

RESEARCH ARTICLE

ROR γ t and ROR α signature genes in human Th17 cells

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Abstract

ROR γ t and ROR α are transcription factors of the RAR-related orphan nuclear receptor (ROR) family. They are expressed in Th17 cells and have been suggested to play a role in Th17 differentiation. Although ROR γ t signature genes have been characterized in mouse Th17 cells, detailed information on its transcriptional control in human Th17 cells is limited and even less is known about ROR α signature genes which have not been reported in either human or mouse T cells. In this study, global gene expression of human CD4 T cells activated under Th17 skewing conditions was profiled by RNA sequencing. ROR γ t and ROR α signature genes were identified in these Th17 cells treated with specific siRNAs to knock down ROR γ t or ROR α expression. We have generated selective small molecule ROR γ t modulators and they were also utilized as pharmacological tools in ROR γ t signature gene identification. Our results showed that ROR γ t controlled the expression of a very selective number of genes in Th17 cells and most of them were regulated by ROR α as well albeit a weaker influence. Key Th17 genes including IL-17A, IL-17F, IL-23R, CCL20 and CCR6 were shown to be regulated by both ROR γ t and ROR α . Our results demonstrated an overlapping role of ROR γ t and ROR α in human Th17 cell differentiation through regulation of a defined common set of Th17 genes. ROR γ t as a drug target for treatment of Th17 mediated autoimmune diseases such as psoriasis has been demonstrated recently in clinical trials. Our results suggest that ROR α could be involved in same disease mechanisms and gene signatures identified in this report could be valuable biomarkers for tracking the pharmacodynamic effects of compounds that modulate ROR γ t or ROR α activities in patients.

Introduction

ROR γ t and ROR α are transcription factors belonging to the RAR-related orphan nuclear receptor (ROR) family [1]. Proteins of the ROR family typically consist of 4 functional domains: an N-terminal (A/B) domain, a conserved DNA binding domain (DBD), a hinge domain, and a C-terminal ligand binding domain (LBD) [1]. ROR γ and ROR γ t are two

preparation of the manuscript were all initiated and generated by the authors. The specific roles of these authors are articulated in the 'author contributions' section.

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isoforms that are transcribed from the RORC gene, and four isoforms, ROR α 1–4, are produced from the RORA gene. These isoforms are generated from their corresponding genes through alternative promoter usage and exon splicing [2–5]. They differ in tissue expression profile and in the amino-terminal A/B domain that is critical for binding to specific ROR binding elements (RORE) to regulate target gene expression [1].

ROR γ t is expressed in distinct immune cell types including thymocytes, Th17 and Tc17 cells, $\gamma\delta$ T cells, ILC3 cells, lymphoid tissue inducer (LTi) cells and NKp46⁺ CD3⁻ NK cells [6–9]. It is induced in activated CD4 T cells under Th17 differentiation conditions, such as the presence of IL-1 β , IL-6, IL-23 and TGF β [10–13]. The other isoform ROR γ is known to be expressed in the liver, adipose, skeletal muscle and kidney, however its expression in T cells is still controversial [14–17]. ROR α is present in a variety of tissues including liver, adipose, kidney, testis and the brain [2,3]. In the immune system, ROR α is expressed in both lymphoid and myeloid cells and is induced during Th17 differentiation [18–20].

ROR transcription factors can work as repressors or activators on target gene transcription depending upon their recruitment of co-repressors or co-activators with their LBD domains, and this recruitment can be modulated by the type of ligands interacting with LBD [1]. Melatonin, cholesterol and cholesterol sulfate have been reported to be ROR α ligands [20–23]. Sterol lipids, such as oxysterols, were recently identified as natural ligands for ROR γ t [24,25]. Small molecule modulators of ROR γ and ROR γ t have been discovered and they bind to the LBD domain that is common for ROR γ and ROR γ t and affect their recruitment of co-activators or co-repressors [26,27].

ROR γ t has been suggested to be important for Th17 differentiation by regulating the expression of Th17 genes [11]. ROR γ t signature genes in mouse T cells have been identified by global transcriptomic analysis of mouse T cells that were either defective in ROR γ t expression or activities as a result of gene targeting or selective inverse agonist treatment respectively [28,29]. The most pronounced effect was decreased expression of Th17 signature genes such as IL-17A, IL-17F, IL-22 and IL-23R, and increased expression of other T cell subset genes such as IL-4 and Tbx21. In human T cells, ROR γ t signature genes were reported in one study on memory CD4 T cells treated with a ROR γ t specific inverse agonist and gene expression profile was performed with gene microarray approach [30]. Further studies in human T cells with other approaches such as siRNA knock down of target genes, global gene expression profile with more sensitive platforms such as RNA-Seq, as well as using multiple ROR γ t compounds to exclude compound non-specific effects are warranted to confirm the findings and to explore the possible species difference between human and mouse in ROR γ t function.

ROR α also contributes to Th17 differentiation, and ROR α deficiency in mouse T cells has been reported to reduce expression of IL-17A, IL-23R, but not IL-17F or IL-22 [31]. Mouse T cells lacking expression of both ROR γ t and ROR α showed a complete blockade in Th17 cell differentiation in contrast to the partial defects in T cells with a deficiency of either one, suggesting a complementary role for ROR γ t and ROR α in Th17 commitment [31]. Global gene profiling to search for ROR α gene signatures has not yet been conducted and information on the overlapping or unique signature genes between ROR α and ROR γ t in Th17 cells is still pending for further investigation.

In this report, we used multiple ROR γ t and ROR α selective siRNAs and ROR γ t inverse agonists to treat human CD4 T cells during Th17 differentiation, and performed RNA-Seq analysis to identify ROR γ t and ROR α signature genes. Overlapping signature genes from ROR γ t and ROR α were identified, and pathway analysis was performed to understand the functional roles of ROR γ t and ROR α in the Th17 differentiation program.

Materials and methods

Materials

Four selective and potent ROR γ t inverse agonists referred to as compound A [32], B, C and D were generated and used as tools in this report.

Human blood samples were provided by the Scripps Research Institute. Blood donors have given consent to participate in this study and the study protocol on human blood samples was submitted by Janssen R&D and approved by the Scripps Research Institute IRB (Institutional Review Board).

Three small interfering RNAs (siRNAs) targeting ROR γ t were used in this study. Two pre-designed ROR γ t specific siRNAs (Stealth siRNA HSS109301, sequence ACAAATTGAAGTGATCCCTTGCAAA, HSS109302, sequence GCTGAGGGCAGAGAGAGCTTCTATA, and HSS109300, GGAGCAATGGAAGTGGTGTGTTA) named as RORC siRNA_1, RORC siRNA_2 and RORC siRNA_4 respectively were purchased from Invitrogen, USA, and one ROR γ t siRNA (Silencer Select s12111, sequence UGCUCUAUCUCUGUCAGGGAGTG) named as RORC siRNA_3 were purchased from Ambion, USA. In addition, two pre-designed siRNAs directed against ROR α (Silencer Select s12103, sequence AUGCGAUUUAGAUUAUUCTG, and s224549, sequence UGUAGUCACAUUUGGUUCTG) labelled as RORA siRNA_1 and RORA siRNA_2 respectively, and three non-targeting siRNA (Stealth negative control medium GC duplex 46–2001 and Silencer negative control #1, AM4611 purchased from Amion, and negative siRNA AF488 from Allstars) labelled as control_1, control_2 and control_3 respectively.

Recombinant human ROR γ , ROR α and ROR β LBD proteins were prepared for ThermoFluor binding assay. The nucleotide sequences encoding the ligand binding domains (LBD) of human ROR γ (Genbank accession no. NP005051, amino acid 237–497), ROR α (Genbank accession no. NP599022, amino acid 304–556), and ROR β (Genbank accession no. NP008845, amino acid 201–452) were PCR-amplified and cloned into pET24a (Novagen) for expression in bacteria. All recombinant proteins contained an additional amino acid sequence of MAHHHHHHAGGAENLYFQGAMD at the N-terminal of LBD. After transformation of the bacteria strain BL21DE3 GOLD (Agilent) with the plasmids, 10 to 20 L cultures were grown to 1 OD and recombinant protein expression was induced overnight at 16°C with 0.2mM isopropyl-beta-D-thiogalactopyranoside (IPTG) (Corning). Bacteria cultures were lysed with microfluidizer in lysis buffer (20mM HEPES, pH 7.8, 500mM NaCl, 20mM imidazole, 2mM TECP). His-tagged proteins were collected with Ni-NTA resin (Qiagen) and purified with a HiTrap Q HP column (GE Life Sciences) eluted with a 40 CV gradient of 50–500mM NaCl.

ThermoFluor[®] binding assay

The ThermoFluor[®] assay was established to measure the change in protein stability upon binding with compounds. 1-Anilinonaphthalene-8-Sulfonic Acid (1,8-ANS) (Invitrogen) was used as a fluorescent dye to label the test proteins. The assay was performed in polypropylene PCR microplates (TF-0384/k, Abgene) with a final assay of 3 μ L reaction mixture per well overlaid with 1 μ L silicone oil (Sigma-Aldrich). Assay plates were robotically loaded onto a thermostatically controlled PCR-type thermal block and then heated at a ramp-rate of 1°C/min. Fluorescence emission was initiated by continuous illumination with UV light (LC6, Hamamatsu) via fiber optic with a band-pass filter (380–400 nm) and detected with a CCD camera (Sensys, Roper Scientific) filtered to detect signal of 500 \pm 25 nm. Reference wells contained recombinant LBD of ROR γ t, ROR α or ROR β alone, and the assay conditions were as follows: 0.065 mg/mL ROR γ t LBD, 60 μ M 1,8-ANS, 100 mM HEPES pH 7.0, 10 mM NaCl, 2.5 mM GSH, 0.002% Tween- 20; 0.1 mg/mL ROR α , 70 μ M 1,8-ANS, 25 mM HEPES pH 7.0, 50

mM NaCl, 0.001% Tween-20; 0.08 mg/mL ROR β , 60 μ M 1,8-ANS, 50 mM HEPES pH 7.0, 100 mM NaCl, 0.001% Tween-20. All 3 assays were performed in the absence and in the presence of 100 μ M co-activator peptide SRC1 (LTERHKILHRLQLQEGSPSD, England Peptide custom synthesis). The binding affinity of test compounds was estimated as described previously [33].

