Myeloid differentiation protein 2 facilitates pollen- and cat dander–induced innate and allergic airway inflammation

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Background: The National Health and Nutrition Examination Survey identified several pollens and cat dander as among the most common allergens that induce allergic sensitization and allergic diseases. We recently reported that ragweed pollen extract (RWPE) requires Toll-like receptor 4 (TLR4) to stimulate CXCL-mediated innate neutrophilic inflammation, which in turn facilitates allergic sensitization and airway inflammation. Myeloid differentiation protein 2 (MD2) is a TLR4 coreceptor, but its role in pollen- and cat dander–induced innate and allergic inflammation has not been critically evaluated.

Objective: We sought to elucidate the role of MD2 in inducing pollen- and cat dander–induced innate and allergic airway inflammation.

Methods: TCM^{Null} (TLR4^{Null}, CD14^{Null}, MD2^{Null}), TLR4^{Hi}, and TCM^{Hi} cells and human bronchial epithelial cells with small interfering RNA–induced downregulation of MD2 were stimulated with RWPE, other pollen allergic extracts, or cat dander extract (CDE), and activation of nuclear factor κB (NF-κB), secretion of the NF-κB–dependent CXCL8, or both were quantified. Wild-type mice or mice with small interfering RNA knockdown of lung MD2 were challenged intranasally with RWPE or CDE, and innate and allergic inflammation was quantified.

Results: RWPE stimulated MD2-dependent NF-κB activation and CXCL secretion. Likewise, Bermuda, rye, timothy, pigweed, Russian thistle, cottonwood, walnut, and CDE stimulated MD2-dependent CXCL secretion. RWPE and CDE challenge induced MD2-dependent and CD14-independent innate neutrophil recruitment. RWPE induced MD2-dependent allergic sensitization and airway inflammation.

Conclusions: MD2 plays an important role in induction of allergic sensitization to cat dander and common pollens relevant to human allergic diseases. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: *Allergic inflammation, antigen, cat dander, pollen, MD2, neutrophil, nuclear factor κB, ragweed, Toll-like receptor 4*

Pollens and cat dander are major causes of allergic airway disorders, such as rhinitis and asthma.[1-3] The National Health and Nutrition Examination Survey identified several pollens and cat dander as among the most common allergens that induce allergic sensitization and allergic diseases.[2] The role of adaptive immune responses in induction of allergic diseases by allergens has been extensively studied. However, relatively little is known about innate immune receptors that contribute to allergic sensitization. Recent studies have identified a role of innate responses mediated by Toll-like receptor 4 (TLR4) in pollen-induced allergic inflammation.[4-6] One study reported that short ragweed pollen induces allergic conjunctivitis by stimulating TLR4-dependent thymic stromal lymphopoietin (TSLP) secretion in sensitized mice.[4] Another study reported that adaptive allergic immune responses to birch pollen extract were reduced in *Tlr4* knockout (KO) mice, thus implying a role of TLR4 in induction of allergic immune responses.[5] Recently, we reported that ragweed pollen extract (RWPE) challenge induces CXCR2-and TLR4-dependent innate recruitment of activated neutrophils to the lungs.[6] We reported that deletion of TLR4 abrogated RWPE-induced allergic sensitization and allergic inflammation.[6] We further demonstrated that passive transfer of neutrophils to *Tlr4*KO recipient mice reconstitutes allergic sensitization and allergic airway inflammation in *Tlr4*KO mice.[6]

Myeloid differentiation protein 2 (MD2) is a 160-amino-acid glycoprotein.[7] MD2 directly binds LPS presented by CD14[8,9] and stimulates TLR4 homodimerization–induced canonical inflammatory signaling.[10] MD2 belongs to the MD2-related lipid recognition domain superfamily, which also includes the mite allergens Der p 2 and Der f 2 in addition to MD1, GM2 activator protein, Niemann-Pick C2 protein (Npc2), and phosphatidylinositol phosphatidylglycerol transfer protein. The structural and functional mimicry of MD2 by Der p 2 stimulates TLR4.[11] However, the role of MD2 in pollen-induced innate and allergic airway inflammatory responses has not been reported. We hypothesized that because RWPE requires TLR4 to induce innate inflammation-mediated allergic sensitization,[6]

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Abbreviations used
BALF: Bronchoalveolar lavage fluid
CDE: Cat dander extract
FACS: Fluorescence-activated cell sorting
HBEC: Human bronchial epithelial cell
KO: Knockout
MD2: Myeloid differentiation protein 2
NF-κB: Nuclear factor κB
RWPE: Ragweed pollen extract
siRNA: Small interfering RNA
TLR4: Toll-like receptor 4
WT: Wild-type

it might also require MD2 to mediate these effects. We further hypothesized that this pathway is shared by other common allergens relevant to human allergic diseases.[2,3]

METHODS

Mice

Eight- to 12-week-old male wild-type (WT) C57BL/10SNJ mice, *Tlr4* knockout (KO) mice (C57BL/10ScNJ), WT mice (C57BL/6J), and *Cd14*KO mice (B6.129S-Cd14tm1Frm/J) were purchased from Jackson Laboratory (Bar Harbor, Me) for these studies. The mice were maintained in a pathogen-free environment at the University of Texas Medical Branch (Galveston, Tex). Animal experiments were performed according to the National Institutes of Health guidelines and were approved by the University of Texas Medical Branch Animal Care and Use Committee.

