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Time-dependent distinct roles of Toll-like receptor 4 in a house-dust-mite-induced asthma mouse model

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Abstract

House dust mites (HDMs) are a common source of allergens that trigger both allergen-specific and innate immune responses in humans. Here we examined the effect of allergen concentration and the involvement of Toll-like receptor 4 (TLR4) in the process of sensitization to house dust mite allergens in an HDM-extract-induced asthma mouse model. Intranasal administration of HDM extract induced an immunoglobulin E response and eosinophilic inflammation in a dose-dependent manner from 2.5 to 30 $\mu\text{g}/\text{dose}$. In TLR4-knockout mice, the infiltration of eosinophils and neutrophils into the lung was decreased compared with that in wild-type mice in the early **phase of inflammation** (total of three doses). However, in the late **phase of inflammation** (total of seven doses), eosinophil infiltration was significantly greater in TLR4-knockout mice than in wild-type mice. This suggests that the roles of TLR4 signaling are different between the early phase and the

later phase of HDM-allergen-induced inflammation. Thus, innate immune response through TLR4 regulated the response to HDM allergens, and the regulation was altered during the **phase of inflammation**.

Keywords: Eosinophils, asthma, Experimental animal, Inflammation, Lipopolysaccharide

Introduction

House dust mites (HDM) are a major source of allergens that induce the development of asthma [1-3]. *Dermatophagoides farinae* (Der f) as well as *Dermatophagoides pteronysnus* (Der p) are the most common species of HDM associated with the induction of asthma [1, 2]. HDMs contain several allergens; however, the HDM allergens to which most HDM-allergic patients are sensitized are Der f 1 and Der f 2. Der f 1 is reported to have serine protease activity, which damages epithelial cell barrier [4-6]. Der f 2 has structural homology with myeloid differentiation factor 2, which is the lipopolysaccharide (LPS)-binding component of the Toll-like receptor 4 (TLR4) signaling complex [7, 8]. Furthermore, HDM allergens react with TLR4 [9, 10]. These findings suggest that sensitization to allergens involves interactions between antigen-specific and innate immune responses. However, the influence of LPS in this process is yet to be fully elucidated [11, 12].

In animal models of asthma, the immunoglobulin E (IgE) response is usually induced via systemic immunization with allergens formulated with alum [13, 14]. However, because some HDM allergens have proteinase activity, which is crucial for sensitization to occur [15, 16], administration of HDM via inhalation has also recently been shown to be useful for inducing airway inflammation [17, 18]. The pattern of sensitization differs depending on the concentration and

composition of HDM extracts from different preparation [19]. HDM extracts differ in inducing barrier dysfunction, but this are not dependent on serine/cysteine protease activity [19]. In addition, the effect of major allergen concentration in HDM extracts on the induction of airway inflammation via inhalation is currently unknown.

Here, we tried to clarify whether allergen concentration and length of administration influenced sensitization and the interactions between the antigen-specific and innate immune responses in an HDM-induced asthma mouse model. We also examined the role of TLR4 in the early and late phases of inflammation in the mouse model.

Materials and methods

Animals

Specific-pathogen-free C57BL6/N female mice (5-6 weeks) were purchased from Sankyo Laboratory (Tokyo, Japan), and TLR4-knockout and **TLR2-knockout mice (both C57BL6/N background)** were purchased from Oriental Bioservice Inc. (Kyoto, Japan). The mice were bred at the animal facilities of Musashino University School of Medicine (Japan) under specific-pathogen-free conditions and with the approval of the Institutional Animal Experiments Committee of Musashino University. Care and use of the animals followed the guidelines in “Principles of Laboratory Animal Care” published in 2006 by the National Society for Medical Research. **Wild type and TLR4-knockout female mice (8-12 weeks old) were used for experiments.**

HDM extract treatment

HDM extract containing Der f 1 (43.5 µg/mL) and Der f 2 (14.1 µg/mL) was kindly provided by the Institute of Tokyo Environmental Allergy, Inc. (Tokyo, Japan). An endotoxin-specific limulus amoebocyte lysate assay (Endospecy; Seikagaku Corporation, Tokyo, Japan) showed that the HDM extract contained 12.8 endotoxin units per microgram of protein.

