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Pharmacological evaluation of TAK-828F, a novel orally available RORγt inverse agonist, on murine chronic experimental autoimmune encephalomyelitis model

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ABSTRACT

We investigated the potency of TAK-828F, a RORγt inverse agonist, in murine experimental autoimmune encephalomyelitis (EAE) model. TAK-828F inhibited the differentiation of Th17 and Th1/17 cells in inguinal lymph node. Increase of these cells in central nervous system (CNS) was also inhibited by TAK-828F. Prophylactic and therapeutic treatments of TAK-828F were efficacious in the model. Plasma concentration of TAK-828F was higher than that in CNS. These results indicate that TAK-828F mainly acts at peripheral and results in the reduction of Th17- and Th1/17-dependent inflammation in CNS. Blocking RORγt may be a promising strategy for treatment of multiple sclerosis.

1. Introduction

Multiple sclerosis (MS) is a chronic demyelinating disease of central nerves system (CNS) mediated by B cells (Hauser et al., 2017; Montalban et al., 2017), $CD4^+$ T cells (Claesson et al., 1996; Fletcher et al., 2010; Kanai et al., 2009; Yoshida et al., 1996), $CD8^+$ T cells (Kaskow and Baecher-Allan, 2018), and myeloid cells (Li et al., 2015). Interactions among these cells underlie the development of MS (Li et al., 2018). Especially, $CD4^+$ T cells are considered to play an important role for the development of experimental autoimmune encephalomyelitis (EAE) which is a widely-used animal model for MS (Frohman et al., 2006; McFarland and Martin, 2007). After stimulation of pathogenic antigens, $CD4^+$ T cells can be differentiated into multiple subsets, such as IFN-γ-producing Th1 and IL-17-producing Th17 cells (Zhu and Paul, 2008; Zou and Restifo, 2010). Of late, Th17 cells are considered to be involved in the pathology of various autoimmune diseases, through the expressions of pro-inflammatory cytokines including IL-17A and IL-17F (Di Cesare et al., 2009; Jiang et al., 2014; Kanai et al., 2012). Furthermore, IL-17 and IFN-γ double-producing Th1/17 cells has been identified and was found to play an important role in the pathophysiology of MS and other inflammatory diseases (Kebir et al., 2009; Rovedatti et al., 2009). In these pathogenic Th cells, retinoic acid-related orphan receptor γt (RORγt) plays an essential role for their differentiation and activation (Kanai et al., 2012; Kebir et al., 2009; Rovedatti et al., 2009). Therefore, RORγt is thought to be an attractive drug target for the treatment of Th17 and Th1/17 cell-related immune diseases.

Recently, several RORγt inverse agonists such as digoxin, SR1001, TMP778 and GSK805, have been reported to show in vivo efficacy in myelin oligodendrocyte glycoprotein peptide ($MOG_{35–55}$)-induced mouse EAE model (Huh et al., 2011; Solt et al., 2011; Xiao et al., 2014). Although these compounds exhibited good protective efficacy and decreased Th17 cell population in the brain and spinal cord of EAE mice, detailed mode of action of RORγt inverse agonists in the peripheral and CNS tissues of EAE model has not been elucidated. Moreover, whether these RORγt inverse agonists are efficacious in therapeutic regimen have not been fully evaluated. We have discovered a novel orally available and selective RORγt inverse agonist TAK-828F (Kono et al.,

Abbreviations: CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; IACUC, Institutional Animal Care and Use Committee; IL-17, interleukin-17; IFN-γ, interferon-γ; mAb, monoclonal antibody; MC, methylcellulose; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; PBMC, peripheral blood mononuclear cell; PD, pharmacodynamics; PE, phycoerythrin; RORγt, retinoic acid-related orphan receptor γt; RT-PCR, reverse transcription-PCR; Th, T helper; Th1/17 cells, IL-17 and IFN-γ double-producing cells.

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2018). In the cell-based functional assay, TAK-828F inhibited the transcriptional activity of human RORγt with IC_{50} of 6.1 nM and TAK-828F showed clear selectivity when tested against the 21 kinds of nuclear receptors including RORα and RORβ (Kono et al., 2018; Nakagawa et al., 2018). TAK-828F, at 10 and 100 nM, suppressed IL-17 production, but not inhibited IFN-γ, from murine splenocytes and human peripheral blood mononuclear cells (PBMCs) (Shibata et al., 2018). TAK-828F also strongly inhibited Th17 and Th1/17 cell differentiation, but not affected Th1 cell differentiation, from primary naive T cells of mouse and human (Shibata et al., 2018). To evaluate the potency of RORγt inverse agonists as potential treatments for MS patients, and elucidate the detailed mode of action in peripheral and CNS tissues, we investigated the pharmacological profile of TAK-828F in $MOG_{35–55}$-induced mouse EAE model at both prophylactic and therapeutic regimen. Additionally, pharmacodynamics (PD) effect of TAK-828F was measured in ex vivo whole blood stimulation assay in mice.

2. Materials and methods

2.1. Animals

C57BL/6J (female, 10-week-old, Charles River Japan) were used for EAE model. BALB/cAnC (female, 7-week-old, Charles River Japan) were used for pharmacodynamics (PD) assay. The mice were bred on a white chip. All procedures were performed in accordance with the standards of humane care, and the treatment of research animal was approved by the Institutional Animal Care and Use Committee (IACUC) in Takeda Pharmaceutical Company, Ltd. (Approval No. 10111).

2.2. Compounds

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

2.3. Experimental autoimmune encephalomyelitis

C57BL/6 mice were subcutaneously immunized with an emulsion containing 200 μg of $MOG_{35–55}$ peptide and 500 μg of killed *Mycobacterium tuberculosis* H37Ra suspended in Freund's incomplete adjuvant on day 0, followed by intraperitoneal injection with 400 ng of pertussis toxin on day 0 and day 2. Clinical signs of EAE in individual mice were assessed using the following score: 0, no clinical signs; 0.5, partially limp tail; 1, paralyzed tail; 2, partially paralysis of hind limb; 3, complete paralysis of both hind limbs: 4, partially paralysis of forelimbs; 5, complete paralysis of forelimbs, death or humane endpoint. The clinical score was assessed under blind fashion on weekdays after the onset. Clinical signs of EAE in the mice were observed from day 12, and peaked at around day 21. These symptoms chronically sustained up to 35 days after immunization.

