**Master of Science**

# Study on the cell function of cereblon protein in psoriasis disease

# 건선 질환 내에서 cereblon 단백의 세포기능 연구

**The Graduate School**
**of the University of Ulsan**

**Department of Medicine**
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# Study on the cell function of cereblon protein in psoriasis disease

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by

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# Study on the cell function of cereblon protein in psoriasis disease

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**Abstract**

**Study on the Cell function of cereblon protein in psoriasis**

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The pro-inflammatory cytokines (IL-17, TNF-α, and IL-6) are known to affect the pathogenesis of psoriasis, no exact cause has yet been found. In this study, we investigated the psoriasis etiology, and relationship between cereblon (CBBN) and psoriasis using wide type (WT) and CRBN knockout (KO) mouse models that stimulated skin with aldara cream (5% Imiquimod (IMQ)) and had similar responses to psoriasis. CRBN KO mice showed a significantly higher inflammatory response compared to WT, especially histologically. The number of immune cells such as macrophages and T cell infiltrated into the epidermis increased, and the thickness of the epidermis, including the back and ear skin, became much thicker. In back skin, expression level of inflammatory cytokines such as IL-6 and TNF-α was considerably higher in CRBN KO than that of WT mice. Moreover, IL-17 known as significant factor in psoriasis etiology was also higher in CRBN deficient mouse. RNA and

protein expression level of CRBN in the skin, decreased when treated with IMQ, which was consistent with the clinical results of CRBN reduction in psoriatic lesions. TNF-α produced by macrophages affects the inflammatory response of psoriasis. So, investigated that changes of psoriasis response are associated with changes of macrophage and CRBN. Directly administered Aldara cream (5% IMQ) to macrophage of WT and CRBN KO mouse, and we find nitric oxide levels were increased when CRBN was absent. These results demonstrate that macrophages can alter immune responses depending on the presence or absence of CRBN. Finally, CRBN can modulate IL-17 and macrophage activation, alleviate the inflammatory response of psoriasis. It also suggests that this role of CRBN may be a key molecule as a new therapeutic agent for psoriasis.

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| | |
|---|---|
| AK | Actinic keratosis |
| BSA | Bovine serum albumin |
| CRBN | Cereblon |
| CUL4A | Cullin-4A |
| DC | Dendritic cell |
| DDB | DNA damage-binding protein 1 |
| DMSO | Dimethyl sulfoxide |
| EDTA | Ethylenediaminetetraacetic acid |
| FACS | Fluorescence activated cell sorter |
| FBS | Fetal bovine serum |
| HaCaT | Human keratinocytes |
| IMQ | Imiquimod |
| iNOS | Inducible nitric oxide synthase |
| IRAK | IL-1 receptor–associated kinase |
| MCP | Monocyte chemotactic protein |
| MM | Multiple myeloma |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium |

| | |
|---|---|
| NHEK | Normal Human Epidermal Keratinocytes |
| PASI | Psoriasis activity and severity index |
| PBS | Phosphate buffered saline |
| PVDF | Polyvinylidene difluoride |
| RIT | Recombinant immunotoxin |
| ROC | Regulator of cullins |
| RPMI 1640 | Roswell Park Memorial Institute 1640 Medium |
| RT-PCR | Real-time polymerase chain reaction |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| TLR | Toll-like receptors |
| TNF-α | Tumor necrosis factor |

## Introduction

Psoriasis is a common chronic inflammatory skin disease affecting 2-3% of the world's population and is considered an immune system mediated inflammatory disease. Histologically, Characteristics of psoriasis is hypo-proliferation and abnormal differentiation of keratinocytes, dilation of blood vessels and infiltration to dermis of immune cells. The phenotype of psoriasis includes erythematous, pruritus, pain, skin tightness, or a silver scale that occurs throughout the body[1)]. The exact etiology mechanism has not been elucidated, the pathogenesis of psoriasis is considered to be a confusion between keratinocytes that produce cytokines, chemokines, and growth factors, and immune cells. Among them, Th1 / Th2 homeostasis, Th17 / Treg balance and IL-23 / Th17 axis are involved[2, 3)]

Five types of psoriasis have been reported: plaque psoriasis, guttate psoriasis, inverse psoriasis, pustular psoriasis, erythrodermic psoriasis. plaque psoriasis is the most common form of the disease, and accounts for about 90% of cases. Typical lesions are monomorphic, sharply demarcated erythematous plaques covered by silvery lamellar scales [4)]. Psoriatic lesions have the following characteristics. (i) The epidermis becomes thicker due to the abrupt proliferation of keratinocytes. (ii) decrease hypogranulosis because of abnormal differentiation of keratinocytes. (iii) dermis blood vessle dilation induced erythematous (iv) in Munro's microabscesses, accumulation of neutrophils. (v) Dermis or CD8+ T cell, infiltration of CD4+

T-helper cell and dendritic cell. These changes are related to inflammatory cytokines such as TNF-α and IL-17 A, C [1)5)].

Various methods have used to establish psoriasis-like model. First, normal Human Epidermal Keratinocytes (NHEK) or human keratinocytes (HaCaT) are used in psoriasis studies in vitro. 3D model is also possible, recent studies have also introduced 3D tissue-engineered human skin-like substances. The psoriasis 3D tissue model provided by MatTek Corporation (MatTek Corporation, Ashland, MA, USA) is very similar to human psoriatic tissue in terms of morphology and cytokines expression, and represents phenotype of multiple psoriasis like IL-6, IL-8, inflammatory cytokines. In vivo animal models are good models for studying various interactions and signal transduction pathways, mainly mice that knock out cytokines or chemokines related to psoriasis. The psoriasis-like model used in this study is also widely used because many psoriasis-like lesions have been created by applying aldara cream (5% Imiquimid) directly to the skin of mice [1, 6, 7)]

Imiquimod(IMQ) is one of the modulators of immune response and is known to stimulate innate / adaptive immune responses and induce cytokine production. The molecular formula of IMQ is C14H16N and the synthetic formula is 1- (2-methylpropyl) -1H-imidazo [4,5-C] quinolin-4-amine. In 1997, IMQ was first approved as a treatment for external genitalia and anus warts, and subsequently received approval for actinic keratosis (AK) and superficial basal cell carcinoma (BCC). Also, IMQ has anti-viral, anti-tumor effects. The IMQ is induce cytokines such as TNF-α, IFN-α, IL-6 or IL-8, and use these cytokines stimulated innate

immune system. IMQ can activate the immune system through the e Toll-like receptors (TLR)-7 / MyD88-depent pathway [8, 9]. The MyD88, protein containing TLR domain, binds to TLR and acts as an adapter to supplement the IL-1 receptor–associated kinase (IRAK) and TNF receptor related factor TRAF6 in the TLR. IMQ-activated dendritic cell (DCs) induce Th cells through regulate of expression of CD80 and CD87, production and release of specific cytokines (IFN-α, TNF- α, IL-12) [10]. In addition to producing IFN and other cytokines, IMQ might be indirectly stimulate the production of IFN-y to enhance adaptive immunity [9].

A multifunctional Cereblon (CRBN) forms the E3 ubiquitin ligase complex with DNA damage-binding protein 1 (DDB1), Cullin-4A (CUL4A) and Regulator of cullins(ROC) regulatory factors [11] and was first known as a gene associated with mental retardation [12]. It is mainly found in organs including brain, cytoplasm, nucleus, endoplasmic reticulum[13]. CRBN is also the target protein of thalidomide, which is also used as a therapeutic agent for multiple myeloma [11]. Thalidomide, which is known to cause anomalies, can bind to DDB1 of CRBN and inhibit the function of the E3 ubiquitin ligase complex [14]. Indeed, the degree of CRBN expression is significantly lower in patients with multiple myeloma [15 16] .

CRBN has also been shown that immunomodulatory drugs play an intermediate role in helping to control immunity and anticancer effects. CRBN is also known as AMPK-binding protein [13] [17]. CRBN interacts directly with the AMPK a1-subunit, inhibiting the phosphorylation of the subunit and reducing the enzyme activity of AMPK [18] [19]. Recent studies indicate that CRBN plays a negative regulatory role in CD4 + T cell activation. CRBN inhibits CD4 + T cell

activation and IL-2 secretion, thereby inhibiting CD4 + Tcell differentiation into Th17. Moreover, T-cell–specific deletion of CRBN also increased the production of IL-17A and IFN-γ cytokines [20] . However, the association with CRBN protein in psoriasis, which is mainly controlled by IL-17, has not yet been reported.

In this study, psoriasis-like model mice using by Aldara cream, which included 5% IMQ were established. We will also analyze mechanisms of TH-17 mediators and macrophages in the initiation and maintenance of psoriasis and understand the role of CRBN in the pathogenesis of psoriasis in the CRBN KO mouse model.

