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Small molecule RORγt antagonists inhibit T helper 17 cell transcriptional network by divergent mechanisms

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Abstract

We identified three RORγt-specific inhibitors that suppress T helper 17 (Th17) cell responses including Th17 cell-mediated autoimmune disease. We systemically characterized RORγt binding

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Conflict of Interest: Vijay K. Kuchroo has a financial interest in Tempero Pharmaceuticals. His interests were reviewed and are managed by the Brigham and Women's Hospital and Partners HealthCare in accordance with their conflict of interest policies. Jianfei Yang, Erkan Baloglu, Darby Schmidt, Radha Ramesh, Mercedes Lobera, Mark S. Sundrud and Shomir Ghosh are employees or former employees and share holders of Tempero Pharmaceuticals, a GSK company. Yonghui Wang, Ling Zhou, Zhijun Xiang, Jinsong Wang, Yan Xu, Xichen Lin, and Zhong Zhongare GSK employees.

in the presence and absence of drug with corresponding whole-genome transcriptome sequencing. RORγt acts both as a direct activator of Th17 cell signature genes and as a direct repressor of signature genes from other T-cell lineages, with the strongest transcriptional effects on *cis*-regulatory sites containing the RORα binding motif. RORγt is central in a densely interconnected regulatory network that shapes the balance of T-cell differentiation. The three inhibitors identified here modulated the RORγt-dependent transcriptional network to varying extents and through distinct mechanisms. Whereas one inhibitor displaced RORγt from its target-loci, the two more potent inhibitors affected transcription predominantly without removing DNA-binding. Our work illustrates the power of a system-scale analysis of transcriptional regulation to characterize potential therapeutic compounds that inhibit pathogenic Th17 cells and suppress autoimmunity.

Introduction

Th17 cells, induced by the “master” transcription factor (TF) RORγt, play an important role in chronic inflammation and autoimmune diseases (Korn et al., 2009). The central role of Th17 cells in human autoimmune diseases has been highlighted by genome-wide association studies that have linked genes preferentially expressed in Th17 cells, including *STAT3* and *IL23R*, to multiple human autoimmune diseases including psoriasis, Inflammatory Bowel Disease (IBD) and ankylosing spondylitis (Cho, 2008; Lees et al., 2011; Nair et al., 2009; Reveille et al., 2010; Zhang et al., 2012). Recent success in clinical trials for the treatment of psoriasis and rheumatoid arthritis with biologics that inhibit the Th17 cell pathway (Ixekizumab and Brodalumab) further underscores the importance of this pathway in human autoimmunity (Genovese et al., 2010; Leonardi et al., 2012; Papp et al., 2012). While blockade of IL-17A alone with Secukinumab, an IL17A monoclonal antibody, proved ineffective in Crohn's patients and may paradoxically worsen disease in a subset of patients (Hueber et al., 2012), Secukinumab has demonstrated potential for treatment of other autoimmune conditions including psoriasis, multiple sclerosis and ankylosing spondylitis (Patel et al., 2013), suggesting variability in response among diseases.

Each of several closely related, but highly functionally specialized $CD4^+$ helper T-cell populations enacts a distinct regulatory program, allowing for their diverse effector functions in the immune response. Accordingly, so-called “master” regulator TFs have been identified that are selectively expressed in each cell population and are required for their proper development and function. RORγt, the “master” TF of Th17 cells, is selectively expressed in Th17 cells, promotes Th17 cell differentiation, and is essential for the development of Th17 cells (Ivanov et al., 2006). Genomic studies have revealed transcriptional targets of key regulatory factors in other $CD4^+$ T-cell populations, including Foxp3 in regulatory T (Treg) cells (Birzele et al., 2011; Marson et al., 2007; Zheng et al., 2007), T-bet in Th1 cells and GATA3 in Th2 cells (Jenner et al., 2009; Wei et al., 2011; Kanhere et al., 2012). A systematic understanding of the genomic targets of RORγt and the transcriptional network that controls differentiation of Th17 cells is beginning to emerge (Ciofani et al., 2012; Yosef et al., 2013), and provides a unique opportunity to instruct the development of small molecular weight compounds that selectively suppress pathogenic effector functions of Th17 cells.

RORγt is an attractive pharmacologic target for the treatment of Th17 cell-mediated immune disorders because it plays a central role in Th17 cell function and is a nuclear receptor with a ligand-binding pocket. Indeed, several small molecular weight compounds have been identified that can inhibit the function of RORγt including Digoxin (Huh et al., 2011) and SR1001 (Solt et al., 2011). Although these molecules inhibit transcription of some genes that are preferentially expressed in Th17 cells, the direct transcriptional effects of RORγt inhibitors have not been analyzed, and no comprehensive examination of the effects of the molecules on RORγt targets and its transcriptional network has been possible.

Here, we report the identification of three small molecule inhibitors of RORγt that potently inhibit the development of Th17 cells and the severity of experimental autoimmune encephalomyelitis (EAE), a murine model of multiple sclerosis. To dissect the molecular mechanism underlying the effect of these compounds, we characterized the direct transcriptional targets of RORγt and report the transcriptional effects of the three small molecules, as well as Digoxin, on gene expression and on RORγt occupancy of its genomic targets. Remarkably, whereas one compound disrupts RORγt binding to genomic DNA, the other two compounds affects transcriptional regulation without globally eliminating RORγt DNA binding. This suggests that compounds can effectively disrupt the RORγt-dependent transcriptional program in Th17 cells either by displacing RORγt or by altering its transcriptional effects without affecting DNA-binding. These studies show the power of using genomic data to guide selection of drug candidates that can selectively inhibit functions of pathogenic Th17 cells and suppress autoimmunity.

Results

Screening for selective RORγt inverse agonists

Using a Fluorescence Resonance Energy Transfer (FRET) assay, consisting of the RORγt ligand-binding domain and cofactor peptide SRC1, we screened a proprietary small molecule library and identified several compounds that bind to RORγt (Supplemental Experimental Procedures). Of particular interest was a scaffold with a benzhydryl amide group that was selected for further chemistry optimization. Extensive SAR studies on the scaffold led to the identification of TMP778 and TMP920 as highly potent and selective RORγt inhibitors (Figure 1A). Both molecules share a benzhydryl amide moiety and an electron rich heterocycle (isoxazole). TMP778 possesses a rigid benzofuran ring in the central portion of the molecule, whereas TMP920 presents higher flexibility in that region with its aryl ether moiety. TMP778 and TMP920 inhibit RORγt binding to the SRC1 peptide in the FRET assay with IC_{50} of 0.005 μM and 0.03 μM, respectively (Figure S1A,B).

We initially confirmed the activity and selectivity of these putative RORγt inhibitors *in vitro* with a cell-based nuclear receptor reporter assay (Supplemental Experimental Procedures). Both compounds potently inhibited RORγt-dependent transactivation. Dose response curves for luciferase activity revealed that the half maximal inhibitory concentration (IC_{50}) of TMP778 was 0.017 μM in RORγ assays. By comparison, the IC_{50} was roughly 100 fold higher for RORα and RORβ, respectively (1.24 μM, 1.39 μM) (Figure S1C). The IC_{50} for TMP920 in RORγ assays was 1.1 μm (Figure S1D). Further highlighting the selective effect of these compounds on RORγt, the IC_{50} for both TMP778 and TMP920 was greater than 10

μM in luciferase assays for 22 other nuclear receptors (Figure S1E). These results indicate that TMP778 and TMP920, identified through the FRET assay, are selective and potent RORγt inhibitors.

RORγt inhibitors suppress Th17 cell differentiation *in vitro*

To determine if RORγt inhibitors affect Th17 cell differentiation *in vitro*, we cultured primary naïve $CD4^+$ T cells under Th17 cell-polarizing conditions in the presence of different doses of TMP778, TMP920, Digoxin, or DMSO (vehicle control). We first measured the effect of the inhibitors on cell proliferation. TMP778 at >2.5 μM and TMP920 and Digoxin at >10 μM started to show toxic effects on cell growth, which however is not RORγt-dependent, since the proliferation of RORγt-deficient T cells (*Rorc*$^{-/-}$; from CD4-Cre$^+$*Rorc*$^{fl/fl}$ mice which specifically do not express RORγ or RORγt in $CD4^+$ T cells) cultured under Th17 cell-polarizing conditions was also decreased (Figure S1F). Otherwise, these inhibitors did not show inhibitory effects on cell proliferation or RORγt expression or its nuclear translocation (RORγt expression was increased by some compounds, such as TMP920, Figure S1F,G), but efficiently inhibited IL-17 production. As reported previously, Digoxin, the first-identified RORγt inhibitor (Fujita-Sato et al., 2011; Huh et al., 2011), specifically inhibited IL-17 production in Th17 cell cultures at 10 μM; however, at <2.5 μM its inhibitory effect on IL-17 production was lost. Similarly, TMP920 lost its IL-17 inhibitory effect at <2.5 μM, while TMP778 had a much broader dose range and efficiently decreased IL-17 production (Figure S1F), consistent with its higher binding affinity for RORγt. These data indicate that TMP778 is the RORγt inhibitor that most potently reduced IL-17 production, followed by TMP920 and Digoxin. Based on the dose-response curves, we chose 2.5 μM of TMP778 and 10 μM of TMP920 and Digoxin for subsequent *in vitro* experiments, because at these concentrations the respective RORγt inhibitors are not toxic to the cells, but maximally inhibit the generation of Th17 cells (Figures 1B & S1F).