2.4. Inhibitory effect of TAK-828F against the population of Th cells in inguinal lymph node

TAK-828F was suspended in 0.5% methylcellulose (0.5% MC) and administered twice a day to the mice by oral gavage at doses of 0.3, 1 and 3 mg/kg from day 0 to day 6 after MOG-immunization. Vehicle-treated control mice were administered with 0.5% MC solution. The inguinal lymph nodes were obtained on day 7 (16 h after final administration of TAK-828F), followed by analyzing the population of Th cells as mentioned below.

2.5. Prophylactic efficacy of TAK-828F in murine EAE model

TAK-828F was administered twice a day from day 0 to day 27 after MOG-immunization and clinical symptoms of EAE were recorded as mentioned above. The cumulative score was calculated by adding the individual score obtained from day 0 to day 28. Twenty eight days after MOG-immunization, the lumber spinal cord was obtained from each mouse and fixed in 10% neutral buffered formalin. Spinal cord (L1) was embedded in paraffin, sectioned in a cross-sectional manner and stained with Luxol Fast Blue and hematoxylin and eosin. Histopathological evaluation was performed independently by two pathologists and demyelination was scored using the following criteria: 0, no demyelination; 1, a few numbers of focal demyelinated lesions were observed mainly at subpial area; 2, more focal demyelinated lesions were observed mainly at subpial area, and focal lesions were also noted in the perivascular area on the inside of the pia mater; 3, demyelinated lesions were noted in almost the entire circumference of white matter at subpial area, and some lesions were also noted in the inner area of the white matter. Sixteen hours after the final administration of TAK-828F, corresponding to trough, blood was collected in a heparinized syringe under pentobarbital anesthesia, and the blood samples were used for measurement of plasma level of TAK-828F. The residual blood sample was centrifuged at 12,000 rpm for 5 min. A volume of 30 μL of 0.01 mol/L $HCOONH_4$ (including 0.2% pH 3 formic acid) was added to 30 μL recovered supernatant and quantitative analysis of TAK-828F in plasma samples were carried out using LC/MS/MS.

In a separate experiment, TAK-828F was again administered to MOG-immunized mice from day 0 to day 27. On the next day after final administration, spinal cord and whole brain were obtained, followed by analyzing the population of T cells determined by flow cytometry as mentioned below.

2.6. Intracellular cytokine staining of CNS tissue and inguinal lymph node

Spinal cord, whole brain, and inguinal lymph node of individual mice were dissociated by passage through a nylon mesh, followed by passing the tissue through a cell strainer. Then, mononuclear cells from CNS tissue were isolated by Percoll gradient centrifugation. Single cell suspensions prepared from CNS tissue and inguinal lymph node were stimulated with phorbol 12-myristate 13-acetate (50 ng/mL, Wako Pure Chemical, Japan) and ionomycin (1 μg/mL, Wako Pure Chemical, Japan), in the presence of transport inhibitor containing monensin (BD Biosciences, USA) for 4 h in RPMI-1640 medium with 10% FBS. After blocking Fcγ receptor by the anti-CD16/CD32 monoclonal antibody (mAb) (clone: 2.4G2, Bio X Cell, USA), these cells were stained with PE-conjugated-anti-CD4 mAb (clone: RM4-5, 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 (clone: XMG1.2, BioLegend, USA) and Alexa Fluor 647-conjugated anti-IL-17A mAb (clone: TC11-18H10.1, BioLegend, USA). Flow cytometry analysis was performed using BD Accuri C6 Flow Cytometer (BD Biosciences, USA). The frequency of Th17, Th1/17 and Th1 cells were defined as follows: Th17, IL-17^+ IFN-γ^- cells gated on $CD4^+$ cells; Th1/17, IL-17^+ IFN-γ^+ cells gated on $CD4^+$ cells; and Th1, IL-17^- IFN-γ^+ cells gated on $CD4^+$ cells.

2.7. Real-time quantitative RT-PCR

For the gene expression study, TAK-828F (0.3, 1, and 3 mg/kg) was again administered to MOG-immunized mice from day 0 to day 28. The spinal cord was collected from EAE mice 4 h after the final administration of TAK-828F. The spinal cord 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 was performed on a ViiA 7 Real-Time PCR System (Life Technologies, USA), using TaqMan Fast Advanced Master Mix (Life Technologies, USA) with specific primers on TaqMan Gene Expression Assays (Life Technologies, USA) according to

