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Original article

Myeloid differentiation-2 is a potential biomarker for the amplification process of allergic airway sensitization in mice

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Abbreviations:
AB/PAS, Alcian Blue/periodic acid Schiff; BALF, bronchoalveolar lavage fluid; HDM, house dust mite; HE, Hematoxylin-Eosin; LPS, lipopolysaccharide; MD-2, myeloid differentiation-2; OD, optical density; OCT, optimal cutting temperature; PBS, phosphate-buffered saline; qRT-PCR, quantitative reverse transcription polymerase chain reaction; TSLP, thymic stromal lymphopoietin; TLR, toll-like receptor

ABSTRACT

Background: Allergic sensitization is a key step in the pathogenesis of asthma. However, little is known about the molecules that are critical regulators for establishing allergic sensitization of the airway. Thus, we conducted global gene expression profiling to identify candidate genes and signaling pathways involved in house dust mite (HDM)-induced allergic sensitization in the murine airway.
Methods: We sensitized and challenged mice with HDM or saline as a control through the airway on days 1 and 8. We evaluated eosinophilia in bronchoalveolar lavage fluid (BALF), airway inflammation, and mucus production on days 7 and 14. We extracted total RNA from lung tissues of HDM- and saline-sensitized mice on days 7 and 14. Microarray analyses were performed to identify up-regulated genes in the lungs of HDM-sensitized mice compared to the control mice. Data analyses were performed using GeneSpring software and gene networks were generated using Ingenuity Pathways Analysis (IPA).
Results: We identified 50 HDM-mediated, stepwise up-regulated genes in response to allergic sensitization and amplification of allergic airway inflammation. The highest expressed gene was myeloid differentiation-2 (MD-2), a lipopolysaccharide (LPS)-binding component of Toll-like receptor (TLR) 4 signaling complex. MD-2 protein was expressed in lung vascular endothelial cells and was increased in the serum of HDM-sensitized mice, but not in the control mice.
Conclusions: Our data suggest MD-2 is a critical regulator of the establishment of allergic airway sensitization to HDM in mice. Serum MD-2 may represent a potential biomarker for the amplification of allergic sensitization and allergic inflammation.

Introduction

Asthma is a respiratory disease with symptoms, such as convulsive wheezing and cough, caused by reversible airway constriction due to allergic airway inflammation, hyperplasia of mucus-producing cells, and airway hyperresponsiveness.[1] The immunological mechanism of asthma has been considered to be of mainly adaptive immune responses and production of T-helper type 2 (TH2) cell-derived cytokines, such as IL-4, IL-5, and IL-13. These cytokines inform asthma phenotypes and aid the development of new therapeutic targets for asthma. Ongoing clinical trials on anti-TH2 cytokine antibodies for asthma therapy have shown therapeutic efficacy in specific populations of asthma patients only.[2–4] This suggests modulation of adaptive immunity is not a sufficient in treating asthma. Currently, the immunological mechanism underlying asthma is increasingly believed to involve the innate immunity at levels upstream of adaptive immunity.

Innate immunity is an immune surveillance system for foreign pathogens. One of the most important innate immune systems is

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the toll-like receptor (TLR) family.[5] The TLR4 ligand, a lipopolysaccharide (LPS), is a structural material on the wall of gram-negative bacteria and acts as an adjuvant and prime allergic sensitization in the airway to promote an asthma-like phenotype.[6,7] Similarly, the TLR5 ligand flagellin, which is a flagellar constituent protein of bacteria, also acts as an adjuvant that induces allergic airway inflammation.[8] These suggest that TLRs may be critical in promoting asthma by priming allergic sensitization to antigens in the airways.

House dust mite (HDM) is a natural allergen, and is a typical environmental factors related to the onset of bronchial asthma; 70%–80% of patients with asthma were found to be sensitive to HDM.[9] Dermatophagoides pteronyssinus, an HDM, has several component allergens such as Der p1 and Der p2[10]. Many of these allergens, such as Der p1, contain protease activity that is highly likely to cause airway damage and initiate allergic airway inflammation.[11] Moreover, a previous study has reported that HDM could be identified by TLR4 on the airway epithelium and could stimulate the production of epithelium-derived cytokines, such as IL-25, IL-33, and thymic stromal lymphopoietin (TSLP), to induce allergic airway inflammation.[12] In this way, allergic sensitization caused by HDM was, at least in part, TLR4-dependent. Furthermore, one of the HDM allergens, Der p2, has structural and functional homology with myeloid differentiation-2 (MD-2, also known as LY96), an LPS-binding component of the TLR 4 signaling complex.[13–15] Thus, the TLR4/MD-2 complex, a component of the innate immune system, is critical for HDM-induced asthma.

Repeated exposure to an allergen is necessary to establish allergic sensitization and this process amplifies the development of allergic airway inflammation. However, little is known about molecular basis of the amplification process of allergic sensitization. In this study, we focused on the sensitization phase of HDM-induced allergic inflammation and performed global gene expression analysis to identify candidate molecules that potentially play important roles in the pathogenesis of asthma.

Methods

Animals

We used 6–8-week-old male, C57BL/6J mice (Charles River Laboratories Japan, Yokohama, Japan). Mice were used in all experiments with 5–8 mice per group.

All studies were approved by the Animal Care and Use Committee at Nihon University School of Medicine.

Experimental protocol

C57BL/6J mice were intratracheally sensitized with HDM, Dermatophagoides pteronyssinus at 100 μg/mouse/treatment (containing approximately 0.1 ng of LPS, 70 ng of Der p2 and 13 ng of Der p1, GREER Laboratories, Lenoir, NC, USA) or with saline as control on days 1 and 8 (n = 5–8 per group). Bronchoalveolar lavage fluid (BALF), serum, and lung tissues were examined on days 7 and 14.

Bronchoalveolar lavage fluid

Mice were intraperitoneally administered pentobarbital at 50 mg/kg (Kyoritsu Pharmaceutical/Schering-Plough Corporation, Tokyo, Japan). Initially, we collected BALF with 1 ml of phosphate-buffered saline (PBS). After adding 1 ml of erythrocyte lysate, we measured the number of cells with a cell counter (Invitrogen, Carlsbad, CA, USA). Using cytospin (Sakura Fine Tech Japan, Tokyo, Japan), cells were sprayed on glass slides (600 spins for 3 min) and were allowed to dry naturally, after which we performed Wright–Giemsa staining by using Diff Quick (Sysmex, Tokyo, Japan). Specimens were sealed with an encapsulant (Matsunami Glass Industry, Tokyo, Japan); we then determined the cell type and number under a microscope.

