House Dust Mite Increases pro-Th2 Cytokines IL-25 and IL-33 via the Activation of TLR1/6 Signaling

Yong Hyun Jang¹,¹⁰, Jin Kyeong Choi²,³,¹⁰, Meiling Jin², Young-Ae Choi², Zae Young Ryoo⁴, Hyun-Shik Lee⁵, Pil-Hoon Park⁵, Sun-Uk Kim⁶, Taeg Kyu Kwon⁷, Myoun Ho Jang⁸, Sin-Hyeog Im⁸,⁹, Sun Young Moon¹, Weon Ju Lee¹, Seok-Jong Lee¹, Do Won Kim¹ and Sang-Hyun Kim¹

House dust mites have been implicated in the etiology and exacerbation of atopic dermatitis. Diverse factors contribute to house dust mite allergenicity through the activation of innate immunity. We investigated whether *Dermatophagoides farinae* extract (DFE) allergens mediate innate immune activation through specific toll-like receptors (TLRs) in epidermal keratinocytes, a DFE-induced murine atopic dermatitis model, and human atopic dermatitis lesions. DFE activated the expression of TLR1, TLR6, IL-25, and IL-33 in human primary keratinocytes and HaCaT cells. Knockdown of TLR6 inhibited DFE-induced upregulation of IL-25 or IL-33. In addition, the suppression of TLR1 inhibited the release of IL-33. DFE induced the expression of IL-25 and IL-33 by upregulation of IL-1 receptor-associated kinase 1, transforming growth factor-β activated kinase-1, IkB kinase, and NF-kB pathways. *Tlr1−/−* mice did not show DFE-induced upregulation of IL-25 and IL-33. Furthermore, DFE-induced upregulation of IL-25 was not induced in *Tlr6−/−* mice. We also identified upregulated mRNA and protein expression of TLR1, TLR6, IL-25, and IL-33 in human atopic dermatitis skin lesions with high house dust mite sensitization. We found that DFE-induced activation of TLR1 and TLR6 may cause polarization toward a T helper type 2 immune response via the release of IL-25 and IL-33.


**INTRODUCTION**

The pathogenesis of atopic dermatitis (AD) is multifactorial and involves a complex immunologic cascade, including skin barrier dysfunction, defects in the cutaneous cell-mediated immune response, IgE dysregulation, genetic susceptibility factors, and environmental factors (Leung and Bieber, 2003). Particularly, defects in epidermal skin barriers lead to elevated sensitivity to atopic aeroallergens including house dust mite (HDM) (De Benedetto et al., 2012).

The innate immune system is a first line of defense against invading microorganisms and other potential threats to the host (Medzhitov, 2001). Toll-like receptors (TLRs) mediate the recognition of a wide range of microbial products including lipopolysaccharide (LPS), lipoproteins, flagellin, and bacterial DNA, and signaling through TLRs leads to the production of inflammatory mediators (Gantner et al., 2003). Activation of innate immunity is a major pathway responsible for the production of innate proallergic type 2 cytokines, such as IL-25 and IL-33. They have been implicated in the stimulation of T helper type 2 (Th2) cytokine production, eosinophils, mast cells, serum IgE levels, and type 2 innate lymphoid cells (ILC) in patients with AD (Cayrol and Girard, 2014; Kuo et al., 2013; Salimi et al., 2013). Human keratinocytes express functional TLR1, TLR2, TLR3, TLR5, and TLR6, whereas there is some controversy regarding the expression of TLR4, TLR7, and TLR9 (Kuo et al., 2013). TLR10 expression has been reported in human keratinocytes, but its function is currently unclear (Kollisch et al., 2005). Only TLR2, TLR3, TLR4, and TLR9 functions and their relevance in AD have been reported (Kuo et al., 2013).

