Supplementary Figure 1. RORγt exhibits strong, ligand-dependent transactivation activity in the *Drosophila* system. a, (Left) S2 cells were transiently transfected with constructs encoding the GAL4 DBD or the fusion protein G4DBD-RORγ, along with the luciferase constructs, and incubated for 3 days prior to Cu^{2+} induction. The ratio of firefly to Renilla luciferase activity (Relative Luciferase Unit or RLU) in the presence of copper was over 100-fold in cells expressing G4DBD-RORγ compared to non-expressing controls. (Right) Copper strongly induced G4DBD-RORγ protein expression as measured by western blot. Equal loading was ensured by analyzing Drice expression. **b**, (Left) The A325F mutation in the putative ligand-binding pocket abolishes RORγ activity. The experiment was carried out as above, using the indicated RORγ constructs. RLU for G4DBD-RORγ wild-type was assigned as 100%, and other values are represented relative to this. The putative ligand binding pocket was predicted based on the previous structural and mutational studies of RORβ[1]. (Right) Western blot analysis indicates that the A325F mutation does not affect protein expression. **c**, RORγ induces potent transcriptional activation, dependent on an intact ligand binding domain, in *Drosophila* 3rd instar larvae. (Top) LacZ staining from the dissected 3rd instar larvae, expressing either the A325F ligand binding domain mutant (left) or wild-type (right) RORγ. Genotypes are *hs*-G4DBD-ROR(A325F)/UAS-lacZ and *hs*-G4DBD-ROR(WT)/UAS-lacZ, respectively. LacZ staining is detected in the salivary glands, fat-body, and gut of larvae expressing G4DBD-RORγ(WT) but not G4DBD-RORγ(A325F), suggesting the presence of RORγ ligands in these organs. (Bottom) GFP expression compared in hemolymph cells isolated from 3rd instar larva. Genotypes for parental strains used to generate larva are as follows; *hs*-G4DBD-ROR(A325F)/*hs*-G4DBD-ROR(A325F) x

UAS-GFP/UAS-GFP (left) and *hs*-G4DBD-ROR(wt)/+ x UAS-GFP/UAS-GFP (right). **d**, Addition of various sterol metabolites led to substantial increases of RORγ transactivation activity in S2 cells grown in serum-free semi-synthetic media. Cholesterol metabolites were added at 2.5 μM. Values on the y-axis depict RLU, with EtOH-treated cell values set to 1. Of note, different metabolites exhibited different effects on RORγ-dependent transcriptional activation. For example, 20-OH-ecdysone or cholesterol (data not shown) did not increase its activity. All error bars indicate standard deviation of multiple experiments.

Supplementary Figure 2. Digoxin, digitoxin, and β-acetyldigoxin selectively inhibit RORγt activity. a, Digoxin (10 μM) inhibits G4DBD-RORγ activity in S2 cells, cultured in serum-free medium and supplemented with 22-OH-cholesterol (1.25 μM). Fold induction of RLU is indicated on the y-axis. **b**, Digoxin does not inhibit transactivation by the nuclear hormone receptors Daf12 and hAR (human androgen receptor) in the *Drosophila* S2 cell luciferase reporter system. Dafachronic acid (10 μM) and R1881 (10 nM) were added as ligands for Daf12 and hAR, respectively. Values on the y-axis depict RLU in logarithmic scale, with DMSO-treated cell values set to 100. Digoxin concentrations (μM) are indicated on the x-axis. **c**, Digoxin (10 μM) has no effect on human liver X receptor (LXR) α activity in S2 cells. 22-OH-cholesterol (10 μM) was used as a ligand for LXRα. **d**, Chemical structure of digitoxin, β-acetyldigoxin, and 20,22-dihydrodigoxin. **e**, RORγ-dependent transcriptional activity in the *Drosophila* S2 cell reporter system in the presence of DMSO, 10 μM digitoxin, or 10 μM β-

acetyldigoxin. Values on the y-axis depict RLU, with DMSO-treated cell values set to 100. The error bars represent standard deviation (a, b, c, and e).

Supplementary Figure 3. Digoxin binds directly to the RORγ LBD. a, Far-ultraviolet circular dichroism thermal melts corresponding to the RORγ LBD bound to digoxin, dig(sal), 25-OH-Cholesterol, or no ligand (apoprotein). The binding of digoxin increased the melting temperature (Tm) of RORγ-LBD by 3.8 °C. Dig(sal) and 25-OH-Chosterol increased the Tm of RORγ-LBD by 4.1 °C. **b,** *In vitro* competition assay and chemical structure of digoxigenin. Digoxigenin does not bind to the RORγ LBD *in vitro*. Recombinant human RORγ LBD was loaded with fluorescently-labeled 25-hydroxycholesterol and addition of digoxigenin failed to displace it. Fluorescence polarization was measured. **c,** Random mutagenesis of the RORγ ligand binding domain led to the identification of RORγ (triple), which is more resistant to digoxin-mediated inhibition of transactivation than the RORγ(wt) control in S2 cells. The graph represents the ratios of luciferase activity, relative to DMSO-treated reporter cells on the y-axis (logarithmic scale), for the ligand domain mutant (triple; L290F/C318S/A494T) and wild type RORγ(wt) upon treatment with different concentrations of digoxin (μM, x-axis). DMSO-treated RORγ(WT) cell values were set to 100. **d**, Digoxin (10 μM) does not inhibit G4DBD-RORγ(triple) activity in S2 cells cultured in serum-free medium and supplemented with 22-OH-cholesterol (1.25 μM). Fold RLU induction is indicated on the y-axis. **e**, (Left) FACS-sorted naïve murine $CD4^+$ T cells from *RORγt*$^{gfp/gfp}$ mice[2] were transduced with retroviral vectors encoding murine RORγt(wt)-IRES-Thy1.1 or RORγt(triple)-IRES-Thy1.1 on day 1 (16 hours after TCR stimulation) and intracellular

cytokine staining was performed on day 5. DMSO or 10 μM digitoxin were added 6–8 hours after viral transduction. Thy1.1$^+$ gated cells are shown. (Right) The graph represents the ratios of Thy1.1$^+$ CD4$^+$ T cells expressing IL-17a, relative to DMSO-treated cells (on the y-axis) upon treatment with different concentrations of digoxin (μM, x-axis). DMSO-treated RORγ(wt)- or RORγ(triple)-transduced cell values were set to 100, respectively. The error bars represent standard deviation (c, d, and e).

Supplementary Figure 4. Digoxin reduces IL-17f and IL-22 expression in Th17 cells, but has no effect on Th1, Th2, or Treg cell differentiation. a, Real-time PCR analysis of relative expression of indicated genes as compared to β-actin in WT T cells grown under Th0 or Th17 conditions. The results represent mean and standard deviation in two independent experiments. For each probe set, one of the DMSO-treated samples under Th17 polarization conditions was set at 100%. **b, c,** Real-time PCR analysis of relative expression of indicated genes compared to β-actin in wild-type T cells grown under Th0 or Th17 conditions, and in *RORγt*$^{gfp/gfp}$ T cells under Th17 conditions. Y-axis represents mean fold induction and standard deviation relative to DMSO-treated samples (Th0 conditions) (n = 2–3). **d,** Immunoblots of RORγt, Batf, and IRF4 expression in CD4$^+$ T cells cultured in Th17 conditions for 40 h. Tubulin immunoblot is presented as a loading control. **e**, Flow cytometry of intracellular staining for IL-17a and IFN-γ in sorted naïve T cell populations activated and expanded in IL-23–induced Th17 polarizing conditions (IL-6, IL-23, IL-1β and anti-TCR stimulation in serum-free XVIVO-20 media). DMSO or 10 μM digoxin was added 16 h after cytokine treatment and intracellular cytokine

staining was performed on day 5. **f, g,** FACS-sorted naïve T cells were cultured in Th1, Th2, and Treg conditions for 4 days in the presence of DMSO or 10 μM digoxin.

