

Mite allergen is a danger signal for the skin via activation of inflammasome in keratinocytes

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Background: Atopic dermatitis (AD) is a chronic inflammatory skin disorder caused by multiple factors. Among them, house dust mite (HDM) allergens are important in the development of AD. In airway allergy, HDM allergens activate innate immunity. However, information regarding the activation of innate immunity by HDM allergens in the skin is limited.

Objectives: The inflammasome is a key regulator of pathogen recognition and inflammation. We investigated whether HDM allergens activate the inflammasome in epidermal keratinocytes.

Methods: Keratinocytes were stimulated with *Dermatophagoides pteronyssinus*, and the activation of caspase-1 and secretion of IL-1 β and IL-18 were examined. Formation of the inflammasome was studied by analyzing the subcellular distributions of inflammasome proteins. The importance of specific inflammasome proteins was studied by knocking down their expression through transfection of keratinocytes with lentiviral particles carrying short hairpin RNAs (shRNAs).

Results: *D pteronyssinus* activated caspase-1 and induced caspase-1-dependent release of IL-1 β and IL-18 from keratinocytes. Moreover, *D pteronyssinus* stimulated assembly of the inflammasome by recruiting apoptosis-associated specklike protein containing a caspase-recruitment domain (ASC), caspase-1, and nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin-domain containing 3 (NLRP3) to the perinuclear region. Finally, infection with lentiviral particles carrying ASC, caspase-1, or NLRP3 shRNAs suppressed the release of IL-1 β and IL-18 from the keratinocytes. Activation of the NLRP3 inflammasome by *D pteronyssinus* was dependent on cysteine protease activity.

Conclusion: House dust mite allergens are danger signals for the skin. In addition, HDM-induced activation of the NLRP3 inflammasome may play a pivotal role in the pathogenesis of AD. (J Allergy Clin Immunol 2011;■■■:■■■-■■■.)

Key words: Keratinocytes, house dust mite, caspase-1, IL-1 β , IL-18, NLRP3, inflammasome, *Dermatophagoides pteronyssinus*, *Der p 1*

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Abbreviations used

AD:	Atopic dermatitis
ASC:	Apoptosis-associated specklike protein containing a caspase-recruitment domain
E-64:	Trans-epoxysuccinyl L-leucylamido(4-guanidine)butane
HDM:	House dust mite
HMGB1:	High-mobility group box protein 1
NF- κ B:	Nuclear factor- κ B
NLR:	Nucleotide-binding oligomerization domain-like receptor
NLRP:	Nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin-domain containing
PAR2:	Protease-activated receptor 2
PMA:	Phorbol 12-myristate 13-acetate
PolyI:C:	Polyinosinic-polycytidylic acid
shRNAs:	Short hairpin RNAs
TLR:	Toll-like receptor
zVAD:	Benzyloxycarbonyl-valine-alanine-aspartate
zYVAD:	Benzyloxycarbonyl-tyrosine-valine-alanine-aspartate

Atopic dermatitis (AD) is a chronic inflammatory skin disorder affecting 10% to 20% of children worldwide.^{1,2} It sometimes persists into adulthood and has a significant impact on the quality of life of patients and their families. Its incidence has increased over recent decades.¹ Its pathogenesis involves interactions among multiple factors including susceptibility genes, environmental factors, skin barrier defects, and immunologic factors.^{2,3}

Among environmental factors, house dust mite (HDM) allergens are important for the development of AD as well as asthma and rhinitis.^{4,6} *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* are the most common types of HDM in temperate climates and are known to contribute to the immunopathogenesis of AD through the induction of IgE binding.⁷ In airway allergy, activation of innate immunity by HDM allergens plays an important role in disease pathogenesis.^{6,8} However, little is known about the activation of innate immunity by HDM allergens in the skin.

The innate immune system senses invading pathogens via evolutionarily conserved pathogen-recognition receptors, such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptors (NLRs),^{9,10} which play a central role in both innate immunity and inflammatory diseases.¹⁰⁻¹² NLR members form a multiprotein complex, the inflammasome, which activates caspase-1 and ultimately leads to the processing and release of the proinflammatory cytokines IL-1 β , IL-18, and IL-33.¹²

There is growing evidence that inflammasomes play important roles in skin inflammation.¹³ Among them, much attention has focused on the nucleotide-binding oligomerization domain,

leucine-rich repeat and pyrin domain containing 3 (NLRP3) (also known as NACHT, LRR, and PYD domains-containing protein 3 [NALP3] or cryopyrin) inflammasome, which is made up of NLRP3, apoptosis-associated specklike protein containing a caspase-recruitment domain (ASC), and caspase-1.¹² Autoinflammatory diseases are characterized by recurrent episodes of fever and skin rash caused by excessive release of IL-1 β , which is a result of active mutations in *NLRP3* and can be alleviated by IL-1 receptor antagonists.¹⁴ In addition, the NLRP3 inflammasome is a key regulator of contact hypersensitivity.¹³ A recent report linked the NLRP3 inflammasome to allergic diseases,¹⁵ revealing associations between 2 *NLRP3* single nucleotide polymorphisms that increase the expression and activity of NLRP3 and susceptibility to food-induced anaphylaxis and aspirin-induced asthma. Elsewhere, it has been shown that the majority of patients with AD are colonized by *Staphylococcus aureus*,¹⁶ and that hemolysins and bacterial lipoproteins from *S aureus* can activate the NLRP3 inflammasome.^{17,18}

The inflammasome can be activated not only by pathogen-associated molecules but also by various other stimuli, including danger-associated molecular pattern molecules such as ATP and urate crystals, environmental stimuli such as silica crystals and aluminum salts,¹⁹ and neurodegenerative stimuli such as amyloid- β fibrils.²⁰

In this study, we investigated whether HDM allergens activate the keratinocyte inflammasome. We present evidence that HDM allergens activate the NLRP3 inflammasome and stimulate keratinocytes to release the proinflammatory cytokines IL-1 β and IL-18. The release of these cytokines may trigger or exacerbate AD-associated inflammation.

