

# *In vivo* regulation of gene expression and T helper type 17 differentiation by ROR $\gamma$ t inverse agonists

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## Introduction

Naive CD4<sup>+</sup> T cells differentiate into a variety of effector and regulatory subsets, including T helper type 1 (Th1), Th2, induced T regulatory (iTreg) cells, and Th17 cells.<sup>1–4</sup> Th1 cells are characterized by transcription factor T-bet and the production of interferon- $\gamma$  (IFN- $\gamma$ ), whereas Th2 cells are characterized by transcription factor GATA3 and the production of interleukin-4 (IL-4). Induced Treg cells, like their thymic-derived 'nTreg' cell counterparts, selectively express the transcription factor Foxp3 and suppress bystander T-cell activation to regulate inflammatory T-cell responses. In contrast, Th17 cells are defined by production of IL-17A, IL-17F, IL-21 and IL-22, and these cells play a central role in autoimmune pathogenesis.<sup>4–7</sup> Human monoclonal antibodies against IL-17A or IL-17 receptor A (IL-17RA) have demonstrated clinical efficacy in both psoriasis and rheumatoid arthritis.<sup>7–9</sup> Differentiation of Th17 cells requires the retinoic acid receptor-related orphan nuclear receptor ROR $\gamma$ t,<sup>10,11</sup> which is

## Summary

The orphan nuclear receptor, retinoic acid receptor-related orphan nuclear receptor  $\gamma$ t (ROR $\gamma$ t), is required for the development and pathogenic function of interleukin-17A-secreting CD4<sup>+</sup> T helper type 17 (Th17) cells. Whereas small molecule ROR $\gamma$ t antagonists impair Th17 cell development and attenuate autoimmune inflammation *in vivo*, the broader effects of these inhibitors on ROR $\gamma$ t-dependent gene expression *in vivo* has yet to be characterized. We show that the ROR $\gamma$ t inverse agonist TMP778 acts potently and selectively to block mouse Th17 cell differentiation *in vitro* and to impair Th17 cell development *in vivo* upon immunization with the myelin antigen MOG<sub>35–55</sub> plus complete Freund's adjuvant. Importantly, we show that TMP778 acts *in vivo* to repress the expression of more than 150 genes, most of which fall outside the canonical Th17 transcriptional signature and are linked to a variety of inflammatory pathologies in humans. Interestingly, more than 30 genes are related with SMAD3, a transcription factor involved in the Th17 cell differentiation. These results reveal novel disease-associated genes regulated by ROR $\gamma$ t during inflammation *in vivo*, and provide an early read on potential disease indications and safety concerns associated with pharmacological targeting of ROR $\gamma$ t.

**Keywords:** autoinflammatory disease; cytokines; T cells.

induced during human and mouse naive T-cell activation in the presence of cytokines such as IL-6, transforming growth factor- $\beta$  (TGF- $\beta$ ) and IL-1 $\beta$ .<sup>4,6,12</sup>

The differentiation of Th17 cells *in vivo* is more complicated, and can lead to functionally distinct pro-inflammatory and anti-inflammatory Th17 cell subsets. Interleukin-23 is critical for both the maintenance of IL-17A expression as well as pathogenic Th17 cell function in experimental autoimmune encephalomyelitis (EAE).<sup>13–15</sup> 'Pathogenic' Th17 cells express Th17 cytokines together with IFN- $\gamma$ ; they express both ROR $\gamma$ t and T-bet, and they are distinguished from non-pathogenic Th17 cells by their selective expression of the multidrug transporter, MDR1.<sup>16</sup> In addition to ROR $\gamma$ t and T-bet, the Runx family transcription factors, namely Runx1 and Runx 3, have been recently shown to control IFN- $\gamma$  production by pathogenic Th17 cells.<sup>17</sup> In mouse and human CD4<sup>+</sup> T cells, as well as in  $\gamma\delta$  T cells, IL-1 $\beta$  and IL-23 are important, both for high-level IL-17A production and development of autoimmune inflammation. Given that

ROR $\gamma$ t is expressed in both pathogenic and non-pathogenic Th17 cells *in vivo*, it is unlikely that the full repertoire of ROR $\gamma$ t-dependent transcriptional activity *in vivo* has been captured by previous transcriptional profiling experiments using Th17 cells generated *in vitro*.

Several small molecule inhibitors targeting ROR $\gamma$ t have been described previously.<sup>11,18–20</sup> These studies provide a proof-of-principle that pharmacological inhibition of ROR $\gamma$ t can regulate Th17 cell differentiation (and IL-17A production) *in vitro*. *In vivo*, administration of these compounds shows variable levels of efficacy in EAE. Previous work from our laboratory has described a novel, potent and selective ROR $\gamma$ t inverse agonist TMP778.<sup>21,22</sup> TMP778 inhibits human Th17 signature gene expression *in vitro*; and targeting of ROR $\gamma$ t in mice via TMP778 administration reduces imiquimod-induced psoriasis-like cutaneous inflammation<sup>21</sup> and severity of EAE progression.<sup>23</sup> Here we demonstrate that TMP778 inhibits mouse Th17 cell development *in vivo* in two different mouse model systems. Further, we show that TMP778 regulates expression of more than 150 genes during inflammatory Th17 cell differentiation. Understanding the broader activity of ROR $\gamma$ t inhibitors on gene expression during physiological inflammation is vital to advancing this class of compounds for clinical development.

## Materials and methods

### Mice

Female C57BL/6 mice (6–8 weeks) were purchased from The Jackson Laboratory and were housed in a pathogen-free barrier facility at Tempero Pharmaceuticals. Green fluorescent protein-tagged IL-17 (IL-17A-GFP) knock-in mice were licensed from Biocytogen, Inc. (Worcester, MA), bred at the Jackson Laboratory (Bar Harbor, ME), and shipped to Tempero for experiments. All studies were conducted in accordance with the GSK Policy on the Care, Welfare and Treatment of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at GSK.

### Mouse T-cell culture

Spleens were isolated from C57BL/6 mice and single-cell suspensions were prepared by passing the spleen through a 70- $\mu$ m filter. Red blood cells were removed by lysis with ammonium chloride. Cells were stimulated for 3 days with anti-CD3 (1  $\mu$ g/ml)/anti-CD28 (0.5  $\mu$ g/ml) monoclonal antibodies (eBioscience, San Diego, CA) plus the indicated cytokines and antibodies (eBioscience) at the following concentrations: IL-6 (10 ng/ml), TGF- $\beta$  (1 ng/ml), IL-12 (5 ng/ml), IL-4 (10 ng/ml), anti-IL-4 monoclonal antibody (2  $\mu$ g/ml), anti-IL-12p40 monoclonal antibody (1  $\mu$ g/ml). Cytokine titres and cell proliferation were

assessed and values of IC<sub>50</sub> were determined using GRAPHPAD PRISM (GraphPad, La Jolla, CA). For mouse Th17 differentiation experiments, naive mouse CD4<sup>+</sup> T cells were stimulated with anti-CD3/anti-CD28 antibodies plus IL-6/TGF- $\beta$  in the presence of TMP778 or Digoxin for 4 days. Cells were then re-stimulated with PMA (10 nM) and ionomycin (1  $\mu$ M) in the presence of brefeldin A (5  $\mu$ g/ml) (all from Sigma, St Louis, MO) for 3 hr before intracellular staining for IL-17 and IFN- $\gamma$  as previously described.<sup>24</sup>

