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Title: Alleviation of imiquimod-induced psoriasis-like symptoms in *Rorα*-deficient mouse skin

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Running Title: Role of RORα in murine psoriasis-like model

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ABSTRACT

Retinoic acid receptor-related orphan receptor α (RORα) plays a vital role in various physiological processes, including metabolism, cancer, circadian rhythm, cerebellar development, and inflammation. Although RORα is expressed in the skin, its role in skin physiology remains poorly elucidated. Herein, Rorα was expressed in the basal and suprabasal layers of the epidermis; however, keratinocyte-specific *Rorα* deletion did not impact normal epidermal formation. Under pathophysiological conditions, *Rorα*-deficient mice exhibited alleviated psoriasis-like symptoms, including relatively intact epidermal stratification, reduced keratinocyte hyperproliferation, and low-level expression of inflammatory cytokines in keratinocytes. Unexpectedly, the splenic population of Th17 cells was significantly lower in keratinocyte-specific RORα deficient mice than in the control. Additionally, *Rorα*-deficiency reduced imiquimod-induced activation of nuclear factor-κB and STAT3 in keratinocytes. Therefore, we expect that RORα inhibitors act on immune cells and keratinocytes to suppress the onset and progression of psoriasis.

INTRODUCTION

Psoriasis is a chronic inflammatory skin disease characterized by deregulated activation of immune cells and keratinocytes (1, 2). The pathophysiology of psoriasis includes hyperproliferation and abnormal differentiation of epidermal keratinocytes and dermal infiltration of multiple immune cells (1, 2). The interplay between immune cells and keratinocytes is crucial for the initiation and progression of psoriasis (3, 4) although detailed molecular mechanisms underlying the development of psoriasis need to be explored. Dendric cells (plasmacytoid and myeloid dendritic cells) contribute to the early stages of psoriasis, and activation of T cells, such as Th1, Th17, and Th22, plays a critical role during the chronic maintenance phase (1, 2).

Furthermore, keratinocytes reportedly participate in the initiation and progression of psoriasis (5, 6). Upon skin damage, keratinocytes secrete self-nucleotides and antimicrobial peptides, activating plasmacytoid dendritic cells (7), and release multiple proinflammatory cytokines via the toll-like receptor (TLR) 3 pathway (8, 9). During psoriasis progression, keratinocytes recruit immune cells by secreting chemokines and enhance inflammation by producing multiple inflammatory mediators (5, 6). Topical application of imiquimod (IMQ), a TLR7 agonist, has been reported to induce psoriasis-like lesions in mouse skin via the interleukin (IL)-23/IL-17 axis (10), which is used as a mouse model for psoriasis. Aldara is an IMQ cream formulation and frequently applied topically (11).

Retinoic acid receptor-related orphan receptor α (RORα) plays a diverse role in physiology, including brain development, circadian rhythm, metabolic pathways, and inflammation, by regulating transcription programs as transcription factors or corepressors (12). Although RORα was initially considered an orphan nuclear receptor, cholesterol, cholesterol sulfate, and other

cholesterol derivatives have been suggested as putative endogenous ligands (13, 14). Subsequently, many natural and synthetic ligands have been developed to modulate RORα activity (15). RORα is highly expressed in the skin epithelium, with a potential role in promoting keratinocyte differentiation (16, 17). Furthermore, cholesterol sulfate was found to induce the expression of filaggrin, a differentiation marker protein, in human keratinocytes by activating RORα (18). SR1001, a synthetic inverse RORα/γ agonist, can alleviate inflammation, atopic dermatitis-related symptoms, and acute irritant dermatitis in mouse models (19). In the present study, we investigated the role of RORα in psoriasis progression using a mouse model with specific deletion of the *Rora* gene in keratinocytes. Notably, we found that a lack of RORα could alleviate psoriasis-like symptoms.

RESULTS

Keratinocyte-specific *Rorα* deletion alleviates IMQ-induced psoriasis-like symptoms in mouse skin

As no systematic analysis regarding the contribution of RORα in skin physiology has been reported, we established a mouse model containing a keratinocyte-specific *Rorα* deletion by crossing *Rorα*-floxed mice with mice expressing Cre recombinase under the control of the keratin 14 promoter (K14-Cre**)** (Fig. 1A). The genotype of the mice was confirmed by PCR (Fig. 1B). We adapted the IMQ-induced psoriasis-like model to examine the role of RORα in pathophysiological conditions (10). IMQ-containing Aldara cream was applied daily to the dorsal surface and ear skin of mice for six days, and psoriasis-like symptoms were examined. The dorsal skin of $Ror\alpha^{+/+}$ (hereafter, $Ror\alpha^{+/+}$*;K14-Cre*) mice exhibited flaky erythema and psoriasis-like lesions six days after IMQ application, whereas $Ror\alpha^{\Delta/\Delta}$ (hereafter, $Ror\alpha^{f/f}$*;K14-Cre*) mice indicated mild symptoms (Fig. 1C). On histological examination of dorsal and ear skin, IMQ-treated mice presented epidermal hyperplasia, resulting in skin thickening, keratosis, and elevated infiltration of the epidermal layer into the dermis in $Ror\alpha^{+/+}$ mice, whereas $Ror\alpha^{\Delta/\Delta}$ mice indicated relatively well-defined epidermal layers (Fig. 1D and 1E). Measurement of epidermal thickness confirmed that the thickness of the epidermis was reduced (approximately 3-fold) in $Ror\alpha^{\Delta/\Delta}$ mice when compared with that of $Ror\alpha^{+/+}$ mice (Fig. 1D). Topical IMQ application can reportedly induce splenomegaly in mice (10, 20). Accordingly, we compared the splenic size and mass before and after IMQ treatment in $Ror\alpha^{+/+}$ and $Ror\alpha^{\Delta/\Delta}$ mice. Topical IMQ application increased the splenic size in both groups; however, the size was relatively smaller (closer to normal) in $Ror\alpha^{\Delta/\Delta}$ mice than in $Ror\alpha^{+/+}$ mice (Fig. 1F). The mean splenic mass indicated a pattern similar to its size assessment (Fig. 1G). We then examined the