Immunohistochemical staining

Lungs were fixed in formalin and embedded in paraffin, and the sections were stained with hematoxylin and eosin (H&E). Inflammation scores were determined in accordance with a previous study.[16] A value from 0 to 3 per criterion was adjudged to each tissue section scored. Two criteria were scored to evaluate pulmonary inflammation: peribronchial inflammation and perivascular inflammation. A value of 0 was adjudged when no inflammation was detectable, a value of 1 for occasional cuffing with inflammatory cells, a value of 2 for most bronchi or vessels surrounded by thin layer (one to five cells) of inflammatory cells and a value of 3 when most bronchi or vessels were surrounded by a thick layer (more than five cells) of inflammatory cells. As 10–15 tissue sections per mouse were scored, inflammation scores could be expressed as a mean value and could be compared between groups. The results are presented as means ± SE. Furthermore, sections were stained with Alcian Blue/periodic acid Schiff (AB/PAS) to identify mucus-producing cells. Mucus-producing cells were measured by mucus scores on a scale of 0–3, in accordance with a previous study.[17] The following were the designations for each mucus score: 0—no mucus, 1—a few cells secreting mucus, 2—many cells secreting mucus, and 3—extensive production.

Global gene expression analysis

Lung tissues were homogenized using the power masher III (Nippi, Tokyo, Japan). Total RNA was extracted using RNeasy Mini Kit (QIAGEN, Hilden, Germany); RNA samples were prepared using The Ambion® WT Expression Kit (Affymetrix, Santa Clara, CA, USA) and GeneChip WT Terminal Labeling Kit. Labeling of second-cycle fragmented RNA and confirmation of DNA fragmentation were performed using Agilent RNA 6000 nano kit (Agilent Technologies, Palo Alto, CA, USA); these processes were conducted according to the manufacturer's protocol. We used GeneChip Mouse Gene 1.0 ST Arrays (Affymetrix) to conduct hybridization of the array. Using Genechip Fluidics Station 450, Genechip Scanner 3000 (Affymetrix), we measured and quantified fluorescence intensity. The microarray images were analyzed using Gene Spring 12.5 software (Agilent Technologies UK, South Queensferry, UK). In addition, we performed pathway analysis using Ingenuity Pathways Analysis (IPA, Ingenuity Systems, Redwood City, CA, USA).

Quantitative reverse transcription polymerraise chain reaction (qRT-PCR)

cDNA libraries were prepared from 10 ng of lung tissue RNA using PrimeScript 1st strand cDNA Synthesis kit (Takara-Bio, Shiga, Japan) according to the manufacturer's protocol. We used TaqMan real-time PCR probes and mouse-specific primers, LY96 and GAPDH, obtained from Applied Biosystems. RT-PCR was performed using a 7500 real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). Data were normalized to GAPDH using the ΔΔCt method.

Immunofluorescence staining

Lung tissues were embedded by optimal cutting temperature (OCT) compound and were cut in frozen sections; anti-mouse MD-

2 rabbit antibody (10 μg/ml; NOVUS BIOLOGICALS, Littleton, CO, USA) and anti-mouse CD31 antibody (NOVUS BIOLOGICALS) were added as primary antibodies. Anti-mouse IgG and anti-rabbit IgG antibodies (Alexa 488,594, life technologies, Carlsbad, CA, USA) were added as secondary antibodies. DAPI was used as a mounting medium (Cell Signaling Technology, Danvers, MA, USA). Staining was assessed using a laser scanning confocal FV1000 microscope (Olympus, Tokyo, Japan) under identical settings between conditions.

Serum MD-2 measurement using an enzyme-linked immunosorbent assay (ELISA)

Each 96-well plate was coated with 2.5ug/ml of anti-mouse MD-2 polyclonal antibody (#NBP1-77201, NOVUS BIOLOGICALS) and incubated overnight at 4 °C. Plates were probed with mouse serum (1:10 dilution) and BALFs and incubated for a further 2 h at room temperature. Next, plates were incubated with 0.25 μg/ml biotin-conjugated anti-mouse MD-2 polyclonal antibody (#H00023643-

