Study on the therapeutic strategies of psoriasis targeting novel regulatory pathways in γδT cells

(γδT 細胞の新規制御経路とこれを標的とした乾癬の治療戦略に関する研究)

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Abbreviations

BSA	bovine serum albumin
CD	cluster of differentiation
DC	dendritic cell
DETC	dendritic epidermal T cells
DMSO	dimethyl sulfoxide
DNase I	deoxyribonuclease I
DR3	death receptor 3
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
γδT17	interleukin-17-producing γδT cells
Gr1	granulocyte receptor-1 antigen
Gapdh	murine glyceradehyde-3-phosphate dehydrogenase gene
H&E	hematoxylin and eosin
IFN	interferon
IL	interleukin
IMQ	imiquimod
INM	ionomycin
MFI	mean fluorescence intensity
qRT-PCR	quantitative reverse transcription-polymerase chain reaction
REV-ERB	reverse orientation c-erb
RFI	relative fluorescence intensity
ROR	retinoic acid receptor-related orphan receptor
TCR	T-cell receptor
TL1A	tumor necrosis factor-like ligand 1A
TLR	toll like receptor
Th17	IL-17-producing helper T cells
PBS	phosphate buffered saline
PMA	phorbol 12-myristate 13-acetate
PCNA	proliferating cell nuclear antigen

Notes

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Preface

Psoriasis is a chronic inflammatory skin disease that causes a rash with itchy and scaly patches most commonly on the knees, elbows, trunk, and scalp. The prevalence reaches around 2–3% of the world population. Recurrent inflammatory flares cause physical and psychological suffering and significantly affect the patient's quality of life (Meneguin et al., 2020; Quinto et al., 2022; Zhang et al., 2021). Long-time management of psoriasis also can be a substantial economic burden on both individuals and society (Vanderpuye-Orgle et al., 2015).

Current clinical treatments for psoriasis encompass a variety of strategies aimed at managing symptoms. Topical treatments with corticosteroids and Vitamin D analogs are common but have side effects including atrophy and striae after long-term use (Menter et al., 2011). Phototherapy also carries the risks of skin irritation and skin cancer (Racz & Prens, 2015). Immunosuppressive drugs such as methotrexate and cyclosporine can be systemically employed but come with long-term complications with liver toxicity and renal impairment (Coates et al., 2020; Kim et al., 2018). Systemic application of biologics, including etanercept, and adalimumab, requires careful monitoring for opportunistic infections (Rodgers et al., 2011). Although other emerging biologics such as interleukin (IL)-17 and IL-23 inhibitors target more specific disease processes (Kim & Krueger, 2017), the risk of fungal infections remains (Bilal et al., 2024; Wu et al., 2023). Therefore, identifying new regulatory mechanisms of psoriasis is essential for developing better treatment strategies.

The skin lesions of psoriasis are characterized by the uncontrolled proliferation of keratinocytes and significant inflammatory infiltrations in both the dermis and epidermis (Michael & Boehncke, 2005). The development of psoriasis involves various immune cells and pathways. In the early stages of psoriasis, innate immune cells such as dendritic cells (DCs) and macrophages in the lesions play a crucial role in triggering

inflammation by producing IL-23. IL-23 subsequently activates IL-17-producing helper T (Th17) cells. Th17-derived cytokines, including IL-17 and IL-22, provoke the recruitment of neutrophils and the proliferation of epidermal keratinocytes, thereby contributing to the inflammation and epidermal hyperproliferation (Nograles et al., 2008). While Th17 cells contribute to psoriasis development as a part of the adaptive immune system, recent studies have highlighted the innate function of γδT cells to produce IL-17 in disease onset. Unlike Th17 cells that reside in lymph nodes until activated and migrate to the site of inflammation, γδT cells can respond rapidly to inflammatory cues. Although γδT17 cells represent a possible therapeutic target, the specific pathways and factors that regulate their function remain poorly understood.

In this thesis, two distinct approaches were explored to modulate γδT cell activity for psoriasis treatment. Chapter I shows that a nuclear receptor called reverse orientation c-erb (REV-ERB) suppresses γδT17 cell activation as in Th17 cells and a REV-ERB agonist ameliorates inflammatory symptoms in a murine psoriasis model (Figure 1). Chapter II demonstrates a member of the tumor necrosis factor superfamily called tumor necrosis factor-like ligand 1A (TL1A) activates γδT cells during psoriasis development in murine models and TL1A neutralization relieves the disease (Figure 2).

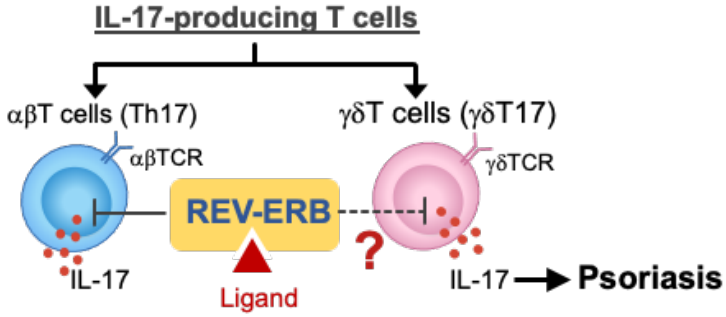

Figure 1. REV-ERBs in γδT17 cell regulation and psoriasis development. It has been reported that REV-ERB agonists reduce the severity of autoimmune diseases by suppressing Th17 cell activation. Chapter I of this thesis shows the role of REV-ERBs in γδT cell regulation and the efficacy of a REV-ERB agonist in psoriatic disease of a mouse model.

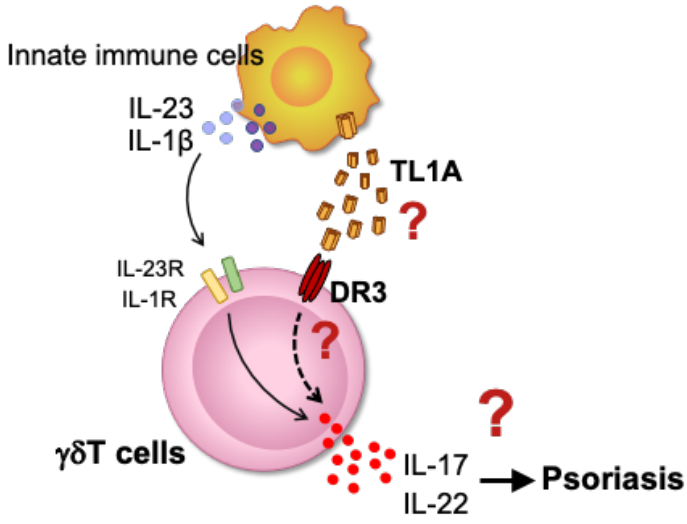

Figure 2. The involvement of TL1A–DR3–IL-17/IL-22 axis in γδT cells and psoriasis. Although inflammatory cytokines IL-23 and IL-1β are known to synergistically activate γδT17 cells, how γδT17 cells sense inflammation to elicit innate function remains fully elucidated. Chapter II demonstrates that TL1A and its receptor DR3 are involved in γδT cell activation and psoriasis development in mouse models.

Chapter I

REV-ERB agonist suppresses IL-17 production in γδT cells and improves psoriatic dermatitis in a mouse model

Introduction

Psoriasis is a chronic inflammatory skin disease characterized by red, raised plaques with adherent silvery scales. Uncontrolled hyper-proliferation, aberrant differentiation of keratinocytes, and neutrophil infiltration are observed in psoriatic skin lesions (Michael & Boehncke, 2005). Previous studies have suggested the critical involvement of interleukin (IL)-17-producing helper T (Th17) cells in psoriasis pathogenesis. Dendritic cells and macrophages in psoriatic lesions produce IL-23, which activates cytokine secretion by Th17 cells (Cai et al., 2011; Van der Fits et al., 2009). Th17-derived cytokines, such as IL-17 and IL-22, play essential roles in disease development by provoking neutrophil recruitment and epidermal keratinocyte proliferation (Elloso et al., 2012; Nograles et al., 2008). Therefore, the IL-23/Th17 axis has attracted attention as a potential biopharmaceutical target in psoriasis patients (Elloso et al., 2012).

T cells are primarily divided into two subpopulations based on the combinational use of α, β, γ, and δ chains to form T cell antigen receptors (TCRs). γδT cells have an innate capacity to respond to inflammatory cues, whereas activation of αβT cells requires antigen stimulation. There is emerging evidence that innate dermal γδT cells contribute to psoriasis development as a primary source of IL-17 in the skin (Cai et al., 2011, 2012, 2014; Gray et al., 2011). Cai et al. showed that psoriatic symptoms are significantly reduced in γδT cell-deficient mice, but not in αβT cell-deficient mice, using psoriasis models generated by applying imiquimod (IMQ) to the skin or by intradermal IL-23 injection (Cai et al., 2011). Dermal γδT cells proliferate and produce IL-17 in response to IL-23, and dermal IL-17 levels in this psoriasis model are drastically decreased in γδT cell-deficient mice (Cai et al., 2011). Moreover, it has been demonstrated that a large number of IL-17-producing γδT (γδT17) cells reside in the dermal lesions in patients with psoriasis (Cai et al., 2011). These findings suggest that innate γδT17 cells are involved in the primary immune responses required to establish the basis of psoriasis onset.

The nuclear receptors REV-ERBs, consisting of α and β isoforms, act as ligand-dependent transcription factors (Kojetin & Burris, 2014; Marciano et al., 2014). Their roles in the regulation of circadian rhythm in combination with other nuclear receptors, retinoic acid receptor-related orphan receptor receptors (RORs), have been broadly appreciated. REV-ERBs and RORs compete for the binding to a shared motif sequence on target gene promoters and regulate the transcription of clock genes (Kojetin & Burris, 2014; Marciano et al., 2014; Solt et al., 2012; Zhang et al., 2015). Because REV-ERBs lack the carboxy-terminal domain required for the interaction with co-activators, they function as transcriptional repressors and negatively regulate the transactivating effect of RORs (Kojetin & Burris, 2014). Similar to the clock genes, the expression of IL-17 in T cells is also regulated by the competition between REV-ERBs and RORs. RORγt is known as a master regulator of Th17 cell differentiation and RORα play a supportive role by synergistically acting with RORγt (Ivanov et al., 2006; Yang et al., 2008). REV-ERBα is specifically expressed in Th17 cells and represses the IL-17 expression through the competition with RORγt (Amir et al., 2018). Deletion of REV-ERBα enhances IL-17 expression and aggravates the symptoms of Th17-mediated autoimmune diseases, including experimental autoimmune encephalomyelitis (EAE) and colitis in mice (Amir et al., 2018). Based on the discovery and characterization of the REV-ERBs' natural ligand: heme, the synthetic ligands have been developed (Amir et al., 2018; Kojetin & Burris, 2014; Solt et al., 2012). It has been shown that the treatment with a REV-ERB agonist reduces the severity of EAE by suppressing Th17 cells (Amir et al., 2018). These findings from the previous report have revealed the REV-ERB-dependent regulation of Th17 cells. However, the involvement of REV-ERBs in the regulation of γδT17 cells remains unclear.

In this study, the regulatory mechanism of γδT17 cells through REV-ERBs was demonstrated. γδT17 cells were significantly increased in the lymphoid organs of REV-ERBα-deficient mice. IL-17 production in γδT cells was repressed by short-term treatment with a synthetic REV-ERB agonist SR9009 either *in vitro* or *in vivo*. Furthermore, the topical application of SR9009 to the skin decreased the inflammatory

symptoms of IMQ-induced psoriasis in mice. Thus, the modulation of REV-ERB activity by synthetic ligands may be a novel therapeutic approach for psoriasis.

Materials and Methods

Mice

The *Nr1d1* knockout mice (B6.Cg-*Nr1d1*^{tmVen}/LazJ) were purchased from Jackson Laboratory (Chomez et al., 2000). The C57BL/6 mice were from Japan SLC (Tokyo). Female mice at 10–15 weeks of age were used for all experiments. The mice were maintained in our animal experimentation facility and the animal experiments were performed under the approval of the Institutional Animal Care and Use Committee of Hokkaido University (approval numbers 16-0131 and 20-0172).

Flow cytometry analysis and cell sorting

Cells were incubated with Fc receptor blocking solution (Miltenyi Biotee) and then stained with fluorochrome-conjugated monoclonal antibodies (all from Biolegend) against cell surface proteins for 30 min. Intracellular staining for interferon (IFN)-γ and IL-17A was performed using a fixation and permeabilization buffer set (Thermo Fisher Scientific) following the manufacturer's instructions. Data were acquired using a FACSVerse flow cytometer (BD Biosciences) and analyzed using FlowJo v.10.3 software (TreeStar). For γδT cell isolation, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-B220, anti-TCRβ, anti-CD11c, anti-CD11b, anti-NK1.1 antibodies (all from Biolegend) and enriched by depleting antibody-bound cells using magnetic beads and columns (Miltenyi Biotec). The cells were then stained with an anti-CD3ε antibody (Biolegend) and FITC^{-} CD3ε^{+} cells were sorted using a FACSAria II instrument (BD Biosciences).

Cell culture

To analyze cytokine production, cells were stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) and 500 ng/mL ionomycin (INM) in the presence of a protein transport inhibitor (Thermo Fisher Scientific) for 3 h. Where indicated, cells

were cultured for 24 h with or without 10 μM SR9009 (Cayman Chemical) and stimulated with PMA and INM for the last 3 h.

Quantitative PCR analysis

Total RNA was extracted using RNeasy Plus Micro kit (Qiagen) and reverse transcribed using PrimeScript RT Master Mix (TaKaRa). Real-time PCR analysis was performed using TB Green Premix Ex Taq II (TaKaRa) on a LightCycler 96 System platform (Roche Diagnostics). Relative mRNA expression was analyzed based on the $\Delta\Delta$Ct method and normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). The primer sequences are listed in Table I-1. The expression levels of target genes were normalized to those of *Gapdh* and compared with the target gene expression levels measured in the control samples.

Preparation of single-cell suspension from the skin

Cells were isolated from the skin based on methods in previous reports with slight modifications (Broggi et al., 2016; Han et al., 2019). Skin samples were washed with phosphate-buffered saline (PBS) and cut into small pieces. The samples were then incubated at 37°C for 1 h with 300 μg/mL Liberase TL (Roche) and 1 mg/mL DNase I (Roche Diagnostics) in RPMI medium supplemented with 5% fetal bovine serum (Sigma-Aldrich). A single-cell suspension was obtained by grinding the samples on a metal mesh and filtration through nylon mesh.

IMQ-induced skin inflammation and REV-ERB against administration

Shaved dorsal skin (6 cm^2) was applied with 42.5 mg of 5% IMQ cream (Mochida Pharmaceuticals) or control Vaseline cream (PROPETO, Maruishi Pharmaceutical) for 2–5 consecutive days. The day of initial treatment was defined as day 0. Where indicated, the mice were intraperitoneally injected with SR9009 (AstaTech) at a dose of 150 mg/kg twice daily on days 0 and 1. For intraperitoneal injection, SR9009 was dissolved in 7.5% dimethyl sulfoxide (DMSO) (Wako), 10% Cremophor EL (Nacalai

Tesque), and 82.5% PBS. In experiments where SR9009 was topically applied, the dorsal skin was smeared with 6 mg of SR9009 dissolved in 50% DMSO and 50% Cremophor EL twice daily from days 0–4.