Mice were intranasally administered HDM extracts (indicated dose in phosphate-buffered saline [PBS]) once every other day for a total of three or seven times while under anesthesia with ketamine (90 mg/kg) and medetomidine (1 mg/kg) administered via intraperitoneal injection. Mice treated with PBS were used as controls.

Bronchoalveolar lavage fluid cell analysis and enzyme-linked immunosorbent assay

Twenty-four hours after the final administration of HDM extract, whole blood was collected via heart puncture into a heparinized syringe, and bronchoalveolar lavage fluid (BALF) cell analysis was performed as reported previously [20]. BALF was obtained by incubating and washing lungs three times with 1 mL sterile saline. Isolated BALF was centrifuged at 450×g for 10 min at 4 °C. The pellets obtained were dissolved in 1 mL PBS and the number of cells counted. Cytospin preparations were stained with Diff-Quik (International Reagents Corporation, Osaka, Japan), and then examined under a microscope.

The IL-13 level in BALF and the IgE level in blood plasma were measured by using specific enzyme-linked immunosorbent assay (ELISA) kits for IL-13 (eBioscience, San Diego, CA) and IgE (Yamasa Shoyu Co. Ltd., Chiba, Japan).

Histological examination

Mice were sacrificed via transcardial perfusion with PBS followed by 4% paraformaldehyde in 100 mM phosphate buffer (pH 7.4). The lungs were removed and immersed in 4% paraformaldehyde for 2 h at 4 °C. After being washed with PBS, the lungs were embedded in paraffin and cut in 5- μ m sections. The specimens were analyzed by means of hematoxylin and eosin and periodic acid–Schiff staining.

Assessment of airway resistance

Airway resistance was examined at 24 h after the final administration of HDM extract by using a flexiVent system (SCIREQ, Montreal, Quebec, Canada). The mice were tracheostomized while under anesthesia with ketamine (90 mg/kg) and medetomidine (1 mg/kg) administered via intraperitoneal injection. The animals were ventilated at a tidal volume of 8 mL/kg and respiratory frequency of 150/min with a positive end-expiratory pressure of 2 cmH₂O. After the mice had been placed on ventilation they were injected with rocuronium bromide. To assess airway responsiveness, methacholine (3.12, 6.25, 12.5, or 25 mg/mL) was administered by means of nebulization. Airway resistance was measured by using the Snapshot-150 perturbation maneuver method of flexiVent system [21].

Isolation of the Gr-1^{int} CD11b^{high} myeloid-derived suppressor-like cells and examination of mRNA expression by using real-time polymerase chain reaction

Bilateral lungs harvested from HDM-sensitized mice were cut into small pieces and digested in HBSS supplemented with 0.2% collagenase (Wako, Osaka, Japan), 0.025% DNase for 60 min at 37°C and a single cell suspension was obtained as previously described [22]. Cells were incubated

with biotinylated anti-Gr-1 Ab and FITC-conjugated anti-CD11b Ab (eBioscience, San Diego, CA) before incubation with streptavidin-PE; Gr-1^{int} CD11b^{high} non-autofluorescent cells were sorted (Cell Sorter SH800, Sony, Tokyo, Japan) as myeloid-derived suppressor-like cells[23].

Total RNA was extracted by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA purity was established by using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). The RNA had an OD₂₆₀/OD₂₈₀ ratio of 1.8, indicating high purity.

For cDNA synthesis, 1.0 µg of total RNA was reverse-transcribed by using PrimeScript (Takara, Tokyo, Japan). The levels of mRNA were determined by means of real-time polymerase chain reaction using SYBR Premix Ex Taq II (Takara) in a total volume of 20 µL. *β-actin* was used as the internal control. The expression of genes of interest was calculated according to the comparative threshold cycle method, using *β-actin* as an internal control.