Supplementary Figure 5. Various cardiac glycosides do not inhibit RORγt activity or Th17 cell differentiation. a, Chemical structures of diverse cardiac glycosides with no inhibitory activity for RORγ in S2 cells at concentrations up to 30 μM. **b**, Flow cytometry of intracellular staining for IL-17a and IFN-γ in sorted naïve T cell populations activated and expanded in the presence of Th17 polarizing cytokines. DMSO or cardiac glycosides (10 μM) were added 16 h after cytokine treatment and intracellular cytokine staining was performed on day 4.

Supplementary Figure 6. Digoxin treatment phenocopies RORγt deficiency and inhibits RORγ, but does not inhibits Ahr activity. a, Two-dimensional hierarchical clustering of the 323 genes (including redundant probe sets and genes of unknown function) identified to be significantly affected (2-way ANOVA test; WT versus KO treament, $p<0.05$). Each row corresponds to a gene and each column corresponds to an experimental sample. Relevant Th17-associated genes are indicated. **b**, FACS-sorted naïve murine $CD4^+$ T cells from *RORγt*$^{gfp/gfp}$ mice were transduced with retroviral vector encoding murine RORγ-IRES-Thy1.1 on day 1 (16 h after TCR stimulation) and intracellular cytokine staining was performed on Day5. DMSO or 10 μM digoxin were added 6–8 h after viral transduction. $Thy1.1^+$ gated cells were shown. **c**, FACS-sorted naïve murine $CD4^+$ T cells were transduced with retroviral vectors encoding murine RORα or RORγt on day 1 (16 hours after TCR stimulation) and intracellular cytokine

staining was performed on day 5. DMSO, 10 μM digitoxin , or 10 μM β-acetyldigoxin were added 6−8 hours after viral transduction. **d,** FACS-sorted naïve murine $CD4^+$ T cells from *RORγt$^{gfp/gfp}$* mice were transduced with retroviral vectors encoding murine RORα-IRES-GFP or RORγt-IRES-GFP on day 1 (16 hours after TCR stimulation) and intracellular cytokine staining was performed on day 5. DMSO, digoxin (10 μM), and 6-formylindolo[3,2-b]carbazole (FICZ, 200 nM) were added 6−8 hours after viral transduction.

Supplementary Figure 7. RORγ transcriptional activity is not required for its nuclear localization and digoxin inhibits its DNA binding *in vitro* . a, Immuno-staining with anti-RORγ (green) and DAPI (blue) of $CD4^+$ T cells cultured in Th17 conditions. RORγ staining was predominantly found in the nucleus in both DMSO- and digoxin-treated cells. The lack of RORγ staining in RORγ knock-out cells demonstrates antibody specificity (middle panel). **b,** *Drosophila* S2 cells expressing EYFP-RORγ (wt) or EYFP-RORγ (A325F) fusion proteins both exhibit nuclear (DAPI) EYFP signals (green). Mutation of the putative RORγt ligand binding pocket did not prevent its nuclear localization. Digoxin treatment also did not change the nuclear localization of RORγ in S2 cells. **c,** RORγ target genes are not enriched in anti-RORγt immunoprecipitated chromatin from RORγt KO cells, demonstrating RORγ antibody specificity. Chromatin obtained from Th17-polarized WT or KO cells was immunoprecipitated with IgG or anti-RORγ antibodies, followed by real-time PCR analysis. A region in the *Il23r* intron 8 served as a negative control. The results represent mean and standard deviation in several independent experiments. **d,** Digoxin inhibits binding of RORγt to target genes.

Chromatin obtained from Th17-polarized cells treated with DMSO or digoxin (at 10 and 20 μM) was immunoprecipitated with IgG or anti-RORγ antibodies, followed by real-time PCR analysis. The int8 region of the *Il23r* locus served as a negative control (CNS stands for **c**onserved **n**on-coding **s**equence). **e,** Electrophoretic mobility shift assay (EMSA) for assessing the effect of digoxin on DNA binding activity of RORγ. Digoxin, but not digoxigenin, reduced RORγ binding to its target *Il17a* sequence. The binding of the digoxin-insensitive RORγ (triple) on *Il17a* was less affected. Competition assays were performed using 70-fold molar excess of unlabeled wild-type or mutated IL-17a oligonucleotides. DNA binding domain mutant (DBDm) RORγt was used as a negative control. 20 or 100 nmol of digoxigenin and 2, 20, or 100 nmol of digoxin were added during the binding reaction (representative of 3 experiments).

Supplementary Figure 8. Digoxin, but not digoxigenin, displaces co-activator peptide and enhances recruitment of co-repressor peptide to the RORγ LBD. a, Digoxin (concentrations indicated on the x-axis), but not digoxigenin, reduced RORγ LBD/SRC3-1b binding in a dose-dependent manner, in the presence of 1 μM 25-hydroxycholesterol. **b,** Digoxin (concentrations indicated on the x-axis), but not digoxigenin, promoted RORγ LBD/NCOR2 binding in a dose-dependent manner. RORγ LBD/SMRT2 binding was not affected.

Supplementary Figure 9. Reduced EAE histopathology in digoxin-treated mice. Representative histological sections of H&E-stained lumbar spinal cords from DMSO- or digoxin-treated animals with different EAE scores on day 14 after disease induction.

Monocytic infiltration is rare in digoxin-treated, asymptomatic animals (clinical score 0).

Supplementary Figure 10. Ectopic expression of murine ATP1a1 confers resistance to digoxin-mediated cytotoxicity and reveals digoxin-mediated suppression of human Th17 cell differentiation. a, Human cord blood $CD4^+$ T cells, transduced with murine ATP1a1, were cultured with anti-CD3/CD28 beads along with IL-2, IL-23, IL-1β, and TGF-β in the presence of lanatoside C or digoxin (10 μM), and intracellular cytokine staining was performed on day 6. **b,** $CD4^+$ T cells isolated from peripheral blood of healthy adult donors were co-transduced with mATP1a1-IRES-HSA and control-IRES-GFP or ROR αd, β, γt -IRES-GFP. After 6 days, cells were intracellularly stained for IFN-γ or IL-17a, and FACS plots gated on HSA and GFP positive cells are shown. Lanatoside C or digoxin (10 μM) was added 6−8 hours after viral transduction.