population of Th17 cells in the spleen in comparison with *Rorα*$^{+/+}$ and *Rorα*$^{Δ/Δ}$ mice before and after IMQ treatment. The spleen population of Th17 cells was increased approximately three-fold by IMQ treatment in *Rorα*$^{+/+}$ mice, whereas it was hardly increased in *Rorα*$^{Δ/Δ}$ mice (Fig. 1H). These findings suggested that *Rorα* deficiency could alleviate the IMQ-induced psoriasis-like symptoms in mice. Thus, *Rorα* may contribute to the progression of psoriasis via its keratinocyte-mediated functions.

The epidermis of *Rorα*$^{Δ/Δ}$ mice maintained a more intact stratification in the IMQ-induced psoriasis-like state

We performed immunostaining to detect *Rorα* expression in the skin epidermis and found that the pattern of *Rorα* immunostaining was in good agreement with that of K14, a marker of the basal epidermal layer involved in cell division (Fig. 2A). *Rorα*$^{Δ/Δ}$ mice displayed normal overall skin appearance and epidermal stratification when compared with those of *Rorα*$^{+/+}$ mice (Fig. 2); this indicated that the lack of *Rorα* expression does not impact the epidermal development. Interestingly, IMQ application increased *Rorα* expression, as indicated by immunostaining (Fig. 2A) and immunoblotting (Fig. 2D) of primary mouse keratinocytes. HaCaT human keratinocytes exhibited increased RORα levels following IMQ treatment (Fig. 2E). Next, we investigated the status of epidermal stratification as a measure of skin integrity. Dorsal skin specimens of *Rorα*$^{+/+}$ and *Rorα*$^{Δ/Δ}$ mice were subjected to immunofluorescence staining for various stratification marker proteins, such as K14 (basal layer and hair follicles), K10 (basal and suprabasal layer), and loricrin (late differentiation marker in the outermost epidermal layer) (10, 20). Herein, K14 and K10 were arranged in uniform and smooth layers in both *Rorα*$^{+/+}$ and *Rorα*$^{Δ/Δ}$ mice without IMQ treatment (Fig. 2B). Upon IMQ treatment, the K14- and K10-

expressing layers thickened and infiltrated into the dermal layer of the skin of *Rorα*$^{+/+}$ mice; however, the epidermis of *Rorα*$^{Δ/Δ}$ mice presented a relatively normal structure approximating that of non-IMQ-treated mice (Fig. 2B). Loricrin staining revealed a disrupted structure of the outermost epidermal layer in IMQ-treated *Rorα*$^{+/+}$ mice when compared with the relatively normal layer of *Rorα*$^{Δ/Δ}$ mice (Fig. 2C). Protein levels of late differentiation markers, including involucrin and loricrin, were similar before and after IMQ treatment in both mouse genotypes (Fig. 2D), indicating that the lack of differentiation markers fails to clarify abnormal stratification. In addition to aberrant stratification, alterations in tight junctions and downregulation of tight junction proteins in the epidermis have been documented in plaque-type psoriasis (21, 22). We analyzed the expression of occludin, a well-known tight junction protein in the epidermis. We noted that occludin expression was reduced in the skin of IMQ-treated *Rorα*$^{+/+}$ mice when compared with that in the untreated control, as determined by immunofluorescence and immunoblotting analyses (Fig. 2F and 2G). Conversely, IMQ-treated *Rorα*$^{Δ/Δ}$ mice maintained occludin expression along the epidermal layer (Fig. 2F and 2G). Another tight junction protein, claudin, indicated a pattern resembling that of occludin in the immunoblotting analysis (Fig. 2G). Collectively, these results suggested that *Rorα* deficiency can favorably maintain the epidermal structure of the skin during IMQ-induced psoriasis-like conditions.

