Oxysterols are agonist ligands of RORγt and drive Th17 cell differentiation

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The RAR-related orphan receptor gamma t (RORγt) is a nuclear receptor required for generating IL-17–producing CD4$^+$ Th17 T cells, which are essential in host defense and may play key pathogenic roles in autoimmune diseases. Oxysterols elicit profound effects on immune and inflammatory responses as well as on cholesterol and lipid metabolism. Here, we describe the identification of several naturally occurring oxysterols as RORγt agonists. The most potent and selective activator for RORγt is 7β, 27-dihydroxycholesterol (7β, 27-OHC). We show that these oxysterols reverse the inhibitory effect of an RORγt antagonist, ursolic acid, in RORγ- or RORγt-dependent cell-based reporter assays. These ligands bind directly to recombinant RORγ ligand binding domain (LBD), promote recruitment of a coactivator peptide, and reduce binding of a corepressor peptide to RORγ LBD. In primary cells, 7β, 27-OHC and 7α, 27-OHC enhance the differentiation of murine and human IL-17–producing Th17 cells in an RORγt-dependent manner. Importantly, we showed that Th17, but not Th1 cells, preferentially produce these two oxysterols. In vivo, administration of 7β, 27-OHC in mice enhanced IL-17 production. Mice deficient in CYP27A1, a key enzyme in generating these oxysterols, showed significant reduction of IL-17–producing cells, including CD4$^+$ and γδ$^+$ T cells, similar to the deficiency observed in RORγt knockout mice. Our results reveal a previously unknown mechanism for selected oxysterols as immune modulators and a direct role for CYP27A1 in generating these RORγt agonist ligands, which we propose as RORγt endogenous ligands, driving both innate and adaptive IL-17–dependent immune responses.

IL-17–producing CD4$^+$ Th17 cells are essential in protective immunity against extracellular bacterial and fungal infections but also, play key pathogenic roles in autoimmune diseases, such as psoriasis and multiple sclerosis. RAR-related orphan receptor gamma t (RORγt) is an orphan nuclear receptor expressed in several immune cell types, including CD4$^+$ Th17 cells. Because of its essential role in driving IL-17 production, RORγt represents a potential target for therapeutic intervention, and a number of antagonists have been published that inhibited Th17 cell differentiation (1–3). However, the nature of RORγt endogenous ligands remains unknown, although accumulating evidence suggested the existence of such ligands. For example, crystal structures of the ligand binding domain of RORγ clearly showed a well-defined pocket that could accommodate a cholesterol or 25-hydroxycholesterol (OHC) molecule (4). Purified naïve CD4$^+$ T cells can be activated in culture to differentiate into IL-17–producing cells, suggesting that these cells have the capacity to produce endogenous RORγt ligand(s). In addition, IL-17–producing mouse RORγt$^+$ innate lymphoid cells, believed to be essential in orchestrating immunity at mucosal sites, including intestine and lung, were shown to be generated in vivo under germfree conditions (5). Identification of the endogenous agonist ligand(s) and understanding of how the ligand engages and activates RORγt may, therefore, offer critical insights into the rational design of RORγt modulators to block pathogenic IL-17–producing cells in disease.

Oxysterols are oxygenated derivatives of cholesterol or by-products of cholesterol synthesis (6–9). Some oxysterols serve as key intermediates for bile acid and steroid synthesis and function as signaling molecules or receptor ligands modulating cell proliferation and apoptosis, lipid and cholesterol synthesis, transportation and metabolism, and immune responses. For example, certain oxysterols have been shown to serve as endogenous ligands of EBI2 receptor (10, 11), activate the liver X receptor (12), function as a selective estrogen receptor modulator (13), or affect Hedgehog signaling by binding to the Smoothened molecule (14). In addition, several oxysterols were previously shown to function as RORγ or RORα inverse agonists (such as 7-hydroxylated oxysterols) or 24(S)-OHC agonists (such as 25-OHC) (4, 15, 16). However, their role as endogenous RORγ, RORγt, or RORα ligands in vivo was not clearly shown.

We have identified a number of naturally occurring oxysterols as RORγt agonists. Although we cannot exclude at present the existence of additional endogenous agonists of RORγt, here we show that two 7, 27-dihydroxycholesterols (i.e., 7β, 27-OHC and 7α, 27-OHC) are strong candidates as endogenous RORγt

Significance

Because of its essential role in driving IL-17 production, the orphan nuclear receptor RAR-related orphan receptor gamma t (RORγt) represents a potential therapeutic target for autoimmune diseases. Here, we present evidence that 7β, 27-dihydroxycholesterol (7β, 27-OHC) and 7α, 27-OHC are RORγt agonist ligands and may serve as potential endogenous RORγt ligands in promoting the differentiation of mouse and human CD4$^+$ Th17 cells. These findings may not only facilitate rational design of RORγt modulators but also, provide insights into additional targets for inhibiting IL-17 production.

