LETTER

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Digoxin and its derivatives suppress T_H17 cell differentiation by antagonizing RORγt activity

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$CD4^+$ T helper lymphocytes that express interleukin-17 (T_H17 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 T_H17 cell differentiation and function with susceptibility to Crohn's disease, rheumatoid arthritis and psoriasis[1–3]. Thus, the pathway towards differentiation of T_H17 cells and, perhaps, of related innate lymphoid cells with similar effector functions[4,5], is an attractive target for therapeutic applications. Mouse and human T_H17 cells are distinguished by expression of the retinoic acid receptor-related orphan nuclear receptor RORγt, which is required for induction of IL-17 transcription and for the manifestation of T_H17-dependent autoimmune disease in mice[6]. By performing a chemical screen with an insect cell-based reporter system, we identified the cardiac glycoside digoxin as a specific inhibitor of RORγt transcriptional activity. Digoxin inhibited murine T_H17 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-dihydrodigoxin-21,23-diol and digoxin-21-salicylidene specifically inhibited induction of IL-17 in human $CD4^+$ T cells. Using these small-molecule compounds, we demonstrate that RORγt is important for the maintenance of IL-17 expression in mouse and human effector T cells. These data indicate that derivatives of digoxin can be used as chemical templates for the development of RORγt-targeted therapeutic agents that attenuate inflammatory lymphocyte function and autoimmune disease.

To identify small molecules that specifically inhibit transcriptional activity of RORγ and RORγt isoforms, we prepared *Drosophila* S2 cells stably expressing fusions of the GAL4 DNA-binding domain (DBD) and the ligand-binding domains (LBDs) of murine RORγ, RORα (mouse homologue of RORγ) and DHR3 (*Drosophila* orthologue for ROR family proteins), as well as the activation domain of the general transcriptional activator VP16. Induction of RORγ and the other fusion proteins led to robust expression of a firefly luciferase reporter (Supplementary Fig. 1a). Next, we investigated whether RORγ 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 pocket[7] of RORγ completely abrogated its function as a transcriptional activator despite a comparable level of protein expression both in S2 cells and in transgenic fly models (Supplementary Fig. 1b, c). In addition, *Drosophila* cells grown in serum-free media completely lacked RORγ activity, unless serum or cholesterol metabolites were supplemented into the cell culture (Supplementary Fig. 1d), indicating that yet-to-be-identified ligands are required for RORγ reporter activity. These data justify utilization of the heterologous system to identify small molecules that modulate RORγ activity.

We next performed a chemical screen with 4,812 compounds and identified digoxin as a specific inhibitor for RORγ transcriptional activity (Fig. 1a). Digoxin inhibited RORγ (Fig. 1b and Supplementary Fig. 2a) with a half-maximum inhibitory concentration (IC_{50}) value of 1.98 μM. Inhibition of RORγ activity by digoxin was specific, as there was no effect on the transcriptional activity of VP16 or of the related nuclear hormone receptors RORα and DHR3 (Fig. 1c). Digoxin did not inhibit the activity of other nuclear hormone receptors, including *Caenorhabditis elegans* DAF-12, human androgen receptor and LXRα (Supplementary Fig. 2b, c). Digitoxin and β-acetyldigoxin also

