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*J Immunol* 2010; 184:6161-6169; Prepublished online 28 April 2010;
doi: 10.4049/jimmunol.0903243
http://www.jimmunol.org/content/184/11/6161

Supplementary Material

http://www.jimmunol.org/content/suppl/2010/04/28/jimmunol.0903243.DC1

References

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RORC2 Is Involved in T Cell Polarization through Interaction with the FOXP3 Promoter

Simone Burgler,* Pierre-Yves Mantel,**† Claudio Bassin,* Nadia Ouaked,* Cezmi A. Akdis,* and Carsten B. Schmidt-Weber‡

The process of Th cell differentiation toward polarized effector T cells tailors specific immunity against invading pathogens while allowing tolerance against commensal microorganisms, harmless allergens, or autologous Ags. Identification of the mechanisms underlying this polarization process is therefore central to understand how the immune system confers immunity and tolerance. The present study demonstrates that retinoic acid receptor-related orphan receptor C2 (RORC2), a key transcription factor in Th17 cell development, inhibits FOXP3 expression in human T cells. Although overexpression of RORC2 in naive T cells reduces levels of FOXP3, small interfering RNA-mediated knockdown of RORC2 enhances its expression. RORC2 mediates this inhibition at least partially by binding to two out of four ROR-responsive elements on the FOXP3 promoter. Knockdown of RORC2 promotes high FOXP3 levels and decreased expression of proinflammatory cytokines β form of pro-IL-1, IL-6, IL-17A, IFN-γ, and TNF-α in differentiating naive T cells, suggesting that the role of RORC2 in Th17 cell development involves not only induction of Th17-characteristic genes, but also suppression of regulatory T cell-specific programs. Together, this study identifies RORC2 as a polarizing factor in transcriptional cross-regulation and provides novel viewpoints on the control of immune tolerance versus effector immune responses. The Journal of Immunology, 2010, 184: 6161–6169.

CD4+ effector cells can be divided into different subsets according to their phenotype and function. Each subset is crucial for mounting an appropriate immune response to a certain type of pathogen (1, 2). Differentiation of naive Th cells toward a specific effector subset is therefore an important process in immune system homeostasis. T cell development is governed by lineage-specific transcription factors that regulate expression of characteristic surface receptors and cytokines. The Th1-specific T box transcription factor (T-bet), for example, coordinates production of IFN-γ and TNF-α (3), whereas the GATA-binding protein 3 (GATA-3) regulates Th2 development including production of their characteristic cytokines IL-4, IL-5, IL-9, and IL-13 (4). Development of Th17 cells and expression of their specific cytokines IL-17A, IL-17F, and IL-26 is promoted by the retinoic acid receptor-related orphan receptor γt/C2 (RORγt/RORC2) (5). Along with Th1 and Th2 cells, Th17 cells promote inflammatory reactions and are important for the induction of neutrophil-attracting chemokines in epithelial or other tissue cells (2). In contrast to the effector subsets, regulatory T cells (Tregs) restrict the strength and duration of the response and establish tolerance to harmless self- and non–self-Ags. Failure in either development or function of Tregs causes severe hyperproliferative diseases and is assumed to underlie autoimmune and allergic disorders. FOXP3 is the master regulator of Tregs. Retroviral gene transfer of FOXP3 is sufficient to convert naive T cells into a Treg phenotype (6) and confers suppressor function on peripheral CD4+ CD25+ T cells (7, 8). Expression of FOXP3 is regulated through interaction of several TCR-induced transcription factors, including NFAT, AP-1, and cAMP response element binding protein (CREB)/activating transcription factor, with the promoter or an enhancer element of the foxp3 gene (9–12). Although TCR stimulation induces basal levels of FOXP3, maximal and sustained FOXP3 expression requires additional stimulation by cytokines such as IL-2, TGF-β, and IL-27 (10, 13–16).

Cross-inhibition of T cell subsets during differentiation is an important mechanism to ensure expansion of the polarized populations. Cytokines produced by one subset inhibit differentiation of other subsets. This antagonism is mirrored on the level of transcription factors, which cross-regulate each other and finally imprint or unlock the transcriptional program on the chromatin. T-bet directly inhibits Th2 differentiation by binding to its master regulator GATA-3, preventing it from DNA binding and activation of Th2-specific genes (17, 18). Likewise, GATA-3 can downregulate STAT4, a transcription factor important for Th1 differentiation (19). By binding to GATA-binding sites in the FOXP3 promoter, GATA-3 also negatively regulates FOXP3 expression and thereby inhibits Treg differentiation (20). The Th17-driving transcription factor RORC2 is expressed by both Treg and Th17 cells, whereas only Tregs express FOXP3 (21–23). In Tregs, FOXP3 suppresses transcriptional activity of RORC2 by direct interaction, thereby inhibiting Th17 differentiation (22, 24).

RORC2 belongs to the family of RORs that consists of three members: RORA (NR1F1, RORα, RZRα), RORB (NR1F2, RORβ,
RZRβ, and RORC (NF1F3, RORγt, RZRγ). All ROR genes generate several isoforms, which differ only in their N termini (25–27). Proteins of the ROR family display a typical nuclear receptor domain structure consisting of four domains: an N-terminal domain, a highly conserved DNA-binding domain (DBD), a hinge domain, and a C-terminal ligand-binding domain. The DBD binds to DNA, containing a so-called ROR response element (RORE), which consists of the DNA sequence GGTC as a core motive, preceded by an A/T-rich sequence (28, 29). In contrast to other nuclear receptors, ROR family proteins bind DNA as monomers (26, 28, 30). They can interact with both coactivators and corepressors to positively or negatively regulate transcription of target genes and are involved in a wide range of differentiation processes. RORC plays a key role in the development of lymph nodes and Peyer’s patches and the regulation of thymopoiesis (27, 31–33).