The HDM, a ubiquitous organism, has been implicated in the etiology and exacerbation of AD (Gavino et al., 2008). HDM worsens AD severity through the following mechanisms: inherent proteolytic enzyme activity, activation of
HDM (Dermaphagoides farinae extract, DFE) activates pattern recognition receptors (PRRs) including TLR1, TLR6, TLR9, and nucleotide-binding oligomerization domain 2, and induces the release of innate proallergic cytokines IL-25 and IL-33 in epidermal keratinocytes

We first analyzed whether HDM could induce the gene expression of various PRRs, and chemical, immunologic, and physical barrier proteins from keratinocytes. HaCaT cells were incubated with standardized/lyophilized DFE (100 µg/ml) for 6, 12, or 24 hours, and gene expression was analyzed by qPCR. DFE application induced a significant increase in gene expression of TLR1, TLR6, TLR9, nucleotide-binding oligomerization domain 2 (NOD2), IL-25, and IL-33 (Figure 1a-c). DFE also induced the expression of chemical mediators (IL-37, human β-defensin3, S100A9, and dermcidin) and physical barrier (claudin-1, claudin-23, and occludin) for 24 hours (Supplementary Figure S1a-c online). HDM contains large amounts of LPS that can activate TLR4 signaling. TLR4 triggering by LPS induces production of proinflammatory cytokines necessary for the activation of potent allergic immune responses (Hammad et al., 2009; Trompette et al., 2009). In addition, LPS activates TLR signaling even at drastically low concentrations. Thus, we investigated the effects of DFE not containing LPS on the innate immune system of keratinocytes. The estimated LPS content of the DFE in our experiment was 0.25 ng in 2.5 µg of DFE. Our results showed that LPS-free DFE also activated innate immunity in keratinocytes. Thus, DFE allergens, in the absence of any microbial compounds such as LPS, can induce innate immune activation in keratinocytes.

We tested the effect of DFE application on the ear of BALB/c mice. Compared with control mice, DFE-induced AD mice showed increased ear thickness and elevated serum IgE (Supplementary Figure S2a-d online). Repeated application of DFE to the ears of BALB/c mice resulted in a significant increase in the expression levels of various PRRs, chemical, immunologic mediators, and physical barriers components (Figures 1d and Supplementary Figure S2e). Similar to the in vitro results (Figure 1a-c), TLR1, TLR6, TLR9, and NOD2 were increased by the DFE application.

Differential role of TLR6 and TLR1 in regulation of IL-25 and IL-33 expression

We hypothesized that the crosstalk between innate immunity and chronic exposure to HDM would elicit a Th2-type response in AD through IL-25 and IL-33. IL-25 or IL-33 might induce Th2 immune response through accumulation of IL-22, a critical source of type 2 cytokines (Hvid et al., 2011). Therefore, we investigated whether DFE induces the activation of IL-25 and IL-33 through specific PRR activation. We examined the knockdown effects of TLR1, TLR6, TLR9, and NOD2 using siRNA on DFE-mediated induction of IL-25 and IL-33. Knockdown of TLR1 or TLR6 inhibited DFE-induced upregulation of IL-25 or IL-33 in HaCaT cells. TLR6 suppression was associated with decreased expression of both IL-25 and IL-33; however, TLR1 inhibition decreased only IL-33. In contrast, TLR9 and NOD2 had no effect on the expression of IL-25 and IL-33 (Figure 2a and b and Supplementary Figure S3 online). The differential gene expression levels were confirmed by relative protein levels (Figure 2c).

TLR1 and TLR6 form heterodimers with TLR2 that sense lipopeptides from bacteria, with TLR1/2 dimers sensing triacylated lipopeptides and TLR2/6 sensing diacylated lipopeptides (Keogh and Parker, 2011). We therefore confirmed whether synthetic TLR1 or TLR6 ligands trigger the expression of IL-25 and IL-33 in HaCaT cells. The expression of IL-25 and IL-33 was significantly increased after stimulation with synthetic triacylated lipopeptides (Pam3CSK4) and/or diacylated lipopeptides (FSL-1) (Supplementary Figure S4a online). However, we hypothesized that TLR2 would affect IL-25/33 production in keratinocytes. Thus, to exclude the effect of differences in the production of IL-25/33 after TLR2 stimulation, we transfected dominant negative (DN)-TLR constructs into HaCaT cells. DN-TLR6 activated with DFE suppressed both IL-25 and IL-33, whereas DN-TLR1 inhibited only IL-33. Interestingly, there were no differences in the production of the IL-25 and IL-33 found in keratinocytes with DN-TLR2, including TLR2 cotransfected with DN-TLR1/2 or DN-TLR2/6 (Figure 2d). Moreover, we used TLR1, TLR2, TLR6, TLR1/2, or TLR2/6 constructs to examine the requirement of IL-25 and IL-33. DFE, Pam3CSK4, and FSL-1 stimulated IL-25 and IL-33 production in keratinocytes, and overexpression of TLR1 or TLR6 significantly increased this response. Similar to DN-TLRs, overexpression of TLR2 alone was insufficient to induce IL-25 or IL-33 (Figure 2e and Supplementary Figure S4b).