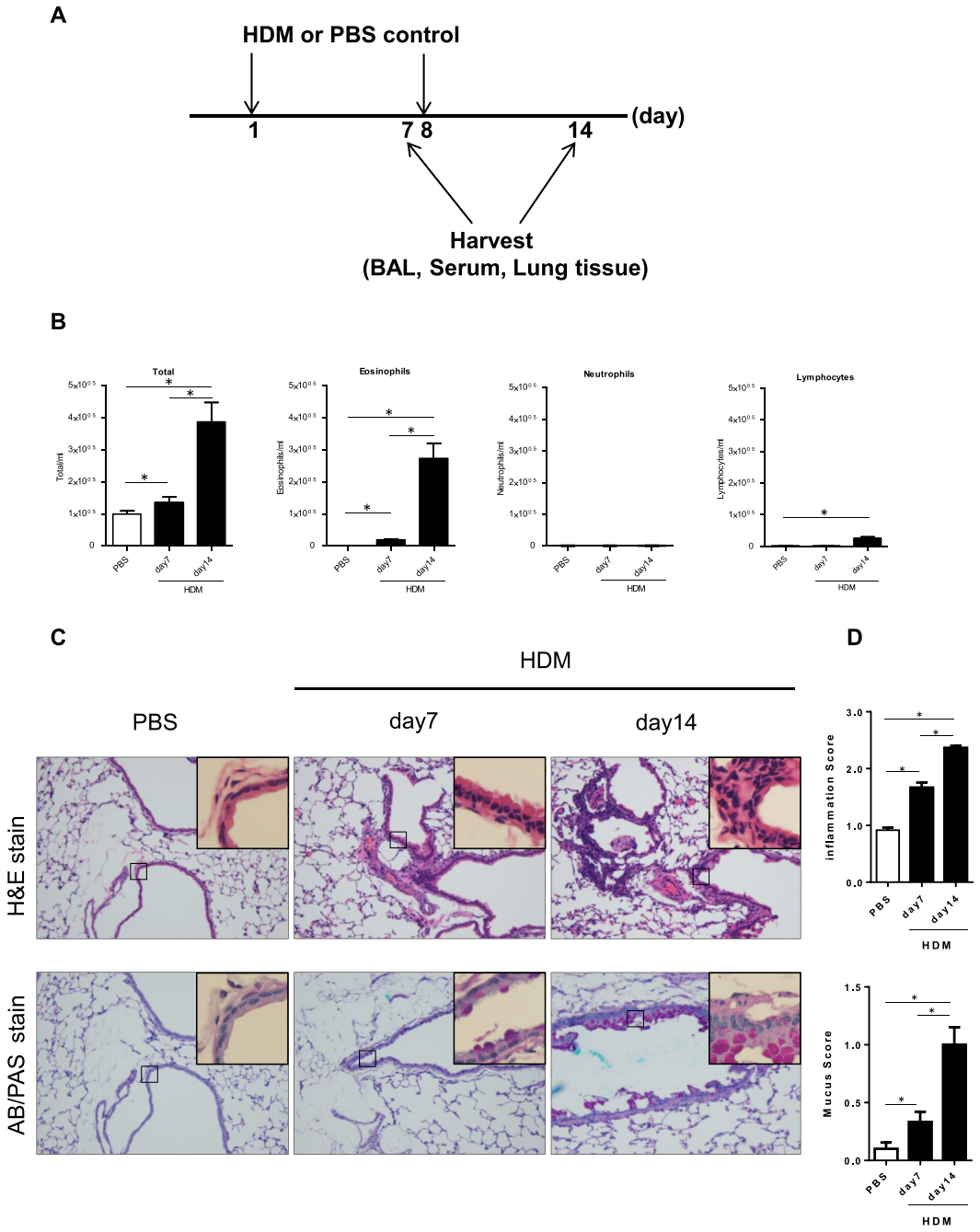

Fig. 1. Allergic airway inflammation and mucus hypersecretion during HDM sensitization in mice. (A) Timeline for sensitization. C57BL/6J mice were sensitized with HDM or saline, as control, on days 1 and 8. BALF and lung tissues were harvested on days 7 and 14. (B) Leukocyte counts (C) Representative low-magnification (×200) and expanded (×1000) images of lung sections that demonstrate the airways and stained mucus producing cells before (left) and after (middle and right) HDM sensitization. (D) Airway inflammation scores and mucus scores. These results are representative of two independent experiments (means ± SEM of 5–8 mice per group), $^{*}P < 0.05$. HDM, house dust mite; BALF, bronchoalveolar lavage fluid; H&E, hematoxylin and eosin; AB/PAS, Alcian Blue periodic acid Schiff.

D01P, NOVUS BIOLOGICALS) for 1 h followed by washing and incubation with streptavidin peroxidase for 30 min. After the addition of 3,3′,5,5′-tetramethyl-benzidine substrate, reactions were stopped with acid, and optical densities (ODs) were determined at a wavelength of 450 nm. Relative serum and BALF levels of MD-2 were expressed as fold change relative to that of controls.

Statistical analysis

All data were described as mean ± standard error values (SEM). Experimental groups were analyzed using Student's *t*-test for data under a normal distribution and Mann–Whitney *U*-test for data indicative of a nonnormal distribution. Statistical significance was set at a P value of <0.05. Data were analyzed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

Results

Amplification of asthma-like responses during HDM sensitization

Our protocol was based on a previous study.[12] We first confirmed that C57BL/6J was responsive to HDM with development