Rorα-deficient keratinocytes exhibited reduced hyperproliferation in IMQ-induced psoriasis-like conditions

Given that epithelial cell hyperproliferation is a typical symptom of psoriasis, we examined keratinocyte proliferation in an IMQ-induced psoriasis-like condition. Keratinocyte proliferation was examined by comparing the epidermal expression of Ki67, a cell proliferation

marker protein, in the dorsal skin of *Rorα*$^{+/+}$ and *Rorα*$^{Δ/Δ}$ mice. IMQ increased the number of Ki67-positive cells more than two-fold in the epidermis of *Rorα*$^{+/+}$ mice, with only a slight increase noted in *Rorα*$^{Δ/Δ}$ mice (Fig. 3A and 3B). To confirm these findings, we knocked out *RORα* in HaCaT cells and examined cell division before and after IMQ treatment. After validating the effective knockout of *RORα* (Fig. 3C), we measured EdU incorporation into newly synthesized DNA as an indicator of cell proliferation. IMQ treatment significantly increased the number of EdU-incorporated HaCaT cells, indicating cellular hyperproliferation. Conversely, *RORα*-deficient cells exhibited a slight increase in EdU incorporation following IMQ treatment (Fig. 3D). Statistical analysis revealed that control HaCaT cells indicated a nearly three-fold increase in EdU-positive cells upon IMQ treatment; however, only a slight increase was documented in *RORα*-deficient cells (Fig. 3E). Collectively, these results suggested that *RORα* deficiency in keratinocytes could suppress their hyperproliferation in IMQ-induced psoriasis-like conditions.

Reduced proinflammatory gene expression and attenuated nuclear factor (NF)-κB and signal transducer and activator of transcription 3 (STAT3) activation in *Rorα*-deficient keratinocytes upon IMQ treatment

We next addressed the inflammatory status of keratinocytes, given that chronic inflammation is another key factor in psoriasis progression. We isolated primary keratinocytes from the skin of newborn *Rorα*$^{+/+}$ and *Rorα*$^{Δ/Δ}$ mice, treated isolated cells with IMQ, and subsequently obtained cell lysates. The production of cytokines, including tumor necrosis factor (TNF)-α, IL-6, IL-23, and chemokine (C-C motif) ligand 20 (CCL20), was evaluated using the Luminex kit (Multiplex chemokine and cytokine assay kit). The amounts of cytokines were relatively

low in *Rorα*-deficient keratinocytes when compared with that of wild-type controls, although fold increases in TNFα and IL-6 by IMQ treatment were similar in each group (Fig. 4A). In a similar set of experiments, IMQ treatment increased the phosphorylation of p65, an NF-κB subunit, in wild-type keratinocytes; only marginal phosphorylation was detected in keratinocytes derived from $Rora^{\Delta/\Delta}$ mice (Fig. 4B). Furthermore, IMQ-induced STAT3 phosphorylation was reduced in *Rorα*-deficient keratinocytes when compared with that in wild-type keratinocytes (Fig. 4C). To confirm whether *RORα* deficiency reduces the activation of proinflammatory signaling, we performed transient knockout of the *RORα* gene in HaCaT cells. As observed in *Rorα*-deficient primary keratinocytes, we confirmed that IMQ-induced activation of NF-κB and STAT3 was reduced in *RORα*-KO HaCaT cells (Fig. 4D and 4E). We then examined the interaction between endogenous RORα and STA3 in keratinocyte cells. When extracts of HaCat cells were immunoprecipitated with an anti-STAT3 antibody, we detected endogenous RORα in the precipitate, and the amount of RORα detected increased upon IMQ treatment (Fig. 4F). Transient RORα knockout diminished this interaction (Fig. 4F). Moreover, IMQ-induced upregulation of proinflammatory gene expression was blunted in *RORα*-knockout HaCaT cells (Fig. 4G). Overall, these results indicated that *RORα* deficiency has a causal relationship with a reduced inflammatory status in IMQ-treated keratinocytes. The reduced inflammation may be responsible for alleviating IMQ-induced psoriasis-like conditions in $Rora^{\Delta/\Delta}$ mice.

DISCUSSION

Psoriasis is an autoimmune skin disease that involves T cell-mediated sustained inflammation during the chronic disease phase (1, 2). Epidermal keratinocytes reportedly play a pivotal role in the progression of psoriasis (5, 6). RORα, along with RORγ, has been identified as a key transcription factor in Th17 differentiation, a critical contributor to psoriasis progression (23). Moreover, a synthetic inverse RORα/γ agonist was found to be effective in treating atopic dermatitis and acute irritant dermatitis in mouse models (19).

It has been reported that RORα controls the expression of some keratinocyte genes, including keratin 1, keratin 10, involucrin, loricrin and flaggrin, and promotes keratinocyte differentiation in human keratinocytes (16). We also detected the decreased expression of certain genes in *Rorα*-deficient keratinocytes, including K10, loricrin, involucrin, and filaggrin. However, despite the low expression of these keratinocyte genes, we did not find any structural problems in the epidermal layer of *Rorα*$^{\Delta/\Delta}$ mice. Thus, in the present study, we focused on the role of RORα in keratinocytes during the development and progression of psoriasis. K14-Cre removes floxed exons in the *Rorα* gene of epidermal keratinocytes but not immune cells; hence, we expected to exclude the contribution of RORα in psoriasis progression through its function in immune cells, including Th17 cells. However, keratinocyte-specific deletion of *Rorα* still significantly reduced the splenic population of Th17 cells upon IMQ treatment. Although we do not know the exact mechanism of how keratinocyte-specific deletion of the *Rorα* gene decrease the development of Th17 cells, we think that this is one reason of alleviated psoriasis-like symptoms in *Rorα*-deficient mouse skin. In addition to the reduced Th17 cells, we found that RORα deficiency reduced cell proliferation and decreased proinflammatory cytokine gene

expression, two well-known aspects of psoriasis progression, in both primary mouse keratinocytes and human HaCaT cells. Accordingly, it can be suggested that RORα plays an important role in keratinocyte hyperproliferation and inflammatory processes in an IMQ-induced psoriasis model, and inhibition of RORα may be valuable for attenuating the progression of psoriasis.

