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Elevated expression of interleukin-6 (IL-6) and serum amyloid A (SAA) in the skin and the serum of recessive dystrophic epidermolysis bullosa: Skin as a possible source of IL-6 through Toll-like receptor ligands and SAA

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Abstract

The effect of persistent skin inflammation on extracutaneous organs and blood is not well studied. Patients with recessive dystrophic epidermolysis bullosa (RDEB), a severe form of the inherited blistering skin disorder, have widespread and persistent skin ulcers, and they develop various complications including anaemia, hyperglobulinaemia, hypoalbuminaemia and secondary amyloidosis. These complications are associated with the bioactivities of IL-6, and the development of secondary amyloidosis requires the persistent elevation of serum amyloid A (SAA) level. We found that patients with RDEB had significantly higher serum levels of IL-6 and SAA compared to healthy volunteers and patients with psoriasis or atopic dermatitis. Both IL-6 and SAA were highly expressed in epidermal keratinocytes and dermal fibroblasts of the skin ulcer lesions. Keratinocytes and fibroblasts surrounding the ulcer lesions are continuously exposed to Toll-like receptor (TLR) ligands, pathogen-associated and damage-associated molecular pattern molecules. In vitro, TLR ligands induced IL-6 expression via NF-κB in normal human epidermal keratinocytes (NHEKs) and dermal fibroblasts (NHDFs). SAA further induced the expression of IL-6 via TLR1/2 and NF-κB in NHEKs and NHDFs. The limitation of this study is that NHEKs and NHDFs were not derived from RDEB patients. These observations suggest that TLR-mediated persistent skin inflammation might increase the risk of IL-6-related systemic complications, including RDEB.

KEYWORDS
epidermolysis bullosa, fibroblasts, IL-6, keratinocytes, serum amyloid A

1 | INTRODUCTION

The skin is one of the human body's largest organs, and it is the first line of defence against the external environment of the body.[1] Therefore, in the skin, the nonspecific quick inflammatory reaction, or the innate immune system, is necessary, and it is comprised of keratinocytes, neutrophils, macrophages and more.[1] Skin inflammation is induced by injury or ulceration when Toll-like receptors (TLRs) in keratinocytes in the lesions recognize molecules derived from microbes that are called pathogen-associated molecular pattern molecules (PAMPs), or molecules derived from injured cells that are called damage-associated molecular pattern molecules (DAMPs), and then the signals induce the expression of inflammatory cytokines and chemokines.[2–5] In fact, the expression and function of TLR2 are enhanced in wounded skin,[5] and TLR3 recognizes self-RNA from damaged cells and mediates inflammatory responses after skin injury or ultraviolet radiation.[3,4] In addition to keratinocytes, another type of resident structural cells, fibroblasts, can also produce inflammatory cytokines and chemokines, the production of which triggers and enhances skin inflammation.[2,6] In skin ulcer lesions, the infiltration of inflammatory cells including lymphocytes, histiocytes, neutrophils and plasma cells is persistently observed, but the effect of chronic skin inflammation on extracutaneous organs and blood is not well studied.

Epidermolysis bullosa (EB), an inherited blistering disorder in the skin and mucosal membranes, is caused by a mutation in keratin or collagen gene, and it has various subtypes.[7] One of the subtypes, dystrophic epidermolysis bullosa (DEB), especially recessive dystrophic epidermolysis bullosa (RDEB) caused by a mutation of the type VII collagen gene *COL7A1*, is a rare and severe form of EB characterized by inherently fragile skin and recurrent blister formation which occurs over the entire body within weeks after birth.[7] Patients with severe types of EB including RDEB persistently have widespread skin ulcers and develop various complications, including oesophageal stricture, loss of function of the hands and feet, renal failure, cutaneous squamous cell carcinoma, secondary amyloidosis (SA) and more.[8,9]

