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Digoxin and its derivatives suppress Th17 cell differentiation by antagonizing ROR γ t activity

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CD4⁺ T helper lymphocytes that express interleukin-17 (Th17 cells) have critical roles in mouse models of autoimmunity, and there is mounting evidence that they also influence inflammatory processes in humans. Genome-wide association studies in humans have linked genes involved in Th17 cell differentiation and function with susceptibility to Crohn's disease, rheumatoid arthritis, and psoriasis¹⁻³. Thus, the pathway towards differentiation of Th17 cells and, perhaps, of related innate lymphoid cells with similar effector functions^{4, 5}, is an attractive target for therapeutic applications. Mouse and human Th17 cells are distinguished by expression of the retinoic acid receptor-related orphan nuclear receptor ROR γ t, which is required for induction of IL-17 transcription and for the manifestation of Th17-dependent autoimmune disease in mice⁶. By performing a chemical screen with an insect cell-based reporter system, we identified the cardiac glycoside digoxin as a specific inhibitor of ROR γ t transcriptional activity. Digoxin inhibited murine Th17 cell differentiation without affecting differentiation of other T cell lineages and was effective in delaying the onset and reducing the severity of autoimmune disease in mice. At high concentrations, digoxin is toxic for human cells, but non-toxic synthetic derivatives, 20,22-

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Full methods are included within the Supplementary information.

Author Contributions J.R.H., J.J.L., H.E.X., D.Y.G., F.R., and D.R.L. designed the experiments. J.R.H. and D.R.L. wrote the manuscript with input from the co-authors. J.R.H. developed the screen and executed it with assistance from J.C. and A.C.. F.R.S. developed the serum-free system for S2 cell culture. M.C. performed the ChIP experiments, J.R.H., N.M., and S.V.K. performed the T cell culture experiments, and J.R.H. and M.W.L.L. did *in vivo* compound tests and the follow-up analyses. P.H. did *in vitro* competition and CD assays, R.M. performed ALPHA screen assays, and D.A.R. and M.R.K. synthesized and purified digoxin derivatives.

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dihydrodigoxin-21,23-diol (Dig(dhd)) and digoxin-21-salicylidene (Dig(sal)), specifically inhibited induction of IL-17 in human CD4⁺ T cells. Using these small molecule compounds, we demonstrate that ROR γ t is important for the maintenance of IL-17 expression in mouse and human effector T cells. These data suggest that derivatives of digoxin can be used as chemical probes for development of ROR γ t-targeted therapeutic agents that attenuate inflammatory lymphocyte function and autoimmune disease.

To identify small molecules that specifically inhibit transcriptional activity of ROR γ and ROR γ t isoforms, we prepared *Drosophila* S2 cells stably expressing fusions of the GAL4 DNA binding domain (DBD) and the ligand binding domains (LBDs) of murine ROR γ , ROR α (mouse homolog of ROR γ), and DHR3 (*Drosophila* orthologue for ROR family proteins), as well as the activation domain of the general transcriptional activator VP16. Induction of ROR γ and the other fusion proteins led to robust expression of a firefly luciferase reporter (Supplementary Fig. 1a). Next, we investigated whether ROR γ activity in the *Drosophila* system is dependent on a functional LBD and is ligand-dependent. A single amino acid change in the putative ligand binding pocket⁷ of ROR γ completely abrogated its function as a transcriptional activator despite comparable level of protein expression both in S2 cells and in transgenic fly models (Supplementary Fig. 1b and c). In addition, *Drosophila* cells grown in serum-free media completely lacked ROR γ activity, unless serum or cholesterol metabolites were supplemented into the cell culture (Supplementary Fig. 1d), suggesting that yet-to-be-identified ligands are required for ROR γ reporter activity. These data justify utilization of the heterologous system to identify small molecules that modulate ROR γ activity.

