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# Identification of a Selective RORγ Ligand That Suppresses $T_H17$ Cells and Stimulates T Regulatory Cells

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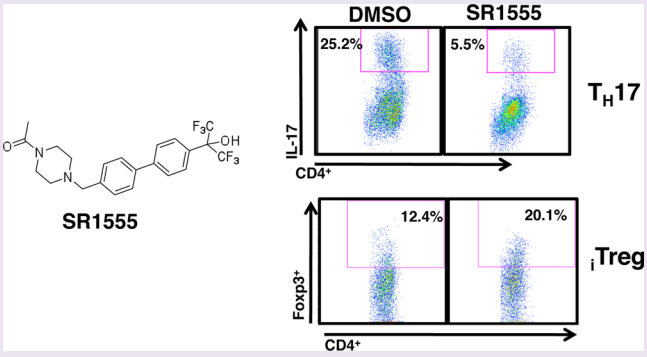
Supporting Information

**ABSTRACT:** Nuclear receptors (NRs) are ligand-regulated transcription factors, many of which are validated targets for clinical purposes. The retinoic acid receptor-related orphan nuclear receptors alpha and gamma t (RORα and RORγt) are considered to be the master regulators of development of $T_H17$ cells, a subset of T cells that have been implicated in the pathology of several autoimmune diseases, including multiple sclerosis (MS) and rheumatoid arthritis (RA). We report here the identification of a novel RORγ-specific synthetic ligand, SR1555, that not only inhibits $T_H17$ cell development and function but also increases the frequency of T regulatory cells. Our data suggests synthetic RORγ ligands can be developed that target both suppression of $T_H17$ and stimulation of T regulatory cells, offering key advantages in development of therapeutics targeting autoimmune diseases.




$T_H17$ cells are a subset of $CD4^+$ T cells that are critically dependent upon the nuclear receptors (NR) retinoic acid receptor-related orphan receptors alpha and gamma t (RORα and RORγt) and have been demonstrated to produce several proinflammatory cytokines, including IL-17A, IL-17F, IL-21, and IL-22.[1−6] While their normal function is mediating immune defense against extracellular bacteria, aberrant $T_H17$ cell function has been implicated in a variety of autoimmune diseases, including multiple sclerosis (MS) and rheumatoid arthritis (RA). Autoimmune disease is thought to arise when the equilibrium between effector and regulatory T cells is disrupted.

$T_H17$ cells can be generated from naïve precursors in the presence of TGFβ and IL-6 or IL-21.[7] However, since TGFβ is also the critical cytokine regulating the development of $Foxp3^+$ T regulatory cells, the development of one lineage over the other is dependent on the environmental milieu. Given this reciprocal relationship between T regulatory cells and $T_H17$ cells, it is still unclear what all of the factors that mediate the expansion of one population over the other may be. Since $T_H17$ cells play a central role in the development of several autoimmune diseases, identification of synthetic modulators that can inhibit $T_H17$ cell differentiation and function would have significant therapeutic value. We recently identified SR1001, a first-in-class, high affinity synthetic RORα/γ inverse agonist. SR1001 was effective at suppressing $T_H17$ cell differentiation and function *in vitro* and *in vivo* through direct inhibition of RORα and RORγ.[8] Given the important role of RORα in the regulation of circadian rhythm and metabolism, there was some concern that a dual ROR-selective inverse agonist could cause undesirable side effects, suggesting that a RORγ-selective compound would be more appropriate.[9]

Concurrent with our characterization of SR1001, several other groups identified RORγ-specific inverse agonists.[10,11] Similar to SR1001, these ligands inhibited $T_H17$ cell development and function. Therefore, in order to investigate the specific effects of RORα versus RORγ in $T_H17$ cell development and alleviate activity at RORα, we performed further modifications to our initial lead compound, T0901317. Previously, we demonstrated that the benzenesulphonamide liver X receptor (LXR) agonist, T0901317, acted as an inverse agonist at RORα and RORγ.[12] Due to its mixed pharmacology, it was unsuitable for specifically evaluating ROR effects. Compounds were identified using a screening approach similar to SR1001 and a RORγ-selective inverse agonist, SR1555, was identified (Figure 1a). A screen of SR1555 in a GAL4-NR chimeric co-transfection assay demonstrated that SR1555 was devoid of LXR, FXR, and RORα activity, but it repressed the activity at RORγ in a dose dependent manner ($IC_{50} \approx 1.5$ μM) (Figure 1b). Next we examined the direct binding of SR1555 to RORα and RORγ in competitive radioligand binding assays. SR1555 was only able to displace [$^3$H]T0901317 from the ligand binding domain (LBD) of RORγ ($IC_{50} = 1$ μM; Figure 1c) and not