STAT3 is considered a crucial transcription factor in the development and progression of psoriasis in terms of the activation of immune cells and keratinocytes (24). STAT3 contributes to the differentiation and activation of Th17 cells, as well as acts as a transcription factor in the response of keratinocytes to cytokines from immune cells (24). Additionally, activation of STAT3 is well known as the cause of hyperproliferation of keratinocytes in psoriasis (24). STAT3 inhibitor has been reported to reduce proliferation of normal human keratinocytes through downregulation of c-Myc and cyclin D1, and STAT3 inhibitor has been considered as a therapeutic target for the treatment of psoriasis (25). It has been reported that constitutive expression of active Stat3 in keratinocytes of mouse skin can induce a psoriasis-like skin phenotype (26). Furthermore, keratinocyte-specific inducible STAT3 knockout can reduce psoriasis-like symptoms and afford greater protection against psoriasis-like dermatitis than that of constitutive deletion of STAT3 in all T cells (27). Herein, we found that STAT3 activation (STAT3 phosphorylation) was significantly reduced in IMQ-treated RORα-deficient keratinocytes. Reportedly, RORα directly interacts with STAT3, and treatment with a RORα antagonist or knockdown of RORα inhibits STAT3 activation in human articular chondrocytes, although the examined cell type varies from that used in the present study (28). We also confirmed the interaction between RORα and STAT3 in keratinocytes, and STAT3 phosphorylation was reduced in the absence of RORα in both mouse primary keratinocytes and HaCat cells. Therefore, inhibition of RORα appears to be effective in both immune cells and

keratinocytes to suppress the development and progression of psoriasis, and inverse RORα agonists may represent a new class of anti-inflammatory compounds to treat inflammatory skin diseases, including psoriasis.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

FIGURE LEGENDS

Figure 1. Evaluation of IMQ-induced psoriasis-like symptoms in skin-specific *Rorα*-deficient mice.

(A) *Rorα* floxed mice were crossed with *K14-Cre* mice leading to keratinocyte-specific deletion of exons 4 and 5 in the *Rorα* gene. (B) Genotyping results for the skin-specific deletion of *Rorα* confirm the successful deletion of exons 4 and 5 in the *Rorα* gene. (C) $Ror\alpha^{+/+}$ and $Ror\alpha^{\Delta/\Delta}$ mice were topically treated with 62.5 mg of Aldara cream (containing 5% IMQ), applied daily on the shaved dorsal skin surface for six days. (D) Representative images of hematoxylin and eosin (H&E) staining of paraffin sections of the dorsal skin of each group of mice, prepared as indicated in (C) (left panel). Scale bar; 100 μm. The mean thickness of the epidermis of each group of mice was measured using H&E stained images (right panel, n=6). Data are presented as mean ± standard error of the mean (SEM). ***; $p<0.001$. (E) Representative images of H&E staining of the ear skin. (F) Representative images of the spleen from each group of mice, prepared as indicated in (C). (G) The spleen mass was calculated from six independent mice for each group. Data are presented as mean ± SEM. *; $p<0.05$. (H) The splenic population of Th17 cells was calculated from four independent mice for each group. Data are presented as mean ± SEM. *; $p<0.05$. IMQ, imiquimod; RORα, Retinoic acid receptor-related orphan receptor α.

Figure 2. Analysis of epidermal stratification and barrier formation of the skin.

(A-C) Skin sections were subjected to immunofluorescence analyses with antibodies against (A) Keratin 14 (K14, green) and Rorα (red), (B) K14 (green) and K10 (red), and (C) K14 (green) and loricrin (red). Scale bar = 100 μm. (D-E) Immunoblot analysis for various stratification marker proteins. Mouse primary keratinocytes (D) or HaCaT cells (E) were treated with IMQ for 24 h, and protein extracts were subjected to immunoblotting for indicated proteins. The equal protein loading was confirmed with GAPDH. (F) Skin sections were subjected to immunofluorescence analyses with antibodies against occludin, a tight junction protein. Scale bar = 100 μm. (G) The expression of occludin and claudin was analyzed as in (D). IMQ, imiquimod.

Figure 3. Assessment of IMQ-induced proliferation of wild-type and *RORα*-deficient human and mouse keratinocytes.