## Material and Method

### 1. Materials

DMEM, fetal bovine serum(FBS), and other tissue culture reagents were purchased from Gibco/ThermoFisher Scientific (Waltham, MA). The CRBN antibody(monoclonal) was obtained from SIGMA (HPA045910; St. Louis, MO). The enhanced chemiluminescence (ECL) western blotting detection reagent was purchased from Supersignal West Pico chemiluminescent substrate (34080; Piercem Rockford IL). All other chemicals, including lipopolysaccharide (LPS; from Escherichia coli 0111:B4) were purchased from Sigma-Aldrich.

### 2. Mice and the induction of skin inflammation by aldara

The generation of CRBN-deficient (CRBN KO) and WT mice on a C57BL/6N background by our group has been described previously[18]. Aldara cream (5% IMQ) was purchased from Vspharm (3M Health care Limited, England). Animals were housed under normal laboratory conditions, i.e., at 21–24 °C and 40–60% relative humidity under a 12 h light/dark cycle with free access to standard rodent food and water. All animals were raised under specific pathogen-free conditions, and the experimental protocol (2017-12-248) was reviewed and approved by the Animal Subjects Committee of Asan Medical Center (Seoul, Republic of Korea). 8-10 week-old CRBN knockout (n=7) and wild-type mice (n=7) were shaved on their back skin and then treated with a daily topical dose of 83.3 mg with back skin and one

ear of a commercial cream containing 5% imiquimod (Aldara™, MEDA Pharma) for 5 consecutive days [2]. To evaluate the severity of skin lesions, we used a scoring system based on the clinical Psoriasis Area and Severity Index (PASI), consisting of the following parameters: erythema, scaling and skin thickness. Those parameters were scored independently (scale from 0 to 4: 0, none; 1, slight; 2, moderate; 3, marked and 4, very marked) and cumulatively as PASI. The experiment ended on day 5 for determination of clinical outcome, for which mice were scored daily for ear thickness, back and ear erythema and back skin scaling.

### 3. Western blot analysis

For western blot analyses, Cell lines and samples (back skin, ear, spleen) were lysed for 1 hours at 4°C using pro prep (Intron bio, Seongnam, Korea) and cleared by centrifugation at 13,000rpm. Protein aliquots were loaded on to each well, electrophotrtically separated on 8% SDS-PAGE gels, and transferred on to polyvinylidene difluoride (PVDF) membranes. Blocking of the membrane was done for 30min using 5% BSA (Biosesang, Seongnam, Korea) and the blots were incubated with antibodies for CRBN (HPA045910; SIGMA, St.Louis, MO) and iNOS (61042; BD biosciences, San Jose, CA), β-actin (sc47778 HRP; santa cruz(Santa Cruz, CA). Blot were developed using the Supersignal West Pico chemiluminescent Substrare (34080; Piercem Rockford IL). Revelation and densitometric blot analysis were performed in

three independent experiments. Membranes were reblotted with anti-β-actin to ensure equal loading.

## 4. RNA preparation and real-time quantitative (RT-PCR)

Gene expression in human psoriasis was determined by mining data in publicly deposited datasets (GDS4602). The total RNA was isolated from cells using TRIzol (Invitrogen, Carlsbad, CA) [21]. The RNA was reverse-transcribed using the IScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Real-time PCR was performed on an iCycler iQ system (Bio-Rad Laboratories) with iQ SYBR Green Supermix (Bio-Rad Laboratories). The following primer sequences were used(amplification temperature): mouse S18 sense (60 °C), 5′- TTTGCGAGTACTCAACACCAACA-3′, and antisense, 5′- CCT CTTGGTGAGGTCAATGTCTG-3′; mouse CRBN sense(60 °C), 5′-AGCATGGTGCGGAACTTAATC-3′, and antisense, 5′-ATCTCTGCTGTTGTCCCAAAC-3′; mouse TNF- α sense(61 °C), 5′-GCCTCTTCTCATTCCTGCTTG-3′ and antisense 5′-CTGATGAGAGGGAGGCCATT-3’; mouse iNOS sence(65 °C), 5’-GGGCTGTCACGGAGATCA-3′ and antisense 5′-CCATGATGGTCACATTCTGC-3’; Human TNF-a sense, 5’-CCCAGGGACCTCTCTCTAATC-3’ and antisense 5’-GCTACAGGCTTGTCACTCGG-3’; Human MCP-1 sense, 5’-CATTGTGGCCAAGGAGATCTG-3’ and antisense 5’-CTTCGGAGTTTGGGTTTGCTT-

3', Human IL-6 sense 5'-GGTACATCCTCGACGGCATCT-3' and antisense 5'-GTGCCTCTTTGCTGCTTTCAC-3'.

**5. Preparation of peritoneal macrophages and cell culture**

Peritoneal macrophages were isolated from mice that had been injected intraperitoneally with 2 ml of 4% (w/v) fluid thioglycollate medium 3 days prior to peritoneal lavage with 10 ml RPMI 1640 medium. The collected cells were washed with RPMI 1640 and cultured in RPMI 1640 supplemented with 10% FBS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells were plated on flat-bottom culture plate and then incubated at 37 °C in a 5% $CO_2$ humidified incubator.

**6. MTT assay**

Cell viability was measured by MTT assay. For MTT assay $1x10^6$ cells were seeded in 24-well plate and incubated at 37°C for 24h. Thereafter, HaCaT, macrophage were treated aldara cream (5% IMQ). Concentration is control, LPS 10ug/ml, aldara cream 5uM, 10uM, 25uM, 50uM. After 24h were treated the cell viability was calculated by means of the MTT assay. All the protein concentrations were tested in triplicate.

**7. Cytokines assay**

The concentration of IFN γ, TNF α, IL 4, IL 6, IL 10 and IL 17 in the serum and back skin tissues of mice was detected by ELISA Kit (eBioscience) according to the manufacturer's

instructions. Absorbance was read at 450 nm within 20 min. using an ELISA reader (Bio Rad Laboratories, Hercules, CA, USA).

**8. ELISA**

Cytokine concentrations in cell supernatants and in homogenised tissue samples were measured by ELISA according to manufacturer's protocols (BioLegend, R&D Systems).

**9. Hematoxylin and eosin stain**

After 5 days, mice were sacrificed to collected dorsal lesional skin for histological examination. Formalin-fixed, paraffin-embedded sections from skin samples were stained with Haematoxylin & Eosin and examined. Histopathologic features of psoriasis, as hyperkeratosis, parakeratosis, microabscesses neutrophils were evaluated.

**10. NO assay**

The cells ($1\times10^6$ cells/ml) prepared as above were cultured in 24-well plates. For in vitro studies, LPS and aldara cream treated in the cells. After 24 h of incubation, nitrite in culture supernatants, the stable reaction product of NO with molecular oxygen, was determined by using Griess reagent [1: 1 mixture (v/v) of 1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 5% $H_3PO_4$]. Nitrite levels were determined colorimetrically at 540 nm.

## 11. Stastatics

Results are expressed as mean ± SD. Differences between groups were evaluated with t-test (for comparisons between two samples) or by a one-way analysis of variance (ANOVA) (for comparisons of multiple samples) using the GraphPad Prism Version 5 software (GraphPad Software, La Jolla, CA, USA). *P <0.05, **P <0.01 were considered to indicate a statistically significant difference.

## Results

### 1. CRBN expression level and IL-17A expression level in psoriasis patients are negatively regulated.

To evaluate the CRBN and other inflammatory cytokines are associated with the clinical manifestations of psoriasis, we compared the levels of gene expression of CRBN, IL-17A, MMP9 in non-lesional skin, lesional skin and normal control skin in psoriatic patients. The control group (n = 64) and the psoriasis patient group were divided into the non-lesion skin (n = 58) and the lesion skin (n = 58) to analysis. In psoriasis patients, CRBN level was higher than control group, and more CRBN was expressed in non-lesional skin than in lesion. In other words, the expression of CRBN in psoriatic-induced skin is significantly reduced. IL-17A, an inflammatory cytokine, showed a significantly higher expression level in psoriasis patients than control, and was more expressed in lesional skin than non-lesion. This means proved that inflammation response of psoriasis is occur by IL-17A and Th-17 was concerned in psoriasis.. In summary, a variety of inflammatory cytokines, including IL-17A, IL-23, MCP-1, IL-6, and TNF-α, can trigger a psoriatic inflammatory response.

## 2. Expression of CRBN proteins reacting in Psoriasis

First, we established the CRBN KO mouse model to investigated the role of CRBN in psoriasis. CRBN known as highly expressed in brain, proved the absent of CRBN by lysed brain. In vivo, WT and CRBN KO models were treated with aldara cream (5% IMQ) daily for about 7 days, and progressed western blot with obtained back skin, left ear, and spleen sample. Aldara non-treated group showed higher CRBN expression than in the treated group, which is consistent with the CRBN expression data in the psoriasis patient shown in Fig. 1 there was.