DFE activates the TLR1- or TLR6-mediated innate immune signaling pathway

HDM is critical in TLR2-mediated proinflammatory cytokine production via the NF-κB-dependent pathway in mouse mast cells (Gregory and Lloyd, 2011). It has been reported that an excrement extract prepared from D. farinae activates NF-κB through TLR4-dependent and CD14/TLR2-dependent mechanisms (Kitajima et al., 2014). In addition, the activation of NF-κB triggers the production of proinflammatory cytokine in AD (Briot et al., 2009). Pam3CSK4 and FSL-1 are synthetic TLR ligands mediated by TLR1 and TLR6, respectively, which cooperate with TLR2 through their cytoplasmic domain to induce the signaling cascade, leading to activation of NF-κB signaling pathways (Kim et al., 2014; West et al., 2011). We therefore compared the roles of DFE with those of TLR ligands, Pam3CSK and FSL-1, in keratinocytes. Similar to Pam3CSK4 and FSL-1, DFE promoted the association of tumor necrosis factor receptor

www.jidonline.org 2355

YH Jang et al.
HDM Induces AD Inflammation via TLR1/6 Signal
TLR1 and TLR6 are key mediators of stimulation of Th2 type immune response through IL-25 and IL-33 in the murine model of AD-like lesions induced by DFE

To clarify the roles of TLR1 and TLR6 in innate immunity activation by HDM in vivo, we used the DFE-induced AD mouse model. DFE was topically applied on the ears of C57BL/6 wild-type (WT), TLR1<sup>−/−</sup> and TLR6<sup>−/−</sup> mice twice per week during 6 weeks. Repeated application of DFE significantly increased ear thickness in WT mice. However, topical application of DFE to both TLR1<sup>−/−</sup> and TLR6<sup>−/−</sup> mice did not alter ear thickness (Figure 4a). The WT mice ear skin showed hyperkeratosis and acute inflammatory cell infiltration after DFE induction. TLR1<sup>−/−</sup> and TLR6<sup>−/−</sup> mice exhibited significant diminution of AD based on epidermal and dermal thickness, and infiltration of inflammatory cells compared with WT mice (Figure 4c). TLR2<sup>−/−</sup> mice showed similar symptoms of AD inflammation with WT mice (Supplementary Figure S5a online).

Th2 cells have been characterized as producers of the AD-associated cytokines, such as IL-4, IL-5, and IL-13 (Leung et al., 2004). IL-33, a pro-Th2 cytokine, has been reported in the lesional atopic skins of AD mice and human patients (Salimi et al., 2013). Repeated application of DFE in WT mice elevated serum IgE; however, this elevation was reduced in TLR1<sup>−/−</sup> and TLR6<sup>−/−</sup> mice (Figure 4b). Although expression levels of IL-4, IL-13, and IL-33 were significantly reduced in TLR1<sup>−/−</sup> mice, IL-25 was unaltered comparing WT mice. On the other hand, levels of IL-4, IL-13, IL-25, and IL-33 were significantly decreased in TLR6<sup>−/−</sup> (Figure 4d). We also performed immunohistochemical analysis and ELISA for the comparison of IL-25 and IL-33 in mouse ears. Compared with WT mice, TLR1<sup>−/−</sup> and TLR6<sup>−/−</sup> mice exhibited a significant decrease in IL-25 or IL-33; however, TLR1 did not affect the expression of IL-25 (Figure 4c and Supplementary Figure S5c). We also confirmed the role of TLR2 in the expression of IL-25 and IL-33 in vivo. As shown in Supplementary Figure S5b and c, TLR2<sup>−/−</sup> mice did not reduce IL-25 and IL-33.

