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The crucial role of IL-22 and its receptor on TARC production and T cell migration by HDM extract

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Abstract

House dust mite (HDM) is known as one of the factors that causes atopic dermatitis (AD). Interleukin (IL)-22 and thymus and activation regulated chemokine (TARC) are related to skin inflammatory disease and highly expressed in AD lesions. However, the effects of HDM on IL-22 production in T cells and on TARC production and IL-22R α receptor expression in keratinocytes are unknown. To identify the role of HDM in keratinocytes and T cells, we investigated IL-22R α expression and TARC production in the human keratinocyte cell line HaCaT and IL-22 production in T cells treated with HDM extract as well as their roles in HDM-induced skin inflammation. HDM extract not only increased IL-22R α expression and TARC production in HaCaT but also enhanced IL-22, tumor necrosis factor (TNF)- α and interferon (IFN)- γ production in T cells. The HDM extract-induced IL-22 from T cells significantly increased the production of IL-1 α , IL-6 and TARC in HaCaT cells. In addition, we found that TARC produced in HDM extract-treated HaCaT induced T cell recruitment. These results suggest that there is a direct involvement of HDM extract-induced IL-22 in TARC production and T cell migration. Taken together, TARC production in HaCaT through the interaction between IL-22 and IL-22R α facilitates T cell migration. These data show one of the reasons for inflammation in the skin lesions of AD patients.

Abbreviations: AD, atopic dermatitis; HDM, house dust mite; IL-22, interleukin-22; IL-22R, interleukin-22 receptor; TARC, thymus and activation regulated chemokine.

Introduction

Atopic dermatitis (AD) is a chronic inflammatory disease, characterized by dry, red and pruritic skin (1-4). The pathogenesis of AD is diverse and complex. The cause of AD is thought to be an abnormal immune response and a dysfunction in keratinocytes (5, 6). The dysfunction of keratinocytes is accompanied with a decline in ceramide and antimicrobial peptides (7-9), and the production of inflammatory chemokines such as macrophage-derived chemokine (MDC), and thymus and activation regulated chemokine (TARC) (10, 11).

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Keratinocyte dysfunction is induced by various environmental factors like house dust mite (HDM) or mold (12). Especially, HDM increases atopic eczema and hypersensitivity in AD patients, and deteriorates the symptoms of AD patients (13-15). HDM is associated with tumor necrosis factor (TNF)- α and interferon (IFN)- γ production in keratinocytes. However, its precise effect of HDM in keratinocytes has not yet been elucidated.

Interleukin (IL)-22 is a member of the IL-10 cytokine family that involves IL-10, -19, -20, -24 and -26 (16). IL-22 is produced by CD4⁺ T cells, natural killer (NK) cells and natural killer T (NKT) cells, and has both pro- and anti-inflammatory effects (17, 18). IL-22 not only regulates keratinocyte proliferation in the skin but also induces epidermal hyperplasia by suppressing terminal keratinocyte differentiation genes (19-22). Several cytokines from type 2 and type 22 helper T (Th2/Th22) cells, such as IL-4, -13 and -22, are dominantly expressed in the skin lesions of AD patients (23-25). IL-22-producing CD4⁺ and CD8⁺ T cells abundantly infiltrate into the skin of AD patients (26). Additionally, IL-22 is regarded to have a critical role in inflammatory diseases with epidermal acanthosis like psoriasis and AD (25, 27). The IL-22 receptor (IL-22R) is a heterodimer consisting of IL-22R α and IL-10R β and highly expressed in the skin, pancreas, kidney, lung and intestine. However, IL-22R α is expressed on the skin and epithelial cells, whereas IL-10R β is expressed in all cell types. IL-22R α has a pathogenic role in psoriasis through the induction of differentiation and proliferation of keratinocytes. Additionally, it causes severe inflammatory responses in the skin (16, 18, 19, 28). Taken together, it suggests that IL-22 and IL-22R α are closely associated with the development and progression of skin diseases.

TARC is a member of the C-C chemokines, and produced by keratinocytes, endothelial cells and dendritic cells (DCs) (10, 11, 29). A previous study reported that TARC was increased in the serum of AD patients (30, 31). It is mainly produced by keratinocytes in the basal layer of the epidermis in the skin lesion of the AD patient. It is also highly expressed in the basal epidermis of the skin lesion in NC/Nga mice, a mouse model for human AD. TARC production is reduced with the healing of skin lesions (27). Thus, TARC is considered to be related with the pathogenesis of AD. TARC accelerates inflammatory responses in AD lesions to recruit T cells that express C-C chemokine receptor type 4 (CCR4) (32, 33). It is also related with the elevated level of serum IgE specific to environmental allergens like HDM and the increase in eosinophil in peripheral blood and tissues (34).