Soring of psoriasis and measurement of skin thickness

The severity of the disease was assessed using a scoring system slightly modified from the clinical psoriasis area and severity index and from previous reports (El Malki et al., 2013; Kim et al., 2018; Peng et al., 2020; Shin et al., 2019). Erythema grades were 0, none; 1, slightly reddish; and 2, red. The scaling grades were 0, none; 1, very fine scales covering an area < 50%; 2, fine scales covering an area ≥ 50%; and 3, coarse scales covering an area ≥ 50%. Thickening was graded according to skin swelling as 0, < 1.0mm; 1, ≥1.0mm; 2, ≥ 1.5mm; and 3, ≥ 2.0 mm. The mouse dorsal skin was lifted using fingers to measure skin thickness, and double-fold thickness was measured at three specific sites using a micrometer caliper (Mitutoyo Corp., Kawasaki, Japan) and averaged (Horváth et al., 2019). Swelling was calculated as the changes in skin thickness between day 0 and the time of measurement. The cumulative score was calculated from scores for erythema, scaling, and thickening.

Histological analysis

Skin samples were collected and fixed with 10% neutral buffered formalin. Next, sections were prepared from the paraffin-embedded tissues and stained with hematoxylin and eosin (H&E). The sectioned samples were scanned using a NanoZoomer 2.0-RS virtual slide scanner and analyzed using NDP.view2 software (Hamamatsu Photonics). Skin dermis and epidermis thicknesses were evaluated following a method described elsewhere and with slight modifications (Shao et al., 2019). The length from the stratum corneum to the basal stratum of the inter-follicular epidermis and the length from right under the inter-follicular epidermis to the top of the adipose tissue layer were measured as the thicknesses of the epidermis and dermis, respectively, using ImageJ software. The number of epidermal layers and the

thicknesses of the dermis and epidermis were measured at eight randomly chosen points in a 1200 μm × 750 μm field, and the average values were adopted as data for each mouse. The sections were deparaffinized and treated with 0.3% pepsin with 0.2 N hydrochloric acid to retrieve target antigens for immunohistochemical analysis. The sections were incubated with blocking serum (10% goat serum) for 1 h at room temperature and then with antimouse Gr1 primary antibody (rat monoclonal) (R&D Systems) overnight at 4°C. After that, the sections were incubated with secondary antibody (Biolegend) and streptavidin-conjugated horseradish peroxidase (SABPO (R) kit; Nichirei) for 30 min each at room temperature. Next, the sections were incubated with 0.01% 3,3′-diaminobenzidine to visualize positive reactions. Counterstaining was performed with Mayer's hematoxylin, and $Gr1^+$ cells were counted in the dermis area along a length of 1 cm.

Statistical analysis

Statistical significance was evaluated using two-tailed unpaired or paired Student's *t*-test. Analysis was performed using GraphPad Prism v.7.0 (GraphPad Software) and Excel (Microsoft). In all figures, *, **, and ***, respectively, denote *P*-values < 0.05, 0.01, and 0.001, respectively.

Table I-1. Sequences of primers used in qRT-PCR analysis

Primers	Sequences
Gapdh F	5'-TTGTCAGCAATGCATCCTGCAC-3'
Gapdh R	5'-GAAGGCCATGCCAGTGAGCTTC-3'
Ifng F	5'- TGGAGGAACTGGCAAAAGGATG -3'
Ifng R	5'- GTTGCTGATGGCCTGATTGTC -3'
Il17a F	5'- AAAGCTCAGCGTGTCCAAAC -3'
Il17a R	5'- TGAGCTTCCCA- GATCACAGA -3'
Il17f F	5'- CCCCATGGGATTACAACATC -3'
Il17f R	5'- TTCATGGTGCTGTCTTCCTG -3'
Il22 F	5'- ATA-CATCGTCAACCGCACCT -3'
Il22 R	5'- GTTGAGCACCTGCTT-CATCA -3'

Results

ROR Increase in γδT17 cells in REV-ERBα-deficient mice

The involvement of REV-ERBα in regulating γδT17 cells was initially examined using lymphocytes from REV-ERBα-deficient (*Nr1d1*$^{-/-}$) mice. The frequency and number of CD3^{+}γδTCR^{+} cells in the spleen and lymph nodes were comparable between *Nr1d1*$^{+/+}$ and *Nr1d1*$^{-/-}$ mice (Figures I-1A and B). Cytokine production was examined in γδT cells stimulated or unstimulated with PMA and INM. As expected, IL-17-producing γδT cell numbers were significantly increased in the spleen and lymph nodes of REV-ERBα-deficient mice compared with control mice (Figures I-1C and D). The IFN-γ-producing counterpart was unaffected by REV-ERBα deficiency. Notably, a low but substantial production of IL-17 was observed in REV-ERBα-deficient mice even under unstimulated conditions (Figures I-1C and D). These data suggested that REV-ERBα negatively regulates IL-17 production by γδT cells.

Synthetic REV-ERB agonist suppresses γδT17 cells in *vitro* and in *vivo*

Given the increase in γδT17 cells in REV-ERBα-deficient mice, whether activating REV-ERBα could inhibit IL-17 production by γδT cells were examined in the next step. SR9009 is a chemical compound that activates REV-ERBs and has been reported to repress IL-17 production by Th17 cells (Amir et al., 2018; Chang et al., 2019). Thus, splenocytes from normal mice were cultured with SR9009 for 24 h, and the cells were stimulated with PMA and INM in the last 3 h to evaluate cytokine production by intracellular staining. The frequency of IL-17-producing γδT cells was significantly reduced after SR9009 treatment, but this was not the case for IFN-γ-producing γδT cells (Figures I-2A and B). SR9009 treatment also resulted in diminished fluorescence intensity derived from IL-17 within IL-17-producing γδT cells, whereas the intensity of IFN-γ staining within IFN-γ-producing γδT cells was increased (Figures I-2C and D). Quantitative reverse transcription PCR analysis of sorted γδT cells showed that

transcription of *Il17a* and *Il17f* was suppressed by SR9009 treatment (Figure I-2E). This was not the case with *Ifng* and *Il22* which are known to be regulated differently from *IL-17* genes in helper T cells (Figure I-2E) (Schulz et al., 2009; Veldhoven et al., 2008).

It has been suggested that IL-17 production by γδT17 cells is crucial for developing IMQ-induced psoriasis-like disease in mice (Cai et al., 2011, 2012). Significantly, the contribution of γδT17 cells in the early stages of the disease has been highlighted (Han et al., 2019). Here, to examine whether SR9009 had suppressive effects on γδT17 cells *in vivo*, IMQ cream was applied on the dorsal skin of mice for two consecutive days. Meanwhile, the mice were intraperitoneally injected with SR9009. The lymphocytes from skin-draining lymph nodes were stimulated with PMA and INM, and cytokine production was analyzed in γδT and $CD4^+$ αβT cells. Production of IFN-γ and IL-17 by γδT cells was detected and, as expected, IL-17 production was significantly suppressed in the SR9009-injected group compared with the vehicle-injected control cohort (Figures I-3A and B, left panels). There was no significant effect on IFN-γ production by γδT cells. Alternatively, cytokine-producing cells were minor in $CD4^+$ αβT cells at the early stages of IMQ-induced disease (Figures I-3A and B, right panels). γδT17 cells were enriched more than Th17 cells in the draining lymph nodes (Figure I-3C) and these T cell subsets produced similar amounts of IL-17 at the single cell level (Figure I-3D). The dermal γδT cells obtained from the IMQ-treated skin produced IL-17 without stimulation by PMA and INM, and the inhibitory effect of SR9009 injection was evident in these dermal γδT17 cells (Figures I-3E and F). These data indicated that SR9009 can suppress the activity of γδT17 cells in the early stages of IMQ-induced dermatitis.

Topical REV-ERB agonist treatment alleviates IMQ-induced psoriatic symptoms

Next, the therapeutic efficacy of SR9009 in IMQ-induced psoriasiform dermatitis was examined. Because REV-ERBs are associated with various biological processes, including the circadian rhythm, long-term systemic application may cause unexpected side effects. Therefore, the effectiveness of topical SR9009 treatment was tested,

assuming the potential for application in human patients with psoriasis. The IMQ cream and SR9009 were applied to the dorsal skin of the mice for five consecutive days. One day after the final treatment, the mice applied with IMQ and a control vehicle exhibited coarse scales over the entire area of treated skin (Figure I-4A). Skin condition was remarkably improved in mice applied with SR9009 and IMQ. Dorsal skin swelling was calculated based on skin thickness before treatment. IMQ-induced skin swelling became evident by day 3 and worsened afterward in both SR9009- and vehicle-treated groups. However, it was significantly improved on days 4 and 5 in the SR9009-treated group compared with the vehicle-treated cohort (Figure I-4B). In mice applied with Vaseline as the control for IMQ, skin swelling was absent after either SR9009 or vehicle treatment. The severity of psoriatic symptoms was assessed by scoring erythema, scales, and skin thickness. Although SR9009 did not affect erythema scores, scales, thickness, and the cumulative scores of all three symptoms of IMQ-induced dermatitis were significantly diminished by SR9009 treatment (Figure I-4C). These data indicated that topical SR9009 application to the skin can improve typical psoriatic symptoms induced by IMQ.

Topical REV-ERB agonist treatment reduces IMQ-induced skin inflammation

To further evaluate the inhibitory effect of topical SR9009 application on skin inflammation induced by IMQ, histopathological analysis was performed on murine dorsal skin samples. Consistent with the clinical scores presented in Figure I-4C, skin hypertrophy with intense inflammatory cell infiltration was evident in mice treated with IMQ and the vehicle (Figure I-5A). Treatment with SR9009 remarkably improved skin hypertrophy and cellular infiltration induced by IMQ.

The number of epidermal cell layers and the thicknesses of the epidermis and dermis in IMQ-treated mice were significantly reduced by topical application of SR9009 (Figures I-5B and C). Other abnormalities found in IMQ-treated mice included an obscure border between the dermis and subcutaneous tissues, and scarce and irregular

collagen fibers were improved by SR9009 administration (Figure I-5D). Similarly, infiltration of $Gr1^+$ granulocytes in the dermis became significantly milder (Figures I-5E and F). Thus, morphological changes to the skin and inflammatory cell infiltration were improved by topical application of SR9009 in IMQ-induced dermatitis.

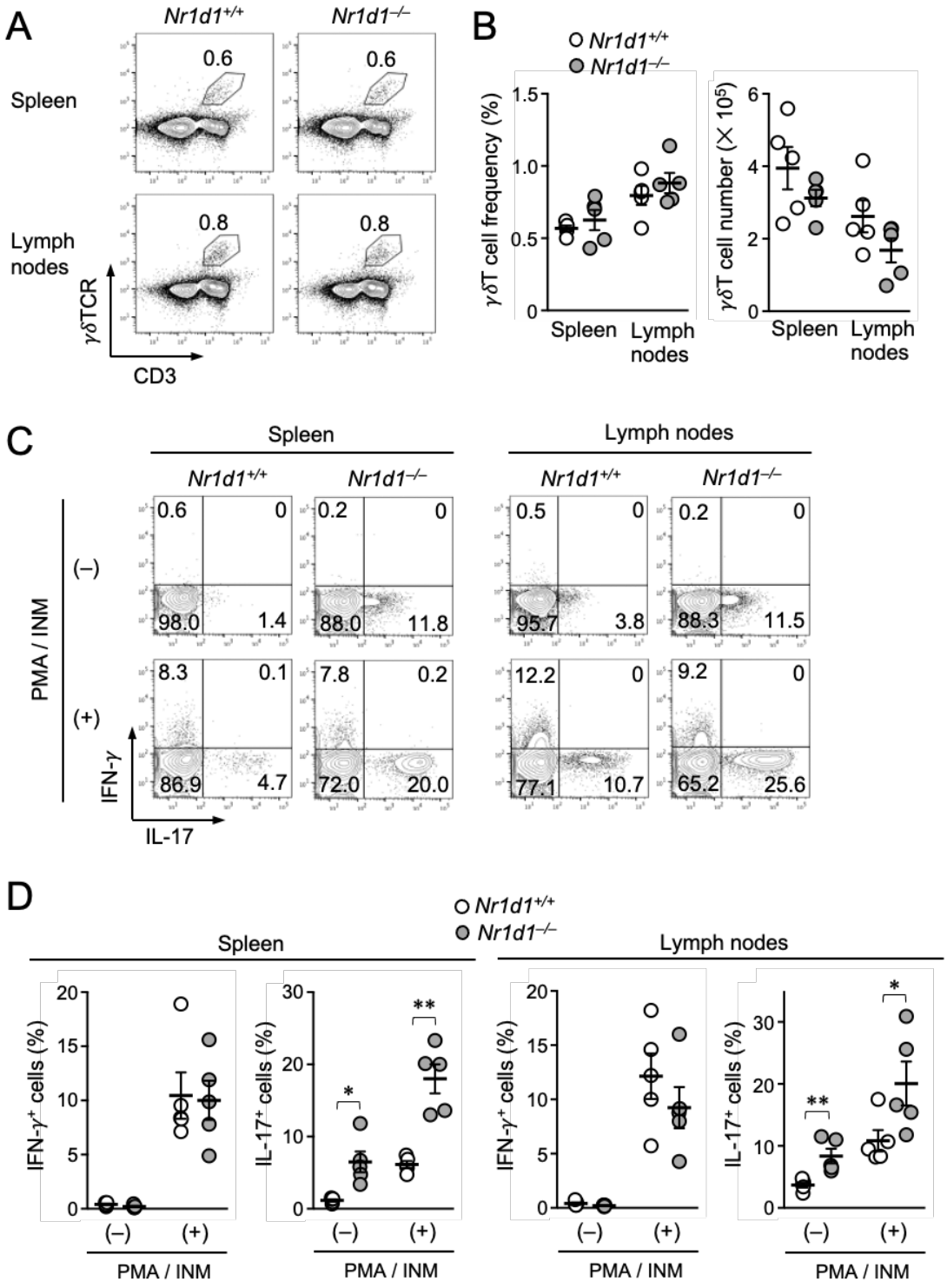

Figure I-1. γδT17 cells are decreased in REV-ERBα-deficient mice. Flow cytometry analysis of γδT cells in the spleen and lymph nodes of *Nr1d1*$^{+/+}$ and *Nr1d1*$^{-/-}$ mice.

(A) Representative flow cytometry profiles indicating the frequency of γδT cells identified by the expression of CD3 and γδTCR. (B) The graphs show the frequencies and absolute numbers of γδT cells. (C, D) The spleen and lymph node cells were stimulated or not with PMA and INM for 3 h in the presence of a protein transport inhibitor. The production of IL-17 and IFN-γ was analyzed by intracellular staining. (C) Representative flow cytometry profiles of γδT cells after culture. (D) The graphs show the frequencies of IFN-γ^{+}IL-17^{-} (IFN-γ^{+}) and IFN-γ^{-} IL-17^{+} (IL-17^{+}) γδT cells. Representative (A, C) and cumulative (B, D) results from three independent analyses (n = 5 per group). Error bars, mean ± SD. *$P < 0.05$, **$P < 0.01$ (unpaired Student's *t*-test).