The following forward and reverse primers were used: *β-actin* (forward, 5'-CTC CTA GCA CCA TGA AGA TCA-3'; reverse, 5'-CCT GCT TGC TGA TCC ACA TC-3'); *IL-5* (forward, 5'-ACA TTG ACC GCC AAA AAG AG-3'; reverse, 5'-ATC CAG GAA CTG CCT CGT C-3'); *IL-10* (forward, 5'-CAG AGC CAC ATG CT CCT AGA-3' ; reverse, 5'-TGT CCA GCT GGT CCT TTG TT-3')

Statistical analyses

Analysis of variance and the Tukey–Kramer honest significant difference test were used to compare multiple groups. The calculations were performed with JMP Pro v.11.2.0 (SAS Institute, Cary, NC). Statistical significance was accepted at $P < 0.05$. Data are expressed as means ± SEM.

Results

HDM extract dose-dependently induces IgE response and airway inflammation

To assess the allergic sensitization induced by inhalation of HDM extract, total IgE levels were measured in serum by means of an ELISA. HDM extract was administered once every other day for a total of seven times. Inhaled HDM significantly induced IgE synthesis in a dose-dependent manner ($P < 0.05$; Fig. 1A). No significant elevation of IgE was observed in mice administered 2.5 μg /dose of HDM extract compared with in controls.

Next, we examined the infiltration of inflammatory cells into the lungs to clarify how the dose of HDM extracts affected the induction of allergic inflammation. Significant infiltration of eosinophils was observed in the mice administered 15 or 30 μg /dose of HDM extract compared with in control mice ($P < 0.05$; Fig. 1B). Significant infiltration of neutrophils was observed in mice administered 30 μg /dose of HDM extract compared with in controls and in mice administered 15 μg /dose of HDM extract (Fig. 1B). No significant infiltration of any cell types was observed in mice administered 2.5 μg /dose of HDM extract compared with controls (Fig. 1B).

Histological examination revealed marked infiltration of eosinophils into the submucosa and surrounding blood vessels in mice administered 15 or 30 μg /dose of HDM extract, compared with controls (Fig. 2A). Periodic acid–Schiff stain-positive mucus-secreting cells were observed in mice administered 15 or 30 μg /dose of HDM extract, whereas they were not observed in controls (Fig. 2B). No significant infiltration of cells was observed in 2.5 μg /dose of HDM extract (data not shown).

Together, these data indicate that intranasal administration of HDM extract induced a dose-dependent IgE response and eosinophilic airway inflammation.

Role of TLR4 in HDM extract-induced immune response

Administration of total seven times of 30 µg/dose HDM extract in TLR4-knockout (TLR4-KO) mice induced accumulation of significantly more eosinophils in BALF than in wild-type mice ($P < 0.05$; Fig. 3A). This tendency was same in 15 µg/dose HDM extract (number of eosinophils 36 ± 3 vs $80 \pm 34 \times 10^4/\text{mL}$, wild type vs TLR4-KO). We bred the TLR2KO mice in the same condition of TLR4-KO. We observed similar eosinophils in BALF in TLR2-KO compared to wild-type mice (number of eosinophils 32 ± 13 vs $39 \pm 15 \times 10^4/\text{mL}$, wild type vs TLR2-KO), suggesting that the origin and breeding condition of the mice until 5-6 weeks influence the experimental data.

Next, we examined three doses of HDM as early **phase of inflammation**. At the earlier **phase of inflammation**, total cell number in the BALF is significantly less in both wild type and TLR4-KO mice compared to later phase at seven doses of administration (30.5 ± 6.0 vs $61.6 \pm 13.9 \times 10^4/\text{mL}$, three doses vs seven doses in wild type mice, $P < 0.05$, Fig. 3A, B). At the earlier **phase of inflammation**, the accumulation of neutrophils and eosinophils were significantly suppressed in TLR4-KO mice compared to wild type mice ($P < 0.05$; Fig. 3B). Thus, TLR4 is critical in early **phase of inflammation**.

In wild-type and TLR4-KO mice, significantly greater IgE expression was observed after seven doses of HDM extract than after three doses ($P < 0.05$; Fig. 3C). In addition, the increase in

IgE synthesis was comparable between TLR4-KO and wild-type mice. IL-13 production at seven doses was greater in TLR4-KO mice compared to wild type mice, however, this result was not statistically significant ($P=0.054$ Fig. 3D).