Accepted Article

It seems that the HDM extract of major factors causes AD, but the exact effect and its related mechanisms have not been clearly elucidated in keratinocytes, so far. Moreover, the effects of HDM on the production of IL-22 and TARC and the expression of IL-22R α have not been reported yet. For this reason, we investigated the effect of HDM extract on TARC production and IL-22R α expression in a human keratinocyte cell line HaCaT in the present study.

Materials and methods

Cell culture

HaCaT was cultured routinely in Roswell Park Memorial Institute 1640 (RPMI1640) medium (WELGENE, Daegu, Korea) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Thermo scientific, Rockford, IL, USA) and antibiotics (100 U/ml of penicillin and 100 μ g/ml streptomycin; WELGENE) at 37°C in a humidified 5% CO₂ incubator.

Confocal microscopy

HaCaT was grown in 1 cm² cover glass for 12 h. After washing with PBS, HaCaT was treated with HDM extract (25 μ g/ml), TNF- α and IFN- γ (10 ng/ml) and cultured for 24 h. HaCaT on the cover glass were fixed with 4% paraformaldehyde (PFA) and pre-incubated with 5% goat serum in PBS-T (0.3% Triton X-100 in PBS) for 1 h. Rabbit developed anti-human IL-22R α antibody (Abcam, Cambridge, UK) was used as primary antibody and Alexa Fluor 633-conjugated anti-rabbit antibody (Invitrogen, Carlsbad, CA, USA) was used as secondary antibody. After mounting with DAPI-containing mounting solution (Immunobioscience, Mukilteo, WA, USA), fluorescence images were captured with inverted confocal microscope (Nikon, Tokyo, Japan).

Depletion of TNF- α and IFN- γ from culture supernatant

To examine the role of TNF- α and IFN- γ produced from T cell by HDM treatment on the expression of IL-22R α , TNF- α and IFN- γ were depleted from culture supernatant of HDM-treated T cells by immunoprecipitation with using of magnetic beads conjugated anti-TNF- α and anti-IFN- γ antibodies (Cell signaling, Danvers, MA, USA). Briefly, culture

supernatants of T cells were collected after incubation in the presence or absence of HDM extract for 72 h, and mixed with magnetic-beads conjugated anti TNF- α and IFN- γ Abs for 15 min. HaCaT was treated with TNF- α and IFN- γ depleted culture supernatant for 24 h, and then IL-22R α was examined with confocal microscopy.

Isolation of T cells

T cells were isolated from peripheral blood mononuclear cells (PBMCs) obtained from health adult donors under protocol approved by an Institutional Review Board (IRB) of Seoul National University Hospital (#1507-117-690). T cells were purified from PBMCs by MACS negative selection system using Pan T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of T cells was determined by flow cytometry, after staining with APC-conjugated anti-CD3 antibody (e-Bioscience, Sandiego, CA, USA). Then, FlowJo software (Tree Star, Ashland, OR, USA) was used for data analysis.

Immunoblotting

HaCaT was treated with HDM (25 μ g/ml) for 5, 15, 30, 60, 120 and 240 min and human T cells were treated with HDM for 5, 15, 30, and 60 min. Cells were homogenized with lysis buffer and total proteins were quantified by bicinchoninic acid (BCA) assay. Equal amounts of protein were resolved over 10% polyacrylamide gel and transferred to a nitrocellulose membrane. Followed blocking with 5% non-fat milk, membranes were incubated with anti-phosphorylated STAT3, anti-STAT3, anti-phosphorylated ERK, anti-phosphorylated AKT, anti-AKT (Cell signaling), anti-ERK, anti-AHR (Santa cruz, TX, USA), or β -actin (Sigma, St. Louis, MO, USA) at 4 °C for overnight. After incubating with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Cell signaling), the immunoreactive proteins were visualized with the ECL detection system (Amersham Biosciences Corp., Piscataway, NJ, USA).

