



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

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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, I κ B kinase, and NF- κ B pathways. *Tlr6*^{-/-} mice did not show DFE-induced upregulation of IL-25 and IL-33. Furthermore, DFE-induced upregulation of IL-25 was not induced in *Tlr1*^{-/-} 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.

Journal of Investigative Dermatology (2017) 137, 2354–2361; doi:10.1016/j.jid.2017.03.042

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

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Abbreviations: AD, atopic dermatitis; DFE, *Dermatophagoides farinae* extract; DN, dominant negative; FSL-1, TLR2/TLR6 agonist, synthetic diacylated lipoprotein; HDM, house dust mite; IKK, I κ B kinase; ILC, innate lymphoid cell; LPS, lipopolysaccharide; NOD2, nucleotide-binding oligomerization domain 2; Pam3CSK4, TLR1/2 agonist, synthetic triacylated lipoprotein; PRR, pattern recognition receptor; Th2, T helper type 2; TLR, toll-like receptor; WT, wild-type

Received 6 November 2016; revised 26 March 2017; accepted 28 March 2017; accepted manuscript published online 4 July 2017; corrected proof published online 23 September 2017

proteinase-activated receptors-2, and IgE binding, leading to increased inflammation (Hostetler et al., 2010). HDM is a carrier of not only allergenic proteins, but also microbial adjuvant compounds, both of which can stimulate innate signaling pathways and lead to allergy (Jacquet, 2013). However, how the innate immunity triggered by HDM contributes to AD by programming and maintaining Th2-bias adaptive immunity and by the recruitment of inflammatory cells is also not investigated.

In the present study, we examined whether HDM activates the innate immune system in keratinocytes activated with HDM allergens, a murine model with AD-like lesions, and human AD skin lesions. In addition, we investigated the contribution of keratinocyte TLR triggering to the Th2-type adaptive immune response through innate immunity activation.

RESULTS

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 (LL-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 immunity 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

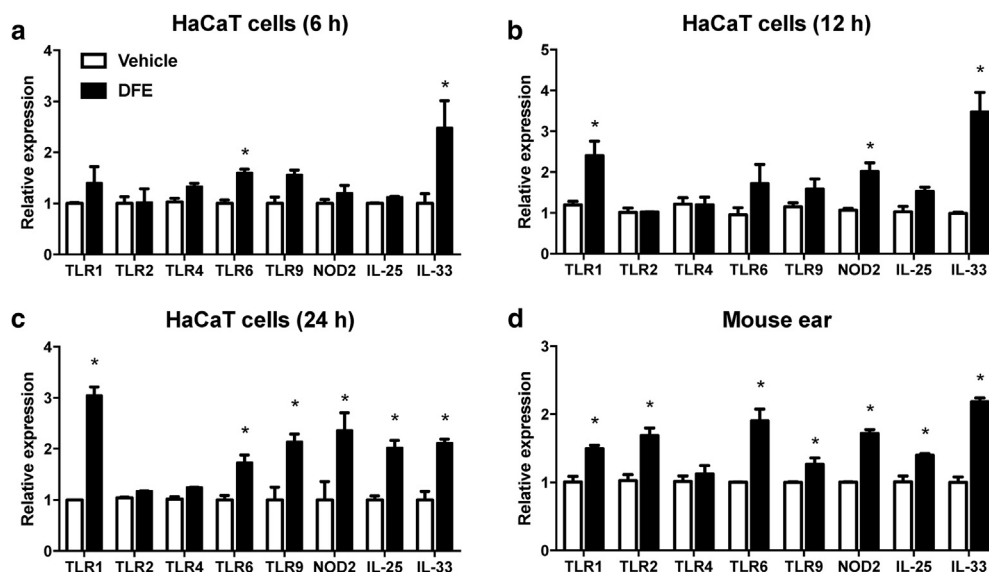
response in AD through IL-25 and IL-33. IL-25 or IL-33 might induce Th2 immune response through accumulation of ILC2, a critical source of type 2 cytokines (Hvid et al., 2011). Therefore, we investigated whether DFE induces the activation of IL-25 and IL-33 through specific PRR activation. We examined 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 of 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 TLR4-mediated proinflammatory cytokine production via the NF-κB-dependent pathway in airway inflammation (Gregory and Lloyd, 2011). It has been reported that an excrement extract prepared from *D. farina* 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, Pam3CSK4 and FSL-1, in keratinocytes. Similar to Pam3CSK4 and FSL-1, DFE promoted the association of tumor necrosis factor receptor

Figure 1. DFE activates the expression of TLR1, TLR6, TLR9, NOD2, IL-25, and IL-33 in vitro and in vivo. (a–c) HaCaT cells were stimulated with DFE (100 µg/ml) for (a) 6, (b) 12, and (c) 24 hours. (d) DFE (10 mg/ml, 20 µl/ear) was topically applied to both ears of BALB/c mice twice per week for 6 weeks ($n = 5$). The ears were excised and gene expression levels of components were analyzed by qPCR. The gene expression levels were normalized to β -actin, and the values of fold changes are represented. Results are presented as the mean $\pm$ SD. * $P < 0.05$. DFE, *Dermaphagoides farinae* extract; NOD2, nucleotide-binding oligomerization domain 2; SD, standard deviation; TLR, toll-like receptor.



associated factor 6 with the IL-1 receptor-associated kinase 1 complex. DFE stimulated the phosphorylation of transforming growth factor- β activated kinase-1 and I κ B kinase, degradation of I κ B α , and nuclear translocation of p65 NF- κ B (Figure 3).

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 $^{-/-}$, and TLR6 $^{-/-}$ 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 $^{-/-}$ and TLR6 $^{-/-}$ mice did not alter ear thickness (Figure 4a). The WT mice ear skin showed hyperkeratosis and acute inflammatory cell infiltration after AD induction. TLR1 $^{-/-}$ and TLR6 $^{-/-}$ mice exhibited significant diminution of AD based on epidermal and dermal thickness, and infiltration of inflammatory cells compared with WT mice (Figure 4c). TLR2 $^{-/-}$ 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 $^{-/-}$ and TLR6 $^{-/-}$ mice (Figure 4b). Although expression levels of IL-4, IL-13, and IL-33 were significantly reduced in TLR1 $^{-/-}$ 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 $^{-/-}$ (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 $^{-/-}$ and TLR6 $^{-/-}$ 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 $^{-/-}$ 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/IFN- γ /IL-1 β /IL-4/IL-13/IL-17A/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 cytokines 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