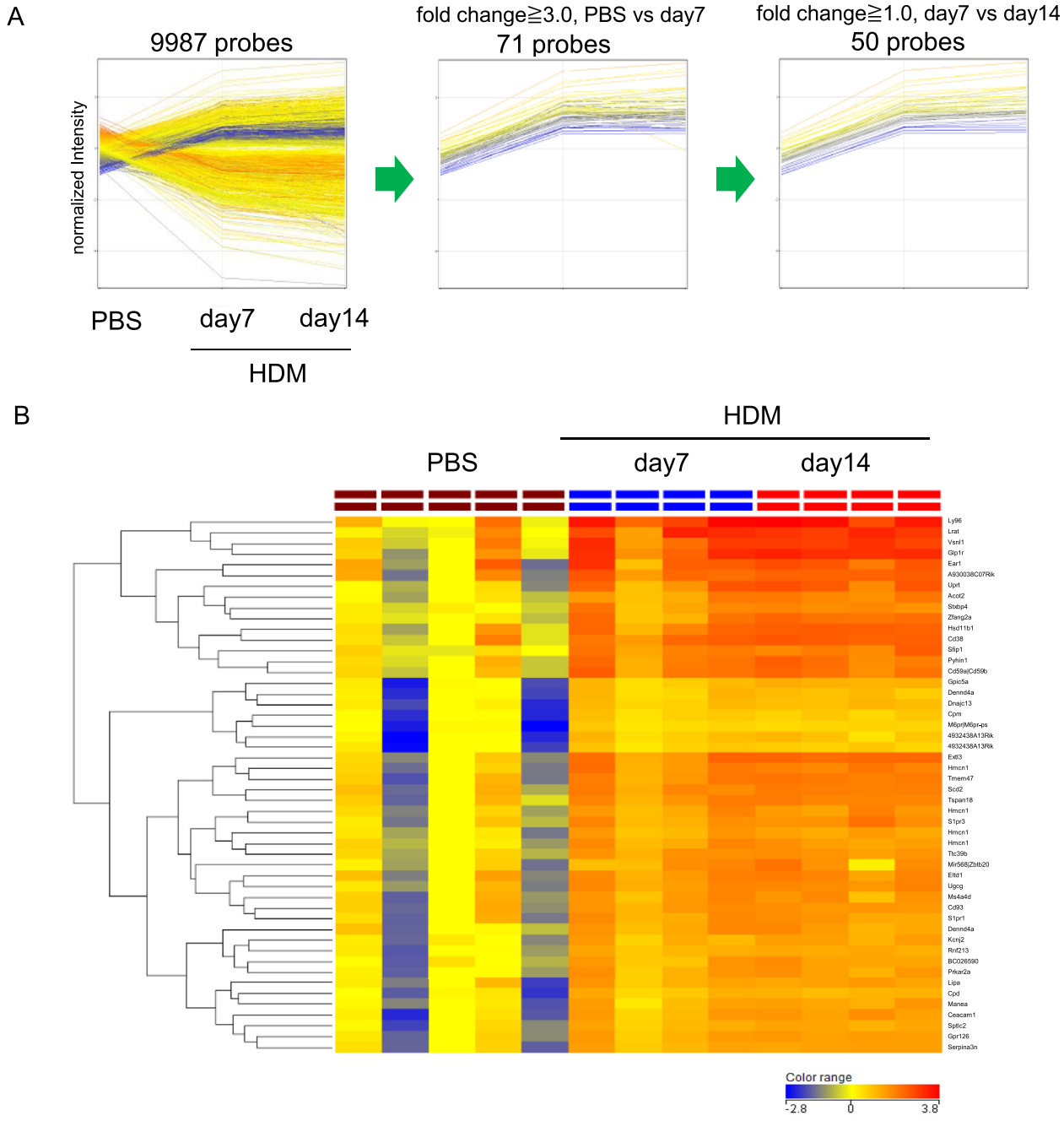

Fig. 2. Global gene expression profiles in the amplification process of HDM sensitization. (A) Microarray data analysis by filtering to identify significantly altered gene expression. Expression profiles were normalized to control (PBS). Data were first filtered by percentile (20–100) and CV<50.0%. We performed fold change analysis by comparing the control and day 7, with a fold change cut-off of ≥3.0 assumed as significant. After identifying 71 genes, we compared day 7 and day 14, with a fold change cut-off of ≥1.0 assumed as significant. (B) Heat map displaying differential gene expression patterns of mRNA by cluster plot. Probe sets are expressed as above average (red), below average (blue), and average (yellow). Hierarchical clustering of the 50 genes identified as being stepwise up-regulated in response to HDM sensitization. The dendrogram provide a qualitative means of assessing the similarity between genes, 4–5 mice per group. CV, coefficent of variation; PBS, phosphate-buffered saline; HDM, house dust mite.

Table 1
Top 20 up-regulated genes in the lung of HDM-sensitized mice.

Gene symbol	GeneBank accession no	Gene description	Fold change PBS vs day7
Ly96	BC116785	lymphocyte antigen 96	5.393773
Glp1r	BC139464	glucagon–like peptide 1 receptor	5.20324
Lrat	AF255061	lecithin–retinol acyltransferase (phosphatidylcholine–retinol–O–acyltransferase)	5.089008
Uprt	BC147845	uracil phosphoribosyltransferase (FUR1) homolog (S. cerevisiae)	4.449313
A930038C07Rik	BC047154	RIKEN cDNA A930038C07 gene	4.207658
Hmcn1		hemicentin 1	4.183091
Extl3	AF083550	exostoses (multiple)–like 3	4.071339
Vsnl1	AY101375	visinin–like 1	3.981668
Ear1	AY316149|BC150991	eosinophil–associated, ribonuclease A family, member 1	3.975323
Tmem47	BC019751	transmembrane protein 47	3.917776
Ceacam1	M77196	carcinoembryonic antigen–related cell adhesion molecule 1	3.67258
Cd59a|Cd59b	U60473	CD59a antigen|CD59b antigen	3.578231
4932438A13Rik		RIKEN cDNA 4932438A13 gene	3.536576
Prkar2a	AF533977	protein kinase, cAMP dependent regulatory, type II alpha	3.455188
Ugcg	BC050828	UDP–glucose ceramide glucosyltransferase	3.429925
S1pr1	BC051023	sphingosine–1–phosphate receptor 1	3.35154
Scd2	BC040384	stearoyl–Coenzyme A desaturase 2	3.350781
Cd38	BC046312	CD38 antigen	3.334364
Hsd11b1	BC132364	hydroxysteroid 11–beta dehydrogenase 1	3.275633
Pyhin1	BC096384	pyrin and HIN domain family, member 1	3.266723

