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# C57BL/6 마우스에서 반복적 자외선 조사에 의해 유도되는 피부 반응에 미치는 Toll-like receptor 2 의 영향

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이 논문을 의학박사 학위논문으로 제출함

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## Abstract

# Toll-like receptor 2 mediates cutaneous reaction induced by repetitive ultraviolet irradiation in C57BL/6 mice

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Toll-like receptors (TLRs) are the most comprehensively investigated and well-characterized pattern recognition receptors, which mediates innate immunity against harmful microbial infection. In addition to microbial organisms, TLRs can also respond to endogenous molecules released from injured tissues or dead cells. TLR-mediated sterile inflammation triggered by endogenous molecules is involved in many pathological processes including tissue injury, repair and remodeling, autoimmune diseases, and tumorigenesis. Recent researches also suggest that TLRs may play a role in the responses to single ultraviolet (UV) exposure. We performed a comparative study regarding the difference of the response which is induced by repetitive UV irradiation between wild-type (WT) and TLR 2 knock-out (KO) mice, to provide *in vivo* evidence on the role of TLRs in mediating repetitive UV-induced response. Repetitive UV-induced

inflammatory responses were less severe in TLR2 KO mice than in TLR2 WT mice after 6 weeks of UV irradiation (three times per week). In gross examination, TLR2 KO mice showed less prominent erythema and scaling. They also showed significantly thinner skin than WT mice, both by caliper measurement and image analysis. In histopathologic findings, TLR2 KO mice showed less inflammatory cell infiltration and heat shock protein 70 (endogenous ligand of TLR2) expression. Quantitative real-time RT-PCR exhibited lower gene expression levels of IL-1β, IL-6, MMP-13 in TLR2 KO mice. They also showed lower protein level of IL-1β in ELISA and MMP-13 in western blot. The present experiment demonstrated that TLR2 is associated with inflammatory responses to repetitive UV irradiation in C57BL/6 mice. However, the involved pathomechanism could not be clearly elucidated through this study. Further studies to investigate associated cell population and mechanism are required.

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Key words: Inflammation, C57BL/6 mouse, Repetitive irradiation, Toll-like receptor 2, Ultraviolet

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## List of abbreviations and symbols

AP-1, activating protein-1

COX, cyclooxygenase

DAMP, danger-associated molecular pattern

H&E, hematoxylin and eosin

HSP, heat-shock protein

IFN, interferon

IKK, IkB kinase

IL, interleukin

IRAK, IL-1R-associated kinase

IRF, IFN regulatory factor

KO, knock out

MAPK, mitogen-activated protein kinase

MD2, myeloid differentiation protein-2

MKK, mitogen-activated protein kinase kinase

MMP, matrix metaloproteinase

MPO, myeloperoxidase

MyD88, myeloid differentiation primary-response protein 88

NF-κB, nuclear factor kappa B

NLRP3, NOD-like receptor protein 3

PAMP, pathogen-associated molecular pattern

PRR, pattern recognition receptors

RIP, receptor interacting protein

ROS, reactive oxygen species

TAB, TAK1-binding protein

TAK, TGF-β activated kinase

TBK, TANK binding kinase

TIR, Toll/interleukin-1

TIRAP, Toll/interleukin-1 receptor domain-containing adaptor protein

TLR, toll like receptor

TRAF, tumour necrosis factor receptor-associated factor

TRAM, TRIF-related adaptor molecule

TRIF, TIR-domain-containing adaptor inducing IFN-β

UV, ultraviolet

WT, wild type

# Introduction

Toll receptors were first identified in *Drosophilia* and shown to be a key in antifungal immunity (1). A homologous family of Toll receptors, the so-called Toll-like receptors (TLRs), also exist in mammals (2). TLR is a recently discovered family of receptors that are important for the immune response to infection. It regulates the innate immune response and provides a rapid protection against harmful organisms. TLRs recognize not only exogenous ligands derived from pathogens (pathogen-associated molecular pattern, PAMP) but also endogenous ligands released from damaged tissues or dead cells (danger-associated molecular pattern, DAMP) (3). They are the most comprehensively investigated and well-characterized pattern recognition receptors (PRRs), which mediate inflammation caused by microbial infection or tissue damage (4). Eleven functional TLRs in human and thirteen in mice have been identified, until now (5). TLRs 1, 2, 4, 5, 6 and 11 are present on the plasma membrane, whereas TLRs 3,7,8,9 and 10 are mainly present on the endolysosomes (4-8). Not much is known about TLR 12 and 13.

Since 1998, TLRs have been shown to recognize various exogenous ligands (9). A variety of exogenous ligands (PAMP) derived from various pathogens, including virus, bacteria, protozoa and fungi are reported. However, recognition of microbial exogenous ligands fails to explain all functions of TLRs (10). In addition to microbial PAMPs, an increasing number of endogenous stimulators are being reported as candidate ligands of TLRs. TLRs can also respond to endogenous molecules released from injured tissues or dead cells, which are often referred to DAMPs. TLR-mediated sterile inflammation

triggered by DAMPs is involved in many pathological processes including tissue injury, repair and remodeling, autoimmune diseases, and tumorigenesis (5). Exogenous and endogenous ligands of TLRs are listed in **Table 1**.

**Table 1. TLRs and their ligands**

| TLR | location | Endogenous ligands | Exogenous ligands |
|---|---|---|---|
| **1** | plasma membrane | not determined | triacyl lipopeptide |
| **2** | plasma membrane | biglycan, endoplasmin, HSP60, HSP70, hyaluronan, human carciac myosin, monosodium urate crystal | lipoprotein/lipopeptides, peptidoglycan, lipoteichoic acid lipoarabinomannan, glycolipids, porins, atypical lipopolysaccharide, zymosan |
| **3** | endolysosome | mRNA | dsRNA |
| **4** | plasma membrane | fibrinogen, fibronectin, heparan sulfate, HSP22, HSP60, HSP70, HSP72 | lipopolysaccharide, taxol, fusion protein, envelop protein, HSP60, hyphae, pertussis toxin |
| **5** | plasma | not determined | flagellin |

| | | | |
|---|---|---|---|
| | membrane | | |
| **6** | plasma membrane | not determined | diacyl lipopeptides, lipoteichoic acid, zymosan |
| **7** | endolysosome | RNA, small interfering RNA | imidazoquinoline, loxoribine, bropirimine, single-stranded RNA |
| **8** | endolysosome | human cardiac myosin, small interfering RNA | imidazoquinoline, single-stranded RNA |
| **9** | endolysosome | hypomethylated CpG-DNA | CpG oligodeoxynucleotides |
| **10** | endolysosome | not determined | not determined |
| **11** | plasma membrane | not determined | profilin-like protein |

Engagement of TLRs by ligands initiates intracellular signaling pathways through Toll/interleukin-1 (TIR) domain containing adaptors, including myeloid differentiation primary response protein 88 (MyD88), TIR domain-containing adaptor inducing IFN-β (TRIF, TICAM-1), MyD88-adaptor-like/TIR-domain-containing adaptor protein (TIRAP/Mal) and TRIF-related adaptor molecule (TRAM) **(Fig. 1)** (4). Each TLR recruits a specific set of adaptors to activate different transcription factors, leading to appropriate inflammatory responses. All TLRs except for TLR3 share MyD88 as a common adaptor protein. TLR2 was of interest and its engagement activates MyD88-dependant pathway. It requires TIRAP/Mal for TLR-MyD88 signaling transduction.

Then MyD88 interacts with IL-1R-associated kinase (IRAK)-4, which activates IRAK-1 and IRAK-2. The IRAKs dissociated from MyD88 interact with TNFR-associated factor 6 (TRAF6). TRAF6 activates a complex of TGF-β-activated kinase 1(TAK1), TAK1-binding protein (TAB)1, TAB2, and TAB3. TAK1 phosphorylates IkB kinase (IKK)-β. Then the IKK complex (IKK-α, IKK-β, and NF-κB essential modulator (NEMO)) phosphorylates IkBα, nuclear factor kappa B (NF-κB) inhibitory protein. Phosphorylated IkBα is degraded and NF-κB translocates into the nucleus and activates the expression of various proinflammatory mediators or cytokines. Activation of MAP kinase cascade, on the other hand, induces AP-1 activation (4). Overactivation or dysregulation of TLR signaling is implicated in the pathogenesis of various inflammatory and autoimmune diseases. For example, lack of negative regulators for TLRs can cause autoimmune disease based on various mouse models. A20 negatively regulates NF-κB activation downstream of TLR2 (11, 12). In the absence of A20, mice develop multi-organ inflammatory disorders that lead to premature death. Another example is TANK. TANK-knocked out mice spontaneously develop autoimmune glomerular nephritis depending on the presence of MyD88 or IL-6 (13).

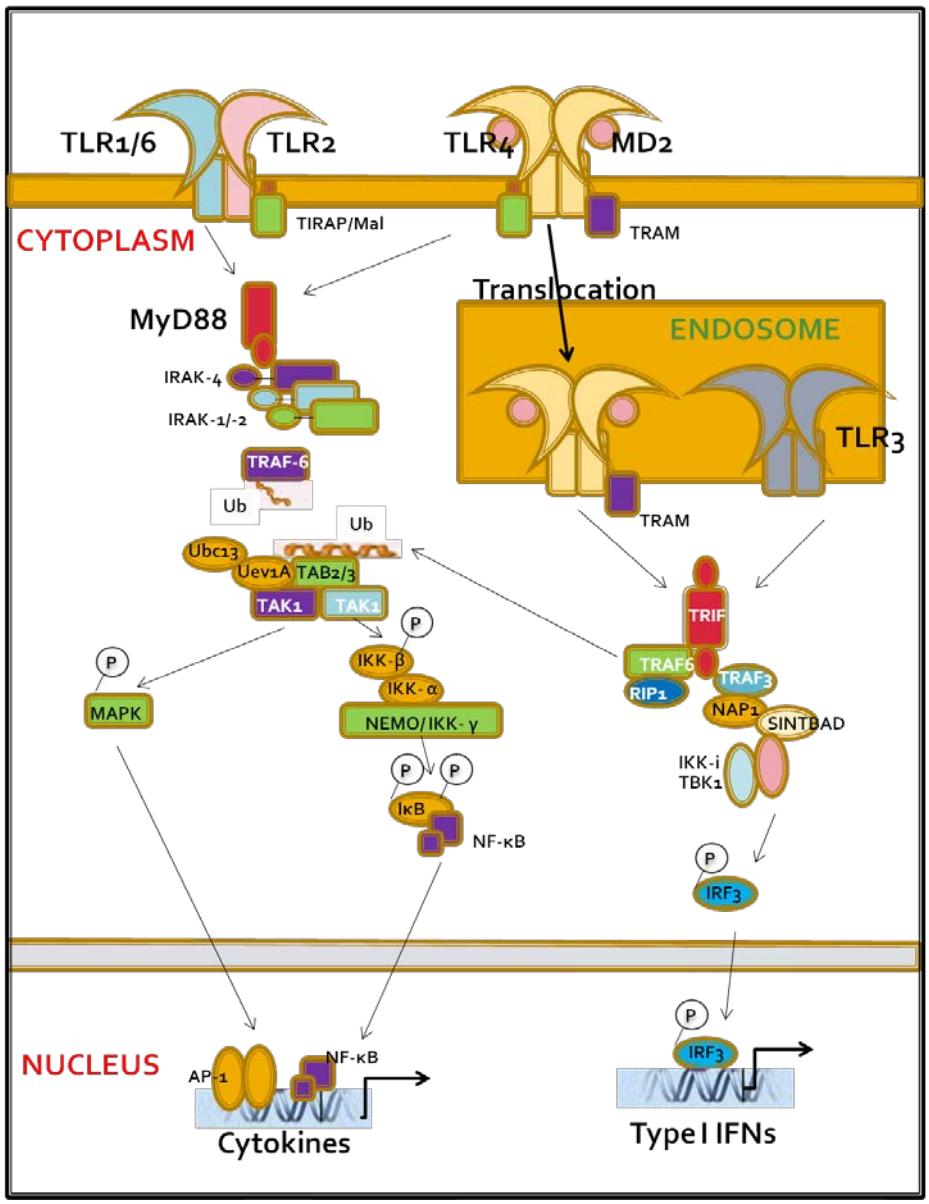



**Fig. 1 TLR signaling (Adapted figure from (4))** Engagement of TLR2 ligands initiates MyD88-dependent pathways. AP-1, activating protein-1; IFN, interferon; IKK, IκB kinase; IRAK, IL-1R-associated kinase; IRF, IFN regulatory factor; MKK, mitogen-activated protein kinase kinase; MD2, myeloid differentiation protein-2; MyD88,

myeloid differentiation primary-response protein 88; RIP, receptor interacting protein; TAB, TAK1-binding protein; TAK, TGF-β activated kinase; TBK, TANK binding kinase; TIRAP, Toll/interleukin-1 receptor domain-containing adaptor protein; TRAF, tumour necrosis factor receptor-associated factor; TRAM, TRIF-related adaptor molecule; TRIF, TIR-domain-containing adaptor inducing IFN-β; NF-κB, nuclear factor kappa B.

