

Muramyl dipeptide mediated activation of human bronchial epithelial cells interacting with basophils: a novel mechanism of airway inflammation

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Summary

Respiratory tract bacterial infection can amplify and sustain airway inflammation. Intracytosolic nucleotide-binding oligomerization domain-containing protein 2 (NOD2) is one member of the nucleotide binding and oligomerization domain (NOD)-like receptor (NLR) family, which senses the conserved structural peptidoglycan component muramyl dipeptide (MDP) in almost all bacteria. In the present study, activation of the NOD2 ligand MDP on primary human bronchial epithelial cells (HBE) co-cultured with human basophils was investigated. Cytokines, NOD2, adhesion molecules and intracellular signalling molecules were assayed by enzyme-linked immunosorbent assay or flow cytometry. The protein expression of NOD2 was confirmed in basophils/KU812 cells and HBE/human bronchial epithelial cell line (BEAS-2B) cells. MDP was found to up-regulate significantly the cell surface expression of intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 on basophils and HBE in the co-culture system with or without basophil priming by interleukin (IL)-33 (all $P < 0.05$). MDP could further enhance the release of inflammatory cytokine IL-6 and chemokine CXCL8, and epithelium-derived anti-microbial peptide β -defensin 2 in the co-culture. HBE cells were the major source for the release of IL-6, CXCL8 and β -defensin2 upon stimulation by MDP in the co-culture system. The expression of ICAM-1 and VCAM-1 and release of IL-6 and CXCL8 were suppressed by various signalling molecule inhibitors, implying that the interaction between basophils and primary human bronchial epithelial cells could be regulated differentially by the mitogen-activated protein kinase pathways and nuclear transcription factors. The results therefore provide a new insight into the functional role of basophils in innate immunity, and the link between respiratory bacteria-mediated innate immunity and subsequent amplification of allergic inflammation in the airway.

Keywords: basophils, epithelial cells, infections, pattern recognition receptors, signalling/signal transduction

Introduction

Allergic asthma is a chronically relapsing inflammatory disease. Respiratory tract bacterial infection can cause allergen sensitization and subsequently amplify and sustain airway inflammation in allergic asthma [1]. Animal experiments have shown that moderate pulmonary bacterial infection such as *Chlamydia pneumoniae* can induce subsequent allergic inflammation upon the activation by inhaled allergens through innate immunity-related dendritic cells

[1]. The innate immune system can recognize bacteria through pattern recognition receptors (PRRs), which detect conserved microbial components called pathogen-associated molecular patterns. One of the key intracytosolic PRRs in inflammatory and immune responses is nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs). Two well-characterized NLRs, NOD1 and NOD2, sense the cytosolic presence of the peptidoglycan fragments γ -D-glutamyl-meso-diaminopimelic acid (iE-DAP) in all Gram-negative and certain Gram-positive bacteria, and

peptidoglycan constituent muramyl dipeptide (MDP) in almost all bacteria, respectively [2,3]. The crucial roles of NOD1,2 in immune responses have been proved from the linkage of NOD1,2 polymorphisms with atopic dermatitis, atopic eczema and allergic asthma [4,5]. In fact, NOD1-mediated recognition of bacterial products in the skin or at mucosal interfaces may regulate T helper type 2 (Th2) polarization and immunoglobulin (Ig)E concentrations [4]. NOD2 expression links to innate immune responses against bacterial pathogens *Streptococcus pneumoniae* [6] and *Mycobacterium tuberculosis* [7]. NOD2 mutations are associated with chronic inflammatory bowel diseases, such as Crohn's disease [8]. Animal studies have shown that the NOD2 ligand induces the expression of allergic inflammation-related cytokine thymic stromal lymphopoietin, interleukin (IL)-25 and OX40 ligand (OX40L) in lung, resulting in subsequent susceptibility to asthmatic lung inflammation [9]. A recent study showed that NOD2 ligand MDP could induce the secretion of CXCL8, regulate expression of CD11b and CD69 and enhance migration of eosinophils, which were mediated via the nuclear factor (NF)- κ B signalling pathway [10].

Basophils are rare, circulating granulocytes, representing <1% of the peripheral blood leucocytes. Circulating basophils can be recruited to the inflammatory tissues in allergic disorders including allergic asthma, atopic dermatitis and allergic rhinitis [11–13]. During asthma exacerbation and in response to allergen inhalation challenge, basophils infiltrate markedly into allergic inflammatory sites [14–16]. Studies investigating allergic late-phase responses have shown that basophils are a significant source of Th2 cytokines IL-4 and IL-13, which are central cytokines for the manifestations of allergic disease [17–19].

Our previous study has shown that the T helper 17 (Th17) cytokine IL-17A cytokine can induce the release of inflammatory cytokines and chemokines upon the interaction of human basophils and bronchial epithelial cells through the differential activation of mitogen-activated protein kinase (MAPK) and NF- κ B signalling pathways [20]. In our study of the link between infections and the exacerbation of allergic inflammation, we have demonstrated that the induction of cytokines, chemokines, superoxides and eosinophilic cationic protein (ECP), and cell migration by Toll-like receptor (TLR)-2, -5 and -7 ligands, are regulated differentially by intracellular focal adhesion kinase (FAK), NF- κ B, extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K)-Akt and p38 MAPK in eosinophils [21,22]. However, the NLR-mediated activation of basophils in allergic inflammation has not yet been studied. Therefore, we hypothesized that NLR are crucial PRRs in the process of airway inflammation, by linking the innate immune response against respiratory bacterial infection with the subsequent allergic inflammation through the activation of basophils interacting with bronchial epithelial cells.

Materials and methods

Reagents

The MDP, MDP-negative control D-D isomer, iE-DAP and iE-DAP-negative control γ -D-Glu-Lys (iE-Lys) were purchased from InvivoGen (San Diego, CA, USA). Recombinant human IL-33 was purchased from R&D Systems (Minneapolis, MN, USA). I κ B α phosphorylation inhibitor BAY11-7082, p38 MAPK inhibitor SB203580 and c-Jun N-terminal protein kinase (JNK) inhibitor SP600125 were purchased from Calbiochem Corporation (San Diego, CA, USA). SB203580 was dissolved in water, while BAY11-7082 and SP600125 were dissolved in dimethylsulphoxide (DMSO). In this study, the final concentration of DMSO was 0.1% (v/v).

Purification of human peripheral blood basophils from buffy coat and basophil culture

Purification of human basophils was performed according to our previous publication [20]. Fresh human buffy coat obtained from non-atopic healthy volunteers of the Hong Kong Red Cross Blood Transfusion Service was diluted 1:1 with phosphate-buffered saline (PBS) at room temperature and centrifuged using Ficoll-Paque Plus solution (GE Healthcare Corp., Allendale, NJ, USA) for 30 min at 500 g. The peripheral blood mononuclear cell (PBMC) fraction was collected and washed twice with cold PBS containing 2% fetal bovine serum (FBS) (Invitrogen Corp., Carlsbad, CA, USA). Basophils were purified from the PBMC fraction using the basophil isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) by magnetic depletion of non-basophils passing through an LS+ column (Miltenyi Biotec) within a magnetic field. With this preparation, the drop-through fraction contained purified basophils with a purity of at least 99%, as assessed by Giemsa staining solution (Sigma-Aldrich Corp., St Louis, MO, USA), and specific basophil cell surface marker CD203c staining (Fig. S1). The isolated basophils were cultured in RPMI-1640 medium (Invitrogen) supplemented with 10% FBS (Invitrogen). The above protocol, using human basophils purified from human buffy coat, was approved by the Clinical Research Ethics Committee of The Chinese University of Hong Kong–New Territories East Cluster Hospitals with written consent from all healthy volunteers of Hong Kong Red Cross Blood Transfusion Service.

Co-culture of primary human bronchial epithelial cells/human bronchial epithelial cell line (BEAS-2B) cells and basophils/KU812 cells

Primary human bronchial epithelial cells were purchased from ScienCell Research Laboratories (San Diego, CA, USA) and maintained in bronchial epithelial cell medium

(ScienCell). Primary human bronchial epithelial cells were incubated at 37°C in a humidified 5% CO₂ atmosphere and cultured for no more than three passages before analysis. The human bronchial epithelial cell line (BEAS-2B) transformed by adenovirus 12-SV40 virus hybrid (Ad12SV40) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), which has been used widely as an *in-vitro* bronchial epithelial cell model [20]. BEAS-2B cells were grown in Dulbecco's modified Eagle's medium nutrient mixture F12 (Invitrogen) with 10% FBS at 37°C in a humidified 5% CO₂ atmosphere until confluence to cell monolayer. The human basophilic leukaemia cell line, KU812 cells (ATCC), was maintained in RPMI-1640 medium (Invitrogen) with 10% FBS. In co-culture, the medium of primary bronchial epithelial cells/BEAS-2B cells was replaced by RPMI-1640 medium containing 10% FBS (Invitrogen) with or without basophils/KU812 cells. For inhibition experiments, primary human bronchial epithelial cells and basophils were pretreated with signalling molecule inhibitors for 1 h.

Co-culture of fixed primary human bronchial epithelial cells and basophils

Confluent primary human bronchial epithelial cells or basophils were pretreated with 1% paraformaldehyde in PBS on ice for 1 h to prevent the release of mediators from cells while preserving the cell membrane integrity to maintain intercellular interaction. After fixation, cells were washed at least 10 times with PBS containing 2% FBS, and fixed or unfixed primary bronchial epithelial cells and basophils were co-cultured in RPMI-1640 medium supplemented with 10% FBS.