RORγt inhibitors suppress IL-17 production from differentiated Th17 cells *in vitro*

We next asked whether TMP778 and TMP920 also inhibit IL-17 production from differentiated Th17 cells. We re-stimulated draining lymph node (LN) cells from WT and RORγt-deficient mice immunized with MOG35-55 plus CFA for the development of EAE with MOG35-55 in the presence of IL-23 and with the different RORγt inhibitors, and measured cytokine production in $CD4^+$ T cells by intracellular cytokine staining. Compared to WT T cells, RORγt-deficient $CD4^+$ T cells showed much lower frequencies of IL-17^+ cells, but increased frequencies of IFNγ^+IL-17^- populations. All compounds inhibited IL-17 production (both IFNγ-IL-$17^+$and IFNγ^+ IL17^+ T cells were reduced) to different degrees in WT, but not RORγt-deficient $CD4^+$ T cells, with TMP778 demonstrating the most potent inhibition (Figure 1C). None of the compounds altered the frequencies of IFNγ^+IL17^- T cells in either WT or RORγt-deficient mice (Figure 1C). Also, all the compounds increased frequencies of IL-2^+ cells in IL-17^+ T cells (Figure S1H). These data suggest that the RORγt inhibitors inhibit Th17 responses not only by reducing IL-17 production, but also by blocking the decrease in IL-2 production that normally occurs as Th17 cells differentiate (McGeachy et al., 2009).

RORγt inhibitors suppress Th17 cell responses *in vivo* and ameliorate EAE

We next examined the *in vivo* effects of the inhibitors on EAE, in which the Th17 cell response plays a crucial role (Bettelli et al., 2006). We induced EAE in C57BL/6 mice with MOG35-55 plus CFA immunization in conjunction with subcutaneous administration of the inhibitors twice daily from day 0. All three compounds delayed the onset of disease and substantially reduced the severity of disease progression compared to control-treated mice (Figure 1D). Consistent with *in vitro* results, TMP778 treatment caused the most pronounced effect on the disease phenotype (by severity and day of onset). This treatment not only decreased the number of mononuclear cells infiltrating the central nervous system (CNS), but also most strongly reduced the percentage of IL-17$^+$ T cells in the CNS (including IL-17$^+$IFNγ$^+$; Figure 1E). There was no significant change in the percentage IFNγ$^+$IL-17$^-$ T cells in the CNS among all groups, indicating that none of the inhibitors affects Th1 responses. These data highlight TMP778 as the most potent RORγt inhibitor among the three tested compounds. TMP778 strongly inhibited Th17 cell generation, reduced IL-17 production from differentiated Th17 cells, and also dramatically ameliorated the progression of EAE.

RORγt inhibitors suppress the Th17 cell transcriptome and promote alternate T-cell subsets

Given the differential effects of the compounds on inhibition of Th17 cells and development of EAE, we proceeded to analyze the specific effects of each compound on gene transcription using RNA-seq. We measured the transcriptome of WT Th17 cells treated with TMP778, TMP920, Digoxin or DMSO, and of RORγt-deficient Th17 cells treated with DMSO. All samples were compared to DMSO-treated WT Th17 cells. We clustered differentially expressed genes (relative to vehicle-treated cells) using K-means clustering (Supplemental Experimental Procedures, Figure 2A & Table S1), and observed five clusters, of which Clusters 1 and 2 were the largest. Cluster 2 consists of genes that are suppressed following all perturbations (chemical or genetic) of RORγt, including many Th17 cell specific genes (e.g., *Il17a*, *Il23r*). Conversely, Cluster 1 genes are induced following all perturbations, and include signature genes from other CD4$^+$ T-cell lineages (e.g., *Il4* and *Tbx21*, which encodes T-bet). Overall, the most pronounced effect of RORγt inhibition is decreased expression of Th17 cell signature genes, but there is also an increase in the expression of genes that are preferentially expressed in other CD4$^+$ T-cell lineages (Figures 2A & S2A).

To further analyze this ‘balanced’ pattern, we examined the effect of each perturbation on sets of signature genes, computationally derived for each CD4$^+$ T-cell lineage using published expression data (Wei et al., 2009) (Figure 2B & Supplemental Information). Indeed, the RORγt inhibitors and genetic ablation strongly suppress the expression of Th17 cell signature genes ($p<10^{-4}$; Table S1), but also increase the expression of signature genes from other CD4$^+$ lineages, most strongly for Th1 cell signature genes (e.g., *Ifng* and *Tbx21*), and more mildly for Th2 cell genes (e.g., *Il4*) (Figures 2A, S2A & Table S1). Consistently, we see significant overlaps between the genes affected by each perturbation and known targets of key TFs, both in Th17 cells (e.g., Batf and IRF4) and in other CD4$^+$ T cells (e.g., STAT4, GATA3, and Foxp3; Table S1; this analysis is based on publically available data of

TF-target interactions, see Supplemental Experimental Procedures). Overall, these results suggest a mode of competition or balance, modulated by the transcriptional activity of RORγt (Bettelli et al., 2006; Yang et al., 2011; Zhou et al., 2008).

To further confirm the potential medical relevance of these observations, we verified the effects of TMP778 and TMP920 on Th17 cell signature genes in human cells (Supplemental Information). We tested the effects on Th17 cell differentiation *in vitro* from naïve T cells and on differentiated Th17 cells re-stimulated with IL-23 (using different doses; Figures S2B-S2K). We found that genes down-regulated following TMP778 treatment of $CCR6^+$ memory human T cells (i.e., population enriched in Th17 cells) are overall up-regulated in Th17 cells (comparing $CCR6^+$ to $CCR6^-$ memory T cells), and vice versa. Furthermore, in a population depleted for Th17 cells ($CCR6^-$), TMP778 has a very minor effect on transcription (no differentially expressed genes with a fold cutoff over 1.5), indicating that its effects are largely restricted to Th17 cells.

TMP778 most closely mimics the effect of RORγt deletion

Although many transcriptional effects are common to all perturbations (chemical inhibitors and *Rorc* gene ablation), there is also substantial variation, suggesting different mechanisms of action (Figure 2C). To estimate the overall extent to which the chemical perturbations recapitulate genetic ablation of RORγt, we computed the overlaps between their affected genes and the genes affected by the RORγt deficiency. Digoxin has the highest specificity rate (a measure of the chance that a gene affected by a compound is affected in the same way in the RORγt deficiency), followed by TMP778 and TMP920. However, TMP778 has the highest sensitivity (a measure of the chance that a gene affected in the RORγt deficiency is affected in the same way by compound), followed by TMP920 and Digoxin. Figure 2C shows the sensitivity and specificity of each compound across a range of different fold changes in transcript levels. A combined measure of specificity and sensitivity (harmonic mean, or F-score) provides an overall estimate by which TMP778 has the highest similarity to the RORγt-deficient effects, especially at genes that show strong differential expression (Figure 2C), which is in agreement with its more potent effects on the Th17 cell phenotype *in vitro* and *in vivo*.

Identification of RORγt binding sites at the Th17 cell genome

Some of the effects of RORγt inhibition (either by chemical agents or by genetic manipulation) may be direct, whereas others may reflect indirect events, either within Th17 cells, or due to changes in the balance of T-cell populations. To better distinguish these possibilities, we used ChIP-Seq to determine the direct transcriptional targets of RORγt in Th17 cells (Figure 3A). Binding events were selectively identified with a RORγt-specific antibody using two controls: an isotype control immunoglobulin and the RORγt-specific antibody in RORγt-deficient cells. Overall, our assay detected 2,257 high-confidence RORγt binding sites (Supplemental Experimental Procedures) The accuracy of the detected binding sites is further supported by a highly significant ($p<10^{-10}$) DNA-binding motif that is present in 58% of the sites (Figure 3B) that is nearly identical ($p<10^{-8}$) to the binding motif of the nuclear receptor RORα. The DNA motif is also found, albeit with considerably more noise, in anti-FLAG ChIP-seq with the epitope-tagged exogenous RORγt in EL4 cells, a murine

lymphoma cell line ($p=2.6*10^{-3}$) (Supplemental Experimental Procedures, Table S2). Interestingly, we also find additional motifs enriched ($p<10^{-5}$) in the RORγt binding sites, including SP1, AP-1, and STAT3. These results are in line with previous findings of RORγt binding in proximity to STAT3, IRF4 and Batf (Ciofani et al., 2012). Taken together, these findings suggest that our ChIP-Seq data reveal high-confidence RORγt binding sites throughout the genome.

