Inflammation, Vol. 42, No. 1, February 2019 (© 2018)
DOI: 10.1007/s10753-018-0875-7

ORIGINAL ARTICLE

Pharmacological Evaluation of TAK-828F, a Novel Orally Available RORγt Inverse Agonist, on Murine Colitis Model

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Abstract— IL-17-producing Th17 cells and IFN-γ and IL-17 double-producing Th1/17 cells have been identified as the pathogenic cells in inflammatory bowel disease (IBD). Retinoic acid-related orphan receptor γt (RORγt) is a master regulator for the differentiation and activation of Th17 and Th1/17 cells. We discovered a novel orally available TAK-828F, a strong and selective RORγt inverse agonist. To assess the potential of RORγt blockade in the therapy for IBD, the efficacy of TAK-828F in activated T cell transfer mouse colitis model was investigated. This model was highly sensitive to the prophylactic treatment of anti-TNF-α monoclonal antibody but partially susceptible to sulfasalazine, tacrolimus, and prednisolone. Oral administration of TAK-828F, at doses of 1 and 3 mg/kg, b.i.d, strongly protected the progression of colitis. TAK-828F decreased the population of Th17 and Th1/17 cells in a dose-dependent manner in the mesenteric lymph node. Moreover, expression of mRNA that are characteristic of the Th17 signature, such as IL-17A and IL-17F in the colon, were inhibited by TAK-828F, while the expression of IL-10, an anti-inflammatory cytokine, was increased. In the therapeutic treatment, TAK-828F lessened disease severity compared to the vehicle control mice. Interestingly, gene expression of zonula occludens-1 (ZO-1) and mucin 2 (Muc2), which play an important role in barrier function of the intestinal mucosa, was recovered by TAK-828F. These results indicate that blocking RORγt has promising pharmacological profile in the colitis model. RORγt blockade may provide a novel therapeutic

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paradigm for treatment of IBD with unique mechanism by which improves imbalance of the immune system.

KEY WORDS: RORγt inverse agonist; TAK-828F; Experimental colitis; Th17 cells; Th1/17 cells.

INTRODUCTION

Incidence of Inflammatory bowel diseases (IBD) is increasing and IBD affects more than 4 million people worldwide [17, 24]. It is known that $CD4^+$ T cells are involved in the pathogenesis of inflammatory diseases, such as IBD [4, 6, 29]. Once naive $CD4^+$ T cells are stimulated with pathological antigen and cytokine, they can differentiate to IFN-γ-producing Th1 cells, IL-4- and IL-13-producing Th2 cells, and IL-17-producing Th17 cells [39]. Recently identified Th17 cells are differentiated under stimulation with T cell receptor (TCR), IL-6, IL-23, and TGF-β and result in producing the pro-inflammatory cytokines containing IL-17A, IL-17F, IL-21, and IL-22 [39]. Th17 cells are thought to contribute to the pathogenesis of inflammatory diseases, including IBD, multiple sclerosis (MS), and psoriasis (Ps), because an increased infiltration of Th17 cells into inflammatory tissues of these patients has been observed [16]. Moreover, it has been demonstrated that Th17 cells are deeply involved in the pathogenesis of several inflammatory disease models, such as colitis and experimental autoimmune encephalomyelitis (EAE) [12, 23, 32].

In addition to the Th17 cells, IFN-γ, and IL-17 co-expressing cells, Th1/17 cells, are recently identified. These cells are increased in the tissues of the patients with IBD, MS, and Bechet's disease, and the number of the cells was correlated with the disease severity [7, 18, 32, 34]. These clinical data suggest that Th1/17 cells may also contribute to the pathophysiology of these diseases. Thus, blocking of Th17 and Th1/17 cells would be a novel therapeutic option to treat patients suffering from these diseases.

Retinoic acid-related orphan receptor γt (RORγt) is the nuclear receptor identified as the master regulator of Th17 cell differentiation and activation [15]. It is expressed in the Th17 cells, γδT cells, innate lymphoid cells, and natural-killer T (NKT) cells and contributes to the secretion of pro-inflammatory cytokines [15, 25, 27, 31]. Moreover, it is reported that Th17 cells can differentiate into Th1/17 cells in the inflammatory condition, and converted Th1/17 cells cause further inflammation in the colon of mice [22, 36]. In this context, RORγt is expected to be the attractive target for the treatment of inflammatory disorders involving in Th17 and Th1/17 cells. In fact, several small-molecule RORγt inverse agonists are discovered as efficacious drug candidate in EAE model and imiquimod-induced Ps-like model [2, 13, 35, 38]. Recently, GSK805, an orally available RORγt inverse agonist, has been reported to show efficacy in a colitis model, but details of pharmacological profile of the compound has not been elucidated [37].

We discovered a novel orally available and selective RORγt inverse agonist, TAK-828F [20]. TAK-828F strongly binds to human RORγt and selectively inhibits RORγt activity in reporter gene assay of human RORγt with IC_{50} of 6.1 nmol/L but do not affect RORα, RORβ, and other 19 kinds of nuclear reporters even at 10 μmol/L [28]. TAK-828F, at 100 nmol/L, also strongly inhibited the Th17 differentiation and the production of RORγt-related pro-inflammatory cytokines in both human and mouse primary cells [33]. To evaluate the therapeutic potential of RORγt inverse agonist for IBD, we investigated the effect of TAK-828F on the activated T cell transfer mouse colitis model. We also investigated the effect of TAK-828F on the T cell population and RORγt-related gene expression in the colon of the colitis model.

MATERIALS AND METHODS

Animals

Balb/c mice (female) were purchased from Charles River Japan (Japan). C.B-17/Icr-scid mice (SCID mice, female) were purchased from CLEA Japan, Inc. (Japan). C.B-17/Icr-scid mice were used as recipient mice and bred individually on white chip (Paperclean, SLC, Japan). Mice were maintained under specific pathogen-free conditions and given food and water *ad libitum*. All procedures were performed in accordance with the standards for humane care, and treatment of research animal was approved by IACUC (Institutional Animal Care and Use Committee) in Takeda Pharmaceutical Company, Ltd. (approval no. 10797).

Chemicals

TAK-828F [20] was synthesized at Takeda Pharmaceutical Company, Ltd. (Japan).

Experimental Colitis

Mouse colitis was induced by adoptive transferring of activated $CD4^+$ T cells of Balb/c to SCID mice [14]. Activated $CD4^+$ T cells (2×10^5 cells) prepared from splenocytes of Balb/c mice were intravenously injected into SCID mice (day 0). In preliminary assay, softening stool, diarrhea, and colon weight gain corresponding to the intestinal inflammation was observed on day 10 to 14 after the adoptive T cells transfer, peaked on day 21, and these parameters chronically continued after then [14].

Prophylactic Treatment of TAK-828F and Representative Drugs for Treatment of IBD

TAK-828F was suspended in 0.5% methyl cellulose (0.5% MC) and orally gavage administered to the mice. Vehicle control mice were administered 0.5% MC. Mice were administered once a day on day 0 and twice a day from day 1 to 21. In the preliminary assay, TAK-828F, at doses of 0.1 to 3 mg/kg, b.i.d., dose-dependently inhibited IL-17A gene expression in the ear of mice caused by single intradermal injection of IL-23 (data not shown). From this data, we selected administration doses of TAK-828F at 0.03 to 3 mg/kg, b.i.d. in the colitis model. Sulfasalazine (3, 10, or 30 mg/kg, Sigma, USA), prednisolone (1 or 5 mg/kg, Sigma, USA), and tacrolimus (1 or 10 mg/kg, ALEXIS Biochemicals, USA) were also suspended in 0.5% MC and orally administered once daily for 21 days. Maximum doses of sulfasalazine, prednisolone, and tacrolimus were selected at 30, 5, and 10 mg/kg, respectively, because these drugs caused severe loss of body weight when their doses were more than those used in the present study (data not shown). Anti-mouse TNF-α mAb (4, 20, and 100 μg/mouse, clone: XT3.11, Bio X Cell, USA) or rat IgG1 isotype control (100 μg/mouse, Bio X Cell, USA) was administered intraperitoneally on days 0, 5, 9, 13 and 17 days after T cell transfer. On day 22, diarrhea score for stool consistency was determined under blind fashion on a scale of 1–4. The diarrhea score for stool consistency was graded on a scale of 1–4 as follows: 1, normal; 2, pasty and formed; 3, pasty and unformed; 4, diarrhea [14]. Then, mice were sacrificed under anesthesia and total colon from each mouse was surgically removed, rinsed with saline, and measured the weight. Mesenteric lymph node (MLN) of each mouse was also collected.

