

House dust mite allergen Der p 1 elevates the release of inflammatory cytokines and expression of adhesion molecules in co-culture of human eosinophils and bronchial epithelial cells

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Abstract

House dust mite (HDM) is a common allergen of allergic asthma. Eosinophils are principal effector cells of allergic inflammation and their adhesion onto human bronchial epithelial cells is mediated by a CD18–intracellular adhesion molecule-1 (ICAM-1)–dependent interaction. We studied the effects of HDM *Dermatophagoides pteronyssinus* (Der p) 1 on the activation of eosinophils and bronchial epithelial BEAS-2B cells. Cytokines and adhesion molecules were measured using flow cytometry. Transcription factor nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1) and signaling molecule p38 mitogen-activated protein kinase (MAPK) were analyzed using electromobility shift assay and western blot, respectively. Der p 1 protein was found to potently induce the release of IL-1 β , IL-6, IL-10, tumor necrosis factor (TNF)- α and granulocyte macrophage colony-stimulating factor from eosinophils. Such induction was further up-regulated for IL-6 and IL-10, and down-regulated for TNF- α and IL-1 β in eosinophil–BEAS-2B cells co-culture. Surface expression of CD18 and ICAM-1 on eosinophils was greatly increased by Der p 1; such inductive effect on ICAM-1 was also found to be more prominent on BEAS-2B cells from the co-culture than BEAS-2B cells alone. Der p 1 was found to activate NF- κ B and AP-1 activity in eosinophils alone and in co-culture and BEAS-2B cells in co-culture. Moreover, Der p 1 could activate p38 MAPK in BEAS-2B cells and eosinophils alone and in co-culture. Selective inhibition of NF- κ B, AP-1 and p38 MAPK resulted in differential suppression of the Der p 1-induced cytokine release and adhesion molecule expression. As an allergen, HDM could therefore induce the release of inflammatory cytokines and expression of adhesion molecules from the interaction of human eosinophils and bronchial epithelial cells.

Introduction

Allergic asthma is a complex and heterogeneous disease that is characterized by intermittent reversible obstruction and chronic inflammation of the airways and bronchial hyperactivity that are mediated by T_H cytokines (1). The inflammatory and airway remodeling processes in allergic asthma are the consequence of highly complex interactions among inflammatory cells such as eosinophils, activated T cells, mast cells

and macrophages with structural tissue cells including the bronchial epithelium, endothelial cells and fibroblasts (1). The chemotaxis and transmigration of eosinophils, eosinophil–epithelial cell interactions and the subsequent eosinophil-mediated damage to the bronchial epithelium by their highly toxic granular proteins such as eosinophilic cationic protein (ECP) together contribute to the pathogenesis of asthma (2, 3).

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Activated airway epithelial cells are a source of hematopoietic cytokines, pro-inflammatory cytokines and chemokines (1). Eosinophils are also capable of producing and releasing a variety of pro-inflammatory cytokines such as IL-3, IL-4, IL-5, IL-6, IL-16, tumor necrosis factor (TNF)- α and granulocyte macrophage colony-stimulating factor (GM-CSF) together with chemokines including eotaxin, IL-8, macrophage inflammatory protein-1 α , monocyte chemoattractant protein and regulated upon activation normal T cell expressed and secreted (4, 5). The interaction of eosinophils and epithelial cells can also result in the induction of inflammatory mediators including cysteinyl leukotrienes (6). Co-culture of IL-5-primed eosinophils and TNF- α - or IFN- γ -treated epithelial cells can enhance ECP release from eosinophilic degranulation through CD18-dependent adhesion (7). Our previous studies also indicated that the interaction of eosinophils and epithelial cells can synergistically elevate the induction of IL-6, chemokines (8, 9) and cell surface expression of intercellular adhesion molecule-1 (ICAM-1) on human bronchial epithelial BEAS-2B cells (our unpublished results). Therefore, the interaction between bronchial epithelial cells and eosinophils can up-regulate the responsiveness and the adhesion of human eosinophils and epithelial cells through the release of cytokines and chemokines and the expression of adhesion molecules, thereby indicating a significant implication in the immunopathological mechanisms of allergic asthma.

House dust mites (HDMs) are a major source of allergens that contribute to the rising incidence of allergic diseases such as allergic asthma (10). Der p 1 protein, the major allergen from the mite *Dermatophagoides pteronyssinus*, is characterized by its cysteine protease activity that amplifies allergen-induced bronchial asthma by increasing the permeability of the allergen in the respiratory tract and disrupts the regulation of IgE synthesis (11). Der p 1 protein is capable of cleaving human proteins with potentially immunomodulatory effects, including α 1-proteinase inhibitor (12), CD23 (low-affinity IgE receptor) (13), CD25 (α subunit of the human IL-2 receptor) (14) and CD40 (15). Der p 1 has been shown to induce the release of GM-CSF, IL-6 and IL-8 because of its proteolytic activity (16) and the activation of nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1) of bronchial epithelial cells (17, 18, 19). However, unlike Der p 3, Der p 1 stimulates the cytokine expression in airway epithelial cells via a proteinase-activated receptor-2-independent mechanism (19). It can also activate NF- κ B of human eosinophils for the production of GM-CSF, TNF- α and IL-8 (20), and selectively recruit airway dendritic cells upon the activation of airway epithelium (21). However, the effect of HDM Der p 1 on the co-culture of epithelial cells and eosinophils has not been well studied.

In an attempt to further elucidate the immunopathological mechanisms of allergen-induced allergic inflammation in asthma, we investigated the effects of Der p 1 on the activation of eosinophils and bronchial epithelial cells upon their interaction in terms of the release of inflammatory cytokine IL-1 β , IL-6, IL-10 and TNF- α and hematopoietic cytokine GM-CSF and expression of adhesion molecule ICAM-1 (CD54) and leukocyte function-associated antigen-1 (CD11a/CD18).

