의학석사 학위논문

이미퀴모드로 유발된 건선 모델에서 인터루킨-22 수용체와 관련 인자 발현 조절을 통한 알로페론의 항염효과에 관한 연구

The anti-inflammatory effect of alloferon on imiquimod-induced psoriasis through the regulation of interleukin-22 receptor expression and its related factors

2023년 8월

서울대학교 대학원

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The anti-inflammatory effect of alloferon on imiquimod-induced psoriasis through the regulation of interleukin-22 receptor expression and its related factors

by

Yoojin Jang

A thesis submitted to the Department of Medicine in partial fulfillment of the requirements for the Degree of Master of Science in Medicine (Anatomy and Cell Biology) at Seoul National University College of Medicine

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ABSTRACT

The anti-inflammatory effect of alloferon on imiquimod-induced psoriasis through the regulation of interleukin-22 receptor expression and its related factors

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Psoriasis is a chronic inflammatory disease that affects 2-3% of the population worldwide. An increase of pro-inflammatory cytokines by excessive T cell activation is a leading cause of psoriasis, characterized by hyperproliferation of keratinocytes that thicken the skin with scaly plaque. Alloferon is a short peptide isolated from *Calliphora vicina*, which has been originally proposed as an anti-viral agent against infection with human papillomavirus (HPV). It also has an anti-inflammatory effect on skin inflammation, but little is known about its anti-inflammatory activity in psoriasis. It is known that interleukin (IL)-22, one of the pro-inflammatory cytokines, plays a role in the development of psoriasis. IL-22 receptor is composed of IL-22Rα and IL-10Rβ, and it is known that IL-22Rα is mainly expressed on the surface of keratinocytes. Therefore, I examined the regulatory role of alloferon on the development and progression of psoriasis in the

imiquimod (IMQ)-induced psoriasis mouse model through the regulation of IL-22Rα expression. I have found that alloferon decreased IL-22Rα expression in the psoriasis-like keratinocyte treated with tumor necrosis factor (TNF)-α. It was observed that alloferon decreased epidermal hyperplasia in the IMQ-induced animal model through the downregulation of IL-22Rα. Although there was no significant difference in redness, a representative symptom of psoriasis, when IMQ was treated and changes in wild-type and IL-22Rα knock-out (KO) mice were compared, a decrease in acanthosis due to the defect of IL-22Rα expression in IL-22Rα KO mice was confirmed. Through this, it was confirmed that IL-22Rα is closely involved in the deterioration of psoriasis symptoms. Interestingly, however, it was also observed that the expression, such as IL-1β, IL-19, IL-33, and β-defensin, was suppressed regardless of IL-22Rα expression when alloferon was treated on IL-22Rα KO mice. Taken together, alloferon is an effective potential drug for the treatment of psoriasis through the down-regulation of IL-22Rα expression and factors related to skin inflammation.

Keywords: IL-22Rα, Alloferon, Psoriasis, Imiquimod, IL-22Rα-deficient mice

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CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

IL: interleukin

TNF: tumor necrosis factor

KO: knock-out

IFN: interferon

Th: T helper

IF: immunofluorescence

PBS: phosphate buffered saline

CCK: cell cytotoxicity kit

PFA: paraformaldehyde

BSA: bovine serum albumin

H&E: hematoxylin and eosin

IHC: immunohistochemistry

RT: room temperature

ABC: avidin-biotin complex

IMQ: imiquimod

IL-22Rα: interleukin 22 receptor alpha

Allo: alloferon

CTRL: control

INTRODUCTION

Psoriasis is an immune-mediated chronic inflammatory skin disease that suffers 2-3% of people worldwide [1]. Psoriasis is characterized by redness, epidermal hyperplasia, and desquamation due to the hyperproliferation and abnormal differentiation of keratinocytes, accompanied by vascular hyperplasia and immune cell infiltration at skin lesions [2-4]. Although psoriasis is not an infectious skin disease, the appearance of the lesions creates prejudice. Thus, psoriasis is a disease that accompanies not only physical but also mental pain.

In psoriasis, excessively activated T cells infiltrate the skin and stimulate keratinocytes by secreting pro-inflammatory cytokines [5]. In turn, pro-inflammatory cytokines activate dendritic cells and activated dendritic cells secrete tumor necrosis factor (TNF)-α, interleukin (IL)-12, and, IL-23, which stimulate T helper cells to enhance the production of pro-inflammatory cytokines such as interferon (IFN)-γ, TNF-α, IL-22, and IL-17. These cytokines stimulate keratinocytes and the pathogenesis related to psoriasis begin. TNF-α binds to the TNF receptor of keratinocytes to cause an inflammatory response and promote the pathogenesis of psoriasis by activating T cells to promote the secretion of inflammatory cytokines through the signaling pathway of NF-κB [6]. IL-23 induces the development and activation of T helper (Th) 17 cells, which secrete IL-17. IL-17 causes keratinocytes to secrete inflammatory

cytokines and induces hyperplasia of keratinocytes [7]. It is widely known that hyperproliferative keratinocytes produce more excessive pro-inflammatory cytokines to induce chronic inflammation [5, 8-10]. Therefore, it could be a therapeutic strategy to alleviate psoriasis to reduce keratinocyte proliferation or excessive inflammation by inflammatory cytokines [11, 12].

In the case of mild psoriasis patients, topical treatments with steroids and vitamin D analogues by cream are the most common treatment [13]. However, for patients with severe symptoms, systemic treatments such as phototherapy or biological treatments are chosen as common treatments. Biological treatments have been developed to prevent the progression of lesions in psoriasis by blocking the cytokine function to prevent an inflammatory signaling cascade [14]. There are four kinds of biological agents, blocking the function of TNF-α, IL-12/23, IL-17, and IL-23, that were used to treat psoriasis [8, 15-18]. However, there are limitations in that treatments are highly expensive and treatments are administered to patients through intramuscular or subcutaneous injection, not cream or ointment.

To compensate for the limitations of biological agents, I focused on alloferon as an adjuvant on the therapy for psoriasis. Alloferon, an immune modulator, is a short peptide isolated from the blood of the *Calliphora vicina* larvae after being infected with bacteria [19-21]. Alloferon was originally developed as a treatment for human papillomavirus (HPV) [22], but several

studies revealed that it also has anti-tumor, anti-viral, and anti-inflammatory activity in asthma, influenza, ulcerative colitis, and pancreatic cancer [19, 20, 23-29]. In the previous study, alloferon inhibited inflammatory responses in the skin exposed to UVB through the suppression of pro-inflammatory cytokines, IL-1α, IL-1β, IL-6, and IL-18 [25].

