IL-17A activation on bronchial epithelium and basophils: a novel inflammatory mechanism

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Abstract: Basophils are the accessory cell type for T helper (Th)2 induction and initiators in IgE-mediated chronic allergic inflammation. Basophils and Th17 cells accumulate at the inflammatory sites such as the airways of allergic asthmatic patients.

We investigated the activation of IL-17A on the primary human basophils/KU812 basophilic cells and primary human bronchial epithelial cells/BEAS-2B bronchial epithelial cells. Cytokines, chemokines, adhesion molecules and intracellular signaling molecules were assayed by ELISA or flow cytometry.

Co-culture of bronchial epithelial cells and basophils could significantly induce the release of epithelial inflammatory cytokine IL-6 and CCL2, a chemokine for basophils, eosinophils and monocytes. Such induction was synergistically enhanced by IL-17A, and direct interaction between these two cells was necessary for IL-17A-induced IL-6 and CCL2 release. Surface expression of intercellular adhesion molecule-1 on bronchial epithelial cells was also up-regulated upon their interaction. The interaction of basophils and bronchial epithelial cells under IL-17A stimulation was differentially regulated by extracellular signal-regulated kinase, c-Jun N-terminal protein kinase, p38 mitogen activated protein kinase and nuclear factor-κB pathways.

The above findings suggest a novel immunopathological role of Th17 cells and basophils in allergic asthma through the activation of granulocytes-mediated inflammation initiated by the direct interaction between basophils and bronchial epithelial cells.

KEYWORDS: adhesion molecules, basophils, bronchial epithelial cells, chemokines, cytokines, signal transduction
INTRODUCTION

Basophils are rare circulating granulocytes that differentiate from CD34+ hematopoietic progenitors in the bone marrow. They are the main effector cells in response to T helper (Th)2-related parasitic infestation and allergic inflammation [1]. Many studies have suggested that basophils could contribute to the development of Th2 immunity by releasing increased quantity of Th2 cytokines including interleukin (IL)-4, IL-13, IL-17E/IL-25 and thymic stromal lymphopoietin (TSLP) in allergic inflammation [2]. Basophils therefore serve as initiators and accessory cells for the Th2 cell polarization in response to protease allergens and by recruiting other effector cells such as eosinophils or neutrophils [3, 4]. This novel type of chronic allergic inflammation requires basophils as initiators, but not mast cells or T lymphocytes, through the interaction of antigen, IgE, and FcRI [3, 4]. Basophils are rarely found in normal tissues. However, their number increases markedly at allergic inflammatory sites in the airways of asthmatic patients, especially during asthma exacerbation and in response to allergen inhalation challenge [5-7]. The allergen-induced increased number of basophils in airway sputum exceeds that of mast cells, and basophils increased by approximately 200-fold from baseline values at 7 h after allergen inhalation challenge [8]. Using immunohistochemical staining, increased basophils in the lungs of patients with severe asthma was found to attach onto the bronchial epithelium, submucosa, and around the vascular walls [5]. Moreover, basophil number in the airway correlates with the bronchial responsiveness of asthmatics [8]. Basophils are one of the main cells that produce IL-4 and IL-13 in the peripheral blood of asthmatic patients after allergen activation [9]. Basophils also produce leukotriene C4 and histamine that can cause the symptoms of acute and chronic allergic inflammation. Activated bronchial epithelial cells are potent sources of a wide variety of proinflammatory cytokines and chemokines such as monocyte
chemoattractant protein (MCP-1/CCL2) [10]. Secretion of inflammatory mediators together with the recruitment and interaction of different immune effector cells such as eosinophils with the bronchial epithelium contribute to bronchial inflammation, tissue damage and remodeling of pulmonary structure in asthma [11]. However, the detailed immunopathological mechanisms of basophils upon the interaction with bronchial epithelium in asthma remain to be elucidated.

Th17 cells is a distinct IL-17-producing Th subset which is different from Th1 and Th2 lineages, producing hallmark cytokines IL-17A, IL-17F and IL-22 [12]. IL-1β, IL-6 and IL-23 can induce the development and effector function of human Th17 cells via the activation of interferon-regulatory factor 4 and transcription factor RORγt [12]. IL-17A can induce the release of IL-6, CXCL-8, GM-CSF and CXCL1 from epithelial and vascular endothelial cells, CXCL8 from fibroblasts, and defensin from keratinocytes [13]. Hyperproduction of IL-17 family cytokines such as IL-17A has been found in allergic asthmatic patients [14]. Apart from the circulation, sputum and bronchial alveolar lavage, elevated IL-17 was also detected in airway eosinophils in asthmatic patients [12, 15].