Quantitative measurement of cytokine release using cytometric bead array (CBA)

Concentrations of inflammatory cytokine IL-6 and CXCL8 in culture supernatant were quantitated by a CBA reagent kit from BD Pharmingen, Inc. (San Diego, CA, USA).

Quantitative analysis of β -defensin 2 release using enzyme-linked immunosorbent assay (ELISA)

Concentrations of human epithelium derived antimicrobial peptide β -defensin 2 in culture supernatant were quantitated by an ELISA reagent kit from Phoenix Pharmaceuticals, Inc. (Burlingame, CA, USA).

Activation profile of nuclear transcription factors in basophils

Nuclear proteins were extracted from basophils upon MDP stimulation for 2 h to study the activation profile of nuclear transcription factors by assessing the DNA binding activity

of 40 nuclear transcription factors using Procarta Human Transcription Factor Plex Assay (Panomics, Inc., Fremont, CA, USA) with the Bio-plex 200 suspension array system (Bio-Rad Corp., Hercules, CA, USA).

Immunofluorescence staining and flow cytometric analysis

To determine the expression of CD203c, intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 on the cell surface, non-adherent basophils were washed and resuspended with cold PBS. Adherent bronchial epithelial cells were harvested using cell dissociation solution. After blocking with 2% human pooled serum for 20 min at 4°C and washing with PBS supplemented with 0.5% bovine serum albumin, cells were incubated with phycoerythrin (PE)-conjugated mouse anti-human CD203c antibody or mouse IgG1 isotype (BioLegend, Inc., San Diego, CA, USA), fluorescein isothiocyanate (FITC)-conjugated mouse anti-human ICAM-1 antibody, PE-conjugated mouse anti-human VCAM-1 antibody or mouse IgG1 κ , mouse IgG2 κ isotype (BD Pharmingen) for 30 min at 4°C in the dark. After washing, cells were subjected to flow cytometric analysis.

The intracellular expression of NOD1,2 and phosphorylated signalling molecules was determined quantitatively using a previously established intracellular staining method with flow cytometry [20]. This quantitative flow cytometric method for analysis of the activation of intracellular signalling molecules by the intracellular staining of phosphorylated signalling molecules is less tedious than Western blot analysis, and the flow cytometric method requires fewer cell numbers and less assay time. To determine the intracellular expression of NOD1,2 and phosphorylated signalling molecules, cells were fixed with prewarmed BD Cytotfix buffer for 10 min at 37°C after previous treatments. After centrifugation, cells were permeabilized in ice-cold methanol for 30 min and then stained with rabbit anti-human NOD1 (Abcam, Inc., Cambridge, MA, USA) or rabbit IgG (BD Biosciences Corp., San Jose, CA, USA), mouse anti-human NOD2 or mouse IgG2a antibodies (BioLegend, Inc., San Diego, CA, USA), mouse anti-human phosphorylated (p)JNK, pp38 MAPK, pI κ B α or mouse IgG1 antibodies (BD Pharmingen) for 60 min followed by allophycocyanin (APC)-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or FITC-conjugated goat anti-mouse secondary antibody (Invitrogen) for another 45 min at 4°C in the dark. Cells were then washed, resuspended and subjected to analysis.

Expression of surface molecules, NOD1,2 and intracellular phosphorylated signalling molecules of 5000 viable cells was analysed by flow cytometry [fluorescence activated cell sorter (FACS) Calibur flow cytometer, BD Biosciences Corp., San Jose, CA, USA] and presented as mean fluorescence intensity (MFI). For the differential analysis of intracellular

MAPK and NF- κ B activity of BEAS-2B cells and KU812 cells, non-adherent KU812 cells were separated from the adherent BEAS-2B cells by washing with PBS after different treatments. Adherent BEAS-2B cells were then harvested using cell dissociation solution. For the flow cytometric analysis of intracellular signalling molecules, we adopted the gating of basophil marker CD203c as a differential cell surface staining marker to ensure that the pure cell population was without other cell contamination, as only KU812 but not BEAS-2B cells express CD203c.

Statistical analysis

The statistical significance of differences was determined by one-way analysis of variance (ANOVA) or unpaired *t*-test. The values were expressed as mean plus standard error of the mean (s.e.m.) from three independent experiments. Any differences with *P*-value < 0.05 were considered significant. When ANOVA indicated a significant difference, Bonferroni's *post-hoc* test was then used to assess the difference between groups. All analyses were performed using SPSS statistical software for Windows (version 16.0; SPSS Inc., Chicago, IL, USA).

Results

Intracellular expression of NOD2 protein

As shown in Fig. 1a–d, the intracellular NOD2 protein was expressed constitutively in basophils, primary human bronchial epithelial cells, KU812 and BEAS-2B cells. However, these expression levels of NOD2 were much lower than that in human peripheral monocytes (Fig. 1e). We also confirmed the gene expression of NOD2 in basophils and KU812 using quantitative real-time polymerase chain reaction (PCR) (data not shown). Therefore, the expression of NOD2 in basophils and bronchial epithelial cells indicates that the NOD2 ligand can exert direct effects on basophils and bronchial epithelial cells either alone or in co-culture. The expression of functional NOD1,2 protein has been reported in human bronchial epithelial cells and BEAS-2B cells [23]. However, we could not detect any significant protein expression of NOD1 in human basophils and KU812 cells (Fig. 1f,g) using eosinophils as positive control cells (Fig. 1h).

Cell surface expression of adhesion molecules on basophils and primary human bronchial epithelial cells activated by MDP

As shown in Fig. 2b,d, compared with MDP-negative control D-D isomer, MDP (10 μ g/ml) could augment the cell surface expression of ICAM-1 and VCAM-1 on basophils, and such up-regulation was enhanced significantly when basophils were co-cultured with bronchial epi-

thelial cells (all *P* < 0.05). Upon NOD2 ligand MDP stimulation, the cell surface expression of ICAM-1 and VCAM-1 on bronchial epithelial cells was enhanced significantly only when they were co-cultured with basophils (all *P* < 0.05, Fig. 2f,h). In concordance with the negative expression of NOD1 in basophils in Fig. 1, NOD1 ligand iE-DAP did not exhibit any significant effect on the expression of ICAM-1 or VCAM-1 on basophils alone or basophils in co-culture (all *P* > 0.05, Fig. 2b,d). As IL-33 has been shown to activate basophils [24], IL-33 was used to prime the basophil for up-regulating adhesion molecule expression. Compared with groups without IL-33 pretreatment, the expressions of ICAM-1 and VCAM-1 on basophils were enhanced significantly by IL-33 priming (all *P* < 0.05, Fig. 2b,d). However, IL-33 did not exhibit any significant effect on the expression of ICAM-1 or VCAM-1 on primary human bronchial epithelial cells (all *P* > 0.05, Fig. 2f,h).

Induction of cytokine, chemokine and β -defensin 2 upon the interaction of basophils and bronchial epithelial cells stimulated by MDP

As shown in Fig. 3a,b, MDP (10 μ g/ml) could promote the release of inflammatory cytokine IL-6 and chemokine CXCL8 from basophils. Upon co-culture, the induction of IL-6, CXCL8 and β -defensin 2 by MDP was found to be significantly higher than those of basophils alone or bronchial epithelial cells alone, in which IL-6 exhibited the most potent synergistic induction (all *P* < 0.05, Fig. 3a–c). However, NOD1 ligand iE-DAP did not affect significantly the release of IL-6 or CXCL8 in the co-culture (all *P* > 0.05, Fig. 3a,b). MDP negative control D-D isomer did not show any significant effect on the induction of IL-6, CXCL8 and β -defensin 2 (all *P* > 0.05), thereby indicating the specific inducing activity of NOD2 ligand MDP.

Bronchial epithelial cells were the main source for the release of IL-6, CXCL8 and β -defensin 2 in co-culture

As shown in Fig. 4, in the co-culture of paraformaldehyde-fixed basophils and unfixed bronchial epithelial cells, the induction of IL-6, CXCL8 and β -defensin 2 with or without MDP stimulation was preserved. However, fixation of bronchial epithelial cells could greatly reduce the secretion of IL-6, CXCL8 or β -defensin 2 in co-culture with or without MDP stimulation. These results indicate that primary bronchial epithelial cells were the main source for releasing IL-6, CXCL8 and β -defensin 2 in co-culture upon interacting with basophils with MDP stimulation.

