Human-induced pluripotent stem cell-derived keratinocyte-like cells for research on protease-activated receptor 2 in non-histaminergic cascades of atopic dermatitis

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Running title: Expression of PAR2 in iKera to model atopic dermatitis

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Discussion: 951 words

Non-Standard Abbreviations:

AD: atopic dermatitis

hiPSC: human induced pluripotent stem cell

iKera: induced pluripotent stem cell-derived keratinocytes-like cells

IL: interleukin

PAR2: protease-activated receptor 2

Th2: helper T cells
TNFα: tumor necrosis factor alpha

TSLP: thymic stromal lymphopoietin

Recommended Section Assignment: Inflammation, Immunopharmacology, and Asthma
Abstract

Keratinocytes are the most abundant cells in the epidermis, and as part of the frontline immunological defense system, keratinocytes function as a barrier to exogenous attacks. Protease-activated receptor 2 (PAR2) is expressed in human keratinocytes and activated in several inflammatory conditions, such as atopic dermatitis (AD). In this study, we demonstrated the differentiation of human induced pluripotent stem cell (iPSC) into keratinocytes by the improved robust procedure, and confirmed that human-induced pluripotent stem cell-derived keratinocyte-like cells (iKera) express PAR2, which is activated by external addition of the ligand peptide and trypsin. The activation of PAR2 led to the release of calcium from intracellular calcium storage, followed by the release of the pro-inflammatory cytokine, tumor necrosis factor-alpha. Moreover, PAR2 antagonist, I-191, inhibited calcium release in a dose-dependent manner. This is the first study to demonstrate that iKera expresses a functional PAR2 protein. Furthermore, our results indicate cross-talk between the PAR2- and IL-4-mediated inflammatory axes in iKera, suggesting that iKera can be used as a platform for a broad range of mechanism-targeted drug screening in AD.
Significance Statement

This is the first study to provide evidence that human-induced pluripotent stem cell-derived keratinocyte-like cells (iKera) express functional protease-activated receptor 2 (PAR2). Furthermore, we demonstrated in iKera that the IL-4 inflammatory axis can cross-talk with the PAR2 mediated inflammatory axis in keratinocytes. To the best of our knowledge, this is the first report to indicate that iKera can be used for research and as a drug screening platform for atopic dermatitis (AD).
Introduction

Theoretically, human-induced pluripotent stem cells are an infinite source of somatic cells that can be used in drug development and toxicity studies. Methodologies for keratinocyte differentiation from pluripotent stem cells have been well established (Itoh et al., 2013; Gledhill et al., 2015; Kim et al., 2018). For Pharmaceutical studies, the HaCaT cell line, a spontaneously immortalized human keratinocyte, is frequently used for in vitro skin-related research and drug screening systems and has a standard epidermal differentiation ability. However, HaCaT cells have a hypotetraploid karyotype (Nagy et al., 2013) and genetic abnormalities, such as mutations in p53 (Lehman et al., 1993). In this study, we aimed to establish a simple and mechanism-targeted drug screening platform by extensively studying the potential of human-induced pluripotent stem cell-derived keratinocyte-like cells (iKera) as an alternative to the HaCaT cell line.

Keratinocytes are the most abundant cell type in the epidermis. They settle on the basement membrane and maintain the keratinized epidermal barrier, which provides protection against exogenous attacks. Keratinocytes not only form a structural barrier, but also act as sensors for external assaults, and in response, secrete various proinflammatory cytokines to initiate different biological responses (Chieosilapatham et al., 2021).

Atopic dermatitis (AD) is characterized by sustained chronic epidermal pruritus caused by an aggravating vicious circle of pruritus, scratches, barrier destruction, chronic inflammation, and reinforcement of
peripheral sensory nerves (Rerknimitr et al., 2017). The quality of life of patients with AD is considerably disrupted; therefore, it is necessary to identify new therapeutic molecular targets. In a study on humans, local antihistamines alleviated itching caused by mast cell degranulation in controls, but not in patients with AD (Steinhoff et al., 2003). This strongly suggests that the inefficacy of the histaminergic cascade in alleviating itching in AD patients is due to the presence of other non-histaminergic targets. Furthermore, the administration of codeine to patients with AD increased endogenous tryptase by four-fold compared to that in healthy controls. Interestingly, intracutaneous injection of endogenous protease-activated receptor 2 (PAR2) agonists provoked enhanced and prolonged itching in patients with AD (Steinhoff et al., 2003).