In contrast to T-bet, GATA-3, and FOXP3, RORC has not been described to participate in cross-regulation of T cell subsets. Based on our observation that GATA-3 inhibits FOXP3 expression (20), we hypothesized that the preference for Th17 cells over Tregs might be mediated through restriction of the foxp3 gene by RORC2. The present study describes a role for RORC2 in T cell cross-regulation by negatively regulating FOXP3 expression, which involves binding of RORC2 to the FOXP3 promoter region.

Materials and Methods

Isolation of naive T cells

PBMCs were isolated from buffy coats of healthy donors using Ficoll (Biochrom, Berlin, Germany) density gradient centrifugation. CD4+ T cells were purified using anti-CD4 magnetic beads and Detach-a-Bead Abs (Dynal, Invitrogen, Carlsbad, CA). CD45RA+ cells were isolated using MACS magnetic beads (Miltenyi Biotec, Auburn, CA).

Differentiation of Treg and Th17 cells

CD45RA+ T cells were stimulated with soluble anti-CD3 and anti-CD28 Abs (4 μg/ml) in serum-free AIM-V medium (Life Technologies, Rockville, MD) supplemented with IL-2 (25 U/ml; Proreo Pharma, Liestal, Switzerland). Anti-CD3 and anti-CD28 Abs were produced using the hybridoma cell line OKT3 (American Type Culture Collection, Manassas, VA) and the hybridoma cell line 15E8 (CLB). Tregs were generated in the presence of TGF-β (5 ng/ml; R&D Systems, Minneapolis, MN) and neutralizing anti-IL-12 (5 μg/ml; R&D Systems) and anti-IFN-γ (1 μg/ml; R&D Systems) Abs. Th17 cells were differentiated as previously described (23) in the presence of TGF-β, IL-6 (20 ng/ml; PeproTech, Rocky Hill, NJ), β form of pro-IL-1 (IL-1β, 10 ng/ml; PeproTech), IL-23 (20 ng/ml; eBioscience, San Diego, CA), and neutralizing anti-IL-12 and anti-IFN-γ Abs. Dose titrations were performed and the optimal dose for each cytokine and Ab was determined in preliminary experiments.

Isolation of RNA, cDNA synthesis, and quantitative RT-PCR

Total RNA was isolated using the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. Reverse transcription was performed with TaqMan reverse transcription reagents (Fermentas, Burlington, Ontario, Canada) using random hexamer primers according to the manufacturer’s protocol.

PCR primers were designed based on the sequences reported in GenBank using the Primer Express software version 1.2 (Applied Biosystems, Foster City, CA). Primers used for quantitative analysis are listed in Supplemental Table I. All primers are spanning intron-exon borders and have been verified for efficacy over a four-log concentration range. The prepared cDNAs were amplified using iTag SYBR Green Supermix with ROX (Bio-Rad, Hercules, CA) according to the manufacturer’s recommendations in an ABI Prism 7900 Sequence Detection System (Applied Biosystems). Relative quantification and calculation of the range of confidence was performed using the comparative ΔΔCT method. EF-1α was used as endogenous control. All amplifications were conducted in triplicates.

Determination of cytokine concentration

T cells were cultured for 5 d under Treg conditions. Before restimulation with soluble anti-CD3 and anti-CD28 Abs (4 μg/ml), the cells were washed to remove the cytokines used for differentiation. Supernatants were collected 48 h after restimulation, and concentrations of IL-1β, IL-2, IL-5, IL-6, IL-13, IL-17A, IFN-γ, and TNF-α were determined by cytometric bead array (Bio-Rad) according to the manufacturer’s protocol.

Western blotting

Protein extraction was performed as follows: the cells were pelleted and resuspended in buffer C (20 mM HEPES [pH 7.9], 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM DTT, protease inhibitors [Roche Diagnostic Systems, Somerville, NJ], and 0.1% Nonidet P-40) and incubated at 4°C with agitation for 15 min. Insoluble material was removed by centrifugation, and the supernatants were diluted with buffer D (as above, but without NaCl). Total protein concentrations were determined by a colorimetric protein assay (Bio-Rad). Samples were loaded next to a prestained protein-mass ladder (Invitrogen-Life Technologies) on a NuPAGE 4–12% bis-Tris gel (Invitrogen/Life Technologies). The proteins were electroblotted onto a polyvinylidene difluoride membrane (Amersham Biosciences, Piscataway, NJ). Unspecific binding was blocked with TBS-Tween 5% BSA. After blocking, the membrane was incubated with an anti-RORγ Ab (Santa Cruz Bio-technology, Santa Cruz, CA) in a 1:500 dilution in blocking buffer overnight at 4°C. Next, the membrane was washed and incubated with an anti-rabbit HRP-labeled Ab (1:3000; Cell Signaling Technology, Beverly, MA) at room temperature for 1 h. The blot was developed using an ECL plus Western blot Detection System (GE Healthcare, Piscataway, NJ) and visualized with an LAS 1000 camera (Fujifilm, Valhalla, NY). To confirm equal sample loading and transfer, membranes were stripped, reblocked and probed using an anti-GAPDH Ab (6C5, Ambion, Austin, TX).