Therapeutic Treatment of TAK-828F

On day 18 after the T cell transfer, diarrhea score for stool consistency was determined on a scale of 1–4 as described above. Mice graded 2 and 3 were selected, and grouping was conducted based on its colitis grades (pre-control, vehicle control, and compound-treated group). Pre-control mice were sacrificed on the grouping day and then colon weight of each mouse was measured. Other mice were administrated with 0.5% MC and TAK-828F at 1 or 3 mg/kg by oral gavage treatment. Those compounds were treated once a day on day 18 and twice a day from day 19 to 31. On day 32, diarrhea score for stool consistency was determined under blind fashion, and colon weight of each mouse was measured as described above. T cell population in the MLN and colonic gene expression of the mouse were also analyzed at this time point.

Intracellular Cytokine Staining

Single cell suspensions prepared from MLNs were stimulated with PMA (50 ng/mL, Wako, Japan) and ionomycin (1 μg/mL, Wako, Japan), in presence of transport inhibitor containing monensin (BD Biosciences, USA) and incubated for 4 h in RPMI-1640 medium containing 10% of FBS. After blocking Fc receptor by anti-CD16/CD32 mAb (clone: 2.4G2, BioXCell, USA), cells were stained with PE-conjugated-anti-CD4 mAb (BioLegend, USA). Stained cells were fixed and permeabilized with fixation/permeabilization solution (BD Biosciences, USA). The intracellular cytokine staining was carried out using FITC-conjugated anti-IFN-γ mAb and Alexa Fluor 647-conjugated anti-IL-17A mAb (BioLegend, USA). Flow cytometry analysis was performed using BD Accuri C6 Flow Cytometer (BD Biosciences, USA). The population of Th17, Th1/17, and Th1 cells were defined as follows: Th17, $IL\text{-}17^+$ $IFN\text{-}\gamma^-$ cells gated on $CD4^+$ cells; Th1/17, $IL\text{-}17^+$ $IFN\text{-}\gamma^+$ cells gated on $CD4^+$ cells; Th1, $IL\text{-}17^-$ $IFN\text{-}\gamma^+$ cells gated on $CD4^+$ cells.

Real-Time Quantitative RT-PCR

Colon was stored in RNAlater (Qiagen, Germany) at 4 °C. Total RNA was isolated using RNeasy Mini Kit (Qiagen, Germany) and DNaseI (Qiagen, Germany) to avoid genomic DNA contamination, according to the manufacturer's instructions. High Capacity cDNA Reverse Transcription Kit (Life Technologies, USA) was used for cDNA synthesis. Quantitative PCR reactions were performed on a ViiA 7 Real-Time PCR System (Life Technologies, USA), using TaqMan Fast Advanced Master Mix (Lifer Technologies, USA) with specific primers on TaqMan Gene Expression Assays (Life Technologies, USA) according to the manufacturer's manual. FAM-probed primers with the following assay identification numbers were

used: *Il17a*, Mm00439618_m1; *Il17f*, Mm00521423_m1; *Ifng*, Mm00801778_m1; *Il10*, Mm00439614_m1; *Tjp1*, Mm00493699_m1; *Muc2*, Mm01276696_m1. The data were normalized by β-actin (*Actb*) gene expression.

Histopathology

Colon tissue of SCID mouse was collected on day 22 after the T cell transfer. Two small pieces from proximal and distal portions of the colon were dissected from each mouse and placed in 10 vol% neutral buffered formalin as described previously [14]. All tissues were embedded in paraffin, sectioned in a cross-sectional manner, and stained with hematoxylin and eosin. Histopathological evaluation was performed under blinded condition by two pathologists, and the following scoring criteria were used.

Histopathological scoring system

	Score	Grade	Criteria
Mononuclear cell infiltration	0	None	Not remarkable
	1	Minimal	Focal or multifocal infiltration of mononuclear cells (mainly lymphocytes) in mucosa and/or submucosa
	2	Mild	Diffuse infiltration of mononuclear cells (mainly lymphocytes in mucosal and/or submucosa
Inflammatory cell infiltration	0	None	Not remarkable
	1	Minimal	Focal infiltrations of inflammatory cells (mainly neutrophils) in mucosa
Crypt abscesses/ cell debris in lumen	0	None	Not remarkable
	1	Minimal	Abscess and/or cell debris in the lumen of the crypts
Mucosal regeneration/ hyperplasia	0	None	Not remarkable
	1	Minimal	Regeneration/ hyperplasia without goblet cells of mucosa in focal area
	2	Mild	Multifocal regeneration/ hyperplasia of mucosa in about half of the area
	3	Moderate	Regeneration/hyperplasia of mucosa in almost all area with doubled mucosal thickness without goblet cells

Histopathological scores of all findings from both proximal and distal sections of the colon were combined to calculate total histopathological score for each animal (maximum score: 14).

Statistics

Data are expressed as mean ± standard error of the mean (S.E.). Results were analyzed using the SAS System for Windows (Release 9.3, SAS Institute, Japan) or the EXSUS statistical analysis system (8.0 ver, CAC EXICARE, Japan). Differences of diarrhea score between normal group and pre-control group were analyzed by Wilcoxon test. Differences except diarrhea score between these groups were made as follows. When equality of variances was indicated by *F*-test, statistical analysis was performed using Student's *t* test. When equality of variances was not indicated, statistical analysis was performed using Aspin-Welch's *t* test. A probability value of $p < 0.05$ was considered statistically significant.

Differences of diarrhea score between vehicle control group and TAK-828F-treated group were analyzed by one-tailed Shirley-Williams' test. The data except diarrhea score between these groups were analyzed as follows. When equality of variances was indicated by Bartlett's test, statistical analysis was performed using one-tailed Williams' test. When equality of variances was not indicated by Bartlett's test, statistical analysis was performed using one-tailed Shirley-Williams' test. A probability value of $p < 0.025$ was considered statistically significant.

RESULTS

The Prophylactic Efficacy of Small Compound Drugs and Anti-TNF-α mAb in Activated T Cell Transfer Colitis Model

To evaluate the sensitivity of the T cell transfer mouse colitis model to the currently used drugs for treatment of IBD, the efficacy of representative clinical medications for IBD was investigated. Firstly, we evaluated protective efficacy of sulfasalazine, prednisolone, tacrolimus, and anti-TNF-α mAb and the results were summarized in Table 1. In the model, modest protective efficacy of these compounds was observed. In contrast, anti-TNF-α mAb strongly inhibited onset of diarrhea score and colon weight gain at doses of 20 and 100 μg/mouse. The inhibition rates of onset of diarrhea and colon weight gain by treatment with anti-TNF-α mAb at these doses were 60–70%. Thus,

Table 1. Effect of Prophylactic Treatment of Representative Oral Drugs and Anti-TNF-αmAb in Activated T Cell Transfer Colitis Model

Treatment	Dose (mg/kg)	Diarrhea score	Colon weight (g)
Normal		1.0 ± 0.0	0.202 ± 0.009
Vehicle control		2.5 ± 0.4	0.401 ± 0.029
Sulfasalazine	3	3.0 ± 0.3	0.417 ± 0.027
	10	3.1 ± 0.2	0.466 ± 0.002
	30	1.8 ± 0.3	0.358 ± 0.024
Normal		1.0 ± 0.0	0.217 ± 0.015
Vehicle control		3.4 ± 0.2	0.614 ± 0.032
Prednisolone	1	2.4 ± 0.4	0.420 ± 0.044*
	5	2.1 ± 0.2#	0.425 ± 0.025*
Tacrolimus	1	2.3 ± 0.5	0.415 ± 0.034*
	10	3.7 ± 0.2	0.400 ± 0.017*
	Dose (μg/mouse)		
Normal		1.0 ± 0.0	0.245 ± 0.007
IgG control		2.9 ± 0.3	0.434 ± 0.030
Anti-TNF-α mAb	4	2.6 ± 0.3	0.435 ± 0.020
	20	1.4 ± 0.3#	0.303 ± 0.020*
	100	1.6 ± 0.2#	0.316 ± 0.027*