(A) Skin sections were subjected to immunofluorescence analysis with an antibody against Ki67 (red), a cell proliferation marker. Scale bar = 100 μm. (B) The number of Ki67-positive keratinocyte cells was counted in four images from each independent mouse skin, expressed as a percentage of total cells. Data are shown as mean ± standard error of the mean (SEM). **; $p<0.01$. (C) HaCaT cells were infected with lentivirus generated from the pLentiCRISPR-E vector or pLentiCRISPR-E-RORα #1 in six-well plates. One day after viral infection, cells were selected with 1 μg/ml puromycin for 2 days. Pools of selected HaCaT cells were treated with 200 μM IMQ for 24 h and subjected to immunoblot analysis for RORα. Control represents HaCaT cells infected with lentivirus generated from the pLentiCRISPR-E vector, and RORα knockout (KO) represents the case of pLentiCRISPR-E-RORα #1. (D) HaCat cells prepared as in (C) were seeded in 12-well plates at a density of 1×10^5 cells per well, treated with IMQ for

24 h, and then EdU labeled for 24 h. EdU-positive cells indicate a red color. Scale bar = 100 μm. (E) The number of EdU-positive cells was counted in four images from each independent experiment, expressed as a percentage of total cells. Data are shown as mean ± SEM. ***; p<0.001. IMQ, imiquimod; RORα, Retinoic acid receptor-related orphan receptor α.

Figure 4. Reduced activation of IMQ-induced inflammatory signals in *RORα*-deficient keratinocyte cells.

(A) Primary keratinocytes from the skin of newborn *Rorα*$^{+/+}$ and *Rorα*$^{\Delta/\Delta}$ mice were treated with 200 μM IMQ for 24 h, and cytokine levels were determined using Luminex-based multiplex assay from keratinocyte cell lysates (n=3, three mice for each group). Data are presented as mean ± standard error of the mean (SEM). *; p<0.05, **; p<0.01, ns; not significant. (B, C) Primary keratinocytes prepared as in (A) were subjected to immunoblot analyses to evaluate the activation (phosphorylation) of NF-κB (B) and STAT3 (C). Equal protein loading was confirmed with GAPDH. (D, E) Knockout of the *RORα* gene was performed as described in Fig. 3C. HaCat cells were treated with 200 μM IMQ for 24 h and subjected to immunoblot analyses to evaluate the activation (phosphorylation) of NF-κB (D) and STAT3 (E). (F) Extracts of HaCat cells were immunoprecipitated with anti-STAT3 antibody and then blotted with antibodies indicated. (G) Control and *RORα* knockout HaCaT cells were treated with 200 μM IMQ for 24 h, and the expression of cytokine genes was evaluated by real-time PCR analysis. Data are presented as mean ± SEM. *; p<0.05, **; p<0.01, ***; p<0.001. IMQ, imiquimod; NF κB, nuclear factor-κB; RORα, Retinoic acid receptor-related orphan receptor α; STAT3, signal transducer and activator of transcription 3.

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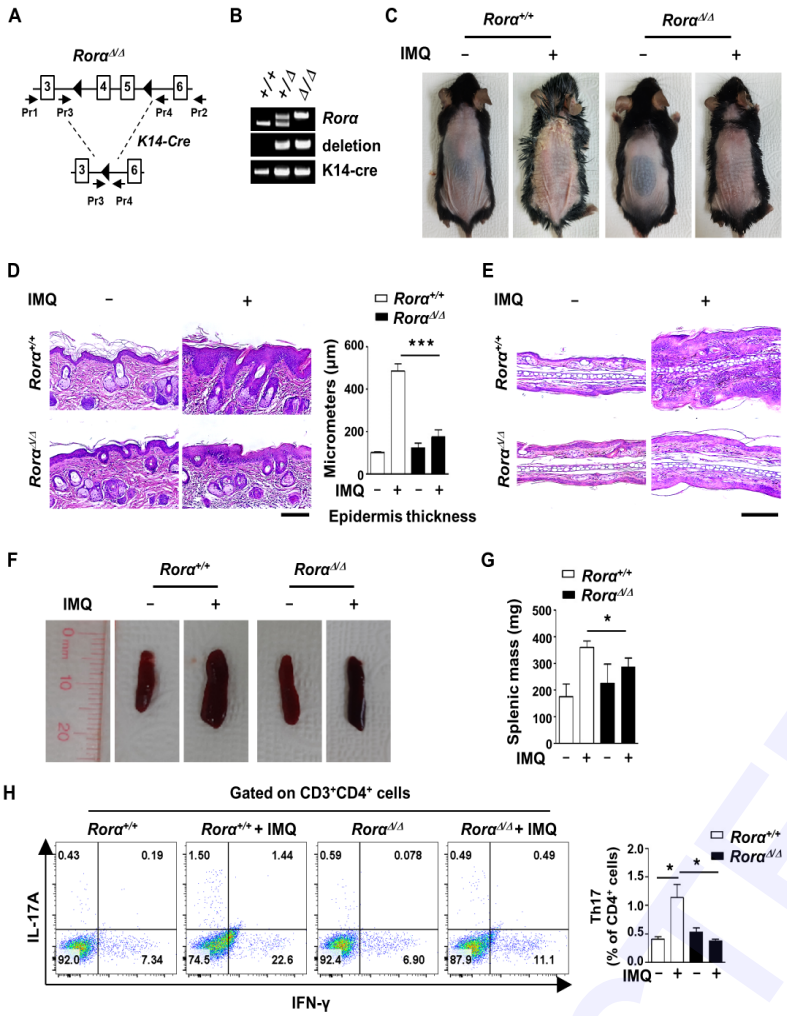
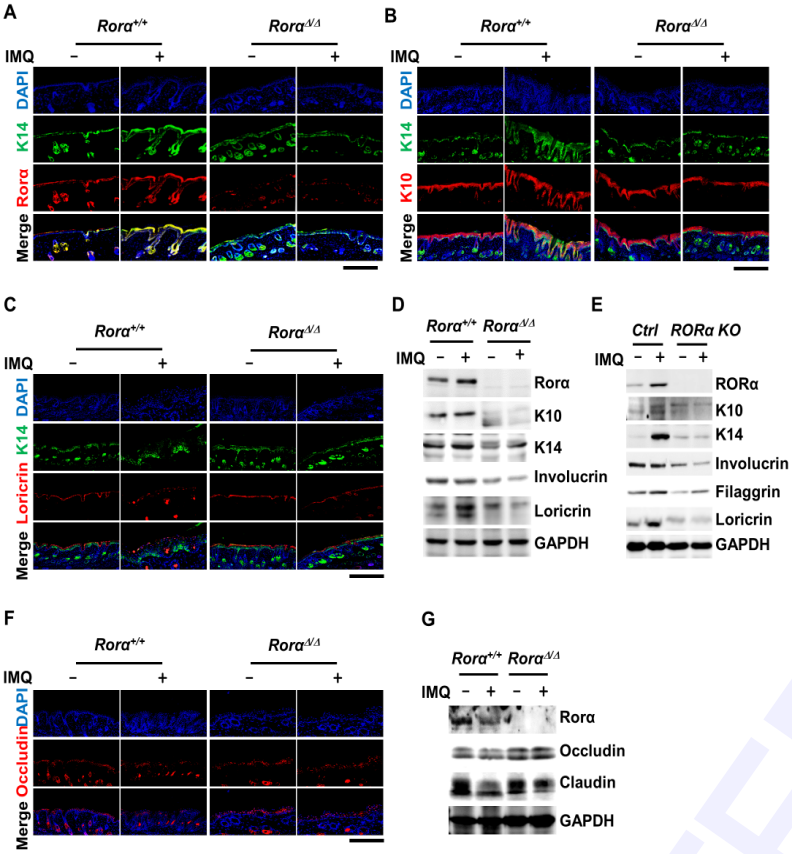


