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Original Article

A novel RORγt inhibitor is a potential therapeutic agent for the topical treatment of psoriasis with low risk of thymic aberrations

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ABSTRACT

Background: Retinoic acid receptor-related orphan receptor gamma t (RORγt) has critical roles in the development, maintenance and function of interleukin (IL)-17-producing cells and is a highly attractive target for the treatment of IL-17-mediated autoimmune disease, particularly psoriasis. On the other hand, RORγt is also critical for controlling apoptosis during thymopoiesis, and genetic RORγt ablation or systematic RORγt inhibition cause progressive thymic aberrations leading to T cell lymphomas.
Objective: We investigated whether topical administration of our novel RORγt inhibitor, S18-000003 has therapeutic potential for psoriasis with low risk of thymic aberrations.
Methods: We evaluated the effect of topical S18-000003 on psoriasis-like skin inflammation and influence on the thymus in a 12-O-tetradecanoylphorbol-13-acetate-induced K14.Stat3C mouse psoriasis model.
Results: S18-000003 markedly inhibited the development of psoriatic skin inflammation via suppression of the IL-17 pathway. In the skin, S18-000003 suppressed all subsets of IL-17-producing cells that we previously identified in this psoriasis model: Th17 cells, Tc17 cells, dermal γδ T cells, TCR$^-$ cells that probably included innate lymphoid cells, and CD4$^-$CD8$^-$ double-negative αβ T cells. Notably, neither reduction of CD4$^+$CD8$^+$ double-positive thymocytes nor dysregulation of cell cycling was observed in S18-000003-treated mice, even at a high dose.
Conclusion: Our topically administered RORγt inhibitor is a potential therapeutic agent for psoriasis with low risk of thymic lymphoma.

1. Introduction

Psoriasis is a common, immune-mediated, chronic inflammatory skin disorder accompanied by erythematous scaly plaques. Psoriatic lesions are characterized by several histological features including epidermal hyperproliferation, abnormal keratinocyte differentiation and excessive infiltration of immune cells [1,2]. Interleukin (IL)-23/IL-17-targeting biologics are highly efficacious for the treatment of psoriasis, so it is clear that the IL-23/IL-17 pathway has a critical role in this disease [3–5]. IL-17 can be produced by several cell types following IL-23 stimulation, including Th17 cells, Tc17 cells, γδ T cells and innate lymphoid cells (ILCs) [6]. The development, maintenance and function of these IL-17-producing cells are regulated by the master transcription factor, retinoic acid receptor-related orphan receptor gamma t (RORγt). Therefore, RORγt is a promising therapeutic target for IL-17-driven inflammatory diseases, including psoriasis. Indeed, clinical trials of several RORγt inhibitors in psoriasis patients are ongoing.

In addition to its critical role in the IL-23/IL-17 pathway, RORγt is also essential in the regulation of thymopoiesis [7,8]. RORγt controls the apoptosis of CD4$^+$CD8$^+$ double-positive (DP)

Abbreviations: BrdU, bromodeoxyuridine; DC, dendritic cell; DMSO, dimethyl sulfoxide; DN αβ T cells, TCRαβ$^+$CD4$^-$CD8$^-$ double-negative T cells; DP, double-positive; IC50, 50% inhibitory concentration; ILC, innate lymphoid cell; IMQ, imiquimod; LN, lymph node; PBMC, peripheral blood mononuclear cell; PMA+IOM, phorbol 12-myristate 13-acetate plus ionomycin; ROR, retinoic acid receptor-related orphan receptor; SALT, skin-associated lymphoid tissue; TPA, 12-O-tetradecanoylphorbol-13-acetate; Treg, regulatory T cell.

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thymocytes, and mice deficient in *Rorc* have been shown to develop thymic lymphoma at high incidence [9]. Recent studies have demonstrated that gene-specific deletion of *Rorc* in adult mice also develop lymphoma, in a similar time frame to embryonic *Rorc* knockouts [10]. Furthermore, some reports have shown that oral administration of a RORγt inhibitor causes progressive thymic aberrations such as downregulation of anti-apoptotic molecule Bcl-xL and DP thymocyte reduction in rats and mice [11,12]. Based on these findings, pharmacological inhibition of RORγt in thymus causes an apparent risk of T cell lymphoma.

Many small molecules targeting RORγt have been developed. Some of them showed efficacy in preclinical *in vivo* models of psoriasis: IL-23-induced dermatitis, imiquimod (IMQ)-induced cutaneous inflammation and 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced ear inflammation in K5.Stat3C transgenic mice [12–15]. However, there have been no reports of RORγt inhibitors demonstrated to have a low effect on the thymus.

In this study, we evaluated the anti-psoriatic potential of a novel, potent RORγt inhibitor, S18-000003, which we previously identified through chemical optimization of ethylsulfonylbenzyl derivatives [16]. To avoid side-effects, especially in the thymus, we topically administered S18-000003 and assessed efficacy on skin inflammation and effects on the thymus, using the TPA-induced K14.Stat3C transgenic mouse model. We previously established this model as a useful animal model of psoriasis that closely resembles the characteristics of human pathology [17]. S18-000003 markedly inhibited psoriasis-like symptoms, by affecting all IL-17-producing cell subsets. Importantly, in the thymus of S18-000003-treated mice, neither abnormal cell cycle progression nor reduction of $CD4^+CD8^+$ DP T cells occurred, even at a high dose. Based on these results, topical treatment with our novel RORγt inhibitor may be a highly attractive therapeutic approach for psoriasis, with low risk of thymic aberrations.

2. Materials and methods

2.1. Human samples

Blood for peripheral blood mononuclear cell (PBMC) preparation was obtained from Shionogi's blood donor program. All procedures were in compliance with Shionogi's ethics committee and conducted under the Declaration of Helsinki principles. Written informed consent was obtained from all subjects.

2.2. Mice

Wild-type C57BL/6 mice were obtained from Charles River Laboratories (Yokohama, Japan). K14.Stat3C transgenic mice were generated as previously reported [17], and heterozygous transgenic mice were used. Mice were kept under specific pathogen-free conditions and provided with food and water *ad libitum*. The experiments were performed in compliance with institutional guidelines and were approved by the institutional animal care and use committee.