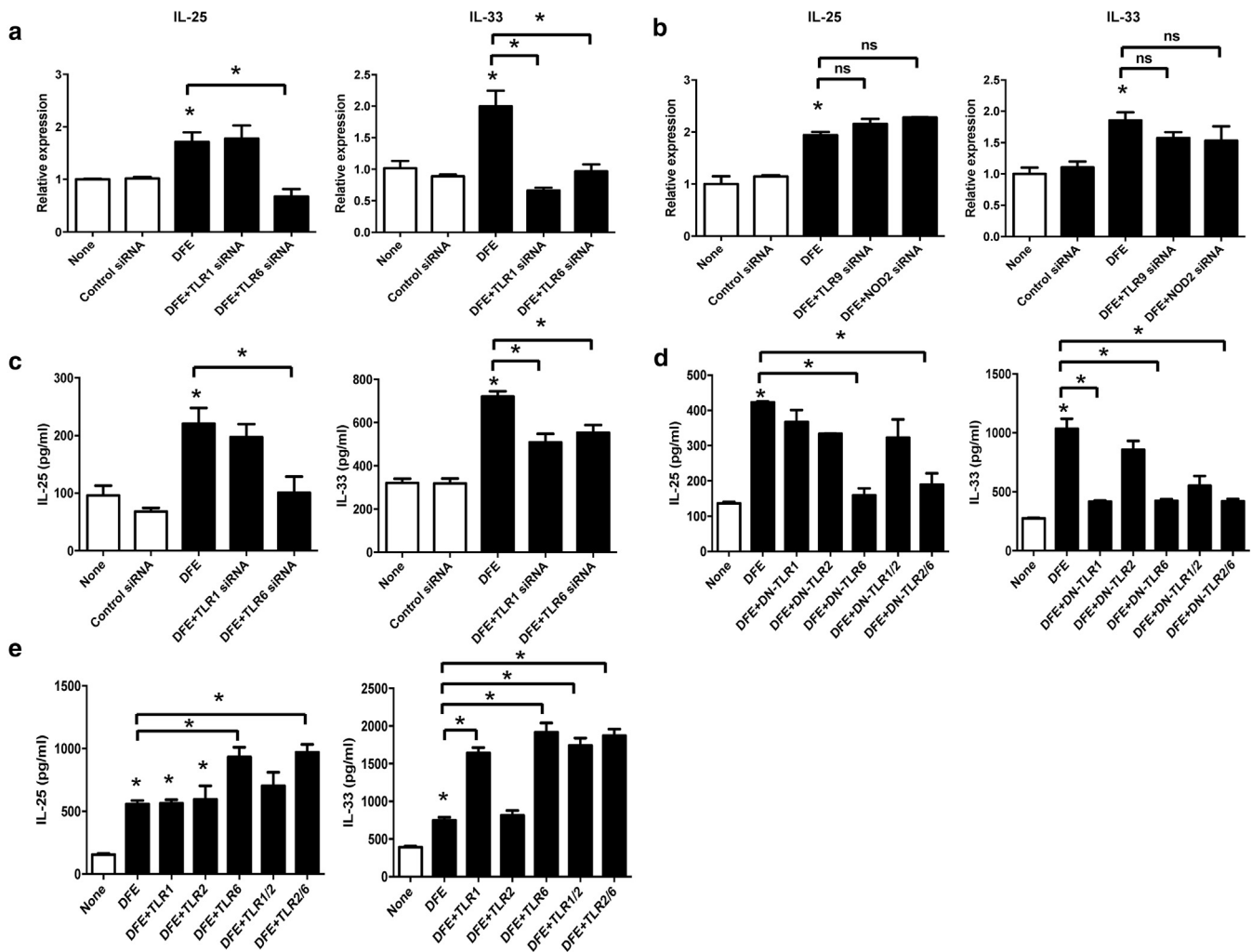


Figure 2. Only TLR1 and TLR6 are related to overexpression of IL-25 and IL-33 by DFE. (a, b) siRNA-transfected *HaCaT* cells were stimulated with DFE (100 μ g/ml) and the expression of IL-25 and IL-33 was measured by qPCR. (c–e) Various types of siRNA, dominant-negative form, or construct were transiently transfected into *HaCaT* cells. After 24 hours of transfection, cells were stimulated with DFE (100 μ g/ml) and secretion of IL-25 and IL-33 was measured by ELISA. The gene expression levels were normalized to β -actin, and the values of fold changes are represented. Results are presented as the mean $\pm$ SD. * P < 0.05. DFE, *Dermaphagoides farinae* extract; SD, standard deviation; siRNA, small interfering RNA; TLR, toll-like receptor.

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.

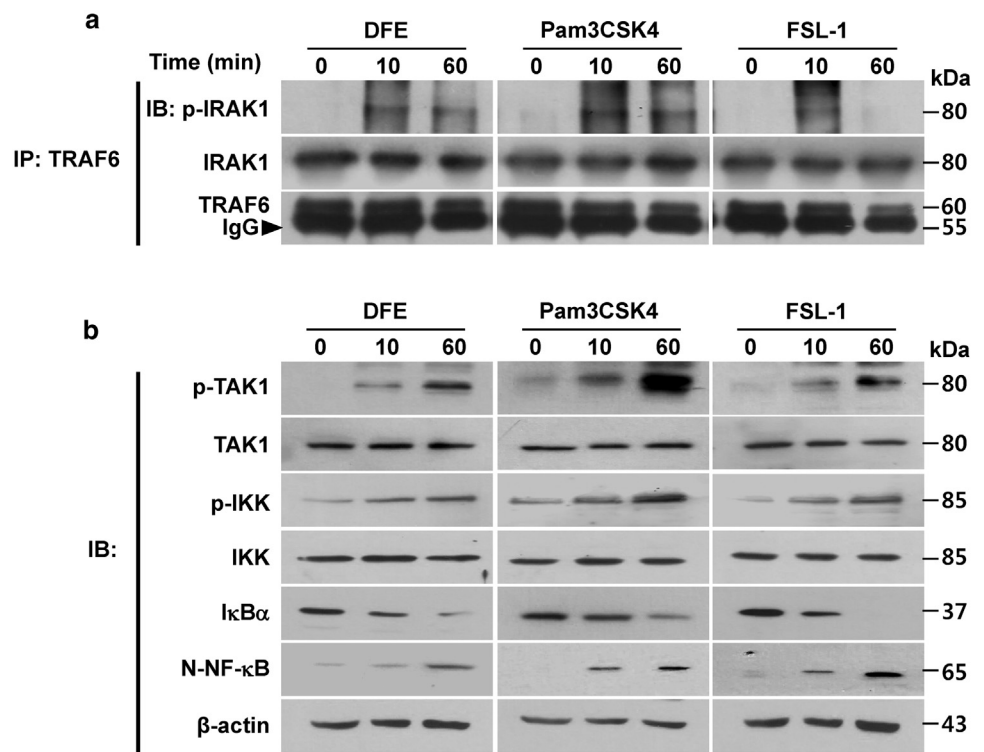
DISCUSSION

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

Figure 3. DFE activates downstream signaling through the TLR1/2 and TLR2/6 signaling pathway. (a) *HaCaT* cells were stimulated with DFE (100 μ g/ml), Pam3CSK4 (1 μ g/ml), and FSL-1 (1 μ g/ml) for the indicated time points and subjected to immunoprecipitation with an anti-TRAF6 antibody. TRAF6 and IRAK1 protein levels from whole-cell lysates and TRAF6 immunocomplexes (IP: TRAF6) were measured by immunoblotting. (b) Activation of TAK1, IKK, and NF- κ B in DFE, Pam3CSK4, and FSL-1 stimulated *HaCaT* cells. Data are representative of two independent experiments. β -Actin served as a loading control.

DFE, *Dermaphagoides farinae* extract; FSL-1, TLR2/TLR6 agonist, synthetic diacylated lipoprotein; IKK, I κ B kinase; IRAK1, IL-1 receptor-associated kinase 1; Pam3CSK4, TLR1/2 agonist, synthetic triacylated lipoprotein; TAK1, transforming growth factor β activated kinase-1; TLR, toll-like receptor; TRAF6, tumor necrosis factor receptor associated factor 6.



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 I κ B kinase complex, which phosphorylates I κ B, releasing NF- κ B for translocation to the nucleus and transcriptional activation (Hoebé 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- κ B 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