Enzyme-linked immunosorbent assay (ELISA)

Human T cells were treated with HDM extract (25 μ g/ml) for 24, 48 and 72 h and TNF- α , IFN- γ and IL-22 concentration in the supernatants was measured by ELISA according to manufacturer's instructions (R&D). The optical density (OD) of each well was determined using the SoftmaxPro software (Molecular Devices). HaCaT was seeded with or

without HDM extract (25 µg/ml), and then cells were treated with culture supernatant of T cells or human IL-22 antibody (R&D)-treated T cells or HDM extract-treated T cells.

Supernatants were harvested after 24, 48 and 72 h and the concentration of TARC, IL-1 α and IL-6 in the culture supernatants was measured by ELISA (Biolegend, San diego, CA, USA).

Transwell migration assay

The migration assay was performed using a microchamber transwell system with 5 µm pores (Corning Costar, Tewksbury, MA, USA). Human T cells were incubated in RPMI1640 medium supplemented with 1% heat-inactivated FBS. T cells were applied to the upper compartment of the insert. The lower compartment of the insert was loaded with HDM extract (25 µg/ml), culture supernatants of HDM extract (25 µg/ml)-treated HaCaT (48 and 72 h). T cells were allowed to migrate to the lower compartment for 3 h. The upper well was removed, and T cells in the lower compartment were counted and expressed as percentage of the total T cells added to the upper well.

Statistical analysis

Data were expressed as mean $\pm$ SD of each group in independent experiments. An unpaired two-tailed t-test was used to compare the two groups. For comparison of three or more groups, data were analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test. *P*-values of < 0.05 were considered statistically significant. Statistical tests were carried out using GraphPad InStat (GraphPad Software, San Diego, CA, USA).

Results

HDM extract increases IL-22R α expression in HaCaT and IL-22 production from T cells

It was recently reported that IL-22 is involved in the proliferation of keratinocytes, we examined the change in IL-22R α expression on the surface HaCaT by the treatment of HDM extract. As shown in Fig. 1a, expression of IL-22R α on HaCaT increased at 24 and 48 h after treatment with HDM extract compared to the control. Next, we investigated whether

HDM extract induces IL-22 production from T cells because IL-22 is mainly produced by CD4⁺ T cells under inflammatory conditions (17, 18). HDM extract enhanced the IL-22 production from T cells in a time-dependent manner (Fig. 1b)

HDM extract and IL-22 increases the production of IL-1 α and IL-6 from HaCaT

It was reported that HDM extract increases the production of pro-inflammatory cytokines such as IL-6 and TNF- α from respiratory epithelial cells and canine epidermal keratinocytes (35, 36). Additionally, IL-22 increases the production of pro-inflammatory cytokines, such as IL-1 α and TNF- α , from keratinocytes in skin with inflammation (37-39). We presumed that IL-22 could also increase the production of pro-inflammatory cytokines in the HDM extract treated HaCaT cells. After cells were exposed to HDM extract, they were incubated another 48 h in the presence or absence of rIL-22. HDM extract or rIL-22 increased the production of IL-1 α and IL-6 from HaCaT. Additionally, the production of IL-1 α and IL-6 was more increased by treatment with rIL-22 in the HDM extract treated HaCaT (Fig. 2a and b).

HDM extract activates STAT3, ERK and AKT in HaCaT and STAT3 and AHR in T cells

There are reports that HDM induces allergic reaction through the activation of signal transducer and activator of transcription 3 (STAT3), extracellular signal-regulated kinase (ERK) and protein kinase B (PKB, also known as AKT) (40-43). In addition, we found the involvement of STAT3, ERK and AKT on IL-22R α expression in our previous study. Therefore, we examined whether HDM activates STAT3, ERK and AKT in HaCaT. As shown in Fig. 3a, STAT3 activation was induced within 15 min after HDM treatment, and then ERK and AKT were activated. It is known that STAT3 and aryl hydrocarbon receptor (AHR) are closely related with the production of IL-22 from T cells (28). And we found that the phosphorylation of STAT3 and AHR expression were increased, when T cells were treated with HDM (Fig. 3b).