## 3. IMQ-induced psoriatic dermatitis is worsens in CRBN KO mice.

We made a psoriasis-like model to compare clinical and histologic differences with WT of the same conditions in the psoriasis environment of CRBN KO mice. In vivo, to establish a psoriasis-like model, 83.3 mg of aldara cream (5% IMQ) was administered about one week in the back skin and left ear of the mouse. Three days after starting to apply Aldara cream, the back skin of the mice began to show signs of red erythema and scale. Inflammation was observed from the fourth day, and the inflammatory response was continuously increased thereafter. Inflammatory response of the lesions is more dramatic in the CRBN KO than in the WT, and similar in the ear thickness. The thickness of the ears was consistently significantly increased.

**4. IMQ treatment results in increased proliferation and altered differentiation of keratinocytes.**

Overexpression and enrichment of the epidermis are characteristic of psoriasis. Lesion epidermal H & E staining analysis of WT and CRBN KO mice treated with Aldara cream (5% IMQ) showed that the epidermis was relatively hypertrophied compared to the control group. Hyperproliferation of keratinocytes is the result of activation of TNF-α or IL-17A. In addition, the density of monocytes infiltration into epidermis was increased in skin treated with IMQ compared to the control. In summary, IMQ treatment can be demonstrated to be consistent with the typical histological pattern of psoriasis. Histologically, IMQ increased the thickness of the epithelium by at least 3 times, especially by a factor of 9 in CRBN KO mouse.

**5. Macrophage data**

Macarphage is another pathway that contributes to the inflammatory response of psoriasis, producing a variety of cytokines including TNF-a. Proceeded to see if the CRBN could regulate the macrophage. Thioglycolate was injected with 1 ml , sacrificed to collect macrophages after two days. 24 wells were plated at 1 × 10 6, aldara cream was treated with 5 uM, 10 uM, 25 uM, and 50 uM concentrations as in HaCaT, and nitric oxide was measured after 24 hours. In the WT mice, about 5 times nitric was generated of control group, but in CRBN KO mice, nitric was generated in the amount of about 20 times. These results

demonstrate that higher levels of inflammatory and immune responses were activated in CRBN KO mice, consistent with in vivo experiments.

**6. mRNA expression of related genes in IMQ-induced psoriasis lesions**

To investigate how the cytokines affecting psoriasis were altered by Aldara cream (5% IMQ) and the difference of presence of CRBN, we measured mRNA expression for key inflammation genes that are known to regulate psoriatic skin inflammation. The changes of cytokines in back skin and serum of Aldara cream (5% IMQ) treated group and control group were investigated. First, IL-17, which is thought to be the most important in psoriasis, has increased approximately 2-fold in CRBN KO mice treated with aldara cream (5% IMQ).

**7. Proinflammatory cytokines of related genes in IMQ-induced psoriasis lesions**

The proinflammatory cytokine IL-6 involved in psoriasis increased about 2.5-fold. TNF-α, which is considered to be an activation of macrophages, is also significantly increased in CRBN KO treated with aldara cream (5% IMQ). Thus, CRBN impacts skin inflammatory changes by regulating important pro-inflammatory cytokine genes such as IL-17 and IL-6.

**8. Change of spleen in IMQ-treated skin of CRBN KO mice**

In order to assess the clinical and histological change of spleen following IMQ treatment, we used H&E stain in WT and CRBN KO mice following induction of psoriasis. We observed scattered staining in the IMQ-treated spleen. The iNOS expression level was measured by

western blotting. Interestingly, the expression level of iNOS in IMQ-treated CRBN KO mice were significantly higher ($P < 0.01$) than that in IMQ-treated WT mice. These data suggest that dermal inflammatry response was inhibited with the presence of CRBN in the psoriasis model.

## Discussion

In this study, CRBN, which plays an important role in the severity of psoriasis by controlling inflammatory cytokine expression in psoriasis disease and participating in the immune system, was investigated. It was confirmed in this study that topical imiquimod treated to skin produces a psoriasis-like response in human phenotype and histologically. This mouse model showed epidermal hyperproliferation and increased thickness, silvery scales, erythema, and high infiltration levels of monocytes. There was a change of inflammatory cytokines in the body during IMQ treatment. The cytokine such as IL-6, TNF-α, including IL-17/23 was measured after treatment with IMQ for approximately one week. These inflammatory cytokines were expressed at high levels in psoriatic lesions and this is consistent with clinical data from psoriasis patients. CRBN, known as the target protein of thalidomide, is known to be expressed in neurons and immune cells, but it has not yet been reported to be expressed in keratinocytes. This study demonstrated the expression of CRBN in the back skin of the psoriasis mouse model. The expression level and correlation of cytokine and CRBN in skin treated with IMQ were investigated. The levels of inflammatory cytokines in the back skin and serum of psoriatic mouse models were increased in lesional skin of CRBN KO mice. As a result, CRBN KO mice showed a significantly higher inflammatory response than WT mice. The other hands, CRBN expression level was significantly decreased in lesional skin of WT mice. This response is consistent with clinical data suggesting a high expression of CRBN in normal skin of psoriatic patients. In summary, CRBN is associated with inflammatory cytokines and negatively

correlated with each other. TNF-α, which is mainly produced in macrophages, is one of the cytokines that induces inflammation of psoriasis, allowing nitric oxide to be produced in macrophages[22]. Extracted macrophage from WT and CRBN KO mice, injected with thioglycolate, were treated by IMQ at different concentrations. Interestingly, macrophages of CRBN KO mice produced high levels of nitric oxide. In other words, CRBN inhibits the production of TNF-α and produces a relatively weak inflammation-related phenomenon. Previous studies reported that IMQ-stimulated macrophages secrete TNF-α and IL-6, induce keratinocyte activation and proliferation, and induce various cytokines including IL-23[23]. In this process, Th-17 was concerned to overexpression of chemokines that are involved in the inflammatory loop of keratinocytes and amplify the production of inflammatory cytokines [24]. IL-17 is a representative cytokine produced by Th-17, and many studies have suggested that the inflammatory pathway of psoriasis is due to IL-23 / IL-17[2]. IL-17 was expressed at a significantly higher level in the back skin of the psoriasis mouse model than in the control group. Among them, the CRBN KO mouse model treated with IMQ showed about three times the IL-17 expression level compared to WT mouse model of the same condition. In addition to IL-17, CRBN KO mouse model showed at least 1.5 times higher expression of TNF-α, IL-6, and MCP-1 than WT mouse. These results prove that CRBN is involved in the inflammatory response of psoriasis while suppressing inflammatory cytokines.

In this study, data were obtained using a mouse model, but further experiments will be required to see whether the data of the mouse model can be applied to human.