Methods

Inhibitors

Recombinant HDM allergen from *Pichia pastoris* eukaryotic yeast, *Dermatophagoides pteronyssinus* (Der p) 1, was purchased from Indoor Biotechnologies Ltd, Manchester, UK. AP-1 inhibitor, curcumin; NF- κ B inhibitor, BAY 11-7082, and p38 mitogen-activated protein kinase (MAPK) inhibitor, SB 203580, were purchased from Calbiochem Corp., San Diego, CA, USA. Curcumin and BAY 11-7082 were dissolved in dimethyl sulfoxide (DMSO) and SB 203580 was dissolved in water. In all studies, the concentration of DMSO was 0.1% (vol/vol).

Endotoxin-free solutions and Der p 1

Cell culture medium (Invitrogen Corp., Carlsbad, CA, USA) and recombinant Der p 1 were found to be free of detectable LPS (<0.1 EU ml $^{-1}$), as determined by the Limulus amoebocyte lyase assay (sensitivity limit 12 pg ml $^{-1}$; Biowhittaker Inc., Walkersville, MD, USA). All other solutions were prepared using pyrogen-free water and sterile polypropylene plasticware. No solution contained any detectable LPS.

Isolation of human blood eosinophils from buffy coat and eosinophil culture

Fresh human buffy coat obtained from the Hong Kong Red Cross Blood Transfusion Service was diluted 1:2 with PBS at 4°C and centrifuged using an isotonic Percoll solution (density 1.082 g ml $^{-1}$; Amersham and Pharmacia Biotech, Uppsala, Sweden) for 30 min at 1000 $\times$ g. The eosinophil-rich granulocyte fraction was collected and washed twice with cold PBS containing 2% certified fetal bovine serum (FBS). Cells were then incubated with anti-CD16 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) at 4°C for 45 min, and CD16-positive neutrophils were depleted by passing through an LS+ column (Miltenyi Biotec) within a magnetic field. With this preparation, the drop-through fraction contained eosinophils with a purity of at least 98% as assessed by Hemacolor rapid blood smear stain (E. Merck Diagnostica, Darmstadt, Germany). The isolated eosinophils were cultured in RPMI 1640 medium (Invitrogen), supplemented with 10% FBS (Invitrogen) and 20 mM HEPES (Invitrogen). All blood samples were ascertained to be collected from subjects sensitized to allergen Der p 1 using specific IgE fluorescence enzyme immunoassay (AutoCAP analyzer, Pharmacia Diagnostics AB, Uppsala, Sweden) (22).

Co-culture of epithelial cells and eosinophils

The human bronchial epithelial cell line (BEAS-2B) transformed by adenovirus 12-SV40 virus hybrid (Ad12SV40) was obtained from the American Type Culture Collection, Manassas, VA, USA, which has been widely used as an *in vitro* bronchial epithelial cell model (8, 9). BEAS-2B cells were grown in DMEM nutrient mixture F12 (Invitrogen) with 10% FBS in a six-well cell culture plates at 37°C with 5% CO $_2$ —95% humidified air until confluence to cell monolayer. The medium was then replaced with RPMI 1640 medium containing 10% FBS (Invitrogen) with or without eosinophils. For inhibition experiments, BEAS-2B cells and eosinophils were pre-treated with SB 203580 or BAY 11-7082 for 1 h. For the assay of p38

MAPK and NF- κ B activity of BEAS-2B cells and eosinophils, non-adherent eosinophils were separated from the adherent BEAS-2B cells by washing with PBS after different treatments.

Co-culture of fixed BEAS-2B cells and eosinophils

Confluent BEAS-2B cells or eosinophils were treated with 1% PFA in PBS for 1 h at room temperature to inhibit the synthesis of cytokines yet maintaining the intercellular interaction. After fixation, cells were washed at least 10 times with wash buffer, and fixed or unfixed BEAS-2B cells and eosinophils were co-cultured in RPMI 1640 medium with 10% FBS.

Assay of chemokines IL-1 β , IL-6, IL-10, TNF- α and GM-CSF in culture supernatant

The four inflammatory cytokines IL-1 β , IL-6, IL-10 and TNF- α in culture supernatant were measured simultaneously by cytometric bead array (CBA) using a four-color FACSCalibur flow cytometer (BD Biosciences Corp., San Jose, CA, USA) (23, 24). In CBA, four bead populations with distinct fluorescence intensities had been coated with capturing antibodies specific for four different cytokines. These bead populations could be resolved in the fluorescence channels of the flow cytometer. After the beads had been incubated with 50 μ l of culture supernatant, different cytokines in the supernatant were captured by their corresponding beads. The cytokine-captured beads were then mixed with phycoerythrin-conjugated detection antibodies to form sandwich complexes. Following incubation, washing and acquisition of fluorescence data, the results were generated in graphical format using the BD CBA software. The concentrations of cytokine IL-1 β , IL-6, IL-10 and TNF- α were measured simultaneously using the human inflammatory cytokine CBA kit (BD Biosciences Pharmingen, San Diego, CA, USA). GM-CSF was assayed by ELISA using reagent kit (R & D Systems Inc., Minneapolis, MN, USA).

Flow cytometric analysis of cell surface adhesion molecules

Eosinophils or BEAS-2B cells after different treatments were washed and re-suspended with cold PBS supplemented with 0.5% BSA. After blocking with 2% human pooled serum for 20 min at 4°C and washed with PBS supplemented with 0.5% BSA, cells were incubated either with FITC-conjugated mouse anti-human adhesion molecule mAb or fluorescein-conjugated mouse IgG₁ or IgG_{2b} isotype for 30 min at 4°C in the dark. After washing, cells were finally re-suspended in 1% PFA in 1 $\times$ PBS as fixative. Expression of surface adhesion molecule was then analyzed by flow cytometry (FACSCalibur, BD) as mean fluorescence intensity.

