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Suppression of T_H17 Differentiation and Autoimmunity by a Synthetic ROR Ligand

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Abstract

T helper cells that produce Interleukin-17 (IL-17) (T_H17 cells) are a recently identified $CD4^+$ T-cell subset with characterized pathological roles in autoimmune diseases[1–3]. The nuclear receptors retinoic acid receptor-related orphan receptors α and γt (RORα and RORγt) have indispensible roles in the development of this cell type[4–7]. Here we present a first-in-class, high-affinity synthetic ligand, SR1001, specific to both RORα and RORγt that inhibits T_H17 cell differentiation and function. SR1001 binds specifically to the ligand binding domains (LBDs) of RORα and RORγt inducing a conformational change within the LBD that encompasses repositioning of helix 12 leading to diminished affinity for coactivators and increased affinity for corepressors resulting in suppression of the receptors transcriptional activity. SR1001 inhibited the development of murine T_H17 cells as demonstrated by inhibition of IL-17A gene expression and protein production. Additionally, SR1001 inhibited the expression of cytokines when added to differentiated murine or human T_H17 cells. Finally, SR1001 effectively suppressed the clinical severity of autoimmune disease in mice. Thus, our data demonstrates the feasibility of targeting the orphan receptors RORα and RORγt to specifically inhibit T_H17 cell differentiation and

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Author Contributions P.R.G and T.P.B. conceived the project. L.A.S., P.R.G., and T.P.B. planned the project. Medicinal chemistry was planned and performed by P.N., T.M.K. and W.R.R. Biochemical and cell based assays were performed by L.A.S., N.S., Y.W., J.L., and M.A.I. Molecular modeling was performed by D.V and S.C.C. The EAE model was designed and performed by J.X., G.W. and P.D.D. HDX studies were performed by J.L.L. The manuscript was written by L.A.S. and T.P.B.

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function and indicates that this novel class of compound has potential utility in the treatment of autoimmune diseases.

T_H17 cells are crucial effector cells implicated in the pathology of numerous autoimmune diseases, including multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease, and systemic lupus erythematosis. These cells produce a number of cytokines, including IL-17, which are known to enhance inflammatory processes[1–3]. The discovery of these cells as critical mediators of autoimmune disorders provides a unique opportunity to develop focused therapeutics that act by inhibiting the function of these cells. An essential role for two nuclear receptors (NRs), RORα and RORγt, has been established in the development of T_H17 cells. Both of these NRs are required for the full differentiation of naïve $CD4^+$ T cells into T_H17 cells[4–7].

Members of the NR superfamily are ligand-dependent transcription factors. A number of drugs utilized in the clinic have been developed that target several NR superfamily members. Therefore, an attractive strategy for the development of novel therapeutics for treatment of T_H17-mediated autoimmune disorders is targeting RORα and RORγt with synthetic ligands that inhibit their activity resulting in decreased T_H17 cell differentiation and/or function. However, RORs are generally characterized as orphan receptors with no well-characterized ligands, thus it is unclear whether this approach is viable.

We recently characterized the benzenesulfonamide liver X receptor (LXR) agonist T0901317 (T1317) as a promiscuous ligand that modulates the activity of several NRs including RORα and RORγ[8]. While T1317 is a very potent and efficacious agonist of LXR, it also acts as an inverse agonist of RORα and RORγ by suppressing their basal transcriptional activity[8]. Using the T1317 scaffold as a lead compound we developed a derivative, SR1001 (Fig. 1a and Supplementary Fig. 1) that was devoid of all LXR activity yet retained its ability to suppress the activity of RORα and RORγ. We found that SR1001 repressed both GAL4-RORα and GAL4-RORγ transcriptional activity in a dose dependent manner (Fig. 1b), but demonstrated no effect on LXRα activity (Fig. 1b). We assessed the specificity of SR1001 in a panel of all 48 human nuclear receptors in a cell-based cotransfection assay [8] and did not observe activity on receptors other than RORα or RORγ (data not shown). We examined the direct binding of SR1001 to RORα and RORγ using competitive radioligand binding assays. SR1001 dose dependently displaced [^{3}H]25-hydroxycholesterol binding to RORα and RORγ (K_i = 172 and 111 nM, respectively) (Fig. 1c) but demonstrated no binding to RORβ (data not shown).

