



House dust mite allergens induce interleukin 33 (IL-33) synthesis and release from keratinocytes via ATP-mediated extracellular signaling

Xiuju Dai^{a,*}, Mikiko Tohyama^b, Masamoto Murakami^a, Ken Shiraishi^a, Shuang Liu^c, Hideki Mori^a, Ryo Utsunomiya^a, Kazutaka Maeyama^c, Koji Sayama^a

^a Department of Dermatology, Ehime University Graduate School of Medicine, Ehime, Japan

^b Department of Dermatology, National Hospital Organization Shikoku Cancer Center, Ehime, Japan

^c Department of Pharmacology, Ehime University Graduate School of Medicine, Ehime, Japan

ARTICLE INFO

Keywords:

Dermatophagoides pteronyssinus
P2Y2 receptor
Calcium intracellular release
Epidermal growth factor receptor
Cysteine protease
Atopic dermatitis

ABSTRACT

In atopic diseases, the epithelium releases cytokines and chemokines that initiate skin inflammation. Atopic dermatitis (AD) is characterized by a disrupted epidermal barrier and is triggered or exacerbated by environmental stimuli such as house dust mite (HDM) allergens. The proinflammatory cytokine interleukin 33 (IL-33) plays an important role in the pathogenesis of AD, but how IL-33 production in keratinocytes is elicited by HDM is unknown. To that end, here we stimulated monolayer-cultured human keratinocytes and human living skin equivalents with *Dermatophagoides pteronyssinus* HDM extract to investigate its effects on IL-33 production from keratinocytes. The HDM extract induced intracellular expression of IL-33 and modulated its processing and maturation, triggering rapid IL-33 release from keratinocytes. Group 1 HDM allergen but not group 2 HDM allergen elicited IL-33 production. An ATP assay of keratinocyte culture supernatants revealed an acute and transient accumulation of extracellular ATP immediately after the HDM extract stimulation. Using the broad-spectrum P2 antagonist suramin, the specific purinergic receptor P2Y2 (P2RY2) antagonist AR-C118925XX, and P2RY2-specific siRNA, we discovered that the HDM extract-induced IL-33 expression was mainly dependent on extracellular ATP/P2Y2 signaling mediated by transactivation of epidermal growth factor receptor, followed by activation of the ERK kinase signaling pathway. Moreover, HDM extract-induced release of 25-kDa IL-33 from the keratinocytes depended on an extracellular ATP/P2 signaling-mediated intracellular Ca^{2+} increase. Our study demonstrates the new mechanism controlling the induction and maturation of keratinocyte-produced IL-33 by HDM allergens, an innate immune process that might play a role in AD development or severity.

1. Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disorder affecting an increasing number of patients [1]. Genetic signals related to skin barrier disruption, environmental allergens, and microbial colonization are integrated and transmitted to diverse immune cells, which initiate and maintain skin inflammation [1]. In addition to classical Th2 cytokines, tissue-derived pro-Th2 cytokines, such as TSLP, IL-25, and IL-33, play active roles in the development of AD [2].

IL-33 belongs to the IL-1 family and was first described as a

chromatin-associated nuclear factor constitutively expressed in the nuclei of endothelial and epithelial cells [3]. IL-33, stored in the nucleus as an alarmin, is rapidly secreted following cell damage and tissue injury [4,5]. IL-33 can also be released from healthy cells by extracellular ATP, or be cleaved by various proteases [3,5]. Extracellular IL-33 acts as a pro-Th2 allergic cytokine and stimulates the innate and acquired immune responses by binding to the membrane-bound IL-33-specific receptor ST2 [3,6]. Collective data suggest a vital role for IL-33 in the development and exacerbation of atopic diseases [7–9]. IL-33 is constitutively expressed in epidermal keratinocytes of the skin and is

Abbreviations: AD, Atopic dermatitis; HDM, house dust mite; Der p, *Dermatophagoides pteronyssinus*; NHEK, normal human epidermal keratinocyte; PAR, protease-activated receptor; GPCR, G-protein coupled receptors; EGFR, epidermal growth factor receptor; MMP, matrix metalloproteinase; TMP, triple membrane passing signaling pathway; HB-EGF, heparin-binding EGF; ADAM17, ADAM metalloproteinase domain 17; TACE, TNF- α -converting enzyme; PLC, phospholipase C; $[\text{Ca}^{2+}]_i$, intracellular calcium concentration; ATP- γ -S, Adenosine 5'-[γ -thio] triphosphate; ABESF, 4-(2-Aminomethyl) benzenesulfonyl fluoride hydrochloride; E-64, trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane; LSE, living skin equivalent; DPSP, Der p serine proteases; rhST2, recombinant human ST2/IL-33R; LDH, Lactate dehydrogenase; CKK-8, cell counting kit-8

* Corresponding author at: Department of Dermatology, Ehime University Graduate School of Medicine, Ehime 791-0295, Japan.

E-mail address: daixiuju@m.ehime-u.ac.jp (X. Dai).

<https://doi.org/10.1016/j.bbadis.2020.165719>

Received 10 October 2019; Received in revised form 1 February 2020; Accepted 5 February 2020

Available online 07 February 2020

0925-4439/© 2020 Elsevier B.V. All rights reserved.

significantly upregulated in inflamed skin samples from patients with AD [9]. Serum IL-33 levels are also elevated in patients with AD, and correlated with disease severity [9,10]. It has been demonstrated that IL-33 produced by epidermal keratinocytes activates ST2⁺ cells (e.g., type 2 innate lymphoid cells, Th2 lymphocytes, mast cells, and basophils), leading to the secretion of Th2 cytokines and eosinophil accumulation in skin [6]. Some skin inflammation-related cytokines, such as IFN- γ , TNF- α , and IL-17A, upregulate IL-33 expression in keratinocytes [11–13]. Nevertheless, the mechanism underpinning the innate immune regulation of IL-33 production in normal human epidermal keratinocytes (NHEKs) remains unclear.

The skin barrier is disrupted in AD and epidermal keratinocytes can be exposed to environmental stimuli, such as house dust mite (HDM) allergens. HDM, a common indoor allergen, is a major external triggering factor for AD [14]. Currently, AD treatment is mainly based on symptomatic treatment and avoidance of irritating/allergenic factors, of which HDM is particularly important [14,15]. The group 1 and 2 molecules are major allergens in HDMs [16]. Group 1 allergens, which are mite cysteine proteases [17], activate NLRP3 inflammasome to produce mature IL-1 β from keratinocytes [18]; and group 2 allergens, without proteolytic activity, could not stimulate IL-1 β secretion but activate IL-8 production [18], probably via binding to TLR2 and TLR4 [19]. Moreover, HDM extract contains weak serine proteases (including group 3, group 6, and group 9 allergens), which also activate keratinocytes to induce inflammatory molecules [20]. HDM allergens can stimulate IL-33 production from airway epithelial cells, which plays a vital role in the development of allergic airway inflammation [21,22]. In the present study, we investigated whether *Dermatophagoides pteronyssinus* (Der p) HDM extract regulate IL-33 production in NHEKs.

We present here that Der p-derived proteases (specifically, cysteine protease Der p1) induced IL-33 expression and stimulated its rapid release from NHEKs through eliciting acute accumulation of extracellular ATP, thereby activating P2 receptors-mediated signal pathways. Moreover, we show that Der p processed extracellular IL-33 to regulate its maturation. Our study suggests that HDM allergens regulate the innate production of IL-33 by keratinocytes, which may contribute to the pathogenesis and exacerbation of AD.

2. Experimental procedures

2.1. Reagents, inhibitors, antagonists and antibodies

The reagents, inhibitors and antagonists used in this study were listed in Table S1. The primary antibodies used for Western blotting and immunofluorescence were listed in Table S2.

2.2. Human subject and keratinocyte culture

This study was conducted according to the principles of the Declaration of Helsinki, and all procedures involving human participants received prior approval from the Ethics Committee at Ehime University School of Medicine, Japan. All participants consented to the study. The human skin samples were obtained after plastic surgery. The skin samples were cut into 3- to 5-mm pieces and incubated with 250 U/ml dispase overnight at 4 °C. After separation of the epidermis from the dermis, the epidermal sheets were incubated in a 0.25% trypsin solution for 10 min at 37 °C and teased with forceps. NHEKs were collected by centrifugation and were further cultured in MCDB153 medium that was supplemented with insulin (0.02 μ M), hydrocortisone (0.5 μ M), ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM), bovine hypothalamic extract (50 μ g/ml), and Ca²⁺ (0.07 mM).

NHEKs that had been passaged four times were treated with Der p or Der p1 with or without inhibitors or antagonists suspended in serum-free medium. HDM extract Der p was obtained from LSL (Tokyo, Japan). Before being used in experiments, the solution of Der p was purified using Pierce High-Capacity Endotoxin Removal Resin (Thermo

Scientific, Yokohama, Japan), according the protocol provided; and no LPS pollution in Der p has been verified in our previous report [18]. The protease activity of Der p was evaluated using Protease Activity Assay Kit (Abcam, Japan) and presented in Fig. S1. To block the proteolytic activity of allergens, Der p was incubated with E-64 or ABESF at 37 °C for 15 min or heated at 65 °C for 30 min, as described previously [18].

2.3. Preparation and stimulation of living skin equivalent (LSE)

To prepare the LSE [23], NHEKs and fibroblasts were isolated from neonatal skin samples from plastic surgery and then cultured. A collagen gel containing the fibroblasts was prepared, and the keratinocytes were seeded onto the concave surface of the contracted gel. When the keratinocytes reached confluence, the LSE was raised to the air–liquid interface, and cornification medium was added. LSE was treated with Der p or heat-treated Der p after air-lifting for 7 days as described previously [24]. Total RNA extracted from the LSE epidermis were subjected to real-time RT-PCR, and concentrated culture medium and protein extracted from the LSE epidermis were subjected to Western blotting.

2.4. RNA preparation and real-time RT-PCR

The probes specific for GAPDH, IL-33, TACE, HB-EGF, TGF- α , IL-8, CCL2, TLR1, TLR2, TLR6 and IL-25 were obtained from Thermo Fisher. Total RNA was isolated and subjected to real-time RT-PCR, and gene expression was analyzed, as described previously [25].

2.5. Protein isolation and Western blotting

Following stimulation, cell extracts and culture supernatants were collected, and supernatants were concentrated using Amicon Ultra-0.5 Centrifugal Filter Devices (Merck Millipore, Ireland). To detect intracellular and extracellular protein levels, cell lysates and concentrated supernatants were separated by SDS/PAGE and transferred to polyvinylidene difluoride membranes. Analyses were performed using a Vistra ECF Kit (Amersham Biosciences, Arlington Heights, IL, USA) and then membranes were scanned using a FluoroImager (Molecular Dynamics, Sunnyvale, CA, USA).

2.6. Transfection of keratinocytes with siRNA

The ON-TARGETplus SMART pool of hPAR-2 siRNA (F2RL1-1 and F2RL1-6) and hTLR2 siRNA, Silencer® pre-designed siRNA for human P2Y2, Stealth siRNA for human IL-33 and human TACE, and relative negative control siRNAs were obtained from Thermo Fisher Scientific K.K. Each set of siRNAs were transfected into NHEKs according to the manufacturer's instructions and as described previously [11].

2.7. Enzyme-linked immunosorbent assay (ELISA)

Cell culture supernatants and Der p-added medium were respectively collected and stored at –20 °C. The levels of HB-EGF, TGF- α , IL-8 and IL-33 were quantified using commercially available ELISA kits (R&D Systems), according to the manufacturer's procedures.

2.8. Measurement of extracellular ATP

Keratinocytes were pretreated with or without ARL67156 for 30 min and then treated with Der p, heat-inactivated Der p, Der p1 or Der p2 for a period of $\leq$ 20 min. Supernatant ATP was measured with an ATP Determination Kit (BioAssay Systems, Hayward, CA) according to the manufacturer's instructions.

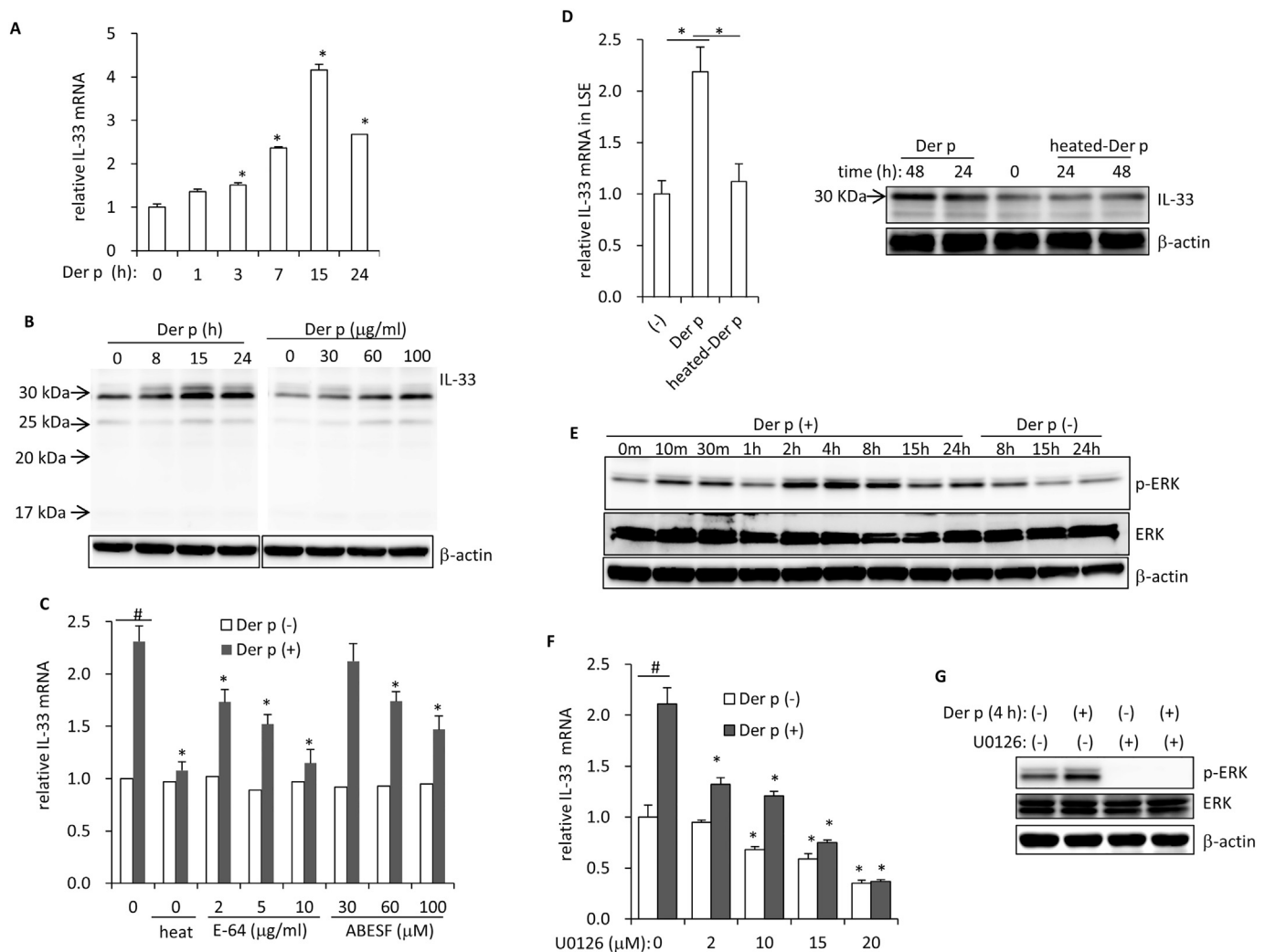


Fig. 1. Der p induces IL-33 expression in keratinocytes. NHEKs were treated with Der p (100 μ g/ml) for the times specified, or were treated with different doses of Der p for 15 h, then (A) IL-33 mRNA levels were measured using real-time RT-PCR, (B) IL-33 protein were analyzed by Western blotting of cell extracts. (C) NHEKs were incubated with Der p, heated-Der p, or E-64-treated or ABESF-treated Der p for 12 h, and IL-33 mRNA was measured. (D) LSE were respectively treated with buffer, Der p and heated Der p for 24 h, then total RNA and protein were extracted from the LSE epidermis. The levels of IL-33 mRNA and IL-33 protein were measured. (E) NHEKs were treated with Der p for the times specified, and the phosphorylation of ERK was examined by Western blotting. (F) Keratinocytes were pretreated with U0126 for 30 min and then stimulated with Der p for 12 h, IL-33 mRNA expression were detected. (F) Keratinocytes were pretreated with U0126 for 30 min and then stimulated with Der p for 4 h. The phospho-ERK levels were evaluated. Relative mRNA levels are expressed as means $\pm$ standard SDs ($n = 3$). (* and * $p < 0.05$).

2.9. Immunofluorescence

Keratinocytes or LSE sections were fixed with 4% paraformaldehyde and incubated overnight at 4 $^{\circ}$ C with anti-EGFR or anti-IL-33. Subsequently, they were treated with an Alexa Fluor 488-conjugated secondary antibody (Thermo Fisher) and DAPI (Invitrogen), as described previously [18]. The images were photographed using a BZ-X710 All-in-One Fluorescence Microscope (Keyence, Osaka, Japan). Control staining with IgG isotype controls or without primary antibodies yielded no positive signal.

2.10. Lactate dehydrogenase (LDH) cytotoxicity assay

Keratinocytes were stimulated with Der p or 1% NP-40 (positive control). Supernatant LDH activity was measured using an LDH Assay Kit (Kyokuto, Tokyo, Japan), according to the manufacturer's instructions.

2.11. Cell counting Kit-8 (CCK-8) assay

To investigate the effect of HDM extract on cell viability, keratinocytes were treated with Der p for the indicated times and then incubated with CCK-8 solution (DOJINDO, Tokyo, Japan) for 2 h. The cell viability was measured and evaluated according to the manufacturer's instructions.

2.12. Measurement of intracellular calcium concentration ($[Ca^{2+}]_i$)

To detect $[Ca^{2+}]_i$, keratinocytes were loaded with 1 μ M fura 2-AM for 1 h at 37 $^{\circ}$ C, prior to stimulation with Der p. The assay was performed as described previously [26]. In some cases, the cells were pretreated with inhibitors prior to Der p stimulation.