RORγt binds genes associated with function of Th17 cells and other CD4$^+$ T cells, acting as a direct activator and repressor, respectively

There is a substantial overlap between the genes affected by inhibition of RORγt (chemical or genetic) and those that are directly bound by it (Figures 2B, 3C & Table S1), with bound targets also highly enriched for both Th17 cells signature genes and for signature genes of other CD4$^+$ T-cell types (Figure 4A). On the one hand, RORγt binds signature Th17 cell cytokines such as *Il17a* and *Il7f*, receptors for cytokines that promote Th17 cell differentiation (e.g., *Il23r*), and key regulators of T-cell activation and Th17 cell differentiation (e.g., *Irf4*, *Junb*, *Ets1*, *Nfatc2*). On the other hand, it binds genes such as that encoding the cytokine IL-2, which induces Th1 cells and inhibits Th17 cell differentiation, and inhibition of RORγt induces *Il2* transcription from Th17 cells in our RNA-seq experiments. This strongly supports our model of a 'balanced' effect of RORγt on both Th17 cell signature genes (positively) and signature genes of other CD4$^+$ T cells (negatively), through a *direct* mechanism. The fact that there is no discernable bias towards up- or down- regulation of bound genes (chi square test, $p>0.1$) suggests that RORγt can act both as an activator at loci that promote Th17 cell differentiation, and as a repressor at other loci associated with other CD4$^+$ T-cell subsets. Interestingly, we find that target genes whose binding sites contain the RORα binding motif tend to show stronger overlap with the Th17 cell signature genes and the genes affected by inhibition of RORγt (Figure S3). These results may suggest that RORγt activity through *cis*-regulatory sites that contain the RORα binding motif could be more relevant to its role in Th17 cell differentiation. Notably, there is no discernable bias towards up- or down-regulation also when considering only binding targets that are associated with the RORα motif.

RORγt co-localizes with other master regulators of CD4$^+$ T-cell subsets

The chemical inhibitors (and genetic perturbation) of RORγt lead both to a decrease in the expression of Th17 cell signature genes and to an increased expression of genes important for the distinct function of other CD4$^+$ T-cell lineages. We hypothesized that RORγt would co-occupy DNA elements with other Th17 cell factors to coordinately activate genes important for Th17 cell function, and also bind to regulatory regions that are targeted by TFs in other lineages to inhibit the expression of genes important for those other lineages. To further explore how RORγt participates in the control of genes important for Th17 cell function and signature genes from other CD4$^+$ T-cell lineages (Figure 4A), we searched for other TFs that share target genes with RORγt (Supplemental Experimental Procedures).

The individual binding sites and genes targeted by RORγt significantly overlap with the specific binding sites and target genes of key Th17 cell regulators ($p<10^{-3}$ for gene targets, $p<10^{-10}$ for individual binding sites; Table S2). In particular, a significant portion of RORγt

binding sites are also occupied by STAT3 or STAT5 (40% of sites; e.g. Figure 4B,C) (Yang et al., 2011), and/or by the pioneering factors IRF4 and Batf (26% and 59% of peaks, respectively) (Glasmacher et al., 2012). This is consistent with recent reports that co-binding of RORγt with other transcriptional regulators promotes the expression of genes that are crucial for Th17 cell function (Ciofani et al., 2012). Notably, the *Rorc* locus is targeted by some of these key factors, including IRF4 and RORγt itself (Figure 4C), forming feed-forward, feedback and auto-regulatory loops. Thus, RORγt is a central node in a densely inter-connected cooperative network for activating Th17 cell genes.

Interestingly, there is also a significant overlap between RORγt binding sites and those of factors controlling other T-cell subsets, consistent with a model of directly opposing transcriptional effects. For example, comparing RORγt binding sites to GATA3 binding sites in Th2 cells (Wei et al., 2011), we discovered a statistically significant overlap (>6.5% of binding sites; >20 fold enrichment), suggesting that divergent transcriptional effects at shared target genes, may be a mode of action of RORγt to promote the Th17 cell state.

The reciprocal relationship between developmental pathways controlling the differentiation of RORγt$^+$ Th17 cells and Foxp3$^+$ Treg cells (Bettelli et al., 2006) raises the hypothesis that similar (or stronger) overlaps may exist with the Treg cell TF Foxp3. Since ChIP-seq data for Foxp3 in induced regulatory T (iTreg) cells was not previously published, we conducted ChIP-seq analysis of Foxp3 in iTreg cells and compared the results to the RORγt binding sites in Th17 cells. We find that >10% of the binding sites are shared between the two factors ($p<10^{-10}$; 28 fold enrichment), covering many key genes. For example, the RORγt and Foxp3 overlapping regions include the *Il17* loci, and the promoters and putative enhancers of genes that characterize either Th17 or iTreg cells, including *Il23r*, *Ctla4*, *Il2*, *Il21*, *Il2ra*, *Il7r*, *Ptpn22*, and *mir-155* (Figures 4B,C). The findings strongly suggest that RORγt and Foxp3 promote reciprocal developmental pathways by acting at a shared set of genomic regions in Th17 and Treg cells, respectively. Furthermore, these findings lend insight into the observation that a subset of Treg cell signature genes tend to be expressed at higher levels in Th17 cells treated with RORγt inhibitors (Figure 2B).

Distinct Effects of Inhibitors on RORγt-DNA Interactions

Whereas small molecule inhibition largely recapitulates the transcriptional effect of genetic ablation of RORγt, including the effect on its direct targets (Figure 2B), this does not clarify the mechanism by which the compounds disrupt the regulatory circuitry. In principal, the compounds could either reduce the occupancy of RORγt at DNA regulatory elements or they could disrupt the transcriptional effects of RORγt without affecting its DNA binding, by affecting protein-protein interactions and suppressing RORγt-dependent gene transcription. To differentiate between the two models, we performed ChIP-seq analysis for RORγt in Th17 cells treated with each compound, or vehicle-control, and in RORγt-deficient cells.

Strikingly, TMP920 significantly reduces the occupancy of RORγt at the majority of its target genomic elements (decreased ChIP signal in 77% of sites; p<1e-10, left-tailed t-test, comparing to fold changes in untreated Th17 cells vs. DMSO-treated Th17 cells), whereas in notable contrast, RORγt binding to the genome is largely preserved in cells treated with

TMP778 (lower ChIP signal in 55% of sites, p>0.5; Figures 5A & S4, S5). The effect on RORγt binding in Digoxin-treated cells is intermediate, but much of the RORγt binding is preserved in these cells as well. To rule out that binding differences were secondary to transcriptional inhibition of *Rorc*, we confirmed that, at the concentrations used here, the respective RORγt inhibitors do not significantly inhibit the mRNA expression of *Rorc* (Figure S2B). These findings suggest that the chemical inhibitors affect the transcriptional network by different mechanisms. In particular, TMP778, the most potent compound, which most closely recapitulates the transcriptional effects of RORγt deficiency, has the least pronounced effect on RORγt DNA-binding, as observed by ChIP-seq analysis.

To confirm these findings and explore them further, we performed ChIP-PCR analysis for a selected set of loci that showed variable RORγt binding depending on chemical treatment. At the panel of loci that we tested, we confirmed the observation that much of RORγt binding is preserved in cells treated with TMP778 and partly preserved in cells treated with Digoxin (Figure 5B & S4). At multiple loci, we observe a dose-related effect on RORγt binding with each of the compounds. However, at the concentrations where we observe potent transcriptional and phenotypic effects with TMP778, RORγt binding to DNA is preserved at most of its target regions, including multiple loci within the *Il17a* and *Il17f* genomic region (Figure 5B).

In addition to examining loss of RORγt binding as a result of chemical treatment, we also examined if any RORγt DNA-binding interactions were further enhanced or stabilized as a result of the chemicals. ChIP-seq experiments have significant noise and well-known potential for false negative results at any particular locus. Nonetheless, we employed a stringent computational approach (using a noise model based on variation between RORγt binding patterns observed in untreated cells and vehicle treated cells) to identify candidate loci where RORγt DNA-occupancy appears to be stabilized by chemical treatment. Remarkably, this approach revealed that treatment with TMP778 led to RORγt occupancy of 179 new binding sites not observed previously in our data in Th17 cells (8.2% of peaks observed with TMP778; using several criteria for peak filtering; see Supplemental Experimental Procedures; Table S3) Notable among them is the TMP778-dependent binding of RORγt to the *Gata3* locus, encoding the “master” TF for Th2 cell differentiation. RORγt binding to two intronic regions within the *Gata3* locus was only observed in cells treated with TMP778, one of which was further confirmed with statistical significance with ChIP-PCR (Figure 5C). Indeed, the RNA-seq data reveal that *Gata3* is expressed at higher levels in cells treated with TMP778 (Figure 2A), and intracellular staining data confirm increased expression of GATA3 protein in cells treated with TMP778 (Figure S6A). Furthermore, genes bound by GATA3 (Wei et al., 2011) were also up-regulated following TMP778 treatment compared to vehicle treatment ($p<10^{-10}$). Interestingly, the *Gata3* locus is also occupied by the Th17 cell pioneering TFs IRF4 and Batf in Th17 cells (Glasmacher et al., 2012), and by the Treg cell TF Foxp3 in iTreg cells. More generally, we find that a substantial percentage of all new TMP778-dependent binding sites is similarly occupied by IRF4 and Batf in Th17 cells (14.5% and 65% respectively), consistent with the published model suggesting that these pioneering factors promote chromatin accessibility (Ciofani et al., 2012). These data suggest that the chemical inhibitors, in addition to inhibiting the Th17

cell transcriptional program, may also promote stabilization of RORγt to unique binding sites to induce transcriptional modules specific to each inhibitor.