Our previous study has shown that the interaction of human eosinophils and bronchial epithelial cells could potently induce IL-6 release through the activation of the intracellular p38 mitogen activated protein kinase (MAPK) and transcription factor nuclear factor-B (NF-κB) cascades [16]. We have further reported that allergen house dust mite (Der p1) protein can induce the release of inflammatory cytokines and expression of adhesion molecules upon the interaction of human eosinophils and bronchial epithelial cells through the differential activation of MAPK and NF-κB [17]. These results may suggest a potential contribution of the interaction of granulocytes and bronchial epithelium to airway inflammation. Based on the previous findings, we hypothesize that Th17 cytokine IL-17A can regulate allergic inflammation in asthma by the
activation of bronchial epithelial cells interacting with basophils, and investigated the modulation of intracellular MAPK and NF-κB activities on regulating the expression of cytokines, chemokines and adhesion molecules under the stimulation of IL-17A in this study.

MATERIALS AND METHODS

Reagents

Recombinant human IL-17A was purchased from R&D Systems, MN, USA. IκB-α phosphorylation inhibitor BAY11-7082, extracellular signal-regulated kinase (ERK) inhibitor U0126, c-Jun N-terminal protein kinase (JNK) inhibitor SP600125, p38 MAPK inhibitor SB203580, phosphoinositide-3 kinase (PI3K) inhibitor LY294002 and Janus kinase (JAK) inhibitor AG490 were purchased from Calbiochem Corp, CA, USA. SB203580 was dissolved in water, while U0126, LY294002, SP600125, AG490 and BAY11-7082 were dissolved in dimethyl sulfoxide (DMSO). In all studies, the concentration of DMSO was 0.1 % (vol/vol).

Isolation of human peripheral blood basophils from buffy coat and basophils culture

Fresh human buffy coat obtained from the non-atopic healthy volunteers of Hong Kong Red Cross Blood Transfusion Service was diluted 1:2 with phosphate buffered saline (PBS) at 4°C and centrifuged using Ficoll-Paque Plus solution (GE Healthcare Corp., NJ USA) for 30 min at 1,000 x g. The peripheral blood mononuclear cell (PBMC) fraction was collected and washed twice with cold PBS containing 2 % fetal bovine serum (FBS) (Invitrogen Corp., CA, USA). Basophils were purified from the PBMC fraction using basophil isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) by magnetic depletion of non-basophils by passing through a 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., MO, USA). The isolated basophils were cultured in RPMI 1640 medium (Gibco) supplemented with 10% FBS and 20 mM Hepes (Gibco).

**Co-culture of primary human bronchial epithelial cells/BEAS-2B cells and human peripheral blood basophils/KU812 cells**

Primary human bronchial epithelial cells were purchased from ScienceCell Research Laboratories, CA, USA and maintained in bronchial epithelial cell medium (ScienceCell). Human primary bronchial epithelial cells were incubated at 37°C in a humidified 5% CO2 atmosphere and cultured for no more than 3 passages before the 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 widely used as an *in vitro* bronchial epithelial cell model [17]. BEAS-2B cells were grown in Dulbecco’s modified Eagle’s medium nutrient mixture F12 (DMEM/F12, Invitrogen) with 10% FBS in 6-well cell culture plates at 37°C in a humidified 5% CO2 atmosphere until confluence to cell monolayer. The human basophilic leukemia cell line, KU812 cells, was purchased from ATCC, USA. KU812 cells were maintained in RPMI1640 medium (Invitrogen) with 10% FBS. For co-culture, the medium of primary bronchial epithelial cells/BEAS-2B cells was replaced with RPMI 1640 medium containing 10% FBS (Invitrogen) with or without basophils/KU812 cells. For inhibition experiments, BEAS-2B and KU812 cells were pretreated with signaling molecule inhibitors for 1 hour.
**Co-culture of fixed primary human bronchial epithelial cells/BEAS-2B cells and basophils/KU812 cells**

Confluent primary human bronchial epithelial cells/BEAS-2B cells or basophils/KU821 cells were treated 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/BEAS-2B cells or basophils/KU812 cells were co-cultured in RPMI 1640 medium supplemented with 10% FBS [17].

**Co-culture of primary human bronchial epithelial cells/BEAS-2B cells and basophils/KU812 cells in the presence of transwell inserts**

To prevent direct interaction between primary bronchial epithelial cells/BEAS-2B cells and basophils/KU821 cells in the co-culture, transwell inserts (pore size: 0.4 µM) (BD Biosciences Corp, San Jose, CA, USA) were used to separate the cells into two compartments. Confluent primary bronchial epithelial cells/BEAS-2B cells and basophils/KU821 cells were cultured together in the presence of transwell inserts, in which basophils/KU821 cells were placed in the upper compartment and primary bronchial epithelial cells/BEAS-2B cells were in the lower one. IL-17A (10 ng/ml) was added at the same time into both the upper and lower compartments for treatment.
Protein array analysis of chemokines and cytokines in culture supernatant

The expression profile of 79 different cytokines in culture supernatant of BEAS-2B cells and KU821 cells was assessed semi-quantitatively using antibody based RayBio™ human cytokine array V (RayBiotech Inc, CA, USA).