We next performed a chemical screen with 4,812 compounds and identified digoxin as a specific inhibitor for ROR γ transcriptional activity (Fig. 1a). Digoxin inhibited ROR γ (Fig. 1b and Supplementary Fig. 2a) with an IC₅₀ (half-maximal inhibitory concentration) value of 1.98 μ M. Inhibition of ROR γ activity by digoxin was specific, as there was no effect on the transcriptional activity of VP16 or of the related nuclear hormone receptors ROR α and DHR3 (Fig. 1c). Digoxin did not inhibit the activity of other nuclear hormone receptors, including *C. elegans* Daf12, human androgen receptor, and LXR α (Supplementary Fig. 2b and c). Digitoxin and β -acetyldigoxin also selectively inhibited ROR γ (Supplementary Fig. 2d and e) with similar IC₅₀ values. Next, we examined if digoxin targets ROR γ directly. 25-Hydroxycholesterol has been shown to bind to the ROR γ LBD⁸, and conjugation of fluorescein to this surrogate ligand did not affect its ability to bind to the human ROR γ LBD (with a K_d of 109 nM). Addition of digoxin led to a dose-dependent decrease in fluorescence polarization values, demonstrating that digoxin can displace the sterol ligand with an IC₅₀ of 4.1 μ M (Fig. 1d). In addition, circular dichroism (CD) analysis showed that digoxin increased the thermal stability of the ROR γ -LBD, indicating that it interacts directly with ROR γ (Supplementary Fig. 3a)⁹. Digoxigenin, the aglycone of digoxin, did not inhibit ROR γ t activity in *Drosophila* cells and did not bind to the ROR γ t LBD in the CD and competition assays (data not shown and Supplementary Fig. 3b). We further investigated whether digoxin binds inside the ligand binding pocket of ROR γ . We performed random mutagenesis on the LBD and screened 200 clones to identify those that were resistant to digoxin-mediated inhibition. Two clones with this property were identified and shared mutation of amino acid 290 (L290P/A494T and L290F/C318S). ROR γ harboring mutations at all three residues (ROR γ / γ t(triple)) exhibited much less sensitivity to digoxin, in spite of transcriptional activity similar to that of the wild-type molecule (Supplementary Fig. 3c and d). Two of the mutations mapped to the ligand binding pocket (L290 and C318) and one to helix 11 (A494)⁸, consistent with digoxin binding inside the pocket.

When naïve mouse CD4⁺ T cells were cultured under Th17 polarizing conditions (IL-6 and TGF- β), treatment with digoxin led to markedly reduced expression of IL-17a protein (Fig.

2a). Transcriptional up-regulation of genes encoding IL-23 receptor (IL-23R), IL-17a, IL-17f, or IL-22, was also strongly inhibited (Supplementary Fig. 4a and b). Expression of ROR γ t-independent Th17 signature genes, such as IL-21, cMaf, ROR α , Batf, and IRF4, was not affected by digoxin (Supplementary Fig. 4c and d). Reduction of Th17 cell differentiation following treatment of wild-type cells with digoxin was similar to that observed upon targeted inactivation of *Rorc(t)* (Fig. 2a). IL-23– induced Th17 cell differentiation¹⁰ was also inhibited in the presence of digoxin (Supplementary Fig. 4e). Importantly, digoxin had no effect on differentiation of naïve CD4⁺ T cells into other lineages, including Th1, Th2, and regulatory T cells (Supplementary Fig. 4f and g). Other cardiac glycosides with structures related to digoxin, including proscillaridin A, deslanoside, erysimoside, oleandrin, ouabain, ouabagenin, digitoxigenin, digoxigenin, and lanatoside C, had no significant effect on ROR γ transcriptional activity or Th17 cell differentiation (Supplementary Fig. 5a and b).