HDM extract increases IL-22R α expression on HaCaT via increasing TNF- α and IFN- γ production from T cells

To increase the production of IL-1 α and IL-6, IL-22 producing T cells should be recruited to IL-22R α expressing keratinocytes *in vivo*. It is known that TARC accelerates the inflammatory response in skin disease to recruit T cells that express the C-C chemokine receptor type 4 (CCR4) (31-33). Moreover, TARC production from keratinocytes is induced by TNF- α and IFN- γ (44, 45). Therefore, we examined whether HDM extract increases the production of TNF- α and IFN- γ from T cells. As we expected, their production remarkably increased by treatment with HDM extract (Fig. S1a and b). Interestingly, we found that IL-22R α expression in HaCaT was increased by the treatment of TNF- α and IFN- γ (Fig. S1c). HaCaT was cultured with TNF- α and IFN- γ depleted culture supernatant to confirm the role of TNF- α and IFN- γ produced from HDM-treated T cells on IL-22R α expression on HaCaT. As shown in Fig. S1d, we found that IL-22R α expression was reduced. It implies that HDM extract directly increases IL-22R α expression on HaCaT as well as indirectly increases IL-22R α expression through the production of TNF- α and IFN- γ from T cells.

IL-22 and IL-22R α induced by HDM extract enhances TARC production from HaCaT

There are reports that TNF- α and IFN- γ -stimulated keratinocytes produce a high level of TARC (39, 44). In addition, we have already shown that HDM extract increased TNF- α and IFN- γ production from T cells in Fig. S1a and b. Therefore, TARC production from HaCaT by HDM extract was examined. Since IL-22R α expression on HaCaT is increased by the treatment with HDM extract, we also examined whether IL-22 could enhance TARC production. As shown in Fig. S2a, TARC production from HaCaT was not only increased at 48 h (left panel) and 72 h (right panel) after treatment with HDM extract or rIL-22; however, it was enhanced by co-treatment of HDM extract with rIL-22. As we expected, co-treatment of HDM extract with rIL-22 enhanced TARC production. It suggests that the interaction between IL-22 and IL-22R α might be closely involved in TARC production under a HDM extract-mediated skin inflammatory response.

Next, we investigated whether the culture medium of HDM extract-treated T cells could increase TARC production from HaCaT because the production of TNF- α , IFN- γ and IL-22 from T cells is increased by HDM extract (Fig. 1b and Fig S1a and b). TARC

production from HDM extract-treated HaCaT was more increased by treatment with the culture medium of the HDM extract-treated T cells compared to the production from HDM extract-non-treated HaCaT (Fig. S2b). This result is due to the interaction between the increased IL-22R α in HDM extract-treated HaCaT and the increased IL-22 from the HDM extract-treated T cells. It was confirmed by IL-22 neutralizing experiment with IL-22 neutralizing Ab (nAb). As shown in Fig. S2c, TARC production was inhibited by the blocking of the interaction between IL-22 and IL-22R α with IL-22 nAb.

HDM extract facilitates T cell migration through the increase of TARC production from HaCaT

Based on the result regarding the increased TARC production shown in Fig. S2, we examined whether T cell migration is increased by HDM extract. When T cells were cultured with HDM extract, we did not observe the migration of T cells (right upper panel of Fig. 4a). However, when T cells were cultured in the presence of the culture supernatant of the HDM-treated HaCaT, the migration of T cells increased (lower panel of Fig. 4a). It suggests that the increased TARC from HaCaT by the treatment of HDM extract recruit T cells to inflammatory sites of the skin.

Discussion

In modern society, AD is a common chronic inflammatory skin disease. It is characterized by the existence of pruritic, red, dry and eczematous lesions (1-4, 38). However, the exact cause and mechanism of AD remain to be elucidated, and it is still an issue of debate. AD appears to have various causes such as environmental factors and immune reactions to environmental allergens like HDM (39, 44). Recent data reported that exposure to HDM causes inflammatory disease and deteriorates the symptoms in AD patients (13-15, 45). Although HDM extract has been known to induce AD, the direct effect of HDM extract on keratinocytes has not been clarified yet.

It is already reported that HDM extract induces proliferation in asthmatic bronchial smooth muscle (BSM) cells (46, 47). However, its role in the proliferation of keratinocytes is largely unknown. To investigate whether HDM extract increases the proliferation of keratinocytes, the human keratinocyte cell line HaCaT was treated with HDM extract for 48 and 72 h, and then proliferation was examined by Cell Counting Kit-8 (CCK-8) assay. From

the results, HDM extract increased the proliferation of HaCaT even though it was not significant in a dose- or time- dependent manner (Fig. S3).