Secondary amyloidosis is a critical complication caused by chronic inflammatory diseases such as rheumatoid arthritis.[10] The insoluble amyloid fibril protein derived from serum amyloid A (SAA) aggregates in various organs throughout the body and disrupts normal functions.[10] SAA is an acute phage protein produced mainly by the liver, and its persistent high concentration in the sera is considered a prerequisite for the development of SA.[11] Our group recently conducted a study demonstrating that TLR ligands induced the expression of SAA in normal human epidermal keratinocytes (NHEKs) and dermal fibroblasts (NHDFs). In addition, SAA induced its own expression in NHEKs and NHDFs.[12,13] IL-6 is a representative inflammatory cytokine that induces SAA expression via STAT3 in various types of cells including hepatocytes.[14,15] Of note, tocilizumab, a humanized anti-IL-6 receptor antibody, has been reported to have the ability to suppress the SAA level and reduce the deposition of amyloid fibrils, which indicates that IL-6 plays a critical role in the pathogenesis of SA.[16,17] Cases of more than 20 patients with severe EB including RDEB who developed SA have been reported to date, whereas patients with chronic inflammatory skin disorders such as atopic dermatitis and psoriasis vulgaris have been rarely reported to be complicated with SA.[18–29] Elevated serum levels of IL-6 have been reported in bullous pemphigoid and EB acquisita[30,31]; however, there is currently no established link of SA with these diseases.

In addition, haematological abnormalities such as anaemia, hyperglobulinaemia, hypoalbuminaemia, and the elevated C reactive protein (CRP), SAA and IL-6 levels have also been observed in patients with RDEB.[18,32,33] Interestingly, all these haematological abnormalities are associated with the bioactivities of IL-6, and they are often seen in patients with multicentric Castleman's disease (MCD).[34] MCD is a rare lymphoproliferative disorder characterized by fever, polyclonal hypergammaglobulinaemia and generalised lymphadenopathy, and tocilizumab is an effective treatment for MCD.[35] Our group previously reported a case of DEB presenting clinical symptoms consistent with MCD.[33] We also demonstrated persistent elevations of serum IL-6 and SAA in two RDEB patients.[36] Here, we report that TLR-mediated persistent skin inflammation increases the risk of IL-6-related systemic complications.

2 | METHODS

2.1 | Patients

Skin and serum samples were collected from patients with RDEB, atopic dermatitis or psoriasis and from normal healthy volunteers under written informed consent at Okayama University Hospital and its affiliated hospitals.

2.2 | Cell culture and stimuli

NHEKs were obtained from Cascade Biologics/Invitrogen, and grown in serum-free EpiLife cell culture media (Cascade Biologics/Invitrogen) containing 0.06 mM Ca^{2+} and EpiLife Defined Growth Supplement (Cascade Biologics/Invitrogen) at 37°C under standard tissue culture conditions. NHDFs were also obtained from Cascade Biologics/Invitrogen and grown in Medium 106S (Cascade Biologics/Invitrogen) containing Low Serum Growth Supplements (Cascade Biologics/Invitrogen). Cultures were maintained for up to eight passages in these media with the addition of 100 U/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B. The cells were stimulated with Pam3Cys-Ser-Lys4 (Pam3CSK4) (10 μg/mL; InvivoGen), poly (I:C) (10 μg/mL; InvivoGen), lipopolysaccharide (10 μg/mL; InvivoGen), flagellin (100 ng/mL; InvivoGen), macrophage-activating lipopeptide-2 (MALP-2) (100 5 ng/mL; Alexis Biochemicals), ODN M362 (2 μg/mL; InvivoGen), recombinant human IL-6 (1–100 ng/mL, R&D systems) or recombinant human SAA (10-8-10-6 M, PeproTech) in 24-well flat bottom plates (Corning Incorporated Life Sciences) for 24 h. After the cell stimulation, the

total cell media was stored at −20°C until analysis. RNA was extracted using TRIzol reagent (Invitrogen) after supernatant collection. RNA was stored at −80°C.

2.3 | siRNA transfection

NHEKs and NHDFs were transfected with siRNA control (SignalSilence Control siRNA #6568S, Cell Signaling Technology) or specific siRNA for NF-κB p65 (Cell Signaling Technology), TLR1 and TLR2 (Santa Cruz Biotechnology) using Lipofectamine RNAiMax (Invitrogen). After 24 h of transfection, the cells were stimulated with TLR ligands or SAA for another 24 h. NF-κB and TLR1 or TLR2 knockdown experiments were each performed three times. Data were given as mean ± standard error of mean (SEM) of triplicate samples and are representative of three independent experiments.