2.13. IL-33 cleavage assay with Der p

Subconfluent keratinocytes were scraped with tips and then the supernatants were immediately collected and centrifuged (in some

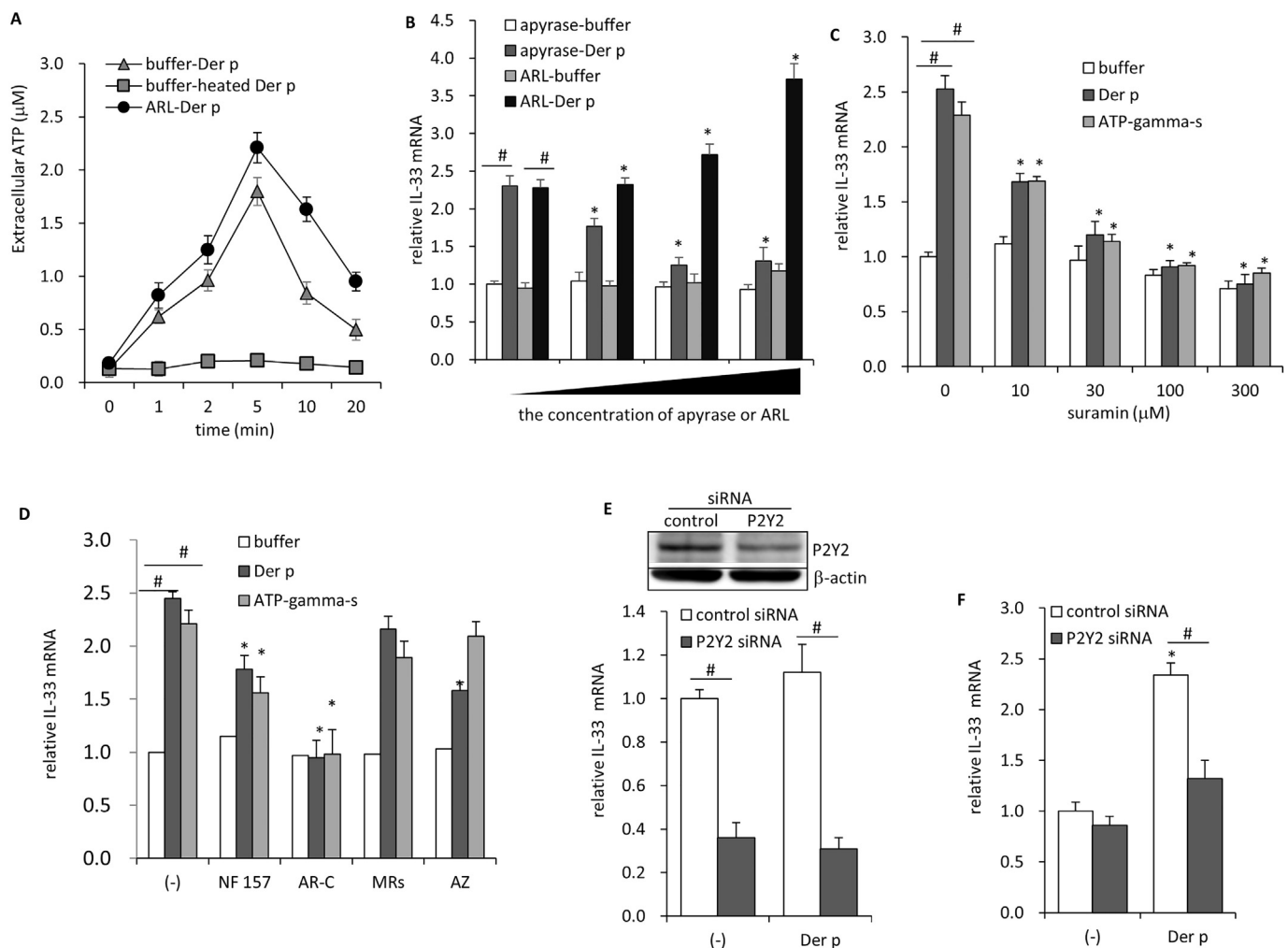


Fig. 2. Der p stimulates IL-33 expression via extracellular ATP. (A) NHEKs were pretreated with ARL67156 (ARL, 100 μM) or buffer for 30 min and then treated with Der p or heat-treated Der p for the indicated times, and ATP levels were measured in the supernatant with an ATP assay. (B) NHEKs were pretreated with apyrase (0, 20, 40, 80 unit/ml) or ARL67156 (ARL; 0, 10, 30, 100 μM) for 30 min and then stimulated with Der p for 12 h, and IL-33 mRNA were detected by real-time RT-PCR. (C) NHEKs were pretreated with different concentrations of suramin for 30 min and then stimulated with Der p or ATP-γ-s. IL-33 mRNA levels were measured. (D) NHEKs were respectively pretreated with specific antagonists for P2Y11 (NF157, 10 μM), P2Y2 (AR-C118925XX (AR-C), 10 μM), P1Y1 (MRs2179 (MRs), 10 μM) and P2X7 (AZ10606120 (AZ), 10 μM) for 30 min and then were stimulated with Der p or ATP-γ-s for 12 h, and IL-33 mRNA levels were measured. (E) NHEKs were transfected with control siRNA or P2Y2 siRNA for 48 h, and the expression of P2Y2 protein and mRNA was detected. (F) NHEKs were transfected with siRNA for 48 h and then stimulated with Der p for 12 h, and IL-33 mRNA was detected. Relative mRNA levels are expressed as means ± SDs ($n = 3$), and ATP levels are expressed as means ± SDs ($n = 5$). ([#] and ^{*} $p < 0.05$).

experiment, the supernatants from thapsigargin or Der p-treated cultures were collected and centrifuged). The cleavage assay of supernatant IL-33 were performed as described previously [5] with minor modulation. The supernatants were incubated with buffer, Der p or heated Der p at 37 °C for 30 min (in some cases, they were incubated with buffer or Der p at 37 °C for 10 min). The cleavage products were concentrated and analyzed by SDS/PAGE to detect the cleavage of extracellular IL-33.

2.14. Analysis of TACE (TNF-α-converting enzyme) activity

Keratinocytes were treated with Der p as described above, in the presence of a fluorogenic TACE substrate (Calbiochem; 10 μM), for the indicated times. Media was collected, and TACE activity was analyzed using a fluorescence plate reader. P2 antagonist suramin was added into cultures 30 min prior to stimulation with Der p.

2.15. Statistical analysis

For each data point, a minimum of three replicates were performed, and each replicate yielded similar results. Data from representative experiments are shown in the figures. Relative mRNA expression, ATP, protease activity, TACE activity, $[Ca^{2+}]_i$ increase, LDH levels and relative cell viability were expressed as the mean ± standard deviations (SDs) ($n \geq 3$). Statistical significance was determined using Student's *t*-tests. A *p* value < 0.05 (indicated as [#] $p < 0.05$ or ^{*} $p < 0.05$ in the figure legends) was considered statistically significant.

3. Results

3.1. Der p-derived proteases induce IL-33 expression in NHEKs

We first investigated whether HDM allergens regulate IL-33 expression in cultured NHEKs. Der p effectively induced IL-33 mRNA expression from 3 h, with an approximately four-fold peak effect after 15 h (Fig. 1A). Fig. 1B illustrates the significant upregulation of IL-33

protein (30-kDa) by Der p after 15 h of stimulation, depending on its concentration. The proteolytic activity of HDM allergens often contributes to their inflammatory function [18]. We found that heat treatment of Der p, which inactivates its proteolytic activity (Fig. S1) [27], almost blocked its induction of IL-33 mRNA (Fig. 1C). Pretreatment with cysteine protease inhibitor E-64, which significantly decreased Der p protease activity (Fig. S1), inhibited IL-33 induction in a dose-dependent manner; and pretreatment with the serine protease inhibitor AEBSF (at a concentration of > 60 μ M), which slightly decreased Der p protease activity (Fig. S1), also partially suppressed Der p-induced IL-33 mRNA (Fig. 1C). We next performed an ex vivo experiment to test the role of Der p in skin and found that Der p, but not heat-treated Der p, induced IL-33 expression in the epidermis of LSE, a 3D skin substitute (Fig. 1D). Thus, Der p-derived proteases are required for IL-33 induction in NHEKs. The activation of ERK, p38, and JAK/STATs contributed to the transcriptional induction of keratinocyte IL-33 [11,12]. After the addition of Der p, the induction of ERK phosphorylation was detected as early as 10 min, which lasted to 24 h and reached a peak at 4 h (Fig. 1E). However, we observed no apparent phosphorylation of p38, STATs or I κ B α by Der p (data not shown). Thus, HDM allergens activate mainly the ERK signaling pathway in NHEKs. Pretreatment of cells with U0126, a specific MEK/ERK inhibitor, suppressed IL-33 expression in a dose-dependent manner, and 20 μ M U0126 not only abolished Der p-induced IL-33 mRNA but also significantly decreased basic IL-33 mRNA (Fig. 1F), consistent with the blocking of ERK activation in both Der p-treated and Der p-untreated cells (Fig. 1G). Therefore, HDM allergens-derived proteases stimulate IL-33 expression in keratinocytes by activating the MEK/ERK signaling pathway.

3.2. Der p triggers acute extracellular accumulation of ATP from NHEKs in response to IL-33 induction by activating P2 receptors (particularly the P2Y2 receptor)

ATP is an energy source within cells, but it functions as an endogenous danger signal in the extracellular environment. Endogenous ATP released from dsRNA-activated bronchial smooth muscle cells [28] and from HMGB1-activated nasal polyp epithelial cells [29] can activate IL-33 transcription. In the cultures of NHEKs, the extracellular levels of ATP increased immediately after the addition of Der p (not heated-Der p), peaked within 5 min, and returned to near-baseline levels 20 min after stimulation (Fig. 2A). The Der p-mediated acute extracellular accumulation of ATP was significantly augmented by ARL67156 (an ecto-ATPase inhibitor) (Fig. 2A). Although mite allergens release ATP from bronchial epithelial cells [30], this is first report that Der p releases ATP from NHEKs. To examine whether extracellular ATP contributes to IL-33 expression, we reinforced the ATP effect by applying ARL67156 and depleted the ATP effect by exogenously administering an ectonucleotidase apyrase. ARL67156 increased while apyrase significantly decreased Der p-induced IL-33 mRNA in NHEKs in a dose-dependent manner (Fig. 2B). Thus, Der p-induced IL-33 expression was dependent on the acute accumulation of extracellular ATP.

P2 purinergic receptors are subtyped into ionotropic P2X1-7 receptors and G-protein coupled P2Y1-14 receptors (GPCRs) [31], most of which are functionally expressed in primary human keratinocytes [32]. Adenosine 5'-[γ -thio] triphosphate (ATP- γ -S) is a relatively stable ATP analog that is commonly used for studies on the effects of ATP binding to P2 receptors. ATP- γ -S significantly increased IL-33 mRNA (Fig. 2C), suggesting that extracellular ATP/P2 signaling can activate IL-33 expression in keratinocytes. To investigate the involvement of P2 receptors in Der p-induced IL-33 expression, keratinocytes were pretreated with suramin, a broad-spectrum P2 antagonist that suppresses ATP binding to P2 receptors in keratinocytes [32,33]. Suramin decreased ATP- γ -S- and Der p-induced IL-33 mRNA in a dose-dependent manner (Fig. 2C). Thus, Der p induces IL-33 expression in NHEKs through

extracellular ATP/P2 signaling. To clarify which P2 receptors are indispensable for IL-33 expression, several P2Y or P2X receptor antagonists were respectively added to the cultures. As shown in Fig. 2D, AR-C118925XX (a specific P2Y2 receptor antagonist) completely blocked both Der p and ATP- γ -S induction of IL-33. NF157 (a potent P2Y11 receptor antagonist) also exhibited a partial inhibitory effect on both; and AZ10606120 (a potent P2X7 receptor antagonist) suppressed IL-33 expression induced by Der p, but not that induced by ATP- γ -S; however, MRS2179 (a specific P2Y1 receptor antagonist) did not reduce IL-33 expression. To characterize the involvement of P2Y2 further, transfection with P2Y2 siRNA, which knocks down P2Y2 expression in NHEKs (Fig. 2E), nearly blocked Der p-induced IL-33 mRNA (Fig. 2F). Thus, extracellular ATP-mediated activation of P2Y2 receptor is essential for Der p-induced IL-33 expression in NHEKs.

3.3. Der p stimulates transactivation of the epidermal growth factor receptor (EGFR) through extracellular ATP/P2Y2 signaling to induce IL-33 expression in NHEKs

Among the metabotropic GPCRs, the P2Y2 receptor is specifically coupled to the G $_{q/11}$ protein in epithelial cells to promote phospholipase C (PLC)-dependent [Ca²⁺]_i mobilization as well as trigger tyrosine kinase receptor EGFR-dependent signal activation through ligand-dependent (triple membrane passing signaling pathway [TMP]) or ligand-independent pathways [34,35]. In keratinocytes, Der p stimulated the phosphorylation of EGFR-tyr1068 in dose-dependent manner, with a peak effect at 4 h (Fig. 3A). Pretreatment with P2 antagonist suramin or transfection with P2Y2 siRNA suppressed Der p-induced EGFR activation and the followed ERK activation, a signal pathway at the downstream of EGFR (Fig. 3B). Pretreatment with AG1478, a specific EGFR inhibitor, abrogated the phosphorylation of both EGFR and ERK in NHEKs (Fig. 3C). Moreover, AG1478 suppressed Der p-induced IL-33 mRNA expression in a dose-dependent manner (Fig. 3D). Therefore, extracellular ATP/P2Y2 signaling-dependent EGFR/ERK activation is responsible for Der p induction of IL-33.

Next, we performed an immunofluorescence assay to evaluate the internalization of EGFR, which occurs after a well-established response to an EGFR ligand [35]. As shown in Fig. 3E, the EGFR was internalized after a 4-h Der p treatment and was punctate in the cytoplasm, which was similar to the pattern observed in HB-EGF-treated keratinocytes. The puncta detected in Der p-treated cells disappeared when the cultures were pretreated with suramin (Fig. 3E), suggesting that ATP signaling is required for Der p-induced EGFR internalization. Internalization of the EGFR indicates TMP-dependent EGFR transactivation [34], a process that includes activation of matrix metalloproteinases (MMPs) followed by ectodomain cleavage and release of the EGFR ligand, which binds to EGFR and induces cell signaling. To verify the involvement of MMP-dependent substrate shedding, batimastat (a broad-spectrum MMP inhibitor) and anti-EGFR (a neutralizing EGFR antibody that prevents binding to EGFR and inhibits EGFR activation [36]) were separately added to the cultures. Both batimastat and anti-EGFR significantly inhibited the Der p-triggered phosphorylation of EGFR and ERK (Fig. 3F) and decreased Der p-induced IL-33 mRNA (Fig. 3G). Thus, TMP-mediated EGFR transactivation is responsible for Der p induction of IL-33. MMP-mediated HB-EGF shedding is involved in P2Y2-mediated EGFR transactivation in melittin-treated keratinocytes [37]. Der p stimulated the rapid secretion of HB-EGF (but not TGF- α) before EGFR activation, which depended on the activation of MMPs by ATP signaling (Fig. S2). Therefore, Der p stimulated MMP-mediated HB-EGF shedding in keratinocytes. CMR197 is a nontoxic diphtheria toxin mutant that specifically prevents the binding of soluble HB-EGF to EGFR [38]. CMR197 not only suppressed Der p-triggered phosphorylation of EGFR and ERK (Fig. 3F) but also inhibited Der p-induced IL-33 mRNA (Fig. 3G), suggesting that HB-EGF is an EGFR ligand that is shed by MMPs in Der p-treated NHEKs and is involved in IL-33 expression. Overall, HDM allergens-induced IL-33 expression in keratinocytes is