It was previously reported that IL-22 not only inhibits epidermal differentiation but also the induction of migration and pro-inflammatory gene expression in keratinocytes (21). In addition, IL-22 induces the proliferation of normal human epidermal keratinocytes (48). Thus, we examined whether HDM extract induces IL-22 production and its receptor expression in the present study. As shown in Fig. 1a, IL-22R α expression was increased by HDM extract in HaCaT. There are reports that HDM induces allergic reaction through the activation of signal transducer and activator of transcription 3 (STAT3), extracellular signal-regulated kinase (ERK) and protein kinase B (PKB, also known as AKT) (40-43). And HDM extract activated STAT3, ERK and AKT in HaCaT (Fig. 3a). It seems that STAT3, ERK and AKT signaling mechanisms are involved in the IL-22R α expression in HDM extract-treated HaCaT. HDM extract also increased IL-22 production from T cells (Fig. 1b). It is reported that IL-22 is produced in T cell through the activation of STAT3 and AHR expression (28). We also found that HDM extract activated STAT3 and increased AHR expression in T cells (Fig. 3b). Therefore, it seems that HDM extract induces IL-22R α expression via the activation of STAT3, ERK and AKT in HaCaT and IL-22 production via the activation of STAT3 and increase of AHR expression.

It was reported that IL-22 increased pro-inflammatory cytokines in the skin of AD patients (35, 49-51). In addition, IL-6 production was increased from peripheral blood T cells of AD patients (45). However, it is unknown whether IL-22 is involved in IL-6 production in AD. As shown in Fig. 2b, there was no difference in IL-6 production from HaCaT by the treatment with HDM or rIL-22 only. However, it was increased by the co-treatment of HDM extract with rIL-22. When we consider that IL-22R α expression is increased by the HDM extract, the increased IL-6 production is caused by an interaction between the increased IL-22R α in HaCaT by the HDM extract and IL-22. We also examined whether IL-22 increases the production of other inflammatory cytokines, such IL-1 β , IL-18 and PGE₂, from HaCaT. Even though it was marginal, we found that the production of IL-1 β , IL-18 and PGE₂ was increased by the treatment with IL-22 (data now shown). Therefore, it seems that HDM has an important role in skin inflammation through an increase in IL-22R α expression. In addition, it means that IL-22 produced from T cells treated with HDM extract could be associated with an inflammatory environment that consists of several pro-inflammatory

cytokines in the skin of AD patients. There is an interesting recent report that IL-22 is predominantly expressed by CD3-c-Kit⁺ cells relative to CD3⁺ T cells in lesional skin of patients with psoriasis and atopic dermatitis. In this report, c-Kit⁺FcεRI⁺ mast cells are the major source of IL-22 in AD patients. The proportion of mast cells that produce IL-22 ranges from 20% to 80% in patients with psoriasis or those with atopic dermatitis (52). It suggests that the IL-22 plays an important role in the interaction between mast cell and keratinocytes during the pathogenesis of AD.

We found that HDM extract increases TARC production from HaCaT (Fig. S4). TARC has a pivotal role in the pathogenesis of AD because of the increased TARC production in AD patients and NC/Nga mice, an animal model for mimicking human AD. It was also reported that keratinocytes are one of the major sources of TARC (10). However, it has still not been reported whether HDM extract induces TARC production. We found that HDM extract induces TARC production from HaCaT. Therefore, this is the first report of HDM extract inducing the production of TARC. This result suggests that TARC production in AD skin lesions might be induced by HDM extract. Because many studies have shown that TNF- α and IFN- γ -stimulated keratinocytes produce a high level of TARC (39, 44), we investigated whether T cells produce TNF- α and IFN- γ in response to HDM extract. HDM extract induced the production of TNF- α and IFN- γ from T cells (Fig. S1a and b). In addition, we found that TNF- α and IFN- γ increased IL-22R α expression in HaCaT (Fig. S1c), but it was decreased by the treatment with TNF- α and IFN- γ depleted culture supernatant (Fig. S1d). Our data indicate that HDM extract has a role in skin inflammatory disease not only directly inducing the production of TNF- α and IFN- γ but also indirectly elevating the expression of IL-22R α in HaCaT.