2.4 | Neutralization of TLR1/2

For the neutralization of TLR1/2, NHEKs and NHDFs were incubated with TLR1- or TLR2-neutralizing antibodies (pab-hstlr1 or pab-hstlr2, 5 μg/mL, InvivoGen) or control IgG for 30 min. The cells were then stimulated with SAA for 24 h. TLR1 or TLR2 neutralization experiments were each performed three times. Data were given as mean ± SEM of triplicate samples and are representative of three independent experiments.

2.5 | Quantitative real-time polymerase chain reaction (qPCR)

cDNA was synthesized from RNA by the iScript cDNA Synthesis Kit (Bio-Rad Laboratories) as described by the manufacturer's protocol. TaqMan Gene Expression Assays (Applied Biosystems) were used to analyse the expression of human IL6 (assay ID: Hs00174131_m1) and SAA1/SAA2 (assay ID: Hs00761940_s1) as described by the manufacturer's instruction (the User Bulletin #2 by Applied Biosystems). GAPDH mRNA was detected by the probe VIC-CATCCATGACAACTTTGGTA-MGB (primers: 5′-CTTAGCACCCCTGGCCAAG-3′ and 5′-TGGTCATGAGTCCTTCCACG-3), and was used as an internal control to validate RNA for each sample. Each mRNA expression was calculated as a relative expression to GAPDH mRNA, and all data are presented as fold change against each control (mean of non-stimulated cells).

2.6 | ELISA

IL-6 and SAA in culture media were measured by commercial sandwich ELISA kits following the manufacturer's instructions (Human IL-6 DuoSet ELISA Development System DY206, R&D Systems and SAA Human ELISA Kit KHA0012, Invitrogen). Culture media were concentrated 20 times by Amicon Ultra-4 (Millipore-Amicon) to detect SAA.

2.7 | Immunohistofluorescence

Formalin-fixed, paraffin-embedded blocks were cut into 4-μm sections and processed for immunohistochemistry. The samples were examined using rabbit polyclonal antibody to IL-6 (ab6672, Abcam) or mouse monoclonal antibody to SAA (ab55720, Abcam). After washing with PBS, Alexa Flour 488-conjugated goat anti-rabbit or anti-mouse IgG antibody (A-11008 or A-11059, Molecular Probes) was used as the second antibody. Nuclei were counterstained with 4′-6-diamidino-2-phenylindole. Images were obtained using a confocal laser scanning microscope (LSM510; Zeiss) at the Central Research Laboratory, Okayama University Medical School.

2.8 | Statistics

Statistical analysis was performed using a two-tailed Student's *t* test or one-way analysis of variance with Prism software (version 4; GraphPad Software). Results are expressed as mean ± SEM. *p* values of less than 0.05 were considered significant.

2.9 | Study approval

This study was approved by the Ethical Committee at Okayama University Hospital (No. 1185) and was performed in accordance with the Helsinki Declaration. Skin and serum samples were collected from patients with RDEB, atopic dermatitis, or psoriasis and from normal healthy volunteers under written informed consent at Okayama University Hospital and its affiliated hospitals.

3 | RESULTS

3.1 | The expression levels of IL-6 and SAA are persistently elevated in the sera and skin lesions of patients with RDEB

We first measured the serum IL-6 and SAA levels of patients with RDEB ($n=7$), psoriasis ($n=33$) or atopic dermatitis ($n=32$), and those of normal healthy volunteers ($n=30$). As shown in Figure 1, the patients with RDEB had significantly higher levels of IL-6 and SAA compared to each of the other groups (Figure 1A,B). The psoriasis patients also had significantly higher levels of SAA compared to the normal healthy volunteers and the atopic dermatitis patients (Figure 1B).

We next hypothesized that the skin could be a source of IL-6 and SAA in patients with RDEB. As expected, the keratinocytes

and dermal fibroblasts in lesional skins from RDEB patients showed abundant expression of IL-6 and SAA (Figure 1C,E). In the normal skin, IL-6 expression was confined to the granular and the spinous layers of epidermis, while SAA expression was completely absent (Figure 1D,F).