PAR2 is a G-protein-coupled receptor whose N-terminal peptide is cleaved by its auto-ligand tryptase (Saifeddine et al., 1996). Mice with epithelial PAR2 overexpression showed AD-like skin inflammation, scratching bouts, and skin remodeling (Smith et al., 2019), with an increased density of nerve fibers (Buhl et al., 2020) when sensitized to house dust. Kawagoe et al. developed PAR2-deficient mice, and showed PAR2-deficiency palliates pathological skin remodeling caused by a topical application of picryl chloride or oxazolone (Kawagoe et al., 2002). These reports strongly suggest a potential role for keratinocyte PAR2 in AD.

Recently, the interleukin (IL)-4/IL-13 receptor blockade using antibodies was found to be an effective therapeutic strategy for the treatment of AD (Kim et al., 2022). IL-4 and IL-13 are primarily secreted by
helper T cells (Th2) and mast cells and directly affect keratinocyte phenotypes. To date, the crosstalk between IL-4/IL-13-related responses and PAR2 remains unclear.

From the results of this study, we suggest the use of iKera as a potentially useful platform that can be applied to drug screening systems focusing on the vicious PAR2-mediated inflammatory circle involving crosstalk with the Th2 cytokine-axis.
Materials and Methods

Human induced pluripotent stem cells (hiPSCs)

The hiPSC lines (RIKEN-2F and 253G1) were obtained from the Laboratory for Pluripotent Cell Studies, RIKEN Center for Developmental Biology, Tukuba-city, Ibaragi, Japan.

Maintenance and keratinocyte differentiation of hiPSCs

The hiPSC lines were maintained on plastic dishes (Corning Inc., NY, USA) coated with 0.5 μg/cm² iMatrix511 Silk (Nippi Inc., Tokyo, Japan) using StemFit AK02N (Ajinomoto, Tokyo, Japan) as the culture medium. The hiPSCs were differentiated into keratinocytes based on the previously reported methods with modifications (Guenou et al., 2009; Kogut et al., 2014). Undifferentiated hiPSCs were seeded at a density of 15,000 cells/cm² on a 6-well plate coated with 0.5 μg/cm² iMatrix511 Silk using StemFit AK02N supplemented with 10 μM Rho kinase inhibitor, Y-27632 (Sellec Inc., Tokyo, Japan), two days before the first day of differentiation (day 1). Upon initiation from epiblast to ectodermal-differentiating-cells, the medium was changed to N2B27 medium consisted with Dulbecco’s modified Eagle medium (DMEM)/F12 (Fuji Film Wako Chemical Inc., Miyazaki, Japan) and Neurobasal medium (1:1; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 0.1 mM non-essential amino acids, 1 mM glutamine, 55 μM 2-mercaptoethanol, 1% N-2 supplement (Thermo Fisher Scientific), 2% B-27 supplement (Thermo Fisher Scientific), 50 μg/mL ascorbic acid 2-phosphate (Merck KGaA, Darmstadt, Germany), 0.05% bovine serum albumin (Merck), and 100 ng/mL FGF-basic (Nacalai Tesque...
Inc., Kyoto, Japan). This process was reported by Kogut et al. (Kogut et al., 2014). Here, we prolonged this process from originally reported 24 hour to 48 hours. We observed that core-part of the colony of human iPSC seems to keep their pluripotency, but the edge of the colonies slightly enlarge their cytoplasmin. We found this process stabilized their future direction in the further ectodermal differentiation processes in avoidance of unexpected sudden death. Therefore, we think this process might change the iPSC internally from pluripotent epiblast-state to ectodermally-directed-state. Depending on the initial iPS states, this process can be optimized between 48 to 72 hours. On day 3, the medium was changed to Defined Keratinocyte-SFM (without adding the attached supplement for on day 3 and with it thereafter on day 5; Thermo Fisher Scientific), supplemented with 0.5 μg/mL hydrocortisone, 1 μM all-trans-retinoic acid, 25 ng/mL hBMP-4 (Thermo Fisher Scientific), 2.4 μg/mL adenine, 1.37 ng/mL triiodothyronine, 0.3 mM ascorbic acid 2-phosphate, and 2 μM forskolin. The above set of the supplements were reported by Guenou et al. (Guenou et al., 2009). Here, we used the similar supplement-compositions (except originally used cholera toxin was replaced by forskolin) added in Keratinocyte-SFM. After the differentiation day 5, the medium was replaced with the same medium added the attached-supplementment every two days. We confirmed this methods can differentiates RIKEN-2F and 253G1 cell lines to keratinocyte-like cells with high reproducibly. Please see the time-table is shown in Supplementary Figure 1.