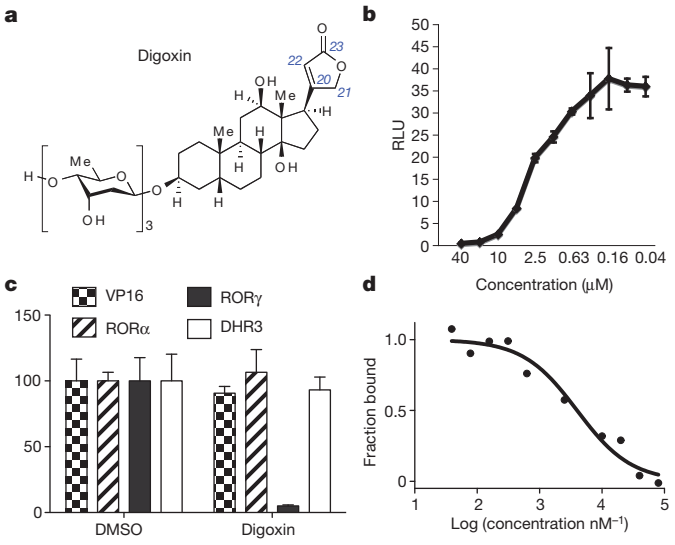

Figure 1 | Digoxin binds to RORγ and inhibits its transcriptional activity. **a**, Chemical structure of digoxin. **b**, Digoxin demonstrates dose-dependent inhibition of RORγ transcriptional activity in the *Drosophila* S2 cell luciferase reporter system. Ratio of firefly to *Renilla* luciferase activity is shown as relative luciferase unit (RLU) on the *y*-axis. **c**, Digoxin (10 μM) selectively inhibits RORγ-dependent transcriptional activity without affecting that of RORα, 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 RORγ LBD was loaded with fluorescently-labelled 25-hydroxycholesterol in the presence of the indicated concentrations of digoxin, and fluorescence polarization was measured.

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selectively inhibited RORγ (Supplementary Fig. 2d, e) with similar IC_{50} values. Next, we examined whether digoxin targets RORγ directly. 25-Hydroxycholesterol has been shown to bind to the RORγ LBD[8], and conjugation of fluorescein to this surrogate ligand did not affect its ability to bind to the human RORγ LBD (with a dissociation constant (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 IC_{50} of 4.1 μM (Fig. 1d). In addition, circular dichroism analysis showed that digoxin increased the thermal stability of the RORγ LBD, indicating that it interacts directly with RORγ (Supplementary Fig. 3a)[9]. Digoxigenin, the aglycone of digoxin, did not inhibit RORγt activity in *Drosophila* cells and did not bind to the RORγt LBD in the circular dichroism and competition assays (data not shown and Supplementary Fig. 3b). We further investigated whether digoxin binds inside the ligand-binding pocket of RORγ. 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). RORγ harbouring mutations at all three residues (RORγ/γt(triple)) exhibited much less sensitivity to digoxin, in spite of transcriptional activity similar to that of the wild-type molecule (Supplementary Fig. 3c, 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 naive mouse $CD4^+$ T cells were cultured under T_H17 polarizing conditions (IL-6 and TGF-β), treatment with digoxin led to markedly reduced expression of IL-17a protein (Fig. 2a). Transcriptional upregulation of genes encoding the IL-23 receptor (IL-23R), IL-17a, IL-17f or IL-22 was also strongly inhibited (Supplementary Fig. 4a, b). Expression of RORγt-independent T_H17 signature genes, such as *Il21*, *Maf*, *Rora*, *Batf* and *Irf4*, was not affected by digoxin (Supplementary Fig. 4c, d). Reduction of T_H17 cell differentiation after treatment of wild-type cells with digoxin was similar to that observed upon targeted inactivation of *Rorc(γt)* (Fig. 2a). IL-23-induced T_H17 cell differentiation[10] was also inhibited in the presence of digoxin (Supplementary Fig. 4e). Importantly, digoxin had no effect on differentiation of naive $CD4^+$ T cells into other lineages, including T_H1, T_H2 and regulatory T cells (Supplementary Fig. 4f, 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 RORγ transcriptional activity or T_H17 cell differentiation (Supplementary Fig. 5a, b).

To investigate if RORγt is the major target of digoxin or if another dominant cellular target exists, we performed gene expression profiling with total RNA samples isolated from dimethylsulphoxide (DMSO)- or digoxin-treated wild-type or RORγt-deficient cells cultured in T_H17 conditions. Treatment with digoxin resulted in changes in gene expression that were very similar to those observed in RORγt-deficient cells: two-way ANOVA analysis of differential gene expression revealed 67 genes that were significantly affected by the compound (DMSO versus digoxin) irrespective of genotype ($P < 0.05$) (Fig. 2b) and 323 that were affected by the genotype (wild type versus knockout) irrespective of compound treatment ($P < 0.05$) (Supplementary Fig. 6a). Ninety-four per cent of genes affected by digoxin treatment were similarly affected by RORγt 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 RORγt. Induction of RORγt messenger RNA and protein expression was not affected by digoxin (Supplementary Fig. 4a, d). To rule out the possibility that digoxin