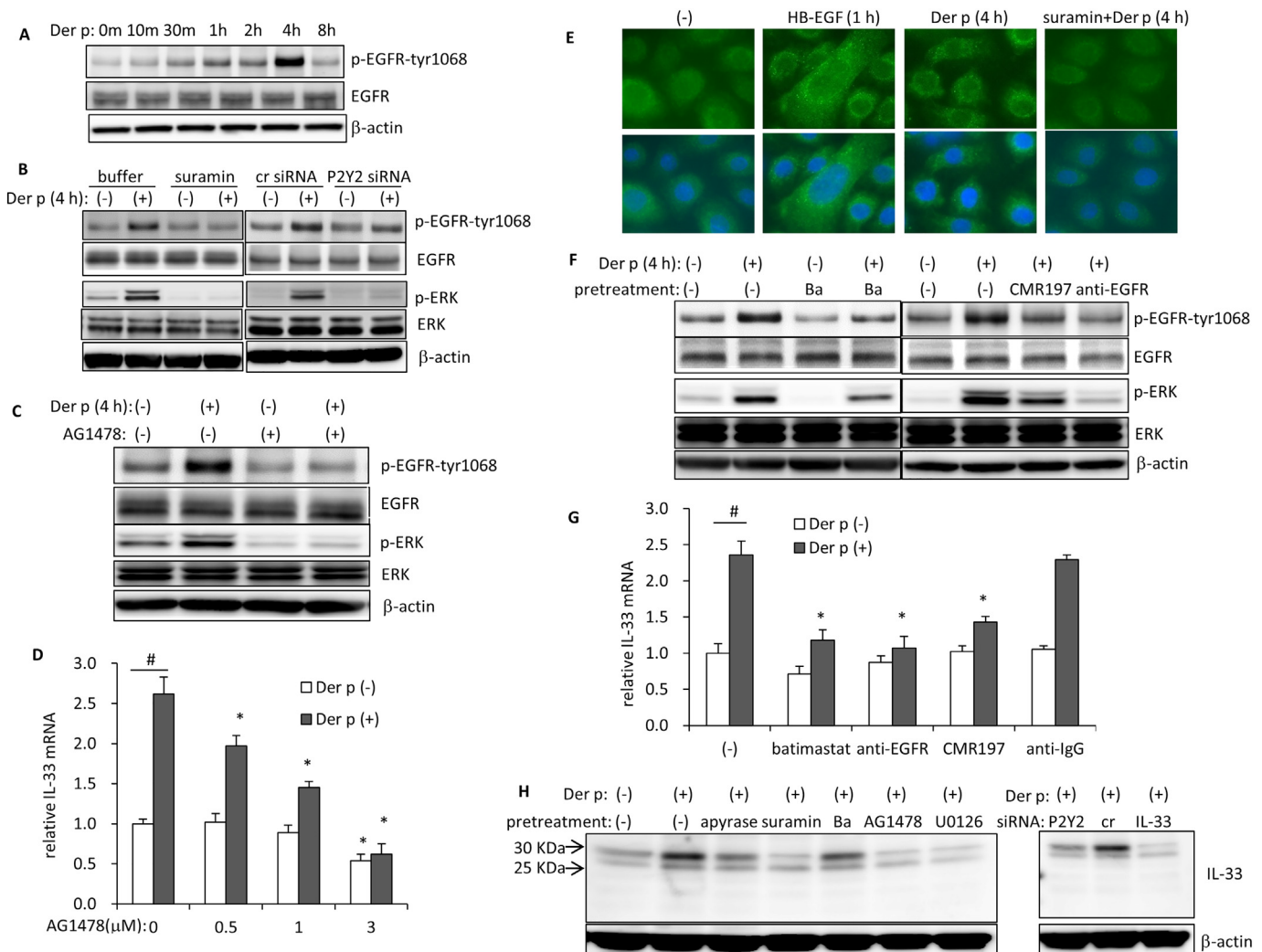


Fig. 3. Der p induces IL-33 expression via extracellular ATP/P2Y2 signaling-dependent EGFR transactivation. (A) NHEKs were stimulated with Der p for the indicated times, and the phosphorylation of EGFR was examined by Western blotting. (B) NHEKs were pretreated with suramin for 30 min or transfected with siRNA for 48 h, then stimulated with Der p for 4 h. The phosphorylation of EGFR and ERK was examined. (C) NHEKs were pretreated with the EGFR inhibitor AG1478 (3 μM) for 30 min and then treated with Der p for 4 h. The phosphorylation of EGFR and ERK was evaluated. (D) NHEKs were pretreated with AG1478 for 30 min and then treated with Der p for 14 h. IL-33 mRNA levels were measured. (E) NHEKs were or were not exposed to suramin for 30 min and then treated with Der p (for 4 h) or HB-EGF (for 1 h). The cells were fixed and the intracellular distribution of EGFR was assessed by immunofluorescence. NHEKs pretreated with the MMP inhibitors batimastat (10 μM), CMR197 (7 μg/ml), anti-EGFR (2 μg/ml) or control were treated with Der p for 4 h (for protein) or for 12 h (for RNA), then the phosphorylation of EGFR and ERK (F) and the expression of IL-33 mRNA (G) were detected. (H) NHEKs were pretreated with inhibitors, or transfected with siRNA, and then stimulated with Der p for 20 h. IL-33 protein levels in NHEKs were evaluated by Western blotting of cell extract. Relative mRNA levels are expressed as means ± SDs ($n = 3$). ($\#$ and $*$ $p < 0.05$; cr: control; Ba: batimastat).

dependent on the serial activation of extracellular ATP/P2Y2 signaling, TMP-mediated EGFR transactivation, and the MEK-ERK signal pathway. This mechanism was further confirmed by the results of IL-33 protein (Fig. 3H).

3.4. Der p-derived proteases stimulate rapid release of stored IL-33 from NHEKs

Circulating IL-33 levels are elevated in patients with AD [10]. We next investigated whether Der p induced IL-33 secretion from keratinocytes. No extracellular release of IL-33 was detected from cytokine-treated keratinocytes, regardless of their upregulation of intracellular IL-33 (Fig. S3); however, immunoblotting of the supernatant showed the rapid release of IL-33 from NHEKs after Der p stimulation (Fig. 4A). Ten minutes after Der-p administration, 25-kDa IL-33 was detected in the supernatant and then gradually increased, reaching a peak at 2–4 h, (much earlier than that of IL-33 induction (Fig. 1)); however, it was

nearly undetectable after 24 h (Fig. 4A, upper). In parallel with the rapid increase in extracellular IL-33 (25-kDa), intracellular 30-kDa IL-33 (full-length) decreased gradually and intracellular ~25-kDa IL-33 was barely detectable during the early phase (30 min–2 h) after Der p stimulation (Fig. 4A, lower), suggesting that Der p triggers rapid extracellular translocation of stored IL-33. IL-33 siRNA transfection and IL-33 immunostaining further confirmed the rapid release of stored IL-33. Transfection with IL-33 siRNA knocked down its intracellular expression in NHEKs and significantly suppressed the extracellular release of IL-33 from Der p-treated cells (Figs. 4B and S4A). Consistent with a previous report [39], IL-33 was strongly detected in the nuclei of resting cells, but became weaker or disappeared after Der p stimulation (Fig. 4C). We next evaluated the requirement of proteolytic activity for Der p-triggered IL-33 release and found that heat-treated Der p hardly released IL-33 from NHEKs (Figs. 4D and S4B), and pretreatment of Der p with either E-64 or ABESF reduced extracellular IL-33 (Fig. 4D). Moreover, we also confirmed the role of HDM-derived proteases in IL-

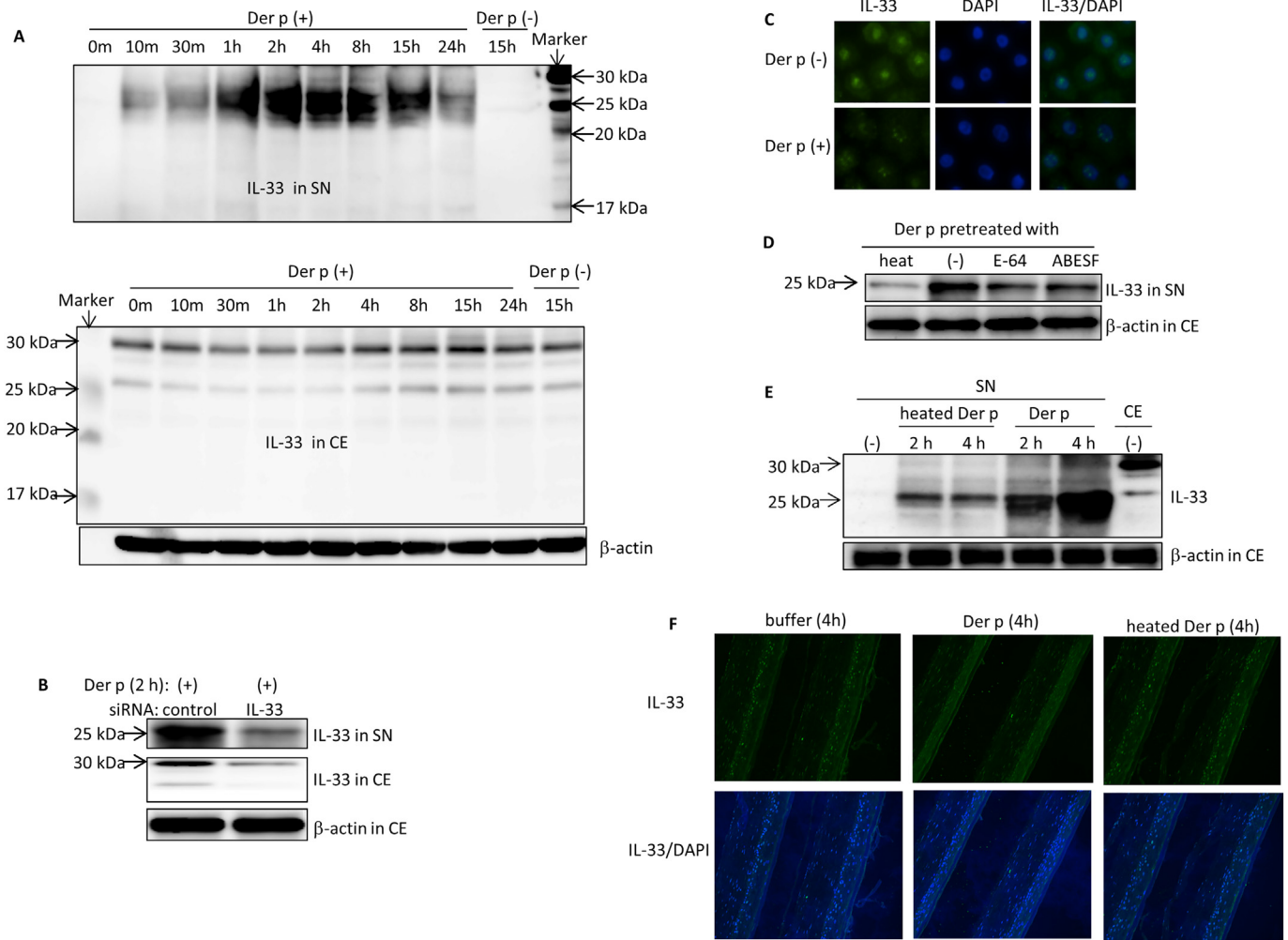


Fig. 4. Der p stimulates the rapid release of stored IL-33. (A) NHEKs were treated with Der p for the indicated times, then the supernatants and cell extracts were collected and the supernatant and intracellular IL-33 protein levels were respectively measured by Western blotting. (B) NHEKs were transfected with control or IL-33 siRNA for 48 h and treated with Der p for 2 h. The supernatant and intracellular IL-33 protein levels were measured. (C) NHEKs were treated with or without Der p for 2 h, and intracellular IL-33 was assessed by immunofluorescence. (D) NHEKs were treated with heated Der p, Der p, ABESF (100 μ M)-treated Der p, or E-64 (5 μ g/ml)-treated Der p for 2 h and the supernatant IL-33 protein levels were measured. (E) LSE were prepared and treated with Der p or heated Der p for 2 h or 4 h. IL-33 protein secreted from LSE was detected by Western blotting of culture medium. (F) LSE treated with Der p or heated Der p for 4 h, and intracellular IL-33 was assessed by immunofluorescence. (SN: supernatant; CE: cell extract).

33 release from 3D skin, as the detection of extracellular IL-33 by immunoblotting (Fig. 4E) and intracellular IL-33 by immunostaining (Fig. 4F), where Der p, but not heat-treated Der p, mediated IL-33 rapid release. Thus, in addition to upregulation of IL-33, Der p-derived proteases stimulated its rapid release from NHEKs.

3.5. ATP/P2 signaling-mediated $[Ca^{2+}]_i$ increase is essential for Der p-stimulated rapid release of IL-33 from NHEKs

Although various events upstream of IL-33 expression in keratinocytes have been reported [11,12], the mechanism responsible for IL-33 secretion from NHEKs is unknown. IL-33 is constitutively expressed in the nucleus and commonly released into the extracellular space as a nuclear alarmin after cell damage [5]. However, no cell damage was detected in Der p-treated NHEKs, as assessed by the detection of cell toxicity and cell viability (Fig. S5). *Alternaria* allergens can trigger the release of IL-33 from healthy bronchial epithelia via extracellular ATP/P2Y2 activation-driven $[Ca^{2+}]_i$ mobilization [40]. The extracellular IL-33 was detected in cultures treated with thapsigargin (an inhibitor of Ca^{2+} pump proteins, which can mobilize $[Ca^{2+}]_i$ in various cells) (Fig. 5A), suggesting that $[Ca^{2+}]_i$ mobilization can trigger IL-33 release

from keratinocytes. To explore the mechanism responsible for Der p-mediated IL-33 release, we measured $[Ca^{2+}]_i$ levels by loading Fura-2 AM into NHEKs. A rapid increase of $[Ca^{2+}]_i$ was detected after the stimulation with Der p (not heated-Der p), which was inhibited by BAPTA-AM (an intracellular Ca^{2+} chelator) (Figs. 5B and S6A); this indicated that HDM-derived proteases trigger $[Ca^{2+}]_i$ mobilization in NHEKs. Moreover, pretreatment of cultures with the PLC inhibitor U73122 increased basic $[Ca^{2+}]_i$ but blocked any increase in response to Der p (Figs. 5C and S6B). Therefore, HDM extract stimulated the rapid $[Ca^{2+}]_i$ mobilization in NHEKs via PLC activation. We noticed that the $[Ca^{2+}]_i$ increase caused by Der p lasted for several minutes, which was not the results of cytolysis (Fig. S5), but probably due to the influx of extracellular calcium [41]. Because this phenomenon disappeared in the cells cultured in a medium without calcium ions (Fig. S6C). The Der p-induced $[Ca^{2+}]_i$ increase was augmented by ARL67156, but nearly blocked by pretreating cells with apyrase or suramin (Figs. 5D and S6D). Therefore, Der p induces $[Ca^{2+}]_i$ increase by activating ATP/P2 signaling. Pretreatment with suramin or BAPTA-AM significantly inhibited Der p-mediated IL-33 release from keratinocytes (Fig. 5E), however, the addition of ARL67156 to Der p-treated cultures, which increased the levels of extracellular ATP (Fig. 2A) and augmented

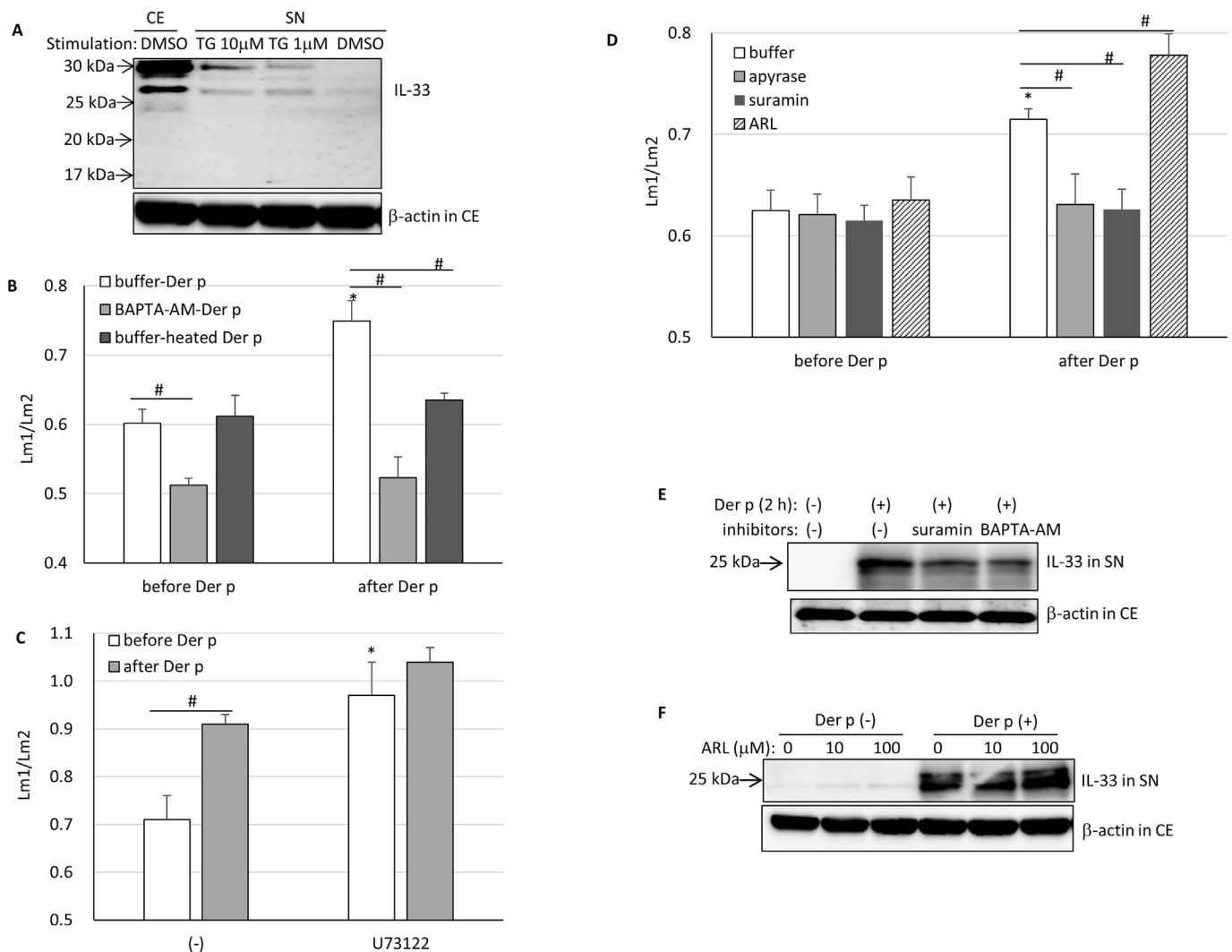


Fig. 5. Extracellular ATP/P2 signaling-mediated increase in [Ca²⁺]_i in Der p-activated keratinocytes is responsible for IL-33 release. (A) NHEKs were treated with DMSO or thapsigargin (TG, 1 μM or 10 μM) for 2 h and then the supernatant was collected and concentrated. The levels of supernatant IL-33 protein were measured by western blotting. (B) After Fura-2 loading, NHEKs were pretreated with buffer or BAPTA-AM (20 μM) for 5 min and then stimulated with Der p or heated Der p. Mobilization of [Ca²⁺]_i was detected by a [Ca²⁺]_i mobilization assay, and the change of [Ca²⁺]_i just before and (2 min) after Der p addition were presented. (C) After Fura-2 loading, NHEKs were pretreated with or without U73122 for 15 min and then stimulated with Der p. [Ca²⁺]_i mobilization was respectively detected; and the change of [Ca²⁺]_i in U73122-treated and U73122-untreated cells before and after Der p addition were presented. (D) After Fura-2 loading, NHEKs were pretreated with buffer, apyrase (40 unit/ml), suramin (100 μM) or ARL67156 (ARL, 100 μM) for 5 min and then stimulated with Der p. A [Ca²⁺]_i mobilization assay was performed, and the change of [Ca²⁺]_i before and after Der p addition were presented. (E) NHEKs were pretreated with buffer, suramin or BAPTA-AM; or (F) NHEKs were pretreated with ARL67156 (ARL), then stimulated with Der p or buffer for 2 h. The levels of supernatant IL-33 protein were measured. [Ca²⁺]_i mobilization is expressed as means ± SDs (n = 3). (# and * p < 0.05; SN: supernatant; CE: cell extract).