Quantitative analysis of IL-6 and CCL2

Concentrations of pro-inflammatory cytokines IL-6, and chemokine CCL2 in culture supernatant were quantitated by ELISA kits from BD Pharmingen Corp, San Diego, CA, USA.

Immunofluorescence staining and flow cytometry

To determine the expression of IL-17RA, IL-17RC and adhesion molecule intercellular adhesion molecule (ICAM)-1 and ICAM-3 on the cell surface, Non-adherent basophils/KU821 cells were washed and resuspended with cold PBS after preceding treatments. Adherent bronchial epithelial cells/BEAS-2B cells were then harvested using 0.05 % trypsin-EDTA. 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 FITC-conjugated mouse anti-human IL-17RA, IL-17RC, ICAM-1 and ICAM-3 antibodies or mouse IgG1κ isotype (BD Pharmingen) for 30 min at 4°C in the dark. After washing, cells were resuspended in 1 % paraformaldehyde as fixative and subjected to analysis.

To determine the intracellular expression of IL-17RA adaptor protein Act1 and phosphorylated signaling molecules, cells were fixed with pre-warmed BD Cytofix Buffer (4 % paraformaldehyde) for 10 min at 37°C after preceding treatments. After centrifugation, cells were permeabilized in ice-cold methanol for 30 min and then stained with mouse anti-human Act1
(Santa Cruz Biotechnology Inc, CA, USA), phosphorylated ERK, phosphorylated-JNK, phosphorylated p38 MAPK, phosphorylated IκB-α or mouse IgG1 antibodies (BD Pharmingen) for 60 min followed by FITC conjugated goat anti-mouse secondary antibody (Invitrogen) for another 45 min at 4°C in dark. Cells were then washed, resuspended and subjected to analysis. Expression of surface molecules and intracellular phosphorylated signaling molecules of 10,000 viable cells was analyzed by flow cytometry (FACSCalibur, BD Biosciences) as mean fluorescence intensity (MFI), which includes both the changes of target molecule expression in individual cell and the percentage of cells expressing the target molecules. For the differential analysis of intracellular MAPK and NF-κB activity of BEAS-2B 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 0.05 % trypsin-EDTA. For the flow cytometric analysis of adhesion molecules and intracellular signaling molecules, we adopted the gating of ICAM-3 as a differential cell surface marker to ensure the pure cell population without other cell contamination since only KU812 but not BEAS-2B cells strongly express ICAM-3 on cell surface.

Statistical analysis

The statistical significance of differences was determined by one-way ANOVA or Student’s t-test. The values were expressed as means ± SD from three independent experiments. Any difference with p values less than 0.05 was considered significant. When ANOVA indicated a significant difference, Bonferroni post hoc test was then used to assess the difference between groups. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) statistical software for Windows, version 16.0 (SPSS Inc, IL, USA).
RESULTS

Protein expression of receptors for IL-17A and IL-17RA adaptor protein Act1 on primary bronchial epithelial cells/BEAS-2B cells and primary blood basophils/KU812 cells

As shown in Fig.1A and B, both primary bronchial epithelial cells/BEAS-2B cells and primary blood basophils/KU812 cells constitutively expressed IL-17RA and IL-17RC on their surfaces. Intracellular staining using flow cytometry showed that adaptor protein Act1 was constitutively expressed in primary bronchial epithelial cells/BEAS-2B cells and basophils/KU812 cells (Fig. 1C).

Cytokine and chemokine release upon the interaction of primary bronchial epithelial cells/BEAS-2B cells and primary blood basophils/KU812 cells activated by IL-17A

The cytokine expression profile was assessed using antibody based human cytokine protein membrane array. KU812 and BEAS-2B cells were cultured either together or separately with or without IL-17A treatment. IL-17A (50 ng/ml) could promote the release of IL-6, CCL2, CXCL8 and CXCL1 from BEAS-2B cells, but no prominent effect was observed on KU812 cells. Upon co-culture, only IL-6 and CCL2 concentrations were found to be higher than those of KU812 cells alone or BEAS-2B cells alone, with or without IL-17A activation. However, co-culture did not enhance the release of CXCL8 and CXCL1, with or without IL-17A activation.