Flow cytometry

Cells were stained with the CD25 mAb (Beckman Coulter, Fullerton, CA) prior to FOXP3 intracellular staining, which was performed according to the manufacturer’s protocol using Alexa Fluor 488 anti-human FOXP3 Flow kit (295D; Biolegend, San Diego, CA). Matched isotype controls were used at the same protein concentration as the respective Abs.

Cell acquisition by flow cytometry was done on a four-color FACS EPICS XL-MCL (Beckman Coulter) using the software Expo32 version for data acquisition and evaluation.

Amplification of FOXP3 promoter fragments

FOXP3 promoter fragments were amplified by conventional PCR using a biotinylated reverse primer (5’-biotin-ACCTACCTCTGGCTGAATCAGC-3’) situated 177 bp downstream of the transcription start site (TSS). Multiple forward primers were designed to generate FOXP3 promoter fragments of different length (Supplemental Table II). The position of the primers are depicted in Fig. 34. The plasmid pGL4 FOXP3 –1210/+177 served as a template. Site-directed mutagenesis in the FOXP3 promoter region was introduced using the QuickChange kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. Primers used for mutagenesis are listed in Supplemental Table III. Reactions were conducted in 75 mM Tris HCl (pH 8.3), 20 mM (NH₄)₂SO₄, 2 mM MgCl₂, 0.2 mM 2’-deoxynucleoside 5’-triphosphate, 0.2 μM of each primer, 6 μg/ml template DNA (pGL4 FOXP3 –1210/+177), and 1.25 U Taq DNA polymerase (Fermentas). The same PCR conditions were used for the amplification of all the products: initial denaturation step (2 min, 94°C), 42 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 60 s, and final elongation step (7 min, 72°C). PCR products were purified by ethanol precipitation. For this purpose, 1/10 volume 3 M sodium acetate and 3 volumes 100% ethanol were added to the PCR products, and samples were mixed, precipitated for 1 h at −80°C, and harvested by centrifugation (30 min, maximum speed, 4°C). The supernatant was removed, residual ethanol was allowed to evaporate, and the pellet was eluted in blocking buffer (PBS, 0.1% BSA, 0.05% Tween 20). To quantify the FOXP3 promoter fragments, samples were loaded next to a MassRuler DNA Ladder Mix (Fermentas) on a 1% agarose gel and quantified using the AIDA software (Raytest, Straubenhardt, Germany). For fragment F11 and competitor oligonucleotides, sense and antisense oligonucleotides (Microsynth, Balgach, Switzerland; sequences in Supplemental Table II) were annealed for 10 min at 95°C.

FOXP3 promoter ELISA

Protein extraction was performed as described for Western blotting, and 384-well flat bottom plates were precoated with streptavidin (Pierce, Rockford, IL) and were washed three times with washing buffer (PBS, 0.05% Tween 20). Biotinylated FOXP3 promoter fragments or consensus sequences were added (1 pmol/well; 50 fmol/μl) and incubated for 1 h at room temperature. Upon three wash cycles, nuclear extracts were added at concentration of 1 μg/μl and incubated overnight at 4°C in the presence of
10 μg poly(dextransinosinic–dextransidylic) acid (Sigma-Aldrich, St. Louis, MO) to block unspecific binding. For competition experiments, the nuclear extracts were preincubated with competitor oligonucleotides for 2 h at 4˚C. The plate was washed three times with buffer C/D and then incubated with the primary Ab (rabbit anti-RORC2; 1:200 in buffer C/D; Biologend) at 4˚C for 2 h. After three wash cycles with buffer C/D, a secondary Ab (anti-rabbit IgG-HRP; 1:3000 in buffer C/D; Cell Signaling Technology) was added and the plate was incubated for 1 h at 4˚C. The wells were washed four times with buffer C/D before substrate reagent was added (R&D Systems). The colorimetric reaction was stopped after 2–5 min by adding 2 M H2SO4. Absorbance at 450 nm was measured using a Mithras microplate reader (Berthold Technologies, Bad Wildbad, Germany).

Transfections

Naive T cells were preactivated overnight in serum-free AIM-V medium (Life Technologies) containing soluble anti-CD3 and anti-CD28 Abs (4 μg/ml). A total of 6–10 × 106 T cells were transfected with 3 μg pcDNA3.1-empty, pcDNA3.1-RORC2FL, or pcDNA3.1-RORC2ΔDBD plasmids in 100 μl Nucleofector solution (Amaxa Biosystems, Cologne, Germany) using the T-23 program. Small interfering RNA (siRNA) oligos (Ambion) were transfected at a concentration of 30 nM. Optimal concentrations were determined in preliminary experiments (Supplemental Fig. 1). Two different siRNA oligos were tested, and oligo siRNA1 was used in subsequent experiments. siRNA sequences are listed in Supplemental Table IV. Eight hours posttransfection with plasmids or siRNA, the medium was replaced with fresh, serum-free AIM-V medium, and cells were stimulated with anti-CD3 and anti-CD28 Abs (4 μg/ml) or as indicated.

HEK cells were plated in six-well dishes and transfected with pcDNA3.1-empty, pcDNA3.1-RORC2FL, or pcDNA3.1-RORC2ΔDBD plasmids at 90–95% confluence using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instruction.