Supplementary Figure 11. Dig(dhd) and dig(sal) selectively inhibit mouse and human RORγ activity and inhibit expression of C-C chemokine receptor 6 (CCR6) in human Th17 cells. a, *In vitro* competition assay. Recombinant human RORγ LBD was loaded with fluorescently-labeled 25-hydroxycholesterol in the presence of the indicated concentrations of Dig(dhd) on the x-axis, and fluorescence polarization was measured. **b**, FACS-sorted naïve murine $CD4^+$ T cells from $ROR\gamma t^{gfp/gfp}$ mice were transduced with retroviral vectors encoding murine RORα-IRES-Thy1.1 or RORγt-IRES-Thy1.1 on day 1 (16 hours after TCR stimulation) and intracellular cytokine staining was performed on Day 5. DMSO, 40 μM Dig(dhd), or 5 μM Dig(sal) was added 6−8 hours after viral transduction. $Thy1.1^+$ gated cells are shown. **c**, Dig(dhd) (40 μM)

and Dig(sal) (10 μM) inhibits G4DBD-human RORγ activity in HEK293t cells. RLU are indicated on the y-axis. **d**, Dig(dhd) (40 μM) and Dig(sal) (10 μM) have no effect on human LXRβ activity in HEK293t cells. 22-OH-cholesterol (10 μM) was used as a ligand for LXRβ. The results represent mean and standard deviation in two independent experiments. **e,** EAE disease course in B6 wild-type mice that were IP injected with either DMSO or Dig(sal) (200 μg/mouse) every day starting from day 2 after disease induction with MOG(35-55)/CFA (complete Freund's adjuvant). Shown is averaged curve shape from eleven DMSO treated– and eight dig(sal) treated– animals. **f**, Flow cytometry of IL-17a and IFN-γ production by cord blood naïve $CD4^+$ T cells ($CD45RO^-$ $CD45RA^+CD3^+CD4^+CD25^-HLA\text{-}DR^-$) transduced with RORαd-IRES-GFP or RORγt-IRES-GFP on day 1 and analyzed on day 6. GFP expressing cells were gated for analysis. DMSO, Dig(dhd) (40μM) or Dig(sal) (10 μM) was added 6–8 h after viral transduction. **g**, Flow cytometric analysis of IL-17a and FoxP3 expression by human naïve cord blood T cells cultured for 6 d in the presence of IL-2, IL-23, and IL-1β, with various concentrations of TGF-β. DMSO or Dig(sal) at 10 μM was added 16h after cytokine addition. **h**, Surface expression of CCR6 by human naïve cord blood T cells ($CD45RO^-$ $CD45RA^+CD3^+CD4^+CD25^-HLA\text{-}DR^-$) after culture for six days in the presence of IL-2, IL-23, and IL-1β with various concentrations of TGF-β (ng/ml). DMSO (open histogram) or 40 μM Dig(dhd) (shaded histograms) were added 16 h after cytokine addition.

Supplementary Figure 12. Digoxin suppresses pre-differentiated Th17 cells both *in vitro* and *in vivo*. **a**, Mononuclear cells were collected from draining lymph nodes of IL-23R-GFP knock-in heterozygous mice 7 days after MOG/CFA injection and cultured for

four days in the presence of IL-23 and MOG(35-55) peptide. IL-23R$^+$ cells were FACS sorted based on GFP expression and cultured for three more days in the presence of IL-23 and DMSO or digoxin (10 μM). Intracellular staining for IL-17a and IFN-γ is shown after stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin for 4 h. **b,** *In vitro* TH17 polarized CD4$^+$ T cells, isolated from IFN-γ-deficient MBP (Myelin Basic Protein) TCR transgenic mice, were transferred to RAG-2 deficient mice, resulting in the development of non-classical EAE. DMSO or digoxin treatment (40 μg/mouse) was given to mice daily starting on day 2 (n=5). Clinical scores from one experiment are shown (representative of two independent experiments).

Supplementary Figure 13. NMR spectra of Dig(dhd). a, ^{13}C NMR spectrum (151 MHz, CD_3OD) of 20,22-dihydrodigoxin-21,23-diol (Dig(dhd)). **b**, ^{1}H NMR spectrum (600 MHz, CD_3OD) of 20,22-dihydrodigoxin-21,23-diol (Dig(dhd)).

Supplementary Figure 14. NMR spectra of Dig(sal). a, ^{13}C NMR spectrum (151 MHz, CD_3OD) of digoxin-21-salicylidene (Dig(sal)). **b**, ^{1}H NMR spectrum (600 MHz, CD_3OD) of digoxin-21-salicylidene (Dig(sal)).

METHODS

Chemicals. The purity of all synthesized compounds was determined by ^{1}H NMR analysis and found to be ≥ 95% of the indicated composition. The following chemicals were purchased from the indicated commercial sources.

Digoxigenin	Sigma	β-acetyldigoxin	Serva
Ouabain octahydrate	Sigma	Digitonin	Sigma
Digitoxin	Sigma	Lanatoside C	Sigma
Proscillaridin A	Sigma	Deslanoside	Avachem Scientific
Digitoxigenin	Sigma	Erysimoside	Latoxan
Digoxin	Sigma	Ouabagenin	Sigma
Oleandrin	TRC	20-Hydroxyecdysone	Sigma
19-Hydroxycholesterol	Sigma	20(R)-Hydroxycholesterol	Sigma
20α-Hydroxycholesterol	Sigma	24(S)-Hydroxycholesterol	Steraloids
20(S)-Hydroxycholesterol	Sigma	25-Hydroxycholesterol	Sigma
22(R)-Hydroxycholesterol	Sigma		

20,22-Dihydrodigoxin-21,23-diol (Dig(dhd)) synthesis. Sodium triethylborohydride (840 μL of a 1 M solution in THF, 0.843 mmol, 10 equiv) was added dropwise to a room temperature solution of digoxin (65.8 mg, 0.0843 mmol, 1.0 equiv) in THF (16.9 mL, 5 mM). After 10 min, the reaction was quenched with the slow addition of methanol (5 mL), stirred for 15 min, and concentrated in vacuo to afford an inseparable mixture of saturated diol (Dig(dhd)) and alkene diol (20,22-alkene) (~3:1 of saturated:alkene) as a

white solid. R_f = 0.15 (9:1 ethyl acetate/methanol). The crude material was used without purification.

To the above mixture of compounds in methanol (4.2 mL, 20 mM) was added 10 wt% palladium on carbon (9.0 mg, 0.0084 mmol, 0.1 equiv). The reaction atmosphere was sparged with hydrogen and the contents were stirred under a balloon of hydrogen for 16 h, at which point the reaction was filtered through Celite and concentrated in vacuo. The crude material was dry-loaded onto Celite and purified by flash chromatography on silica gel (19:1 → 9:1 → 85:15 dichloromethane/methanol) to afford Dig(dhd) (63.7 mg, 0.0809 mmol, 96% yield over two steps) as a white solid. Dig(dhd) was obtained as a ~1.5:1 mixture of diastereomers that were not separated.

R_f = 0.20 (85:15 dichloromethane/methanol); ^{1}H NMR (600 MHz, CD_3OD): δ 4.94–4.90 (comp m, 3H), 4.25–4.23 (m, 2H), 4.02–4.00 (m, 2H), 3.88–3.75 (comp m, 2.5H), 3.68–3.61 (m, 1H), 3.62 (app t, J = 7.0 Hz, 1H), 3.54 (dd, J = 10.8, 6.1 Hz, 0.6H), 3.48 (dd, J = 10.9, 5.8 Hz, 0.6H), 3.44 (dd, J = 11.1, 6.9 Hz, 0.4H), 3.31–3.21 (comp m, 4.8H), 3.15 (dd, J = 9.6, 3.1 Hz, 1H), 2.30–2.23 (m, 1H), 2.18 (ddd, J = 14.2, 7.2, 7.2 Hz, 0.6H), 1.95–1.44 (comp m, 22.6H), 1.33–1.20 (comp m, 4H), 1.24 (d, J = 6.2 Hz, 3H), 1.22 (d, J = 6.2 Hz, 3H), 1.20 (d, J = 6.2 Hz, 3H), 0.96 (s, 1.2H), 0.95 (s, 3H), 0.91 (s, 1.8H); ^{13}C NMR (151 MHz, CD_3OD): δ 100.7, 100.5, 96.9, 87.3, 87.0, 83.8, 83.6, 79.3, 78.7, 74.4, 74.2, 70.8, 69.5, 69.4, 69.1, 68.5, 68.3, 66.9, 65.1, 62.6, 62.1, 54.6 (two lines), 48.2, 42.5, 42.1, 41.0, 39.8, 39.4, 38.9, 38.5, 37.9, 37.0, 36.1, 33.7, 32.8 (two lines), 31.4, 31.0, 30.9, 30.7, 27.9, 27.8, 27.5, 24.5, 24.3 (two lines), 22.9, 22.8, 18.7, 18.5, 18.4, 9.6, 9.4; IR (Neat Film, NaCl): 3406 (br), 2933, 1641, 1406, 1370, 1166, 1130, 1068, 1014, 869 cm^{-1}; HRMS (ESI+) m/z calc'd for $C_{41}H_{70}O_{14}Na$ $[M + Na]^+$: 809.4663, found

809.4662; $[\alpha]_D^{18.4}$ +16.4° (c = 0.33, CH_3OH). The carbon and proton spectra are provided (Supplementary Fig. 13a and b).