METHODS

Cell culture

Primary human keratinocytes were isolated from neonatal skin samples discarded after surgery. This study was conducted according to the principles of the Declaration of Helsinki, and all procedures involving human subjects received previous approval from the ethics committee at the Ehime University School of Medicine, Japan. Written consent was provided by patient guardians before experiments were initiated. Keratinocytes were cultured in MCDB153 medium (Nissui, Tokyo, Japan) as described previously.²¹

Reagents and cell stimulation

Phorbol 12-myristate 13-acetate (PMA) and polyinosinic-polycytidylic acid (polyI:C) were obtained from Invivogen (San Diego, Calif). Standardized lyophilized extracts of HDM *D pteronyssinus* and *D farinae* were obtained from COSMO BIO (Tokyo, Japan). Affinity chromatography-purified natural allergens (Der p 1 and Der f 1) and the recombinant allergen Der p 2 were obtained from Indoor Biotechnologies (Cardiff, United Kingdom).

Keratinocytes were stimulated with allergens suspended in serum-free medium. Trans-epoxysuccinyl L-leucylamido(4-guanidine)butane (E-64; Sigma-Aldrich, St Louis, Mo) was used as a specific cysteine protease inhibitor.^{22,23} Before being added to the cultures, E-64 was incubated at 37°C for 15 minutes in medium containing allergens. To inactivate allergen proteases, HDM extract was incubated at 65°C for 30 minutes.²² In a subset of experiments, the pan-caspase inhibitor benzyloxycarbonyl-valine-alanine-aspartate (zVAD) and the caspase-1-specific inhibitor benzyloxycarbonyl-tyrosine-valine-alanine-aspartate (zYVAD; Enzo Life Sciences, Polymouth Meeting, Pa) were added to cultures before the application of allergens.

RNA preparation and real-time RT-PCR

Total RNA was isolated by using Isogen (Nippon Gene, Tokyo, Japan). Real-time RT-PCR was performed by using an ABI PRISM 7700 sequence

detector (Applied Biosystems, NJ). Primers and probes specific for GAPDH (Glyceraldehyde-3-phosphate dehydrogenase), IL-1 β , and IL-8 were obtained from Applied Biosystems. PCR analysis was performed by using a TaqMan RT-PCR Master Mix Reagent Kit (Applied Biosystems) according to the manufacturer's protocol. Target gene expression was normalized to the GAPDH signal. Levels of gene expression in allergen-treated cells were quantified relative to those in untreated cells.

ELISA

After the incubation of cells with allergens, cell culture supernatants were collected and stored at -20°C. The release of IL-1 β , IL-8, IL-1 α and caspase-1 was quantified by using ELISA kits from R&D Systems (Minneapolis, Minn) and that of IL-18 by using an ELISA kit from MBL (Nagoya, Japan).

Protein isolation and Western blotting

After stimulation of cells, cell lysates and supernatants were separated by SDS-PAGE and analyzed by using a Vistra ECF Kit (GE, Tokyo, Japan). Membranes were scanned by using a FluoroImager (Molecular Dynamics, Sunnyvale, Calif). The following primary antibodies were used: rabbit anti-I κ B α (inhibitor of kappa B- α), anti-phospho-I κ B α , anti-caspase-1, and anti-IL-1 β (Cell Signaling, Danvers, Mass)²⁴; rabbit anti-IL-18 (MBL); rabbit anti-ASC; mouse anti-NLRP1 and anti-NLRP3 (Abnova, Walnut, Calif); rabbit anti-high-mobility group box protein 1 (HMGB1) (Abcam, Cambridge, Mass) and rabbit anti-actin (Santa Cruz Biotechnology, Santa Cruz, Calif).

Immunofluorescence microscopy

Keratinocytes were fixed with 4% paraformaldehyde and incubated overnight at 4°C with antibodies raised against caspase-1, ASC, and NLRP3. They were then treated with an Alexa Fluor 488-conjugated secondary antibody and DAPI (Invitrogen, Carlsbad, Calif). The cells were washed with PBS and mounted by using VECTASHIELD (Vector, Burlingame, Calif). Images were acquired by using a Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss, Jena, Germany).

Lentiviral transfection

Keratinocytes were transfected with lentiviral particles (at a multiplicity of infection of 0.4) carrying control, ASC, caspase-1, or NLRP3 shRNAs (Santa Cruz Biotechnology) in the presence of 4 μ g/mL polybrene (Santa Cruz Biotechnology), according to the manufacturer's instructions. Knockdown of ASC, caspase-1, and NLRP3 was verified in pooled cell populations by nested RT-PCR and Western blotting.

Statistical analysis

For each analysis, at least 3 independent experiments were performed, all of which yielded similar results. Data from representative experiments are shown. Relative mRNA expression and levels of secreted cytokines are presented as the means $\pm$ SDs ($n > 3$). Statistical significance was determined by using Student *t* tests. A *P* value $< .05$ was considered statistically significant.

RESULTS

HDM extracts induce caspase-dependent secretion of IL-1 β and IL-8 from keratinocytes

Because nuclear factor- κ B (NF- κ B) is a central mediator of inflammatory responses, we first studied whether HDM allergens activate NF- κ B signaling and regulate cytokine expression in keratinocytes. PMA and polyI:C were used as positive controls.²⁵ Whereas PMA and polyI:C triggered rapid phosphorylation of I κ B α , *D pteronyssinus* and *D farinae* did not (Fig 1, A). Furthermore, they did not influence IL-1 β mRNA levels. In contrast, PMA and polyI:C increased IL-1 β mRNA levels as soon as 3 hours after stimulation (Fig 1, B). Although *D pteronyssinus*