[Ca²⁺]_i concentrations (Fig. 5D), caused an additional extracellular release of IL-33 (Fig. 5F). Thus, HDM allergens stimulate the rapid release of stored IL-33 from keratinocytes via an extracellular ATP/P2 signaling-mediated [Ca²⁺]_i increase.

Despite the roles of extracellular ATP and [Ca²⁺]_i in IL-33 secretion, suramin and BAPTA-AM failed to completely inhibit extracellular IL-33, suggesting that other mechanisms may be involved in HDM-mediated rapid release of epidermal IL-33.

3.6. Der p1 but not Der p2 induces IL-33 expression and stimulates IL-33 release from NHEKs by activating extracellular ATP/P2 signaling

Next, we investigated the roles of Der p1 and Der p2 in the production of IL-33 by NHEKs. Both Der p1 and Der p2 induced IL-8 expression; however, Der p1, but not Der p2, upregulated IL-33 expression and stimulated its rapid release (Fig. 6A), suggesting specific roles of mite cysteine proteases in the production of keratinocyte IL-33. We

further explored the mechanism involved in Der p1-mediated IL-33 production. Der p1 (but not Der p2) stimulated the acute accumulation of extracellular ATP (Fig. 6B). Der p1 stimulated the activation of EGFR/ERK pathway, and blocking EGFR/ERK activation using the specific inhibitors AG1478 and U0126 notably suppressed IL-33 induction (Fig. 6C, D). Moreover, pretreatment with apyrase or suramin, or knockdown of P2Y2 expression, significantly inhibited the activation of EGFR/ERK and decreased the expression of IL-33 induced by Der p1 (Fig. 6C, D). Thus, Der p1 induces IL-33 expression in NHEKs via extracellular ATP/P2Y2 signaling-mediated EGFR/ERK activation, similar to Der p. Der p1 also triggered an increase in [Ca²⁺]_i, depending on its dose (Fig. S7A) and entirely on its cysteine protease activity (Figs. 6E and S7B). Cysteine protease inhibitor E-64 inhibited the function of Der p1 to release IL-33 from NHEKs (Fig. 6F, upper). And pretreatment with BAPTA-AM or suramin prevented the increase of [Ca²⁺]_i triggered by Der p1 (Figs. 6E and S7B) and inhibited the release of IL-33 from activated cells (Fig. 6F, lower). Thus, HDM allergen Der p1, but not Der

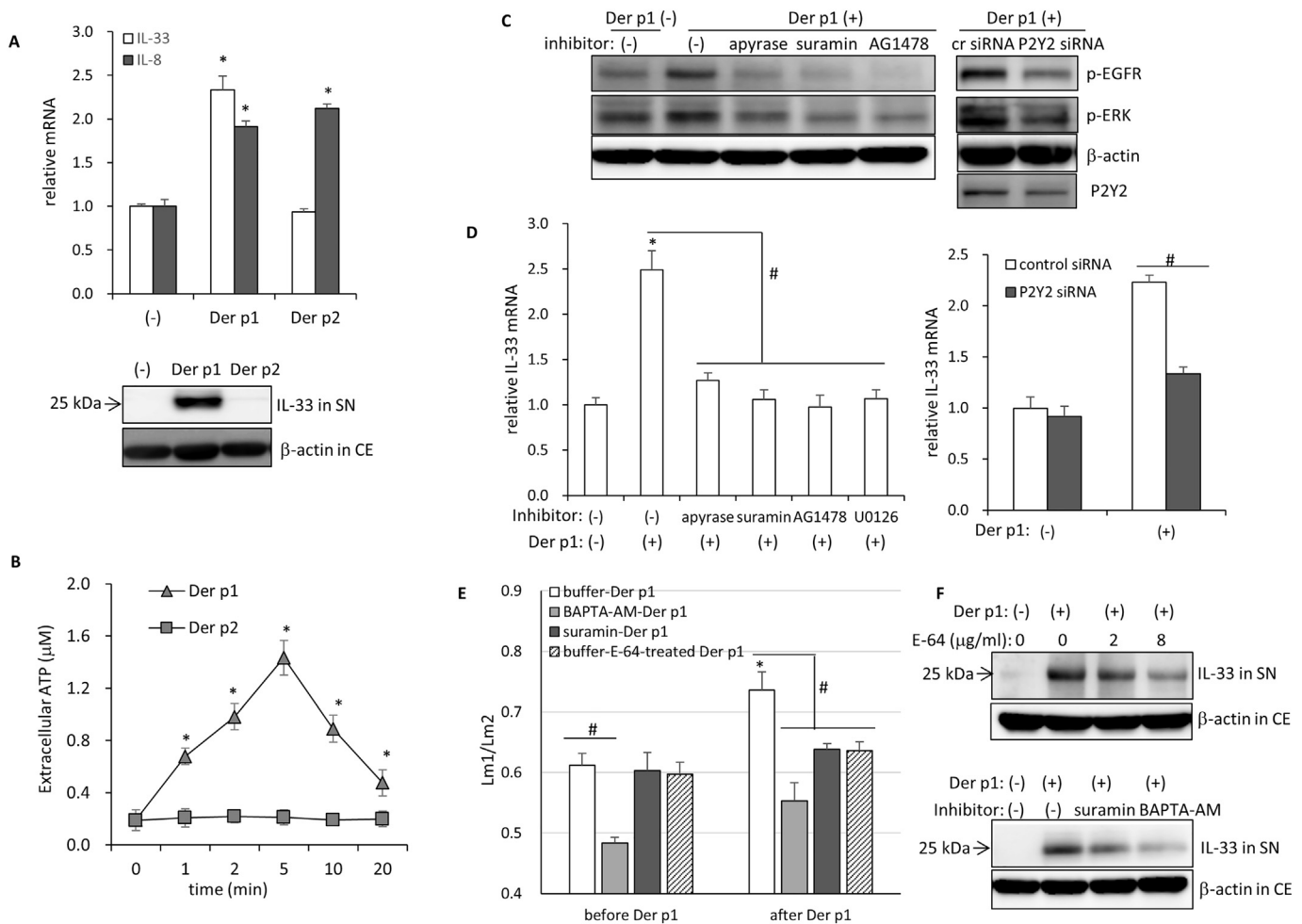


Fig. 6. Der p1 stimulates IL-33 expression and induce its release via extracellular ATP-mediated signal pathways. (A) NHEKs were treated with buffer, Der p1 (10 μg/ml) or Der p2 (10 μg/ml) for 12 h. IL-33 and IL-8 mRNA levels were measured using real-time RT-PCR; NHEKs were treated with buffer, Der p1 or Der p2 for 2 h, then the supernatant IL-33 protein levels were measured by Western blotting. (B) NHEKs were treated with Der p1 or Der p2 for the indicated times, and ATP levels were measured in the supernatant with an ATP assay. (C) NHEKs were pretreated with apyrase, suramin or AG1478 for 30 min and then stimulated with Der p1 for 4 h; or NHEKs were transfected with control (cr) or P2Y2 siRNA then stimulated with Der p1 for 4 h. The phosphorylation of EGFR and ERK was examined, and P2Y2 protein levels were evaluated. (D) NHEKs were respectively pretreated with specific inhibitors for 30 min; or transfected with control siRNA or P2Y2 siRNA for 48 h, and then stimulated with Der p1 for 15 h. IL-33 mRNA levels were measured using real-time RT-PCR. (E) After Fura-2 loading, NHEKs were pretreated with buffer, suramin or BAPTA-AM, then stimulated with Der p1 or E-64-treated Der p1. Mobilization of $[Ca^{2+}]_i$ was detected by a $[Ca^{2+}]_i$ mobilization assay, and the change of $[Ca^{2+}]_i$ before and after Der p addition were presented. (F) Der p1 were pretreated with E-64 or not, then added to the cultures for 2 h; or NHEKs were pretreated with buffer, suramin, BAPTA-AM and then stimulated with Der p1 or not for 2 h. The levels of supernatant IL-33 protein were measured. Relative mRNA levels and $[Ca^{2+}]_i$ mobilization are expressed as means $\pm$ SDs ($n = 3$), and ATP levels are expressed as means $\pm$ SDs ($n = 5$). (* and # $p < 0.05$; SN: supernatant; CE: cell extract).

p2, stimulates the production of IL-33 from NHEKs by activating ATP signaling.

In addition to Der p1 and Der p2, Der p serine proteases (DPSP) also produce inflammatory molecules from keratinocytes. We detected a significant induction of IL-8, a slight upregulation of IL-33, and a negligible extracellular IL-33 in DPSP-treated cultures (Fig. S8), which were not comparable to the effects of Der p1. However, DPSP synergistically augmented the effects of Der p1 on IL-33 release (Fig. S8B), which may account for the considerable role of serine proteases in Der p-triggered IL-33 release (Fig. 4D). Further studies are required to determine how DPSP regulates IL-33 production from keratinocytes.

3.7. Der p mediates the processing and maturation of extracellular IL-33

Alternaria stimulated full-length IL-33 release from airway epithelia [40], whereas Der p induced 25-kDa IL-33 release from NHEKs, suggesting IL-33 cleavage occurs in Der p-treated cultures. Because intracellular IL-33 is mainly observed as a 30-kDa band (full-length IL-33) in both stable and active NHEKs (Fig. 4A), and the IL-33 isoforms

released from thapsigargin-treated NHEKs were larger than 25-kDa (Fig. 5A). We hypothesized that in Der p-treated cultures, $[Ca^{2+}]_i$ mobilization stimulated extracellular IL-33 (> 25-kDa), which was then immediately cleaved into a 25-kDa band by extracellular proteases. To test this theory, we performed an IL-33 cleavage assay by treating the supernatants collected from thapsigargin-treated cultures with Der p, and found that Der p cleaved the thapsigargin-induced extracellular IL-33 bands (> 25-kDa) into 25-kDa fragments (Fig. 7A). To further clarify the role of Der p in IL-33 processing, the extracellular IL-33 released from damaged cells was treated with Der p. We detected the 30-kDa band, together with a relatively weak ~25-kDa band in the supernatants of necrotic keratinocytes (a pattern similar to that of intracellular IL-33) (Fig. 7B). Treating the supernatants with Der p for 10 or 30 min cleaved extracellular IL-33, generating more 25-kDa and less 30-kDa IL-33 (Fig. 7C), suggesting that Der p processes full-length IL-33 secreted from damaged NHEKs into 25-kDa fragments in extracellular space. However, this processing disappeared when the supernatants were treated with protease-inactive Der p (heated-Der p) (Fig. 7D). Our data suggest that Der p-derived proteases process extracellular IL-33,

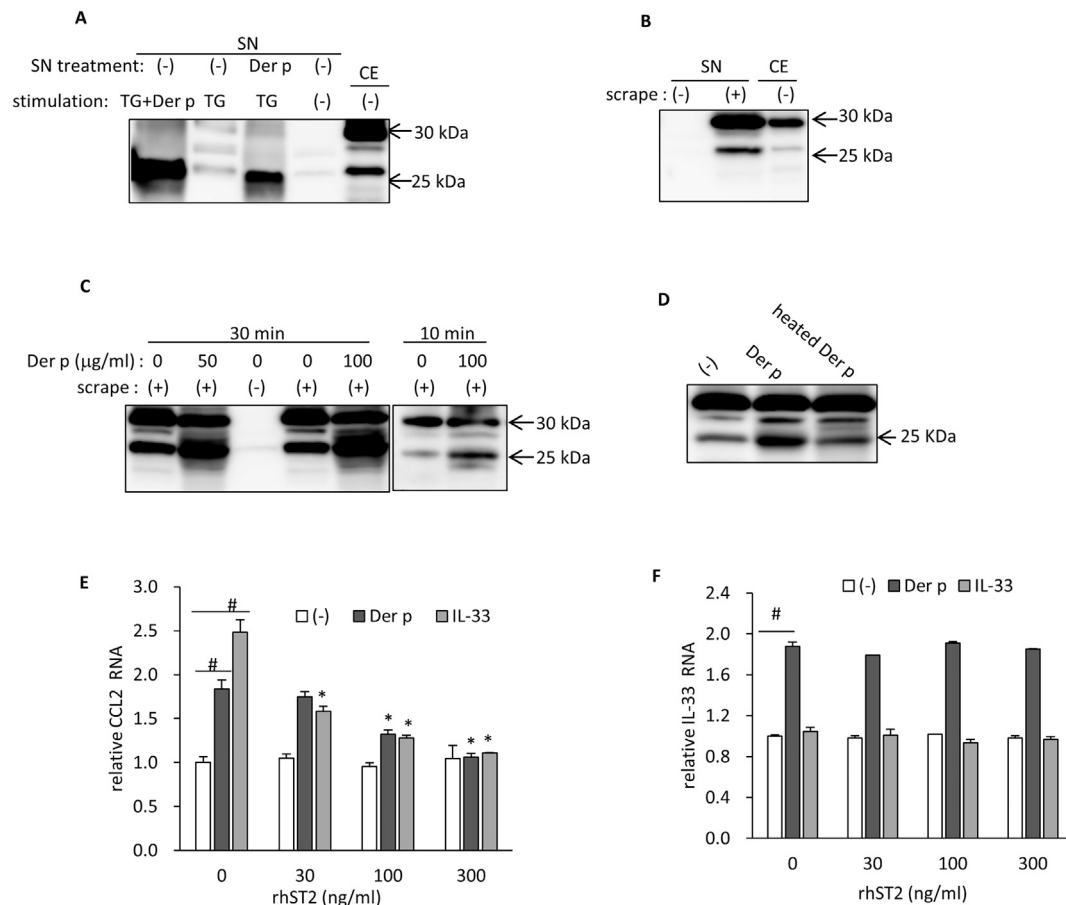


Fig. 7. Der p-derived proteases process IL-33 in the extracellular space, generating active 25-kDa fragments. (A) NHEKs were treated with buffer, thapsigargin (TG, 10 μM) or Der p together with TG for 2 h and then the supernatant were collected. To perform the cleavage assay, the supernatant collected from thapsigargin-treated cultures were incubated with or without Der p before concentration. These concentrated supernatants and cell extracts were subjected to Western blotting to detect the IL-33 protein. (B) NHEKs were or were not scraped, and the supernatants and cell extracts were collected and subjected to Western blotting to detect the IL-33 protein. (C) To perform the cleavage assay, the supernatants collected from scraped NHEKs were treated with or without Der p for 10 or 30 min; they were then concentrated and subjected to Western blotting to detect the IL-33 protein. (D) The supernatants collected from scraped NHEKs were incubated with buffer, Der p or heated Der p for 30 min to perform the IL-33 cleavage assay. The mixture was concentrated and subjected to Western blotting to detect the IL-33 protein. NHEKs were pretreated with different doses of rhST2 then stimulated with Der p (for 8 h) or with rhIL-33 (for 6 h). The mRNA expression of CCL2 (E) and IL-33 (F) were detected. Relative mRNA levels are expressed as means ± SDs ($n = 3$). (# and * $p < 0.05$; SN: supernatant; CE: cell extract).

producing 25-kDa IL-33.

Through heterotrimeric binding to ST2 and IL-1RAcP, IL-33 exerts an influence over innate and adaptive type-2 immune responses. Mature IL-33 can activate epidermal keratinocytes to produce cytokines [42,43] and interfere with differentiation [44]. To evaluate the bioactivity of HDM-elicited extracellular 25-kDa IL-33 from NHEKs, we treated cultures with recombinant human ST2/IL-33R (rhST2), which functions as a soluble decoy receptor that blocks the ability of IL-33 to signal through transmembrane ST2. Der p, as well as recombinant human IL-33, induced CCL2 mRNA expression in keratinocytes, and the addition of rhST2 significantly inhibited the effects of both Der p and IL-33 in a dose-dependent manner, with complete blockage in 300 ng/ml rhST2-treated cultures (Fig. 7E). These data indicate that Der p-elicited extracellular IL-33 can signal into keratinocytes to induce CCL2 expression. Epithelial IL-33 stimulates its own expression in airway epithelium by binding to IL-33R [45]. However, IL-33 auto-induction does not occur in NHEKs, because recombinant human IL-33 failed to induce IL-33 (Fig. 7F); and epidermal IL-33 released from Der p-treated NHEKs is not responsible for the subsequent IL-33 induction, as sequestering extracellular IL-33 showed no effect on IL-33 mRNA (Fig. 7F). Thus, HDM allergens not only stimulate IL-33 release but also regulate its maturation as extracellular proteases. This is the first report to demonstrate the role of HDM proteases in the secretion and

processing of keratinocyte IL-33.