3.2 | TLR ligands induce the expression of IL-6 in epidermal keratinocytes and dermal fibroblasts

We then sought to identify the mechanisms of IL-6 production in keratinocytes and fibroblasts of patients with RDEB. Patients with RDEB have widespread persistent skin ulcers, and keratinocytes and fibroblasts surrounding the ulcer lesions are continuously exposed to PAMPs and DAMPs. We thus investigated the effects of TLR ligands on the expression of IL-6 in NHEKs and NHDFs. Some TLR signalling has been reported to induce IL-6 production in keratinocytes,[4] but here we demonstrated that among a panel of TLR ligands, poly (I:C) (TLR3), flagellin (TLR5) and MALP-2 (TLR2/6) significantly induced IL-6 expression at both the transcript and protein synthesis levels in NHEKs (Figure 2A,B). In NHDFs, among a panel of TLR ligands, Pam3CSK4 and MALP-2 significantly induced IL-6 expression (Figure 2C,D).

TLR signalling has been reported to induce inflammatory cytokines through NF-κB.[37] The knockdown of p65 (a subunit of NF-κB) using siRNA significantly suppressed the induction of IL-6 by poly (I:C), flagellin, and MALP-2 in NHEKs (Figure 2E). As with NHEKs, the knockdown of p65 significantly suppressed the IL-6 induction by Pam3CSK4 and MALP-2 in NHDFs (Figure 2F). These data indicate that the induction of IL-6 by TLR ligands in NHEKs and NHDFs is NF-κB-dependent.

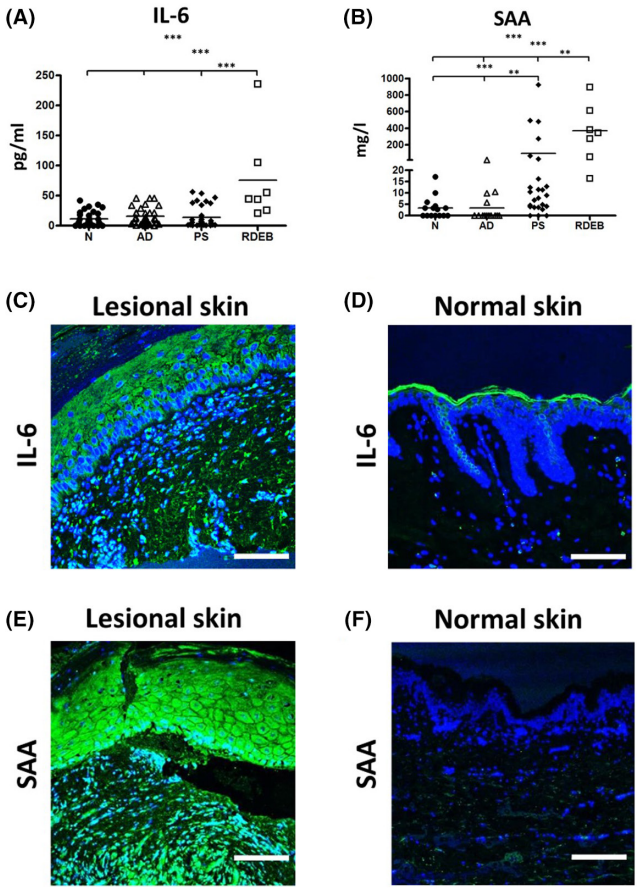

FIGURE 1 The expression levels of IL-6 and SAA are increased in the sera of RDEB patients. (A, B) IL-6 and SAA levels in the sera were measured by ELISA. N, normal healthy volunteer ($n=30$); AD, patients with atopic dermatitis ($n=32$); PS, patients with psoriasis ($n=33$); RDEB, patients with recessive dystrophic epidermolysis bullosa ($n=7$). $^{**}p<0.01$; $^{***}p<0.001$. IL-6 and SAA are highly expressed in the skin of RDEB patients. (C–F) The expression of IL-6 and SAA1 was examined by immunofluorescence in the skin ulcer lesions of RDEB patients or normal skin. Data are representative of three samples. Scale bar = 100 μm. SAA, serum amyloid A.