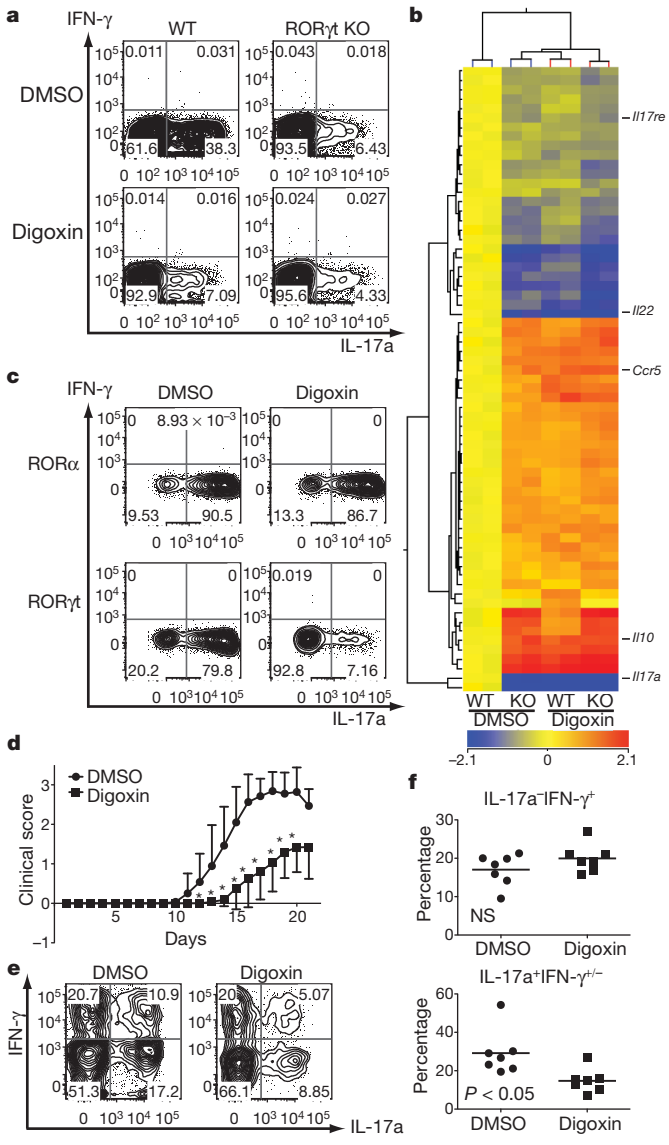

Figure 2 | Digoxin inhibits mouse T_H17 cell differentiation and ameliorates T_H17-mediated autoimmune disease. **a**, Flow cytometry of intracellular staining for IL-17a and IFN-γ in sorted naive T cell populations (from *Rorγt*$^{fl/fl}$ mice after transduction with control IRES–GFP (wild type (WT)) or CRE-IRES–GFP (knockout (KO)) retrovirus) activated and expanded in the presence of mouse T_H17 polarizing cytokines. DMSO or 10 μM digoxin was added at 6 h 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 two-way ANOVA analysis (DMSO versus digoxin treament, $P < 0.05$). Each row corresponds to a gene and each column corresponds to an experimental sample. **c**, FACS-sorted naive T cells were transduced with retroviral vectors encoding murine RORα–IRES–GFP or RORγt–IRES–GFP on day 1 (16 h after TCR stimulation) and GFP-expressing cells were gated for anyalsis on day 5. DMSO or 10 μM digoxin was added 6−8 h after transduction. **d**, EAE disease course in B6 wild-type mice that were injected i.p. with either DMSO or digoxin (40 μg per mouse) every day starting from day 2 after disease induction with myelin oligodendrocyte glycoprotein peptide 35–55 (MOG_{35-55}) in complete Freund's adjuvant (CFA). Shown is averaged curve shape from seven experiments (10 or 20 mice were used per trial). *$P < 0.05$. Error bars represent standard deviation. **e**, **f**, T_H1 and T_H17 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 4 h with phorbol myristate acetate (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 (**e**). 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 $P = 0.014$ (**f**).