Further investigation by ELISA confirmed that co-culture could synergistically induce the release of IL-6 and CCL2 while IL-17A could significantly enhance IL-6 and CCL2 release from both primary bronchial epithelial cells/BEAS-2B cells alone and co-culture with basophils/KU812 cells in a dose-dependent manner (Fig. 2). However, in co-culture of primary cells, much more IL-6 was found to be induced by IL-17A than that of cell lines (Fig. 2C).
Together and in concordance with previous study [18], ELISA results demonstrated that IL-17A could actually induce the release of IL-6, CXCL8, CCL2, CXCL1 and G-CSF from bronchial epithelial cells. However, we have observed that co-culture with basophils could only significantly enhance the release of CCL2 and IL-6 (p < 0.05). Other cytokines such as IL-4, IL-13 and TSLP did not increase in the co-culture system. IL-17A could further significantly augment the release of IL-6 and CCL2 from co-culture (p < 0.05). Similar to that of IL-17A, another Th17 cytokine IL-17F (10 ng/ml) could also induce the release of IL-6, CCL2, CXCL8, CXCL1 and G-CSF from bronchial epithelial cells and further significantly augment the release of IL-6 and CCL2 from co-culture of human bronchial epithelial cells and basophils (data not shown).

Source of IL-6 and CCL2 released in the co-culture system

In the co-culture of 1% paraformaldehyde fixed KU812/basophils and unfixed BEAS-2B/primary bronchial epithelial cells, the stimulatory effects of co-culture on the release of IL-6 and CCL2, and the IL-17A-induced stimulation were preserved. However, fixation of primary bronchial epithelial cells/BEAS-2B cells alone could almost completely abolish the secretion of IL-6 and CCL2 in co-culture with or without IL-17A stimulation. These results indicated that primary bronchial epithelial cells/BEAS-2B cells were the main source for releasing IL-6 and CCL2 in co-culture upon IL-17A stimulation (Fig. 3A - D).
Direct interaction between primary bronchial epithelial cells/BEAS-2B cells and basophils/KU812 cells is required for IL-6 and CCL2 release in IL-17A-treated co-culture

Fig. 3E - H show that the presence of transwell inserts could significantly suppress the IL-17A-induced secretion of IL-6 and CCL2 in co-culture. Without IL-17A stimulation, induction of IL-6 and CCL2 release in co-culture was also significantly abolished in the presence of transwell inserts (Fig. 3E – H), implying that both IL-6 and CCL2 release in co-culture might depend on direct interaction between primary bronchial epithelial cells/BEAS-2B cells and basophils/KU812 cells.

Effect of IL-17A on adhesion molecule expression on primary bronchial epithelial cells/BEAS-2B cells in co-culture system

As shown in Fig. 4, IL-17A could not significantly up-regulate the surface expression of ICAM-1 on primary bronchial epithelial cells/BEAS-2B cells alone or in co-culture of primary bronchial epithelial cells/BEAS-2B cells and basophils/KU812 cells. Upon co-culture with basophils/KU812 cells, ICAM-1 expression on primary bronchial epithelial cells/BEAS-2B cells was significantly increased. However, surface expression of ICAM-1 was not significantly up-regulated on basophils/KU812 cells by IL-17A, with or without the co-culture with primary bronchial epithelial cells/BEAS-2B cells (data not shown). Moreover, the expression of other adhesion molecules such as ICAM-3 and vascular cell adhesion molecule-1 on primary bronchial epithelial cells/BEAS-2B cells and ICAM-3, CD18 and L-selectin on basophils/KU812 cells remained unchanged upon co-culture and the addition of IL-17A (data not shown).
**Activation of ERK, JNK, p38 MAPK and NF-κB pathways in co-culture of BEAS-2B and KU812 cells upon IL-17A stimulation**

Since basophils represent less than 1% of peripheral blood leukocytes and only about $2.5 \times 10^6$ cells can be purified from $4 \times 10^8$ peripheral blood mononuclear cells, it is not feasible to obtain enough number of basophils for the signaling experiments. Therefore, for the subsequent signaling mechanistic study, we have used the representative BEAS-2B bronchial epithelial cells and KU812 basophilic cells. To investigate the underlying signaling mechanism(s), intracellular staining by multiparametric flow cytometry was applied. After fixation and permeabilization, BEAS-2B cells and KU812 cells were gated separately on the basis of ICAM-3 expression on KU812 cells only but not BEAS-2B cells (Fig. 5A). Fig. 5B-E shows that IL-17A could activate ERK, p38 MAPK and NF-κB activity of BEAS-2B cells, and ERK activity of KU812 cells at 15 minutes. Upon co-culture, ERK, JNK, p38 MAPK and IκB-α in BEAS-2B cells, and ERK in KU812 cells were significantly phosphorylated. In the presence of IL-17A, the phosphorylation of ERK, JNK, p38 MAPK and IκB-α in BEAS-2B cells and ERK in KU812 cells was further enhanced in the co-culture.