ROR γ t cellular reporter assay

This reporter assay was used to determine the agonistic or antagonist/inverse agonist activity of ROR γ t modulatory compounds on transcriptional activation driven by the ROR γ t LBD. HEK293T cells at 80% confluent were transfected with DNA constructs A and B using Fugene 6 (E2691, Invitrogen) at a 1:6 ratio of DNA and Fugene 6. DNA construct A is pBIND-ROR γ t LBD containing the wild type human ROR γ t LBD fused to the DNA binding domain of the GAL4 protein, and construct B is pGL4.31 (C935A, Promega) containing multiple GAL4 responsive DNA elements upstream of firefly luciferase. The pBIND vector contains the renilla luciferase gene under control of the SV40 promoter and Renilla luciferase expression is served as a control for transfection efficiency and cell viability. As a background control, cells were similarly co-transfected with an AF2 mutated ROR γ t LBD construct (LYKELF to LFKELF, pBIND-ROR γ t-AF2). The AF2 mutation has been shown to prevent co-activator binding to the ROR γ t LBD, thus preventing transcription of firefly luciferase.

Transiently transfected cells were plated into 96-well plates at 50,000 cells/well in phenol-red free DMEM, containing 5% lipid reduced FCS and Pen/Strep and treated with compounds for 24 hours. Media was removed and cells were lysed with 50 μ L 1x Glo Lysis Buffer (Promega). Dual Glo Luciferase Reagent (50 μ L/well) was then added and firefly luciferase luminescence was read on an Envision multilabel plate reader, after ten minutes incubation. Finally, Stop and Glo reagent (50 μ L/well) was added and renilla luciferase luminescence was read on an Envision after ten minutes incubation. To calculate the effect of compounds on ROR γ t activity, the ratio of firefly to renilla luciferase was determined and plotted against compound concentration. Agonist compounds increase ROR γ t-driven luciferase expression, and antagonist or inverse agonist compounds decrease luciferase expression. IC₅₀ values were determined using Graphpad Prism.

Human CD4 T cell purification

Human PBMC were purified from two healthy donor blood using Ficoll-Paque (GE Healthcare) in density centrifugation. CD4 T cells were purified from human PBMC by negative selection with human CD4 T cell isolation kit (Miltenyi Biotec). After treatment with antibodies and magnetic beads, CD4 T cells were purified with SuperMacs using XS column (Miltenyi Biotec) and grown in culture medium. T cell culture medium was composed of IMDM, 2 mM L-glutamine, 1X NEAA, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μ g/ml streptomycin, plus 10% KnockOut Serum Replacement (all from ThermoFisher Scientific).

Compound IC₅₀ determination in Th17 and Th1 differentiation assays

Effects of ROR γ t inverse agonists on human Th17 or Th1 differentiation was determined in T cell assays. CD4 T cells at 1.5×10^5 cells per well in 96-well plate were pre-treated with compounds for 1 hour before activation with anti-CD3/anti-CD28 coated beads (Miltenyi Biotec) at 2:1 bead to cell ratio in RPMI-1640 culture medium containing 10% FCS, 2 mM glutamine, 1mM sodium pyruvate, 10mM HEPES, 1mM MEM nonessential amino acid solution, and 100 U/ml each of penicillin G and streptomycin (all culture reagents from Life Technologies). Th17 differentiation was initiated by supplementing culture medium with 20 U/ml IL-2, 10 ng/ml IL-1 β (R&D), 10 ng/ml IL-23 (R&D), 50 ng/ml IL-6, 3 ng/ml TGF β 1 (R&D), 10 μ g/ml

of anti-IL-4 and anti-IFN γ (eBioscience). Th1 differentiation was driven by supplementing culture medium with 20 U/ml IL-2, 10 ng/ml IL-12 and 10 μ g/ml anti-IL4 (eBioscience). After 3 days of incubation, culture supernatants were collected and the accumulated IL-17A in Th17 cultures and IFN γ in Th1 cultures were measured using MSD MULTI-SPOT[®] Cytokine Plate following manufacturer's instructions (Meso Scale Discovery). The plate was read using Sector Imager 6000, and IL-17A or IFN γ concentrations in samples were calculated from the standard curve. Proliferation of T cells in day 3 cultures was measured by 5 hours pulsing with 1 μ Ci/well of 3H-thymidine (Perkin Elmer). Cells with incorporated radioactive thymidine were harvested onto glass fiber filter plates (Perkin Elmer). Filter plates were soaked with scintillant (Perkin Elmer) and radioactivity was counted using a Topcount (Packard). Compound IC₅₀ values in proliferation and cytokine production were determined using GraphPad Prism.

Compound or siRNA treatment of T cells in Th17 assay for gene expression study

Human CD4 T cells from 2 healthy donors were used in studies. 5.0×10^6 human CD4 T cells were used per sample and cell samples were collected at 0, 24 and 48 hours of Th17 differentiation for gene expression studies with RNA-Seq and RT-PCR.

For compound treatment, CD4 T cells were pre-treated with compounds or DMSO vehicle control at 37°C for 1 hour before T cell activation. ROR γ t inverse agonists compound A, B and C were tested at two concentrations and DMSO treatments were used as controls in human Th17 differentiation assays as shown in the study plan (S1 Table).

For siRNA treatment, three ROR γ t siRNAs, two ROR α siRNAs, two control siRNAs were tested at 1 μ M, and mock transfected samples were included as controls (S1 Table). siRNA treatment was performed by Amaxa electroporation of human CD4 T cells with siRNAs. Human CD4 T cells were taken up in 100 μ L Nucleofector solution (P3 primary cell 4D Nucleofector Kit, reference V4XP-3024, Amaxa Biosciences) at 5.0×10^6 cells/100 μ L. Cell suspensions were transfected with siRNA by electroporation using the FI-115 program in the Amaxa 4D-Nucleofector. T cells after electroporation were rinsed with 500 μ L culture medium, transferred to 24-well culture plates and kept at 37°C cell incubator before use in T cell assays. It was noticed that Amaxa electroporation caused an increased expression of some genes in human T cells and this elevated gene expression lasted up to 8 hours after electroporation. To avoid gene expression study interfered by electroporation, human CD4 T cells were rested in culture medium at 37°C for 8 hours after siRNA electroporation before the use of these cells to initiate Th17 differentiation for transcriptome profile.

T cells after compound or siRNA treatments were stimulated with anti-CD3/anti-CD28 coated beads (Miltenyi Biotech) at 2:1 bead to cell ratio in culture medium containing 10 ng/ml IL1 β (R&D), 10 ng/ml IL23 (R&D), 30 ng/ml TGF β 1 (R&D), 10 μ g/ml of anti-IL4 and anti-IFN γ (eBioscience). T cell cultures were collected at different time points for RNA extraction and gene expression studies.

RNA extraction

Total RNA was extracted from CD4 T cells using the RNeasy mini kit (Qiagen) following the manufacture's instruction. CD4 T cells were lysed in RLT solution supplemented with β -mercaptoethanol and cell lysates were loaded in RNeasy mini spin column. DNA was removed by on-column DNase digestion and RNA was eluted from column with 50 μ l nuclease-free water and stored at -80°C until analysis. RNA integrity was assessed with LabChip GX (PerkinElmer, USA).

RNA-Seq

RNA-Seq based transcriptome profiling was conducted by Beijing Genomics Institute (Hong Kong) using the Illumina HiSeqTM 2000 platform. The detailed procedures of RNA-Seq have been described previously [17]. In brief, RNA sequencing was performed for each sample to obtain about 40 million QC clean reads that were pair-end sequenced and with an average insert size of 160 base pairs and a typical read-length of 90 base pairs. Raw reads were mapped to the human reference genome (Reference Human.B37.3) and the expression of individual gene was quantified as the count of the mapped reads using RefGene20121217 as the gene model. The read alignment and gene expression quantification were conducted using Array Studio (www.omicsoft.com). Expression profiles of 25011 genes were generated for a total of 178 samples representing the independent cell cultures from the two donors under various treatments (S1 Table).

Quantitative RT-PCR of ROR α and ROR γ t signature genes

Expression of ROR α , ROR γ t and IL-17A genes in siRNA or compound treated human Th17 cells was measured by quantitative RT-PCR using the TaqMan RNA-to-Ct 1 step kit (Applied Biosystems) following the manufacturer's instructions. Validated TaqMan PCR primers and probes for detection of human IL-17A (Hs00936345_m1), ROR α (Hs00536545_m1) and GAPDH (Hs02758991_g1), and custom made primers and probe for detection of ROR γ t (forward primer TGGACCACCCCTGCTGAGAA GG, reverse primer CTTCAATTTGTGTTCTCAT GACT, and probe GGGAGCCAAGGCCGG) were purchased from ThermoFisher. Real time PCR was performed using 600ng total RNA as template in the ABI PRISM 7500 Sequence Detection System (Applied Biosystems), with reaction starting at 48°C for 15 minutes, then 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C.

ROR γ t signature genes in Th17 cells identified in RNA-Seq analysis were confirmed by quantitative RT-PCR. Duplicate samples from donor 1 treated with high concentrations of ROR γ t inverse agonists or DMSO were used in RTPCR confirmation. RNA samples were converted to cDNA using the high capacity cDNA reverse transcription kit (ThermoFisher) following the manufacturer's instructions. TaqMan PCR primers and probes for target genes were purchased from ThermoFisher (S2 Table). Real time PCR was performed using cDNA samples as templates in the TaqMan array micro fluidic cards (Applied Biosystems) and ran in the QuantStudio 12K Flex System (Applied Biosystems). PCR reaction started at 50°C for 2 minutes, then 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C.