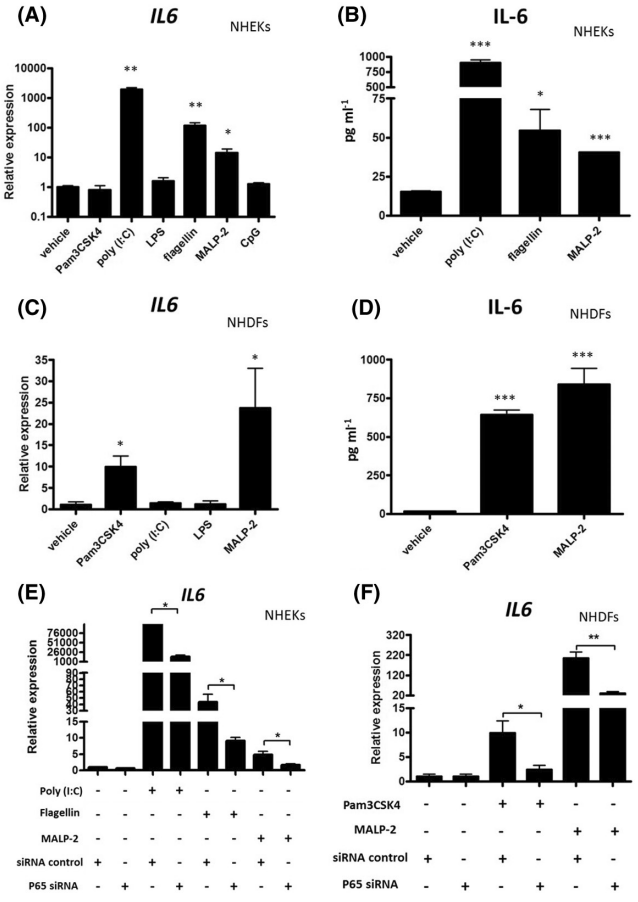

FIGURE 2 TLR ligands induce the expression of IL-6 through NF-kB in keratinocytes and fibroblasts. (A–D) NHEKs or NHDFs were stimulated with TLR ligands for 24 h. The relative *IL6* mRNA abundance was analysed by qPCR, and the protein expression of IL-6 was measured by ELISA. (E, F) After the knockdown of p65 (a subunit of NH-kB) using siRNA, NHEKs or NHDFs were stimulated with TLR ligands for 24 h. The relative *IL6* mRNA abundance was analysed by qPCR. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$. Data are mean ± SEM of triplicate samples and representative of three independent experiments. NHDFs, normal human dermal fibroblasts; NHEKs, normal human epidermal keratinocytes; TLR, Toll-like receptor.

3.3 | SAA induces the expression of IL-6 via TLR1/2 in keratinocytes and fibroblasts

SAA, a precursor of amyloid protein, potently activates immunocytes and induces inflammatory cytokines and chemokines.[38] We therefore investigated whether SAA could enhance inflammatory responses in an autocrine/paracrine manner in keratinocytes and fibroblasts. Like other types of cells, SAA induced the expression of IL-6 in NHEKs and NHDFs in a dose-dependent manner (Figure 3A,B). We also investigated whether IL-6 could induce the expression of IL-6 itself and SAA in an autocrine/paracrine manner in NHEKs or NHDFs, but this cytokine did not induce their expression (Figure S1A,B).

We next sought to clarify the mechanism by which keratinocytes and fibroblasts respond to SAA. Since SAA is also reported to activate NF-κB signalling,[39] we knocked down p65, a subunit of NF-κB, and confirmed that the IL-6 induction by SAA was significantly inhibited in NHEKs and NHDFs (Figure 4A,B). Cell viabilities in post-transfected NHEKs and NHDFs did not change. Dermal fibroblasts were recently reported to induce IL-6 through TLR2.[40] In monocytes, SAA induces several cytokines and chemokines via TLR1/2 heterodimer.[41] Our present experiments revealed that TLR1 and TLR2 siRNA significantly inhibited the IL-6 induction by SAA in NHEKs and NHDFs (Figure 4C,D). Cell viabilities in post-transfected NHEs and NHDFs did not change. There was a statistical difference of IL-6 expression in NHEKs between TLR1 siRNA- and TLR2 siRNA-treated groups (Figure 4C).