**Effects of signaling molecule inhibitors on IL-6 and CCL2 release in co-culture upon IL-17A stimulation**

Based on the results of cytotoxicity assay using MTT assay (data not shown), we used the optimal concentrations of JAK inhibitor AG490 (5 μM), NF-κB inhibitor BAY11-7082 (1 μM), PI3K inhibitor LY294002 (5 μM), ERK inhibitor U0126 (10 μM), p38 MAPK inhibitor SB203580 (20 μM) and JNK inhibitor SP600125 (5 μM) with significant inhibitory effects without any cell toxicity. As shown in Fig. 5F and H, ERK inhibitor U0126, p38 MAPK
inhibitor SB203580 and NF-κB inhibitor BAY11-7082 could significantly suppress the induction of IL-6 and CCL2 by IL-17A in BEAS-2B cells alone. U0126, BAY11-7082 and SB203580 could significantly suppress the release of IL-6 in co-culture with or without IL-17A stimulation (Fig. 5G), while U0126, BAY11-7082, SP600125 and SB203580 could significantly suppress the release of CCL2 release in co-culture with or without IL-17A stimulation (Fig. 5I). In addition, AG490 and LY294002 did not exert any inhibitory effect on the release of IL-6 and CCL-2, and all tested inhibitors did not exert any significant effects on IL-6 and CCL2 induction from KU812 cells alone with or without IL-17A stimulation (all p > 0.05, data not shown). DMSO solvent control did not have any significant effects in all inhibition experiments (all p > 0.05).

Effect of signaling molecule inhibitors on ICAM-1 expression on the cell surface of BEAS-2B cells in co-culture

Fig. 6 shows that p38 MAPK inhibitor SB203580 (20 µM) and NF-κB inhibitor BAY11-7082 (1 µM) could significantly suppress the up-regulation of ICAM-1 surface expression on BEAS-2B cells co-cultured with KU812 cells.

DISCUSSION

In the present study, we have adopted BEAS-2B and KU812 cells which are widely used cell models. BEAS-2B cells maintain the characteristics of primary bronchial epithelial cells including expression of adhesion molecule ICAM-1, eotaxin and CXCL8 [19-21], while KU812 cells constitute the phenotypes of basophils such as histamine release and FcγRII expression [22]. We also used primary bronchial epithelial cells and peripheral blood basophils
to confirm our findings of BEAS-2B and KU821 cells. We have examined the protein expression of IL-17RA, IL-17RC, and IL-17RA adaptor protein Act1 and found that both primary bronchial epithelial cells/BEAS-2B cells and basophils/KU812 cells constitutively expressed the above proteins for IL-17 receptors. IL-17RA and IL-17RC form a heterodimer for the binding of both IL-17A and IL-17F [23]. IL-17RC binds to both IL-17A and IL-17F with the same affinity, while IL-17RA binds to IL-17A with approximately ten-fold higher affinity than to IL-17F [23]. Previous reports have provided evidences for the essential involvement of an adaptor protein Act1 in IL-17RA signalling and hence the immune responses elicited by both IL-17A and IL-17F [24]. In line with Th17 cytokines fostering subsequent cytokine and chemokine profiles in allergic lung diseases [15], we found that IL-17A could induce IL-6, CXCL8, CCL2, CXCL1 and G-CSF release from primary bronchial epithelial cells/BEAS-2B cells. Importantly, co-culture of primary bronchial epithelial cells/BEAS-2B cells and basophils/KU812 cells could increase the release of IL-6 and CCL2, probably related to the direct intercellular contact with bronchial epithelial cells, up-regulation of adhesion molecule ICAM-1 expression and the activated intracellular signaling mechanisms such as MAPK and NK-κB pathways in bronchial epithelial cells upon basophil contact. In addition, IL-17A could dose-dependently induce the release of IL-6 and CCL2 from co-culture. The induction of IL-6 indicated that IL-17A could be involved in mediating inflammation and Th2 immune responses in allergic asthma [25]. Previous study has demonstrated that the expression of IL-17 in the sputum of patients with persistent asthma correlated with the influx of airway neutrophils [12]. Besides, IL-17A has been shown to induce the human airway smooth muscle cells and bronchial fibroblasts to release IL-6 and CXCL8 [12]. CXCL8 and CXCL1 are neutrophil chemoattractants, whereas IL-6 and G-CSF can involve in neutrophil development and granulopoiesis [12]. CCL2 can mediate the
activation and recruitment of monocytes, mast cells, basophils, eosinophils and Th2 lymphocytes in allergic inflammation [26]. Therefore, IL-17A may play a pathological role in promoting and sustaining neutrophils and other immune effector cells-mediated inflammation in asthma [27, 28], initiated by the interaction between basophils and bronchial epithelial cells. Our results further support the pathological role of Th17 cells, basophils and epithelial cells in local inflammatory sites of allergic asthma. Basophils were found to act as an accessory cell type essential for Th17-mediated inflammation upon interacting with bronchial epithelial cells.