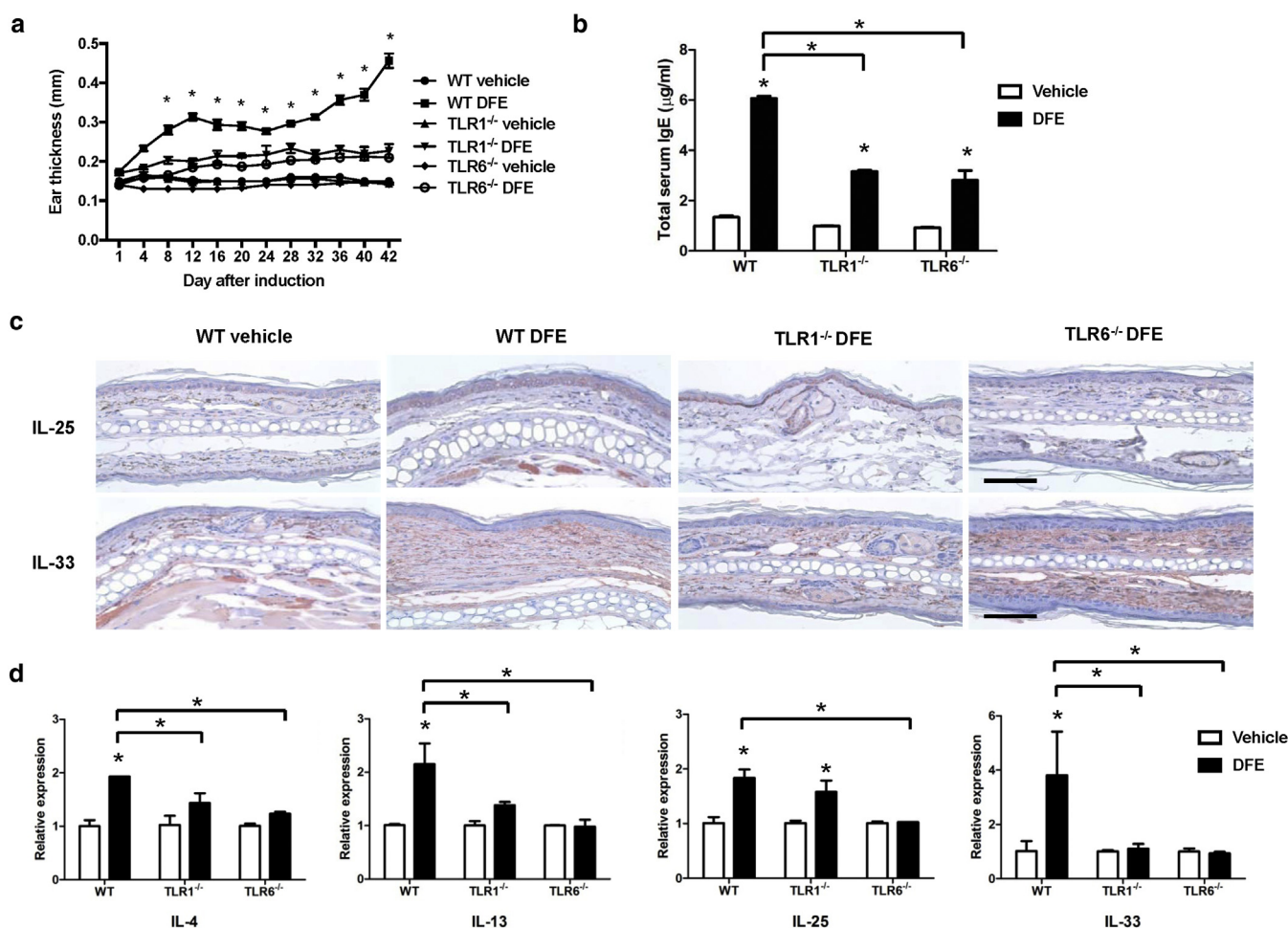


Figure 4. TLR1 and TLR6 are necessary and sufficient for aggravation of Th2 responses and allergic inflammation through IL-25 and IL-33 in the murine model of AD-like lesions induced by DFE. Wild-type (WT, C56BL/6), TLR1^{-/-}, and TLR6^{-/-} mice were used in DFE-induced AD. DFE (10 mg/ml, 20 μl/ear) or vehicle was alternatively applied to both ears twice per week for 6 weeks. (a) Ear thickness of DFE-induced mice at the indicated days. Ear thickness was measured 24 hours after DFE application with a dial thickness gauge. (b) Serum IgE levels in AD mice were measured by ELISA. (c) Representative photomicrographs of ear sections were stained with hematoxylin and eosin, IL-25, and IL-33 (scale bar = 20 μm). (d) The expression of cytokines was analyzed by qPCR. The gene expression levels were normalized to β-actin, and the values of fold changes are represented. Results are presented as the mean ± SD. **P* < 0.05. AD, atopic dermatitis; DFE, *Dermaphagoides farinae* extract; SD, standard deviation; TLR, toll-like receptor.

(Cayrol and Girard, 2014). 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? 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

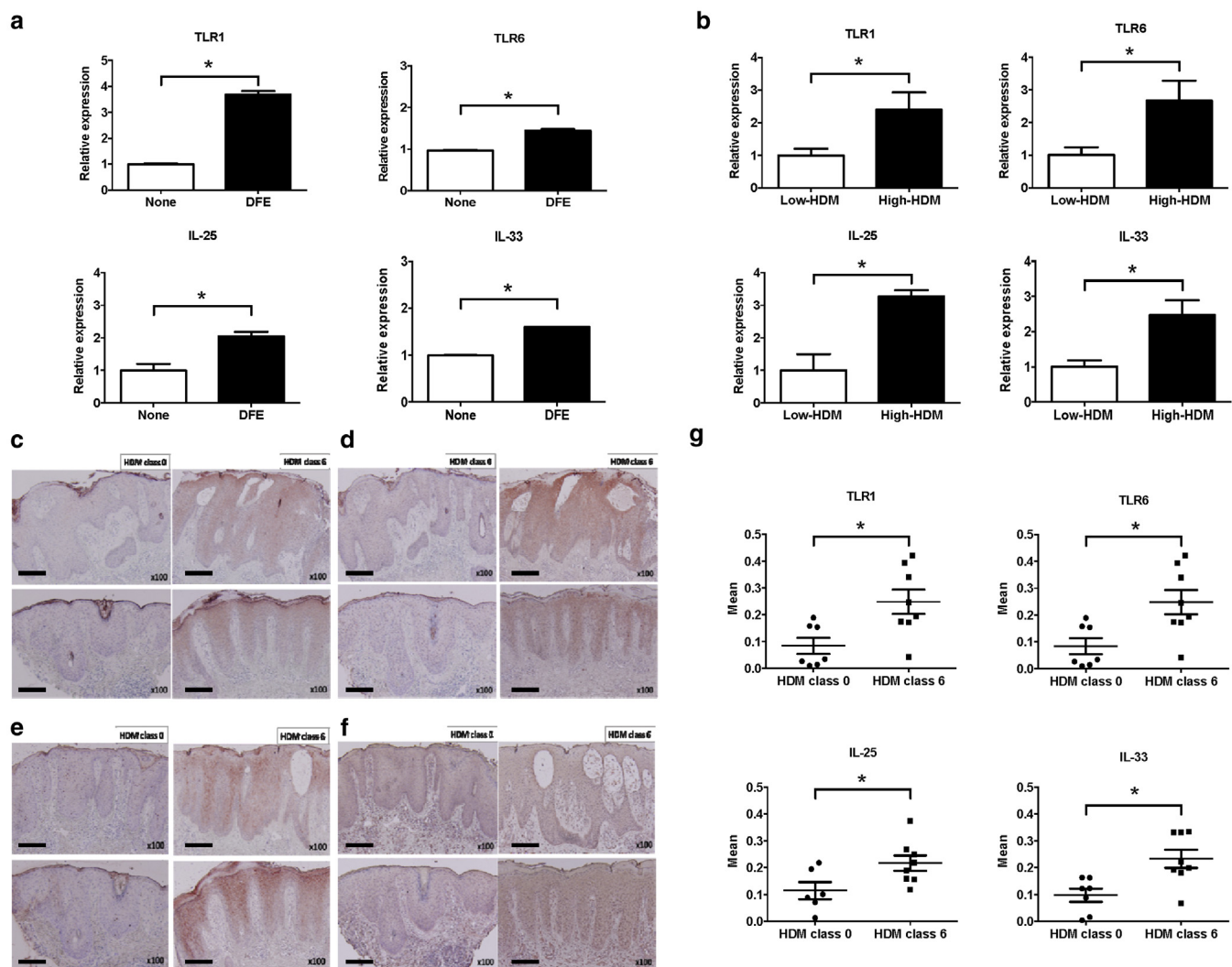


Figure 5. Expressions of TLR1, TLR6, IL-25, and IL-33 were increased in human AD skin with high HDM sensitization. (a) Human primary keratinocytes were stimulated with DFE (100 µg/ml) for 24 hours and the expression of TLR1, TLR6, IL-25, and IL-33 was measured by qPCR. (b) TLR1, TLR6, IL-25, and IL-33 expression as assessed by qPCR from HDM-exposed patients with AD skin. The gene expression levels were normalized to β-actin, and the values of fold changes are represented. Expression of (c) TLR1, (d) TLR6, (e) IL-25, and (f) IL-33 was examined by immunohistochemical staining in the AD skin with high HDM sensitization (class 6, DFE-specific IgE level > 100 IU/ml) and that with no HDM sensitization (class 0, DFE-specific IgE level < 0.35 IU/ml). Scale bar = 20 µm. (g) Quantitative image analysis of TLR1, TLR6, IL-25, and IL-33 expression was performed using Image-Pro Plus software. Results are presented as the mean ± SD. *P < 0.05. AD, atopic dermatitis; DFE, *Dermaphagoides farinae* extract; HDM, house dust mite; SD, standard deviation; TLR, toll-like receptor.

(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>.

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Supplemental information for methods

Cell culture

A human keratinocyte cell line, *HaCaT* cells were cultured in Dulbecco's modified Eagle's medium (Thermo Scientific, Waltham, MA) supplemented with 10% fetal bovine serum and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin) at 37°C in 5% CO₂. The cells were passaged by incubating the monolayer of the cells with prewarmed trypsin-EDTA solution (Thermo Scientific) for 5 to 10 min at room temperature. Trypsin-EDTA solution was added to the cell suspension, and cells were formed into pellets by means of centrifugation at 1200 *g* for 2 min. After this, they were resuspended in culture medium, counted by means of trypan blue exclusion to determine the cell number and viability. The cells were used for experiments at the 3 to 8 passages. The protocol for human primary keratinocytes culture was from a previous report (Nat Protoc 2010;5(2):371-82). Passage 3-seeded cells (2×10^5) were stimulated with DFE (100 µg/mL) for 24 h. After 24 h, gene expression levels were determined by qPCR.

qPCR

To measure cytokine expression, qPCR was carried out using Thermal Cycler Dice TP850 (Takara Bio Inc., Shiga, Japan) according to the manufacturer's protocol. At the end of *in vivo* experimental period, the ears were excised, and total RNA was isolated. *HaCaT* cells were stimulated with DFE, Pam3CSK4, or FSL-1 for 6 - 24 h. For qPCR, 2 µL of cDNA, 1 µL of sense and antisense primer solution, 12.5 µL of SYBR Premix Ex Taq (Takara Bio Inc.), and 9.5 µL of dH₂O were mixed together to obtain a final 25 µL reaction mixture in each reaction tube. The respective primers were chosen by the Primer3 program (Whitehead Institute, Cambridge, MA) and all primers were obtained from Genotech (Daejeon, Korea). List of primers is shown in

Table S1. Levels of gene expression were quantified by using the TP850 software supplied by the manufacturer. The mRNA level was normalized to the gene expression of β -actin and is shown relative to the control group.

ELISA

After the incubation of cells with allergens, culture supernatants were collected and stored at -20°C. The release of human or mouse IL-25 and IL-33 were quantified by using ELISA kits from R&D systems (Minneapolis, MN).