Quantitative polymerase chain reaction (qPCR) analysis
Total RNA was extracted from the cells using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer’s instructions. Reverse transcription of 50 ng of RNA was performed using ReverTra Ace™ (Toyobo, Osaka, Japan) with oligo (dT) primers. To investigate keratinocyte differentiation and PAR2 ligand- and IL-4-stimulated induction of tumor necrosis factor alpha (tnfα) gene and other gene expression, a quantitative polymerase chain reaction was performed using Power SYBR™ Green PCR Master Mix (Thermo Fisher Scientific) with gene-specific primer sets designed using Primer3. All experiments were performed independently-prepared three samples. All gene expression levels were normalized to internal 18S ribosomal RNA expression levels. Primer pair sequences are listed in Table 1.

**Enzyme-linked immunosorbent assay (ELISA)**

Quantification of TNF-α in the medium was performed using the TNF alpha ELISA kit (#88-7346-22; Thermo Fisher Scientific), following the manufacturer’s instructions. Quantification of thymic stromal lymphopoietin (TSLP) in the medium was performed using Human TSLP ELISA Kit (ab155444, Abcam, Cambridge, UK), following the manufacturer’s instructions.

**Immunofluorescent staining**

Cells were fixed in 4% paraformaldehyde for 5 min at 25°C. The cells were then washed twice with Tris-buffered saline containing 0.2% Tween-20 (TBS-T) and subsequently treated with a blocking solution (Nacalai Tesque) for 30 min at room temperature. The first antibody-containing blocking agent was added and incubated overnight at 4°C with paraffin sealing to prevent evaporation. The cells were then washed thrice with TBS-T and immersed in the second antibody-containing blocking agent for 1 h at room temperature. After washing thrice, fluorescent signals were observed using a fluorescence microscope (Nikon Instruments, Tokyo, Japan). The primary and secondary antibodies used are listed in
Table 2.

**Observation of PAR2 stimulation and intracellular calcium release**

Calcium imaging was performed using a Nikon Eclipse Ti2 inverted microscope (Nikon Instruments) with a Fluo-4 AM Ester (Biotium Inc., Fremont, CA, USA). Fluo-4 AM was loaded onto the iKera for 30 min at 37 °C. After washing with a pre-warmed Defined Keratinocyte-SFM medium with the addition of the attached supplement, we started time-lapse recordings and added the PAR2 ligand peptide or trypsin. The strength of fluorescence in randomly selected cells (n=8) was evaluated using NIS Elements software (Nikon Instruments).

**Interleukin 4 (IL-4) stimulation and PAR2 protein upregulation**

IL-4 (10 or 50 ng/mL) was added to the culture medium treated with iKera on the differentiation day 30 for three days. The cells were washed once with phosphate-buffered saline (PBS)(-) and lysed using RIPA buffer with a protease inhibitor cocktail (#08714; Nacalai Tesque). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 1.0 mm thick 4–12% gradient gel with Bolt™ electrophoresis System (Thermo Fisher Scientific) was used. Proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (Thermo Fisher Scientific). The primary and secondary antibodies used are listed in Table 2. The chemiluminescent reaction was induced by the addition of horseradish peroxidase substrates (Cytive, Tokyo, Japan) and visualized and quantified using Fusion Solo S (M&S Instruments Inc., Osaka, Japan).
**Statistical analysis**

Statistical analyses were performed using EZR software (Jichi Medical University, Japan) (Kanda, 2013).