IL-22 is a pro and anti-inflammatory cytokine, a member of the IL-10 cytokine family [30]. It is known that IL-22 induces the differentiation and proliferation of keratinocytes, leading to hyperplasia of the epidermis [30-32]. As mentioned earlier, IL-22 plays a role in the pathogenesis of psoriasis, but a treatment for the regulation of the activity of IL-22 has not yet been developed. IL-22 is secreted by IL-23, stimulating Th 17 cells, which is known to play an important role in psoriasis pathogenesis [33-40]. In addition, elevated IL-22 concentrations were measured in plasma from psoriasis patients and IL-22 levels correlated positively with the severity of the disease [33, 36-38, 41]. IL-22 binds to the heterodimeric receptor, which consists of IL-22Rα and IL-10Rβ [33, 40]. IL-22/IL-22Rα complex leads to the activation of Janus kinase, followed by the activation of signal transducer and activator of transcription 3 (STAT3) [42]. IL-10Rβ is expressed everywhere, but in the case of IL-22Rα, it is known that it is widely expressed in epidermis. Thus, to control the biological process of IL-22, it is necessary to regulate the expression of IL-22Rα. Therefore, regulation of IL-22Rα has the potential for a therapeutic strategy for

the treatment of psoriasis.

Imiquimod (IMQ) is generally used to induce psoriasis in a mouse model. Because it is an agonist of toll-like receptor 7 (TLR7) [12, 39, 43-47], it occurs inflammation like psoriatic pathogenesis on mouse skin. It is known that psoriasis is a unique disease that occurs only in human, not in animals. Since psoriasis is caused by various pro-inflammatory cytokines secreted by activated T cells, psoriasis induced by IMQ in animal experiments mimicked the pathogenesis of psoriasis in human. Therefore, I investigated the anti-inflammatory effect of alloferon in IMQ-induced psoriasis model through the regulation of IL-22Rα and inflammation-related factors in this study.

MATERIALS AND METHODS

Cell culture Human skin keratinocyte cell line, HaCaT was provided by Dr. N.E. Fusenig, DKFZ, Heidelberg, Germany. HaCaT was maintained in RPMI 1640 medium (WELGENE, Kyungsan, Korea) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin; WELGENE) at 37°C in a humidified 5% CO_2 incubator.

CCK-8 assay To examine the cytotoxicity of alloferon and TNF-α, the CCK-8 assay was performed. HaCaT (7 x 10^3 cells/well) was seeded in a 96-well culture plate and stabilized for overnight. Cells were washed with phosphate-buffered saline (PBS) at one time and then treated with 100 μl of TNF-α (10 and 20 ng/ml) (Peprotech, East Windsor, NJ, USA), that diluted in serum-free RPMI 1640 and incubated another for 24 hrs at 37°C in a humidified 5% CO_2 incubator. After incubation for 24 hrs, media was aspirated, and then 100 μl of alloferon (2 and 4 μg/ml) that diluted serum free-RPMI 1640 for 24 hrs at 37°C in a humidified 5% CO_2 incubator. To examine the cell proliferation, media was suctioned and then 100 μl of PBS containing 10 μl of CCK-8 reagent (Cell proliferation & cytotoxicity assay kit, EZ-Cytox; Dogen, Seoul, Korea) was added to each well of the plate. The plate was incubated for 1 hr at 37°C

away from the light. The absorbance was measured at 450 nm using the SpectraMax iD3 and normalized with Softmax Pro software (Molecular Devices, San Jose, CA, USA). The cell viability ratio was calculated from the following equation: % cell viability = (absorbance of test group/ absorbance of control) x 100.

Animal experiment All mice were maintained in a specific pathogen-free condition at the Seoul National University College of Medicine animal facility (IACUC no. SNU-220921-6-3). ICR mice were purchased from OrientBio (OrientBio Inc., Seongnam, Korea). IL-22Rα knock-out (KO) mice were purchased from Jackson Laboratories (stock no. 031003, Strain: C57BL/6J; Jackson Laboratory, Sacramento, CA, USA). C57BL/6J mice maintained internal reproduction at the animal facility as wild-type (WT). ICR mice (male, 12 weeks old) were randomly divided based on weights into 3 groups (n=5): normal control + vehicle cream, IMQ (83.3 mg/day) + vehicle cream, IMQ + alloferon (2 μg/ml/day). WT and IL-22Rα KO mice (male, 5 months old) were randomly divided into 4 groups (n=4): WT normal control, WT + IMQ, IL-22Rα KO normal control, IL-22Rα KO + IMQ. In addition, IL-22Rα KO mice (male, 5 months old) were divided into 3 groups (n=4): normal control + vehicle cream, IMQ + vehicle cream, IMQ + alloferon. The dorsal hair of the mice was shaved on day 0. IMQ (a topical dose of 83.3 mg 5% Aldara cream; 3M

Pharmaceuticals, Long Beach, CA, USA) was applied on the dorsal area and ear daily starting from day 1 to day 7. Ear thickness is measured every day with vernier calipers. Treatment group mice were applied vehicle cream or alloferon cream every day after 6 hrs of IMQ application. After 7 days, mice were sacrificed and dorsal skin and ear tissues were collected. Animal experiment was performed in three independent experiments.

Immunofluorescence (IF) analysis To examine the expression of IL-22Rα on HaCaT, immunofluorescence analysis was performed. HaCaT (4×10^4 cells/well) was seeded on 12 mm coverslips in a 24-well plate and stabilized for overnight. Cells were washed with PBS at one time and then treated with TNF-α 20 ng/ml that was diluted in serum-free media for 24 hrs at 37°C in a humidified 5% CO_2 incubator. After incubation, media was aspirated, alloferon 2 and 4 μg/ml diluted serum free-RPMI 1640 and incubated for another 24 hrs. After incubation, cells were washed twice with PBS and fixed with 4% paraformaldehyde (PFA) at 4°C for 15 mins. Cells were washed twice with 0.5% bovine serum albumin (BSA) in PBS for 5 mins each time and permeabilized 0.3% Triton X-100 in PBS for 15 mins at room temperature (RT). After washing with washing buffer (0.5% BSA, 0.3% Triton X-100 in PBS), cells were incubated with blocking buffer (0.5% BSA, 0.3% Triton X-100, 5% normal goat serum in PBS) for 1 hr at RT. Cells were incubated with rabbit-developed

anti-human IL-22Rα (1:150; Abcam, Cambridge, UK) diluted with blocking buffer for 2 hrs at RT. After washing cells with washing buffer 3 times and incubated with a goat-developed anti-rabbit IgG conjugated with Alexa fluor 488 (1:1500, Invitrogen, Carlsbad, CA, USA) as a secondary antibody diluted with blocking buffer for 30 mins at RT. After washing with washing buffer 3 times, cells were mounted with a mounting medium and counter stained with 4',6-diamidino-2-phenylindole (DAPI; ImmunoBioscience, Mukilteo, WA, USA). Signals were visualized by fluorescence microscopy (EVOS M5000, Invitrogen). Celleste 5 software (Invitrogen) was used for measuring fluorescent intensity.