We next investigated the effect of IL-22 on TARC production from HaCaT. IL-22 increased TARC production from HDM extract-treated HaCaT (Fig. S2a). Furthermore, the culture supernatant of HDM extract-treated T cells, which contains TNF- α , IFN- γ and IL-22, elevated TARC production from HaCaT (Fig. S2b). In addition, we found that TARC production from HaCaT by treatment with the culture supernatant of HDM extract-treated HaCaT was inhibited by the neutralization of IL-22 with anti-IL-22 neutralizing Ab (Fig. S2c). These data suggest that IL-22 and IL-22R α are closely related with TARC production from HaCaT. It is already known that TARC promotes T cells migration (31-33). And we confirmed that TARC production was increased by treatment with HDM extract. Moreover, we observed the migration of T cells by the culture supernatant of HDM extract-treated

HaCaT (Fig. 4). Therefore, we concluded that the increase in T cell migration by the culture supernatant of HDM extract-treated HaCaT could be mediated by TARC.

Taken together, this study demonstrates that HDM extract affects not only an increase in IL-22R α expression and TARC production in HaCaT but also an increase in IL-22 production and migration of T cells. These results provide specific mechanisms that are related to the deterioration of inflammatory responses in the skin of AD patients.

Author Contribution

Mirim Jang and Ye-jin Kim performed original research. Hyemin Kim performed additional research for revision. Jiyea Choi and Jane Jeon contributed to support experiment, Young-il Hwang and Wang Jae Lee contributed to analyse data. Jae Seung Kang contributed to study design and study supervision.

Conflict of Interest

The authors have declared no conflicting interests

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Figure legends

Figure 1. The effect of HDM extract on IL-22R α expression on HaCaT and IL-22

production from T cells. (a) After HaCaT was treated with HDM extract (25 $\mu\text{g/ml}$) for 24 and 48 h and then fixed with 4% PFA. Fixed cells were stained with anti-human IL-22R α antibody (1:100) and Alexa Fluor 633-conjugated anti-rabbit antibody (1:1500). Nuclei were counter-stained with DAPI. The distribution of IL-22R α was examined with confocal microscopy (magnification $\times 1000$). (b) T cells were cultured with or without HDM extract (25 $\mu\text{g/ml}$) for 48 and 72 h, and then culture supernatants were collected. The concentration of IL-22 in the culture supernatant was determined by ELISA as described in *Materials and Methods*. Data are presented as the mean $\pm$ SD. *** $p < 0.001$, N.D: Not detected.

Figure 2. The production of IL-1 α and IL-6 from HaCaT by the treatment of HDM and

rIL-22. HaCaT was cultured in the absence or presence of HDM extract (25 $\mu\text{g/ml}$), rIL-22 (50 ng/ml) and combination of HDM and rIL-22 for 48 h. The culture supernatant was collected, and the production of (a) IL-1 α and (b) IL-6 was measured by ELISA as described in *Materials and Methods*. Data are presented as the mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, # $p < 0.05$, ## $p < 0.01$.

Figure 3. The activation of STAT3, ERK and AKT in HaCaT and STAT3 and AHR in

T cells by the treatment with HDM. (a) After HaCaT was treated with HDM extract (25 $\mu\text{g/ml}$) for 5, 15, 30, 60, 120 and 240 min, the phosphorylation of STAT3, ERK and AKT was examined by immunoblotting. β -actin was used as an internal control. (b) T cells were treated with HDM extract (25 $\mu\text{g/ml}$) for 5, 15, 30, and 60 min, and the expression of phosphorylated STAT3 and AHR was determined with immunoblot.

Figure 4. The increase of T cell migration by treatment with the culture supernatant of HDM

extract-treated HaCaT. (a) T cells isolated from human peripheral blood and placed in the upper compartment of the microchamber transwell with 5 μm pores. T cells were exposed to control medium, medium with HDM extract (25 $\mu\text{g/ml}$), culture supernatant of HDM extract-

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treated HaCaT for 48 h and 72 h. Microscopic pictures were captured at 3 h after T cells application. Representative images are shown (magnification $\times 400$). (b) Migrated T cells are expressed as the percentage of the total T cells applied to the upper well. Data are presented as the mean $\pm$ SD. ** $p < 0.01$, *** $p < 0.001$.