Effects of signalling inhibitors on MDP-induced cytokines and adhesion molecules

Different signalling molecule inhibitors were applied to investigate the activation of relevant intracellular signalling

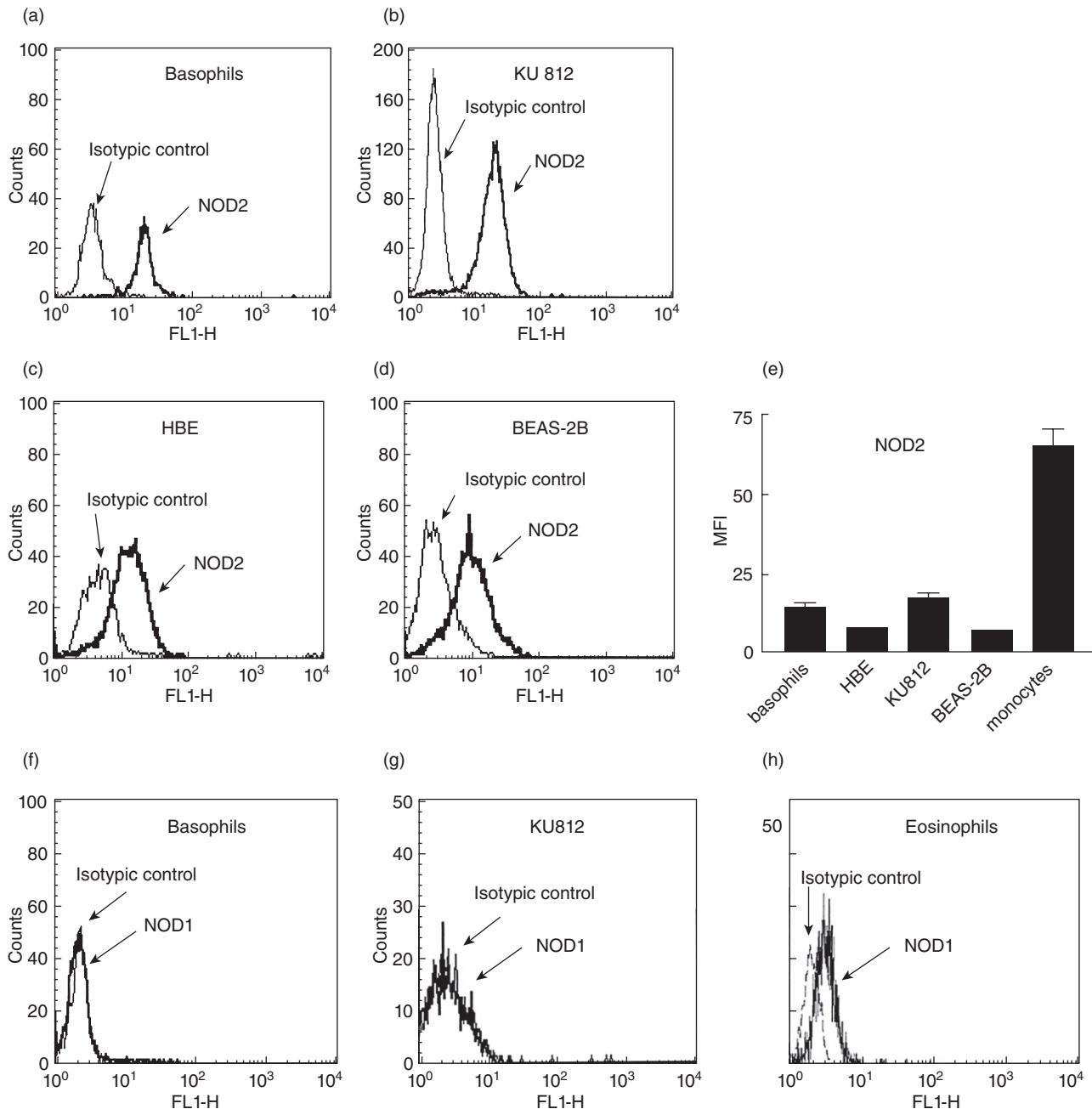


Fig. 1. Protein expression of nucleotide-binding oligomerization domain-containing protein (NOD)1,2 in basophils, KU812 cells, primary human bronchial epithelial cells (HBE) and human bronchial epithelial cell line (BEAS)-2B cells. Representative histograms of intracellular expression of NOD2 in (a) basophils, (b) KU812, (c) HBE and (d) BEAS-2B cells were determined by flow cytometry from triplicate experiments with essentially identical results. (e) Quantitative results of flow cytometric analysis of intracellular expression of NOD2 in basophils, KU812 cells, HBE, BEAS-2B cells and human monocytes (positive control cells) are presented with arithmetic mean plus standard error of the mean of mean fluorescence intensity (MFI) of three independent experiments in the bar chart. Results were normalized by subtracting appropriate isotypic control. Representative histograms of intracellular expression of NOD1 in (f) basophils, (g) KU812 cells and (h) eosinophils (positive control cells) were determined by flow cytometry from triplicate experiments with essentially identical results.

pathways mediated by the interaction between basophils and bronchial epithelial cells upon MDP stimulation. Based on the results of cytotoxicity assay using MTT assay (data not shown) and previous publications [20–22], we used the

optimal concentrations of $\text{I}\kappa\text{B}\alpha$ inhibitor BAY11-7082 (1 μM), p38 MAPK inhibitor SB203580 (7.5 μM) and JNK inhibitor SP600125 (5 μM) with significant inhibitory effects without any cell toxicity. In co-culture, BAY11-7082,

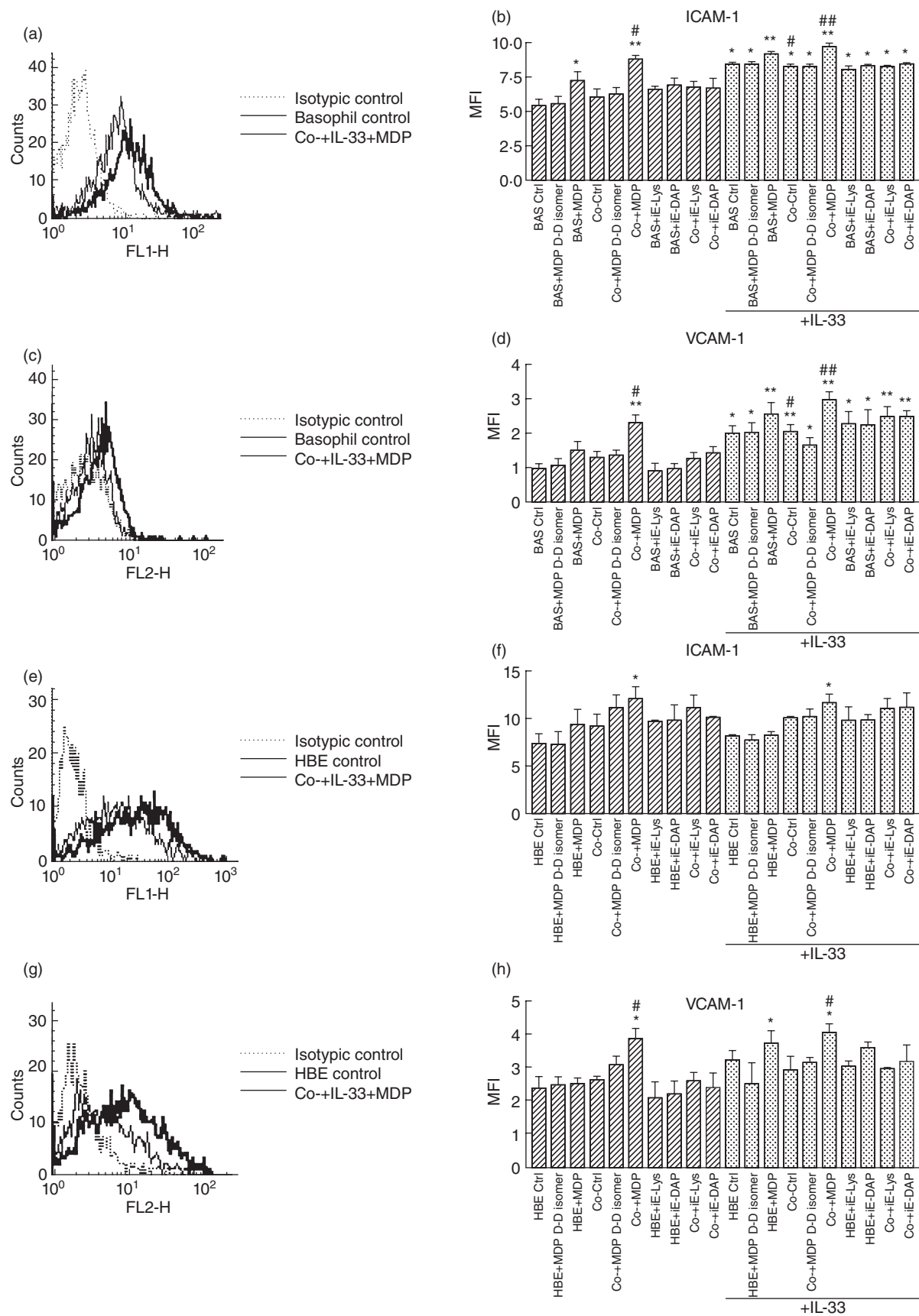


Fig. 2. Effect of muramyl dipeptide (MDP) γ -D-glutamyl-meso-diaminopimelic acid (iE-DAP) on the cell surface expression of intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 on basophils (BAS) and primary human bronchial epithelial cells (HBE), together with interleukin (IL)-33 or alone. Expressions of ICAM-1 and VCAM-1 on (a–d) basophils and (e–h) primary HBE cells in the co-culture with or without MDP or iE-DAP stimulation are presented with representative histograms (a,c,e,g) and bar charts (b,d,f,h). Basophils were pretreated with or without IL-33 (50 ng/ml) for 4 h, then the basophils (3×10^5 cells) and confluent primary HBE (1×10^5 cells) were cultured either together or separately with or without MDP (10 μ g/ml) or iE-DAP (10 μ g/ml) for a further 24 h. As shown in the bar charts, surface expressions of ICAM-1 and VCAM-1 on 5000 basophils or primary HBE cells are expressed as the mean plus standard error of the mean of mean fluorescence intensity (MFI) and normalized by subtracting appropriate isotypic control of three independent experiments. * $P < 0.05$; ** $P < 0.01$ compared with corresponding cells alone without treatment. # $P < 0.05$; ## $P < 0.01$ compared with corresponding co-culture of basophils and HBE cells without treatment. γ -D-Glu-Lys (iE-Lys) and MDP D-D isomer were negative controls of nucleotide-binding oligomerization domain-containing protein (NOD)1 and NOD2 ligand, respectively. Ctrl: medium control, Co-: co-culture.