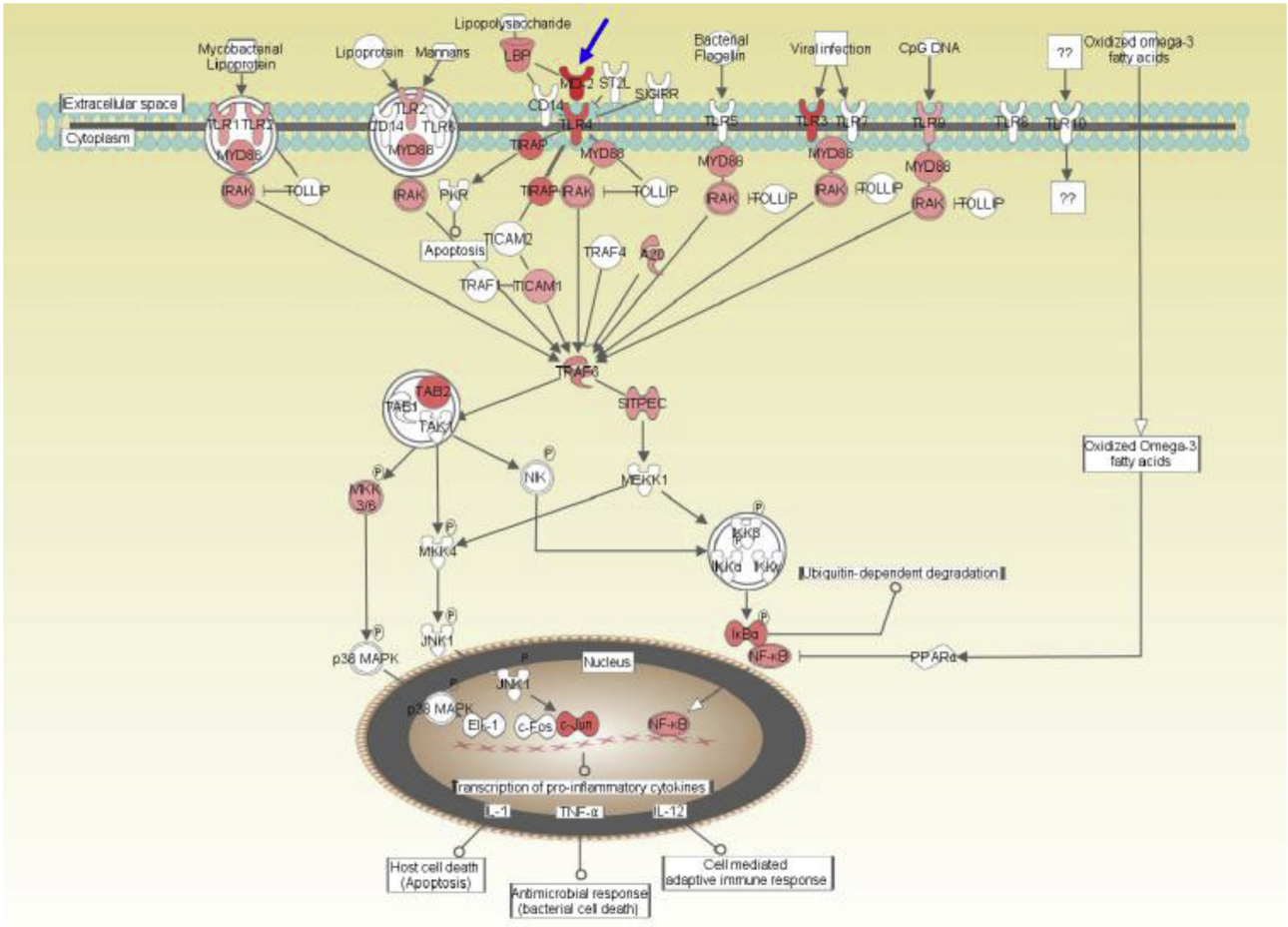

Fig. 3. Interactive network of MD-2 with known TLR4-related genes using IPA. Overexpressed genes are shown in red, whereas down-expressed genes are shown in green. Interactions among the different genes in the network are displayed as solid lines (direct interaction) or dotted (indirect interaction) lines that connect the different genes. Blue arrow indicates MD-2.

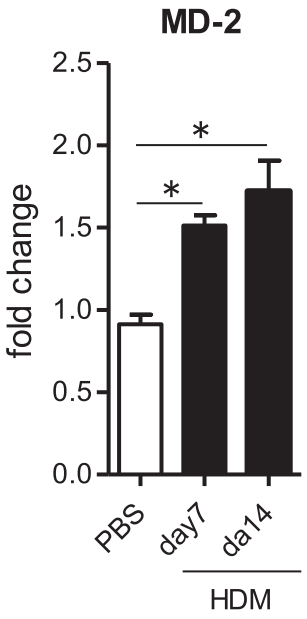

Fig. 4. MD-2 gene expression in lungs of HDM-sensitized mice. Validation of microarray analysis by qRT-PCR analysis of RNA expression in the lungs of HDM-sensitized mice. Fold change calculated compared with PBS control after normalization for GAPDH. These results are representative of two independent experiments (means ± SEM of 3 mice per group), $^{*}P < 0.05$.

allergic inflammation, mucus production and AHR to HDM when using the originally reported protocol[12] (Supplementary Fig. 1). We next focused on the HDM sensitization phase and conducted an animal model of asthma, in which twice-repeated intratracheal administration of HDM amplifies asthma-like responses in mice (Fig. 1A). The number of eosinophils and lymphocytes were significantly increased in the BALF of HDM-sensitized mice compared with the control mice, but not neutrophils. Hammad *et al.* reported that HDM exposure, at a dose identical to that used in this study, is known not to induce neutrophilia in the BALF because there is no induction of KC or G-CSF.[12] In addition, allergic airway inflammation on day 14 (1 week after the second HDM administration) was significantly higher than that on day 7 (1 week after the first HDM administration) (Fig. 1B). Similarly, H&E and AB/PAS staining showed that histological changes in the lung, such as inflammatory cell infiltration and goblet cell hyperplasia, on day 14 were increased compared with those on day 7 (Fig. 1C, D). On day 14, AHR was significantly increased in HDM-exposed mice stimulated by 50 mg/ml of methacholine, but on day 7, there was a trend that did not reach statistical significance (Supplementary Fig. 2A, B). Total IgE and HDM-specific IgG_1 in the serum and IL-4 in the BALF were significantly increased on day 14 (Supplementary Fig. 3). IL-5 displayed a slight trend; however, this difference did not reach statistical significance (data not shown). We were unable to measure IL-13. Levels of these cytokines were found to be too low to be accurately measured. These results indicate that twice-repeated intratracheal administration of HDM amplified allergic airway inflammation, mucus production and AHR.

Global gene expression profiles of sensitization phase

To identify the molecule relevant to the amplification of allergic sensitization to HDM, we performed global gene expression analysis using RNAs isolated from lung tissues of mice (Fig. 1). We first excluded genes, which had poor reproducibility or had wide variability, and selected genes which had <50% variability (CV value) in expression values in at least one of three conditions (control group, day 7, day 14). After normalization to controls, 9987 genes were identified (Fig. 2A). As we focused on the amplification process of HDM-sensitization, we performed fold change analysis by comparing the control and day 7 with a fold change cut-off of ≥3.0 assumed as significant. After identifying 71 genes, we compared days 7 and 14 with a fold change cut-off of ≥1.0 assumed as significant. From our stepwise analysis during allergic sensitization, we identified 50 HDM-mediated up-regulated genes (Fig. 2A). Heat maps demonstrated differential gene expression patterns of mRNA from three different conditions (PBS control, HDM day 7 and HDM day 14) (Fig. 2B). The highest expressed gene was LY96, known as MD-2, an LPS-binding component of TLR4 signaling complex (Table 1).