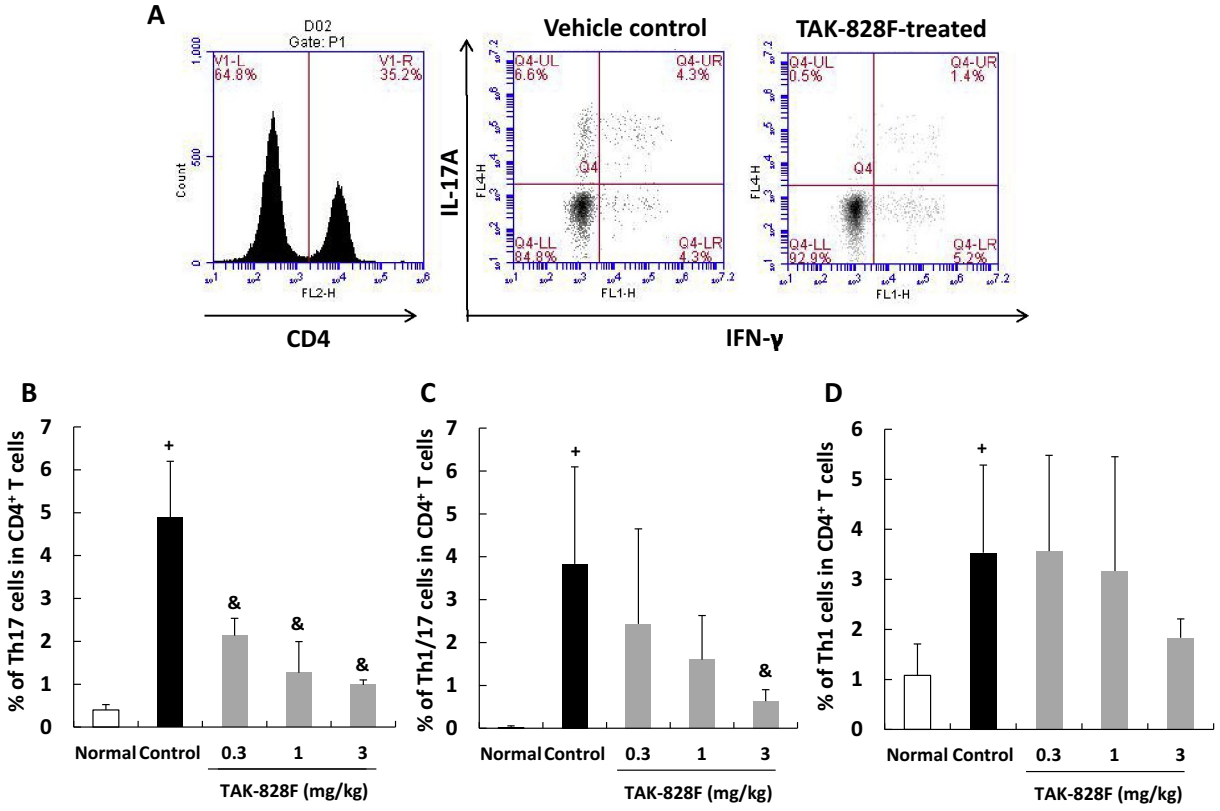

Fig. 1. TAK-828F inhibited Th17 and Th1/17 cells differentiation in the inguinal lymph node of EAE mice.
TAK-828F (0.3, 1 and 3 mg/kg) or vehicle (0.5% methylcellulose) was orally administrated to mice twice a day for 7 days. The frequencies of Th17, Th1/17 and Th1 cells in inguinal lymph node were determined by intracellular staining. (A) Representative data of flow cytometry analysis of $CD4^+$ T cell population in inguinal lymph node. (B–D) The frequency of (B) Th17, (C) Th1/17 and (D) Th1 cells gated on $CD4^+$ T cells. Data are presented as the mean ± S.E. ($n = 6$). $^+p < .05$ (Welch's *t*-test) vs. normal group. $^\&p < .025$ (one-tailed Shirley-Williams' test) vs. vehicle-treated control group.

the manufacturer's manual. The TaqMan Gene Expression Assays used were: *Il17a*, Mm00439618_m1; *Ifng*, Mm00801778_m1; *Il10*, Mm00439614_m1; *Gzmb,* Mm00442837_m1; *Csf2*, Mm01290062_m1. The gene expressions were calculated by relative expression rate against β-actin (*Actb*) as the quantitative control.

2.8. Therapeutic efficacy of TAK-828F in murine EAE model

In the therapeutic dose regimen, the clinical sign of EAE of each mouse was determined on a scale of 1–5 as described above on day 21. Grouping of each group was performed at day 21 as following mean EAE score: pre-control, 3.7; vehicle control, 3.4; 1 mg/kg, 3.5; 3 mg/kg, 3.4. The spinal cord and brain of pre-control group were collected immediately after grouping, followed by performing the flow cytometry as described above. TAK-828F at doses of 1 and 3 mg/kg was orally administered to the mice twice a day from day 21 to day 34. The cumulative score was calculated by summing the individual score obtained from day 21 to day 35. On day 35, spinal cord and whole brain were collected, followed by analyzing the population of Th cells with flow cytometry as described above.

2.9. Pharmacodynamics assay in normal mice

TAK-828F was suspended in 0.5% MC and administered twice to the normal BALB/c mice by oral gavage at time 0 and 8 h. Sixteen hours after the final administration, blood was collected in a heparinized syringe under pentobarbital anesthesia, and the samples were immediately used for ex vivo whole blood stimulation assay. This time point was defined as trough level of TAK-828F in the experiment. Mouse whole blood (350 μL) was mixed with 280 μL of 10% FBS RPMI-1640 medium (Thermo Fisher, USA), and IL-23 (R&D Systems, USA) was added to the final concentration of 30 ng/mL (final volume of 700 μL). The mixture (200 μL) was plated in 96 well assay block, pre-coated with anti-CD3 mAb (10 μg/mL) and anti-CD28 mAb (5 μg/mL) over night at 4 °C, and the plate was then cultured for 20 h at 37 °C. After incubation, the culture supernatant was collected, and the amount of IL-17 in the supernatant was measured by using a mouse IL-17 ELISA kit (R&D Systems, USA). The concentration of IL-17 from mouse whole blood in control was 185.8 pg/mL. The residual blood sample was used for the measurement of plasma level of TAK-828F as described above. BALB/c mice were used in the PD assay, though C57BL/6 mice were used in the EAE model, because in vivo efficacy studies of TAK-828F were conducted not only in EAE model but also in IL-23-induced skin inflammation in BALB/c (under submission) and murine colitis model induced by adoptive transferring of T cells of BALB/c to SCID mice (Igaki et al., 2019b)

2.10. Statistics

Statistical analysis was performed using SAS System for Windows (Release 8.2, SAS Institute), or the EXSUS statistical analysis system (8.0 ver, CAC EXICARE, Tokyo, Japan). Differences in EAE clinical score and histopathological score between the vehicle-treated control group and the TAK-828F-treated group were analyzed by means of the one-tailed Shirley-Williams' test. The difference between the population of Th17 and Th1/17 cells between normal and vehicle-treated control group was determined using the Student's *t*-test or Welch's *t*-test. Differences in the population of Th cells and PD effect between vehicle control group and TAK-828F-treated group were analyzed by way of the one-tailed Williams' test or one-tailed Shirley-Williams' test. A

probability value of $p < .05$ (Student's t-test, and Welch's t -test), or $p < .025$ (Shirley-Williams' test, and Williams' test) was considered statistically significant.

3. Results

3.1. TAK-828F inhibited the differentiation of Th17 and Th1/17 cells in inguinal lymph nodes of mice

Firstly, to confirm pharmacological effect of TAK-828F in vivo, the influence of TAK-828F on the effector T cell differentiation was investigated in the inguinal lymph nodes of the mice at early stage after MOG-immunization. Inguinal lymph node was used for the experiment, because increase in Th17, Th1/17 and Th1 number in the lymph node was observed 7 days after MOG-immunization in our preliminary experiments. TAK-828F was administered twice a day at 0.3, 1 and 3 mg/kg, from day 0 to day 6 after MOG-immunization. After the administration of TAK-828F for 7 days, the increase in Th17 and Th1/17 cell populations were strongly inhibited in a dose-dependent manner, whereas Th1 cell population was not significantly influenced (Fig. 1). Thus, TAK-828F specifically inhibited Th17 and Th1/17 cell differentiation in inguinal lymph nodes.