The skin is the largest organ of our body, provides a first line of defense against the outer environment. Skin cells include variable cells, which act to maintain integrity of the skin, to provide barrier against microbial pathogens and physical or chemical insults. Especially, the major cell types of the epidermis, keratinocytes are primary sentinels that detect foreign matters or dangerous agents using PRRs and mediate immune responses by producing antimicrobial peptides, cytokines, and chemokine (14). TLRs are the best studied receptors of RPRs and ligation of TLRs by PAMPs or DAMPs can activate host cell signal pathways which are described earlier in the TLR signaling part. Epidermal keratinocytes express several TLRs, located either on the cell surface (plasma membrane) or in endolysosomes (15). TLR2 of our interest is expressed on the cell surface of epidermal keratinocytes. It is also expressed on Langerhans cells, melanocytes, and fibroblasts.

Skin response to ultraviolet (UV) and its association with TLRs has been also studied. The UV spectrum include three sections, UVA (320-400 nm), UVB (280-320 nm), and UVC (200-280 nm) (16). Acute exposure to UV induces inflammatory responses leading to skin irritations or sunburn, while chronic UV exposure can cause immunosuppression, skin cancers, and photoaging (17). UVC is a strong carcinogen,

but it is filtered through ozone layer and rarely arrives to the earth. UVB effectively causes erythema and epidermal damage via apoptosis or inflammatory responses. UVA less effectively causes erythema and its penetration depth is limited.

The mechanisms by which UVB induces inflammation on the skin are complicated and have not been fully established. UV activates mitogen-activated protein kinase (MAPK) family (18). Then AP-1 is activated and stimulates the production of matrix metalloproteinases (MMPs) such as MMP-1, 3, 9, and 12. Mice have MMP-13 instead of MMP-1 gene of humans. MMPs degrade collagen and elastic fibers. They are also implicated in inflammation, cell proliferation, and remodeling of damaged tissues (19).

UV irradiation can induce various proinflammatory cytokines including interleukin (IL)-1β, IL-6, and TNF-α through transcription factor NF-κB (20). After UV irradiation, keratinocytes produce and release IL-1β. The production of IL-1β is regulated by synthesis of pro-IL-1β mRNA in a TLR-dependent manner and by cleavage of pro-IL-1β by inflammasome, in a TLR-independent manner (4). IL-1 receptor activation can induce IL-6 (21), which then induces keratinocyte proliferation, skin inflammation, and production of MMPs (22). By contrast, UV-induced TNF-α is associated with sunburn cells, contact hypersensitivity suppression (18).

If UVB is directly absorbed by nuclear DNA, it leads to the formation of cyclobutane-pyrimidine dimer and pyrimidine (6-4) photoproducts. Additionally, UVB-induced reactive oxygen species (ROS) cause oxidative damage to DNA and other cellular components. In a ROS-regulated pathways of UVB-exposed cells, UVB-induced NF-κB activation may result in either suppression or stimulation of apoptosis and inflammation.

It depends on its specific subunit composition and mechanisms that are influenced by the availability of growth and survival factors in the surrounding microenvironments (23).

These days, emerging evidence suggests that TLRs may play a role in the response to UV exposure (22, 24-26). A recent study showed that expression of TLR2/ MyD88 was increased in photoaged human skin compared to in intrinsically aged skin of the same elderly individuals. In an *in vitro* study with human epidermal keratinocytes, single UV irradiation (100mJ/cm$^2$) increased expression of IL-6 and MMP-1 through MyD88 (22). TLR2 also positively regulated UV-induced expression of cyclooxygenase (COX)-2 and IL-6 (unpublished data). Additionally, administration of TLR2 agonist increased expression of MMP-1 and MMP-9 in human epidermal keratinocytes via NF-κB (24). Some scholars also reported that TLR3 is associated with inflammation after single UVB radiation. They showed that RNA was released from human keratinocytes after UVB exposure (15 mJ/cm$^2$), which stimulated production of TNF-α and IL-6 from human non-irradiated keratinocytes and peripheral blood mononuclear cells, dependent on TLR3 and TRIF. Additionally, wild type (WT) mice upregulated TNF-α and IL-6 in the skin against UVB irradiation (500 mJ/m$^2$) or injection of UVB-damaged self RNA, whereas TLR3 knock-out (KO) mice did not. These findings establish that UVB damage is detected by TLR3 and that self-RNA serves as a DAMP (25). The most recent *in vitro* study reported that protein extract of UV-irradiated (UVB 30 mJ/cm$^2$) human keratiocytes activated TLR2 of Langerhans cells and induced the gene expression of MAPK, NF-κB, and IRF 3 (26). Taken together, some TLRs appear to be associated with skin response to UV. However, most of the previous reports were of *in vitro* studies focusing on single UVB irradiation.

# Objective

We performed a comparative study regarding the difference of the response which is induced by repetitive UV irradiation between WT and TLR 2 KO mice, to provide *in vivo* evidence on the role of TLRs in mediating repetitive UV-induced response.

# Materials and Methods

## Mice

For comparisons of repetitive UV-induced inflammatory responses between TLR2-deficient (TLR2 KO) mice and WT counterparts, C57BL/6 mice and TLR2-deficient mice (C57BL/6 background) were purchased from Oriental Bioservice (Kyoto, Japan). Animals were bred under specific pathogen-free conditions in AAALAC accredited facility in Seoul National University Hospital. Eight-week-old female mice with a telogen hair cycle were used. All animal experiments were approved by the institutional animal care and use committee (IACUC) of Seoul National University Hospital.

**Table 2. Classification of mice**

| **Condition** | **UV-** | | **UV+** | |
|---|---|---|---|---|
| **Genotype** | WT | KO | WT | KO |
| **Number** | n=5 | n=5 | n=6 | n=6 |

## UV irradiation to mice

Mice were irradiated with a custom-made UV device equipped with TL 20W/12 RS fluorescent sunlamp (Philips, Amsterdam, The Netherlands) with an emission spectrum between 275 and 380 nm (peak at 310-315 nm). A TA401/407 Kodacel filter (Kodak, NY, USA) was used to remove UVC (wavelengths below 290 nm). Irradiance was measured

using a Waldmann UV meter (Model 585100; Herbert Waldmann GmbH & Co. KG. Villingen-Schwenningen, Germany). In a telogen period, back skin was shaved with an electric clipper and depilated using thioglycolic acid and potassium hydroxide-containing chemical depilatory cream (Veet, Oxy-Reckitt Benckiser, Seoul, Korea) 48 hours before UV irradiation. When mice entered an anagen period, shaving with an electric clipper only was done right before every UV irradiation.

The experiment was initiated when all the mice were synchronized in a telogen period. The UVB irradiation dose started from 75mJ/$cm^2$ and gradually increased. The dorsal skins of mice were irradiated with dose up to 250 mJ/$cm^2$ in telogen period and 400 mJ/$cm^2$ in anagen period. The irradiation was performed three times a day over 6 weeks. Dorsal skin thickness using a digital caliper (Mitutuyo, Kawasaki, Japan) and body weight were measured repeatedly when the mice were in telogen synchronization, at week 0 and 6, 24 hours after the previous irradiation. Photographs of dorsal skin were acquired at the same time point.

## Histopathological analysis of the skin

Skin samples were obtained 24 hours after the last irradiation. Formalin-fixed paraffin embedded tissue blocks sectioned and stained. Histopathologic findings were interpreted with an image analysis program. Four-μm thick sections of dorsal skin were mounted onto silane-coated slides (Dako, Glostrup, Denmark) and either subject to hematoxylin and eosin (H&E) staining or immunostaining to compare morphological and cellular

changes (27). For myeloperoxidase (MPO) and Ki-67 staining, paraffin samples were fixed in 10% neutralized formalin for 48 hours before processing into paraffin wax. Sections allowed to dry at 58°C for 1 hour, were dewaxed, and rehydrated through graded alcohols to water. Sections were boiled to antigen retrieval in TRS solution (S2031, Dako, Denmark) for 15 minutes. Sections were incubated with blocking solution for 1 hour and then stained with the monoclonal Ki-67 (1:50, M7249, Dako, Denmark), MPO (1:100, Abcam, MA, USA) overnight at 4 °C. After rinsing in phosphate buffered solution, sections were visualized using an LSAB kit (Golden Bridge International, Inc., WA, USA), which uses a biotinylated secondary antibody and horseradish-streptavidin conjugate; 3-amino-9-ethylcarbazole was used as the chromogenic substrate. Sections were counter-stained briefly in Mayer's hematoxylin. Immunohistochemistry with a mouse monoclonal antibody against HSP70 (1:700, Santa Cruz, CA, USA) was conducted on 4-μm-thick slices of formalin-fixed paraffine-embedded tissues. An automated immunostaining was performed using the BenchMark XT immunostainer (Ventana Medical Systems, Tucson, AZ, USA) according to the manufacturer's protocol. Images of each section were taken at a magnification of 100x or 200x with a 12.5 megapixel digital camera (DP70, NA=0.5, Olympus Optical Co., Japan) connected to a light microscope (BX51, Olympus Optical Co.). The images were analyzed with an image analysis software (IMAGE J, NIH, USA). Degree of inflammatory cell was evaluated by the modified criteria from the previous study (28). Mild inflammatory cell infiltration was defined by 0-49 dermal inflammatory cells per field (×200, average number of 3 random measurements), moderate 5-99, and severe >100. The number of MPO-positive cells in 10,000 $\mu m^2$ was acquired from the average number of three

measurements. The number of Ki-67-positive cells per field (×200) in the strip of 100 $\mu$ m epidermis was determined by average of three times of manual counting.

## TUNEL staining

Apoptotic cells were detected by the TUNEL staining, using the ApopTag in situ apoptotic detection kit (Serologicals, Norcross, GA, USA). The number of TUNEL-positive cells per field (×200) was determined by the same method applied to the evaluation of Ki-67 positive cells.

## Quantitative real-time RT-PCR

Total RNA was isolated from tissue using a RNAiso Plus (Takara Bio Inc., Shiga, Japan) and 1 μg of total RNA was converted to cDNA using a First Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania) Quantitation of mIL-1β, mIL-6, mTNF-α, mIL-10, mMMP-9, mMMP-13, mouse procollagen and endogenous reference m36B4 cDNA was performed using the SYBR Premix Ex Taq II kit (Takara Bio) with a 7500 Real time PCR System (Applied Biosystems, Foster City, CA). Sequence specific primer sets were purchased by Bioneer (Daejeon, Korea) **(Table 3)**. PCR conditions were 50°C for 2 min, 95°C for 2 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Data were analyzed using the $2^{-\Delta\Delta CT}$ methods, with the data presented as the fold number in gene expression normalized to levels of m36B4 transcript and relative to the

average of control samples from WT mice without UV irradiatdion.

**Table 3. Primer sequences for real-time RT-PCR**

| Gene | forward sequence | reverse sequence |
|---|---|---|
| **m36B4** | TGGGCTCCAAGCAGATGC | GGCTTCGCTGGCTCCCAC |
| **mIL-1β** | GACTCATGGGATGATGATGATAAC | CCATACTTTAGGAAGACACGGATT |
| **mIL-6** | GCTACCAAACTGGATATAATCAGGA | CCAGGTAGCTATGGTACTCCAGAA |
| **mTNF-α** | GGCAGGTCTACTTTGGAGTCATTGC | ACATTCGAGGCTCCAGTGAATTCGG |
| **mIL-10** | CAGAGCCACATGCTCCTAGA | TGTCCAGCTGGTCCTTTGTT |
| **mMMP-13** | CATCCATCCCGTGACCT | GCATGACTCTCACAATG |
| **mMMP-9** | AGACGACATAGACGGCATCC | GGGACACATAGTGGGAGGTG |

*m36B4 was used as an endogenous control gene.

## ELISA

Mouse IL-1β, IL-6, IL-10, and TNF-$\alpha$ secretions were measured by ELISA or mouse Fluorokine MultiAnalyteProfiling (MAP) bead-based assays (R&D systems, MN, USA) using Versamax ELISA reader (Molecular Devices, CA, USA) or Bioplex200 (Bio-rad) system, according to the manufacturers' protocols.