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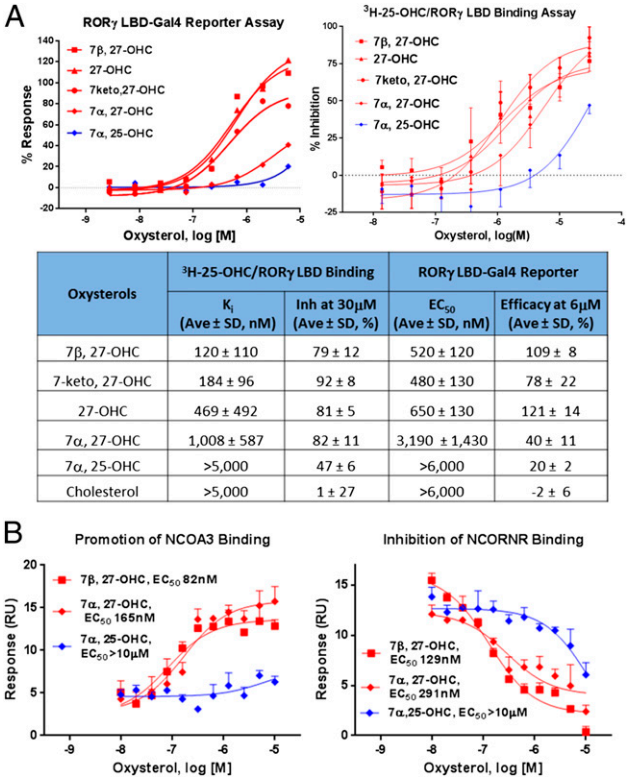
agonists driving IL-17 production in $CD4^+$ Th17 cells as well as other IL-17–producing innate cells, such as $\gamma\delta^+$ T cells.

Results

RORγt Agonist Activity of Selected Oxysterols. To identify potential RORγt agonists, we screened a panel of naturally occurring oxysterols and a few other compounds (*SI Appendix*, Tables S1 and S2) for their agonist activity in reversing the inhibitory effect of ursolic acid (UA), an RORγt antagonist (3). We first used a cell-based RORγ-dependent reporter assay, in which the DNA-binding domain of GAL4 was fused to the ligand binding domain (LBD) of human RORγ. This construct was transiently expressed in HEK293T cells and showed constitutive activity in driving luciferase reporter gene expression. Because RORγt and the related isotype, RORγ, share the same LBD, such an assay potentially allowed detection of agonists for both receptors. A number of oxysterols showed significant agonist activity, with 27-OHC, 7β, 27-OHC, and 7-keto, 27-OHC being the most potent and efficacious (Fig. 1*A* and *SI Appendix*, Tables S1 and S2). In comparison, 7α, 25-OHC, an endogenous ligand for EBI2 receptor, was inactive. Several previously reported RORγ or RORα inverse agonists or agonists including 7α-OHC, 7β-OHC, 7-KC, 25-OHC, 24(S), 25-EC, and 24(S)-OHC (15, 16) showed weak to moderate agonist activity. Cholestenoic acid derivatives of the 27-hydroxylated sterols displayed minimal agonist activity (*SI Appendix*, Table S1), indicating that the hydroxyl group at carbon 27 is required for RORγt agonism. We next tested selected oxysterols, including all four 27-OHCs, in cell-based full-length human RORγ- or RORγt-dependent reporter assays. One clear exception was 7-keto, 27-OHC, which was largely inactive in those two assays, whereas other 27-OHCs were active, and there was no apparent difference between RORγ- and RORγt-dependent assays (*SI Appendix*, Fig. S1).



Oxysterols	^{3}H-25-OHC/RORγ LBD Binding		RORγ LBD-Gal4 Reporter	
	K_i (Ave ± SD, nM)	Inh at 30μM (Ave ± SD, %)	EC_{50} (Ave ± SD, nM)	Efficacy at 6μM (Ave ± SD, %)
7β, 27-OHC	120 ± 110	79 ± 12	520 ± 120	109 ± 8
7-keto, 27-OHC	184 ± 96	92 ± 8	480 ± 130	78 ± 22
27-OHC	469 ± 492	81 ± 5	650 ± 130	121 ± 14
7α, 27-OHC	1,008 ± 587	82 ± 11	3,190 ± 1,430	40 ± 11
7α, 25-OHC	>5,000	47 ± 6	>6,000	20 ± 2
Cholesterol	>5,000	1 ± 27	>6,000	-2 ± 6

Fig. 1. Agonist activity of oxysterols in a cell-based RORγ reporter assay and direct binding to RORγ LBD. (*A*) Effect of 27-OHCs in reversing the inhibitory effect of UA in a cell-based chimeric RORγ reporter assay and inhibition of binding of ^{3}H-25-OHC to the RORγ ligand binding domain. Shown in *Upper* are dose titration curves from representative experiments. For the reporter assay, oxysterols (starting at 6 μM; 1:3 serial dilutions) were tested in duplicates in the presence (0% of response) or absence (100% of response) of 1 μM UA. For the ^{3}H-25-OHC/RORγ LBD competition binding assay, oxysterols (starting at 30 μM; 1:3 serial dilutions) were also tested in duplicates. Percent inhibition values were calculated based on without RORγ LBD protein as 100% and DMSO only as 0%. EC_{50} or IC_{50} values were calculated using GraphPad Prism 5. K_i values were calculated based on $K_i = IC_{50}/([^3\text{H-25-OHC}]/K_d + 1)$. Average ± SD ($n$ = or > 3) of EC_{50} or K_i values and efficacy values at the highest tested concentration are shown in the table. (*B*) Effect of 7β/α, 27-OHC vs. 7α, 25-OHC on binding of coactivator NCOA3 and corepressor NCORNR peptides by RORγ LBD in a surface plasmon resonance binding assay.