### *Ex vivo* myelin oligodendrocyte glycoprotein recall studies

C57BL/6 mice were immunized with myelin oligodendrocyte glycoprotein peptide 35–55 (MOG<sub>35–55</sub>) in complete Freund's adjuvant (CFA), and 20 mg/kg TMP778 or Vehicle (3% dimethylacetamide, 10% solutol, and 87% saline) was administered twice daily via subcutaneous injection. On day 6, draining lymph node cells were isolated and stimulated with 50  $\mu$ g/ml MOG<sub>35–55</sub> in T-cell culture medium as described previously.<sup>21</sup> Cells were harvested 20 hr later for RNA isolation and gene expression analysis, and cell supernatants were harvested 5 days after MOG stimulation for detection of IL-17 by Meso Scale Discovery (Rockville, MD). Interleukin-17A-GFP mice were immunized and treated with or without TMP778 as described above. Draining lymph node cells were isolated on day 9, and total mononuclear cells were stimulated with PMA/ionomycin for 3 hr in the presence of brefeldin A. Cells were then stained and analysed. The integrated mean fluorescence intensity MFI (iMFI) was calculated at the percentage of positive cells multiplied by the MFI. For each experiment, at least 10 mice were used for vehicle treatment at least 10 mice for each compound treatment.

### Cell proliferation

Cell proliferation was measured using CellTiter-Glo as instructed by the manufacturer (Promega, Madison, WI), with luminescence read on a FLUOstar OPTIMA (BMG Labtech, Cary, NC).

### Quantification of secreted cytokines

Interleukin-17A and Th1/Th2 cytokines were measured using electrochemiluminescent assays from Meso Scale Discovery as instructed by the manufacturer.

### RNA extraction, quantitative RT-PCR

Total RNA was extracted using RNeasy mini kits including the optional DNaseI digestion (Qiagen, Valencia, CA). Complementary DNA synthesis and TaqMan Real Time PCR were performed as described previously.<sup>25,26</sup>

TaqMan quantitative PCR was performed on a 7900HT Real Time PCR System (Applied Biosystems, Foster City, CA). All TaqMan reagents were purchased from Applied Biosystems.

### Microarray

After extraction of total RNA using an RNeasy mini kit, microarray assays were performed at the Boston University Microarray Resource Facility (Boston, MA). Briefly, the RNA samples were amplified and labelled following Ambion<sup>®</sup> WT Expression Kit Protocol (Life Technologies, Grand Island, NY) and GeneChip<sup>®</sup> Whole Transcript (WT) Sense Target Labeling Assay Manual (Affymetrix, Santa Clara, CA). The cRNA samples were then hybridized to Affymetrix mouse 1-0ST gene chips. Affymetrix data were extracted, normalized and analysed using both in-house-developed MULTIPLEX software based on GENE-PATTERN software of Broad Institute (Cambridge, MA) and INGENUITY IPA software (<http://www.ingenuity.com>). Transcriptional factor enrichment analysis was also conducted using IPA. Significance is defined as absolute value of  $Z$ -score  $\geq 2$  and  $P$ -value  $< 10^{-3}$ . The network model was reconstructed using Advanced Network Analysis Tool, a tool for constructing and analysing functional networks using inference algorithms based on physical and regulatory associations.<sup>27</sup>

### Calculation of percent of expected composition and statistics

Percentage of Expected Composition equals  $(N_{\text{obs}} / N_{\text{exp}}) \times 100$  in which  $N_{\text{obs}}$  represents the number of genes in the signature that belong to a given family; and  $N_{\text{exp}}$  is the number of genes expected to be present in the list if the list were taken at random. Different groups were evaluated for statistical significance using the two-tailed unpaired Student's  $t$ -test for Figs 2 and 3. The  $P$ -value in Fig. 4 is calculated using Fisher's Exact Test.

## Results and Discussion

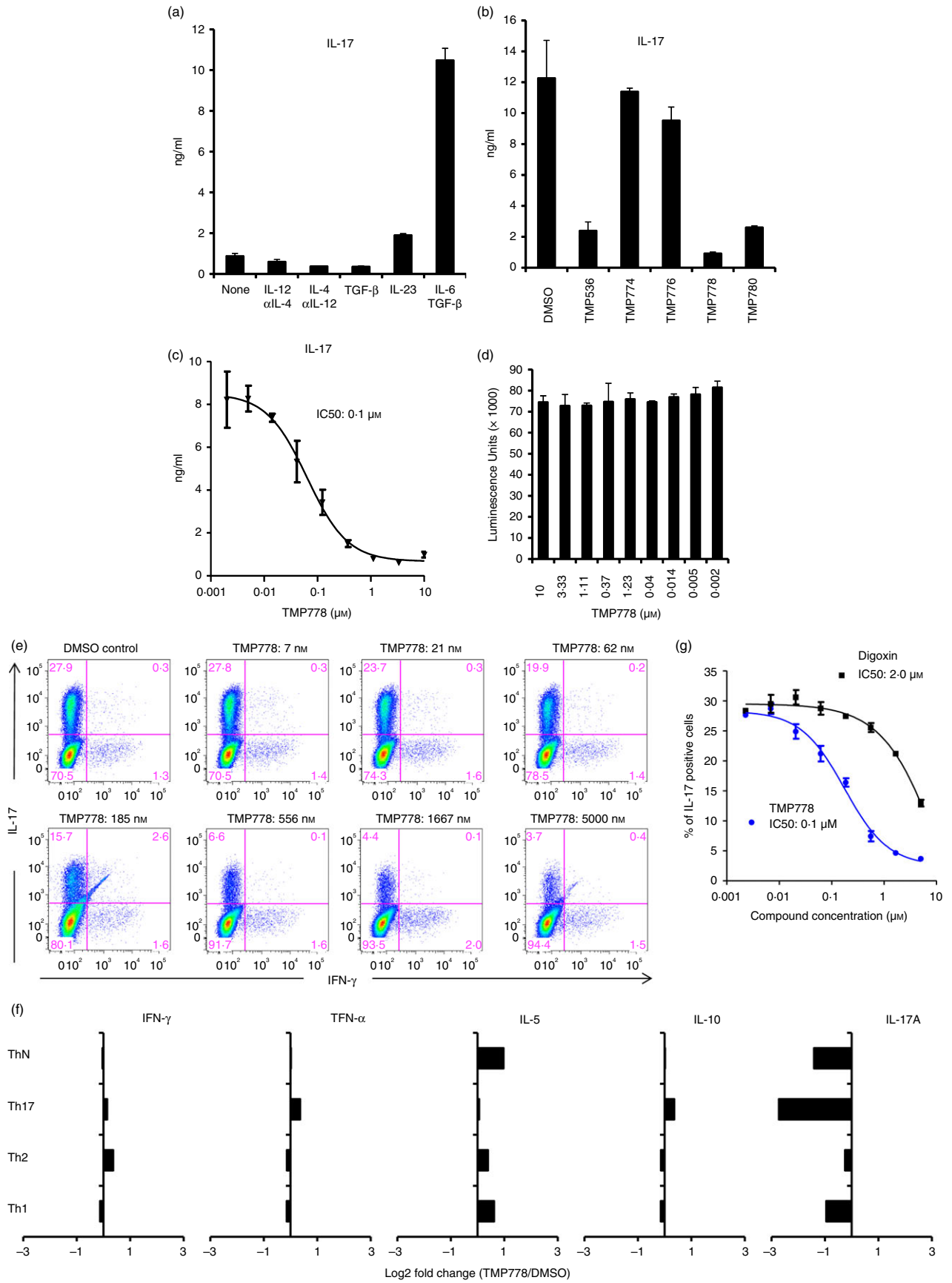
### TMP778 inhibits mouse Th17 cell differentiation *in vitro*