Activated T cells (2×10^5 cells/mouse) were transferred to SCID mice. Then, test compounds were orally administered once daily for 21 days. Anti-TNF-α mAb or isotype rat IgG1 mAb was intraperitoneally administrated at the day 0 and 5, 9, 13, 17 days after T cell transfer. Diarrhea score and colon weight were assessed on day 22. Data were represented as the mean ± S.E. of 3 (normal) and 7 or 8 (transferred mice) animals
#$p < 0.025$ (one-tailed Shirley-Williams' test); *$p < 0.025$ (one-tailed Williams' test) *vs.* vehicle control group

this colitis model was highly sensitive to anti-TNF-α mAb, though efficacy of sulfasalazine, prednisolone, and tacrolimus was modest.

The Effect of Prophylactic Treatment with TAK-828F on Activated T Cell Transfer Colitis Model

Next, we investigated the protective efficacy of TAK-828F in the T cell transfer colitis model at the doses of 0.03, 0.3 and 3 mg/kg, b.i.d. Oral gavage administration of TAK-828F prevented the onset of diarrhea and increase of colon weight in a dose-dependent manner (Fig. 1a, b), and TAK-828F, at a dose of 3 mg/kg, significantly ameliorated both parameters. In order to clarify the mode of action of TAK-828F in the colitis model, we analyzed the population of Th17, Th1/17, and Th1 cells in MLNs of T cell transferred SCID mice on day 22. The populations of Th17 and Th1/17 cells were decreased by the treatment of TAK-828F in a dose-dependent manner (Fig. 1c, d). On the other hand, the population of Th1 cells was not affected at any doses (Fig. 1e). Next, gene expressions of IL-17A, IL-17F, IFN-γ, and IL-10 in the colon of mice were measured. Colonic gene expressions of IL-17A and IL-17F were suppressed in a dose-dependent manner by treatment with TAK-828F (Fig. 2a, b), whereas expression of IFN-γ was not affected (Fig. 2c). The expression of IL-10, an anti-inflammatory cytokine, was significantly upregulated by TAK-828F at 3 mg/kg (Fig. 2d). In summary, TAK-828F treatment resulted in the decrease of population of Th17 and Th1/17 cells in MLN and improved imbalance of pro-inflammatory and anti-inflammatory cytokine in the colon.

Histopathological Study in Activated T Cell Transfer Colitis Model

Additionally, we conducted histopathological analysis in the colon of the mice after treatment of TAK-828F in the separate experiment. TAK-828F, at doses of 1 and 3 mg/kg, prevented onset of diarrhea and colon weight gain in the model (Fig. 3a, b). The inhibition rate of TAK-828F, at doses of 1 and 3 mg/kg, against progression of diarrhea and colon weight gain was around 70 and 55%, respectively. In the mice, TAK-828F, at doses of 1 and 3 mg/kg, significantly reduced total histopathological scores in the colon (Fig. 3c). TAK-828F treatment reduced incidence of infiltration of mononuclear and inflammatory cells, mucosal regeneration/hyperplasia, and crypt abscesses/debris in the lumen (Fig. 3d–g). Therefore, TAK-828F, at 1 and 3 mg/kg, protected the progression of the colitis in the model.

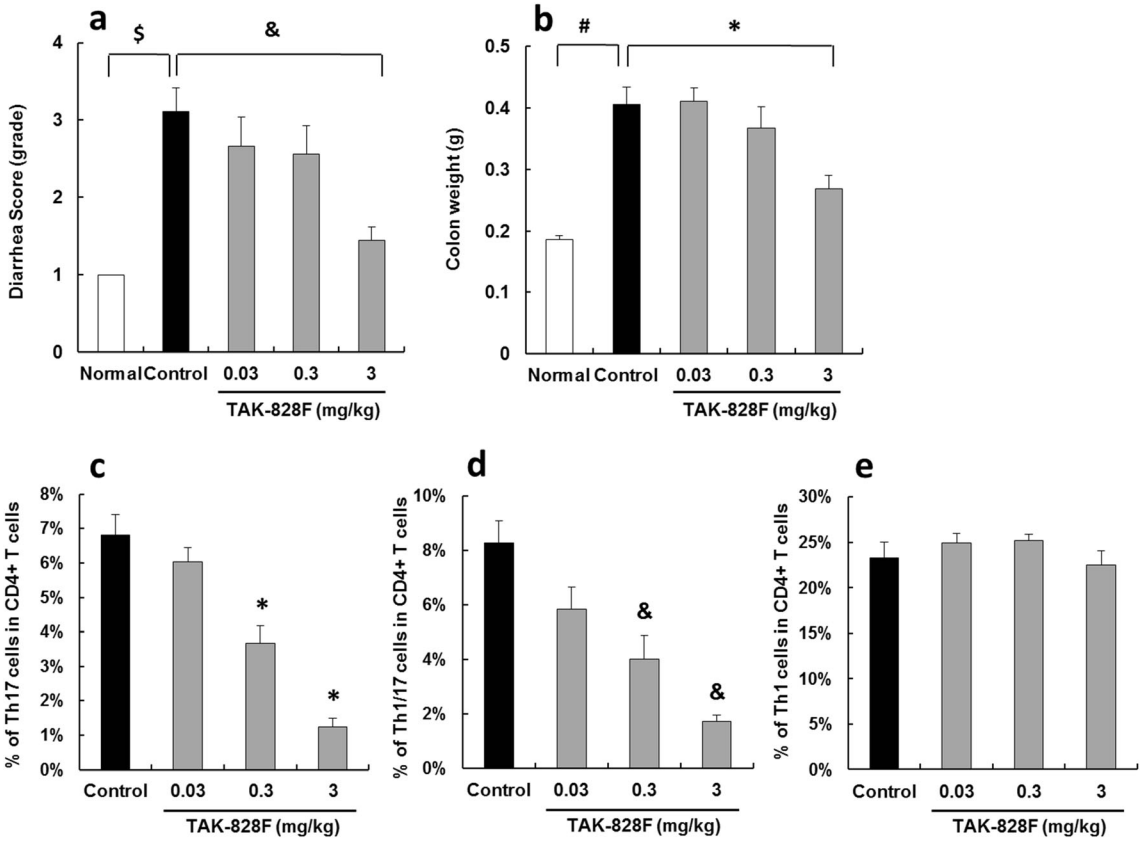

Fig. 1. Effect of prophylactic treatment of TAK-828F on activated T cell transfer colitis model. TAK-828F (0.3, 1 and 3 mg/kg) or vehicle control (0.5% methylcellulose) were orally administrated to the SCID mice twice a day for 22 days. **a** Colon weight and **b** diarrhea score were analyzed in a blind fashion. The population of Th17, Th1/17, and Th1 cells in MLNs was determined by intracellular staining. The populations of **c** Th17 cells, **d** Th1/17 cells, and **e** Th1 cells gated on $CD4^+$ T cells were analyzed by flow cytometry. Data were represented as the mean ± S.E. of 3 (normal) or 9 (transferred mice) animals. #$p < 0.05$ (Student's *t* test) and \$$p < 0.05$ (Aspin-Welch's *t* test) *vs.* normal group. &$p < 0.025$ (one-tailed Shirley-Williams' test) and *$p < 0.025$ (one-tailed Williams' test) *vs.* vehicle control group.