To investigate if ROR γ t is the major target of digoxin or if another dominant cellular target exists, we performed gene expression profiling with total RNA samples isolated from DMSO- or digoxin-treated wild-type or ROR γ t-deficient cells cultured in Th17 conditions. Treatment with digoxin resulted in changes in gene expression that were very similar to those observed in ROR γ t-deficient cells: 2-way ANOVA analysis of differential gene expression revealed 67 genes that were significantly affected by the compound (DMSO vs. digoxin) irrespective of genotype ($p < 0.05$) (Fig. 2b) and 323 that were affected by the genotype (WT vs. KO) irrespective of compound treatment ($p < 0.05$) (Supplementary Fig. 6a). 94% of genes affected by digoxin treatment were similarly affected by ROR γ t deficiency. Importantly, no genes were significantly affected by the combination of gene inactivation and digoxin treatment. These results indicate that the effects of digoxin are predominantly mediated through ROR γ t. Induction of ROR γ t mRNA and protein expression was not affected by digoxin (Supplementary Fig. 4a and d). To rule out the possibility that digoxin blocks steps downstream of ROR γ t activity during Th17 cell differentiation (e.g. IL-17a production), we examined its effect upon ectopic expression of ROR γ t or ROR α in naïve CD4⁺ T cells. Both nuclear receptors were previously shown to be sufficient to induce IL-17a expression¹¹, presumably by binding to the same cis-acting elements¹². Digoxin suppressed ROR γ - and ROR γ t-, but not ROR α -mediated induction of IL-17a (Fig. 2c and Supplementary Fig. 6b), confirming that it acts selectively on ROR γ t in mouse T cells. However, digoxin (10 μ M) failed to inhibit ROR γ t(triple) mutant- mediated IL-17a production (Supplementary Fig. 3e). Digitoxin and β -acetyldigoxin also selectively inhibited ROR γ t-dependent Th17 cell differentiation (Supplementary Fig. 6c). The aryl hydrocarbon receptor (AHR) is another ligand-dependent transcription factor that augments Th17 responses¹³. Its activity was unaffected by digoxin, as addition of the AHR ligand FICZ increased ROR α -dependent IL-17a expression even in the presence of digoxin (Supplementary Fig. 6d). ROR γ t is predominantly found in the nucleus of Th17 cells¹⁴. Digoxin treatment did not inhibit its nuclear localization in *Drosophila* cells or in *in vitro* differentiated Th17 cells (Supplementary Fig. 7a and b). These data raise the question of how digoxin suppresses ROR γ t transcriptional activity. Chromatin immunoprecipitation-sequencing (ChIP-Seq) analysis with an anti-ROR γ t antibody (Supplementary Fig. 7c) was used for genome-wide identification of its transcriptional target sites in Th17 cells (M. Ciofani and D.R.L., unpublished results). We evaluated the effect of digoxin on binding of ROR γ t to sites in two relevant loci, *Il17a/f* and *Il23r*. ROR γ t binding to these sites was substantially reduced upon treatment with digoxin (Supplementary Fig. 7d), demonstrating one mode of its activity. *In vitro*, digoxin not only reduced the binding of ROR γ t onto its target, but also displaced SRC3-1b co-activator peptides (IC₅₀ of 1.8 μ M) from the ROR γ t LBD and facilitated its interaction with co-repressor NCOR2 peptides (IC₅₀ of 3.9 μ M) (Supplementary Fig. 7e, 8a, and b).

We next examined if digoxin can exert an anti-inflammatory effect in mice. We induced experimental autoimmune encephalomyelitis (EAE), a Th17-mediated autoimmune inflammatory disease, in C57BL/6 wild-type mice^{15, 16} in conjunction with intra peritoneal (IP) injections of digoxin or carrier each day from day 2. Digoxin treatment not only delayed onset, but also reduced severity of EAE progression (Fig. 2d). Also, the total number of mononuclear cells infiltrating the spinal cord was markedly reduced in mice treated with digoxin (Supplementary Fig. 9). Importantly, the percentage of IL-17-producing T cells infiltrating the spinal cord in digoxin-treated mice was reduced by more than 50%, as compared to DMSO-treated mice, whereas that of IFN- γ -producing Th1 cells was approximately the same (Fig. 2e and f). Administration of digoxigenin had no effect on progression of EAE (data not shown), indicating that the cardiac glycoside activity¹⁷ has no role in the observed amelioration of disease.

Digoxin, an inhibitor of the Na⁺/K⁺-ATPase, has long been used for treatment of congestive heart failure^{17, 18}, but is toxic for human cells at concentrations (>300 nM)¹⁹ well below those required for ROR γ t inhibition. Expression of the catalytic α 1 subunit of murine Na⁺/K⁺-ATPase, which binds digoxin poorly, rendered human cells much less sensitive to digoxin-mediated cytotoxicity^{20, 21}. Thus, we ectopically expressed in human cord blood CD4⁺ T cells the α 1 subunit of murine Na⁺/K⁺-ATPase in the presence of cardiac glycosides. Lanatoside C (Supplementary Fig. 5a), which has inhibitory activity on Na⁺/K⁺-ATPase similar to digoxin¹⁷, but does not inhibit ROR γ t activity (data not shown), had no effect on IL-17a expression. However, digoxin suppressed IL-17a production (Supplementary Fig. 10a). Next, human T cells expressing the murine Na⁺/K⁺-ATPase were further transduced with lentivirus encoding human ROR α d, β , or γ t, all of which are sufficient to induce IL-17 expression²². Digoxin inhibited only ROR γ t-mediated induction of IL-17a (Supplementary Fig. 10b), demonstrating its direct and selective activity on human ROR γ t.