3.2. TAK-828F prevented disease progression in the MOG-induced EAE mice

Next, protective efficacy of TAK-828F was investigated in the MOG-induced EAE mice. TAK-828F was administered from day 0 to day 27, and the progression of clinical signs of EAE was monitored daily. TAK-828F dose-dependently attenuated the development of clinical symptoms of EAE (Fig. 2A) and cumulative scores of 1 and 3 mg/kg treatment groups were significantly lower than that of the vehicle-treated group (Fig. 2B). Histopathologically, representative observations in spinal lesions were demyelination characterized by vacuolation or decrease in Luxol Fast Blue-positive myelin area accompanied by inflammatory cell infiltration. Treatment of TAK-828F at 3 mg/kg significantly attenuated the degree of demyelination (Fig. 2C–E). Trough concentration of TAK-828F, at doses of 0.3, 1 and 3 mg/kg, in the plasma of the EAE mice at day 28 was 212.9, 462.3 and, 1498.6 ng/mL, respectively. In contrast, concentration of TAK-828F in the spinal cord at doses of 0.3, 1 and 3 mg/kg was < 0.1, 38.2 and 89.7 ng/g in this order, indicated that TAK-828F mainly distributes to the peripheral.

3.3. TAK-828F inhibited increase in population of Th17 and Th1/17 cells in CNS tissues in the MOG-induced EAE mice

Effect of TAK-828F on the T cell population in CNS tissues of the MOG-induced EAE mice was investigated. For this purpose, a second efficacy study of TAK-828F in the EAE mice was performed, and influence of TAK-828F on the population of effector T cells in brain and spinal cord was measured. The protective effect on clinical scores of TAK-828F at 1 and 3 mg/kg was reproduced in this study (Fig. 3A). A significant reduction of the population of Th17 and Th1/17 cell, but not Th1 cell, was observed in brain and spinal cord on day 28 after MOG immunization (Fig. 3B–D). Cell population of the 0.3 mg/kg group was not analyzed, due to its insignificant effect on the clinical score.

3.4. TAK-828F inhibited IL-17A and other pro-inflammatory cytokine gene expression in spinal cord

To investigate the effect of TAK-828F on the effector function of T cells in CNS tissue, a third efficacy study of TAK-828F in the EAE mice was conducted. We also found the significant efficacy of TAK-828F at 1 and 3 mg/kg for 28 days treatment as shown in Figs. 2 and 3 (data not

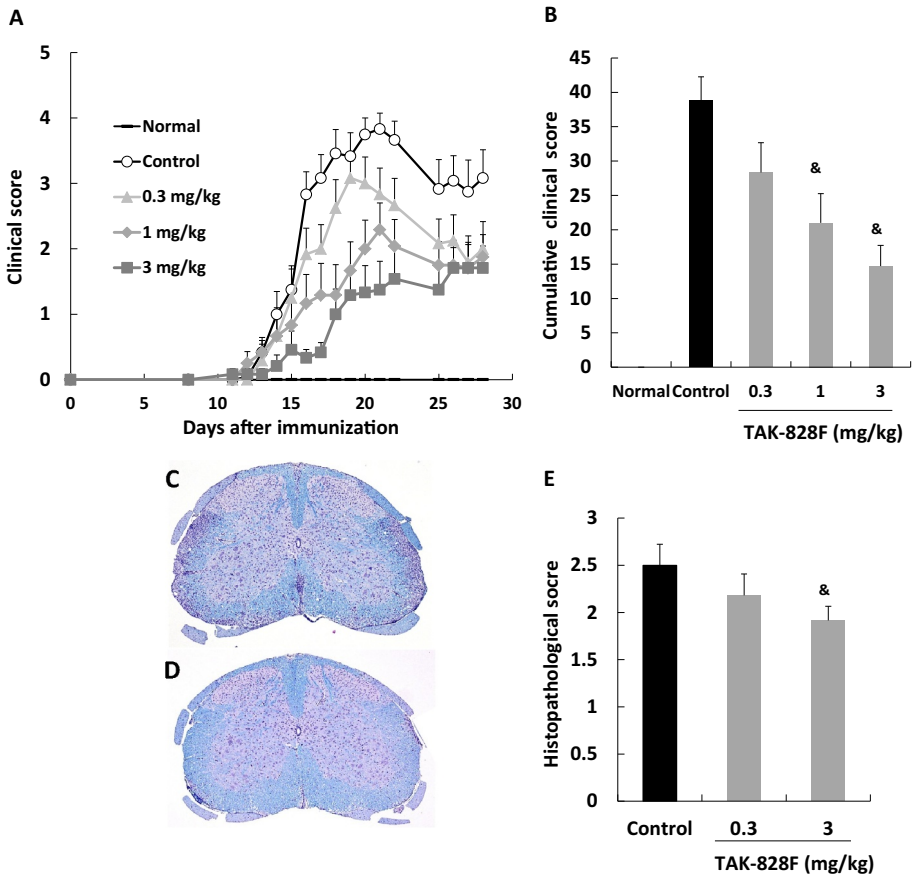

Fig. 2. TAK-828F protected disease progression and demyelination of EAE.
TAK-828F (0.3, 1 and 3 mg/kg) or vehicle (0.5% methylcellulose) was orally administrated to mice twice a day for 28 days. (A–B) EAE clinical score of (A) time course, and (B) cumulative clinical score from day 0 to day 28. Data are presented as mean ± S.E. (n = 12). (C–D) Representative image of the spinal cord stained with Luxol Fast Blue, and hematoxylin and eosin of (C) vehicle-treated control, and (D) TAK-828F-treated mouse at 3 mg/kg. (E) Histopathological score of demyelination in spinal cord. Data are presented as mean ± S.E. (n = 10, vehicle-treated control; n = 11, TAK-828F at 0.3 mg/kg; n = 12, TAK-828F at 1 and 3 mg/kg). $^{\&}p < .025$ (one-tailed Shirley-Williams' test) vs. vehicle-treated control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