blocks steps downstream of RORγt activity during T_H17 cell differentiation (for example, IL-17a production), we examined its effect on ectopic expression of RORγt or RORα in naive $CD4^+$ T cells. Both nuclear receptors were previously shown to be sufficient to induce IL-17a expression[11], presumably by binding to the same *cis*-acting elements[12]. Digoxin suppressed RORγ- and RORγt- but not RORα-mediated induction of IL-17a (Fig. 2c and Supplementary Fig. 6b), confirming that it acts selectively on RORγt in mouse T cells. However, digoxin (10 μM) failed to inhibit RORγt(triple)-mutant-mediated IL-17a production (Supplementary Fig. 3e). Digitoxin and β-acetyldigoxin also selectively inhibited RORγt-dependent T_H17 cell differentiation (Supplementary Fig. 6c). The aryl hydrocarbon receptor (AhR) is another ligand-dependent transcription factor that augments T_H17 responses[13]. Its activity was unaffected by digoxin, as addition of the AhR ligand 6-formylindolo[3,2-b]carbazole (FICZ) increased RORα-dependent IL-17a expression even in the presence of digoxin (Supplementary Fig. 6d). RORγt is predominantly found in the nucleus of T_H17 cells[14]. Digoxin treatment did not inhibit its nuclear localization in *Drosophila* cells or in *in vitro* differentiated T_H17 cells (Supplementary Fig. 7a, b). These data raise the question of how digoxin suppresses RORγt transcriptional activity. Chromatin immunoprecipitation-sequencing (ChIP-Seq) analysis with an anti-RORγt antibody (Supplementary Fig. 7c) was used for genome-wide identification of its transcriptional target sites in T_H17 cells (M. Ciofani and D.R.L., unpublished results). We evaluated the effect of digoxin on binding of RORγt to sites in two relevant loci, *Il17a/f* and *Il23r*. RORγt binding to these sites was substantially reduced on treatment with digoxin (Supplementary Fig. 7d), demonstrating one mode of its activity. *In vitro*, digoxin not only reduced the binding of RORγt onto its target, but also displaced SRC3-1b co-activator peptides (IC_{50} of 1.8 μM) from the RORγt LBD and facilitated its interaction with co-repressor NCOR2 peptides (IC_{50} of 3.9 μM) (Supplementary Figs 7e, 8a, b).

We next examined if digoxin can exert an anti-inflammatory effect in mice. We induced experimental autoimmune encephalomyelitis (EAE)—a T_H17-mediated autoimmune inflammatory disease—in C57BL/6 wild-type mice[15,16] in conjunction with intraperitoneal (i.p.) injections of digoxin or carrier each day from day 2. Digoxin treatment not only delayed the onset, but also reduced the 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 T_H1 cells was approximately the same (Fig. 2e, f). Administration of digoxigenin had no effect on the progression of EAE (data not shown), indicating that the cardiac glycoside activity[17] 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 RORγt inhibition. Expression of the catalytic α1 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 the αl subunit of murine Na^+/K^+-ATPase in human cord blood $CD4^+$ T cells in the presence of cardiac glycosides. Lanatoside C (Supplementary Fig. 5a, b), which has inhibitory activity on the Na^+/K^+-ATPase similar to digoxin[17] but does not inhibit RORγt 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 RORαd, β, or γt, all of which are sufficient to induce IL-17 expression[22]. Digoxin inhibited only RORγt-mediated induction of IL-17a (Supplementary Fig. 10b), demonstrating its direct and selective activity on human RORγt.

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^+-ATPase[17]. 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 RORγ activity in the S2 reporter system (data not shown). As 20,22-dihydrodigoxin was still cytotoxic for human cells at 2.5 μM, digoxin was further modified by complete reduction of the butenolide to generate 20,22-dihydrodigoxin-21,23-diol (Dig(dhd)) (Fig. 3a). Dig(dhd) lacked cytotoxic activity on human cells at concentrations up to 40 μM, but it still possessed RORγ inhibitory activity and displaced the sterol ligand from the RORγ LBD (IC_{50} of 12 μM) (Supplementary Fig. 11a). Additional derivatization of digoxin was achieved by aldol condensation of the butenolide with salicylaldehyde to produce digoxin-21-salicylidene (Dig(sal)) (Fig. 3a), which, similarly to digoxin, bound directly to RORγ in the circular dichroism assay (Supplementary Fig. 3a). These compounds selectively inhibited both mouse and human RORγt activities without affecting those of mouse RORα and human LXRβ (Supplementary Fig. 11b–d). Moreover, Dig(sal) treatment reduced the severity of EAE progression (Supplementary Fig. 11e). When tested on human $CD4^+$ T cells transduced with viruses encoding RORαd or RORγt, Dig(dhd) or Dig(sal) treatment selectively suppressed RORγt-mediated IL-17a induction (Supplementary Fig. 11f). Intriguingly, addition of either compound blocked T_H17 cell differentiation[22] (Fig. 3b) and induced reciprocal increases of IFN-γ or FOXP3 expression in T cells (Fig. 3b and Supplementary Fig. 11g), indicating that functional RORγt or its