We examined whether SR1001 would affect RORα- and RORγ-dependent regulation of an *Il17* promoter-driven luciferase reporter[9]. HEK293 cells were transfected with the *Il17* reporter and either full-length RORα or RORγ and treated with SR1001 or vehicle. As illustrated in Fig. 1d, SR1001 dose-dependently suppressed the *Il17* promoter driven activity by each of the receptors. Since SR1001 bound RORα and RORγ, resulting in suppression of each receptors' transcriptional activity, we expected that SR1001 would inhibit coactivator binding to the receptors. SR1001 reduced the interaction of a coactivator TRAP220 NR box 2 peptide with RORγ in a dose dependent manner (Fig. 1e) (IC_{50} value ~117 nM).

Collectively, these data demonstrate that SR1001 function as an inverse agonist ligand of RORα/RORγ.

Next, we determined whether SR1001 affected endogenous *Il17a* gene expression. The EL4 murine tumor cell line constitutively expresses RORα (*Rora*), RORγt (*Rorc*), and IL-17A (*Il17a*)[10]. EL4 cells were treated with either control siRNA or a mixture of RORα/γ siRNA followed by treatment with either vehicle or SR1001. Reduction in the expression of RORα and RORγt significantly reduced the expression of IL-17A mRNA as measured by quantitative PCR (Fig. 2a). More importantly, treatment of cells with SR1001 suppressed *Il17a* mRNA expression whereas treatment of RORα/γ depleted cells displayed a significantly blunted response indicating that SR1001 suppression of *Il17a* mRNA expression is RORα/RORγ dependent (Fig. 2a). Furthermore, SR1001 suppressed the expression of the RORα and RORγ target gene *G6Pase* in HepG2 cells, a human hepatocellular carcinoma cell line, providing further proof that the effect of SR1001 is mediated by RORα and RORγ (Supplementary Fig. 2)[11, 12].

We hypothesized that SR1001 would inhibit binding of the coactivator SRC2 to either RORα or RORγ when these receptors are occupying the *Il17* promoter. We performed a sequential chromatin immunoprecipitation assay (ChIP-reChIP) assessing the relative amount of SRC2 associated with either RORα or RORγ resident at the *Il17* promoter in EL4 cells. SR1001 suppressd the ability of SRC2 to bind to RORα and RORγ at the *Il17* promoter and increased the recruitment of the corepressor NCoR (Fig. 2b, lanes 3 and 4 and Fig. 2c, lanes 3 and 4). Thus, SR1001 suppresses *Il17a* expression by directly inhibiting coactivator binding and promoting the recruitment of corepressors to RORα and RORγ.

To understand how ligand mediates the transcriptional activation of RORγ, we performed comprehensive differential hydrogen-deuterium exchange mass spectrometry (HDX) analysis of the RORγ LBD in the presence and absence of SR1001. This approach provides a measure of the localized ligand-induced perturbation in the conformational ensemble of the receptor. HDX kinetics of peptic peptides derived from the RORγ LBD were measured and the average difference in percentage of incorporated deuterium between *apo* RORγ LBD and SR1001 bound RORγ LBD are presented in Supplementary Figure 3. A negative value represents an increase in protection to exchange (more stable, less dynamic) in that region of the LBD when bound to ligand as compared to *apo* whereas a positive value represents a decrease in protection to exchange (less stable, more dynamic). HDX kinetics are sensitive to hydrogen bond networks and perturbations in these networks upon ligand binding can be determined using differential HDX. The differential HDX induced by SR1001 binding to RORγ correlates with the co-crystal structure of RORγ complexed with the sterol ligand, 25-hydroxycholesterol 25-OHC (PDB:3LOL)[13] (Supplementary Fig. 4). In the RORγ/25-OHC structure, the C25 hydroxyl tail is oriented towards helix 11 (H11) and the A ring toward H1/H2. As can be inferred from PDB:3LOL, the hydroxyl group at the C1 position of 25-OHC is hydrogen bonded to Qln-286 (H1) and the 25-hydroxyl is hydrogen bonded to His-479 (H11). The regions within the RORγ LBD that show increased protection to exchange upon binding of SR1001 include portions of H1 and H11. To highlight this, the HDX data in Supplementary Fig.3 is represented graphically by overlay onto PDB:3LOL with SR1001 docked (Fig. 2d). Consistent with the differential HDX data, docking of

SR1001 to PDB:3LOL suggests a similar binding mode for SR1001 to RORγ (Supplementary Fig. 4).

