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# RIPK1 downregulation in keratinocyte enhances TRAIL signaling in psoriasis☆

Nao Saito*, Masaru Honma, Takashi Shibuya, Shin Iinuma, Satomi Igawa, Mari Kishibe, Akemi Ishida-Yamamoto

*Department of Dermatology, Asahikawa Medical University, Japan*

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ABSTRACT

*Background:* Psoriasis, a common inflammatory skin disorder characterized by scaly erythema and plaques, is induced by dysregulation of dendritic cell- and T cell-mediated immune reaction. Receptor-interacting protein kinase 1 (RIPK1) regulates inflammatory signaling in response to stimuli such as TNF-α, TRAIL, and TLRs, resulting in apoptosis, necroptosis and NF-κB activation. However, the physiological relevance in human epidermis remains elusive.

*Objective:* In this study, we examined whether RIPK1 is involved in the pathogenesis of psoriasis vulgaris.

*Methods:* Skin samples of eight patients with psoriasis vulgaris were investigated by western blotting and immunohistochemistry. The functions of RIPK1 in keratinocytes were examined by RT-PCR and ELISA *in vitro*. TRAIL-neutralization-experiment was employed in an imiquimod-induced murine psoriasis model.

*Results:* In lesional psoriatic epidermis, RIPK1-expression was decreased compared with that in normal epidermis. Cytokines involved in the pathomechanism of psoriasis, such as IL-1β, IL-17A, IL-22 and TRAIL, reduced RIPK1-expression in normal human epidermal keratinocytes (HEK) *in vitro*. In addition, RIPK1-knockdown enhanced TRAIL-mediated expression of psoriasis-relating cytokines, such as IL-1β, IL-6, IL-8, TNF-α, in HEK. Numerous TRAIL-positive cells were detected in the dermis of lesional psoriatic skin, and TRAIL receptors were expressed in psoriatic epidermis and HEK in conventional cultures. Moreover, TRAIL-neutralization in an imiquimod-induced murine psoriasis model remarkably improved skin phenotypes, such as ear thickness, and TNF-α expression in lesional skin.

*Conclusions:* These results lead us to conclude that RIPK1-downregulation in keratinocytes increases their susceptibility to TRAIL stimulation, and plays a role in the pathogenesis of psoriasis vulgaris.



## 1. Introduction

Psoriasis, a common inflammatory skin disorder characterized by scaly erythemas and plaques, affects around 2% of the population [1]. Dysregulated interactions of innate and adaptive immunities are deeply involved in the pathomechanism. Complexes of epidermis-derived antimicrobial peptide, LL-37, and host DNA are thought to act as an initiating factor via stimulating IFN-α-production from dermal plasmacytoid dendritic cells [2]. Consequently, activated myeloid dendritic cell (DC) populations release TNF-α and IL-23, and stimulate Th1 cells and T helper cell 17 (Th17) to produce IFN-γ, IL-17 and IL-22 [3,4]. These cytokines induce proliferation of epidermal keratinocytes and mobilize immune cells into lesional skin [5]. In the exacerbating inflammatory chain, DC/Th17 axis is a major research focus as well as being the therapeutic target in the pathomechanism of psoriasis [6,7].

Receptor-interacting protein kinase 1 (RIPK1), one of the receptor-interacting serine/threonine-protein kinases, was originally identified as a death domain-containing kinase. It is a key regulator of inflammation, apoptosis and necroptosis downstream of tumor necrosis factor (TNF)-α, TRAIL, and Toll-like receptors (TLRs). Expression or function of RIPK1 is regulated in complex and intricate

*Abbreviations:* HEK, human epidermal keratinocytes; RIPK1, receptor-interacting protein kinase 1; RIPK1-KD-NHK, RIPK1 knockdown keratinocyte; Th17, T helper cell 17; TRAIL, TNF-related apoptosis-inducing ligand; TNF, tumor necrosis factor.

☆ Work was done in Asahikawa, Japan.

* Corresponding author at: Department of Dermatology, Asahikawa Medical University, 2−1−1−1 Midorigaoka, Higashi, Asahikawa, Hokkaido, 078−8510, Japan.
*E-mail address:* bird09160078@yahoo.co.jp (N. Saito).

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mechanisms including ubiquitylation, deubiquitylation, and phosphorylation [8]. Recent studies have shown that RIPK1 is also involved in oncogenicity of melanoma, axonal degeneration of amyotrophic lateral sclerosis, and apoptosis in breast cancer [9–11]. Moreover, epidermis-specific RIPK1-knockout mice developed significant inflammatory skin reaction characterized by thickened epidermis with various types of cell death [12].

Given this background, we examined whether RIPK1 is involved in the pathogenesis of psoriasis vulgaris and found that keratinocytes become highly sensitive to TRAIL signaling by RIPK1-downregulation.

## 2. Materials and methods

### 2.1. Patient samples

A total of eight patients with psoriasis vulgaris at Asahikawa Medical University Hospital participated in this study. The collection of samples was performed with approval by the local ethical committee and the institutional review board of Asahikawa Medical University, and each patient gave written informed consent.