Allergenic extracts

We have previously reported that lyophilized RWPE containing a very low amount of endotoxin (<0.1 pg of LPS/1 μg of allergen protein; Greer Laboratories, Lenoir, NC) induces TLR4-dependent innate neutrophilic inflammation and allergic sensitization.[6] For the present study, we purchased lyophilized RWPE, Bermuda grass, timothy grass, rye grass, firebush, pigweed, Russian thistle, black walnut, eastern cottonwood, mountain cedar, and cat dander extract (CDE) from Greer Laboratories. Like our previous study[6] all tested allergenic extracts had very low (<0.1 pg of LPS/1 μg) levels of allergen protein, as determined by using the LAL chromogenic endotoxin quantitation kit (Thermo Scientific, Hudson, NH).

Protocols used for animal studies

Mice were sedated with low-dose intraperitoneal xylazine-ketamine anesthetic mixture for intranasal sensitization or challenge and euthanized by a lethal anesthetic mixture overdose.[12]

Single-challenge model (see Fig E1, *A*, in this article's Online Repository at www.jacionline.org). WT mice were intranasally challenged with a single dose of RWPE or CDE (100 μg/60 μL) and euthanized after 16 hours. In additional experiments, 1 hour before RWPE challenge, WT mice were treated with or without intranasal administration of a nuclear factor κB (NF-κB) inhibitor that selectively irreversibly blocks IκBα phosphorylation with BAY 11-7082 (10 mg/kg body weight; Calbiochem, San Diego, Calif)[13] or NEMO-binding domain binding peptide (25 μg per mouse, Calbiochem).[14] These treated mice were challenged with RWPE and euthanized, as described above.

Single-challenge model after single small interfering RNA treatment (see Fig E1, *B*). Two HPLC-purified predesigned small interfering RNAs (siRNAs) against *Md2* (catalog no. s69441; Ambion Silencer, Thermo Fisher) and *Tlr4* (catalog no. s75206; Ambion Silencer) and control nonspecific siRNA oligos (catalog no. 12935-100; stealth RNAi Negative Control duplexes; Ambion) were diluted in 5% glucose mixed with *in vivo* JET-PEI (Polyplus-transfection, New York, NY). We selected the intravenous route of siRNA administration because it has been shown to suppress specific gene expression by 80% in airway epithelial cells and has minimal toxicity, unlike intranasal administration.[15] Forty micrograms of each siRNA was administered to WT mice on day 0. The mice were challenged intranasally with 100 μg of RWPE or CDE on day 2 and euthanized 16 hours after challenge.

Repeated-challenge allergy model after repeated siRNA treatment (see Fig E1, *C*). WT mice were administered control siRNA oligos or siRNAs against *Md2*, as described above, on days −2, 1, and 9. These mice were administered 5 intranasal doses of RWPE (100 μg/60 μL) on days 0, 1, 2, 3, 4, and 11 to mimic chronic exposure of human subjects to RWPE[6,16] and euthanized on day 14, as described above.

Processing of mouse fluid and tissue samples

Total and differential bronchoalveolar lavage fluid (BALF) cell counts were performed.[16] Lungs were perfused and fixed with Zn fixative (BD Biosciences, San Jose, Calif), and sections were stained with periodic acid–Schiff for mucus staining.

Quantitative RT-PCR of mouse lung mRNA

RNA from mouse lung tissue was reverse transcribed and amplified by using real-time PCR in an ABI 7000 (Applied Biosystems, Foster City, Calif). The primer sequences of MD2 were as follows: forward, 5′-AGCTCTG CAAAAAGAATAGTCATC-3′; reverse, 5′-ATAAGACTGAGGGGAACCA ATG-3′. This primer were purchased from Integrated DNA Technologies (Coralville, Iowa).

Mucin production

Mucin production was assessed by 2 investigators who were blind to the treatment groups by using a modification of a method reported[6,16] on a subjective scale of 0, 1, 2, 3, and 4 corresponding to none, mild, moderate, marked, or severe mucin deposition, respectively. Data were expressed as means of scores recorded by 2 blinded investigators.[6,16]

Measurement of IL-5, IL-13, IL-33, and TSLP levels in BALF

BALF from WT mice in a repeated-challenge allergy model were assayed for IL-5, IL-13, IL-33, and TSLP by using a DuoSet ELISA development kit (R&D Systems, Minneapolis, Minn), according to the manufacturer's instructions.

Measurement of RWPE-specific serum IgE levels

RWPE-specific IgE levels were measured by using a previously described method.[6,16] Briefly, 96-well plates were coated with 100 μg/mL RWPE protein overnight. After washing 3 times, the plates were blocked with SEA BLOCK Blocking Buffer (Pierce Biotechnology, Rockford, Ill). Diluted sera from mice were added to the plates and incubated overnight. After washing, the plates were incubated with biotin-conjugated rat IgE (clone R35-72; BD Biosciences, San Jose, Calif) for 2 hours at room temperature, washed, and incubated with avidin-conjugated alkaline phosphatase for 45 minutes at 4°C. After washing, fluorometric values for each well were measured after addition of AttoPhos substrate solution (Promega, Madison, Wis).

Studies involving HEK 293 cell lines and human bronchial epithelial cells

Three HEK 293 cell lines, TCM^{Null} ($TLR4^{Null}$, $CD14^{Null}$, $MD2^{Null}$), $TLR4^{Hi}$ ($TLR4^{Hi}$, $CD14^{Null}$, $MD2^{Null}$) and TCM^{Hi} ($TLR4^{Hi}$, $CD14^{Hi}$, $MD2^{Hi}$; InvivoGen, San Diego, Calif), were used. In some experiments