In order to examine the role of SR1001 in modulation of this interaction between SRC2 and RORγ, we performed differential HDX on RORγ LBD in the presence and absence of the receptor interaction domain (RID) of SRC2 (Fig. 2d & Supplementary Fig.3), which contains three NR boxes (~18kDa). Several regions of the LBD demonstrate reduced HDX kinetics in the presence of SRC2 RID, indicating an interaction between the two proteins. One region stabilized is H12, containing the AF2 domain of the receptor, which has been shown to be important for NR interaction with coactivators. Furthermore, differential HDX analysis of the RORγ:SRC2 complex in the presence and absence of SR1001 clearly demonstrates that ligand disrupts the receptors interaction with SRC2 RID (Fig. 2d). These data provide strong mechanistic insight into how inverse agonists such as SR1001 repress transcriptional output of RORγ target genes.

Since RORα and RORγt activity is required for optimal T_H17 cell development[4], we explored whether SR1001 would inhibit T_H17 cell differentiation. Splenocytes were cultured under T_H17 polarizing conditions (TGF-β and IL-6) with SR1001 or vehicle control for 5 days. The combination of TGF-β and IL-6 increased the mRNA expression of *Il17a*, *Il17f*, *Il21*, and *Il22*, in vehicle treated cells whereas SR1001 treated cells failed to significantly upregulate these cytokines (Fig. 3). Propidium iodide staining indicated that SR1001 was not toxic and did not induce cell death (Supplementary Fig. 5). T_H17 cells and inducible T regulatory cells (iT_{reg}) are both dependent on TGFβ for their differentiation. We evaluated whether expression of the T_{reg}-specific transcription factor Foxp3, was affected by SR1001 treatment. Similar to vehicle control, *Foxp3* mRNA expression was unaffected by SR1001 treatment suggesting that inhibition of T_H17 cell differentiation by SR1001 did not drive the cells into an iT_{reg} phenotype (Supplementary Fig. 6)[9]. Furthermore, suppression of T_H17 cell development with SR1001 treatment did not drive the splenocyte cultures into any of the other T helper lineages, T_H1 or T_H2, as indicated by the decrease in *Tbx21* (T-bet) and *Gata3* mRNA expression, respectively (Supplementary Fig. 6).

Finally, we explored whether SR1001 would inhibit IL-17 protein production and secretion. Splenocytes were cultured under T_H17 polarizing conditions and analyzed for IL-17 expression by intracellular flow cytometry. Treatment with SR1001 inhibited the expression of IL-17 from $CD4^+$ T cells at Day 4, 5, and 6 (Fig. 4a). Similar to splenocyte cultures, intracellular flow cytometry demonstrated that SR1001 significantly repressed IL-17 expression in purified differentiated murine $CD4^+$ T cells ($CD4^+CD25^-$ $CD62L^{hi}CD44^{lo}$) (Fig. 4b). Next we assessed the effect of SR1001 on IL-17 secretion from splenocyte cultures by ELISA. Treatment with SR1001 inhibited IL-17 secretion over a three-day time course, when SR1001 was added at either the initiation of T_H17 cell differentiation (initiation) or 48 hours post initiation of differentiation (post) (Fig. 4c). SR1001 was also effective at inhibiting intracellular IL-17 expression in human peripheral blood mononuclear cells (hPBMCs) (Fig. 4d). Finally, we examined the effects of SR1001 on other T helper cell lineages. Differentiation of T_H1, T_H2, and iT^{reg} cells was unaffected by SR1001 treatment as similar amounts of IFNγ, IL-4, or Foxp3, respectively, were expressed compared to

vehicle controls, indicating that SR1001 specifically targets T_H17 cells (Supplementary Fig. 7).