Effect of Therapeutic Treatment with TAK-828F on Activated T Cell Transfer Colitis Model

To evaluate therapeutic efficacy of TAK-828F in the model, the compound at doses of 1 and 3 mg/kg was administered after the onset of colitis as described in the methods. For 14 days treatment of TAK-828F, TAK-828F dose-dependently ameliorated diarrhea score and inhibited colon weight gain and significant inhibition of these parameters compared with vehicle treated control group was observed at 3 mg/kg (Fig. 4a, b). The populations of Th17 and Th1/17 cells in the MLNs of mice were dose-dependently decreased by the administration of TAK-828F (Fig. 4c, d). On the other hand, TAK-828F did not affect the population of Th1 cells at any doses (Fig. 4e). In addition, colonic mRNA expressions of IL-17A and IL-17F were suppressed in a dose-dependent manner by the treatment with TAK-828F (Fig. 5a, b), whereas expression of IFN-γ was partially affected (Fig. 5c). As observed in the protective dose regimen study, the expression of IL-10 was significantly upregulated by TAK-828F at dose of 3 mg/kg (Fig. 5d). Colonic gene expression of zonula occludens-1 (ZO-1) and mucin 2 (Muc2), the major component of mucosal barrier, significantly decreased at the timing of onset of colitis (on day18, Fig. 5e, f). Interestingly, TAK-828F recovered these gene expressions after 14 days treatment in the therapeutic dose regimen. Finally, TAK-828F showed therapeutic efficacy in the colitis model.

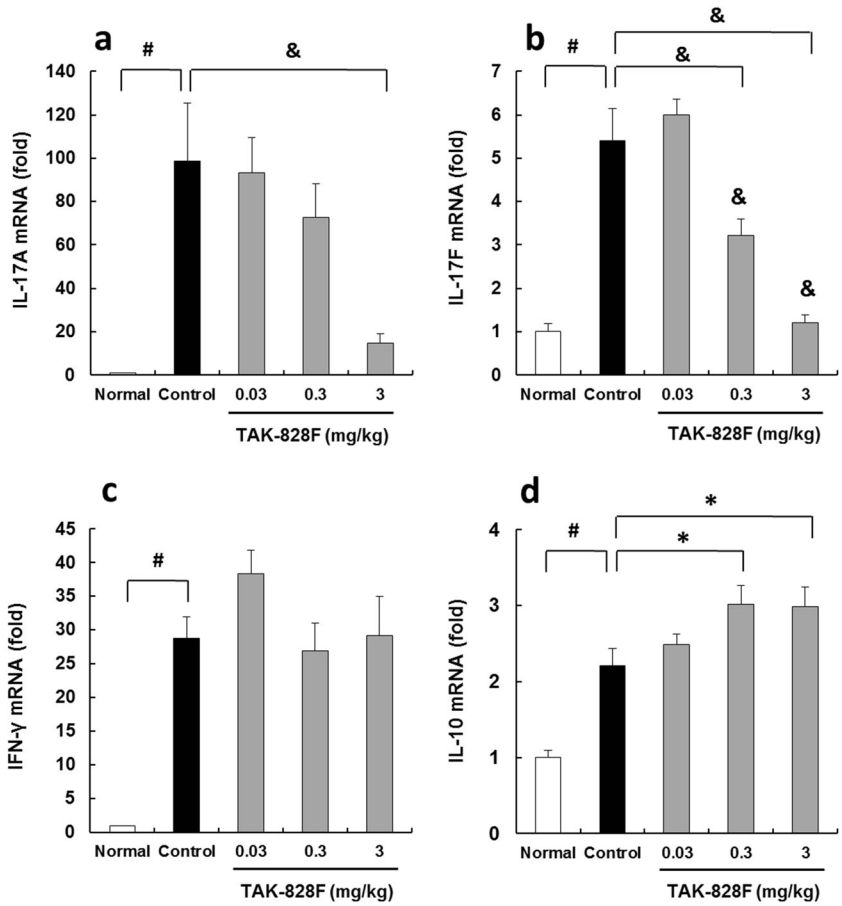

Fig. 2. Effect of prophylactic treatment of TAK-828F on colonic gene expressions in activated T cell transferred mice. TAK-828F (0.03, 0.3, and 3 mg/kg) or vehicle control (0.5% methylcellulose) were orally administrated to the SCID mice twice a day for 22 days. Total RNA was isolated from colonic tissue on day 22, and relative mRNA expression of **a** IL-17A, **b** IL-17F, **c** IFN-γ, and **d** IL-10 in colon were measured by real-time RT-PCR, normalized with the expression of β-actin. Data were represented as the mean ± S.E. of 3 (normal) or 9 (transferred mice) animals. #$p < 0.05$ (Student's *t* test) *vs.* normal group. &$p < 0.025$ (one-tailed Shirley-Williams' test) and *$p < 0.025$ (one-tailed Williams' test) *vs.* vehicle control group.

DISCUSSION

In these experiments, TAK-828F showed efficacy both in prophylactic and therapeutic dose regimen in the activated T cell transfer mouse colitis model.

Before conducting efficacy study of TAK-828F, we have evaluated the sensitivity of the activated T cell transfer colitis model to currently used drugs for treatment of IBD. As shown in Table 1, anti-TNF-α mAb showed strong efficacy in the colitis model. In contrast, oral administration of sulfasalazine, corticosteroid, and tacrolimus showed modest efficacy in the colitis model. Thus, the sensitivity of our colitis model to the representative drugs currently used for the patients with IBD looks similar to that in IBD patients. These results suggest that the activated T cell transfer colitis model is useful to evaluate efficacy of candidate compounds and biologics for IBD treatment.

In the prophylactic dose regimen, TAK-828F showed protective efficacy at 1 and 3 mg/kg in the colitis model (Figs. 1 and 3). At these doses, TAK-828F dose-dependently reduced population of Th17 and Th1/17 cells but did not affect population of Th1 cells in the MLN at efficacy doses (Fig. 1). In addition, colonic gene expressions of IL-17A and IL-17F, but not IFN-γ, were specifically inhibited by the treatment with TAK-828F (Fig. 2). As described in the "INTRODUCTION" section, similar pharmacological effects, such as specific inhibition of Th17 differentiation from naive T cells and IL-17A and IL-17F production in peripheral blood cells, were observed *in vitro* mouse and human primary cell assay [33]. In