Western blot analysis

Cells were lysed with lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 2 mM sodium orthovanadate, 20 mM sodium pyrophosphate, 1% sodium deoxycholate and 1 $\times$ protease inhibitors, Assay Designs Inc., Ann Arbor, MI, USA). Cell lysate was centrifuged at 14 000 $\times g$ for 15 min and the protein in supernatants was quantified using Micro BCATM Protein Assay Reagent kit (Pierce Chemical Co., Rockford, IL, USA). Total protein (10 μ g) was diluted 1:2 with Laemmli sample buffer (Bio-Rad Inc., CA, USA) and boiled for 5 min. The

resultant protein was then separated on 10% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (Amersham Biosciences Corp., Piscataway, NJ, USA). The membrane was blocked with 5% skimmed milk in Tris-buffered saline with 0.05% Tween 20, pH 7.6, for 1 h at room temperature, and probed with primary rabbit anti-human phospho-p38 MAPK antibody (Cell Signaling Technology Inc., Beverly, MA, USA) at 4°C overnight. After washing, membranes were incubated with secondary donkey anti-rabbit antibody coupled to HRP (Amersham Biosciences) for 1 h at room temperature, and developed with ECL chemiluminescent detection reagent (Amersham Biosciences).

Electrophoretic mobility shift assay of AP-1 and NF- κ B activity

Non-adherent eosinophils were separated from adherent BEAS-2B cells by washing. Cells were then harvested and washed and nuclear proteins extracted with NE-PERTM nuclear and cytoplasmic extraction reagents (Pierce) according to the manufacturer's instructions. Equal amounts of nuclear protein extracts were subjected to a test for AP-1 protein or NF- κ B protein/DNA binding using LightShiftTM chemiluminescent electrophoretic mobility shift assay (EMSA) kit (Pierce) with a biotin end-labeled AP-1 oligonucleotide (5'-TTC CGG CTG ACT CAT CAA GCG-3') and NF- κ B oligonucleotide (5'-AGT TGA GGG GAC TTT CCC AGG C-3') (Research Genetics Invitrogen Co., Huntsville, AL, USA), respectively. Briefly, nuclear extracts were incubated with biotin end-labeled AP-1 or NF- κ B oligonucleotide for 20 min at room temperature to allow DNA-protein binding. The DNA-protein complexes were then resolved by a 6% native PAGE and transferred to a Hybond-N+ membrane (Amersham and Pharmacia Biotech). The biotin end-labeled DNA was detected using a streptavidin-HRP conjugate and a chemiluminescent substrate (4).

In contrast to eosinophils, BEAS-2B cells do not require sonication for protein extraction using lysis buffer (8, 9). We did not use any sonication for the protein extraction in all our experiments. Therefore, the measured activity of NF- κ B and AP-1 and the phosphorylation of p38 MAPK of BEAS-2B cells were actually located at the BEAS-2B cells but not due to the contamination of eosinophils.

Statistical analysis

All data were expressed as mean $\pm$ standard error of the mean. Differences between groups were assessed by the non-parametric Kruskal-Wallis test. A probability $P < 0.05$ was considered significantly different. All analyses were performed using the statistical software GraphPad Prism for Windows, Version 3.00 (GraphPad Software, San Diego, CA, USA).

Results

Release of IL-6, IL-10, TNF- α and IL-1 β upon the interaction of BEAS-2B cells and eosinophils activated by Der p 1

Der p 1 (5 μ g ml⁻¹) was adopted for the experiments based on the concentrations used in previous studies (16, 18, 20) as well as our preliminary activation studies without causing any toxicities on eosinophils and BEAS-2B cells. As shown in

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Fig. 1(A–D), Der p 1 ($5 \mu\text{g ml}^{-1}$) could significantly activate eosinophils to release IL-6, IL-10, TNF- α and IL-1 β (all $P < 0.005$). The induction was further up-regulated for IL-6 and IL-10 and down-regulated for TNF- α and IL-1 β in eosinophil-bronchial epithelial cell co-culture.

To investigate the source of cytokines in the culture supernatant, we have compared the cytokine release from the co-culture of normal cells and PFA-fixed cells. The co-culture of fixed BEAS-2B cells and unfixed eosinophils exhibited similar induction of IL-6 and IL-10 but suppressed induction of TNF- α and IL-1 β compared with eosinophils alone with or without Der p 1 activation, and suppressed the induction of IL-6 and IL-10 compared with Der p 1-activated unfixed eosinophil-BEAS-2B cell co-culture (Fig. 1A–D). However, the fixation of eosinophils could largely abolish the Der p 1-induced release of IL-6 and IL-10, TNF- α and IL-1 β in co-culture. The co-culture of fixed BEAS-2B cells and eosinophils actually exhibited similar effects on the induction of TNF- α and IL-1 β compared with the co-culture of normal unfixed BEAS-2B cells and eosinophils with or without the Der p 1 activation (Fig. 1C and D).

Release of GM-CSF upon the interaction of BEAS-2B cells and eosinophils activated by Der p 1

Der p 1 ($5 \mu\text{g ml}^{-1}$) was found to significantly activate eosinophils (5×10^5 per well) but not BEAS-2B cells (1.5×10^5

per well) to release GM-CSF (BEAS-2B cells: 1.8 ± 0.7 versus $3.6 \pm 0.3 \text{ pg ml}^{-1}$, $P > 0.05$; eosinophils: 0.5 ± 0.4 versus $242.9 \pm 31.0 \text{ pg ml}^{-1}$, $P < 0.05$). The induction of GM-CSF was significantly less in co-culture with Der p 1 ($191.5 \pm 23.6 \text{ pg ml}^{-1}$) or without Der p 1 ($51.7 \pm 10.5 \text{ pg ml}^{-1}$) for 18 h compared with Der p 1-treated eosinophils ($242.9 \pm 31.0 \text{ pg ml}^{-1}$) (both $P < 0.05$).

Release of IL-6, IL-10, TNF- α and IL-1 β upon the interaction of BEAS-2B cells and eosinophils activated by Der p 1 with or without transwell inserts

A control experiment was performed using transwell inserts (pore size $0.4 \mu\text{M}$) to separate the BEAS-2B and eosinophils into two separated compartments in the co-culture using a 24-well plate. It was used to ascertain if direct interaction was essential for the cytokine release. Transwell insert experiments in the presence of Der p 1 showed that transwell inserts could significantly enhance the induction of IL-1 β and TNF- α (Fig. 2C and D) with no significant effect on the induction of IL-6 and IL-10 (Fig. 2A and B).