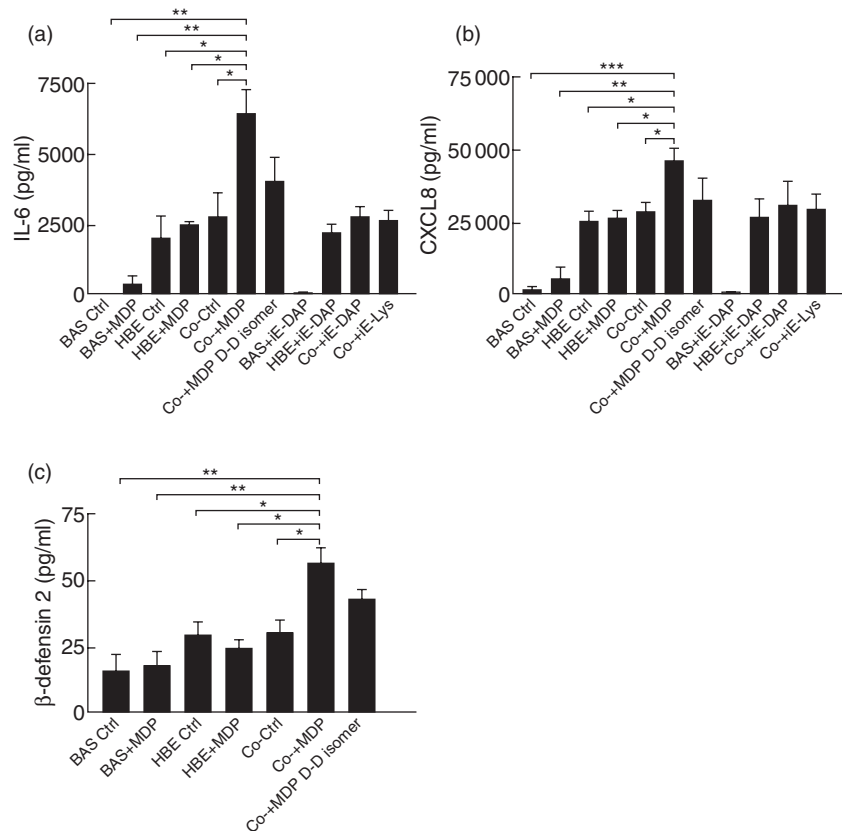
SP600125 and SB203580 could suppress MDP-induced release of IL-6, CXCL8 and β -defensin 2 (Fig. 5) significantly, while only BAY11-7082 and SB203580 could reduce the MDP-induced expression of cell surface adhesion molecule ICAM-1 and VCAM-1 significantly on primary human bronchial epithelial cells, but not on basophils (Fig. 6). In order to study the activation profile of nuclear transcription factors, we investigated the DNA binding activity of 40 transcription factors in basophils upon MDP stimulation. The DNA-binding activities of nine nuclear transcription factors were up-regulated in basophils upon MDP stimulation at 2 h, including NF- κ B, octamer binding proteins (OCT), peroxisome proliferator-activated receptor (PPAR) and cAMP response element-binding protein

(CREB) (>1.4-fold, Table 1). However, the other 31 nuclear transcription factors seemed not to be affected by MDP at 2 h (data not shown).

Differential activation of intracellular signalling pathways involved in the interaction of KU812 and BEAS-2B upon MDP stimulation

Because basophils represent 1% of peripheral blood leucocytes and only 2.5×10^6 cells can be purified from 4×10^8 PBMC, it is not feasible to obtain a large enough number of basophils for the investigation of the activation of signalling molecules. According to our previous experiments, we found that the representative BEAS-2B bronchial epithelial

Fig. 3. Effect of muramyl dipeptide (MDP) or γ -D-glutamyl-meso-diaminopimelic acid (iE-DAP) on the release of (a) interleukin (IL)-6, (b) CXCL8 and (c) β -defensin 2 in the co-culture of basophils (BAS) and primary human bronchial epithelial cells (HBE). Confluent human bronchial epithelial cells (1×10^5 cells) and basophils (3×10^5 cells) were cultured either together or separately with or without MDP (10 μ g/ml) or iE-DAP (10 μ g/ml) for 24 h. Release of IL-6 and CXCL8, and β -defensin 2 in culture supernatants was determined by cytometric bead array (CBA) and enzyme-linked immunosorbent assay (ELISA), respectively. Results are expressed as the mean plus standard error of the mean of three independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ when compared between the denoted groups. γ -D-Glu-Lys (iE-Lys) and MDP D-D isomer were negative controls of nucleotide-binding oligomerization domain-containing protein (NOD)1 and NOD2 ligand, respectively.



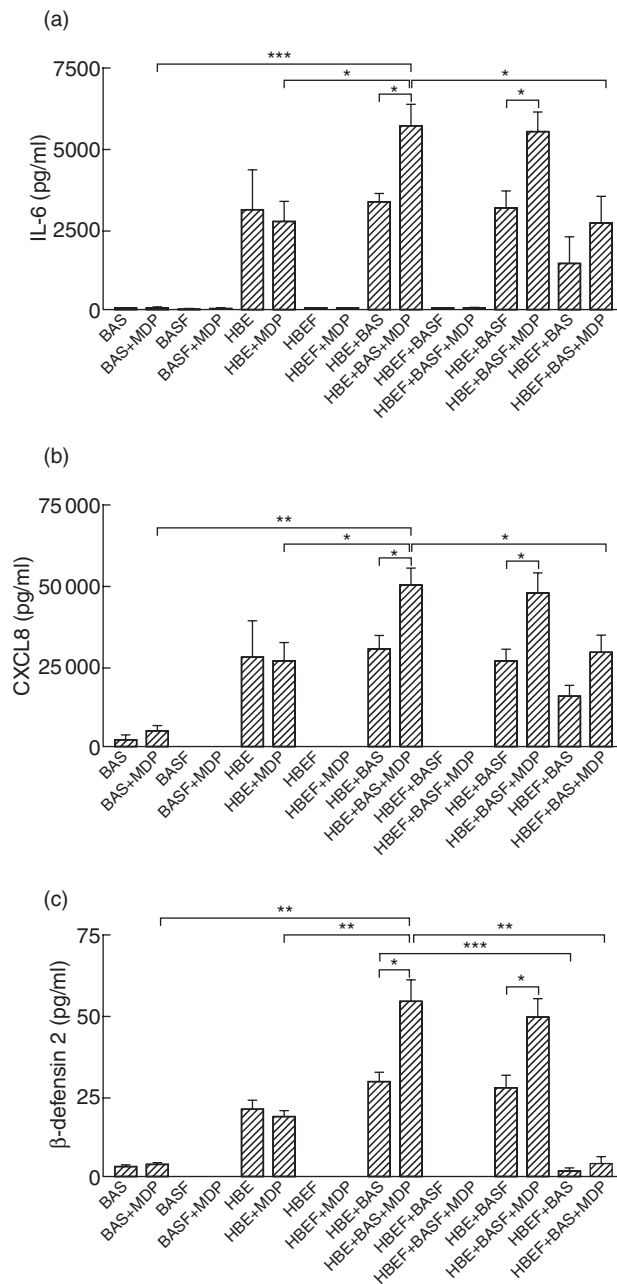


Fig. 4. Source of the release of interleukin (IL)-6, CXCL8 and β-defensin 2 in co-culture of basophils (BAS) and bronchial epithelial cells (HBE). Basophils (3×10^5 cells) and confluent primary HBE cells (1×10^5 cells) were treated with or without 1% paraformaldehyde for 1 h in ice prior to being cultured together with or without muramyl dipeptide (MDP) (10 µg/ml) for 24 h. Release of (a) IL-6 and (b) CXCL8, and (c) β-defensin 2 in culture supernatants was determined by cytometric bead array (CBA) and enzyme-linked immunosorbent assay (ELISA), respectively. Results are expressed as the mean plus standard error of the mean of three independent experiments. HBEF: fixed primary HBE; BASF: fixed basophils. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ when compared between denoted groups.