For multiple comparisons, significant differences were determined using one-way analysis of variance (one-way ANOVA), followed by post-hoc testing using the Tukey–Kramer test. A Student’s t-test was performed for comparisons between the two samples. Statistical significance was set at $P < 0.05$. 
Results

1. Confirmation of keratinocyte differentiation

Keratinocyte differentiation was confirmed using fluorescent immune staining of keratin 10 and 14 proteins and mRNA expression of keratin 1, 10, and 14 using the primers listed in Table 1. Between differentiation days 30 and 40, the cells were fixed and fluorescent immunostaining was performed (Figure 1A) using the antibodies listed in Table 2. Furthermore, we observed that iKera expressed PAR2 (Figure 1B). The expression of par2 and keratin 1, 10 and 14 mRNA in iKera, was also confirmed, as shown in Figure 1C.

2. PAR2 agonist-induced intracellular calcium spark and its chemical inhibition

We observed intracellular calcium sparks following the addition of 100 μM of the PAR2 agonist peptide, SLIGKV, using two iPSC lines, RIKEN2F (Figure 2A and Supplemental Video 1) and 253G1 (Figure 2B and Supplemental Video 2). We also observed the immediate elevation of intracellular calcium concentration by the addition of 0.25 mg/mL of trypsin into the culture medium using RIKEN2F (Figure 2C and Supplemental Video 3). The calcium sparks induced by the addition of SLIGKV in 253G1 cells were dose-dependently inhibited by I-191 (Figure 2D–G).

3. Induction of TNFα expression and release via PAR2 stimulation
We challenged iKera with a 100 and 200 μM PAR2 agonistic peptide and investigated the resulting TNFα secretion and mRNA expression. We found that TNFα concentration was significantly increased by PAR2 stimulation (Figure 3A); qPCR analysis quantified the significant increase in \( \text{tnf}\alpha \) mRNA levels using the primers listed in Table 1. (Figure 3B).

4. Alteration of other mRNA expression levels by PAR2 stimulation

We also investigated other significant mRNA expression levels by qPCR using the primers listed in Table 1. Strong itchy-mediating cytokines, thymic stromal lymphopoietin (tslp), and the principal structural proteins of skin, keratin 1, 10, and 14, were significantly increased (Figure 3C). The attractant and repellant of peripheral sensory nerves, nerve growth factor (ngf) and semaphorin-3A (sema-3a) showed an increasing tendency (Figure 3C).

5. Up-regulation of PAR2 protein and mRNA expression levels by IL-4 treatment

We conducted IL-4 treatment in iKera and investigated PAR2 protein levels by western blotting, and found a significant upregulation. We also measured \( \text{par2} \) mRNA expression levels by qPCR and found a significant dose-dependent increase in expression (Figure 4A).

6. Alteration of other mRNA expression levels by IL-4 stimulation
Other AD-related mRNA expression levels were investigated by the use of qPCR, and significant increases in the mRNA levels of *ngf* and *sema-3a* in a dose-dependent manner were found (Figure 4B).

7. **PAR2 and IL-4, IL-13 co-stimulation increased secretion of thymic stromal lymphopoietin (TSLP) from iKera**

We challenged 200 μM PAR2 agonistic peptide to iKera for 5 days, however failed to detect any change of TSLP secretion. On the other hand, each 50 ng/ml IL-4 and IL-13 treatment significantly enhanced TSLP secretion. Interestingly, we found PAR2 co-stimulation with IL-4 and IL-13 further enhanced the TSLP secretion than that of IL-4 and IL-13 (Figure 4C).
Discussion

Atopic dermatitis (AD) is sustained chronic inflammation of the epidermis caused by an aggravating vicious circle consisting of scratches, barrier destruction, chronic inflammation, and peripheral sensory nerve remodeling (Rerknimitr et al., 2017). In this study, we focused on the significance of keratinocytes in this cycle as an upper acceptor of scratching effects and an amplifier of cell-cell interactions. Various layered culture-based skin models using human pluripotent stem cells have been reported; however, they involve complex processes and require more time before they can be applied in testing. We believe that a highly reproducible screening system would require a simple platform. In this study, we demonstrated that iKera is a potential platform that can be applied for high-throughput screening to target PAR2-based mechanisms in AD.

To date, PAR2 expression in iKera has not yet been reported. In this study, we demonstrated that administration of the PAR2 agonist peptide, as well as trypsin treatment, induced intracellular calcium sparks, suggesting that iKera expresses functional PAR2 protein and the corresponding downstream signal transducers. To the best of our knowledge, this study is the first to demonstrate this relationship.