2.3. Human and mouse T helper differentiation

Human naïve $CD4^+$ T cells were isolated from healthy donors' PBMCs using a Naïve $CD4^+$ T Cell Isolation Kit II (Myltenyi Biotec, Auburn, CA, USA). Mouse naïve $CD4^+$ T cells were isolated from pooled splenocytes of C57BL/6 mice using a $CD4^+$ $CD62L^+$ T Cell Isolation Kit II (Myltenyi Biotec). Isolated cells were activated with plate-bound anti-CD3 and soluble anti-CD28 under Th17, Th1, Th2 or Treg polarizing conditions as shown in Supplementary Table S1. Compounds or the vehicle, dimethyl sulfoxide (DMSO, Sigma) were also included in the cultures. After 7 days (human) or 4 days (mouse) of culture, cytokine-producing cells were analyzed by intracellular staining and flow cytometry following re-stimulation with phorbol 12-myristate 13-acetate plus ionomycin (PMA + IOM).

2.4. Human PBMC cultures and cytokine analysis

PBMCs from healthy donors were activated with plate-bound anti-CD3 and soluble anti-CD28 in the presence or absence of compounds for 3 days. Culture supernatants were collected and the accumulated cytokines were measured by Bio-Plex Pro Human Cytokine Assay (Bio-Rad, Hercules, CA, USA). Cell proliferation was evaluated with a WST-8 Kit (Kishida Chemical, Osaka, Japan).

2.5. Induction of K14.Stat3C psoriasis-like mouse model and administration of compound

Psoriasis-like skin inflammation was induced in the TPA-treated K14.Stat3C mice as described previously [17]. We topically treated the shaved dorsal skin with 100 μL of 2 nmol TPA every second day over 12–14 consecutive days. The skin was administrated topically with 100 μL of S18-000003 (0.1–8%), betamethasone (0.01 μg/mL) or vehicle (acetone) once a day from day 0 until the end of the study (0.5–3 h before TPA treatment). Mice were sacrificed 3 or 24 h after the final TPA treatment, and blood, skin, inguinal lymph nodes (LNs) and thymus samples were obtained.

2.6. Mouse PBMC cultures and cytokine analysis

PBMCs from normal or TPA-treated K14.Stat3C mice were activated with plate-bound anti-CD3 and soluble anti-CD28 in the presence or absence of compounds for 3 days. Culture supernatants were collected and the accumulated cytokines were measured using ELISA kits (R&D systems). Cell proliferation was evaluated with a WST-8 Kit.

2.7. Scoring the severity of skin inflammation

An objective scoring system was developed to score the severity of inflammation induced on the back of mice, based on the clinical Psoriasis Area and Severity Index. The extents of erythema, scaling and thickening were blindly scored on a scale from 0 to 6 as follows: 0, none; 1, very slight; 2, slight; 3, mild; 4, moderate; 5, severe; 6, very severe. The three index scores were added together to obtain a total score.

2.8. Cytokine production in skin

3 h after the final TPA challenge, skin samples were taken with 8.0 mm biopsy punches and homogenized in T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Tokyo, Japan) supplemented with a Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific). After centrifugation, the supernatant was assayed. The concentrations of IL-17 and IL-22 in the extract were determined using ELISA kits (R&D Systems).

2.9. Flow cytometric analysis

All antibodies (Abs) were obtained from BioLegend, eBioscience and BD Biosciences (San Diego, CA, USA).

Single-cell suspensions from the skin were prepared 24 h after the final TPA challenge as previous described [17].

Prepared cells were incubated with Leukocyte Activation Cocktail with BD GolgiPlug (BD Biosciences) for PMA + IOM stimulation or with 100 ng/mL IL-23 and BD GolgiPlug (BD Biosciences) for IL-23 stimulation, for 4–6 h at 37 °C. Cells were

stained with Fixable Viability Dye (eBiosciences) and fluorescence-labeled antibodies for surface markers, then fixed/permeabilized using a BD Cytofix/Cytoperm Kit (BD Biosciences) and stained with antibodies for intracellular cytokines. For Foxp3 staining, cells were fixed/permeabilized using a transcription factor buffer set (eBioscience). Cell cycling of thymocytes was analyzed using a BrdU Flow Kit (BD Biosciences) after bromodeoxyuridine (BrdU) incorporation for 45 min. Cells were fixed/permeabilized, treated with DNase, and stained with anti-BrdU and 7-aminoactinomycin D. Cells were analyzed using a BD Biosciences FACS Canto II flow cytometer.

2.10. Reverse transcription–polymerase chain reaction (PCR)

Total RNA was extracted from the thymus three hours after the final TPA challenge using an RNeasy Mini Kit (QIAGEN) and reverse transcribed with the PrimeScrip RT Master Mix (TaKaRa, Shiga, Japan). PCR was performed using SYBR Premix Ex Taq II (TaKaRa). Sequences of the PCR primers and amplification conditions are shown in Supplementary Table S5. Gene expression was normalized to the *GAPDH* housekeeping gene, and data are presented as -fold differences determined using the $2^{-\Delta\Delta Ct}$ method.

2.11. Statistics

Statistical analysis of significance was performed using Dennett's test. A p value < 0.05 was considered significant, and all data are shown as mean ± standard error of mean (SEM).

3. Results

3.1. S18-000003 is a novel, potent RORγt inhibitor

We identified S18-000003 in a structure–activity relationship study following a high-throughput screening campaign [16]. The chemical structure is shown in Fig. 1. As we previously reported, S18-000003 binds to the ligand binding domain of RORγt but not RORα (Supplementary Table S3). Furthermore, S18-000003 inhibited human and mouse RORγt-dependent transactivation in cell-based GAL4 promoter reporter assays, although the inhibitory potency towards mouse RORγt was approximately ten-fold lower than towards human RORγt. It did not repress human RORβ transcriptional activity.

3.2. Selective inhibition of Th17 cell differentiation and function

As we previously reported [16], S18-000003 inhibits Th17 cell differentiation. Flow cytometric data are shown in Fig. 2. S18-000003 dose-dependently inhibited Th17 cell differentiation from human naive $CD4^+$T cells with a 50% inhibitory concentration (IC_{50}) of 0.024 μM (Fig. 2(a), (b)). However, S18-000003 had little effect on Th1, Th2 and Treg cell differentiation, even at 1 μM (Fig. 2(c)). S18-000003 also inhibited the differentiation of mouse Th17 cells from splenic naïve $CD4^+$ T cells (Fig. 2(d), (e)). In this experiment, IL-17 and IL-22 production in the culture supernatants were also suppressed (Supplementary Fig. S4). Consistent with the RORγt reporter assays, inhibitory potency towards mouse Th17 cell differentiation was approximately ten-fold lower than towards human Th17 cell differentiation (IC_{50} = 0.20 μM). S18-000003 also did not inhibit the differentiation of other Th cells from mouse naïve $CD4^+$ T cells (data not shown).