Given that RORα and RORγt are required for development of T_H17-mediated autoimmune diseases[4,5] and SR1001 inhibits the activity of both of these receptors leading to suppressed T_H17 cell development *in vitro*, we evaluated the effects of SR1001 treatment in an animal model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), a well characterized model of T_S17 cell-mediated autoimmune disease[14, 15]. After myelin oligodendrocyte glycoprotein ($MOG_{35–55}$) immunization at day 0, mice were treated with 25 mg kg^{-1} of SR1001 b.i.d. i.p. for the duration of the study. As shown in Figure 4e, SR1001 treatment delayed the onset and clinical severity of EAE. Further analysis of spinal cords from mice harvested at day 18 post-immunization revealed that SR1001 repressed *Il17a* mRNA expression by ~60%, as well as reduced *Il21*, and *Il22* mRNA expression (Fig. 4f). Intracellular cytokine analysis of splenocytes indicated a significant reduction in IL-17 expression and reduced total $CD4^+$ T cells with no effect on $CD8^+$ T cells. mRNA expression of IL-4 and IFNγ was unaffected in both spleen and spinal cords (Supplementary Fig. 8). These data are consistent with our interpretation that SR1001 suppresses EAE through its effects on T_H17 cell function *in vivo*. Further optimization of SR1001 may yield compounds with greater activity.

While RORα and RORγt expression and activity are essential for full T_H17 cell development, it is important to note that RORα and RORγ have roles outside of the immune system and are critical regulators of hepatic metabolism. Administration of SR1001 to C57BL/6 mice suppressed the expression of hepatic ROR target genes, *Cyp7b1*, *Rev-erb*α, and *Serpine 1* (Pai-1) suggesting that this class of compound may have metabolic effects; however, we noted no obvious toxicity in animals treated with SR1001 (Supplementary Fig. 9)[16–18].

In summary, we describe a novel, first-in-class, highly selective drug targeting the orphan NRs RORα and RORγ that effectively suppresses T_H17 cell differentiation and cytokine expression and reduces the severity of disease in an animal model of multiple sclerosis. Our data indicates that the targeting of T_H17 cells, by blocking RORα/γ function with a synthetic ligand, is a tractable approach for potential therapeutic intervention. Current treatments for T_H17-mediated autoimmune diseases, including multiple sclerosis, utilize agents that are general immunosuppressant's and thus the side effect profile is significant. Clearly, our data demonstrates that by targeting RORα and RORγ one can specifically inhibit T_H17 cells without affecting other T helper cell lineages thereby providing a more focused therapy that will not be a general immunosuppressant. Therefore, SR1001 and derivatives of this compound may represent a novel class of superior drugs to not only treat T_H17-mediated autoimmune disorders, but ROR-mediated metabolic disorders as well.

Methods Summary

Synthesis of SR1001 (N-(5-(N-(4-(1,1,1,3,3,3-hexafluoro-2-hydroxypropan-2-yl)phenyl)sulfamoyl)-4-methylthiazol-2-yl)acetamide). A solution of 2-(4-aminophenyl)-1,1,1,3,3,3-hexafluoropropan-2-ol (0.88 g, 3.4 mmol), 2-acetamido-4-