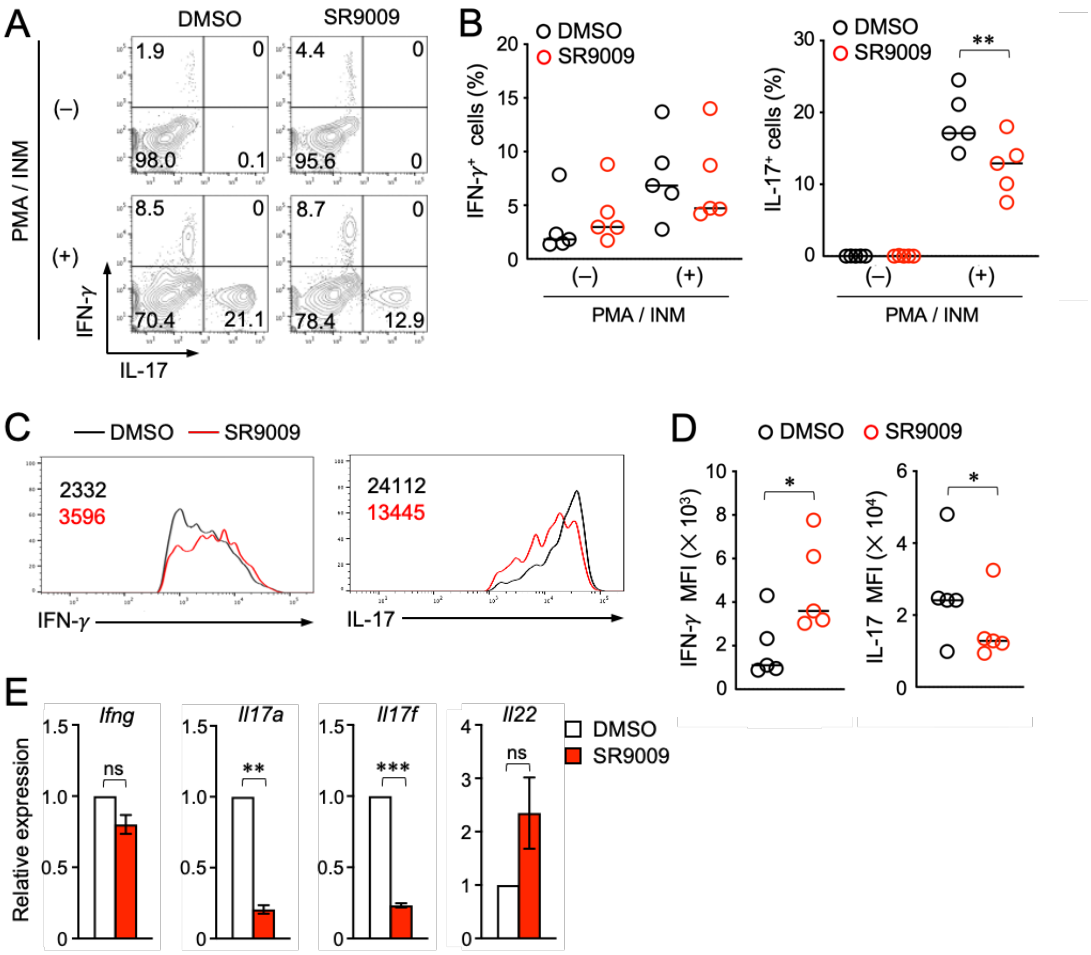

Figure I-2. Effects of a REV-ERB agonist on γδT cells i*n vitro*. Spleen cells from normal C57BL/6 mice were cultured with SR9009 or a solvent (DMSO) for 24 h. In the last 3 h of culture, the cells were stimulated or not with PMA and INM in the presence of a protein transport inhibitor. (A–D) The production of IL-17 and IFN-γ was analyzed by intracellular staining (n = 5 per condition). (A) Representative flow cytometry profile of γδT cells after culture. (B) The graphs show the frequencies of IFN-γ^{+}IL-17^{-} (IFN-γ^{+}) and IFN-γ^{-} IL-17^{+} (IL-17^{+}) γδT cells. (C, D) The expression levels of IFN-γ and IL-17 in IFN-γ^{+}IL- 17^{-} and IFN-γ^{-} IL-17^{+} gates were compared between DMSO- and SR9009-treated γδT cells. (C) Representative histograms. The numbers in the histograms indicate the mean fluorescence intensity (MFI) (black, DMSO-treated cells; red, SR9009-treated cells). (D) The graphs show MFI comparison between the two groups. (E) Spleen cells cultured in the presence or

absence of SR9009 were stimulated with PMA and INM, and γδT cells were sorted. The mRNA expression of indicated cytokine genes was analyzed by qPCR (n = 3 per condition). Representative (A, C) and cumulative (B, D) results of five independent culture experiments. Cumulative results of three independent experiments (E). Error bars, mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (paired Student's *t*-test). ns, not significant. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

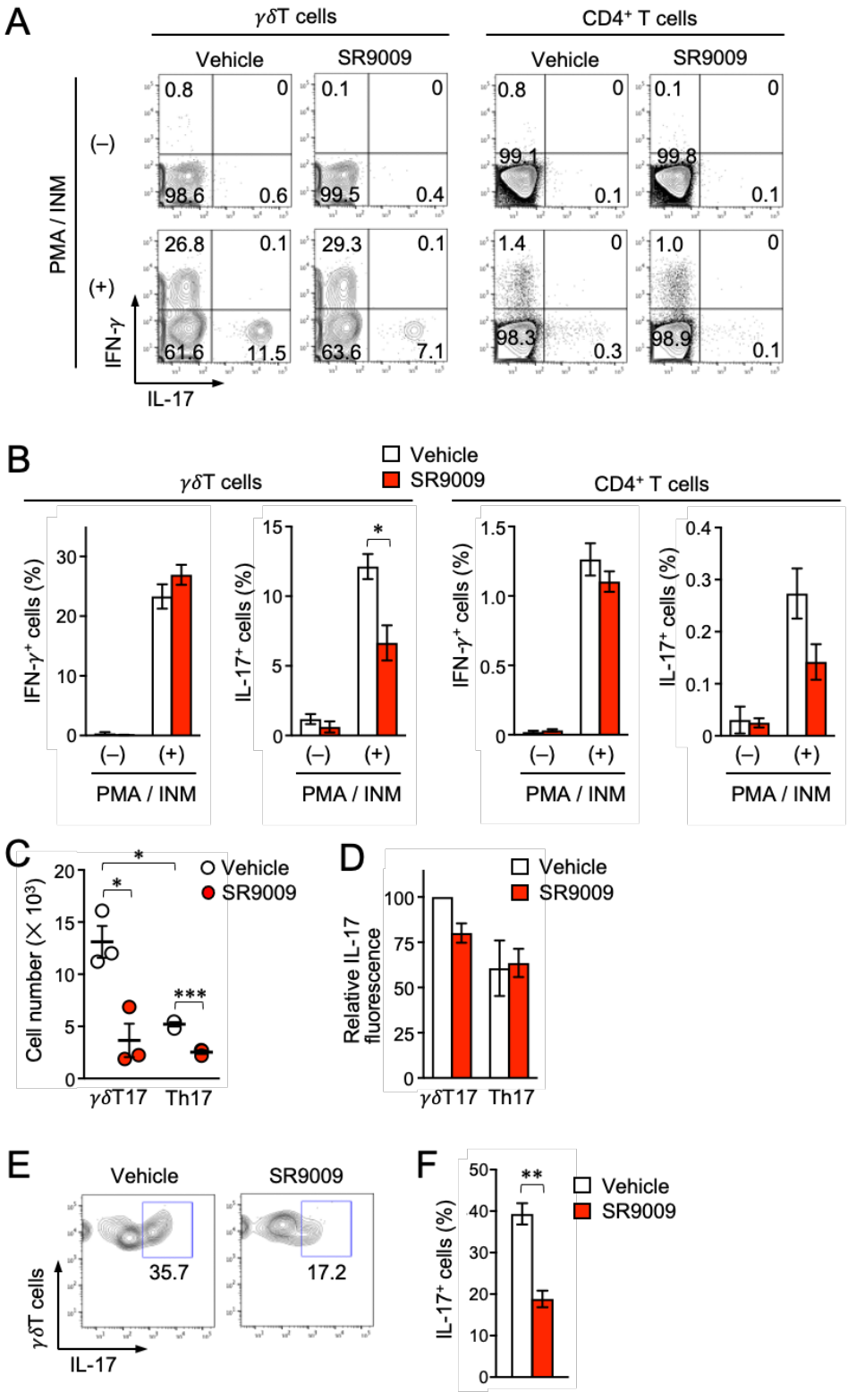

Figure I-3. Effects of a REV-ERB agonist on γδT17 cells *in vivo*. C57BL/6 mice were treated on their shaved dorsal skin with IMQ cream for two consecutive days (0

and 1). Meanwhile, the mice were intraperitoneally injected with SR9009 twice per day. One day after the last treatment (day 2), the skin-draining lymph nodes and the dorsal skin were harvested. (A–D) Lymphocytes isolated from the skin-draining lymph nodes were stimulated or not with PMA and INM in the presence of a protein transport inhibitor. (A, B) Production of IFN-γ and IL-17 in γδT cells ($CD3^+\gamma\delta TCR^+$) and $CD4^+$ T cells ($TCR\beta^+CD4^+$) was analyzed by intracellular staining. (C) Absolute numbers of γδT17 and Th17 cells in the draining lymph nodes were calculated. (D) Expression levels of IL-17 were compared by normalizing the mean fluorescence intensity of the indicated population to that of γδT17 cells from the vehicle-injected control mouse (defined as 100) in each experiment. (E, F) γδT cells ($CD45.2^+CD3^+\gamma\delta TCR^+$) isolated from the dorsal skin were analyzed for IL-17 production without stimulation. Representative (A, E) and cumulative (B–D, F) results of three independent experiments (n = 3 for each treatment group). Error bars, mean ± SD. *$P < 0.05$, **$P < 0.01$, **$P < 0.001$ (unpaired Student's *t*- test).

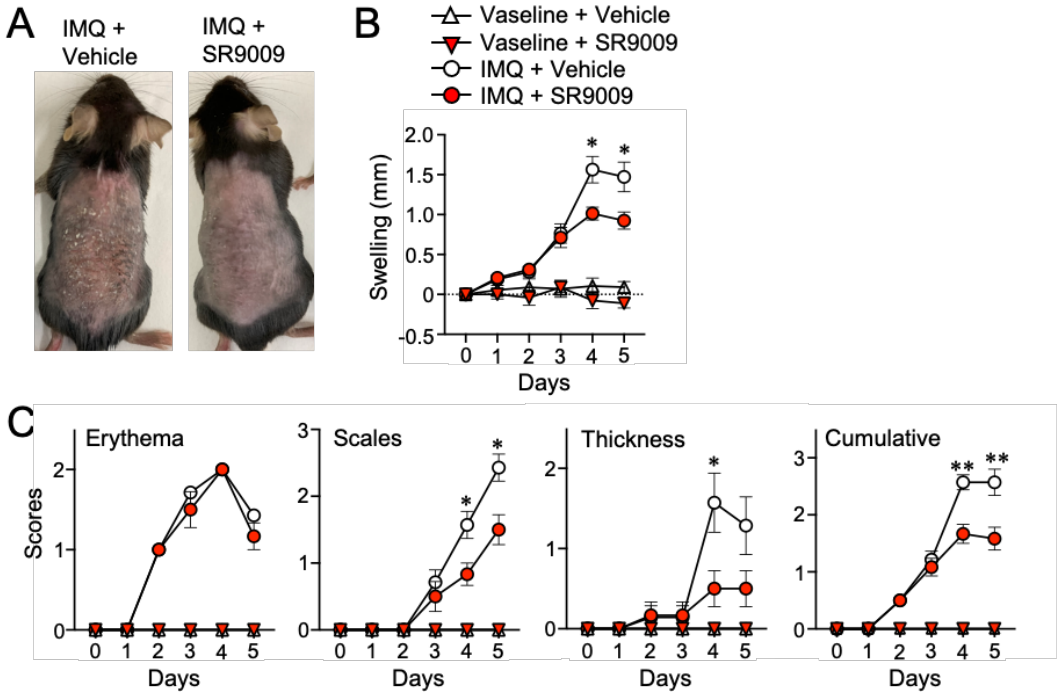

Figure I-4. Topical treatment with a REV-ERB agonist attenuates the clinical manifestations of psoriasiform dermatitis. The dorsal skin of C57BL/6 mice was smeared with IMQ cream or control Vaseline for five consecutive days (from days 0–4). Meanwhile, SR9009 or vehicle was topically applied twice daily. (A) Representative images on day 5 (one day after the last treatment) illustrate the effects of SR9009 on IMQ-treated mouse skin. (B) The graph shows dorsal skin swelling calculated as changes in skin thickness from day 0. Asterisks indicate the statistical significance of comparing the "IMQ + vehicle" and "IMQ + SR9009″ groups. (C) Clinical scores for disease severity were determined as described in Materials and Methods. Representative (A) and cumulative (B, C) data of two independent experiments (Vaseline + vehicle, n = 3; Vaseline + SR9009, n = 3; IMQ + vehicle, n = 7; IMQ + SR9009, n = 6). Error bars, mean ± SD. *$P < 0.05$, **$P < 0.01$ (unpaired Student's *t*-test).

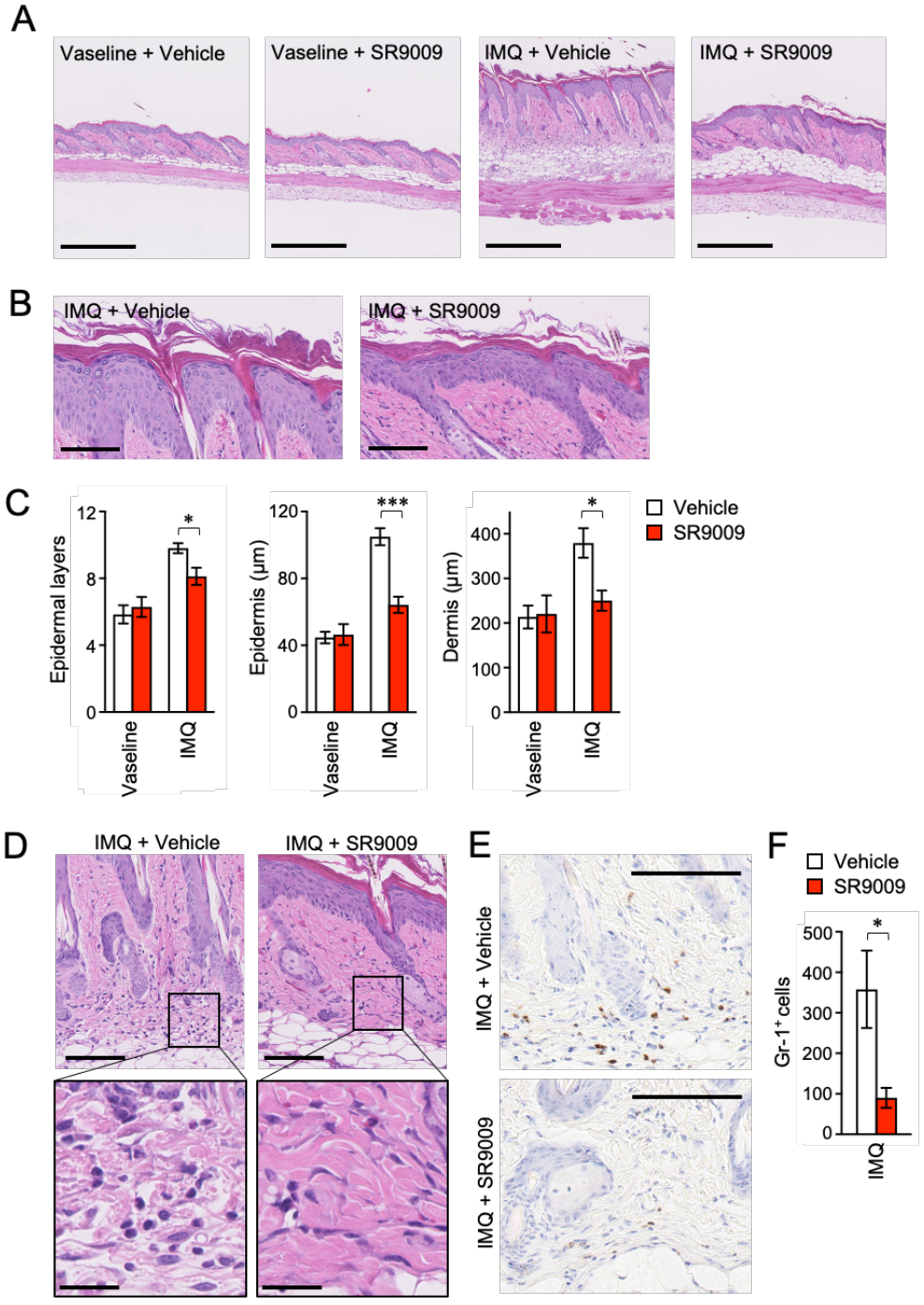

Figure I-5. Topical REV-ERB agonist treatment attenuates cellular responses with dermal tissue inflammation. The dorsal skin of C57BL/6 mice was treated with IMQ cream or control Vaseline for four consecutive days (from day 0 to day 3). Meanwhile, SR9009 or vehicle was topically applied twice daily. Dorsal skin samples were obtained on day 4. (A, B, D) Representative view of histological analyses after H&E-staining.