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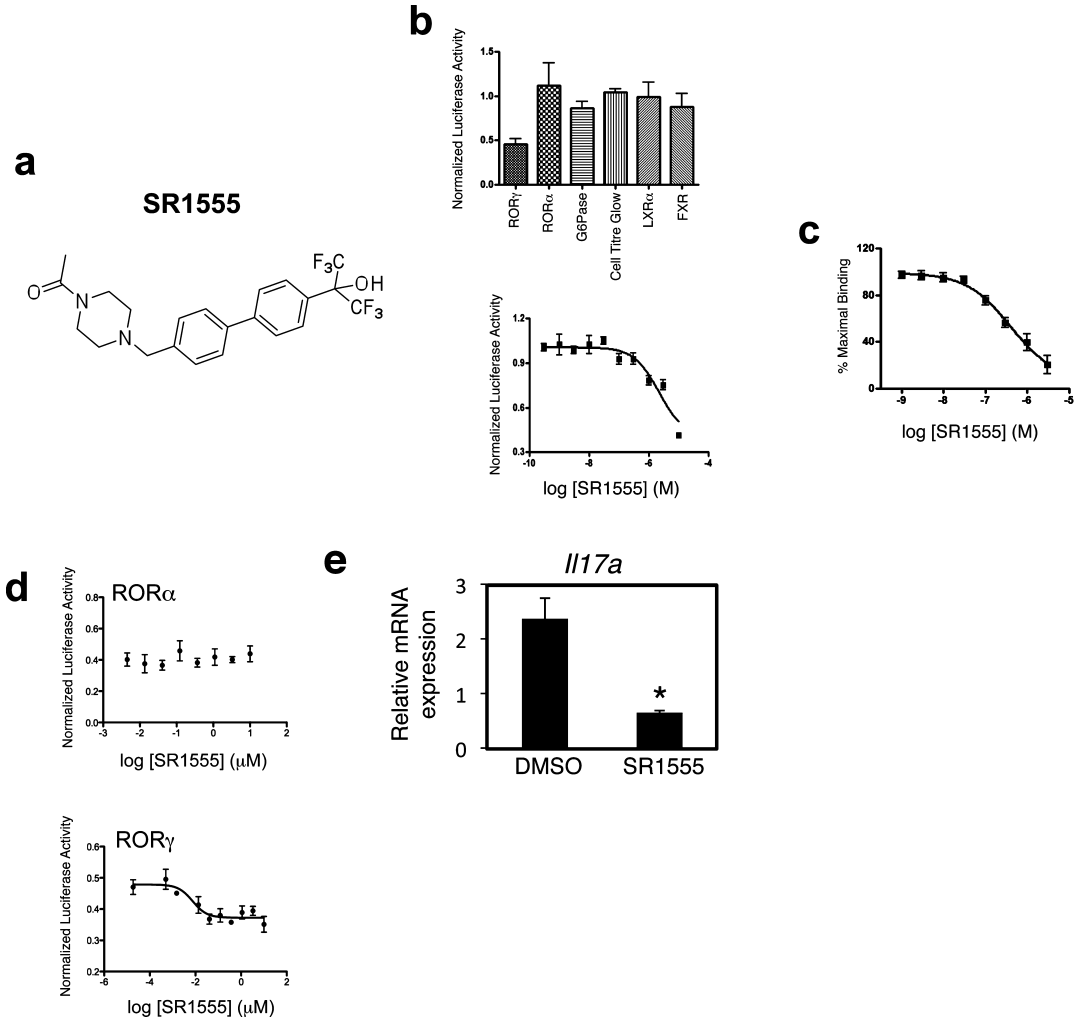


**Figure 1.** Characterization of SR1555. (a) Structure of SR1555. (b) Full length G6 Pase and GAL4-LXR$\alpha$, ROR$\alpha$, ROR$\gamma$, and FXR co-transfection assays in HEK293 cells illustrating the specificity SR1555 ($n$ = 8). (c) Competition radiolabeled binding assays illustrating the direct binding of SR1555 to the LBD of ROR$\gamma$ only. C.P.M., counts per minute ($n$ = 4). (d) SR1555 dose-dependently inhibits an IL-17 promoter driven luciferase construct only in the presence of ROR$\gamma$ ($n$ = 4). (e) EL4 cells were treated with DMSO or SR1555 (10 $\mu$M) for 24 h followed by mRNA analysis of IL-17A by RT-PCR ($n$ = 4). (*$p$ < 0.05)

ROR$\alpha$ (data not shown) confirming that SR1555 was indeed specific for ROR$\gamma$.