Orally available compound GSK805 inhibits the RORγt-dependent transcriptional network to treat Th17 cell-mediated autoimmunity

Although two inhibitors presented here potently inhibit Th17 cell responses, especially TMP778, their potential clinical usage is limited by their required subcutaneous administration. Further screening with a FRET-based assay, we obtained a new RORγt inhibitor GSK805 (Figure 6A). At a dose of 0.5 μM, compound GSK805 showed comparable inhibition of IL-17 production as TMP778 at 2.5 μM, during Th17 cell differentiation (Figure 6B), suggesting that GSK805 is an even more potent inhibitor of Th17 cell responses than TMP778. Strikingly, when orally administrated into the hosts starting at the time of the disease induction, the compound GSK805 could efficiently ameliorate the severity of EAE (Figure 6C). Analysis of CNS samples after 14 days of GSK805 treatment revealed that the treatment strongly inhibits Th17 cell responses (reduced both IFNγ$^-$IL-17$^+$ and IFNγ$^+$IL-17$^+$ T cells) in the CNS without significant alteration in the frequency of TNF-α$^+$ T cells) (Figure 6D & S6B).

We then determined the global effects of GSK805 on transcription in Th17 cells using a cost efficient RNA-seq protocol. We see an overall high degree of similarity in the effects of GSK805 and the other compounds (Figure 6E; r^2>0.5; p<1e-10). ChIP-PCR data suggest that similar to TMP778, compound GSK805 did not affect RORγt binding to DNA in many gene loci, and also the compound induced RORγt binding to *Gata3* locus and was associated with increased GATA3 protein expression (Figure S6A,C)

Taken together, these results indicate that GSK805, an orally administered compound, inhibits RORγt transcriptional effects and Th17 cell function through mechanisms that overlap with those of TMP778. The potency and oral bioavailability of GSK805 suggest that it could be a promising lead compound for the treatment of Th17 cell-mediated diseases.

Discovery of a densely inter-connected regulatory network downstream of RORγt

Our data collectively reveal that RORγt plays a central role in a transcriptional network shaping CD4$^+$ T-cell identity. We discovered that the genomic binding sites of RORγt neighbor the binding sites of other key TFs in Th17 cells and TFs in other T-cell subsets, as assessed by DNA motif analysis and ChIP-Seq. By computing the overlaps between the RORγt-bound genes and targets of other TFs, we find many cases of substantially overlapping TFs, including STAT4, GATA3, and Foxp3. The transcriptional data from RORγt-deficient cells provides further support: the affected genes also overlap with the targets of a similar set of TFs (including STAT4, GATA3, and Foxp3). Together these results provide functional support for the RORγt-centric network presented in Figure 7 and place RORγt as a regulatory hub that affects not only Th17 cell signature genes but can also directly or indirectly affect the regulatory program of other T-cell subsets. These data suggest a transcriptional regulatory network where an important set of genes, encoding proteins with roles in T-cell differentiation and effector function, are coordinately regulated by a core set of “master” TFs that control CD4$^+$ T-cell lineage differentiation. These TFs

could be acting cooperatively to enforce appropriate gene expression programs and pharmaceutical compounds could disrupt necessary protein-protein interactions and thus modulate T-cell differentiation. Indeed, we highlight the potent modulation of this network by compounds that inhibit RORγt-dependent Th17 cell differentiation and affect the network in a similar manner to genetic ablation of RORγt.

Discussion

We report here three small molecule inhibitors of RORγt, a nuclear receptor that is essential for Th17 cell development. We provide *in vitro* and *in vivo* evidence that these molecules repress the development of Th17 cells and have a substantial effect in ameliorating the autoimmune disease EAE, a murine model of multiple sclerosis. Strikingly, the compound GSK805 is not only more potent in inhibiting Th17 cell responses than other compounds, it can also be orally administrated for treatment of Th17 cell-mediated autoimmune diseases, such as EAE. To better characterize these inhibitors, we have analyzed their function within the context of the transcriptional network that is controlled by RORγt in Th17 cells. We discover that RORγt directly controls the expression of a set of genes that lie at the core of Th17 cell identity and also contributes the repression of signature genes of other $CD4^+$ T-cell lineages. The chemical molecules largely recapitulate the transcriptional effects of genetic ablation of RORγt on these target genes. Furthermore, we use ChIP-seq to identify the DNA regulatory elements directly occupied by RORγt and to assess the effects of the small molecules on DNA-binding. In addition to providing insight into these compounds that could serve a therapeutic role in the treatment of human autoimmune disease, these studies also provide a unique paradigm for combining drug discovery efforts with mechanistic, genome-wide analysis of transcriptional regulation in defined primary T cells that mediate tissue inflammation.

Importantly, the RORγt network targeted by the identified compounds includes “master” regulators in other $CD4^+$ T-cell populations that are either transcriptionally affected by RORγt perturbation, bound by RORγt, or share target genes with RORγt. These findings offer insight into our observation that perturbation (by either genetic ablation or by targeting compounds) of RORγt not only inhibits the expression of Th17 cell signature genes, but also contributes to the activation of signature genes from other T-cell linages. Specifically, we demonstrate that the “master” Treg TF Foxp3 binds in iTreg cells a significant percentage of the regulatory elements occupied by RORγt in Th17 cells. This is consistent with a model where STAT3, which promotes Th17 cell differentiation, and STAT5, which promotes Treg development, may compete for genomic binding sites (Yang et al., 2011), where RORγt binds. Our discovery of the overlap between RORγt and Foxp3 further suggests that the reciprocal development of Th17 and Treg cells is driven, at least in part, by differential regulation of key set of common target genes. This appears to be a general feature of the regulatory circuitry controlling the closely related $CD4^+$ T-cell lineages. For instance, T-bet and GATA3 occupy a shared set of promoter elements to regulate differentiation towards Th1 and Th2 lineages, respectively (Jenner et al., 2009).

One of the major limitations in developing therapeutic agents is the challenge of identifying compounds that affect a specific biologic target and downstream network with minimal off-

target affects. In the studies described here, the biologic target of interest is a transcriptional regulator. Here, we establish a proof of principle that systematic, quantitative studies of the DNA binding and transcriptome of cells treated with the identified compounds can provide insight into their biologic effect. The ideal is to discover a compound that would mirror all of the RORγt-dependent transcriptional effects (which we describe as the drug's 'sensitivity' in hitting the desire transcriptional targets), with no collateral effect on genes not controlled by RORγt (which we measure as the drug's 'specificity'). Using the genomic analysis described here, it may even possible to selectively inhibit pro-inflammatory modules activated by RORγt while leaving other modules activated by RORγt intact. We use an F-score to integrate the 'sensitivity' and 'specificity' of the studies compounds. Using this index, TMP778 performs better than Digoxin and TMP920, which is consistent with its potent role in abrogating the *in vivo* and *in vitro* differentiation of Th17 cells. Furthermore, specific transcriptional modules that are selectively induced by a compound of interest, such as the GATA3-dependent module induced by TMP778 and GSK805, may ultimately provide insights into unexpected effects of candidate therapeutics. This systematic approach is likely to prove useful as a growing number of compounds targeting transcriptional regulators become available as potential therapeutic agents for a wide range of human diseases.

We further characterized the molecular mechanism of the identified compounds using ChIP-Seq. This approach unexpectedly revealed significant divergence in the mechanisms by which the various compounds affected the transcriptional network downstream of RORγt. Notably, the compound with highest F-score as discussed above, had the least profound effect on RORγt-DNA binding. This finding underscores that occupancy of the genome at key regulatory elements is not sufficient for "master" regulators to exert their transcriptional effects. TFs, including nuclear receptors such as RORγt, depend on contact with co-regulatory molecules to control gene expression. All three compounds described here, TMP778, TMP920 and GSK805 were identified as an inverse agonists that interact physically with the putative ligand-binding domain of RORγt. Through its interaction with this domain, TMP920 appears to also disrupt RORγt binding to DNA, while TMP778 and GSK805 interactions with RORγt ligand binding domain exert less pronounced effects on DNA binding. This raises the possibility that these compounds exert their pharmacological effects by disrupting RORγt interaction with a currently unidentified ligand, which may affect its ability to recruit co-regulators or the RNA-polymerase machinery independent of whether or not DNA-binding is disrupted.