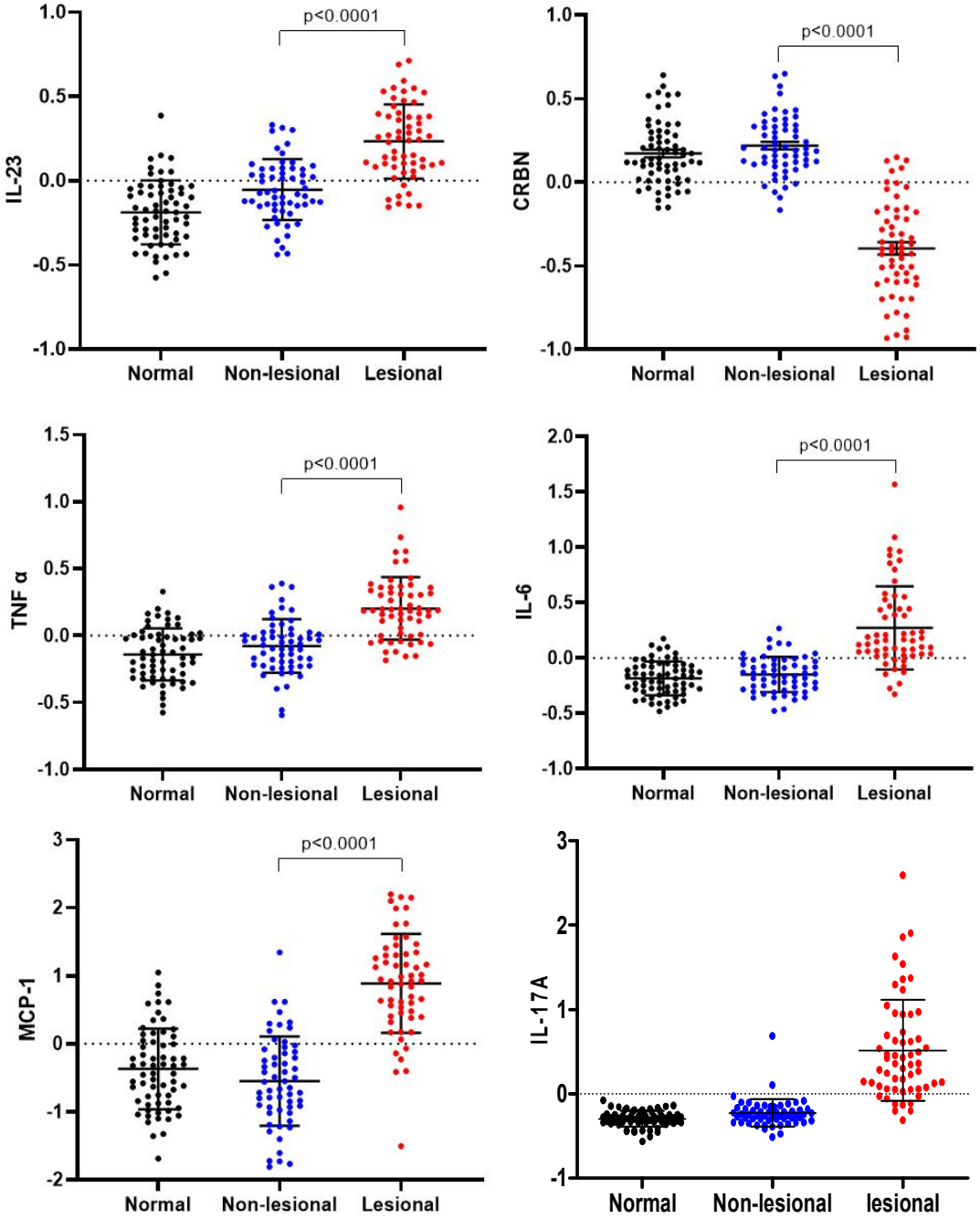
**Table**

| | | Back skin CRBN KO-Con /WT-Con | | Serum CRBN KO-Con /WT-Con | | Back skin CRBN KO-aldara/WT-aldara | | Serum CRBN KO-aldara/WT-aldara | | Back skin WT-aldara /WT-Con | | Serum WT-aldara /WT-Con | | Back skin CRBN KO-aldara /CRBN KO-Con | | Serum CRBN KO-aldara /CRBN KO-Con | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| IL-1a | 1 | 0.000 | | 0.617 | | 0.000 | | 1.131 | | 0.743 | | 0.505 | | 0.000 | | 0.925 | |
| IL-1b | | 1.089 | | 0.391 | | 1.476 | | 0.532 | | 1.594 | | 1.014 | | 2.160 | | 1.378 | |
| IL-2 | 2 | 1.396 | | 0.498 | | 2.276 | | 0.145 | | 1.117 | | 0.813 | | 1.822 | | 0.237 | |
| IL-3 | | 1.046 | | 0.663 | | 2.001 | | 1.700 | | 1.320 | | 0.573 | | 2.526 | | 1.469 | |
| IL-4 | 3 | 0.873 | | 0.900 | | 1.133 | | 0.552 | | 1.226 | | 0.754 | | 1.591 | | 0.463 | |
| IL-5 | | 1.328 | | 0.964 | | 1.887 | | 0.187 | | 1.414 | | 0.928 | | 2.010 | | 0.180 | |
| IL-6 | 4 | 2.008 | | 2.147 | | 2.439 | | 0.837 | | 1.424 | | 3.384 | | 1.730 | | 1.320 | |
| IL-7 | 5 | 0.738 | | 0.668 | | 0.848 | | 0.645 | | 1.511 | | 0.884 | | 1.738 | | 0.855 | |
| IL-10 | 6 | 2.045 | | 0.702 | | 2.268 | | 0.864 | | 1.335 | | 0.942 | | 1.48 | | 1.159 | |
| IL-12p70 | 7 | 6.958 | | 0.957 | | 2.109 | | 0.840 | | 3.557 | | 0.981 | | 1.078 | | 0.861 | |
| IL-13 | 8 | 0.813 | | 0.749 | | 1.269 | | 1.021 | | 1.337 | | 1.073 | | 2.087 | | 1.463 | |
| IL-15 | | 0.526 | | 0.705 | | 2.418 | | 0.741 | | 1.254 | | 1.069 | | 5.768 | | 1.123 | |
| IL-17 | 9 | 1.319 | | 0.917 | | 2.069 | | 0.890 | | 1.358 | | 1.191 | | 2.13 | | 1.157 | |
| IL-21 | | 0.868 | | 0.553 | | 2.924 | | 0.697 | | 1.529 | | 0.646 | | 5.149 | | 0.814 | |
| KC | 10 | 1.455 | | 3.637 | | 2.548 | | 3.082 | | 1.710 | | 3.227 | | 2.994 | | 2.735 | |
| Leptin | 11 | 2.377 | | 1.085 | | 2.754 | | 2.023 | | 3.644 | | 1.020 | | 4.222 | | 1.903 | |
| LIX | 12 | 0.000 | | 0.908 | | 0.249 | | 1.066 | | 1.019 | | 1.981 | | 0.000 | | 2.326 | |
| MCP-1 | 13 | 1.063 | | 2.027 | | 1.821 | | 0.851 | | 1.229 | | 9.413 | | 2.106 | | 3.953 | |
| TIMP-1 | 14 | 1.463 | | 1.392 | | 1.200 | | 1.158 | | 2.124 | | 2.405 | | 1.743 | | 2.000 | |
| TNFa | 15 | 0.680 | | 0.869 | | 2.035 | | 0.792 | | 1.504 | | 1.011 | | 4.500 | | 0.922 | |
| TNF RI | | 0.547 | | 0.940 | | 1.409 | | 1.257 | | 1.647 | | 0.923 | | 4.246 | | 1.234 | |
| TNF RII | 16 | 1.238 | | 0.913 | | 1.532 | | 1.205 | | 1.394 | | 1.289 | | 1.725 | | 1.701 | |
| TNFa | | 0.680 | | 0.869 | | 2.035 | | 0.792 | | 1.504 | | 1.011 | | 4.500 | | 0.922 | |
| TNF RI | 17 | 0.547 | | 0.940 | | 1.409 | | 1.257 | | 1.647 | | 0.923 | | 4.246 | | 1.234 | |
| TNF RII | | 1.238 | | 0.913 | | 1.532 | | 1.205 | | 1.394 | | 1.289 | | 1.725 | | 1.701 | |

**Table 1. Imiquimod induces various cytokines.** Check the change of cytokine about aldara cream treat group and control group. Cytokines from back skin and serum is analyzed. Data is comparison of each group : (i)CRBN KO-control group and WT-control group. (ii) CRBN KO-aldara cream(5% IMQ) treated group and WT-aldara cream(5% IMQ) treated group. (iii)

WT-aldara cream(5% IMQ) and WT-control. (vi) CRBN KO-aldara cream(5% IMQ) treated group and CRBN KO control group.

## Figures

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https://www.ncbi.nlm.nih.gov/geoprofiles/100686855
https://www.ncbi.nlm.nih.gov/geoprofiles/100698089

**Figure 1. The cytokines expression level in human psoriasis patients.** Skin biopsy samples from healthy control skin(n=64), and non-lesional skin (n=58) or lesional skin (n=58) from psoriasis patients, were assessed for inflammaion cytokines expression (mean ± SEM). Expression of IL-23 and CRBN, TNF- α, IL-6, IL-17 and MCP-1 in skin samples, including normal control skin (black symbols), non-lesional skin from psoriasis patients (blue symbols) or psoriatic lesions (red symbols).