Histological analysis To examine the epidermal thickness and the infiltration of immune cells in the dermis, hematoxylin and eosin (H&E) staining was performed. Dorsal skin and ear tissues were fixed in 4% PFA at 4°C for overnight. Tissues were dehydrated and embedded in paraffin. Embedded tissues were sectioned with 4 μm thickness. After deparaffinization and hydration, the sections were stained with hematoxylin and eosin. Tissue sections were mounted (Sigma, St. Louis, MO, USA) and images were acquired on a microscope (EVOS M5000, Invitrogen). Epidermal thickness was measured in three parts per image by Celleste 5 software (Invitrogen).

Immunohistochemistry (IHC) To examine the expression of IL-22Rα on mice dorsal skin, immunohistochemical analysis was performed. Dorsal skin tissues were fixed in 4% PFA at 4°C for overnight. Tissues were dehydrated and embedded in paraffin. Embedded tissues were sectioned with 4 μm thickness. After deparaffinization and hydration, an epitope of the antigen was retrieved by microwave heating with citrated buffer (pH 6.0). Endogenous peroxidase was blocked the treatment with using 0.3% H_2O_2. The sections were blocked with 5% goat serum in PBS for 1 hr at RT, and the sections were incubated with rabbit-developed anti-mouse IL-22Rα antibody (1:250, Millipore, St. Louis, MO, USA) for overnight at 4℃ in the humid chamber. After incubation, the sections were incubated with biotinylated goat-developed anti-rabbit IgG for 1 hr at RT and then Avidin-Biotin Complex (ABC) solution (Vector Laboratories, Burlingame, CA, USA) was treated on the sections for 40 mins at RT. DAB kit (Vector Laboratories) was used for chromogenic detection. And then hematoxylin was used as counterstaining. After dehydration and clearing, the sections were mounted using a mounting medium (Sigma). Images were acquired on a microscope (EVOS M5000, Invitrogen).

Microarray analysis Gene expression by IL-22Rα KO mice treated with IMQ and alloferon was analyzed using Affymetrix GeneChip® Mouse Gene 2.0 ST arrays. The array chip has more than 33,000 probe sets of gene level. Total

mRNA was extracted from the dorsal skin using TRIzol (Invitrogen). Hundred nanogram of each RNA sample was subjected to the Affymetrix analytical procedure, as recommended by the manufacturer. The data were summarized and normalized with a robust multi-average (RMA) method implemented in Affymetrix® Power Tool. Gene-Enrichment and Functional Annotation analysis for a significant probe list was performed using Gene Ontology. All data analysis and visualization of differentially expressed genes were conducted using R 3.3.2.

Statistical analysis Experimental data are presented as mean ± standard deviation (SD). Comparisons between three or more groups were used one-way ANOVA. P values less than 0.05 were used to indicate a statistically significant difference. Statistical analysis was carried out using GraphPad InStat version 8.0.2 (GraphPad Software, La Jolla, CA, USA).

Results

1. Alloferon decreased the expression of IL-22Rα in TNF-α induced psoriasis-like model in HaCaT

Even though several cytokines, such as TNF-α, IFN-γ, IL-23, and IL-17 are involved in psoriasis pathology [5], TNF-α particularly plays an important role in. TNF-α is used to stimulate the human keratinocyte cell line, HaCaT, to establish a psoriasis-like model in various studies [48-50]. To determine the optimal concentration of TNF-α on the induction of psoriasis like model in HaCaT and the cytotoxicity of alloferon and TNF-α, CCK-8 assay was performed. As shown in Figure 1, there was no cytotoxic effect with TNF-α (10 and 20 ng/ml) and alloferon (2 and 4 μg/ml). Therefore, I used TNF-α at 20 ng/ml in the subsequent experiment.

Next, I investigated IL-22Rα expression in TNF-α treated psoriasis-like model in HaCaT in the presence or absence of alloferon by immunofluorescence staining (Figure. 2A). The relative fluorescent intensity was measured by Celleste and presented as a graph in Figure 2B. As shown in Figure 2A, IL-22Rα expression in TNF-α-treated HaCaT increased when it compared to control. And this increase was suppressed when the cells were simultaneously treated with alloferon.

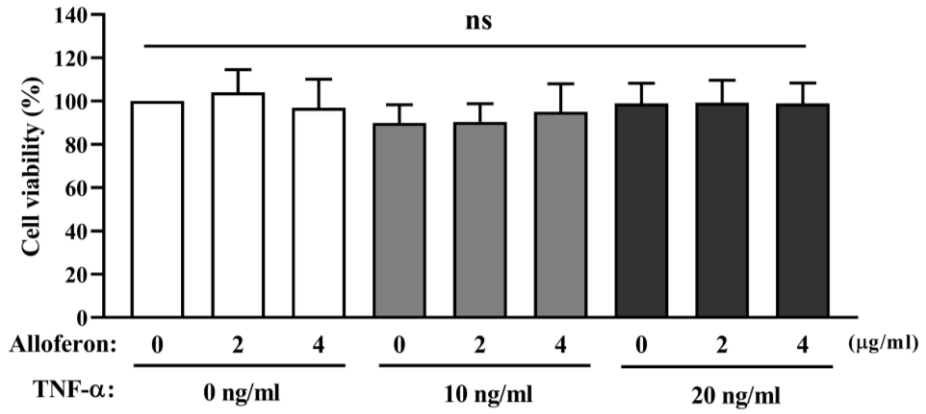


Figure 1. Examination of cytotoxic effect of TNF-α and alloferon on HaCaT: CCK-8 assay

HaCaT (7 x 10^3 cells/well) was seeded in the 96-well plate and treated with 10 or 20 ng/ml of TNF-α for 24 hrs. And then alloferon was treated with 2 or 4 μg/ml for 24 hrs. Cytotoxic effect was measured by CCK-8 assay as described in materials and methods. Data were collected from three independent experiments and presented as mean ± SD. One-way ANOVA with Tukey's multiple comparisons test was performed. ns; non significance