In regard to IL-5 mRNA expressions at seven doses of HDM extract, TLR4-KO mice exhibited greater expression than wild type mice (IL-5mRNA, 5.7 ± 0.4 vs 9.5 ± 0.3 fold induction, wild type vs TLR4-KO), which is comparable to greater infiltration of eosinophils in TLR4-KO mice than in wild type mice.

Airway resistance was also examined. Later phase at seven doses of HDM extract administration, wild type mice administered exhibited a significantly greater response to inhaled methacholine at doses of 12.5, and 25 mg/mL than did controls and three doses of HDM as early **phase of inflammation** ($P < 0.05$; Fig. 4A, left panel). TLR4-KO mice exhibited the similar tendency ($P<0.05$; Fig. 4A right panel). There is no statistical difference between wild type and TLR4-KO in airway resistance.

To investigate the cause of more recruitment of eosinophils and Th2 responses in TLR4-KO mice with seven doses of HDM extract, we focused on Gr-1^{int} CD11b^{high} non-autofluorescent myeloid-derived suppressor-like cells. These cells are lung-resident cells activated by lipopolysaccharide and produce IL-10, leading to the suppression of lung-dendritic cell-mediated Th2 effector function. In our seven doses of HDM extract model, the number of these cells in the lung isolated by cell sorter was comparable between wild-type and TLR4-KO mice (Fig. 4B). However, IL-10 mRNA expression of these cells was significantly suppressed in TLR4-knockout mice (Fig. 4B). These data suggest TLR4 may involve in activation of these suppressive cells in the late **phase of inflammation**.

Discussion

Here, we showed that repeated exposure to HDM extract induced allergic airway inflammation in a dose-dependent manner and that this response was regulated by TLR4. However, the regulation through TLR4 differed depending on the **phase of inflammation**. In the early **phase of inflammation** (after three doses of HDM extract), a deficiency of TLR4 meant that little inflammation was induced, as reported previously [7]. However, in the late **phase of inflammation** (after seven doses of HDM extract), eosinophil-induced inflammation was increased in TLR4-KO mice.

Repeated intra-tracheal exposure to HDM in mice induces the asthma phenotype [17, 18, 24]. However, the induction activity of each allergen is different [19, 25]. **Here, we found that the HDM extract dose-dependently induced allergic inflammation and a Type 2 helper-T-cell cytokine response (IL-13 and IL-5).** However, doses above 30 µg HDM extract/dose (i.e., 60 µg HDM extract/dose) did not significantly increase inflammation (data not shown).

The molecular similarity of Der f 2 and myeloid differentiation factor 2 suggests the importance of TLR4 in the immune response to this allergen [9, 10]. TLR4 is crucial for allergen sensitization [9, 10]. However, it has been shown that TLR4 expressed by hematopoietic cells and epithelial cells has different roles—that is, TLR4 expressed by hematopoietic cells is involved in neutrophilic inflammation whereas TLR4 expressed by epithelial cells is involved in eosinophilic inflammation, which is examined after three times administration of HDM extracts [26]. We also observed reduced inflammation after the administration of three doses of HDM extract; however, after seven doses, eosinophilic inflammation was increased in the absence of TLR4.

The major ligand of TLR4 is the endotoxin LPS, substantial amounts of which are present in HDM extract. The role of LPS in allergen sensitization and allergic inflammation is currently unclear. It has been reported that low-dose LPS induces a Type 2 helper-T-cell response, whereas high-dose LPS induces a Type 1 helper-T-cell response [27, 28]. Administration of 10 µg of LPS suppresses eosinophil inflammation and induces neutrophil inflammation [29]. In an ovalbumin-sensitized model, administration of LPS attenuated eosinophilic inflammation, and this attenuation was found to be mediated by TLR4-expressing hematopoietic cells [30]. This suppressive effect of lipopolysaccharide is also observed in a HDM model, and the CD11b⁺Gr-1^{int} suppressive cells may be relevant because these cells are activated by lipopolysaccharide and suppress lung dendritic cell-mediated effector Th2 cell function [23]. In our model, these cells isolated from the lung of mice subject to seven doses of HDM extract showed significantly lower IL-10 expression in TLR4-KO mice. We can speculate lipopolysaccharide included in HDM extract may have partial suppressive effect in allergic inflammation and TLR4-KO mice fail to receive this effect. This is relevant to our finding that long period of intranasal administration of HDM extract increased eosinophil accumulation in the lungs of TLR4-knockout mice.