PCR results were analyzed with the ddCT algorithm [34] to generate data on relative quantitation and changes in gene expression.

Selection criteria on differentially expressed genes from ROR α or ROR γ t siRNA treatment

For each of the two donors and at each of the two time points (24 and 48 hours) after Th17 activation, four comparisons (two ROR α siRNAs against two scrambled siRNAs) and six comparisons (three ROR γ t siRNAs against two scramble siRNAs) were generated using general linear model to identify the differentially expressed genes in response to ROR α or ROR γ t siRNA treatment respectively. In each comparison, differentially expressed genes were selected based on $PV < 0.01$ and fold change > 1.3 . To further minimize the off-target effect associated with individual siRNA, genes that were differentially expressed in response to ROR α siRNAs at each time point in each donor were selected on those that met the selection criteria in at

least three of the all four comparisons; while genes that were differentially expressed in response to ROR γ t siRNA at each time point in each donor were selected on those that met the selection criteria in at least four of the six comparisons.

Selection criteria on differentially expressed genes from treatments with ROR γ t inverse agonists

To analyze differential gene expression in response to ROR γ t compounds, 6 comparisons were generated using general linear model for each donor at each time point, including every compound at every concentration compared to the DMSO vehicle control. In each comparison, differentially expressed genes were selected based on false discovery rate (FDR) < 0.05 and fold change > 1.5. Genes that were differentially expressed in response to compound treatment at each time point were further selected on those fulfilling the selection criteria in both donors.

IPA analysis

Upstream regulators and canonical pathways that were enriched by ROR γ t regulated genes were identified by Ingenuity Pathway Analysis [35].

Result

Characterization of ROR γ t inverse agonists used in gene signature study

ROR γ t compounds were identified from a high-throughput screen (HTS) of approximately 300,000 proprietary compounds using the ThermoFluor[®] assay that measures binding to the human ROR γ t ligand binding domain (LBD) as a function of thermal stabilization [24]. Four ROR γ t inverse agonists referred to as compound A, B, C and D in this report were generated from optimization of quinolone tertiary alcohol HTS hits and they were used in gene signature studies [32]. These ROR γ t inverse agonists were shown to bind potently and selectively to ROR γ t LBD when compared to LBDs from other family members ROR α and ROR β in the ThermoFluor[®] assay (Fig 1). Evaluation of these compounds in a broader panel of 52 protein targets including receptors, ion channels and transporters confirmed the selectivity of these compounds which did not show more than 50% inhibition at 1 μ M on any test targets (S3 Table). All these inverse agonists inhibited ROR γ t transcriptional activity in a reporter assay that measures ROR γ t driven luciferase expression (Fig 1). To validate their potency and efficacy in blocking ROR γ t mediated cellular response, compounds were tested in human Th17 and Th1 cell differentiation assays *in vitro* and shown to achieve significant inhibition of Th17 differentiation in IL-17A production (Fig 1). In contrast, these compounds were ineffective in blocking Th17 cell proliferation or IFN γ production in Th1 cell differentiation assay as described in Materials and Methods.

Delayed effect of ROR γ t inverse agonists and siRNAs on IL-17A target gene expression

The effect on ROR γ t inverse agonists and siRNAs on human Th17 cell differentiation was tested in a time course study. ROR γ t specific siRNAs (RORC siRNA_2 & 4) and control scramble siRNA (control_3) as described in Methods and Materials were used to transfect human CD4 T cells before activation. ROR γ t and IL-17A transcripts were significantly induced in T cells at 2, 4 and 6 hour time points following anti-CD3/CD28 coated bead activation (Fig 2). In ROR γ t siRNAs transfected T cells, ROR γ t knock down was observed

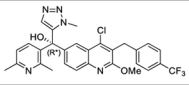
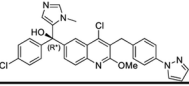
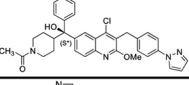
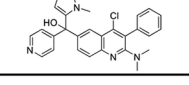
Compounds	ThermoFlour Binding Assay (K _d , nM)			ROR γ / γ t Cellular Reporter Assay (IC ₅₀ , nM)	Th17 Differentiation (IL-17A production) (IC ₅₀ , nM)	Th17 Proliferation (IC ₅₀ , nM)	Th1 Differentiation (IFN γ production) (IC ₅₀ , nM)
	ROR γ / γ t	ROR α	ROR β				
A 	0.1	166	4940	13.3	3.7	>240	>240
B 	15.1	>19574	>29941	83.7	84.5	>240	>240
C 	4.4	>70000	>70000	28.9	18.3	>240	>240
D 	2.5	192	1244	10.0	22.2	>240	>240

Fig 1. ROR γ t inverse agonists used in gene signature studies. Compound structures and their potencies in binding assay, cellular reporter assay, and T cell differentiation assay are shown. These compounds were selective in binding to ROR γ / γ t over ROR α and ROR β , effective in blocking ROR γ t driven luciferase expression, inhibiting Th17 differentiation but not proliferation, and had no effect on Th1 differentiation.

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throughout the entire time course from 2 hours to 6 days, with a maximal effect of 86% at 6 and 72 hours, and 43% maintained on day 6 (Fig 2A). In contrast, levels of IL-17A transcript were not significantly reduced by ROR γ t siRNAs at early time points, with only 23% reduction at 4 hours after T cell activation (Fig 2B). However significant ROR γ t siRNA effects on IL-17A transcript were shown 1 day after T cell activation, with 77% and 95% reduced IL-17A levels at 24 and 72 hours, respectively, and 79% decrease maintained on day 6. We also explored the effect of ROR γ t inverse agonists in these studies. Unlike the siRNA knock down effects, ROR γ t compound D tested at 0.1 and 0.5 μ M did not affect ROR γ t expression in T cells throughout the time course of the experiment (Fig 2C). However, a delayed effect on IL-17A transcripts similar to siRNA treated cells was observed. As shown in Fig 2D, compound D at 0.1 and 0.5 μ M had similar effects on IL-17A transcripts and T cells with 0.5 μ M compound D treatment resulted in 79% and 73% reduction of IL-17A transcripts at 24 and 72 hours, respectively, and 67% decrease maintained on day 6. Based on this finding, we decided to focus our search of ROR γ t signature genes at later time points, specifically on day 1 and day 2 of human Th17 differentiation.

ROR γ t and ROR α signature gene identification in human T cells

We took two different approaches to identify ROR α and ROR γ t signature genes in human Th17 cells. We used ROR γ t inverse agonists to inhibit ROR γ t activity, and pursued the knock down approach using siRNAs specific for ROR γ t or ROR α to remove their gene expression in human T cells. Human Th17 cells generated in the presence of compounds or siRNAs were then profiled in global gene expression using RNA-Seq to identify ROR α and ROR γ t signature genes.

The study plan to identify ROR γ t and ROR α signature genes in human T cells is as described in Materials and Methods (S1 Table). Three ROR γ t inverse agonists, namely compound A, B and C were tested at two concentrations that were higher than their IC₅₀ values in human Th17 differentiation assays to ensure significant inhibition of ROR γ t transcriptional control on target genes (Fig 1). DMSO treated samples were used as controls to compare with compound treated samples. Three ROR γ t siRNAs, two ROR α siRNAs, two control siRNAs, and mock transfected samples were also tested in same studies. Human CD4 T cells from 2