(C) The epidermal layers of keratinocytes (left graph), epidermal thickness (middle), and dermal thickness (right) were measured as indicated in Materials and Methods. (E) Immunohistochemical staining for Gr1 in the dermis of mice treated with IMQ + vehicle or IMQ + SR9009. (F) Numbers of infiltrating $Gr1^+$ cells in the dermis were counted as indicated in Materials and Methods. Representative (A, B, D, E) and cumulative (C, F) results from two independent experiments (Vaseline + vehicle, n = 3; Vaseline + SR9009, n = 3; IMQ + vehicle, n = 6; IMQ + SR9009, n = 5). Error bars, mean ± SD. *$P < 0.05$, ***$P < 0.001$ (unpaired Student's *t*-test). Scale bars represent (A) 500 μm, (B, D upper, and E) 100 μm, and (D lower) 25 μm.

Discussion

This study revealed that IL-17 production by γδT cells was enhanced in the absence of REV-ERBα and, in contrast, suppressed by REV-ERB agonist SR9009 treatment. REV-ERBs contain α and β isoforms partially sharing target genes as transcriptional regulators, and SR9009 acts on REV-ERBα and REV-ERBβ. Although the results demonstrated the involvement of REV-ERBα in the regulation of γδT17 cells, it remains unclear whether REV-ERBβ similarly contributes to regulating γδT17 cells. REV-ERBβ-deficient mice can be examined, yet leave the possibility that functional redundancy involving REV-ERBα and REV-ERBβ may compensate for the possible effects of REV-ERBβ deficiency on γδT17 cells. Likewise, it is difficult to clarify whether the effect of SR9009 on γδT17 cells is mediated through REV-ERBα or REV-ERBβ. Because the conventional double-knockout of REV-ERBα and REV-ERBβ results in embryonic lethality (Ikeda et al., 2019), the contribution of REV-ERBβ and the cooperation of REV-ERBα and REV-ERBβ need to be ultimately assessed in conditional knockout mice where only γδT cells lack REV-ERBs.

The results demonstrated that SR9009 has inhibitory effects on γδT17 cells, but not on IFN-γ-producing γδT cells, both in vitro and in vivo. Notably, in the IMQ-induced dermatitis model, γδT17 cells were enriched more than Th17 cells in the draining lymph nodes and were affected by SR9009 injection even before overt symptoms, such as skin scaling and swelling, appeared. These observations are in agreement with a previous report suggesting that γδT17 cells play a primary role in the establishment of IMQ-induced psoriasiform dermatitis because the disease is remarkably mild in γδT cell-deficient mice but not in αβT cell-deficient mice (Cai et al., 2011). Differentiation of Th17 cells requires antigen stimulation and subsequent differentiation into effector cells to gain the capacity to produce specific cytokines. In contrast, γδT17 cells rapidly produce IL-17 in response to inflammatory cues, such as IL-23, and constitute the early immune response in inflammatory diseases. Although the results demonstrated the repression of γδT17 cell function by SR9009 injection in the early phase of IMQ-

induced dermatitis, it remains unclear whether the therapeutic effect of topical SR9009 treatment involves Th17 cells. Further investigations using mice deficient in γδT or αβT cells are required.

γδT17 cells are involved in the pathogenesis of various inflammatory and autoimmune diseases in humans and mice, including multiple sclerosis, experimental autoimmune encephalomyelitis (EAE, a murine model of multiple sclerosis), rheumatoid arthritis (RA), and inflammatory bowel disease (Blink et al., 2014; Cai et al., 2011; Do et al., 2011; Paul, 2015; Roark et al., 2007; Sutton et al., 2009). The crucial roles of $V\gamma4^+$ γδT17 cells have been shown in the disease development of EAE, RA, and psoriasis (Blink et al., 2014; Paul, 2015; Roark et al., 2007). Therefore, REV-ERB agonists can similarly improve the symptoms of these autoimmune diseases by manipulating γδT17 cell activity. The effectiveness of REV-ERB agonists in treating EAE has been demonstrated (Amir et al., 2018; Chang et al., 2019). Although this therapeutic effect is currently understood in terms of inhibitory effects on Th17 cells (Amir et al., 2018; Chang et al., 2019), it is possible that γδT17 cells are also involved. It was recently reported that developing EAE depends on IL-17 produced by $V\gamma4^+$ γδT17 cells in the early stages of disease induction (McGinley et al., 2020). The present study showed that the REV-ERB agonist repressed cytokine production by γδT17 cells in the early stages of IMQ-induced dermatitis before clinical symptoms appeared. In many inflammatory or autoimmune diseases, relapsing-remitting cycles are repeated after disease onset, which limits patient quality of life. REV-ERB agonist treatment targeting γδT17 cells during pre-symptomatic periods may improve control of these diseases.

In the experiments with IMQ-induced dermatitis, a REV-ERB agonist was effective even by topical dermal application. Because REV-ERBs are systematically expressed and exert tissue-specific functions (Wang et al., 2020), topical administration of REV-ERB agonists may reduce the risk of unexpected side effects. Currently, the topical medications available for psoriasis treatment include corticosteroids, non-specific immunosuppressants, and active vitamin D, which acts on keratinocytes and suppresses

abnormal cell proliferation and keratosis (Armstrong & Read, 2020). Alternatively, current therapy targeting IL-17-mediated immune responses by anti-IL-17A and anti-IL-17 receptor antibodies can disturb systemic immunocompetence, increasing susceptibility to opportunistic infections, such as candidiasis (Lønnberg et al., 2014; Saunte et al., 2017). The topical application of REV-ERB agonists may be an additional therapeutic option that avoids severe systemic side effects and exclusively targets the pathogenic cytokine. Adjuvant therapeutic effects by combining REV-ERB agonist treatment with conventional therapies, including anti-IL-17 antibodies, should also be tested in the future.

Summary

It had been unclear whether REV-ERBs regulate γδT17 cells. In this study, REV-ERB-mediated control of γδT17 cell activity was demonstrated by the findings that REV-ERBα deficiency augmented IL-17 production by γδT cells and the pharmacological activation of REV-ERBs by a synthetic agonist, SR9009, suppressed the activity of γδT17 cells. This study also showed that topical application of a REV-ERB agonist to the skin relieved the clinical symptoms of IMQ-induced psoriasiform dermatitis in mice. Thus, this study proposes a novel therapeutic approach for psoriasis by targeting REV-ERBs in γδT17 cells.

Chapter II

Roles of tumor necrosis factor-like ligand 1A in γδT-cell activation and psoriasis pathogenesis

Introduction

γδT cells bridge innate and adaptive immune systems through their innate-like potential to rapidly secrete a large amount of cytokines under inflammation (Chien et al., 2014; Ribot et al., 2009). γδT cells are particularly abundant in barrier tissues, such as mucosa and skin, and predominate in the early stage of immune responses against microbial infections (Chien et al., 2014). γδT cells are largely segregated into interferon-γ (IFN-γ) and interleukin (IL)-17 producers functionally committed during intrathymic development. These γδT-cell subsets can be distinguished by the expression or lack of markers, including CD27, CC-chemokine receptor type 6, and IL-1 receptor (IL-1R) (Akitsu & Iwakura, 2018; Cua & Tato, 2010; Haas et al., 2009; Ribot et al., 2009). IL-17-producing γδT (γδT17) cells play crucial roles in the body's protection against fungi and bacteria, but they are also involved in the pathogenesis of inflammatory and autoimmune diseases as a major source of IL-17 and IL-22 in inflamed tissues (Cua & Tato, 2010). It is generally known that inflammatory cytokines IL-23 and IL-1β synergistically induce IL-17 and IL-22 production by γδT17 cells without T-cell receptor (TCR) stimulation (Akitsu & Iwakura, 2018). In addition to IL-23 and IL-1β, other environmental signals that can activate γδT17 cells have been reported (Lalor et al., 2011; Martin et al., 2009). How γδT17 cells sense inflammation to elicit innate function has not been fully elucidated.

Psoriasis is a chronic inflammatory skin disease grossly characterized by inflamed plaques with adherent silvery scales (Michael & Boehncke, 2005). The skin lesions show hyperproliferation and aberrant differentiation of keratinocytes and large neutrophil infiltration. IL-17-producing helper T (Th17) cells in psoriasis pathogenesis have been intensely studied, suggesting a model in which IL-23 derived from dendritic cells and macrophages activates the cytokine secretion of Th17 cells and that Th17-derived IL-17 and IL-22 provoke neutrophil recruitment and epidermal keratinocyte proliferation in the skin (Eberle et al., 2016; Nograles et al., 2008). In contrast, there is growing evidence that γδT cells contribute to psoriasis development as a primary source

of IL-17 and IL-22 in skin lesions (Cai et al., 2011; Mabuchi et al., 2013; Qi et al., 2021; Sandrock et al., 2018). Deficiency of γδT, but not αβT, cells attenuates IL-17 production and relieves psoriasiform dermatitis in mice intradermally injected with IL-23 (Cai et al., 2011). Massive infiltration of γδT17 cells has also been found in skin lesions of patients with psoriasis (Cai et al., 2011).

Tumor necrosis factor (TNF)-like ligand 1A (TL1A) and its receptor, death receptor 3 (DR3), belong to the TNF/TNF receptor superfamily (Valatas et al., 2019). TL1A is initially expressed as a membrane-bound form, and its extracellular domain is released as a soluble protein through cleavage by metalloproteinases (Migone et al., 2002; Zhan et al., 2009). TL1A expression is upregulated by stimulation of Fcγ receptors and Toll-like receptors in dendritic cells and macrophages known as major sources of TL1A under inflammatory conditions (Meylan et al., 2008; Prehn et al., 2007; Siakavellas & Bamias, 2015). DR3 is expressed in various immune cells, including activated T cells, natural killer cells, and innate lymphoid cells (Valatas et al., 2019), and TL1A-DR3 interaction triggers proinflammatory responses through nuclear factor-kB activation and mitogen-activated protein kinase (Castellanos et al., 2018; Migone et al., 2002; Siakavellas & Bamias, 2015). Genetic studies have revealed the association of TL1A gene (Tnfsf15) polymorphisms with psoriasis (Dand et al., 2017; Képíró et al., 2014). Elevated TL1A levels in the serum and increased TL1A and DR3 expression in skin lesions have been reported in psoriasis patients (Bamias et al., 2011; Li et al., 2014). Li et al. recently reported that anti-TL1A antibody injection alleviates psoriasis-like symptoms in mice treated with imiquimod (IMQ), indicating the involvement of TL1A in the pathogenesis of this animal model (Li et al., 2020). However, the role of the TL1A-DR3 pathway in γδT17 cells and its relevance with psoriasis pathogenesis has not been investigated.

This study reports the crucial role of TL1A in γδT17 cell activation and psoriasis pathogenesis. Anti-TL1A antibody injection inhibited cytokine production by γδT17 cells in IMQ-treated skin. DR3 was constitutively expressed in splenic and dermal γδT cells. TL1A induced cytokine production by γδT17 cells synergistically with IL-23. The

effect of TL1A was especially striking in the enhancement of IL-22 production. TL1A exacerbated the symptoms of the murine psoriasis model generated by intradermal IL-23 injection, and TL1A-dependent γδT17 cell activation was crucial in the early pathogenesis of this disease. These findings suggest a novel regulatory mechanism of γδT17 cells through TL1A and provide insights into psoriasis pathogenesis.

Materials and Methods

Mice

C57BL/6 mice were purchased from Japan SLC. TCR γ-chain (Tcrd) knockout (KO) mice (B6.129P2-Tcrdtm1Mom/J) were obtained from The Jackson Laboratory (Itohara et al., 1993). Mice were maintained under specific pathogen-free conditions in the animal facility. All animal experiments were performed under the approval of the Institutional Animal Care and Use Committee of Hokkaido University (approval number 20-0172). When data from some independent experiments were accumulated, each experiment was conducted to include all groups consisting of one or more mice per condition. In some experiments, test groups were set to contain more mice than control groups, assuming that the drug treatment produces larger variation among individuals.

Preparation of single-cell suspension from ear

To prepare single-cell suspensions, spleens were ground on a metal mesh by a syringe plunger and filtrated through nylon mesh. Cells isolated from ear skin based on methods in previous reports with slight modifications (Lou et al., 2020). Ear samples were treated with enzymes as follows before the mechanical digestion. Ear pinnae were cut into small pieces and incubated at 37°C for 1 h with 250 mg/mL Liberase TL (Roche Diagnostics) and 1 mg/mL DNase I (Roche Diagnostics) in RPMI 1640 containing 5% fetal bovine serum (Sigma-Aldrich).

Flow cytometry and cell sorting

The single-cell suspensions were incubated with antimouse CD16/CD32 (Biolegend) for the blockade of Fc receptors and stained with fluorochrome-labeled monoclonal antibodies (all from Biolegend) against cell surface proteins for 30 min. Intracellular cytokine staining was performed using a fixation and permeabilization buffer set (Thermo Fisher Scientific) according to the manufacturer's directions. Data were obtained using FACSVerse, LSRFortessa, or FACSAria II flow cytometer (BD Biosciences) and analyzed using FlowJo version 10.3 (TreeStar). To isolate γδT cells, cells were incubated with fluorescein isothiocyanate (FITC)-labeled anti-B220, anti-TCRb, anti-CD11c, anti-CD11b, and anti-NK1.1 antibodies (all from Biolegend) and enriched by depleting antibody-bound cells using anti-FITC-conjugated magnetic beads and columns (Miltenyi Biotec). Cells were stained with anti-CD3e and anti-CD27 antibodies (Biolegend), and $CD27^+$ and $CD27^-$ fractions in $FITC^-CD3e^+$ cells were sorted using a FACSAria II (BD Biosciences).

Cell culture

To induce cytokine production, cells were cultured with 20 ng/mL IL-23, IL-1β, and TL1A (all from Biolegend) alone or in different combinations for 24 h. A protein transport inhibitor (Thermo Fisher Scientific) was added during the last 4 h to analyze intracellular cytokines.

Quantitative polymerase chain reaction analysis

Total RNA was extracted from whole ear samples or specific subsets of splenic γδT cells using the RNeasy Plus Micro kit (Qiagen) and reverse transcribed using the PrimeScript RT master mix (Takara Bio). Real-time PCR was performed using TB Green Premix Ex Taq II (Takara Bio) on a LightCycler 96 System (Roche Diagnostics). The primer sequences are listed in Table II-1. Relative mRNA expression was analyzed using the $\Delta\Delta$Ct method and normalized to glyceraldehyde 3- phosphate dehydrogenase.