methylthiazole-5-sulfonyl chloride (0.79 g, 3.1 mmol) in acetone (15 mL) and 2,6-lutidine (0.73 mL, 6.2 mmol) was warmed to 60°C for 18 h. The reaction was judged complete by analytical HPLC (starting materials consumed).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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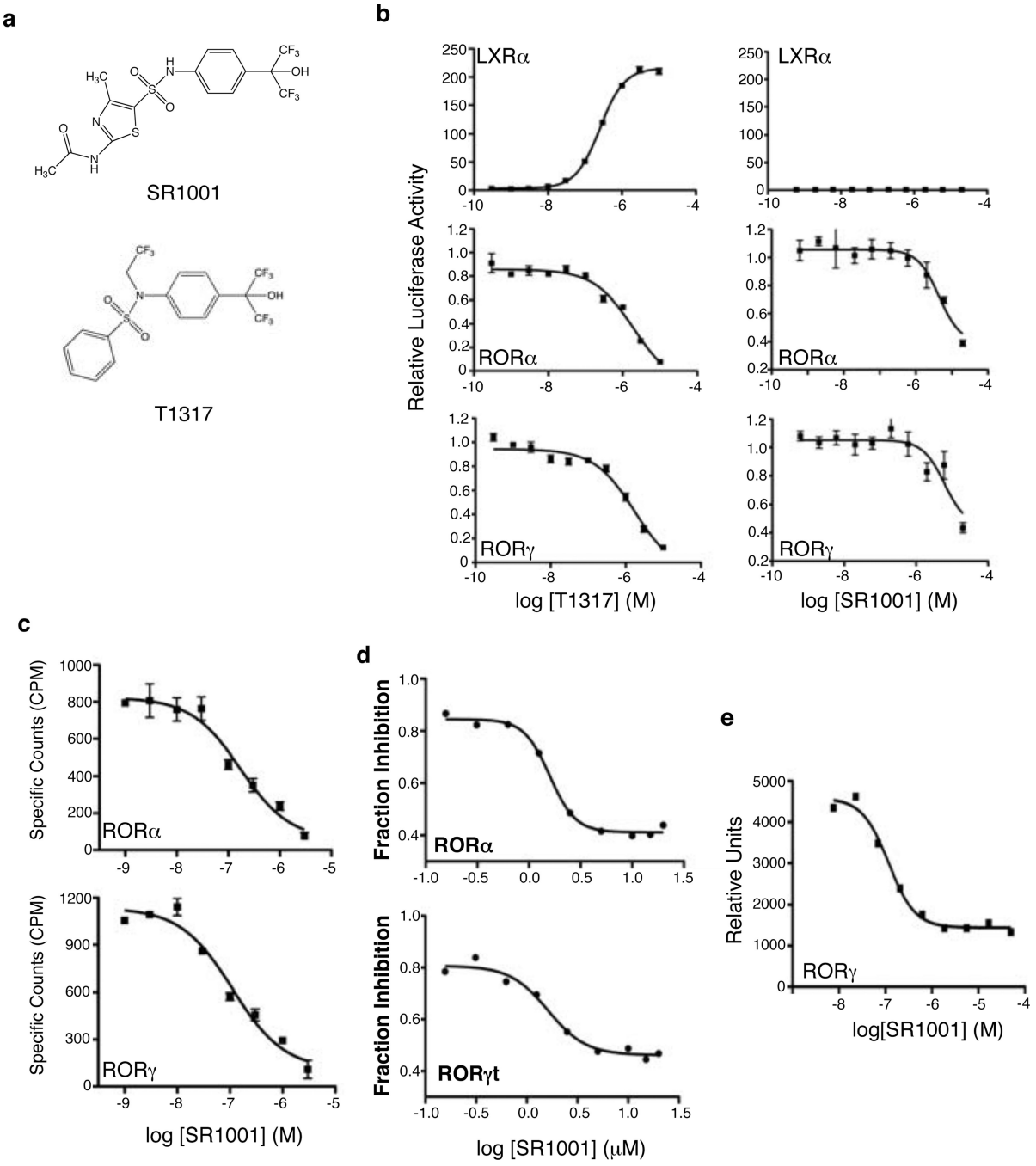

Figure 1. SR1001 is a selective RORα and RORγ inverse agonist

a, Structure of SR1001 and T0901317 (T1317). **b,** GAL4-LXRα, GAL4-RORα, and GAL4-RORγ cotransfection assays in HEK293 cells comparing T1317 to SR1001 (*n=8*). **c,** Competition radioligand binding assays illustrating the direct binding of SR1001 to the LBD of RORα and RORγ relative to [^{3}H]25-hydroxycholesterol (*n=4*). **d,** SR1001 dose-dependently inhibits an *Il17* promoter-driven luciferase construct in the presence of RORα or RORγt in HEK293 cells. Results are normalized to vehicle (DMSO) control (*n=4*). **e,**

AlphaScreen assay indicating SR1001 dose-dependently inhibits the recruitment of a TRAP220 NR box 2 peptide to the LBD of RORγ (*n=3*). Error bars denote sem.