Fig. 1. Figure 1



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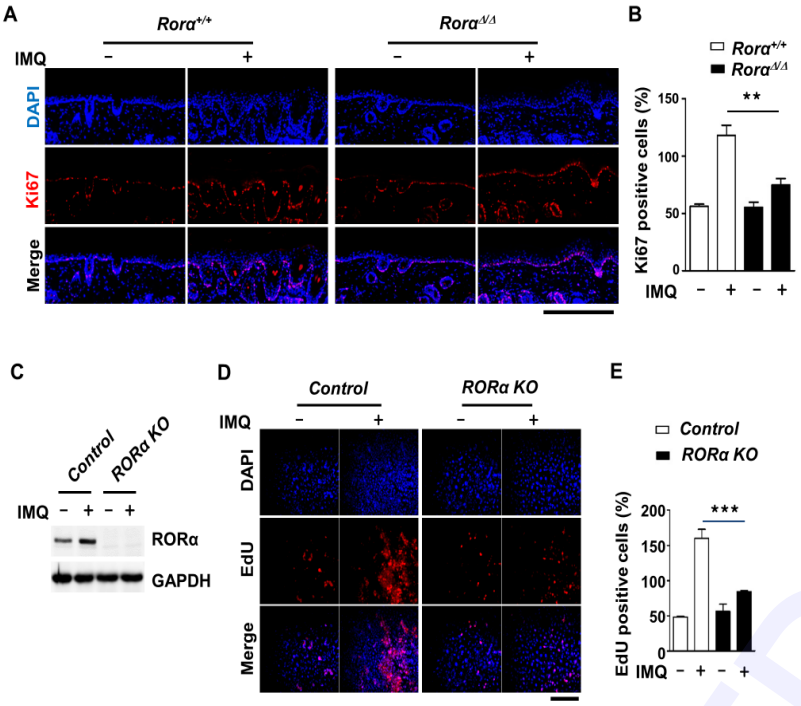