IMQ treatment

Each ear was applied daily with 10 mg 5% IMQ cream (Mochida Pharmaceuticals) or control Vaseline cream (PROPETO), defining the initial treatment day as day 0. Where indicated, mice were intraperitoneally injected once daily with 20 mg/kg anti-TL1A monoclonal antibodies (clone 5G4.6; Bio X Cell) from day −1 until a day before analysis (Meylan et al., 2011). Single-cell suspensions were prepared from ears on day 3 for intracellular cytokine staining. Ear thickness was measured daily at two specific sites using a micrometer caliper (Mitutoyo) and averaged. Swelling was calculated as changes in the thickness between day 0 and measurement time. Ear pinnae were obtained for histopathological analysis on day 4.

Intradermal cytokine injection

Either side of mouse ears was injected intradermally with 0.5 mg recombinant mouse IL-23 (Biolegend) alone or combined with 0.5 mg recombinant mouse TL1A (Biolegend) in 20 ml volume (Rizzo et al., 2011). To analyze IL-17 and IL-22 production, single-cell suspensions were prepared from ears 22 h after cytokine injection. To induce psoriasis-like dermatitis, cytokine injection was repeated daily for 4 days, defining the initial treatment day as day 0. Ear thickness was monitored as described above. Ear pinnae were obtained for histological analysis or qPCR at the indicated time points.

Histological analysis

Ear samples were fixed with 10% neutral buffered formalin. Paraffin-embedded tissues were sectioned and stained with hematoxylin and eosin (H&E). The sections were scanned using a NanoZoomer 2.0-RS virtual slide scanner (Hamamatsu Photonics). Dermis and epidermis thicknesses were measured as described elsewhere with minor modifications (Shao et al., 2019; Shangyi Wang et al., 2021). The length from the stratum corneum to the basal stratum of the interfollicular epidermis and the

length from right under the interfollicular epidermis to the top of the cartilage layer was measured as the epidermis and dermis thicknesses, respectively, using ImageJ software. The dermis and epidermis thicknesses and the number of epidermal layers were measured at eight randomly chosen points in two 600 × 400 mm fields and averaged. For immunohistochemical analysis, deparaffinized sections were treated with 0.3% hydrogen peroxide-methanol. After antigen retrieval, the sections were incubated with blocking 10% goat serum and antimouse cytokeratin-5 (rabbit polyclonal; Biolegend), antimouse proliferating cell nuclear antigen (PCNA) (clone PC10; Calbiochem), or antimouse Gr1 (clone RB6-8C5; R&D Systems) primary antibodies. The sections were incubated with biotinylated secondary antibodies and streptavidin-conjugated horseradish peroxidase (Nichirei). The sections were finally reacted with 0.01% 3,3′-diaminobenzidine. $PCNA^+$ and $Gr1^+$ cells were counted manually in two randomly chosen 200-mm-long epidermal and dermal areas, respectively, and averaged for statistical evaluation.

Statistical analysis

All data were assessed for normality using the Shapiro-Wilk test. Statistical significance was evaluated using the two-tailed unpaired or paired Student′s *t*-test. Mann-Whitney U test, or Wilcoxon signed-rank test based on the result of normality test. Analysis was performed with Prism 9.0 (GraphPad Software) and Excel (Microsoft). In all figures, *p*-values less than 0.05, 0.01, and 0.001 are shown as *, **, and ***, respectively. The abbreviation “ns” denotes “not significant” in the graphs.

Table II-1. Sequences of primers used in qRT-PCR analysis

Primers	Sequences
Gapdh F	5’-TTGTCAGCAATGCATCCTGCAC-3’
Gapdh R	5’-GAAGGCCATGCCAGTGAGCTTC-3’
Rorc F	5’-TGCAAGACTCATCGACAAGG-3’
Rorc R	5’-AGGGGATTCAACATCAGTGC-3’
Tnfrsf25 F	5’-CATGTCTGGCAGGTGTGACT-3’
Tnfrsf25 R	5’-ACTTTGCCGAGCAGTTCTCA-3’
Tbx21 F	5’-GTCCAAGTTCAACCAGCACC-3’
Tbx21 R	5’-GTTGGTGAGCTTTAGCTTCC-3’
Il1r1 F	5’-TGTGGCTGAAGAGCACAGAG-3’
Il1r1 R	5’-CGATCGTCTCATTCCGAGGG-3’
Il23r F	5’-GGCAACATGACATGCACCTG-3’
Il23r R	5’-AGCCCTGGAAATGATGGACG-3’
S100A7 F	5’-CCTCGCTTCATGGACACCTT-3’
S100A7 R	5’-TTCACCAGCTTGCCCAAGAT-3’
S100A8 F	5’-AGGAAATCACCATGCCCTCT-3’
S100A8 R	5’-ATCACCATCGCAAGGAACTC-3’
S100A9 F	5’-TCATCGACACCTTCCATCAA-3’
S100A9 R	5’-TTTGTGTCCAGGTCCTCCAT-3’
Ccl2 F	5’-CCCAATGAGTAGGCTGGAGA-3’
Ccl2 R	5’-TCTGGACCCATTCCTTCTTG-3’
Ccl20 F	5’-CTGCTCTTCCTTGCTTTGGC-3’
Ccl20 R	5’-TGGATCAGCGCACACAGATT-3’

Results

Involvement of TL1A in γδT-cell activation in IMQ-treated skin

Topical application of IMQ, a ligand of Toll-like receptor 7 (TLR7) and TLR8, to the skin, induces psoriasis-like manifestations in mice (Van der Fits et al., 2009), where γδT cells are associated with disease development (Cai et al., 2011). IL-17- and IL-22-producing lymphocytes were identified in mouse ears after IMQ treatment for 3 consecutive days (Figures II-1A and B). Remarkably, γδT cells occupied 60%–80% of cytokine-producing lymphocytes in IMQ-treated ears (Figures II-1C and D). αβT and TCR^{-} cells appeared minor compared to γδT cells. These data indicated that γδT cells are the major producers of IL-17 and IL-22 in the skin during the early phase of the IMQ-induced psoriasis model. Neutralizing anti-TL1A antibodies were injected into IMQ-treated mice. IL-17 and IL-22 production by $CD3^{int}$γδTCR^{int} dermal γδT17 cells (Gray et al., 2011) was significantly repressed when TL1A availability was limited (Figures II-1E and F), suggesting the contribution of TL1A to dermal γδT-cell activation in this psoriasis model. Anti-TL1A injection relieved inflammatory symptoms, including skin swelling and cellular infiltrations, after IMQ treatment for 4 consecutive days (Figures II-1G and H). The number of epidermal cell layers and the epidermis and dermis thicknesses in anti-TL1A-injected mice were also significantly reduced (Figure II-1I). Notably, the effect of anti-TL1A to ease ear swelling was evident even at the early time point after IMQ treatment for 2 consecutive days when IL-17 and IL-22 are mainly produced by γδT cells (Figures II-1C, D, and G). These data suggested the involvement of TL1A in dermal γδT-cell activation and early disease development in IMQ-induced dermatitis.

TL1A activates γδT17 cells in synergy with IL-23

Expression of DR3, a TL1A receptor, in different γδT-cell subsets, was examined using spleen cells from C57BL/6 mice. Splenic γδT cells had four different subsets according to CD27 and Vγ4 expression (Figure II-2A). $CD27^+$ γδT cells are mostly IFN-γ producers, and IL-17 production is limited to $CD27^-$ γδT cells (Qi et al., 2021). γδT17 cells include $V\gamma6^+$ and $V\gamma4^+$ cells that can roughly correspond to natural and inducible γδT cells, respectively (Akitsu & Iwakura, 2018), and $V\gamma4^+$ γδT17 cells are mainly associated with psoriasis development (Cai et al., 2014). DR3 expression was apparent in most $CD27^-$ γδT cells regardless of Vγ4 expression, whereas only a small fraction of $CD27^+$ γδT cells weakly expressed DR3. Type I IL-1R was expressed exclusively in $CD27^-$ γδT cells. Weak IL-23R expression was detected in all γδT-cell subsets, although $CD27^-$ γδT cells tended to express slightly higher levels than $CD27^+$ γδT cells (Figures II-2B and C).

Expression of genes encoding these cytokine receptors was assessed in $CD27^-$ and $CD27^+$ γδT cells isolated from the spleen. Tbx21 and Rorc encode transcription factors functionally characterizing IFN-γ-producing γδT and γδT17 cells, respectively (Akitsu & Iwakura, 2018). qPCR analysis verified that $CD27^-$ γδT cells express higher levels of Rorc and lower levels of Tbx21 than the $CD27^+$ counterpart (Figure II-2D). As expected, Tnfrsf25 (encoding DR3), Il1r1, and Il23r were highly expressed in $CD27^-$ γδT cells as Rorc whose expression characterizes γδT17 cells (Figure II-2D). These data suggested that DR3 is preferentially expressed in γδT17 cells in the spleen.

Cytokine production by γδT cells was examined in vitro by culturing spleen cells with different combinations of IL-23, IL-1β, and TL1A. Stimulation with IL-23 alone induced substantial IL-17 and IL-22 production by $CD27^-$ γδT cells but not $CD27^+$ γδT cells. Although the response to either IL-1β or TL1A alone was poor, they enhanced cytokine production when mixed with IL-23 (Figures II-2E and F). Regarding the enhancement of IL-17 production, synergistic effects with IL-23 were similar between IL- 1β and TL1A (Figure II-2F). In contrast, TL1A was significantly stronger than IL-1β in IL-22 induction when used with IL-23 (Figure II-2F). This striking effect

of TL1A enhancing IL-22 production was evident in IL-22^{+} cell frequency (Figure II-2F) and fluorescence intensity (Figure II-2G).

Dermal γδT cells are ready to respond to TL1A

γδT cells are abundantly present in the skin and provide a barrier against microorganisms. They include γδT TCR^{hi}Vγ5^{+} dendritic epidermal T cells (DETCs) and γδTCR^{int}Vγ5^{-} γδT cells resident in the dermis (Gray et al., 2011). The latter, but not the former, γδT cells can produce IL-17 and IL-22 (Gray et al., 2011). CD3^{hi}γδTCR^{hi} DETCs and CD3^{int}γδTCR^{int} dermal γδT cells were identified in the single-cell suspension prepared from untreated mouse ear pinnae (Figure II-3A). DR3 expression was seen in CD3^{int}γδTCR^{int} dermal γδT cells but not CD3^{hi}γδTCR^{hi} DETCs (Figure II-3B). Vγ4^{+} and Vγ4^{-} dermal γδT cells expressed DR3 at similar levels. Although IL-1R and IL-23R expression in dermal γδT cells was not so clear as DR3, Vγ4^{-} dermal γδT cells expressed slightly higher IL-1R and IL-23R than DETCs (Figures II-3B and C).

Given the remarkable DR3 expression in dermal γδT cells in a steady state, cytokine production by these γδT cells upon exposure to IL-23 and TL1A *in vivo* was tested. Mouse ears were intradermally injected with IL-23 alone or combined with TL1A, and IL-17 and IL-22 production was examined. DETCs produced little IL-17 and IL-22 in any conditions. The overall IL-17 and IL-22 production by dermal γδT cells were predominant in the Vγ4^{+} population and less in the Vγ4^{-} counterpart (Figures II-3D and E). IL-17 was produced at similar levels upon injection with IL-23 alone or combined with TL1A (Figure II-3D). IL-22 production was only slightly induced in the group injected with IL-23 alone and remarkably enhanced (more than fourfold) by the combined injection with TL1A (Figure II-3E). These results suggested an essential role of the TL1A-DR3 pathway in inducing immediate IL-22 production by dermal γδT cells.

TL1A exacerbates the symptoms in the IL-23-induced murine psoriasis model

IL-23 injection into the mouse skin is a popular psoriasis model that shares various manifestations with human patients, including epidermal hyperplasia (acanthosis), parakeratosis, and cellular infiltration (Cai et al., 2011; Riol-Blanco et al., 2014; Rizzo et al., 2011; Zheng et al., 2007). Intradermal IL-23 administration into IL-22-deficient mouse ears has demonstrated the requirement of IL-22 for acanthosis and neutrophil infiltration (Zheng et al., 2007). Given that TL1A was crucial for IL-22 production by dermal γδT cells in vivo (Figure II-3E), how IL-23-induced psoriatic symptoms are affected by the combined administration with TL1A was examined. Mouse ears were injected daily with IL-23 alone or combined with TL1A for 3 consecutive days. As reported, mice injected with IL-23 alone showed overt ear swelling and increased over time (Figure II-4A). Additional TL1A injection significantly enhanced the response (Figure II-4A). Notably, this effect of TL1A appeared only a day after the initial cytokine injection (Figure II-4A), implying the involvement of immune cells, such as dermal γδT cells, ready to respond to these cytokines *in situ*.

Histological analysis revealed that TL1A massively enhances epidermal hyperplasia and cellular infiltration in this psoriasis model (Figures II-4B and C). In mice injected with IL-23 plus TL1A, epidermal hyperplasia was accompanied by increased epidermal cell layers and hypertrophy of each cell in basal and spinosum layers (Figures II-4C and D). Enlarged nucleoli indicating robust RNA synthesis was also observed in these epidermal cells (Figure II-4D). Signs of hyperkeratosis and parakeratosis (remaining nuclei in the thickened stratum corneum as indicated in Figure II-4D, arrowheads) were obvious after the combined injection with IL-23 and TL1A. These mice also showed neutrophil infiltration into the epidermal layer (Figure II-4D, closed arrows) and the collection of neutrophils in the stratum corneum (Figure II-4D, open arrow), similar to Munro microabscesses characteristic of psoriasis patients (Michael & Boehncke, 2005). Microabscesses were absent in all mice injected with phosphate-buffered saline (PBS) or IL-23 alone under the experiment condition that gives the treatment only four times (Figure II-4E). Keratin-5 expression in suprabasal

layers, a hallmark of keratinocyte hyperproliferation in psoriasis (Zhang et al., 2019), was remarkable in mice injected with IL-23 and TL1A (Figure II-4F). Similarly, $PCNA^+$ epidermal cells undergoing proliferation increased in those mice. Gr1 signals mainly existed in the reticular layer of the lower dermis in mice injected with IL-23 alone (Figure II-4F). However, in mice given IL-23 and TL1A, infiltration of $Gr1^+$ neutrophils was more intense and extended to the papillary layer of the upper dermis and even into the epidermis, resulting in microabscess formation (Figures II-4F and G).

TL1A-mediated γδT-cell activation is pivotal for the early development of psoriasis

The disease caused by intradermal IL-23 injection is significantly attenuated in mice deficient in γδT cells compared to wild-type (WT) mice (Cai et al., 2011). Based on observations that dermal γδT cells were ready to respond to TL1A (Figure II-3) and TL1A promoted IL-23-induced skin swelling only within a day (Figure II-4A), the contribution of TL1A-dependent γδT-cell activation to the early inflammation was assessed by analyzing the ear samples one day after single dose cytokine injection. IL-17-producing lymphocytes were detected at comparable levels between the groups injected with IL-23 alone and in combination with TL1A. In contrast, IL-22-producing lymphocytes were evident only in the combined injection group at this early time point (Figures II-5A and B). γδT cells occupied most IL-17- or IL-22-producing lymphocytes (Figures II-5C and D). To directly examine the involvement of γδT cells in early disease development, WT and γδT cell-deficient mice were similarly injected with IL-23 alone or combined with TL1A once, and manifestations that appeared within a day were compared. The effect of TL1A enhancing IL-23-induced skin swelling was absent in γδT cell-deficient mice (Figure II-5E). Histological analysis also showed that γδT-cell deficiency abrogated TL1A-dependent epidermal responses, including the increase of layers and thickness (Figures II-5F and G). γδT-cell deficiency did not affect dermal thickness at statistically significant levels (Figure II-5G). Finally, the effect of γδT-cell

deficiency on the expression of genes associated with psoriatic skin lesions a day after cytokine injection was examined. Expression of antimicrobial proteins, such as S100A7, S100A8, and S100A9, are induced in psoriatic keratinocytes (Boniface et al., 2005; Wolk et al., 2006). S100A8 and S100A9 mRNA expression in ear tissues was significantly elevated by the combined cytokine injection compared to IL-23 alone in WT mice, but this TL1A-dependent response was absent in γδT cell-deficient mice (Figure II-5H). Likewise, the effect of TL1A enhancing the mRNA expression of keratinocyte-derived chemokine CCL20 (Behfar et al., 2018; Tohyama et al., 2018) was γδT cell–dependent. S100A7 and CCL2 mRNA expression showed nonsignificant but similar results. These data indicated that TL1A-dependent γδT-cell activation contributes to the psoriasis development in the very early stage of the disease.