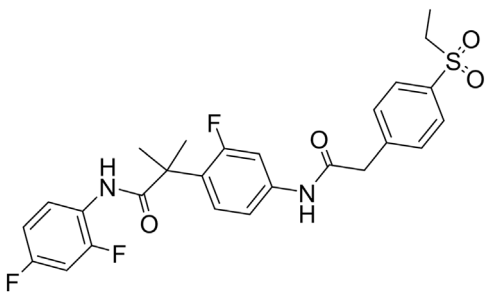


Fig. 1. Structure of S18-000003.

We next evaluated the effect of S18-000003 on the function of Th17 cells from a healthy human donor (Fig. 3(a)). When human PBMCs were stimulated with anti-CD3/CD28 antibody, IL-17 was mainly produced from $CD4^+$ T cells (Supplementary Fig. S5(a)). S18-000003 dose-dependently reduced the IL-17 production. On the other hand, S18-000003 did not inhibit either the production of other cytokines (IL-2, IL-4, IL-10 and IFN-γ) or cell proliferation, whereas the immunosuppressant, cyclosporine A reduced all cytokine production and cell proliferation. These results suggest that S18-000003 selectively inhibited Th17 cell function.

Next, we investigated the effect of S18-000003 on the function of Th17 cells from psoriatic mice (Fig. 3(b)). We induced psoriatic inflammation in the back skin of K14.Stat3C transgenic mice by TPA treatment. When PBMCs isolated from TPA-treated K14.Stat3C (Psoriasis) mice were stimulated with anti-CD3/CD28 antibody, a large amount of IL-17 was produced compared with PBMCs from TPA-non-treated (Normal) mice. This IL-17 was mainly derived from $CD4^+$ T cells (Supplementary Fig. S5(b)). S18-000003 dose-dependently reduced IL-17 and IL-22 production in PBMCs from psoriatic mice, suggesting that it functionally inhibited pathogenic Th17 cells.

3.3. An ameliorating effect on psoriasis-like lesions in TPA-induced K14.Stat3C transgenic mice

To investigate the anti-psoriatic potential of S18-000003, TPA-treated mice were topically administered S18-000003 (0.1%–8% in acetone solution) on a daily basis for 14 days, and several representative symptoms were measured. As shown in Fig. 4a and b, S18-000003 dose-dependently ameliorated clinical symptoms such as erythema, scaling and skin thickening. The highest-dose group we tested (8%) showed remarkable reduction of all index scores to the extent of equaling normal skin. Histopathologically, epidermal thickness was dramatically suppressed as well (Fig. 4(a), (c)). IL-17 and IL-22 secretion in the skin were dose-dependently decreased by S18-000003 treatment (Fig. 4(d)), which suggests that the improved efficacy of S18-000003 is probably dependent on the IL-17 pathway.

Although betamethasone also attenuated skin inflammation, we found signs of cutaneous side-effects in betamethasone-treated mice. We observed skin thinning and histological loss of basal keratinocytes (Supplementary Fig. S6), which relate to skin atrophy. Furthermore, betamethasone decreased LN size and reduced the expression of IFN-γ as well as IL-17-related cytokines in the skin (data not shown), which relate to immunosuppression leading to cutaneous infections. In contrast, S18-000003 did not induce these responses.

3.4. Suppression of IL-17-producing cells in the skin and LNs

To investigate whether S18-000003 decrease IL-17-producing cells in TPA-treated K14.Stat3C mice, we isolated single-cell suspensions from the skin and draining LNs and analyzed IL-17-producing cells by flow cytometry. As shown in Fig. 5(a)–(c), topical administration of S18-000003 reduced IL-17-producing cells to the level of normal mice. We analyzed subsets of these cells