Cardiac glycosides of the cardenolide class have three common structural motifs, namely a central steroidal core fused with a butenolide and various sugars^{23, 24}. The glycans are dispensable, as digoxigenin still inhibits Na⁺/K⁺-ATPase¹⁷. 20,22-Dihydrodigoxin (Supplementary Fig. 2d), which was derived upon butenolide reduction of digoxin by the intestinal commensal *Eubacterium lentum*²⁵, has weak cardiac glycoside activity with much lower binding affinity than that of digoxin for Na⁺/K⁺-ATPase^{17, 26}, yet it inhibited ROR γ activity in the S2 reporter system (data not shown). Since 20,22-dihydrodigoxin was still cytotoxic for human cells at 2.5 μ M, digoxin was further modified by complete reduction of the butenolide to generate Dig(dhd) (Fig. 3a). Dig(dhd) lacked cytotoxic activity on human cells at concentrations up to 40 μ M, but it still possessed ROR γ inhibitory activity and displaced the sterol ligand from the ROR γ LBD (IC₅₀ of 12 μ M) (Supplementary Fig. 11a). Additional derivatization of digoxin was achieved by aldol condensation of the butenolide with salicylaldehyde to produce Dig(sal) (Fig. 3a), which, similarly to digoxin, bound directly to ROR γ in the CD assay (Supplementary Fig. 3a). These compounds selectively inhibited both mouse and human ROR γ t activities without affecting those of mouse ROR α and human LXR β (Supplementary Fig. 11b, c, and d). Moreover, Dig(sal) treatment reduced severity of EAE progression (Supplementary Fig. 11e). When tested on human CD4⁺ T cells transduced with viruses encoding ROR α d or ROR γ t, Dig(dhd) or Dig(sal) treatment selectively suppressed ROR γ t-mediated IL-17a induction (Supplementary Fig. 11f). Intriguingly, addition of either compound blocked Th17 cell differentiation²² (Fig. 3b) and induced reciprocal increases of IFN- γ or FoxP3 expression in T cells (Fig. 3b and Supplementary Fig. 11g), suggesting that functional ROR γ t or its downstream events may normally suppress development into other T cell lineages. Expression of another human Th17 cell-associated surface marker, CCR6, was also reduced in Dig(dhd) treated cells (Supplementary Fig. 11h).

We next investigated if digoxin can inhibit IL-17 production from pre-differentiated Th17 cells. *In vitro* digoxin treatment of expanded mouse Th17 cells derived from immunized mice inhibited both IL-23R (Fig. 4a) and IL-17a expression without affecting IFN- γ expression (Fig. 4b). We also purified GFP-positive Th17 cells from MOG-immunized *Il23r^{gfp/+}* mice²⁷ after 4-day *in vitro* culture with IL-23 and MOG peptide. More than 70% of the sorted GFP-positive cells expressed IL-17a (Supplementary Fig. 12a, day 0). GFP-positive cells were then treated with DMSO or digoxin for an additional 3 days. Digoxin treatment reduced IL-17a-expressing cells by more than 70% (Supplementary Fig. 12a, day 3), confirming that mouse Th17 cells generated *in vivo* and expanded *in vitro* require continuous ROR γ t activity to maintain their identity. To test if digoxin suppresses the activity of pre-existing Th17 cells *in vivo*, we transferred IFN- γ -deficient, MBP-specific Th17 cells into lymphopenic RAG-2-deficient mice and assessed EAE manifestation following daily administration of digoxin. Since the transferred cells lack IFN- γ , the EAE phenotype observed in these mice is entirely attributed to the function of Th17 cells. Digoxin treatment from day 2 delayed onset and reduced severity of Th17 cell transfer-mediated EAE, which further confirms a requirement for continuous ROR γ t activity in Th17 cells (Supplementary Fig. 12b). We then examined if ROR γ t activity is also important for sustained expression of IL-17a in human CD4⁺ T cells. Human memory Th17 cells were purified from peripheral blood samples and enriched by *in vitro* culture. Naïve CD4⁺ T cells cultured in the same cytokine conditions did not produce IL-17a (Fig. 4c, left plot). Dig(dhd) treatment led to 40–50% reduction of IL-17a-expressing cells with little effect on IFN- γ -expressing cells (Fig. 4c and d). These data demonstrate that human ROR γ t activity plays an important role in maintaining the human Th17 cell population.