### 2.2. Keratinocyte culture and stimulation

Primary keratinocytes were cultured in CnT-PR medium (CELLnTEC, Bern, Switzerland) at 37 °C with 5% $CO_2$, and used for the assay with no more than fourth passages. Recombinant human TRAIL (100 ng/ml, Peprotech, Rocky Hill, NJ), recombinant human IL-1β (R&D systems, Minneapolis, MN), recombinant human IL-17A (Peprotech), recombinant human IL-22 (Peprotech), recombinant human IFN-γ (Peprotech), recombinant human TNF-α (100 ng/ml, Peprotech) and recombinant LL37 (2.56 μM, 7.68 μM, Eurogentec, Seraing, Belgium) were added into the culture medium.

### 2.3. siRNA transfection

Normal human keratinocytes (NHK) were transfected with si-RNA of RIPK1 (40 pM) or si-control (40 pM) (*Silencer*® Select Negative Control No.1 siRNA, Ambion, Austin, TX) using the electroporation system Nucleofection™ (Lonza, Basel Schweiz). At 72 h following transfection, cells were used in experiments.

### 2.4. Quantitative real-time PCR

Total RNA extracted using RNeasy Kit (Qiagen, Hilden, Germany) was applied for quantitative real-time PCR (RT-PCR) performed by a LightCycler 480 Instrument (Roche, Basel Schweiz) or TaqMan gene expression assays (Applied Biosystems, Carlsbad, CA). Primer and probe sets used were TNFRSF10A (Hs00269492_m1), TNFRSF10 B (Hs00366278_m1), TNFRSF10D (Hs00174664_m1), GAPDH (Hs02758991_g1), mIL17A (Mm00439618_ m1), mIL17F (Mm00521423_ m1), mIL-22 (Mm01226722_ g1) and mGAPDH (Mm99999915_g1). For each data point, similar results were obtained at least in two independent experiments.

### 2.5. Immunohistochemistry

Immunostaining on human and mouse skin was performed with antibodies against RIPK1 (Thermo Fisher Scientific, Waltham, MA), human CD4, CD8, CD11c (BD Biosciences, San Jose, CA), DR4, DR5, DcR2 (abcam, Cambridge, UK), and TRAIL (Cell signaling, Danvers, MA). Fluorescence staining was detected using a confocal laser scanning fluorescence microscope (Fluoview FV1000, Olympus, Tokyo, Japan).

### 2.6. ELISA

Human IL-1β and human TNF-α ELISA on culture supernatants of keratinocytes was performed using ELISA kits from R&D systems in accordance with the manufacturer's instructions.

### 2.7. Mice

C57BL/6J female mice (six weeks old) were purchased from Oriental Yeast (Tokyo, Japan). All the animal experiments were performed with the approval of the ethical committee for animal studies of Asahikawa Medical University.

### 2.8. IMQ-induced psoriasis model and Ab treatment

The skin of the back and the right ear of each mouse was treated daily for seven days with 62.5 mg and 31.25 mg of 5% IMQ cream (Beselna cream; Mochida Pharmaceutical, Tokyo, Japan). Vaseline petroleum jelly was used as control. In some experiments, mice received ip injections with 250 μg of anti-TRAIL (clone N2B2) or control rat IgG2a (Biolegend, San Diego, CA) per mouse before application of IMQ cream on days 0, 2, 4, and 6.

### 2.9. Evaluation of skin inflammation severity and measurement of ear thickness

To evaluate severity of mouse skin inflammation, an objective scoring system based on the clinical Psoriasis Area and Severity Index (PASI) was employed. Severity of erythema and scaling was rated independently as score 0 to 4: 0, none; 1, slight; 2, moderate; 3, marked; 4, severe. The ear thickness was measured using a caliper (Peacock Ozaki MFG. CO., LTD., Tokyo, Japan).

### 2.10. Statistics

In all figures, data are presented as mean ± SD from pooled data of at least 3 independent experiments. $P$ values were calculated with one-way analysis of variance (*post hoc* Tukey or Dunnett) and two-tailed independent Student's $t$-tests, and $P < 0.05$ was considered significant.

## 3. Results

### 3.1. Decreased RIPK1 expression in lesional epidermis of psoriasis

To clarify the function of RIPK1 in the epidermis, we first performed immunohistochemistry for RIPK1. In healthy control epidermis, atopic dermatitis and erythema multiforme, abundant RIPK1-expression was detected throughout whole layers of the epidermis. In contrast, it was minimal in psoriatic epidermis (Fig. 1A). Moreover, protein expression levels of RIPK1 was significantly decreased in lesional and non-lesional epidermis of psoriasis as compared with that in healthy control (Figs. 1B, C and S1). In lesional skin of IMQ-treated mice, an animal model recapitulating key features of psoriasis [13], qRT-PCR and western blotting revealed 80 to 97% decrease of RIPK1-expression in psoriasiform skin (Fig. 1D). To further clarify the regulatory mechanism, RIPK1-expession was analyzed in HEK treated with psoriasis-relating cytokines. IL-1β, IL-17A, IL-22, TRAIL, and IL-17A + IL-22 significantly reduced RIPK1-expression in HEK (Fig. 1E). These cytokines are produced by immune cells relating to both innate and adaptive immunities, and they are deeply involved in the pathogenesis of psoriasis. In addition to IL-1β and Th17 cytokines, TRAIL is expressed in inflammatory DC in psoriatic lesions, and can directly act on keratinocytes [14]. RIPK1 mainly regulates inflammatory signaling relating to NF-κB, apoptosis, and

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