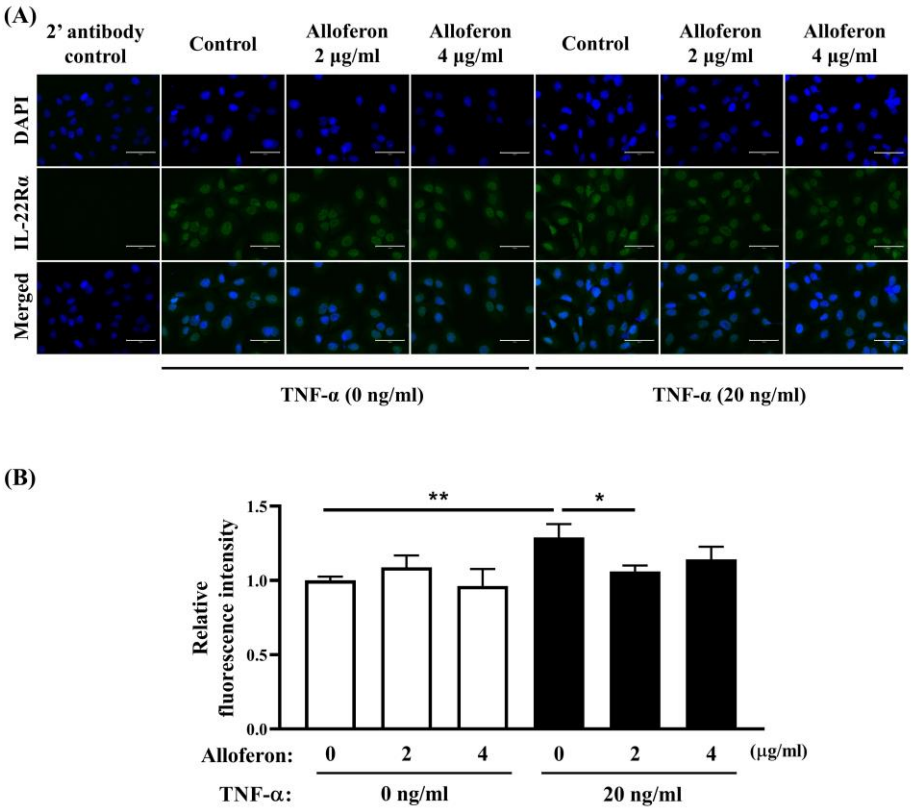


Figure 2. Down-regulation of IL-22Rα expression on TNF-α-induced psoriasis-like HaCaT and its down-regulation by the treatment of alloferon: IF staining

HaCaT (4 x 10^4 cells/well) was seeded on 12 mm coverslips in the 24-well plate and treated with 0 ng/ml or 20 ng/ml of TNF-α for 24 hrs. Then, alloferon was added at a concentration of 2 or 4 μg/ml for another 24 hrs. (A) And then the expression of IL-22Rα was examined using of Alexa fluor 488 (green) conjugated anti-IL-22Rα antibody as described in materials and methods. Nuclei were counter-stained with DAPI (blue). Images were acquired

at 400x magnification. (B) Relative comparison of IL-22Rα expression changes in TNF-α or alloferon-treated experimental group to the control group. One-way ANOVA with Tukey's multiple comparisons test was performed. Data were collected from three independent experiments and presented as mean ± SD. $^{**}P<0.01$; $^{*}P<0.1$.

2. Alloferon alleviated psoriatic symptoms in IMQ-induced psoriasis mouse model

Based on previous results in several reports regarding anti-inflammatory effect of alloferon on skin disease, I examined the therapeutic effect of alloferon on psoriasis in IMQ-induced psoriasis mouse model. ICR mice were topically applied with alloferon 6 hrs after imiquimod (IMQ) treatment on the shaved dorsal skin and ears for 7 days. The experimental scheme is shown in Figure 3A. It was observed that psoriasis accompanied with redness and scales were severely developed by IMQ treatment (Figure 3B). However, it was remarkably suppressed by the treatment of alloferon (Figure 3B). Next, histological changes by alloferon treatment on IMQ-induced psoriatic skin lesion were examined by H&E staining. I found that epidermal thickness of the dorsal skin increased by IMQ treatment with a value of 86.16 ± 13.21 μm, compared to that of control mice which was 19.63 ± 3.30 μm-thickness. However, the thickness of the dorsal skin dramatically decreased by the treatment of alloferon; 44.25 ± 4.01 μm (Figure 3C, D). Ear thickness was also measured every day during the experiment. As shown in Figure 3E, IMQ significantly increased ear thickness, but I could not find any changes with the treatment of alloferon (Figure 3C, E).

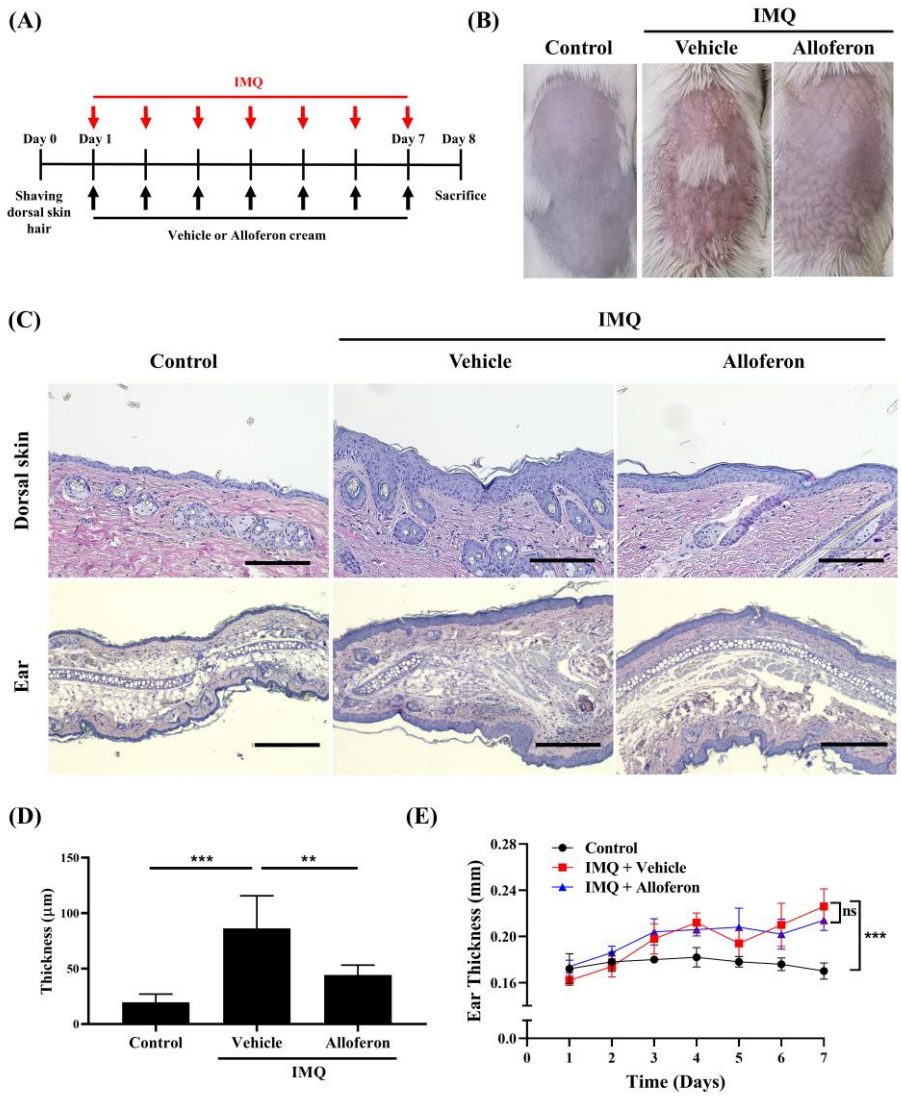


Figure 3. The effect of alloferon in IMQ-induced psoriasis mice

ICR mice (male, 12 weeks old) were used in this experiment. Mice were divided based on weights into 3 groups (n=5): control + vehicle cream, imiquimod (IMQ; 83.3 mg/day) + vehicle cream, IMQ + alloferon (2 μg/ml/day). (A) All the experimental schedules were conducted as follows. The day before starting the experiments, day 0, the dorsal skin of all mice was