TLR1 and TLR2-neutralizing antibodies also significantly suppressed the IL-6 induction by SAA in NHEKs and NHDFs (Figure 4E,F). Despite the absence of statistical differences, TLR2-neutralizing antibodies exhibited a more pronounced inhibition of IL-6 expression in both NHEKs and NHDFs compared to TLR1-neutralizing antibodies (Figure 4E,F). These results indicate that SAA acts through TLR1/2 heterodimer to induce the expression of IL-6 and SAA in keratinocytes and fibroblasts, especially through TLR2.

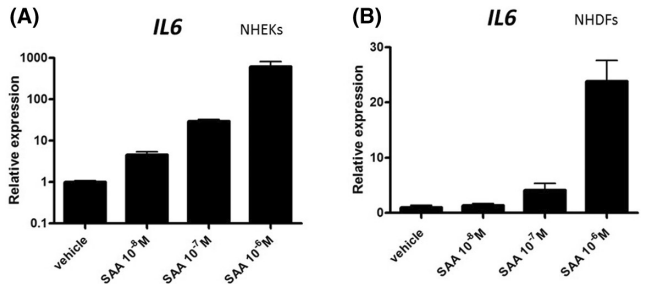

FIGURE 3 SAA induces the expression of IL-6 in keratinocytes and fibroblasts. (A, B) NHEKs or NHDFs were stimulated with SAA for 24 h. The relative *IL6* mRNA abundance was analysed by qPCR. Data are mean ± SEM of triplicate samples and representative of three independent experiments. NHDFs, normal human dermal fibroblasts; NHEKs, normal human epidermal keratinocytes; SAA, serum amyloid A.

4 | DISCUSSION

Recently, accumulating evidence suggests that RDEB can be a systemic inflammatory disease and there are many aspects of systemic immunological defects in RDEB.[42] Among them, IL-6, collagen VII and transforming growth factor β (TGF-β) activity are especially important to elucidate the systemic immunological abnormalities of RDEB.

We observed markedly elevated serum levels of IL-6 in RDEB patients compared to patients with other chronic inflammatory disorders, such as atopic dermatitis or psoriasis. A previous study indicated that the IL-6-to-IL-10 ratio in serum could be used as a prognostic marker of disease severity in RDEB.[43] Another investigation revealed that the disease severity of DEB was significantly