Direct Binding of Oxysterols to RORγ LBD. We next measured direct binding of oxysterols to recombinant RORγ LBD protein in the absence or presence of a peptide from the steroid receptor coactivator-1 (SRC1) using the thermal shift readout ThermoFluor (17–19) (*SI Appendix*, Fig. S3*A*). Direct binding of oxysterols to recombinant LBD of RORα and RORβ in the presence of SRC1 peptide was also examined (*SI Appendix*, Fig. S3*A*). Consistent with their activities in the cell-based chimeric RORγ-dependent reporter assay, 27-OHCs were among the most potent binders to RORγ LBD, with a rank order of 7β, 27-OHC > 7-keto, 27-OHC > 27-OHC > 7α, 27-OHC. The presence of SRC1 peptide did not affect binding to RORγ LBD by 27-OHCs except 27-OHC, which showed reduced affinity, suggesting that this most abundant oxysterol in the body is unlikely to be an endogenous RORγ agonist. When tested against the LBDs of RORα and RORβ in the presence of SRC1, some selectivity was observed: 7α, 27-OHC was inactive in binding to RORα and RORβ, and 7β, 27-OHC was inactive in binding to RORα. In contrast, 7-keto, 27-OHC bound to all three ROR LBD proteins and showed the highest affinity for RORα in the presence of SRC1 peptide. Taken together, these findings suggest that 7β, 27-OHC and 7α, 27-OHC are more selective agonist ligands for RORγt than 27-OHC or 7-keto, 27-OHC.

25-OHC was previously shown to form a complex with RORγ LBD, and ^{3}H-labeled 25-OHC exhibited specific binding to recombinant RORγ LBD in vitro (4), which showed a K_d of 10 nM in a saturation binding assay (*SI Appendix*, Fig. S3*B*). Consistent with their activities in the cell-based RORγ reporter assay, the RORγ-active oxysterols effectively competed with ^{3}H-25-OHC for binding to recombinant RORγ LBD (Fig. 1*A*). The same rank of potency for the four 27-OHCs was observed as that determined using the ThermoFluor readout, with 7β, 27-OHC being most potent in competing with ^{3}H-25-OHC for binding to RORγ LBD. Moreover, we found that RORγ-active oxysterols (i.e., 7β, 27-OHC and 7α, 27-OHC but much less for 7α, 25-OHC) enhanced binding of RORγ LBD to NCOA3 while decreasing binding to NCORNR (Fig. 1*B*) using surface plasmon resonance as readout.

7β, 27-OHC and 7α, 27-OHC Induce IL-17 Production by Mouse and Human Th17 Cells. Addition of 7β, 27-OHC and 7α, 27-OHC but not 7α, 25-OHC (Fig. 2*A* and *SI Appendix*, Figs. S4*A* and S5) to

mouse total or naïve CD4$^+$ T cells under Th17 condition increased the number of IL-17A–producing cells with minimal effect or increasing number of IFN-γ–producing cells. This finding became more obvious when 7β, 27-OHC or 7α, 27-OHC was added to T-cell cultures in the presence of UA (Fig. 2*A* and *SI Appendix*, Figs. S4*A* and S6). As observed for murine cells, 7β, 27-OHC and 7α, 27-OHC but not 7α, 25-OHC promoted differentiation of human naïve CD4$^+$ T cells to IL-17–producing Th17 cells in culture and reversed the inhibitory effect of UA (Fig. 2*B* and *SI Appendix*, Fig. S4*B*). It was unclear whether 27-OHC affected IL-17 production, because it showed strong cytotoxicity for mouse and human T cells in culture. 7-Keto, 27-OHC was much less efficacious than 7α/β, 27-OHC in promoting the appearance of IL-17–producing mouse or human Th17 cells (*SI Appendix*, Fig. S7), which was expected from the findings with the full-length RORγt reporter assay. The effect of 7β, 27-OHC or 7α, 27-OHC on Th17 cell polarization seems to depend on RORγt, because no increase in the number of IL-17$^+$ cells was observed when RORγt-deficient mouse CD4$^+$ T cells were cultured in the presence of these oxysterols (Fig. 2*A*). Because RORα expression was intact in the RORγt-deficient CD4$^+$ T cells, the lack of any apparent effect of 7β, 27-OHC or 7α, 27-OHC in those cells further suggested that they were not functional RORα agonists, consistent with ThermoFluor binding data.

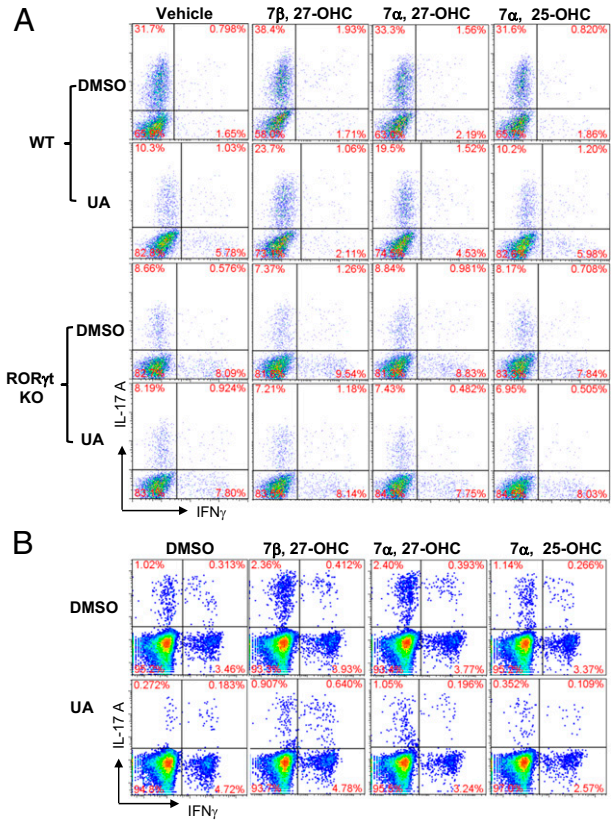


Fig. 2. 7β, 27-OHC and 7α, 27-OHC but not 7α, 25-OHC promoted IL-17 production of mouse and human Th17 cells in vitro. Shown are data from representative flow cytometry intracellular staining analysis of IL-17A and IFN-γ in mouse or human CD4$^+$ T cells. (*A*) Purified total CD4$^+$ T cells from WT and RORγt KO mice were activated under Th17-polarizing condition for 3 d. (*B*) Purified human naïve CD4$^+$ T cells (CD45RO$^-$, CCR6$^-$) activated under Th17-polarizing condition for 10 d. DMSO (vehicle) or 1 μM UA was added 2 h before cell stimulation. Oxysterols (6 μM for mouse T cells and 0.3 μM for human T cells) were added at the start of culture. Statistical analyses of data from several independent experiments are shown in *SI Appendix*, Fig. S4.