Previously, we have reported that TMP778 blocks human Th17 cell differentiation *in vitro*.<sup>21</sup> To study the effect of TMP778 in animal models, we first assessed whether TMP778 also inhibits mouse Th17 cell differentiation *in vitro*. Mouse CD4<sup>+</sup> T cells in splenocytes differentiate to Th1, Th2, Treg or Th17 cells under different conditions. We stimulated mouse splenocytes under Th1 (IL-12/anti-IL-4), Th2 (IL-4/anti-IL-12), Treg (TGF- $\beta$ ) or Th17 (IL-23 or IL-6/TGF- $\beta$ ) polarizing conditions for 3 days and then examined IL-17 production. The IL-6/TGF- $\beta$

stimulation induced the highest level of IL-17 (Fig. 1a). Next, we asked whether TMP778 could inhibit IL-6/TGF- $\beta$  induced IL-17 production. TMP778 blocked IL-17 production in a dose-dependent manner with an IC<sub>50</sub> of 0.1  $\mu$ M (Fig. 1b,c). In addition, TMP536, the parental compound of TMP778, and TMP870, its diastereomer, also suppressed IL-17 production (Fig. 1b). In contrast, the diastereomers TMP774 and TMP776 had no significant effect on mouse IL-17 production, consistent with a lack of activity displayed by these compounds in an IL-17F reporter system.<sup>21</sup> The inhibition of IL-17 could be a result of the non-specific inhibition of T-cell proliferation. However, TMP778 treatment did not impair T-cell proliferation (Fig. 1d). TMP778 regulation of Th17 cell development was also evident by intracellular IL-17A staining of purified naive mouse CD4<sup>+</sup> T cells stimulated with plate-bound anti-CD3/anti-CD28 antibodies and cultured for 4 days with IL-6 and TGF- $\beta$  (Fig. 1e). Here, TMP778 had no effect on residual IFN- $\gamma$ -producing Th1 cells in Th17 cultures (Fig. 1e), nor did it affect production of Th1 or Th2 cytokines by Th1 or Th2 cells that were polarized *in vitro* (Fig. 1f). The IC<sub>50</sub> for inhibition of mouse Th17 cell differentiation by TMP778 was 0.1  $\mu$ M (Fig. 1g). In contrast to the single nanomolar potency of TMP778, another reported ROR $\gamma$ t inhibitor, digoxin, showed much weaker activity against mouse Th17 cell differentiation (IC<sub>50</sub> = 2.0  $\mu$ M) (Fig. 1g). There are a variety of small molecule ROR $\gamma$ t inhibitors that have been recently discovered, such as digoxin, SR2211, SR1001 and ursolic acid.<sup>18–20</sup> Although these compounds do inhibit Th17 cell differentiation, they all have far less potency than TMP778. In addition, they are not genome-wide profiled *in vivo*. Hence, these compounds can only be used as tool compounds but not possible clinical inhibitors.

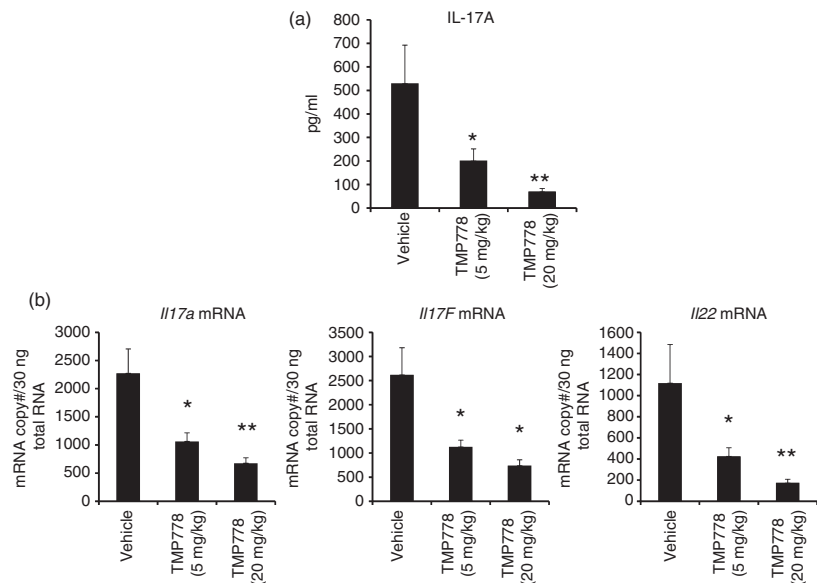
### ROR $\gamma$ t inverse agonist TMP778 inhibits mouse Th17 cell differentiation *in vivo*

Given its potent and selective activity in blocking mouse Th17 cell differentiation *in vitro*, we next asked whether TMP778 also regulates Th17 cell development during inflammatory T-cell activation *in vivo*. EAE models have recently been shown to be largely caused by the induction of pathogenic Th17 cells.<sup>6,13,28</sup> Mice develop EAE within 10–14 days after immunization with MOG<sub>35–55</sub> in CFA with pertussis toxin. *In vivo* administration of TMP778 for 3 weeks significantly delayed the onset of EAE and substantially reduced the severity of disease progression.<sup>23</sup> Here, we modified the model where C57BL/6 mice were immunized with MOG in CFA without pertussis toxin and the draining lymph nodes were harvested to assess their function *ex vivo*. Lymph node cells from naive mice did not produce IL-17A upon stimulation with MOG, whereas lymph node cells from mice immunized with



**Figure 1.** Retinoic acid receptor-related orphan nuclear receptor  $\gamma$ t (ROR $\gamma$ t) inverse agonist inhibits mouse interleukin-17 (IL-17) expression and T helper type 17 (Th17) cell differentiation *in vitro*. (a) Mouse splenocytes were stimulated with anti-CD3/anti-CD28 monoclonal antibody (mAb) plus the indicated cytokine and antibody for 3 days. The IL-17 titres in the supernatants were then determined by Meso Scale Discovery (MSD). (b) Mouse splenocytes were stimulated with anti-CD3/anti-CD28 mAb plus IL-6 and transforming growth factor- $\beta$  (TGF $\beta$ ) in the presence of 1  $\mu$ M of indicated ROR $\gamma$ t inverse agonists for 3 days. The IL-17 titres in the supernatants were then determined by MSD. (c) Different doses of TMP778 were added into the culture as described in Fig. 2(b) for 3 days. The IL-17 titres in the supernatants were then determined and the IC<sub>50</sub> was calculated. (d) Cell proliferation from (c) was determined using CellTiter-Glo. (e) Naive mouse CD4<sup>+</sup> T cells were stimulated with anti-CD3/anti-CD28 plus IL-6/TGF- $\beta$  in the presence of small molecule compound for 4 days. Cells were then re-stimulated with PMA/ionomycin for 3 hr before intracellular staining for IL-17 and interferon- $\gamma$  (IFN- $\gamma$ ). (f) Mouse splenocytes were stimulated under ThN (anti-CD3/28 mAb), Th17 (anti-CD3/28 mAb, IL-6, TGF $\beta$ ), Th2 (anti-CD3/28 mAb, IL-4, anti-IL12 mAb), or Th1 (anti-CD3/28 mAb, IL-12, anti-IL4) skewing conditions with or without 1  $\mu$ M TMP778 for 3 days. The Th1/Th2/Th17 cytokines were then determined by Meso Scale Discovery. Data shown are the log<sub>2</sub>-fold change between TMP778 and DMSO control. (g) The percentage of IL-17-positive T cells after treatment with TMP778 or digoxin as in (e) was used to calculate their IC<sub>50</sub>. Data are representative of two to five separate experiments.

