 naive T cells activated with anti-CD3/anti-CD28 Abs were coinfected with ROR\(_{\gamma}\)/pMIG, FOXO1TM/pMIT, or FOXO1TM\(_{\Delta}\)DBD/pMIT. GFP\(^+\)Thy1.1\(^+\) or GFP\(^+\)Thy1.1\(^{\text{high}}\) cells were then analyzed for IFN-γ and IL-17A expression. Data are representative of three independent experiments.
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 iTTh17 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 Foxo1<sup>Tko</sup> 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 naïve T cells had a reduced capacity to differentiate into iTTh17 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 iTTh17 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 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).

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<sup>T</sup> 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<sup>+</sup> 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<sup>+</sup> memory T cells, a similar mechanism might take place, as it has been recently reported that overexpression 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 Foxo1<sup>Cre</sup> and Foxo3<sup>Cre</sup> 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 Lambolze 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.
References

1. Noack, M., and P. Miossec. 2014. Th17 and regulatory T cell balance in auto-
2. Bettelli, E., M. Oukka, and V. K. Kuchroo. 2007. Th17–17 cells in the circle of
5. Abbas, A. K., C. Benoist, J. A. Bluestone, D. J. Campbell, S. Ghosh, S. Hori,
R. A. Flavell, and J. Craft. 2009. Thymic self-reactivity selects natural inter-
leukin 17-producing T cells that can regulate peripheral inflammation. Nat.
8. Ouyang, W., S. L. McDevitt, L. Zhou, C. E. Tadokoro, A. Lepelley, J. J. Laffaille,
J. D. Cua, and D. R. Littman. 2006. The orphan nuclear receptor RORgammat
directs the differentiation program of proinflammatory IL-17+ T helper cells.
Cell 126: 1121–1133.
9. Yang, X. O., B. P. Pappu, R. Nurieva, A. Akimzhanov, H. S. Kang, Y. Chung,
B. P. Pappu, R. Nurieva, A. Kizhmanov, H. S. Kang, Y. Chung, B. P. Pappu,
R. Nurieva, A. Kizhmanov, H. S. Kang, Y. Chung, B. P. Pappu, R. Nurieva,
A. Kizhmanov, H. S. Kang, Y. Chung, B. P. Pappu, R. Nurieva, A. Kizhmanov,
H. S. Kang, Y. Chung, B. P. Pappu, R. Nurieva, A. Kizhmanov, H. S. Kang,
Y. Chung, B. P. Pappu, R. Nurieva, A. Kizhmanov, H. S. Kang, Y. Chung,
B. P. Pappu, R. Nurieva, A. Kizhmanov, H. S. Kang, Y. Chung, B. P. Pappu,
R. Nurieva, A. Kizhmanov, H. S. Kang, Y. Chung, B. P. Pappu, R. Nurieva,
A. Kizhmanov, H. S. Kang, Y. Chung, B. P. Pappu, R. Nurieva, A. Kizhmanov,
H. S. Kang, Y. Chung, B. P. Pappu, R. Nurieva, A. Kizhmanov, H. S. Kang,
Y. Chung, B. P. Pappu, R. Nurieva, A. Kizhmanov, H. S. Kang, Y. Chung,
B. P. Pappu, R. Nurieva, A. Kizhmanov, H. S. Kang, Y. Chung, B. P. Pappu,
R. Nurieva, A. Kizhmanov, H. S. Kang, Y. Chung, B. P. Pappu, R. Nurieva,
A. Kizhmanov, H. S. Kang, Y. Chung, B. P. Pappu, R. Nurieva, A. Kizhmanov,
H. S. Kang, Y. Chung, B. P. Pappu, R. Nurieva, A. Kizhmanov, H. S. Kang,
Y. Chung, B. P. Pappu, R. Nurieva, A. Kizhmanov, H. S. Kang, Y. Chung,
B. P. Pappu, R. Nurieva, A. Kizhmanov, H. S. Kang, Y. Chung, B. P. Pappu,
R. Nurieva, A. Kizhmanov, H. S. Kang, Y. Chung, B. P. Pappu, R. Nurieva,
A. Kizhmanov, H. S. Kang, Y. Chung, B. P. Pappu, R. Nurieva, A. Kizhmanov,
H. S. Kang, Y. Chung, B. P. Pappu, R. Nurieva, A. Kizhmanov, H. S. Kang,
Y. Chung, B. P. Pappu, R. Nurieva, A. Kizhmanov, H. S. Kang, Y. Chung,
B. P. Pappu, R. Nurieva, A. Kizhmanov, H. S. Kang, Y. Chung, B. P. Pappu,
R. Nurieva, A. Kizhmanov, H. S. Kang, Y. Chung, B. P. Pappu, R. Nurieva,
A. Kizhmanov, H. S. Kang, Y. Chung, B. P. Pappu, R. Nurieva, A. Kizhmanov,
H. S. Kang, Y. Chung, B. P. Pappu, R. Nurieva, A. Kizhmanov, H. S. Kang,
Y. Chung, B. P. Pappu, R. Nurieva, A. Kizhmanov, H. S. Kang, Y. Chung,
B. P. Pappu, R. Nurieva, A. Kizhmanov, H. S. Kang, Y. Chung, B. P. Pappu,
R. Nurieva, A. Kizhmanov, H. S. Kang, Y. Chung, B. P. Pappu, R. Nurieva,
A. Kizhmanov, H. S. Kang, Y. Chung, B. P. Pappu, R. Nurieva, A. Kizhmanov,