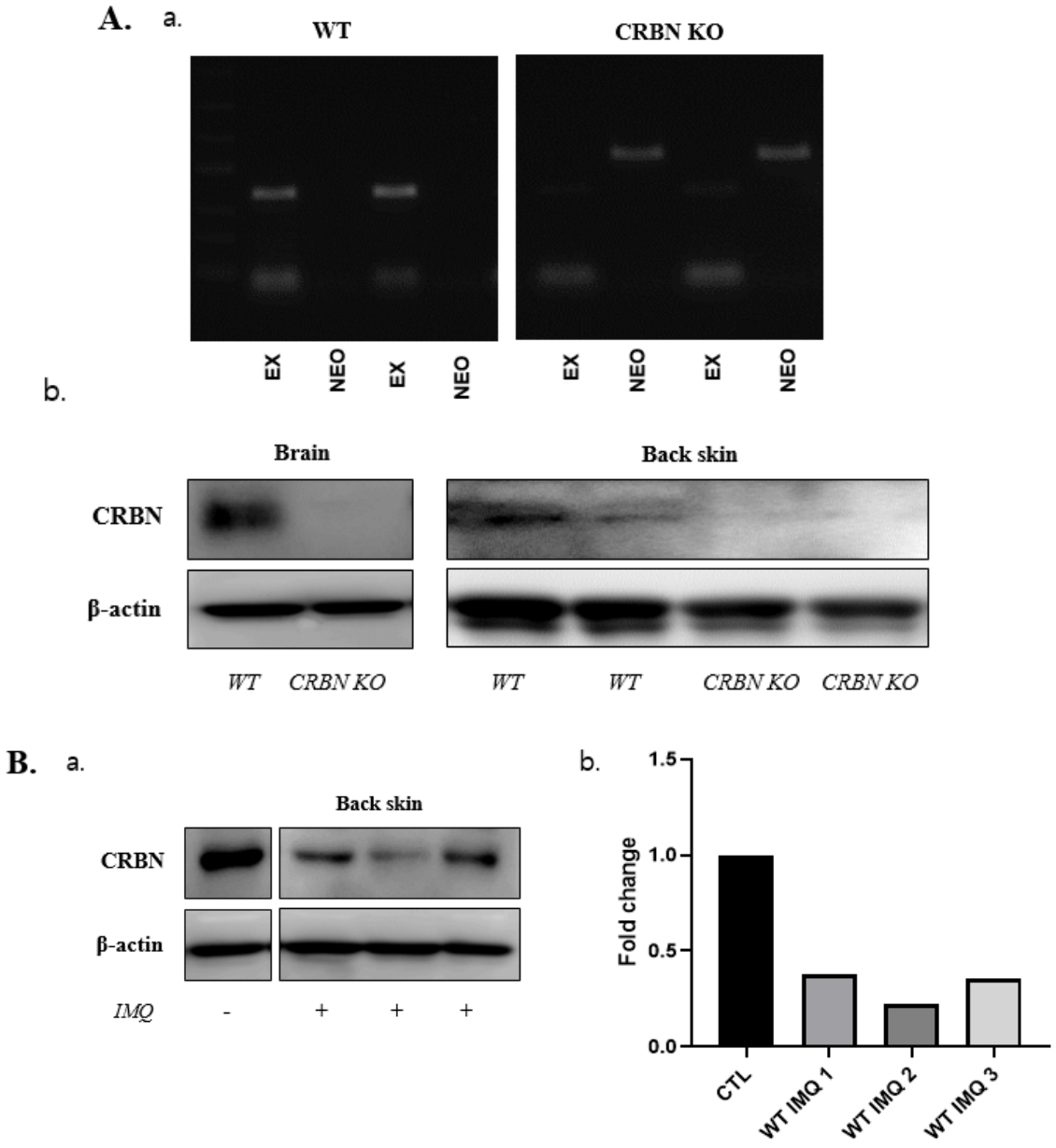


**Figure 2.** The 8-10 week old C57BL/6 Female mice were used for psoriasis mouse model. (A) (a)Prove to WT, CRBN KO by genotyping (b) Prove of CRBN absent by brain lysis. (B) Protein extracts from back skins and spleen of aldara cream(5% IMQ) treatment mouse and analyzed by Western blotting. The CRBN level in the untreated group is increased compared to the aldara cream treated group.

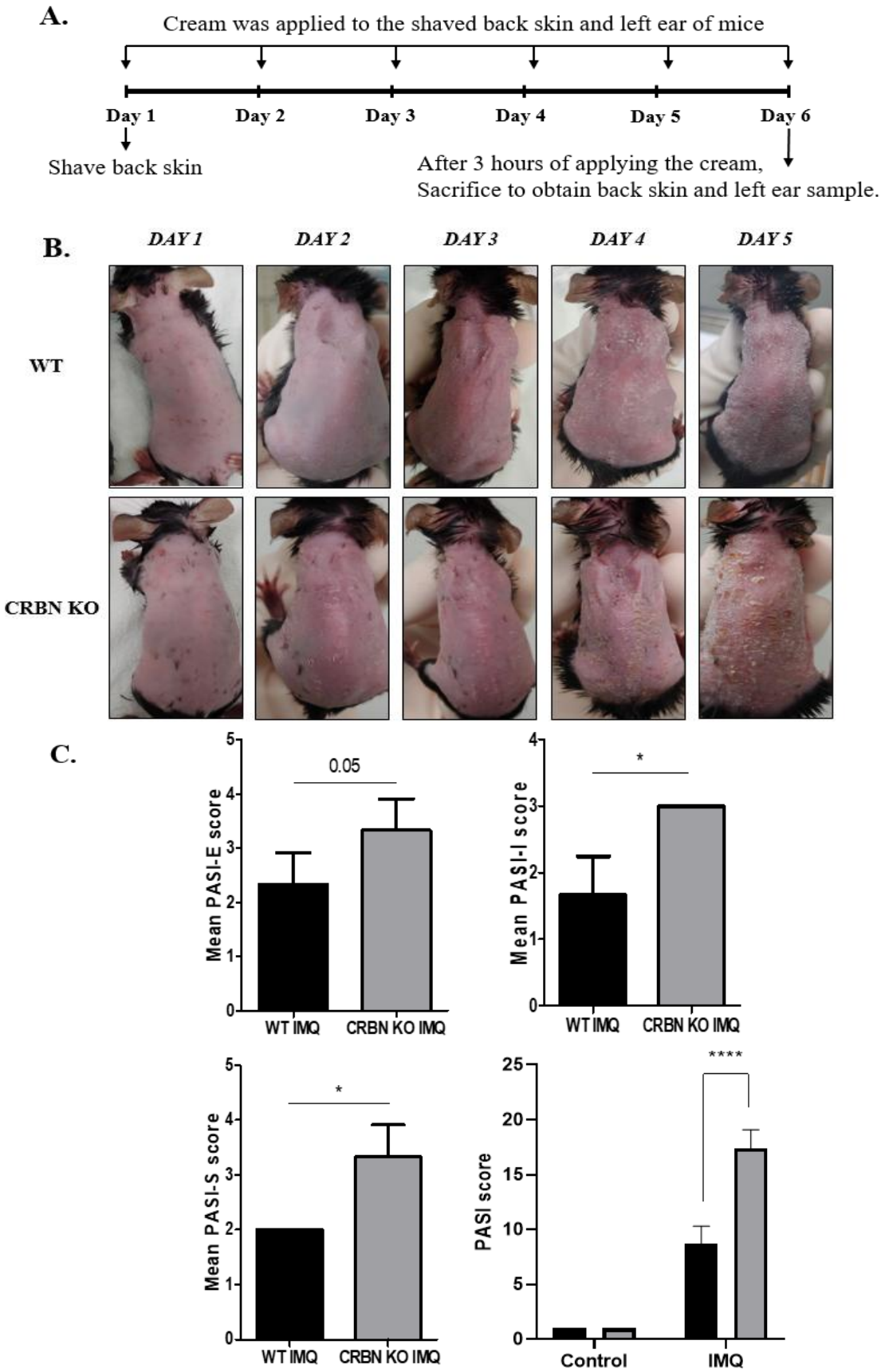
A.
Cream was applied to the shaved back skin and left ear of mice
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Shave back skin
After 3 hours of applying the cream,
Sacrifice to obtain back skin and left ear sample.
B.
DAY 1
DAY 2
DAY 3
DAY 4
DAY 5
WT
CRBN KO
C.
Mean PASI-E score
0.05
WT IMQ
CRBN KO IMQ
Mean PASI-I score
*
WT IMQ
CRBN KO IMQ
Mean PASI-S score
*
WT IMQ
CRBN KO IMQ
PASI score
****
Control
IMQ

**Figure 3. Psoriasis-like model in WT and CRBN KO mouse.** Mice treated with 83.3mg IMQ for 1 weeks. (A) Experimental schedule and duration. (B) Changes in back skin and ear of mice over time. To evaluate the severity of inflammation in the ears and back skin of mice, we used an objective scoring system 32 based on the clinical Psoriasis Area and Severity Index (PASI). (C) PASI scores of IMQ-induced psoriasis. Erythema(E), induration(I), Scales(S) were scored independently on a scale from 0 to 4: 0, none; 1, slight; 2, moderate; 3, marked; or 4, very marked. P-value scores for PASI scores were calculated by t-test. Mean ± S.D. values are indicated (*P < 0.05, **P < 0.01, ***P < 0.0001).