Since ROR$\alpha$ and ROR$\gamma$ are critical regulators of IL-17 gene expression, we examined whether SR1555 would affect ROR$\alpha$- and ROR$\gamma$-dependent regulation of an IL-17 promoter driven luciferase reporter construct. HEK293 cells were transiently transfected with an IL-17 reporter, either full-length ROR$\alpha$ or ROR$\gamma$, and treated with an increasing concentration of SR1555.[8] In a dose-dependent manner, SR1555 suppressed IL-17 promoter driven activity by ROR$\gamma$ but not ROR$\alpha$, further confirming that SR1555 is indeed a ROR$\gamma$-specific inverse agonist (Figure 1d). Finally, using EL4 cells, a murine thymoma which endogenously express ROR$\alpha$, ROR$\gamma$t, and IL-17A, we examined whether SR1555 affected endogenous IL-17 gene expression.[8] EL4 cells were treated with DMSO or SR1555 for 24 h and analyzed for *Il17a* gene expression by quantitative real-time PCR. SR1555 inhibited *Il17a* gene expression by greater than 70%, demonstrating that SR1555 can inhibit the expression of this $T_H17$ mediated cytokine (Figure 1e). Together, these data indicate that SR1555 specifically targets ROR$\gamma$ and inhibits its transcriptional activity leading to suppression of IL-17 gene expression.

Given that SR1555 was an effective inhibitor of endogenous IL-17 gene expression in EL4 cells, we next examined whether SR1555 would affect the differentiation of $T_H17$ cells *in vitro*. Murine splenocytes were cultured under $T_H17$ polarizing conditions (TGF$\beta$ and IL-6) for 3 days in the presence of SR1555 or vehicle control (DMSO). Real-time RT-PCR analysis revealed that while SR1555 inhibited the mRNA expression of *Il17a*, it was less effective at inhibiting *Il17f*, *Il21*, and *Il22* (Figure 2a). While the effects of SR1555 are distinct from those observed with our dual ROR$\alpha/\gamma$ inverse agonist, SR1001, or other previously published ROR$\gamma$ ligands, our data suggests that ROR ligands with differing ROR$\alpha/\gamma$ selectivity may display differential effects on $T_H17$ cytokine gene expression. It may also be the case that differential effects may be observed with different chemical classes of ROR ligands as has been observed with other nuclear receptor ligands.

Next, we examined the effects of SR1555 on IL-17 protein expression. Again, splenocytes were cultured under $T_H17$ polarizing conditions for 4 days in the presence of SR1555 or vehicle control for the full time course (Figure 2b) or without ligand until 24 h prior to completion of the experiment (compound added after day 3, Figure 2c). Using intracellular