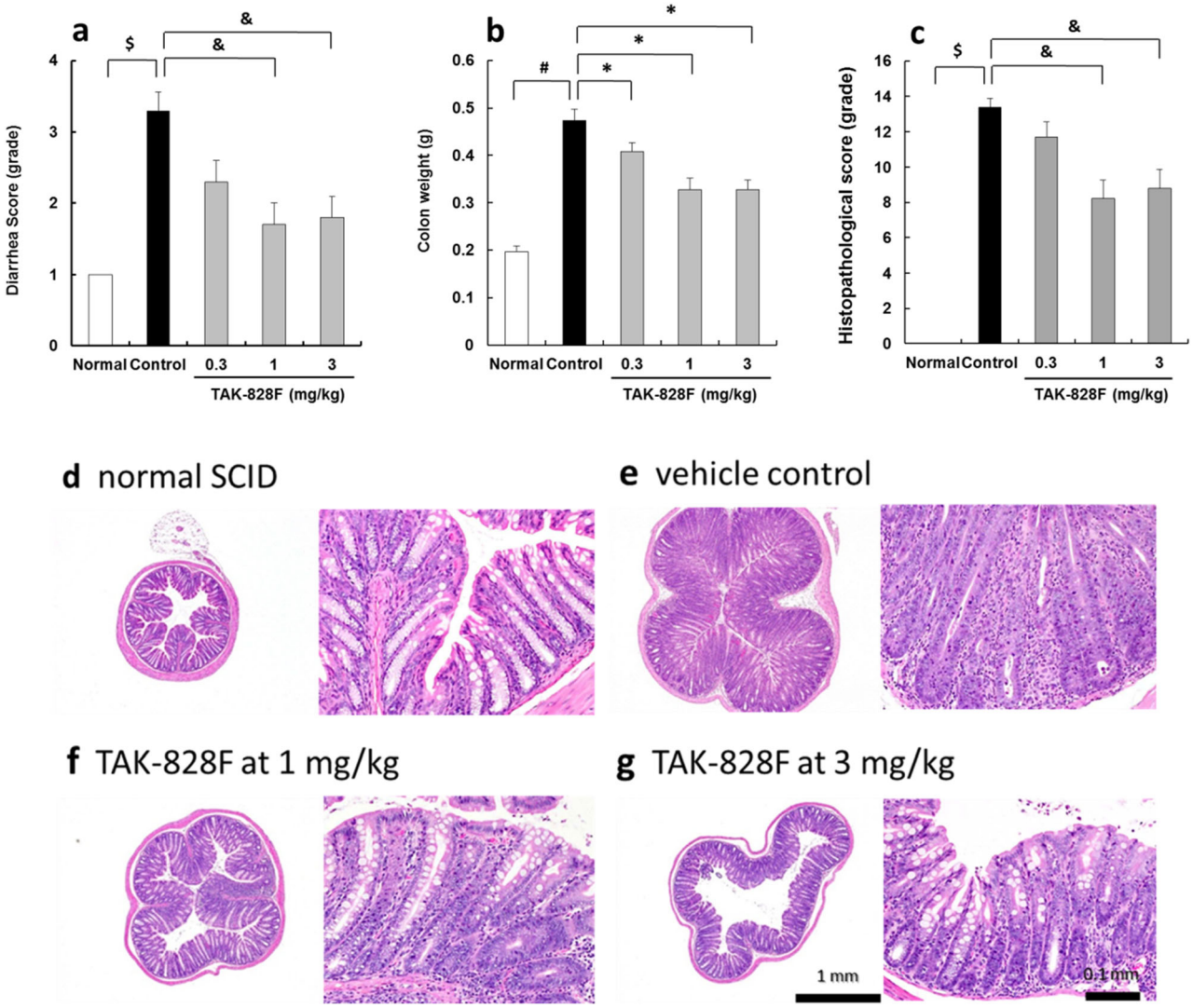

Fig. 3. Effect of prophylactic treatment of TAK-828F on activated T cell transfer colitis model. TAK-828F (0.3, 1, and 3 mg/kg) or vehicle (0.5% methylcellulose) was orally administrated to the SCID mice twice a day for 22 days. **a** Diarrhea score, **b** colon weight, and **c** total histopathological score were analyzed. Histopathological score of the colon was determined by staining with H&E as **d** normal SCID mice, **e** vehicle control group, **f** TAK-828F at 1 mg/kg-treated group, and **g** TAK-828F at 3 mg/kg-treated group. Data were represented as the mean ± S.E. of 5 (normal) or 10 (transferred mice) animals. $p < 0.05$ (Wilcoxon test) and #$p < 0.05$ (Student's *t* test) *vs.* normal group. &$p < 0.025$ (one-tailed Shirley-Williams' test) and *$p < 0.025$ (one-tailed Williams' test) *vs.* vehicle control group.

addition, *in vitro* Th1/17 differentiation from human memory $CD4^+$ T cells was also inhibited by treatment with TAK-828F [33]. These results indicate that TAK-828F showed the efficacy in the colitis model by modulating Th17 and Th1/17 cell differentiation and activation of these cells *via* inhibition of RORγt.

IL-23 has critical role of differentiation and maintenance of Th17 [26]. Recently, anti-IL-12/23p40 mAb and anti-IL-23 mAb showed marked efficacy in biologic-naive or anti-TNF-α mAb-refractory patients with moderate and severe CD [3, 19]. These facts suggest that inhibition of Th17 cell differentiation is a validated strategy to treat IBD. Thus, the blocking RORγt activity may lead to an alternative strategy to treat moderate and severe IBD patients before or after use of anti-TNF-α mAbs. Blocking Th1/17 cells may lead to the beneficial effect of RORγt inverse agonist in human as well, because Th1/17 cells which express

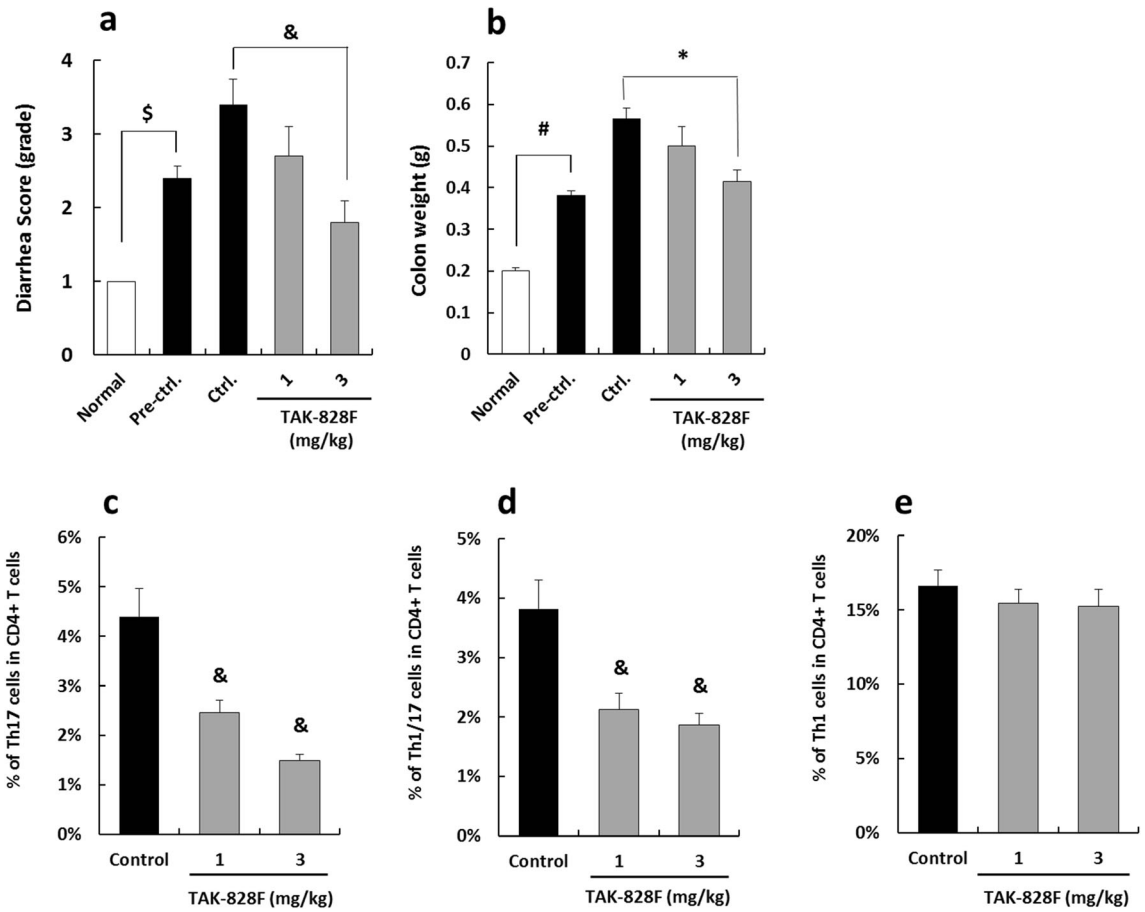

Fig. 4. Effect of therapeutic treatment with TAK-828F on activated T cell transfer colitis model. TAK-828F (1 and 3 mg/kg) or vehicle (0.5% methylcellulose) was orally administrated to the SCID mice twice a day from day 18 to day 31. Colon weight of normal and pre-control mice were measured on day 18. **a** Diarrhea score and **b** colon weight were analyzed in a blind fashion on day 32. The populations of Th17, Th1/17, and Th1 cells in MLNs were determined by flow cytometry. The population of **c** Th17 cells, **d** Th1/17 cells, and **e** Th1 cells gated on $CD4^+$ T cells. Data were represented as the mean ± S.E. of 5 (normal) or 10 (transferred mice) animals. $\$p < 0.05$ (Wilcoxon test) and $\#p < 0.05$ (Student's *t* test) *vs.* normal group. $\&p < 0.025$ (one-tailed Shirley-Williams' test) and $*p < 0.025$ (one-tailed Williams' test) *vs.* vehicle control group.