T cells and innate lymphocytes that produce IL-17a and IL-22 are recognized as having key roles in maintenance of barrier function at mucosal surfaces and also in the pathophysiology of autoimmune disease. All such cells, which include Th17 cells, other TCR $\alpha\beta$ cells, TCR $\gamma\delta$ cells, lymphoid tissue inducer cells, and NK-like cells (also referred to as NK22 cells) share in the property of requiring expression of ROR γ t for their differentiation. Abrogation of ROR γ t expression results in marked reduction or complete depletion of these cell types and in resistance to Th17-mediated autoimmune disease in mouse models^{4, 6, 28}. Therefore, ROR γ t antagonists digoxin, Dig(dhd), and Dig(sal) may serve as good chemical templates for the development of potent therapeutic compounds to treat various diseases associated with inflammatory lymphocyte dysfunction.

The digitalis-like compounds were originally identified in plants. A body of evidence indicates the presence of endogenous digitalis-like compounds in mammals including humans^{18, 29}. Identification of digoxin as a ROR γ t antagonist suggests that related molecules in mammals may modulate ROR γ - and ROR γ t-mediated functions. However, it would be derivative compounds with better IC₅₀ values that would have such roles. In light of recent findings of the roles of microbiota in generation of Th17 cells in the small intestine³⁰, it is interesting that *Eubacterium lentum*, another commensal bacterium, has the capacity to metabolize digoxin into dihydrodigoxin. The possibility of the existence of endogenous digitalis-like compounds in host organisms and of their modification by microbes may present additional opportunities for modulating the function of ROR γ t and Th17 cell differentiation.

Methods Summary

Chemical screen

10,000 *Drosophila* stable S2 cells with genomic integration of the Cu²⁺-inducible G4DBD-mouse ROR γ construct were transfected with 5 ng of pUAST-firefly luciferase and 7 ng of Pol III-Renilla luciferase and dispensed into white bottom-tissue culture 384-well plates

(Corning). Two days later, small compounds (total 4,812 compounds from the ICCB chemical libraries, including Bioactives and Prestwick collections) were added and, after 6 hours, Cu^{2+} was added to the wells (700 μM). The following morning, Stop-glo luciferase substrates (Promega) were used to measure luciferase activity. Initial hits including digoxin were tested against three different control S2 reporter cell lines.

Cell culture

Mouse and human CD4^+ T cell culture and viral transduction were performed as described previously^{6, 22}, unless indicated otherwise in the text.

Identification of non-toxic digoxin derivatives

Various digoxin derivatives were synthesized and first tested for toxicity on human embryonic kidney 293T cells at various concentrations. Compounds exhibiting reduced toxicity compared to digoxin were further tested for their ROR γ inhibitory activities with the insect cell reporter lines.