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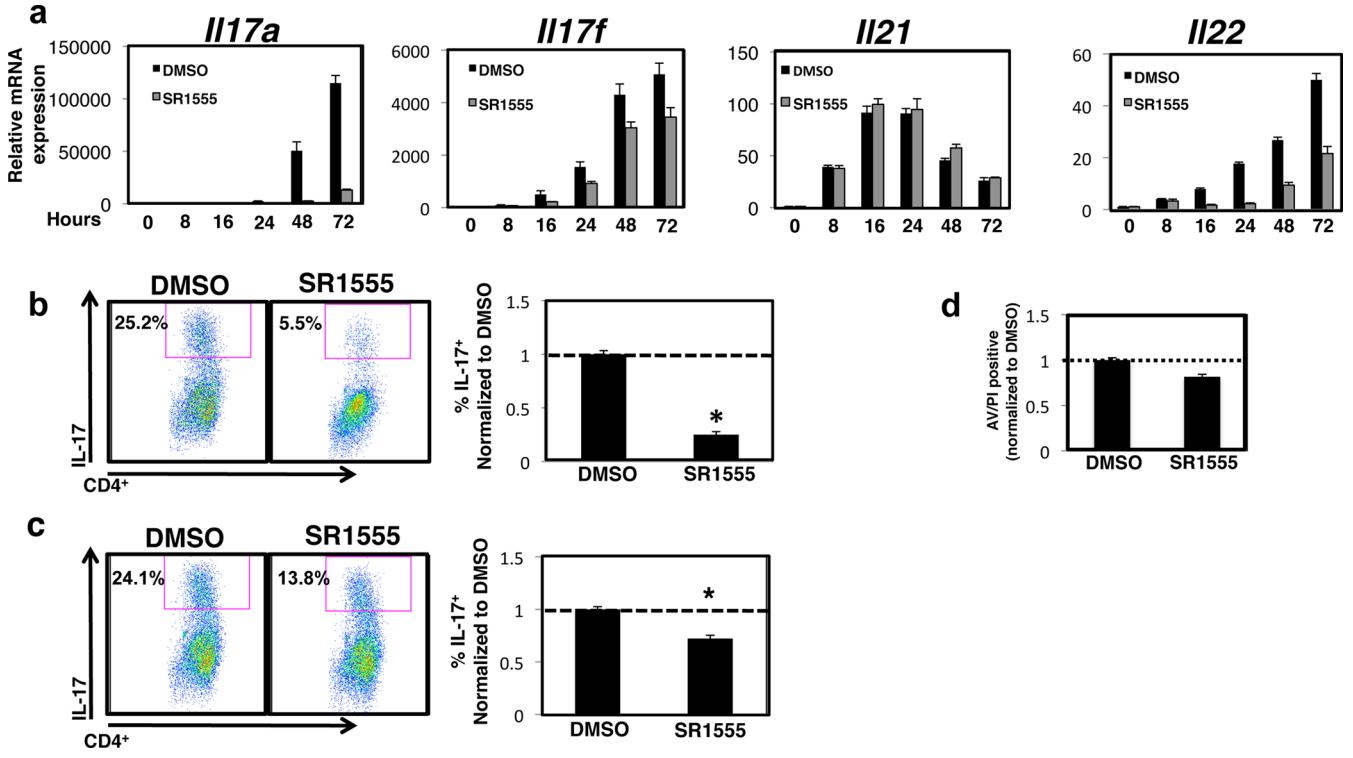


**Figure 2.** Effects of SR1555 during $T_H17$ cell differentiation. (a) Real time RT-PCR analysis of splenocytes cultured under $T_H17$ polarizing conditions in the presence of DMSO or SR1555 (10 $\mu$M) for the duration of the time course. Data are normalized to GAPDH ($n$ = 3). (b) The effect of SR1555 (10 $\mu$M) on IL-17A cytokine expression during murine $T_H17$ cell differentiation. Intracellular cytokine staining on splenocytes treated with DMSO or SR1555 for the duration of the experiment. Cells were grated on $CD4^+$ T cells. Graph on the right summarizes IL-17 expression normalized to DMSO ($n$ = 3, *$p$ < 0.05). (c) Intracellular cytokine staining on splenocytes treated with DMSO or SR1555 (10 $\mu$M) for 24 h prior to FACS analysis. Cells were grated on $CD4^+$ T cells. Graph on the right summarizes IL-17 expression normalized to DMSO ($n$ = 3, *$p$ < 0.05). (d) The effect of SR1555 (10 $\mu$M) on the viability of $T_H17$ cells. Cells were gated on AnnexinV and propidium iodide positive cells. Graph depicts percent of positive cells normalized to DMSO ($n$ = 3).

cytokine FACS analysis, we determined that SR1555 was effective at inhibiting IL-17 protein expression when added at the initiation of $T_H17$ cell differentiation (Figure 2b) and when SR1555 was added 24 h prior to the completion of the experiment (Figure 2c). In order to clarify that the effects observed in the cultures were not due to toxicity of the compounds, we analyzed the cultures for the expression of Annexin V and propidium iodide, markers of cell death, using flow cytometry. SR1555 did not induce cell death during $T_H17$ cell differentiation relative to the DMSO control (Figure 2d). To date, most ROR$\gamma$-specific compounds have been evaluated as "preventative" measures. Currently, there is little data evaluating the efficacy of ROR-selective drugs once the initiation of $T_H17$ development has begun. Our data suggests that ROR$\gamma$-specific inverse agonists will be effective at inhibiting $T_H17$ cell-mediated cytokine responses once the differentiation process has commenced (Figure 2c).