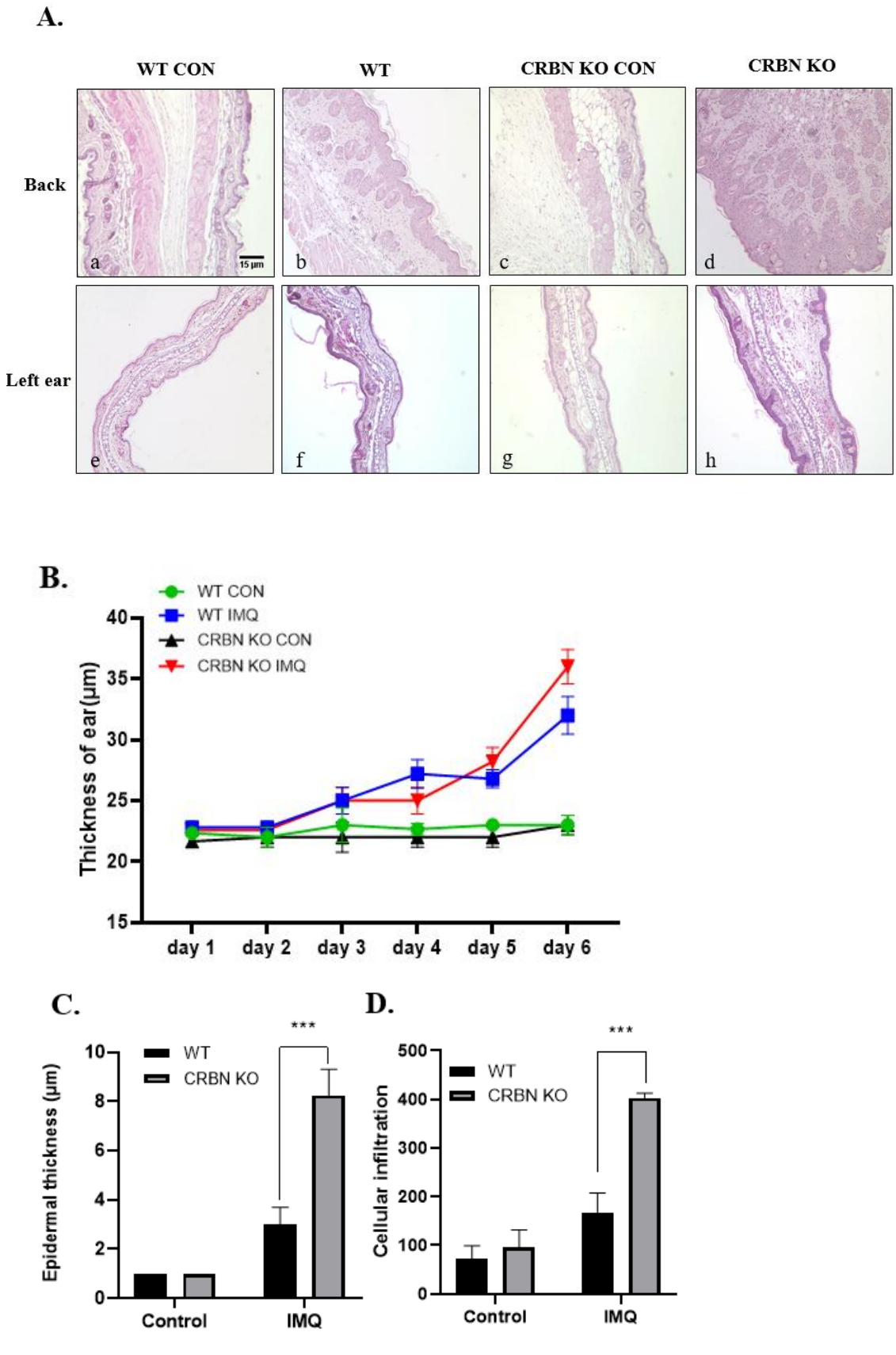
A.
WT CON
WT
CRBN KO CON
CRBN KO
Back
Left ear
a
b
c
d
e
f
g
h
15 μm
B.
WT CON
WT IMQ
CRBN KO CON
CRBN KO IMQ
Thickness of ear(μm)
40
35
30
25
20
15
day 1
day 2
day 3
day 4
day 5
day 6
C.
Epidermal thickness (μm)
10
8
6
4
2
0
WT
CRBN KO
***
Control
IMQ
D.
Cellular infiltration
500
400
300
200
100
0
WT
CRBN KO
***
Control
IMQ

**Figure 4. IMQ treatment alters keratinocyte proliferation and differentiation**

Epidermal hyperplasia following IMQ treatment. (A) Formalin-fixed paraffin-embedded tissue sections were examined by H&E staining. 100X magnification (scale bar = 15 μm). Staining of skin from control and aldara cream(5% IMQ) treated mice in each group on the final day. Many monocytes infiltrated the epidermis. (B) Graph of IMQ treated ear thickness. (C) The epidermal thickness measured. (D) Measurements of monocytes infiltrating the epidermis. ( * P<0.05, ** P<0.01, ***P<0.0001) Statistical testing included t-test for epidermal thickness, one-way ANOVA followed. Mean ± S.D. values are indicated (*P < 0.05, **P < 0.01, ****P < 0.0001).

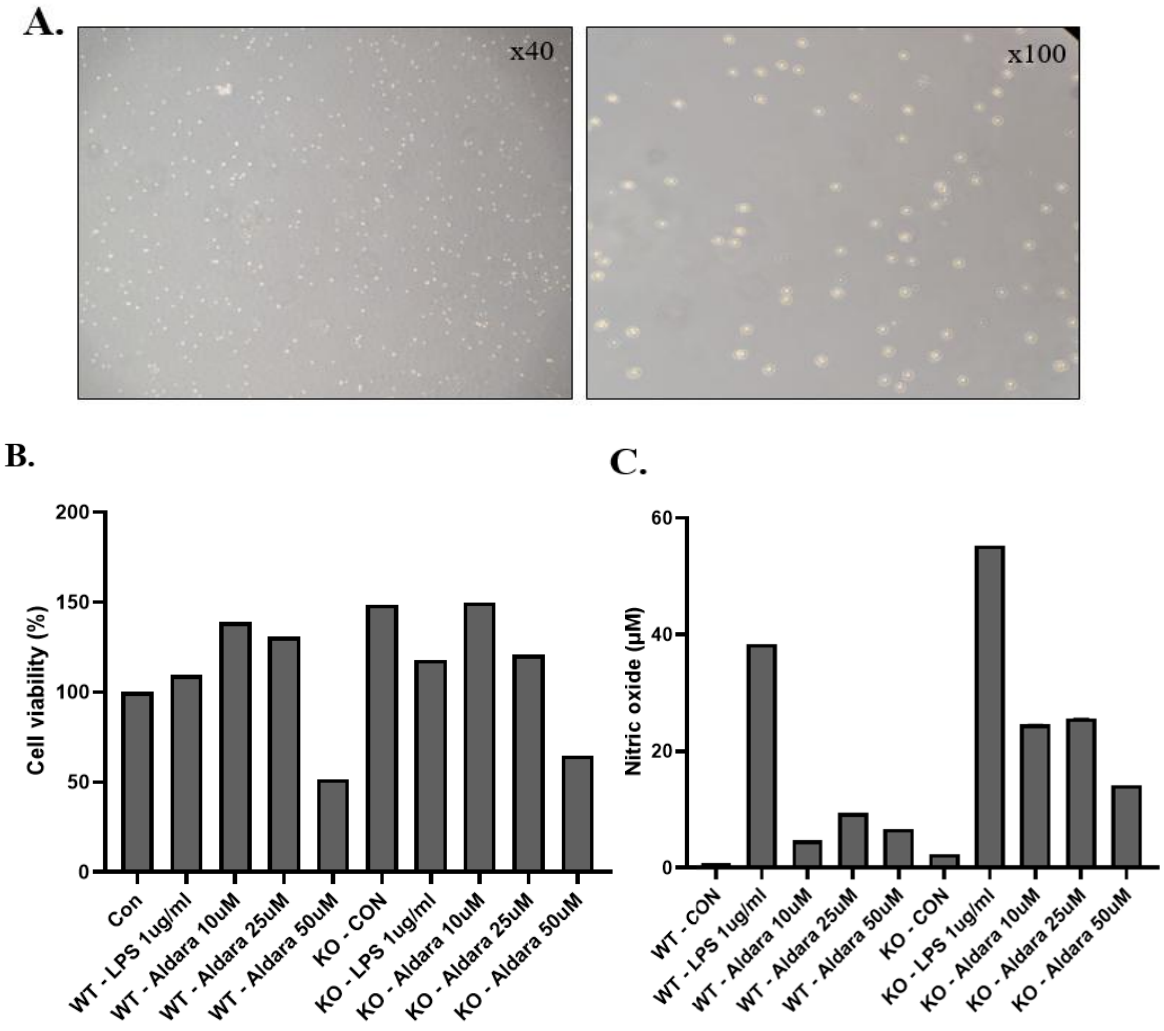


**Figure 6. macrophage is modulated by CRBN.** After injection of thioglycolate 1ml, extract peritoneal macrophage of WT mouse and CRBN KO mouse. (A) Raw 264.7 cell picture (B) Peritoneal macrophage viability was measured by MTT assays and Nitric oxide assay (C).

