House Dust Mite Extract Induces Interleukin-9 Expression in Human Eosinophils

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ABSTRACT
Background: Eosinophils play a pivotal role in allergic inflammation. Recent evidence suggests that they not only function as terminal effector cells but have potential to interact with allergen and initiate immune responses. We investigated cytokine production from eosinophils through direct interaction with a major allergen, house dust mite (HDM).

Methods: Purified eosinophils from HDM-sensitized or non-sensitized donors were cultured with HDM extract or lipopolysaccharide (LPS) for 18 or 40 h. A panel of cytokine gene expression in eosinophils was examined by means of real-time RT-PCR. Released cytokines in the culture supernatants were assessed with a specific ELISA. In some experiments, HDM was pretreated with protease inhibitors, then added to the culture. Cytokines tested for gene expression were interleukin (IL)-2, 4, 6, 7, 8, 9, 10, 11, 12, 13, 16, 17, 18, TGF-β1 and GM-CSF.

Results: LPS induced small enhancement of GM-CSF gene expression at 18 h. At 40 h, HDM induced about 60-fold enhancement of IL-9 gene expression. IL-9 protein was also detected in the culture supernatants at 60 h. Those reactions were observed regardless of HDM sensitization status of the donors. HDM-induced IL-9 expression was completely inhibited with a serine protease inhibitor, AEBSF, not with a cysteine protease inhibitor, E-64.

Conclusions: Accumulated eosinophils in the airways in asthma may directly react with HDM and produce IL-9 to further promote Th2-type immune responses. Protease-activated receptor 2, a ligand for serine proteases, which contained in HDM, may be involved in the reaction.

KEY WORDS
allergens, eosinophils, house dust mites, interleukin-9, proteinase activated receptor 2

INTRODUCTION
Massive eosinophil infiltration in the airway mucosa is a prominent feature of the pathology of bronchial asthma and multiple evidence suggest that eosinophils are the major effector cells in the pathogenesis of bronchial hyperresponsiveness and airway remodeling in asthma.¹ Among various mediators from eosinophils, major basic protein (MBP) in specific granules has pleiotropic functions to cause bronchial epithelial cell damage,² airway hyperresponsiveness,³ and activation of other inflammatory cells.⁴ Cysteinyl leukotrienes cause acute bronchoconstriction, hypersecretion, and promote airway inflammation.⁵ Transforming growth factor β (TGF-β) from eosinophils⁶ has been shown to be involved in airway remodeling.⁷

Conventional understanding of the role of eosinophils in asthma is the terminal effectors as described above under control of Th2 cells,⁸ natural killer T cells,⁹ mast cells,¹⁰ and monocytes.¹¹ Recent evidence, however, suggests that eosinophils not only function as terminal effector cells but as immunomodulatory cells in innate immunity. Eosinophils in the airways have a potential to traffic to regional lymphnodes for antigen presentation to T cells.¹²,¹³

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Eosinophil-derived neurotoxin (EDN), one of eosinophil granule proteins, induces dendritic cell migration.\(^{14}\) Eosinophils also directly interact with allergens; house dust mite (HDM) and birch pollen allergens induce chemotaxis and degranulation,\(^{15}\) pollen-associated lipid mediators induce chemotaxis and CD11b expression,\(^{16}\) and an environmental fungus Alternaria protein product induces intracellular calcium mobilization, cell surface expression of CD63 and CD11b, and degranulation.\(^{17}\) These observations indicate that eosinophils can initiate or modulate allergic immune responses by interacting with exogenous molecules such as allergens.

Thus, to further clarify the potential of eosinophils as initiators of allergic inflammation, we investigated a panel of cytokine production from eosinophils through direct interaction with a major allergen, HDM. Here, we found that eosinophils produce significant amount of IL-9, one of the pivotal Th2-type cytokines in the pathogenesis of asthma, in response to HDM extract.

**METHODS**

**REAGENTS**

House dust mite (Dermatophagoides pteronyssinus) extract was purchased from GREER Laboratories (Lenoir, NC, USA). E-coli-derived lipopolysaccharide (LPS) was from Sigma (St. Louis, MO, USA). Protease inhibitors, trans-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane (E-64) and 4-(2-Aminoethyl)-benzenesulfonyl fluoride (AEBSF) were also purchased from Sigma.

**ISOLATION OF EOSINOPHILS**

Heparinized peripheral blood was obtained from HDM-sensitized or non-sensitized donors. The former subjects had mild allergic rhinitis and the latter were healthy non-atopic individuals. Sensitization to HDM was defined as CAP-RAST titer to Dermatophagoides pteronyssinus >0.7 UA/ml (Phadia, Tokyo, Japan). Eosinophils were isolated by negative selection using anti-CD16 bound micromagnetic beads (MACSTM, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) as previously described.\(^{18}\) The purity of eosinophils was more than 99%. This work was approved by the ethical committee of Mic National Hospital and written informed consents were obtained from all subjects.