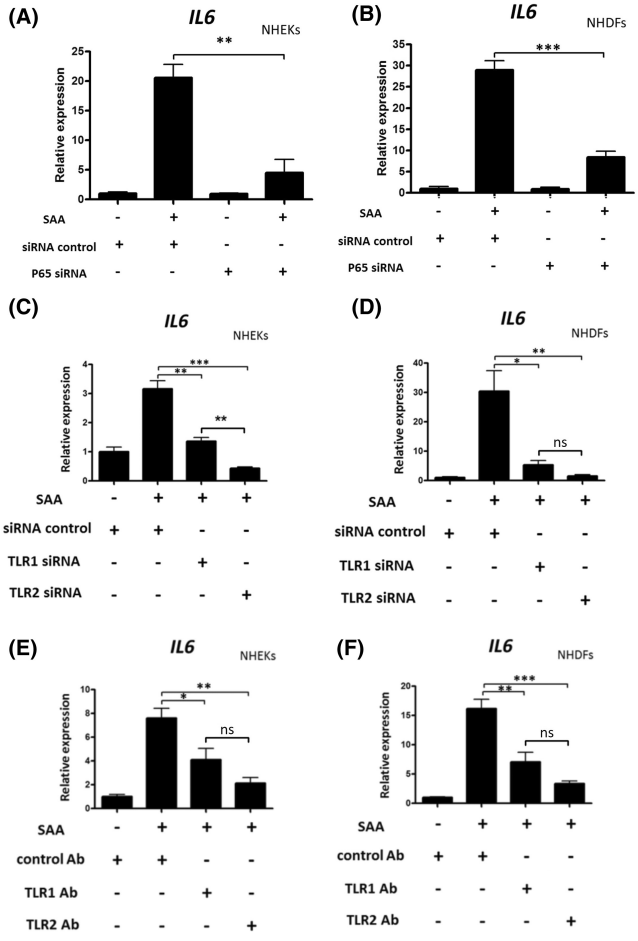

FIGURE 4 SAA induces the expression of IL-6 via NF-κB or TLR1/2 in keratinocytes and fibroblasts. (A–D) After the knockdown of p65 (a subunit of NH-kB), TLR1 or TLR2 using siRNA, NHEKs or NHDFs were stimulated with SAA or IL-6 for 24 h. The relative *IL6* mRNA abundance was analysed by qPCR. (E, F) After the neutralization of TLR1/2 with TLR1- or TLR2-neutralizing antibodies for 30 min, NHEKs or NHDFs were stimulated with SAA for 24 h. The relative *IL6* mRNA abundance was analysed by qPCR. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Data are mean ± SEM of triplicate samples and representative of three independent experiments. ns, not significant; SAA, serum amyloid A; TLR, Toll-like receptor.

correlated with serum levels of IL-6 and IL-12.[44] IL-6 is a pluripotent cytokine with multiple functions inducing fatigue, fever and lymph node swelling. Furthermore, it is linked to abnormal laboratory findings such as anaemia, thrombocytosis, hypoalbuminaemia, hypergammaglobulinaemia, and increase in acute-phase proteins including CRP and SAA,[45] all of which were observed in a previous study of RDEB patients.[32,36] Consequently, IL-6 is a central cytokine of inflammatory conditions of RDEB.

Then, a question arises regarding the source of IL-6 in RDEB. Chronic wounds of EB are exposed to various pathogens, so we therefore speculated that skin is a primary source of IL-6 in RDEB. Our study showed that IL-6 and SAA are expressed in the lesional skin of RDEB patients. Besides keratinocytes and fibroblasts, IL-6 is synthesized by a variety of cell types, including lymphoid and non-lymphoid cells, such as T cells, B cells, monocytes, endothelial cells and mesangial cells.[46] In lymph nodes, dendritic cells in the T zone and monocyte/macrophages in the medullary cords are the main IL-6 mRNA producers influencing survival of plasma cells.[47] Given the presence of peripheral lymphadenopathy in two out of five cases of EB with severe clinical forms,[32] it raises the possibility that lymph nodes might be another candidate source of IL-6 in RDEB.

Previously, our group demonstrated IL-6 expression in NHEKs following the co-stimulation with interferon-gamma (IFN-γ) and TLR3 ligand poly (I:C) (TLR3 ligand), a synthetic analogue of double stranded RNA.[48] Additionally, we demonstrated that Pam3CSK4 (TLR1/2 ligand), flagellin (TLR5 ligand) and MALP-2 (TLR2/6 ligand) significantly induced IL-6 expression at both the transcript and protein synthesis levels. Pam3CSK4 (TLR1/2 ligand) is a bacterial lipopeptide.[49] MALP-2 (TLR2/6 ligand) is derived from *Mycoplasma fermentans*, a wall-less bacteria that occurs as commensals or pathogens in animals and humans.[50] Flagellin (TLR5 ligand) is obtained from bacterial flagella.[49] It is noteworthy that RDEB is frequently colonized with *Staphylococcus aureus* even in the uninvolved skin,[51] suggesting that RDEB skin is widely exposed to external stimuli than we have anticipated. Thus, external pathogens might be involved in the stimulation of IL-6 production in RDEB through TLR ligands. Meanwhile, IL-6 expression was restricted to the granular and spinous layers of the normal epidermis in the present study. Disruption of skin barrier by tape-stripping induced IL-6 expression in the whole layers of epidermis in mice, whereas its expression diminished with time in the basal layer.[52] Therefore, IL-6 expression restricted to the granular and spinous layers of the normal epidermis in the normal skin might reflect the unrecognized previous external stimuli.