## Western blotting

Preparation of protein lysates was performed as described previously (22). Briefly, biopsied skin samples were homogenized in 1X RadioImmunoPrecipitation Assay lysis buffer (50 mM Tris-HCl (pH 8), 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate) containing a phosphatase inhibitor, protease inhibitor cocktail (Roche, Indianapolis, IN, USA) and phenylmethylsulphonyl fluoride. Lysates were centrifuged at 12,000 g, 4°C for 25 min, and supernatants were collected for protein quantification using bicinchoninic acid assay. Equal amounts of proteins were loaded onto 8%, 10%, or 12% (where appropriate) Tris-Glycine gels, and then electrophoretically transferred to a polyvinylidene difluoride membrane. Membranes were subsequently blocked with 5% skim milk in Tris-buffered saline/T (20 mM Tris-HCl (pH 7.6), 137 mM NaCl, 0.1% Tween-20) at $4^{o}$C for 1 hr. Membranes were then incubated with appropriate antibodies and horseradish peroxidase-conjugated secondary antibody. Polyclonal anti-MMP-9 antibody was purchased from Cell Signaling Technology (Beverly, MA, USA). Normal anti-rabbit IgG and β-actin were from Santa Cruz Technology (Santa Cruz, CA, USA). Monoclonal anti-MMP-13 antibody was from Neomarkers (Fremont, CA, USA). Bands were visualized by enhanced chemiluminescence (Amersham, Buckinghamshire, England). The relative signal strengths as normalized by β-actin were quantified using a densitometry program (TINA; Raytest Isotopen-mebgerate, Straubenhardt, Germany).

## Statistical analyses

Experimental values represent the mean ± standard error of mean of five or six mice

per group. Statistical significances in all experiments were determined using Mann-Whitney test for continuous variables and Fisher's extract test for categorical variables with less than 5 counts (SPSS, IBM, Chicago, USA). A P-value of less than 0.05 was regarded as statistically significant.

# Results

## 1. Gross response

Repetitive UV-induced inflammatory responses were less severe in TLR2 KO mice than in TLR2 WT mice at 6 weeks after UV irradiation (**Fig. 2**).

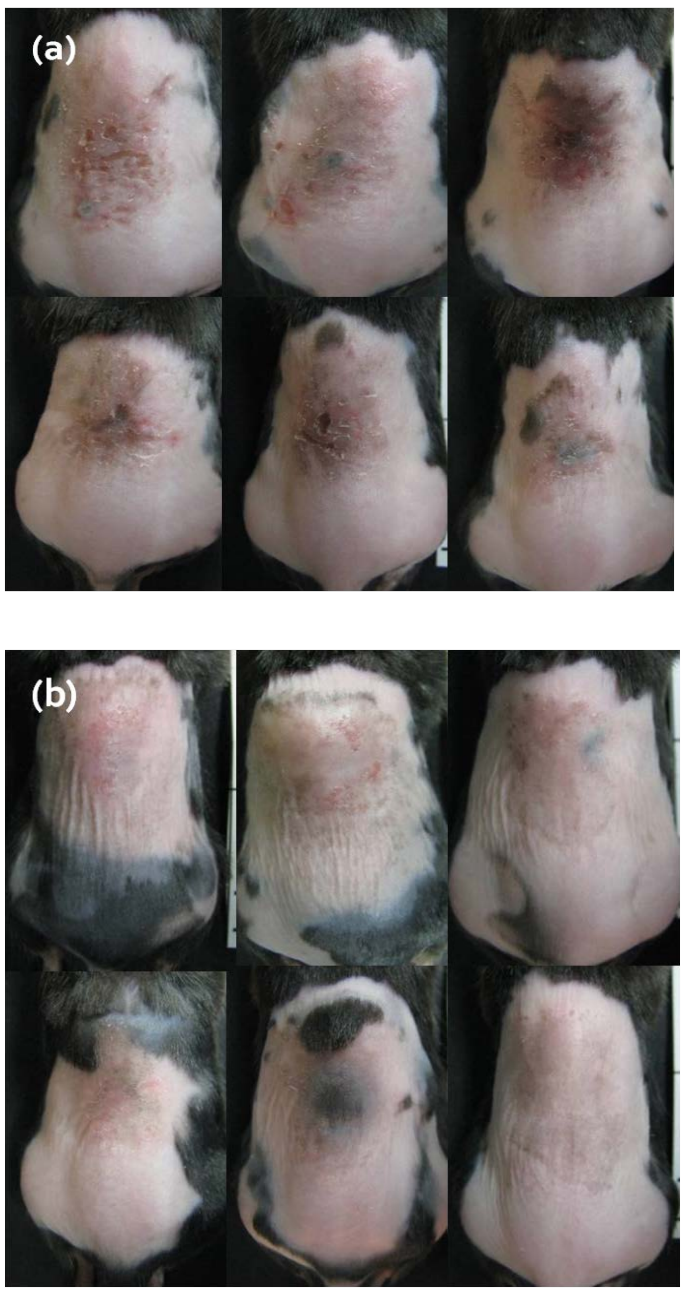



**Fig. 2 Less prominent erythema and scaling in UV+ KO mice**

(a) UV+ WT mice

(b) UV+ KO mice

## 2. Skin thickness by a caliper

TLR2 KO mice showed significantly thinner skin than WT mice after repetitive UV irradiation of 6 weeks **(Table 4)**, when there was no significant difference in body weight between groups **(Fig. 3)**.

**Table 4. Skin thickness measured by a caliper** (*:P<0.05, by Mann-Whitney test)

| | week 0 | week 6 | Δ (week 6-week 0) | |
|---|---|---|---|---|
| UV- WT | 0.88±0.14 | 0.87±0.03 | -0.01±0.12 | |
| UV- KO | 0.86±0.12 | 0.89±0.06 | 0.03±0.07 | |
| UV + WT | 0.87±0.09 | 2.02±0.20 | 1.15±0.23 | * |
| UV + KO | 0.90±0.14 | 1.38±0.19 | 0.47±0.29 | |

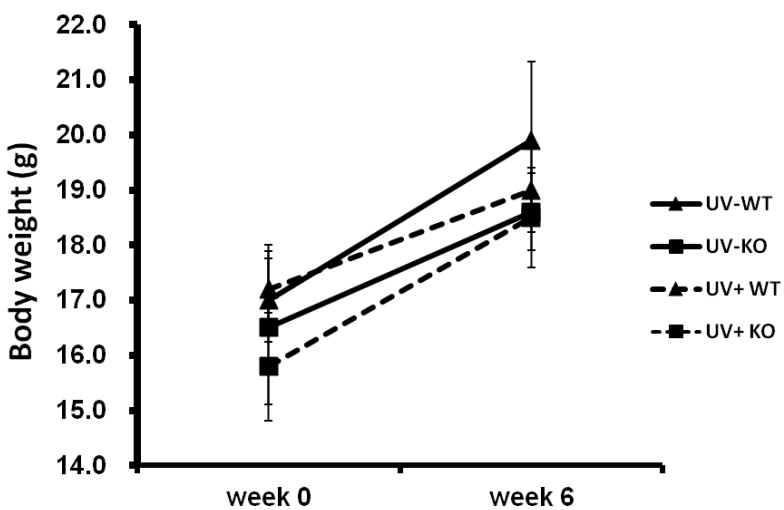
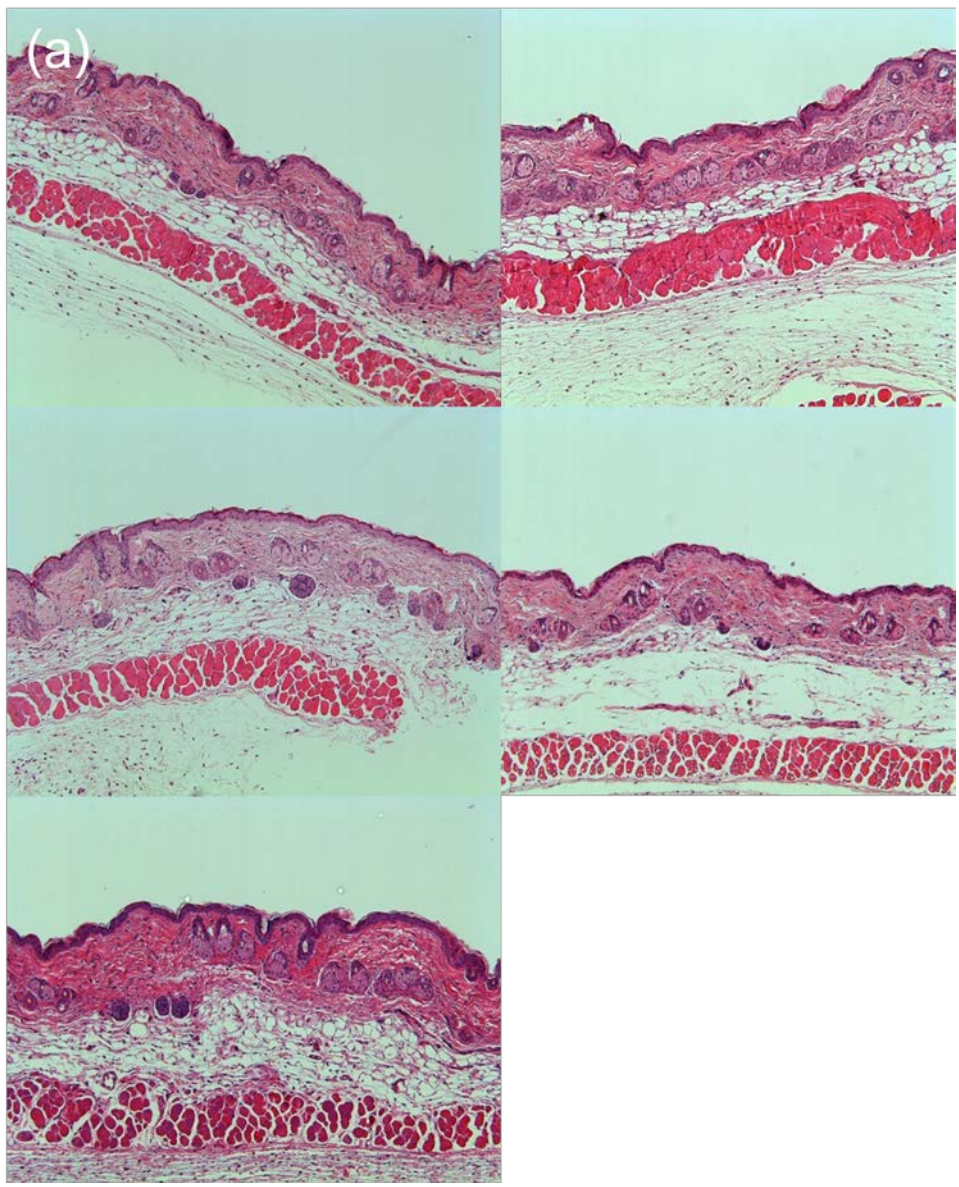
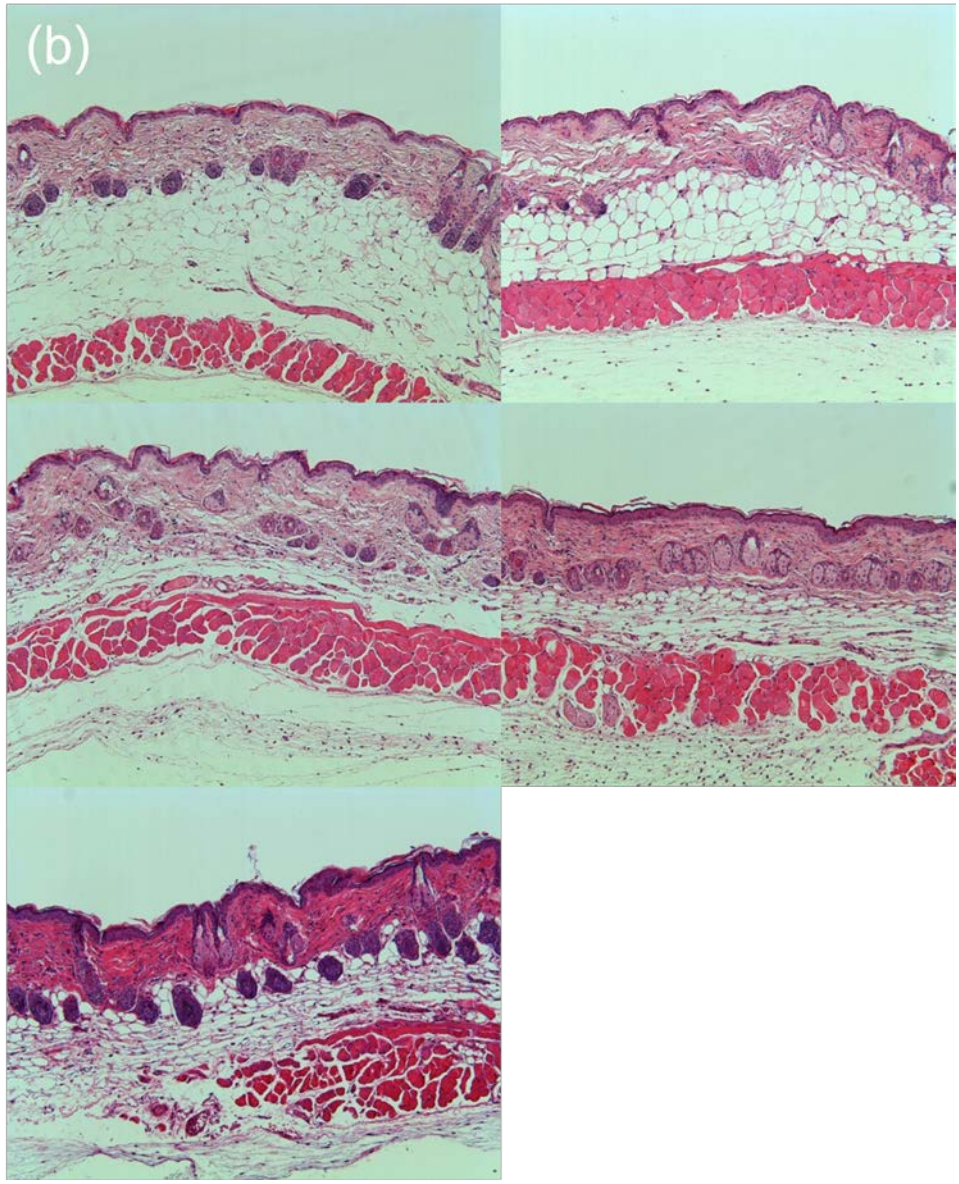