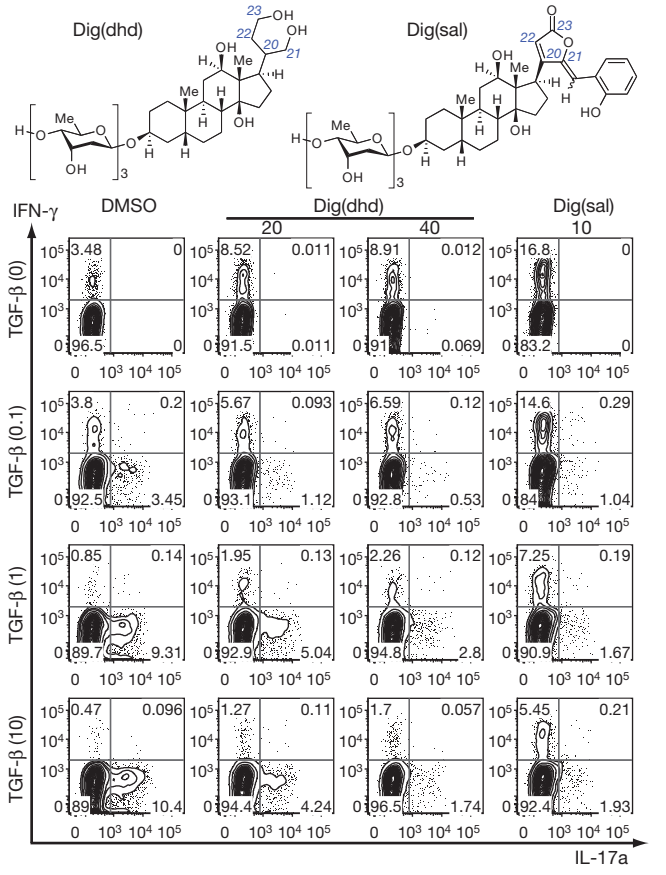

Figure 3 | Dig(dhd) and Dig(sal) inhibit human T_H17 cell differentiation. **a**, Chemical structures of Dig(dhd) and Dig(sal). **b**, Flow cytometry of the production of IL-17a and IFN-γ by human naive cord blood T cells cultured for six days in the presence of IL-2, IL-23 and IL-1β, with various concentrations of TGF-β (ng ml^{-1}). DMSO, Dig(dhd) or Dig(sal) at indicated concentrations (μM) was added 16 h after cytokine addition.

downstream events may normally suppress development into other T cell lineages. Expression of another human T_H17 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 T_H17 cells. *In vitro* digoxin treatment of expanded mouse T_H17 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 T_H17 cells from MOG-immunized *Il23r*$^{gfp/+}$ mice[27] 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 T_H17 cells generated *in vivo* and expanded *in vitro* require continuous RORγt activity to maintain their identity. To test if digoxin suppresses the activity of pre-existing T_H17 cells *in vivo*, we transferred IFN-γ-deficient, MBP-specific T_H17 cells into lymphopenic RAG2-deficient mice and assessed EAE manifestation after daily administration of digoxin. Because the transferred cells lack IFN-γ, the EAE phenotype observed in these mice is entirely attributable to the function of T_H17 cells. Digoxin treatment from day 2 delayed onset and reduced severity of T_H17 cell transfer-mediated EAE, which further confirms a requirement for continuous RORγt activity in T_H17 cells (Supplementary Fig. 12b). We then examined if RORγt activity is also important for sustained expression of IL-17a in human CD4$^+$ T cells. Human memory T_H17 cells were purified from peripheral blood samples and enriched by *in vitro* culture. Naive CD4$^+$ T cells cultured in the same cytokine conditions did not produce IL-17a (Fig. 4c, left plot). Dig(dhd) treatment led to a 40–50% reduction of IL-17a-expressing cells with little effect on IFN-γ-expressing cells (Fig. 4c, d). These data demonstrate that human RORγt activity has an important role in maintaining the human T_H17 cell population.