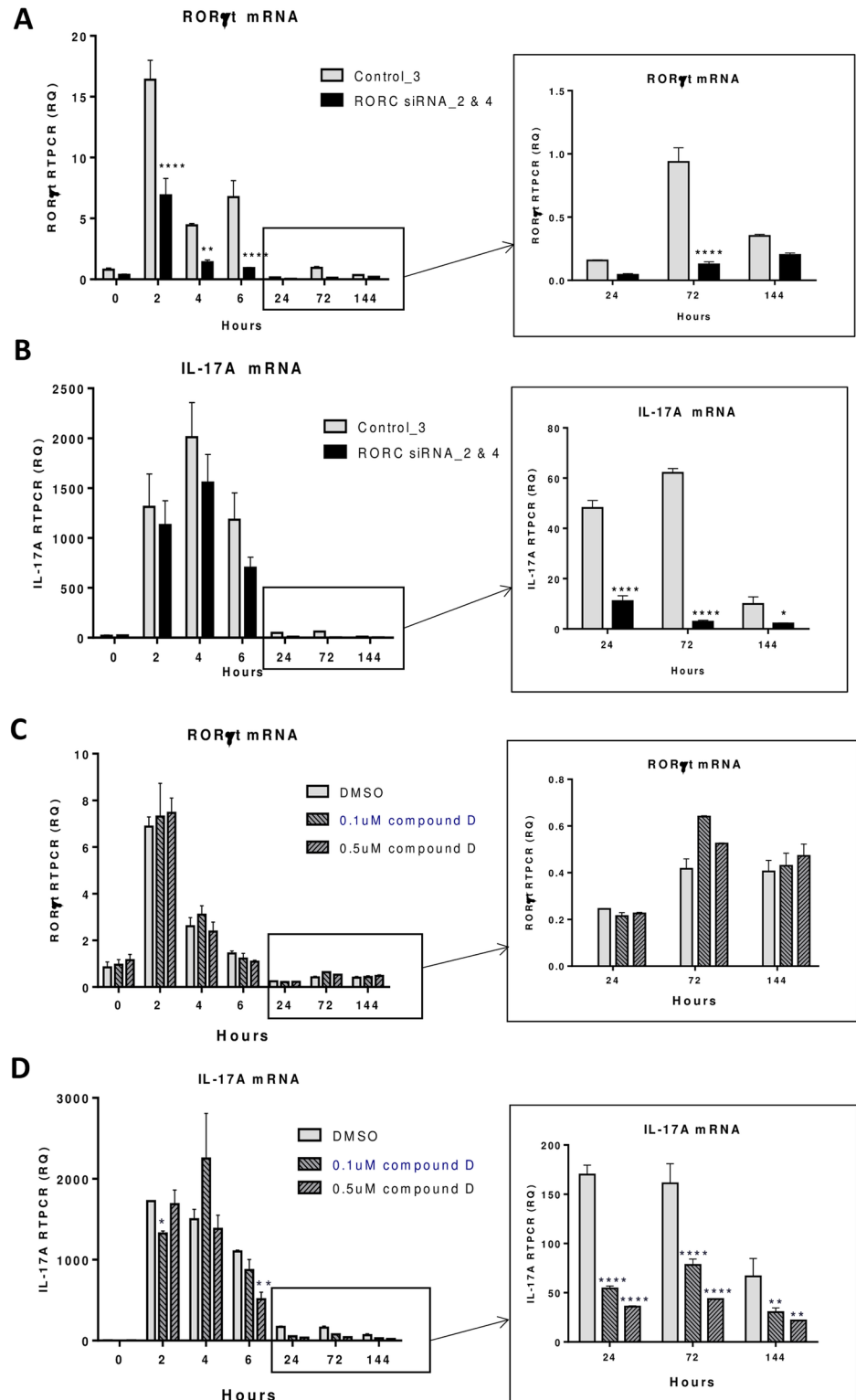


Fig 2. IL-17A transcript in human CD4 T cells reduced by ROR γ t siRNA or inverse agonist at later but not early stage of Th17 differentiation. Human CD4 T cells were activated under Th17 differentiation conditions as described in Material and Methods. ROR γ t siRNAs (RORC siRNA_2 & 4) and control scramble siRNA (control_3) were transfected into CD4 T cells prior to Th17 differentiation and samples were collected at different time points and the expression of ROR γ t (A) and IL-17A (B) transcripts was measured by RT-PCR

as described in Materials and Methods. ROR γ t compound D at 0.1 and 0.5 μ M and DMSO vehicle control were tested in a similar manner in Th17 differentiation. ROR γ t (C) and IL-17A (D) transcripts were measured by RT-PCR at different time points. Statistical significance of RORC siRNA or compound treated samples in reducing ROR γ t or IL-17A mRNA at different time points was analyzed with 2-way ANOVA and P values were indicated as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$.

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healthy donors were used and cell samples were collected for RT-PCR or RNA-Seq studies at 0, 24 and 48 hours of Th17 differentiation.

T cells transfected with ROR γ t siRNAs showed a significant knockdown of ROR γ t transcripts with 50% decrease at 24 hours, and up to 70% decrease at 48 hours (Fig 3A). ROR α transcripts in T cells transfected with ROR α siRNAs were reduced by 40% and 60% at 24 and 48 hours respectively (Fig 3B). The effect on IL-17A transcripts was 70–80% reduction by ROR γ t siRNAs and 40–50% by ROR α siRNAs (Fig 3C). Measurement of IL-17A protein in supernatants of 2 day cultures showed a 70% and 50% reduction by ROR γ t and ROR α siRNAs respectively (Fig 3D).

IL-17A mRNA levels in T cells treated with ROR γ t inverse agonists A, B and C were detected by RT-PCR and compared to DMSO treated controls at different time points. Significant decrease in IL-17A transcripts was observed in all the inverse agonist treated samples with an average of 61% reduction at 24 hours and 83% at 48 hours (Fig 4A). IL-17A released in 2-day cell culture supernatants were reduced by an average of 69% in cell samples treated with inverse agonists (Fig 4B). In summary, expression of IL-17A in human Th17 cells was reduced by ROR γ t siRNAs or inverse agonists. ROR α siRNA also decreased IL-17A expression but to an extent much less than that from ROR γ t siRNAs or inverse agonists.

The effects of ROR γ t and ROR α siRNAs or inverse agonists on target gene IL-17A validated this study approach in signature gene identification. Global gene expression profile was conducted in all the T cell samples from siRNA and compound studies by RNA-Seq. ROR γ t and ROR α gene signatures were identified through differential gene expression analysis as described in the following sections.

ROR γ t and ROR α 4 isoforms expressed in human Th17 cells

Expression of specific isoforms of the RORA and RORC genes from alternative splicing was identified from RNA-Seq analysis of human T cell samples. As shown in Fig 5, RORC gene expression was minimal and below detection levels in CD4 T cells at the resting stage. Strong induction of RORC gene expression was detected in T cells at 24 and 48 hours after activation under Th17 polarizing conditions and only transcripts of the ROR γ t isoform were detected, as demonstrated with both donors (Fig 5A). In contrast, expression of the RORA gene was found in T cells at both resting and activated stages and only transcripts of the ROR α 4 isoform were detected, with expression levels higher in resting T cells and slightly reduced in activated T cells, as shown at 24 and 48 hours in both donors (Fig 5B).

ROR γ t signature genes in human Th17 cells identified with siRNAs or inverse agonists

Three siRNAs specific for the RORC gene were used in signature gene studies in human CD4 T cells. Although the sequences of these siRNAs were in the common region utilized by the ROR γ and ROR γ t splice variants, signature genes are expected to be from ROR γ t, since it was the only isoform detected in human T cells (Fig 5A).

During the time course of Th17 differentiation, expression of 12 genes, including RORC, IL-17A, IL-17F, IL-22, IL-26, ABCA1, C2CD4A, CCL20, COL5A3, CTSH, IQCG, and

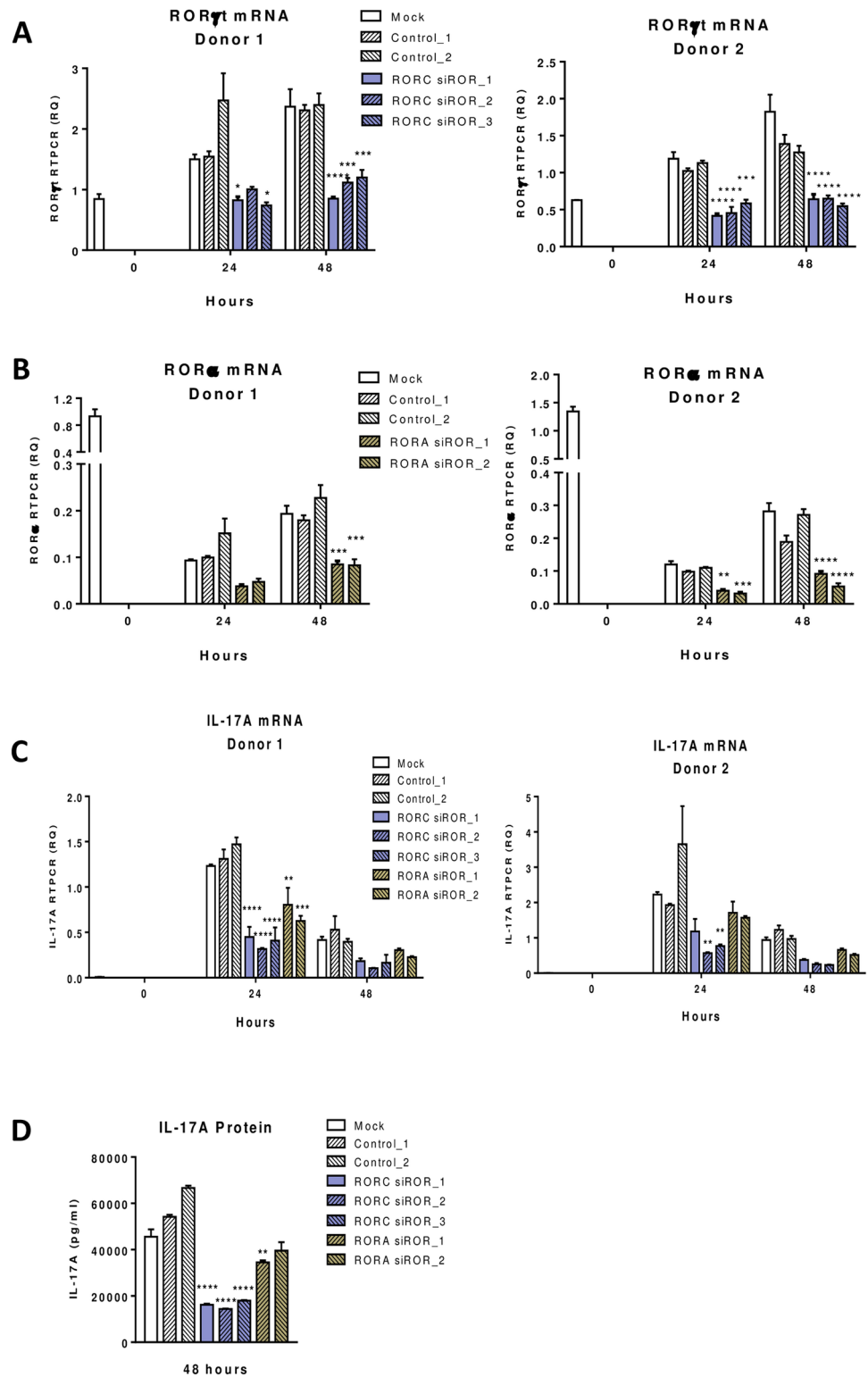


Fig 3. ROR γ t or ROR α siRNA effect on IL-17A transcript in human CD4 T cells during Th17 differentiation. Human CD4 T cells purified from 2 donors were activated under Th17 differentiation condition as described in Material and Methods. ROR γ t and ROR α siRNAs, as well as control siRNAs were transfected into CD4 T cells prior to Th17 differentiation and samples were collected at different time points as indicated. Expression of ROR γ t (A), ROR α (B) and IL-17A (C) transcripts was measured by RT-PCR. IL-17A

cytokine released in culture supernatants (D) was measured by ELISA. Statistical significance of the difference between RORC or RORA siRNA treated samples compared to mock samples at different time points was analyzed with 2-way ANOVA and P values were presented as *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001.