shaved. IMQ was applied with the dorsal skin and ear every day starting from day 1 to day 7. Treatment group mice were applied vehicle cream or alloferon cream 6 hrs after IMQ application. All mice were sacrificed on day 8. (B) The images to examine the effect of alloferon on IMQ-induced psoriasis on day 7, the last day of the experiment. (C) The skin and ear tissues were fixed in 4% PFA at 4°C. Paraffin-embedded tissues were sectioned with 4 μm thickness. The sections were stained using hematoxylin and eosin. Scale bar, 150 μm. (D) The epidermal thickness of dorsal skin measured in three parts per histological sections by Celleste 5 software. (E) The ear thickness measured every day with vernier calipers. One-way ANOVA with Tukey's multiple comparisons test was performed; ***P<0.0001, **P<0.001, **P<0.01, ns; not significance

3. Alloferon decreased the expression of IL-22Rα on IMQ-induced psoriatic skin lesions

As shown in Figure 2A, alloferon down-regulated IL-22Rα expression in TNF-α-induced psoriasis-like HaCaT. So, I examined whether alloferon could also suppress the IL-22Rα expression in skin lesions of IMQ-treated mice. IL-22Rα expression is increased by the treatment of IMQ, but the increased expression is remarkably suppressed by the treatment of alloferon (Figure 4). In contrast, alloferon-treated mice decreased IL-22Rα expression compared to IMQ-treated mice.

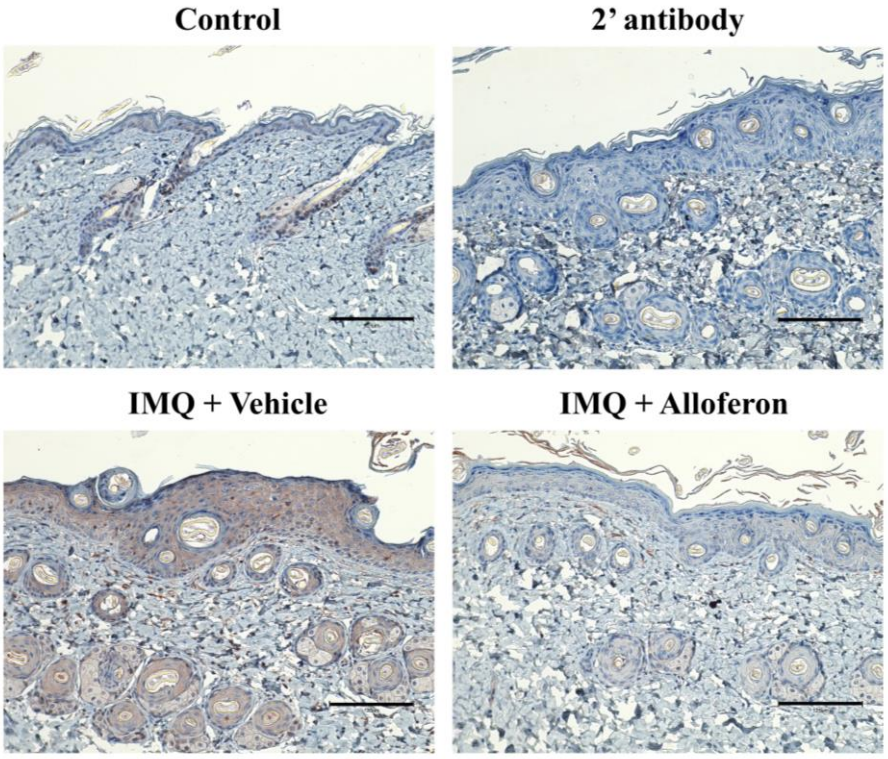


Figure 4. Suppressive effect of alloferon IMQ-induced IL-22Rα expression: IHC staining

The skin was fixed in 4% PFA at 4℃ for overnight and embedded in paraffin. The tissues were sectioned with 4 μm thickness. The sections were incubated with anti-IL-22Rα antibody (1:250) for overnight at 4℃ in a humified chamber. After incubation, the sections were incubated with biotinylated goat anti-rabbit IgG antibody (1:150) for 1 hr at RT. ABC complex was treated for 40 mins color reaction was performed using DAB kit. Scale bar, 125 μm.

4. IL-22Rα-deficient mice showed reduced skin peeling in IMQ-induced psoriatic skin lesions

Since previous data showed IL-22Rα expression was down-regulated and psoriatic symptoms were decreased by treatment of alloferon, I investigated the psoriasis pathogenesis using IL-22Rα knock-out (KO) mice. Mice were treated with IMQ for inducing psoriasis dermatitis. As shown in Figure 5A, the scales and plaques were decreased but the redness remained in IMQ-treated IL-22Rα deficient mice compared to IMQ-treated wild-type (WT) mice. H&E staining showed that epidermal thickness was reduced in IL-22Rα KO mice compared to WT, being 57.97 ± 7.22 μm in KO mice and 81.24 ±5.06 μm in WT mice respectively (Figure 5B, C). However, ear thickness was not changed in IL-22Rα KO mice compared to WT when treated with IMQ (Figure 5B, D).