General

All DNA constructs were generated by PCR-based methodology and confirmed by sequencing. Retroviral production and transduction, EAE experiments, and gene chip analysis were performed as described previously⁶. IL-17a, IFN- γ , IL-4, Foxp3, and CCR6 protein expression was examined by intracellular or surface staining according to the manufacturer's protocol.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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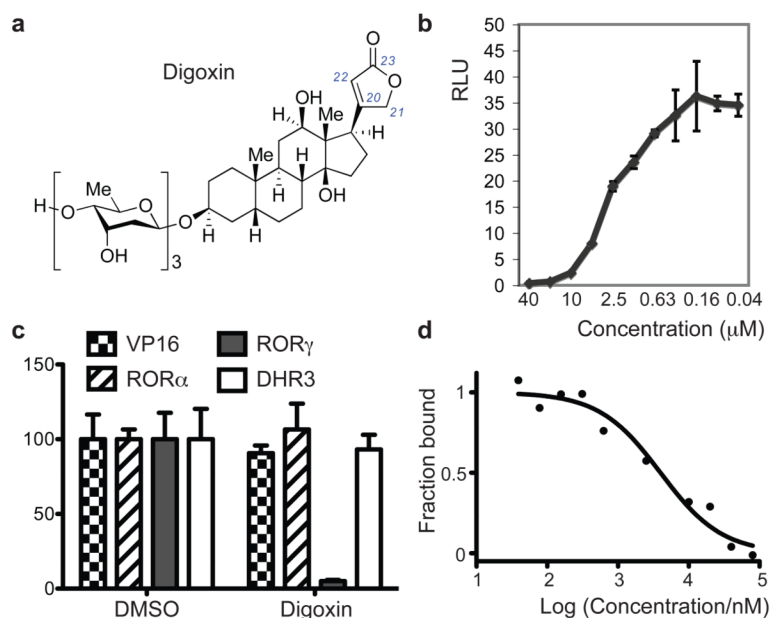


Figure 1). Digoxin binds to ROR γ and inhibits its transcriptional activity

a, Chemical structure of digoxin. **b**, Digoxin demonstrates dose-dependent inhibition of ROR γ transcriptional activity in the *Drosophila* S2 cell luciferase reporter system. Ratio of firefly to Renilla luciferase activity is shown as relative luciferase unit (RLU) on the y-axis. **c**, Digoxin (10 μ M) selectively inhibits ROR γ dependent transcriptional activity without affecting that of ROR α , DHR3, or VP16. Percentages of relative luciferase units compared to DMSO-treated reporter cells are shown on the y-axis. Error bars indicate standard deviation. **d**, *In vitro* competition assay. Recombinant human ROR γ LBD was loaded with fluorescently-labeled 25-hydroxycholesterol in the presence of the indicated concentrations of digoxin, and fluorescence polarization was measured.

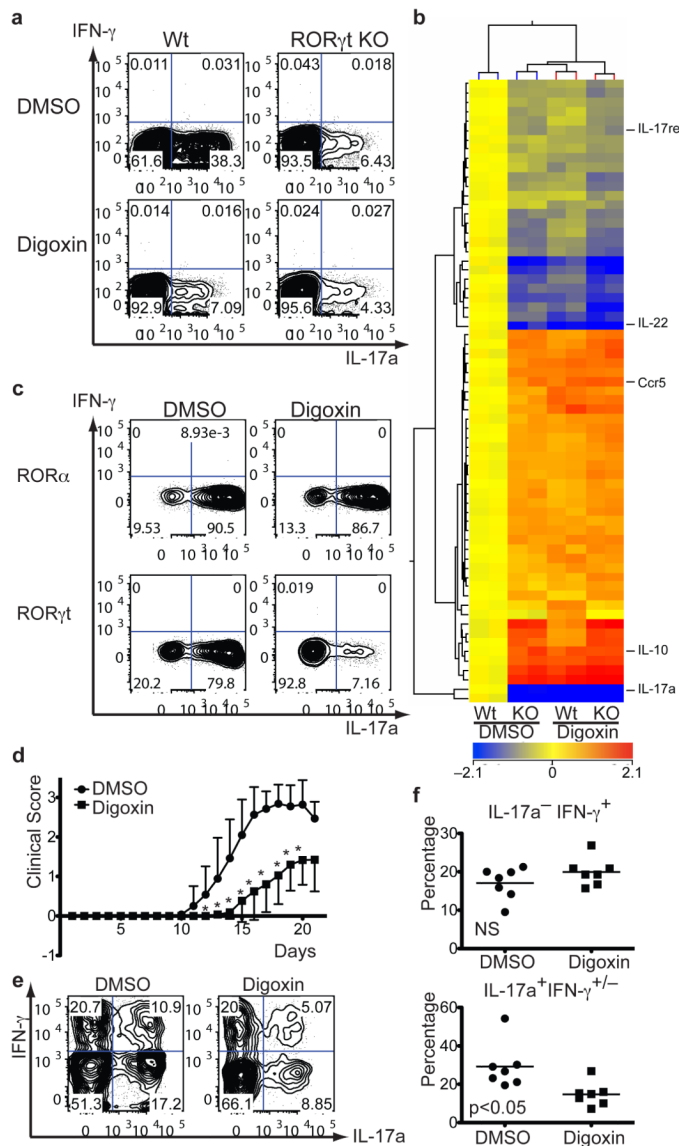


Figure 2). Digoxin inhibits mouse Th17 cell differentiation and ameliorates Th17-mediated autoimmune disease