Mice Deficient in CYP27A1 Show Reductions in IL-17 Production. Mitochondrial sterol 27-hydroxylase (CYP27A1) (*SI Appendix*, Fig. S8) is a key enzyme in bile acid synthesis and the production of 27-OHCs in vivo (6, 19–21). Impaired formation of 27-OHC in *Cyp27a1* KO mice was previously reported (22). We have confirmed this observation by liquid chromatography (LC) MS/MS analysis and extended previous findings by detecting reductions in 7β, 27-OHC, and 7α, 27-OHC in *Cyp27a1* KO mice (Fig. 3*A* and *SI Appendix*, Fig. S9 and Table S4). In comparison, the levels of 7-OHCs and 25-OHCs (e.g., 7α-OHC, 7β-OHC, 7α, 25-OHC, and 7β, 25-OHC) were elevated in *Cyp27a1* KO mice (Fig. 3*A* and *SI Appendix*, Fig. S10 and Table S4). We observed a reduction in the number of IL-17$^+$ cells in KO mice (Fig. 3*B*), including spleen CD4$^+$ and γδ$^+$ T cells. *Cyp27a1* KO splenocytes showed diminished mRNA levels for IL-17 and other Th17-related genes, such as IL-17F and IL-22, by RT-PCR (Fig. 3*B*). Similar observations with RORγt KO splenocytes (*SI Appendix*, Fig. S11) suggest that CYP27A1 may, indeed, affect IL-17 production through an RORγt-dependent mechanism. Although by FACS analysis, the number of spleen or peritoneal IFN-γ$^+$ CD4$^+$ or γδ$^+$ T cells in *Cyp27a1* KO seemed to be similar to WT, RT-PCR analysis indicated that there was an increase in IFN-γ mRNA in *Cyp27a1* KO splenocytes, which was observed in RORγt KO splenocytes (Fig. 3*B* and *SI Appendix*, Fig. S11).

We next examined whether CYP27A1 was required for differentiation of highly purified mouse naïve CD4$^+$ T cells into Th17 cells in vitro. *Cyp27a1* KO-naïve CD4$^+$ T cells showed a dramatically reduced capacity for Th17 differentiation and increased Th1 differentiation in culture (Fig. 3*C*). Different from RORγt-deficient CD4$^+$ T cells (Fig. 2*A*), addition of 7β, 27-OHC to naïve CD4$^+$ T cells from *Cyp27a1* KO mice restored IL-17–producing cells. To determine whether CYP27A1 was also required for in vivo differentiation of antigen-specific Th17 cells, we immunized mice with ovalbumin/complete Freund's adjuvant (OVA/CFA) followed by ex vivo restimulation of draining lymph node cells with ovalbumin in culture. The draining lymph node cells from *Cyp27a1* KO mice showed reduced numbers of both IL-17$^+$ and IL-22$^+$ CD4$^+$ T cells and increased numbers of IFN-γ$^+$ cells as observed in vitro (Fig. 3*D*), which was also confirmed by determination of the levels of IL-17A, IL-22, and IFN-γ in culture supernatants by ELISA (Fig. 3*D*). In addition, in vivo dosing of synthetic 7β, 27-OHC through s.c. injections in mice increased the number of IL-17$^+$ cells in both CD4$^+$ and γδ$^+$ T cells, while showing no effect on IFN-γ$^+$ cells (Fig. 4*A*). Dosing of synthetic 7β, 27-OHC also boosted in vivo differentiation of Th17 cells and production of IL-17 in culture in the OVA/CFA immunization model, reversing the effect of UA dosed in those same animals (Fig. 4*B*). In this particular study, production of IFN-γ by T cells in ex vivo culture was also increased but to a smaller extent than that of IL-17 (~2× increase for IFN-γ compared with >35× increase for IL-17 production). Taken together, these findings suggest a role for *Cyp27a1* and 7β/α, 27-OHC in driving IL-17 production in vivo in mice. In comparison, the number of CD4$^+$ IL-17$^+$ cells was normal in splenocytes deficient in CH25H, a key enzyme catalyzing the formation of 25-OHC from cholesterol (6, 7) (*SI Appendix*, Fig. S10). Because the levels of 25-OHCs were elevated in *Cyp27a1* KO mice (*SI Appendix*, Figs. S9 and S10 and Table S4), these results indicate that 25-OHCs are unlikely to function as endogenous RORγt agonists.

7β, 27-OHC and 7α, 27-OHC Are Preferentially Produced by Mouse Th17 Cells. Because highly purified CD4$^+$ T cells can be differentiated into IL-17–producing cells in culture, we reasoned that differentiating Th17 cells had the capacity to synthesize endogenous RORγt agonist ligands. Interestingly, we noted that both 7β-OHC and 7α-OHC, when tested in mouse CD4$^+$ T-cell cultures, were active in reversing the inhibitory effect of UA on IL-17 production, with similar efficacy to their 27-dihydroxylated analogs (*SI Appendix*, Fig. S12*A*). This finding was in contrast to the observation in the RORγt reporter assay, where 7-OHCs