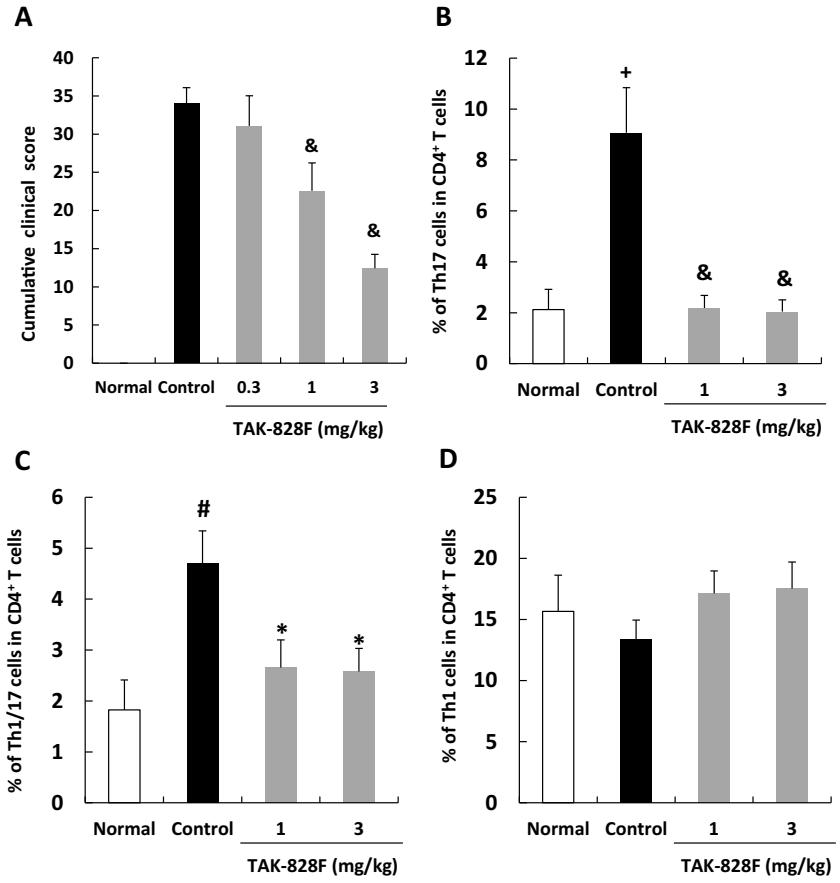

Fig. 3. TAK-828F decreased the population of Th17 and Th1/17 cells in brain and spinal cord.
A second efficacy study of TAK-828F in the EAE model was performed with same condition as shown in Fig. 2. (A) Cumulative clinical score from day 0 to day 28. Data are presented as mean ± S.E. ($n = 12$). (B–D) Spinal cord and brain of mice were collected on day 28 after MOG immunization and used for the flow cytometry analysis as described in Materials and methods. The population of (B) Th17, (C) Th1/17 and (D) Th1 cells gated on $CD4^+$ T cells in the CNS tissue. For the quantification study of effector T cell, 4 (normal) or 6 (control and TAK-828F-treated group) mice were randomly selected. Data are presented as the mean ± S.E. ($n = 4$, normal; $n = 6$, vehicle-treated control and TAK-828F-treated group). $^{+}p < .05$ (Welch's t-test) vs. normal group. $^{\#}p < .05$ (Student's t-test) vs. normal group. $^{\&}p < .025$ (one-tailed Shirley-Williams' test) vs. vehicle-treated control group. $^{*}p < .025$ (one-tailed Williams' test) vs. vehicle-treated control group.

shown), and the spinal cord was collected from EAE mice 4 h after the final administration of TAK-828F as described in Materials and methods. Gene expressions of IL-17A, IFN-γ, IL-10, granulocyte macrophage colony-stimulating factor (GM-CSF) and granzyme B were investigated in the spinal cord of EAE mice. Gene expression of IL-17A in the spinal cord was strongly suppressed by the treatment of TAK-828F at 0.3, 1 and 3 mg/kg, whereas gene expression of IL-10 was not affected (Fig. 4A and B). TAK-828F treatment induced partial but significant reduction of gene expression of IFN-γ, GM-CSF and granzyme B (Fig. 4C–E).

3.5. TAK-828F was effective in therapeutic dose regimen

In order to evaluate the potency of TAK-828F in the treatment of MS, the therapeutic effect of TAK-828F was evaluated. In this experiment, administration of TAK-828F was started from day 21 after MOG-immunization when the clinical signs of EAE were apparently observed and plateaued, and dosing continued to day 34. In the regimen, TAK-828F, at 1 and 3 mg/kg, improved the clinical sign of EAE after initiation of the treatment (Fig. 5A). Importantly, the cumulative score from day 21 to day 35 significantly decreased after treatment with TAK-828F (Fig. 5B). The tendency of reduction of Th17 and Th1/17 cell population in the CNS tissues, but not Th1 cell population, was observed in the mice on day 35 (Fig. 5C–E).

3.6. Pharmacodynamics effect of TAK-828F in mice

Finally, to elucidate the relation between RORγt inhibition by TAK-828F in the peripheral whole blood and concentration of the compound in the plasma of mice, PD effect of TAK-828F was investigated. TAK-828F inhibited production of IL-17 in the ex vivo whole blood stimulation assay in a dose-dependently manner with inhibition rate at 1 and 3 mg/kg was 86% and 88%, respectively (Fig. 6A). In addition, trough level of TAK-828F in the plasma of mice linearly increased (Fig. 6B). PD effects of TAK-828F were well-correlated to the trough level of the compound in the plasma at 0.03 to 1 mg/kg and reached a plateau at 3 mg/kg.

4. Discussion

We demonstrated that a small molecular RORγt inverse agonist, TAK-828F, prophylactically and therapeutically suppressed the progression of clinical symptoms in the MOG-induced EAE model in mice. These efficacies corresponded to the decrease in the population of Th17 and Th1/17 cells in both peripheral lymph node and CNS tissues in the EAE mice. Moreover, TAK-828F inhibited the gene expressions of Th17- and Th1/17-related molecules in spinal cords.

TAK-828F, at 0.3 to 3 mg/kg, dose-dependently inhibited the increase in Th17 and Th1/17 cell population in inguinal lymph nodes, but did not significantly influence the Th1 cell population at early stage of MOG-immunized mice (Fig. 1). In addition, TAK-828F protected progression of symptoms at 1 and 3 mg/kg in the MOG-induced EAE mice (Fig. 2) and reduced the populations of Th17 and Th1/17 cells in the CNS tissue on day 28 (Fig. 3). RORγt transgenic mice developed more severe EAE than wild-type mice (Martinez et al., 2014). In addition, when $CD4^+$ T cells from $ROR\gamma^{-/-}$ mice were transferred to the RAG2-deficient mice, a decrease in disease severity of EAE and a reduction of Th17 and Th1/17 cell populations in the spinal cord were observed (Ivanov et al., 2006). These results indicate that TAK-828F inhibits Th17 and Th1/17 cell differentiation in the inguinal lymph node of the mice by selectively blocking RORγt, and it results in the reduction of Th17 and Th1/17 cells in the CNS and exert efficacy in the EAE mice. Higher concentration of TAK-828F in the plasma than that of the spinal cord, also indicating that TAK-828F mainly act in the peripheral