Digoxin-21-salicylidene (Dig(sal)) synthesis. Sodium carbonate (15.0 mg, 0.142 mmol, 1.1 equiv) was added to a suspension of digoxin (99.8 mg, 0.128 mmol, 1.0 equiv) and salicylaldehyde (77.81 μL, 0.639 mmol, 5.0 equiv) in methanol (5.1 mL, 25 mM), at which point the contents turned yellow in color. A reflux condenser was affixed to the flask and the contents were warmed to reflux in a 125 °C oil bath. The reaction was cooled to room temperature after 23 h and concentrated in vacuo. The crude material was dry-loaded onto Celite and purified by flash chromatography on silica gel (1:0 → 19:1 ethyl acetate/methanol) to afford Dig(sal) (30.3 mg, 0.0342 mmol, 27% yield) as a pale yellow solid in ~8:1 dr. Mixed fractions containing the desired compounds were repurified by flash chromatography on silica gel (1:0 → 19:1 → 9:1 dichloromethane/methanol) to yield an additional 30.1 mg (0.0340 mmol, 27% yield) of Dig(sal) in ~3:1 dr. In some cases, different batches of Dig(sal) displayed varying toxicity with human embryonic kidney (HEK) 293t cells, presumably due to minor contamination with digoxin or a digoxin-related compound. Material obtained from the conditions described above can be further purified by preparative thin-layer chromatography, developing the plate thrice with either 9:1 dichloromethane/methanol or 19:1 ethyl acetate/methanol, to afford pure Dig(sal) that does not exhibit cytotoxicity up to 16 μM with HEK 293t cell.

R_f = 0.38 (19:1 ethyl acetate/methanol), 0.34 (9:1 dichloromethane/methanol); ^{1}H NMR (600 MHz, CD_3OD): δ 8.05 (dd, J = 7.9, 1.3 Hz, 1H), 7.22 (app dd, J = 9.7, 7.6 Hz,

0.6H), 7.20 (s, 1H), 7.14 (ddd, *J* = 8.3, 8.3, 1.4 Hz, 1H), 6.87–6.82 (comp m, 3H), 6.50 (d, *J* = 1. Hz, 0.3H), 6.18 (s, 1H), 4.94–4.86 (comp m, 4H), 4.25–4.22 (comp m, 3H), 4.02 (app dd, *J* = 6.5, 3.2 Hz, 2.6H), 3.97 (br s, 0.4 H), 3.86–3.75 (comp m, 4.5H), 3.59 (dd, *J* = 9.7, 5.4 Hz, 1H), 3.52 (dd, *J* = 10.0, 6.0 Hz, 0.4H), 3.49 (dd, *J* = 11.9, 4.1 Hz, 1H), 3.32 (ddd, *J* = 9.7, 9.7, 2.9 Hz, 2.8H), 3.20–3.19 (comp m, 0.3H), 3.15 (dd, *J* = 9.6, 3.0 Hz, 1.6H), 2.92 (dd, *J* = 11.7, 4.1 Hz, 0.4H), 2.35–2.31 (m, 1.2H), 2.25–2.17 (comp m, 0.6H), 2.08–2.01 (comp m, 5.3H), 1.95–1.68 (comp m, 21H), 1.53–1.41 (comp m, 5.8H), 1.35–1.18 (comp m, 18H), 0.95 (s, 3H), 0.89 (s, 1H), 0.80 (s, 3H), 0.68 (s, 1H); ^{13}C NMR (151 MHz, CD_3OD): δ 172.7, 172.0, 168.4, 166.2, 157.9, 157.1, 152.3, 151.1, 132.8, 132.3, 131.5, 131.4, 121.9, 121.7, 121.5, 120.7, 120.1, 116.6, 116.2, 115.6, 114.5, 107.4, 100.7, 100.5, 96.9, 87.6, 87.0, 83.8, 83.6, 76.2, 75.9, 74.4, 74.3, 70.8, 69.5 (two lines), 69.1, 68.5, 68.3, 57.5, 57.4, 49.6, 45.0, 43.6, 42.3, 42.2, 39.5, 38.9, 38.5, 38.0, 36.2, 36.1, 33.8, 33.5, 33.3, 33.0, 31.4, 31.3, 31.1 (two lines), 30.9, 30.7, 27.8, 27.7, 27.5, 24.2 (two lines), 22.8, 22.7, 18.7, 18.5, 18.4, 10.0, 9.9; IR (Neat Film, NaCl): 3417 (br), 2933, 1734, 1602, 1584, 1454, 1406, 1164, 1068, 1015, 956, 868 cm^{-1}; HRMS (ESI+) *m/z* calc'd for $C_{48}H_{68}O_{15}Na$ $[M + Na]^+$: 907.4456, found 907.4471; $[\alpha]_D^{18.5}$ +43.2° (*c* = 0.51, CH_3OH). The carbon and proton spectra are provided (Supplementary Fig. 14a and b).

Development of S2 cell reporter cell lines. The *Drosophila* S2 cell line was originally purchased from Invitrogen and was maintained in Schneider's *Drosophila* medium supplemented with 10% heat-inactivated bovine fetal calf serum and 1x antibiotic-antimycotic solution (Invitrogen). The Gal4 DNA binding domain (G4DBD),

corresponding to amino acids 1 through 147 of the Gal4 protein, was PCR amplified to make the fusion construct with murine RORγ (amino acids 79 to the carboxyl terminal end) lacking its DNA binding domain. The resulting chimeric gene was subcloned into the copper inducible pMT/V5-His A vector (Invitrogen) and into the constitutive pAc vector. Similar methods yielded fusion constructs between G4DBD and murine RORα (amino acids 142 to end), *Drosophila* DHR3 (amino acids 120 to end), *C. elegans* Daf12 (amino acids 184 to end), and herpes simplex virus VP16 (amino acids 413 to end). Coding sequences for firefly luciferase (Promega) were PCR amplified and subcloned into the pUAST vector containing five Gal4 binding enhancer sequences. The Renilla luciferase construct under a pol III promoter and the pAc vector were obtained from R. Dasgupta (NYU School of Medicine). To generate cell lines stably expressing various chimeric proteins upon copper addition, S2 cells were co-transfected with pMT plasmids encoding individual G4DBD chimeric constructs and the pCoHygro plasmid (Invitrogen), before screening for resistant colonies in the presence of hygromycin (0.3 mg/mL). Clones with genomic integration of G4DBD-RORγ, G4DBD-RORα, G4DBD-DHR3, and G4DBD-VP16 were further transfected with pUAST-firefly luciferase, polIII-Renilla luciferase, and pCoPuro[3], and resistant clones were selected with puromycin (2.5 μg/mL). Upon copper treatment in 384 well plates, all cell lines described above specifically increased firefly luciferase activity versus normalized Renilla luciferase activity, validating the method for high-throughput screens. Interestingly, unlike RORα and RORγ, the ROR family member RORβ, did not transactivate firefly luciferase in *Drosophila* cells (data not shown), suggesting that the ligand for RORβ is not produced in this system due to either enzymatic deficiencies in fly cells or precursor deficiencies in

serum. The human androgen receptor (hAR) expression plasmid pMK33-hAR and its reporter TAT3-Luc were obtained from M. Garabedian (NYU School of Medicine). The human LXRα and RXR expression constructs were obtained from D. Mangelsdorf (UT Southwestern) and co-transfected into S2 cells for reporter analysis. Fusion constructs were made between eYFP and wild-type or mutant (A325F) RORγ coding sequences, sub-cloned under the pMT promoter, and used for generating stable S2 cell lines as described above. R1881 (10 nM), Dafachronic acid (10 μM), and 22-hydroxycholesterol (10 μM) were used as ligands for hAR, Daf12, and hLXRα, respectively.