In conclusion, the involvement of TLR4 in HDM induced murine model is time dependent and we must consider the phenomenon in order to target TLR4 pathway to modify asthma.

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Conflict of interest

Neither of authors declares any conflict of interest.

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Figure legends

Figure 1 HDM-induced IgE response and airway inflammation. (A) Serum total IgE concentrations. Serum IgE was measured by means of an ELISA. Data are presented as means $\pm$ SEM, $n = 3-7$ mice. $*P < 0.05$. (B) BALF cell analysis. Cells were counted under a microscope. Control mice were treated with phosphate-buffered saline. Mice were administered the indicated dose of HDM extract (micrograms of HDM extract/per day) every other day for a total of seven doses. Data are presented as means $\pm$ SEM, $n = 3-7$ mice. $*P < 0.05$.

Figure 2 Histological analysis. (A) Histological analysis was performed with hematoxylin and eosin (HE). (B) Histological analysis was performed with and periodic acid–Schiff (PAS) staining. The arrow indicated the PAS positive epithelial cells. In each figure, left panels are pictures using x4 optical lens and right panels are pictures using x40 optical lens. Square in the left panel indicated region in the right panel. The arrows in (B) indicate PAS positive epithelial cell.

Figure 3 Time-dependent role of TLR4 in HDM-induced airway inflammation. (A) BALF cell analysis in wild-type (WT) and Toll-like receptor 4-knockout (TLR4KO) mice after administration of seven doses of HDM extract (30 µg/dose). HDM was administered every other day. Data are presented as means ± SEM, $n = 6$ mice. $*P < 0.05$. (B) BALF cell analysis in WT and TLR4KO mice after administration of three doses of HDM extract (30 µg/dose). HDM was administered every other day. * Data are presented as means ± SEM, $n = 4$ mice. $*P < 0.05$. (C) IgE production in WT and TLR4KO mice. Plasma IgE were measured by using specific ELISAs. 30×3; 30 µg HDM extract/dose every other day for a total of three doses, 30×7; 30 µg HDM extract/dose every other day for a total of seven doses. Control means mice administered PBS * Data are presented as means ± SEM, $n = 4-6$ mice. $*P < 0.05$. (D) Interleukin (IL)-13 production in WT and TLR4KO mice. IL-13 concentrations in BALF were measured by using specific ELISAs. 30×3; 30 µg HDM extract/dose every other day for a total of three dose, 30×7; 30 µg HDM extract/dose every other day for a total of seven doses. Control means mice administered PBS * Data are presented as means ± SEM, $n = 4-6$ mice. $*P < 0.05$.

Figure 4 Role of TLR 4 in HDM-induced response. (A) Airway response. Airway response was examined by exposing mice to increasingly higher doses of inhaled methacholine (3.12–25 mg/mL). Total resistance of the respiratory system (Rrs) was measured by using a Snapshot-150 perturbation maneuver of a flexiVent system. Left panel; Wild type mice, 30×7($n=10$), 30×3($n=3$), control ($n=5$). Right panel; TLR4KO mice ($n=3$ each group). 30×3; 30 µg HDM extract/dose every other day for a total of three doses, 30×7; 30 µg HDM

extract/dose every other day for a total of seven doses. Data are presented as means $\pm$ SEM.

*:30 $\times$ 7 vs Control $\circ$:30 $\times$ 7 vs 30 $\times$ 3 * $\circ$ $P < 0.05$. (B) The role of Gr-1^{int} CD11b^{high}

myeloid-derived suppressor-like cells in mice subject to seven doses of HDM extract. Bilateral lungs from WT or TLR4-KO mice were harvested 24h after last sensitization and processed for cell sorting. Left panel; the cell number of Gr-1^{int} CD11b^{high} myeloid-derived suppressor-like cells per bilateral lungs. Right panel; IL-10 mRNA expression in these cells measured by qPCR. Data are presented as means $\pm$ SEM, $n = 4-5$ mice. * $P < 0.05$.