Upregulated expression of TLR1, TLR6, IL-25, and IL-33 in DFE-stimulated human primary keratinocytes and human AD skin with high HDM sensitization

We evaluated the effects of HDM on the activation of the TLR1/6-IL-25/33 axis in human primary keratinocytes. As expected, the mRNA expressions of TLR1, TLR6, IL-25, and IL-33 were increased after DFE application (Figure 5a). To further prove the involvement of the TLR1/6-IL-25/33 axis in human AD skin, we tested the gene expression profile of various PRRs (TLR1/TLR2/TLR4/TLR6/TLR9/NOD2) and cytokines associated with Th2 and other immune axes (IL-25/IL-33/IL-17/IL-22) in human AD samples (Supplementary Figures S6, S7, and Supplementary Table S2 online). As shown in Figure 5b, gene expression TLR1, TLR6, IL-25, and IL-33 was increased in human AD skin with high HDM sensitization. Likewise, upregulation of Th2 cytokine mRNA such as IL-4 and IL-13 was also observed in human AD skin with high HDM sensitization. However, IFN-γ, IL-17, and IL-22 were not increased in human AD skin with high HDM sensitization. These data suggest that the activation of the TLR1/6-IL-25/33 axis in keratinocytes by HDM is not a general inflammatory response. Biopsy specimens were also obtained from both lesional skin of eight patients with AD showing high HDM sensitization (class 6, HDM-specific IgE level > 100 IU/ml) and seven patients with AD with no HDM sensitization (class 0, HDM-specific IgE level < 0.35 IU/ml). Demographic and laboratory data of patients are summarized in Supplementary Table S3 online. In the lesional epidermis of patients with AD with high HDM sensitization, the expression of TLR1 and TLR6 was significantly increased compared with skin without HDM sensitization (Figure 5c, d, and g). Moreover, IL-25 and IL-33 expression was significantly increased in the epidermis of the AD skin with high HDM sensitization (Figure 5e–g). These results suggest that the TLR1/6-IL-25/33 axis in
keratinocyte may have some role in the pathogenesis of AD with high HDM sensitization. As shown in Supplementary Tables S2 and S3, the group with high HDM sensitization had a higher IgE value and eosinophil counts than the group with low or no HDM sensitization. In skin samples showing a high IgE value and eosinophil counts, that is, high HDM sensitization, gene and protein expression of TLR1, TLR6, IL-25, and IL-33 were increased.

**DISCUSSION**

Keratinocytes comprise the first line of defense against airborne substances such as HDM allergens and endotoxins (Kuo et al., 2013). We hypothesized that HDM enhances the innate immune system of keratinocytes to cause complex allergic diseases, specifically AD. In the present study, we found TLR1 and TLR6 activation on keratinocytes and the subsequent production of innate proallergic cytokines IL-25 and IL-33, which might potentially promote Th2 cell responses through ILC2 activation.

Recent studies have focused on a variety of genetic and acquired defects in the innate immune system in AD. These abnormalities include epithelial barrier disruptions, reduced antimicrobial peptide release, genetic polymorphisms, dysfunction in PRRs, and diminished recruitment of innate immune cells to the skin (Wollenberg et al., 2011). Some of these defects promote disease development, whereas others are developed as a consequence of the disease process and affect AD severity (De Benedetto et al., 2009). However, few studies have been reported regarding the aggravation of AD due to activation of innate immunity (Dai et al., 2011). In airway allergy, HDM activates TLR4 via its endotoxin contents and subsequently induces the production of IL-25 and IL-33 (Hammad et al., 2009). The role of TLR6 activation has been reported in IL-23 production and Th17 responses, which both regulate the allergic inflammatory response in chronic fungal-induced asthma (Moreira et al., 2011). Moreover, clinical approaches in the field of asthma immunotherapy to target TLRs are already being tried to targeted therapy tailored to the patient’s need (Zeyer et al., 2016). In the present study, we tried to...
found the definite evidence that innate allergic cytokines derived from epidermal keratinocytes (e.g., TSLP, IL-25, and IL-33) induced activation of the innate immune system by HDM that is also involved in the pathogenesis of AD. Consistent with the previous studies in asthma, our results showed that HDM activated the expression of proallergic cytokines, IL-25 and IL-33. In contrast, TLR1 and TLR6 signaling pathways rather than TLR4 played a central role in activating innate immunity in AD. Recent meta-analysis of genome-wide association study also has suggested an association between common allergen sensitivities including HDM and susceptibility loci 4p14 near TLR1 and TLR6 (Hinds et al., 2013).