Chromatin immunoprecipitation

CD4+ T cells were transfected with pcDNA3.1-RORC2FL and cultured under Th17 conditions for 48 h. Alternatively, naive T cells were differentiated toward Th17 cells. The chromatin immunoprecipitation (ChIP) assay was performed as described (34). Protein–DNA complexes were cross-linked with formaldehyde at a final concentration of 1.42% for 15 min. Formaldehyde was quenched with 125 mM glycine for 5 min, and cells were harvested. Cells were lysed with immunoprecipitation buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.5], 5 mM EDTA, Nonidet P-40 [0.5% v/v]; R&D Systems) containing phosphatase and protease inhibitor mixtures (both Roche, Basel, Switzerland); the chromatin was sheared by sonication and incubated with anti-RORC2 Abs (H-190 X; Santa Cruz Biotechnology) overnight at 4˚C. Rabbit IgG isotype controls (Santa Cruz Biotechnology) overnight at 4˚C. As a positive control, anti-human RNA polymerase II Abs and corresponding control IgG (both from SA Biosciences, Frederick, MD) were used. The cleared chromatin was then incubated with protein A agarose beads, and the DNA was isolated with a 10% (w/v) Chelex 100 resin. Samples were treated with proteinase K at 55˚C for 30 min. The proteinase K was then inactivated by boiling the samples for 10 min. Real-time PCR was performed using specific primers for the FOXP3 promoter (Supplemental Table V) and control GAPDH primer (SA Biosciences) for the RNA polymerase II. A negative control PCR for each immunoprecipitation using IgG1A negative control primer targeting open reading frame-free intergenic DNA (SA Biosciences) was performed. The fold enrichment in site occupancy was calculated in independent experiments. MFI, median fluorescence intensity.

Reporter gene assays

A total of 6–10 × 106 naive T cells were cotransfected with 3 μg pGL4-empty or the pGL4-FOXP3 promoter luciferase reporter vector and 2 μg pcDNA3.1-empty, pcDNA3.1-RORC2FL or pcDNA3.1-RORC2ΔDBD plasmids in 100 μl Nucleofector solution (Amaxa Biosystems). Electroporation was performed using the T-23 program. After 8 h, the medium was replaced with fresh serum-free AIM-V medium, and cells were stimulated with anti-CD3 and anti-CD28 Abs (4 μg/ml) for another 12 h until luciferase activity in cell lysates was measured using the dual luciferase assay system (Promega, Madison, WI) in a Berthold Lumat LB 9507 luminometer (Berthold Technologies) according to the manufacturer’s instructions.

Suppression assays

Naive T cells were transfected with a scrambled siRNA or siRNA against RORC2. Cells were cultured under Treg conditions for 10 d and then mixed with 2 × 106 autologous irradiated PBMCs that were used as APCs and autologous CFSE-labeled CD4+ T cells. Cells were stimulated with 2.5 μg/ml anti-CD3 mAb and cultured in a 96-well plate, and proliferation of effector cells was determined by analyzing the CFSE dilution by flow cytometry after 5 d of culture. Gating on the CD4+CFSE+ T cells enabled the exclusion of APCs and Tregs.

FIGURE 1. Overexpression of RORC2 inhibits FOXP3 expression. A, Naive CD4+ T cells were transfected with RORC2ΔDBD and RORC2FL and expression of the proteins was analyzed by Western blot. B and C, Naive CD4+ T cells were transfected with RORC2FL and RORC2ΔDBD. Eight hours posttransfection, the cells were stimulated with anti-CD3 and anti-CD28 Abs in the presence of IL-2 and TGF-β. B, FOXP3 mRNA levels were determined by RT-PCR after 48 h and 5 d, and levels in untreated cells were set to 100%. C, FOXP3 protein expression was measured by FACS after 5 d. Bars/numbers indicate mean and SD of at least three independent experiments. p ≤ 0.005.

FIGURE 2. siRNA-mediated knockdown of RORC2 increases FOXP3 expression. Naive CD4+ T cells were transfected with siRNA oligonucleotides targeting RORC2. Eight hours posttransfection, the cells were stimulated with anti-CD3 and anti-CD28 Abs in the presence of IL-2 and TGF-β. A, mRNA levels of RORC2 (left panel) and FOXP3 (right panel) were determined by RT-PCR after 48 h and 5 d. Levels in untreated cells were set to 100% or to 1, respectively. Bars represent mean and SD of at least 3 independent experiments. B, FOXP3 protein expression was measured by FACS after 5 d. Numbers indicate mean and SD of at least three independent experiments. MFI, median fluorescence intensity. p ≤ 0.001.
Plasmids

The human FOXP3 promoter was cloned into the pGL4 vector (Promega) to generate the pGL4 FOXP3 -1246/+177 as previously described (9). Human RORC2FL and RORC2ΔDBD were cloned into a pcDNA3.1 vector (Invitrogen) using the primers listed in Supplemental Table VI.

Bioinformatics

Alignments of the human FOXP3 promoter were performed using the Genomatix software (www.genomatix.de).