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PXDC1, were reduced by treatment of T cells with ROR γ t siRNAs (Fig 6A). All these genes except for COL5A3 were genes with induced expression in Th17 cells at 24 and 48 hours of differentiation. IL-9 was an induced gene in Th17 cells and interestingly its expression was enhanced by ROR γ t siRNAs (Fig 6C).

Studies with ROR γ t inverse agonists identified 20 genes with expression reduced by any one of the three ROR γ t inverse agonists in both donor T cells (Fig 7A). Most of them except for MATN2 were also down-regulated by ROR γ t siRNAs, and 14 of them were induced genes in Th17 cells at 24 and 48 hours of differentiation. There were 20 genes with expression induced by any one of the three ROR γ t inverse agonists in both donor T cells in a dose-

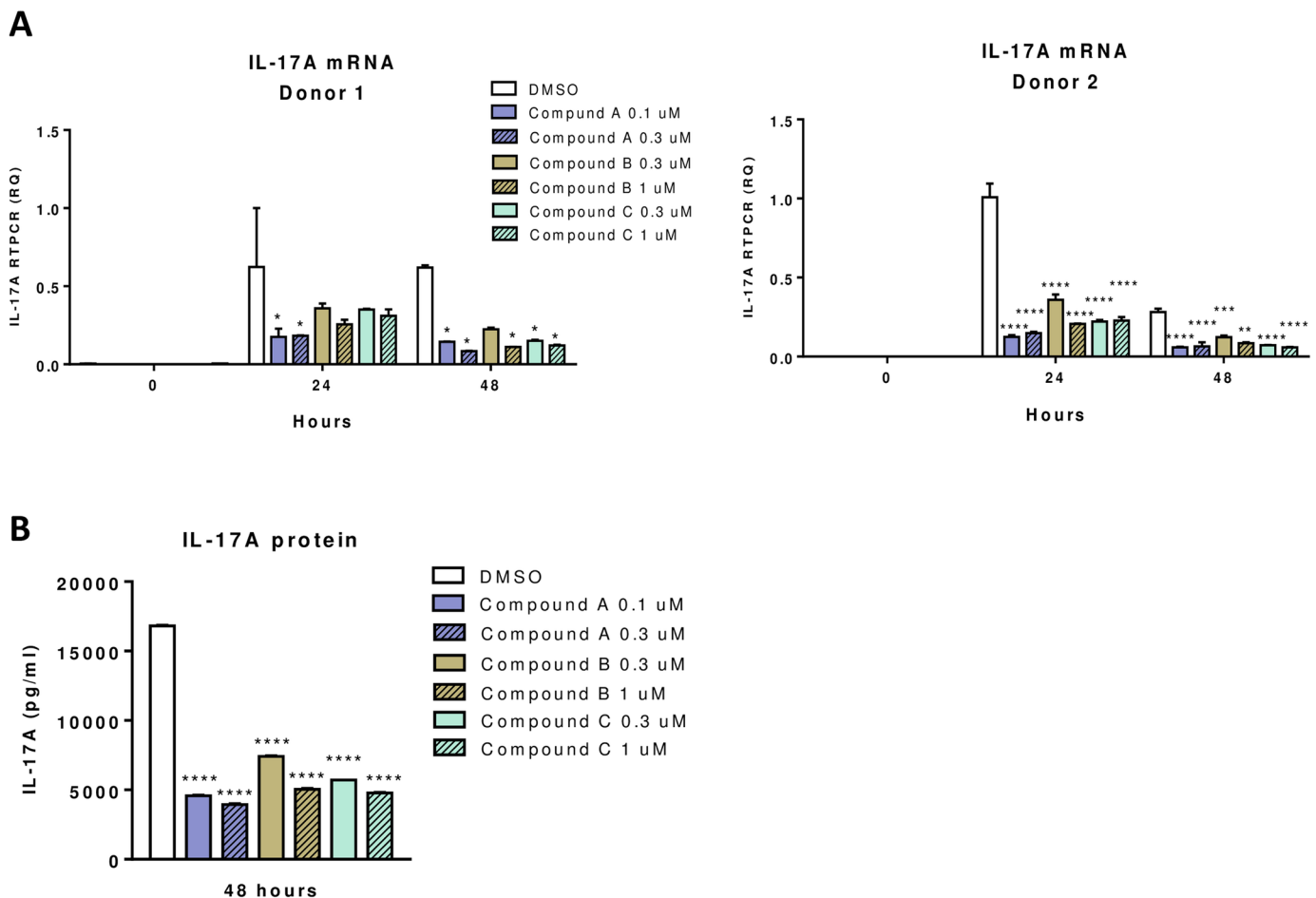
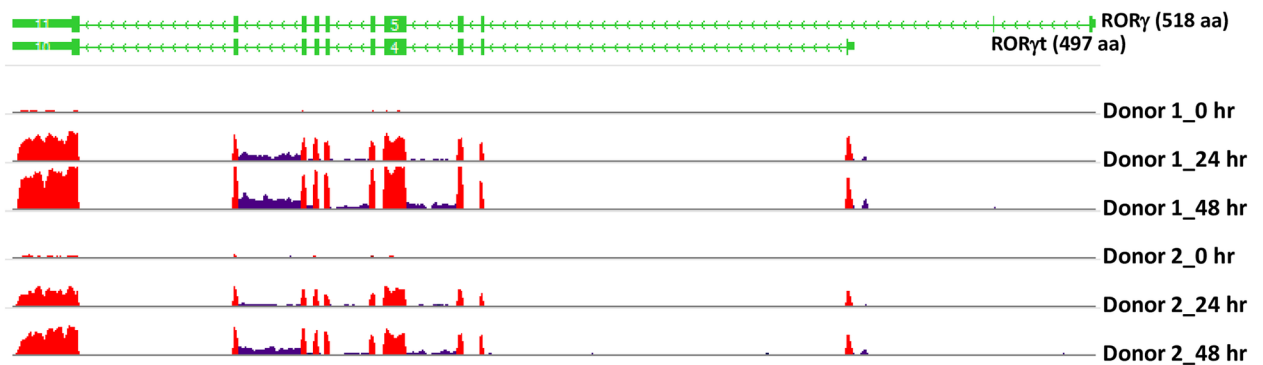


Fig 4. ROR γ t inverse agonist effect on IL-17A transcript in human CD4 T cells during Th17 differentiation. Human CD4 T cells purified from 2 donors were activated under Th17 differentiation condition as described in Material and Methods. CD4 T cells were treated with three ROR γ t inverse agonists at two different concentrations as well as DMSO vehicle control. Expression of IL-17A mRNA in different treated samples was measured by RT-PCR at time points as indicated (A). IL-17A cytokine released in culture supernatants (B) was measured by ELISA. Statistical significance of the difference between compound treated to DMSO samples at different time points was analyzed with 2-way ANOVA and P values were indicated as *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

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A RORC gene isoform expression



B RORA gene isoform expression

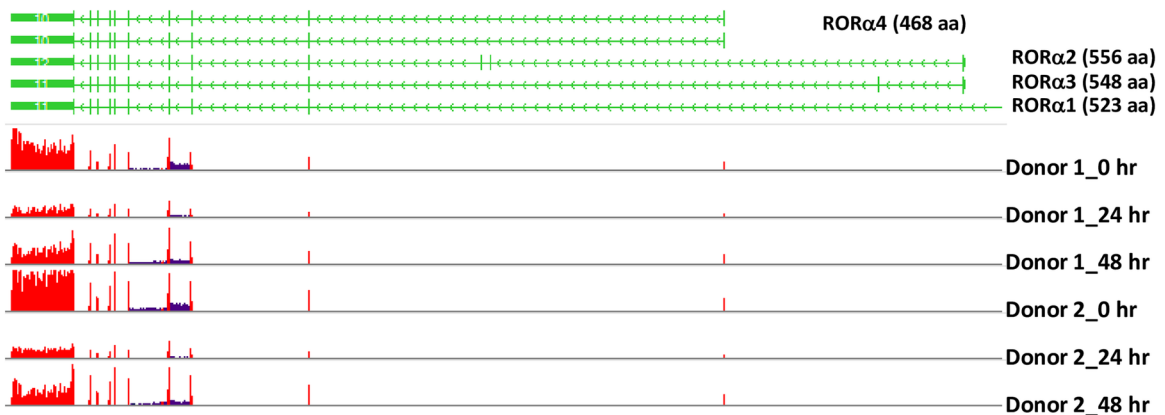


Fig 5. Expression of ROR γ t and ROR α 4 in human CD4 T cells during Th17 differentiation. Expression of ROR γ t isoform from the RORC gene (A) and ROR α 4 isoform from the RORA gene (B) was determined from detection of isoform specific exon sequences in RNA-Seq data analysis. Exons utilized by different isoforms of the RORC and RORA genes are shown in green, and the frequency of exon sequences in RNA-Seq data are shown in red in the histograms. DMSO treated human CD4 T cell samples from 2 donors at different time points of activation under Th17 differentiation conditions were used for isoform expression analysis.

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dependent fashion (Fig 7B). Among them 8 were induced genes and 4 were suppressed genes in activated Th17 cells at 24 and 48 hours of culture. The differential gene expression in response to these inverse agonists was more evident in donor 1 than in donor 2. Genes down-regulated by ROR γ t inverse agonists identified from RNA-Seq analysis were further confirmed by RTPCR on donor 1 samples (Fig 7C). In summary, ROR γ t signature genes were identified with two different approaches using ROR γ t specific siRNAs or inverse agonists.