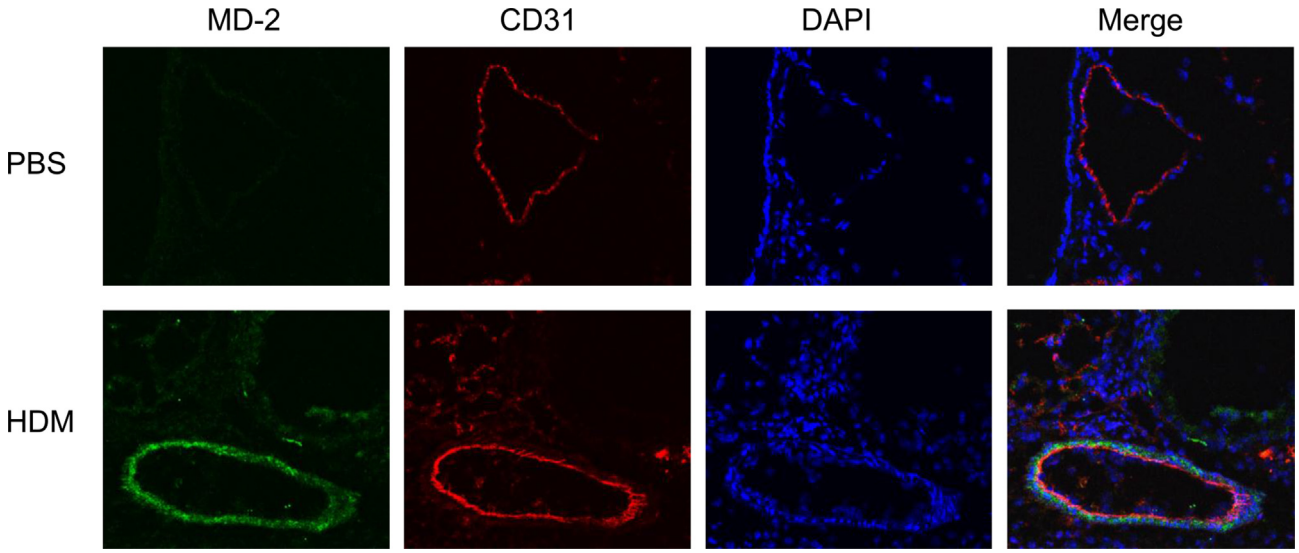

Fig. 5. MD-2 protein expression in lung vascular endothelial cells of HDM-sensitized mice. Immunofluorescence staining of lung tissues with anti-MD2, anti-CD31 antibodies, DAPI, and merge in the HDM-sensitized (day 14) and control mice.

Interactive network of MD-2 with TLR-4 related genes during sensitization

MD-2 is one of the molecules that binds to TLR4 and activates innate immune reaction. Thus, we performed pathway analysis of the genes which were associated with TLR4, including MD-2, using IPA. In the pathway analysis, we found that there were no down-regulated TLR4-related genes during HDM sensitization; most of the expressions in the TLR4-related genes were up-regulated (Fig. 3). These data suggest MD-2 and other TLR4 signaling pathways were activated by HDM sensitization.

Validation of MD-2 gene expression

On qRT-PCR to validate gene expression of MD-2 in microarray data, we found that MD-2 expression was up-regulated in a stepwise manner from control to day 7 and day 14 in HDM-sensitized lung (Fig. 4).

MD-2 expression in lung vascular endothelial cells of HDM-sensitized mice

To clarify the localization of MD-2 during HDM sensitization phase, we conducted immunofluorescence staining of the lung tissue using anti-mouse MD-2 antibody. On immunofluorescence staining, we found that MD-2 was histologically expressed on lung blood vessels in HDM-sensitized mice on day 14 (Fig. 5, left, lower) but not the control mice (Fig. 5, left, upper). In order to confirm the localization of MD-2, we used CD31, a marker for vascular endothelial cells. Moreover, we found that MD-2 and CD31 were expressed at the same location in the lung of HDM-sensitized mice (Fig. 5, right, lower). These suggested that MD-2 is expressed in lung vascular endothelial cells during HDM sensitization throughout the airway.

Serum level of MD-2 in HDM-sensitized mice

Because MD-2 was expressed in the lung vascular endothelium during HDM sensitization, we hypothesized that MD-2 would be released into the blood.

To address this, we measured MD-2 in the serum using the ELISA method. MD-2 protein expression in the serum significantly increased on day 14 compared with the control in OD values (Fig. 6A). No difference was observed in the MD-2 protein level in BALF of HDM-sensitized mice when compared with that of the control mice in OD values (Fig. 6B). We evaluated the correlation between the numbers of eosinophils in the BALF and MD-2 levels in serum on day 14 but not MD-2 levels in the BALF; however, there was a trend that did not reach statistical significance (Fig. 6C). We also measured the numbers of eosinophils in BALF and MD-2 in serum on day 21 (7 days following the third HDM exposure on day 15) (Supplementary Fig. 4A, B, C) and evaluated this correlation. On day 21, MD-2 serum levels correlated significantly with the number of eosinophil in the BALF (Supplementary Fig. 4D)

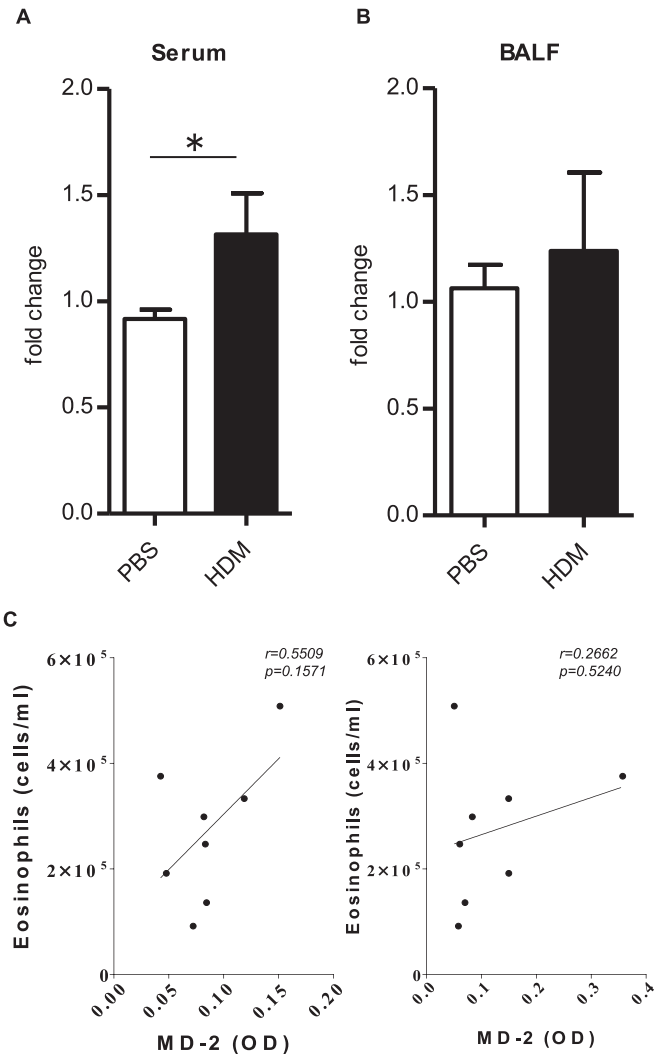

Fig. 6. MD-2 protein levels in serum and BALF of HDM-sensitized mice. We collected serum and BALF from HDM-sensitized mice or control mice on day 14 and measured serum (A) and BALF (B) MD-2 protein using ELISA in OD values. Serum and BALF levels of MD-2 are expressed as fold change relative to controls. Data are represented as means ± SEM, 5–9 per group, $^{*}P < 0.05$ vs PBS control. (C) The correlation between the number of eosinophils in BALF and serum MD-2 levels in HDM-exposed mice on day 14. Pearson's correlation coefficient was used to evaluate the correlation between two sets of variables. Statistical significance was set at a P value of <0.05. Data were analyzed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