Results

Overexpression of RORC2 reduces FOXP3 expression

Although RORC2 activity is inhibited by FOXP3 in Tregs, this inhibitory function appears to be abolished in Th17 cells. Considering the key roles of lineage-specific transcription factors in mutual inhibition of T cell subsets, RORC2 itself represents a candidate for this unidentified suppressor of FOXP3. To test whether RORC2 has the capacity to suppress FOXP3 expression, RORC2 was cloned as a full-length construct (RORC2FL) as well as a truncated version lacking the DBD (RORC2ΔDBD), which was used as negative control. Naive CD4+ T cells were transfected with these plasmids, and Western blot analysis was performed to confirm recombinant expression of the proteins (Fig. 1A). The molecular mass of the proteins observed on the gel were in accordance with the calculated molecular mass of ∼56 kDa for RORC2FL and 48 kDa for RORC2ΔDBD. The additional faint band at ∼50 kDa was due to unspecific binding of the Ab. To test the influence of RORC2 on FOXP3 expression, transfected T cells were stimulated with anti-CD3 and anti-CD28 Abs in the presence of IL-2 and TGF-β, and FOXP3 levels were determined by RT-PCR (Fig. 1B). Consistent with the results obtained by RT-PCR, RORC2FL-transfected cells showed a markedly decreased FOXP3 staining (14.0 ± 3.2%) compared with mock-transfected cells (22.0 ± 1.9%) or RORC2ΔDBD-transfected cells (23.2 ± 1.8%). Taken together, these results demonstrate an inhibitory role for RORC2 on FOXP3 mRNA and protein expression by a DNA binding-dependent mechanism.

Knockdown of RORC2 increases FOXP3 levels

The observation that high RORC2 levels result in a downregulation of FOXP3 led us to the hypothesis that a decrease in RORC2 might enhance the expression of FOXP3. To test this, RORC2 was knocked down using siRNA oligonucleotides. Two different oligonucleotides were tested, and a dose titration as well as a time kinetic was performed to validate RORC2 knockdown (Supplemental Fig. 1). Cells were transfected with siRNA against RORC2 or with a scrambled siRNA as negative control and then cultured with anti-CD3 and anti-CD28 Abs for 48 h. mRNA levels of RORC2 and FOXP3 were determined by RT-PCR (Fig. 2A). In cells transfected with siRNA against RORC2 or with a scrambled siRNA as negative control and then cultured with anti-CD3 and anti-CD28 Abs in the presence of IL-2 and TGF-β. After 48 h, mRNA levels of RORC2 and FOXP3 were determined by RT-PCR (Fig. 2A). In cells transfected with siRNA against RORC2, levels of RORC2 were decreased, and FOXP3 levels were increased.

No FIGURE 3. The FOXP3 promoter contains conserved ROREs. A, Sequence of the FOXP3 promoter from bp −1246 to bp +223. ROREs are marked in blue; NFAT binding sites are marked in green. CAAT, GC, and TATA boxes are indicated with orange boxes. Primers used for generation of the promoter fragments are marked with black arrows. B, FOXP3 promoters of different species were aligned using the Genomatix software. Conserved bases are highlighted in bold; RORE core sequences are marked with blue boxes.
knocked down by 76.1 ± 14% compared with untransfected cells, whereas no effect was observed with scrambled siRNA, confirming the specificity of the assay. In cells with reduced RORC2 levels due to siRNA treatment, a 3-fold increase in FOXP3 was observed. To analyze FOXP3 expression on protein level, cells were cultured for 5 d under the same conditions and then subjected to FACS analysis (Fig. 2B). Consistent with the results obtained by RT-PCR, numbers of FOXP3-positive cells increased in the presence of siRNA against RORC2 compared with scrambled siRNA-transfected cells (from 20.1 ± 2.0% to 28.5 ± 4.0%). The median fluorescence intensities of FOXP3 were also slightly higher, suggesting that levels of FOXP3 increased on a per-cell basis as well. Together, these data reveal that downregulation of RORC2 increases FOXP3 expression and are in agreement with the results above showing reduced FOXP3 expression after RORC2 overexpression.

RORC2 binds to the FOXP3 promoter at RORE −942 and RORE +115

Transcription factors of the nuclear receptor family like RORC2 control gene expression by binding to the promoter of their target genes. To investigate the mechanism by which RORC2 controls FOXP3 expression, we first analyzed the FOXP3 promoter sequence for the presence of ROREs, putative binding sites for RORC2 (Fig. 3A). The sequence from bp −1242 until bp +223 contains four ROREs: three upstream (at −1096, −942, and −847) and one downstream of the TSS (at +115). These putative binding sites for ROR family members are conserved among the FOXP3 promoters of different species (Fig. 3B). Direct binding of RORC2 to the FOXP3 promoter was assessed using a FOXP3 promoter ELISA system (Fig. 4A). In this assay, either consensus sequences of 30 bp containing one RORE or promoter fragments spanning bp −1116 to +177 were coated on ELISA plates. Plates were incubated with extracts of HEK cells transfected with RORC2 or an empty vector as a control, and binding of RORC2 was quantified by chemiluminescence following incubation with the appropriate Abs. Binding of RORC2 to the wild-type but not to the mutated consensus sequence was observed, validating the specificity of the assay. Absorbance was even slightly higher when extracts of HEK cells transfected with RORC2 were incubated with the FOXP3 promoter sequence, whereas incubation with extracts from empty vector-transfected cells produced only background levels of absorbance. These results suggest that RORC2 binds to one or more of the ROREs on the FOXP3 promoter. The sequence specificity of the binding was further confirmed by addition of soluble oligonucleotides, which abolished binding by competition when they contained the wild-type but not a mutated consensus sequence.