Transfection of keratinocytes with plasmid DNA

pcDNA3-TLR1-YFP, pcDNA3-TLR2-YFP and pcDNA3-TLR6-YFP were purchased from Addgene plasmid (Cambridge, MA). Dominant negative (DN) constructs of TLR1- Δ TIR, TLR2- Δ TIR and TLR6- Δ TIR are TIR-less form of the TLR1, TLR2 and TLR6 gene generated by deleting the TIR domain. Deleted, TLR1, TLR2 and TLR6 constructs were generated by the EZchange™ Site-directed Mutagenesis Kit (Enzymomics, Daejeon, Korea) as recommended by the manufacturer. TLR1, TLR2 and TLR6 DN constructs were confirmed by PCR with the following primers: TLR1- Δ TIR F 5'-CTC GAG ATG GTG AGC AAG GG-3C, R 5'-AAG AAC TCC AAA GAA ATC TC-3G, TLR2- Δ TIR F 5'-CTC GAG ATG GTG AGC AAG GG-3C, R 5'-CCA GCA GGA ACA TCT GCT AT-3A, TLR6- Δ TIR F 5'-CTC GAG ATG GTG AGC AAG GG-3C, R 5'-AAG AAC TCC AAA GAA ACC TC-3G, pcDNA3 F 5'-GAC GAC GGC AAC TAC AAG AC-3C, R 5'-GAC GAC GGC AAC TAC AAG AC-3C. Constructs of TLR in *HaCaT* cells were transiently transfected using lipofectamine 2000 (Invitrogen) according to manufacturer's instructions.

Transfection of keratinocytes with siRNA

Cells were cultured until 70% to 80% confluent; then the medium was changed to basal medium without the supplements, and the cells were incubated for 6 h. Keratinocytes were transfected with the following Stealth siRNAs using lipofectamine 2000: TLR1-siRNA, TLR6-siRNA, TLR9-siRNA, NOD2-siRNA, and negative control siRNA were purchased from Santa Cruz Biotechnology. Lipofectamine 2000 (2 μ L) was mixed with 2 μ L of a 20 pmol/L siRNA solution and 100 μ L of serum free medium. After incubation for 25 min at room temperature, basal medium without the supplements was added to a total volume of 600 μ L. Then 600 μ L of solution was added per well to the 12-well plates, respectively. After cultivation with siRNAs for 6 h, the serum free medium was changed to serum add medium. After further cultivation for 18 h, keratinocytes were stimulated with DFE. The expression of mRNA or protein levels at 24 h after stimulation relative to that in the medium was analyzed by means of qPCR and ELISA.

Murine model of AD-like lesions induced by DFE

Both surfaces of the ear lobes were very gently stripped three times with surgical tape (Nichiban, Tokyo, Japan). After stripping, 20 μ L of DFE (10 mg/mL) was painted on each ear. Treatment of DFE was repeated twice a week alternatively for 6 weeks. Two weeks after the first induction, tail bleeding was performed to check the serum IgE level. Ear thicknesses were measured 24 h after DFE application with a dial thickness gauge (Mitutoyo, Co., Tokyo, Japan). On day 42, blood samples were collected by celiac artery before euthanasia. The serum was stored at -70°C for further analysis. After blood collection, ears were removed and used for a histopathological analysis. Serum IgE level was measured using an ELISA kit (BD Biosciences, Oxford, UK)

according to the manufacturer's instructions. The DFE-specific and lipoprotein-specific IgE levels were indicated by the O.D. value.

Histological observation

The patients and mouse skin fixed with 4% formaldehyde and embedded in paraffin. Staining was performed using the avidin-biotin-horseradish peroxidase methods (ABC standard; Vector Laboratories, Burlingame, CA). Antigen retrieval was performed by autoclaving in a 0.01 M citrate buffer (pH 6.0) for 10 min. The following primary antibodies were used: anti-TLR1, anti-TLR6, anti-IL-25, and anti-IL-33 (mouse IgG1 κ , eBioscience, San Diego, CA). Color was developed using 3,3-diaminobenzidine as a substrate solution (KPL, Gaithersburg, MD). For quantitative evaluation of the immunoreactivity of TLR1, TLR2, TLR6, IL-25 and IL-33 in human and mouse skin, computer-based software (Image-Pro Plus; Media Cybernetics, Silver Spring, MD) was used.

Immunoprecipitation and immunoblotting

Cells were lysed in non-denaturing lysis buffer (50 mM Tris-HCl [pH 7.4], 5 mM EDTA, 1% Triton X-100, 0.02% Sodium azide) supplemented with a protease inhibitor mixture composed of leupeptin, pepstatin A, aprotinin, and PMSF. The lysates were cleared of insoluble materials by centrifugation at 12,000 *g* for 20 min. For immunoprecipitation, the lysates equalized for total protein amounts were incubated with appropriate antibodies for overnight and then 2 h with protein G–agarose beads at 4°C. The beads were washed three times with wash buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 0.02% Sodium azide), and the immunoprecipitated proteins were eluted by mild denaturation in the SDS sample buffer for 5

min at 95°C. Proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with the indicated antibodies. The following primary antibodies were used: rabbit anti-TRAF6, anti-phospho-IRAK1, anti-IRAK1, anti-I κ B α , anti-NF- κ B (p65), and mouse anti- β -actin (Santa Cruz Biotechnology); rabbit anti-phospho-TAK1, anti-TAK1, anti-phospho IKK, and anti-IKK (Cell Signaling, Danvers, MA). The immunodetection was performed using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

Statistical analysis

Statistical analyses were performed using Prism 5 (GraphPad Software, San Diego, CA). Treatment effects were analyzed using a one-way ANOVA, followed by Tukey's multiple comparisons testing. $p < 0.05$ were considered statistically significant.

Table S1. List of primers for qPCR experiments

Gene		Forward primer (5' -3')	Reverse primer (5' -3')
Human	TLR 1	GGG TCA GCT GGA CTT CAG AG	GGA ACG GGC TAA TTT TGG AT
	TLR 2	GGG TTG AAG CAC TGG ACA AT	TTG GAC AGG TCA AGG CTT TT
	TLR 4	TAC AGA AGC TGG TGG CTG TG	CCG CAA GTC TGT GCA ATA AA
	TLR 6	AGT AGC TGG GCT TGC ATT GT	TTA TTG GAG GGC CTT GAG TG
	TLR 9	AAG GGG TGA AGG AGC TGT CT	ACA GCA GCT ACA GGG AAG GA
	NOD2	AAG TTC AGG TTC ACG GAT CG	CCT GTT CAG AGA AGC CCT TG
	LL-37	CTA GAG GGA GGC AGA CAT GG	AGG AGG CGG TAG AGG TTA GC
	β -defensin-1	CGC CAT GAG AAC TTC CTA CC	CAC TTG GCC TTC CCT CTG TA
	β -defensin-3	TGT TTG CTT TGC TCT TCC TG	TTT CTT TCT TCG GCA GCA TT
	S100A7	GGC ATG ATC GAC ATG TTT CA	CCC AGC AAG GAC AGA AAC TC
	S100A8	ATT TCC ATG CCG TCT ACA GG	TGG CTT TCT TCA TGG CTT TT
	S100A9	CAG CTG GAA CGC AAC ATA GA	TCA GCT GCT TGT CTG CAT TT
	Dermcidin	CCT CTT CCT GAC AGC TCT GG	CTC CGT CTA GGC CTT TTT CC
	IL-25	TAG GGC CAG TGA AGA TGG AC	GCC GGT AGA AGA CAG TCT GG

IL-33	TAG GGC CAG TGA AGA TGG AC	GCC GGT AGA AGA CAG TCT GG
TSLP	GAA AGC TCT GGA GCA TCA GG	AGG GAA CAT ACG TGG ACA CC
Kallikrein 5	AAA AGG TGC GAG GAT GCT TA	TGA ACT TGC AGA GGT TCG TG
PAR2	ATC CCA CGT CAC TGG AAA AG	ATC ACA GCA GGG TGC TTC TT
Claudin-1	CGA TGA GGT GCA GAA GAT GA	AAG GCA GAG AGA AGC AGC AG
Claudin-23	CTT GCC ATG CAA ACT CTC AA	TCG ACT TTC ACC GCA AAA AT
Occludin	CCG ACA CAC CAC ACC TAC AC	CTG GAT GAC ATG GCT GAT TG
IFN- γ	CTT TAA AGA TGA CCA GAG CAT C	ATC TCG TTT CTT TTT GTT GCT ATT GA
IL-4	ACT TTG AAC AGC CTC ACA GAG	TTG GAG GCA GCA AAG ATG TC
IL-13	TGA GGA GCT GGT CAA CAT CA	CAG GTT GAT GCT CCA TAC CAT
IL-17A	CAA CCG ATC CAC CTC ACC TT	GGC ACT TTG CCT CCC AGA T
IL-22	GCA GGC TTG ACA AGT CCA ACT	GCC TCC TTA GCC AGC A TGA A
IL-1 β	AGC ACC TCT CAA GCA GAA AAC A	TTG CAT GGT GAA GTC AGT TAT ATC C
β -actin	GGA CTT CGA GCA AGA GAT GG	AGC ACT GTG TTG GCG TAC AG