RORγt are significantly increased in blood from patients with various chronic inflammatory disorders including IBD and have been considered as the most pathogenic T cell subset [7, 18, 32, 34].

In contrast to the reduction of IL-17A and IL-17F, gene expression of IL-10, an anti-inflammatory cytokine, in the colon was increased by the treatment with TAK-828F (Fig. 3). Since Treg and Th17 cell differentiation is reciprocally regulated [5, 30] and IL-10 is one of the cytokines produced by Treg cells [8, 9], the upregulation of IL-10 in the colon suggests the induction of Treg cells by TAK-828F in the colitis model. In the *in vitro* studies by using mouse and human primary cells, TAK-828F inhibited Th17 cell differentiation and upregulated FOXP3 positive cell population under the Th17 differentiation condition [33]. These results indicate that TAK-828F has a unique mode of action to restore immune balance in the intestine of IBD. Although we have not yet detected increase in Treg cell population in the MLN of colitis model by treatment with TAK-828F, Treg cell population might be increased in the lamina propria, because upregulation of IL-10 gene expression was observed in the colon tissue. Further investigation is needed to detect increase of the population of Treg cells in the colitis model.

In the therapeutic dose regimen, TAK-828F also showed efficacy in the colitis model and treatment of TAK-828F resulted in the reduction of Th17 and Th1/17 cell population, but not Th1, in MLN (Fig. 4). In addition, reduction of IL-17A and IL-17F and upregulation of IL-10 gene expression in the colon were observed (Fig. 5). These

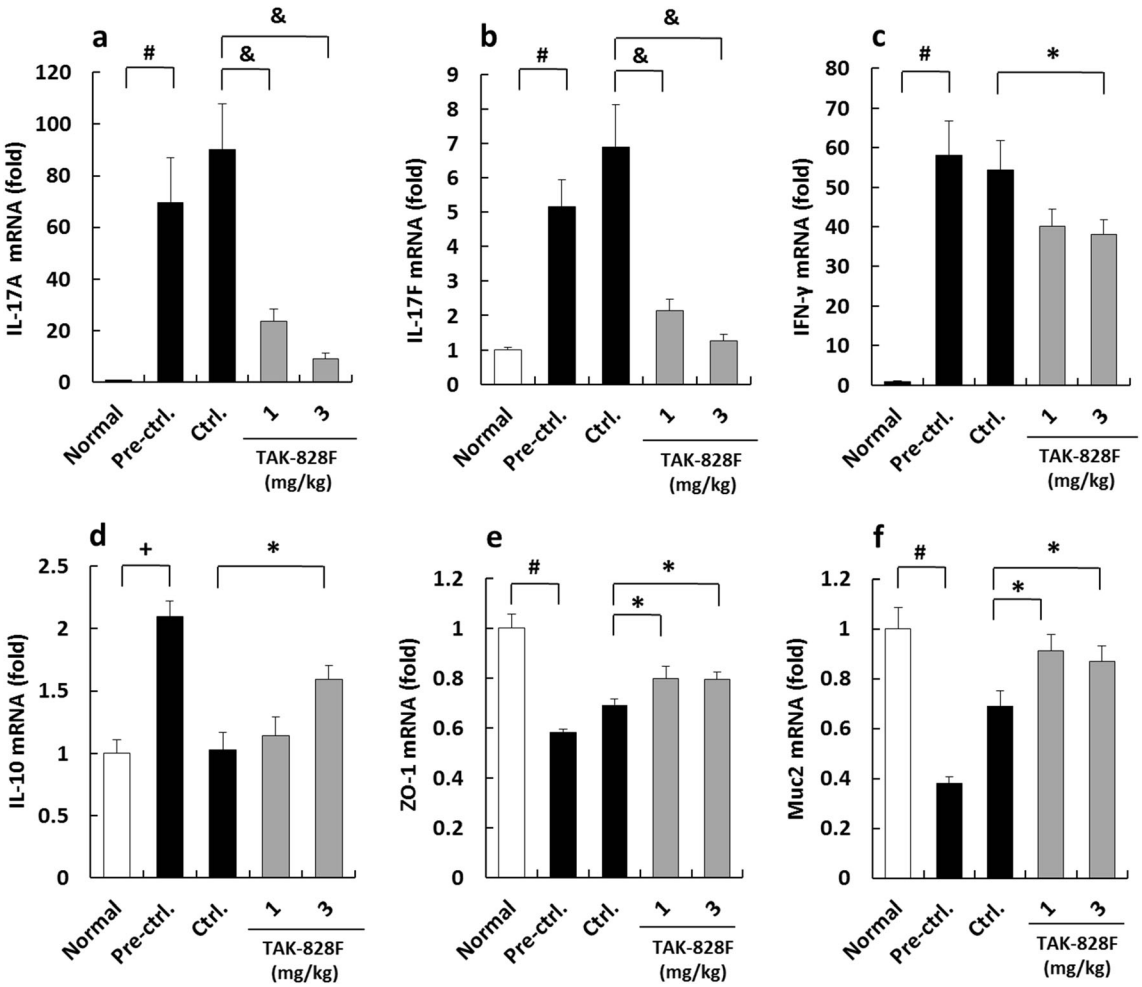

Fig. 5. Therapeutic efficacy of TAK-828F against colonic gene expressions of T cell-transferred mice. Total RNA was isolated from colon of T cell-transferred mouse and relative mRNA expression of **a** IL-17A, **b** IL-17F, **c** IFN-γ, **d** IL-10, **e** ZO-1, and **f** Muc2 in colons were measured by real-time RT-PCR, normalized with the expression of β-actin. Data were represented as the mean ± S.E. of 5 (normal) or 10 (transferred mice) animals. $+p < 0.05$ (Student's *t* test) and $\#p < 0.05$ (Aspin-Welch's *t* test) *vs.* normal group. $\&p < 0.025$ (one-tailed Shirley-Williams' test) and $*p < 0.025$ (one-tailed William's test) *vs.* vehicle control group.

results were consistent with the results obtained in the protective dose regimen and thus strongly support that TAK-828F is efficacious in the colitis model by inhibiting RORγt. Significant inhibition of IFN-γ at dose of 3 mg/kg is considered due to the reduction of Th1/17 cell population, since Th1/17 cells produce IFN-γ.

TAK-828F treatment significantly recovered gene expression of ZO-1 and Muc2 in the colon at the therapeutic dose regimen (Fig. 5e, f). Both ZO-1 and Muc2 have an important role for the maintenance of mucosal barrier and the mucosal homeostasis [1, 21]. Actually, disruption of mucosal barrier function in the intestine of the colitis model was observed after the onset of colitis in permeability assay of mucosa by use of a fluor-labeled dextran (data not shown). Clinically, gut inflammation disrupts barrier function of epithelium in the intestine, and therefore serum level of LPS and 1, 3-β-D-glucan significantly increases in CD patients (10). These events are positively correlated with the severity of the disease [10]. Treatment with anti-TNF-α mAb alleviates the intestinal inflammation and promotes to restore the gut epithelial barrier [11]. Therefore, recovery of ZO-1 and Muc2 in the colon by treatment with TAK-828F suggests the beneficial effect of blocking RORγt activity leads to amelioration of gut barrier function and acceleration of mucosal healing in patients of IBD.

The minimum efficacy dose of TAK-828F in our colitis model was 2 mg/kg/day. As regards the other RORγt inverse agonist, we could not find reports that was effective

less than 10 mg/kg by oral administration, though GSK-805 was efficacious at 10 mg/kg/day in mouse model of colitis (37). The lower efficacy dose of TAK-828F than the other RORγt inverse agonists might be due to the good pharmacokinetics profile of TAK-828F in mice (20).