To investigate which of the four ROREs are important for binding of RORC2 to the FOXP3 promoter, serial deletions of the FOXP3 promoter were constructed, and fragments were coated on an ELISA plate (Fig. 4B). Binding of RORC2 to the promoter fragment lacking RORE −1096 was equal to binding observed using the fragment containing all four ROREs. However, when RORC2 was incubated with a construct lacking RORE −942, a drop in absorbance was detected, suggesting that RORE −942 is relevant for binding of RORC2 to the promoter. Deletion of RORE −847 did not substantially alter the absorbance of the promoter ELISA, whereas a clear drop in absorbance was found in the fragment also lacking RORE +115. RORC2 has been described to compete with NFAT for DNA binding (35). As three putative NFAT-binding sites are located on the FOXP3 promoter (Fig. 3A), a construct lacking these sites was analyzed for RORC2 binding (Fig. 4B). However, no difference in binding was observed.

To confirm these results, all ROREs were individually mutated, and promoter constructs carrying these mutations were investigated for RORC2 binding (Fig. 4C). A decreased absorbance in the ELISA was observed when analyzing constructs containing a mutation in RORE −942 or RORE +115, whereas binding of RORC2 to the promoter was not affected by mutations in RORE −1096 and RORE −847. These results confirm that RORE −942 and RORE +115 are involved in the binding of RORC2 to the FOXP3 promoter. Human RORC2 seems not to recognize the corresponding mouse ROREs, as only human but not mouse site-specific competitor oligonucleotides reduced binding of RORC2 to the FOXP3 promoter (Supplemental Fig. 2). This suggests that binding of RORC2 to the FOXP3 promoter is species specific.

To assess whether RORC2 binds to the FOXP3 promoter in living T cells, a ChIP assay was performed (Fig. 5A). T cells were transfected with RORC2 or alternatively differentiated toward Th17 cells. Using anti-RORC2 Abs, the FOXP3 promoter was immunoprecipitated, confirming that RORC2 binding to the FOXP3 promoter occurs in T cells. In contrast, no significant changes in site occupancy were observed when negative control primer targeting open reading frame-free intergenic DNA (IGX1A) were used.

To find out whether binding to the FOXP3 promoter is required for RORC2-mediated inhibition, a FOXP3 promoter luciferase assay was performed (Fig. 5B). Cotransfection of RORC2FL and a luciferase plasmid containing the FOXP3 promoter resulted in...
Knockdown of RORC2 shifts Th17 cells toward T cells with a less proinflammatory but more regulatory phenotype

To investigate whether these findings have an implication in T cell differentiation, especially in the balance between Treg and Th17 cells, we analyzed whether lack of RORC2 shifts Th17 cells toward a Treg phenotype. Therefore, RORC2 was downregulated in naive T cells using siRNA, and the cells were cultured under either Treg or Th17 differentiation conditions. As expected, in control settings (i.e., scrambled siRNA-transfected cells), FOXP3 mRNA levels were clearly higher in Treg than in Th17 (Fig. 6A). However, in Th17 cells with downregulated RORC2 levels, FOXP3 expression was significantly increased, almost reaching levels in siRNA-treated Tregs. Similarly, frequencies of FOXP3-positive cells in siRNA-transfected Th17 cells were comparable to frequencies in cells cultured under Treg conditions, whereas in cells transfected with control siRNA, a considerably smaller fraction of Th17 cells was FOXP3 positive (Fig. 6B). We further analyzed the cytokine profile of Th17 cells carrying reduced RORC2 levels during differentiation due to siRNA treatment (Fig. 6C). As expected and previously reported (36, 37), IL-17A levels were markedly reduced in these cells. Moreover, these cells also showed a decreased expression of other proinflammatory cytokines like IL-1β, IL-6, IFN-γ, and TNF-α (Fig. 6C, left panel), whereas no changes in IL-5 and IL-13 levels were observed (Fig. 6C, right panel), suggesting a specific effect.

Finally, we investigated whether siRNA-mediated RORC2 knockdown leads to a higher suppressive capacity of Tregs. Naive T cells were transfected with a scrambled siRNA or RORC2-specific siRNA. The cells were then cultured under Treg conditions, and their capacity to suppress autologous effector cells was analyzed (Fig. 7). Cells with decreased RORC2 levels and thus higher FOXP3 levels were slightly more suppressive than scrambled siRNA-treated cells. The results, however, did not reach statistical significance. Taken together, these results indicate that Th17 cell differentiation is impaired in RORC2-depleted cells and that these cells instead differentiate toward a less proinflammatory but more tolerating subset.

Discussion

The current study demonstrates repression of FOXP3 by the Th17 cell-promoting transcription factor RORC2 and thus identifies a novel role for RORC2 in T cell polarization.