Keratinocytes, as part of the innate immune defense, contribute to the inflammatory reactions and immune responses in AD by regulating the release of cytokines, chemokines, proteases, and bioactive lipids (Gutowska-Owsiak and Ogg, 2012). On stimulation by various allergens, toxins, or infectious agents, keratinocytes are capable of beginning a cross-talk between the innate and adaptive immune responses by stimulating T cells in patients with AD through the release of key mediators (Cevikbas and Steinhoff, 2012). Cytokines such as IL-33, IL-25, and thymic stromal lymphopoietin or chemokines such as CCL27 have critical roles in this interactive network (Carmi-Levy et al., 2011). We found that the activation or inhibition of TLR1 and TLR6 modulated the IL-25/33-mediated Th2 type response. In addition, we showed differential functionality of TLR1 and TLR6 in the regulation of IL-25 and IL-33. The activation of TLR6 was shown to be essential to the secretion of both IL-25 and IL-33; on the other hand, TLR1 activation was only related to IL-33.

Among the innate immune receptors, TLR2 has received the greatest attention, as it is crucial for protective immune responses to a number of microbes, such as *Staphylococcus aureus* and herpes simplex virus, that more commonly colonize and/or infect the skin of patients with AD (Boguniewicz and Leung, 2010). Each TLR1 and TLR6 heterodimerizes with TLR2 to form receptors for different ligand types (Takeuchi et al., 1999). Because of this heterodimerization of TLR2 with TLR1 or TLR6, we initially predicted that defects in TLR2 would affect the induction of innate immunity via TLR1 and TLR6 triggering of keratinocytes by HDM. However, the activation or inhibition of TLR2 did not affect the susceptibility to AD. These data indicate that there is unique action of TLR1 and TLR6 in the innate immunity activation by HDM.

The activated TLR1/2 and TLR2/6 heterodimer transduces the signal via the adapter protein MyD88, which recruits and activates several IL-1 receptor-associated kinases and tumor necrosis factor receptor associated factor 6 (Akira and Takeda, 2004). Their functions lead to the activation of the IkB kinase complex, which phosphorylates IkB, releasing NF-κB for translocation to the nucleus and transcriptional activation (Hoebe et al., 2006). We examined the molecular mechanism of innate immunity activation by HDM in AD and examined whether TLR1/6-dependent signaling pathways are involved in releasing IL-25 and IL-33. Interestingly, HDM activated the well-known downstream signaling pathways of TLR1/6. These results demonstrate that the stimulatory role of HDM in the production of IL-25 and IL-33 is regulated by TLR1/6-mediated activation of the tumor necrosis factor receptor associated factor 6, IL-1 receptor-associated kinase 1, transforming growth factor β activated kinase-1, and NF-κB pathways in keratinocytes.

IL-33 appears to act by activating ILC2 for the production of large amounts of type 2 cytokines, IL-5 and IL-13
The strong activity of IL-33 on ILC2 and the crucial role of these cells in the initiation of airway allergic inflammation are likely to explain the dominant role of the IL-33/ST2 axis in genetic susceptibility to human asthma (Cayrol and Girard, 2014). However, important questions in understanding the IL-33/ST2 pathway in cutaneous inflammation and immune regulation remain. What physiological and pathophysiological conditions in the skin are related to the activation of IL-33 and ST2 signaling? What physiological and pathophysiological conditions in the skin are related to the activation of IL-33 and ST2 signaling? Our data suggest that IL-33 plays a role in the induction of cutaneous inflammation by stimulating a prompt expression of IL-13-producing ILC2. TLR1 or TLR6 are therefore considered to play a role in regulating ILC2.