<i>Mouse</i>	<i>Tlr 1</i>	CTG CCC TGG TAT GTG AGG AT	TCC TCT CAT GGA GGC AAA TC
	<i>Tlr 2</i>	AAG AGG AAG CCC AAG AAA GC	CGA TGG AAT CGA TGA TGT TG
	<i>Tlr 4</i>	AAC ATG GCA GTT TCT GAG CA	CGA GGC TTT TCC ATC CAA TA
	<i>Tlr 6</i>	AAC AGG ATA CGG AGC CTT GA	CCA GGA AAG TCA GCT TCG TC
	<i>Tlr 9</i>	TTC CTG CCG CTG ACT AAT CT	GCC AGG CTA AGG CTG TGT AG

<i>Nod 2</i>	AGT GGC TCA GCT TGG TAG GA	AGT GGC TCA GCT TGG TAG GA
<i>Il-37</i>	GTC TTG GGA ACC ATG CAG TT	CAG GTC CAG GAG ACG GTA GA
<i>S100a7</i>	CAG GCA GTC TCT CAT CAC CA	CCC AAA ACG ATG TTC CTG AT
<i>S100a8</i>	GGA AAT CAC CAT GCC CTC TA	TCC TTG TGG CTG TCT TTG TG
<i>S100a9</i>	CAG CAT AAC CAC CAT CAT CG	GCT CAG CTG ATT GTC CTG GT
<i>Il-25</i>	CTC AAC AGC AGG GCC ATC TCT C	CTG TCT GTA GGC TGA CGC AGT GTG
<i>Il-33</i>	CTG GCC TCA CCA TAA GAA AGG A	AGG GAG GCA GGA GAC TGT GTT AAA
<i>Tslp</i>	CGA CAG CAT GGT TCT TCT CA	CGA TTT GCT CGA ACT TAG CC
<i>Kallikrein 5</i>	GGG GAT GTT TCC TCT TGT GA	GCC CCA CAG TAC AGC TTG TT
<i>Par2</i>	TCT CTG CAC CAA TCA CAA GC	CTT AGC CTT CTT GCC AGG TG
<i>Claudin-1</i>	TTT TCC CGA TGA CCT TTC TG	AGT TTG CAG GAT CTG GGA TG
<i>Claudin-23</i>	CTC CCT GCT TCA GTC TCC AG	GGT ATC TCG GGA ACA GGA CA
<i>Occludin</i>	CCT CCA ATG GCA AAG TGA AT	CTC CCC ACC TGT CGT GTA GT
<i>β-actin</i>	TAG ACT TCG AGC AGG AGA TG	TTG ATC TTC ATG GTG CTA GG

Table S2. Clinical and laboratory findings in patients with AD for real-time PCR.

	High HDM sensitization* (n=6)	Low HDM sensitization* (n=5)	<i>p</i> [‡]
Sex	Male (n=4), Female (n=2)	Male (n=2), Female (n=3)	
Age	21.5 ± 2.7	31.4 ± 5.9	0.107
AD Severity (by EASI [†])	38.5 ± 3.7	30.2 ± 4.1	0.772
Combined allergic diseases	Allergic rhinitis & Asthma (n=1)	None	
Total IgE (kU/L)	3015.3 ± 835.3	807.2 ± 288.9	0.015
Total eosinophil count(/mm ³)	764.0 ± 219.7	377.5 ± 153.1	0.035

*The HDM-specific IgE level was classified into seven quantitative classes by the following criteria: class 0, below 0.35 IU/mL; class 1, 0.35 to 0.69 IU/mL; class 2, 0.7 to 3.49 IU/mL; class 3, 3.5 to 17.49 IU/mL; class 4, 17.5 to 49.99 IU/mL; class 5, 50 to 99.99 IU/mL; and class 6, above 100 IU/mL. The patients were divided into two groups, the low sensitization group, composed of HDM-specific IgE classes 0&1, and the high sensitization group, composed of classes 5&6.

[†]Eczema area and severity index

[‡]Statistical analysis was done using Mann-Whitney U test.

Table S3. Clinical and laboratory findings in patients with AD for immunohistochemical analysis.

	High HDM sensitization* (n=8)	No HDM sensitization* (n=7)	p [‡]
Sex	All males	All males	
Age	22.9 ± 0.7	22.9 ± 4.03	0.516
AD Severity (by EASI [†])	34.1 ± 1.4	33.1 ± 2.60	0.904
Combined allergic diseases	Allergic rhinitis & Asthma (n=3)	None	
	Allergic rhinitis only (n=3)		
Total IgE (kU/L)	4549.8 ± 400.1	749.2 ± 468.9	0.012
Total eosinophil count(/mm ³)	912.9 ± 253.6	472.8 ± 111.3	0.038

*The HDM-specific IgE level was classified into seven quantitative classes by the following criteria: class 0, below 0.35 IU/mL; class 1, 0.35 to 0.69 IU/mL; class 2, 0.7 to 3.49 IU/mL; class 3, 3.5 to 17.49 IU/mL; class 4, 17.5 to 49.99 IU/mL; class 5, 50 to 99.99 IU/mL; and class 6, above 100 IU/mL. The patients were divided into two groups, no sensitization group and the high sensitization group, composed of classes 5&6.

[†]Eczema area and severity index

[‡]Statistical analysis was done using Mann-Whitney U test.

Legends for Supplementary Figures

Fig. S1. HDM activates chemical, immunologic, and physical barriers in *HaCaT* cells. Cells were stimulated with HDM (100 µg/mL) for 6 **(a)**, 12 **(b)**, and 24 **(c)** h. Gene expression was analyzed by qPCR. The gene expression levels were normalized to β-actin, and the values of fold-changes are represented. Results are presented as the mean ± SD. * $p < 0.05$

Fig. S2. Application of HDM induces innate and Th2 adaptive immunity responses in AD mice. HDM (10 mg/mL, 20 µL/ear) was topically applied to both ears of female BALB/c mice twice per week during six weeks ($n = 5$). **(a)** Ear thickness of HDM-induced mice at the indicated days. Ear thickness was measured 24 h after HDM application with a dial thickness gauge. **(b)** Representative photomicrographs of ear sections were stained with hematoxylin and eosin (scale bar = 20 µm). **(c)** Serum total IgE, HDM-specific IgE, and lipoprotein-specific IgE levels were measured using ELISA. **(d)** Epidermal and dermal thickness and numbers of infiltrated eosinophils. **(e)** Mice ear skin barrier components were analyzed by qPCR. Results are presented as the mean ± SD. * $p < 0.05$

Fig. S3. Analysis of negative control siRNA-transfected silencing of IL-25 and IL-33 in DFE-stimulated *HaCaT* cells by qPCR. The gene expression levels were normalized to β-actin, and the values of fold-changes are represented.

Fig. S4. Synthetic TLR1/2 and TLR2/6 ligands induce the production of IL-25 and IL-33 in *HaCaT* cells. **(a)** Cells were stimulated with synthetic TLR1/2 and TLR2/6 ligands, Pam3CSK4 (1 µg/mL) and FSL-1 (1 µg/mL), respectively. The expression of IL-25 and IL-33 were

measured by qPCR. **(b)** Cells were transiently transfected with various types of TLR constructs. After 24 h, the cells were stimulated with Pam3CSK4 (1 µg/mL) and FSL-1 (1 µg/mL). Secretion of IL-25 and IL-33 was measured by ELISA. The gene expression levels were normalized to β-actin, and the values of fold-changes are represented. Results are presented as the mean ± SD. * $p < 0.05$

Fig. S5. DFE-induced of IL-25 and IL-33 upregulation did not found in *Tlr6*^{-/-} and *Tlr1*^{-/-} mice.