Overexpression of RORC2 decreased the levels of FOXP3 on both the mRNA and protein levels. This relationship was confirmed by siRNA-mediated knockdown of RORC2, which increased FOXP3 expression, indicating an inverse correlation of the two factors. Binding to the promoter of FOXP3 is necessary for RORC2-mediated repression, as overexpression of RORC2 lacking the DBD did not inhibit FOXP3 expression. Moreover, no decrease in luciferase activity was observed upon cotransfection with the DBD-deficient construct. To further investigate the mechanism of repression, we analyzed the FOXP3 promoter sequence from bp −1242 until bp +223 and identified four putative RORC2-binding sites. In agreement with the fact that
ROR transcription factors bind DNA as monomers (26, 28, 30), all ROREs identified consist of a single independent motif. Three of these ROREs are located in a poorly characterized part of the FOXP3 gene, directly upstream of the core promoter and the region, which we previously identified as an inductive area of the promoter containing TCR-inducible signals such as NFAT and AP-1 (9, 38). A fourth RORE is situated downstream of the TSS. Using an ELISA-based system, we detected binding of RORC2 to RORE_{2942} and to a lesser extent to RORE_{+115}. ROREs consist of the DNA sequence GGTCA as a core motive and are often preceded by an A/T-rich sequence (28, 29). This A/T-rich sequence of RORC2 binding sites is also found upstream of RORE_{2942}. Using a ChIP assay, we demonstrate that interaction of RORC2 and the promoter region of FOXP3 occurs in vivo. Altogether, these experiments reveal that repression of FOXP3 by RORC2 involves physical binding of RORC2 to two ROREs on its promoter, a mechanism commonly observed for ROR family members.

ROR family transcription factors induce repression of target genes by recruiting corepressors, which induce chromatin deacetylation and therefore gene repression (39–41). Several corepressors have been described to interact with ROR family members, and it remains to be determined which of them mediates the repression of the foxp3 gene observed in this study. Retinoic acid has been reported to be a key factor in regulating the balance of Treg versus Th17 cells (42–45). Although retinoic acid seems to mediate these effects by its cognate receptor retinoic acid receptor α (46, 47), the mechanism possibly also involves RORC2, as retinoic acid has been found to bind to RORC2 and inhibit its transcriptional activity (48). Therefore, retinoic acid might favor Treg cell differentiation by binding and inhibiting RORC2, leading to an increased FOXP3 expression. However, addition of retinoic acid did not change NF binding to the ROREs on the FOXP3 promoter (data not shown).

Beside corepressor-mediated suppression, RORC2 might also inhibit FOXP3 expression by competition with NFAT, which is important for TCR-induced foxp3 gene activation (9), as RORC2 has been shown to bind to NFAT binding sites and inhibit its transcriptional activity (35). We did not detect binding of RORC2 to NFAT binding sites, suggesting an alternate mechanism of repression in context of the foxp3 gene. Because IL-2 plays a major role in FOXP3 induction (13, 14), and RORC2 has been shown to suppress IL-2 production (27), it may exert its inhibitory function through negative regulation of autocrine IL-2 secretion. In fact, levels of IL-2 dropped significantly upon siRNA-mediated RORC2 knockdown (data not shown).

Because IL-2 plays a major role in FOXP3 induction (13, 14), and RORC2 has been shown to suppress IL-2 production (27), it may exert its inhibitory function through negative regulation of autocrine IL-2 secretion. In fact, levels of IL-2 dropped significantly upon siRNA-mediated RORC2 knockdown (data not shown).

The siRNA-induced RORC2 knockdown during differentiation of Th17 cells led to a phenotype characterized not only by high FOXP3 expression, but also by low levels of proinflammatory Th17 and Th1 cytokines such as IL-1β, IL-6, and IL-17A as well as IFN-γ and TNF-α, respectively. This suggests an important role for RORC2 in the inhibition of Treg differentiation under inflammatory conditions. Reduced levels of IL-17A are presumably a direct consequence of RORC2 knockdown, which is known to regulate IL-17A expression on a transcriptional level (49). In contrast, the decrease in IFN-γ is a novel observation and might be caused indirectly by the increased FOXP3 expression, as FOXP3 has been shown to repress IFN-γ production (50). Tregs with reduced RORC2 levels tended to be more suppressive, although the results did not reach statistical significance. The small increase in suppressive activity might be explained by the fact that the siRNA-mediated RORC2 knockdown is maximal after 48 h and might be less pronounced at the time point of suppression. Furthermore, it has been shown that RORC2 expression does not completely exclude suppressive activity (51, 52). The extent to which RORC2 reduces suppressive activity is likely to depend on the relative levels of FOXP3 and RORC2.
We observed a relatively high frequency of FOXP3+ cells in Th17 cells treated with control siRNA (13%). Recent studies suggest that Th17 cells and Tregs are related in ontogeny and show some plasticity (21, 23, 51–53). The high numbers of FOXP3+ cells under Th17 conditions might therefore be explained with the existence of a population of FOXP3+IL-17+ T cells. Furthermore, the in vitro differentiated cells are likely to exist not purely of Th17 cells, but some Tregs might be found as well.

Crossover inhibition of T cell subsets during differentiation is an important mechanism to ensure polarization and clonal expansion of the most efficient subset. The presented data highlight a new role of some Tregs might be found as well.

Differentiated cells are likely to exist not purely of Th17 cells, but plasticity (21, 23, 51–53). The high numbers of FOXP3+ cells under IL-12beta2 chain or Tbet. Immunity 18: 415–428.