Activation of NF- κB and AP-1 activity in BEAS-2B cells and eosinophils in co-culture upon the activation by Der p 1

Co-culture of BEAS-2B cells and eosinophils for 19 h was shown to activate NF- κB and AP-1 activity in eosinophils because there was a significant increase in intensity of band

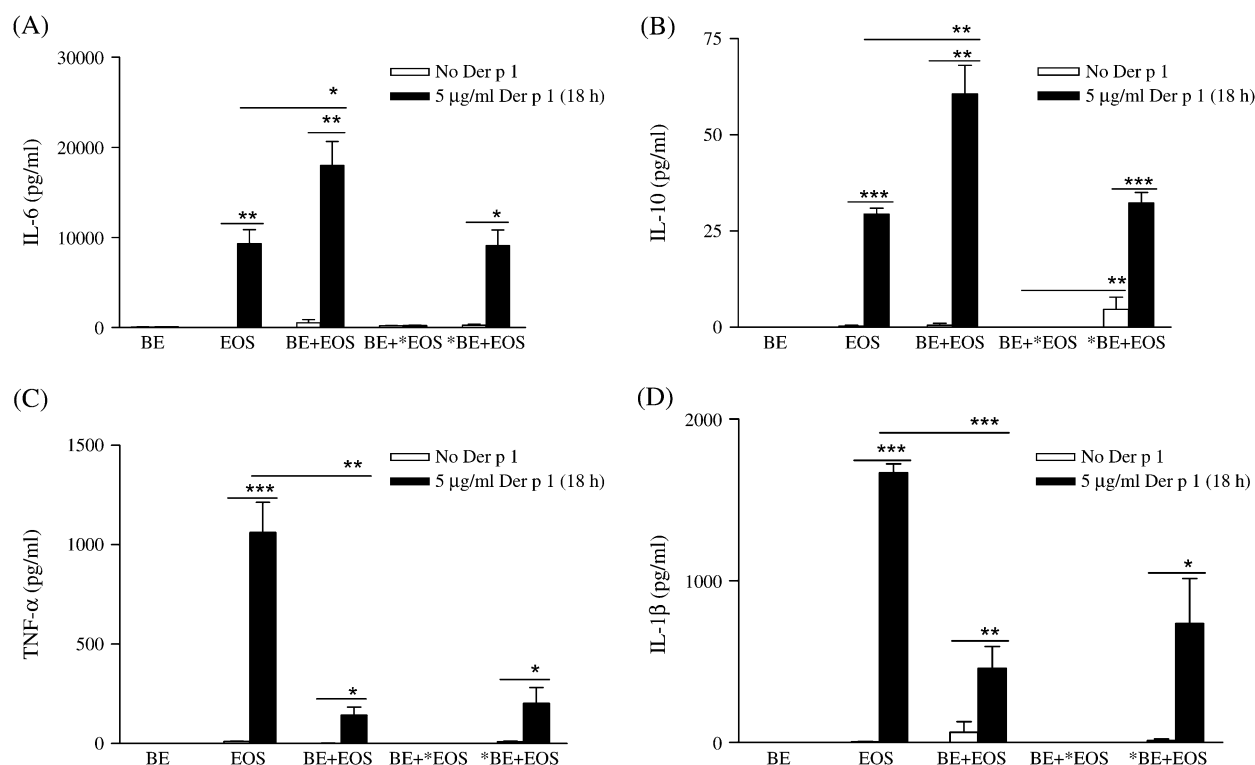


Fig. 1. Effect of Der p 1 on the induction of the release of (A) IL-6, (B) IL-10, (C) TNF- α and (D) IL-1 β upon the interaction of eosinophils and BEAS-2B cells. Confluent BEAS-2B cells (1.5×10^5 per well) were cultured with or without eosinophils (5×10^5 per well) for 18 h in 24-well plate with or without the presence of Der p 1 ($5 \mu\text{g ml}^{-1}$). Cytokines released in culture supernatant was determined by human inflammatory cytokine CBA kit using flow cytometry. Results are expressed as the arithmetic mean $\pm$ standard error of the mean from three independent experiments. * $P < 0.05$, ** $P < 0.005$, and *** $P < 0.0005$ when compared between groups denoted by the horizontal lines. BE, BEAS-2B cells; EOS, eosinophils; *BE, PFA-fixed BEAS-2B cells; *EOS, PFA-fixed eosinophils.

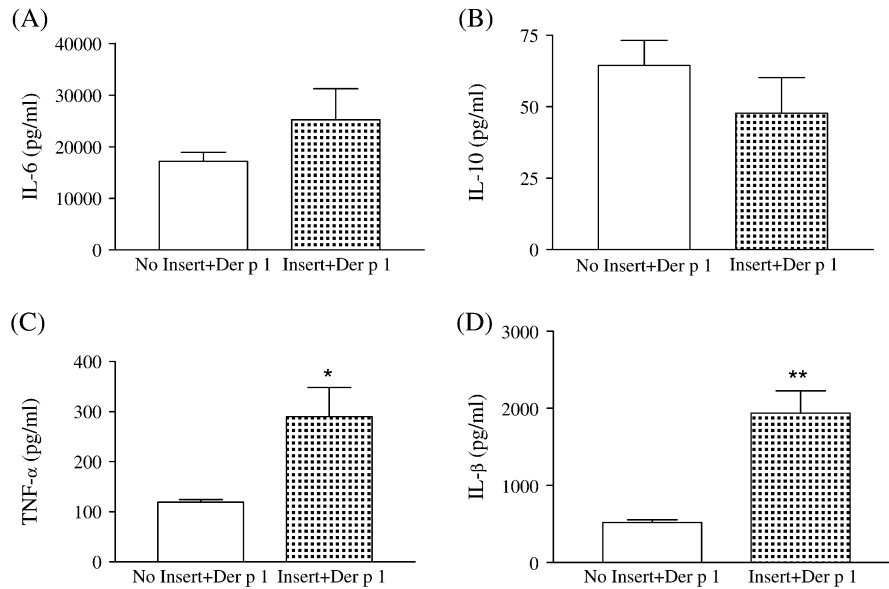


Fig. 2. Effect of transwell inserts on the induction of the release of (A) IL-6, (B) IL-10, (C) TNF- α and (D) IL-1 β upon the co-culture of eosinophils and BEAS-2B cells. Confluent BEAS-2B cells (1.5×10^5 per well) were cultured with eosinophils (5×10^5 per well) for 18 h in a 24-well plate in the presence of Der p 1 ($5 \mu\text{g ml}^{-1}$) with or without transwell insert. Cytokines released in culture supernatant was determined by human inflammatory cytokine CBA kit using flow cytometry. Results are expressed as the arithmetic mean $\pm$ standard error of the mean from three independent experiments. * $P < 0.05$ and ** $P < 0.005$ when cultures with or without transwell inserts are compared.