ROR α 4 signature genes in human Th17 cells identified with ROR α siRNAs

Two siRNAs specific for the RORA gene were used to knock down expression of ROR α transcripts in human CD4 T cells. Although the sequences of these siRNAs were in the region common for all splice variants of the RORA gene, signature genes identified in this study are expected to be from ROR α 4, since it was the only isoform detected in human T cells (Fig 5B).

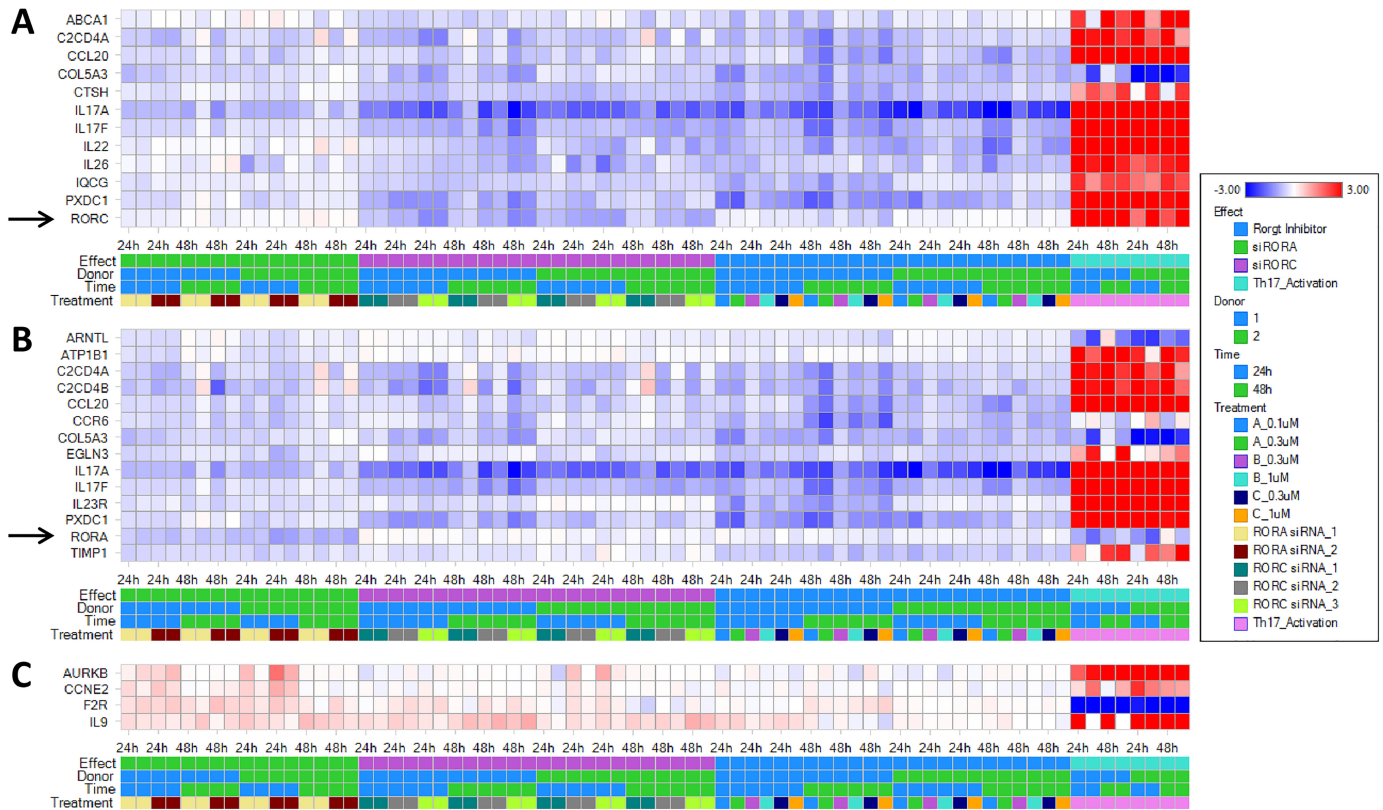


Fig 6. Global gene expression profile with RNA-Seq identified genes differentially regulated by ROR γ t or ROR α siRNA in human CD4 T cells during Th17 differentiation. RNA sequencing profile was performed on RNA samples extracted from human CD4 T cells treated with ROR γ t or ROR α siRNA, or ROR γ t inverse agonists as described in S1 Table. Genes down-regulated by ROR γ t (A) and ROR α (B) siRNAs, or up-regulated by ROR γ t or ROR α siRNAs (C) were identified in analysis of RNA-Seq data using the criteria described in Materials and Methods. Each column represents log₂ ratios of selected genes in one of the 72 comparisons, including comparison of untreated Th17 cells with ROR γ t or ROR α siRNA transfected cells or ROR γ t inverse agonist treated cells at two time points and T cells from 2 independent donors.

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Most of the ROR α 4 signature genes identified in this study were up-regulated in Th17 cells and reduced by ROR α siRNA treatments. As shown in Fig 6B, expression of ROR α 4 and 13 additional genes were reduced by treatment of ROR α siRNAs in both donor T cells. Among the 13 signature genes, 11 of them were induced genes in Th17 cells. There were 3 genes AURKB, CCNE2, and F2R moderately induced by ROR α siRNAs in T cells from both donors (Fig 6C). AURKB and CCNE2 were induced genes in Th17 cells whereas F2R was a Th17 suppressed gene.

ROR α 4 and ROR γ t common signature genes in human Th17 cells

Results from our study showed that ROR α 4 and ROR γ t have shared signature genes including IL-17A, IL-17F, C2CD4A, C2CD4B, CCL20, CCR6, COL5A3, PXDC1, and TIMP1 (Figs 6A, 6B and 7A). Although IL23R and EGLN3 were identified with ROR α siRNAs, and CTSH, IL-26, ADAM12, COL5A1, GNLY, PKHD1, RAPGEF3, CYP1A1 and CYP1B1 were from ROR γ t signature search, careful review of these genes in expression heat maps revealed a similar trend of differential gene expression with approaches targeting ROR α 4 or ROR γ t (Figs 6 and 7), suggesting that they could be common signatures for ROR γ t and ROR α 4 as well. In general, the effect of ROR α siRNAs on signature gene expression was less than ROR γ t siRNAs or inverse

ROR γ t inverse agonist treated cells at two time points and T cells from 2 independent donors. Genes down-regulated by ROR γ t inverse agonists in Th17 cells identified from RNA-Seq analysis were confirmed by RTPCR of donor 1 samples as described in Materials and Methods (C). Heat map of log₂ ratios of gene expression in response to treatment of ROR γ t inverse agonists determined by RNA-Seq and RTPCR were compared.

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agonists. In summary, ROR α 4 and ROR γ t shared some of their signature genes in Th17 cells and ROR γ t had a more significant role than ROR α 4 in regulating signature gene expression.

ROR γ t pathways and upstream regulators identified in Ingenuity Pathway Analysis

Most of the genes with expression down-regulated by ROR γ t siRNA overlapped with genes reduced by ROR γ t inverse agonists (Figs 6A & 7A). A total of 24 ROR γ t signature genes that were down-regulated by either siRNAs or compounds were generated from differential gene expression analysis (Table 1). These genes were used to conduct Ingenuity Pathway Analysis (IPA) to identify the corresponding upstream regulators (Table 2) and canonical pathways (Table 3). As shown in Table 2, the RORC and RORA genes, as well as the ROR γ t inverse agonist TMP778 were identified by IPA in the list of top 20 upstream regulators, validating our approach in ROR γ t and ROR α signature gene identification, and confirming the overlapping functions of these two transcription factors. In addition, regulators and pathways involved in

Table 1. A total of 24 genes were down-regulated by ROR γ t siRNAs or inverse agonists in human Th17 cells. The change in gene expression resulted from treatment of ROR γ t siRNAs or ROR γ t inverse agonists was presented in fold change of the geomean average calculated from samples of the two donors.

Gene	Down-regulated by ROR γ t siRNAs	Down-regulated by ROR γ t inverse agonists	GeoMean Fold Change by ROR γ t siRNAs	GeoMean Fold Change by ROR γ t inverse agonists
ABCA1	Yes		-1.3	-1.3
ADAM12		Yes	-1.4	-1.5
C2CD4A	Yes	Yes	-1.6	-1.7
C2CD4B		Yes	-1.7	-1.8
CCL20	Yes	Yes	-1.4	-1.8
CCR6		Yes	-1.4	-1.9
COL5A1		Yes	-1.2	-1.7
COL5A3	Yes	Yes	-1.6	-1.9
CTSH	Yes	Yes	-1.6	-1.7
GNLY		Yes	-1.7	-1.3
IL17A	Yes	Yes	-3.4	-4.3
IL17F	Yes	Yes	-1.9	-2.0
IL22	Yes	Yes	-1.6	-2.0
IL26	Yes		-1.7	-1.6
IQCG	Yes		-1.5	-1.5
MATN2		Yes	-1.0	-2.5
NOL3		Yes	-1.1	-1.2
PKHD1		Yes	-2.8	-3.6
PPARG		Yes	-1.0	-1.3
PXDC1	Yes	Yes	-1.8	-2.3
RAPGEF3		Yes	-1.4	-1.9
RORC	Yes		-1.9	-1.2
SH3D21		Yes	-1.1	-1.3
TIMP1		Yes	-1.2	-1.6

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Table 2. Upstream regulators of the 24 genes down-regulated by ROR γ t siRNAs or inverse agonists in human Th17 cells were predicted from IPA analysis and the top 20 regulators are shown. These regulators were ranked by their P values which reflect the enrichment of the 24 genes regulated directly or indirectly by these upstream regulators. The activation status of upstream regulators was determined from the treatment effect on the involved genes and presented as activation z-scores, with positive value for activated status, and negative value for inhibited status.