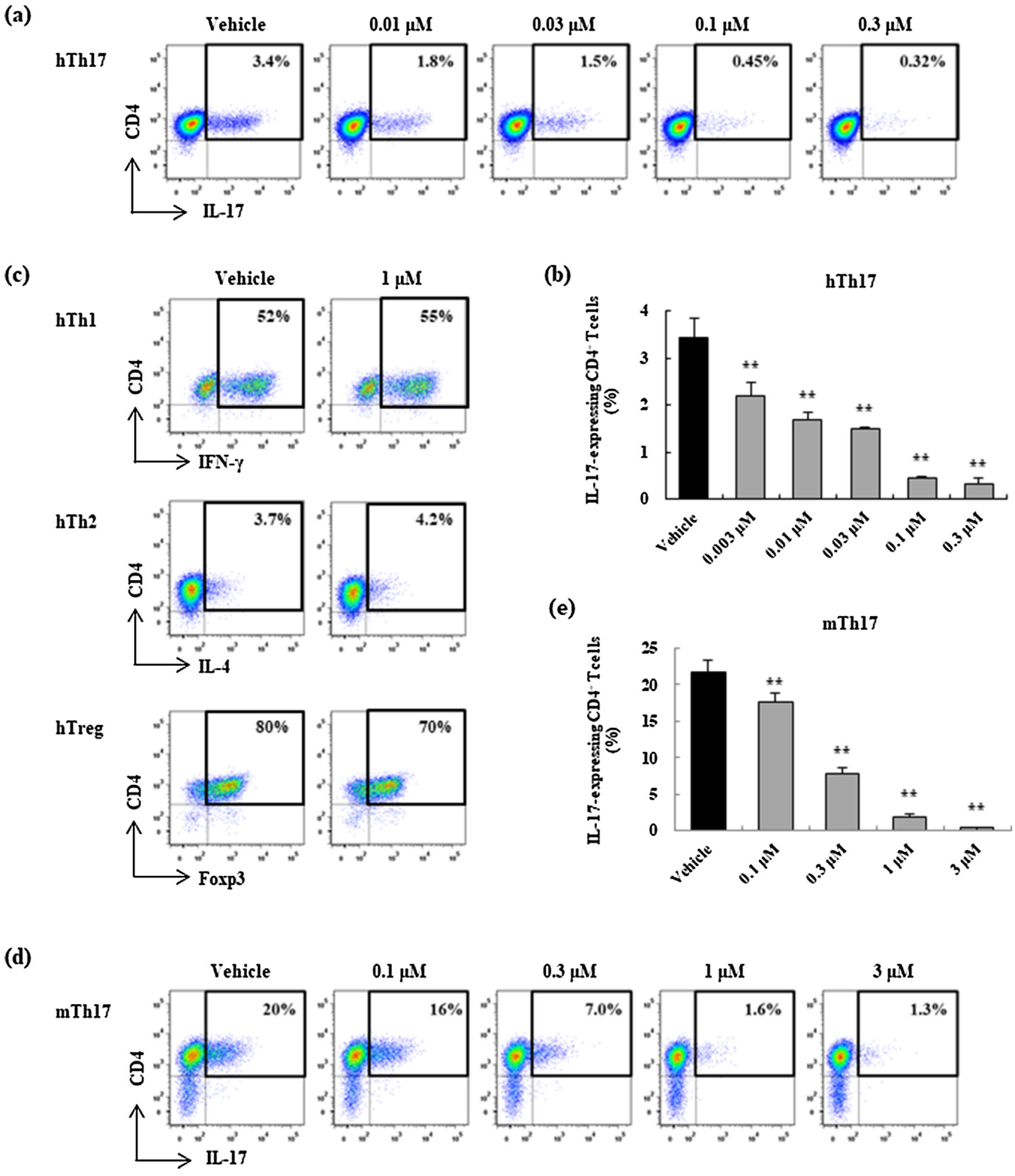
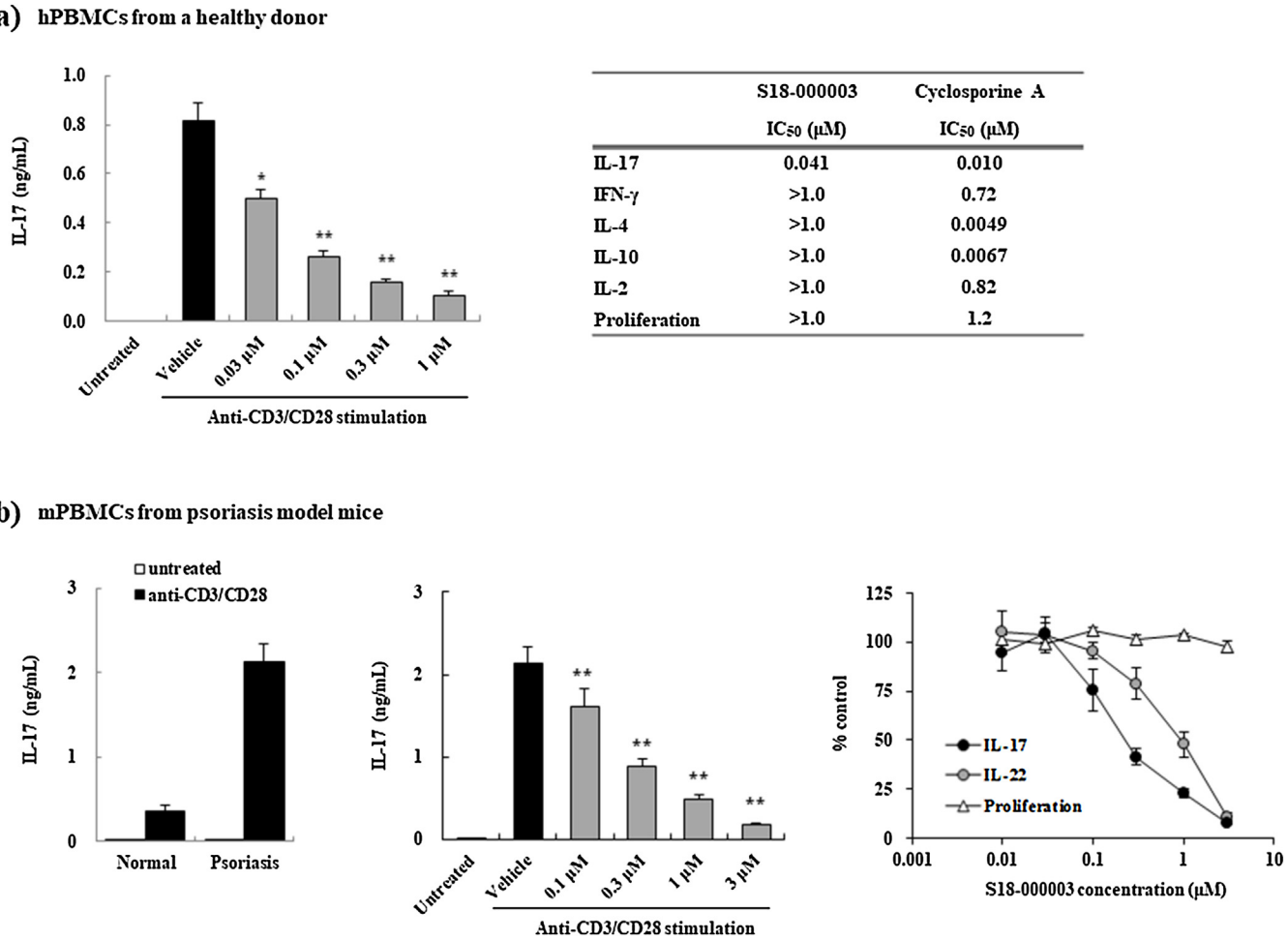


Fig. 2. S18-000003 specifically inhibited human/mouse Th17 differentiation. (a–c) Human naïve $CD4^+$ T cells isolated from the blood of a healthy donor were differentiated towards Th17, Th1, Th2 and Treg lineages for 7 days in the presence of vehicle (DMSO) or titrated S18-000003. (a) Representative flow cytometry plots showing IL-17-expressing $CD4^+$ T cells among differentiated Th17 cells. (b) The mean percentage of IL-17-expressing $CD4^+$ T cells among differentiated Th17 cells. * $p < 0.05$, ** $p < 0.01$ versus vehicle. Values represent the mean ± SEM of triplicate assays. (c) Representative flow cytometry plots showing IFN-γ-, IL-4- or Foxp3-expressing $CD4^+$ T cells among differentiated Th1, Th2 or Treg cells. (d–e) Mouse naïve $CD4^+$ T cells purified from the spleens of C57BL/6 mice were differentiated towards the Th17 lineage for 4 days in the presence of vehicle (DMSO) or titrated S18-000003. (d) Representative flow cytometry plots showing IL-17-expressing $CD4^+$ T cells. (e) The mean percentages of IL-17-expressing $CD4^+$ T cells. ** $p < 0.01$ versus vehicle. Values represent the mean ± SEM of triplicate assays.