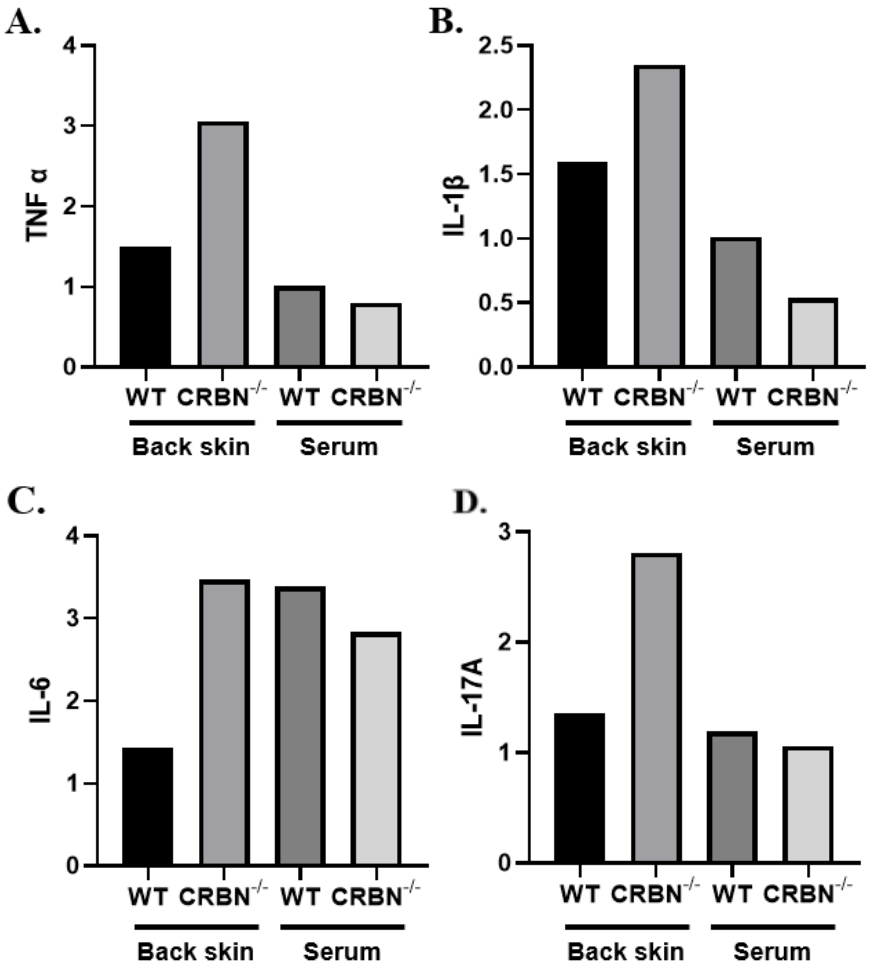


**Figure 7. Cytokine levels in lesional skin** Transcriptional mRNA expression of psoriasis-related genes in lesions. Relative skin mRNA expression of IL-1β, IL-6, IL-17, and TNF-α in CRBN KO mice/WT mice treated with IMQ were analyzed and quantified.

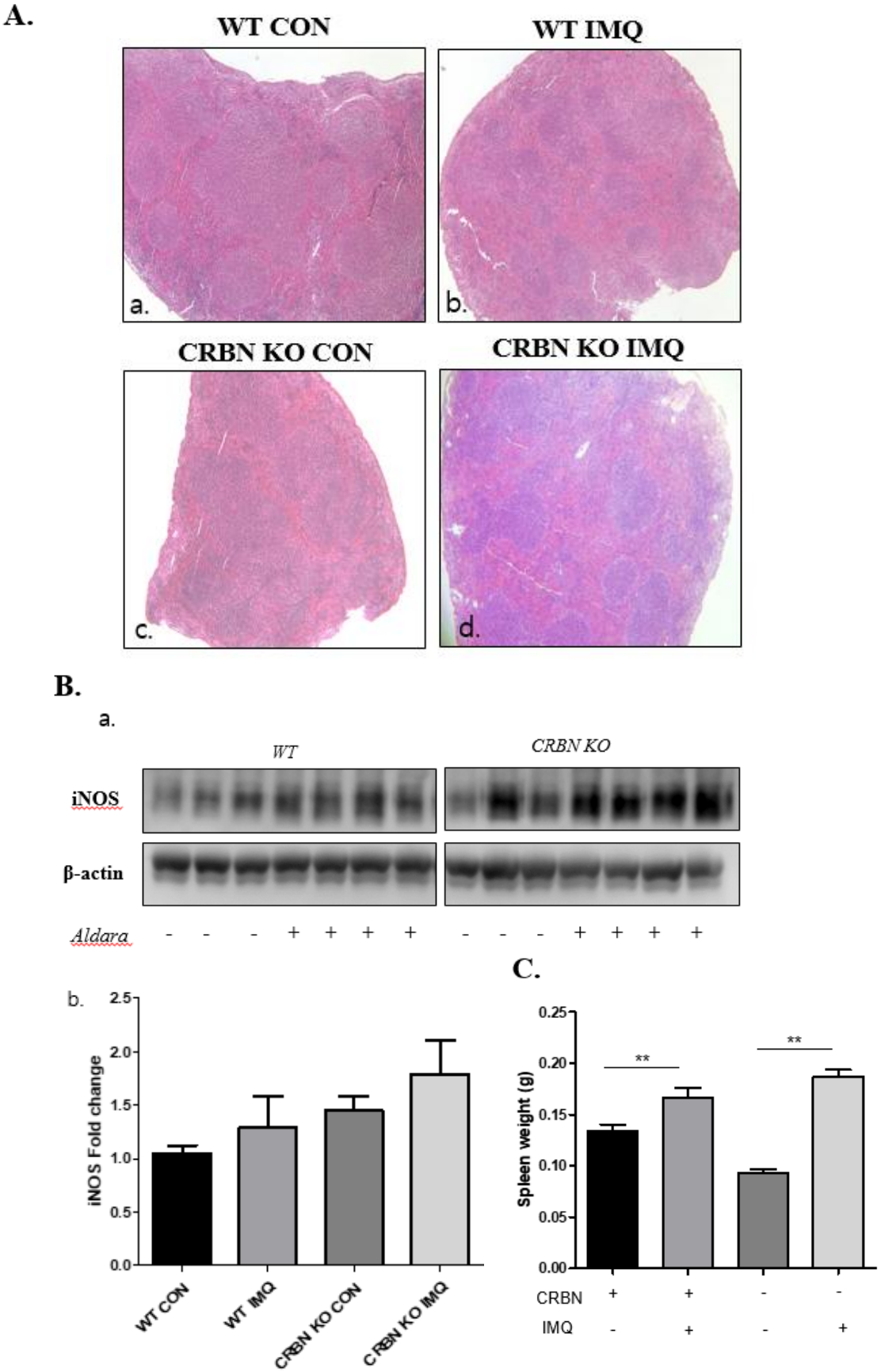
A.
WT CON
WT IMQ
a.
b.
CRBN KO CON
CRBN KO IMQ
c.
d.
B.
a.
WT
CRBN KO
iNOS
β-actin
Aldara - - - + + + + - - - + + + +
C.
b.
iNOS Fold change
2.5
2.0
1.5
1.0
0.5
0.0
WT CON
WT IMQ
CRBN KO CON
CRBN KO IMQ
Spleen weight (g)
0.25
0.20
0.15
0.10
0.05
0.00
**
**
CRBN + + - -
IMQ - + - +

**Figure 8. Expression of iNOS in the spleen of psoriasis induced by IMQ can induce inflammation exacerbations.** Spleen was extracted 3 hours after IMQ treatment. (A) H & E staining for histological analysis. (B) Quantitative iNOS changes are shown for CRBN KO mice and WT mice by western blot. (C) Spleen weight changes of WT and CRBN KO mice. Statistical testing included t-test for spleen weight. Mean ± S.D. values are indicated (*P < 0.05, **P < 0.01, ****P < 0.0001).

## 국문요약

### 건선 질환 내에서 cereblon 단백의 세포기능

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의학과

IL-17을 비롯하여 TNF- α, IL-6 등이 건선의 병인에 영향을 미치는 것으로 알려져 있지만, 정확한 원인은 아직까지 밝혀지지 않았다. 이 연구에서는 알다라 크림(5% IMQ)으로 피부를 자극하여 사람의 건선 피부와 유사한 반응을 일으킨 WT과 CRBN KO 마우스 모델을 사용하여 건선의 병인 및 CRBN - 건선 간의 상관성에 대하여 알아보고자 하였다. CRBN KO 마우스의 경우 WT과 비교했을 때 몸 전체에서 현저하게 높은 염증 반응을 보였으며, 특히 조직학적으로 표피에 침윤한 immune cell의 밀집도, 등 피부를 비롯한 귀 표피의 두께가 모두 증가하였다. 등 피부에서 IL-6, TNF-a 와 같은 염증성 사이토카인들의 발현 수준 역시 CRBN KO이 WT에 비해 확연하게 높았다. 건선의 병인에 중요한 요소로 알려져 있는 IL-17 또한 CRBN이 결여된 마우스에서 높아졌다. 다르게 말하면, CRBN은 IL-17을 비롯한 다양한 염증성 사이토카인들과 negative하게 작용하여 염증 반응을 조절하고 있음을 의미한다. 피부에서의 CRBN 단백질 발현 수준은 IMQ를 처리했을 때 감소하였고 이는 건선 환자의 병변에서 CRBN이 감소했던 임상 결과와 일치함을 보였다. Macrophage에서 생성되는 TNF-α는 건선의 염증 반응에 영향을 미친다.