**Fig. 3 Change of body weight of mice during the experimental period**

### 3. Histopathologic findings of dorsal skin

TLR2 KO mice showed less severe parakeratosis, acanthosis, inflammatory cell infiltration than WT mice did, after repetitive UV irradiation of 6 weeks. Although proportion of severe infiltration was lower in UV+KO group (0/6) compared to UV+WT (2/6), the difference was not statistically different **(Fig. 4).**

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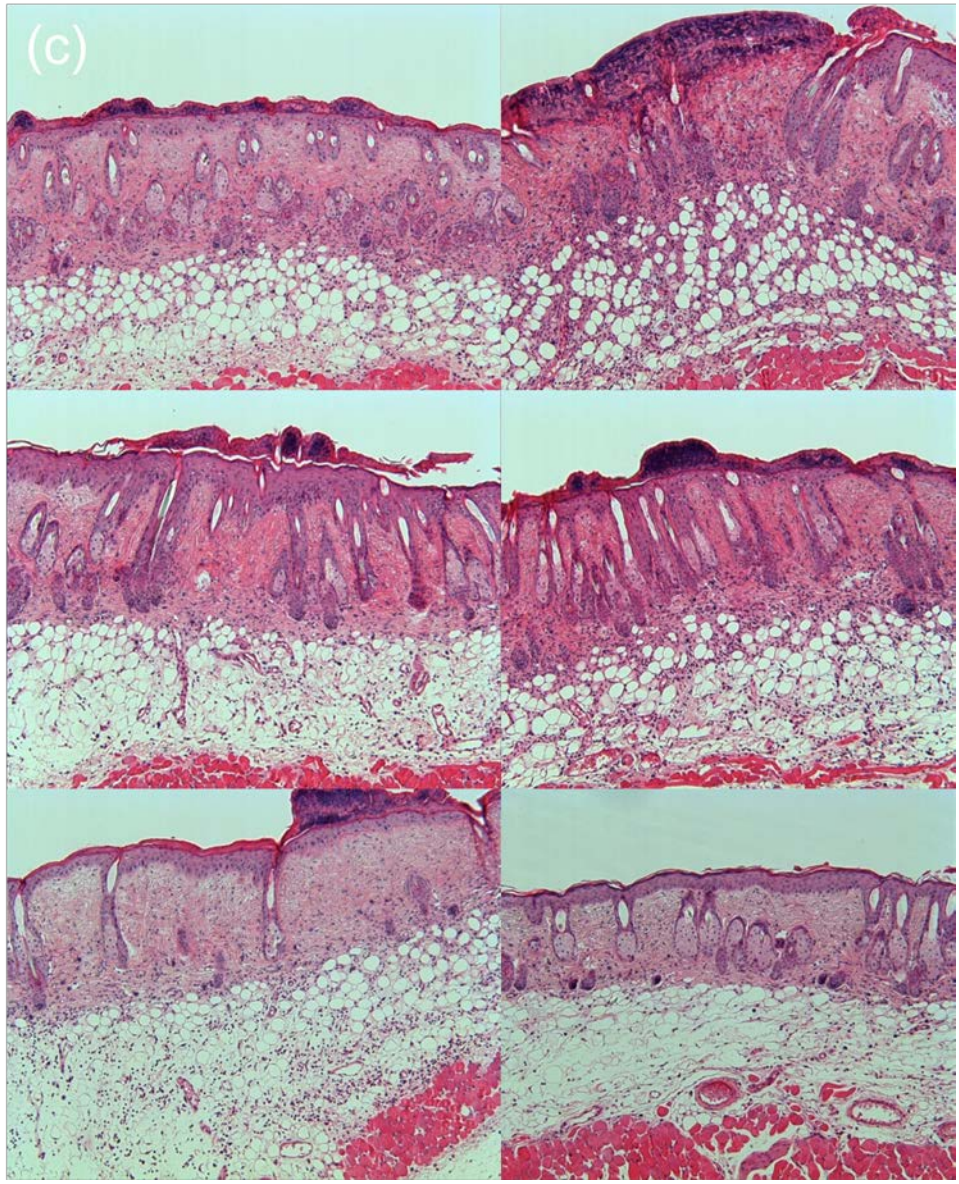
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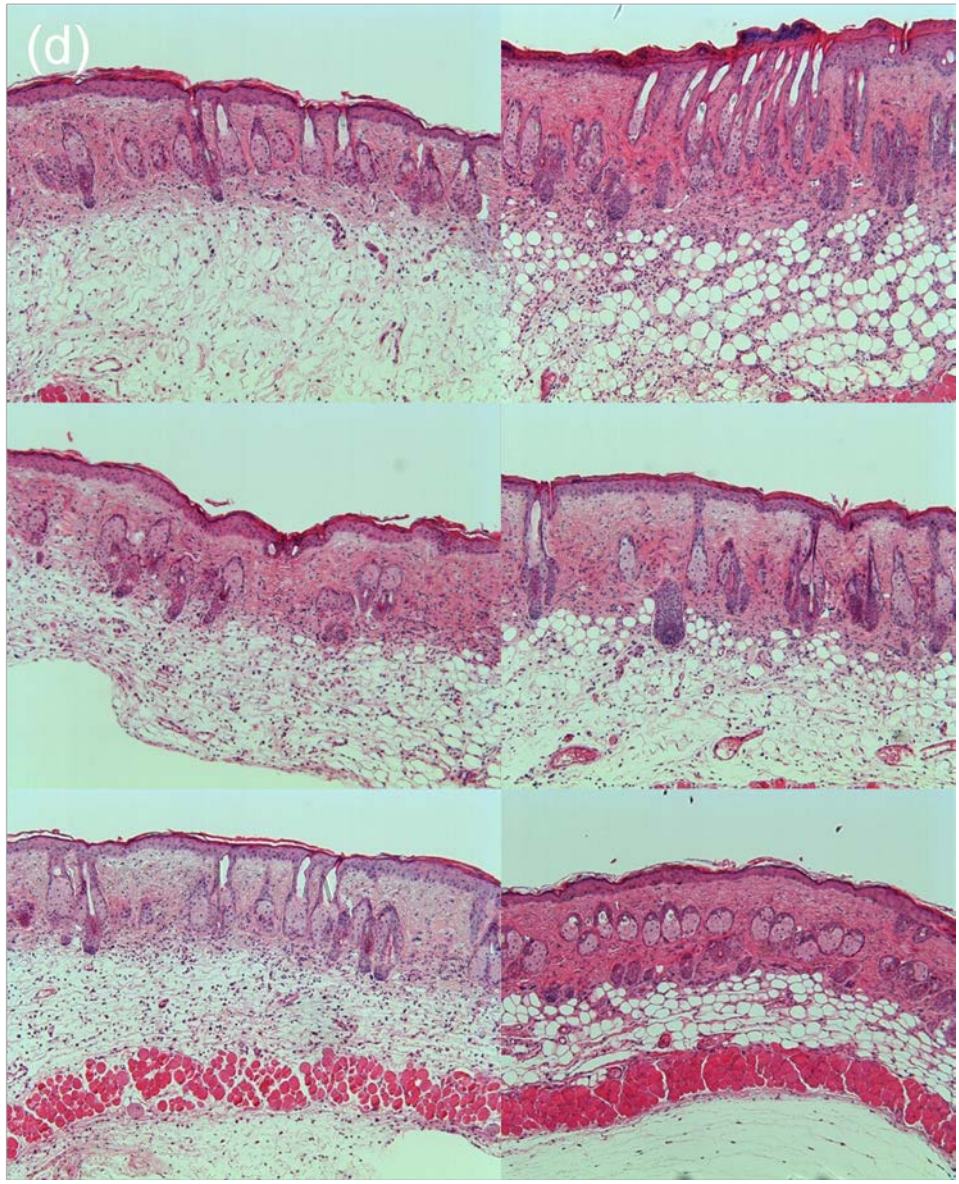
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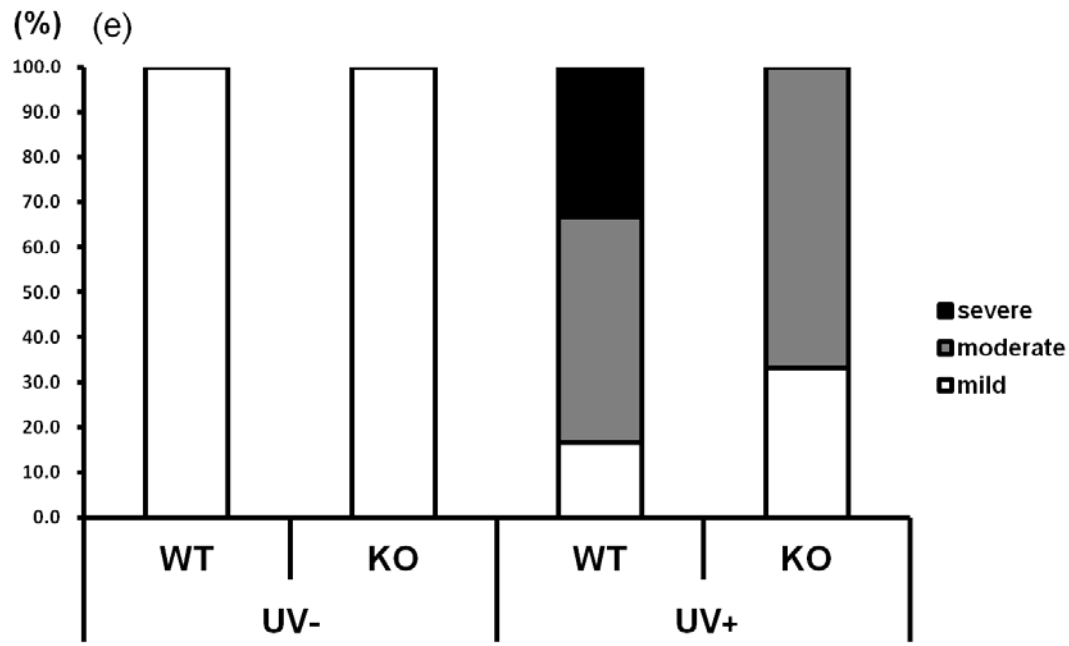



**Fig. 4 Histopathologic findings of dorsal skin after 6 weeks of UV irradiation (H&E × 100)** (a) UV- WT (b) UV- KO (c) UV+ WT (d) UV+ KO (e) degree of inflammatory cellular infiltration in each group (Fisher's exact test, P=0.455) Degree of inflammation was evaluated by the number of inflammatory cells. Mild inflammatory cell infiltration was defined by 0-49 dermal inflammatory cells per field (×200, average number of 3 random measurements), moderate 5-99, and severe >100.

### 4. Skin thickness by image analysis of H&E stained specimens

The thickness of epidermis and dermis was not much different between the two groups before UV irradiation (UV-KO 19.2±1.9 $\mu$m vs. UV-WT 19.7±1.4 $\mu$m). The epidermis and dermis were significantly thinner in TLR2 KO mice than those in WT at week 6 of UV irradiation (UV+KO 230.7±1.4 $\mu$m vs. UV+WT 270.0±30.1 $\mu$m).