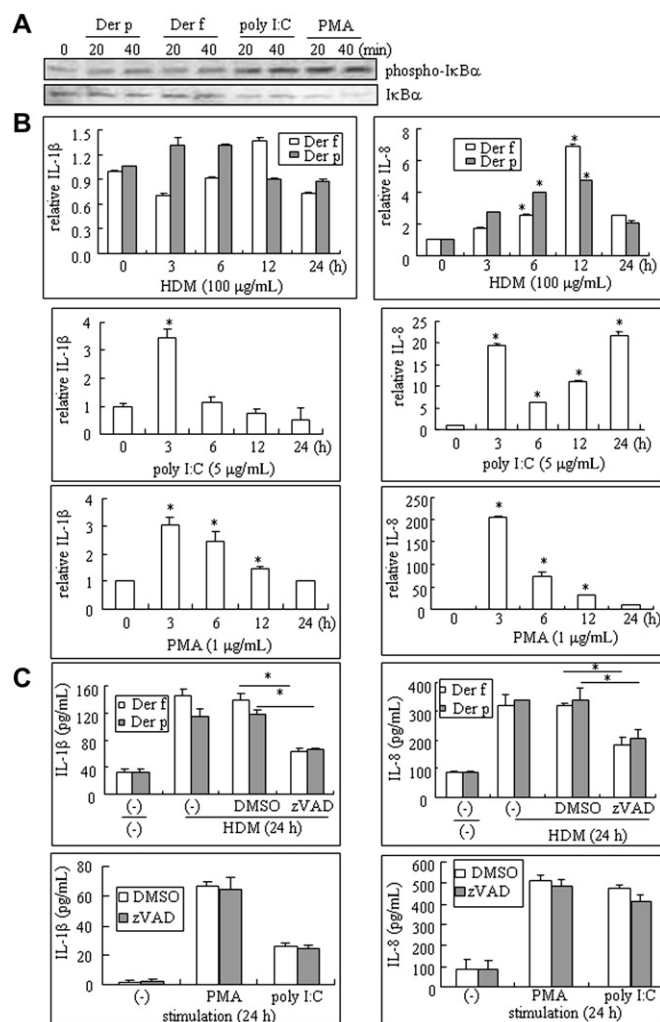


FIG 1. HDM extracts induce caspase-dependent secretion of IL-1 β and IL-8. Keratinocytes were treated with *D pteronyssinus* (Der p), *D farinae* (Der f), PMA and polyI:C. **A**, Cellular I κ B α and phospho-I κ B α levels were detected by Western blotting. **B**, Levels of IL-1 β and IL-8 mRNA were measured by real-time RT-PCR. Relative mRNA levels are presented as the means $\pm$ SDs (n = 5); *P < .05. **C**, Keratinocytes were stimulated with HDM extracts, PMA, and polyI:C in the presence or absence of zVAD (10 μ mol/L). Levels of secreted IL-1 β and IL-8 were measured by ELISA. Concentrations are presented as the means $\pm$ SDs (n = 4); *P < .05. DMSO, Dimethyl sulfoxide.

and *D farinae* elicited approximately 4-fold and 7-fold increases, respectively, in IL-8 mRNA expression, with peak induction occurring 12 hours after stimulation (Fig 1, B), their effects were modest and gradual compared with those of polyI:C and PMA.

We next analyzed the release of IL-1 β and IL-8 from cultured keratinocytes. Although HDM did not alter keratinocyte expression of IL-1 β mRNA, it significantly enhanced the release of IL-1 β and IL-8 from cultured cells (Fig 1, C), as did PMA and polyI:C (Fig 1, C).

Pretreatment of keratinocytes with the pan-caspase inhibitor zVAD reduced HDM-induced IL-1 β release and IL-8 release by approximately 60% and 40%, respectively (Fig 1, C), but did not modulate the responses to PMA and polyI:C. Thus, HDM allergens stimulate keratinocyte production of IL-1 β and IL-8 in a caspase-dependent manner, despite their inability to activate NF- κ B signaling.

Because keratinocytes respond similarly to *D farinae* and *D pteronyssinus*, henceforth we present data obtained by using only *D pteronyssinus*.

D pteronyssinus triggers the activation of caspase-1 and cleavage of pro-IL-1 β and pro-IL-18 in keratinocytes

Although *D pteronyssinus* did not enhance the expression of pro-IL-1 β mRNA (Fig 1, A), primary keratinocytes expressed detectable amounts of pro-IL-1 β (Fig 2, A, upper), the likely source of mature IL-1 β . In parallel with a gradual decrease in intracellular pro-IL-1 β levels after *D pteronyssinus* stimulation, supernatant levels of mature IL-1 β increased in a time-dependent fashion, as detected by Western blotting (Fig 2, A, upper), with cleaved p17 transiently detected in cellular lysates (Fig 2, A, upper). In contrast, the supernatant pro-IL-1 β levels were not increased after stimulation with *D pteronyssinus* (Fig 2, B, upper). These data suggest that the time-dependent secretion of IL-1 β from *D pteronyssinus*-treated keratinocytes, as detected by ELISA (Fig 2, B, lower), largely relates to mature IL-1 β and not inactive pro-IL-1 β . Like IL-1 β , the cytosolic precursor of IL-18 was converted to its biologically active form in HDM-treated keratinocytes. After *D pteronyssinus* stimulation, active, mature IL-18 was rapidly secreted, as shown by Western blotting and ELISA analyses of cell culture supernatants, with intracellular levels of pro-IL-18 decreasing in parallel (Fig 2, B).

Caspases are proteases with regulatory roles in inflammation and apoptosis. There are 11 known caspases in human beings, but only caspase-1 has been shown to mediate the processing of pro-IL-1 β and pro-IL-18.²⁶ We investigated the role of caspase-1 in HDM-induced activation of IL-1 β . Caspase-1 is initially expressed as an inactive precursor that, after stimulation, is cleaved, yielding active p10 and p20 subunits.²⁷ Intracellular active p20 was detected as soon as 3 hours after *D pteronyssinus* stimulation (Fig 2, C, upper). ELISA analysis of supernatants from *D pteronyssinus*-treated keratinocytes further revealed time-dependent release of active caspase-1 p10 and p20 (Fig 2, C, lower). Thus, *D pteronyssinus* stimulates the activation of caspase-1 in keratinocytes. The kinetics of caspase-1 activation, which was initially detected 3 hours after stimulation, and the activation of IL-1 β and IL-18, which increased subsequently, indicate the dependence of cytokine release on caspase-1.

Concomitant exposure to the caspase-1-specific inhibitor zVAD blocked the activation of caspase-1 (Fig 2, D) and significantly inhibited the processing and secretion of IL-1 β (Fig 2, E) and IL-18 (Fig 2, F). Thus, *D pteronyssinus* stimulates caspase-1 activation, which is necessary for the activation and secretion of IL-1 β and IL-18 from keratinocytes.