**Figure 2.** *In vivo* differentiation of T helper type 17 (Th17) cells was blocked by administration of TMP778. Mice were immunized with myelin oligodendrocyte glycoprotein peptide 35–55 (MOG<sub>35–55</sub>) in complete Freund's adjuvant (CFA). TMP778 (20 mg/kg or 5 mg/kg) or Vehicle was subcutaneously injected twice a day for 6 days. (a) The draining lymph node cells were isolated and stimulated with MOG<sub>35–55</sub> for 5 days and the supernatants were harvested to detect interleukin-17 (IL-17) titres by Meso Scale Discovery. (b) Draining lymph node cells were isolated and stimulated with MOG<sub>35–55</sub> for 20 hr. Cells were harvested for RNA extraction and real time RT-PCR analysis for mRNA expression of *Il17a*, *Il17f* and *Il22*. \**P* < 0.05 TMP778 versus Vehicle; \*\**P* < 0.01 TMP778 versus Vehicle. Data are representative of three separate experiments.



MOG for 10 days produced detectable levels of IL-17A upon *ex vivo* re-stimulation with MOG (data not shown). Further, we performed a time-course study to determine the length of immunization required for the development of naive T cells into Th17 cells as measured by IL-17A production from draining lymph node cells upon stimulation with MOG<sub>35–55</sub> specific antigen. Lymph node cells produced a high level of IL-17A after immunization for only 6 days (data not shown), indicating differentiation of Th17 cells. After determining the pharmacokinetics and exposure levels of TMP778, we asked whether TMP778 regulates the development of Th17 cells in response to immunization with MOG<sub>35–55</sub> in CFA. MOG/CFA-immunized mice were dosed with TMP778 (20 mg/kg or 5 mg/kg) or vehicle for 6 days. Whereas TMP778 did not influence the overall numbers or proportions of CD4<sup>+</sup>, CD8<sup>+</sup>, B220<sup>+</sup>, NK1.1<sup>+</sup> or CD11c<sup>+</sup> cells in draining lymph nodes (see Supporting information, Fig. S1), re-stimulation of *ex vivo*-isolated draining lymph

node cells with MOG showed that cells from immunized mice treated with TMP778 produced substantially less IL-17A than cells dosed with vehicle alone (Fig. 2a). The effects of TMP778 on MOG recall-induced IL-17A production was dose-dependent (Fig. 2a), and TMP778 treatment also reduced *Il17a*, *Il17f* and *Il22* mRNA expression in *ex vivo*-stimulated draining lymph node cells, as measured by RT-PCR (Fig. 2b).

Similar to TMP778, the parental compound TMP536<sup>21</sup> inhibited mouse Th17 cell differentiation *in vitro* (IC<sub>50</sub> = 0.3  $\mu$ M) (see Supporting information, Fig. S2a); dosing of MOG/CFA-immunized mice with TMP536 also inhibited MOG recall-induced IL-17A production (Fig. S2b). A third ROR $\gamma$ t inverse agonist, TMP934, which is structurally distinct from TMP778 and TMP536 (Fig. S2c), also inhibited mouse Th17 cell differentiation *in vitro* (IC<sub>50</sub> = 1.1  $\mu$ M) (Fig. S2d). TMP934 also reduced *ex vivo* IL-17A production upon dosing in MOG/CFA-immunized mice (Fig. S2e). These results suggest that



ROR $\gamma$ t inverse agonists block the *in vivo* development of effector cells capable of expressing IL-17A upon recall stimulation.

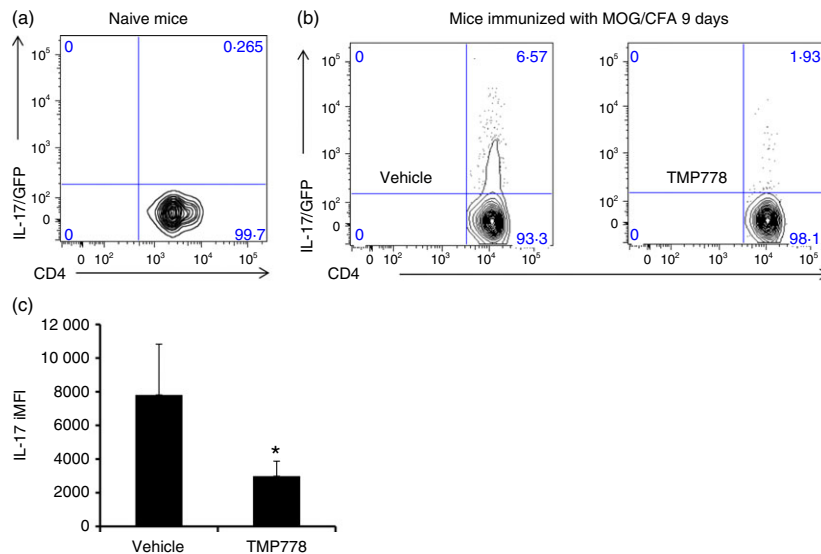
To gain further insight into the effects of ROR $\gamma$ t inhibition on Th17 cell differentiation *in vivo*, we next tracked the development of Th17 cells upon MOG/CFA immunization using a transgenic IL-17A-GFP reporter system and flow cytometry.<sup>29</sup> We immunized IL-17A-GFP mice with MOG/CFA, treated these animals +/- TMP778, and then analysed the development of IL-17A-GFP<sup>+</sup> effector (CD4<sup>+</sup> CD25<sup>-</sup> CD44<sup>hi</sup> CD62L<sup>lo</sup>) T cells from draining lymph nodes after 9 days by *ex vivo* FACS analysis. Detectable GFP expression in effector T cells from IL-17A-GFP mice required immunization with MOG/CFA (Fig. 3a,b), and treatment of these immunized mice with TMP778 almost completely abolished GFP expression (Fig. 3b). The effects of TMP778 on *in vivo* Th17 cell development in this model were evident, both in terms of the number of GFP<sup>+</sup> effector T cells (Fig. 3b) and the level of GFP expression iMFI observed in residual GFP<sup>+</sup> effector cells (Fig. 3c). Collectively, these data indicate that pharmacological inhibition of ROR $\gamma$ t potently blocks Th17 cell development *in vivo*.