4. Discussion

IL-33 is a novel pro-allergic cytokine that promotes Th2-type immune responses and plays an important role during development and exacerbation of AD [7,8]. Skin barrier dysfunction of AD patients allows HDM allergens to easily penetrate through the epidermis to directly act on keratinocytes, stimulating the production of inflammatory molecules, including IL-33. A previous report has shown that Der p exposure increases the levels of IL-33 mRNA in the skin of patients with AD or in filaggrin-deficient mice [46]. We show here that Der p not only mediate IL-33 production from monolayer-cultured NHEKs but also regulate IL-33 expression and elicit its rapid release from LSE, suggesting exposure to HDM allergens could stimulate the innate production of keratinocyte IL-33 in vivo. The molecular mechanisms underlying HDM allergens-induced IL-33 production of keratinocytes are summarized in Fig. 8. Der p stimulation triggers acute extracellular accumulation of ATP, which binds to P2 receptors to signal into cells. The extracellular ATP/P2 signaling not only activates TMPs-dependent EGFR transactivation to induce IL-33 expression, a step involving MMPs-mediated ligand shedding; but also promotes PLC-dependent $[Ca^{2+}]_i$ mobilization to impel the release of stored IL-33. Our finding provided the knowledge

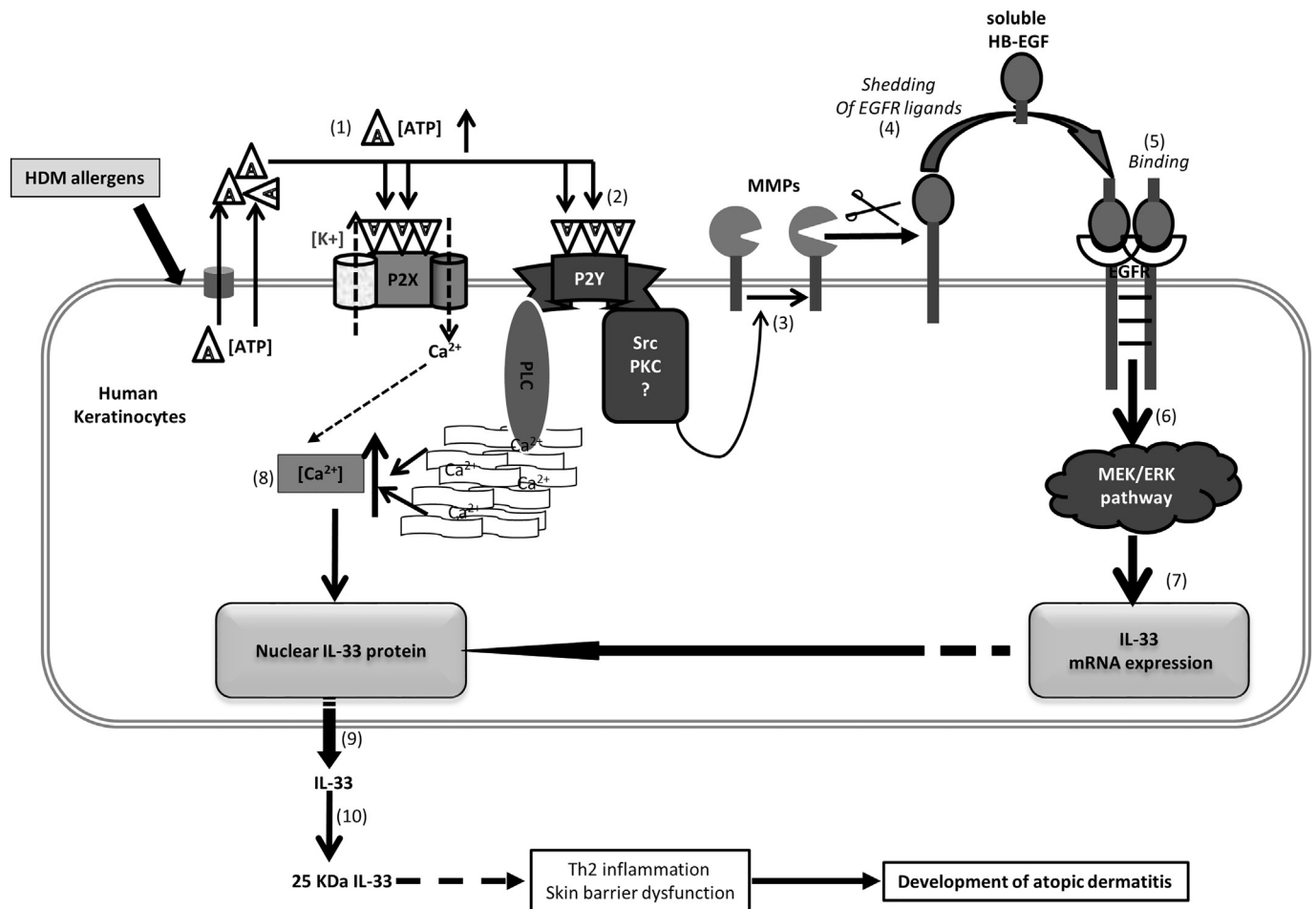


Fig. 8. Summary of the molecular mechanisms involved in HDM allergens-induced IL-33 synthesis and release in NHEKs. After Der p stimulation, keratinocytes rapidly release large amounts of extracellular ATP (1). Through an interaction with P2Y receptors (2), extracellular ATP induces the activation of MMPs (3) followed by the shedding of EGFR ligands (such as HB-EGF) (4). These ligands bind to EGFR, resulting in EGFR transactivation (5). Activated EGFR in turn activates the downstream MEK/ERK signaling pathway (6), which induces IL-33 expression (7) in Der p-treated keratinocytes. In addition, Der p-mediated the activation of extracellular ATP/P2 signaling rapidly mobilizes $[Ca^{2+}]_i$ (8). And the rapid and sustained $[Ca^{2+}]_i$ increase impels extracellular release of the stored IL-33 from activated keratinocytes (9). Extracellular IL-33 released from NHEKs is rapidly cleaved into 25-kDa forms (10).

about the innate production of epidermal IL-33 by environmental allergens, which may contribute to the initiation and exacerbation of AD.

The group 1 and 2 molecules are major allergens in HDMs [16]. We found that cysteine protease Der p1 but not Der p2 play a vital role in the production of IL-33 from keratinocytes. Der p1-elicited IL-33 release from NHEKs occurs much earlier than the activation of NLRP3 inflammasome, suggesting inflammasome activation mediate the secretion of IL-1 family members IL-1 α , IL-1 β and IL-18 from keratinocytes [18] but not that of IL-33. Jang et al. have reported that Der p induces the production of both IL-33 and IL-25 in HaCaT cells and in mouse primary keratinocytes through increasing TLR1/6 expression thereby activating TLR1/6 signaling-mediated NF- κ B signal pathway [47]. However, Der p did not increase the expression of IL-25 and TLR1/6 (Fig. S9A) and failed to activate NF- κ B [18] in NHEKs. The distinct reactions responsive to Der p might be due to the difference of keratinocytes used. We found the upregulation of TLR2 by Der p (Fig. S9A), however, which is not required for IL-33 production (Fig. S9B). Moreover, Der p-mediated PAR2 activation in keratinocyte is responsive for IL-8 transactivation but not involved in IL-33 upregulation (Fig. S10). Thus, HDM proteases stimulate IL-33 production from NHEKs via a *novus* signal pathway.

We show here that Der p-induced IL-33 expression is dependent on acute accumulation of extracellular ATP. Since nucleotides released from cells would be diluted in the culture supernatant and rapidly

metabolized, the ATP concentration at the surface of a neighboring cell after Der p stimulation is likely to be substantially higher than that detected by ATP assay of the culture supernatant. We speculate that sufficient extracellular ATP was released to activate P2 purinergic receptors of the neighboring keratinocytes after Der p stimulation. P2Y receptors often signal into cells through G-coupled proteins, while P2X receptors usually act as ligand-gated cation channels, mediating the influx and efflux of various cations. Epidermal keratinocytes functionally express most P2X and P2Y receptors [32], and several P2Y receptors have been showed to produce inflammatory molecules from keratinocytes [33,48,49]. Through treating cells with various specific P2 antagonists and knocking down P2Y2 expression in NHEKs, we have identified that P2Y receptors are important for IL33 induction by HDM, among which P2Y2 receptor is essential. In addition, P2X7 is also involved in the upregulation of IL-33 by Der p. Compared to other P2 receptors, P2X7 has unique characteristics [50,51]. It not only participates in intracellular inflammatory signaling, but also facilitates ATP release from cells through interaction with membrane hemichannels [33]. Signal activation of P2X7 requires millimolar levels of extracellular ATP, much higher than the detected ATP levels in the supernatants of Der p-treated cultures. We reasonably presumed that P2X7 contribute to IL-33 induction in Der p-activated NHEKs through facilitating ATP release but not directly activating intracellular signaling, as its role for the UVA-produced IL-6 in keratinocytes [33]. Thus, the

extracellular ATP released from Der p-activated NHEKs induces IL-33 expression through binding to P2 receptors (particularly P2Y2).

EGFR participates in cytokines-induced transcriptional activation of IL-33 in keratinocyte [11,12]. We identified the extracellular ATP/P2Y2 signaling-dependent EGFR/ERK activation in Der p-treated NHEKs, which triggers IL-33 synthesis. Knockdown of P2Y2 did not completely block Der p-induced EGFR activation and IL-33 induction (Fig. 3). This may be due to partial expression of P2Y2 in P2Y2 siRNA-transfected cells (Fig. 2E) and other P2Y receptors' possible contributions to EGFR activation [34] and IL-33 expression (Fig. 2D). In the downstream of P2Y2 receptor, ligand-independent pathway can initiate EGFR activation via Src-mediated phosphorylation of EGFR-tyr845 in keratinocytes [52], which seems to be not responsible for the activation of EGFR by Der p on account that no phospho-EGFR-tyr845 was detected in NHEKs (data not shown). However, we detected EGFR-tyr1068 phosphorylation and identified EGFR internalization in activated NHEKs, suggesting the involvement of free EGFR ligands. Using a broad-spectrum inhibitor of MMPs (batimastat) and an EGFR neutralizing antibody, we further demonstrated the role of MMPs-dependent shedding of EGFR ligands for Der p-induced IL-33 expression. Among MMPs, TACE, also called ADAM17 (ADAM metallopeptidase domain 17), is preferentially involved in the shedding of EGFR ligands in various cells, resulting in EGFR transactivation [35,53], and has been showed to involve various skin inflammation [37,54]. We found that Der p rapidly stimulated ATP/P2 signaling-dependent TACE activation, and knockdown of TACE suppressed the activation of EGF/ERK and the induction of IL-33 caused by Der p (Fig. S11). These data suggest that TACE is a major MMP involved in Der p-stimulated keratinocyte immune response, and TACE may promote AD development by regulating IL-33 expression. We noticed that blocking MMP activation by bamastat did not completely prevent Der p-induced ERK activation and IL-33 induction (Fig. 3), which may be due to the partial activation of ERK relying on an increase in $[Ca^{2+}]_i$ rather than EGFR activation (Fig. S12) [55]. Elevated HB-EGF has been detected in the skin of AD patients [56], suggesting a role of HB-EGF in the pathogenesis of AD. In Der p-treated NHEKs, HB-EGF is at least one of the EGFR ligands that MMPs cleaved and released, and participates in IL-33 expression. Thus, in addition to NLRP3 [18] and PAR2 [20], nucleotide receptor P2Y2 plays active roles in HDM-mediated keratinocyte immune response through activating TMPs-mediated EGFR/ERK signal pathways and upregulating IL-33 expression.

Circulating IL-33, which can trigger or exacerbate skin inflammation through amplifying Th2 immune response, is highly detected in AD patients [10]. In this study, in addition to increased IL-33 expression, we identified rapid IL-33 release from NHEKs after Der p stimulation. By knocking down IL-33 or blocking Der p protease activity, we confirmed that Der p-derived proteases rapidly stimulate IL-33 release from keratinocytes even within 10 min (Figs. S4A and B). IL-1 family members are released through unconventional secretory mechanisms, and IL-1 β and IL-18 residue in the cytosol and are cleaved and released in an inflammasome-dependent manner; however, IL-33 is constitutively expressed in the nucleus of epithelial cells and can be released into the extracellular space after cell damage [5]. A disrupted skin barrier and cutaneous microtrauma, both of which can lead to cell necrosis, are believed to result in IL-33 release in patients with AD and psoriasis [8–10]. No cell damage was detected in Der p-treated NHEKs and in airborne *Alternaria*-stimulated airway epithelial cells, which release IL-33 via extracellular ATP/P2 activation-driven sustained concentration of $[Ca^{2+}]_i$ [40]. Extracellular ATP/P2 signaling-mediated IL-33 release has been also detected in corneal epithelial cells [57] and in astrocytes [58]. We demonstrate here that extracellular ATP/P2 signaling-mediated $[Ca^{2+}]_i$ mobilization prompts Der p-activated NHEKs to rapidly release stored IL-33. One recent study has reported that Der p-mediated EGFR/ERK signal activation not only induces IL-33 expression but also stimulates IL-33 release from the airway epithelia [59]. And another group has shown that Der p stimulates IL-33

production from HaCaT cells [47]. They both analyzed extracellular IL-33 by ELISA, showing the extracellular release of IL-33 from Der p-treated cells is dependent on its intracellular induction [59]. We evaluated the levels of extracellular IL-33 by immunoblotting of supernatants, and found that neither the inhibition of EGFR/ERK signaling pathway nor the blocking of MMP activation can effectively prevent the rapid release of IL-33 caused by Der p (Fig. S4C). Although we cannot completely exclude the involvement of certain cellular molecules or enzymes in Der p-mediated secretion and processing of IL-33, MMPs and MMPs-dependent activation of EGFR/ERK is not required. In this study, we have also analyzed IL-33 release using a commercial IL-33 ELISA kit (R&D) and detected high “IL-33” not only in the supernatants of Der p-treated cultures but also in the Der p-added medium (without cells) (Fig. S13A), suggesting that Der p itself interferes with this reaction. The ELISA kit detected different sizes of IL-33, but the reaction was most sensitive to the 18-kDa form (the size of recombinant human IL-33) and less sensitive to the 25-kDa form (Fig. S7B). Therefore, this commercial IL-33 ELISA kit cannot be used to detect extracellular IL-33 in our system. These data suggest that HDM extract stimulate IL-33 release from different cells via specific mechanisms.

In this study, we not only detect IL-33 production from NHEKs but also verify its processing and maturation by HDM. Several intracellular and extracellular proteases can regulate the processing and maturation of IL-33. Apoptotic caspases process IL-33 and produce an inactive 6.5-kDa band [60], and calpains, which are Ca-dependent intracellular cysteine proteases, cleave full-length IL-33 into a 20-kDa band in IFN- γ /TNF- α -stimulated keratinocytes [11]. During inflammation, several serine proteases secreted from activated neutrophils [61] or activated mast cells [62] have been reported to cleave IL-33 within central domain and generate different mature IL-33 isoforms (sized as 18–25/26-kDa) with 10–30 fold increased bioactivity [62]. Scratching stimulated the production of ~25-kDa IL-33, indicating that some intracellular proteases process IL-33. Full-length IL-33 associates with chromatin through a short chromatin-binding motif, which is easily cleaved during cell damage and stress [62], producing a band of about ~25-kDa. Scratching might cause the cleavage of IL-33 nuclear domain. Although we cannot figure out or exclude their role in the processing of IL-33 caused by scraping, intracellular proteases do not appear to be involved in the cleavage of IL-33 by HDM. Because no additional intracellular IL-33 bands can be detected in Der p-treated keratinocytes compared to Der p-untreated cells (Fig. 4A). To date, the identified IL-33 intracellular proteases produce the bands below 20-kDa, and most are inactive [5], whereas Der p releases bioactive 25-kDa IL-33 from keratinocytes (Fig. 7E). According to one recent publication, environmental allergens (including HDM serine and cysteine proteases) can cleave recombinant human pro-IL-33 to produce mature bands that can be detected after 5–10 min of incubation [4]. We clearly demonstrate the processing of mite proteases on extracellular IL-33 (bigger than 25-kDa) released from thapsigargin-activated cells and scraped cells (Fig. 7). Ten minutes after interacting Der p with the culture supernatants collected from scraped cells, the newly produced 25-kDa IL-33 can be detected (Fig. 7C). We also verified the proteolytic maturation of IL-33 by HDM-derived proteases. Several environmental protease allergens can induce airway allergic inflammation through proteolytic maturation of IL-33 [5]. Using rhST2, a soluble decoy receptor that specifically blocks the binding of free IL-33 to transmembrane ST2, we verified the biological activity of IL-33 released from Der p-treated NHEKs (Fig. 7E). Confirmed, mite proteases can cause the processing and proteolytic maturation of epidermal IL-33.

In vivo, epidermal IL-33 is regulated by various inflammatory molecules associated with AD. The induction of IL-33 by cytokines failed to stimulate its secretion, however, it could contribute to IL-33 release caused by HDM exposure and cell damage. Similar to IL-1 α , IL-33 is a dual-function protein, displaying both nuclear effects (as a regulator of transcription factor) and extracellular effects (as an inflammatory cytokine) [11,63]. Nuclear expression of IL-33 in keratinocytes not only

suppresses inflammation [11] but also promotes wound healing [64] by blocking NF- κ B signal. That Th1 and Th17 increase IL-33 expression in keratinocytes [11,12] could explain the elevated IL-33 levels in chronic lesions of AD [9,46], which may play a negative role in the progression of dermatitis via suppressing inflammation and promoting tissue repair. However, upon exposure to mite allergens or mechanical injury (scratching behavior), nuclear IL-33 could be released from epidermis to promote the transition from Th1 to Th2 inflammation by activating ST2⁺ cells and to affect skin barrier homeostasis by downregulating filaggrin expression in keratinocytes, resulting the exacerbation of AD [9,10]. Therefore, the contribution of IL-33 to skin inflammation is decided by a distinct microenvironment involving various cytokines and environmental stimuli.

5. Conclusion

The defective barrier of AD skin can facilitate environmental allergens to directly stimulate epidermal keratinocytes to initiate immune responses. Our findings demonstrate that HDM allergens not only induce the expression and release of pro-allergic cytokine IL-33 from epidermal keratinocytes but also increase IL-33 activity, which may trigger or exacerbate AD. The role of extracellular ATP/P2 signaling in the innate production of IL-33 demonstrates that nucleotides could contribute to skin inflammation via autocrine or paracrine signaling.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank Teruko Tsuda, Eriko Tan and Erika Takamasa for excellent technical assistance. We thank JSPS KAKENHI Grant Number JP18K09485 for the funding.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbdis.2020.165719>.