Chemical screen. Transfections of firefly and Renilla reporter constructs were performed using the Effectene kit from Qiagen according to the manufacturer's protocol with some modifications. Briefly, 5 ng of pUAST-firefly luciferase and 7 ng of Pol III-Renilla luciferase were mixed with Enhancer/Effectene reagents in EC buffer (total volume: 6 μL), incubated for 10 to 15 minutes, and then the entire transfection mix was homogenously mixed with the stable G4DBD-RORγt cell line in Schneider's Drosophila medium supplemented with heat inactivated fetal bovine serum, hygromycin, and Pen/Strep antibiotics (Invitrogen). A total volume of 30 μL, containing 6 μL of transfection mix and 10,000 cells, was dispensed automatically into white bottom-tissue culture 384-well plates (Corning) with a high-speed, 8-channel microplate dispenser (Wellmate). Two days later, robot-mediated pin-transfer of small compounds (4,812 total compounds from the ICCB-Longwood chemical libraries, including Maybridge5, Bioactives and Prestwick collections) was performed, and after 6 h Cu^{2+} was added to the wells (700 μM). The following morning, cell culture medium was reduced to the final

volume of 10 μL, and 10 μL of Dual-glo and 10 μL of Stop-glo luciferase substrates were sequentially added (Promega). Firefly and Renilla luciferase activities were determined by measuring luminescence signals, using an automated 384-well plate reader (Analyst GT).

Drosophila. Coding sequences for G4DBD-RORγ (wild-type) or G4DBD-RORγ (A325F) were sub-cloned into the heat-shock inducible pCaSpeR-hs-act vector (obtained from C. Thummel, University of Utah). The resulting constructs were used to generate transgenic *Drosophila* lines (Rainbow Transgenic Flies, Inc). RORγ (WT) and RORγ (A325F) transgenic flies were crossed to the GAL4 reporter lines UASt-LacZ or UASt-GFP (Bloomington *Drosophila* Stock center) and were later heat-shocked at 37 °C for 30 minutes on three consecutive days. LacZ activity was measured using a beta-gal assay kit (Invitrogen), and GFP signals were analyzed by flow cytometry (BD LSRII).

Mice. For the conditional targeting of RORγt, we have generated a line, $ROR\gamma t^{fl/fl}$, in which exons 3,4,5,6 are flanked by LoxP sites. Cre-mediated recombination results in an out-of-frame mutation, abrogating productive protein translation (Kim and Littman, unpublished data). The IL-23R-GFP reporter line was provided by M. Oukka and V. Kuchroo (Harvard Medical School)[4]. $ROR\gamma t^{gfp/gfp}$ mice have been previously described[2].

S2 cell culture in serum-free media. S2 cells were maintained in Express Five SFM media (Invitrogen) and passaged in semi-synthetic (SSM-1) media (Santori et al., unpublished results) in the absence of sterol lipids[5] 2–4 days prior to transfection. 1.5 x

10^5 cells/well were plated in 96 well plates and incubated 1 h at 25 °C. After incubation, media was exchanged for 75 μl/well transfection mix. The transfection mix (in SSM-1) included 10 ng of G4DBD-RORγ(wt) or G4DBD-RORγ(triple) (in pAc or pMT vectors), 5 ng of destabilized firefly luciferase (Promega; pUAST), 2 ng of Pol III-Renilla luciferase and 0.5 μl of Cellfectin reagent (Invitrogen) per well. Following 4–5 h incubation at 25 °C, the SSM-1 media was replaced with fresh media. Various cholesterol metabolites were added 24 h later and, after overnight incubation cell lysate was prepared for analysis with the Dual Luciferase Reporter Kit (Promega). Luciferase activity was measured using the Viktor3 system (Perkin-Elmer).

Mammalian luciferase reporter assay. 50,000 human embryonic kidney 293t cells per well were plated in 96 well plates in antibiotic-free DMEM media containing 1% FCS. Cells were transfected with a DNA mix containing 0.5 μg/ml of firefly luciferase reporter plasmid (Promega pGL4.31), 2.5 ng/ml of plasmid containing Renilla luciferase (Promega pRL-CMV), and G4DBD-RORγ (0.2 μg/ml) or G4DBD-LXRβ (1 μg/ml) under regulation by the CMV promoter. The human LXRβ construct was obtained from D. Mangelsdorf (UT Southwestern). Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Compounds or vehicles were added 24 h after transfection and luciferase activity was measured 16 h later using the dual luciferase reporter kit (Promega).

***In vitro* mouse T cell culture.** T cells were purified from lymph nodes and spleens of six to eight week old C57BL/6 mice (Taconic), by magnetically depleting $B220^+$ cells

(autoMACS, Miltenyi), and then sorting $CD8^-DAPI^-CD19^-CD4^+CD25^-$ $CD62L^+CD44^{low/Int}$ naïve T cells on a FACSAria (BD). Cells were cultivated at 37 °C and 5% CO_2 in T cell media: RPMI 1640 (Invitrogen) supplemented with 10% (vol/vol) heat-inactivated FBS (Hyclone), 50 U penicillin-streptomycin (Invitrogen), 2 mM glutamine, and 50 μM β-mercaptoethanol. For T cell polarization, 200 μl cells were seeded at $0.4x10^5$ cells per mL in 96-well plates pre-coated with anti-CD3ε (5 μg/mL) and anti-CD28 (10 μg/mL). Cells were cultured for 4−5 days in Th17 polarizing conditions (TGF-β (0.3 ng/mL), IL-6 (20 ng/mL), and anti-IFN-γ and anti-IL-4 (2 ng/mL)), Th1 conditions (IL-12 (10 ng/mL), IL-2 (10 U/mL) and anti-IL-4 (2 ng/mL)), Th2 (IL-4 (10 ng/mL), and anti-P35 and anti-IFN-γ (2 ng/mL)), or Treg conditions (TGF-β (5 ng/mL)). For IL-23-induced Th17 cell differentiation[6], cells were cultured for 4–5 days in XVIVO-20 (Lonza) media, supplemented with IL-6 (20 ng/mL), IL-23 (50 ng/mL), and IL-1β (20 ng/mL). For ectopic expression ROR proteins, native $CD4^+$ T cells were infected with retroviral MIGr-control, MIGr-RORα or MIGr-RORγ 24 h after activation, and cultured without exogenous cytokines or only with IL-6. At day 1, compounds dissolved in DMSO were added. For cytokine analysis, cells were incubated for 5 h with phorbol 12-myristate 13-acetate (50 ng/mL; Sigma), ionomycin (500 ng/mL; Sigma) and GolgiStop (BD). Intracellular cytokine staining was performed according to the manufacturer's protocol (Cytofix/Cytoperm buffer set from BD or the FoxP3 staining buffer set from eBioscience) with alexa647-conjugated anti-IL-17a (eBioscience), PE-conjugated anti-IFN-γ or anti-IL-4 (eBioscience), and PE-conjugated anti-FoxP3 (eBioscience). An LSR II (BD Biosciences) and FlowJo software (Tree Star) were used

for flow cytometry and analysis. Dead cells were excluded using the Live/Dead fixable aqua dead cell stain kit (Invitrogen).