이러한 내용과 연관 지어서 macrophage와 CRRN의 변화에 따라서 건선의 반응에 변화가 있을지 알아보고자 하였다. WT 마우스와 CRBN KO 마우스의 macrophage에 직접적으로 알다라 크림(5% IMQ)을 처리하였고, CRBN이 결여된 상태일 때 nitric oxide 수준이 증가하는 것을 확인하였다. 이러한 결과는 macrophage가 CRBN의 유무에 따라서 면역 반응에 변화를 일으킬 수 있음을 입증한다. 이러한 양상은 인간 각화 세포(HaCaT)에서도 동일하게 작용한다. 결론적으로, CRBN은 건선의 염증 반응에서 중요한 역할을 담당하고 있으며, 특히 IL-17과 macrophage는 CRBN과 밀접한 관계가 있다. 또한 CRBN의 이러한 역할은 건선의 새로운 치료제로써 key molecule이 될 수 있음을 시사한다.

**Uncategorized References**

[1] K. Bochenska, E. Smolinska, M. Moskot, J. Jakobkiewicz-Banecka, M. Gabig-Ciminska, Models in the Research Process of Psoriasis, Int J Mol Sci, 18 (2017).

[2] L. van der Fits, S. Mourits, J.S. Voerman, M. Kant, L. Boon, J.D. Laman, F. Cornelissen, A.M. Mus, E. Florencia, E.P. Prens, E. Lubberts, Imiquimod-induced psoriasis-like skin inflammation in mice is mediated via the IL-23/IL-17 axis, J Immunol, 182 (2009) 5836-5845.

[3] L. Puig, The role of IL 23 in the treatment of psoriasis, Expert Rev Clin Immunol, 13 (2017) 525-534.

[4] W.H. Boehncke, M.P. Schon, Psoriasis, Lancet, 386 (2015) 983-994.

[5] Y. Cai, C. Fleming, J. Yan, New insights of T cells in the pathogenesis of psoriasis, Cell Mol Immunol, 9 (2012) 302-309.

[6] K. Nakajima, S. Sano, Mouse models of psoriasis and their relevance, J Dermatol, 45 (2018) 252-263.

[7] S. Costa, O. Marini, D. Bevilacqua, A.L. DeFranco, B. Hou, S. Lonardi, W. Vermi, P. Rodegher, A. Panato, F. Tagliaro, C.A. Lowell, M.A. Cassatella, G. Girolomoni, P. Scapini, Role of MyD88 signaling in the imiquimod-induced mouse model of psoriasis: focus on innate myeloid cells, J Leukoc Biol, 102 (2017) 791-803.

[8] M. Urosevic, R. Dummer, C. Conrad, M. Beyeler, E. Laine, G. Burg, M. Gilliet, Disease-independent skin recruitment and activation of plasmacytoid predendritic cells following imiquimod treatment, J Natl Cancer Inst, 97 (2005) 1143-1153.

[9] E. Hanna, R. Abadi, O. Abbas, Imiquimod in dermatology: an overview, Int J Dermatol, 55 (2016) 831-844.

[10] H. Hemmi, T. Kaisho, O. Takeuchi, S. Sato, H. Sanjo, K. Hoshino, T. Horiuchi, H. Tomizawa, K. Takeda, S. Akira, Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway, Nat Immunol, 3 (2002) 196-200.

[11] K.M. Kortum, Y.X. Zhu, C.X. Shi, P. Jedlowski, A.K. Stewart, Cereblon binding molecules in multiple myeloma, Blood Rev, 29 (2015) 329-334.

[12] T. Ito, H. Handa, Cereblon and its downstream substrates as molecular targets of immunomodulatory drugs, Int J Hematol, 104 (2016) 293-299.

[13] Q. Shi, L. Chen, Cereblon: A Protein Crucial to the Multiple Functions of Immunomodulatory Drugs as well as Cell Metabolism and Disease Generation, J Immunol Res, 2017 (2017) 9130608.
[14] Y.X. Zhu, E. Braggio, C.X. Shi, K.M. Kortuem, L.A. Bruins, J.E. Schmidt, X.B. Chang, P. Langlais, M. Luo, P. Jedlowski, B. LaPlant, K. Laumann, R. Fonseca, P.L. Bergsagel, J. Mikhael, M. Lacy, M.D. Champion, A.K. Stewart, Identification of cereblon-binding proteins and relationship with response and survival after IMiDs in multiple myeloma, Blood, 124 (2014) 536-545.
[15] Y.X. Zhu, K.M. Kortuem, A.K. Stewart, Molecular mechanism of action of immune-modulatory drugs thalidomide, lenalidomide and pomalidomide in multiple myeloma, Leuk Lymphoma, 54 (2013) 683-687.
[16] Y.X. Zhu, E. Braggio, C.X. Shi, L.A. Bruins, J.E. Schmidt, S. Van Wier, X.B. Chang, C.C. Bjorklund, R. Fonseca, P.L. Bergsagel, R.Z. Orlowski, A.K. Stewart, Cereblon expression is required for the antimyeloma activity of lenalidomide and pomalidomide, Blood, 118 (2011) 4771-4779.
[17] K.M. Lee, S. Jo, H. Kim, J. Lee, C.S. Park, Functional modulation of AMP-activated protein kinase by cereblon, Biochim Biophys Acta, 1813 (2011) 448-455.
[18] K.M. Lee, S.J. Yang, Y.D. Kim, Y.D. Choi, J.H. Nam, C.S. Choi, H.S. Choi, C.S. Park, Disruption of the cereblon gene enhances hepatic AMPK activity and prevents high-fat diet-induced obesity and insulin resistance in mice, Diabetes, 62 (2013) 1855-1864.
[19] H.K. Kim, T.H. Ko, B. Nyamaa, S.R. Lee, N. Kim, K.S. Ko, B.D. Rhee, C.S. Park, B. Nilius, J. Han, Cereblon in health and disease, Pflugers Arch, 468 (2016) 1299-1309.
[20] J.A. Kang, S.H. Park, S.P. Jeong, M.H. Han, C.R. Lee, K.M. Lee, N. Kim, M.R. Song, M. Choi, M. Ye, G. Jung, W.W. Lee, S.H. Eom, C.S. Park, S.G. Park, Epigenetic regulation of Kcna3-encoding Kv1.3 potassium channel by cereblon contributes to regulation of CD4+ T-cell activation, Proc Natl Acad Sci U S A, 113 (2016) 8771-8776.

[21] M. Grabmuller, B. Madea, C. Courts, Comparative evaluation of different extraction and quantification methods for forensic RNA analysis, Forensic Sci Int Genet, 16 (2015) 195-202.
[22] S.G. Fonseca, P.R. Romao, F. Figueiredo, R.H. Morais, H.C. Lima, S.H. Ferreira, F.Q. Cunha, TNF-alpha mediates the induction of nitric oxide synthase in macrophages but not in neutrophils in experimental cutaneous leishmaniasis, Eur J Immunol, 33 (2003) 2297-2306.
[23] J.R. Chan, W. Blumenschein, E. Murphy, C. Diveu, M. Wiekowski, S. Abbondanzo, L. Lucian, R. Geissler, S. Brodie, A.B. Kimball, D.M. Gorman, K. Smith, R. de Waal Malefyt, R.A. Kastelein, T.K. McClanahan, E.P. Bowman, IL-23 stimulates epidermal hyperplasia via TNF and IL-20R2-dependent mechanisms with implications for psoriasis pathogenesis, J Exp Med, 203 (2006) 2577-2587.
[24] B. Flutter, F.O. Nestle, TLRs to cytokines: mechanistic insights from the imiquimod mouse model of psoriasis, Eur J Immunol, 43 (2013) 3138-3146.
[25] Z.J. Li, K.C. Sohn, D.K. Choi, G. Shi, D. Hong, H.E. Lee, K.U. Whang, Y.H. Lee, M. Im, Y. Lee, Y.J. Seo, C.D. Kim, J.H. Lee, Roles of TLR7 in activation of NF-kappaB signaling of keratinocytes by imiquimod, PLoS One, 8 (2013) e77159.
[26] M.P. Schon, M. Schon, K.N. Klotz, The small antitumoral immune response modifier imiquimod interacts with adenosine receptor signaling in a TLR7- and TLR8-independent fashion, J Invest Dermatol, 126 (2006) 1338-1347.