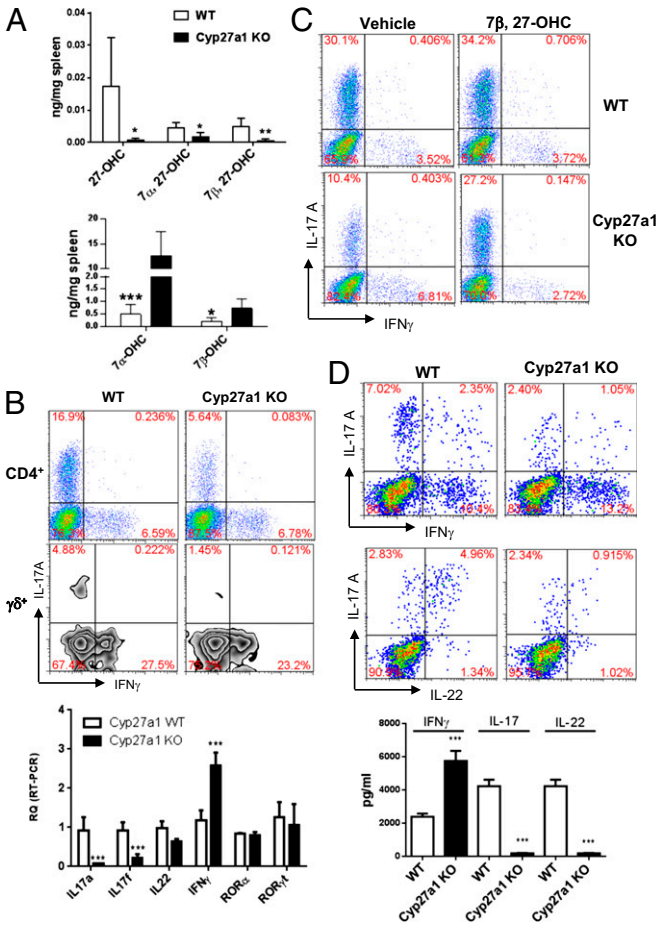


Fig. 3. Defective production of 27-OHCs and IL-17–producing cells in *Cyp27a1* KO mice. (*A*) Spleen levels of 27-OHCs and 7-OHCs of *Cyp27a1* KO vs. WT mice. Oxysterols in spleen samples (n = 5) were measured using deuterated oxysterols as internal controls as described in *SI Appendix*. (*B*) Flow cytometry intracellular staining analysis for IL-17A and IFN-γ in spleen CD4$^+$ and γδ$^+$ T cells from *Cyp27a1* KO vs. WT mice. Total splenocytes were activated under Th17-polarizing condition for 3 d. RT-PCR analyses were performed on selected IL-17 pathway genes from mRNA samples (n = 2–3). RQ, relative quantification. (*C*) Defective in vitro Th17 differentiation of naive CD4$^+$ T cells from *Cyp27a1* KO mice. Purified naive CD4$^+$ T cells of *Cyp27a1* KO and WT controls were activated under Th17-polarizing condition for 6 d. DMSO (vehicle) or 7β, 27-OHC (6 μM) was added at the beginning of the culture. (*D*) Defective in vivo Th17 differentiation in *Cyp27a1* KO mice. *Cyp27a1* KO and WT mice were immunized with ovalbumin/complete Freund's adjuvant (OVA/CFA); 7 d later, draining lymph node (LN) cells were harvested and restimulated with OVA in vitro for 2 d. IL-17A, IFN-γ, and IL-22 production from cultures of draining LN cells were determined by flow cytometry intracellular staining analysis and ELISA. Statistics by two-tailed, unpaired Student t test. *P < 0.05; **P < 0.01; ***P < 0.001.

were, in general, much weaker agonists. This observation may be because of conversion of 7-OHCs to 7, 27-OHCs by T cells in culture, because neither 7β-OHC nor 7α-OHC enhanced IL-17 production in CYP27A1-deficient CD4$^+$ T cells (*SI Appendix*, Fig. S12*B*). To directly test this hypothesis, we investigated whether mouse Th17 cells could convert exogenously added ^{3}H-7α-OHC or 7β-OHC into 7, 27-OHCs in culture. Mouse Th17 cells but not Th1 cells significantly converted ^{3}H-7β-OHC into more hydrophilic ones (Fig. 5*A*). Similarly, ^{3}H-7α-OHC was converted by Th17 cells to a lower extent but not by Th1 cells. HPLC analysis of the product from ^{3}H-7β-OHC resulted in its separation into peaks A and B (Fig. 5*B*). Peak B is likely 7β, 27-OHC, because it was absent from the converted product of cultured *Cyp27a1* KO CD4$^+$ T cells; also, its identity was confirmed by MS analysis. Peak A was complex as suggested by MS studies. Its generation seems to be partially dependent on CYP27A1: this peak was reduced but not absent from the converted product in *Cyp27a1* KO CD4$^+$ T cells. Similar products were observed when ^{3}H-7β-OHC was added to cultures of COS7 cells transiently expressing human CYP27A1 (Fig. 5*C*). These findings suggest that mouse Th17 but not Th1 cells preferentially produce 7β, 27-OHC from exogenously added 7β-OHC. To a lesser extent, they likely produced 7α, 27-OHC from exogenously added 7α-OHC in a CYP27A1-dependent manner. Interestingly, the expression of *Cyp27a1* was enriched in mouse Th17 cells, whereas the expression of *Ch25h* was higher in mouse Th1 cells (*SI Appendix*, Fig. S13*A*). Expected from this observation, higher levels of endogenous 25-OHCs were observed in in vitro differentiated Th1 cells (~50% IFN-γ$^+$ at harvest) (*SI Appendix*, Fig. S13*B*). Among 27-OHCs, in vitro differentiated Th17 cells (~30% IL-17$^+$ at harvest) produced significantly higher levels of 7β, 27-OHC relative to Th1 cells. No apparent difference in the levels of 7α, 27-OHC and higher levels of 27-OHC were seen in Th1 cells. Whether the lower level of 27-OHC in Th17 was caused by conversion to 7β, 27-OHC is unclear, although neither Th17 nor Th1 cells showed much capacity for conversion of ^{3}H-27-OHC in vitro (*SI Appendix*, Fig. S14). Taken together, our findings suggest that Th17 cells have higher capacity to produce 7β, 27-OHC, although it is less clear whether the same applies for 7α, 27-OHC.