Upstream Regulator	Activation Z-score	P-value of Overlap	Involved Genes
TMP778 (ROR γ t inverse agonist)	2.39	4.25E-18	CCL20,CCR6,IL17A,IL17F,IL22,IL26
IL23	-2.57	5.80E-15	CCL20,CCR6,IL17A,IL17F,IL22,IL26,RORC
IL23R	-1.98	3.03E-11	IL17A,IL17F,IL22,RORC
AMG 827 (anti-IL-17RA)	2.00	8.61E-10	CCL20,IL17A,IL17F,IL22
TGFB1	-2.38	1.18E-09	ABCA1,ADAM12,CCL20,CCR6,COL5A1,CTSH,IL17A,IL17F,IL22,PPARG,RAPGEF3,RORC,TIMP1
IL2	-2.14	2.24E-09	ABCA1,CCR6,IL17A,IL17F,IL22,IL26,PPARG,RORC,TIMP1
IL27	1.48	3.04E-09	CCL20,CCR6,IL17A,IL17F,IL22,RORC
NFATC2	1.00	6.99E-09	ABCA1,IL17A,IL17F,IL22,PPARG,RORC
RORC	-2.16	8.88E-09	CCL20,CCR6,IL17A,IL17F,IL22,RORC
Secretase gamma prostaglandin E2	-1.96	9.08E-09	IL17A,IL17F,IL22,RORC
ZBTB16	-2.21	1.40E-08	CCL20,CCR6,IL17A,IL17F,IL22,PPARG,RORC
IL6	-2.57	1.49E-08	CCR6,IL17A,IL17F,IL22,RORC
NFATC1	-2.17	1.62E-08	ABCA1,CCL20,CCR6,IL17A,IL17F,IL22,PPARG,RORC,TIMP1
PLP1	-2.00	1.81E-08	IL17A,IL17F,IL22,PPARG,RORC
AHR	0.48	2.68E-08	CCL20,CCR6,IL17A,TIMP1
IL1	-1.56	2.72E-08	COL5A1,IL17A,IL17F,IL22,MATN2,PPARG,RORC
RORA	-2.40	3.22E-08	CCL20,CCR6,IL17A,IL22,PPARG,RORC,TIMP1
IL23A	-1.99	3.24E-08	ABCA1,CCR6,IL17A,IL17F,IL22,RORC
IL17A	-1.99	3.48E-08	IL17A,IL17F,IL22,RORC
	-0.70	5.24E-08	CCL20,IL17A,IL17F,IL22,PPARG,TIMP1

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Table 3. Top 10 canonical pathways in IPA associated with the 24 genes that were down-regulated by ROR γ t siRNAs or inverse agonists in human Th17 cells. The top 10 canonical pathways were selected by the p-value of overlap of the 24 genes and the genes in each pathway.

Ingenuity Canonical Pathways	-log(p-value)	Involved Genes
Role of Cytokines in Mediating Communication between Immune Cells	6.24	IL26,IL22,IL17F,IL17A
T Helper Cell Differentiation	4.03	RORC,IL17F,IL17A
IL-17 Signaling	4.01	TIMP1,IL17F,IL17A
Role of IL-17A in Psoriasis	3.94	CCL20,IL17A
Differential Regulation of Cytokine Production in Macrophages and T Helper Cells by IL-17A and IL-17F	3.65	IL17F,IL17A
Differential Regulation of Cytokine Production in Intestinal Epithelial Cells by IL-17A and IL-17F	3.43	IL17F,IL17A
IL-17A Signaling in Gastric Cells	3.36	CCL20,IL17A
Inhibition of Matrix Metalloproteases	2.97	ADAM12,TIMP1
Hepatic Fibrosis / Hepatic Stellate Cell Activation	2.83	COL5A1,COL5A3,TIMP1
Role of IL-17A in Arthritis	2.69	CCL20,IL17A

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Th17 cell differentiation and IL-17 mediated effects were pulled out from IPA analysis (Tables 2 and 3). The results are consistent with other reports on the role of ROR γ t and ROR α in modulating Th17 cell generation and functions [11,12,29,31].

Discussion

ROR γ t is a key transcription factor playing an important role in Th17 cell differentiation [11]. Although expression of ROR γ has been reported in T cells [16], we only detected ROR γ t but not ROR γ in our previous report on RNA-Seq analysis of human CCR6⁺ CD4 memory T cells [17]. RNA-Seq data from this study also confirmed our earlier finding that only ROR γ t but not ROR γ was expressed in human Th17 cells. ROR α 2 and ROR α 4 are the two isoforms from the RORA gene and their expression in T cells have been reported previously [18]. RNA-Seq data from this study however only detected ROR α 4 but not the other isoforms from the RORA gene in human Th17 cells.

We conducted a time course study to track the effects of ROR γ t siRNAs or inverse agonists on human Th17 differentiation. ROR γ t target gene IL-17A was highly induced at early time points within the first day of T cell activation. However neither ROR γ t specific siRNAs nor inverse agonists had significant impacts on IL-17A gene expression on the first day of T cell activation. The effect on IL-17A gene expression was observed only on the second day after the peak level of IL-17A gene expression. The results suggest that ROR γ t may be more involved in sustaining the late phase expression of target genes than the initial phase of gene induction. The delayed effect of ROR γ t on target gene expression also highlights its function on Th17 cell differentiation which takes place later in the course of T cell activation.

Although ROR γ t signature genes have been characterized in mouse Th17 cells, information on human T cells from global transcriptional profile was reported only in one study [28–30]. ROR α has been suggested to play a role in regulation of Th17 gene expression [18–20,31], however study of ROR α signature genes from global gene profile has not been reported previously. To identify ROR γ t and ROR α signature genes in human T cells, we conducted the global gene expression profile in human CD4 T cells under Th17 differentiation conditions after treatment with ROR γ t or ROR α siRNAs, or small molecule ROR γ t inverse agonists. To build the confidence in signature gene data, multiple siRNAs and inverse agonists were used with test samples in replicates and experiments repeated with CD4 T cells from 2 healthy donors. Global gene expression was measured with RNA-Seq to ensure high detection sensitivity. Conservative analytical algorithms were utilized to determine signature genes for ROR γ t and ROR α 4, the two isoforms expressed in human Th17 cells. Using this approach we were able to identify ROR α 4 and ROR γ t signature genes in human Th17 cells. It is also the first report on ROR α 4 signature genes in Th17 cells from global transcription analysis.

The majority of ROR α 4 and ROR γ t signature genes were induced genes in Th17 cells and positively regulated by these transcription factors, as demonstrated by their expression being reduced by siRNA or inverse agonist treatments. Furthermore, expression of these signature genes was not completely blocked by siRNAs or inverse agonists. IL-17A was the most significantly affected gene amongst all signature genes, and yet the reduction of transcript and protein levels was not complete at maximal inhibition effects. The effect of ROR α 4 in regulation of target gene expression was more subtle than ROR γ t. Our study also showed an overlapping role of ROR α 4 and ROR γ t in regulating a small number of key Th17 genes including IL-17A, IL-17F, IL-22, CCL20 and CCR6. These genes have been reported to be important for Th17 differentiation and transcriptionally regulated by ROR γ t in human and mouse Th17 cells [28–30]. We demonstrated in this report that ROR α 4 also participated in the regulation of these genes in Th17 cells. In addition, other genes such as C2CD4A, PXDC1, TIMP1 and COL5A3

were found to be regulated by both ROR α 4 and ROR γ t in Th17 cells as well and the function of these genes remain unclear and warrant further investigations.

Th17 cells play a key role in the pathogenesis of many autoimmune diseases such as psoriasis, rheumatoid arthritis, inflammatory bowel disease and multiple sclerosis [36,37]. Antibodies targeting IL-17A and the IL-17 receptor IL-17RA have shown clinical efficacy in psoriasis, rheumatoid arthritis, and uveitis [38–40]. ROR γ t compound can selectively regulate Th17 signature gene expression in mononuclear cells isolated from both the blood and affected skin of psoriasis patients and inhibited IL-23 induced IL-17A from psoriasis patient PBMC [30]. Since ROR γ t and ROR α play a significant role in Th17 differentiation and IL-17A production, identification of their target genes in Th17 cells may lead to discovery of candidate genes with potential value for new therapeutic approaches.

Supporting information

S1 Table. ROR γ t and ROR α gene signature study plan. Numbers of cell culture samples with different treatments at multiple time points in gene expression analysis are shown. The various culture conditions are defined by pairs of treatments (rows) and time points (columns) after activation of CD4 T cells from two donors.

(DOCX)

S2 Table. ROR γ t signature genes identified in ROR γ t compound treated Th17 cells were confirmed by RTPCR using TaqMan PCR primers and probes from ThermoFisher and their catalogue IDs are shown.

(DOCX)

S3 Table. Selectivity profile of ROR γ t inverse agonists A, B, C and D. Compounds were evaluated at 1 μ M and 10 μ M in a panel of assays against 50 receptors, ion channels and transporters (Profile service by Cerep Inc.).