	S18-000003 IC_{50} (μM)	Cyclosporine A IC_{50} (μM)
IL-17	0.041	0.010
IFN-γ	>1.0	0.72
IL-4	>1.0	0.0049
IL-10	>1.0	0.0067
IL-2	>1.0	0.82
Proliferation	>1.0	1.2

Fig. 3. S18-000003 selectively suppressed human/mouse Th17 cell function. (a) Human PBMCs isolated from a healthy donor were stimulated with anti-CD3/CD28 antibody for 3 days. Accumulated cytokines in cell culture supernatants and cell proliferation were measured. The left graph shows IL-17 production in the presence of vehicle (DMSO) or titrated S18-000003. ** $p < 0.01$ versus vehicle. Values represent the mean ± SEM of triplicate assays. The right table shows the concentrations of S18-000003 or cyclosporine A required to achieve 50% inhibition (IC_{50}), for production of each cytokine and for cell proliferation. (b) Mouse PBMCs isolated from untreated or TPA-treated K14.Stat3C mice were stimulated with anti-CD3/CD28 antibody for 3 days. Accumulated cytokines in cell culture supernatants and cell proliferation were measured. The left graph shows IL-17 production by PBMCs of TPA-non-treated (Normal) or TPA-treated (Psoriasis) K14.Stat3C mice. The middle graph shows IL-17 production by PBMCs of TPA-treated K14.Stat3C mice in the presence of vehicle (DMSO) or titrated S18-000003. ** $p < 0.01$ versus vehicle. The right graph shows dose–response curves for S18-000003, plotted as percentages of the vehicle control. Values represent the mean ± SEM of triplicate assays.

by multi-color staining. S18-000003 suppressed $CD4^+$ Th17 cells, $CD8^+$ Tc17 cells, dermal γδ T cells and TCR^- cells in the skin and LNs (Fig. 5(d), Supplementary Fig. S7). Interestingly, IL-17-producing $TCR\alpha\beta^+$ $CD4^-CD8^-$ double-negative T cells (DN αβ T cells), which we have previously identified in this model, were also suppressed by S18-000003.

More importantly, IL-17-producing cells that respond to IL-23 stimulation were also reduced by S18-000003 treatment (Fig. 5(e)). Because IL-23 is an upstream regulator of IL-17 production and has a critical role in psoriatic inflammation, both in human patients and in mice, IL-17-producing cells that respond to IL-23 stimulation are implicated in the pathogenesis of psoriasis. Thus, S18-000003 impacts on the pathogenic IL-17-producing cells in psoriatic skin.

3.5. Little impact on the thymus

It has been reported that *Rorc* deficiency causes various thymocyte changes prior to T cell lymphoma development [7,8]. Some of these alterations were observed in our analysis of *Rorc* deficient mice as well: the expression of Bcl-xL (an anti-apoptotic molecule) and $p27^{kip1}$ (a negative cell cycle regulator) were reduced, thymocytes in the S-phase of the cell cycle were increased and $CD4^+CD8^+$ DP T cells were decreased (Supplementary Fig. S8). We investigated whether S18-000003 causes similar changes to the thymus of TPA-induced psoriatic K14.Stat3C mice after 2 weeks topical administration. Although treatment with 8% S18-000003 slightly reduced the expression of Bcl-xL and $p27^{kip1}$, these changes were not induced by treatment with 2% S18-000003 (Fig. 6(a)). The reductions of Bcl-xL and $p27^{kip1}$ expression in the thymus were not observed when 8% S18-000003 was topically administrated on the back skin of wild-type BALB/c mice (data not shown). In addition, S18-000003 did not affect either cell cycling or T cell populations in the thymus, even at a dose of 8% (Fig. 6(b), (c)). Based on these results, it is expected that topical administration of S18-000003 has strong effects on the skin with low risk of side-effects in the thymus.

4. Discussion

In this study, we showed that topical administration of our novel RORγt inhibitor, S18-000003 markedly inhibited the development of psoriatic skin inflammation with low risk of thymus side-effects in a mouse model of psoriasis.