**QUANTITATIVE RT-PCR**

Purified eosinophils at 1 × 10⁶/ml in RPMI 1640 medium (Sigma) were cultured with HDM extract at 100 µg/ml or LPS at 1 µg/ml in the presence of 5% heat-inactivated fetal bovine serum (FBS; Sigma) or 5% non-processed FBS for 18 or 40 h. Total RNA was extracted from the cells and reverse transcribed. Real-time PCR was carried out for IL-2, IL-4, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-13, IL-17, IL-18, and GM-CSF on ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Primer sets for each cytokine were designed using Primer Express software (Applied Biosystems). Relative gene expression was calculated with the 2^{-ΔΔCT} method reported elsewhere.\(^{19}\) In some experiments, HDM was incubated with a cysteine protease inhibitor, E-64 or a serine protease inhibitor, AEBSF, at 37°C for 30 min then added to the culture.

**ELISA FOR IL-9**

Eosinophils at 1 × 10⁶/ml in RPMI 1640 medium with 5% FBS in the presence or absence of HDM were cultured for 60 h and the supernatants were tested for IL-9 protein by a sandwich ELISA. Each well of a 96-well microplate (Immuo Module F8 Maxisorp, Nunc, Roskilde, Denmark) was filled with 50 µl of anti-human IL-9 monoclonal antibody (clone MH9A4, BioLegend, San Diego, CA, USA) at 5 µg/1 in carbonate buffer (pH 9.6) and incubated for 18 h at 4°C. After removal of the antibody solution, the wells were washed four times with phosphate-buffered saline containing 0.5 ml/1 Tween 20 (PBS-T). PBS containing 0.1% bovine serum albumin (BSA; Sigma) was added to each well, then incubated for 1 h at 25°C. After aspiration, aliquots of human recombinant IL-9 (PeproTech, New Jersey, USA) standards or samples (25 µl each) were added to the wells and incubated for 16 h at 4°C. Each well was washed four times with PBS-T, 50 µl of biotin-conjugated anti-human IL-9 (clone MH9A3, BioLegend) was added and incubated for 1 h at 25°C. After washing with PBS-T, 100 µl of streptavidin-HRP (GIBCO Industries, Langley, OK, USA) in PBS containing 0.1% BSA was added to each well and incubated for 1 h at 25°C. Following four washing of the assay plates, the immunoreactivity was visualized by addition of 100 µl/well of substrate solution (TMB solution, Roche Diagnostics GmbH, Mannheim, Germany) for 15 min at 25°C. The reaction was stopped by the addition of 50 µl of 1 g/1 sodium dodecyl sulfate to each well, and absorbance was measured at 405 nm. The IL-9 levels were calculated based on the standard curve on each assay plate.

**STATISTICAL ANALYSIS**

The data were expressed as mean ± SEM of indicated numbers of experiments and p values were determined with ANOVA for multiple groups with Bonferroni post test.

**RESULTS**

**CYTOKINE GENE EXPRESSION OF EOSINOPHILS BY HDM EXTRACT AND LPS**

First, we tested whether HDM extract directly induces cytokine gene expression from eosinophils. Because HDM extract may contain LPS and LPS activates eosinophils via TLR-4 and CD14,\(^{20}\) which is not
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**Fig. 1** Cytokine gene expression by HDM extract (open bar), LPS with heat-inactivated FBS (horizontal hatched bar), and LPS with non-processed FBS (diagonal hatched bar). **A.** 19 h culture **B.** 40 h culture. The data represent geometric means of fold changes in gene expression. \(n = 4 - 5\). * \(p < 0.05\)

only expressed on the cell surface but present as soluble form in the serum,\(^{22}\) we also cultured eosinophils with LPS in FBS-containing medium. In addition, to fully induce activation potential of LPS, FBS with no heat inactivation process was also used since complements may be involved in LPS-induced activation.\(^{22,23}\) At 18 h of incubation, HDM extract did not induce more than 3-fold increase in gene expression of a panel of cytokine tested (Fig. 1A). LPS in combination with non-processed serum induced significant gene expression of GM-CSF (Fig. 1A). At 40 h, surprisingly, HDM induced about 60-fold enhancement of IL-9 gene expression while LPS did not cause significant gene expression of any cytokine (Fig. 1B). We also confirmed with a specific ELISA that HDM extract, not LPS, induced significant IL-9 protein production at 60 h of incubation (Fig. 2).

**HDM EXTRACT INDUCED IL-9 PRODUCTION REGARDLESS OF HDM SENSITIZATION STATUS**

Since HDM is a major IgE sensitizing allergen, we sought the possible relationship between the presence of HDM-specific IgE antibody and HDM-induced IL-9 gene expression in the subjects. There was, however, no difference in IL-9 gene expression between HDM-sensitized and non-sensitized donors (Fig. 3).