A number of groups have discovered small molecule inhibitors targeting ROR $\gamma$ t.<sup>18–20</sup> These molecules inhibited Th17 cell differentiation and demonstrated efficacy in EAE. The efficacy of these molecules *in vivo* could be due to either inhibition of Th17 cell differentiation or acute

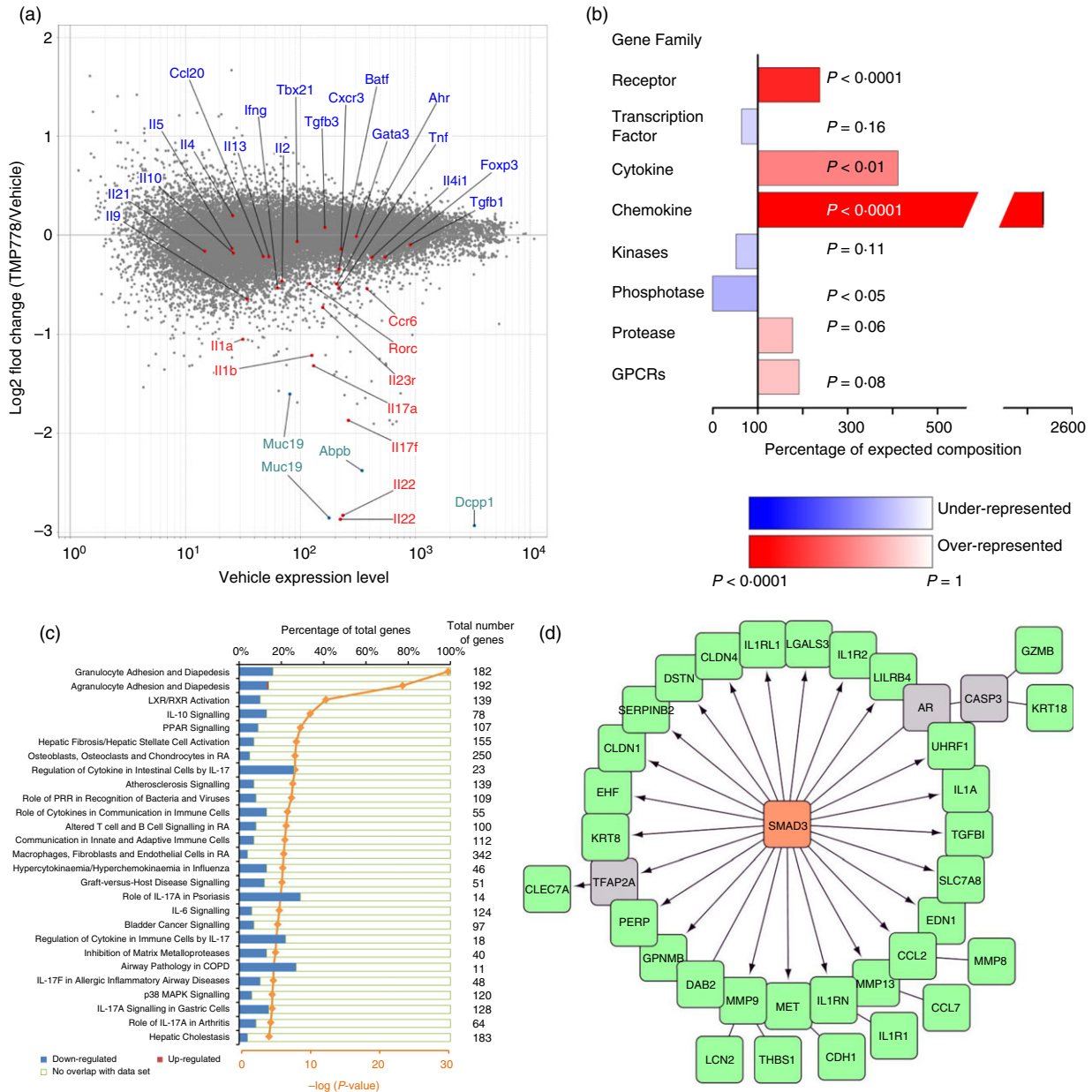
regulation of Th17 signature gene expression by mature Th17 cells, or both. Here, we demonstrated that ROR $\gamma$ t inverse agonist TMP778 inhibited *in vivo* Th17 cell differentiation in two different models, indicating the important role of ROR $\gamma$ t inverse agonists in treating Th17-related autoimmune inflammatory diseases.

### Whole genome transcriptional profile of *in vivo* ROR $\gamma$ t inhibition by TMP778

We next sought to analyse the broader effects of ROR $\gamma$ t inhibition on gene expression during Th17 cell differentiation *in vivo*. For these experiments, we analysed the RNA of lymph node cells from vehicle- or TMP778-treated mice, prepared as in Fig. 2(b) above, by microarray. As expected, expression of Th17-signature genes, including *Il17a*, *Il17f*, *Il22*, *Il23r*, *Rorc* and *Ccr6* were reduced in cells from TMP778-treated mice (Fig. 4a). In addition, a large number of genes outside the established Th17 transcriptional signature were significantly different in cells from TMP778-treated versus vehicle-treated mice, with the vast majority of these differentially regulated genes being reduced by TMP778 treatment. For example, expression of *Il1a* and *Il1b* was reduced by TMP778 treatment *in vivo*. Expression of other, non-canonical Th17-related genes, namely *Ifng*, *Il2*, *Tnf*, *Il9* and *Ahr*, which can be expressed in pathogenic and non-pathogenic Th17 cells at varying levels, were modestly decreased by



**Figure 3.** Retinoic acid receptor-related orphan nuclear receptor  $\gamma$ t (ROR $\gamma$ t) inverse agonist inhibits the generation of interleukin-17 (IL-17) - producing CD4<sup>+</sup> T cells in an IL-17/GFP transgenic model. (a) IL-17/GFP expression from naive lymph nodes. (b) IL-17-IRES-GFP transgenic mice were immunized with myelin oligodendrocyte glycoprotein peptide 35–55 (MOG<sub>35–55</sub>) in complete Freund’s adjuvant (CFA). TMP778 (20 mg/kg) or Vehicle was subcutaneously injected twice a day for 9 days. The draining lymph node cells were purified and stimulated with PMA/ionomycin in the presence of brefeldin A for 3 hr before staining for mouse CD4. (c) Average of integrated MFI (iMFI) of individual animal CD4<sup>+</sup> IL-17/GFP<sup>+</sup> quadrant, as in Figure 4(b). \**P* < 0.05 TMP778 20 mg/kg versus Vehicle. Data are representative of two separate experiments.



**Figure 4.** The effect on genome-wide transcription by administration of retinoic acid receptor-related orphan nuclear receptor  $\gamma$ t (ROR $\gamma$ t) inverse agonist TMP778 *in vivo*. Total RNA from Fig. 2(b) samples were used for microarray analysis using an AFFYMETRIX MOUSE GENE 1.0 ST Array. Each RNA sample was the pool of four individual mouse RNA samples. (a) Data are shown as the  $\log_2$ -fold change of TMP778/vehicle versus the expression level, indicating genes increased or decreased by TMP778 treatment *in vivo*. Data shown are representative of microarray data of one RNA sample set (vehicle sample versus TMP778 sample) from two RNA sample sets. (b) Gene family signature enrichment. Genes up-regulated or down-regulated at least 1.5-fold with an expression level of 50 or greater were analysed. Each gene family enrichment is displayed as a percentage of the expected number of genes for a list of that size. When more genes than expected are found, the family is over-represented/enriched in the list (red bars). If the list contains fewer members of that family than expected, then that family is under-represented (blue bars).  $P$ -value is calculated using Fisher Exact Test and the value is used in colouring the bars (the more significant the  $P$ -value, the darker blue/red a bar is). All of the enriched genes in the figure happened to be down-regulated genes. (c) Canonical pathway analysis by INGENUITY. Genes from (b) were analysed for their role in canonical pathways by IPA of INGENUITY. Each canonical pathway has a different total number of genes as indicated. Pathways shown are the percentage of our down-regulated or up-regulated genes. Graphs were selected from data of which  $-\log(P\text{-value})$  is equal or higher than 4.  $P$ -value was also calculated using right-tailed Fisher Exact test. (d) Transcriptional factor enrichment analysis was conducted using IPA. The network model was reconstructed using the Advanced Network Analysis Tool (ANAT). SMAD3 was discovered by transcriptional factor enrichment analysis, the green nodes were genes down-regulated by TMP778 treatment, and the grey nodes were predicted genes connecting SMAD3. The arrow indicates direct regulation and the straight line indicates correlation between two nodes.