D pteronyssinus activates the NLRP3 inflammasome in keratinocytes

Primary keratinocytes stably expressed the inflammasome proteins caspase-1 (Fig 2, A), ASC (Fig 3, A), and NLRP3 (Fig 3, A), but not NLRP1 (data not shown). Keratinocyte expression of ASC and NLRP3 was upregulated by treatment with *D pteronyssinus* (Fig 3, A). Caspase-1 is activated as a result of inflammasome assembly, which occurs in the cytosol.²⁸ To investigate whether *D pteronyssinus* triggers the formation of inflammasomes in keratinocytes, we examined the subcellular localization of inflammasome proteins by immunofluorescence.

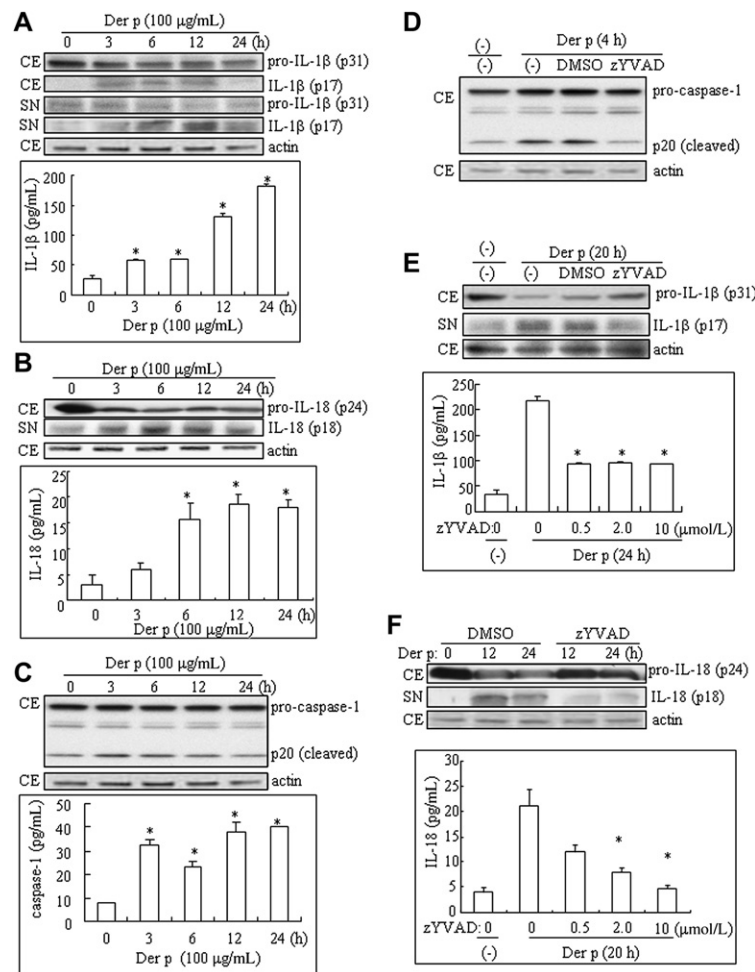


FIG 2. *D pteronyssinus* (*Der p*) induces caspase-1–dependent secretion of IL-1β and IL-18. Keratinocytes were treated with *Der p* for the indicated periods. **A**, Levels of pro-IL-1β and active mature IL-1β in cell lysates and supernatants. **B**, Levels of pro-IL-18 (intracellular) and active mature IL-18 (in supernatants). **C**, Levels of active caspase-1 in cell lysates (as measured by Western blotting) and supernatants (by ELISA). Keratinocytes were incubated with *Der p* in the presence of zYVAD (10 μmol/L), and cellular caspase-1 levels (**D**) and supernatant IL-1β levels (**E**) were measured by Western blotting. The effects of different doses of zYVAD on IL-1β release were assessed by ELISA (**E**) and the effects of zYVAD on the activation and release of IL-18 by Western blotting and ELISA (**F**). Concentrations are presented as the means ± SDs (n = 4); *P < .05. CE, Cell extracts; DMSO, Dimethyl sulfoxide; SN, supernatants.

ASC is sequestered in the nucleus of resting phagocytic cells and only becomes available for the bridging of NLRs and caspase-1 in the cytosol.²⁸ In resting keratinocytes, ASC predominantly localized to the nucleus, as determined by nuclear counterstaining (Fig 3, B, left). However, treatment with *D pteronyssinus* stimulated the rapid redistribution of ASC to the cytosol. At 4 hours post-treatment, ASC was detected throughout activated cells but was predominantly localized to the perinuclear region (Fig 3, B, left). Caspase-1 staining showed diffuse expression in the cytosol of resting cells and perinuclear aggregation in *D pteronyssinus*-treated cells (Fig 3, B, middle). Specific staining for NLRP3 revealed its diffuse distribution throughout resting cells. After stimulation, NLRP3 rapidly translocated to the cytosol and accumulated in the perinuclear region (Fig 3, B, right). Thus, in keratinocytes, ASC, caspase-1, and NLRP3 accumulate in the perinuclear region in response to *D pteronyssinus* stimulation, suggesting that *D pteronyssinus* activates NLRP3 inflammasomes in keratinocytes.

To test whether inflammasome proteins are involved in caspase-1/IL-1β activation, we infected keratinocytes with lentiviral particles carrying ASC, caspase-1, and NLRP3 shRNAs, which efficiently knocked down protein expression, as assessed by Western blotting (Fig 3, C). After transfection, supernatant levels of active caspase-1/IL-1β were measured. *D pteronyssinus* treatment significantly increased supernatant levels of active caspase-1 (Fig 3, D) and secreted IL-1β (Fig 3, E) and IL-18 (Fig 3, F). These responses were greatly attenuated in cells expressing ASC, caspase-1, and NLRP3 shRNAs, but not in others expressing a control shRNA. Thus, ASC, caspase-1, and NLRP3 are apparently all essential for the release of IL-1β and IL-18.