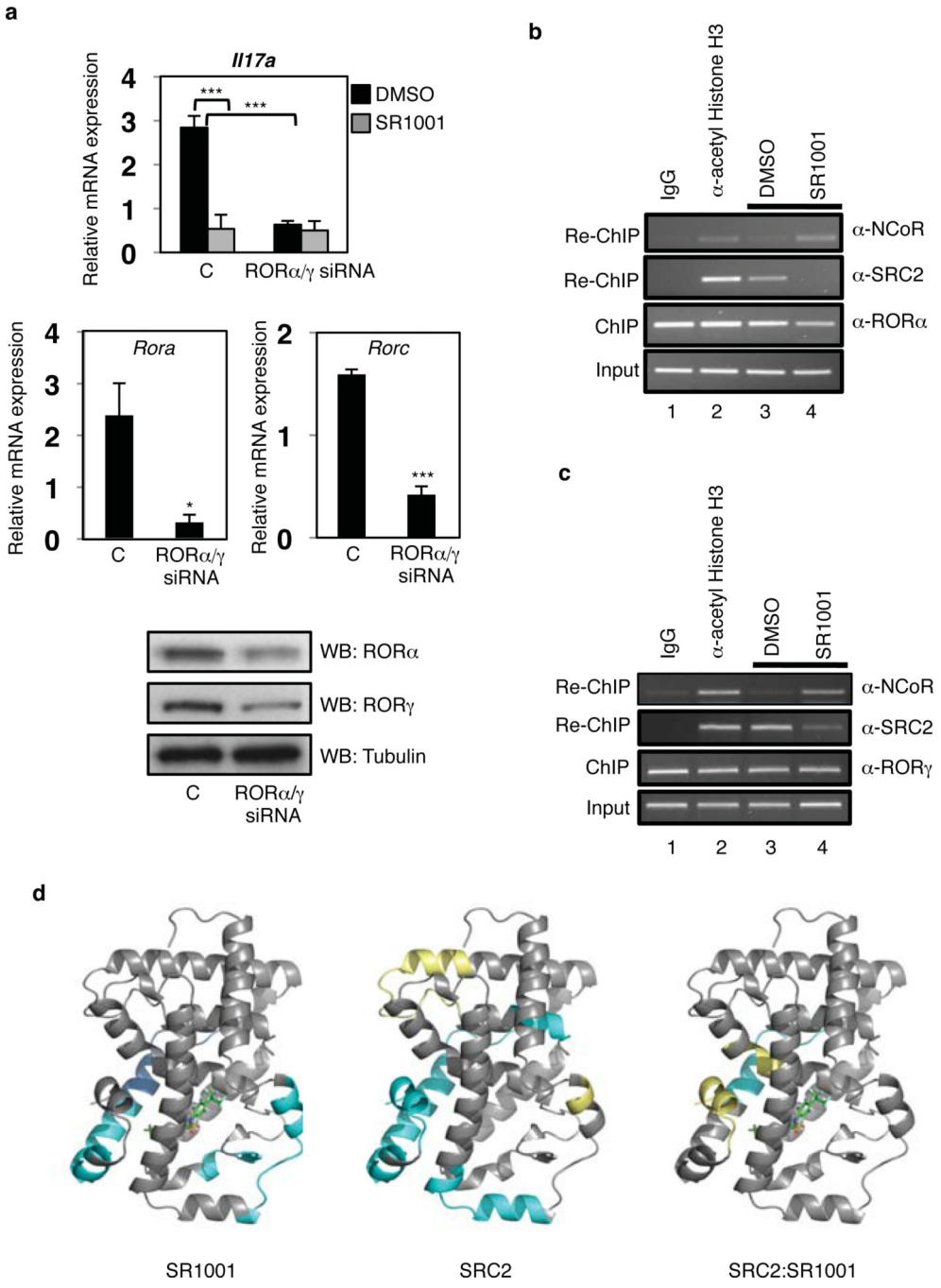

Figure 2. SR1001 modulates the expression of ROR target genes by decreasing coactivator recruitment

a, *Il17a*, *Rora*, and *Rorc* mRNA expression in EL4 cells treated with control (C), or mouse RORα/γ siRNA, vehicle (DMSO), or SR1001 (10μM, 24 hours) (*n=3*). Protein expression of RORα and RORγ is shown. **P<0.05*; ****P<0.005*. **b,** ChIP-reCHIP assay in EL4 cells illustrating that SR1001 reduces **b,** RORα- and **c,** RORγ-dependent recruitment of SRC-2 and promotes recruitment of NCoR to the *Il17* promoter. **d,** Illustration of the HDX kinetics

of peptic peptides derived from the RORγ LBD.. Cyan indicates an increase in protection to exchange; yellow represents a decrease in protection to exchange. Error bars denote sem.

