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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-