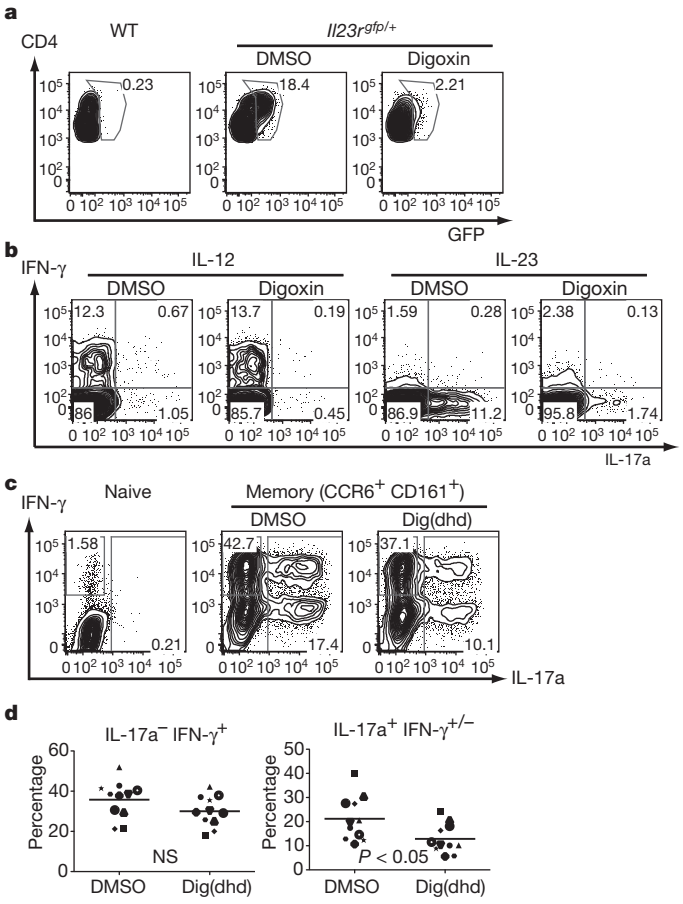

Figure 4 | RORγt activity is important for maintenance of mouse and human T_H17 cells. a, **b**, Flow cytometry of intracellular staining for IL-17a and IFN-γ by CD4$^+$ T cells. Mononuclear cells were collected from draining lymph nodes of wild-type or IL-23R–GFP knock-in heterozygous mice 7 days after $MOG_{35–55}$/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 μM digoxin. Without pre-immunization, addition of IL-23 and $MOG_{35–55}$ peptide to culture did not lead to *de novo* T_H17 cell differentiation (data not shown). Digoxin treatment suppressed expansion of *in vivo* differentiated T_H17 cells, assayed by IL-23R reporter GFP expression (**a**) or by IL-17a production (**b**). **c**, **d**, Human naive (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-1β, IL-23 and IL-2 for 6 days with or without 40 μM Dig(dhd). Representative FACS plots from one donor are shown (**c**). Each symbol indicates a separate donor ($n = 11$). Statistical analysis was by a two-tailed unpaired Student's *t*-test; IL-17a$^-$ IFN-γ$^+$, NS, and IL-17a$^+$ IFN-γ$^{+/-}$, $P = 0.02$ (**d**).

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 T_H17 cells, other TCRαβ cells, TCRγδ cells, lymphoid tissue inducer cells and NK-like cells (also referred to as NK22 cells) share in the property of requiring expression of RORγt for their differentiation. Abrogation of RORγt expression results in marked reduction or complete depletion of these cell types and in resistance to T_H17-mediated autoimmune disease in mouse models[4,6,28]. Therefore, RORγt 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 RORγt antagonist suggests that related molecules in mammals may modulate RORγ- and RORγt-mediated functions. However, it would be derivative compounds with better IC_{50} values that would have such roles. In light of recent findings of the roles of microbiota in the generation of T_H17 cells in the small intestine[30], 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 further opportunities for modulating the function of RORγt and T_H17 cell differentiation.

METHODS SUMMARY

Chemical screen. Ten thousand *Drosophila* stable S2 cells with genomic integration of the Cu^{2+}-inducible G4DBD-mouse RORγ 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 h, 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 RORγ 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 previously[6]. IL-17a, IFN-γ, IL-4, FOXP3 and CCR6 protein expression was examined by intracellular or surface staining according to the manufacturer's protocol.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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