Supplementary figure 1. (Related to Fig. 1) 
(A) Foxo1 and β-actin protein expression in B220+ , CD4+ and CD8+ cells from lymph nodes of Foxo1ctrl and Foxo1Tko mice. 
(B) Gating strategy for tT_h17 cells. Thymocytes from 4-15 weeks-old Foxo1ctrl or Foxo1Tko mice were stimulated with PMA (20 ng/ml) and ionomycin (0.5 µg/ml) plus Golgiplug for 4 h and stained with the indicated antibodies. The gating strategy to analyze tT_h17 is shown. Data are representative of four independent experiments.
(C) Quantification of tT_h17 cells from Foxo1ctrl vs. Foxo1Tko mice. Data are represented as means ± SD. 
(D) No defect in pT_h17 development in the absence of Foxo3. Cells from mesenteric or peripheric LNs from 5-11 weeks-old Foxo3Tko or Foxo3ctrl mice were stimulated with PMA (20 ng/ml) and ionomycin (0.5 µg/ml) plus Golgiplug for 4 h. CD4+ TCRβ+ CD44hi IL-17A+ cells were analyzed by LSRII. 
(E) Schematic representation of the mixed bone marrow (BM) reconstitution experiment.
(F) Recovery (%) of WT and Foxo1Tko effector memory CD4+ TCRβ+ Foxp3- CD44hi cells upon four weeks of reconstitution of irradiated C57BL/6 CD3εko mice mixed bone marrow from WT and Foxo1Tko mice (ratio 1:4).
(G) Analysis of Foxp3+ Treg cells from the mixed bone marrow chimeras. Representative panels from mLNs and quantification of Foxp3+ cells among CD4+ TCRβ+ cells from mLNs and pLNs (n=10 mice). The graph represents means ± SEM.
Supplementary figure 2. (Related to Figs. 2 and 3)

(A) Foxo3 expression in B and T cells. Splenocytes from Foxo3ctrl or Foxo3Tko mice were stained for B220, CD4 and CD8. B220+, CD4+ or CD8+ cells were then sorted by FCS ARIA and analyzed for Foxo3 expression by immunoblot. ß-actin is used as a loading control. (B) Foxo3 is not required for iT\(_{H17}\) differentiation. CD4 naïve T cells (CD44\(^{lo}\) CD25\(^{-}\) TCR\(_{\gamma\delta}^{-}\) NK1.1\(^{-}\)) from Foxo3ctrl and Foxo3Tko mice were sorted by FACS ARIA and stimulated by anti-CD3 plus anti-CD28 antibodies (2 \(\mu\)g/ml each) in the presence or the absence of IL-6 (10 ng/ml) and increasing concentrations of TGF\(_{\beta}\) (0.015-8 ng/ml, as indicated). Three days later, cells were stimulated with PMA and ionomycin (0.5 \(\mu\)g/ml each) plus Golgiplug for 2 h and analyzed for IL-17A expression by intracellular staining and the FACS LSRII. The data shown are representative of two independent experiments. (C) Low IL-6R\(_{\alpha}\) expression in the absence of Foxo1 (representative panel of Fig. 2B). CD4*CD44\(^{lo}\) naïve T cells from peripheral lymph nodes from three Foxo1Tko (#1-3, green and yellow curves) or one Foxo1ctrl (blue curve) mice were analyzed for IL6R\(_{\alpha}\) expression. The isotype control (red curve) was performed on Foxo1ctrl LN CD4\(^{+}\) T cells. This panel is representative of five independent experiments. (D) No defect in TCR/CD28 signaling events in Foxo1Tko CD4 naïve T cells. CD4 naïve T cells (CD44\(^{lo}\) CD25\(^{-}\) TCR\(_{\gamma\delta}^{-}\) NK1.1\(^{-}\)) from Foxo1Tko and Foxo1ctrl mice were stimulated with anti-CD3 plus anti-CD28 antibodies (10 \(\mu\)g/ml each) for the indicated time points. Cell lysates were analyzed using anti-Foxo1, -phospho-tyrosine (p-Tyr), -phospho-Erk1/2 and -Erk1/2 antibodies. The data shown are representative of two independent experiments. (E) Inhibition of Akt does not affect ROR\(_{yt}\) nuclear expression. CD4 naïve T cells (T\(_{N}\), CD44\(^{lo}\) CD25\(^{-}\) TCR\(_{\gamma\delta}^{-}\) NK1.1\(^{-}\)) were cultured in either T\(_{0}\) (no cytokines) or iT\(_{H17}\) (TGF\(_{\beta}\) 0.062 ng/ml plus IL-6 10 ng/ml) conditions either in the presence of Akt\(_{1/2}\) inhibitor (0.75 \(\mu\)M) or absence (DMSO). Three days later, cells were subjected to a nuclear fractionation and lysates were analyzed for the presence of ROR\(_{yt}\) by immunoblot. Tubulin is used as a control for the cytosolic fraction.
Supplementary figure 3. (Related to Figs. 4 and 5)