TMP778 treatment. The vast majority of other T helper cell signature genes were not influenced by *in vivo* inhibition of ROR $\gamma$ t (Fig. 4a).

Outside the conventional T helper cell-related genes, more than 150 genes were found to be reduced at least 1.5-fold in TMP778-treated mice compared with control animals, whereas 13 genes were up-regulated. Among them, *Dcpp*, *Muc19* and *Abpb* were markedly repressed by *in vivo* TMP778 treatment. In defining gene families among the non-Th17 signature genes significantly influenced by *in vivo* ROR $\gamma$ t inhibition, we found that chemokines, cytokines and receptors were highly enriched, and all were down-regulated by *in vivo* TMP778 treatment (Fig. 4b). We next analysed TMP778-modulated genes for functional pathways using IPA. TMP778-modulated genes were classified into at least 51 distinct pathways, where the  $-\log(P\text{-value})$  is equal to or is higher than 2.0 or  $P \leq 0.01$  (data not shown). Twenty-seven of these

pathways showed  $-\log(P\text{-value}) \geq 4.0$  or  $P \leq 0.0001$  (Fig. 4c). Only one gene, *Glycam1*, was up-regulated in these 27 pathways. Interestingly, the top two pathways modulated by *in vivo* TMP778 treatment are involved in granulocyte/agranulocyte adhesion and diapedesis (Fig. 4c and Table 1). Other pathways regulated by TMP778 treatment are those involved in cell activation, cytokine signalling and regulation, and are also associated with autoimmune inflammatory diseases such as rheumatoid arthritis, atherosclerosis, psoriasis, chronic obstructive pulmonary disease and airway allergic inflammation. Other classes of TMP778-modulated pathways were those associated with bacterial/viral infection, liver function, cancer signalling and graft-versus-host disease signalling. The individual genes of these pathways are summarized in Table 1.

Using IPA's transcriptional factor enrichment analysis, we found that several transcription factors were correlated

**Table 1.** Down-regulated molecules in the top canonic pathways

Canonical pathways	Down-regulated molecules
Granulocyte adhesion and diapedesis	IL1A, IL1RL1, MMP14, CXCL 1, CCL17, CCL22, SDC3, CLDN7, CXCL 10, IL1R2, CXCL3, IL36G, CLDN4, MMP8, Ccl2, PPBP, MMP12, Ccl6, C5AR1, PF4, IL1 R1, FPR1, Ccl9, ITGAM, CCL7, IL1 RN, CLDN1, IL1 B, MMP9
Agranulocyte adhesion and diapedesis LXR/RXR activation	IL1A, C5AR1, PF4, MMP14, CXCL1, CCL 17, CCL22, IL1R1, CLDN7, Ccl9, CXCL10, CXCL3, IL36G, CCL7, CLDN4, CLDN1, IL1RN, MMP8, Ccl2, PPBP, IL1 B, MMP12, Ccl6, MMP9
IL-10 signalling PPAR signalling	IL1A, MSR1, IL1RL1, CD36, ARG2, IL1R1, IL1R2, IL36G, CCL7, IL1RN, IL 1B, S100A8, PTGS2, MMP9, IL1R2, HMOX1, FOS, IL36G, IL1A, IL 1RN, IL1RL1, IL 1B, ARG2, IL1 R1
Hepatic fibrosis I Hepatic stellate cell activation osteoblasts, osteoclasts and chondrocytes in RA regulation of cytokine in intestinal cells by IL-17 atherosclerosis signalling	IL1R2, FOS, IL36G, IL1A, PDGFA, IL 1RN, IL1RL1, IL 1B, IL1R1, PTGS2, IL1R2, MET, CXCL3, IL1A, EDNRB, EDN1, PDGFA, IL1RL1, IL 1B, IL1R1, MMP9, IL1R2, FOS, IL36G, IL1A, IL 1RN, IL1RL1, MMP8, MMP14, IL1B, IL1R1, CSF1R, LRP1, IL17A, IL1A, LCN2, CXCL 1, IL1B, IL17F, IL17A
Role of PRR in recognition of bacteria and viruses role of cytokines in communication in immune cells altered T-cell and B-cell signalling in RA communication in innate and adaptive immune cells	IL36G, IL1A, MSR1, PDGFA, IL1RN, CD36, IL1B, S100A8, PLA2G7, MMP9, CLEC7A, OAS1, IRF7, C5AR1, OAS2, IL1B, CLEC6A, OAS3, C3AR1, IL36G, IL1A, IL1RN, IL1B, IL22, IL17F, IL17A, IL36G, IL1A, IL1RN, FCER1G, Tlr13, IL 1B, IL22, IL17A
Macrophages, fibroblasts and endothelial cells in RA hypercytokinaemia/hyperchemokinaemia in influenza graft-versus-host disease signalling	CXCL10, IL36G, IL1A, IL1RN, FCER1G, Tlr13, IL1B, Ccl9
Role of IL-17A in psoriasis IL-6 signalling	IL1R2, FOS, IL36G, IL1A, C5AR1, IL1RN, IL1RL1, PDGFA, IL1B, Tlr13, IL1R1, LRP1, IL17A, CXCL10, IL36G, IL1A, IL1RN, IL1B, IL17A
Bladder cancer signalling	IL36G, IL1A, IL1RN, FCER1G, IL1B, GZMB, CXCL3, CXCL1, S100A8, IL17A, IL1R2, FOS, IL36G, IL1A, IL1RN, IL1RL1, IL1B, IL1R1
Regulation of cytokine in immune cells by IL-17 inhibition of matrix metalloproteases	CDH1, THBS1, MMP14, MMP8, CDKN1A, MMP12, MMP9, CXCL1, IL1B, IL17F, IL17A
Airway pathology in COPD	MMP14, MMP8, MMP12, MMP9, LRP1, CXCL3, MMP8, MMP9
IL-17F in allergic inflammatory airway diseases p38 MAPK signalling	CXCL10, CCL7, CXCL1, IL1B, IL17F, IL1R2, IL36G, IL1A, IL1RN, IL1RL1, IL1B, IL1R1, CXCL10, FOS, CXCL1, IL17A
IL-17A signalling in gastric cells role of IL-17A in arthritis hepatic cholestasis	CXCL3, CCL7, CXCL1, PTGS2, IL17A, IL1R2, IL36G, IL1A, IL1RN, IL1RL1, IL1B, IL1R1

The table lists the specific down-regulated gene names in Fig. 4(c) except *Glycam1*, which is up-regulated in agranulocyte adhesion and diapedesis pathway.



with the 150 genes regulated by our ROR $\gamma$ t inverse agonist (data not shown). These data were further analysed to generate a network model using the Advanced Network Analysis Tool.<sup>27</sup> Interestingly, more than 30 genes were downstream of or related with transcription factor SMAD3 (Fig. 4d). As we know, TGF- $\beta$  is critical for the differentiation of Th17 cells and Th17-mediated pathology.<sup>30</sup> SMAD3 is required for TGF- $\beta$ -induced gene expression. After phosphorylation by TGF- $\beta$  signalling, SMAD3 binds SMAD4, which then cooperates with c-Jun/c-Fos to mediate TGF- $\beta$ -induced transcription.<sup>31–33</sup> Recently, Dong and colleagues discovered that SMAD3 interacts with ROR $\gamma$ t and decreases its transcriptional activity. Furthermore, Smad3-deficiency led to enhanced Th17 differentiation *in vitro* and *in vivo*.<sup>34</sup> Here, we discovered that ROR $\gamma$ t inverse agonist inhibited more than 30 SMAD3 downstream genes (Fig. 4d), indicating that ROR $\gamma$ t could in turn regulate the function of SMAD3. The precise mechanism remains to be determined.