Although allergen-specific Th2 cells orchestrate the HDM allergic response, notably through induction of IgE directed toward mite allergens, recent studies have demonstrated that innate immunity activation also plays a critical role in HDM-induced allergy pathogenesis (Jacquet, 2011). The current study provides evidence that HDM allergens trigger activation of TLR1 and TLR6, thus stimulating keratinocyte release of IL-25 and IL-33. Our results demonstrate an array of immunomodulation and proinflammatory activities of HDM on keratinocytes, which may be considered essential factors for their allergenicity. An enhanced keratinocyte function characterized by increased generation of proinflammatory Th2 cells may represent an important mechanism for initiating and amplifying allergic inflammation and sensitization. However, further studies utilizing different HDM types are needed.

**MATERIALS AND METHODS**

**Reagents**

Lyophilized DFE (100 μg/ml, endotoxin level < 625,000 EU/vial) was purchased from Greer Laboratories (Lenoir, NC). To inactivate allergen proteases, DFE was incubated at 65°C for 30 minutes (Dai et al., 2011). In addition, polymyxin B (dissolved at 50 μg/ml and stored for 18 hours at 4°C, Sigma, St. Louis, MO) was used to inactivate endotoxin in the DFE before stimulating the cells. Pam3CSK4 (TLR1/2 agonist) and FSL-1 were used to activate TLR1/6 and TLR2/6, respectively.
(TLR2/6 agonist) were obtained from Invitrogen (Carlsbad, CA). Lipoprotein was purchased from Sigma.

Animals
BALB/c and C57BL/6 wild-type mice were purchased from SLC (Hamamatsu, Japan). TLR1\(^{-/-}\), TLR2\(^{-/-}\), and TLR6\(^{-/-}\) mice on a C57BL/6 background were purchased from the Jackson Laboratory (Bar Harbor, ME). All animals were housed in a specific pathogen-free facility at Kyungpook National University (KNU), and all animal experiments were approved by the Ethics Committee of KNU in accordance with the institutional, regional, and national guidelines (KNU-2015-00501).

Murine model of AD-like lesions induced by DFE
The induction of AD-like lesions by DFE was performed based on our previous research (Kwon et al., 2010; Ryu et al., 2013). Both surfaces of the ear lobes were very gently stripped three times with surgical tape (Nichiban, Tokyo, Japan). After stripping, 20 µl of DFE (10 mg/ml) was painted on each ear. Application with DFE was repeated twice per week for 6 weeks. Two weeks after the first induction, tail bleeding was performed to determine the serum IgE level. Ear thicknesses were measured 24 hours after DFE application with a dial thickness gauge (Mitutoyo, Tokyo, Japan). For details, see the Supplementary Methods online.

Human AD skin
Clinical and demographic data were retrieved from the patient files, and all cases were reviewed by two dermatologists. Skin samples were collected under written, informed patient consent at the Kyungpook National University Hospital from patients with AD in accordance with the approved IRB protocol (KNUH 2015-09-029). See the Supplementary Methods for details on cell culture, transfections, immunoblotting, immunohistochemistry, immunoprecipitation, and other methods (Supplementary Figure S8, Supplementary Table S1 online).
CONFLICT OF INTEREST
The authors state no conflict of interest.

ACKNOWLEDGMENTS
This work was supported by the National Research Foundation of Korea Grant funded by the Korean Government (2014R1A5A2009242, 2012M3A9B6055416, 2016R1A2B4008513, and 2015R1D1A3A01016229), and grants from the KRIBB Research Initiative Program (KGM4251723).

SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2017.03.042.

REFERENCES


Jacquet A. Innate immune responses in house dust mite allergy. ISRN Allergy 2013;2013:735031.


Moreira AP, Cavassani KA, Ismailoglu UB, Hullinger R, Dunleavy MP, Knight DA, et al. The protective role of TLR6 in a mouse model of asthma is mediated by IL-23 and IL-17A. J Clin Invest 2011;121:4420–32.