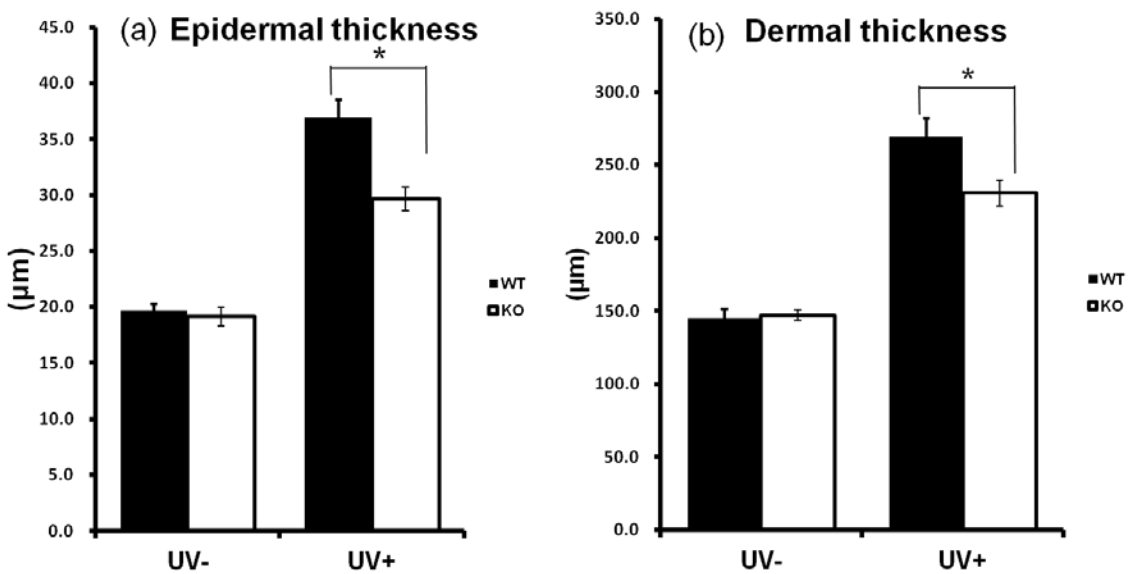



**Fig. 5 Skin thickness by image analysis** (a) significantly thicker epidermal skin in UV+ WT mice than in UV+ KO mice (*:P<0.05, by Mann-Whitney test) (b) thicker dermal skin in UV+ WT mice than in UV+ KO mice (*:P<0.05, by Mann-Whitney test, error bars demonstrate standard error of the mean)

## 5. Analysis of TUNEL-positive apoptotic cells

The number of TUNEL-positive cells before UV irradiation was not much different between the two groups (UV-KO 6.9±10.4 cells vs. UV-WT 8.7±5.4). Less TUNEL-positive cells were observed in TLR2 KO mice than in WT after repetitive UV irradiation, although the difference was not statistically significant (UV+KO 35.9±6.2 cells vs. UV+WT 39.0±8.2).

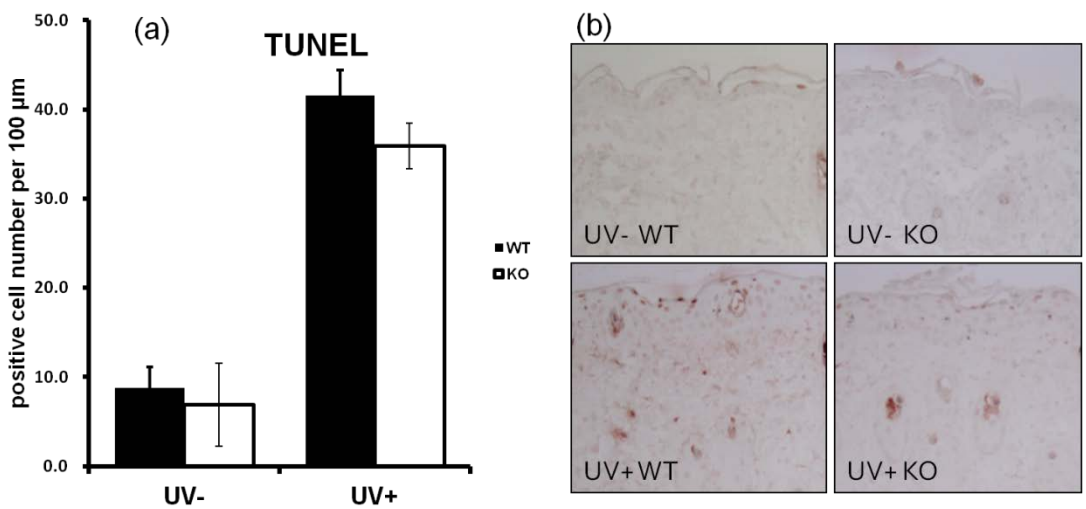



**Fig. 6 UV-irradiated TLR2 KO mice showed less TUNEL-positive apoptotic cells than WT mice.** (a) number of apotopic cells by image analysis (error bars demonstrate standard error of the mean) (b) TUNEL staining in each group (representative image of five or six mice in each group)

## 6. Analysis of inflammatory cells by immunohistochemistry for MPO

The number of MPO-positive cells before UV irradiation was not much different between the two groups (UV-KO 28.5±16.8 cells vs. UV-WT 23.8±17.7). Less MPO-positive cells were observed in TLR2 KO mice than in WT after repetitive UV irradiation (UV+KO 44.1±12.8 cells vs. UV+WT 61.9±16.5).

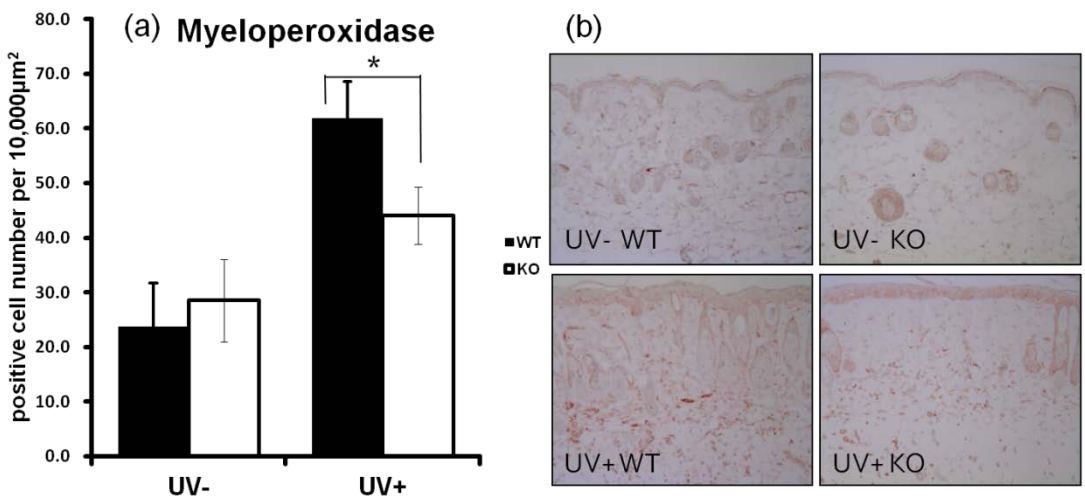



**Fig. 7 UV-irradiated TLR2 KO mice showed less MPO-positive cells than WT mice.** (a) number of MPO-positive cells by image analysis (*:P<0.05, by Mann-Whitney test, error bars demonstrate standard error of the mean) (b) MPO staining in each group (representative image of five or six mice in each group)

## 7. Analysis of proliferative cells by immunohistochemistry for Ki-67

The number of Ki-67-positive cells before UV irradiation was slightly different between the two groups, although it was not significant (UV-KO 23.2±12.4 cells vs. UV-WT 30.3±7.8). More Ki-67-positive cells were observed in TLR2 KO mice than in WT after repetitive UV irradiation (UV+KO 45.3±5.8 cells vs. UV+WT 41.5±9.6), although the difference was not statistically significant.

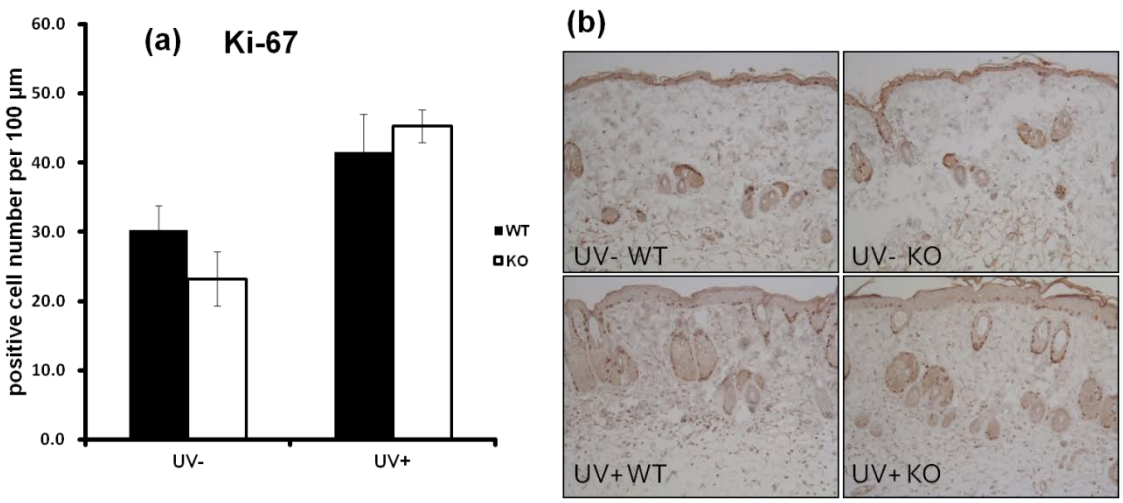
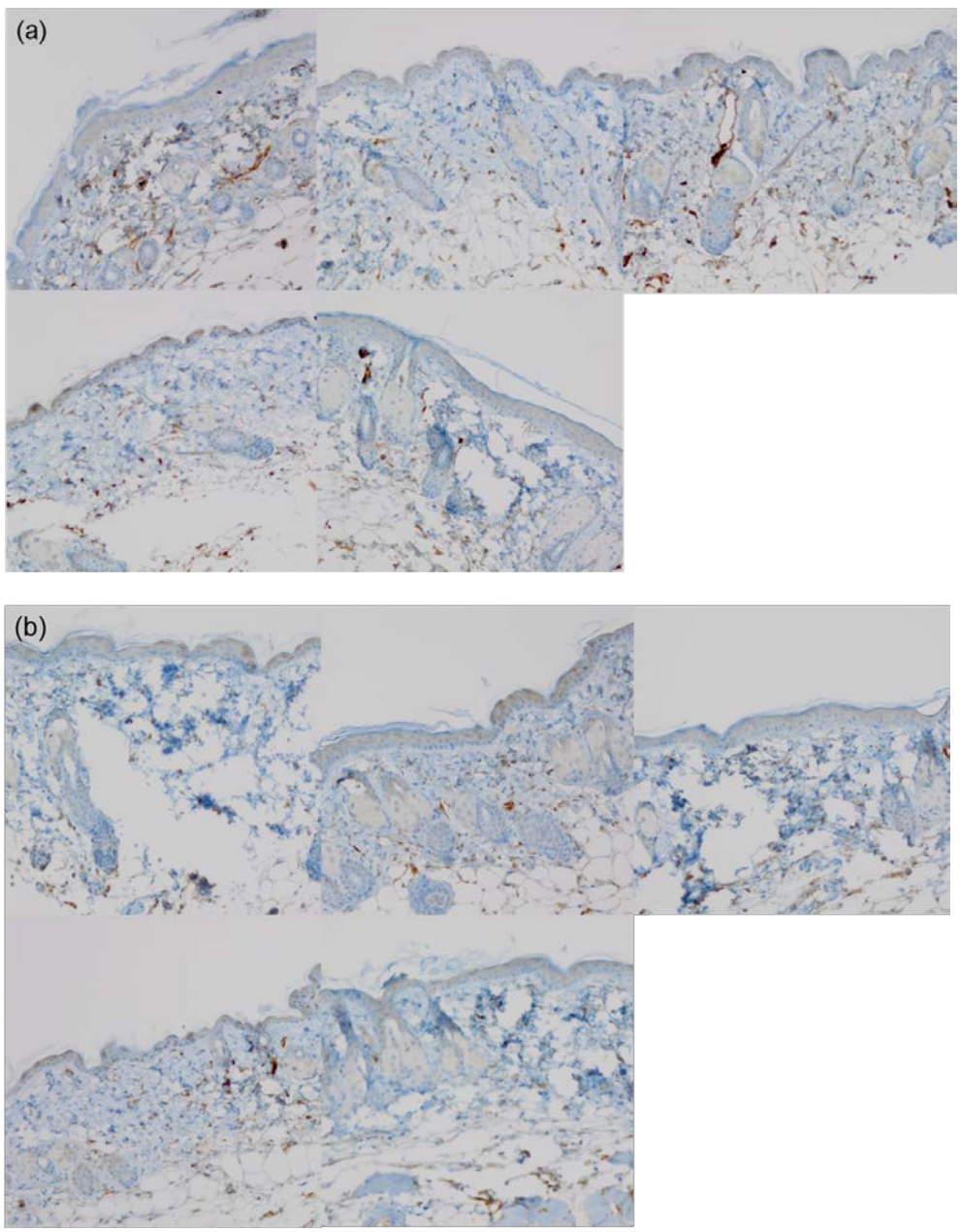
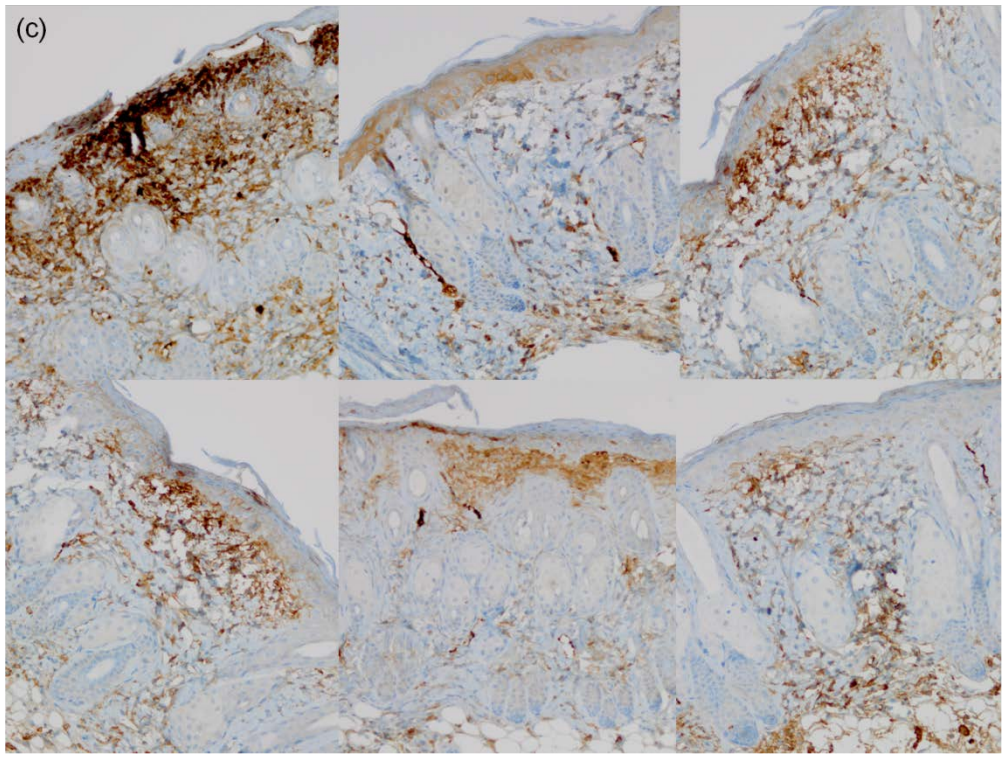