References


Ayyoub, M., F. Deknuydt, I. Raimbaud, C. Dousset, L. Leveque, G. Bisley, and D. Valmori. 2009. Human memory FOXP3+ Tregs secrete IL-17 ex vivo and constitutively express the Th17 lineage-specific transcription factor ROR- 


## Supplemental data

### Supplemental table 1. Primers used for RT-PCR

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<tr>
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<tbody>
<tr>
<td>EF-1α fw</td>
<td>CTGAACCATCCAGGCCAAAT</td>
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<tr>
<td>EF-1α rv</td>
<td>GCCGTGTGGCAATCCAAT</td>
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<tr>
<td>FOXP3 fw</td>
<td>GAAACAGCACAT CCCAGAGTTC</td>
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<tr>
<td>FOXP3 rv</td>
<td>ATGGCCCAGCGGATGAG</td>
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<tr>
<td>RORC2 fw</td>
<td>CAGTCATGAAGAACACAAAT GTAAGTG</td>
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<tr>
<td>RORC2 rv</td>
<td>CAGGTGATAAACC CGTAGTGGAT</td>
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### Supplemental table 2. Primers and oligonucleotides used for promoter ELISA

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<td>P-1116fw</td>
<td>GACATGTTCAGCAC ACT GTATCTG ACC</td>
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<td>P-1006fw</td>
<td>AGTAAAAGAC CCCAA AGGCTGAGG</td>
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<td>P-511fw</td>
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<td>P+1fw</td>
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<td>P+176rv-bio</td>
<td>bio-ACCTTACCTGGCTGGAATCACAG</td>
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<td>F11 sense</td>
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<td>Competitor MUT sense</td>
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<td>AGAGTACTATGAGACAGACTGT TCATTCAAGGCT</td>
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7 **Supplemental table 3.** Primers used for mutagenesis

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<td>GTTTATCTTGAATTTTTCCATGGGCCAGATACAGTGCTGAAC</td>
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<td>CCCATTGGGACCCCTGGGCTCTTTGTTTCAG</td>
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<td>-847mutfw</td>
<td>GACAGTGCGCTGGGCCAGGGCAGACTCAAG</td>
</tr>
<tr>
<td>-847mutrv</td>
<td>CTTGAGTGCCCTGGCCAGCCACTGTC</td>
</tr>
<tr>
<td>+115mutfw</td>
<td>GGCAGGGGGGCGCCAGGGGCTCTCTG</td>
</tr>
<tr>
<td>+115mutrv</td>
<td>CAGAGGGCCCCTGGCCCCCCGC</td>
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8

9 **Supplemental table 4.** SiRNA oligos

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<tr>
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<tr>
<td>SiRNA1 antisense</td>
<td>UCAAGGAUAAAGAGAUUGtg</td>
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<tr>
<td>SiRNA2 sense</td>
<td>CCCUGACAGAGAUAGAGCAAtt</td>
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<tr>
<td>SiRNA2 antisense</td>
<td>UGCUCUAUCUCUGACGGGag</td>
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10

11 **Supplemental table 5.** Primers used for ChIP

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<td>ChIPFOX3-1fw</td>
<td>ATCGTGAGGATGGATGCATTAATA</td>
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<td>ChIPFOX3-1rv</td>
<td>CCACTGGGAAGGTCCCTAGC</td>
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13 **Supplemental table 6.** Primers used for cloning

<table>
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<tr>
<td>RORC2FLfw</td>
<td>GAGAGGTACCGACATGAGAACACAAATTGAAGTGATCCC</td>
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<td>RORC2ΔDBDfw</td>
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<td>RORC2rv</td>
<td>ATATGCGGGCGCTCAGTGGACAGCCACAGGT</td>
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14

15
Figure legends

Supplemental FIGURE 1. Dose-titration and time-kinetic of siRNA oligonucleotides against RORC2. A, Naïve CD4⁺ T cells were transfected with two different siRNA oligonucleotides against RORC2 at increasing concentrations. After 48 h RORC2 mRNA expression was determined by RT-PCR. Levels in scrambled siRNA transfected cells was set to 100%. Bars represent mean and standard deviation of 3 independent experiments. B, Naïve CD4⁺ T cells were transfected with siRNA1 at a concentration of 30 nM. RORC2 mRNA levels were determined by RT-PCR after different time points. Expression at time-point 0 h was set to 100% and levels at each time points were normalized to levels in scrambled siRNA transfected cells at the same time point. Bars represent mean and standard deviation of 3 independent experiments. (siScram = scrambled siRNA)

Supplemental FIGURE 2. Species-specificity of RORC2 binding. FOXP3 promoter sequences were incubated with nuclear extracts of HEK cells transfected with RORC2 in the presence or absence of human or mouse soluble competitor oligonucleotides corresponding to RORE-942 and RORE+115. Binding of RORC2 was quantified using the FOXP3 promoter ELISA assay. Bars show mean and standard deviations of triplicates representative of three independent experiments.

Supplemental FIGURE 3. Mutual regulation of the transcription factors FOXP3 and RORC2. TGF-β induces both FOXP3 and RORC2. Under non-inflammatory conditions, mediators like IL-2, retinoic acid (RA) or IL-27 enhance TGF-β-induced FOXP3 expression. FOXP3 inhibits
DNA-binding of RORC2 and thereby its transcriptional activity through physical interaction. As FOXP3 levels predominate under these conditions, no active RORC2 is available to repress the *foxp3* gene and to activate Th17 cell programs. FOXP3 levels further increase, promoting the development of Treg cells. During inflammation, Th17-differentiating cytokines such as IL-6, IL-21 and IL-23 enhance RORC2 expression and/or inhibit FOXP3 expression, thereby favoring RORC2 over FOXP3. RORC2 further inhibits FOXP3 expression and induces transcriptional programs leading to Th17 cell development.