To test whether K⁺ efflux is required for *D pteronyssinus*-induced activation of the NLRP3 inflammasome, we stimulated keratinocytes with *D pteronyssinus* in the presence of KCl (1–100 mmol/L) or identical concentrations of NaCl (control). The activation and release of caspase-1 (Fig 4, A), IL-1β (Fig 4, B), and IL-18 (Fig 4, C) in response to treatment with *D*

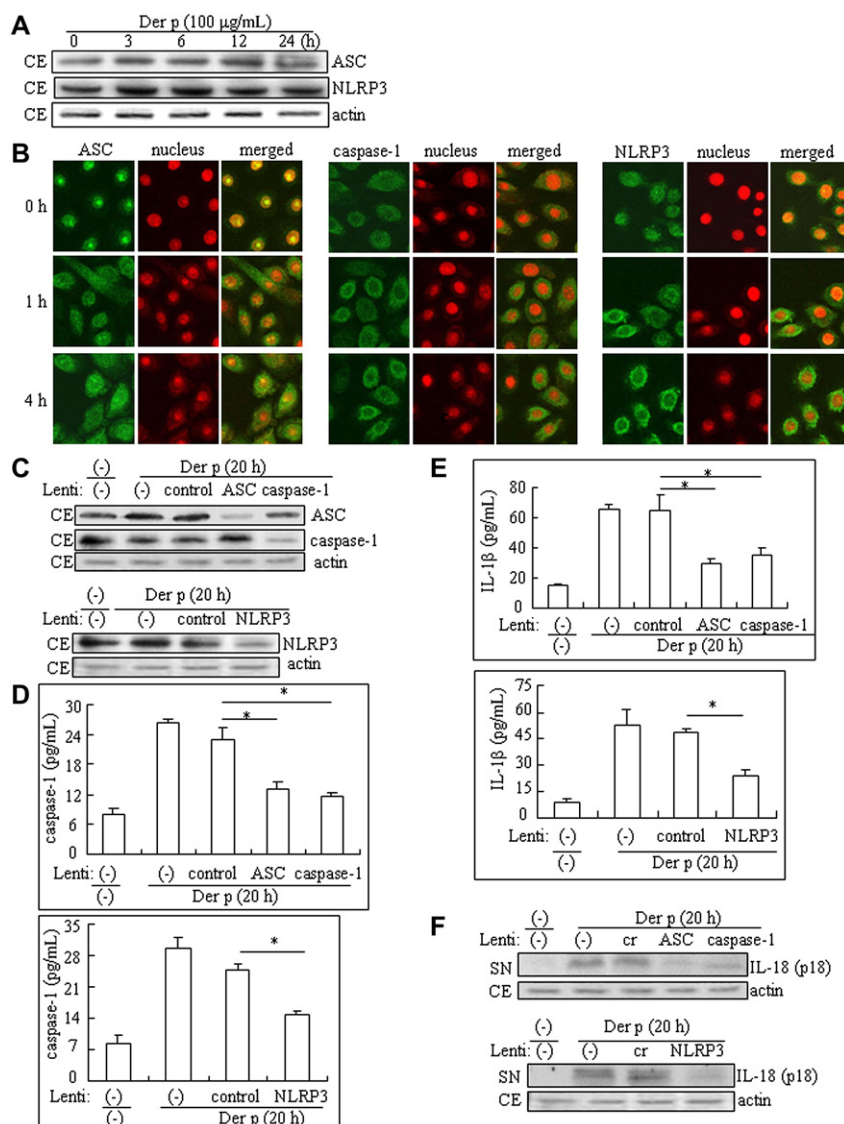


FIG 3. *D pteronyssinus* (*Der p*) activates the NLRP3 inflammasome in keratinocytes. **A**, Levels of cellular ASC and NLRP3 were estimated by Western blotting. **B**, Subcellular distribution of ASC, caspase-1, and NLRP3 in keratinocytes treated with *Der p* (for 0, 1, and 4 hours), assessed by immunofluorescence. Keratinocytes were infected with lentiviral particles (*Lenti*) carrying ASC, caspase-1, NLRP3, and control (*cr*) shRNAs, and then treated with *Der p*. Levels of ASC, caspase-1, and NLRP3 were determined by Western blotting (**C**), and the release of caspase-1 (**D**), IL-1β (**E**), and IL-18 (**F**) was assessed by ELISA or Western blotting. Concentrations are presented as the means ± SDs ($n = 4$); * $P < .05$. CE, Cell extracts; SN, supernatants.

pteronysinus was blocked when the extracellular KCl concentration was increased to 100 mmol/L. Therefore, *D pteronyssinus*-mediated activation of the NLRP3 inflammasome depends on the efflux of K^+ ions.

Thus, *D pteronyssinus* activates the NLRP3 inflammasome in keratinocytes, which is in turn required for the activation of caspase-1 and release of IL-1β and IL-18.

***D pteronyssinus*-induced caspase-1/IL-1β activation depends on cysteine protease activity**

We next investigated whether HDM proteases are involved in inflammasome activation. Heat treatment of *D pteronyssinus*,

which inactivates its proteolytic activities,^{22,29} almost completely blocked IL-1β release (Fig 5, A), suggesting that proteolytic activity is indeed required for NLRP3 inflammasome-mediated IL-1β secretion. We further tested the effects of specific protease inhibitors. Preincubation of *D pteronyssinus* with the cysteine protease inhibitor E-64 not only impaired the activation of caspase-1 (Fig 5, B) but also inhibited the release of IL-1β (by approximately 60%; Fig 5, C) and IL-18 (Fig 5, D). In contrast, the specific serine protease inhibitor 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride²³ did not block the release of IL-1β (data not shown). These data indicate that the activation of the NLRP3 inflammasome by *D pteronyssinus* was, at least in part, dependent on HDM-derived cysteine protease activity.