**Fig. 8 UV-irradiated TLR2 KO mice showed more Ki-67positive cells than WT mice.**
(a) number of Ki-67 positive cells by image analysis (error bars demonstrate standard error of the mean) (b) Ki-67 staining in each group (representative image of five or six mice in each group)

## 8. Analysis of expression of HSP70 by immunohistochemistry

The expression of HSP70 was induced after UV irradiation in both WT and KO mice. However, the degree of expression was lower in UV+KO group.




(c)

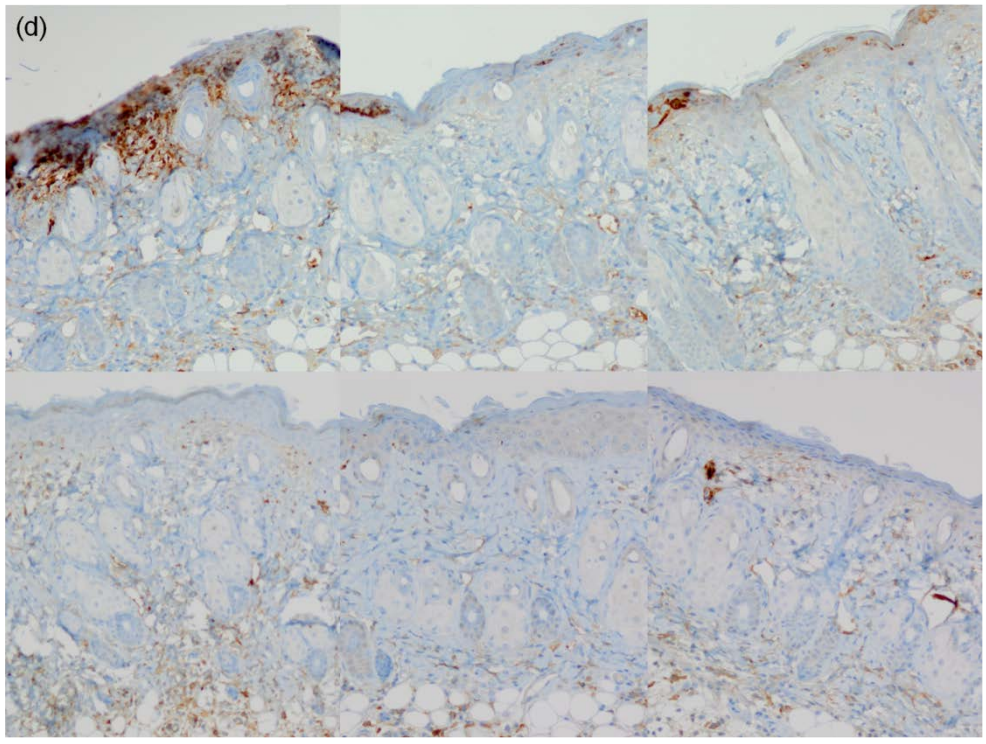

(d)

**Fig. 9 UV-irradiated TLR2 KO mice showed less positivity to HSP70 than WT mice did.** (a) UV- WT (b) UV-KO (c) UV+ WT (d) UV+KO

## 9. Gene level quantitation of cytokines, MMPs, and procollagen using real-time RT-PCR

### A. Proinflammatory or immunoregulatory cytokine expression

The expression of IL-1β, IL-6, TNF-α, and IL-10 was measured by quantitative real-time RT-PCR. Average basal expression of UV-WT group was standardized as 1 fold and expression of other groups was compared. Baseline expression of those cytokines was not much different between UV-WT and UV-KO group. However, UV-induced expression was different between UV+WT and UV+KO groups, according to cytokines. UV-induced expression of IL-1β (UV+KO 8.9±0.7 fold vs. UV+WT 28.4±0.7) and IL-6 (UV+KO 7.5±0.7 vs. UV+WT 24.7±0.7) was significantly less increased in TLR2 KO mice than in WT mice. Expression of TNF-α was not significantly different between the two groups (UV+KO 0.5±0.9 vs. UV+WT 1.2±0.9). Baseline expression of IL-10 was not much different between UV-WT and UV-KO group. UV-induced expression of IL-10 was increased in UV+KO group compared to that in UV+WT group (UV+KO 3.0±0.7 fold vs. UV+WT 1.7±0.9), but the difference was not significant.

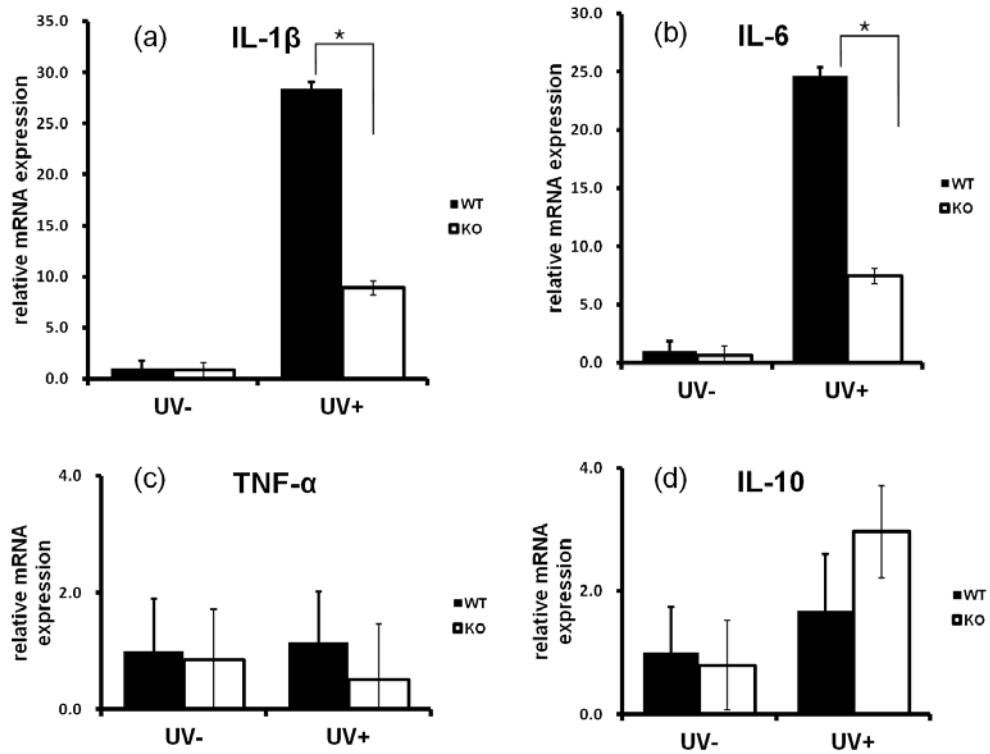



**Fig. 10 UV-induced cytokine expressions including IL-1β and IL-6 were significantly less increased in TLR2 KO mice.** (a) IL-1β (b) IL-6 (c) TNF-α (d) IL-10 (*:P<0.05, by Mann-Whitney test, error bars demonstrate standard error of the mean)

## B. MMPs and procollagen

Average basal expression of UV-WT group was standardized as 1 fold and expression of other groups was compared. Baseline expression of MMPs and

procollagen was not much different between UV-WT and UV-KO group. UV-induced expression of MMP-13 was significantly lower in UV+KO group (UV+KO 2.8±0.7 fold vs. UV+WT 9.2±0.5). UV-induced expression of MMP-9 (UV+KO 1.0±0.8 fold vs. UV+WT 1.1±0.6) and procollagen (UV+KO 1.6±0.9 fold vs. UV+WT 1.3±0.8) was not much different between the two groups.

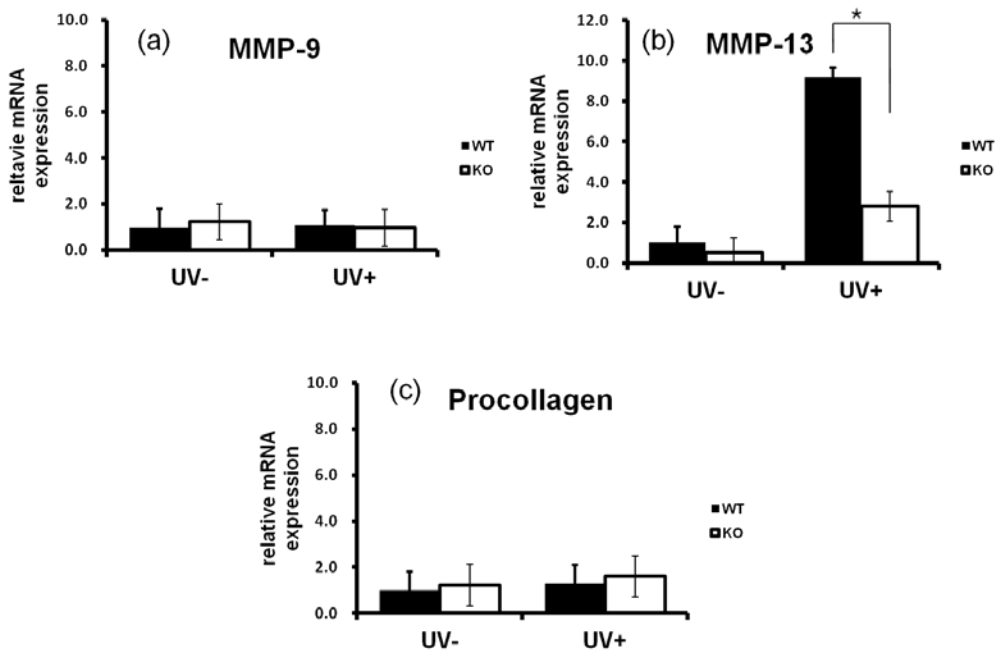



**Fig. 11 RT-PCR results showing basal and UV-induced expression of** (a) MMP-9 (b) MMP-13 (c) Procollagen (*:P<0.05, by Mann-Whitney test, error bars demonstrate standard error of the mean)

## 10. Protein level quantitation of proinflammatory cytokines and other associated cytokines using ELISA

Baseline levels of various cytokines were not so different between UV-KO and UV-WT groups. UV-induced level of IL-1β was significantly lower in UV+KO group (UV+KO 13.1±2.7 pg/ml vs. UV+WT 29.2±6.2 pg/ml). UV-induced IL-10 level was slightly higher in UV+KO group (UV+KO 5.3±1.0 pg/ml vs. UV+WT 4.2±0.5 pg/ml), but the difference was not significant. IL-6, TNF-α and IL-10 showed similar levels in both groups.