Stimulation of PAR2 induces iKera to release TNFα, suggesting that iKera have a functional PAR2-mediated inflammatory response. Furthermore, IL-4 treatment augmented par2 mRNA and protein levels in iKera cells. These results suggest that PAR2 plays an important role as an enhancer in the aggravating cycle of AD via cross-talk with Th2 cytokines.
Skin hypersensitivity to physical stimulation causes an unrelenting urge to scratch. This is a significant contributing factor in the development and aggravation of AD. Keratinocytes secrete nerve growth factor (NGF) and semaphorin-3A, which attract and repel itch-sensing neurons called type-C fibers, respectively, which are believed to cause sensory neuron remodeling (Tominaga and Takamori, 2014; Tominaga et al., 2009). However, there are no reports indicating a relationship between PAR2 stimulation or IL-4 treatment and the above peripheral sensory nerve regulating factors. In our study, IL-4 treatment significantly and PAR2 agonistic peptide treatment tended to augment mRNA expression levels of NGF and semaphorin-3A in iKera, suggesting the possible existence of cross-talk between PAR2 and the Th2 cytokine-axis in keratinocytes.

So far, no report showed that iKera can secrete TSLP. We showed iKera can secrete TSLP by IL-4/IL-13 receptor activation. Furthermore, PAR2 and IL-4/IL-13 receptor co-stimulation further enhanced the TSLP secretion. This strongly suggests that the PAR2 mediated inflammatory and itchy axis is a co-player with Th2 cytokine-axis in vicious circle of AD pathology.

Damaged barrier function in AD skin lesions is sustained by skin remodeling, which is partly due to alterations in keratinocyte phenotypes (Totsuka et al., 2017). Keratinocytes alter the expression of keratin subtype genes in accordance with the symptoms of AD. In our study, each iKera transiently stimulated with a PAR2 agonist or IL-4 altered the mRNA expression levels of different keratin subtype genes. However, most of these gene regulations are not the same as those observed in primary keratinocytes.
from AD lesions (Totsuka et al., 2017); further studies using iKera are required to mimic AD pathology.

Some studies suggested that keratinocyte PAR2 plays a role in the development and aggravation of AD. However, few studies have been conducted to determine its role in human pathology and pathological significance. Therefore, further investigations using various chemical inhibitors of keratinocyte PAR2 as research tools are required to explore new drugs for the treatment of AD. In this study, we proposed a simple and efficient drug screening system that targets keratinocytes and PAR2. We believe that PAR2-targeted drugs could be potential breakthroughs in AD treatment.
Authorship Contributions

Participated in research design: Hattori F.

Conducted experiments: Nishimoto R, Kodama C, Yamashita H, Hattori F.

Performed data analysis: Nishimoto R, Kodama C, Yamashita H, Hattori F.

Wrote or contributed to the writing of the manuscript: Hattori F.
References


Kanda Y (2013) Investigation of the freely available easy-to-use software “EZR” for medical statistics. 

*Bone Marrow Transplant* **48**:452–458.


Factor, Semaphorins, in *Itch: Mechanisms and Treatment* (Carstens E, and Akiyama T eds) p,

CRC Press/Taylor & Francis, Boca Raton (FL).


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**Figure Legends**

Figure 1. Confirmation of keratinocyte differentiation. (A) Phase-contrast image of human-induced pluripotent stem cell-derived keratinocyte-like cells (iKera); immunohistochemical images for Keratin 10 (K10: Red), Keratin 14 (K14: Green), and merged image with nuclear staining with DAPI (Blue). (B) Immunohistochemical staining for protease-activated receptor 2 (PAR2) in lower (left) and higher (right) magnification images. Scale bars indicate 200 μm in (A) and (B) except for (B) - right indicates 100 μm. (C) Comparison of mRNA levels of *keratin 1 (k1), 10 (k10), 14 (k14)* and *par2* with undifferentiated human induced pluripotent stem cells (iPSCs) (RIKEN2F).

Figure 2. Calcium sparks induced by PAR2 activation in iKera. (A) RIKEN2F-derived iKera stimulated by PAR2 agonistic peptide SLIGKV. (B) 253G1-derived iKera stimulated by SLIGKV. (C) 253G1-derived iKera stimulated by trypsin. (D), (E), (F) 253G1-derived iKera stimulated by SLIGKV in the presence of 0, 2.5, 10 ng/mL PAR2 antagonist, I-191, respectively. (G) Inhibitory effects of I-191 against PAR2-agonistic stimulation measured by the fluorescence change from the base line.