shift observed in eosinophils (Fig. 3). Moreover, Der p 1 could activate both NF- κ B and AP-1 activity in eosinophils with or without co-culture with BEAS-2B cells. It was found that Der p 1 could activate NF- κ B and AP-1 activity more significantly in BEAS-2B cells co-cultured with eosinophils than BEAS-2B cells alone. Competitive control using excess unlabeled p50 NF- κ B or AP-1-binding DNA confirmed the specificity of NF- κ B/AP-1-DNA interaction.

Activation of p38 MAPK activity in BEAS-2B cells and eosinophils in co-culture upon the activation by Der p 1

Figure 4 shows that Der p 1 could rapidly induce the phosphorylation of p38 MAPK at 20 min in BEAS-2B and eosinophils with or without co-culture, thereby indicating the activation of p38 MAPK activity. Total p38 MAPK was served as control for the normalization of protein amount.

Effect of BAY 11-7082, SB 203580 and curcumin on the release of cytokines in co-culture of eosinophils and BEAS-2B

As shown in Fig. 5, NF- κ B inhibitor BAY 11-7082 and p38 MAPK inhibitor SB 203580 could significantly inhibit the release of IL-1 β , IL-6, IL-10 and TNF- α release from eosinophils alone and co-culture of eosinophils and BEAS-2B cells activated by Der p 1, while AP-1 inhibitor curcumin could only significantly suppress the release of IL-10 but not IL-1 β , IL-6 and TNF- α in Der p 1-activated co-culture. Curcumin could also significantly suppress the release of IL-10, TNF- α and IL-1 β but not IL-6 from eosinophils culture alone activated by Der p 1.

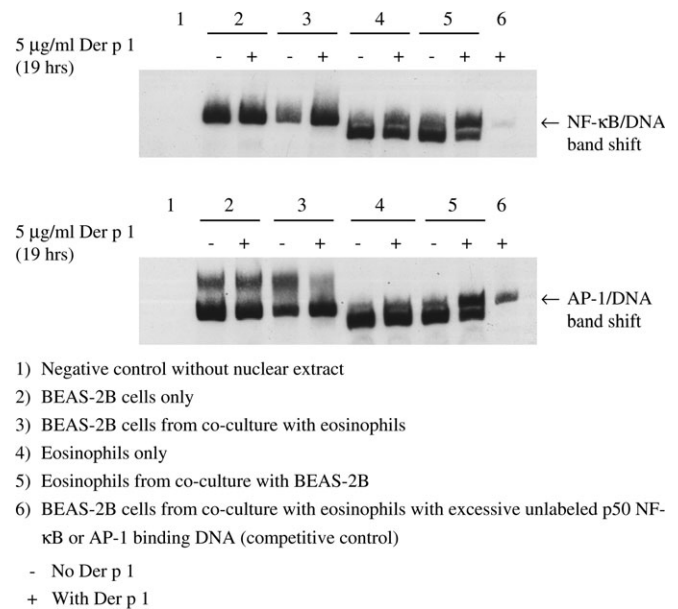


Fig. 3. Activation of NF- κ B and AP-1 activity in eosinophils and BEAS-2B cells by Der p 1. Confluent BEAS-2B cells (6×10^5 per well) were co-cultured with eosinophils (4×10^6) in a 6-well plate for 19 h with or without Der p 1 ($5 \mu\text{g ml}^{-1}$), eosinophils were removed by washing with ice-cold PBS. Nuclear proteins were extracted from eosinophils or BEAS-2B cells and 5 μg protein of each sample was then analyzed by EMSA for AP-1 and NF- κ B protein/DNA-binding activity using EMSA. Experiments were performed in three independent experiments with essentially identical results, and representative results are shown. 1, Negative control without nuclear extract; 2, BEAS-2B cells only; 3, BEAS-2B cells from co-culture with eosinophils; 4, eosinophils only; 5, eosinophils from co-culture with BEAS-2B; 6, BEAS-2B cells from co-culture with eosinophils with excessive unlabeled p50 NF- κ B or AP-1-binding DNA (competitive control). '-', No Der p 1; '+', with Der p 1.

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Effect of Der p 1, BAY 11-7082, SB 203580 and curcumin on the expression of adhesion molecules in co-culture of BEAS-2B cells and eosinophils

As shown in Fig. 6(A and B), co-culture of BEAS-2B cells with eosinophils could significantly enhance the cell surface

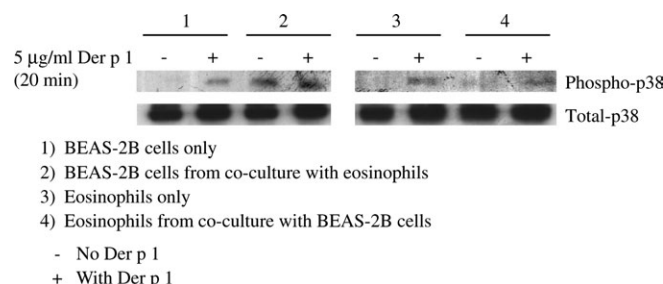


Fig. 4. Activation of p38 MAPK activity in BEAS-2B cells and eosinophils by Der p 1. Confluent BEAS-2B cells (6×10^5 per well) or eosinophils (4×10^6 per well) in a 6-well plate cultured alone or together for 20 min in the presence or absence of Der p 1 (5μ g ml^{-1}). Total cellular proteins were extracted from BEAS-2B cells or eosinophils for the detection of phosphorylated and total p38 MAPK protein level by western blot analysis. Experiments were performed in three independent experiments with essentially identical results, and representative results are shown. 1, BEAS-2B cells only; 2, BEAS-2B cells from co-culture with eosinophils; 3, eosinophils only; 4, eosinophils from co-culture with BEAS-2B cells. '–', No Der p 1; '+', with Der p 1.

expression of adhesion molecules CD18 and ICAM-1 on BEAS-2B cells in the presence or absence of Der p 1, and the effect of Der p 1 on ICAM-1 induction was also found to be more prominent on BEAS-2B cells from the co-culture than BEAS-2B cells alone.