In summary, we have identified compounds that antagonize the transcriptional effects of RORγt. These compounds block Th17 cell differentiation and help to limit Th17 cell-mediated diseases. Furthermore, we gain insight into the mechanism of these compounds by examining in detail the transcriptional regulatory circuitry of Th17 cells. The network model provided here highlights the transcriptional effects of the compounds on genes that lie at the core of Th17 cell function. In turn, these data also serve as valuable resource for those interested in studying genes that are under direct transcriptional control of RORγt in Th17 cells, including genes encoding effector molecules, cell signaling components, transcriptional regulators, cytokines and cell surface molecules. These studies represent a

unique approach of combining drug discovery efforts with systematic genomic investigations of transcriptional regulation, which can predict specific, off-target and new-target effects induced by a drug candidate. This approach can be very instructive in selecting lead candidates and has considerable potential to aid in the drug discovery and identification of new effective therapeutic agents for human diseases.

Experimental Procedures

Mice and Reagents

C57BL/6 mice and *RORγt*^{GFP} mice, and *Rorc*$^{fl/fl}$ mice were purchased from The Jackson Laboratory. *Rorc*$^{fl/fl}$ mice were bred with *CD4-Cre* transgenic mice to obtain T cell-specific RORγ/RORγt null mice. Mice were maintained and all animal experiments were done according to the animal protocol guidelines of Harvard Medical School and GSK. MOG_{35-55} was synthesized by Quality Controlled Biochemicals. Digoxin and DMSO were purchased from Sigma-Aldrich. TMP778 and TMP920 and GSK805 were synthesized by Tempero Pharmaceuticals and GSK, respectively. All fluorescence-conjugated Abs were obtained from Biolegend, eBioscience and BD Biosciences. All cytokines were purchased from eBioscience and R&D Systems.

Naïve CD4$^+$ T-cell isolation and stimulation

$CD4^+CD62L^{high}CD25^-$ naive $CD4^+$ T cells were purified by FACS sorting following a MACS bead isolation of $CD4^+$ cells as previously described (Xiao et al., 2008). Naive $CD4^+$ cells were activated with plate-bound anti-CD3 (2 μg/ml) and anti-CD28 (2 μg/ml). For Th17 cell differentiation, cultures were supplemented with IL-6 (20 ng/ml) plus TGF-β1 (1 ng/ml), and IL-23 (10 ng/ml) was added after 48 h. RORγt inhibitors or vehicle control DMSO was also included in the cultures, or as indicated in figure legends. After 96 h, cells were collected for further experiments.

EAE induction and treatment with TMP778, TMP920 and Digoxin

Female C57BL/6 mice (8–12 wk old) were immunized s.c. in the flanks with an emulsion containing MOG_{35-55} (100 μg/mouse) and *M. tuberculosis* H37Ra extract (3 mg/ml, Difco Laboratories) in CFA (100 μl/mouse). Pertussis toxin (100 ng/mouse, List Biological Laboratories) was administered i.p. on days 0 and 2. RORγt inhibitor (TMP778, 200 μg per injection; TMP920, 500 μg per injection; Digoxin, 50 μg per injection, >100 μg caused mouse death) were subcutaneously injected twice daily starting from day 0 throughout the period of the experiments. Mice were monitored and assigned grades for clinical signs of EAE as previously described (Xiao et al., 2008).

Flow cytometry

For intracellular cytokine staining, cells were stimulated in culture medium containing phorbol 12-myristate 13-acetate (PMA, 30 ng/ml, Sigma-Aldrich), ionomycin (500 ng/ml, Sigma-Aldrich), and GolgiStop (1 μl/ml, BD Biosciences) in a cell incubator with 10% CO_2 at 37°C for 4 h. After staining surface markers, cells were fixed and permeabilized using Cytofix/Cytoperm and Perm/Wash buffer (BD Biosciences) according to the manufacturer's instructions. Then, cells were stained with fluorescence-conjugated cytokine Abs at 25°C for

30 min before analysis. 7-aminoactinomycin D (BD Biosciences) was also included to gate out the dead cells. All data were collected on a FACSCalibur or an LSR II (BD Biosciences) and analyzed with FlowJo software (TreeStar).

ChIP-seq and RNA-seq

Antibodies used for ChIP were anti-RORγt (Clone AFKJS-9, eBioscience), anti-Foxp3 (Zheng et al., 2007), anti-FLAG (clone M2), and IgG control. Purified ChIP DNA was used to prepare ChIP-seq libraries using the Illumina TruSeq DNA Sample Preparation v2 kit. Total RNA was used to prepare RNA-seq libraries using the Illumina TruSeq RNA Sample Preparation Kit. Libraries were sequenced with single-end 36 bp reads on an Illumina GAII. Sequencing data were analyzed as described in Supplemental Experimental Procedures.

Detailed experimental procedures can be found in Supplemental Experimental Procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Identification of three RORγt inhibitors, one of which is orally bioavailable.
- RORγt inhibitors are effective in animal models of autoimmunity.
- Integration of drug discovery with Th17 cell genomics.
- Chemical inhibitors have distinct effects on RORγt binding.

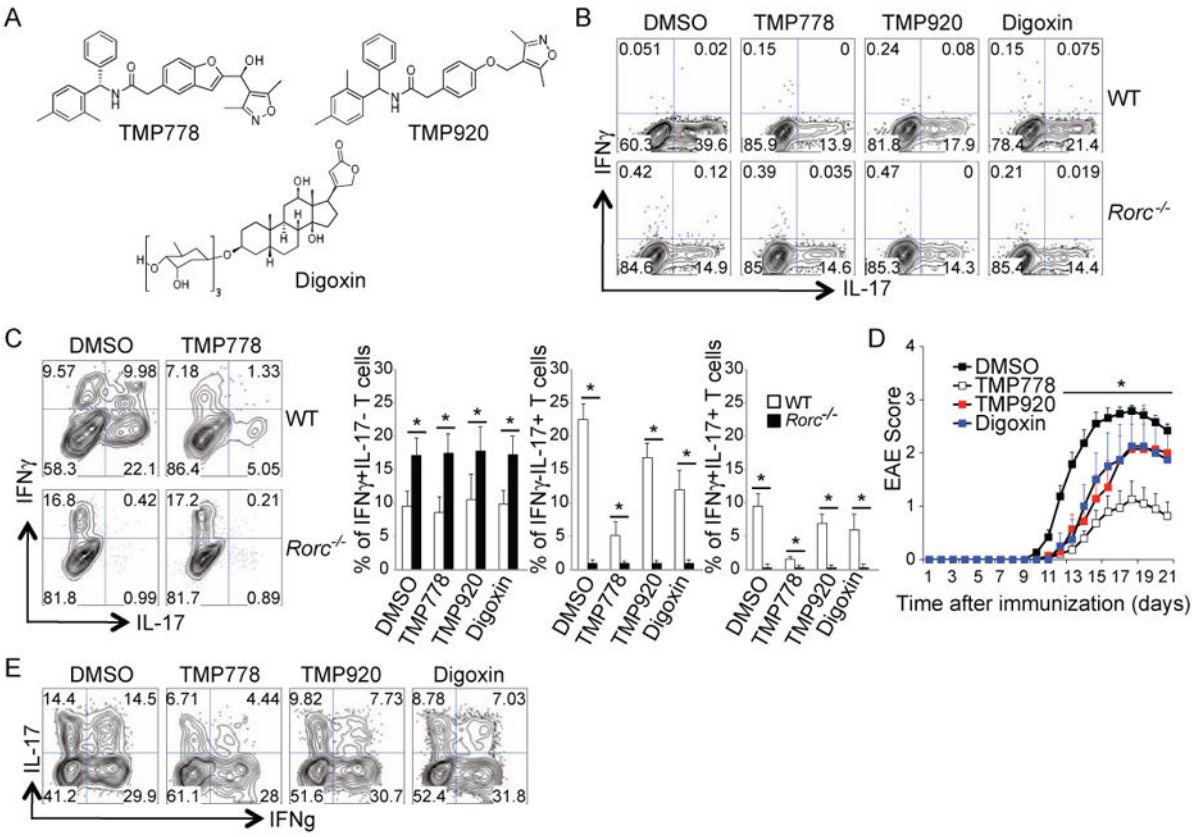

Figure 1. RORγt inhibitors suppress Th17 cell differentiation and maintenance and ameliorate EAE

(**A**) Chemical structures of TMP778, TMP920, and Digoxin. (**B**) Naïve CD4$^+$ T cells were activated with anti-CD3 and CD28 under Th17 cell-polarizing conditions in the presence of optimal doses (not toxic but with maximal IL-17 inhibition) of TMP778 (2.5 μM), TMP920 (10 μM), Digoxin (10 μM), or DMSO. After 4 days, IL-17 and IFNγ production were measured by intracellular cytokine staining. Data are representative of 5-8 experiments. (**C**) Draining LN cells from mice immunized with MOG35-55 plus CFA for the development of EAE were re-stimulated with MOG35-55 in the presence of IL-23 plus TMP778, TMP920, Digoxin, or DMSO. After 4 days, production of IL-17 and IFNγ in CD4$^+$ T cells was determined by intracellular cytokine staining. Left panel shows representative FACS plots the frequencies of IFNγ and IL17 producing cells in gated CD4$^+$ T cells from samples treated with DMSO and TMP778; right panel shows the statistical data (n=5). Error bars represent the mean ± SD. * $p<0.01$. (**D**) C57BL/6 mice were immunized with MOG35–55 plus CFA, and RORγt inhibitor (TMP778, 200 μg per injection, n=19; TMP920, 500 μg per injection, n=7; Digoxin, 50 μg per injection, n=5, >100 μg caused mouse death; DMSO, n=19) were subcutaneously injected twice daily starting from day 0. Mice were evaluated daily for signs of EAE. Error bars represent the mean ± SD. * $p<0.05$ when 11 days after groups of mice treated with different RORγt inhibitors were compared with the group of mice with DMSO (vehicle control) treatment. (**E**) CNS-infiltrating mononuclear cells were isolated from brains and spinal cords of the mice on day 21 after EAE induction. IL-17 and

IFNγ production of CNS-infiltrating $CD4^+$ T cells were determined by intracellular staining. Data are representative of 4-5 mice in each group.