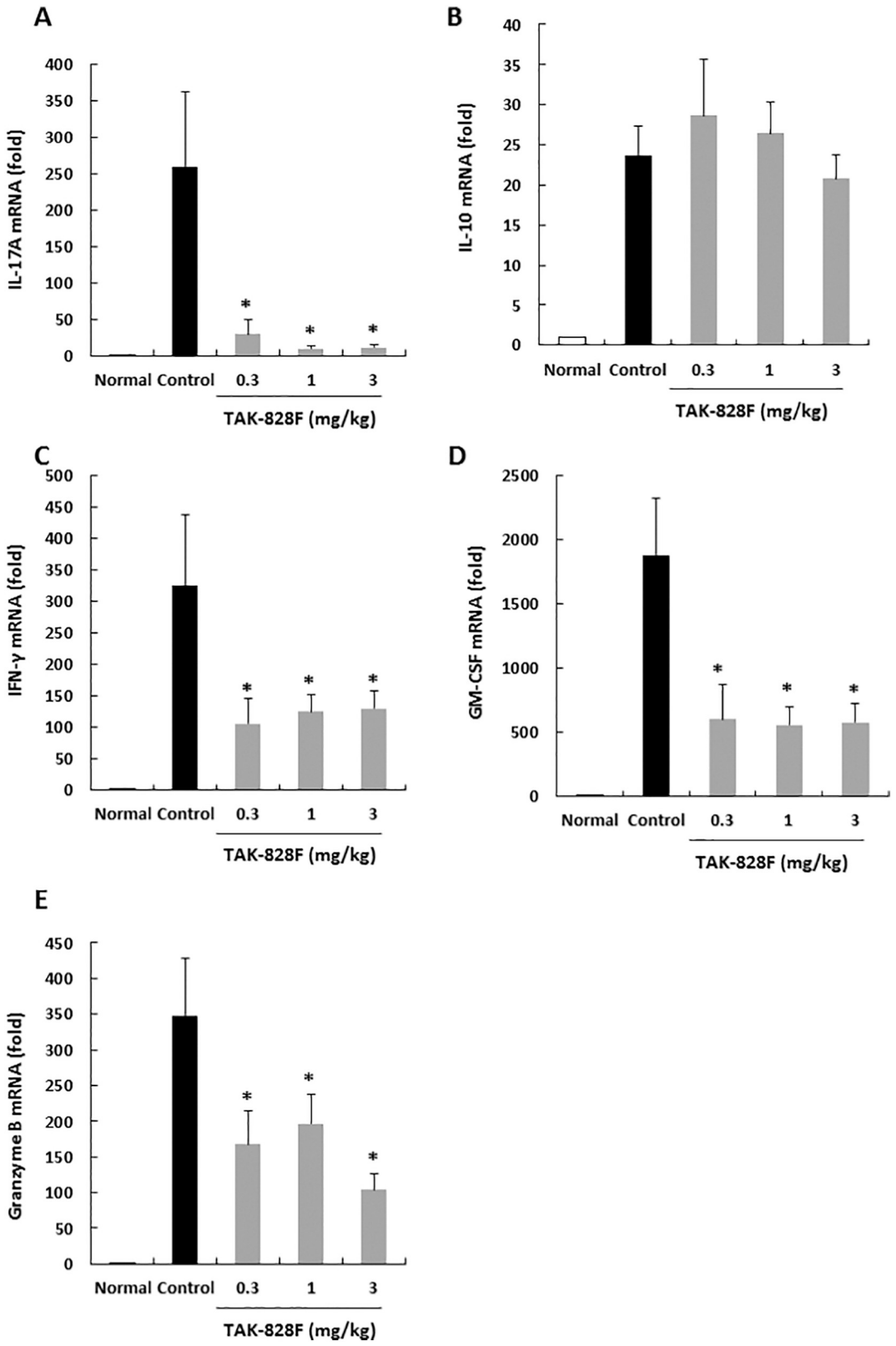

Fig. 4. TAK-828F inhibited the gene expression of IL-17A and other pro-inflammatory cytokines in spinal cord.
A third efficacy study of TAK-828F in the EAE model was performed with same condition as shown in Fig. 2. The spinal cord was collected from EAE mice 4 h after the final administration of TAK-828F at day 28. (A–E) The gene expressions of pro- and anti-inflammatory cytokines in spinal cord of EAE mice. (A) IL-17A, (B) IL-10, (C) IFN-γ, (D) GM-CSF and (E) granzyme B. Data are presented as mean ± S.E. ($n = 4$, normal; $n = 12$, MOG-immunized mice). $^{\&}p < .025$ (one-tailed Shirley-Williams' test) vs. vehicle-treated control group. $^{*}p < .025$ (one-tailed Williams' test) vs. vehicle-treated control group.