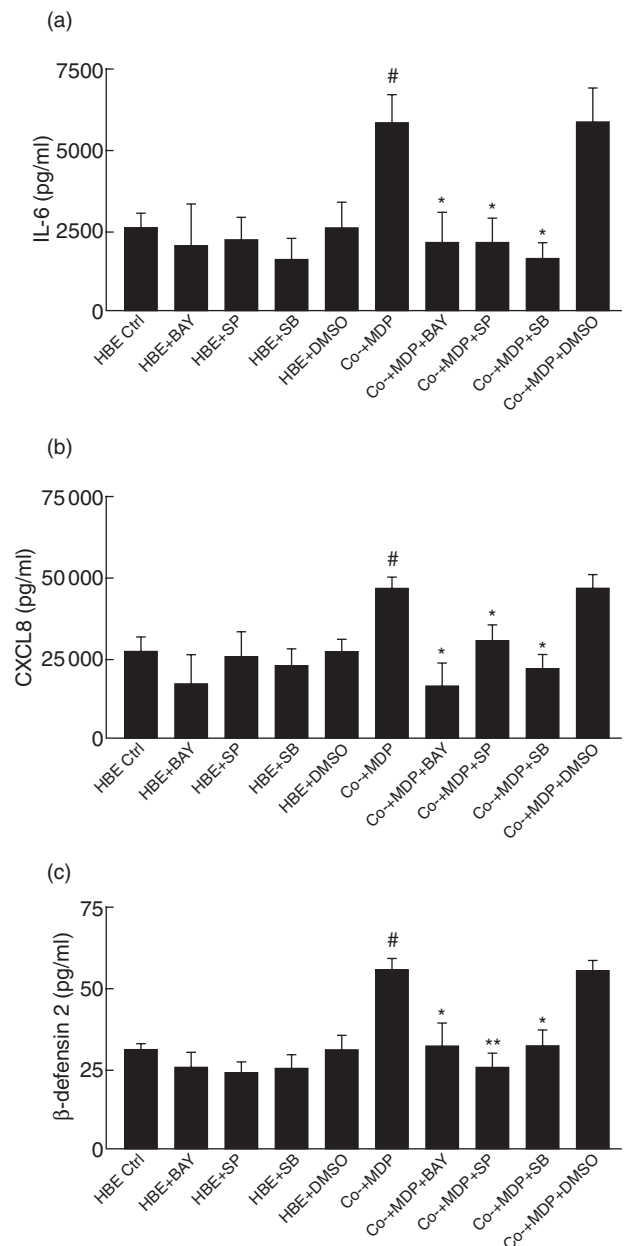


Fig. 5. Effect of signalling molecule inhibitors on the release of interleukin (IL)-6, CXCL8 and β-defensin 2 from bronchial epithelial cells (HBE) cells and co-culture of HBE cells and basophils with or without treatment with muramyl dipeptide (MDP). Basophils (3×10^5 cells) and confluent HBE cells (1×10^5 cells) cultured either separately or together were pretreated with BAY11-7082 (BAY, 1 µM), SP600125 (SP, 5 µM) or SB203580 (SB, 7.5 µM) for 1 h, followed by incubation with or without MDP (10 µg/ml) in the presence of inhibitors for further 24 h. Release of IL-6 and CXCL8, and β-defensin 2 in culture supernatants, were determined by cytometric bead array (CBA) and enzyme-linked immunosorbent assay (ELISA), respectively. Results are expressed as mean plus standard error of the mean of three independent experiments. Ctrl: control; Co-: co-culture. Dimethyl sulphoxide (DMSO, 0.1%) was used as the vehicle control. * $P < 0.05$; ** $P < 0.01$ when compared with MDP-activated co-culture DMSO control. # $P < 0.05$ when compared with HBE control.

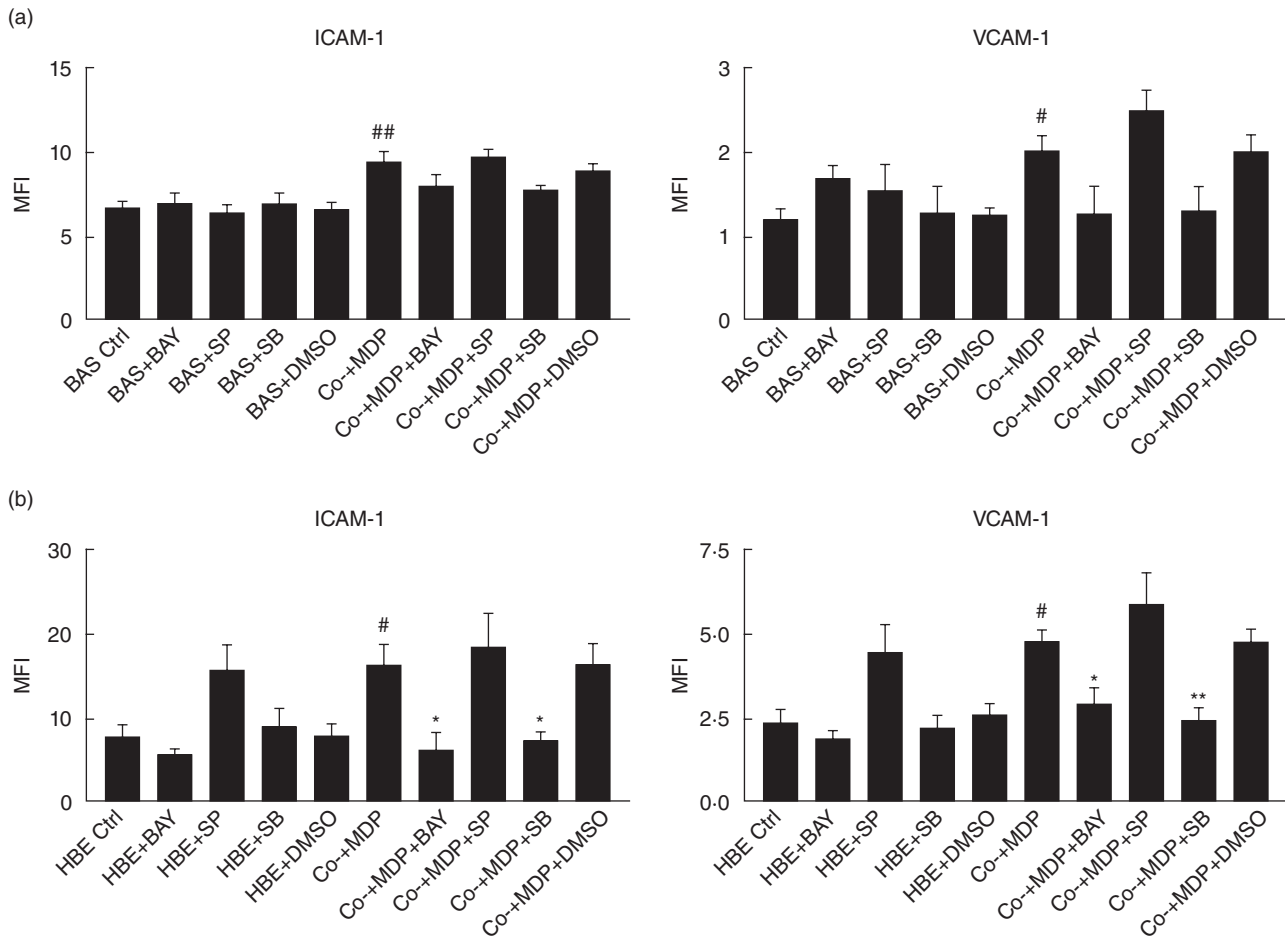


Fig. 6. Effect of signalling molecule inhibitors on the cell surface expression of intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 on (a) basophils (BAS) and (b) primary human bronchial epithelial (HBE) cells in the co-culture. Basophils (3×10^5 cells) and confluent HBE cells (1×10^5 cells) cultured either separately or together were pretreated with BAY11-7082 (BAY, 1 μ M), SP600125 (SP, 5 μ M) or SB203580 (SB, 7.5 μ M) for 1 h, followed by incubation with or without muramyl dipeptide (MDP) (10 μ g/ml) in the presence of inhibitors for further 24 h. Cell surface expression of ICAM-1 on 5000 cells was analysed by flow cytometry as mean fluorescence intensity (MFI). Results are expressed as mean plus standard error of the mean and normalized by subtracting appropriate isotypic control of three independent experiments in bar charts. Ctrl: control; Co-: co-culture. Dimethyl sulphoxide (DMSO, 0.1%) was used as the vehicle control. * $P < 0.05$; ** $P < 0.01$ when compared with MDP activated co-culture DMSO control. # $P < 0.05$; ## $P < 0.01$ when compared with BAS or HBE control.

cells and KU812 basophilic cells showed similar results for the expression of ICAM-1 and VCAM-1, and induction of cytokines and β -defensin-2 in co-culture (data not shown). Therefore, similar to our previous publication, we adopted the representative BEAS-2B bronchial epithelial cells and

KU812 basophilic cells for the subsequent signalling mechanistic study [20]. Figure 7 shows that JNK in KU812 cells was phosphorylated significantly upon co-culture at 10 min. With treatment of MDP (10 μ g/ml) for 10 min, phosphorylation of JNK, p38 MAPK and I κ B α in both

Table 1. Up-regulated activities of human nuclear transcription factors in basophils upon MDP stimulation.

NF- κ B	OCT	NF-E2	E2F1	PPAR	RUNX	CREB	STAT-1	NFAT
++	++	+	+	++	+	++	+	+

Basophils (3×10^5 cells) were cultured with or without muramyl dipeptide (MDP) (10 μ g/ml) for 2 h. Activation profile of nuclear transcription factors was analysed by assessing the DNA binding activity of 40 nuclear transcription factors using Procarta human Transcription Factor Plex Assay (Panomics). + denotes the ≤ 1.4 -fold increase of mean fluorescence intensity (MFI) for MDP-treated cells compared with untreated cells; ++ denotes the > 1.4 -fold increase of mean fluorescence intensity (MFI) for MDP-treated cells with untreated cells. CREB: cAMP response element-binding protein; NFAT: nuclear factor of activated T cells; NF-E2: nuclear factor erythroid-derived 2; OCT: octamer binding proteins; PPAR: peroxisome proliferator-activated receptor; RUNX: runt box; STAT: signal transducers and activators of transcription.