(A) Panel related to fig. 4B. Infected CD4 naïve T cells from experiment shown in fig. 4B were analyzed for IL-17A and IFNγ expression on GFP+ Thy1.1+ or GFP- Thy1.1+ cells. (B) Foxo1 TM decreases RORγt-induced IL-23R promoter activity. 293T cells were transfected in triplicates with RORγt-Myc (1 µg) and/or Myc-Flag-Foxo1TM (0.05 and 0.5 µg) along with IL-23R promoter luciferase (IL-23R Prom-Luc) reporter (1 µg) and renilla (0.1 µg). Forty-eight hours later, cells were lysed and the ratio firefly/renilla was measured and the graph shows means ± SD. Expression of the transfected proteins in nucleus was detected by immunoblot using the Myc antibody (inset). Immunoblotting with Ku80 antibodies served as a loading control of the nuclear fractions. The data shown are representative of three independent experiments. (C) Activity of Foxo1TM H212R mutant. 293T cells were transfected in triplicates with increasing doses of Myc-Flag-Foxo1TM (0.05, 0.1 and 0.5 µg) along with Forkhead Responsive Element-Luciferase (FRE-Luc) reporter (0.5 µg) and Renilla (0.1 µg). Twenty-four hours later, cells were lysed and the ratio firefly/renilla was measured. The graph shows means ± SD. Expression of the transfected proteins in the whole cell lysate was detected by immunoblot using the Myc antibody (inset). Immunoblotting with Ku80 antibodies served as a loading control of the nuclear fractions. (D) Binding of Foxo1 TM, Foxo1 mutants and RORγt in the nucleus. 293T cells were co-transfected with RORγt-Myc (10 µg) and either Flag-Foxo1TM, Flag-Foxo1TM H212R or Flag-Foxo1TM AKEAA (10 µg each). Twenty-four hours later, anti-Myc immunoprecipitates from the nuclear fraction were analyzed for the presence of Flag-Foxo1 and RORγt-Myc by immunoblotting using the indicated antibodies. (E) Activity and localization of the truncated mutants of Foxo1TM. 293T cells were transfected in triplicates with either Flag-Foxo1TM, Flag-Foxo1TMΔDBD or Flag-Foxo1TMΔTAD mutants (0.5 µg each) along with the reporter construct FRE-Luc (0.5 µg) and renilla (0.1 µg). Twenty-four hours later, cells were lysed and the ratio firefly/renilla was measured. The graph represents means ± SD. Expression of Foxo1TM and the mutants was analyzed in the cytosol and the nucleus by immunoblot using an anti-Flag antibody. Tubulin was used as a marker of the cytosolic fraction. The data shown are representative of three independent experiments. (F) The binding of Foxo1 and RORγt in the nucleus requires the DBD of Foxo1. 293T cells were co-transfected with RORγt-Myc and Flag-Foxo1TM, Flag-Foxo1TMΔDBD or Flag-Foxo1TMΔTAD (10 µg each). Twenty-four hours later, the anti-Myc immunoprecipitates (left panel) from the nuclear fraction and the nuclear lysates (right panel) were analyzed using the indicated antibodies.
Supplementary figure 4. (Related to discussion) (A) RORγt antagonizes Foxo1TM activity. 293T cells were transfected with Flag-Foxo1TM (0.5 µg) and/or RORγt-Myc (0.05, 0.1 and 0.5 µg) along with FRE-Luc reporter (0.5 µg) and renilla (0.1 µg). Twenty-four hours later, the ratio firefly/renilla was measured and protein nuclear expression was verified by immunoblot using anti-Myc and anti-Ku80 as a loading control. The graph shows means ± SD. Data are representative of three independent experiments. (B-C) No change in Foxo1-deficient iT\(_{\gamma}17\) differentiation in the presence of anti-IFNγ and anti-IL-4 neutralizing antibodies. CD4 naïve T cells (CD4\(^{+}\)CD25\(^{-}\)TCR\(^{\gamma\delta}\)NK1.1\(^{-}\)) from Foxo1\(^{Tko}\) and Foxo1\(^{dfl}\) mice were stimulated with anti-CD3 plus anti-CD28 antibodies (2 µg/ml each), IL-6 (10 ng/ml) and increasing doses of TGFβ (0.015-2 ng/ml) for 3 days in the presence (blue curves) or absence (black curves) of anti-IFNγ and anti-IL-4 antibodies (10 µg/ml each). CD4\(^{+}\) cells expressing IFNγ (B), IL-17A (C) were analyzed by intracellular staining and the FACS LSRII. The data shown are representative of two independent experiments.