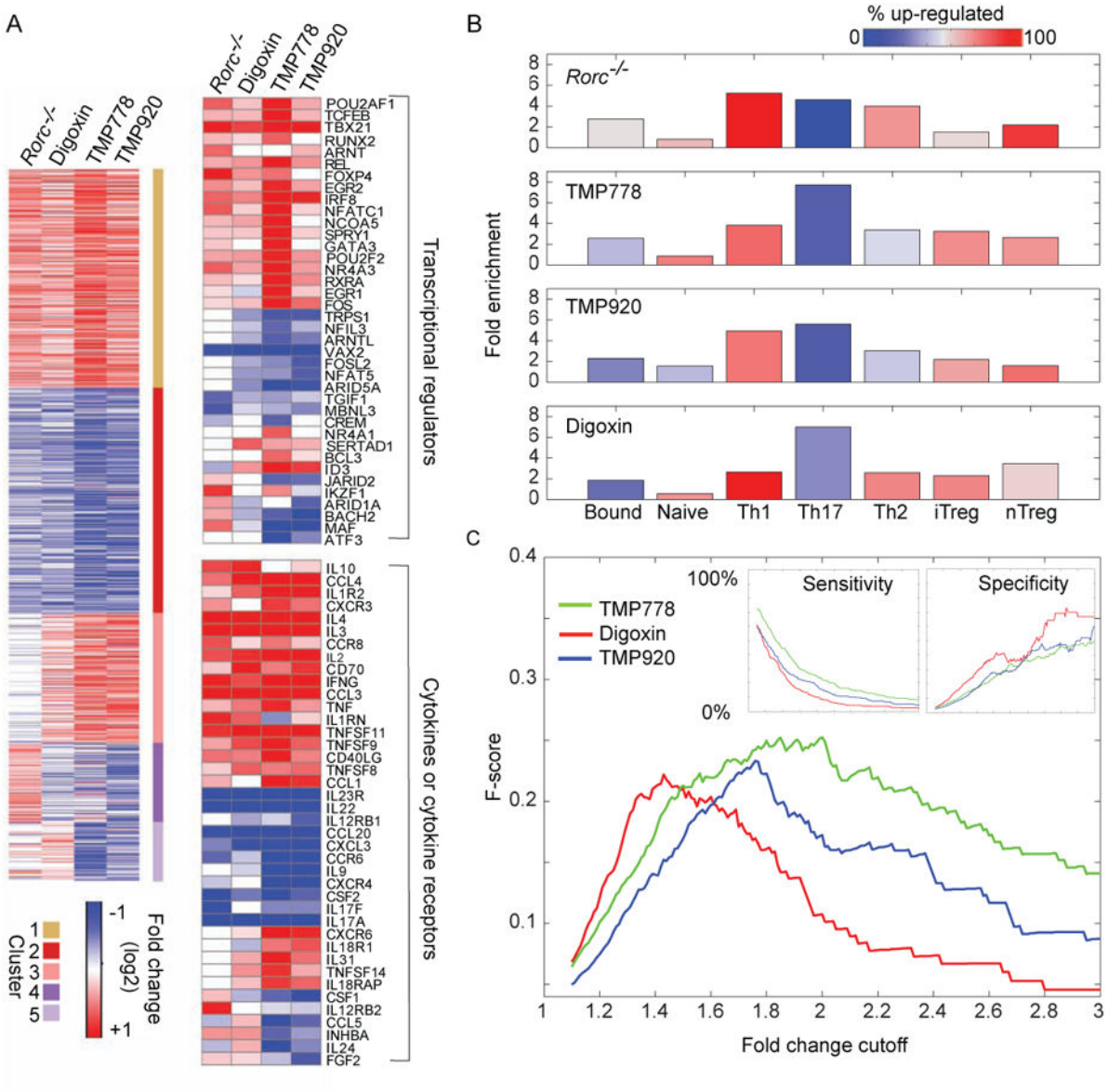

Figure 2. Effects of RORγt inhibitors on the Th17 cell transcriptome

(**A**) Heat map displaying the fold changes of genes (rows) in the various perturbations (columns). Displayed are only genes that were differentially expressed (fold>1.5) in at least one condition. On the right, differential expression of selected genes encoding transcriptional regulators (above) and cytokines or cytokine receptors (below). (**B**) Enrichment of the differentially expressed genes in gene signatures of different T-cell subsets. The height of the bars indicates fold enrichment; the color of the bars indicates the percentage of genes in the overlap (i.e. genes that are both differentially expressed and belong to the respective signature) that are over expressed (from blue to red). (**C**) The overlap between the sets of genes affected by each compound and the sets of genes affected by RORγt deficiency is evaluated using an F-score: the harmonic mean of their specificity (% of compound-affected genes that are affected by RORγt deficiency) and sensitivity (% of

RORγt deficiency-affected genes that are affected by the compound). Results are presented for different fold change cutoffs for calling differential expression in the compounds.

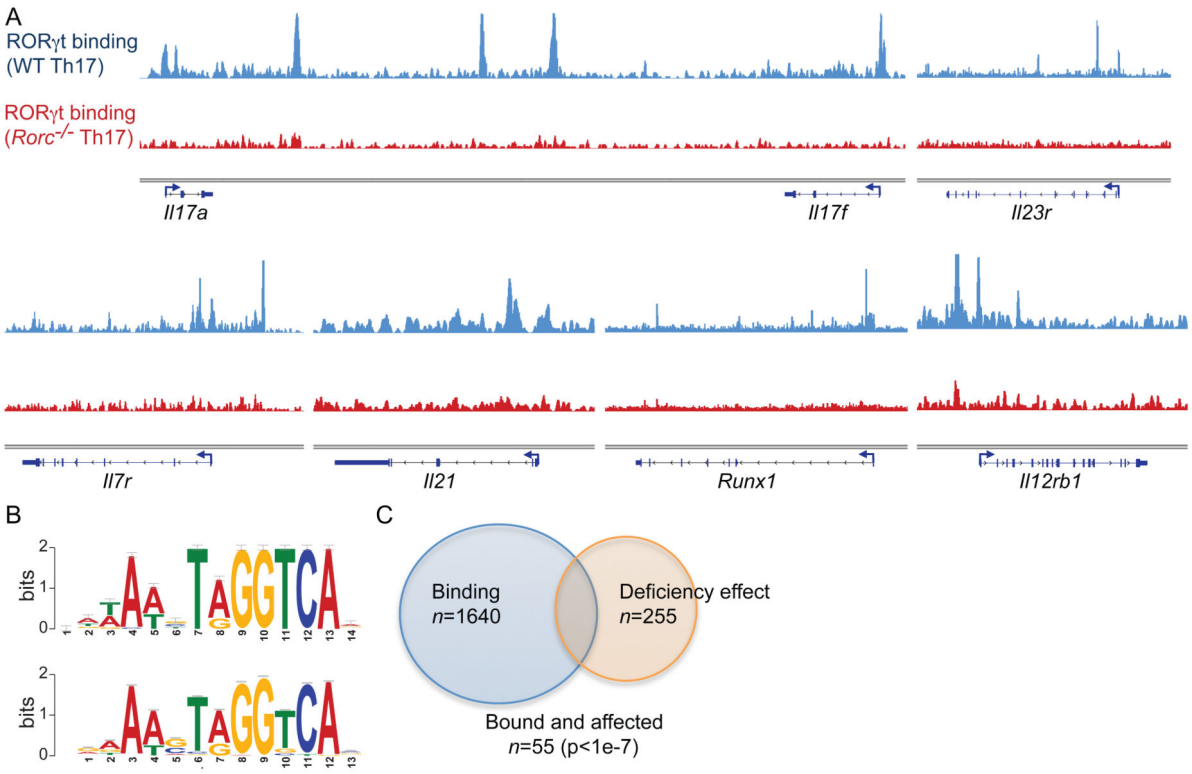

Figure 3. The binding landscape of RORγt in Th17 cells

(**A**) RORγt binding at key Th17 cell gene loci. (**B**) RORγt binding motif (bottom) highly matches the known RORα binding motif (top). (**C**) Overlap between the set of genes bound by RORγt and the genes affected by RORγt deficiency.