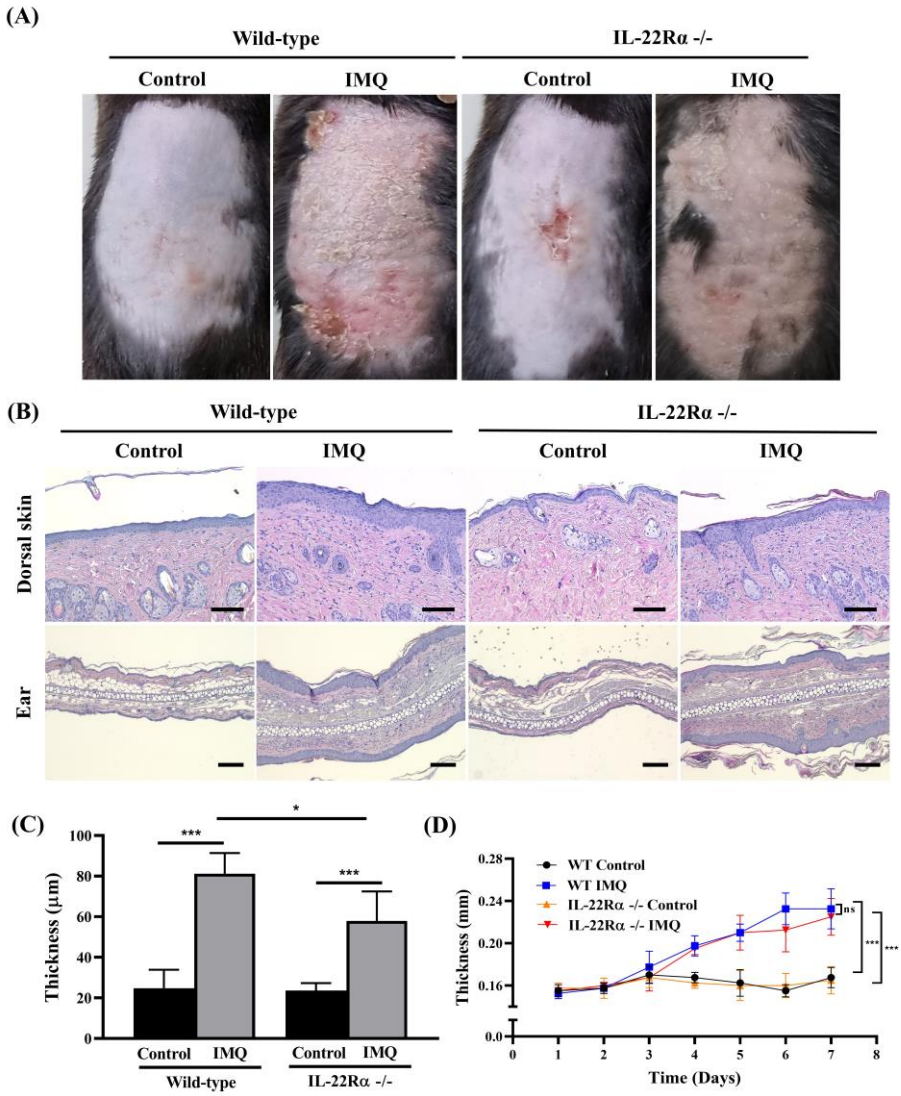


Figure 5. Differences of skin lesions in IMQ-treated WT and IL-22Rα KO mice

WT and IL-22Rα KO mice (male, 5 months old) were used in this experiment. Mice were divided into 4 groups (n=4): WT control, WT IMQ, IL-22Rα KO control, IL-22Rα KO IMQ. (A) The images were taken on day 7, the last day of the experiment. (B) The dorsal skin and ear tissues were fixed in 4%

PFA at 4°C. The paraffin-embedded tissues were sectioned with 4 μm thickness. Sections were stained using hematoxylin and eosin. Skin tissue's scale bar, 100 μm, and ear tissue's scale bar, 150 μm. (C) The epidermal thickness of the dorsal skin measured in three parts per histological sections by Celleste 5 software. (D) The ear thickness measured every day measured with vernier calipers. Two-way ANOVA with Sidak's multiple comparisons test was performed; *P<0.05; ***P<0.0001; ns, not significance

5. Alloferon effectively inhibited IMQ-induced skin inflammation related to psoriasis symptoms in IL-22Rα deficient mice

To investigate the therapeutic effect of alloferon in IL-22Rα depletion, alloferon was treated in IMQ-induced psoriatic IL-22Rα deficient mice. As shown in Figure 6A, alloferon reduced psoriatic symptoms, especially redness, in IL-22Rα KO mice compared to IMQ only-treated mice. H&E staining showed increased immune cell infiltration and epidermal thickness in IMQ-treated mice compared to the control group with 23.79 ± 2.80 μm-thickness. However, alloferon treatment effectively decreased epidermal thickness and immune cell recruitment, with a value of 34.02 ± 2.46 μm, while IMQ-treated mice exhibited 60.33 ± 4.19 μm-thickness (Figure 6B, C). The ear thickness showed the same results with changes in the skin (Figure 6B, D).

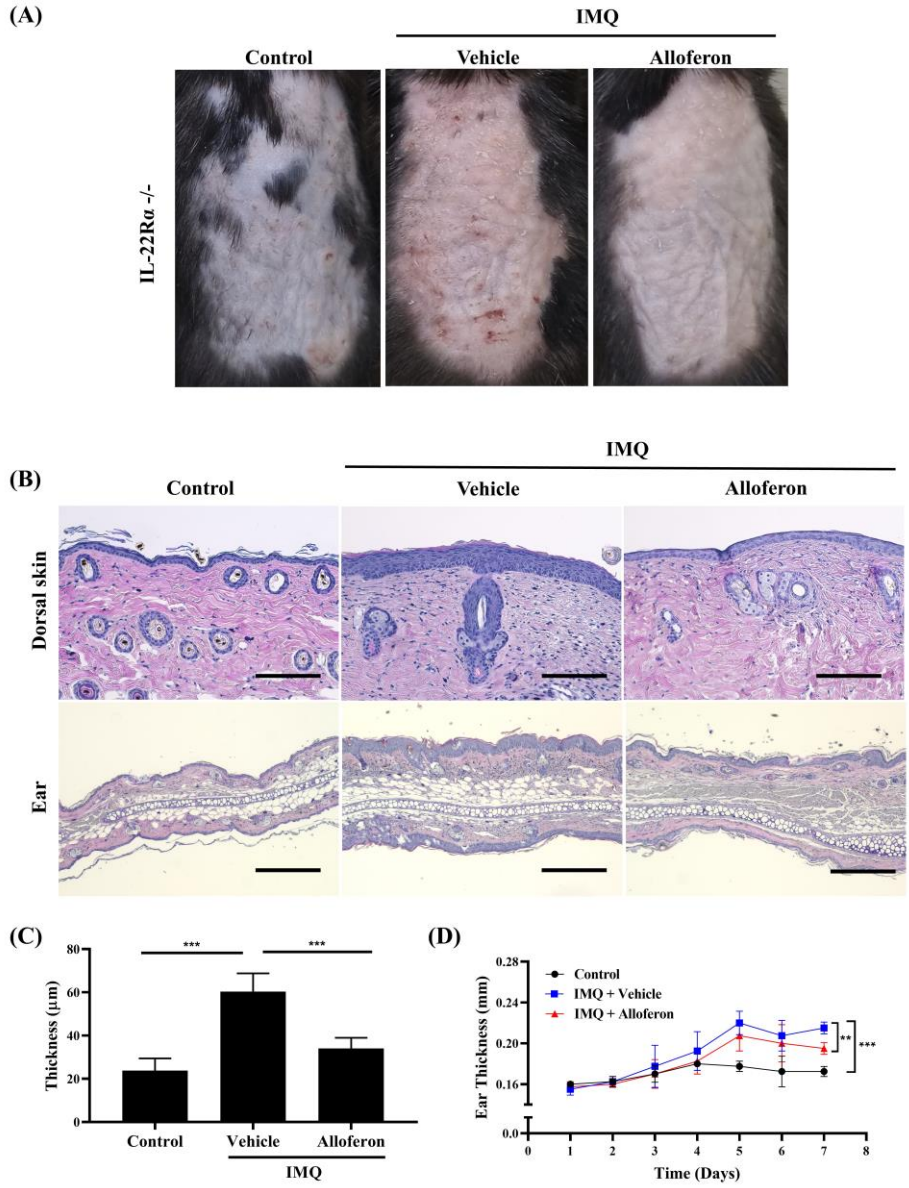


Figure 6. The effect of alloferon in IMQ-induced psoriasis IL-22Rα KO mice skin and ear