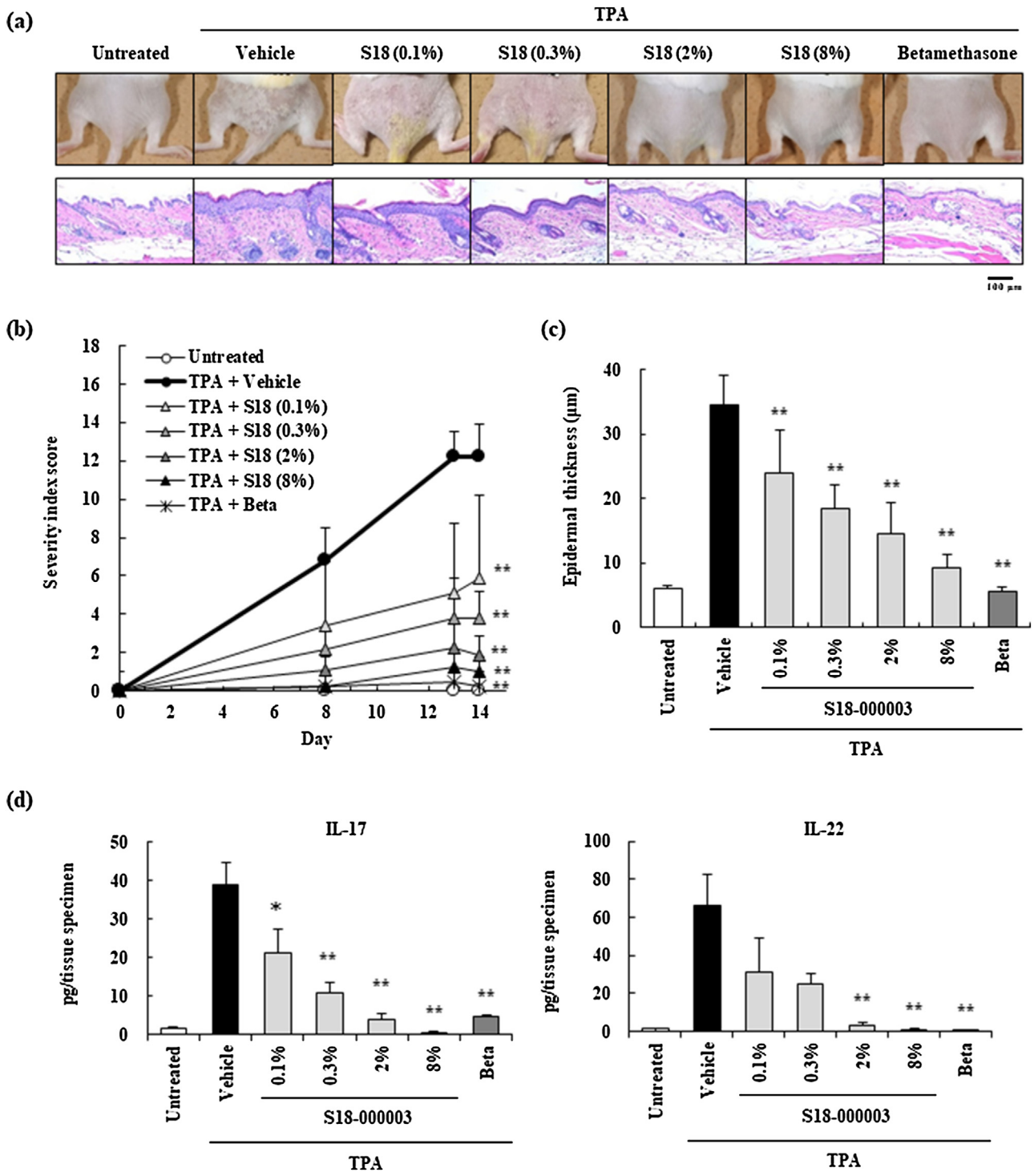


Fig. 4. Topical S18-000003 attenuated TPA-induced psoriatic skin inflammation in K14.Stat3C mice. K14.Stat3C mice were treated with TPA on their shaved back skin every second day over 14 consecutive days. S18-000003 (0.1–8%), betamethasone (0.01 μg/mL) or vehicle (acetone) was administrated topically at 100 μL/mouse on the same skin area, once a day for all 14 days. (a) Representative microscopic views (upper) and skin sections stained with hematoxylin and eosin (lower) at day 14. (b) Time course of severity index scores. (c) Epidermal thickness was quantified at day 14 by averaging the values of 3–5 independent fields per section. (d) Protein levels of IL-17 and IL-22 in homogenized skin tissue were measured at day 14. Values represent the mean ± SEM of 4–15 mice per group. * $p < 0.05$, ** $p < 0.01$ versus vehicle.

To avoid side-effects, especially in the thymus, we evaluated the anti-psoriatic potential of topically administered S18-000003 using the TPA-induced K14.Stat3C transgenic mouse model. S18-000003 dramatically ameliorated psoriasis-like symptoms without the skin atrophy seen in betamethasone-treated mice. The maximum efficacy of S18-000003 was as great as that of an anti-IL-12/23p40 antibody that we described in a previous report [17], and also that of betamethasone.

The prevention of disease development by S18-000003 was associated with reduced IL-17 and IL-22 production in the skin, suggesting that disease amelioration was caused via suppression of the IL-17 pathway. Flow cytometric analysis demonstrated a