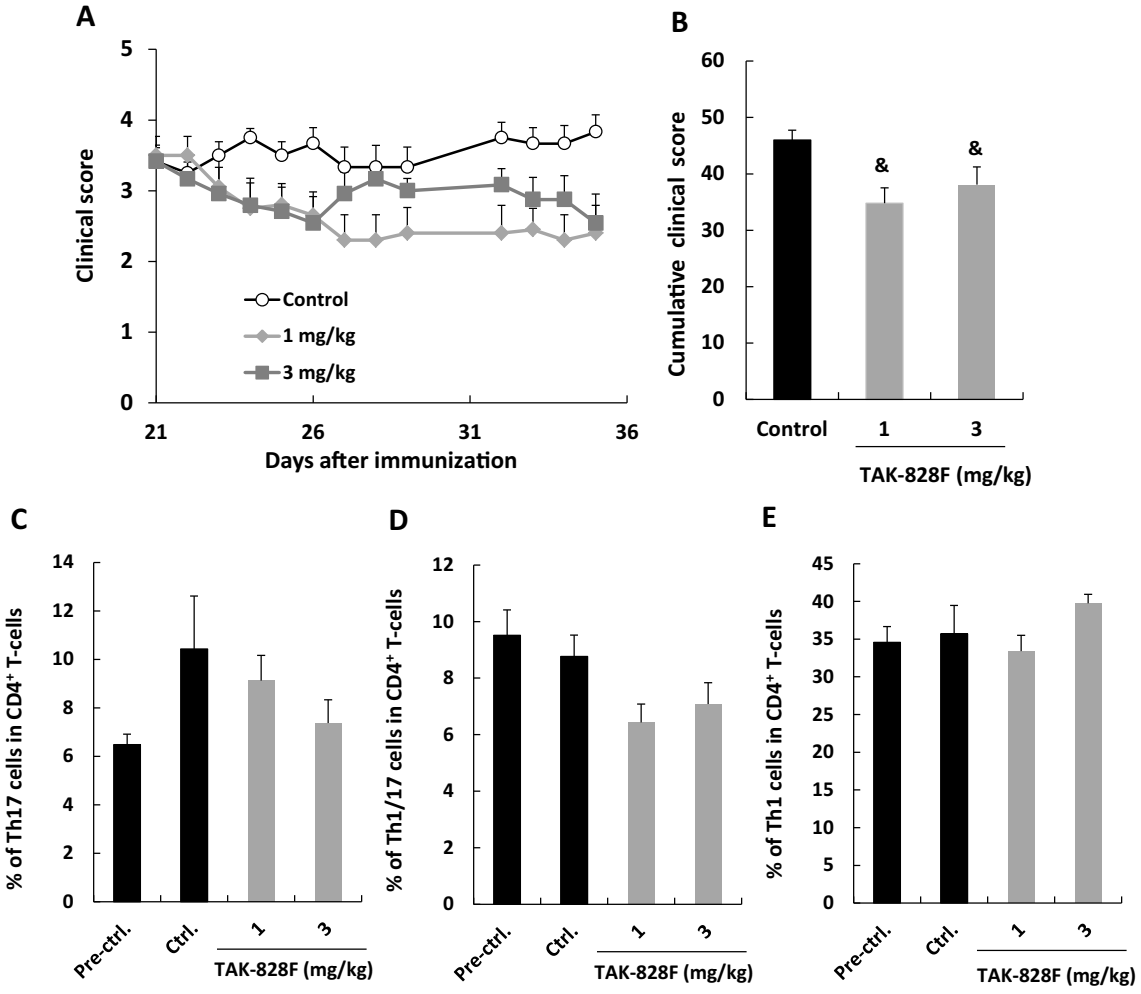

Fig. 5. TAK-828F showed efficacy in therapeutic regimen in EAE mice.
Grouping of each group was performed at day 21 as following mean EAE score: pre-control, 3.7; vehicle control, 3.4; 1 mg/kg, 3.5; 3 mg/kg, 3.4. TAK-828F (1 and 3 mg/kg) or vehicle (0.5% methylcellulose) was orally administrated to mice twice a day from day 21 to day 34. Data are presented as mean ± S.E. ($n = 12$). (A) Time course of clinical score. (B) Cumulative clinical score from day 21 to day 35. (C–E) The population of (C) Th17, (D) Th1/17 and (E) Th1 cells gated on $CD4^+$ T cells in the CNS tissue. For the quantification study of effector T cell, 6 mice were randomly selected. Data are presented as mean ± S.E. ($n = 6$). $^{\&}p < .025$ (one-tailed Shirley-Williams' test) vs. vehicle-treated control group.

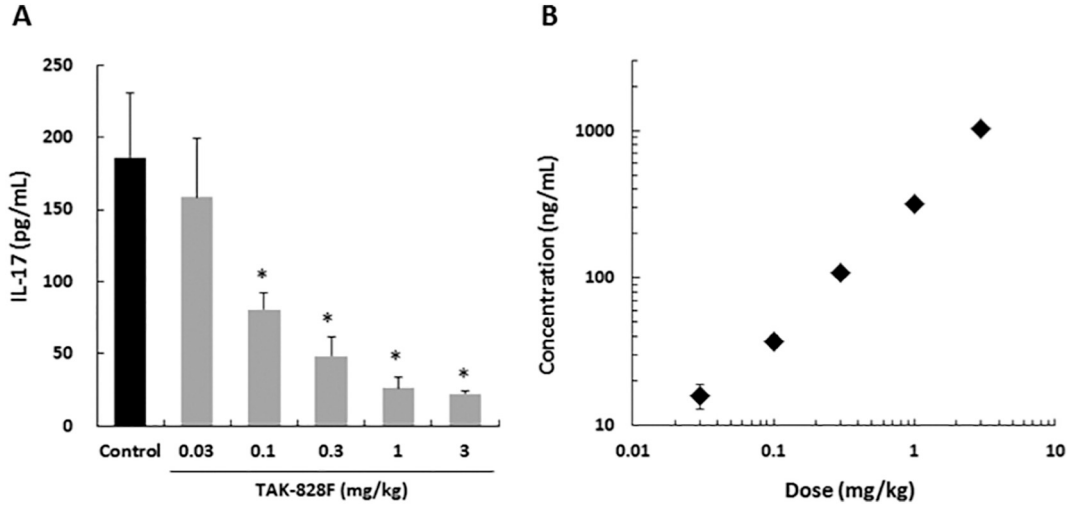

Fig. 6. TAK-828F showed good pharmacodynamics efficacy in normal mice.
TAK-828F (0.03 to 3 mg/kg) or vehicle (0.5% methylcellulose) was orally administrated to BALB/c mice twice, and blood sample was collected 16 h after final administration. (A) IL-17A concentration in the supernatants of ex vivo whole blood stimulation assay. (B) Concentration of TAK-828F in the plasma of mice which correspond to the trough level. Data are presented as mean ± S.E. ($n = 5$). $^{*}p < .025$ (one-tailed Williams' test) vs. vehicle-treated control group.

including inguinal lymph node.

Modulation of pathogenic T cells in the regional lymph node is important to control of disease condition of MS, since sphingosine-1-phosphate receptor modulator, fingolimod, is used to treat MS and its mechanism of action is considered to inhibit egress of pathogenic T cells from the lymph nodes (Cyster and Schwab, 2012). Thus, clinical efficacy of RORγt inverse agonist in MS might be expected through the unique pharmacological effect blocking the differentiation of Th17 and Th1/17 cells in the regional lymph node.