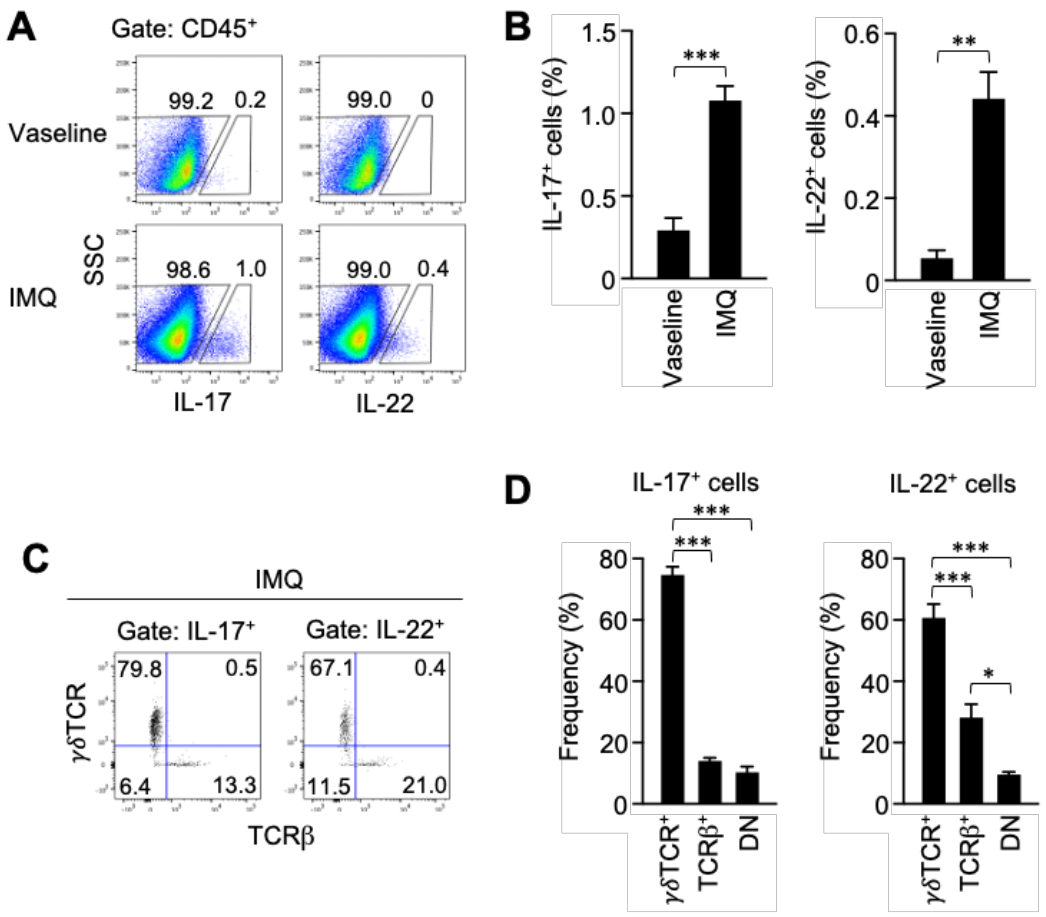

Figure II-1. Imiquimod (IMQ) treatment activates dermal γδT cells through TL1A. (A–D) Mouse ears were smeared with IMQ cream or control Vaseline for 3 consecutive days. One day after the last treatment, single-cell suspensions from ears were analyzed for cytokine production (n = 5). (A, B) Frequencies of cytokine-producing cells in the $CD45^+$ gate. (C, D) TCR usage of $IL\text{-}17^+$ and $IL\text{-}22^+$ lymphocytes in IMQ-treated mice. DN, double negative.

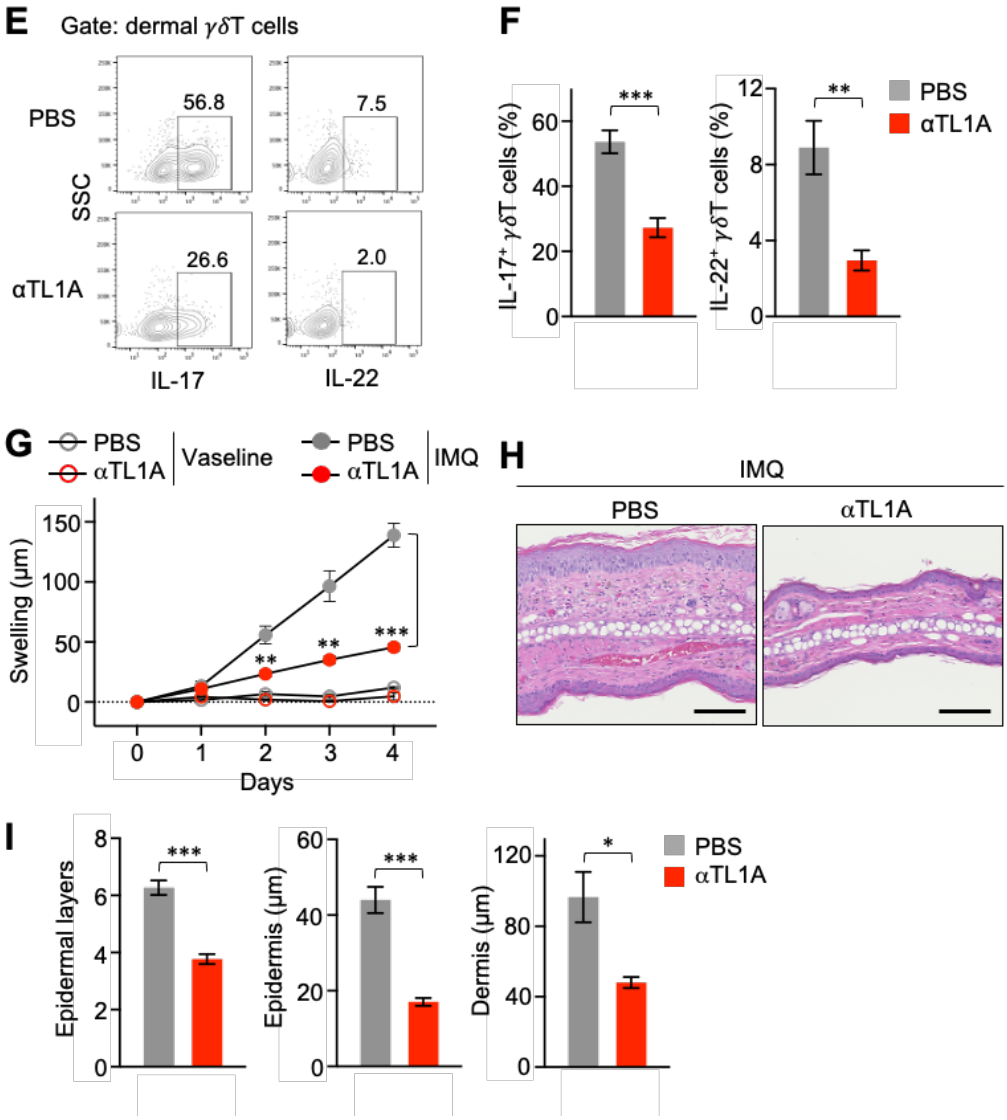

Figure II-1. Imiquimod (IMQ) treatment activates dermal γδT cells through TL1A.

(E–I) Ears were smeared with IMQ for 3 (E, F) or 4 (G–I) consecutive days. Anti-TL1A antibodies were injected from day −1 until the day before the analysis. (E, F) $CD3^{int}\gamma\delta TCR^{int}$ cells from ears on day 3 were analyzed for cytokine production (n = 5). (G) Ear swelling was calculated as changes in thickness from day 0 (IMQ n = 6; Vaseline n = 3). (H) H&E staining sections were prepared from ears on day 4. Scale bar, 100 μm. (I) Epidermal keratinocyte layers, epidermal and dermal thicknesses were measured in the sections. Representative of three (A, C, E) and two (H) independent experiments. Accumulated from three (B, D, F) and two (G, I) independent experiments. Error bars, mean ± standard error (SE). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

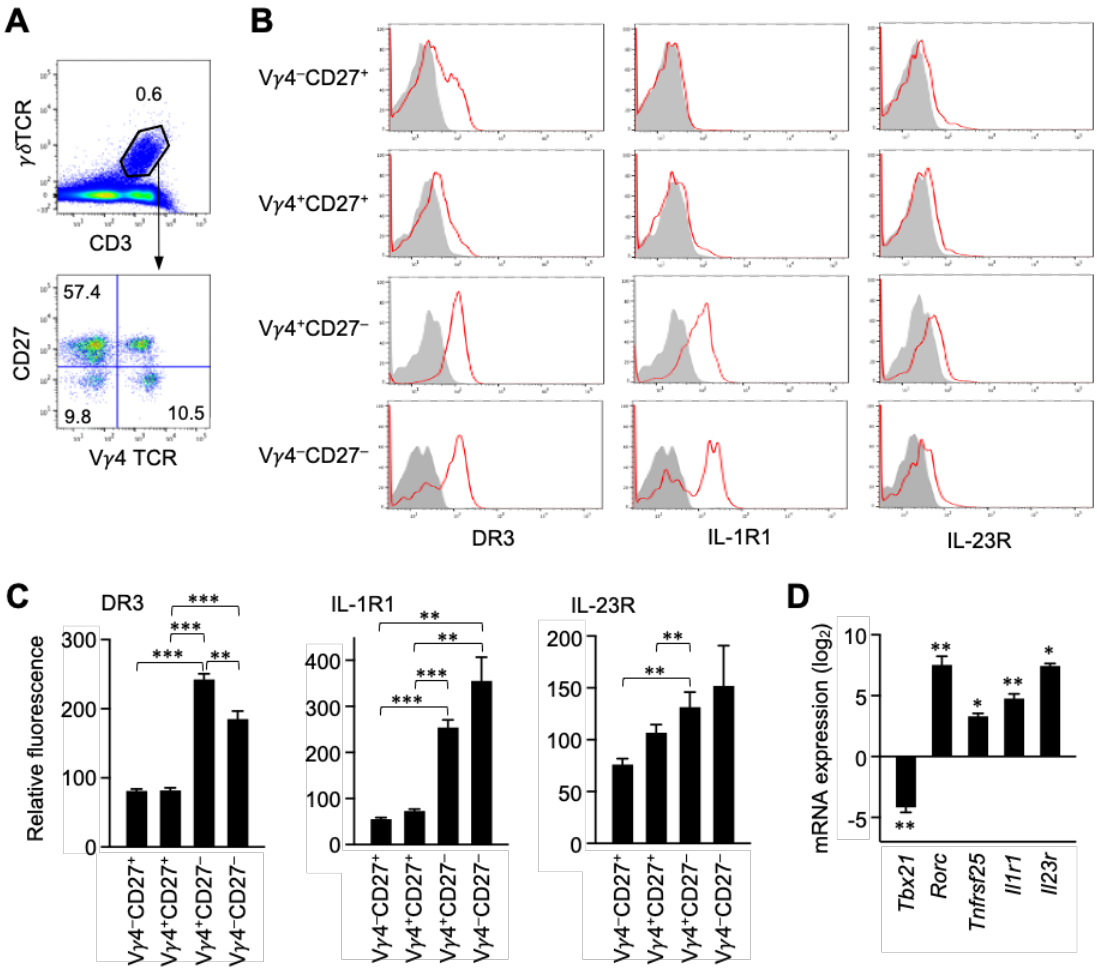

Figure II-2. TL1A activates splenic γδT17 cells synergistically with IL-23. (A) $CD3^{+}\gamma\delta TCR^{+}$ cells were subdivided into four fractions based on CD27 and Vγ4 expression. (B) Expression of DR3, IL-1 receptor (type I), and IL-23 receptor in indicated γδT-cell subsets. (C) Expression levels of cytokine receptors in each γδT-cell subset are shown as relative fluorescence intensities normalized with the mean fluorescence intensity in total γδT cells defined as 100 (n = 6). (D) $CD27^{+}$ and $CD27^{-}$ γδT cells isolated by sorting were analyzed by qPCR for the expression of the indicated genes (n = 3). mRNA expression in $CD27^{-}$ γδT cells is shown as relative levels in $CD27^{+}$ γδT cells defined as 1. The statistical significance of the expression levels between $CD27^{+}$ and $CD27^{-}$ cells is shown by asterisks.

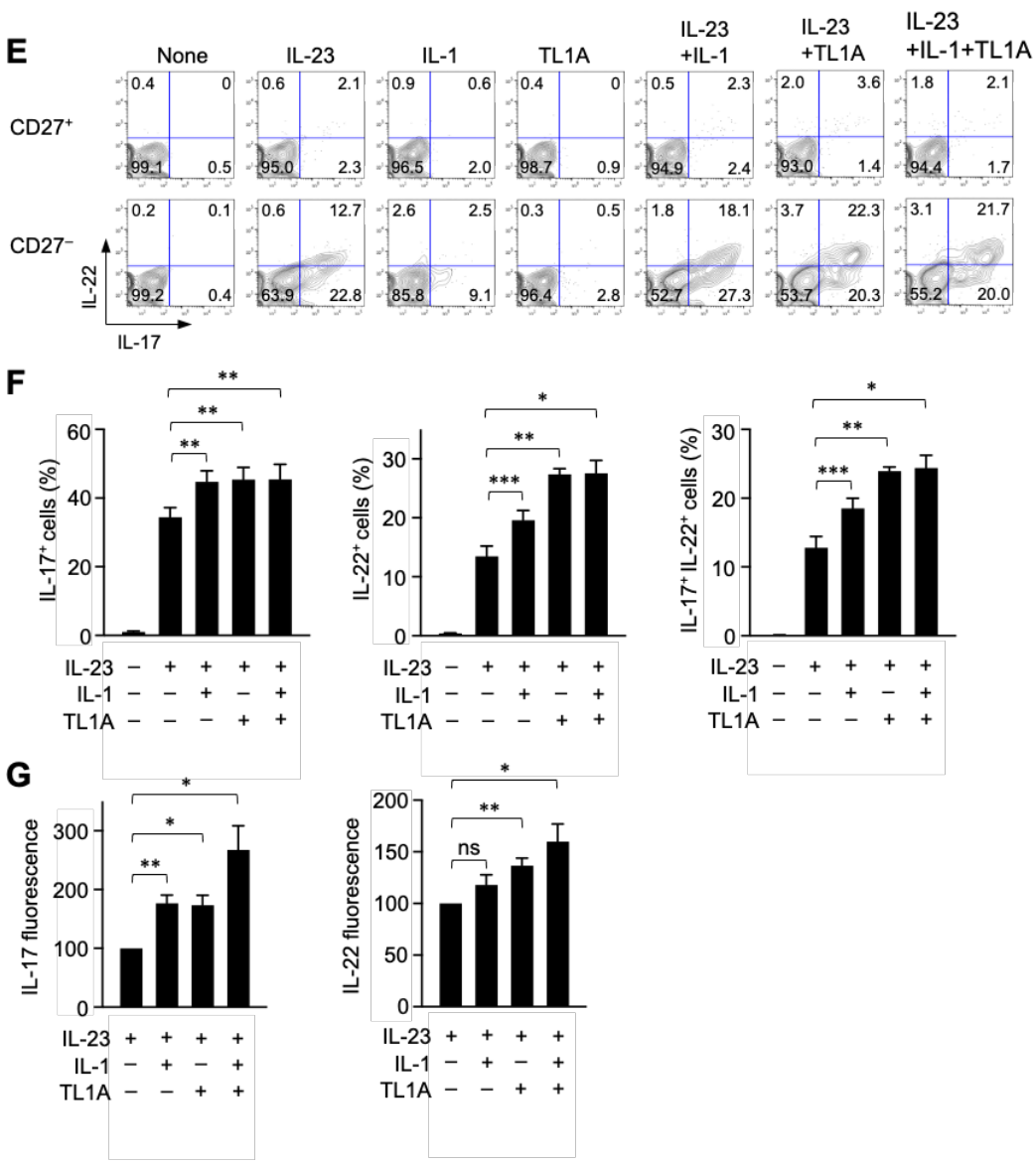

Figure II-2. TL1A activates splenic γδT17 cells synergistically with IL-23. (E–G) Spleen cells were cultured with the indicated cytokines for 24 h. A protein transport inhibitor was added for the last 4 h. IL-17 and IL-22 production was analyzed by intracellular staining. (E) Representative flow cytometry profiles of γδT cells at the end of culture. (F) Frequencies of IL-17$^+$, IL-22$^+$, and IL-17$^+$IL-22$^+$ γδT cells (n = 5). (G) Expression level of IL-17$^+$ and IL-22$^+$ γδT cells (n = 5). Representative of three (A, B) and four (E) independent experiments. Cumulative results from three (C, D) and four (F) independent experiments. Error bars, mean ± SE. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