Upon antigen exposure and in the presence of TGF$\beta$, both *Foxp3* and *Ror$\alpha$/$\gamma$t* are upregulated. Therefore, the environmental cues associated with the concentration of TGF$\beta$ dictates the balance between $T_H17$ and T regulatory cell differentiation. In light of the reciprocal relationship between these two cell types, we assessed whether SR1555 affected the development of T regulatory cells *in vitro*. Splenocytes were cultured under T regulatory cell polarizing conditions (TGF$\beta$ and IL-2) for 3 days. Quantitative RT-PCR analysis revealed that SR1555 effectively increased the gene expression of *Foxp3* while simultaneously suppressing the gene expression of *Ror$\gamma$t* (Figure 3a). To determine whether the gene expression data would correlate with protein expression, we again cultured splenoyctes for 5 days under T regulatory cell polarizing conditions in the presence of DMSO or SR1555 and examined the expression of Foxp3 by intracellular cytokine FACS analysis. Similar to the gene expression data, SR1555 treatment increased the frequency of T regulatory cells as evidenced by the almost 2-fold increase in the expression of $Foxp3^+$ T cells in the splenocyte culture (Figure 3b). This result was surprising given that all previously described ROR$\gamma$ ligands have no effect on T regulatory cell populations *in vitro*.[8] Furthermore, these effects were not mediated through RAR$\alpha$ or RXR$\alpha$, given that activation of RAR has been demonstrated to be a mediator of inducible T regulatory cells (Supplementary Figure 1).[13] Therefore, our data suggest that all ROR$\gamma$-selective ligands are not created equally. While ligands such as digoxin and ursolic acid may be touted as ROR$\gamma$-selective and look similar in reporter gene assays, compounds of different chemical classes may have differential effects.[10,11] Thus, while inhibition of ROR$\gamma$ alone may be sufficient to inhibit $T_H17$ responses, unique ROR$\gamma$-specific ligands like SR1555 may hold utility in the generation of T regulatory cells.

Since the nuclear receptors ROR$\alpha$ and ROR$\gamma$t are considered the master regulators of $T_H17$ cell development, generation of synthetic ligands designed to modulate the activity of the RORs is a promising strategy for therapeutics


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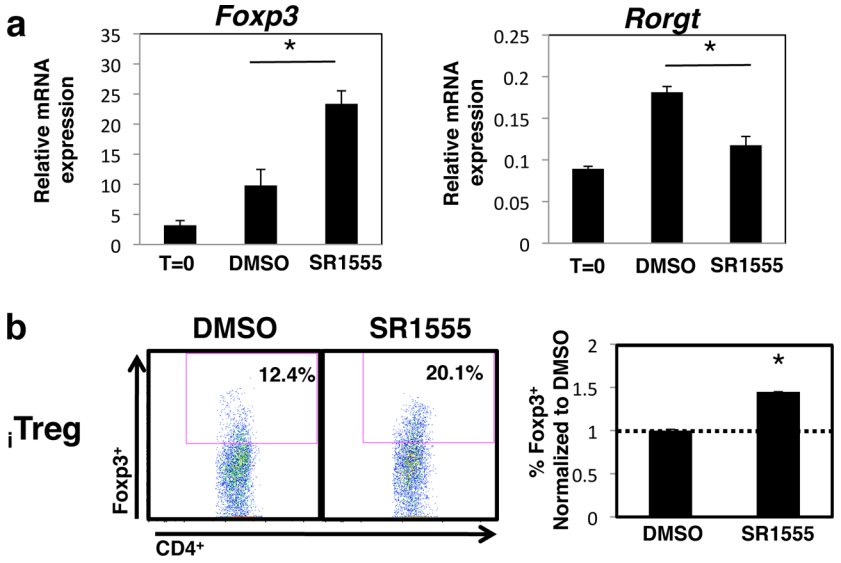
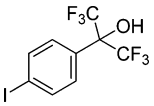