BAY 11-7082, SB 203580 and curcumin could significantly suppress ICAM-1 but not CD18 expression on BEAS-2B cells upon co-culture (Fig. 6A and B). As shown in Fig. 6(C and D), Der p 1 could significantly enhance the cell surface expression of CD18 and ICAM-1 on eosinophils alone or co-culture with BEAS-2B cells, and BAY 11-7082 and SB 203580 could significantly suppress the Der p 1-induced expression of CD18 and ICAM-1 on eosinophils.

There was no significant difference on the up-regulation of ICAM-1 on BEAS-2B cells and CD18 on eosinophils with or without using transwell inserts in the presence of Der p 1 in co-culture (data not shown, all $P > 0.05$).

Discussion

Interaction of bronchial epithelium with eosinophils represents a crucial mechanism of initiating local inflammation in allergic asthma. In this study, we examined the release of inflammatory cytokines and the expression of adhesion molecules upon the interaction of airway epithelial cells and eosinophils that mimic *in vivo* conditions in bronchial allergic asthma stimulated by HDM allergen. Although the *in vitro*

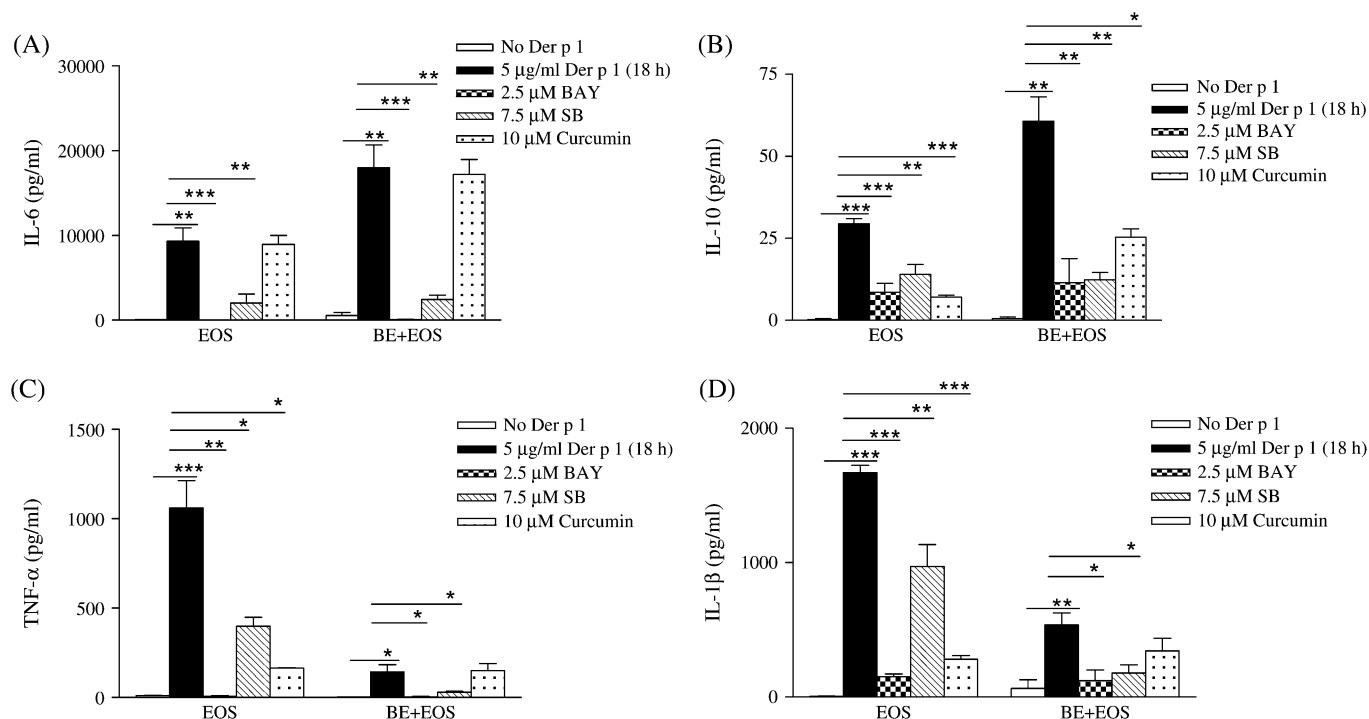


Fig. 5. Effect of BAY 11-7082, SB 203580 and curcumin on the release of (A) IL-6, (B) IL-10, (C) TNF- α and (D) IL-1 β upon the interaction between eosinophils and BEAS-2B cells in the presence or absence of Der p 1. Confluent BEAS-2B cells (1.5×10^5 per well) were pre-treated with BAY 11-7082 (2.5 μ M), SB 203580 (7.5 μ M) or curcumin (10 μ M) for 1 h followed by culture with eosinophils (5×10^5 per well) in a 24-well plate for further 18 h in the presence of BAY 11-7082, SB 203580 or curcumin. Cytokines released in culture supernatant were determined by human inflammatory cytokine CBA kit using flow cytometry. Results are expressed as the arithmetic mean $\pm$ standard error of the mean from three independent experiments. The eosinophil and BEAS-2B single culture and co-culture of eosinophils and BEAS-2B cells contained 0.1% DMSO as a DMSO control. * $P < 0.05$, ** $P < 0.005$, and *** $P < 0.0005$ when compared between groups denoted by the horizontal lines. BE, BEAS-2B cells; EOS, eosinophils.