In addition to the reduction in Th17 and Th1/17 cells population, gene expression of IL-17A in the spinal cord was strongly inhibited by the treatment with TAK-828F (Fig. 4). As described in the introduction, RORγt has an essential role in both the differentiation and activation of Th17 and Th1/17 cells (Kanai et al., 2012; Kebir et al., 2009; Rovedatti et al., 2009). Indeed, TAK-828F inhibits the differentiations of Th17 and Th1/17 cells from human memory $CD4^+$ T cells and suppresses IL-17A secretion from human PBMCs (Shibata et al., 2018). Therefore, we consider that protective efficacy of TAK-828F in the EAE mice is attributed the regulation of both differentiation and effector function of Th17 and Th1/17 cells via the inhibition of RORγt. Only partial efficacy was observed in the EAE mice by treatment with 0.3 mg/kg of TAK-828F, despite the strong inhibition of IL-17A gene expression in the spinal cord as shown in Fig. 4. In the assay, the spinal cords were collected 4 h after the final administration of TAK-828F. As shown in Figs. 1 and 6, inhibitory effect of TAK-828F at 0.3 mg/kg, measured 16 h after the final administration, on Th17 and Th1/17 differentiation in the inguinal lymph nodes and whole blood IL-17A production was slightly weak compared with 1 and 3 mg/kg. We consider that TAK-828F did not show significant efficacy at 0.3 mg/kg probably because of weak inhibitory activity against RORγt in the peripheral of EAE mice. In spite of ineffectiveness of TAK-828F on Th1 cell population in the CNS (Fig. 3), the gene expression of IFN-γ in the spinal cord was decreased by the treatment with TAK-828F (Fig. 4). The reduction rate of IFN-γ was relatively weaker than that of IL-17A. We consider that inhibition of IFN-γ gene expression by TAK-828F is due to the reduction of population and the inhibition of activity in Th1/17 cell in the CNS, since Th1/17 cells could produce IFN-γ (Kebir et al., 2009).

Interestingly, TAK-828F decreased the gene expression of GM-CSF and granzyme B in the spinal cord (Fig. 4), suggesting that blockage of RORγt is different therapeutic concept from anti-IL-17A monoclonal antibody that specifically neutralizes IL-17A. GM-CSF is reported as an encephalitogenic factor produced by helper T cells and its expression depends on the activity of RORγt (Codarri et al., 2011; Ponomarev et al., 2007). In addition, a recent report showed that Th1/17 cells potently produce GM-CSF in murine EAE model (Ronchi et al., 2016). Thus, TAK-828F has the potential to inhibit the gene expression of GM-CSF by suppressing the differentiation and activation of Th1/17 cells. On the other hand, there is no evidence that RORγt is involved in the expression of granzyme B. However, one report indicated that human Th17 cells express granzyme B and have cytotoxicity against human neuron in in vitro co-culture assay (Kebir et al., 2007). Another report indicated that human PBMC-derived $CD4^+$ T cells expressed both IL-17 and granzyme B by stimulation of IL-23 in vitro (Matsui et al., 2015). Therefore, decreased gene expression of granzyme B in the spinal cord might be resulted from the reduction of Th17 cell population in CNS by the treatment with TAK-828F. Further investigations are required for the precise transcriptional regulation and identification of granzyme-producing cells in the spinal cord of EAE mice.

TAK-828F at 1 and 3 mg/kg also showed therapeutic efficacy when treated after the development of EAE (Fig. 5). When TAK-828F at 1 and 3 mg/kg was administered from day 15 to day 29 after MOG-immunization, the significant efficacies were again observed in both doses, indicating that therapeutic effect on TAK-828F is reproducible (data not shown). In the intervention study, TAK-828F improved symptoms of EAE after initiation of the treatment and induced a tendency of reduction of Th17 and Th1/17 cells population, but not Th1 cell population, in the CNS tissue of mice (Fig. 5). These results suggest that TAK-828F exhibits therapeutic efficacy by reducing Th17 and Th1/17 cells in the CNS lesions, despite therapeutic intervention from the peak of disease severity. This is the first report that shows blocking RORγt by small molecule leads to therapeutic efficacy in the EAE model. These efficacies of TAK-828F in EAE model in protective and therapeutic dose regimen suggest that the potential effect of RORγt blocking for the treatment of relapsing-remitting MS with the new mode of action.

TAK-828F showed good correlation between PD effect of mice and plasma concentration of the compound (Fig. 6). TAK-828F was efficacious in the EAE model at doses of 1 and 3 mg/kg, which correspond to the inhibition rate over 80% in the PD assay. Interestingly, TAK-828F also showed efficacy in two types of mouse colitis models that are responsive and non-responsive to anti TNF-α mAb at doses of 1 and 3 mg/kg (Igaki et al., 2019a, Igaki et al., 2019b). These results indicate that keeping over 80% inhibition of IL-17 production in the peripheral blood is necessary to exert significant efficacy of TAK-828F in vivo. The PD efficacy reached a plateau at 3 mg/kg, despite of higher plasma level of TAK-828F than that of 1 mg/kg. The ceiling PD effect of TAK-828F at 3 mg/kg might be due to the occurrence of RORγt saturation by TAK-828F at this dose. TAK-828F has been administered to healthy volunteers, and pharmacokinetics and pharmacodynamics data of TAK-828F will be published elsewhere.

Importantly, an anti-IL-12/IL-23p40 monoclonal antibody, ustekinumab, failed in the relapsing-remitting MS (Segal et al., 2008). Ustekinumab inhibits the differentiation of Th1 and Th17 cells from naïve T cells by binding to p40 subunit of IL-12 and IL-23. We consider, however, blockage of IL-12/IL-23p40 does not directly inhibit the expression of pathological cytokines from Th1 and Th17 cells. Therefore, treatment of ustekinumab after many years of disease which naïve T cells have already been differentiated into Th1 and Th17 cells is likely too late to show clinical effect, as Longbrake and Racke discussed (Longbrake and Racke, 2009). In contrast to block IL-12/IL-23p40, RORγt inverse agonist, TAK-828F, directly inhibits the expressions of Th17-related cytokines (Fig. 4), as well as the inhibition of Th17 and Th1/17 cell-differentiation (Figs. 1 and 3). In addition, TAK-828F was efficacious despite therapeutic intervention condition from the peak of disease severity (Fig. 5). Thus, we expect that RORγt inverse agonist is efficacious in EAE and MS with a different mode of action from anti-IL-12/IL-23p40 antibody.

5. Conclusions

We found that TAK-828F mainly acts at peripheral and results in the reduction of Th17 and Th1/17 dependent immune reaction in CNS of EAE mice. In addition, TAK-828F showed significant efficacy in the EAE model in the condition of therapeutic dose regimen. Orally available RORγt inverse agonist would be an attractive drug candidate to treat MS as a modulator of Th17 and Th1/17 cells.

Declaration of Competing Interests

The authors declare no conflicts of interest.

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