**Figure 3.** SR1555 increases the frequency of iTregs. (a) RT-PCR analysis of splenocytes cultured under T regulatory cell polarizing conditions in the presence of DMSO or SR1555 (10 $\mu$M) for the duration of the time course. Data are normalized to GAPDH ($n$ = 3). (b) The effect SR1555 (10 $\mu$M) on the expression of Foxp3$^+$ cells during the differentiation of iTregulatory cells. Intracellular cytokines staining on splenocytes differentiated under iTreg conditions for 5 days treated with vehicle control (DMSO) or SR1555 for the duration of the experiment. Cells were gated on CD4$^+$ T cells. Graphs to the right of the FACS plots summarize the Foxp3$^+$ expression normalized to DMSO ($n$ = 3, *$p$ < 0.05).

aimed at $T_H17$-mediated diseases. Although several NRs, including RAR$\alpha$ (retinoic acid receptor alpha), VDR (vitamin D receptor), and GR (glucocorticoid receptor), have been demonstrated to regulate the effector functions of fully differentiated T cells, these NRs are not required for T cell development as ROR$\alpha$ and ROR$\gamma$t are for $T_H17$ cells.[14] In fact, in the absence of RAR$\alpha$ and VDR, T cells develop normally.[15,16] Furthermore, direct targeting of these NRs can lead to a global inhibition of T cell function as GR and VDR are present in all T cell types, not just $T_H17$ cells.[14,16] Therefore, the ultimate goal for the treatment of autoimmune diseases is not only to inhibit the cell type(s) responsible for the disease state but also to help the body regain its immune homeostasis, which may involve increasing the T regulatory cell population. Recently, we and others have reported the identification and characterization of ligands specific to ROR$\alpha$ and/or ROR$\gamma$t that inhibit $T_H17$ cell differentiation and function.[8,10,11,17] None of these compounds altered T regulatory cell function. In this report, we identified a ROR$\gamma$-specific inverse agonist, SR1555, with unique features. Similar to the other ROR-ligands published, SR1555 inhibits $T_H17$ cell differentiation and function. However, SR1555 treatment also enhances the induction of inducible T regulatory cells *in vitro*. Interestingly, SR1555 is structurally similar to the most recent ROR$\gamma$ selective ligand we recently reported, SR2211, but this compound does not alter T regulatory cell proliferation (Supplementary Figure 2). These data suggest that the $T_H17$ vs T regulatory effects of ROR$\gamma$ ligands are separable and can likely be optimized individually. Of course, a ROR ligand that affects the $T_H17$/Treg cell ratio by directly targeting both cell types could be of particular benefit in treatment of autoimmunity. Our data suggest that each ROR ligand is unique and may have differential effects at ROR$\alpha$ and/or ROR$\gamma$, which could result in some ligands being considered more attractive candidates for therapeutics aimed at treating $T_H17$-mediated diseases.

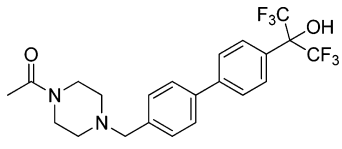
## METHODS

**Synthesis of SR1555.** *Step 1. 1,1,1,3,3,3-Hexafluoro-2-(4-iodophenyl)propan-2-ol.*




To 2-(4-amino-phenyl)-1,1,1,3,3,3-hexafluoropropan-2-ol (commercially available) in DMF was added a solution of sodium nitrite (1.2 equiv) in water and 6 M hydrochloric acid (3 equiv), while maintaining the temperature between 0−5 °C in an ice bath. The reaction mixture was stirred for 30 min, and then potassium iodide (1.5 equiv) was added in portions. The resulting reaction mixture was stirred overnight at rt. The reaction mixture was diluted with $Et_2O$, washed several times with a solution of saturated sodium thiosulphate and treated with $Na_2SO_4$. The solvent was evaporated in vacuo, leaving a crude oil, and the product, 1,1,1,3,3,3-hexafluoro-2-(4-iodophenyl)-propan-2-ol, was isolated by flash chromatography on silica gel (~5% EtOAc/Hex) to obtain the title compound.