In summary, TAK-828F demonstrated strong efficacy in the activated T cell transfer mouse colitis model by both protective and therapeutic dose regimen. In addition, reduction of Th17 and Th1/17 population in MLN was observed along with the decrease of IL-17A and IL-17F and the upregulation of IL-10 gene expression in the colon. We conclude that RORγt blockade may provide a novel therapeutic paradigm for treatment of IBD with unique mechanism by which improves imbalance of the immune system.

ACKNOWLEDGMENTS

The authors thank the following employees of Takeda Pharmaceutical Company Ltd.: Yasushi Fujitani, Chihiro Akimoto, Keiko Koga and Keiko Ishigami, and Hikaru Saitou for their contribution to pharmacological studies and discussion and Tsuneo Oda, Atsuko Ochida, Mitsunori Kono, Junya Shirai, and Satoshi Yamamoto for their contribution to compound synthesis.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest.

REFERENCES

1. Allen, A., D.A. Hutton, and J.P. Pearson. 1998. The MUC2 gene product: a human intestinal mucin. *The International Journal of Biochemistry & Cell Biology* 30 (7): 797–801.
2. Fauber, B.P., O. Rene, Y. Deng, J. DeVoss, C. Eidenschenk, C. Everett, A. Ganguli, et al. 2015. Discovery of 1-{4-[3-fluoro-4-((3s,6r)-3-methyl-1,1-dioxo-6-phenyl-[1,2]thiazinan-2-ylmethyl)-phenyl]-piperazin-1-yl}-ethanone (GNE-3500): a potent, selective, and orally bioavailable retinoic acid receptor-related orphan receptor C (RORc or RORgamma) inverse agonist. *Journal of Medicinal Chemistry* 58 (13): 5308–5322. https://doi.org/10.1021/acs.jmedchem.5b00597.
3. Feagan, Brian G., William J. Sandborn, Geert D'Haens, Julián Panés, Arthur Kaser, Marc Ferrante, Edouard Louis, Denis Franchimont, Olivier Dewit, Ursula Seidler, Kyung-Jo Kim, Markus F. Neurath, Stefan Schreiber, Paul Scholl, Chandrasena Pamulapati, Bojan Lalovic, Sudha Visvanathan, Steven J. Padula, Ivona Herichova, Adina Soaita, David B. Hall, and Wulf O. Böcher. 2017. Induction therapy with the selective interleukin-23 inhibitor risankizumab in patients with moderate-to-severe Crohn's disease: a randomised, double-blind, placebo-controlled phase 2 study. *The Lancet* 389 (10080): 1699–1709. https://doi.org/10.1016/s0140-6736(17)30570-6.
4. Fuss, I.J., M. Neurath, M. Boirivant, J.S. Klein, C. de la Motte, S.A. Strong, C. Fiocchi, and W. Strober. 1996. Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. *Journal of Immunology* 157 (3): 1261–1270.
5. Galvez, J. 2014. Role of Th17 Cells in the Pathogenesis of Human IBD. *ISRN Inflamm* 2014: 928461–928414. https://doi.org/10.1155/2014/928461.
6. Gerner, R.R., A.R. Moschen, and H. Tilg. 2013. Targeting T and B lymphocytes in inflammatory bowel diseases: lessons from clinical trials. *Digestive Diseases* 31 (3–4): 328–335. https://doi.org/10.1159/000354687.
7. Globig, A.M., N. Hennecke, B. Martin, M. Seidl, G. Ruf, P. Hasselblatt, R. Thimme, and B. Bengsch. 2014. Comprehensive intestinal T helper cell profiling reveals specific accumulation of IFN-gamma+IL-17+coproducing CD4+ T cells in active inflammatory bowel disease. *Inflammatory Bowel Diseases* 20 (12): 2321–2329. https://doi.org/10.1097/MIB.0000000000000210.
8. Gregori, S., K.S. Goudy, and M.G. Roncarolo. 2012. The cellular and molecular mechanisms of immuno-suppression by human type 1 regulatory T cells. *Frontiers in Immunology* 3: 30. https://doi.org/10.3389/fimmu.2012.00030.
9. Groux, H., A. O'Garra, M. Bigler, M. Rouleau, S. Antonenko, J.E. de Vries, and M.G. Roncarolo. 1997. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389 (6652): 737–742. https://doi.org/10.1038/39614.
10. Guo, Y., G. Zhou, C. He, W. Yang, Z. He, and Z. Liu. 2015. Serum Levels of Lipopolysaccharide and 1,3-beta-D-Glucan Refer to the Severity in Patients with Crohn's Disease. *Mediators of Inflammation* 2015: 843089–843089. https://doi.org/10.1155/2015/843089.
11. Hanauer, S.B. 2010. The expanding role of biologic therapy for IBD. *Nature Reviews. Gastroenterology & Hepatology* 7 (2): 63–64. https://doi.org/10.1038/nrgastro.2009.238.
12. Harbour, S.N., C.L. Maynard, C.L. Zindl, T.R. Schoeb, and C.T. Weaver. 2015. Th17 cells give rise to Th1 cells that are required for the pathogenesis of colitis. *Proceedings of the National Academy of Sciences of the United States of America* 112 (22): 7061–7066. https://doi.org/10.1073/pnas.1415675112.
13. Huh, J.R., and D.R. Littman. 2012. Small molecule inhibitors of RORgammat: targeting Th17 cells and other applications. *European Journal of Immunology* 42 (9): 2232–2237. https://doi.org/10.1002/eji.201242740.
14. Igaki, K., Y. Komoike, Y. Nakamura, T. Watanabe, M. Yamasaki, P. Fleming, L. Yang, D. Soler, E. Fedyk, and N. Tsuchimori. 2018. MLN3126, an antagonist of the chemokine receptor CCR9, ameliorates inflammation in a T cell mediated mouse colitis model. *International Immunopharmacology* 60: 160–169. https://doi.org/10.1016/j.intimp.2018.04.049.
15. Ivanov, I.I., B.S. McKenzie, L. Zhou, C.E. Tadokoro, A. Lepelley, J.J. Lafaille, D.J. Cua, and D.R. Littman. 2006. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* 126 (6): 1121–1133. https://doi.org/10.1016/j.cell.2006.07.035.
16. Jiang, W., J. Su, X. Zhang, X. Cheng, J. Zhou, R. Shi, and H. Zhang. 2014. Elevated levels of Th17 cells and Th17-related cytokines are associated with disease activity in patients with inflammatory bowel

disease. *Inflammation Research* 63 (11): 943–950. https://doi.org/10.1007/s00011-014-0768-7.