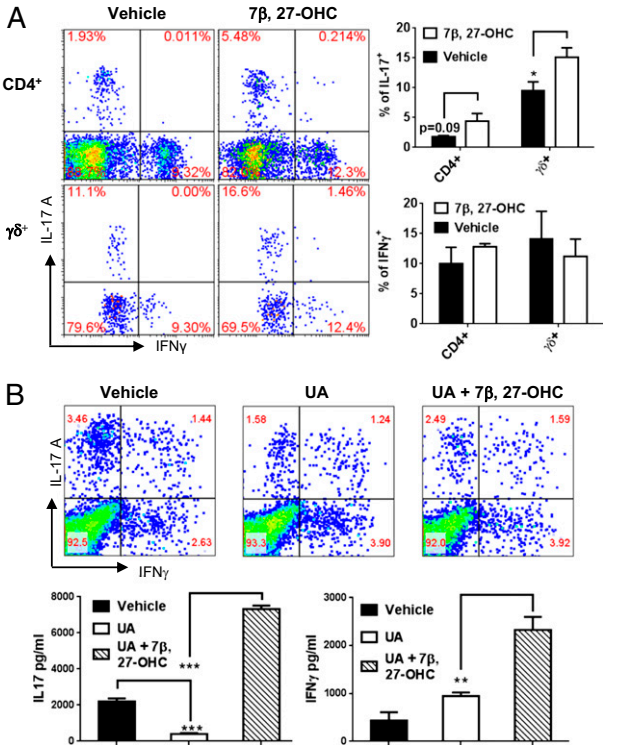


Fig. 4. Dosing of 7β, 27-OHC in mice enhanced IL-17 production in vivo. (*A*) Effect on LN IL-17$^+$ CD4$^+$ and γδ$^+$ T cells. B6 mice (n = 3) were dosed with vehicle or 7β, 27-OHC [60 mg/kg s.c. bis in die (BID)] for 3 d. Inguinal lymph node (LN) cells were harvested and stimulated with phorbol myristate acetate/inomycin for 4 h before intracellular staining. Data in the bar graph are averages ± SEMs of three mice per group. (*B*) Effect on IL-17 production in the OVA/CFA priming model. Three groups of B6 WT mice (n = 3) were immunized with OVA/CFA and dosed with vehicle, UA, (150 mg/kg i.p. every other day), or UA + 7β, 27-OHC (60 mg/kg s.c. BID); 7 d later, draining LN cells were harvested and restimulated with OVA in vitro for 2 d. IL-17A and IFN-γ production from cultures of draining LN cells were determined by flow cytometry intracellular staining analysis and ELISA. Data in bar graph are averages ± SEMs. Statistics by two-tailed, unpaired Student t test. *P < 0.05; **P < 0.01; ***P < 0.001.

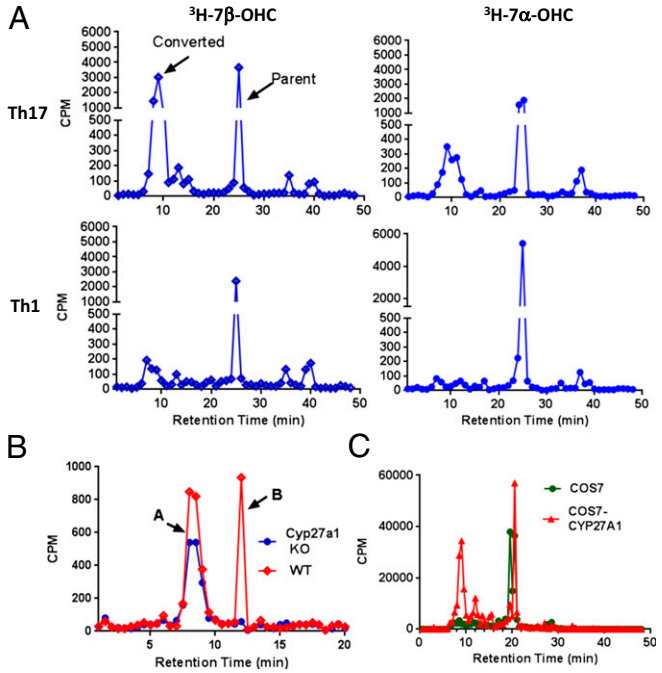


Fig. 5. ^{3}H-7β/α-OHC were preferentially converted into ^{3}H-7β/α, 27-OHC in mouse Th17 but minimally in Th1 T cells. (*A*) Conversion of ^{3}H-7β-OHC or 7α-OHC by mouse Th17 and Th1 cells in culture (purified total CD4$^+$ T cells activated under Th17 or Th1 conditions for 3 d). Conditioned media were fractionated using HPLC as described in *SI Appendix*. Radio activities of 50 μL 1-min fractions were determined. Data shown were from a representative experiment of three independent experiments. (*B*) Conversion of ^{3}H-7β-OHC by cultured Th17 cells differentiated from WT and *Cyp27a1* KO naïve CD4$^+$ T cells. Conditioned media were purified as above. Fractions containing the converted products were pooled and further purified by another HPLC; 30-s fractions were collected to resolve converted species, which resulted in peaks A and B. MS analysis revealed that peak B was consistent with being 7β, 27-OHC. (*C*) HPLC fractionation of conditioned medium from ^{3}H-7β-OHC–spiked culture of untransfected COS7 cells or COS7 cells transiently expressing human CYP27A1 protein. CPM, counts per minute.