We have previously reported that TMP778 does not affect the gene expression of *Il1a* and *Il1b* in human CD4<sup>+</sup> T cells *in vitro*.<sup>21</sup> Here, *Il1a* and *Il1b* gene expression was significantly inhibited by TMP778 treatment *in vivo*. As CD4<sup>+</sup> T cells express a low level of *Il1*, the *Il1a* and *Il1b* we observed may be mainly expressed in macrophages within the draining lymph nodes after the MOG immunization. The inhibition of *Il1a* and *Il1b* gene expression by TMP778 treatment may be an indirect effect such as through the change of SMAD3 activity (Fig. 4d); the exact mechanism remains to be determined. Importantly, as IL-1 signalling is critical for the differentiation of Th17 cells and IL-17 production by  $\gamma\delta$  T cells, inhibition of mRNA expression of *Il1a* and *Il1b* could result in a synergistic effect on the inhibition of Th17 cell differentiation and IL-17 production.

Here, we discovered that *Dcpp*, *Abpb* and *Muc19* were expressed in lymph node cells from mice immunized with MOG in CFA and their expression was down-regulated by TMP778 treatment. However, their roles in the immune system have not yet been determined. *Dcpp* gene is expressed in embryo-containing oviduct.<sup>35</sup> Hence, ROR $\gamma$ t might have some role in embryo development. *Abpb* is the  $\beta$ -subunit of the androgen-binding protein and is expressed in salivary gland and lacrimal glands;<sup>36</sup> its function in the immune system needs to be explored. Mucus covers the epithelial surface of various tissues, providing lubrication and protection. The gel-like properties of mucus are generally attributed to the physical properties and structural features of gel-forming mucins, including Muc19.<sup>37</sup> Mucus has a protective function for the intestine,<sup>38</sup> and genome-wide association studies have shown an association between Muc19 and Crohn's disease.<sup>39</sup> As Muc19 is down-regulated by TMP778 administration *in vivo*, ROR $\gamma$ t inverse agonist should be evaluated in animal models of inflammatory bowel

diseases such as Crohn's disease. In addition, digestive system symptoms should be monitored when treating other diseases by ROR $\gamma$ t small molecule inverse agonists in clinic.

Most of the works in this study were carried out in mixed cell population (spleen cells or lymph node cells). We purposely designed the study to use mixed cell populations so that the compound data we obtained could be one step closer to the clinic because a ROR $\gamma$ t inverse agonist would eventually be administered to human patients. The ROR $\gamma$ t inverse agonist affect could be due to its inhibition of ROR $\gamma$ t function in different cell types not just CD4<sup>+</sup> Th17 cells. ROR $\gamma$ t is expressed not only in CD4<sup>+</sup> T cells, but also CD8<sup>+</sup> Tc17 cells,<sup>21,40</sup> innate lymphocytes,<sup>41,42</sup>  $\gamma\delta$  T cells,<sup>41,42</sup> and macrophage.<sup>43</sup> Hence, ROR $\gamma$ t inverse agonists should also affect the function of these cells.

This whole genomic approach has led us to discover that *in vivo* administration of TMP778 inhibited a variety of gene expression in addition to the Th17 signature gene, which is different from *in vitro* studies. These transcriptomic results provide new and important insight into the broader impact of targeting ROR $\gamma$ t *in vivo*. Further, these studies provide an unbiased view of potential disease indications and side-effects for ROR $\gamma$ t inhibitors, and so may facilitate clinical development.

In summary, we have demonstrated that the ROR $\gamma$ t inverse agonist TMP778 blocks Th17 cell differentiation *in vivo*. This transcriptomics study provided critical information for understanding the possible mechanisms of ROR $\gamma$ t inhibitors *in vivo*. This study should enable disease indication identification for ROR $\gamma$ t inhibitors, and may also help to predict adverse effects in clinic and hence improve clinical trials for the ROR $\gamma$ t small molecule inverse agonists.

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## Disclosures

All authors are present or former employees of Tempero Pharmaceuticals, Inc. ML is an employee of GlaxoSmithKline.