a, Flow cytometry of intracellular staining for IL-17a and IFN- γ in sorted naïve T cell populations (from *ROR γ t^{fl/fl}* mice following transduction with control-IRES-GFP (WT) or CRE-IRES-GFP (KO) retrovirus) activated and expanded in the presence of mouse Th17 polarizing cytokines. DMSO or 10 μ M digoxin was added at 6 hours after viral transduction on day 1 and GFP expressing cells were gated for analysis on day 5. **b**, Two-dimensional hierarchical clustering of the 67 genes (including redundant probe sets and genes of unknown function) identified to be significantly affected by 2-way ANOVA analysis (DMSO versus digoxin treatment, $p < 0.05$). Each row corresponds to a gene and each column corresponds to an experimental sample. **c**, FACS-sorted naïve T cells were transduced with retroviral vectors encoding murine ROR α -IRES-GFP or ROR γ t-IRES-GFP on Day 1 (16 h after TCR stimulation) and GFP expressing cells were gated for analysis on Day 5. DMSO or 10 μ M digoxin was added 6–8 h after transduction. **d**, EAE disease course in B6 wild-type mice that were IP injected with either DMSO or digoxin (40 μ g/mouse) every day starting from day 2 after disease induction with MOG(35-55)/CFA. Shown is averaged

curve shape from seven experiments (10 or 20 mice were used per trial). * indicates statistical significance ($p < 0.05$). Error bars represent standard deviation. **e, f**, Th1 and Th17 cells in spinal cord of EAE mice treated with DMSO or digoxin. Lymphocytes were isolated on day 21 after disease induction. The cells were stimulated for 4h with PMA/Ionomycin and stained for surface markers and intracellular cytokines. Representative FACS plots (gated on $CD45^+CD11b^-CD4^+$ cells) from mice from each group are shown (top). T cells isolated from spinal cords of DMSO ($n=7$) or digoxin treated mice ($n=7$) were stained intracellularly for IFN- γ or IL-17a. Statistical analysis was by a two-tailed unpaired Student's t test; NS, not significant and $p=0.014$ (bottom).

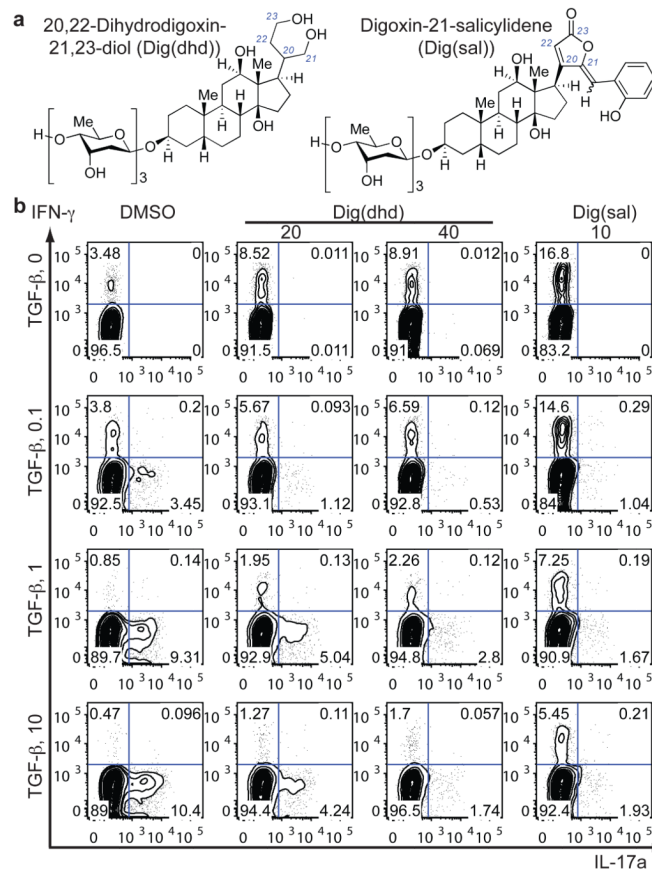


Figure 3). 20,22-Dihydrodigoxin-21,23-diol and digoxin-25-salicylidene inhibit human Th17 cell differentiation