Discussion

We have shown that 7β, 27-OHC and 7α, 27-OHC are RORγt agonist ligands and provided evidence suggesting their role as endogenous drivers of Th17 differentiation. Both bound to recombinant RORγ LBD protein directly and were selective against other nuclear receptors tested. They were active in enhancing RORγt-dependent reporter expression and IL-17 production in primary mouse and human Th17 cells in vitro. In vivo dosing of 7β, 27-OHC in mice increased the number of IL-17$^+$ cells. Production of these two oxysterols in vivo requires CYP27A1, a key bile acid synthesis enzyme. Mice deficient in CYP27A1 showed reduced levels of 7β, 27-OHC and 7α, 27-OHC and importantly, decrease in IL-17–producing cells. Thus, naïve *Cyp27a1* KO CD4$^+$ T cells are defective in their ability to differentiate into Th17 cells in vitro, which could be rescued by addition of 7β, 27-OHC. In addition, in vivo antigen-specific generation of Th17 cells was also defective in CYP27A1-deficient mice in a priming model. The overall profile of 7α, 27-OHC seemed to be less favorable than that of 7β, 27-OHC; however, we cannot exclude it as a potential candidate endogenous RORγt agonist ligand.

Although a deficiency in IL-17–producing cells was consistently observed in younger *Cyp27a1* KO mice (<8 wk), one intriguing observation was that, as these KO mice age, the deficiency became less obvious. Some animals even seemed to develop a phenotype resembling autoinflammation (e.g., splenomegaly was observed on necropsy, and production of cytokines, including IL-17A and IFN-γ, increased). When tested in immune disease models of longer duration, such as collagen-induced arthritis, our preliminary data indicated no difference between WT and *Cyp27a1* KO mice or even some exacerbation in KO mice. Although these observations seem to contradict our finding that these mice were deficient in IL-17 production, it is worth noting that certain IL-17– or IL-22–producing innate lymphocytes might play a regulatory role, and dysregulation of those cells could account, in part, for the apparent autoinflammatory phenotype in aged KO animals. Nonetheless, the observed IL-17 production in aged CYP27A1-deficient mice suggests the existence of endogenous RORγt agonists other than 27-OHCs, exogenous RORγt ligands, or RORγt-independent mechanisms. These alternative hypotheses require additional investigation.

Endogenous levels of 7β, 27-OHC and maybe 7α, 27-OHC seem to be in the same ranges of binding affinity values determined using the ^{3}H-25-OHC/RORγ LBD competitive binding assay. Based on our LC/MS/MS studies, the levels of endogenous 7β, 27-OHC and 7α, 27-OHC in total spleen or in vitro-generated Th17 cells are in single to double digit nanomolar ranges (~3–12 nM for 7β, 27-OHC and 1–35 nM for 7α, 27-OHC). In the ^{3}H-25-OHC/RORγ LBD competitive binding assay, 7β, 27-OHC showed higher affinity (K_i = 120 nM) and 7α, 27-OHC showed lower affinity (K_i = 1,008 nM) than unlabeled 25-OHC (K_i = 280 nM) (Fig. 1*A*). Based on the K_d value of 10 nM for ^{3}H-25-OHC in this assay (*SI Appendix*, Fig. S3*B*), we estimate that K_d values of 7β, 27-OHC and 7α, 27-OHC are ~4 and ~36 nM, respectively. Additional studies will be needed to confirm this estimation.

We have further showed that cultured mouse Th17 cells but not Th1 cells showed enhanced capability to convert exogenously added 7β-OHC or 7α-OHC to 7, 27-OHCs in culture. This finding explained why 7-OHCs were also active in reversing the inhibitory effect of UA in mouse T cells and indicates that T cells themselves can synthesize these endogenous RORγt agonists. Addition of ^{3}H-labled 27-OHC to Th17 or Th1 cells did not support generation of 7β, 27-OHC or 7α,27-OHC from exogenously added 27-OHC (*SI Appendix*, Fig. S13). It may be due to low expression of CYP7B1 in mouse T cells, which is the enzyme required for converting 27-OHC into 7α,27-OHC.

Because of its high cytotoxicity in primary mouse and human T cells in culture, we could not show whether 27-OHC had any agonist activity in enhancing IL-17 production. However, there are a number of findings suggesting that 27-OHC might not be an endogenous RORγt agonist. In the presence of SRC1, 27-OHC bound to RORγ LBD with reduced affinity. In addition, 27-OHC also showed partial agonist activity against LXRα, LXRβ, and ERβ in cell-based reporter assays. We cannot rule out that 27-OHC may still have a role in modulating RORγt function in cells because of binding RORγ LBD in the absence of SRC1 with relatively high affinity and because of its abundance at steady state.

Among other naturally occurring oxysterols, we showed that 25-OHCs are unlikely endogenous agonists for RORγt. First, they were less potent or efficacious than their 27-hydroxylated derivatives in the RORγ/γt reporter assays and the RORγ LBD binding assay. Second, none of the 25-OHCs were active in enhancing IL-17 production in T cells. Furthermore, in *Cyp27a1* KO mice, which had decreased IL-17–producing cells, the levels of all 25-OHCs were increased. Finally, in *Ch25h* KO mice, in which 25-OHCs production was defective, normal or slightly increased numbers of IL-17–producing cells were observed.