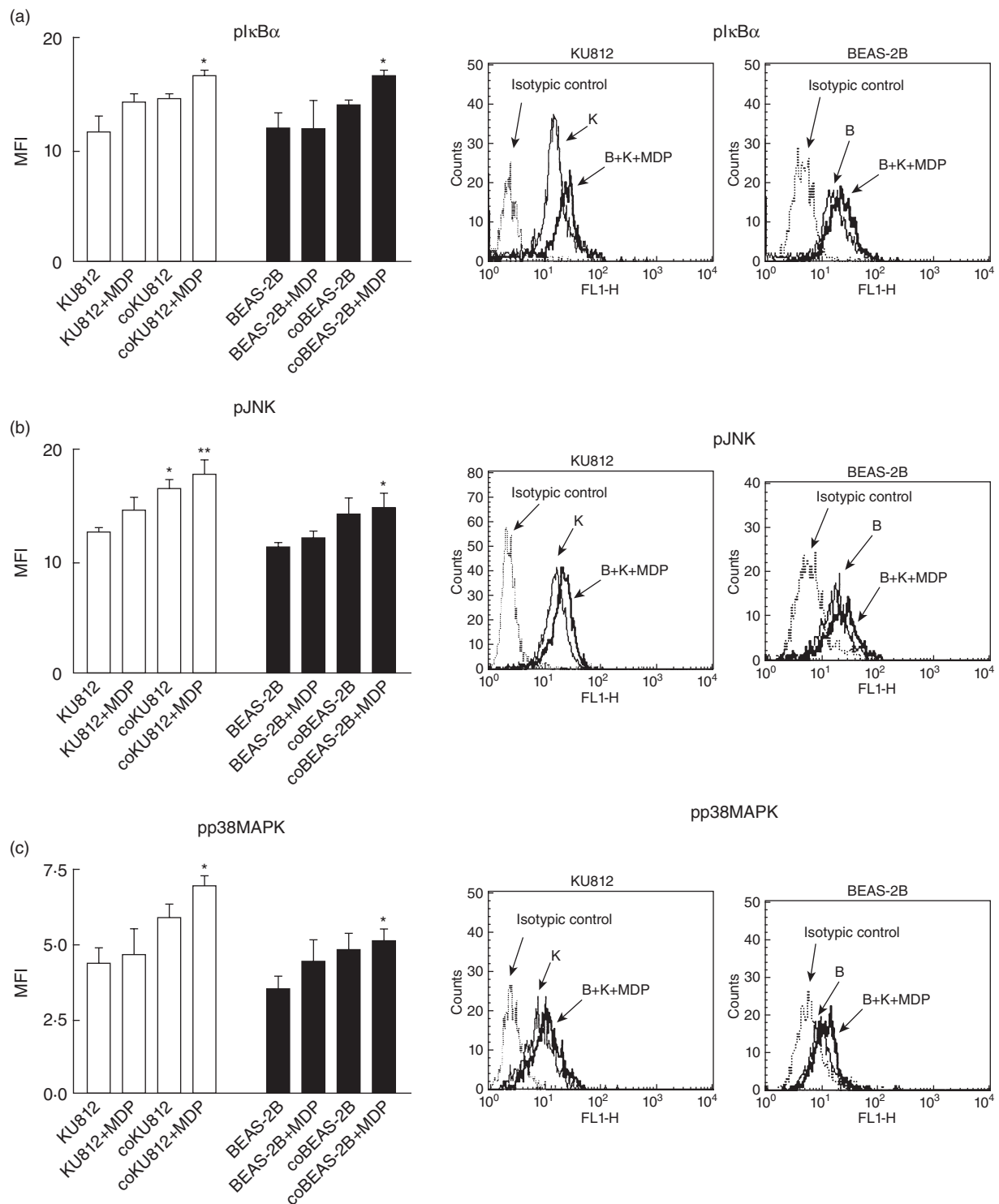


Fig. 7. Activation of c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK) and nuclear factor (NF)- κ B in co-culture of KU812 cells and human bronchial epithelial cell line (BEAS)-2B cells upon muramyl dipeptide (MDP) stimulation. KU812 cells (3×10^5 cells) and confluent BEAS-2B cells (1×10^5 cells) were cultured either together or separately with or without MDP (10 μ g/ml) stimulation for 10 min. The intracellular expression of (a) phosphorylated (p)I κ B α , (b) pJNK and (c) pp38 MAPK of permeabilized KU812 cells (white bars) and BEAS-2B cells (black bars) were measured by intracellular immunofluorescence staining using flow cytometry. Results are shown as the mean plus standard error of the mean of mean fluorescence intensity (MFI) and normalized by subtracting appropriate isotypic control of three independent experiments. Representative histograms of intracellular expression of (a) pI κ B α , (b) pJNK and (c) pp38 MAPK in KU812 and BEAS-2B cells are shown. K: KU812 cells; B: BEAS-2B cells; coBEAS-2B: BEAS-2B cells in co-culture; coKU812: KU812 cells in co-culture. * $P < 0.05$; ** $P < 0.01$ compared with corresponding cells alone without treatment.

BEAS-2B cells and KU812 cells was enhanced significantly in the co-culture (Fig. 7).

Discussion

Expression of functional NOD1,2 has been reported in different cells, including epithelial cells, eosinophils and neutrophils [10,23,25,26]. NOD in various epithelial cells are functional receptors that mediate anti-bacterial responses by producing anti-microbial peptides [25], while NOD2 ligand could induce the release of inflammatory chemokine CXCL8 and granular toxic protein eosinophil-derived neurotoxin from eosinophils [10]. However, the expression and functional studies of NLR of airway basophils has not yet been examined. In this study of the bacteria-mediated innate immune response of basophils interacting with bronchial epithelial cells, we found that basophils could express intracytosolic NOD2 but not NOD1 (Fig. 1), and NOD1 ligand iE-DAP up to 10 µg/ml could not exhibit any significant effect on the expression of adhesion molecules on basophils and the release of IL-6 or CXCL8 from the co-culture of basophils and bronchial epithelial cells (Figs 2 and 3). These two well-characterized NLRs, NOD1 and NOD2, actually detect different bacterial cell wall components, the peptidoglycan fragments iE-DAP in all Gram-negative and certain Gram-positive bacteria, and MDP in almost all Gram-positive and Gram-negative bacteria, respectively [2,3]. Therefore, NOD1 has a more restricted structural specificity in the recognition of bacteria.

In the present study, we found that basophils, primary human bronchial epithelial cells, KU812 basophilic cells and BEAS-2B epithelial cells expressed NOD2 protein constitutively. Activation of NOD2 can provoke the induction of inflammatory cytokines and other anti-microbial genes, which contributes to host defence [27]. Co-culture of basophils and primary human bronchial epithelial cells could promote the release of IL-6, CXCL8 and β -defensin 2 significantly, related probably to the up-regulation and interaction of cell surface adhesion molecules ICAM-1 and VCAM-1 on basophils and epithelial cells (Figs 2 and 3), which play an immunological role in cell attachment and the subsequently activated intracellular signalling pathways [28,29]. This up-regulation profile of adhesion molecule expression is in concordance with our previous study of IL-17A activation on basophils interacting with bronchial epithelial cells [20]. In fact, ICAM-1 and VCAM-1 are two crucial adhesion molecules for the cell adhesion, chemotactic and transendothelial migration of leucocytes to local inflammatory sites in allergic asthma [30,31]. ICAM-1 is an adhesion molecule expressing on epithelial cells and plays an essential role in cell adherence and migration by interacting with the highest affinity to the integrin family member, lymphocyte function-associated antigen-1 (CD11a/CD18), a hallmark of allergic inflammation [28]. Moreover, the expression of ICAM-1 and VCAM-1 was

found to be elevated in patients with allergic asthma, showing a positive correlation with disease activity [32,33]. In our present study, MDP was found to up-regulate significantly the cell surface expression of ICAM-1 and VCAM-1 on basophils and human bronchial epithelial cells in the co-culture system with or without basophil priming by IL-33 (Fig. 2, all $P < 0.05$). Therefore, MDP plays crucial roles for the intercellular interaction between basophils and bronchial epithelial cells.

In the present study, synergistic induction of inflammatory cytokine IL-6 from co-culture of basophils and bronchial epithelial cells may induce tissue remodelling and fibrosis in pulmonary disease [34]. IL-6 also induces the synthesis of acute-phase proteins, and mediates various inflammatory responses such as airway inflammation [35]. Furthermore, IL-6 has been reported as a critical inducer for the polarization of a novel subset of T helper lymphocytes, Th17, which has been suggested to play a pathogenic role in allergic asthma [36]. CXCL8 is a potent chemoattractant for neutrophils and basophils [37]. Over-expression of epithelial CXCL8 can be induced by respiratory bacteria and viruses, resulting in the neutrophilic infiltration of the airways [38,39]. MDP-induced release of CXCL8 from bronchial epithelial cells, eosinophils and basophils can therefore mediate the recruitment, infiltration and activation of the above immune effector cells at the microenvironment of the inflammatory airway, thereby amplifying the inflammatory responses in allergic asthma [7].

β -defensin 2, an arginine-rich cationic host defence peptide secreted from leucocytes, keratinocytes and epithelium, was first identified in psoriatic skin [40]. It plays an important role in the defence against bacterial infection by effective killing of Gram-negative bacteria, including *Escherichia coli* and *Pseudomonas aeruginosa* [41], but exhibiting relatively low bacteriostatic activity on Gram-positive bacteria such as *Staphylococcus aureus* [41]. It indicates that β -defensin 2 is effective in the defence against Gram-negative bacteria [42,43]. Our present results show that bronchial epithelial cells were the major source for the release of IL-6, CXCL8 and β -defensin2 in co-culture upon MDP stimulation. For innate immunity against microbial infection, activated bronchial epithelial cells are potent sources of a wide variety of proinflammatory cytokines and chemokines, such as CXCL8 [44,45]. Apart from MDP, Gram-negative bacterial cell wall component lipopolysaccharide and human rhinovirus can also activate epithelial cells to release anti-bacterial peptide β -defensin 2 [46,47]. Although epithelial cells are the main source for the release of IL-6, CXCL8 and β -defensin 2 upon stimulation, the direct interaction with circulating inflammatory granulocytes, such as basophils, may optimize the production of IL-6, CXCL8, β -defensin 2 from epithelial cells, leading to more effective innate immunity against bacterial infection. Our results therefore indicate that basophils play a novel

role for mediating anti-bacterial innate immunity by inducing epithelial-derived IL-6, CXCL8 and β -defensin 2.