a. Chemical structures of 20,22-dihydrodigoxin-21,23-diol and digoxin-21-salicylidene. **b.** Flow cytometry of the production of IL-17a and IFN- γ by human naïve cord blood T cells cultured for six days in the presence of IL-2, IL-23, and IL-1 β , with various concentrations of TGF- β . DMSO, Dig(dhd), or Dig(sal) at indicated concentrations (μ M) was added 16h after cytokine addition.

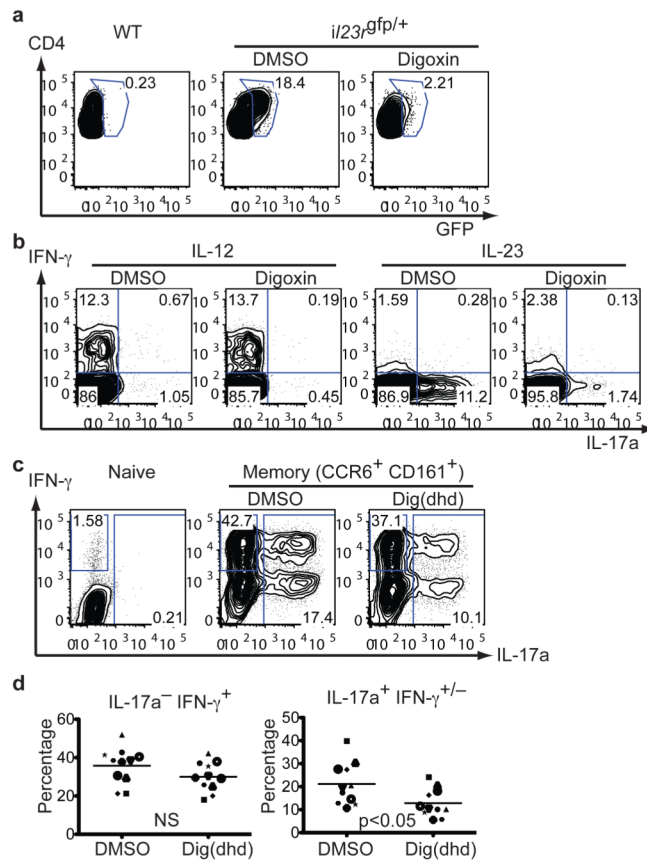


Figure 4). ROR γ t activity is important for maintenance of mouse and human Th17 cells
a, b, Flow cytometry of intracellular staining for IL-17a and IFN- γ by CD4⁺ T cells. Mononuclear cells were collected from draining lymph nodes of wild-type or IL-23R-GFP knock-in heterozygous mice 7 days after MOG(35-55)/CFA injection. Cells were cultured for four more days with MOG(35-55) peptide and exogenous IL-23 or IL-12, in the presence of DMSO or 10 μ M digoxin. Without pre-immunization, addition of IL-23 and MOG(35-55) peptide to culture did not lead to *de novo* Th17 cell differentiation (data not shown). Digoxin treatment suppressed expansion of *in vivo* differentiated Th17 cells, assayed by IL-23R reporter GFP expression (a) or by IL-17a production (b). **c, d**, Human naïve (CD45RA⁺CD3⁺CD4⁺) or memory (CD45RO⁺CD45RA⁻CD3⁺CD4⁺CCR6⁺CD161⁺) cells were purified from healthy donor peripheral blood samples and were cultured in the presence of IL-1 β , IL-23 and IL-2 for 6 days with or without 40 μ M Dig(dhd). Intracellular staining for IFN- γ or IL-17a in memory CD4⁺ T cells from multiple donors (n=11) in the presence of IL-1 β , IL-23, and IL-2, assessed on day 6. **c**, Representative FACS plots from one donor are shown. **d**, Each symbol indicates a separate donor. Statistical analysis was by a two-tailed unpaired Student's *t* test; IL-17a⁻ IFN- γ ⁺, not significant and IL-17a⁺IFN- γ ^{+/-}, p=0.02.