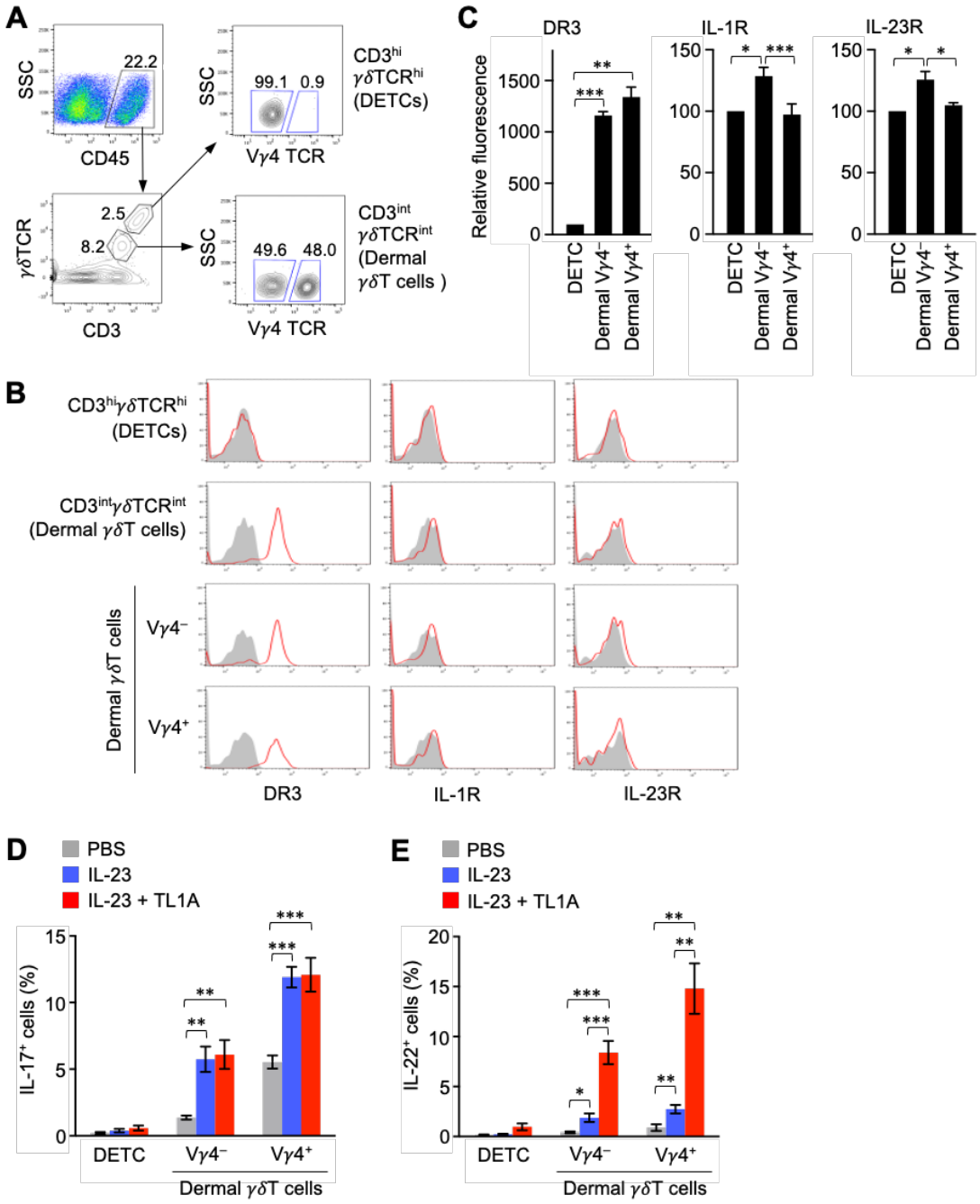

Figure II-3. Dermal γδT cells are ready to respond to IL-23 and TL1A. (A) $CD45^+$ gate in single-cell suspensions from ears contained $CD3^{hi}\gamma\delta TCR^{hi}$ dendritic epidermal T cells (DETCs) and $CD3^{int}\gamma\delta TCR^{int}$ conventional dermal γδT cells. $CD3^{int}\gamma\delta TCR^{int}$ dermal γδT cells were divided into $V\gamma4^+$ and $V\gamma4^-$ cells. (B) Expression of DR3, type I IL-1R, and IL-23R in DETCs and dermal γδT cells. (C) Expression levels of cytokine receptors in $V\gamma4^+$ and $V\gamma4^-$ γδT cells are shown as relative fluorescence intensities normalized with the mean fluorescence intensity in DETCs defined as 100 (n = 4). (D,

E) Mouse ears were intradermally injected with IL-23 alone or combined with TL1A. Twenty-two hours later, DETCs and γδT cells were analyzed for IL-17 (D) and IL-22 (E) by intracellular staining (n = 7 per group). Representative data of three independent experiments (A, B). Cumulative results from three (C) and four (D, E) independent experiments. Error bars, mean ± SE. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

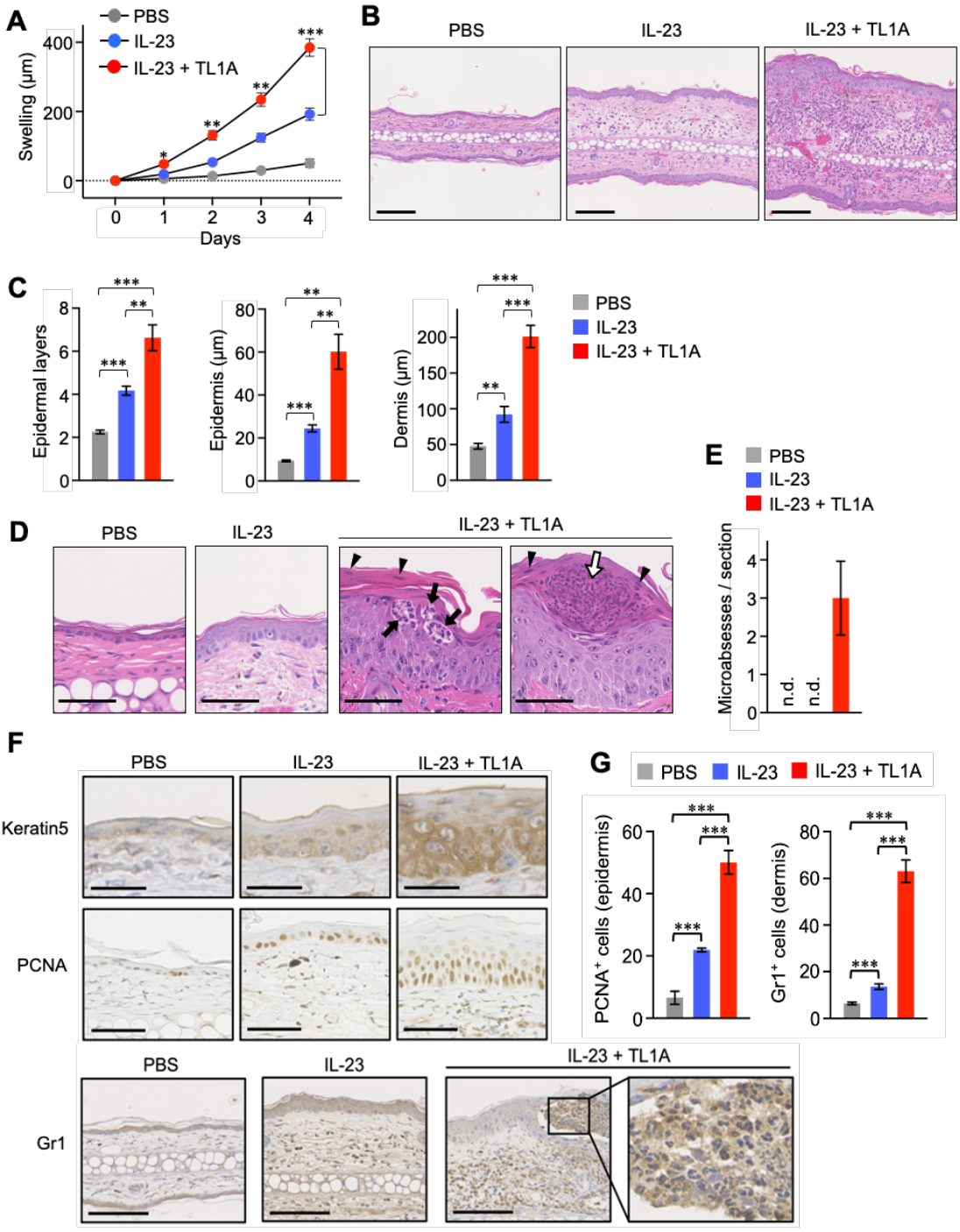

Figure II-4. TL1A exacerbates symptoms in the IL-23-induced murine psoriasis model. Mouse ears were intradermally injected with PBS, IL-23 alone, or the combination of IL-23 and TL1A for 4 consecutive days from days 0 to 3. (A) Thickness of ears was monitored (n = 4). The swelling was calculated as changes in thickness before the treatments. (B–E) H&E staining sections were prepared on day 4. (B) Representative view with low magnification. Scale bar, 100 μm. (C) Epidermal layers

of keratinocytes and epidermal and dermal thicknesses were measured (n = 6). (D) Magnified view of the epidermis. Scale bar, 50 μm. (E) Number of microabscesses per section (n = 6). nd, not detected. (F) Immunohistochemical analysis. Scale bar, 25 μm (top), 50 μm (middle), and 100 μm (bottom). (G) $PCNA^+$ and $Gr1^+$ cells were counted in epidermal and dermal areas, respectively (n = 6). Cumulative results from two (A) and three (C, E, G) independent experiments. Representative data of three independent experiments (B, D, F). Error bars, mean ± SE. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

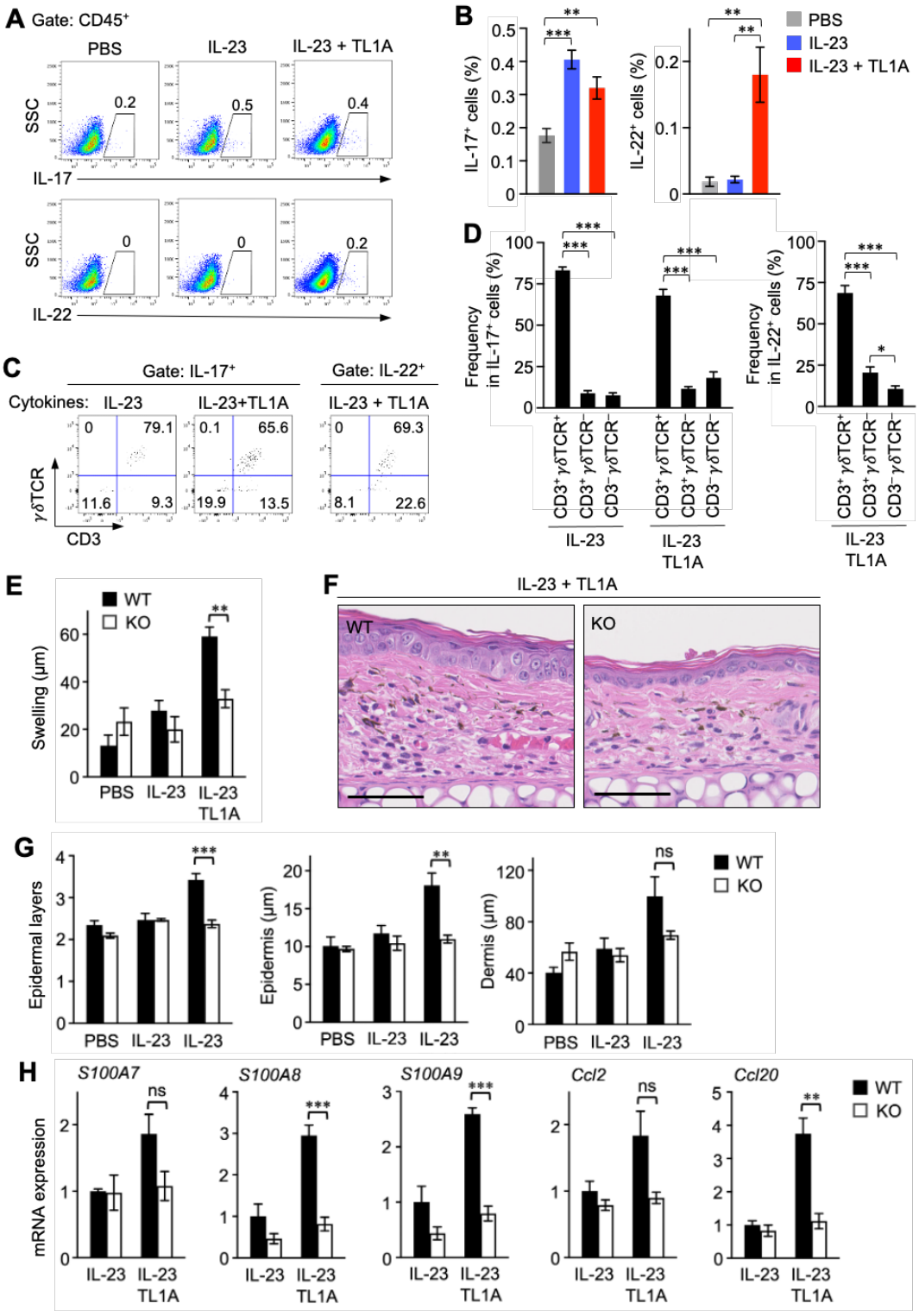

Figure II-5. Involvement of TL1A-mediated γδT-cell activation in early psoriasis. Mouse ears were intradermally injected with indicated cytokines. One day later, ears were harvested. (A–D) Single-cell suspensions were prepared for flow cytometry analysis (n = 7). (A and B) Frequencies of cytokine-producing cells in the $CD45^+$ gate.