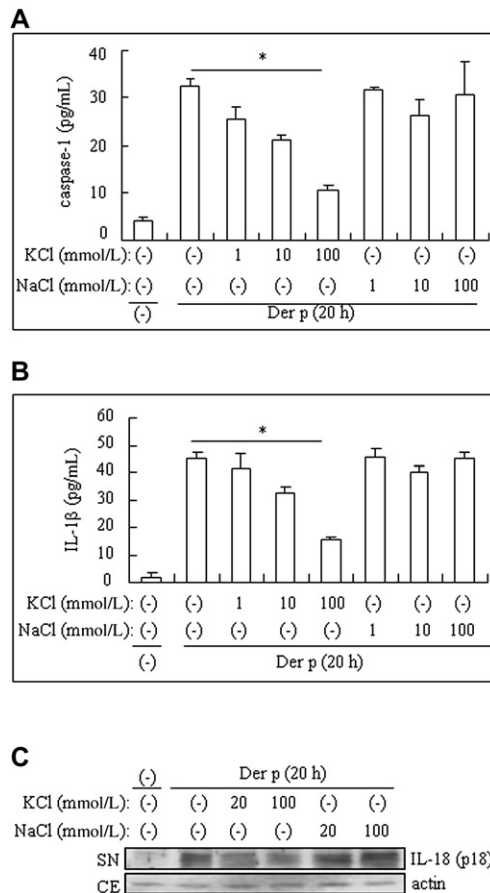


FIG 4. Extracellular K^+ inhibits *D pteronyssinus* (Der p)-induced activation of the NLRP3 inflammasome. Keratinocytes were treated with Der p for 20 hours in the presence of KCl or NaCl, and supernatant levels of active caspase-1 (A), IL-1 β (B), and IL-18 (C) were measured. Concentrations are presented as the means $\pm$ SDs (n = 4); * P < .05. CE, Cell extracts; SN, supernatants.

Der p 1 activates caspase-1 and induces the release of IL-1 β and IL-18

Because cysteine protease activity is essential for *D pteronyssinus*-mediated inflammasome activation (Fig 5), we investigated whether the group 1 allergens, which exhibit cysteine protease activity,^{23,30} were capable of activating the inflammasome. Purified natural Der p 1 caused significant activation of caspase-1 (Fig 6, A) and the release of considerable amounts of IL-1 β (Fig 6, B) and IL-18 (Fig 6, C). These responses were nearly fully abrogated by the preincubation of Der p 1 with E-64. Furthermore, we showed, by using zYVAD, that the Der p 1-induced release of IL-1 β and IL-18 from keratinocytes depended on caspase-1 activity (Fig 6, D and E). Stimulation with Der f 1 yielded similar results (data not shown). These data indicate that group 1 allergens stimulate inflammasome activation and induce the release of IL-1 β and IL-18, responses that depend on their cysteine protease activity.

DISCUSSION

The current study provides the first evidence that HDM allergens trigger assembly of the NLRP3 inflammasome, activate caspase-1, and thus stimulate the processing and release of IL-1 β and IL-18. The secretion of IL-1 β usually requires two separate

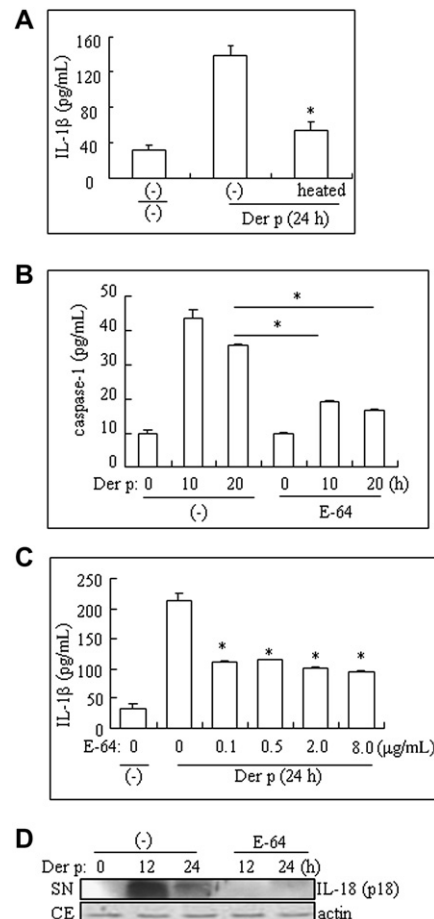


FIG 5. Cysteine protease activity is required for *D pteronyssinus* (Der p)-mediated inflammasome activation. A, Der p was heat-treated and then applied to cultures. IL-1 β release was measured. Der p was or was not preincubated with E-64 (2 μ g/mL) before being added to cultures. Supernatant levels of active caspase-1 (B), IL-1 β (C), and IL-18 (D) were subsequently measured. Concentrations are presented as the means $\pm$ SDs (n = 4); * P < .05. CE, Cell extracts; SN, supernatants.

signals. The first, from a pathogen-associated molecular pattern molecule, promotes intracellular production of the immature cytokine. The second, derived from a danger signal, triggers inflammasome assembly, caspase-1 activation, and secretion of mature IL-1 β .²⁶ However, the IL-1 β and IL-18 released from *D pteronyssinus*-treated keratinocytes appear to originate from pre-stored pools of precursor cytokines, because no priming of immature cytokine production was detected. The response to *D pteronyssinus* bears some similarities to necrosis-induced NLRP3 activation, in which inflammasome activation and the release of caspase-1 do not depend on LPS priming or other proinflammatory stimuli.³¹ However, cell necrosis was not observed in our cultures.

K^+ efflux seems to be the best common and specific trigger of NLRP inflammasome activation,³² including the *D pteronyssinus*-mediated activation of the NLRP3 inflammasome in keratinocytes. Other important intracellular compounds include reactive oxygen species, which are often required for activation of the NLRP3 inflammasome.³³ However, preincubation of cells with the broad-spectrum NADPH oxidase inhibitor diphenyleneiodonium failed to impair *D pteronyssinus*-induced IL-1 β