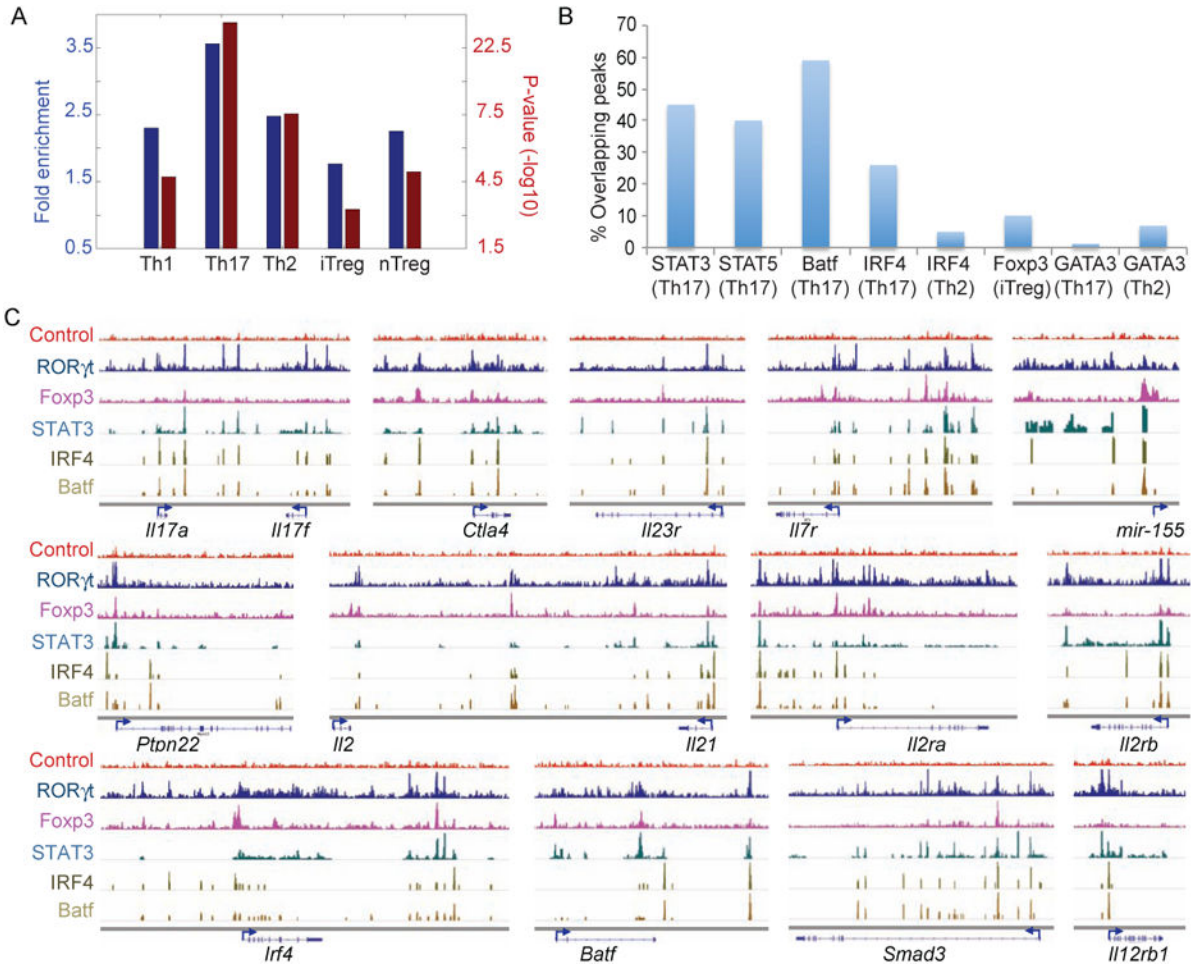

Figure 4. RORγt selectively targets genes associated with Th17 cell function and signatures of other CD4$^+$ T cells

(**A**) Enrichment (blue bars) and significance (brown bars) of RORγt target genes in signatures of different subsets of T cells. (**B**) Percentage of RORγt binding sites that are also occupied by other TFs in Th17 cells and other CD4$^+$ T-cell subsets. (**C**) RORγt binding sites overlap with STAT3 (Yang et al., 2011), IRF4, and Batf in Th17 cells (Glasmacher et al., 2012), and Foxp3 in iTreg cells at selected key target gene loci.

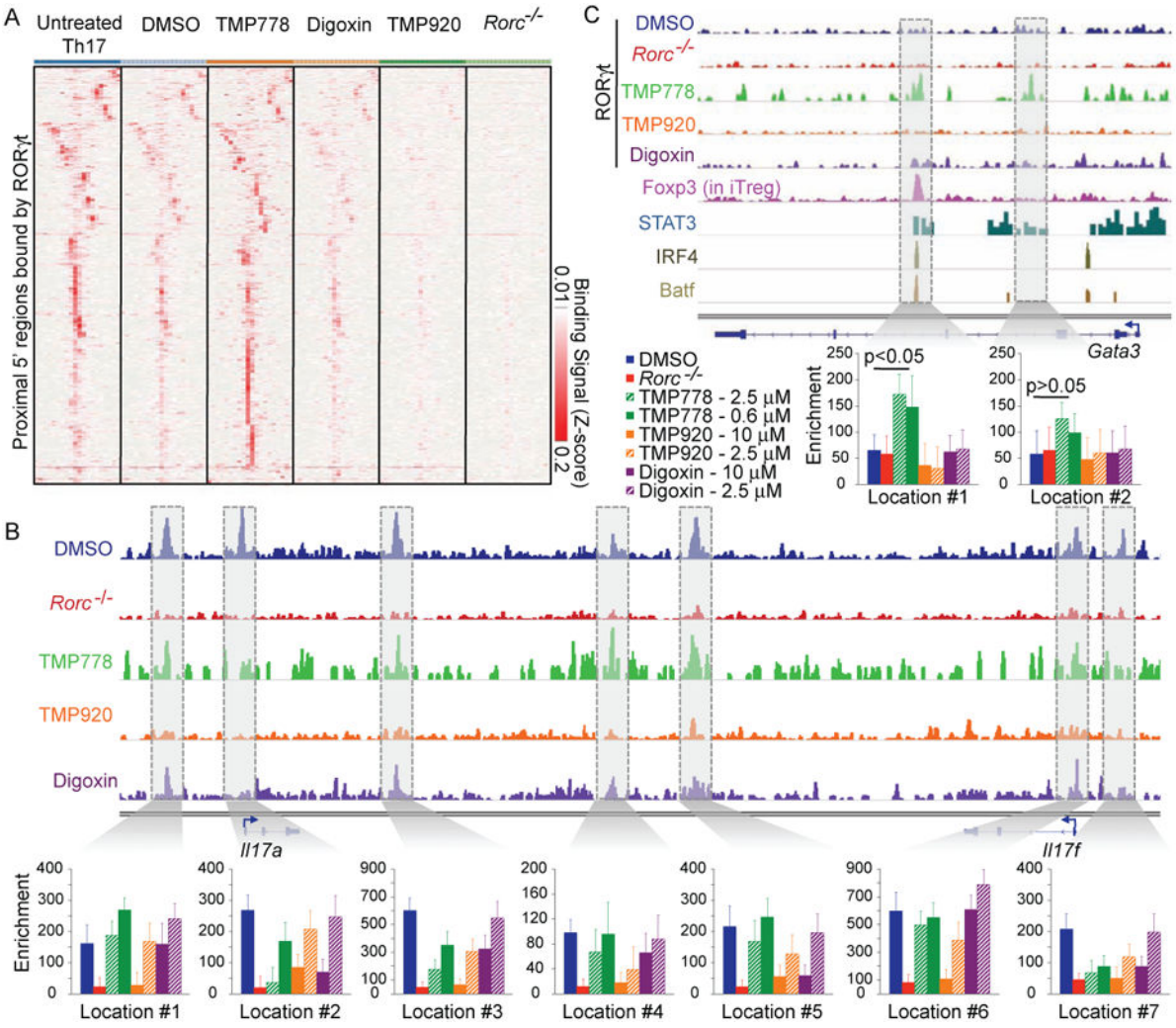

Figure 5. Distinct effects of inhibitors on RORγt-DNA Interactions

(A) RORγt binding near transcription start sites (TSS). Every line depicts the 6kb region around a TSS (center) in 300bp windows. Shown are the 1544 TSS that contain a binding peak ($p<10^{-8}$; Methods) in at least one condition (compounds, DMSO, untreated Th17 cells) and do not contain any signal (Z score>0.1) in the control (RORγt-deficient) cells. Color intensity is proportional to the number of reads mapped to each window (normalized separately for each condition using Z-scores). Effects of RORγt inhibitors on RORγt occupancy at *Il17a* and *Il17f* (**B**) and *Gata3* (**C**) loci were validated by ChIP-PCR. Naïve $CD4^+$ T cells were cultured under Th17 cell-polarizing conditions in the presence of indicated doses of RORγt inhibitors. After 96 h, ChIP was performed with anti-RORγt, followed by real-time PCR analysis. Th17 cells for ChIP-seq were cultured in the presence of 2.5 μM TMP778, 10 μM TMP920, 10 μM Digoxin, or DMSO for 96 h. The RORγt binding sites in *Il17a* and *Il17f* and *Gata3* loci are as indicated in the ChIP-seq binding tracks. ChIP-PCR was used to confirm binding at selected sites (shown below ChIP-Seq tracks) and the RORγt occupancy (% of input) is shown as "Enrichment". Data are representative of two experiments. TMP778-caused RORγt binding site in *Gata3* locus in Th17 cells overlaps with STAT3, IRF4, Batf in Th17 cells and Foxp3 in iTreg cells.