***In vitro* human T cell culture.** Blood samples were obtained from the New York Blood Center. Mononuclear cells were prepared from buffy coats from healthy adult donors or from cord blood, using FicollPAQUE gradients. $CD4^+$ T cells were magnetically selected (autoMACS, Miltenyi). Adult memory $CD4^+$ T cell subsets were further purified as $CD3^+CD4^+CD25^-$ $CD45RO^+CCR6^+CD161^+$ by cell sorting on a FACSAria (BD). Naïve $CD4^+$ T cells were isolated from cord blood as $CD3^+CD4^+CD45RA^+$HLA-DR^-CD25^- $DAPI^-$. Memory cells were activated with anti-CD3/CD28 coated beads (1 bead/cell) in T cell media (see above) supplemented with 100 U/mL IL-2 (Peprotech), 10 ng/mL IL-1β and 10 ng/mL IL-23 (eBioscience). Naïve cells were activated with anti-CD3/CD28 beads and cultured with the same cytokines, and TGF-β (0, 0.1, 1, or, 10 ng/mL) (Peprotech), in XVIVO-20 (Lonza) media. For ectopic expression of ATP1A1, cells were first transduced with a lentiviral GFP vector, HDVIRESGFP, encoding murine ATP1A1 (Origene clone image 4950746). On day 2, cells were transduced with the empty HDVIRESHSA vector or constructs encoding human RORαd, RORβ or RORγT[7], and compounds were added at 10 or 20 μM. On day 6, cells were treated with PMA, ionomycin and GolgiStop, surface stained with anti-HSA-PE, and intracellularly stained with anti-IL-17a-Alexa 647 and anti-IFN-γ-PECy7. Viral supernatants were produced by transient transfection of HEK293T cells with HDVIRESGFP or HDVIRESHSA vectors, a VSV-G expression plasmid and the packaging plasmid pCMVR8.9. Naive $CD4^+$ T cells, cultured in XVIVO-20 media supplemented with100 U/mL IL-2 (Peprotech), were

transduced with RORαd-IRES-GFP or RORγt-IRES-GFP on day 1 and analyzed on day 6. GFP-expressing cells were gated for analysis. DMSO or compounds were added 6–8 h after the viral transduction. For flow cytometry, the following human specific antibodies were used: CCR6-biotin (11A9 BD), CD3-Alexa750Cy7 (UCHT1 eBioscience), CD4-PacBlue (OKT4 eBioscience), CD25-APC (555434 BD), CD45RA-PE (HI100 eBioscience), CD45RO-APC (UCHL1 eBioscience), IL-17a-APC (eBio64CAP17 eBioscience), IFN-γ-PECy7 (45.B3 eBioscience), FoxP3-PacBlue (236A/E7 eBioscience), HLA-DR-FITC (555558 BD), CD3 purified (UCHT1 eBioscience), and CD28 purified (CD28.2 eBioscience).

qPCR analysis. Complementary DNAs (cDNAs) were synthesized from TRIzol (Invitrogen) isolated RNA, using M-MLV Reverse Transcriptase (Promega). Real-time RT-PCR was performed with SYBRgreen master mix (Roche) and the Roche real-time PCR system (Roche480). Gene specific values were normalized to *Actb* for each sample. Primer sequences are the following: Actb (TACAGCTTCACCACCACAGC and TCTCCAGGGAGGAAGAGGAT), IL-17a (CTCCAGAAGGCCCTCAGACTAC and AGCTTTCCCTCCGCATTGACACAG), RORγt (CCGCTGAGAGGGCTTCAC and TGCAGGAGTAGGCCACATTACA), IL-23R (GCCAAGAAGACCATTCCCGA and TGCAGGAGTAGGCCACATTACA), IL-17f (GAGGATAACACTGTGAGAGTTGAC and GAGTTCATGGTGCTGTCTTCC), IL-22 (CATGCAGGAGGTGGTGCCTT and CAGACGCAAGCATTTCTCAG), IL-21 (ATCCTGAACTTCTATCAGCTCCAC and GCATTTAGCTATGTGCTTCTGTTTC), RORα (TCTCCCTGCGCTCTCCGCAC and TCCACAGATCTTGCATGGA), cMaf (GAGGAGGTGATCCGACTGAA and TGGTTCTTCTCCGACTCCAG).

Gene chip Analysis. RNA was prepared from *in vitro* cultured and GFP$^+$ sorted T cells as described[8]. For microarray analysis, RNA was labeled and hybridized to GeneChip Mouse Genome 430 2.0 arrays according to Affymetrix protocols. Data were analyzed with GeneSpring GX11.5 software. Significant genes with p-values smaller than 0.05 (2-way ANOVA) and fold changes greater than 1.7 were selected.

EMSA. Double-stranded oligonucleotides containing wild-type (AGTCAGTTGCTGACCTTGATTCTAAG) ROR binding sequences of the *il17a* locus and four base-pair sequence tags (gatc) were labeled with dCTP-P^{32} using Klenow enzyme, purified on Microspin G-25 columns (GE Healthcare), and used as probes for an electrophoretic mobility shift assay (EMSA). Recombinant mouse RORγt (wild-type), RORγt ((DNA binding domain mutant)(R35A/R36G)), and RORγt (triple) proteins were synthesized using the TNT T7 quick translation-transcription system (Promega). All binding reactions were performed at 20 °C in 20 μl (1 μl of protein lysate, 5% (vol/vol) glycerol, 10 mM Tris-HCl (pH 7.6), 1 mM dithiothreitol, 200 μg/ml of bovine serum albumin, 0.5 mM EDTA, 50 mM NaCl, 1 μg poly(dI-dC), and 15 ng of probe). For competition assays, a 70 molar excess of unlabeled oligonucleotide (both wild-type and mutated (AGTCAGTTGCTGAGGTTGATTCTAAG)) was included in the reaction mixture. The effects of compounds on RORγt DNA binding activities were assessed by pre-incubating protein lysates with digoxigenin or digoxin. Gels were dried after electrophoresis and analyzed on a Phosphorimager (GE healthcare).

Mouse EAE inductions. For induction of active EAE, mice were immunized subcutaneously on day 0 with 70 μg MOG 35-55 peptides, emulsified in CFA (CFA supplemented with 200 mg/mL Mycobacterium tuberculosis), and injected (IP) on days 0 and 2 with 100 ng/mouse of pertussis toxin (Calbiochem). Alternatively, the MOG_{35-55}/CFA Emulsion PTX kit (Hooke Laboratories) was used. The EAE scoring system was as follows: 0-no disease, 1-limp tail; 2-weak/partially paralyzed hind legs; 3-completely paralyzed hind legs; 4-complete hind and partial front leg paralysis; 5-complete paralysis/death. For induction of passive EAE, splenocytes derived from MBP transgenic, IFN-γ knock-out mice[9] were cultured under Th17 conditions (T cell media, 20 ng/mL IL-6, 0.9 ng/mL TGF-β, 10 μg/mL anti-IL-4 antibodies). After 4 days, cells were re-stimulated with 10 ng/mL IL-23 and irradiated APCs (3000 rad) from TCRαβ−deficient mice. On day 7, $2x10^6$ MBP-specific $CD4^+$ T cells were transferred into B10.PL RAG-1 deficient recipients. Non-classical EAE was scored as follows: level 2-head tilt; level 3-ataxia; level 4-uncontrollable rolling; level 5-moribund. DMSO or digoxin (40 μg/mouse) prepared in PBS and DMSO or Dig(sal) (200 μg/mouse) prepared in PEG300 (Sigma) (due to the negligible solubility of Dig(sal) in PBS) were injected I.P. daily starting on day 2. Body weight and EAE scores were monitored daily.