(C and D) IL-17^{+} and IL-22^{+} lymphocytes were analyzed for CD3 and γδTCR expression. (E–G) Comparison of wild-type and *Tcrd*-knockout mice. (E) Ear thickness was measured before and a day after the treatments (n = 4–5). (F, G) Histological analysis of H&E sections. (F) Representative views from mice injected with IL-23 plus TL1A. Scale bar, 50 μm. (G) Epidermal layers and epidermal and dermal thicknesses (n = 4–5). (H) Ear samples were subjected to qPCR analysis (n = 4–6). mRNA expression is shown relative to the average values in wild-type mice injected with IL-23 alone defined as 1. Representative of two (F) and four (A, C) independent experiments. Accumulated from two (E, G, H) and four (B, D) independent experiments. Error bars, mean ± SE. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, not significant.

Discussion

This study demonstrated the involvement of the TL1A-DR3 pathway in γδT17 cell activation. TL1A in cooperation with IL-23, but not by itself, induced IL-17 and IL-22 production. This effect of TL1A was similar to that of IL-1β, a major cytokine regulating γδT17 cell activation (Sutton et al., 2009). However, there was a clear difference between DR3 and IL-1R in their expression pattern. Among γδT- cell subsets in the spleen and skin, IL-1R expression was clearly detected only in splenic $CD27^{-}$ γδT17 cells. This result aligned with the observation in a previous study that IL-1R expression in γδT17 cells varies depending on anatomic sites, including the intestine and the lung (Duan et al., 2010). In contrast, DR3 was more broadly expressed in various γδT-cell populations distinct in phenotype and anatomic location. Notably, dermal γδT cells in steady state expressed high levels of DR3, suggesting a role of TL1A signaling for the immediate response of dermal γδT cells in situ. γδT cell activation through IL-1β has been shown critical for the disease development in IMQ-induced psoriasis model (Cai, et al., 2019). Although IL-1β and TL1A can similarly activate γδT cells, they might act on different γδT cell populations (e.g. skin-resident versus migratory) and/or in different stages (e.g. inflammation trigger versus progression) during psoriasis development. How these cytokines cooperate for psoriasis development is an intriguing question for future studies.

Intradermal IL-23 and TL1A injection demonstrated that dermal γδT cells strongly depend on TL1A for IL-22 production. IL-22 acts on epidermal keratinocytes and promotes the release of antimicrobial proteins (Boniface et al., 2005; Wolk et al., 2006). Hence, TL1A-DR3 signaling in skin-resident γδT17 cells may contribute to the host defense by maintaining the barrier against microbial invasion. Additionally, IL-22 also supports the renewal of the skin epidermis by downregulating the molecules associated with the terminal differentiation of keratinocytes and promoting their proliferation (Boniface et al., 2005; Wolk et al., 2006). IL-22 also has prosurvival effects on intestinal epithelial cells by inducing antiapoptotic genes (Keir et al., 2020). These

regenerative activities of IL-22 suggest that TL1A-dependent dermal γδT-cell activation is important for the homeostatic renewal and wounded tissue repair of the epidermis. The possible role of TL1A and γδT cells in skin homeostasis is an issue that can be addressed in the future.

The function of dermal γδT17 cells immediately producing cytokines in response to inflammation is beneficial for early pathogen clearance, but this characteristic can underlie inflammatory skin diseases. Results showed that γδT cells produce most IL-17 and IL-22 in murine psoriasis models before overt clinical manifestations appear. In the intradermal IL-23-injection model, TL1A significantly accelerated skin swelling only within a day in a γδT cell-dependent manner. A recent study showed that IL-17 derived from γδT cells is primarily required to induce experimental autoimmune encephalomyelitis (McGinley et al., 2020). Therein, the early wave of IL-17 from $V\gamma4^{+}$ γδT cells within several hours after autoantigen immunization was critical for recruiting IL-1β-secreting inflammatory monocytes and neutrophils, in turn promoting the priming of pathogenic T cells (McGinley et al., 2020). Likewise, dermal γδT17 cell activation through TL1A may initially condition the inflammatory environment for subsequent immune responses, including the recruitment of conventional αβT cells. However, whether and how this TL1A-dependent γδT17 cell activation in the acute phase of inflammation eventually results in chronic manifestations of psoriasis remains to be clarified. It is possible that TL1A also modulates immune reactions in the later stages of the disease. TL1A exacerbates inflammatory bowel disease by synergistically acting with IL-23 on Th17 cells and enhancing their IL-17 production (Takedatsu et al., 2008). Similarly, the progression of psoriatic disease with repetitive cytokine injections and IMQ treatments in the experiments may involve the action of TL1A on αβT cells.

IL-17 and IL-22 are cooperatively associated with psoriasis pathogenesis. Deficiency of either of these cytokine signals attenuates but does not fully abolish psoriatic disease development in mice (Malki et al., 2013; Zheng et al., 2007). These cytokines are derived from the same sources (γδT17 and Th17 cells) similarly triggered by IL-23. Therefore, upstream factors that differentially activate T cells toward

secreting IL-17 or IL-22 have been unclear. Although the involvement of Th22 cells producing IL-22 but not IL-17 has been reported in psoriasis patients (Luan et al., 2014), the γδT-cell population with such functional characteristics was not observed in this study. In a recent study, anti-TL1A antibody injection was shown to relieve the disease in the IMQ-induced psoriasis model (Li et al., 2020). TL1A neutralization represses IL-17 and IL-22 production by γδT cells in IMQ-treated skin. Although anti-IL-17 and anti-IL-17 receptor antibodies are effective for psoriasis (Lønnberg et al., 2014; Saunte et al., 2017), the blockade of TL1A-DR3 interaction can also be an alternative therapeutic approach by repressing IL-22 overproduction.

Genome-wide association studies have revealed the link of TL1A gene (Tnfsf15) variants with various autoimmune and inflammatory diseases, including psoriasis (Dand et al., 2017; Képíró et al., 2014), IBD (Baskaran et al., 2014; Thiebaut et al., 2009; Yamazaki et al., 2013; Yang et al., 2008), Graves' disease (Zhang et al., 2020), uveitis (Li et al., 2015), Behcet's disease (Jiang et al., 2017), and systemic lupus erythematosus (Wang & Tu, 2018). Increased TL1A levels in the serum or inflamed tissues from patients have also been reported in psoriasis (Bamias et al., 2011; Li et al., 2014), rheumatoid arthritis (Bamias et al., 2008; Cassatella et al., 2007), and IBD (Bamias et al., 2010). The pathogenic roles of TL1A in these diseases have been experimentally demonstrated in animal models or patient lymphocytes (Calder & Wang, 2012; Castellanos et al., 2018; Li et al., 2020; Takedatsu et al., 2008; Wang et al., 2014). Accumulating evidence suggests the role of γδT17 cells in these diseases (Cai et al., 2011; Cui et al., 2009; Roark et al., 2007; Sutton et al., 2009), but the significance of TL1A-mediated γδT17 cell activation in pathogenesis has not been directly shown. Thus, fundamental knowledge from this study can aid in elucidating the pathogenesis of various inflammatory and autoimmune diseases.

Summary

This study revealed that TL1A activates γδT17 cells synergistically with IL-23, and this regulatory pathway is associated with the early pathogenesis of psoriatic disease in mice. These findings provide insights into psoriasis pathogenesis and aid in developing therapeutics targeting γδT cells.

Conclusion

Psoriasis, characterized by hyperproliferation of keratinocytes and an inflammatory infiltration in the dermis and epidermis, involves various immune cells, including γδT cells. γδT cells play critical roles in the disease's pathogenesis due to their ability to produce IL-17 and other pro-inflammatory cytokines. This thesis demonstrated novel therapeutic strategies for psoriasis by targeting regulatory mechanisms of γδT cells.

Chapter I focused on the potential of REV-ERB agonists in psoriasis treatment. The study demonstrated that γδT17 cells were significantly increased in the secondary lymphoid organs of REV-ERBα-deficient mice. IL-17 production by γδT cells was significantly suppressed by a synthetic REV-ERB agonist, SR9009, both *in vitro* and *in vivo*. Furthermore, topical application of SR9009 to the skin improved the symptoms of imiquimod-induced psoriasiform dermatitis in mice. These results suggest that γδT17 cells contribute to psoriasis pathogenesis through REV-ERBs, and targeting REV-ERB suppression by synthetic ligands may offer a new approach to control γδT cell-mediated inflammation in psoriasis.

Chapter II examined the role of an inflammatory cytokine TL1A and its receptor DR3 in γδT cell activation and their involvement in psoriasis pathogenesis. The study revealed that TL1A blockade reduced cytokine production by γδT cells in a murine psoriasis model. Moreover, TL1A, in synergy with IL-23, exacerbated psoriatic symptoms by significantly increasing the production of IL-22 which inhibits terminal keratinocyte differentiation. Enhancement of inflammation and keratinocyte proliferation due to TL1A was significantly repressed in γδT cell-deficient mice. These results indicate that targeting the TL1A–DR3 pathway could modulate γδT cell activity, offering another therapeutic approach for psoriasis.

Taken together, these findings provide valuable insights into the regulatory mechanisms of γδT cells and therapeutic strategies for psoriasis. By focusing on REV-ERB and TL1A, this research aims to develop effective treatments that modulate the immune response, reduce inflammation, and improve the quality of life for patients with

psoriasis. Diagnosis and treatment of psoriasis in companion animals such as cats and dogs is an emerging field in veterinary medicine. Given the high prevalence of psoriasis in humans reaching 2–3% of the world population, the clinical cases in animals are possibly underreported. Novel therapeutics suggested from this study could also be adapted to those potential psoriasis patients in companion animals. Future research should continue to explore the details of these regulatory pathways at the molecular level and investigate their safety and effectiveness for clinical applications.

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Abstract in English

Psoriasis is a chronic inflammatory skin disease characterized by epidermal hyperproliferation and cellular infiltration. Previous studies have shown that psoriasis development depends on proinflammatory cytokines, such as interleukin (IL)-23 and IL-17. IL-23 produced by innate immune cells, such as dendritic cells and macrophages, in psoriatic lesions activates a subset of helper T cells to release IL-17, promoting neutrophil recruitment and epidermal keratinocyte proliferation. However, recent studies have revealed that innate γδT cells are involved in psoriasis pathogenesis as the primary source of IL-17. The purpose of the present study was to unravel the regulatory mechanisms of IL-17-producing γδT (γδT17) cells and establish the basis of psoriasis therapies targeting γδT17 cells.

In Chapter 1, γδT17 cell regulation through nuclear receptors, REV-ERBs, and the therapeutic strategy of psoriasis targeting this regulatory pathway were shown. REV-ERBs are ligand-dependent transcription factors and are generally known to regulate circadian rhythm. Although a recent study revealed that REV-ERBs suppress the function of IL-17-producing helper T cells, the role of REV-ERBs in γδT17 cell regulation had been unclear. γδT17 cells were significantly increased in the secondary lymphoid organs of REV-ERB-deficient mice. IL-17 production by γδT cells was suppressed by short-term treatment with a synthetic REV-ERB agonist, SR9009, both *in vitro* and *in vivo*. Furthermore, topical application of SR9009 to the skin reduced the inflammatory symptoms of imiquimod (IMQ)-induced psoriasiform dermatitis in mice. These results demonstrate that γδT17 cells associated with psoriasis development are regulated by REV-ERBs and that modulation of REV-ERB activity in γδT17 cells using synthetic ligands may become a novel therapeutic approach for psoriasis.

Next in Chapter 2, the role of an inflammatory cytokine called tumor necrosis

factor-like ligand 1A (TL1A) and its receptor, death receptor 3 (DR3), in γδT17 cell activation and its relevance with psoriasis pathogenesis were examined. Neutralization of TL1A by anti-TL1A antibody injection inhibited the cytokines production by γδT17 cells in IMQ-treated mouse skin. DR3 was constitutively expressed in splenic and dermal γδT17 cells. TL1A induced cytokine production by splenic γδT17 cells synergistically with IL-23. TL1A exacerbated the dermal symptoms of the murine psoriasis model generated by intradermal IL-23 injection in wild-type but not in γδT cell-deficient mice. These findings suggest a novel regulatory mechanism of γδT17 cells through TL1A and DR3 and its involvement in psoriasis pathogenesis as a possible therapeutic target.

In conclusion, the present study on the novel regulatory mechanism of γδT17 cells may provide insights into psoriasis pathogenesis and therapeutics.

Abstract in Japanese

乾癬は、表皮の過剰増殖と細胞浸潤を主徴とする慢性炎症性皮膚疾患であり、その発症にはインターロイキン(IL)-23 や IL-17 などの炎症性サイトカインが関与すると考えられている。乾癬病変部では、樹状細胞やマクロファージなどの自然免疫細胞によって産生される IL-23 が、ヘルパーT 細胞に作用して IL-17 を放出させ、好中球の動員と表皮角化細胞の増殖を促進する。一方、近年の研究から、自然免疫機能を有する γδT 細胞が病変部での IL-17 の主たる産生源であることが明らかにされ、乾癬発症における γδT 細胞の役割が注目されている。本研究は、この IL-17 産生 γδT（γδT17)細胞の制御機構を解明し、γδT17 細胞を標的とした乾癬治療の基盤を確立することを目的とした。

第 1 章では、核内受容体 REV-ERB を介した γδT17 細胞の制御機構の解明に取り組むとともに、この制御経路を標的とした乾癬の新たな治療法を動物モデルで試みた。REV-ERB はリガンド依存性の転写因子であり、概日リズムを調節することが広く知られている。最近、REV-ERB が IL-17 産生ヘルパーT 細胞の機能を抑制することが明らかにされたが、γδT17 細胞における REV-ERB の役割は不明であった。本研究ではまず、REV-ERB 欠損マウスの二次リンパ器官において γδT17 細胞が有意に増加することを明らかにした。合成 REV-ERB アゴニストのひとつである SR9009 は、γδT 細胞の IL-17 産生を *in vitro* および *in vivo* で抑制した。さらに、イミキモド誘発乾癬様皮膚炎モデルマウスの皮膚に SR9009 を塗布することにより炎症症状の軽減が認められた。これらの結果から、乾癬の発症に関与する γδT17 細胞の機能が REV-ERB を介して制御され、合成リガンドによる REV-ERB 活性の調節が乾癬の新たな治療法となり得ることが示された。

つづいて第 2 章では、炎症性サイトカインである腫瘍壊死因子様リガンド 1A （TL1A） と、その受容体であるデスレセプター3 （DR3） が γδT17 細胞の活性化と乾癬の発症に果たす役割を検討した。イミキモド誘発乾癬様皮膚炎モデルマウスに抗 TL1A 抗体を投与したところ、病変部位に存在する γδT17 細

胞のサイトカイン産生が阻害された。脾臓および真皮の γδT17 細胞には DR3 の発現が認められ、脾臓の γδT17 細胞を TL1A と IL-23 で刺激したところ、サイトカインの産生が誘導された。IL-23 を野生型マウスに皮内投与することで誘導される乾癬様病変は、TL1A の追加投与により顕著に悪化した。一方、γδT 細胞欠損マウスでは、TL1A による病変の悪化は認められなかった。これらの結果から、TL1A-DR3 経路を介した γδT17 細胞の調節機構が乾癬の発症に関与し、新たな治療標的となる可能性が示唆された。

以上のように、本研究を通じて得られた γδT17 細胞の新たな制御機構に関する知見は、乾癬の病因解明と治療法の開発に寄与するものである。