Figure 3. Effect of PAR2-agonistic peptide treatment in iKera. (A) TNFα secretions from iKera measured by ELISA assay. (B) Elevation of mRNA expression levels of TNFα. (C) The other significant gene expression levels under PAR2 stimulation.
Figure 4. Effect of IL-4 treatment in iKera. (A) PAR2 protein expression levels were increased by IL-4 dose-dependently. (B) par2 and other significant gene expression levels under IL-4 stimulation. (C) TSLP secretions from iKera measured by Elisa assay (n=3). The cells were treated with 200μM SLIGKV and/or 50ng/ml each IL-4 and IL-13 for 5-days.
### Table 1. Primer pairs for qPCR

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<td><strong>par2</strong></td>
<td>Fw TGCCTGAGCAGCTCTTGTTGGAGA</td>
</tr>
<tr>
<td></td>
<td>Rv AGGCAGAGGGCTTGAGGAGGCTGG</td>
</tr>
<tr>
<td><strong>kertin1</strong></td>
<td>Fw GCATCACTAACAATATGGCTTGGC</td>
</tr>
<tr>
<td></td>
<td>Rv AAACCTCATTGGGAAACAGCAAA</td>
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<tr>
<td><strong>keratin 10</strong></td>
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</tr>
<tr>
<td></td>
<td>Rv TCCATAGACCATCAAGACAGAAGGT</td>
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<tr>
<td><strong>keratin 14</strong></td>
<td>Fw GAGATGCAGATGGAGCGCTGAAG</td>
</tr>
<tr>
<td></td>
<td>Rv CCTCTGTCTTTGGTAAGAACCATT</td>
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<tr>
<td>18s ribosomal RNA</td>
<td>Fw TCAACTTTCGATGGTAGCTGCGGT</td>
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<tr>
<td></td>
<td>Rv TCCTTGATGTGGTAGCCGTTTCT</td>
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<tr>
<td><strong>sema-3a</strong></td>
<td>Fw ACCCAACTATCAATGGGTCCTTA</td>
</tr>
<tr>
<td></td>
<td>Rv AACACTGATGTACATGCGTGA</td>
</tr>
<tr>
<td><strong>tnf α</strong></td>
<td>Fw CCGAGGACTGACTCATCTTT</td>
</tr>
<tr>
<td></td>
<td>Rv AGCTGCCCTCAGCTTTGA</td>
</tr>
<tr>
<td><strong>tsp</strong></td>
<td>Fw TAGCAATGGCCACATTGCC</td>
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<td></td>
<td>Rv CTGAGTTCCGAAATAGCCTG</td>
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<tr>
<td><strong>ngf</strong></td>
<td>Fw TGTGGTTGGGATAAGACCA</td>
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<td>Rv GCTGTCACGGGATTGGGT</td>
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Table 2. Antibody list

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<tr>
<td>Keratin 10</td>
<td>donkey anti-mouse IgG(H+L) Alexafluor 488</td>
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<tr>
<td>Keratin 14</td>
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<td>PAR-2</td>
<td>SC-1615 Santacruz</td>
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Figure 1

(A) Phase contrast and PAR2 DAPI images. 

(B) K10 and K14 DAPI images. 

(C) Graph showing arbitrary units for k1, k10, k14, and par2.
Figure 3

(A) TNFα (pg/ml) vs. SLIGKV (μM)

(B) tnfα

(C) tslp, ngf, sema-3a

keratin 1, keratin 10, keratin 14, par2
Figure 4

(A) PAR2 and b-Actin protein expression levels with IL-4 treatment.

(B) Expression levels of par2, ngf, sema-3a, and tslp with IL-4 treatment.

(C) TSLP expression levels with different treatments.
Human-induced pluripotent stem cell-derived keratinocyte-like cells for research on protease-activated receptor 2 in non-histaminergic cascades of atopic dermatitis

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Supplementary Figure 1

Differentiation day
-2 1 3 5 30
Pluripotent Ectodermal directed epithelial iKera
Stem fit
N2B27 Medium (Kogut et al.) w/o attached supplement
K-SFM With supplement-set (Guenou et al.) with attached supplement

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