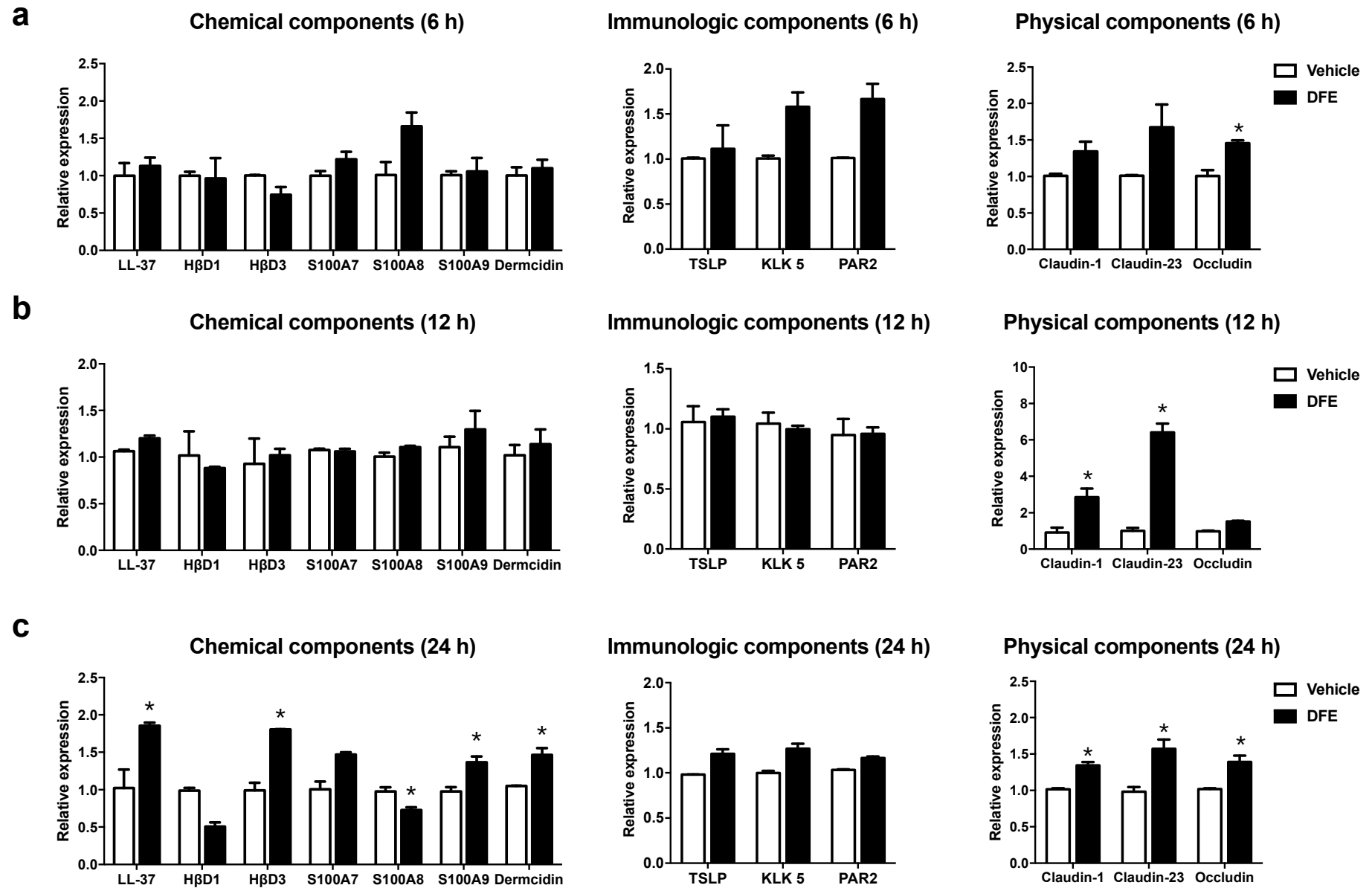
(a) Representative photomicrographs of ear sections stained with hematoxylin and eosin (scale bar = 20 µm). **(b)** Representative photomicrographs of TLR2 knockout mice ear sections stained with IL-25 and IL-33 (scale bar = 10 µm). **(c)** The serum levels of IL-25 and IL-33 were measured by ELISA. Results are presented as the mean ± SD. * $p < .05$

Fig. S6. TLR2, TLR4, TLR9, and NOD2 expression as assessed by qPCR analysis from skin samples of HDM-sensitized and non-sensitized AD patients. The gene expression levels were normalized to β-actin, and the values of fold-changes are represented.

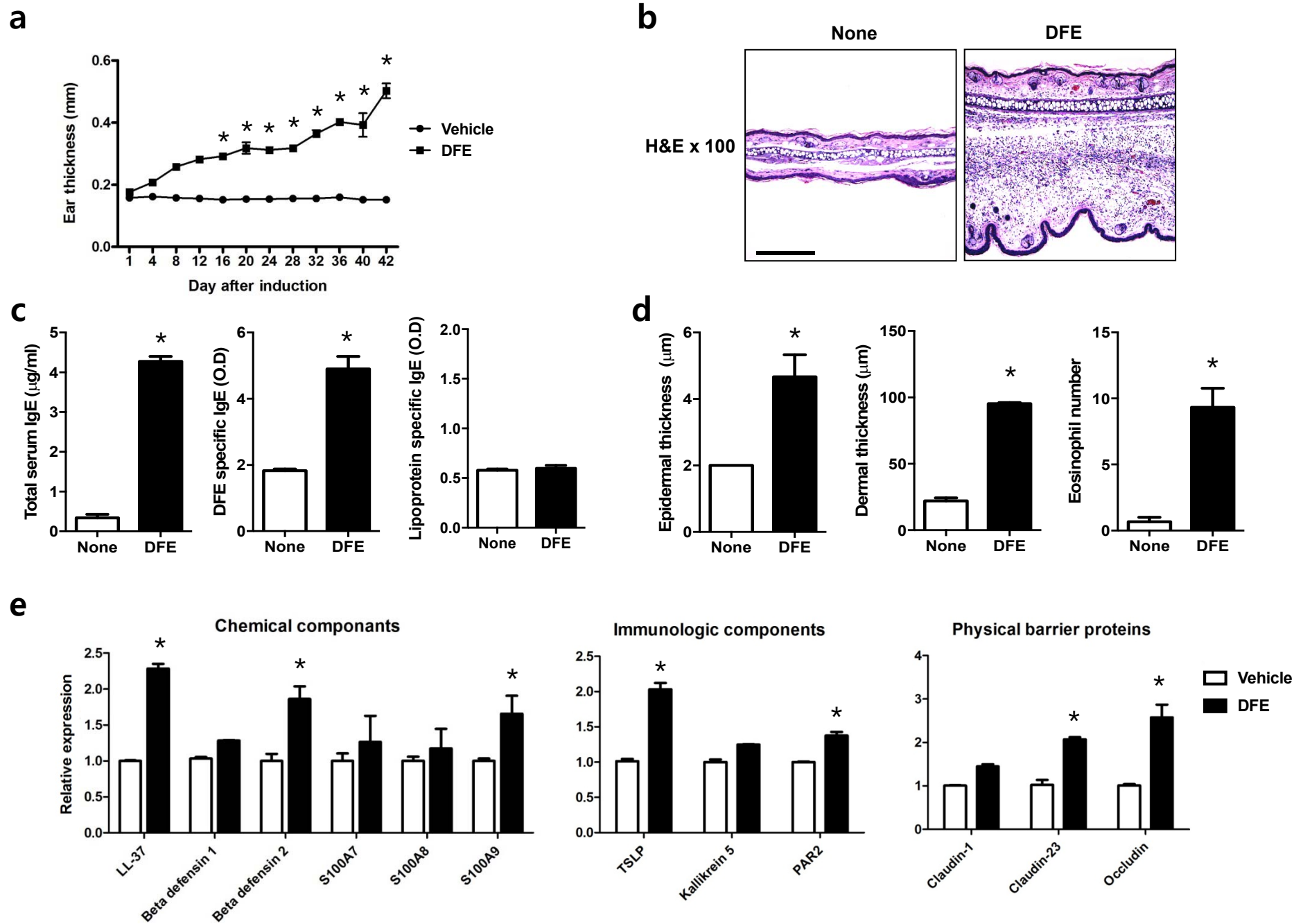
Fig. S7. AD patients with high HDM sensitization only showed activation of Th2 immune axis not, Th1, Th17, and Th22 response. Expression of cytokines was measured by qPCR. The gene expression levels were normalized to β-actin, and the values of fold-changes are represented. Results are presented as the mean ± SD. * $p < .05$

Fig. S8. IL-1α increased in DFE-stimulated human primary keratinocytes. Human primary keratinocytes were stimulated with DFE (100 µg/mL) for 24 h and the expression of IL-1α and

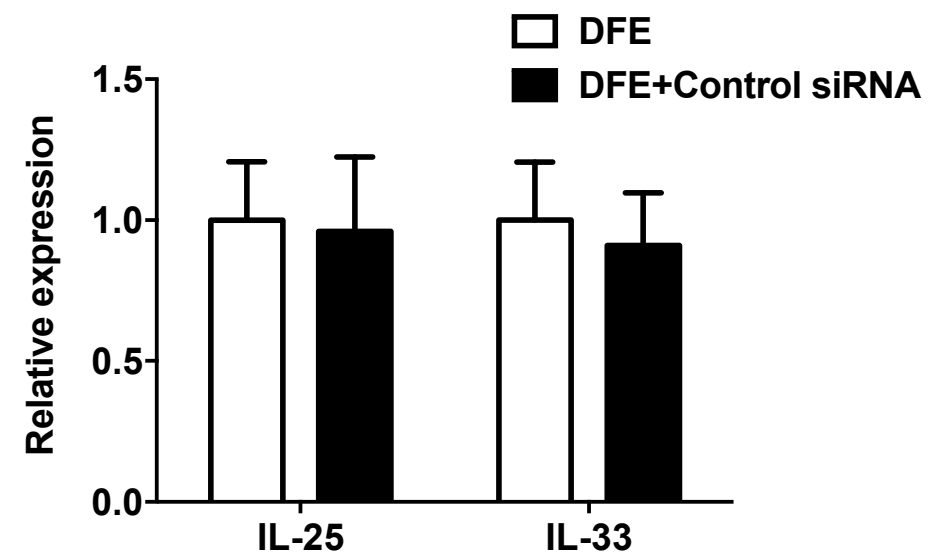
IL-1 β were measured by qPCR. The gene expression levels were normalized to β -actin, and the values of fold-changes are represented. Results are presented as the mean $\pm$ SD. * $p < .05$