In the present study of intracellular signal transduction, we adopted the representative cell lines KU812 and BEAS-2B cells. Our previous study has shown consistency between BEAS-2B and primary human bronchial epithelial cells, as well as KU812 and basophils, in terms of the expression of cell surface adhesion molecules and the release of inflammatory cytokines and chemokines upon IL-17A stimulation [20]. In our recent study of the link between the infections and the exacerbation of allergic inflammation, we demonstrated that the induction of IL-6 and CCL2 upon the interaction of basophils and bronchial epithelial cells under IL-17A stimulation was regulated differentially by ERK, JNK, p38 MAPK and NF- κ B pathways [20]. In the present study, to investigate the signalling pathways involved in the interaction of basophils and human bronchial epithelial cells, several signalling molecule inhibitors were used to block the pathways. I κ B α inhibitor BAY11-7082, JNK inhibitor SP600125 and p38 MAPK inhibitor SB203580 could suppress significantly MDP-induced release of IL-6, CXCL8 and β -defensin 2, while only BAY11-7082 and SB203580 could reduce significantly the MDP-induced expression of cell surface adhesion molecules ICAM-1 and VCAM-1 on bronchial epithelial cells, but not on basophils (Figs 5 and 6). Together with the results in Fig. 7, regarding the MDP-mediated activation of JNK, p38 MAPK and I κ B α in both BEAS-2B cells and KU812 cells in co-culture, the results therefore indicate that the release of cytokines and β -defensin 2 and expression of adhesion molecules in MDP-activated co-culture were regulated differentially by NF- κ B, JNK and p38 MAPK, and NF- κ B and p38 MAPK, respectively. These results are actually in concordance with our previously published results, that the cytokines/chemokines and adhesion molecules expression in co-culture of basophils and bronchial epithelial cells are regulated differentially by distinct activation profiles of signalling molecules [20]. Many previous studies have indicated that different signalling molecules play differential regulatory roles for the expression of various cytokines and adhesion molecules [48].

NOD2 can initiate signals through intracellular MAPK and NF- κ B for the production of inflammatory cytokines tumour necrosis factor (TNF)- α and IL-6 [26,49,50]. The adaptor protein caspase recruitment domain-containing protein 9 (CARD9) is important for the activation of p38 and JNK downstream of NOD2, but it is dispensable for NF- κ B activation [51]. NOD2 promotes the membrane recruitment of receptor interacting protein kinase 2 (RIP2), the serine–threonine kinase involved in NF- κ B activation downstream of NOD2 [52], and the NOD2–RIP2 complex also stimulates JNK and p38 MAPK pathways [53]. MDP-mediated activation of the NOD2–RIP2 complex can modulate both innate and adaptive immune responses by inducing the release of cytokines and chemokines [53]. We

also found that a panel of nuclear transcription factors including NF- κ B, CREB and OCT may be involved in the regulation gene transcription of basophils stimulated by MDP (Table 1). The detailed gene transcription regulatory mechanism, including microRNA machinery, therefore requires further investigation.

In summary, upon bacterial peptidoglycan MDP stimulation, the expression of adhesion molecules and the release of inflammatory cytokines were up-regulated only moderately by basophils. However, as the main source of the release of cytokines and anti-bacterial peptides, bronchial epithelial cells could be provoked significantly for the production of inflammatory cytokines and anti-bacterial peptides upon interacting with basophils under MDP stimulation. Our present results indicate that basophils alone may not act as major immune effector cells for host defence against bacterial infection, but airway basophils could facilitate and optimize the innate immunity of bronchial epithelial cells against bacterial infection. Our results therefore provide a novel pathophysiological mechanism by which bacterial infection can provoke inflammatory cascades in the airway, and new insights into the crucial role of innate immunity played by granulocyte basophils via distinct intracellular signalling pathways. The results therefore provide a biochemical basis for the development of a novel treatment for basophil-mediated allergic pulmonary diseases.

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Disclosure

No conflicts of interest have been declared by the authors.

References

- 1 Schroder NW, Crother TR, Naiki Y *et al.* Innate immune responses during respiratory tract infection with a bacterial pathogen induce allergic airway sensitization. *J Allergy Clin Immunol* 2008; **122**:595–602.
- 2 Martinon F, Mayor A, Tschopp J. The inflammasomes: guardians of the body. *Annu Rev Immunol* 2009; **27**:229–65.
- 3 Girardin SE, Boneca IG, Viala J *et al.* Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J Biol Chem* 2003; **278**:8869–72.
- 4 Hysi P, Kabesch M, Moffatt MF *et al.* NOD1 variation, immunoglobulin E and asthma. *Hum Mol Genet* 2005; **14**:935–41.
- 5 Rosenstiel P, Till A, Schreiber S. NOD-like receptors and human diseases. *Microbes Infect* 2007; **9**:648–57.

- 6 Opitz B, Puschel A, Schmeck B *et al.* Nucleotide-binding oligomerization domain proteins are innate immune receptors for internalized *Streptococcus pneumoniae*. *J Biol Chem* 2004; **279**:36426–32.
- 7 Ferwerda G, Girardin SE, Kullberg BJ *et al.* NOD2 and Toll-like receptors are nonredundant recognition systems of *Mycobacterium tuberculosis*. *PLoS Pathog* 2005; **1**:279–85.
- 8 Hugot JP, Chamaillard M, Zouali H *et al.* Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* 2001; **411**:599–603.
- 9 Duan W, Mehta AK, Magalhaes JG *et al.* Innate signals from Nod2 block respiratory tolerance and program T(H)2-driven allergic inflammation. *J Allergy Clin Immunol* 2010; **126**:1284–93.
- 10 Kvarnhammar AM, Petterson T, Cardell LO. NOD-like receptors and RIG-I-like receptors in human eosinophils: activation by NOD1 and NOD2 agonists. *Immunology* 2011; **134**:314–25.
- 11 Voehringer D. Basophils in allergic immune responses. *Curr Opin Immunol* 2011; **23**:789–93.
- 12 Karasuyama H, Obata K, Wada T, Tsujimura Y, Mukai K. Newly appreciated roles for basophils in allergy and protective immunity. *Allergy* 2011; **66**:1133–41.
- 13 Ito Y, Satoh T, Takayama K, Miyagishi C, Walls AF, Yokozeki H. Basophil recruitment and activation in inflammatory skin diseases. *Allergy* 2011; **66**:1107–13.
- 14 Kepley CL, McFeeley PJ, Oliver JM, Lipscomb MF. Immunohistochemical detection of human basophils in postmortem cases of fatal asthma. *Am J Respir Crit Care Med* 2001; **164**:1053–8.
- 15 Macfarlane AJ, Kon OM, Smith SJ *et al.* Basophils, eosinophils, and mast cells in atopic and nonatopic asthma and in late-phase allergic reactions in the lung and skin. *J Allergy Clin Immunol* 2000; **105**:99–107.
- 16 Gauvreau GM, Evans MY. Allergen inhalation challenge: a human model of asthma exacerbation. *Contrib Microbiol* 2007; **14**:21–32.
- 17 Schroeder JT, Lichtenstein LM, Roche EM, Xiao H, Liu MC. IL-4 production by human basophils found in the lung following segmental allergen challenge. *J Allergy Clin Immunol* 2001; **107**:265–71.
- 18 Nouri-Aria KT, Irani AM, Jacobson MR *et al.* Basophil recruitment and IL-4 production during human allergen-induced late asthma. *J Allergy Clin Immunol* 2001; **108**:205–11.
- 19 Schroeder JT, Bieneman AP, Chichester KL, Breslin L, Xiao H, Liu MC. Pulmonary allergic responses augment interleukin-13 secretion by circulating basophils yet suppress interferon- α from plasmacytoid dendritic cells. *Clin Exp Allergy* 2010; **40**:745–54.
- 20 Wong CK, Cao J, Yin YB, Lam CW. Interleukin-17A activation on bronchial epithelium and basophils: a novel inflammatory mechanism. *Eur Respir J* 2010; **35**:883–93.
- 21 Wong CK, Cheung PF, Ip WK, Lam CW. Intracellular signaling mechanisms regulating Toll-like receptor-mediated activation of eosinophils. *Am J Respir Cell Mol Biol* 2007; **37**:85–96.
- 22 Cheung PFY, Wong CK, Ip WK, Lam CWK. FAK-mediated activation of ERK for eosinophil migration: a novel mechanism for infection-induced allergic inflammation. *Int Immunol* 2008; **20**:353–63.
- 23 Barton JL, Berg T, Didon L, Nord M. The pattern recognition receptor Nod1 activates CCAAT/enhancer binding protein β signalling in lung epithelial cells. *Eur Respir J* 2007; **30**:214–22.
- 24 Pecaric-Petkovic T, Didichenko SA, Kaempfer S, Spiegl N, Dahinden CA. Human basophils and eosinophils are the direct target leukocytes of the novel IL-1 family member IL-33. *Blood* 2009; **113**:1526–34.
- 25 Uehara A, Fujimoto Y, Fukase K, Takada H. Various human epithelial cells express functional Toll-like receptors, NOD1 and NOD2 to produce anti-microbial peptides, but not proinflammatory cytokines. *Mol Immunol* 2007; **44**:3100–11.
- 26 Ekman AK, Cardell LO. The expression and function of Nod-like receptors in neutrophils. *Immunology* 2009; **130**:55–63.
- 27 Kobayashi KS, Chamaillard M, Ogura Y *et al.* Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science* 2005; **307**:731–4.
- 28 Canonica GW, Ciprandi G, Pesce GP, Buscaglia S, Paolieri F, Bagnasco M. ICAM-1 on epithelial cells in allergic subjects: a hallmark of allergic inflammation. *Int Arch Allergy Immunol* 1995; **107**:99–102.
- 29 Yago T, Tsukuda M, Tajima H *et al.* Analysis of initial attachment of B cells to endothelial cells under flow conditions. *J Immunol* 1997; **158**:707–14.
- 30 Smith CH, Barker JN, Lee TH. Adhesion molecules in allergic inflammation. *Am Rev Respir Dis* 1993; **148**:S75–S78.
- 31 Wardlaw AJ, Walsh GM, Symon FA. Adhesion interactions involved in eosinophil migration through vascular endothelium. *Ann NY Acad Sci* 1996; **796**:124–37.
- 32 Gosset P, Tillie-Leblond I, Janin A *et al.* Expression of E-selectin, ICAM-1 and VCAM-1 on bronchial biopsies from allergic and non-allergic asthmatic patients. *Int Arch Allergy Immunol* 1995; **106**:69–77.
- 33 Gorska-Ciebiada M, Ciebiada M, Gorska MM, Gorski P, Grzelewska-Rzymowska I. Intercellular adhesion molecule 1 and tumor necrosis factor α in asthma and persistent allergic rhinitis: relationship with disease severity. *Ann Allergy Asthma Immunol* 2006; **97**:66–72.
- 34 Gomes I, Mathur SK, Espenshade BM, Mori Y, Varga J, Ackerman SJ. Eosinophil–fibroblast interactions induce fibroblast IL-6 secretion and extracellular matrix gene expression: implications in fibrogenesis. *J Allergy Clin Immunol* 2005; **116**:796–804.
- 35 Kopf M, Baumann H, Freer G *et al.* Impaired immune and acute-phase responses in interleukin-6-deficient mice. *Nature* 1994; **368**:339–42.
- 36 Wang YH, Liu YJ. The IL-17 cytokine family and their role in allergic inflammation. *Curr Opin Immunol* 2008; **20**:697–702.
- 37 Rossi D, Zlotnik A. The biology of chemokines and their receptors. *Annu Rev Immunol* 2000; **18**:217–42.
- 38 Ratner AJ, Bryan R, Weber A *et al.* Cystic fibrosis pathogens activate Ca²⁺-dependent mitogen-activated protein kinase signaling pathways in airway epithelial cells. *J Biol Chem* 2001; **276**:19267–75.
- 39 Subauste MC, Jacoby DB, Richards SM, Proud D. Infection of a human respiratory epithelial cell line with rhinovirus. Induction of cytokine release and modulation of susceptibility to infection by cytokine exposure. *J Clin Invest* 1995; **96**:549–57.
- 40 Metz-Boutigue MH, Shooshtarizadeh P, Prevost G, Haikel Y, Chich JF. Antimicrobial peptides present in mammalian skin and gut are multifunctional defence molecules. *Curr Pharm Des* 2010; **16**:1024–39.
- 41 Harder J, Bartels J, Christophers E, Schroder JM. A peptide antibiotic from human skin. *Nature* 1997; **387**:861.
- 42 Schroder JM, Harder J. Human β -defensin-2. *Int J Biochem Cell Biol* 1999; **31**:645–51.