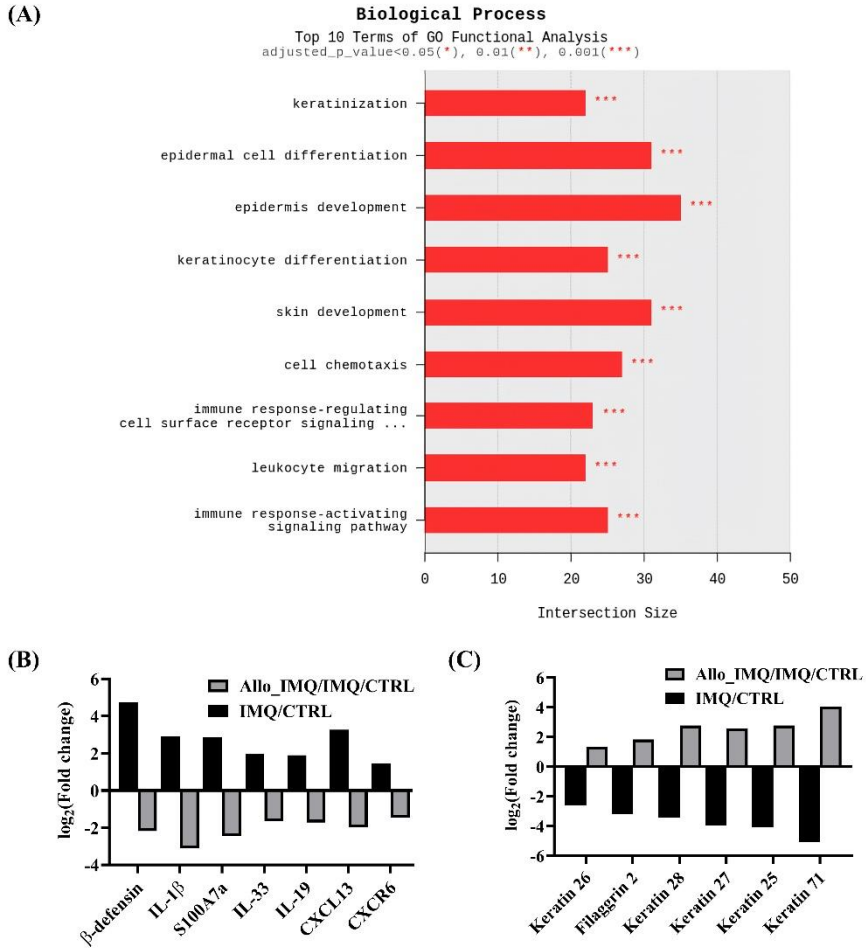
IL-22Rα KO mice (male, 5 months old) were used in this experiment. Mice were divided into 3 groups (n=4): control + vehicle cream, IMQ + vehicle

cream, IMQ + alloferon (2 μg/ml). (A) The images to examine the effect of alloferon on IMQ-induced psoriasis on day 7, the last day of the experiment. (B) The dorsal skin and ear tissues were fixed in 4% PFA at 4°C. The paraffin-embedded tissues were sectioned with 4 μm thickness. The sections were stained using hematoxylin and eosin. Scale bar, 150 μm. (C) The epidermal thickness of the dorsal skin measured in three parts per histological sections by Celleste 5 software. (D) The ear thickness measured every day with a vernier caliper. One-way ANOVA with Tukey's multiple comparisons test was performed; ***P<0.001, ns; not significance

6. Alloferon regulated psoriasis pathogenesis-related genes in IL-22Rα-deficient mice

Microarray analysis was performed to investigate whether alloferon regulated other factors related to inflammation in the absence of IL-22Rα.

Gene ontology (GO) analysis sorted all data by biological process and showed the top 10 terms of functional classification. Alloferon regulated psoriasis symptoms-related genes level, such as keratinization, epidermal cell differentiation, and keratinocyte differentiation. Moreover, alloferon regulated psoriasis pathology-associated genes level, such as cell chemotaxis, leukocyte migration, immune response-regulating cell surface receptor, and immune response-activating signaling pathway (Figure 7A). As shown in Figure 7B and 7D, genes related to psoriatic inflammation and pathology, such as β-defensin, IL-1β, S100A7a, IL-33, IL-19, CXCL13, and CXCR6 were upregulated by IMQ treatment, but alloferon reduced them. Also, genes related to skin barriers, such as filaggrin and keratin were decreased by IMQ administration, but alloferon increased them (Figure 7C, E).



(D)

Gene	Fold change	
	IMQ/Control	IMQ_Allo/IMQ
β-defensin 3	26.54	-4.54
β-defensin 14	9.84	-2.55
IL-1β	7.62	-8.62
S100A7a	7.37	-5.44
IL-33	3.88	-3.11
IL-19	3.72	-3.28
CXCL13	9.65	-3.90
CXCR6	2.75	-2.73

(E)

Gene	Fold change	
	IMQ/Control	IMQ_Allo/IMQ
Keratin 26	-2.58	1.35
Filaggrin 2	-3.19	1.84
Keratin 28	-3.41	2.77
Keratin 27	-3.98	2.56
Keratin 25	-4.10	2.78
Keratin 71	-5.07	4.06

Figure 7. Gene ontology (GO) analysis of alloferon-treated IL-22Rα-deficient mice bearing IMQ-induced psoriasis: microarray analysis

Gene expression by IL-22Rα KO mice treated with IMQ and alloferon was analyzed using Affymetrix GeneChip® Mouse Gene 2.0 ST arrays. Total

mRNA was extracted from the dorsal skin using TRIzol (Invitrogen). A 100 ng of each RNA sample was subjected to the Affymetrix procedure, as recommended by the manufacturer. (A) The biological process-related mRNA was compared between IMQ-treated and alloferon and IMQ co-treated IL-22Rα KO mice. (B, C) Data were presented as $\log_2$ fold change. (E, F) Data presented specific number of fold change in genes.

DISCUSSION

Psoriasis is an immune-mediated skin inflammation disease, which is exacerbated by pro-inflammatory cytokines caused by excessive activation of T cells [5]. Alloferon, an immune modulator, was revealed therapeutic effect on asthma and ulcerative colitis by regulating inflammation factors [21]. Also, alloferon alleviated UVB-induced skin inflammation by reducing pro-inflammatory cytokines such as IL-1α, IL-1β, IL-6, and IL-18 [25]. Therefore, this study was conducted whether alloferon, which regulates immune related factors, would relieve psoriasis by regulating inflammatory factors secreted by T cells. In particular, IL-22 is known to play a role in the pathology of psoriasis [40], but the pathology of psoriasis with increased IL-22Rα expression is unknown. If the expression of the receptor to which IL-22 is bound is reduced, the signaling of IL-22 is not initiated, so it is important to regulate the expression of IL-22Rα. Therefore, this study was conducted on the assumption that alloferon, an immune modulator, would regulate the expression of IL-22Rα to prevent the pathology of psoriasis.