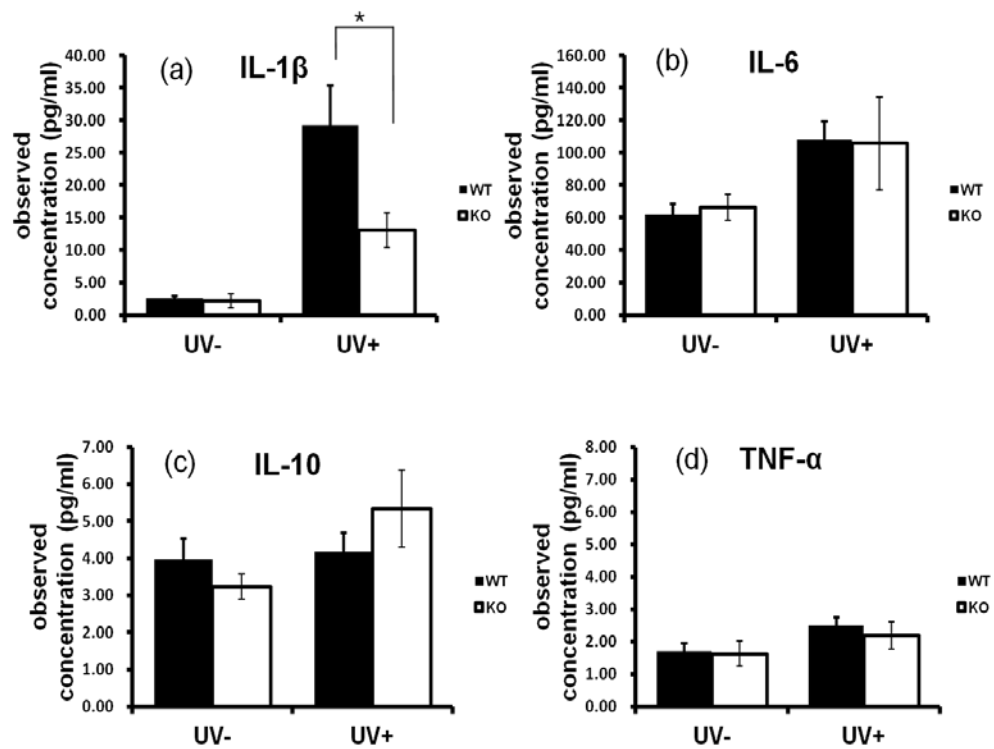
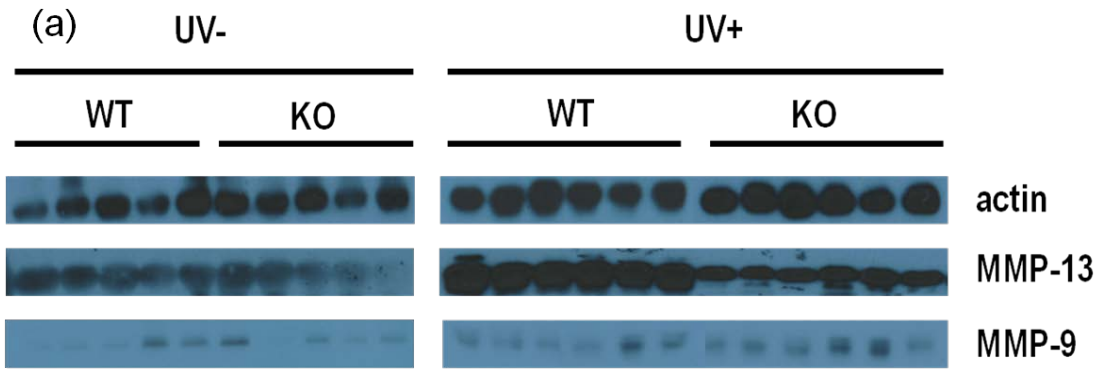


**Fig. 12 ELISA showing expression of IL-1β, IL-6, TNF-α and IL-10 in WT and TLR2 KO mice** (*:P<0.05, by Mann-Whitney test, error bars demonstrate standard error of the mean)

## 11. Protein level quantitation of MMPs using western blot

Baseline levels of MMP-13 were not so different between groups. UV-induced level of MMP-13 was significantly lower in UV+KO than that of UV+WT. (UV+KO 350.5±59.9 % vs. UV+WT 859.8±123.8%). Baseline and UV-induced MMP-9 levels were not so different between groups.



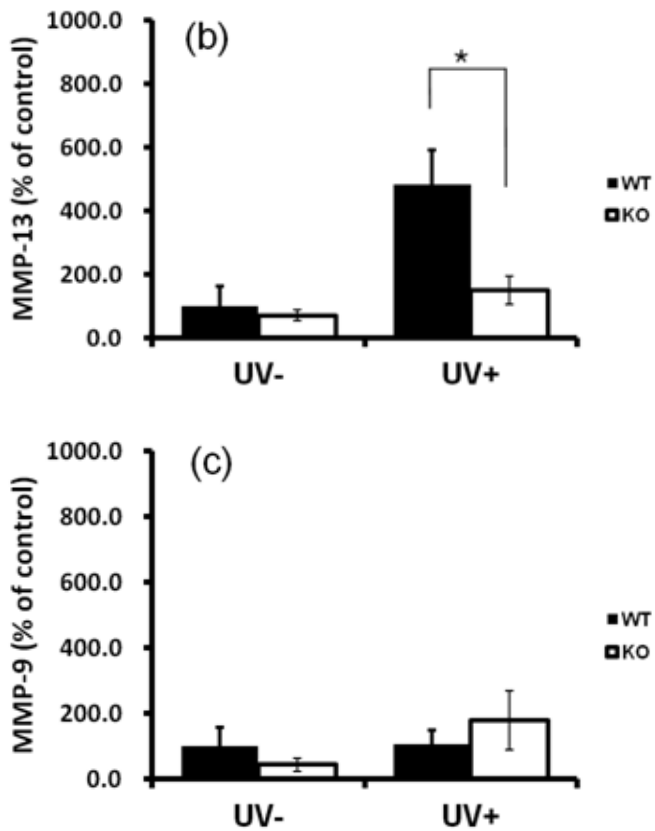



**Fig. 13 Western blot demonstrating levels of MMP-13 and MMP-9 in WT and TLR2 KO mice** (a) western blot (b) quantitative comparison of MMP-13 (c) quantitative comparison of MMP-9 (*:P<0.05, by Mann-Whitney test, error bars demonstrate standard error of the mean)

# Discussion

TLRs, expressed on sentinel cells of the immune system, including keratinocytes, macrophages and dendritic cells, are the key sensors of pathogen invasion (14, 29). They play a major role of the initiator of the innate immune responses to protect a host against pathogens including bacteria or viruses (30). In addition to their role in host defense from infection, they are involved in tissue homeostasis, such as wound healing, tissue repair, and regeneration even under sterile conditions (31). Recently, TLR has been also shown to have a role in carcinogenesis (31, 32). TLR is like a two-edged sword and it has an opposing function in reaction to pathogens, carcinogenesis, wound healing, and tissue regeneration (32-34). The exact pathomechanism of TLR paradox has not been elucidated yet. However, involving organs (organs like skin or colon with normal colonies *vs*. sterile heart) (31), types of ligands (peptide bound *vs*. membrane bound) (35, 36), species (human *vs.* mouse) (30) or participating cells (macrophage *vs.* dendritic cells) (37) are suggested to make differences. For example, the same pathogen, *Staphylococcus aureus*, can show either proinflammatory or modulatory reaction through TLR2. Proinflammatory signals are induced through dendritic cells, whereas IL-10 immunomodulatory responses are through macrophages (38).

Inflammation is an adaptive response that is triggered by abnormal conditions, such as infection (the best-understood trigger of inflammation) and tissue injury. TLRs are the best characterized PRRs which can initiate an inflammatory response (4) and their role, not only key sensors of pathogen invasion but also key regulators of acute and chronic

inflammatory processes has been emerged (39). UV can cause sterile inflammation without microbial pathogens and TLRs are thought to be involved in the inflammatory response induced by UV irradiation (14, 30). In an *in vitro* study with human epidermal keratinocytes, single UVB irradiation (100 mJ/cm$^2$) increased expression of IL-6 and MMP-1 through MyD88, which is a common adaptor protein of TLRs (22) and administration of TLR2 agonist increased expression of MMP-1 and MMP-9 in human epidermal keratinocytes via NF-κB (24). However, the role of TLR in acute single UV irradiation has been also paradoxical. In another *in vivo* study which was performed in our laboratory, TLR2 negatively regulated an inflammatory response after single dose UV irradiation (200 mJ/cm$^2$) through activation of regulatory T-cells and induction of IL-10 (unpublished data). Some authors also reported that TLR3 is associated with inflammation after single UVB radiation. RNA released from human keratinocytes after UVB irradiation exposure (15 mJ/cm$^2$) stimulated TNF-α and IL-6 from non-irradiated human keratinocytes and peripheral blood mononuclear cells, in a TLR3-dependent manner. In the same study, WT mice up-regulated TNF-α and IL-6 in the skin in response to single high dose UVB irradiation (500 mJ/cm$^2$) whereas TLR3 KO mice did not (25). The most recent *in vitro* study reported that protein extracts of single UVB-irradiated (30 mJ/cm$^2$) human keratinocytes activated TLR2 on Langerhans cells and induced the gene expression of MAPK, NF-κB, and interferon regulatory factor-3 (26). Compared to the previous reports which were mostly of *in vitro* studies focusing on single UVB irradiation, the present study aimed to investigate the role of TLR2 in repetitive moderate dose UVB- induced inflammation *in vivo* that has not been previously studied.

This repetitive irradiation pattern was not available in previous studies, considering the nature of cell-based *in vitro* experiments. Furthermore, in this study, we titrated the UVB irradiation dose to avoid severe burn, but to maintain reasonable erythema, which appears to better simulate the UV exposure pattern experienced in everyday life. There is no satisfying explanation why TLRs demonstrate opposing functions and it remains to be answered. Different UV doses (15-500mJ/$cm^2$), participating cell types, and microenvironment or conditions between various experiments might be one of the causes of paradoxical responses. We wanted to investigate not only acute inflammation but also chronic responses if possible, but unfortunately, carcinogenesis or skin aging was not observed during 6 weeks of repetitive UV irradiation in the present study. However, unfortunately, carcinogenesis or skin aging was not observed during the 6 weeks of repetitive UVB irradiation in this study. In our preliminary studies (data not presented), mice irradiated with low or moderate doses did not survive long enough to generate any wrinkles or skin cancers. Higher dose UV irradiation to shorten the experiment period was not successful either, because it caused burn and hindered repetitive irradiation. Backcrossing of TLR2 KO mice with C57BL/6 background onto the hairless mice may make these experiments easier and shorten the time necessary to achieve the required responses.

Although much progress is achieved, we do not know the exact pathomechanism of UV responses yet. The most recent study suggested that UV-damaged self noncoding RNA was detected by TLR3 and it was a DAMP which served as an endogenous signal of UV injury (25). It stimulated keratinocytes to produce proinflammatory cytokines including

IL-6 and TNF-$\alpha$. However, TLR3 did not influence IL-1 secretion. In our study, levels of TNF-$\alpha$ and IL-6 were not much different between WT and TLR2 KO mice whereas IL-1 and MMP-13 were. TLR2 might influence IL-1 and MMP-13 when TLR3 involved TNF-$\alpha$ and IL-6. Further studies upon UV pathway and UV-associated TLR pathway are required.

As a damaged self noncoding RNA released from UV-irradiated keratinocytes was an endogenous ligand of TLR3 and activated proinflammatory signals of UV injury (25), one of endogenous ligands of TLR2 may be associated with different responses against UV injury. There are many endogenous ligands of TLR2, including biglycan, endoplasmin, HMGB1, HSP60, HSP70, human cardiac myosin, hyaluronan, and monosodium urate crystal (5). Among them, HSP family is most likely to be associated with UV response. Mammalian cells increase HSPs against several biological stress factors, including heat, high pressure, ischemia, oxidative stress, heavy metal and UV irradiation (40, 41). In early 1960's, HSPs were first discovered as highly conserved proteins present in both prokaryotes and eukaryotes (42). These are essential molecular chaperones because they assist the correct folding of misfolded proteins and prevent aggregation (43). HSPs can be classified according to their molecular size: HSP110, HSP90, HSP70, HSP60, and the small HSPs (44). They are either constitutively expressed or inductively regulated. For example, HSP90 is constitutively abundantly expressed in the cells whereas HSP70 and HSP27 are highly induced in response to stress (44, 45).