Supplementary Fig. S1

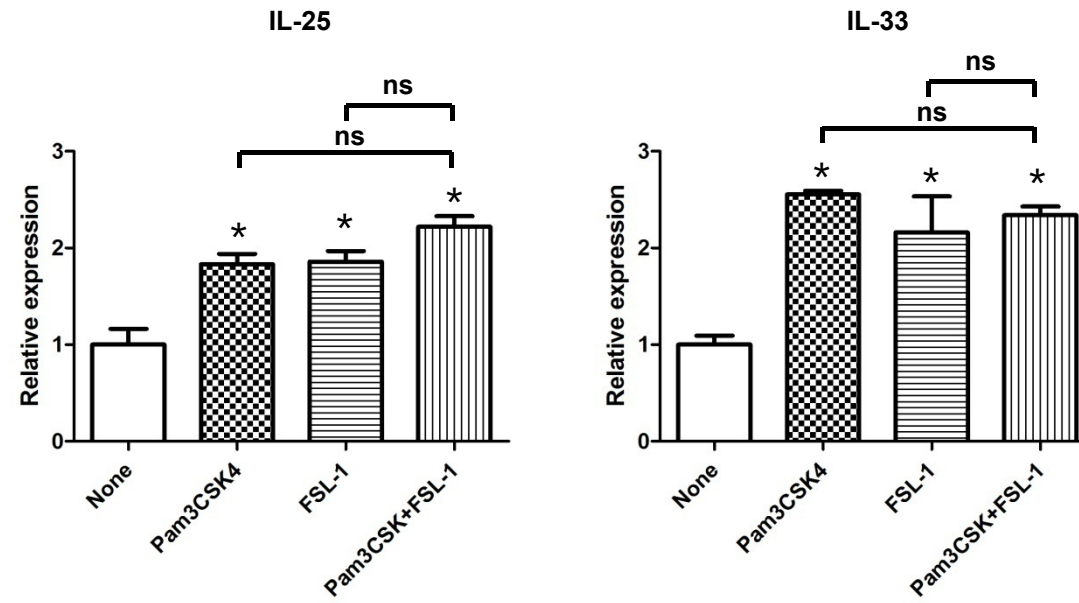


Supplementary Fig. S2

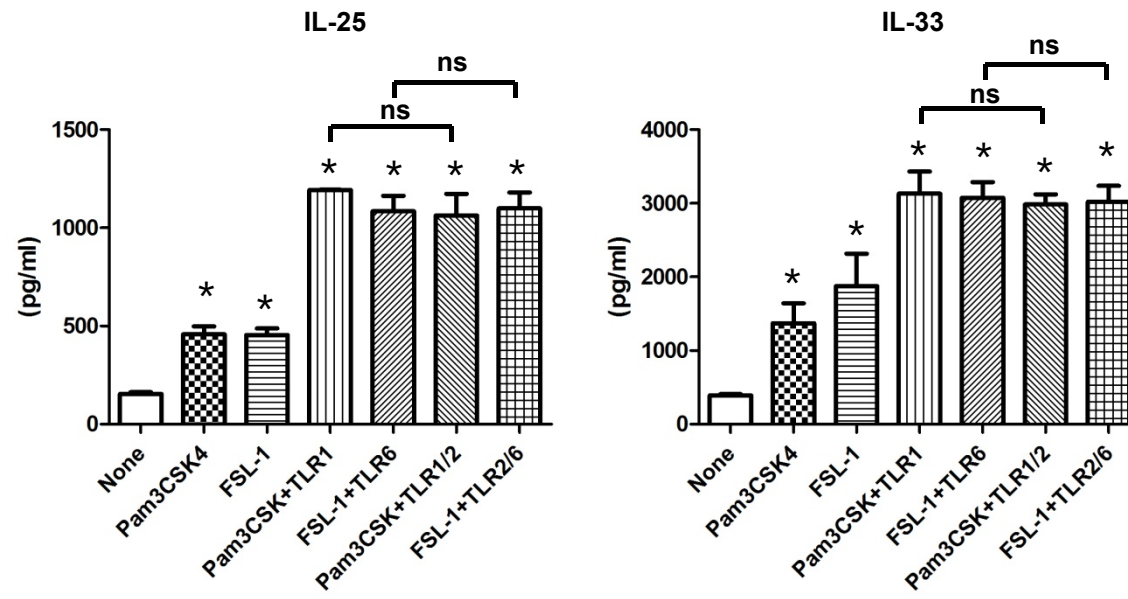


Supplementary Fig. S3

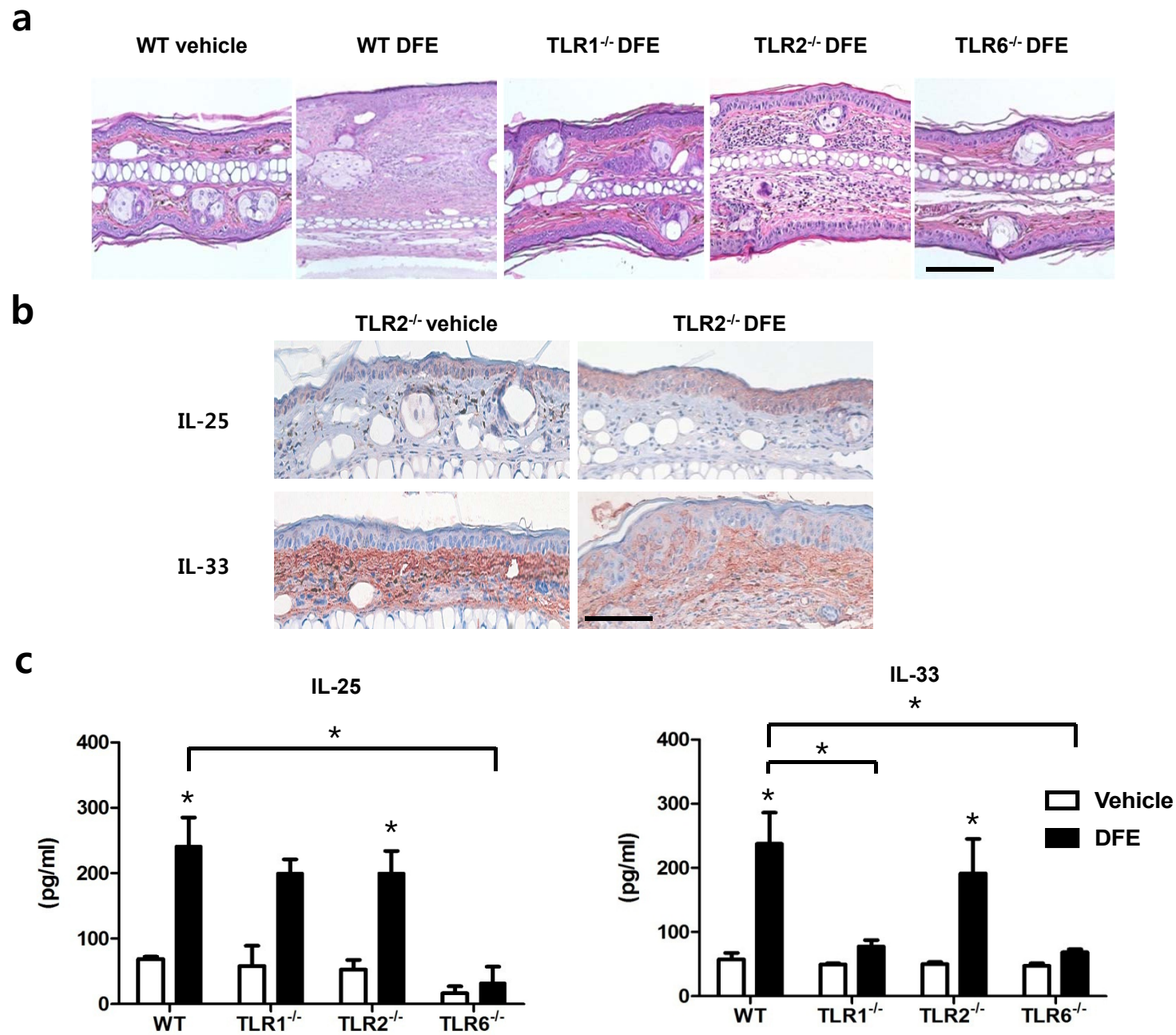
a



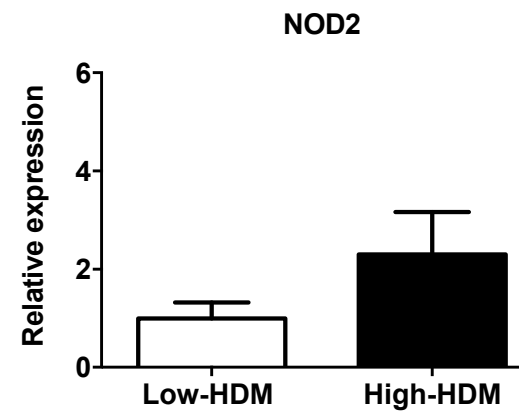
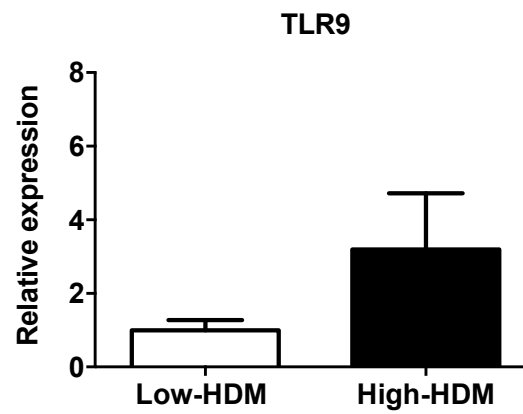
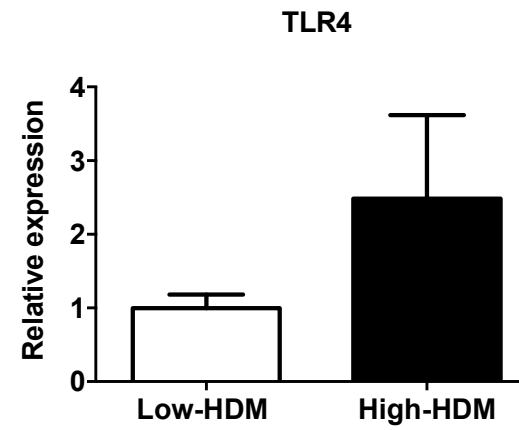
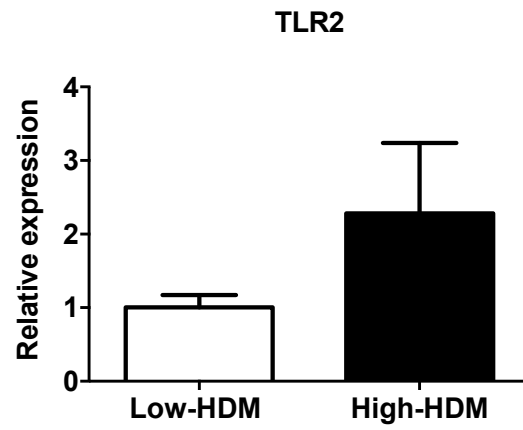
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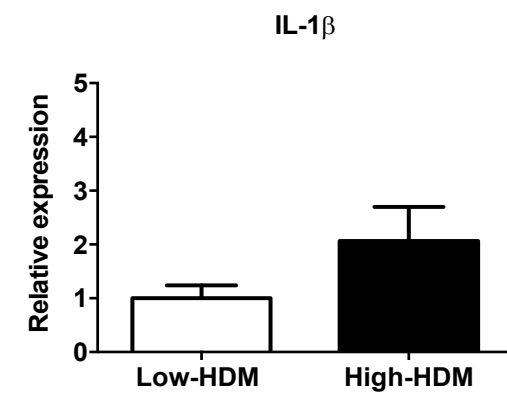