**SERINE PROTEASE INHIBITOR ABOLISHED HDM-INDUCED IL-9 GENE EXPRESSION**

Many allergens including HDM are found to be proteases. We then examined the effect of protease in-
Fig. 2  IL-9 protein concentrations in the supernatants of eosinophils after 60 h of culture with HDM extract (open bar), LPS with heat-inactivated FBS (horizontal hatched bar), and LPS with non-processed FBS (diagonal hatched bar). The data represent geometric means. (n = 3). *p < 0.05

Fig. 3  HDM-induced IL-9 gene expression in HDM-sensitized (HDM-IgE (+)) and non-sensitized (HDM-IgE (−)) individuals. We compared the two groups by Man-Whitney test. N.S.; not significant

DISCUSSION

In the present study, we showed that eosinophils directly reacted with a major allergen, HDM, and produced significant amount of a pleiotropic Th2-type cytokine, IL-9. Since the stimulatory effect of HDM was completely abolished by pretreatment with a serine protease inhibitor, AEBSF, completely abolished the subsequent IL-9 gene expression with HDM (Fig. 4). On the other hand, a cysteine protease inhibitor, E-64, did not have any effect on the reaction even at the higher concentration. Because of known specificity of proteases to PAR, it is suggested that eosinophils may respond to HDM via PAR-2.

IL-9 is classified as a Th2-type cytokine originally identified in activated CD4+ T cells.25 It promotes the proliferation and differentiation of mast cells and hematopoietic progenitors, stimulates the proliferation of activated T cells, and enhances the production of immunoglobulins by B cells.26 In the context of Th2-type inflammation, IL-9 was reported to induce eotaxin production from airway smooth muscle cells, IL-5 receptor expression from eosinophils,27,28 and goblet cell hyperplasia.29 Selective overexpression of the IL9 gene within the lungs of transgenic mice resulted in massive airways inflammation with eosinophils and lymphocytes as predominant infiltrating cells.30 Blockade of IL-9 inhibited the development of airway inflammation in a mouse model of asthma.31 In humans, a close association between the IL9 gene and bronchial hyperresponsiveness has been suggested.32 Collectively, IL-9 may play an important role in the pathogenesis of asthma and the potential of eosinophils to produce IL-9 adds another mechanism that underlie exacerbating nature of Th2-type inflammation.

Environmental allergens including house dust mites, fungi, and pollens contain various kinds of proteases and it has been demonstrated that these proteases directly cause activation of epithelial cells and inflammatory cells leading to inflammatory cytokine production24 and degradation of epithelial tight junctions facilitating further allergen entry33. Thus, the proteolytic activities characterize the pathogenic nature of the allergen molecules independent of their IgE-binding epitopes. HDM, the most common allergen in Japan and other area of the world with warm and humid climate, contains cysteine proteases and serine proteases as well as a number of other uncharacterized proteases. Der p 1 and Der f 1, the major allergens in HDM, are cysteine proteases and Der p 3, Der f 3, Der p 9, and Der f 9 are serine proteases.33-36 Proteases stimulate cells via PARs, 7-transmembrane G protein-coupled receptors. They cleave the amino acids at a specific site of the extracellular N-terminus of the PARs to expose a new N-terminal ligand do-
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main that binds to another site on the same molecule, thereby activating the receptor. Four PARs have been identified and amino acid sequences of each cleavage site is specific for the particular PAR. Serine proteases are PAR-2 agonists and we speculate that HDM extract stimulated eosinophils to produce IL-9 via PAR-2 because a serine protease inhibitor, not a cysteine protease inhibitor, blocked the reaction although the results presented here were still insufficient to prove PAR-2-mediated IL-9 production.

We also examined the possibility that HDM-induced IL-9 production from eosinophils was IgE-mediated. Eosinophils express low affinity IgE receptors, CD23, and may express high affinity Fc epsilon receptors as well. If the latter is the case, isolated eosinophils from peripheral blood of HDM-sensitized individuals have membrane-bound HDM-specific IgE antibody and have potentials to be activated upon binding of IgE to the allergen. We found, however, that HDM induced the activation was independent of specific IgE to HDM. We also confirmed that that effect was not from contaminated LPS in HDM extract because LPS did not induce IL-9 production even in the presence of the serum.

In the present experiments, we employed the concentrations of HDM and LPS based on a previous study in which the ability of various allergens to cause eosinophil activation and chemotaxis in vitro was investigated. One drawback of our study is that the concentration of HDM used in the experiments was rather high and we did not find significant production of IL-9 on stimulation with lower concentrations of HDM (data not shown) and the present findings may not be relevant to clinical situations. However, exposure levels of HDM vary widely and actual concentrations found in the airways are not known. Alternatively, our experimental model may represent other PAR-2 and PAR-2 agonist interactions such as tryptase from mast cells.

In summary, we have shown that eosinophils are capable of interacting with HDM and producing IL-9. These findings suggest that eosinophils may play a role in innate immunity. When eosinophils encounter with an allergen that has serine-specific enzymatic activity, they may promote Th2-type immune response by producing IL-9.

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