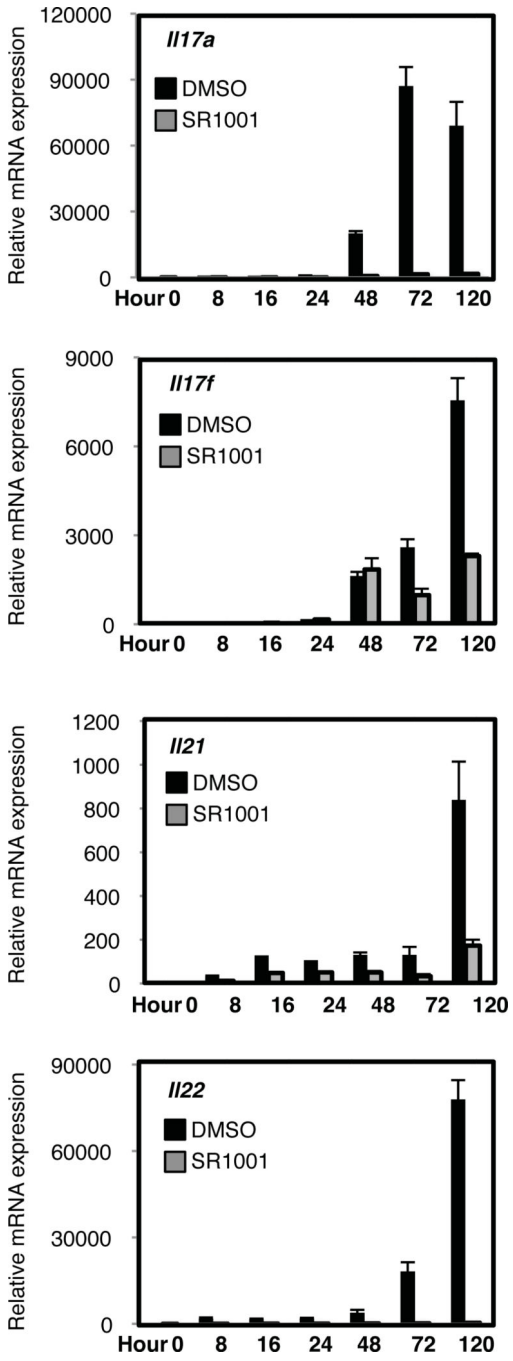

Figure 3. SR1001 inhibits the expression of cytokines expressed by T_H17 cells

Il17a, *Il17f*, *Il21*, and *Il22* mRNA expression in splenocytes differentiated under T_H17 polarizing conditions in the presence of vehicle (DMSO) or SR1001 (5μM) for 5 days. mRNA expression levels are normalized to *Gapdh* (*n*=3). Error bars denote sem.