References

- [1] T. Bieber, Atopic dermatitis, *Ann. Dermatol.* 22 (2010) 125–137.
- [2] E.B. Brandt, U. Sivaprasad, Th2 cytokines and atopic dermatitis, *Journal of Clinical & Cellular Immunology* 2 (2011).
- [3] C. Cayrol, J.P. Girard, Interleukin-33 (IL-33): a nuclear cytokine from the IL-1 family, *Immunol. Rev.* 281 (2018) 154–168.
- [4] C. Cayrol, A. Duval, P. Schmitt, S. Roga, M. Camus, A. Stella, O. Burlet-Schiltz, A. Gonzalez-de-Peredo, J.P. Girard, Environmental allergens induce allergic inflammation through proteolytic maturation of IL-33, *Nat. Immunol.* 19 (2018) 375–385.
- [5] E. Lefrancais, C. Cayrol, Mechanisms of IL-33 processing and secretion: differences and similarities between IL-1 family members, *Eur. Cytokine Netw.* 23 (2012) 120–127.
- [6] Y. Imai, Interleukin-33 in atopic dermatitis, *J. Dermatol. Sci.* 96 (2019) 2–7.
- [7] R. Saluja, M.E. Ketelaar, T. Hawro, M.K. Church, M. Maurer, M.C. Nawijn, The role of the IL-33/IL-1RL1 axis in mast cell and basophil activation in allergic disorders, *Mol. Immunol.* 63 (2015) 80–85.
- [8] T. Nabe, Interleukin (IL)-33: new therapeutic target for atopic diseases, *J. Pharmacol. Sci.* 126 (2014) 85–91.
- [9] A. Balato, A. Raimondo, N. Balato, F. Ayala, S. Lembo, Interleukin-33: increasing role in dermatological conditions, *Arch. Dermatol. Res.* 308 (2016) 287–296.
- [10] R. Tamagawa-Mineoka, Y. Okuzawa, K. Masuda, N. Katoh, Increased serum levels of interleukin 33 in patients with atopic dermatitis, *J. Am. Acad. Dermatol.* 70 (2014) 882–888.
- [11] J. Meephansan, H. Tsuda, M. Komine, S. Tominaga, M. Ohtsuki, Regulation of IL-33 expression by IFN- γ and tumor necrosis factor- α in normal human epidermal keratinocytes, *J Invest Dermatol* 132 (2012) 2593–2600.
- [12] J. Meephansan, M. Komine, H. Tsuda, M. Karakawa, S. Tominaga, M. Ohtsuki, Expression of IL-33 in the epidermis: the mechanism of induction by IL-17, *J. Dermatol. Sci.* 71 (2013) 107–114.
- [13] A. Balato, R. Di Caprio, L. Cantà, M. Matti, S. Lembo, A. Raimondo, M. Schiattarella, N. Balato, F. Ayala, IL-33 is regulated by TNF- α in normal and psoriatic skin, *Arch. Dermatol. Res.* 306 (2014) 299–304.
- [14] B.B. Tan, D. Weald, I. Strickland, P.S. Friedmann, Double-blind controlled trial of effect of housedust-mite allergen avoidance on atopic dermatitis, *Lancet.* 347 (1996) 15–18.
- [15] E. Ridolo, I. Martignago, G.G. Riario-Sforza, C. Incorvaia, Allergen immunotherapy in atopic dermatitis, *Expert. Rev. Clin. Immunol.* 14 (2018) 61–68.
- [16] W.R. Thomas, W.A. Smith, B.J. Hales, K.L. Mills, R.M. O'Brien, Characterization and immunobiology of house dust mite allergens, *Int. Arch. Allergy Immunol.* 129 (2002) 1–18.
- [17] R.J. Simpson, E.C. Nice, R.L. Moritz, G.A. Stewart, Structural studies on the allergen Der p1 from the house dust mite *Dermatophagoides pteronyssinus*: similarity with cysteine proteinases, *Protein Seq Data Anal.* 2 (1989) 17–21.
- [18] X. Dai, K. Sayama, M. Tohyama, Y. Shirakata, Y. Hanakawa, S. Tokumaru, L. Yang, S. Hirakawa, K. Hashimoto, Mite allergen is a danger signal for the skin via activation of inflammasome in keratinocytes, *The Journal of Allergy and Clinical Immunology*, 127 806–814 (2011) e801–e804.
- [19] Y.L. Chiou, C.Y. Lin, Der p2 activates airway smooth muscle cells in a TLR2/MyD88-dependent manner to induce an inflammatory response, *J. Cell. Physiol.* 220 (2009) 311–318.
- [20] T. Kato, T. Takai, T. Fujimura, H. Matsuoka, T. Ogawa, K. Murayama, A. Ishii, S. Ikeda, K. Okumura, H. Ogawa, Mite serine protease activates protease-activated receptor-2 and induces cytokine release in human keratinocytes, *Allergy.*, 64 (2009) 1366–1374 Epub 2009 Mar 1323.
- [21] K. Oboki, T. Ohno, N. Kajiura, K. Arae, H. Morita, A. Ishii, A. Nambu, T. Abe, H. Kiyonari, K. Matsumoto, K. Sudo, K. Okumura, H. Saito, S. Nakae, IL-33 is a crucial amplifier of innate rather than acquired immunity, *Proc. Natl. Acad. Sci. U. S. A.* 107 (2010) 18581–18586.
- [22] C. Porsbjerg, K. Baines, P. Gibson, A. Bergqvist, J.S. Erjefelt, A. Sverrild, V. Backer, IL-33 is related to innate immune activation and sensitization to HDM in mild steroid-free asthma, *Clinical and Experimental Allergy: Journal of the British Society for Allergy and Clinical Immunology* 46 (2016) 564–574.
- [23] L. Yang, Y. Shirakata, K. Tamai, X. Dai, Y. Hanakawa, S. Tokumaru, Y. Yahata, M. Tohyama, K. Shiraishi, H. Nagai, X. Wang, S. Murakami, K. Sayama, Y. Kaneda, K. Hashimoto, Microbubble-enhanced ultrasound for gene transfer into living skin equivalents, *J. Dermatol. Sci.* 40 (2005) 105–114.
- [24] M. Tohyama, L. Yang, Y. Hanakawa, X. Dai, Y. Shirakata, K. Sayama, IFN- α enhances IL-22 receptor expression in keratinocytes: a possible role in the development of psoriasis, *J Invest Dermatol* 132 (2012) 1933–1935.
- [25] X. Dai, K. Sayama, K. Yamasaki, M. Tohyama, Y. Shirakata, Y. Hanakawa, S. Tokumaru, Y. Yahata, L. Yang, A. Yoshimura, K. Hashimoto, SOCS1-negative feedback of STAT1 activation is a key pathway in the dsRNA-induced innate immune response of human keratinocytes, *J Invest Dermatol.*, 126 (2006) 1574–1581 Epub 2006 Apr 1520.
- [26] S. Liu, T. Kiyoi, E. Takemasa, K. Maeyama, Systemic lentivirus-mediated delivery of short hairpin RNA targeting calcium release-activated calcium channel 3 as gene therapy for collagen-induced arthritis, *J. Immunol.* 194 (2015) 76–83.
- [27] H.F. Kauffman, M. Tamm, J.A. Timmerman, P. Borger, House dust mite major allergens Der p 1 and Der p 5 activate human airway-derived epithelial cells by protease-dependent and protease-independent mechanisms, *Clin Mol Allergy.* 4 (2006) 5.
- [28] J. Calven, H. Akbarshahi, M. Menzel, C.K. Ayata, M. Idzko, L. Bjermer, L. Uller, Rhinoviral stimuli, epithelial factors and ATP signalling contribute to bronchial smooth muscle production of IL-33, *J. Transl. Med.* 13 (2015) 281.
- [29] G. Paris, T. Pozharskaya, T. Asempta, A.P. Lane, Damage-associated molecular patterns stimulate interleukin-33 expression in nasal polyp epithelial cells, *International Forum Of Allergy & Rhinology* 4 (2014) 15–21.
- [30] S. Post, M.C. Nawijn, M.R. Jonker, N. Kliphuis, M. van den Berge, A.J. van Oosterhout, I.H. Heijink, House dust mite-induced calcium signaling instigates epithelial barrier dysfunction and CCL20 production, *Allergy* 68 (2013) 1117–1125.
- [31] M.P. Abbracchio, G. Burnstock, Purinoreceptors: are there families of P2X and P2Y purinoreceptors? *Pharmacol. Ther.* 64 (1994) 445–475.
- [32] K. Inoue, J. Hosoi, M. Denda, Extracellular ATP has stimulatory effects on the expression and release of IL-6 via purinergic receptors in normal human epidermal keratinocytes, *J Invest Dermatol* 127 (2007) 362–371.
- [33] A. Kawano, R. Kadomatsu, M. Ono, S. Kojima, M. Tsukimoto, H. Sakamoto, Autocrine regulation of UVA-induced IL-6 production via release of ATP and activation of P2Y receptors, *PLoS One* 10 (2015) e0127919.
- [34] Z. Wang, Transactivation of epidermal growth factor receptor by G protein-coupled receptors: recent progress, Challenges and Future Research, *International Journal of Molecular Sciences* 17 (2016).
- [35] I. Boucher, A. Kehasse, M. Marcincin, C. Rich, N. Rahimi, V. Trinkaus-Randall, Distinct activation of epidermal growth factor receptor by UTP contributes to epithelial cell wound repair, *Am. J. Pathol.* 178 (2011) 1092–1105.
- [36] S. Tokumaru, K. Sayama, Y. Shirakata, H. Komatsuzawa, K. Ouhara, Y. Hanakawa, Y. Yahata, X. Dai, M. Tohyama, H. Nagai, L. Yang, S. Higashiyama, A. Yoshimura, M. Sugai, K. Hashimoto, Induction of keratinocyte migration via transactivation of the epidermal growth factor receptor by the antimicrobial peptide LL-37, *J. Immunol.* 175 (2005) 4662–4668.
- [37] A. Sommer, A. Fries, I. Cornelsen, N. Speck, F. Koch-Nolte, G. Gimml, J. Andra, S. Bhakdi, K. Reiss, Melittin modulates keratinocyte function through P2 receptor-dependent ADAM activation, *J. Biol. Chem.* 287 (2012) 23678–23689.
- [38] S. Dateoka, Y. Ohnishi, K. Kakudo, Effects of CRM197, a specific inhibitor of HB-EGF, in oral cancer, *Medical molecular morphology* 45 (2012) 91–97.

- [39] C. Moussion, N. Ortega, J.P. Girard, The IL-1-like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells in vivo: a novel 'alarmin'? *PLoS One* 3 (2008) e3331.
- [40] H. Kouzaki, K. Iijima, T. Kobayashi, S.M. O'Grady, H. Kita, The danger signal, extracellular ATP, is a sensor for an airborne allergen and triggers IL-33 release and innate Th2-type responses, *J. Immunol.* 186 (2011) 4375–4387.
- [41] A. Jairaman, C.H. Maguire, R.P. Schleimer, M. Prakriya, Allergens stimulate store-operated calcium entry and cytokine production in airway epithelial cells, *Sci. Rep.* 6 (2016) 32311.
- [42] P. Li, H. Ma, D. Han, K. Mou, Interleukin-33 affects cytokine production by keratinocytes in vitiligo, *Clin. Exp. Dermatol.* 40 (2015) 163–170.
- [43] W.I. Ryu, H. Lee, J.H. Kim, H.C. Bae, H.J. Ryu, S.W. Son, IL-33 induces Egr-1-dependent TSLP expression via the MAPK pathways in human keratinocytes, *Exp. Dermatol.* 24 (2015) 857–863.
- [44] W.I. Ryu, H. Lee, H.C. Bae, H.J. Ryu, S.W. Son, IL-33 down-regulates filaggrin expression by inducing STAT3 and ERK phosphorylation in human keratinocytes, *J. Dermatol. Sci.* 82 (2016) 131–134.
- [45] C.A. Christianson, N.P. Goplen, I. Zafar, C. Irvin, J.T. Good Jr., D.R. Rollins, B. Gorenfla, W. Liu, M.M. Gorska, H. Chu, R.J. Martin, R. Alam, Persistence of asthma requires multiple feedback circuits involving type 2 innate lymphoid cells and IL-33, *J. Allergy Clin. Immunol.* 136 (2015) 59–68 (e14).
- [46] T. Savinko, S. Matikainen, U. Saarialho-Kere, M. Lehto, G. Wang, S. Lehtimäki, P. Karisola, T. Reunala, H. Wolff, A. Lauerma, H. Alenius, IL-33 and ST2 in atopic dermatitis: expression profiles and modulation by triggering factors, *J. Invest. Dermatol.* 132 (2012) 1392–1400.
- [47] Y.H. Jang, J.K. Choi, M. Jin, Y.-A. Choi, Z.Y. Ryoo, H.-S. Lee, P.-H. Park, S.-U. Kim, T.K. Kwon, M.H. Jang, S.-H. Im, S.Y. Moon, W.J. Lee, S.-J. Lee, D.W. Kim, S.-H. Kim, House dust mite increases pro-Th2 cytokines IL-25 and IL-33 via the activation of TLR1/6 signaling, *J. Investig. Dermatol.* 137 (2017) 2354–2361.
- [48] E. Takai, M. Tsukimoto, H. Harada, S. Kojima, Involvement of P2Y6 receptor in p38 MAPK-mediated COX-2 expression in response to UVB irradiation of human keratinocytes, *Radiat. Res.* 175 (2011) 358–366.
- [49] E. Satoh, M. Tsukimoto, S. Kojima, Involvement of P2Y receptors in the protective effect of ATP towards the cell damage in HaCaT cells exposed to H₂O₂, *J. Toxicol. Sci.* 36 (2011) 741–750.
- [50] B.S. Khakh, G. Burnstock, C. Kennedy, B.F. King, R.A. North, P. Seguela, M. Voigt, P.P. Humphrey, International union of pharmacology. XXIV. Current status of the nomenclature and properties of P2X receptors and their subunits, *Pharmacol. Rev.* 53 (2001) 107–118.
- [51] A. Baroja-Mazo, M. Barbera-Cremades, P. Pelegrin, The participation of plasma membrane hemichannels to purinergic signaling, *Biochim. Biophys. Acta* 1828 (2013) 79–93.
- [52] J. Liu, Z. Liao, J. Camden, K.D. Griffin, R.C. Garrad, L.I. Santiago-Perez, F.A. Gonzalez, C.I. Seye, G.A. Weisman, L. Erb, Src homology 3 binding sites in the P2Y2 nucleotide receptor interact with Src and regulate activities of Src, proline-rich tyrosine kinase 2, and growth factor receptors, *J. Biol. Chem.* 279 (2004) 8212–8218.
- [53] F. Cattaneo, G. Guerra, M. Parisi, M. De Marinis, D. Tafuri, M. Cinelli, R. Ammendola, Cell-surface receptors transactivation mediated by G protein-coupled receptors, *Int. J. Mol. Sci.* 15 (2014) 19700–19728.
- [54] M.C. Chang, C.P. Chan, Y.J. Chen, H.C. Hsien, Y.C. Chang, S.Y. Yeung, P.Y. Jeng, R.H. Cheng, L.J. Hahn, J.H. Jeng, Areca nut components stimulate ADAM17, IL-1 α , PGE2 and 8-isoprostane production in oral keratinocyte: role of reactive oxygen species, EGF and JAK Signaling, *Oncotarget* 7 (2016) 16879–16894.
- [55] Y.Y. He, J.L. Huang, C.F. Chignell, Delayed and sustained activation of extracellular signal-regulated kinase in human keratinocytes by UVA: implications in carcinogenesis, *J. Biol. Chem.* 279 (2004) 53867–53874.
- [56] J. Malaisse, V. Bourguignon, E. De Vuyst, C. Lambert de Rouvroit, A.F. Nikkels, B. Flamion, Y. Poumay, Hyaluronan metabolism in human keratinocytes and atopic dermatitis skin is driven by a balance of hyaluronan synthases 1 and 3, *J. Invest. Dermatol.* 134 (2014) 2174–2182.
- [57] L. Zhang, R. Lu, G. Zhao, S.C. Pflugfelder, D.Q. Li, TLR-mediated induction of pro-allergic cytokine IL-33 in ocular mucosal epithelium, *Int. J. Biochem. Cell Biol.* 43 (2011) 1383–1391.
- [58] C.A. Hudson, G.P. Christophi, R.C. Gruber, J.R. Wilmore, D.A. Lawrence, P.T. Massa, Induction of IL-33 expression and activity in central nervous system glia, *J. Leukoc. Biol.* 84 (2008) 631–643.
- [59] M. Hristova, A. Habibovic, C. Veith, Y.M. Janssen-Heininger, A.E. Dixon, M. Geiszt, A. van der Vliet, Airway epithelial dual oxidase 1 mediates allergen-induced IL-33 secretion and activation of type 2 immune responses, *The Journal of Allergy and Clinical Immunology*, 137 e1511 (2016) 1545–1556.
- [60] A.U. Luthi, S.P. Cullen, E.A. McNeela, P.J. Duriez, I.S. Afonina, C. Sheridan, G. Brumatti, R.C. Taylor, K. Kersse, P. Vandenabeele, E.C. Lavelle, S.J. Martin, Suppression of interleukin-33 bioactivity through proteolysis by apoptotic caspases, *Immunity* 31 (2009) 84–98.
- [61] E. Lefrancais, S. Roga, V. Gautier, A. Gonzalez-de-Peredo, B. Monsarrat, J.P. Girard, C. Cayrol, IL-33 is processed into mature bioactive forms by neutrophil elastase and cathepsin G, *Proc. Natl. Acad. Sci. U. S. A.* 109 (2012) 1673–1678.
- [62] E. Lefrancais, A. Duval, E. Mirey, S. Roga, E. Espinosa, C. Cayrol, J.P. Girard, Central domain of IL-33 is cleaved by mast cell proteases for potent activation of group-2 innate lymphoid cells, *Proc. Natl. Acad. Sci. U. S. A.* 111 (2014) 15502–15507.
- [63] V. Carriere, L. Roussel, N. Ortega, D.A. Lacorre, L. Americh, L. Aguilar, G. Bouche, J.P. Girard, IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo, *Proc. Natl. Acad. Sci. U. S. A.* 104 (2007) 282–287.
- [64] T. Oshio, M. Komine, H. Tsuda, S.I. Tominaga, H. Saito, S. Nakae, M. Ohtsuki, Nuclear expression of IL-33 in epidermal keratinocytes promotes wound healing in mice, *J. Dermatol. Sci.* 85 (2017) 106–114.