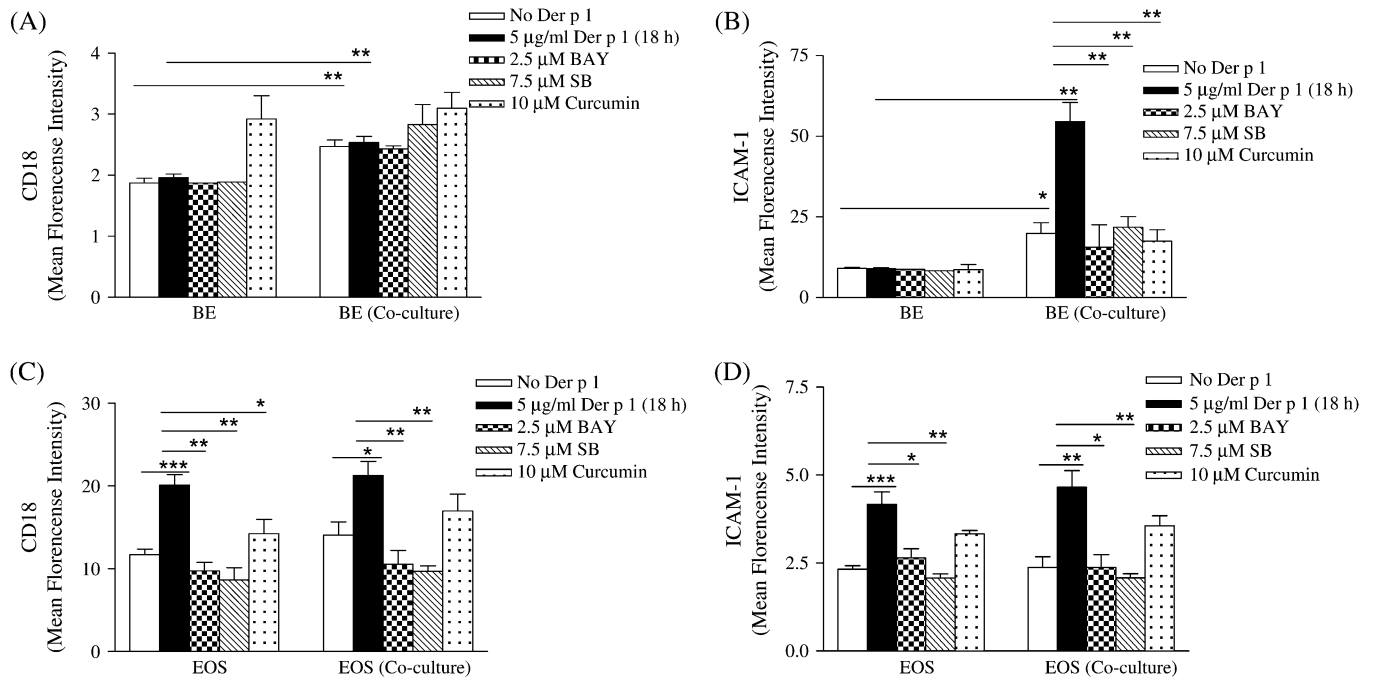


Fig. 6. Effects of Der p 1, signaling molecule inhibitors and co-culture of BEAS-2B cells and eosinophils on the cell surface expression of adhesion molecule CD18 (A and C) and ICAM-1 (B and D) on BEAS-2B cells and eosinophils. Confluent BEAS-2B cells ($1.5 \times 10^5 \text{ ml}^{-1}$) were cultured with Der p 1 ($5 \mu\text{g ml}^{-1}$), eosinophils ($5 \times 10^5 \text{ ml}^{-1}$) or eosinophils ($5 \times 10^5 \text{ ml}^{-1}$) with or without BAY 11-7082 ($2.5 \mu\text{M}$), SB 203580 ($7.5 \mu\text{M}$) or curcumin ($10 \mu\text{M}$) for 18 h (A and B). Eosinophils ($5 \times 10^5 \text{ ml}^{-1}$) were cultured with Der p 1 ($5 \mu\text{g ml}^{-1}$), confluent BEAS-2B cells ($1.5 \times 10^5 \text{ ml}^{-1}$) or BEAS-2B cells ($1.5 \times 10^5 \text{ ml}^{-1}$) with or without BAY 11-7082 ($2.5 \mu\text{M}$), SB 203580 ($7.5 \mu\text{M}$) or curcumin ($10 \mu\text{M}$) for 18 h (C and D). Results are expressed in the bar chart showing the arithmetic mean $\pm$ standard error of the mean of three independent experiments with essential identical results for the cell surface expression of CD18 and ICAM-1. * $P < 0.05$, ** $P < 0.005$, and *** $P < 0.0005$ when compared between groups denoted by the horizontal lines. BE, BEAS-2B cells; EOS, eosinophils

concentration ($5 \mu\text{g ml}^{-1}$) of Der p 1 being used in the present study are orders of magnitudes higher than that of inhaled Der p 1 used in asthma human subjects from a previous study (25), the local concentrations of Der p 1 accumulated at the bronchial inflammatory sites could be much higher than the concentration of inhaled Der p 1 in human subjects to reach levels which are similar to the concentration used in the present study. A previous study has indicated that HDM extracts could activate NF- κ B of eosinophil to produce cytokines (20). In the present study, Der p 1 allergen could activate eosinophils to release IL-6, IL-10, TNF- α and IL-1 β much more potently than that from BEAS-2B cells. Moreover, such induction was further up-regulated for IL-6 and IL-10 and down-regulated for TNF- α and IL-1 β in eosinophil-bronchial epithelial cell co-culture. Since Der p 1 was free of any detectable endotoxin ($<0.1 \text{ EU ml}^{-1}$), the activation of Der p 1 in the culture of eosinophils and epithelial cells was not due to the endotoxin contamination. According to the manufacturer's specification of rDer p 1, it is a kind of mature rDer p 1 that is secreted without the pro-peptide and has cysteine protease activity. We observed that the rDer p 1 protein could suppress the activation effect of recombinant IL-18 on human lymphocytes for cytokine release after the incubation of rDer p 1 with IL-18 at 37°C for 4 h. This abolishing effect is probably due to the cleavage of IL-18 protein into biological inactive fragments by the proteolytic activity of rDer p 1, which is in concordance with the previous results of cysteine proteinase inactivating IL-18