(DOCX)

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References

1. Jetten AM (2009) Retinoid-related orphan receptors (RORs): critical roles in development, immunity, circadian rhythm, and cellular metabolism. *Nucl Recept Signal* 7: e003. <https://doi.org/10.1621/nrs.07003> PMID: 19381306
2. Giguere V, Tini M, Flock G, Ong E, Evans RM, et al. (1994) Isoform-specific amino-terminal domains dictate DNA-binding properties of ROR alpha, a novel family of orphan hormone nuclear receptors. *Genes Dev* 8: 538–553. PMID: 7926749
3. Matysiak-Scholze U, Nehls M (1997) The structural integrity of ROR alpha isoforms is mutated in staggerer mice: cerebellar coexpression of ROR alpha1 and ROR alpha4. *Genomics* 43: 78–84. PMID: 9226375
4. Carlberg C, Hooft van Huijsduijnen R, Staple JK, DeLamarter JF, Becker-Andre M (1994) RZR, a new family of retinoid-related orphan receptors that function as both monomers and homodimers. *Mol Endocrinol* 8: 757–770. PMID: 7935491
5. Villey I, de Chasseval R, de Villartay JP (1999) RORgammaT, a thymus-specific isoform of the orphan nuclear receptor RORgamma / TOR, is up-regulated by signaling through the pre-T cell receptor and binds to the TEA promoter. *Eur J Immunol* 29: 4072–4080. PMID: 10602018
6. Eberl G, Marmon S, Sunshine M-J, Rennert PD, Choi Y, et al. (2004) An essential function for the nuclear receptor ROR[gamma]t in the generation of fetal lymphoid tissue inducer cells. *Nat Immunol* 5: 64–73. PMID: 14691482
7. He YW, Deftos ML, Ojala EW, Bevan MJ (1998) RORgamma t, a novel isoform of an orphan receptor, negatively regulates Fas ligand expression and IL-2 production in T cells. *Immunity* 9: 797–806. PMID: 9881970
8. Eberl G, Littman DR (2003) The role of the nuclear hormone receptor RORgammat in the development of lymph nodes and Peyer's patches. *Immunol Rev* 195: 81–90. PMID: 12969312
9. Luci C, Reynders A, Ivanov II, Cagnet C, Chiche L, et al. (2009) Influence of the transcription factor RORgammat on the development of NKp46+ cell populations in gut and skin. *Nat Immunol* 10: 75–82. <https://doi.org/10.1038/ni.1681> PMID: 19029904
10. Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F (2007) Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol* 8: 942–949. PMID: 17676045
11. Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, et al. (2006) The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* 126: 1121–1133. PMID: 16990136
12. Ivanov II, Zhou L, Littman DR (2007) Transcriptional regulation of Th17 cell differentiation. *Semin Immunol* 19: 409–417. <https://doi.org/10.1016/j.smim.2007.10.011> PMID: 18053739
13. Manel N, Unutmaz D, Littman DR (2008) The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgammat. *Nat Immunol* 9: 641–649. <https://doi.org/10.1038/ni.1610> PMID: 18454151
14. Hirose T, Smith RJ, Jetten AM (1994) ROR gamma: the third member of ROR/RZR orphan receptor subfamily that is highly expressed in skeletal muscle. *Biochem Biophys Res Commun* 205: 1976–1983. PMID: 7811290
15. Ortiz MA, Piedrafita FJ, Pfahl M, Maki R (1995) TOR: a new orphan receptor expressed in the thymus that can modulate retinoid and thyroid hormone signals. *Mol Endocrinol* 9: 1679–1691. PMID: 8614404
16. Ruan Q, Kameswaran V, Zhang Y, Zheng S, Sun J, et al. (2011) The Th17 immune response is controlled by the Rel–ROR γ –ROR γ T transcriptional axis. *The Journal of Experimental Medicine* 208: 2321–2333. <https://doi.org/10.1084/jem.20110462> PMID: 22006976
17. Zhao S, Fung-Leung WP, Bittner A, Ngo K, Liu X (2014) Comparison of RNA-Seq and microarray in transcriptome profiling of activated T cells. *PLoS One* 9: e78644. <https://doi.org/10.1371/journal.pone.0078644> PMID: 24454679

18. Du J, Huang C, Zhou B, Ziegler SF (2008) Isoform-specific inhibition of ROR alpha-mediated transcriptional activation by human FOXP3. *J Immunol* 180: 4785–4792. PMID: [18354202](#)
19. Dzhagalov I, Giguere V, He YW (2004) Lymphocyte development and function in the absence of retinoic acid-related orphan receptor alpha. *J Immunol* 173: 2952–2959. PMID: [15322153](#)
20. Dzhagalov I, Zhang N, He YW (2004) The roles of orphan nuclear receptors in the development and function of the immune system. *Cell Mol Immunol* 1: 401–407. PMID: [16293208](#)
21. Kallen J, Schlaeppi JM, Bitsch F, Delhon I, Fournier B (2004) Crystal structure of the human RORalpha Ligand binding domain in complex with cholesterol sulfate at 2.2 Å. *J Biol Chem* 279: 14033–14038. PMID: [14722075](#)
22. Kallen JA, Schlaeppi JM, Bitsch F, Geisse S, Geiser M, et al. (2002) X-ray structure of the hRORalpha LBD at 1.63 Å: structural and functional data that cholesterol or a cholesterol derivative is the natural ligand of RORalpha. *Structure* 10: 1697–1707. PMID: [12467577](#)
23. Becker-Andre M, Wiesenberg I, Schaeren-Wiemers N, Andre E, Missbach M, et al. (1994) Pineal gland hormone melatonin binds and activates an orphan of the nuclear receptor superfamily. *J Biol Chem* 269: 28531–28534. PMID: [7961794](#)
24. Soroosh P, Wu J, Xue X, Song J, Sutton SW, et al. (2014) Oxysterols are agonist ligands of ROR γ t and drive Th17 cell differentiation. *Proceedings of the National Academy of Sciences* 111: 12163–12168.
25. Santori FR, Huang P, van de Pavert SA, Douglass EF Jr, Leaver DJ, et al. (2015) Identification of natural RORgamma ligands that regulate the development of lymphoid cells. *Cell Metab* 21: 286–297. <https://doi.org/10.1016/j.cmet.2015.01.004> PMID: [25651181](#)
26. Yang J, Sundrud MS, Skepner J, Yamagata T (2014) Targeting Th17 cells in autoimmune diseases. *Trends Pharmacol Sci*.
27. Fauber BP, Magnuson S (2014) Modulators of the nuclear receptor retinoic acid receptor-related orphan receptor-gamma (RORgamma or RORc). *J Med Chem* 57: 5871–5892. <https://doi.org/10.1021/jm401901d> PMID: [24502334](#)
28. Xiao S, Yosef N, Yang J, Wang Y, Zhou L, et al. (2014) Small-molecule RORgamma δ antagonists inhibit T helper 17 cell transcriptional network by divergent mechanisms. *Immunity* 40: 477–489. <https://doi.org/10.1016/j.immuni.2014.04.004> PMID: [24745332](#)
29. Ciofani M, Madar A, Galan C, Sellars M, Mace K, et al. (2012) A validated regulatory network for Th17 cell specification. *Cell* 151: 289–303. <https://doi.org/10.1016/j.cell.2012.09.016> PMID: [23021777](#)
30. Skepner J, Ramesh R, Trocha M, Schmidt D, Baloglu E, et al. (2014) Pharmacologic inhibition of RORgamma δ regulates Th17 signature gene expression and suppresses cutaneous inflammation in vivo. *J Immunol* 192: 2564–2575. <https://doi.org/10.4049/jimmunol.1302190> PMID: [24516202](#)
31. Yang XO, Pappu BP, Nurieva R, Akimzhanov A, Kang HS, et al. (2008) T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. *Immunity* 28: 29–39. <https://doi.org/10.1016/j.immuni.2007.11.016> PMID: [18164222](#)
32. Xue X, Soroosh Pejman, De Leon-Tabaldo Aimee, Luna-Roman Rosa, Sablad Marciano, Rozenkrants Natasha, Y J, Castro Glenda, Banie Homayon, Fung-Leung Wai-Ping, Santamaria-Babi Luis, S T, Albers Michael, Leonard Kristi, Budelsky Alison L, Fourie Anne M (2016) Pharmacologic modulation of ROR γ t translates to efficacy in preclinical and translational models of psoriasis and inflammatory arthritis. *Scientific Reports*. in press.
33. Clemente JC, Nulton E, Nelen M, Todd MJ, Maguire D, et al. (2012) Screening and characterization of human monoglyceride lipase active site inhibitors using orthogonal binding and functional assays. *J Biomol Screen* 17: 629–640. <https://doi.org/10.1177/1087057112441012> PMID: [22496098](#)
34. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2^{(-Delta Delta C(T))} Method. *Methods* 25: 402–408. <https://doi.org/10.1006/meth.2001.1262> PMID: [11846609](#)
35. Kramer A, Green J, Pollard J Jr, Tugendreich S (2014) Causal analysis approaches in Ingenuity Pathway Analysis. *Bioinformatics* 30: 523–530. <https://doi.org/10.1093/bioinformatics/btt703> PMID: [24336805](#)
36. McGeachy MJ, Cua DJ (2008) Th17 cell differentiation: the long and winding road. *Immunity* 28: 445–453. <https://doi.org/10.1016/j.immuni.2008.03.001> PMID: [18400187](#)
37. Miossec P, Korn T, Kuchroo VK (2009) Interleukin-17 and type 17 helper T cells. *N Engl J Med* 361: 888–898. <https://doi.org/10.1056/NEJMr0707449> PMID: [19710487](#)
38. Papp KA, Leonardi C, Menter A, Ortonne JP, Krueger JG, et al. (2012) Brodalumab, an anti-interleukin-17-receptor antibody for psoriasis. *N Engl J Med* 366: 1181–1189. <https://doi.org/10.1056/NEJMoa1109017> PMID: [22455412](#)

39. Leonardi C, Matheson R, Zachariae C, Cameron G, Li L, et al. (2012) Anti-interleukin-17 monoclonal antibody ixekizumab in chronic plaque psoriasis. *N Engl J Med* 366: 1190–1199. <https://doi.org/10.1056/NEJMoa1109997> PMID: 22455413
40. Hueber W, Patel DD, Dryja T, Wright AM, Koroleva I, et al. (2010) Effects of AIN457, a fully human antibody to interleukin-17A, on psoriasis, rheumatoid arthritis, and uveitis. *Sci Transl Med* 2: 52ra72. <https://doi.org/10.1126/scitranslmed.3001107> PMID: 20926833