Regarding the mechanism of synergistic induction of IL-6 and CCL2, TNF-α has been shown to synergize with IL-17 for the release of cytokine and chemokine from epithelial cells by activating the NF-κB pathway (19). However, we observed that anti-human p75TNFR and anti-human p55TNFR monoclonal antibodies could not significantly block the elevated induction of IL-6, CCL2 and ICAM-1 upon interaction between basophils and bronchial epithelial cells activated by IL-17A (data not shown). The augmenting mechanism for IL-6 and CCL2 production in this study was thus not due to TNF-α. Apart from TNF-α, Th2 cytokine IL-4 and IL-13 in basophils also cannot account for this synergistic effect in co-culture of human bronchial epithelial cells and human basophils, since it has been demonstrated that the combination of IL-17 and IL-4 or IL-13 could not augment the expression of IL-6 in bronchial epithelial cells while comparing with bronchial epithelial cells stimulated with individual cytokine (20). It is possible that other unidentified cytokines that were not included in the initial screening by protein array may be up-regulated in this model. Further studies are therefore needed to explore the detailed mechanisms for the synergistic induction of IL-6 and CCL2 in the co-culture of bronchial epithelial cells and basophils activated by IL-17A.
To investigate the source of IL-6 and CCL2 released in the co-culture system, we compared their levels in co-culture of normal cells with cells fixed with 1% paraformaldehyde. Our findings suggested that primary bronchial epithelial cells/BEAS-2B cells, instead of basophils/KU812 cells, were the main source for releasing IL-6 and CCL2 in co-culture upon IL-17A stimulation. Actually, IL-17 has been previously shown to induce IL-6 in human bronchial epithelial 16HBE cells [28]. The presence of transwell inserts could significantly suppress the release of IL-6 and CCL2 in co-culture, with or without IL-17A stimulation. Direct interaction between primary bronchial epithelial cells/BEAS-2B cells and basophils/KU812 cells should at least partially contribute to the induction of IL-6 and CCL2. Since the direct interaction between their surface adhesion molecules is the possible mechanism for the induction of IL-6 and CCL2 in co-culture, we further investigated the expression of adhesion molecules on primary bronchial epithelial cells/BEAS-2B cells and basophils/KU812 cells in co-culture upon IL-17A stimulation. ICAM-1 is a crucial adhesion molecule present on epithelial cells and plays an essential role in cell adherence by interacting with the highest affinity to the integrin family member LFA-1 (CD11a/CD18), a hallmark of allergic inflammation [29]. We found that ICAM-1 expression on the surface of primary bronchial epithelial cells/BEAS-2B cells was significantly enhanced upon the interaction with basophils/KU812 cells and IL-17A could not further upregulate the expression of ICAM-1. Such ICAM-1/CD18 interaction might provide a potential stimulation for the release of IL-6 and CCL2 upon the interaction of basophils and bronchial epithelial cells.

Using multiparametric flow cytometry with cell surface ICAM-3 gating for differential analysis of the intracellular phosphorylation levels of different signaling molecules [30], we found that ERK, JNK, p38 MAPK and NF-κB pathways were activated in BEAS-2B cells, while
only ERK was phosphorylated in KU812 cells in the co-culture system. We further elucidated the differential activation of above signaling pathways in the induction of IL-6, CCL2 and ICAM-1 expression using specific inhibitors. Results from inhibition experiments demonstrated that the production of IL-6 induced by IL-17A in BEAS-2B alone and in co-culture was mediated by ERK, p38 MAPK and NF-κB pathways. Results were in concordance with those of previous studies suggesting that ERK, p38 MAPK and NF-κB pathways are differentially activated by IL-17A and IL-17F in eosinophils [30] and IL-17 in bronchial epithelial cells [28]. Regarding CCL2, in addition to ERK, p38 MAPK and NF-κB, JNK also plays a role for CCL2 release in co-culture. Moreover, the intracellular signaling mechanisms regulating ICAM-1 expression on BEAS-2B involved NK-κB and p38 MAPK. The results from inhibition experiments were therefore consistent with those of intracellular staining by flow cytometry. Since only ERK activity of KU812 cells was activated in co-culture with or without IL-17A activation, it may account for that basophils/KU812 cells were not the main source for producing IL-6 and CCL2. AG490 and LY294002 did not exert any inhibitory effect, JAK-STAT and PI3K-Akt pathways therefore did not play any regulatory roles in IL-6, CCL2 and ICAM-1 expression in the co-culture system.