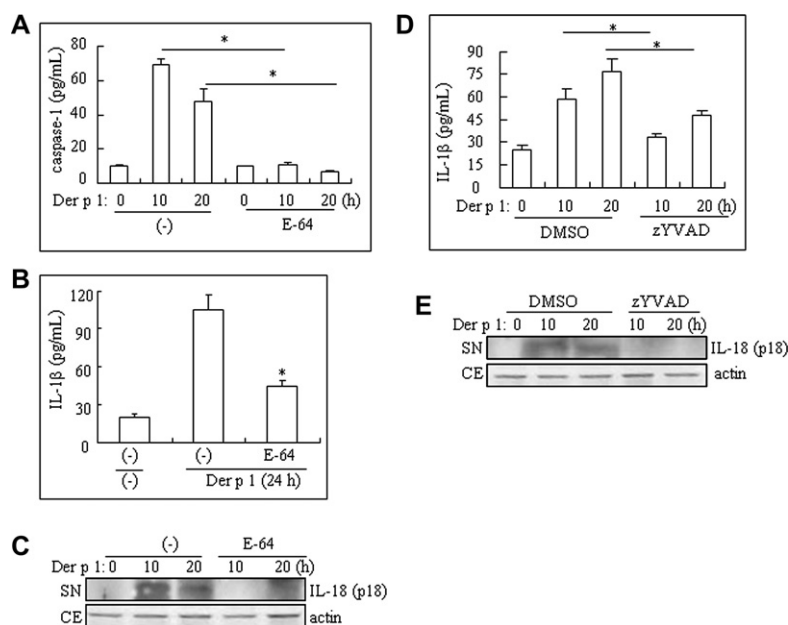


FIG 6. Der p 1 activates the inflammasome in keratinocytes. Keratinocytes were treated with Der p 1 (10 μ g/mL) preincubated or not preincubated with E-64 (2 μ g/mL), and the release of active caspase-1 (**A**), IL-1 β (**B**), and IL-18 (**C**) was assessed. Cells were treated with Der p 1 in the presence or absence of zYVAD (10 μ mol/L). Supernatant levels of mature IL-1 β (**D**) and IL-18 (**E**) were subsequently measured. Concentrations are presented as the means $\pm$ SDs (n = 4); *P < .05. CE, Cell extracts; DMSO, Dimethyl sulfoxide; SN, supernatants.

activation (data not shown), suggesting that *D pteronyssinus* activates the NLRP3 inflammasome independently of reactive oxygen species. ATP is an activator of inflammasomes³⁴ and may mediate *D pteronyssinus*-induced inflammasome activation in keratinocytes. However, extracellular ATP failed to trigger the release of IL-1 β from *D pteronyssinus*-nontreated³⁵ or *D pteronyssinus*-treated keratinocytes (data not shown), suggesting that ATP is not involved in activation of the inflammasome by *D pteronyssinus*, at least in keratinocytes.

High-mobility group box protein 1 (HMGB1) is a nuclear protein that acts as a powerful proinflammatory cytokine when released from cells.³⁶ According to a recent report, HMGB1 activates the inflammasome in dendritic cells.³⁷ We performed western blotting of supernatants and detected the release of HMGB1 from *D pteronyssinus*-treated keratinocytes (see this article's Fig E1 in the Online Repository at www.jacionline.org). Thus, it is possible that HMGB1 is involved in *D pteronyssinus*-mediated inflammasome activation in keratinocytes. Further studies are needed to confirm whether danger molecules such as HMGB1 are involved in *D pteronyssinus*-mediated activation of the NLRP3 inflammasome.

Skin barrier dysfunction is one of the major manifestations of AD and a key contributor to its pathogenesis.³ Barrier disturbances resulting from genetic defects,^{38,39} abnormal expression of epidermal proteins caused by T_H2-type cytokines,^{40,41} or protease allergens^{42,43} may increase the risk of sensitization to allergens and contribute to the development of AD. However, how physical defects result in immunologic abnormalities remains unclear. Barrier defects may allow environmental allergens, such as HDM allergens, to penetrate the underlying tissues and increase the likelihood of epidermal keratinocytes being exposed to them.

Although Gutgesell et al⁴⁴ reported that HDM elimination strategies do not improve disease activity in patients with AD,

other studies have described the beneficial effects of HDM avoidance in limiting eruptions.^{45,46} We suggest that HDM allergens contribute to the development of AD by activating the NLRP3 inflammasome and triggering the release of the proinflammatory cytokines IL-1 β and IL-18. Although most clinical features of AD are a direct consequence of T_H2-skewed acquired immune responses,¹⁶ patients with AD appear to develop a biphasic T_H-cell pattern, characterized by T_H2 cytokine production early during the acute phase, followed by a switch to a more T_H1-like profile during the chronic phase. IL-1 β and IL-18, both members of the IL-1 family,⁴⁷ are important contributors to T_H1 responses⁴⁸ and also regulate T_H2 responses.⁴⁹ IL-18 has been shown to regulate allergic inflammation by inducing T_H2 cytokine production and eosinophilia.^{50,51} HDM-induced release of IL-18 from keratinocytes may, therefore, be important in the pathogenesis of AD.

Recent studies using mouse models have further indicated the importance of IL-1 β and IL-18 for the development of AD. Yamanaka et al⁵² demonstrated that skin-specific caspase-1-transgenic mice maintained under specific pathogen-free conditions spontaneously develop chronic dermatitis, accompanied by abnormally elevated skin and serum IL-18 and IL-1 β levels. Elsewhere, an *Il18*-transgenic mouse that exhibited oversecretion of IL-18 from epidermal cells developed AD-like skin eruptions, which improved after deletion of the *Il18* gene.⁵³ Of potential interest is the fact that the skin phenotypes of *Il18*-transgenic mice closely resemble those of *Nlrp3*-knockin mice. Furthermore, in an intrinsic AD mouse model⁵⁴ generated through the daily application of protein A (an *S aureus* surface molecule and virulence factor), administering a neutralizing anti-IL-18 antibody and knocking out the *Il18* gene completely ameliorated the AD-like skin eruptions.

In addition to its effects on IL-1 β and IL-18, *D pteronyssinus* stimulated the release of IL-1 α (an unconventional leadless

protein but not a protease substrate⁵⁵), at least partially in a caspase-1-dependent manner (see this article's Fig E2 in the Online Repository at www.jacionline.org). Thus, HDM allergen-mediated activation of the NLRP3 inflammasome and subsequent release of IL-1 proteins may be important in the development of dermatitis.