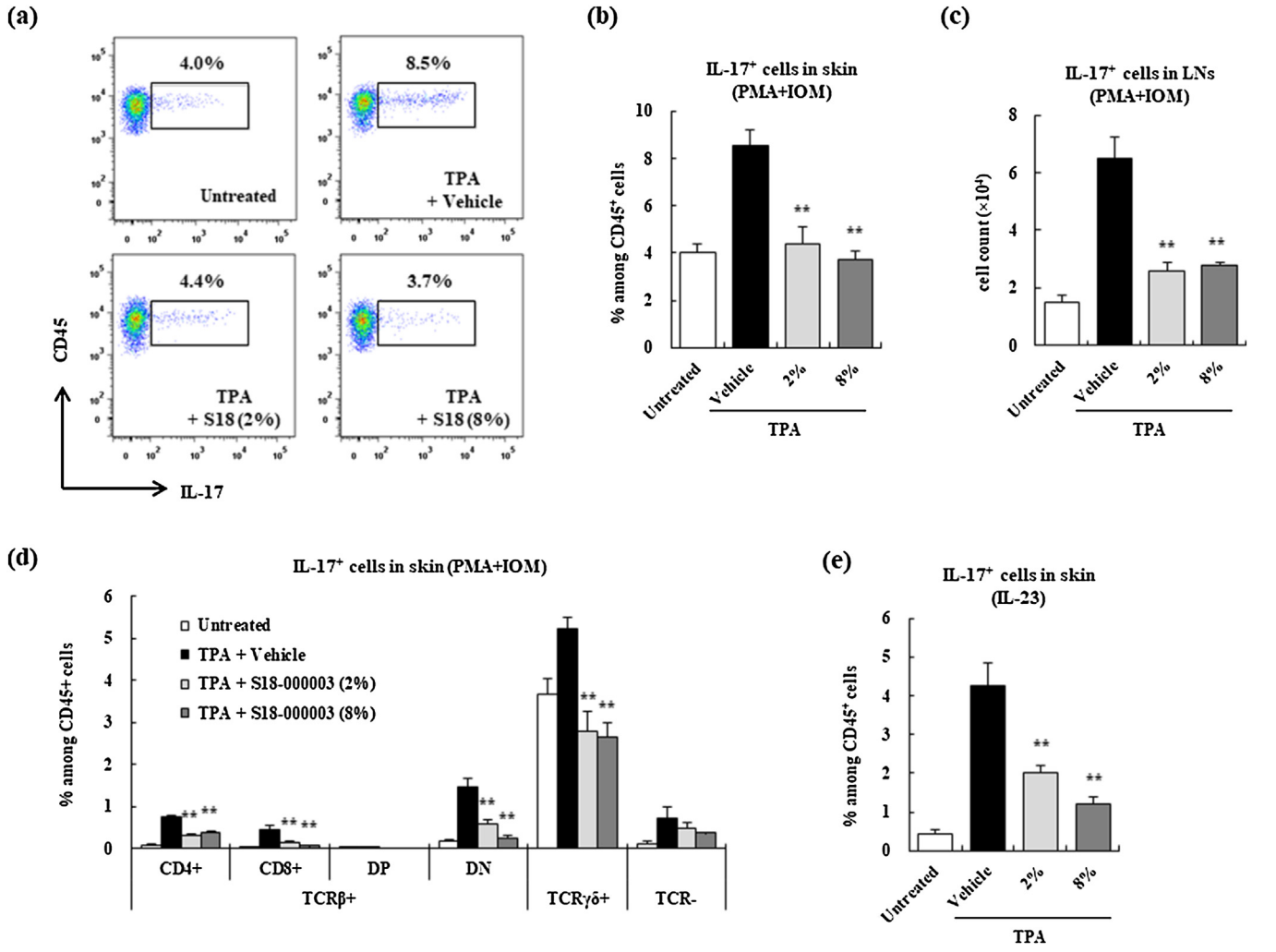


Fig. 5. IL-17-producing T cell subsets involved in TPA-induced skin inflammation in K14.Stat3C mice were suppressed by topical S18-000003. Mice were topically administered with vehicle or S18-000003 (2% or 8%). (a) Cell suspensions prepared from the skin of TPA-treated K14.Stat3C mice 24 h after the final TPA challenge were stimulated with PMA + IOM and analyzed for intracellular IL-17 expression by flow cytometry. Cells were gated on live CD45$^+$ leukocytes and the percentages of IL-17$^+$ cells were determined. Representative flow cytometric data for skin cells are shown. (b) Mean frequencies of IL-17-producing skin cells following PMA + IOM treatment are shown in a histogram. (c) Total numbers of IL-17-producing cells in the draining LNs were analyzed. (d) Multi-color analysis was performed using combinations of antibodies for IL-17 and various T cell surface markers. Mean frequencies of each IL-17$^+$ subset in lesional skin following PMA + IOM stimulation were determined. (e) Mean frequencies of IL-17-producing skin cells following IL-23 stimulation are shown in a histogram. Values represent the mean ± SEM of four mice per group. * $p < 0.05$, ** $p < 0.01$ versus vehicle.

significant decrease of IL-17-producing cells in the skin and draining LNs of S18-000003-treated mice. Importantly, the pathogenic IL-17-producing cells that immediately respond to IL-23 stimulation in lesional skin were also decreased by S18-000003 treatment. As we previously reported, heterogeneous cell types are responsible for IL-17 production in this mouse model [17]. IL-17-producing Th17 cells, Tc17 cells, dermal γδ T cells, and TCR$^-$ cells probably including ILCs all participate in skin inflammation, which is similar to the clinical features of human psoriasis. Notably, S18-000003 was effective against not only Th17 cells but also against all of these IL-17-producing cell populations.

Interestingly, S18-000003 also decreased IL-17-producing DN αβ T cells. We previously found that IL-17-producing DN αβ T cells are also involved in psoriatic inflammation in this model and that they are likely to express RORγt [17]. Some reports showed that the IL-17-producing DN αβ T cells involved in infectious or autoimmune disease express RORγt as well [18–21]. However, to our knowledge, this is the first report that demonstrates the functional importance of RORγt in IL-17-producing DN αβ T cells. Technical difficulty prevented us from performing *in vitro* studies using purified DN αβ T cells. Further analysis is necessary to examine directly whether our RORγt inhibitor can suppress the function and development of IL-17-producing DN αβ T cells.

In addition to critical roles for the development and function of IL-17-producing cells, RORγt is also essential for controlling apoptosis during thymopoiesis [7,8], and mice deficient in *Rorc* have been shown to develop thymic lymphoma at high incidence [9]. Recent studies have demonstrated that adult mice with a gene-specific deletion of *Rorc* also develop lymphoma, in a similar time frame to embryonic *Rorc* knockouts [10]. We have also confirmed the development of thymic lymphoma in adult mice when the *Rorc* gene was knocked down at the age of 10–20 weeks (unpublished data; in preparation). Furthermore, some reports have shown that oral administration of a RORγt inhibitor causes progressive thymic aberrations in rats and mice [11,12]. In one of these studies, thymic alterations such as Bcl-xL downregulation and DP thymocyte reduction were caused only 72 h after *Rorc* deletion or pharmacological RORγt inhibition. However, our study showed that topical treatment with S18-000003 had minimal influence on the thymus after 2 weeks repetitive administration, despite its strong effects