Our study also demonstrated SAA-induced expression of IL-6 in NHEKs and NHDFs. SAA expression was induced by TLR1/2, 3, 5, and 2/6 ligands in NHEKs, whereas in NHDFs, it exhibited increased expression upon TLR1/2 and TLR2/6 ligand stimulation. Notably, SAA induced its own expression both in NHEKs and NHDFs through TLR1/2 ligands.[12] Therefore, the pathogenesis of the persistent expression of IL-6 and SAA in RDEB patients[36] could be linked to continuous production of SAA based on positive inflammatory feedback loop in keratinocytes and/or fibroblasts (Figure S2). Additionally, SAA–receptor interactions are seen with SR-B1, formyl peptide receptor-like 1 (FPRL1), FPR2, receptor for advanced glycation end products (RAGE), and bacteria, suggesting that SAA can activate various kind of cells.[53] In the normal skin, IL-6 expression was confined to the granular and spinous layers of epidermis, while SAA expression was completely absent. Our previous investigation showed that IL-6 did not induce SAA expression in NHEKs.[13] The discrepancy of in vivo localization in the normal skin between IL-6 and SAA might be explained by the lack of IL-6 to stimulate SAA expression. Likewise, our experiments with IL-6 stimulation did not induce IL-6 expression in NHEKs or NHDFs (Figure S1A,B), although murine or human keratinocytes were shown to express IL-6 receptors (IL-6R).[52,54] Other mediators might be required to function IL-6 signalling through IL-6R in NHEKs or NHDFs.

The phenotypic presentation of RDEB is determined not only by the causative mutations but also is influenced by external factors. In monozygotic twins of RDEB with the same *COL7A1* mutations, their clinical phenotype began to diverge during adolescence and the lesional fibroblasts of a more severe phenotype displayed higher expression of IL-6 and constitutive TGF-β signalling activation.[55] Transcriptomic profiling of RDEB showed aberrant expression of cytokine–cytokine interactions, TLR signalling and JAK–STAT signalling pathways.[56] In silico prediction for compounds that reverse gene expression signatures highlighted methotrexate as a leading candidate.[56] Recent advance in molecular targeted therapy for inflammatory diseases has been influenced by our improved understanding of the underlying pathways involved in these diseases. Our study showed that the induction of IL-6 and SAA by TLR ligands in NHEKs and NHDFs is NF-κB-dependent. These findings might help to find another candidate to modulate inflammatory conditions of RDEB. Whether or not inflammatory conditions in RDEB play a protective role is still uncertain. Since sera of EB patients contained higher anti-staphylococcal IgG levels than those of healthy individuals,[57] protective roles of hypergammaglobulinaemia against bacteraemia should not be ignored. However, excessive amounts of IgG or SAA are harmful for our body and lead to SA. Therefore, these inflammatory conditions in RDEB should be controlled appropriately.

In summary, we report that the serum levels of IL-6 and SAA are continuously elevated in patients with RDEB, and both proteins are expressed in the epidermis and dermis of skin ulcer lesions. We also show that TLR ligands induce the expression of IL-6 and SAA via NF-κB in epidermal keratinocytes and dermal fibroblasts, and SAA further enhances the expression of IL-6 and SAA itself via TLR1/2 and NF-κB in these cells. A limitation of this study is that NHEKs and NHDFs were not derived from RDEB patients. Our observations might help uncover the molecular mechanisms for the induction of IL-6-related systemic complications by TLR-dependent excessive skin inflammation including RDEB.

AUTHOR CONTRIBUTIONS

Dr. Shin Morizane had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of data analysis. *Study concept and design*: Drs. Keiji Iwatsuki, Yoshio

Kawakami and Shin Morizane. *Acquisition and interpretation of the data*: Drs. Shin Morizane, Yoshio Kawakami, Ai Kajita, Ken-Ichi Hasui and Yoshihiro Matsuda. *Drafting of the manuscript*: Dr. Yoshio Kawakami.

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CONFLICT OF INTEREST STATEMENT

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DATA AVAILABILITY STATEMENT

Raw data were generated at Department of Dermatology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences. Derived data supporting the findings of this study are available from the corresponding author S.M. on request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1. IL-6 does not induce the expression of IL-6 in keratinocytes and fibroblasts. (A, B) NHEKs or NHDFs were stimulated with IL-6 for 24 h. The relative *IL6* mRNA abundance was analysed by qPCR. Data are mean ± SEM of triplicate samples and representative of three independent experiments.

Figure S2. Summarized mechanism of IL-6-related systemic complication in epidermolysis bullosa.

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