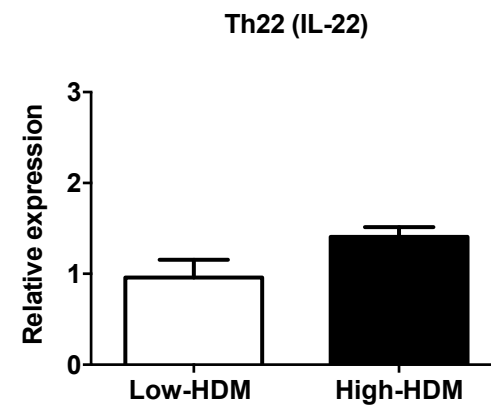
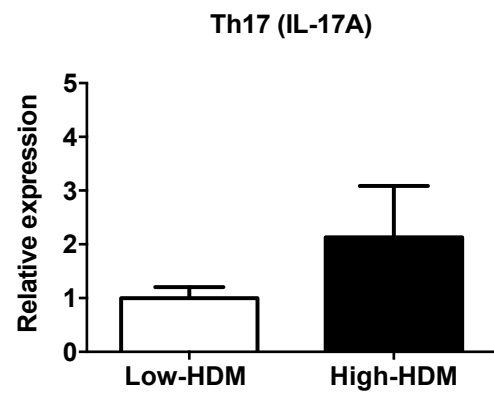
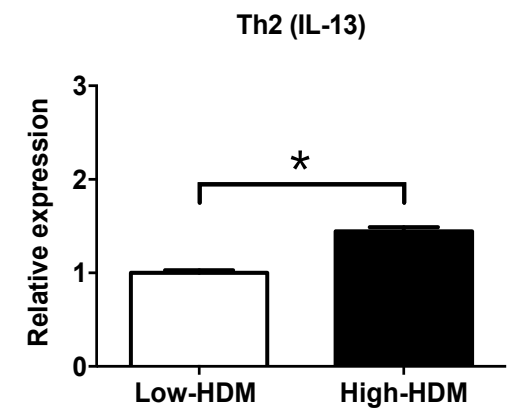
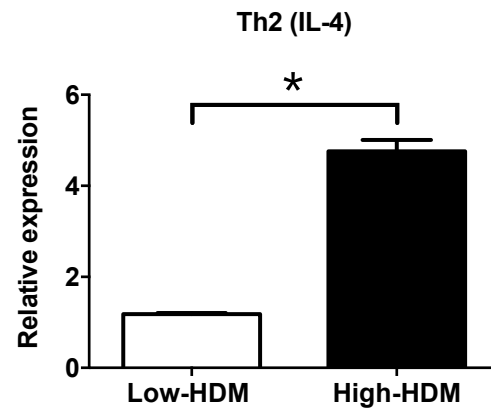
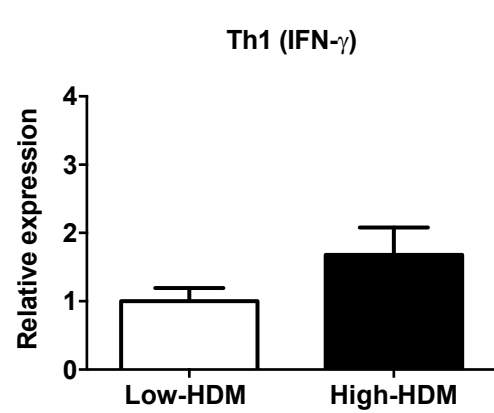
Supplementary Fig. S4



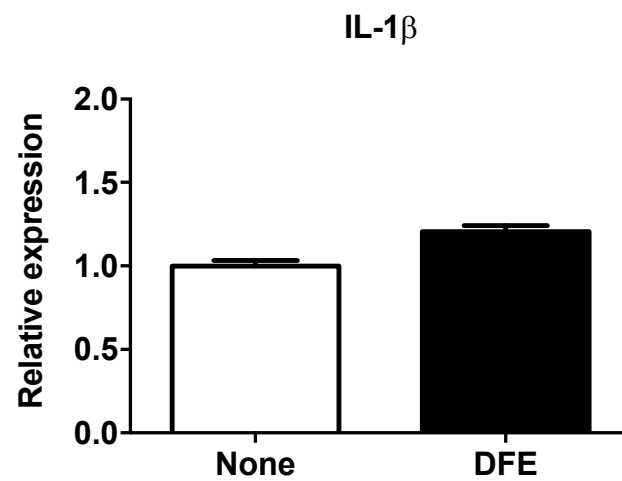
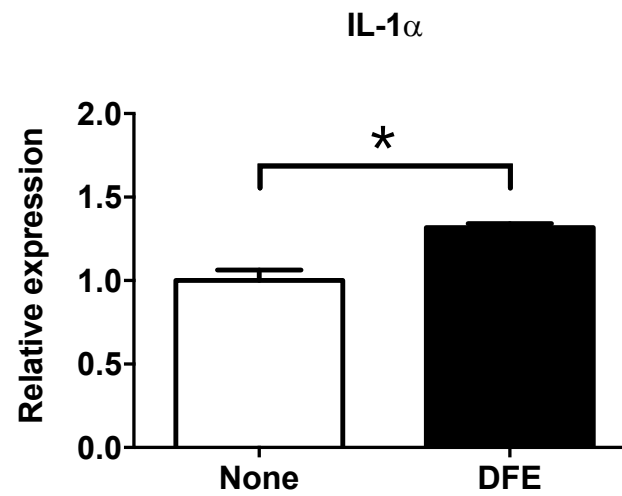
Supplementary Fig. S5



Supplementary Fig. S6



Supplementary Fig. S7



Supplementary Fig. S8