Discussion

In this study, we explored the molecules that amplify allergic inflammation in the HDM sensitization phase by global gene expression analysis of murine lung tissue. We found that the MD-2 gene was significantly up-regulated in the lungs during airway sensitization to HDM and activation of the TLR4 signaling cascade was important for this sensitization phase. In addition, MD-2 protein was expressed in lung vascular endothelial cells and increased in the serum during HDM sensitization.

Shimazu *et al.* first reported that MD-2 binds to TLR4 on the cell surface and enables TLR4 response to LPS.[18] MD-2 contains a hydrophobic pocket that is suitable for accommodating endotoxic lipid A.[19] The lipid A portion corresponds to a conserved molecular pattern of LPS and is the main inducer of biological responses to LPS.[20] In an in vivo study using MD-2 deficient mice, MD-2 was found to be essential for LPS response via TLR4 and protected against bacterial infection.[21]

In murine models of asthma, allergic responses to HDM are strain-dependent due to differing genetic backgrounds.[22,23] C57BL/6J, used in this study, is a responder to HDM through airway sensitization.[12] Hammad *et al.* have demonstrated a TLR4-dependent innate immune response underlies the asthma phenotype induced by airway sensitization to HDM.[12] Similar to the allergen used in this study, this HDM contained Der p2, LPS, and Der p1[24]. Der p2, a protein that has a lipid-binding cavity and the

highest-scoring functional homologue of MD-2 in a crystal structure,[14] facilitates signaling through direct interactions with the LPS-binding component of the TLR4 complex.[15] Der p2 contains low levels of LPS which can act as an adjuvant.[6,15] One possible mechanism by which airway sensitization to HDM is established is that Der p2 mimics MD-2, binds to TLR4, and acts as an autoadjuvant that promotes asthma-like phenotype in mice.[15] These findings suggest the TLR4/MD-2 pathway may be critical for allergic airway sensitization. In human studies, single nucleotide polymorphisms of MD-2 were found to be significantly associated with hospital admissions for asthma exacerbations[25] and with occupant endotoxin exposure and wheezing in agricultural workers,[26] suggesting that MD-2 may also be a key molecule in the pathogenesis of inflammatory lung disease, such as asthma, in humans. Based on our microarray data, the TLR4/MD-2 pathway plays a critical role in the amplification process of airway sensitization to HDM.

It has been shown that MD-2 is co-expressed in endothelial cells with TLR4[27]. In addition, MD-2 expression in vascular endothelial cells was increased by bacterial sepsis.[28,29] Vascular endothelial cells are main sources of soluble MD-2 (sMD-2) when stimulated by LPS. In contrast to endothelial cells, bronchial, corneal, and intestinal epithelial cells were found to express low levels of MD-2 in a steady state.[30–32] We found that MD-2 protein levels were potently induced by HDM sensitization, in vascular endothelial cells. These data suggest repeated exposure to HDM induced the expression of MD-2 in lung vascular endothelial cells and may amplify TLR4/MD-2-mediated allergic airway inflammation.

In human studies, it has been shown that sMD-2 increased in the plasma of patients with sepsis,[28,29] active tuberculosis,[33] and HIV.[34] Plasma levels of sMD-2 rapidly decline during tuberculosis treatment; thus, sMD-2 could potentially serve as a complimentary biomarker when evaluating initial response to tuberculosis therapy.[33] We demonstrated that HDM-mediated induction of sMD-2 in the serum was increased and correlated with eosinophilia in the BALF in mice. HDM-induced serum MD-2 may become a biomarker for bronchial asthma.

We speculate that airway sensitization to HDM induced MD-2 expression in lung vascular endothelial cells and release sMD-2 in blood because of an innate immune response to pathogens containing HDM. Further studies are needed to validate the role of MD-2 in HDM-induced allergic sensitization of the airways in mice and in human asthma.

In conclusion, to the best of our knowledge, we demonstrate for the first time that the innate immune-regulated gene, MD-2, was induced by allergic airway sensitization to HDM. Serum MD-2 is a potential biomarker of amplification of allergic sensitization and of the severity of allergic inflammation in human asthma.

Acknowledgments

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Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

DK, SM designed the study and wrote the manuscript. DK, SM, YS, TS, HH, SS and KS performed animal experiments. DK, SM and IT performed RT-PCR. ET performed immunofluorescence staining. DK, SM and KS performed global gene expression analysis. KK performed confocal microscope. SH, YG gave advice on the study design, conduct of the study and interpretation of the data. All authors read and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.alit.2015.05.011.