After 27-OHC, 7α-OHC and 24(S)-OHC are the next two most abundant oxysterols in vivo. In the RORγ reporter assay, both seemed to show partial efficacy at highest noncytotoxic concentrations compared with the efficacy observed for 27-OHCs. 7α-OHC failed to show significant agonist activity in enhancing IL-17 production in *Cyp27a1* KO CD4$^+$ T cells, and its level was highly increased in *Cyp27a1* KO mice, suggesting that it is not an RORγt agonist in vivo. Both 7α-OHC and 24(S)-OHC were previously reported by others (15, 16) to function as inverse agonists for RORγ. It is possible that they may play a role in regulating the effect of full endogenous agonists, such as 7β, 27-OHC

and 7α, 27-OHC, by competing for binding to RORγ or RORγt. We hypothesize that the balance between the abundant oxysterols, such as 27-OHC, 7α-OHC, and 24(S)-OHC, and the low levels of 7β, 27-OHC and 7α, 27-OHC at steady state maintains the homeostatic state of IL-17–producing innate cells. However, T-cell activation under Th17 conditions may favor generation of high levels of 7β, 27-OHC and 7α, 27-OHC, which serve as RORγt agonists to program Th17 differentiation and drive IL-17 production.

Nonetheless, we must point out that, although our data support that 7β, 27-OHC and 7α, 27-OHC are strong candidates as endogenous RORγt ligands, additional investigations are needed in many aspects. Because of the observations associated with aged *Cyp27a1* KO mice and the labile nature of oxysterols, such as 7β, 27-OHC, it has been difficult to explore these tools in more relevant in vivo IL-17–dependent models, such as experimental allergic encephalomyelitis or susceptibility to *Citrobacter* infection. Moreover, we cannot exclude the possibility that endogenous molecules other than the oxysterols that we studied or pathogen-derived factors may also act as exogenous RORγt ligands in activating or modulating RORγt in IL-17–producing cells. In addition, although we have focused on examining effects of oxysterols in RORγt-dependent functions in IL-17–producing cells, we also need to point out that the same oxysterols may serve as endogenous RORγ agonists. To this end, it will be of interest to investigate whether some of the abnormalities observed in CYP27A1-deficient humans or mice might be partially caused by RORγ functional defects.

Materials and Methods

Reagents. Oxysterols, antibodies, cytokines, and other chemicals are described in *SI Appendix*.

Recombinant LBD of RORγ, RORα, and RORβ. The generation of the LBD proteins of human *RORs* are described in *SI Appendix*.

RORγ- or RORγt-Dependent Luciferase Reporter Assays. Cell-based reporter assays were performed by transiently transfecting HEK293T cells as described in *SI Appendix*.

Nuclear Receptor Reporter Assays for Selectivity. Cell-based reporter assay kits were purchased from Indigo Biosciences, Inc. or Invitrogen and performed according to the manufacturer's instructions as described in *SI Appendix*.

^{3}H-25-OH/RORγ LBD Scintillation Proximity Assay. ^{3}H-25-OHC was purchased from Perkin-Elmer. The homogenous scintillation proximity assay was run as described in *SI Appendix*.

RORγ LBD Surface Plasmon Resonance Assay. Biacore studies were performed by Biosensor Tools as described in *SI Appendix*.

ThermoFluor Binding Assay. ThermoFluor experiments were carried out as described in *SI Appendix*.

Synthesis of 7α/β/keto, 25-OHC, and 7β, 27-OHC. Detailed synthesis methods are described in *SI Appendix*.

Extraction and Quantification of Oxysterols with LC/MS/MS. Detailed methods are described in *SI Appendix*.

Mice. *Ch25h* KO mice, *Cyp27a1* KO mice, RORγt KO mice, and C57BL/6J control mice, as described in *SI Appendix*, were purchased from The Jackson Laboratory.

Mouse Th17 Cell Activation and Differentiation in Vitro. Isolation and in vitro Th17 differentiation of total or naïve mouse CD4$^+$ T cells were carried as described in *SI Appendix*.

Gene Expression Studies in Mouse Splenocytes or Th17 Cells. RNA extraction using the RNeasy Plus Mini Kit (Qiagen) and real-time RT-PCR using the commercial Taqman RNA-to-Ct 1-Step Kit (Life Technologies) were carried out as described in *SI Appendix*.

Conversion of ^{3}H-7α-OHC, ^{3}H-7β-OHC, or ^{3}H-27-OHC by Mouse Th1 or Th17 Cells in Culture. ^{3}H-7α-OHC or 7β-OHC (American Radiolabeled Chemicals) or ^{3}H-27-OHC (synthesized by Quotient Bioresearch) was added to in vitro differentiated mouse Th1 or Th17 cells, and conditioned media were collected and analyzed by HPLC as described in *SI Appendix*.

Conversion of ^{3}H-7α-OHC or ^{3}H-7β-OHC to Dihydroxycholesterols by COS7 Cells Transiently Expressing Recombinant Human CYP27A1 Protein. COS7 cells transiently transfected with human CYP27A1 cDNA in pcDNA3.1$^+$ (Invitrogen) or vector alone were spiked with 5 μCi ^{3}H-7α-OHC or ^{3}H-7β-OHC. Conditioned media of 24-h culture were collected and analyzed by HPLC described in *SI Appendix*.

Human Th17 Cell Activation and Differentiation in Vitro. Total or naïve CD4$^+$ T cells were isolated from peripheral blood mononuclear cells of healthy donor using the CD4 T-Cell Isolation Kit II or the Naïve CD4 T Cells Isolation Kit II, respectively (Miltenyi Biotec). Th17 cell differentiation in culture was carried out as described in *SI Appendix*.

In Vivo Generation of Antigen-Specific Th17 Cells. Induction of Th17 cells in vivo and restimulation of draining lymph nodes cells in vitro are described in *SI Appendix*.

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