Fig. 3. Figure 3

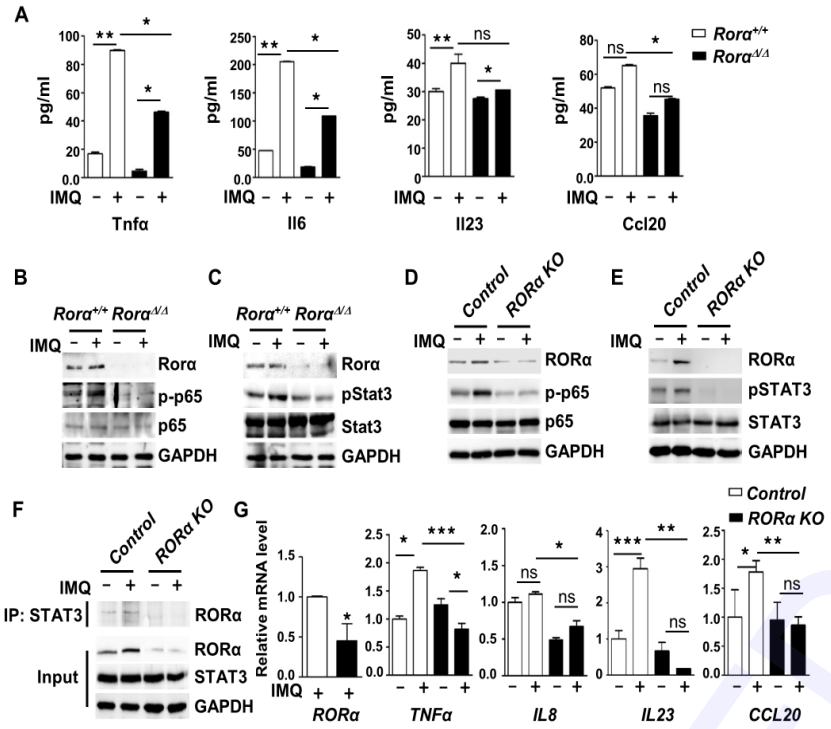

Fig. 4. Figure 4

SUPPLEMENTARY MATERIALS AND METHODS

Skin-specific deletion of the *Rorα* gene in mice

Rorα-floxed mice were provided by Professor Sung Hee Baek (Seoul National University, Republic of Korea) (1). Skin-specific deletion of *Rorα* was achieved by crossing *Rorα*-floxed mice with *K14-Cre* mice (Jackson Laboratory, USA), in which exons 4 and 5 of the *Rorα* gene were removed from keratinocytes. The mouse genotype was confirmed by PCR using specific primer sets for *Rorα* and *K14-Cre* (Supplementary Table 1). The mice were handled according to the Sookmyung Women's University Animal Handling Guidelines, and all experimental procedures were approved by the Institutional Animal Care and Use Committee of the University (approval number: SMWU-IACUC-1708-016-01).

Generation of IMQ-induced psoriasis-like condition

Eight-to-nine-week-old $Ror\alpha^{+/+}$ and $Ror\alpha^{\Delta/\Delta}$ mice were treated topically with 62.5 mg of Aldara cream (containing 5% IMQ), applied to the shaved backs and right or left ears daily for six days. Control mice were treated with the same dose of vehicle cream (Vaseline) for six days. Following a six-day treatment, mice were sacrificed, and the skin and spleen were harvested.

Hematoxylin and eosin (H&E) and immunofluorescence staining of mouse skin

Histological analyses of mouse skin were performed by Histoire (Seoul, Republic of Korea). For immunostaining, primary antibodies against the following proteins were used: Ki67, RORα, K14 (Abcam, Cambridge, UK), K10 (Covance, Princeton, USA), loricrin (BioLegend, San Diego, USA), claudin, and occludin (Invitrogen, Massachusetts, USA). Appropriate secondary antibodies labeled with Alexa Fluor 488 or 594 (Invitrogen, Carlsbad, CA, USA) were

subsequently applied, and sections were mounted on Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, USA).

Flow cytometry

Splenocytes were treated with Red Blood Cell (RBC) lysing buffer (Sigma-Aldrich, St. Louis, MO) for the depletion of RBCs. Cells were stimulated with 500 ng/ml PMA (Sigma-Aldrich), 0.5 μg/ml ionomycin (Sigma-Aldrich), and Brefeldin A (eBioscience, San Diego, CA) for 5 hours. Harvested cells were stained with CD3 (145-2C11; BD Biosciences, San Jose, CA), CD4 (RM4-5; BD Biosciences), and CD8 (53-6.7; eBioscience) antibodies at 4ºC for 30 min. For intracellular staining, the cells were treated with the Intracellular Fixation & Permeabilization Buffer (eBioscience) according to the manufacturer's protocol. The cells were then stained with IFN-γ (XMG1.2; BD Biosciences) and IL-17A (eBio17B7; eBioscience) antibodies. All samples were analyzed by FACSCantoII™ flow cytometer (BD Biosciences) and quantified by FlowJo V.10 software (Tree Star, Ashland, OR).

Antibodies for immunoblot analysis

Antibodies were purchased as follows. Antibodies against p65, involucrin, and GAPDH were purchased from Santa Cruz Biotechnology (Dallas, TX, USA); p-STAT3, STAT3, p-ERK, ERK, p-IKK, and p-p65 were from Cell Signaling Technology (Danvers, USA); RORα, MyD88, K14, and filaggrin were procured from Abcam (Cambridge, UK); K10 was obtained from Covance (Princeton, USA); loricrin was obtained from BioLegend (San Diego, California, USA); occludin and claudin were from purchased Invitrogen (Carlsbad, USA).

Culture of mouse primary keratinocytes

Primary mouse keratinocytes were prepared and cultured as described previously (2). Keratinocytes were isolated from the skin of embryos (E18.5~E19.5) and cultured in serum-free keratinocyte growth medium (Defined Keratinocyte SFM, Gibco, USA). Keratinocytes were stimulated with 200 μM IMQ for 24 h unless otherwise specified.

Measurement of cytokines in mouse primary keratinocyte lysates by Luminex analysis

Primary keratinocytes from the epidermis of *Rorα*$^{+/+}$ and *Rorα*$^{\Delta/\Delta}$ mice were cultured *in vitro*, with and without IMQ treatment. Cell lysates (25 μl) were collected from mouse primary keratinocytes and immediately frozen at −70ºC until analysis. Multiple cytokine analyses were performed using a Luminex kit (Merck Millipore, Milliplex MaP Mouse Th17 Magnetic Bead Panel) according to the manufacturer's instructions. Plates were measured using a Luminex 100/200 Milliplex Analyzer (Merck Millipore, Germany).