*Step 2. 1,1,1,3,3,3-Hexafluoro-2-(4′-((1-(acetyl) piperazine) methyl)-[1,1′-biphenyl]-4-yl)propan-2-ol.* To 4-bromomethylphenylboronic acid was added 1,4-dioxane, followed by addition of $K_2CO_3$




(4.0 equiv), 1-acetyl-piperazine (1.1 equiv), and potassium iodide (2% weight). The mixture was allowed to stir overnight at rt (~23 °C) under an argon balloon. To the mixture were then added water (1:4, water/dioxane) and $Pd(PPh_3)_4$ (5 mol %). The mixture was then purged of air *via* vacuum line and argon balloon, fitted with an air-cooled condenser, and heated at reflux under an argon balloon for 1.5 h. The reaction mixture was allowed to cool and was then subsequently extracted with excess EtOAc, keeping the organic phase, which was washed with brine, dried over $Na_2SO_4$, and concentrated in vacuo. The crude residue was purified by reverse-phase preparative HPLC to provide the TFA salt of the title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compound as a colorless solid. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 7.92−7.65 (m, 7 H), 7.66 (d, $J$ = 8.0 Hz, 2 H), 4.61−4.49 (m, 3 H), 4.11−4.09 (m, 1 H), 3.50−2.91 (m, 6 H), 2.08 (s, 3H); $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 168.53. 158.49 (q, $J$ = 33.7 Hz, $CF_3COOH$), 140.86, 140.08, 131.96, 130.23, 129.12, 127.43, 127.25, 126.96, 122.91, 117.97 ($CF_3COOH$), 76.78 (q, $J$ = 30 Hz, $CF_3$), 58.43, 50.71, 50.33, 42.49, 37.71, 20.85; ESI-MS ($m/z$): 460.94 $[M + 1]^+$.

**Mice.** Male C57BL/6 mice (8 weeks old) were purchased from Jackson Laboratory (Bar Harbor, ME). All mice were maintained and experiments were performed in specific pathogen free environments in accordance with protocols approved by the Institutional Animal Care and Use Committee.

**Cell Culture and Co-transfections.** Co-transfection assays were performed as previously described in HEK293 cells (Gal4 co-transfection assay or IL-17 luciferase reporter).[8] HEK293 and the mouse lymphoma EL4 (ATCC) cells were maintained in DMEM supplemented with 10% FBS plus antibiotics (penicillin and streptomycin; Invitrogen).

**Splenocyte Differentiation.** Splenocytes were differentiated under the following conditions. iTreg: 10 $\mu$g mL$^{-1}$ anti-IFN$\gamma$, 10 $\mu$g mL$^{-1}$ anti-IL-4, and 2 ng mL$^{-1}$ TGF$\beta$ (R&D Systems); 20 $\mu$g mL$^{-1}$ anti-IFN$\gamma$, 20 $\mu$g mL$^{-1}$ anti-IL-4, 1 ng mL$^{-1}$ TGF$\beta$, and 10 ng mL$^{-1}$ IL-6 (R&D Systems) for $T_H$17 conditions. All cultures were stimulated with 1 $\mu$g mL$^{-1}$ anti-CD3 (eBiosciences) and 1 $\mu$g mL$^{-1}$ anti-CD28 (eBiosciences). Four to five days after activation, all cells were restimulated with 5 ng mL$^{-1}$ phorbol-12-myristate-13-acetate (PMA) (Sigma) and 500 ng mL$^{-1}$ ionomycin (Sigma) for 2 h with the addition of GolgiStop (BD Bioscience) for an additional 2 h before intracellular staining. Cells were cultured in RPMI 1640 medium (Invitrogen) with 10% FBS and antibiotics.

**Flow Cytometry and Antibodies.** Single cell suspensions prepared from spleen were stained with fluorophore-conjugated monoclonal antibodies: FITC anti-CD4 (GK1.5, eBioscience), phycoerythrin-conjugated antimouse IL-17A (eBio17B7, eBioscience), and Alexafluor 647 anti-Foxp3 (FJK-16s, eBioscience) along with the Foxp3 staining buffer set (eBioscience). Annexin V and propidium iodide staining was performed using the FITC AnnexinV Apoptosis Detection Kit II (BD Pharmingen). Flow cytometric analysis was performed on a BD LSRII (BD Biosciences) instrument and analyzed using FlowJo software (TreeStar).

**Quantitative RT-PCR.** RNA extraction and QRT-PCR analysis was performed as previously described.[8]

**Statistical analysis.** All data are expressed as the mean ± sem ($n$ = 3 or more). Statistical analysis was performed using an unpaired Student's $t$-test.

## ■ ASSOCIATED CONTENT

### Supporting Information

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### Notes

The authors declare no competing financial interest.

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