A previous study demonstrated that exposure to UVB radiation resulted in the induction of inflammation in the skin, accompanied by an upregulation of IL-22Rα expression in keratinocytes [51]. Therefore, I investigated the up-regulation of IL-22Rα in a psoriasis model upon

inflammation. In both a TNF-α-induced psoriasis-like HaCaT model and an IMQ-induced psoriasis mouse model, I observed an increase in IL-22Rα expression through immunofluorescence and immunohistochemistry (Figure 2, 4). Furthermore, I obtained the results that alloferon reduced IL-22Rα expression both in in vitro and in vivo psoriasis model (Figure 2, 4). In animal experiments, I also observed alleviation of hyperplasia, a characteristic symptom of psoriasis, indicating that alloferon mitigated psoriasis symptoms (Figure 3). These results suggested that alloferon ameliorates IMQ-induced hyperproliferation of keratinocytes in the psoriasis mouse model. However, ear thickness was not changed by the treatment of alloferon. The ear thickness was measured by vernier calipers including the dermis and cartilage of the ear as well as changes in the ear epidermis. Based on the high concentration of Aldara cream applied to the thin ear skin of mice, it appears that there is a difference in the observed changes in thickness between the dorsal skin and the ear skin.

Based on previous data, which demonstrated that alloferon reduced the increased IL-22Rα expression and alleviated symptoms of psoriasis, it was hypothesized that a decrease in IL-22Rα expression would lead to the amelioration of psoriasis. However, the induction of psoriasis in IL-22Rα knock-out mice using IMQ did not completely palliate psoriasis symptoms (Figure 5). As previously mentioned, psoriasis is influenced by various factors, including IL-22 as well as cytokines such as TNF-α, IL-23, and IL-17 [5],

which play roles in inflammation. Therefore, it is evident that solely depleting IL-22Rα is insufficient to fully prevent the development of psoriasis, as multiple pathways and factors are involved in its pathogenesis, requiring a more comprehensive approach.

Thus, I elucidated the therapeutic effect of alloferon in the absence of IL-22Rα. Alloferon significantly prevented the occurrence of psoriasis as revealed by reduced redness as well as plaque and epidermal hyperplasia in IMQ-treated IL-22Rα deficient mice (Figure 6). These results suggest that alloferon alleviates psoriasis by regulating not only the expression of IL-22Rα but also various factors that cause inflammation in psoriasis.

Through microarray analysis data, it has been suggested that alloferon alleviated psoriasis by suppressing the expression of inflammation-related cytokines such as IL-1β, IL-19, and IL-33 (Figure 7). Particularly, IL-19, known to be part of the IL-10 cytokine family, is secreted by keratinocytes in response to stimulation by IL-23 and IL-17, which are involved in the same mechanism as IL-22 in psoriasis. IL-19 has been implicated in inducing inflammation in keratinocytes, and there is currently active research being conducted on its relevance to psoriasis [31, 52]. Therefore, the hypothesis that alloferon regulates not only IL-22Rα expression but also inflammatory factors to alleviate psoriasis has been proven.

In this study, it is meaningful that alloferon showed an anti-

inflammatory effect on psoriasis dermatitis by regulating the expression of IL-22α and genes related to psoriasis inflammation and pathology. Therefore, these results suggested that alloferon has therapeutic potential for psoriasis.

Currently, biological agents such as Stelara® are being used as treatment options for psoriasis. However, these treatments have limitations, including high cost and the need for periodic administration. To address these limitations, it is suggested that a combination therapy approach, involving the co-administration of alloferon and biological agents, could be beneficial.

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국 문 초 록

이미퀴모드로 유발한 건선 모델에서 인터루킨-22 수용체 및 관련 인자 발현 조절을 통한 알로페론의 항염효과에 관한 연구

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건선은 만성적인 피부 염증 질환으로 전 세계 인구의 2-3%가 앓고 있다. 한번 발병하게 되면 완치가 어렵고 호전과 재발이 반복되어 육체적 고통뿐 만 아니라 정신적인 고통도 함께 수반된다. 현재는 건선의 원인이 되는 사이토카인을 억제함으로써 질환의 악화를 막는 치료제가 개발되어 있다. 인터루킨-22는 염증성 사이토카인으로 건선의 원인이 되는 사이토카인 중 하나이나 아직 인터루킨-22와 인터루킨-22 수용체를 타겟으로 하는 치료법은 없는 실정이다. 알로페론은 곤충에게서 발견된 면역 조절제로 항바이러스, 항암, 항염작용을 하는 물질로 알려져 있으며, 피부 염증 질환에서도 항염효과를 본 선행 연구가 존재한다. 그리하여 이번 연구에서는 건선에서 알로페론의 인터루킨-22 수용체의 조절과 함께 건선 완화 효과를 보고자 하였다.

종양괴사인자-α로 건선을 유도한 HaCaT과 이미퀴모드로 건선을 유도한 마우스에서 알로페론을 처리하였을 때, 인터루킨-22 수용체의 발현이 줄어드는 것을 확인하였고, 건선의 증상이 완화되는 것을 확인하였다. 이미퀴모드로 건선을 유도한 인터루킨-22 수용체 결핍 마우스에서는 야생형 마우스에 비해 건선 유도가 덜 되는 것을 확인하였으나 홍반 증상의 경우에는 야생형 마우스와 유사한 정도로 나타났다. 이미퀴모드로 건선을 유발한 인터루킨-22 수용체가 결핍된 마우스에 알로페론을 처리함으로써 유의미하게 건선의 증상이 완화되는 것을 확인하였고, 이는 알로페론이 인터루킨-1β, 인터루킨-19, 인터루킨-33와 같은 건선을 유발하는 염증과 관련된 유전자들을 조절함으로써 건선 완화 효과를 가지는 것임을 발견하였다.

이번 연구에서는 알로페론이 인터루킨-22 수용체 조절과 더불어 건선을 유발하는 염증과 관련된 유전자를 조절함으로써 건선 완화에 효과를 보인다는 것에 의의가 있다. 이러한 결과는 알로페론이 건선의 잠재적인 치료제가 될 수 있음을 시사하며, 알로페론을 생물학적 제제와 더불어 보조적 치료제로 사용한다면 치료 효과를 더 극대화시킬 수 있을 것으로 보인다.

주요어: 인터루킨-22, 알로페론, 건선, 이미퀴모드, 인터루킨-22 수용체 결핍 마우스

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