17. Kaplan, G.G. 2015. The global burden of IBD: from 2015 to 2025. *Nature Reviews. Gastroenterology & Hepatology* 12 (12): 720–727. https://doi.org/10.1038/nrgastro.2015.150.
18. Kebir, H., I. Ifergan, J.I. Alvarez, M. Bernard, J. Poirier, N. Arbour, P. Duquette, and A. Prat. 2009. Preferential recruitment of interferon-gamma-expressing TH17 cells in multiple sclerosis. *Annals of Neurology* 66 (3): 390–402. https://doi.org/10.1002/ana.21748.
19. Khanna, R., J.C. Preiss, J.K. MacDonald, and A. Timmer. 2015. Anti-IL-12/23p40 antibodies for induction of remission in Crohn's disease. *Cochrane Database of Systematic Reviews* 5: CD007572. https://doi.org/10.1002/14651858.CD007572.pub2.
20. Kono, M., A. Ochida, T. Oda, T. Imada, Y. Banno, N. Taya, S. Masada, T. Kawamoto, K. Yonemori, Y. Nara, Y. Fukase, T. Yukawa, H. Tokuhara, R. Skene, B.C. Sang, I.D. Hoffman, G.P. Snell, K. Uga, A. Shibata, K. Igaki, Y. Nakamura, H. Nakagawa, N. Tsuchimori, M. Yamasaki, J. Shirai, and S. Yamamoto. 2018. Discovery of [cis-3-({(5 R)-5-[(7-Fluoro-1,1-dimethyl-2,3-dihydro-1 H-inden-5-yl)carbamoyl]-2-methoxy-7,8-dihydro-1,6-naphthyridin-6(5 H)-yl}carbonyl)cyclobutyl] acetic Acid (TAK-828F) as a Potent, Selective, and Orally Available Novel Retinoic Acid Receptor-Related Orphan Receptor gammat Inverse Agonist. *Journal of Medicinal Chemistry* 61 (7): 2973–2988. https://doi.org/10.1021/acs.jmedchem.8b00061.
21. Lee, S.H. 2015. Intestinal permeability regulation by tight junction: implication on inflammatory bowel diseases. *Intest Res* 13 (1): 11–18. https://doi.org/10.5217/ir.2015.13.1.11.
22. Lee, Y.K., H. Turner, C.L. Maynard, J.R. Oliver, D. Chen, C.O. Elson, and C.T. Weaver. 2009. Late developmental plasticity in the T helper 17 lineage. *Immunity* 30 (1): 92–107. https://doi.org/10.1016/j.immuni.2008.11.005.
23. Leppkes, M., C. Becker, I.I. Ivanov, S. Hirth, S. Wirtz, C. Neufert, S. Pouly, A.J. Murphy, D.M. Valenzuela, G.D. Yancopoulos, B. Becher, D.R. Littman, and M.F. Neurath. 2009. RORgamma-expressing Th17 cells induce murine chronic intestinal inflammation via redundant effects of IL-17A and IL-17F. *Gastroenterology* 136 (1): 257–267. https://doi.org/10.1053/j.gastro.2008.10.018.
24. M'Koma, A.E. 2013. Inflammatory bowel disease: an expanding global health problem. *Clin Med Insights Gastroenterol* 6: 33–47. https://doi.org/10.4137/CGast.S12731.
25. Martin, B., K. Hirota, D.J. Cua, B. Stockinger, and M. Veldhoen. 2009. Interleukin-17-producing gammadelta T cells selectively expand in response to pathogen products and environmental signals. *Immunity* 31 (2): 321–330. https://doi.org/10.1016/j.immuni.2009.06.020.
26. Morishima, N., I. Mizoguchi, K. Takeda, J. Mizuguchi, and T. Yoshimoto. 2009. TGF-beta is necessary for induction of IL-23R and Th17 differentiation by IL-6 and IL-23. *Biochemical and Biophysical Research Communications* 386 (1): 105–110. https://doi.org/10.1016/j.bbrc.2009.05.140.
27. Muir, R., M. Osbourn, A.V. Dubois, E. Doran, D.M. Small, A. Monahan, C.M. O'Kane, et al. 2016. Innate Lymphoid Cells Are the Predominant Source of IL-17A during the Early Pathogenesis of Acute Respiratory Distress Syndrome. *American Journal of Respiratory and Critical Care Medicine* 193 (4): 407–416. https://doi.org/10.1164/rccm.201410-1782OC.
28. Nakagawa, H., Koyama, R., Kamada, Y., Ochida, A., Kono, M., Shirai, J., Yamamoto, S et al. 2018. Biochemical properties of. TAK-828F, a potent and selective RORγt inverse agonist. *Pharmacology*. (in press).
29. Parronchi, P., P. Romagnani, F. Annunziato, S. Sampognaro, A. Becchio, L. Giannarini, E. Maggi, C. Pupilli, F. Tonelli, and S. Romagnani. 1997. Type 1 T-helper cell predominance and interleukin-12 expression in the gut of patients with Crohn's disease. *The American Journal of Pathology* 150 (3): 823–832.
30. Quintana, F.J., A.S. Basso, A.H. Iglesias, T. Korn, M.F. Farez, E. Bettelli, M. Caccamo, M. Oukka, and H.L. Weiner. 2008. Control of T (reg) and T (H)17 cell differentiation by the aryl hydrocarbon receptor. *Nature* 453 (7191): 65–71. https://doi.org/10.1038/nature06880.
31. Rachitskaya, A.V., A.M. Hansen, R. Horai, Z. Li, R. Villasmil, D. Luger, R.B. Nussenblatt, and R.R. Caspi. 2008. Cutting Edge: NKT Cells Constitutively Express IL-23 Receptor and ROR t and Rapidly Produce IL-17 upon Receptor Ligation in an IL-6-Independent Fashion. *The Journal of Immunology* 180 (8): 5167–5171. https://doi.org/10.4049/jimmunol.180.8.5167.
32. Rovedatti, L., T. Kudo, P. Biancheri, M. Sarra, C.H. Knowles, D.S. Rampton, G.R. Corazza, G. Monteleone, A. Di Sabatino, and T.T. Macdonald. 2009. Differential regulation of interleukin 17 and interferon gamma production in inflammatory bowel disease. *Gut* 58 (12): 1629–1636. https://doi.org/10.1136/gut.2009.182170.
33. Shibata, A., K. Uga, T. Sato, M. Sagara, K. Igaki, Y. Nakamura, A. Ochida, M. Kono, J. Shirai, S. Yamamoto, M. Yamasaki, and N. Tsuchimori. 2018. Pharmacological inhibitory profile of TAK-828F, a potent and selective orally available RORgammat inverse agonist. *Biochemical Pharmacology* 150: 35–45. https://doi.org/10.1016/j.bcp.2018.01.023.
34. Shimizu, J., K. Takai, N. Fujiwara, N. Arimitsu, Y. Ueda, S. Wakisaka, H. Yoshikawa, F. Kaneko, T. Suzuki, and N. Suzuki. 2012. Excessive CD4+ T cells co-expressing interleukin-17 and interferon-gamma in patients with Behcet's disease. *Clinical and Experimental Immunology* 168 (1): 68–74. https://doi.org/10.1111/j.1365-2249.2011.04543.x.
35. Skepner, J., R. Ramesh, M. Trocha, D. Schmidt, E. Baloglu, M. Lobera, T. Carlson, J. Hill, L.A. Orband-Miller, A. Barnes, M. Boudjelal, M. Sundrud, S. Ghosh, and J. Yang. 2014. Pharmacologic inhibition of RORgammat regulates Th17 signature gene expression and suppresses cutaneous inflammation in vivo. *Journal of Immunology* 192 (6): 2564–2575. https://doi.org/10.4049/jimmunol.1302190.
36. Sujino, T., T. Kanai, Y. Ono, Y. Mikami, A. Hayashi, T. Doi, K. Matsuoka, T. Hisamatsu, H. Takaishi, H. Ogata, A. Yoshimura, D.R. Littman, and T. Hibi. 2011. Regulatory T cells suppress development of colitis, blocking differentiation of T-helper 17 into alternative T-helper 1 cells. *Gastroenterology* 141 (3): 1014–1023. https://doi.org/10.1053/j.gastro.2011.05.052.
37. Withers, D.R., M.R. Hepworth, X. Wang, E.C. Mackley, E.E. Halford, E.E. Dutton, C.L. Marriott, V. Brucklacher-Waldert, M. Veldhoen, J. Kelsen, R.N. Baldassano, and G.F. Sonnenberg. 2016. Transient inhibition of ROR-gammat therapeutically limits intestinal inflammation by reducing TH17 cells and preserving group 3 innate lymphoid cells. *Nature Medicine* 22 (3): 319–323. https://doi.org/10.1038/nm.4046.
38. Xiao, S., N. Yosef, J. Yang, Y. Wang, L. Zhou, C. Zhu, C. Wu, E. Baloglu, D. Schmidt, R. Ramesh, M. Lobera, M.S. Sundrud, P.Y. Tsai, Z. Xiang, J. Wang, Y. Xu, X. Lin, K. Kretschmer, P.B. Rahl, R.A. Young, Z. Zhong, D.A. Hafler, A. Regev, S. Ghosh, A. Marson, and V.K. Kuchroo. 2014. Small-molecule RORgammat antagonists inhibit T helper 17 cell transcriptional network by divergent mechanisms. *Immunity* 40 (4): 477–489. https://doi.org/10.1016/j.immuni.2014.04.004.
39. Zhu, J., and W.E. Paul. 2008. CD4 T cells: fates, functions, and faults. *Blood* 112 (5): 1557–1569. https://doi.org/10.1182/blood-2008-05-078154.