Knockout of the *RORα* gene in human keratinocytes using the CRISPR-Cas9 system

RORα was silenced in HaCaT human keratinocytes using the CRISPR-Cas9 gene editing system. Guide RNA sequences (Supplementary Table 2) were cloned into the pLentiCRISPR-E vector (Addgene #78852) according to the protocol presented by Addgene and introduced into HaCaT cells using lentiviral transduction. Lentiviruses were generated by transfecting guide RNA constructs (pLentiCRISPR-E-RORα #1 and pLentiCRISPR-E-RORα #2) and packaging plasmids (psPAX2 and pMD2.G) into 293T cells. After 2 days, the culture supernatant containing the virus was collected, and HaCaT cells were infected by centrifugation for 1 h at 3,000 rpm (Beckman Coulter Allegra X-15R) in the presence of 8 μg/ml polybrene. One day after viral infection, HaCaT cells were selected with puromycin at 1 μg/ml for 48 h. Unless indicated otherwise, HaCaT cells were stimulated with IMQ (200 μM)

for 24 h. Two guide RNAs showed nearly similar knockout efficiency in our experiments, and HaCaT cells knocked out by each guide RNA presented the same response to IMQ treatment. We presented the data obtained from guide RNA #1.

Cell proliferation assay

Proliferating cells were visualized by performing an EdU incorporation assay using Click-iT Plus EdU Cell Proliferation imaging kits (Invitrogen, USA). Briefly, cells were seeded in 12-well plates at a density of 1×10^5 cells/well. HaCaT cells were labeled with EdU for 24 h before fixation. After fixation and permeabilization, the cells were incubated with the Click-iT Plus reaction cocktail for 30 min at 25°C and stained with DAPI for nuclear visualization. EdU-positive cells were observed using a fluorescence microscope (Olympus IX71, Japan).

Quantitative real-time PCR analysis

Total RNA (0.5 μg) isolated using Trizol reagent (Invitrogen, USA) was converted to cDNA using a superscript first-strand cDNA synthesis kit (Fermentas, USA) according to the manufacturer's instructions. PCR amplification was performed using SYBR Green PCR Master Mix (Biosystems, Spain) with 10 pmol primers, 125 ng cDNA, and nuclease-free water according to the manufacturer's protocol. The samples were analyzed in triplicate for three separate runs. Primer sequences used in the present study are listed in Supplementary Table 3.

Statistical analysis

Statistical differences in test and control samples were determined non-parametrically using the Mann-Whitney test with GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). Data were pooled from three independent experiments, and results are presented as mean ± standard

error of the mean.

REFERENCES

1. Kim K, Boo K, Yu YS et al (2017) RORα controls hepatic lipid homeostasis via negative regulation of PPARγ transcriptional network. Nat Commun 8, 162
2. Park KC, Lee M, Jeon Y et al (2017) Skin-specific deletion of Mis18α impedes proliferation and stratification of epidermal keratinocytes. J Invest Dermatol 137, 414-421

Supplementary Table 1. Primer sequences for mouse genotyping

	Forward	Reverse
RORα	GCTTGGGGTTCTCCTACA	GCAGCAAGTGTGTCTCCCA
RORα deleted	CATAGCACCCAACACAGACGAG	CCTTTTGGCAAAGCGAGCCC
K14-Cre	GTCGATGCAACGAGTGATGA	TCATCAGCTACACCAGAGAC

Supplementary Table 2. Sequences for *RORα* CRISPR-Cas9 guide RNA

	Sequence
RORα CRISPR Guide RNA #1	TTACAGAAATGCCTTGCCGT
RORα CRISPR Guide RNA #2	AAGAGTGCCGTGGTCAATCA

Supplementary Table 3. Primer sequences for real-time qPCR analysis (human genes)

	Forward	Reverse
RORα	AGGGATGTCTCGAGATGCTGTAA	GCATACAAGCTGTCTCTCTGCTT
TNF-α	TCCTTCAGACACCCTCAACC	AGGCCCCAGTTTGAATTCTT
IL-8	GTCCTTGTTCCACTGTGCCT	GCTTCCACATGTCCTCACAA
Ccl20	AATCTGTGTGCGCTGATCCA	CCTTGGGCTGTGTCCAATTC
IL23	CCCGTATCCAGTGTGAAGATG	CCCTTTGAAGATGTCAGAGTCA
Keratin10	GGCAAAATCAAGGAGTGGTATG	GAAGCAGGATGTTGGCATTATC
Involucrin	ACTGAGGGCAGGGGAGAG	TCTGCCTCAGCCTTACTGTG
Filaggrin	GTTTCCAAACACATGGATCAAAT	TTTGAATCTTGTTGGTGTCTGTG
Loricrin	TCCTTCCCTCACTCATCTTCC	CTCCTCCACCAGAGGTCTTT
GAPDH	TGTTGCCATCAATGACCCCTT	CTCCACGACGTACTCAGCG