House dust mite allergens induce interleukin 33 (IL-33) synthesis and release from keratinocytes via ATP-mediated extracellular signaling

Xiuju Dai^{a¶}, Mikiko Tohyama^b, Masamoto Murakami^a, Ken Shiraishi^a, Shuang Liu^c, Hideki Mori^a, Ryo Utsunomiya^a, Kazutaka Maeyama^c, and Koji Sayama^a

^aDepartment of Dermatology, Ehime University Graduate School of Medicine, Ehime, Japan

^bDepartment of Dermatology, National Hospital Organization Shikoku Cancer Center, Ehime, Japan

^cDepartment of Pharmacology, Ehime University Graduate School of Medicine, Ehime, Japan

¶To whom correspondence should be addressed: Xiuju Dai, Department of Dermatology, Ehime University Graduate School of Medicine, Ehime 791-0295, Japan
E-mail: daixiuju@m.ehime-u.ac.jp; Tel: +81 (89) 960-5350; Fax: +81 (89) 960-5352

Table S1

Names and description of reagents, inhibitors and antagonists	Provider
Standardized lyophilized extracts of Der p	LSL (Tokyo, Japan)
Thapsigargin (an inhibitor of Ca ²⁺ pump proteins)	Wako (Tokyo, Japan)
Affinity chromatography–purified natural Der p 1	Indoor Biotechnologies (Cardiff, United Kingdom)
recombinant allergen Der p 2	Indoor Biotechnologies (Cardiff, United Kingdom)
natural DPSP (Der p Serine Proteases)	Indoor Biotechnologies (Cardiff, United Kingdom)
ATP- γ -S (a relatively stable ATP analog)	Sigma-Aldrich (Saint Louis, Missouri, USA)
suramin (a broad-spectrum P2 antagonist)	Sigma-Aldrich (Saint Louis, Missouri, USA)
Apyrase (a highly active ATP-diphosphohydrolase)	Sigma-Aldrich (Saint Louis, Missouri, USA)
ARL67156 (an ecto-ATPase inhibitor)	Sigma-Aldrich (Saint Louis, Missouri, USA)
ABESF (serine protease inhibitor)	Sigma-Aldrich (Saint Louis, Missouri, USA)
E-64 (cysteine protease inhibitor)	Sigma-Aldrich (Saint Louis, Missouri, USA)
<i>PAR-2-AP</i> (a PAR-2 activating peptide)	Sigma-Aldrich (Saint Louis, Missouri, USA)
BAPTA-AM (an intracellular calcium chelator)	Sigma-Aldrich (Saint Louis, Missouri, USA)
U73122 (a pharmacological inhibitor of PLC)	Sigma-Aldrich (Saint Louis, Missouri, USA)
1% Nonidet P-40 ((NP-40))	Sigma-Aldrich (Saint Louis, Missouri, USA)
NF157 (a potent P2Y ₁₁ receptor antagonist)	Tocris Bioscience (Bristol, United Kingdom)
MRS2179 (a specific P2Y ₁ receptor antagonist)	Tocris Bioscience (Bristol, United Kingdom)
AZ10606120 (a potent P2X ₇ receptor antagonist)	Tocris Bioscience (Bristol, United Kingdom)
AR-C118925XX (a specific P2Y ₂ receptor antagonist)	Tocris Bioscience (Bristol, United Kingdom)
AG 1478 (a specific EGFR inhibitor)	Calbiochem (Tokyo, Japan)
Batimastat (a broad-spectrum MMP inhibitor)	Calbiochem (Tokyo, Japan)
CMR197 (a nontoxic diphtheria toxin mutant)	Calbiochem (Tokyo, Japan)
U0126 (a specific MEK/ERK inhibitor)	R&D Systems (Minneapolis, MN, USA)
recombinant human HB-EGF	R&D Systems (Minneapolis, MN, USA)
recombinant human IL-33	R&D Systems (Minneapolis, MN, USA)
recombinant human ST2/IL-33R Fc chimera protein	R&D Systems (Minneapolis, MN, USA)
anti-EGFR (GR01, a neutralizing EGFR antibody)	R&D Systems (Minneapolis, MN, USA)

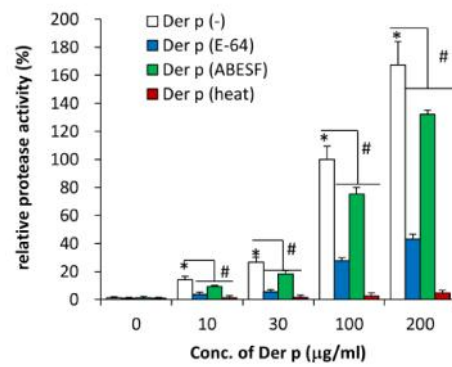
Supplementary Table 1 (Table S1). List of reagents, inhibitors and antagonists used for experiments

Table S2

Name of antibodies	Catalog Number	Provider
p44/42 MAPK (Erk1/2)	#9102	cell signaling (Danvers, MA, USA)
Phospho-p44/42 MAPK (Erk1/2)	#9101	cell signaling (Danvers, MA, USA)
Phospho-EGF Receptor (Tyr1068)	#3777	cell signaling (Danvers, MA, USA)
Phospho-EGF Receptor (Tyr845)	#6963	cell signaling (Danvers, MA, USA)
P2Y2	SC-518019	Santa Cruz Biotechnology (Dallas, TX, USA)
EGFR	SC-373746	Santa Cruz Biotechnology (Dallas, TX, USA)
β -actin	SC-37778	Santa Cruz Biotechnology (Dallas, TX, USA)
IL-33	PM033	MBL (Tokyo, Japan)
TACE	SC-390859	Santa Cruz Biotechnology (Dallas, TX, USA)

Supplementary Table 2 (Table S2). List of primary antibodies used for immunoblotting and immunostaining in experiments

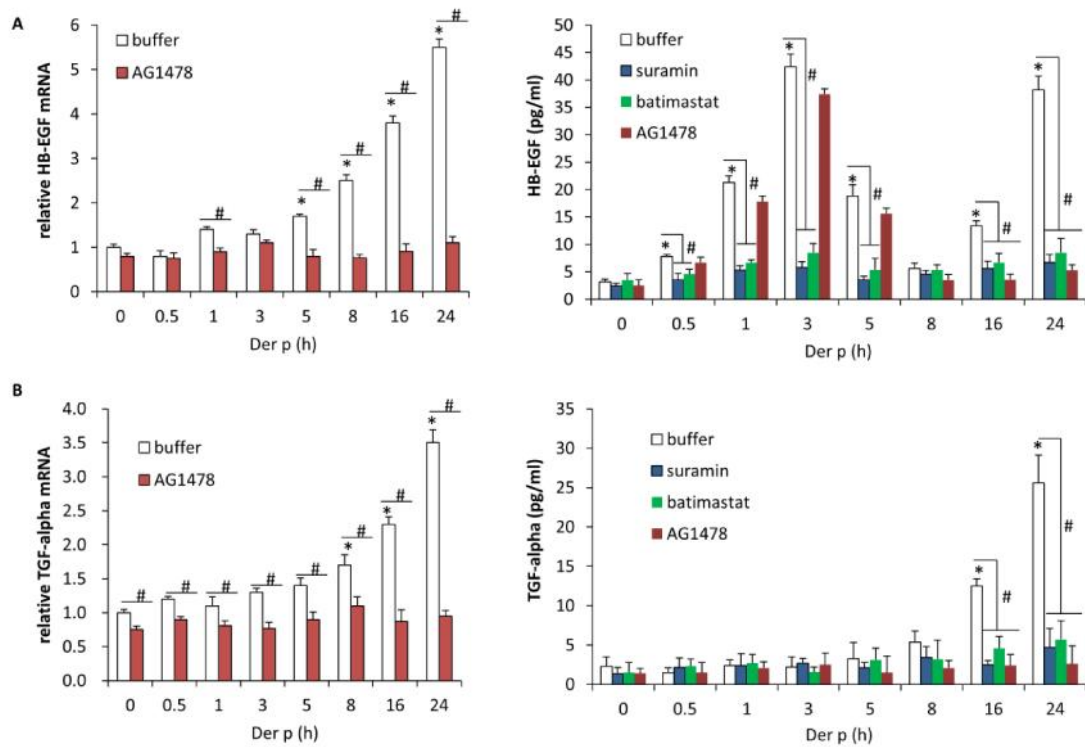
Fig. S1



Supplemental Figure 1 (Fig. S1). Der p shows cysteine and serine protease activity.

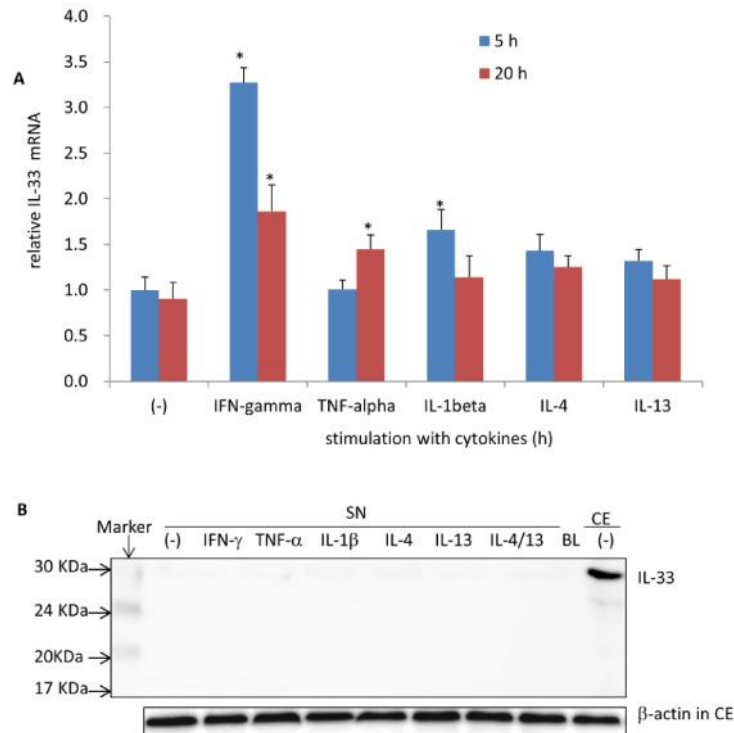
Different concentration of Der p (after purification) were prepared, pretreated with E-64 (8 µg/ml) or ABESF (100 µM) for 15 min, or heated for 30 min; then the protease activity of Der p solution was evaluated using Protease Activity Assay Kit. Relative protease activity of Der p solution was present as means ± SDs ($n = 4$). (# and * $p < 0.05$)

Fig. S2



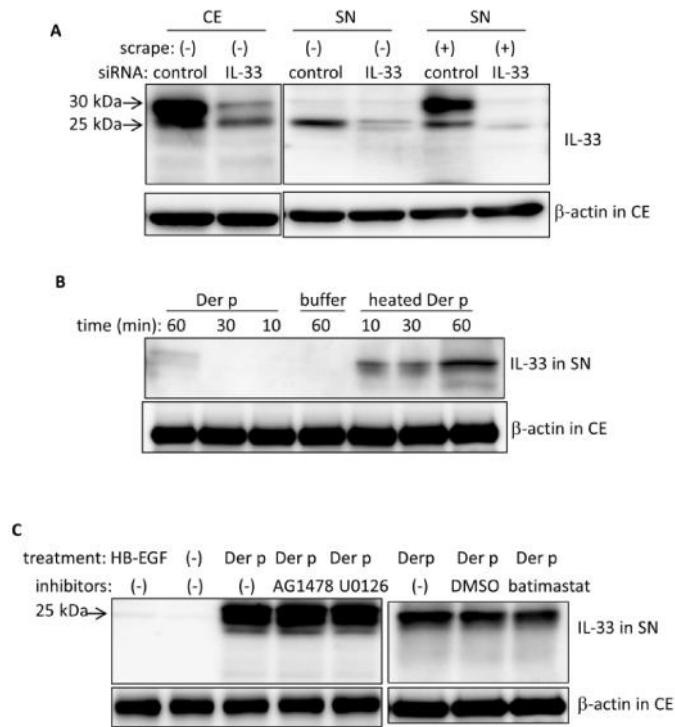
Supplemental Figure 2 (Fig. S2). Der p stimulates MMPs-dependent HB-EGF (not TGF- α) shedding and release through activating ATP signaling. (A and B) Keratinocytes were pretreated with inhibitors for 1 h then stimulated with Der p for the indicated times. The mRNA levels and the supernatant protein levels of HB-EGF and TGF- α were respectively examined. Relative mRNA levels and protein levels are expressed as means $\pm$ SDs ($n = 3$). ([#] and ^{*} $p < 0.05$)

Fig. S3



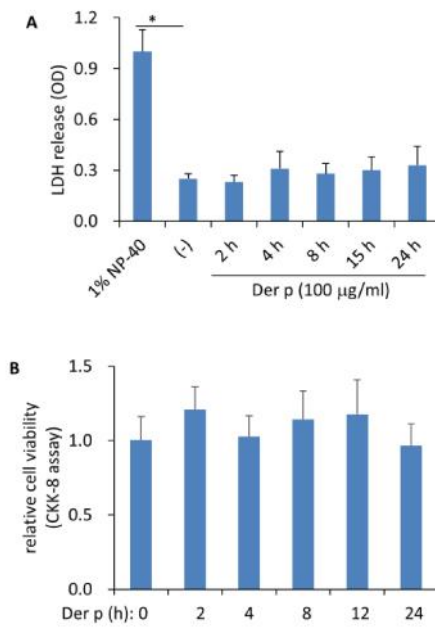
Supplemental Figure 3 (Fig. S3). No cytokines stimulate the release of IL-33 from keratinocytes. **(A)** NHEKs were treated with IFN- γ (100 ng/ml), THF- α (100 ng/ml), IL-1 β (30 ng/ml), IL-4 (100 ng/ml), and IL-13 (100 ng/ml) (All the recombinant human cytokines were obtained from R&D) for the indicated times, and the IL-33 mRNA levels were determined by real-time PCR. Relative mRNA levels are expressed as means $\pm$ SDs ($n = 3$). (* $p < 0.05$) **(B)** NHEKs were treated with IFN- γ , THF- α , IL-1 β , IL-4, IL-13 or IL-4 plus IL-13 for 12 h, and supernatants were collected. The concentrated supernatants were loaded by SDS-PAGE together with cell extracts collected from control cells, and the levels of IL-33 protein were detected by Western blotting. (SN: supernatant; CE: cell extract)

Fig. S4



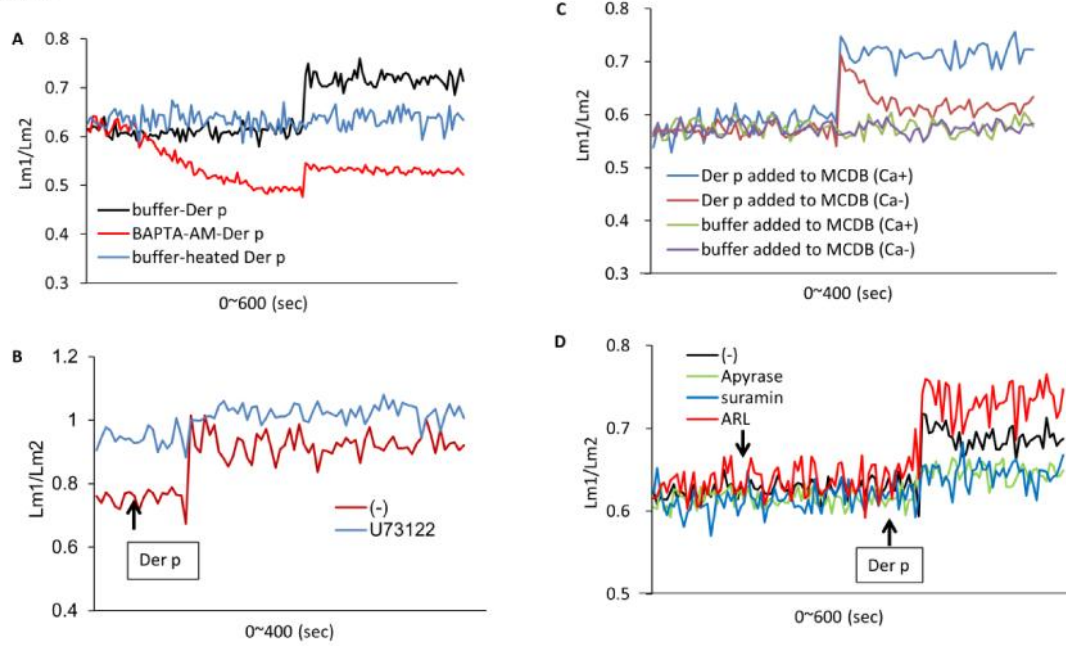
Supplemental Figure 4 (Fig. S4). Der p-derived proteases mediated IL-33 release, which not is dependent on the activation of MMPs, EGFR and ERK. **(A)** Keratinocytes were transfected with control or IL-33 siRNA and treated with Der p for 10 min, then the cells were scraped or not. The levels of IL-33 protein in cell extract and cell supernatants were respectively detected. **(B)** Keratinocytes were treated with Der p or heated-Der p for 10, 30, or 60 min. The supernatants were collected and IL-33 protein levels were evaluated. **(C)** Keratinocytes were pretreated with inhibitor or not, then stimulated with HB-EGF or Der p for 2 h. The supernatants were collected and IL-33 protein levels were evaluated. (SN: supernatant; CE: cell extract)

Fig. S5



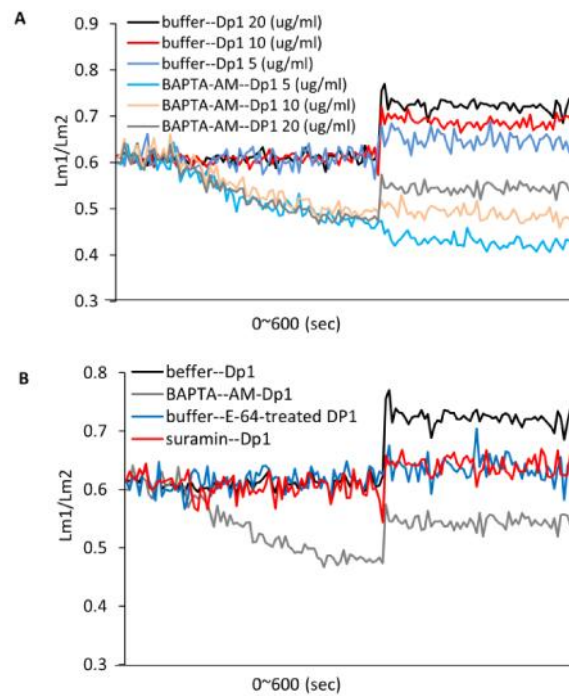
Supplemental Figure 5 (Fig. S5). Cell damage is not detected in Der p-treated NHEKs **(A)** NHEKs were exposed to Der p for the indicated times, or treated with 1% NP-40 for 5 min, and the supernatant was subjected to a LDH cytotoxicity assay. **(B)** NHEKs were exposed to Der p for the indicated times and CKK-8 assay were performed to evaluated the cell viability. ($n = 5$; * $p < 0.05$)