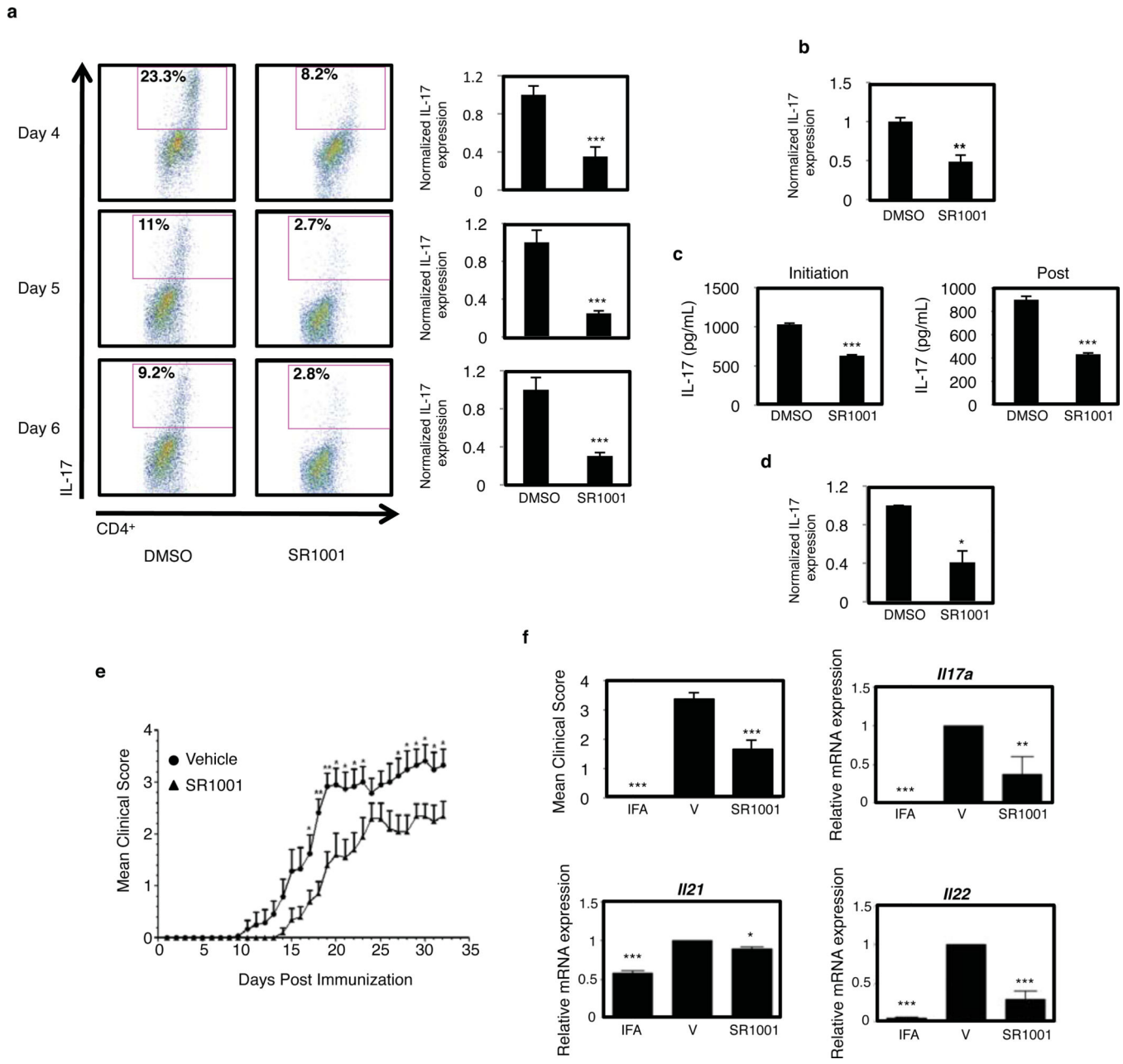

Figure 4. SR1001 inhibits T_H17 cell development and IL-17A secretion

a, IL-17 expression in splenocytes cultured under T_H17 polarizing conditions with vehicle control (DMSO) or SR1001 (5μM). Graphs represent the average percentage of IL-17A expressing cells normalized to vehicle control (*n=3*). **b,** IL-17 expression in differentiated purified naïve murine $CD4^+$ T cells normalized to vehicle control *(n=3)*. **c,** IL-17A secretion from splenocytes cultured under T_H17 polarizing conditions with SR1001 (5μM) for 3 days *(n=3)*. **d,** Intracellular IL-17A expression in hPBMCs cultured for 24 hours with vehicle or SR1001 (5μM) (*n=3*). **e,** Treatment with SR1001 suppresses the clinical severity of EAE: vehicle (●, *n*=12) or SR1001 (25 mg kg^{-1}) (▲, *n*=10). **f,** *Il17a*, *Il21*, and *Il22* mRNA

expression from spinal cords of sham-operated (IFA), vehicle control (V), or drug treated (SR1001) mice. *(n=4)*. Error bars denote sem. ***$P < 0.001$; **$P < 0.01$; and *$P < 0.05$.