Histology protocol. Animals were perfused with PBS + 5 mM EDTA before spinal cord removal. Spinal cords were treated with 10% formalin for 24–48 hours and placed into histology cassettes. Samples were quickly washed 3x with PBS, then with 50% EtOH for 30 min on a shaker. Samples were placed in 70% EtOH until processed by the NYU core histology unit.

***In vitro* binding and competition assay.** Human RORγ LBD (residues 262–518), flanked by an N-terminal 6xHis-tag and a thrombin cleavage site, was expressed from a pET46 Ek/Lic vector in BL21-CodonPlus(DE3)-RIL E. coli cells (Stratagene). The cells were induced with 0.5 mM IPTG at 16 °C overnight, then collected and lysed in 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 20 mM imidazole and 10% glycerol. Supernatant was loaded onto a HisTrapTM FF crude 5 mL column on an AKTAxpress HPLC system. The protein was eluted with a 20–500 mM imidazole gradient, dialyzed overnight against 20 mM Tris-HCl (pH 8.0) and 200 mM NaCl, and further purified on a Q Sepharose^{TM} Fast Flow anion exchange column (Amersham Biosciences). The flow-through fraction containing pure RORγ LBD protein was collected, and DTT was added to 10 mM immediately after calculating protein concentration by OD_{280}. For the binding assay, 2 nM Fluorescein-labeled 25-hydroxycholesterol was incubated with purified RORγ LBD protein for 2 h at room temperature. Protein concentration was varied by serial dilution in binding buffer (20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 10 mM DTT). Fluorescent polarization signals were measured with a Beacon 2000 (PanVera). The data were later converted to fluorescent anisotropy values, and the curve was fitted using the equation A=Af+(Ab-Af)*[Protein]/([Protein]+Kd), where Af and Ab are the anisotropy values of the free and bound fluorescein-labeled 25-hydroxycholesterol, respectively, and Kd is the dissociation constant. For competition assays, 109 nM RORγ LBD protein and 2 nM Fluorescein-labeled 25-hydroxycholesterol were added to a dilution series of individual chemical compounds. After 3h at room temperature, the data were recorded and IC_{50} was calculated by GraphPad Prism 5 software using the fitting equation Y=Bottom+(Top-

Bottom)/(1+10^(X-LogIC50)), where X is log molar concentration of the synthetic compound.

Circular dichroism experiments. 0.2 mg/mL purified RORγ-LBD protein samples in 20 mM Tris pH 8.0, 200 mM NaCl and 10 mM DTT were incubated with 60 μM digoxin or digoxin derivative compounds, and 20 μM 25-hydroxycholesterol, at 4 °C overnight. Samples were passed through a Sephadex G-25 column (GE Healthcare) for buffer exchange prior to circular dichroism (CD) experiments. The final buffer contained 20 mM sodium borate pH 8.5, 100 mM NaCl and 40 μM of each compound (5 μM for 25-hydroxycholesterol). Melting temperature CD was measured on an AVIV circular dichroism spectrometer (Model 420) using a 10–80 °C gradient with 2 °C jumps and 2 min equilibration time. CD signals were monitored at 225 nM and the collected data points were fitted to a curve to calculate the melting temperatures (Tm) using KaleidaGraph[10]. LBDs consist of twelve α-helices, and recording the helical content signal at ~222 nm while raising the temperature allows one to monitor the thermal unfolding transition of LBDs and determine the melting temperature (Tm), the midpoint of the transition at which 50% of the protein is unfolded. Previous studies have well demonstrated that occupancy of a lipophilic ligand within the hydrophobic pocket of an LBD is manifested in changes observed in the protein thermal stability, showing CD thermal denaturation assays to be a good method to assess the ligand binding[11-13]. The change in the Tm, when an LBD has a bound ligand, has been shown to increase by 2–6 °C for other nuclear receptors, including PPAR, ERR and Rev-Erb[11-13], consistent with our findings here for RORγ ligands tested.

ALPHA Screen assay. The hRORγ ligand binding domain was cloned downstream of the T7 promoter in the pET24a (Invitrogen) vector, along with an N-terminal 6×His tag followed by an *E. coli* GST tag and a thrombin cleavage site. The pET24a plasmid with the H6 GST RORγ LBD was transformed into BL21 (DE3) cells (Stratagene). An average size colony was inoculated into 500 mL LB with 100 μg/mL ampicillin and grown overnight at 37 °C. 160 mL of the above cells were added into 3×2-liter of LB with 100 μg/mL ampicillin and grown at 30 °C to an OD_{600} of 0.8–1.0 before induction of protein expression with 0.1mM (100 μM) of IPTG. Cells were then cultured overnight at 16 °C, centrifuged at 4000rpm for 20 min and resuspended in 400 ml GST buffer A (10 mM Tris, 100 mM NaCl, 10% glycerol at pH 8.0). 50 mg of lysozyme powder and 250ul of saturated PMSF (Phenylmethylsulphonyl fluoride) isopropanol solution was added to the cell suspension before 3 passages through a French press at 900Pa pressure. The lysate was centrifuged at 20,000 rpm for 30 min, and the supernatant was loaded onto a GST column. The column was washed 1x with 40mL GST Wash Buffer (0.1% Triton-X 100 in GST buffer A) and 1x with 200 mL GST Buffer A, before elution with a 300 ml gradient to buffer B (25 mM Tris, 100 mM NaCl, 20 mM Glutathione (GSH), 10% glycerol at pH 8.0). The eluted protein was further purified on a Nickel column. The preliminary binding efficiencies of the cofactor peptides to H6 GST hRORγ LBDs were determined by AlphaScreen® assay (PerkinElmer). Experiments were performed with 100 nM receptor LBD and 100 nM biotinylated co-activator or co-repressor motif peptides, in the presence of 5 μg/mL donor and acceptor beads in a buffer containing 50

mM MOPS, 50 mM NaF, 0.05 mM CHAPS, and 0.1 mg/mL bovine serum albumin, all adjusted to pH 7.4. The reaction mixture was equilibrated for 90 min and read using a Wallac 2140 EnVision^{TM} multilabel plate reader (384 well plate format). The photon count intensity is directly proportional to the binding efficiency of the protein to the peptide and thus the relative binding affinities of the peptides to the protein. The initial screen suggested that bSRC3-1b (GHKKLLQLLTS-COOH) synthetic peptide has the highest affinity for the apo protein. Co-activator displacement experiments, with bSRC3-1b peptide (100 nM), H6GST-RORγ (100 nM), and varying concentrations of digoxin, were performed in the presence of 1 μM 25-hydroxycholesterol. NCOR2 (ADPASNLGLEDIIRKALMGSFD-COOH) and SMRT2 (ASTNMGLEAIIRKALMGKYDQ) peptides were used as co-repressor peptides. Co-repressor recruitment experiments performed in the absence of exogenous 25-hydroxycholesterol.

Random mutagenesis. The ligand binding domain of RORγt in G4DBD-RORγt was PCR amplified (primers: tgccccagaggtaccatatgc and tcactttgacagcccctcagg) in error prone conditions using the GeneMorph® II EZClone Domain Mutagenesis kit (Stratagene), and products were individually subcloned into the pMT vector. Their transcriptional activities in the presence or absence of 15 μM digoxin were compared, and clones exhibiting ≥ 20-fold decreases in digoxin inhibition, compared to wild-type RORγ, were selected for full sequencing and further analysis.