Mite cysteine proteases (group 1 allergens) and serine proteases (group 3, 6, and 9 allergens) both stimulate cells, but by different mechanisms. Der p 3 and Der p 9 induce the release of IL-8 and GM-CSF from keratinocytes²³ and airway epithelial cells^{56,57} by activating protease-activated receptor 2 (PAR2) signaling. In contrast, Der p 1 and Der f 1, instead of activating PAR2 signaling, stimulate the inflammasome in keratinocytes.^{23,56} Because epidermal keratinocytes and airway epithelial cells respond to HDM allergens in similar ways,^{23,56,57} it is possible that *D pteronyssinus* and Der p 1 may trigger activation of the NLRP3 inflammasome in airway epithelial cells. This should be studied further.

The group 2 allergens are major antigens for IgE and lack protease activity.⁵⁸ Unlike Der p 1, Der p 2 did not stimulate the release of IL-1 β from keratinocytes but did stimulate IL-8 release (see this article's Fig E3 in the Online Repository at www.jacionline.org) via a nonproteolytic mechanism, presumably involving the TLR2/myeloid differentiation primary response gene 88 (MyD88)-dependent activation of NF- κ B and mitogen-activated protein kinase signaling pathways.^{59,60} Thus, keratinocytes are able to sense HDM allergens directly and to initiate local inflammatory responses, perhaps through the cooperation of PAR2, inflammasomes, and even TLR pathways.

In conclusion, HDM allergens are novel, effective activators of the inflammasome in epidermal keratinocytes. Notably, HDM allergen-mediated activation of the NLRP3 inflammasome, and consequent release of IL-1 family proteins, may be important in the development of atopic dermatitis.

Clinical implications: Although mite allergens have previously been shown to activate TLRs, this is the first study to show that mite allergens directly activate the inflammasome in epidermal keratinocytes.

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METHODS

Cell cultures

Primary human keratinocytes were isolated from neonatal skin samples discarded after surgery. This study was conducted according to the principles of the Declaration of Helsinki, and all procedures involving human subjects received previous approval from the ethics committee at the Ehime University School of Medicine, Japan. Written consent was provided by patient guardians before experiments were initiated. Keratinocytes were cultured in MCDB153 medium as described previously.^{E1}

Reagents and cell stimulation

Standardized lyophilized extract of HDM *D pteronyssinus* was obtained from LSL (Japan), and the recombinant allergen Der p 2 was obtained from Indoor Biotechnologies Ltd (Cardiff, United Kingdom). Keratinocytes were stimulated with allergens suspended in serum-free medium. To inhibit the activation of caspase-1, the specific caspase-1 inhibitor zYVAD (Alexis, Pa) was added to cultures before the application of allergens.

ELISA

After incubating cells with allergens, cell culture supernatants were collected and stored at -20°C . The release of IL-1 α , IL-1 β , and IL-8

was quantified by using commercially available ELISA kits (R&D Systems).

Protein isolation and Western blotting

After stimulation of cells for the indicated times, cell lysates and supernatants were separated by SDS-PAGE and analyzed by using a Vistra ECF Kit (Amersham Biosciences, Ill), and membranes were scanned by using a FluoroImager (Molecular Dynamics, Calif). The antibodies used were HMGB1 from Abcam and antiactin from Santa Cruz Biotechnology.

Statistical analysis

For each analysis, at least 3 independent experiments were performed, all of which yielded similar results. Data from representative experiments are shown. Statistical significance was determined by using Student *t* tests. A *P* value $<.05$ was considered statistically significant.

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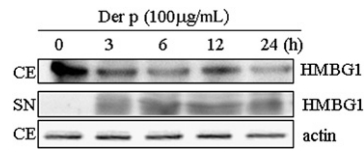


FIG E1. *D pteronyssinus* (*Der p*) stimulates the release of HMGB1 from keratinocytes. Keratinocytes were treated with Der p (100 µg/mL) for the indicated times. The levels of intracellular and supernatant HMGB1 were assessed by Western blotting. *CE*, Cell extracts; *SN*, supernatants.

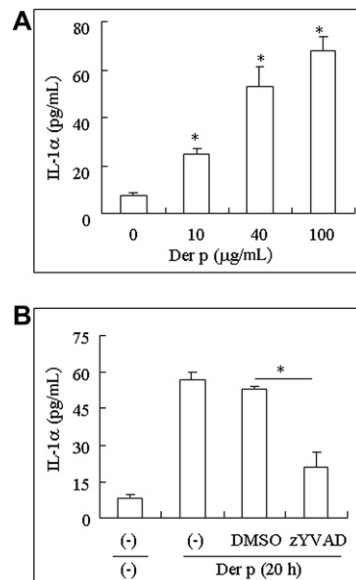


FIG E2. *D pteronyssinus* (*Der p*) induces caspase-1–dependent secretion of IL-1 α . **A**, Keratinocytes were stimulated with increasing concentrations of *Der p* for 20 hours. The release of IL-1 α was detected by ELISA. **B**, Keratinocytes were incubated with *Der p* (100 μ g/mL) in the presence or absence of zYVAD (10 μ mol/L). The supernatant levels of IL-1 α were measured by ELISA. Concentrations represent means $\pm$ SDs ($n = 4$); * $P < .05$. *DMSO*, Dimethyl sulfoxide.

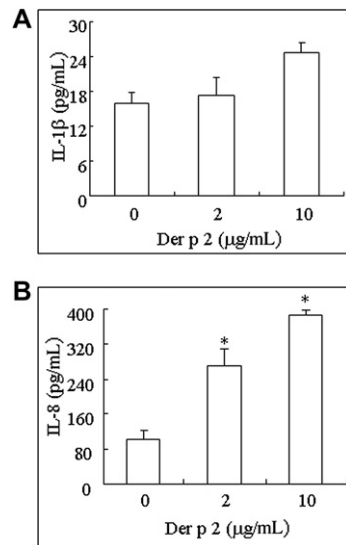


FIG E3. Der p 2 does not stimulate the release of IL-1 β from keratinocytes. Keratinocytes were treated with increasing concentrations of Der p 2 for 24 hours, and the secretion of IL-1 β (**A**) and IL-8 (**B**) was detected by ELISA. Concentrations represent means $\pm$ SDs ($n = 4$); * $P < .05$.