The role of HSPs is controversial. Recently, novel immunoregulatory, anti-apototic and cytoprotective function of HSPs were reported. High levels of inducible HSPs suppress NF-κB, decrease proinflammatory cytokines, and ameliorate tissue inflammatory injury (46, 47). Contrary to the finding that inducible HSP protects further cellular injury, several other groups noted that induction of HSP lead to apoptosis in endothelial cells and enterocytes (48, 49). Therefore, DeMeester and his colleagues coined the term 'heat shock paradox' and suggested that the expression of HSPs before a proinflammatory stimulus protects cells but after the inflammation precipitates cell death. Still, there is neither appropriate explanation nor mechanism which can clearly address this paradox (44). Some scientists try to explain this paradox according to the location of HSPs. HSPs have been known to exist in the cells under normal physiologic conditions. However, some HSPs can be released into the extracellular compartment when cells undergo necrotic deaths, deliver a maturation signal for dendritic cells, and activate the NF-κB pathway (50). Additionally, HSP70 and HSP90 have been found in the extracellular location, bound to the plasma membrane. However, the mechanism of transport to the plasma membrane, the membrane anchorage, and the export remains unclear yet (51). An interaction between HSP60 or 70 and TLR2 or 4 on antigen presenting cells activates TIR pathway and induces subsequent proinflammatory response (52, 53). Therefore, Chen *et al.* (44) suggested that heat shock pre-treatment induces intracellular HSPs inhibiting inflammation whereas extracellular HSPs released from damaged or stressed cells after inflammatory injury or compromised cell integrity aggravate cell injury.

Extracellular HSPs are released from necrotic dead cells and can act as DAMPs to the immune system (54, 55). And even without immunogenic peptides, extracellular HSP70 and HSP90 can provide danger signals (56). Recent studies suggested that extracellular HSPs activated innate immunity and triggered an inflammatory response. Most of the studies were performed *in vitro* using cells in culture and exogenous HSPs. For example, HSP70 activated NF-κB and up-regulated proinflammatory cytokines (TNF-α, IL-1β and IL-6) in human monocytes (dendritic cells) (52). However, Tsan and Gao (57) criticized previous studies which used recombinant HSPs because they may have been contaminated by LPS, and increased cytokines may be the result of LPS but not due to HSPs themselves. Therefore, Basu *et al.* (50) used HSP70 derived from mouse liver in which LPS was undetectable and reported that HSP70 simulated macrophages to secrete TNF-α, IL-12, IL-1β and granulocyte macrophage colony stimulating factor.

Extracellular HSPs should interact with specific receptors to activate intracellular pathways. So far, various HSP receptor candidates have been identified, such as CD91, lectin-like oxidized low-density lipoprotein receptor-1, scavenger receptor-A, TLR2, and TLR4 (44). The immunogenic role of extracellular HSPs is dependent on specific receptors. If HSPs make a complex with peptides, they interact with receptors such as CD91 and lectin-like oxidized low-density lipoprotein receptor-1 (35, 36). Then these antigens are processed through major histocompatibility complex and stimulate CD4+ or CD8+ T cell responses. In a peptide independent consequence, HSPs interact with TLRs, CD36, and CD40 receptors to activate p38, NF-κB, and AP-1 (35, 36, 58). This activation of innate immunity is dependent on MyD88 and IRAK4 (59-61).

Many studies demonstrated that HSP70s are constitutively expressed in skin (40, 62, 63). Skin may demonstrate constant presence of HSP70s because it is exposed to multiple environmental stressors (41). As previously mentioned, various stresses can induce expression of HSPs, protecting cells from injury and promoting refolding of denatured proteins (64). Not only heat but also UV irradiation can also result in further enhanced expression of HSP70 in keratinocytes (62, 65, 66). Increased expression of HSP has been known to have protective function because HSP70-overexpression in cultured keratinocytes prevented UVB radiation-induced damage by inhibiting NF-κB and suppressing proinflammatory cytokines. Also, transgenic mice overexpressing human HSP70 showed less inflammatory response against 180mJ/cm$^2$ of UV irradiation (low MPO activity, less infiltration of inflammatory cells, and less TUNEL positive cells) compared to wild type mice (67). Similary, HSP70 demonstrated a disease suppressive role in experimental models of autoimmunity (68, 69). These immunoregulatory functions of HSP70 was through induction of regulatory T-cells producing IL-10 (30, 64). However, further exact pathomechanism should be elucidated and role of HSPs can be either immune-regulatory or proinflammatory according to the circumstances as previously mentioned in the discussion.

In the present study, depending on the presence of TLR2, C57BL/6 mice showed different inflammatory responses against repetitive UVB irradiation. TLR2 KO mice demonstrated less cell infiltration and lower number of neutrophils with MPO activity compared to TLR2 WT mice. They also exhibited lower levels of gene expression of IL-1β, IL-6, and MMP-13 on RT-PCR, lower protein level of IL-β on ELISA and MMP-13

on western blot. In summary, TLR2 KO mice demonstrated less inflammatory response against repetitive UVB irradiation. HSP70 staining was also weaker in TLR2 KO mice, but it is hard to conclude through the present study whether it directly caused the difference of inflammatory response or was merely a result. Although HSP70 may not be the initial cause of different inflammatory response, it consequentially seems to cause more proinflammatory response through interaction with TLR2 to activate TIR pathways depending on MyD88 pathways, considering repetitive nature of the present study. Further experiments involving injection of purified HSP70 from necrotic cells after UVB irradiation into TLR2 KO mice or application of HSP70 inhibitors are required to establish this hypothesis.

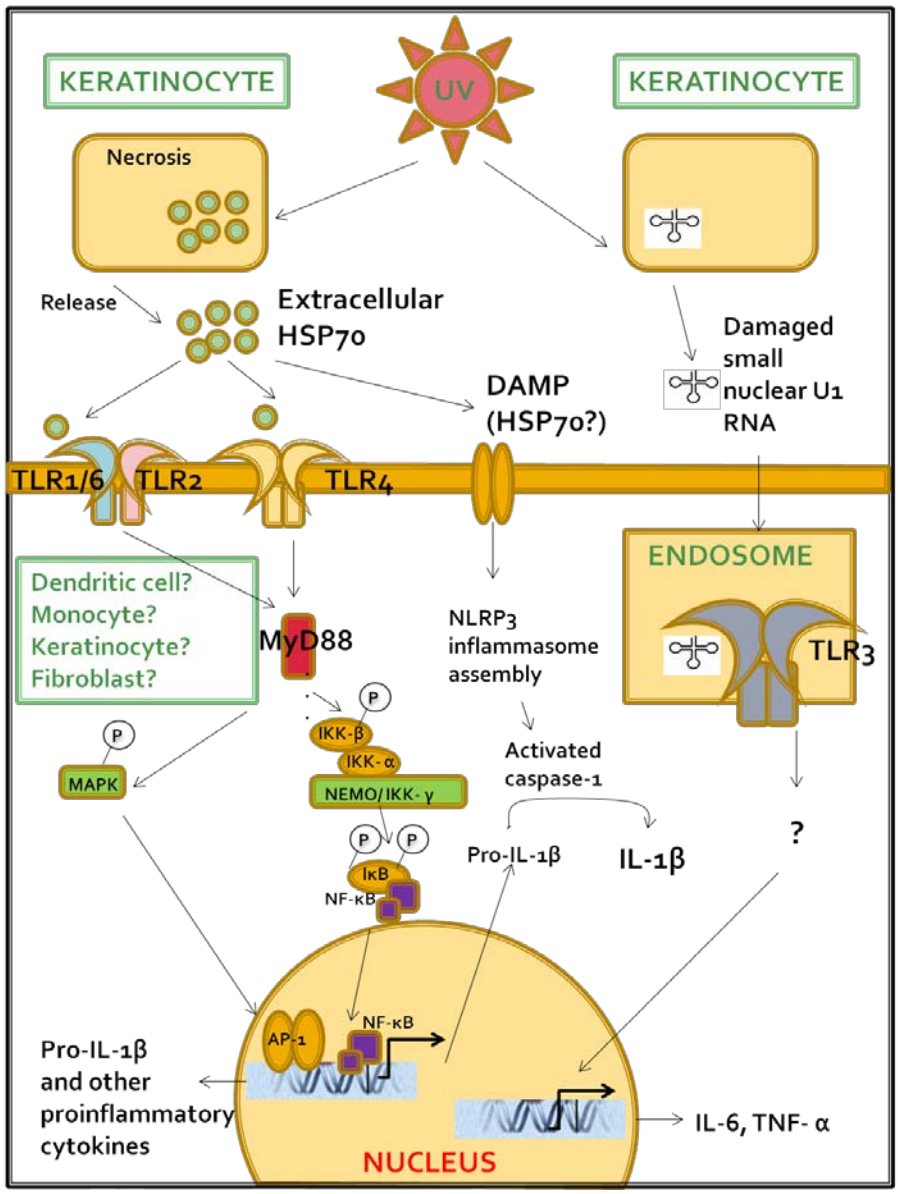



**Fig. 14 Signal pathways which is supposed to be associated with an interaction between UV radiation and TLR**

A chain of reactions after UV irradiation in the present study can be summarized as **Fig. 14**, which may explain the different responses between single and repetitive UV

irradiation. In an acute stress situation caused by single UV irradiation, an immunoregulatory effect rather than a proinflammatory cascade was predominant through TLR2. However, in a chronic stress environment with repetitive UV irradiation, immune system may be worn out and HSP70s released not before but after the inflammation precipitates cell death and proinflammatory responses, like suggested in HSP paradox hypothesis. This vicious cycle might contribute to the different inflammatory response. Also, it is possible that different outcomes can ensue depending on the form of extracellular HSP70 and the target cell/tissue microenvironment (64). Furthermore, it is an interesting feature of TLR2 that it can either induce proinflammatory or anti-inflammatory response depending on the nature of the ligand and the population of target cells. Further studies are required to verify the conflicting results between experiments and exact pathomechanisms need to be elucidated.

Our study demonstrated that TLR2 mediated inflammatory responses to repetitive UVB irradiation in C57BL/6 mice, which suggested that the role of TLR2 in the cutaneous response of UV irradiation and in developing new agents for modulating the effects of UV irradiation should be considered. However, the present study has some important limitations. Target cell population making the difference according to the presence of TLR2 was not clearly elucidated in the present study. Keratinocytes, monocytes, fibroblasts and dendritic cells are possible candidates expressing TLR2. Also, the present study showed only major differences and did not clearly indentify the pathways involved in the different responses. Therefore, further experiments with primary cultures of different cell group or investigating the mechanisms are required.

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# 초 록

Toll-like receptor 는 가장 잘 알려진 pattern recognition receptor 의 하나로, 외부 병원체의 침입으로부터 개체를 보호하는 선천 면역 기능을 갖는다. 이뿐 아니라, 상처 치유, 발암 현상 등 병원체의 침입이 없는 상황에서도 개체의 생체 반응에 관여한다고 알려져 있다. 최근의 연구에서는 일회의 자외선 조사와 관련된 다양한 반응들에도 Toll-like receptor 가 관여한다고 보고된다. 본 연구에서는 Toll-like receptor 2가 있는 C57BL/6 마우스와 Toll-like receptor 2가 없는 C57BL/6 마우스에서 반복적인 자외선 조사에 의한 반응이 차이가 있는지 비교 실험을 시행하였다. 주 3회 6주 간의 반복적인 자외선 조사에 대해 Toll-like receptor 2 가 없는 마우스에서 더 약한 염증 반응을 보였다. 즉, 자외선 조사 후 Toll-like receptor 2 가 없는 마우스에서 피부 두께가 더 얇았으며, 염증 세포의 침윤도 적게 관찰되었다. 또한 반복적인 자외선 조사 후 채취한 피부 조직을 이용한 real time PCR 실험에서 Toll-like receptor 2 가 없는 마우스가 정상 마우스에 비해 IL-1β, IL-6, MMP-13 유전자 발현이 낮았다. 동일한 반복적인 자외선 조사 후 채취한 피부 조직을 이용한 ELISA 에서는 IL-1β가, western blot 에서는 MMP-13 의 단백질 발현이 Toll-like receptor 2 가 없는 마우스에서 정상 마우스 보다 낮게 관찰되었다. 면역 조직화학 염색 실험에서 Toll-like receptor 2 의 endogenous ligand 로 알려져 있는 heat shock protein 70의 발현이 반복적인 자외선 조사 후Toll-like receptor 2 가 없는 마우스에서 정상 마우스보다 낮게 관찰되었다.

본 실험을 통해 C57BL/6 마우스에서 Toll-like receptor 2 가 반복적인 자외선 조사에 의해 나타나는 염증반응에 관여함을 알 수 있었으나, 관련된 정확한 경로는 본 실험에서 밝힐 수 없는 한계가 있었다. 향후의 연구를 통해, Toll-like receptor 2 가 매개하는 반복적 자외선 조사에 의한 염증 반응과 연관된 정확한 세포 집단과 그 기전을 밝히는 것이 필요하다.

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주요어: 반복 조사, 염증, 자외선, C57BL/6 마우스, Toll-like receptor 2

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