RORγ antibody generation. The region corresponding to amino acids 110–212 of the RORγ linker region was cloned into pGex-4T1 (Amersham Biosciences) to generate a GST fusion. The RORγ(110-212)-GST fusion was produced in Rosetta-gami B(DE3)pLysS bacterial cells (Novagen, Madison WI), and purified by binding to Glutathione Sepharose 4B beads (GE) overnight at 4 °C. Beads were washed 3 times with PBS + 1% Triton X-100, and RORγ(110-212)-GST was eluted with 40 mM glutathione, 200 mM NaCl, pH 8.5. Polyclonal antibody was raised in rabbits using the antibody production service at Covance (Denver, PA). Antibody was purified from serum using the NAB Protein A spin kit (Pierce).

Chromatin immuno-precipitation (ChIP). Th17 polarized cells were crosslinked with 1% formaldehyde for 10 min at RT. Crosslinking was stopped by adding glycine to 0.125 M, and samples were washed once in cold PBS. Nuclei were prepared by re-suspending cells in lysis buffer (5 mM PIPES pH 8, 85 mM KCl, 0.5% NP-40, Complete Protease Inhibitor (Roche)) and gently rocking at 4 °C for 10 min. Nuclei were pelleted, and resuspended in RIPA buffer (1x PBS, 1% NP-40, 0.5% Na-Deoxycholate, 0.1% SDS, Complete Protease Inhibitor). Nuclear lysates were sonicated using a VibraCell VCX 130PB (Soncis and Materials, Inc; CT USA) to obtain chromatin fragments ranging from 100–400 bp and samples were spun at 14000 rpm for 10 min to pellet debris. 1% of each sample was set aside to normalize input. For IP, sheared chromatin (10^6 cell equivalents) was incubated with 50 µl of protein G Dynabeads (Dynal, Invitrogen), which were pre-bound with 5 µg of anti-RORγ or rabbit IgG, in 500 µl RIPA buffer, at 4 °C overnight. Beads were washed 5x with LiCl wash buffer (100 mM Tris-HCl pH 7.5, 500 mM LiCl,

1% NP-40, 1% Na-Deoxycholate), and once with TE. Immunoprecipitated chromatin was subsequently eluted with 200 μl elution buffer (1% SDS, 0.1 M NaHCO3) at 65 °C for 1 h. Eluted ChIP samples and input were transferred to new tubes and protein-DNA crosslinks were reversed by overnight incubation at 65 °C. Samples were treated with RNase, followed by Proteinase K, and DNA was purified by phenol/chlorophorm extraction and EtOH precipitation. ChIP'ed and input DNA were analyzed by quantitative real time PCR using the BioRad iCycler and the Roche LightCycler 480II. Locus specific primers were *il17a* pro (GGTGGTTCTGTGCTGACCTCATTT and TGTGAGGTGGATGAAGAGTAGTGC), *il17a* CNS2 (TTGCATGCGCCTCCTAACACATAG and TTTCTAGGTGGGTTCCTCACTGGT), *il17a* 3'UTR (AGCAAGGAATGTGGATTCAGAGGC and ACAAACACGAAGCAGTTTGGGACC), *il17f* pro (CCTGGGTATGTCAAACAGCAGTAG and GAGTAGAAACCCTGCATTCGTCAG), *il23r* pro (GCAAATGGAAATGTCAGCAGAGCC and GCAGCTCACTTTCAGTAATCTGGG), *il23r* int3 (ATATCCTGTATCAAAGCGGCCCAG and CTGGGTTCTTGGCAAACTTCCTTC), and *il23r* int8 (TCCAGATTGCCTGACTGTATACCC and GAAGATGCACTTCTAGAAACCCGC). Enrichement was calculated as percent of input for each genomic region.

Immunoblot and histochemistry. For immunoblot analysis, wild-type or *RORγt*$^{gfp/gfp}$ knock-out T cells cultured in Th17 conditions were lysed with cell lysis buffer (20 mM HEPES-KOH/7.6, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 1x

protease inhibitor (Roche) and 1 mM DTT). 70 μg protein was used for the immunoblot analysis with 1:2,000 rabbit anti-RORγt (M. Ciofani and D.R. Littman, unpublished), 1:100 anti-Batf (Santa Cruz), 1:1,000 anti-IRF4 (Santa Cruz), and 1:1,000 anti-tubulin (The Developmental Studies Hybridoma Bank). For the immunostaining, T cells were plated on 8-well glass slides (Lab-Tek II Chamber Slide System) and fixed for 15 min in 2% paraformaldehyde in PBS. Cells were blocked and permeabilized in PBSt (5% BSA (Sigma) in PBS containing 0.3% Triton X-100) for 30 minutes at room temperature. Cells were then incubated for 12–16 h at 4 ^{o}C with 1:50 rabbit anti-RORγt. After three washes in PBSt, the cells were incubated for 1 h at room temperature with Cy5-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch Laboratory) (1:500 in PBSt). After 3 washes in PBSt, samples were mounted in Vectashield mounting medium supplemented with DAPI (Vector lab). Samples were examined with a Zeiss ZMD510 microscope with a CCD camera, and images were processed with Zeiss LSM Image Browser 4.0 and Adobe Photoshop 7.0.

1. Stehlin, C. *et al.* X-ray structure of the orphan nuclear receptor RORbeta ligand-binding domain in the active conformation. *Embo J* **20**, 5822-5831 (2001).
2. Eberl, G. *et al.* An essential function for the nuclear receptor RORgamma(t) in the generation of fetal lymphoid tissue inducer cells. *Nat Immunol* **5**, 64-73 (2004).
3. Iwaki, T., Figuera, M., Ploplis, V.A. & Castellino, F.J. in BioTechniques, Vol. 35 482-484, 4862003).
4. Awasthi, A. *et al.* Cutting edge: IL-23 receptor gfp reporter mice reveal distinct populations of IL-17-producing cells. *J Immunol* **182**, 5904-5908 (2009).
5. Silberkang, M., Havel, C.M., Friend, D.S., McCarthy, B.J. & Watson, J.A. Isoprene synthesis in isolated embryonic Drosophila cells. I. Sterol-deficient eukaryotic cells. *J Biol Chem* **258**, 8503-8511 (1983).
6. Ghoreschi, K. *et al.* in Nature, Vol. 467 967-9712010).

7. Manel, N., Unutmaz, D. & Littman, D.R. 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 (2008).
8. Zhou, L. *et al.* IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat Immunol* **8**, 967-974 (2007).
9. Wensky, A.K. *et al.* IFN-gamma determines distinct clinical outcomes in autoimmune encephalomyelitis. *J Immunol* **174**, 1416-1423 (2005).
10. Greenfield, N.J. Using circular dichroism collected as a function of temperature to determine the thermodynamics of protein unfolding and binding interactions. *Nat Protoc* **1**, 2527-2535 (2006).
11. Wang, L. *et al.* X-ray crystal structures of the estrogen-related receptor-gamma ligand binding domain in three functional states reveal the molecular basis of small molecule regulation. *J Biol Chem* **281**, 37773-37781 (2006).
12. Raghuram, S. *et al.* Identification of heme as the ligand for the orphan nuclear receptors REV-ERBalpha and REV-ERBbeta. *Nat Struct Mol Biol* **14**, 1207-1213 (2007).
13. Yu, C. *et al.* Binding analyses between Human PPARgamma-LBD and ligands. *Eur J Biochem* **271**, 386-397 (2004).