(26). Therefore, Der p 1 could exhibit the effect in eosinophil-bronchial epithelial cell co-culture by its cysteine protease activity (11, 19). As also found by us, incubation of PFA-fixed eosinophils with BEAS-2B cells could largely abolish the Der p 1-induced release of IL-6, IL-10, TNF- α and IL-1 β in co-culture. The co-culture of fixed BEAS-2B cells and eosinophils actually exhibited similar effects on the induction of TNF- α and IL-1 β but lower induction of IL-6 and IL-10 compared with the co-culture of normal unfixed BEAS-2B cells and eosinophils with or without Der p 1 activation. Results therefore indicated that eosinophils but not BEAS-2B cells is the main source for the release of IL-6, IL-10, TNF- α and IL-1 β upon activation by Der p 1. However, the optimal induction of IL-6 and IL-10 but not TNF- α and IL-1 β in co-culture required viable BEAS-2B cells, probable via the soluble mediator release from BEAS-2B cells but not direct interaction. This mechanism was further supported by the results of using transwell inserts to separate eosinophils and BEAS-2B cells. This exhibited no inhibitory effect on the Der p 1 induction of IL-6 and IL-10 in co-culture. In summary, HDM allergen Der p 1 could enhance the T_H2 cytokine IL-6 and regulatory cytokine IL-10 (27, 28) but suppress the T_H1 -related cytokines TNF- α and IL-1 β upon the interaction of eosinophils and epithelium in allergic inflammation. The up-regulation of T_H2 cytokine IL-6 and regulatory cytokine IL-10 may require the mediators release from bronchial epithelial cells. Many studies have demonstrated a lower T_H1/T_H2 ratio and the elevation of IL-6 and IL-10 at local inflammatory site

in allergic asthmatic patients (29–31). Our *in vitro* co-culture system has further confirmed the increase in T_H2 cytokine IL-6 and regulatory cytokine IL-10 but decrease in T_H1 cytokines in the bronchial airway upon allergen provocation. Regarding the investigation of intracellular signaling mechanisms, we used the optimal concentration of BAY 11-7082 (2.5 μ M), SB 203580 (7.5 μ M) and curcumin (10 μ M) with the highest inhibitory effect without any cell toxicity following previous studies (9, 32). Results therefore indicated that p38 MAPK, NF- κ B and AP-1 are involved in the IL-10 release and only p38 MAPK and NF- κ B are involved in IL-1 β , IL-6 and TNF- α release in Der p 1-activated eosinophils-epithelium system.

Bronchial epithelial cells have been demonstrated to act as immunoregulators through the cell surface expression of adhesion molecules including ICAM in allergic airway diseases (33, 34). These adhesion molecules have been proposed to be involved in the release of ECP (35), chemotaxis and transendothelial migration of eosinophils (36). Such processes can be mediated and enhanced by several cytokines (37) and chemokines (38). Regarding the expression of adhesion molecules, Der p 1 played an important role for the induction of adhesion molecule ICAM-1 on BEAS-2B cells and CD18 on eosinophils upon co-culture (Fig. 6). Since transwell inserts did not have any significant effect on the cell surface expression of ICAM-1 on BEAS-2B cells and CD18 on eosinophils in the presence of Der p 1, the Der p 1 effect on the up-regulation of ICAM-1 may also be due to release of soluble mediators such as hematopoietic cytokine GM-CSF (39). In fact, we observed that Der p 1 could significantly activate eosinophils to release GM-CSF. Regarding intracellular mechanisms, Der p 1-induced expression of ICAM-1 on BEAS-2B cells was regulated by p38 MAPK, NF- κ B and AP-1 while only p38 MAPK and NF- κ B were involved in the regulation of ICAM-1 on eosinophils upon co-culture. Moreover, p38 MAPK and NF- κ B were involved in Der p 1-induced expression of both CD18 and ICAM-1 on eosinophils (Fig. 6). It has been shown that Der p 1 is capable of significantly up-regulating ICAM-1 on human endothelial cells (40), and CD18-ICAM-1-dependent adhesion of eosinophils to bronchial epithelial cells has also been demonstrated (41). Moreover, the stimulation of human eosinophil respiratory burst, degranulation and release of leukotriene C4 by platelet-activating factor has been demonstrated to be dependent on CD18-mediated cellular adhesion (42). Eotaxin-induced selective eosinophil transendothelial migration also relies on Mac-1 (CD11b/CD18)-ICAM-1 interaction (36). Therefore, the up-regulated surface expression of adhesion molecules such as ICAM-1 and CD18 on epithelial cells and eosinophils by Der p 1 is crucial for their activation, adhesion and release of chemokines and cytokines.

In summary, our present study has elucidated the immunopathological roles of HDM in allergic inflammation by the activation of eosinophils-epithelium system via the shift of T_H2 predominance and up-regulation of the expression of crucial adhesion molecules such as ICAM-1 and CD18 through differential regulation of intracellular p38 MAPK, AP-1 and NF- κ B. Results provide new clues for the immunopathological mechanisms of HDM-mediated allergic inflammation and the abnormally large number of leukocytes including eosinophils, neutrophils, lymphocytes, basophils and macrophages in the lungs of allergic pulmonary disorders (43). Since eosinophil

interaction to bronchial epithelium and T_H and inflammatory cytokines release are dynamically stimulated by Der p 1, this process might be a target for therapeutic intervention in the treatment of allergic asthma. In view of the recent advances in the application of p38 MAPK and NF- κ B inhibitors as potential anti-inflammatory agents in asthma (44, 45), our findings should facilitate the future development of more effective agents for allergic inflammation.

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Abbreviations

AP-1	activator protein-1
CBA	cytometric bead array
DMSO	dimethyl sulfoxide
ECP	eosinophilic cationic protein
EMSA	electrophoretic mobility shift assay
FBS	fetal bovine serum
GM-CSF	granulocyte macrophage colony-stimulating factor
HDM	house dust mite
ICAM	intracellular adhesion molecule
MAPK	mitogen-activated protein kinase
NF- κ B	nuclear factor- κ B
TNF	tumor necrosis factor

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