## References

- Murphy KM, Reiner SL. The lineage decisions of helper T cells. *Nat Rev Immunol* 2002; **2**:933–44.
- Ouyang W, Kolls JK, Zheng Y. The biological functions of T helper 17 cell effector cytokines in inflammation. *Immunity* 2008; **28**:454–67.
- Weaver CT, Harrington LE, Mangan PR, Gavrieli M, Murphy KM. Th17: an effector CD4 T cell lineage with regulatory T cell ties. *Immunity* 2006; **24**:677–88.
- McGeachy MJ, Cua DJ. Th17 cell differentiation: the long and winding road. *Immunity* 2008; **28**:445–53.
- Nurieva R, Yang XO, Martinez G et al. Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature* 2007; **448**:480–3.
- Miossec P, Korn T, Kuchroo VK. Interleukin-17 and type 17 helper T cells. *N Engl J Med* 2009; **361**:888–98.
- Hueber W, Patel DD, Dryja T et al. Effects of AIN457, a fully human antibody to interleukin-17A, on psoriasis, rheumatoid arthritis, and uveitis. *Sci Transl Med* 2010; **2**:52ra72.
- Leonardi C, Matheson R, Zachariae C et al. Anti-interleukin-17 monoclonal antibody ixekizumab in chronic plaque psoriasis. *N Engl J Med* 2012; **366**:1190–9.
- Papp KA, Leonardi C, Menter A et al. Brodalumab, an anti-interleukin-17-receptor antibody for psoriasis. *N Engl J Med* 2012; **366**:1181–9.
- Ivanov II, McKenzie BS, Zhou L et al. The orphan nuclear receptor ROR $\gamma$ t directs the differentiation program of proinflammatory IL-17 $^{+}$  T helper cells. *Cell* 2006; **126**:1121–33.
- Yang J, Sundrud MS, Skepner J, Yamagata T. Targeting Th17 cells in autoimmune diseases. *Trends Pharmacol Sci* 2014; **35**:493–500.
- Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F. Interleukins 1 $\beta$  and 6 but not transforming growth factor- $\beta$  are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol* 2007; **8**:942–9.
- Langrish CL, Chen Y, Blumenschein WM et al. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med* 2005; **201**:233–40.
- McGeachy MJ, Chen Y, Tato CM et al. The interleukin 23 receptor is essential for the terminal differentiation of interleukin 17-producing effector T helper cells *in vivo*. *Nat Immunol* 2009; **10**:314–24.
- Lee Y, Awasthi A, Yosef N et al. Induction and molecular signature of pathogenic Th17 cells. *Nat Immunol* 2012; **13**:991–9.
- Ramesh R, Kozhaya L, McKevitt K et al. Pro-inflammatory human Th17 cells selectively express P-glycoprotein and are refractory to glucocorticoids. *J Exp Med* 2014; **211**:89–104.
- Wang Y, Godec J, Ben-Aissa K et al. The transcription factors T-bet and Runx are required for the ontogeny of pathogenic interferon- $\gamma$ -producing T helper 17 cells. *Immunity* 2014; **40**:355–66.
- Huh JR, Leung MW, Huang P et al. Digoxin and its derivatives suppress TH17 cell differentiation by antagonizing ROR $\gamma$ t activity. *Nature* 2011; **472**:486–90.
- Solt LA, Kumar N, Nuhant P et al. Suppression of TH17 differentiation and autoimmunity by a synthetic ROR ligand. *Nature* 2011; **472**:491–4.
- Xu T, Wang X, Zhong B et al. Ursolic acid suppresses interleukin-17 (IL-17) production by selectively antagonizing the function of ROR $\beta$  $\gamma$ t protein. *J Biol Chem* 2011; **286**:22707–10.
- Skepner J, Ramesh R, Trocha M et al. Pharmacologic inhibition of ROR $\gamma$ t regulates Th17 signature gene expression and suppresses cutaneous inflammation *in vivo*. *J Immunol* 2014; **192**:2564–75.
- Yang J, Skepner J, Mark T, Ghosh S. Small molecule inhibitors targeting the Th17 cell transcription factor ROR $\gamma$ t for the treatment of autoimmune diseases. *J Clin Cell Immunol* 2013; **4**:e111.
- Xiao S, Yosef N, Yang J et al. Small-molecule ROR $\gamma$ t antagonists inhibit T helper 17 cell transcriptional network by divergent mechanisms. *Immunity* 2014; **40**:A77–89.
- Carlson TJ, Pellerin A, Djuretic IM et al. Halofuginone-induced amino acid starvation regulates STAT3-dependent Th17 effector function and reduces established autoimmune inflammation. *J Immunol* 2014; **192**:2167–76.
- Yang J, Castle BE, Hanidu A et al. Sphingosine kinase 1 is a negative regulator of CD4 $^{+}$  Th1 cells. *J Immunol* 2005; **175**:6580–8.
- Yang J, Yang M, Htut TM et al. Epstein-Barr virus-induced gene 3 negatively regulates IL-17, IL-22 and ROR $\gamma$ t. *Eur J Immunol* 2008; **38**:1204–14.
- Yosef N, Zalckvar E, Rubinstein AD et al. ANAT: a tool for constructing and analyzing functional protein networks. *Sci Signal* 2011; **4**:11.
- Betelli E, Korn T, Oukka M, Kuchroo VK. Induction and effector functions of Th17 cells. *Nature* 2008; **453**:1051–7.
- Murakami M, Okuyama Y, Ogura H et al. Local microbleeding facilitates IL-6- and IL-17-dependent arthritis in the absence of tissue antigen recognition by activated T cells. *J Exp Med* 2011; **208**:103–14.
- McGeachy MJ, Bak-Jensen KS, Chen Y et al. TGF- $\beta$  and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain Th17 cell-mediated pathology. *Nat Immunol* 2007; **8**:1390–7.
- Zhang Y, Feng XH, Derynck R. Smad3 and Smad4 cooperate with c-Jun/c-Fos to mediate TGF- $\beta$ -induced transcription. *Nature* 1998; **394**:909–13.
- Derynck R, Zhang Y, Feng XH. Smads: transcriptional activators of TGF- $\beta$  responses. *Cell* 1998; **95**:737–40.
- Massague J, Xi Q. TGF- $\beta$  control of stem cell differentiation genes. *FEBS Lett* 2012; **586**:1953–8.
- Martinez GJ, Zhang Z, Chung Y et al. Smad3 differentially regulates the induction of regulatory and inflammatory T cell differentiation. *J Biol Chem* 2009; **284**:35283–6.
- Lee KF, Xu JS, Lee YL, Yeung WS. Demilune cell and parotid protein from murine oviductal epithelium stimulates preimplantation embryo development. *Endocrinology* 2006; **147**:79–87.
- Remington SG, Nelson JD. Secretoglobins: sexually dimorphic expression of androgen-binding protein mRNA in mouse lacrimal glands. *Invest Ophthalmol Vis Sci* 2005; **46**:31–8.
- Chen Y, Zhao YH, Kalaslavadi TB et al. Genome-wide search and identification of a novel gel-forming mucin MUC19/Muc19 in glandular tissues. *Am J Respir Cell Mol Biol* 2004; **30**:155–65.
- Johansson ME, Ambort D, Pelaseyed T et al. Composition and functional role of the mucus layers in the intestine. *Cell Mol Life Sci* 2011; **68**:3635–41.
- Kumar V, Mack DR, Marciel V et al. Genome-wide association study signal at the 12q12 locus for Crohn's disease may represent associations with the MUC19 gene. *Inflamm Bowel Dis* 2013; **19**:1254–9.
- Curtis MM, Way SS, Wilson CB. IL-23 promotes the production of IL-17 by antigen-specific CD8 T cells in the absence of IL-12 and type-I interferons. *J Immunol* 2009; **183**:381–7.
- Pantelyushin S, Haak S, Ingold B et al. ROR $\gamma$ t $^{+}$  innate lymphocytes and gammadelta T cells initiate psoriasisform plaque formation in mice. *J Clin Invest* 2012; **122**:2252–6.
- Becher B, Pantelyushin S. Hiding under the skin: interleukin-17-producing  $\gamma\delta$  T cells go under the skin? *Nat Med* 2012; **18**:1748–50.
- Gu Y, Yang J, Ouyang X et al. Interleukin 10 suppresses Th17 cytokines secreted by macrophages and T cells. *Eur J Immunol* 2008; **38**:1807–13.

## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** No change of cell surface markers by administration of TMP778. C57BL/6 mice were immunized with MOG<sub>35–55</sub> in complete Freund's adjuvant. TMP778 (20 mg/kg or 5 mg/kg) or Vehicle was subcutaneously injected twice a day for 6 days as in Fig. 2. Draining lymph node cell were harvested, stained for cell surface markers, and analysed by flow cytometry.

**Figure S2.** *In vivo* differentiation of T helper type 17 (Th17) cells was blocked by administration of TMP536 or TMP934. (a and d) C57BL/6 mouse splenocytes were stimulated with anti-CD3/anti-CD28 monoclonal antibody plus interleukin-6 (IL-6) and transforming growth factor- $\beta$  (TGF- $\beta$ ) in the presence of the indicated concentration of ROR $\gamma$ t inverse agonist TMP536 (a) or TMP934 (d) for 3 days. The IL-17 titres in the supernatants were then determined and the IC<sub>50</sub> were calculated. (b and e) C57BL/6 mice were immunized with MOG<sub>35–55</sub> in CFA. TMP536 (20 or 50 mg/kg) (b) or TMP934 (50 mg/kg) (e) were subcutaneously injected twice a day for 6 days. The draining lymph node cells were isolated and stimulated with MOG<sub>35–55</sub> for 5 days, after which the supernatants were harvested to detect IL-17 titers by Meso Scale Discovery. (c) Chemical structure of TMP934.