Figure 1

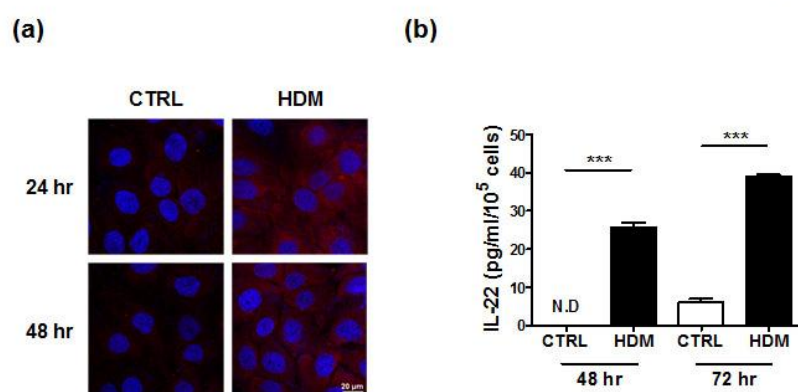


Figure 2

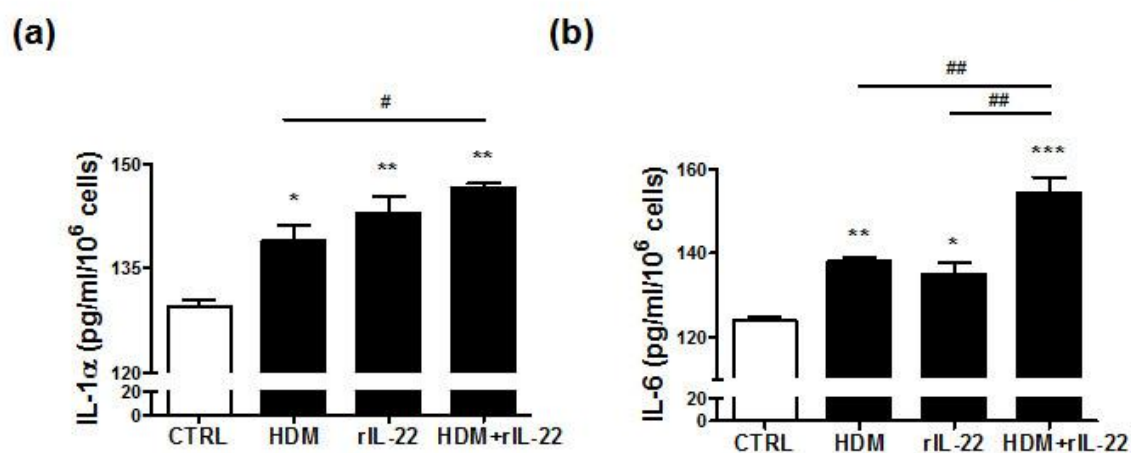


Figure 3

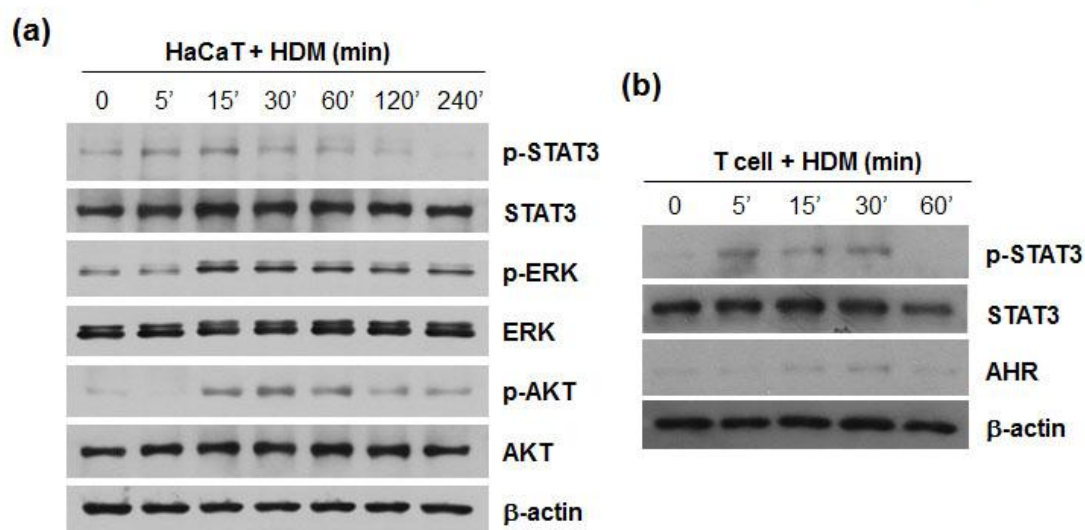


Figure 4