Fig. S6



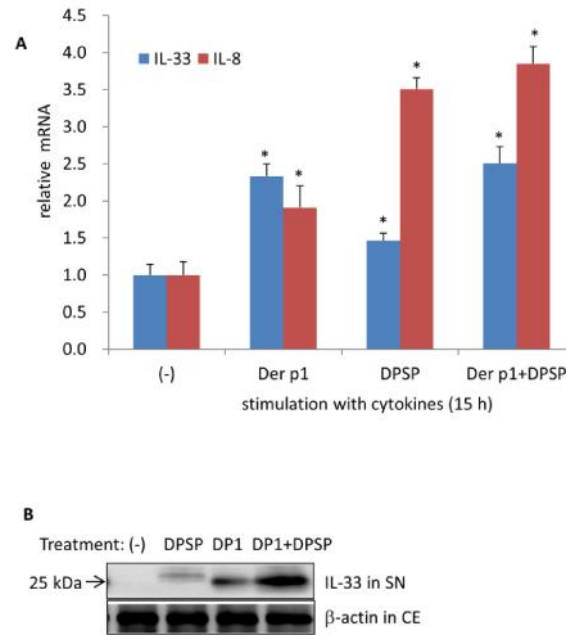
Supplemental Figure 6 (Fig. S6). Der p stimulates $[Ca^{2+}]_i$ increase through activating ATP/P2 signal pathway. **(A)** After Fura-2 loading, NHEKs were pretreated with buffer or BAPTA-AM (20 μ M) for 5 min and then stimulated with Der p or heated Der p. Mobilization of $[Ca^{2+}]_i$ was detected by a $[Ca^{2+}]_i$ mobilization assay. **(B)** After Fura-2 loading, NHEKs were pretreated with or without U73122 for 15 min and then stimulated with Der p. $[Ca^{2+}]_i$ mobilization in U73122-treated and U73122-untreated cells was respectively detected. **(C)** Keratinocytes were cultured in MCDB (-) with or without calcium ions (Ca- or Ca+) just before Fura-2 loading. Der p or buffer were respectively applied into the cultures, and the mobilization of $[Ca^{2+}]_i$ was detected by a $[Ca^{2+}]_i$ mobilization assay. **(D)** After Fura-2 loading, NHEKs were pretreated with buffer, apyrase (40 unit/ml), suramin (100 μ M) or ARL67156 (ARL, 100 μ M) for 5 min and then stimulated with Der p. A $[Ca^{2+}]_i$ mobilization assay was performed.

Fig. S7



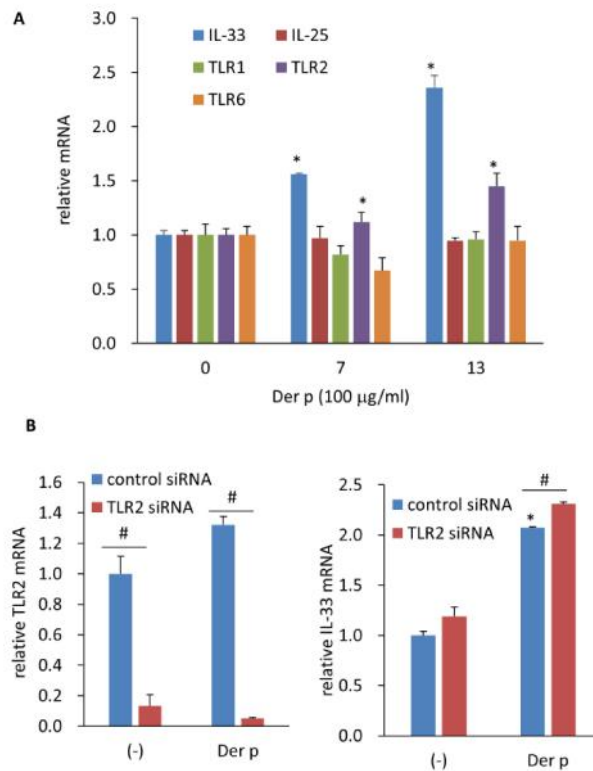
Supplemental Figure 7 (Fig. S7). Der p1 stimulates $[Ca^{2+}]_i$ increase through activating ATP/P2 signal pathway. **(A)** After Fura-2 loading, NHEKs were treated with buffer or BAPTA-AM (20 μ M) and then with different concentrations of Der p1. Mobilization of $[Ca^{2+}]_i$ was detected by a $[Ca^{2+}]_i$ mobilization assay. **(B)** After Fura-2 loading, NHEKs were pretreated with buffer, BAPTA-AM or suramin and then stimulated with Der p1 (20 μ g/ml) or E-64-treated Der p1. Mobilization of $[Ca^{2+}]_i$ was detected by a $[Ca^{2+}]_i$ mobilization assay. (Dp1: Der p1)

Fig. S8

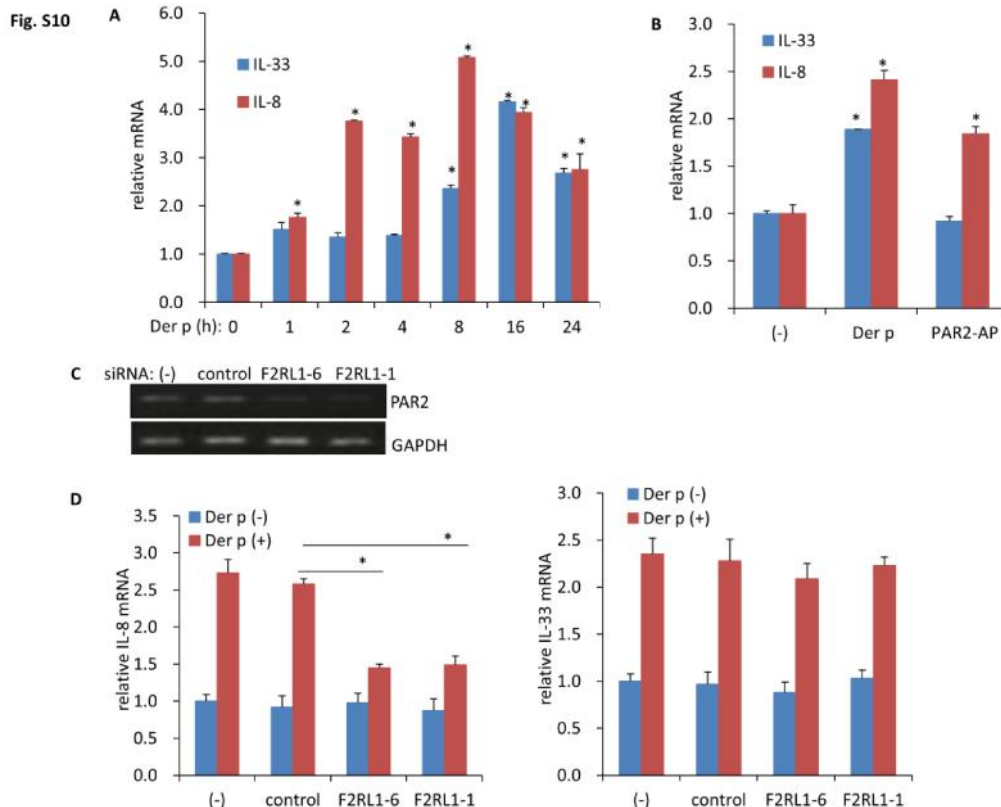


Supplemental Figure 8 (Fig. S8). DPSP slightly induces IL-33 expression and synergistically increases the levels of Der p1-released extracellular IL-33 from keratinocytes. **(A)** NHEKs were treated with buffer, Der p1 (10 µg/ml), DPSP (10 µg/ml), or Der p1 (10 µg/ml) together with DPSP (10 µg/ml) for 12 h, and the mRNA levels IL-33 and IL-8 were determined by real-time PCR. Relative mRNA levels are expressed as means $\pm$ SDs ($n = 3$). (* $p < 0.05$) **(B)** NHEKs were treated with buffer, Der p1 (DP1; 10 µg/ml), DPSP (10 µg/ml), or DP1 (DP1; 10 µg/ml) together with DPSP (10 µg/ml) for 2 h, and supernatants were collected. The concentrated supernatants were loaded by SDS-PAGE, and the supernatant levels of IL-33 protein were evaluated. (SN: supernatant; CE: cell extract)

Fig. S9

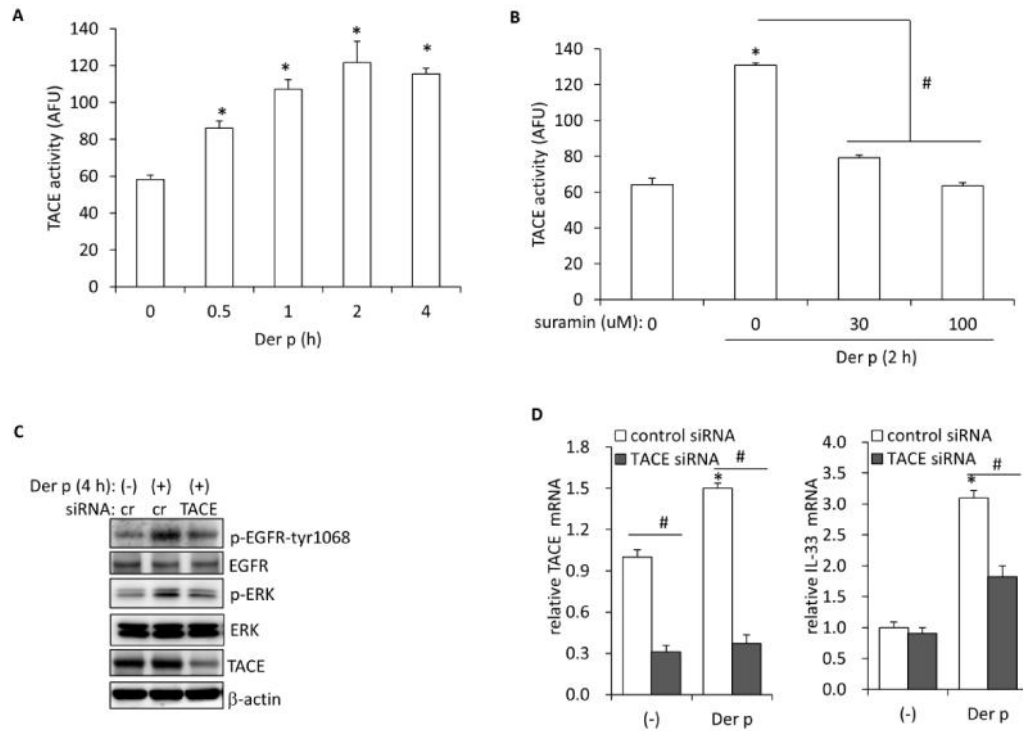


Supplemental Figure 9 (Fig. S9). No upregulation of IL-25 and TLR1/6 was detected in Der p-treated NHEKs; and TLR2 signal could not stimulate the production of IL-33. **(A)** NHEKs were treated with Der p for 0, 7 or 13 h, and the expression of IL-33, IL-25, TLR1, TLR2, and TLR6 mRNA was detected by real-time RT-PCR. **(B)** NHEKs were transfected with control or TLR2 siRNA for 48 h then simulated with Der p for 12 h, and the mRNA levels of TLR2 and IL-33 were evaluated by real-time RT-PCR. The relative mRNA levels are expressed as means $\pm$ SDs ($n = 3$). (* $p < 0.05$)



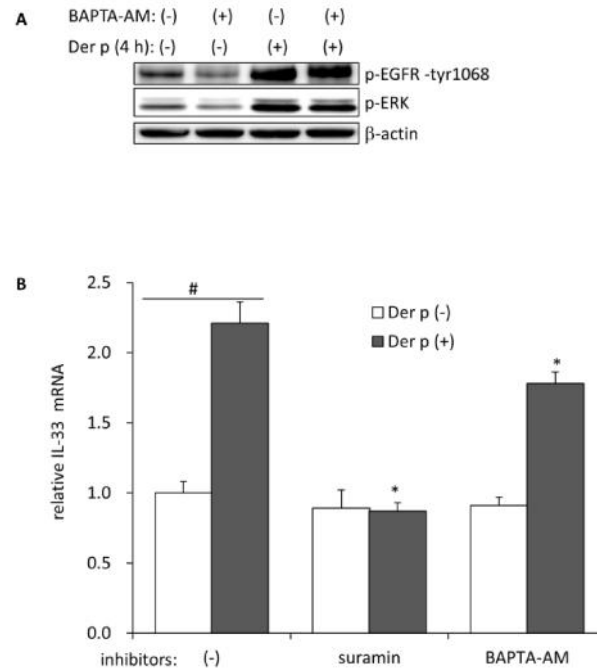
Supplemental Figure 10 (Fig. S10). The PAR2 signal is not required for Der p-induced IL-33 expression in keratinocytes. **(A)** Der p-stimulated IL-33 increase in keratinocytes occurred much later than its upregulation of IL-8. NHEKs were treated with Der p for the indicated times, and the expression of IL-33 and IL-8 mRNA was detected by real-time RT-PCR of total RNA. **(B)** Signal activation of keratinocyte PAR2 by PAR2 agonist upregulates IL-8 expression but not IL-33 expression. NHEKs were treated with Der p or SLIGKIV-NH(2) (an agonist peptide of PAR2, PAR2-AP) for 12 h, and IL-33 and IL-8 mRNA levels were measured by real-time RT-PCR of total RNA. **(C)** The knockdown of PAR2 expression by siRNA transfection. NHEKs were transfected with control or PAR2 siRNA (F2RL1-1 and F2RL1-6) for 24 h, and PAR2 mRNA expression was detected by RT-PCR. **(D)** PAR2 knockdown decreased Der p induction of IL-8 but not that of IL-33. NHEKs were transfected with control or PAR2 siRNA for 48 h and then treated with Der p for 10 h. The IL-8 and IL-33 mRNA expression levels were detected by real-time RT-PCR. The relative mRNA levels are expressed as means $\pm$ SDs ($n = 3$). (* $p < 0.05$)

Fig. S11



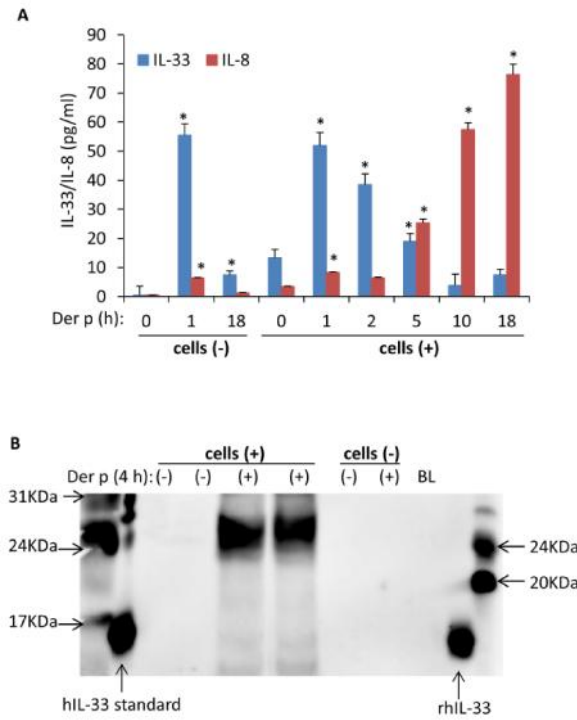
Supplemental Figure 11 (Fig. S11). Der p activates TACE, which is required for activating EGFR/ERK and inducing IL-33. **(A)** NHEKs were treated with Der p for the indicated times, and TACE activity was detected by TACE activity assay of the supernatant. **(B)** NHEKs were pretreated with different concentrations of suramin for 30 min and then with Der p for 2 h. TACE activity in the supernatant was detected. NHEKs were transfected with control (cr) or TACE siRNA for 48 h. The cells were treated with Der p for 4 h, the phosphorylation of EGFR and ERK and TACE protein levels were evaluated **(C)**; or the cells were treated with Der p for 12 h, the mRNA levels of TACE and IL-33 were determined by real time-PCR **(D)**. Relative mRNA levels are expressed as means $\pm$ SDs ($n = 3$), and TACE activity is expressed as means $\pm$ SDs ($n = 5$). (# and * $p < 0.05$).

Fig. S12



Supplemental Figure 12 (Fig. S12). Der p-mediated $[Ca^{2+}]_i$ increase slightly regulates ERK activation and IL-33 induction. **(A)** NHEKs were pretreated with BAPTA-AM or not, then stimulated with Der p for 4 h, the phosphorylation of EGFR and ERK was evaluated. **(B)** NHEKs were pretreated with buffer, suramin or BAPTA-AM for 30 min and then with Der p for 15 h. The IL-33 mRNA levels were determined by real time-PCR. Relative mRNA levels are expressed as means $\pm$ SDs ($n = 3$). ($^{\#}$ and $^* p < 0.05$)

Fig. S13



Supplemental Figure 13 (Fig. S13). Human IL-33 ELISA kit could not be used in the detection of extracellular IL-33 in our system. **(A)** NHEKs were incubated in serum-free medium with Der p at 37°C for the indicated times and then the supernatant were collected, or serum-free medium applied with Der p (without cells) were incubated at 37°C for the indicated times and then collected. They were subjected to IL-33 ELISA and IL-8 ELISA. ($n = 3$; $* p < 0.05$). **(B)** NHEKs were treated with or without Der p for 4 h and then supernatants were collected and concentrated. The IL-33 standard of human IL-33 ELISA kit (hIL-33 standard), as well as recombinant human IL-33 (rhIL-33), together with concentrated culture supernatants were subjected to Western blotting for the detection of IL-33 protein (serum-free medium applied with or without Der p (cells (-)) were used as negative controls). (BL: Blank; SN: supernatant; CE: cell extract))