In summary, this is the first report elucidating the regulatory mechanisms for the elevated production of IL-6, CCL2 and ICAM-1 expression upon the interaction of human primary bronchial epithelial cells/BEAS-2B cells and basophils/KU812 cells activated by hallmark Th17 cytokine IL-17A (Fig. 7). Together with previous findings of the participation of IL-17A, IL-17E and IL-17F in Th2 responses and airway hyperresponsiveness [12, 30], our results therefore support the crucial immunopathological role of the IL-17 family and Th17 lymphocytes in the amplification of allergic diseases such as allergic asthma. Although basophils could not be
activated to release IL-6 and CCL2 upon interaction with bronchial epithelial cells, it can facilitate the activation of bronchial epithelial cells by IL-17A to synergistically release IL-6 and CCL2, probably through the up-regulation of adhesion molecule ICAM-1 expression and the activation of intracellular ERK, JNK, p38 MAPK and NK-κB pathways of bronchial epithelial cells. Such synergistic induction of inflammatory cytokine IL-6 and chemokine CCL2 for the chemoattraction of neutrophils, monocytes, mast cells, basophils, eosinophils and Th2 lymphocytes can together amplify inflammatory and Th2 immune responses in allergic inflammation [26]. Further study will be performed using basophil-deficient mice to investigate the potential role of basophils in the pathogenesis of allergic inflammation [2, 4]. Development of the application of ERK, p38 MAPK and NF-κB inhibitors as potential anti-inflammatory agents in asthma is on-going [31]. Animal studies indicated that NF-κB and MAPK inhibitors can suppress the allergic inflammation and lessen the severity of asthma by suppressing the expression of inflammatory cytokines and adhesion molecule, and attenuating lung tissues eosinophilia and airway mucus production [32, 33]. Our present study may therefore provide further biochemical basis for the development of new agents for treating allergic diseases by modulating the intracellular signaling mechanism in immune effector cells.

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Figure 1. Protein expression of IL-17RA, IL-17RC and IL-17RA adaptor protein Act1 of primary bronchial epithelial cells/BEAS-2B cells and basophils/KU812 cells. Cell surface expression of (A) IL-17RA and (B) IL-17RC, and (C) intracellular expression of Act1 of primary bronchial epithelial cells/BEAS-2B cells and basophils/KU812 cells (5 x 10^5 cells) was determined by flow cytometry. The results are shown as mean fluorescence intensity (MFI) histograms. Experiments were performed in three independent replicates with essentially identical results and representative figures are shown.

HBE: human primary bronchial epithelial cells
Fig. 1

(A)

(B)

(C)
**Figure 2.** Effect of IL-17A on the induction of IL-6 and CCL2 upon the interaction of primary bronchial epithelial cells/BEAS-2B cells and basophils/KU812 cells. (A, B) KU812 cells (5 x 10^5 cells) and confluent BEAS-2B cells (1 x 10^5 cells) were cultured either together or separately with or without IL-17A (0 – 100 ng/ml) for 24 h. (C, D) Basophils (5 x 10^5 cells) and confluent bronchial epithelial cells (1 x 10^5 cells) were cultured either together or separately with or without IL-17A (10 ng/ml) for 24 h. IL-6 and CCL2 released in culture supernatants were determined by ELISA. Results are expressed as the arithmetic mean plus SD of three independent experiments.

B: BEAS-2B cells; K: KU812 cells; HBE: human primary bronchial epithelial cells; BAS: basophils; 17A (10), (20), (50), (100): concentration of IL-17A (10 – 100 ng/ml)

Multiple comparisons were done between control and treated samples by one-way ANOVA with Bonferroni post hoc test. *p<0.05, **p<0.01, ***p<0.001 when compared between groups denoted by horizontal lines; #p<0.05 when compared with corresponding BEAS-2B alone treated with the same concentrations of IL-17A.
Figure 3. Source and effect of transwell inserts on the induction of IL-6 and CCL2 in co-culture of primary bronchial epithelial cells/BEAS-2B cells and basophils/KU812 cells under IL-17A stimulation. (A, B, C, D) Basophils/KU812 cells (5 x 10^5 cells) and confluent primary bronchial epithelial cells/BEAS-2B cells (1 x 10^5 cells) were treated with or without 1% paraformaldehyde for 1 h on ice prior to being cultured together with or without IL-17A (10 ng/ml) for 24 h. (E, F, G, H) Basophils/KU812 cells (5 x 10^5 cells) and confluent primary bronchial epithelial cells/BEAS-2B (1 x 10^5 cells) were cultured together with or without IL-17A (10 ng/ml) at both upper and lower compartments in the presence or absence of transwell inserts for 24 h. IL-6 and CCL2 released in culture supernatants were determined by ELISA. Results are expressed as the arithmetic mean plus SD of three independent experiments.