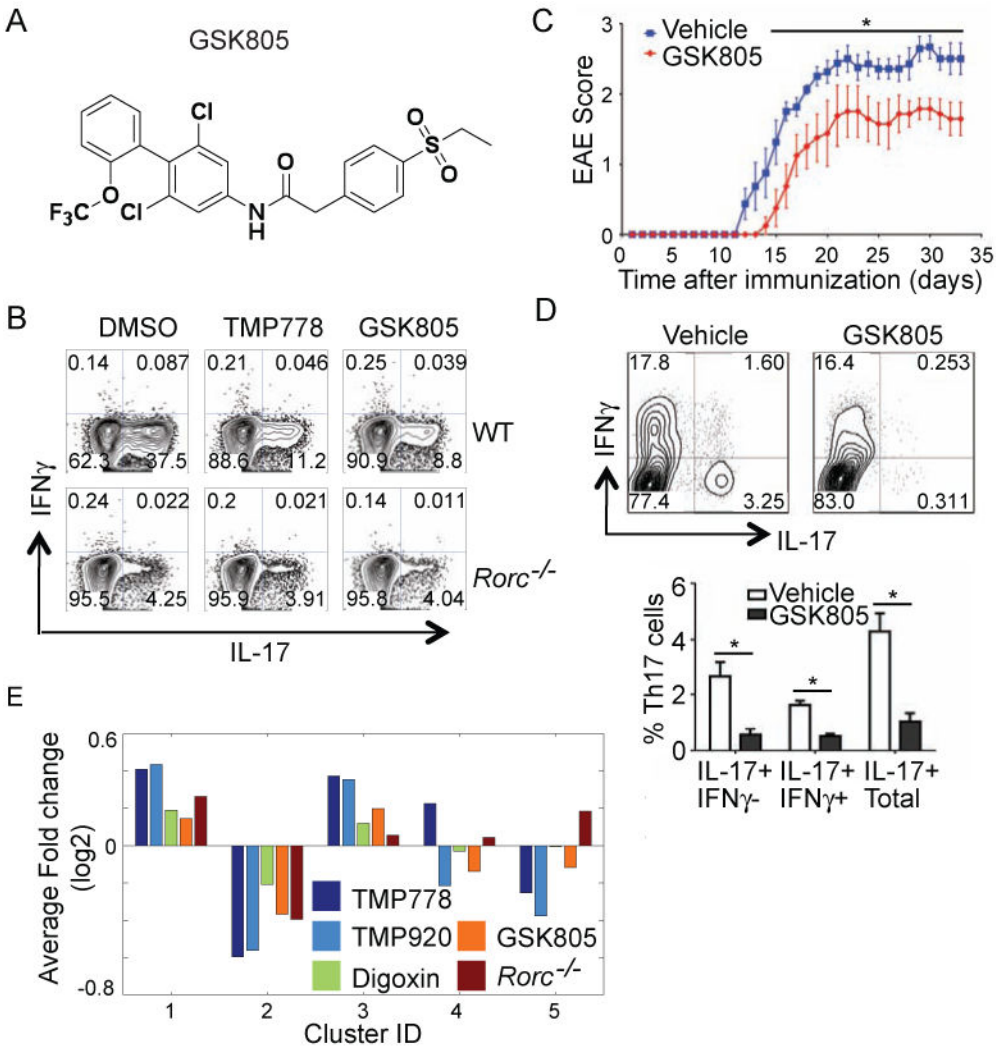

Figure 6. Effects of RORγt inhibitor GSK805 on Th17 cells and Th17 cell-mediated autoimmune diseases

(**A**) Chemical structure of GSK805. (**B**) Naïve $CD4^+$ T cells were activated under Th17 cell-polarizing conditions in the presence of GSK805 (0.5 μM), TMP778 (2.5 μM), or DMSO. After 4 days, IL-17 and IFNγ production were measured by intracellular cytokine staining. Data are representative of 3 experiments. (**C**) C57BL/6 mice were immunized with MOG35-55 plus CFA, and RORγt inhibitor GSK805 (10 mg/kg) were orally given daily starting from day 0. Mice (n=8) were evaluated daily for signs of EAE. Error bars represent the mean ± SD. * $p<0.01$ by repeated ANOVA test. (**D**) C57BL/6 mice were induced for EAE and treated with GSK805 (30 mg/kg). On day 14, CNS-infiltrating cells were isolated and measured for IL-17 and IFNγ production by intracellular staining. Error bars represent the mean ± SD. * $p<0.001$. **(E)** Comparison of gene expression under the various perturbations (indicated in the legend) with WT DMSO. The figure depicts the average fold change of the genes in clusters #1-#5 from Figure 2A. Results were obtained by profiling transcripts 3′ end.

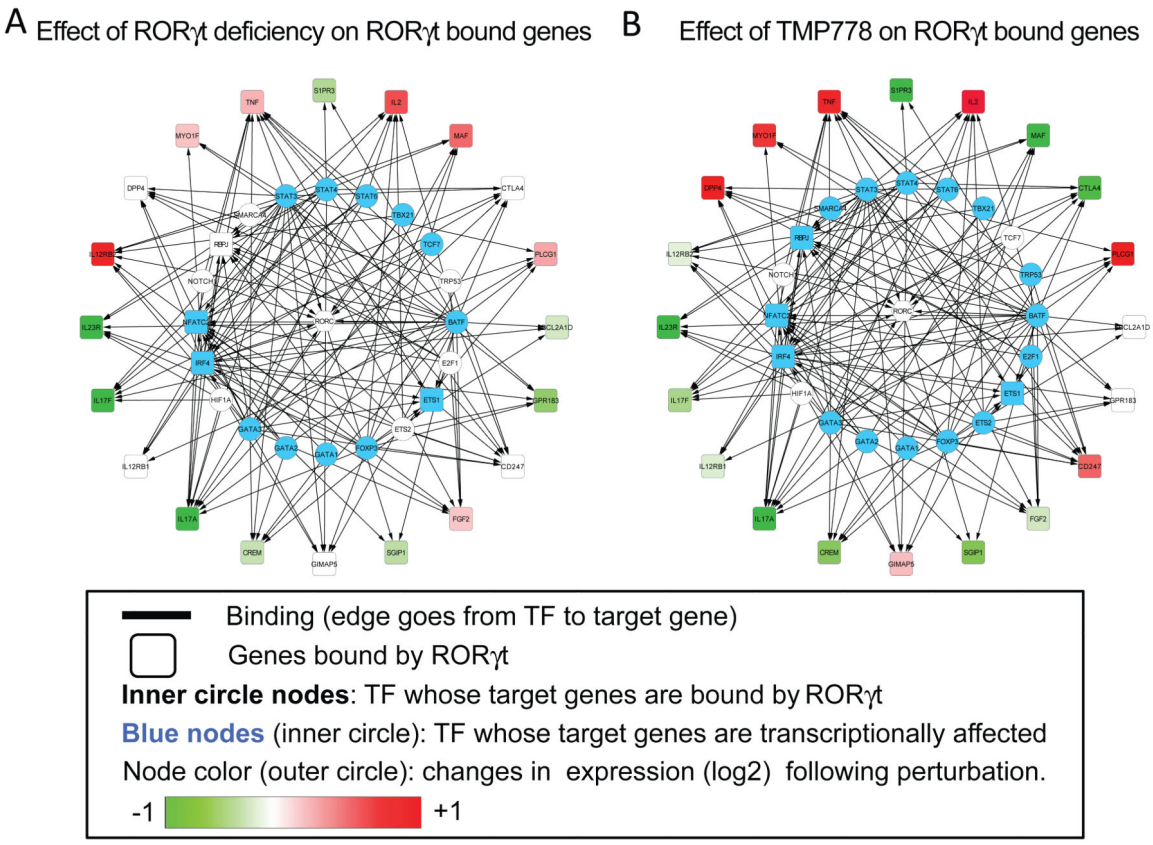

Figure 7. RORγt is a regulatory hub in a densely inter-connected network of CD4$^+$ T-cell regulation

Depicted are TFs that share a significant ($p<10^{-3}$) number of common targets with RORγt (inner circle), and a subset of these common targets that are also differentially expressed under perturbation of RORγt (outer circle; shown are only genes that are associated with immune response; see Tables S1 and S2 for the complete lists). Edges indicate TF binding in a target gene. Node colors reflect the modulation of mRNA levels in RORγt-deficient cells (**A**) or under TMP778 treatment (**B**).