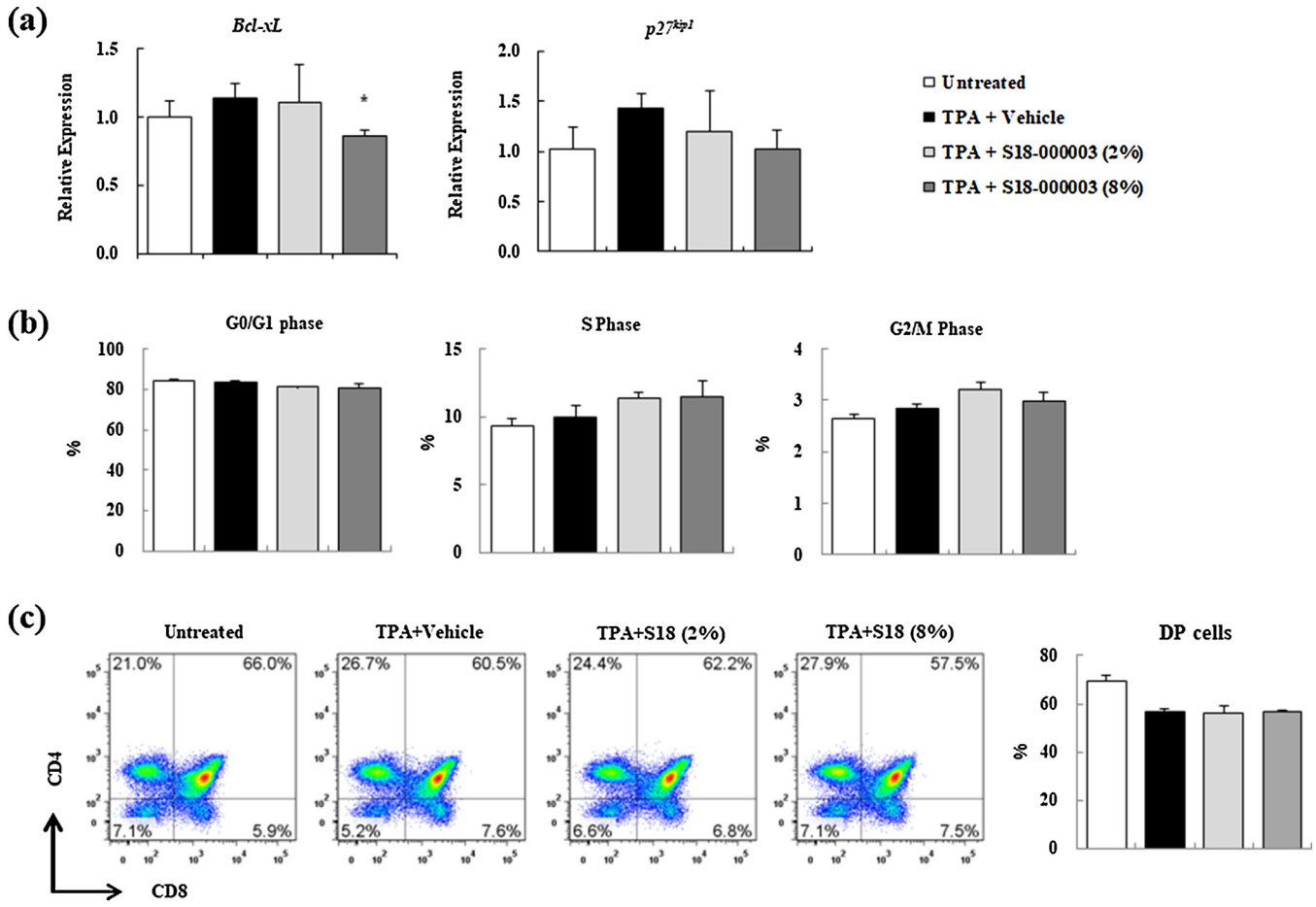


Fig. 6. Influence of topical S18-000003 on the thymus. Thymus samples were taken from TPA-treated K14.Stat3C mice after 2 weeks repetitive topical administration of S18-000003 (2% or 8%) or vehicle. (a) mRNA expression of *Bcl-xL* and *p27^{kip1}* in the thymus. (b) Cell cycle analysis of thymocytes. After incubation with BrdU, cells were stained for BrdU and 7-aminoactinomycin D and analyzed by flow cytometry. Cell cycle phases were determined by the levels of incorporated BrdU and total DNA. (c) Thymocytes were stained for CD4 and CD8 and analyzed by flow cytometry. The left panels show representative flow cytometry plots following treatment with vehicle or S18-000003. The right graph shows mean frequencies for the $CD4^+CD8^+$ DP cell population. Values represent the mean ± SEM of four mice per group. ** $p < 0.01$ versus vehicle.

on psoriatic skin. Neither abnormal cell cycle progression nor a reduction of DP thymocytes was seen in S18-000003-treated mice, even at a dose of 8%. Although expression of Bcl-xL and $p27^{kip1}$ was slightly suppressed, only at a high dose, the extent of their suppression is likely to be smaller than that in *Rorc* heterozygous knockout mice (Supplementary Fig. S8). It has been reported that no *Rorc* heterozygous knockout mice developed thymic lymphomas over 1 year [9], and we have verified this observation (data not shown). Hence, topical S18-000003 possibly would have a low risk of thymic lymphoma even at a high dose. However, further tests are needed to fully evaluate its safety.

One of the possible reasons why topical treatment with S18-000003 had a small impact on the thymus despite strong effects on psoriatic skin may be that S18-000003 has high skin-retentivity. S18-000003 has high lipophilicity with low aqueous solubility. Furthermore, S18-000003 is easy to crystallize. Because of these physical properties, it is presumed that much of the applied S18-000003 might remain in the skin and the amount of S18-000003 distributed to the blood capillaries might be low, leading to strong effects on psoriatic skin and a small influence on the thymus. This probably means that action in the skin is important for the anti-psoriatic effect of RORγt inhibitors. The skin is thought to be not only a peripheral site of inflammation, but also an essential component of the lymphatic system known as skin-associated lymphoid tissue (SALT). It has been reported that the priming of naïve T cells by antigen-presenting cells and the efficient activation of effector T cells can occur in the skin, as it does in other secondary lymphoid tissues [21,22]. With regard to psoriasis, conventional dendritic cells (DCs) and Langerhans cells in the dermis have been identified as the critical pathogenic DC populations initiating chronic skin inflammation in mice via IL-23 production [23,24]. In humans, a few 6-sulfo LacNAc DCs, which can produce Th17-programming cytokines, can be identified in healthy skin, and increased numbers of IL-23-expressing 6-sulfo LacNAc DCs are found in lesional psoriatic skin [25]. Based on these facts, it seems that the inhibition of RORγt function in skin by topical S18-000003 directly suppresses the development and activation of pathogenic IL-17-producing cells involved in psoriatic inflammation, leading to a strong impact on ameliorating psoriasis.

Topical steroids are commonly used for a wide range of skin disorders, including psoriasis. The anti-inflammatory and anti-proliferative actions of topical steroids result in their therapeutic effect, but also produce various cutaneous side-effects such as skin atrophy and cutaneous infections [26]. Our *in vivo* study also found signs of these adverse events following betamethasone treatment. We observed skin thinning and histopathological changes as signs of skin atrophy, and LN shrinkage and reduced IFN-γ expression in the skin (data not shown) as signs of immunosuppression in betamethasone-treated mice. However, topical S18-000003 did not cause these adverse effects. Considering these points, topical

S18-000003 is potentially a therapeutic treatment for psoriasis that would not produce cutaneous side-effects.

In summary, our novel RORγt antagonist, S18-000003 exhibited strong effects on psoriatic skin inflammation in a TPA-induced K14. Stat3C transgenic mouse model following topical administration, which compares favorably with betamethasone. In lesional skin, S18-000003 treatment suppressed the development of all subsets of pathogenic IL-17-producing cells. On the other hand, topical S18-000003 had little effect on the thymus, and did not cause the cutaneous side-effects seen in betamethasone-treated mice. S18-000003 inhibited human RORγt approximately ten-fold more potently than mouse RORγt, so it should be possible to produce a powerful effect on human psoriasis, not inferior to IL-23/IL-17-targeting biologics, while using a lower dose. Topical treatment with our novel RORγt inhibitor is expected to be a highly attractive therapeutic approach for psoriasis with a low risk of thymic aberrations.

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Conflict of interest

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jdermsci.2019.03.002.

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