References

1. Bateman ED, Hurd SS, Barnes PJ, Bousquet J, Drazen JM, FitzGerald M, et al. Global strategy for asthma management and prevention: GINA executive summary. *Eur Respir J* 2008;**31**:143–78.
2. Holgate ST. Innate and adaptive immune responses in asthma. *Nat Med* 2012;**18**:673–83.
3. Ingram JL, Kraft M. IL-13 in asthma and allergic disease: asthma phenotypes and targeted therapies. *J Allergy Clin Immunol* 2012;**130**:829–42. quiz. 43–4.
4. Nair P, Pizzichini MM, Kjarsgaard M, Inman MD, Efthimiadis A, Pizzichini E, et al. Mepolizumab for prednisone-dependent asthma with sputum eosinophilia. *N Engl J Med* 2009;**360**:985–93.
5. Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* 2011;**34**:637–50.
6. Eisenbarth SC, Piggott DA, Huleatt JW, Visintin I, Herrick CA, Bottomly K. Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J Exp Med* 2002;**196**:1645–51.
7. Wilson RH, Whitehead GS, Nakano H, Free ME, Kolls JK, Cook DN. Allergic sensitization through the airway primes Th17-dependent neutrophilia and airway hyperresponsiveness. *Am J Respir Crit Care Med* 2009;**180**:720–30.
8. Wilson RH, Maruoka S, Whitehead GS, Foley JF, Flake GP, Sever ML, et al. The Toll-like receptor 5 ligand flagellin promotes asthma by priming allergic responses to indoor allergens. *Nat Med* 2012;**18**:1705–10.
9. Nelson Jr RP, DiNicolo R, Fernandez-Caldas E, Seleznick MJ, Lockey RF, Good RA. Allergen-specific IgE levels and mite allergen exposure in children with acute asthma first seen in an emergency department and in nonasthmatic control subjects. *J Allergy Clin Immunol* 1996;**98**:258–63.
10. Thomas WR, Hales BJ, Smith WA. House dust mite allergens in asthma and allergy. *Trends Mol Med* 2010;**16**:321–8.
11. Jacquet A. The role of innate immunity activation in house dust mite allergy. *Trends Mol Med* 2011;**17**:604–11.
12. Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat Med* 2009;**15**:410–6.
13. Miyake K. Innate recognition of lipopolysaccharide by Toll-like receptor 4-MD-2. *Trends Microbiol* 2004;**12**:186–92.
14. Gangloff M, Gay NJ. MD-2: the Toll 'gatekeeper' in endotoxin signalling. *Trends Biochem Sci* 2004;**29**:294–300.
15. Trompette A, Divanovic S, Visintin A, Blanchard C, Hegde RS, Madan R, et al. Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. *Nature* 2009;**457**:585–8.
16. Tournoy KG, Kips JC, Schou C, Pauwels RA. Airway eosinophilia is not a requirement for allergen-induced airway hyperresponsiveness. *Clin Exp Allergy* 2000;**30**:79–85.
17. Wittke A, Weaver V, Mahon BD, August A, Cantorna MT. Vitamin D receptor-deficient mice fail to develop experimental allergic asthma. *J Immunol* 2004;**173**:3432–6.
18. Shimazu R, Akashi S, Ogata H, Nagai Y, Fukudome K, Miyake K, et al. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J Exp Med* 1999;**189**:1777–82.
19. Park SH, Kim ND, Jung JK, Lee CK, Han SB, Kim Y. Myeloid differentiation 2 as a therapeutic target of inflammatory disorders. *Pharmacol Ther* 2012;**133**:291–8.
20. Kim HM, Park BS, Kim JI, Kim SE, Lee J, Oh SC, et al. Crystal structure of the TLR4-MD-2 complex with bound endotoxin antagonist Eritoran. *Cell* 2007;**130**:906–17.
21. Nagai Y, Akashi S, Nagafuku M, Ogata M, Iwakura Y, Akira S, et al. Essential role of MD-2 in LPS responsiveness and TLR4 distribution. *Nat Immunol* 2002;**3**:667–72.
22. Kelada SN, Wilson MS, Tavarez U, Kubalanza K, Borate B, Whitehead GS, et al. Strain-dependent genomic factors affect allergen-induced airway hyperresponsiveness in mice. *Am J Respir Cell Mol Biol* 2011;**45**:817–24.
23. Sahu N, Morales JL, Fowell D, August A. Modeling susceptibility versus resistance in allergic airway disease reveals regulation by Tec kinase Itk. *PloS One* 2010;**5**:e11348.
24. Post S, Nawijn MC, Hackett TL, Baranowska M, Gras R, van Oosterhout AJ, et al. The composition of house dust mite is critical for mucosal barrier dysfunction and allergic sensitisation. *Thorax* 2012;**67**:488–95.
25. Kljaic-Bukvic B, Blekic M, Aberle N, Curtin JA, Hankinson J, Semic-Jusufagic A, et al. Genetic variants in endotoxin signalling pathway, domestic endotoxin exposure and asthma exacerbations. *Pediatr Allergy Immunol* 2014;**25**:552–7.
26. Smit LA, Heederik D, Doekes G, Koppelman GH, Bottema RW, Postma DS, et al. Endotoxin exposure, CD14 and wheeze among farmers: a gene–environment interaction. *Occup Environ Med* 2011;**68**:826–31.
27. Pugin J, Stern-Voeffray S, Daubeuf B, Matthay MA, Elson G, Dunn-Siegrist I. Soluble MD-2 activity in plasma from patients with severe sepsis and septic shock. *Blood* 2004;**104**:4071–9.

28. Tissieres P, Dunn-Siegrist I, Schappi M, Elson G, Comte R, Nobre V, et al. Soluble MD-2 is an acute-phase protein and an opsonin for Gram-negative bacteria. *Blood* 2008;**111**:2122–31.
29. Wolfs TG, Dunn-Siegrist I, van't Veer C, Hodin CM, Germeraad WT, van Zoelen MA, et al. Increased release of sMD-2 during human endotoxemia and sepsis: a role for endothelial cells. *Mol Immunol* 2008;**45**:3268–77.
30. Jia HP, Kline JN, Penisten A, Apicella MA, Gioannini TL, Weiss J, et al. Endotoxin responsiveness of human airway epithelia is limited by low expression of MD-2. *Am J Physiol Lung Cell Mol Physiol* 2004;**287**:L428–37.
31. Vamadevan AS, Fukata M, Arnold ET, Thomas LS, Hsu D, Abreu MT. Regulation of Toll-like receptor 4-associated MD-2 in intestinal epithelial cells: a comprehensive analysis. *Innate Immun* 2010;**16**:93–103.
32. Zhang J, Kumar A, Wheater M, Yu FS. Lack of MD-2 expression in human corneal epithelial cells is an underlying mechanism of lipopolysaccharide (LPS) unresponsiveness. *Immunol Cell Biol* 2009;**87**:141–8.
33. Feruglio SL, Troseid M, Damas JK, Kvale D, Dyrhol-Riise AM. Soluble markers of the Toll-like receptor 4 pathway differentiate between active and latent tuberculosis and are associated with treatment responses. *PloS One* 2013;**8**:e69896.
34. Troseid M, Lind A, Nowak P, Barqasho B, Heger B, Lygren I, et al. Circulating levels of HMGB1 are correlated strongly with MD2 in HIV-infection: possible implication for TLR4-signalling and chronic immune activation. *Innate Immun* 2013;**19**:290–7.