- 43 Schneider JJ, Unholzer A, Schaller M, Schafer-Korting M, Korting HC. Human defensins. *J Mol Med (Berl)* 2005; **83**:587–95.
- 44 Meyer-Hoffert U, Lezcano-Meza D, Bartels J, Montes-Vizuet AR, Schroder JM, Teran LM. Th2- and to a lesser extent Th1-type cytokines upregulate the production of both CXC (IL-8 and gro-alpha) and CC (RANTES, eotaxin, eotaxin-2, MCP-3 and MCP-4) chemokines in human airway epithelial cells. *Int Arch Allergy Immunol* 2003; **131**:264–71.
- 45 Wong CK, Wang CB, Ip WK, Tian YP, Lam CW. Role of p38 MAPK and NF- κ B for chemokine release in coculture of human eosinophils and bronchial epithelial cells. *Clin Exp Immunol* 2005; **139**:90–100.
- 46 Chadebecq P, Goidin D, Jacquet C, Viac J, Schmitt D, Staquet MJ. Use of human reconstructed epidermis to analyze the regulation of beta-defensin hBD-1, hBD-2, and hBD-3 expression in response to LPS. *Cell Biol Toxicol* 2003; **19**:313–24.
- 47 Proud D, Sanders SP, Wiehler S. Human rhinovirus infection induces airway epithelial cell production of human beta-defensin 2 both *in vitro* and *in vivo*. *J Immunol* 2004; **172**:4637–45.
- 48 Wong CK, Ip WK, Lam CW. Biochemical assessment of intracellular signal transduction pathways in eosinophils: implications for pharmacotherapy. *Crit Rev Clin Lab Sci* 2004; **41**:79–113.
- 49 Park JH, Kim YG, McDonald C *et al.* RICK/RIP2 mediates innate immune responses induced through Nod1 and Nod2 but not TLRs. *J Immunol* 2007; **178**:2380–6.
- 50 Sabbah A, Chang TH, Harnack R *et al.* Activation of innate immune antiviral responses by Nod2. *Nat Immunol* 2009; **10**:1073–80.
- 51 Hsu YM, Zhang Y, You Y *et al.* The adaptor protein CARD9 is required for innate immune responses to intracellular pathogens. *Nat Immunol* 2007; **8**:198–205.
- 52 Lecine P, Esmiol S, Metais JY *et al.* The NOD2–RICK complex signals from the plasma membrane. *J Biol Chem* 2007; **282**:15197–207.
- 53 Ting JP, Duncan JA, Lei Y. How the noninflammasome NLRs function in the innate immune system. *Science* 2010; **327**:286–90.

Supporting information

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Fig. S1. Cell surface expression of CD203c on basophils. Representative histograms of cell surface expression of basophil surface marker CD203c on cell population (A) before and (B) after purification using magnetic cell sorting were determined by flow cytometry from triplicate experiments with essentially identical results.

Supplementary Fig. S1

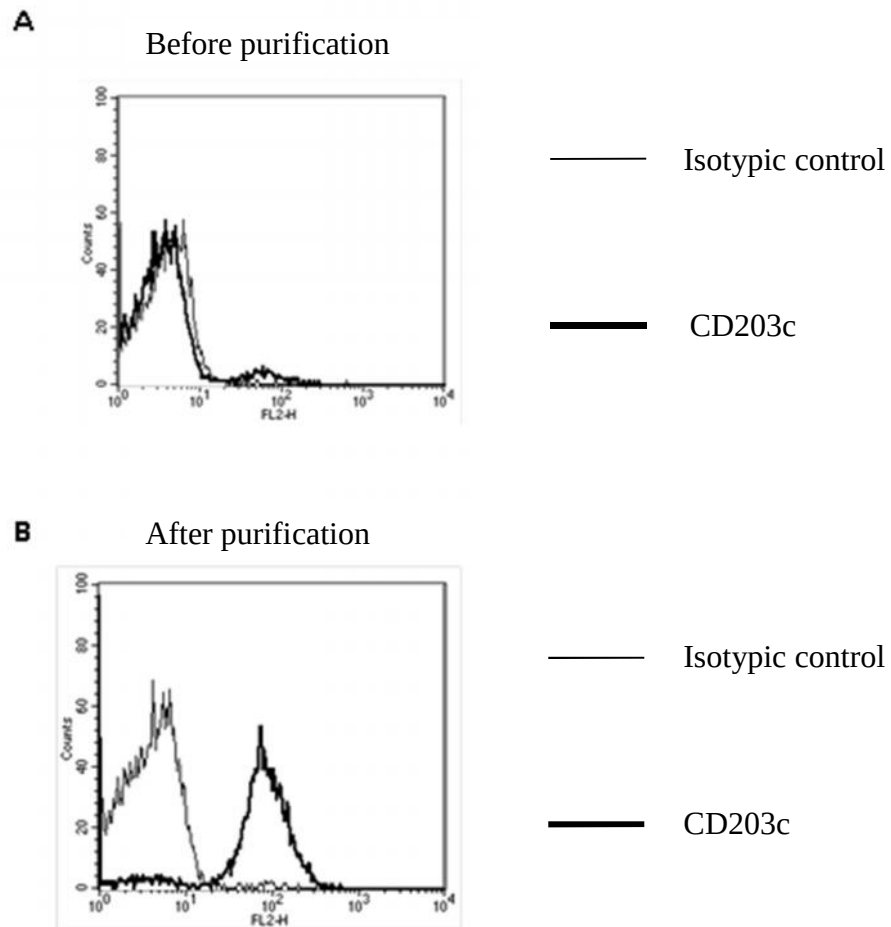


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