B: unfixed BEAS-2B cells; B^: fixed BEAS-2B cells; K: unfixed KU812 cells; K^: fixed KU812 cells; HBE: unfixed human primary bronchial epithelial cells; HBE^: fixed primary bronchial epithelial cells; BAS: unfixed basophils; BAS^: fixed basophils.

In Figure 3A-D, multiple comparisons were done between control and treated samples by one-way ANOVA with Bonferroni post hoc test; Student’s t-test was used in Figure 3E-H. *p<0.05, **p<0.01, ***p<0.001 when compared between groups denoted by horizontal lines.
Figure 4. Effect of IL-17A on the surface expression of ICAM-1 on primary bronchial epithelial cells/BEAS-2B cells in co-culture. (A, C) Representative MFI histogram of ICAM-1 expression on primary bronchial epithelial cells/BEAS-2B cells with or without co-culture with basophils/KU812 cells. (B, D) Bar chart of ICAM-1 expression on primary bronchial epithelial cells/BEAS-2B cells in co-culture with basophils/KU812 cells with or without treatment of IL-17A. Basophils/KU812 cells (5 x 10^5 cells) and confluent primary bronchial epithelial cells/BEAS-2B cells (1 x 10^5 cells) were cultured either together or separately with or without IL-17A (10 ng/ml) for 24 h. Surface expression of ICAM-1 on 10,000 primary bronchial epithelial cells/BEAS-2B cells was analyzed by flow cytometry as MFI. Results have been normalized by subtracting appropriate isotypic control and are expressed as the arithmetic mean plus SD of three independent experiments. Multiple comparisons were done between control and treated samples by one-way ANOVA with Bonferroni post hoc test. ** p<0.01 when compared between groups denoted by horizontal lines B: BEAS-2B cells; K: KU812 cells; HBE: human primary bronchial epithelial cells; BAS: basophils;
Figure 5. Activation of ERK, JNK, p38 MAPK and NF-κB pathways regulating the release of IL-6 and CCL2 in co-culture of KU812 cells and BEAS-2B cells under IL-17 stimulation. KU812 cells (5 x 10^5 cells) and confluent BEAS-2B cells (1 x 10^5 cells) were cultured either together or separately with or without IL-17A (10 ng/ml) stimulation for 15 min. (A) Representative MFI histogram of ICAM-3 expression on BEAS-2B cells and KU812 cells. After separation, fixation and permeabilization, KU812 cells (5 x 10^5 cells) and BEAS-2B cells (1 x 10^5 cells) were gated based on the cell expression of ICAM-3. Dotted line: isotypic control, grey solid line: ICAM-3 expression. The intracellular contents of phosphorylated (B) ERK, (C) JNK, (D) p38 MAPK and (E) IκB-α of permeabilized KU812 and BEAS-2B cells were measured by intracellular immunofluorescence staining using flow cytometry. Results are shown in MFI subtracting corresponding isotypic control and are expressed as the arithmetic mean plus SD of three independent experiments. (F, G, H, I) Effect of signaling molecule inhibitors on the release of IL-6 from (F) BEAS-2B cells and (G) co-culture of BEAS-2B cells and KU812 cells with or without treatment with IL-17A, and CCL2 from (H) BEAS-2B cells and (I) co-culture of BEAS-2B cells and KU812 cells with or without treatment with IL-17A. KU812 cells (5 x 10^5 cells) and confluent BEAS-2B cells (1 x 10^5 cells) either cultured separately or together were pretreated with BAY11-7082 (1 µM), SB203580 (20 µM), SP600125 (5 µM) or U0126 (10 µM) for 1 h, followed by incubation with or without IL-17A (10 ng/ml) in the presence of inhibitors for further 24h. Concentrations of IL-6 and CCL2 released in culture supernatants were determined by ELISA. Results are expressed as the arithmetic mean plus SD of three independent experiments. DMSO (0.1%) was used as the vehicle control.
B: BEAS-2B cells only; coB: BEAS-2B cells in co-culture; K: KU812 cells only; coK: KU812 cells in co-culture; CTL: control, BAY: BAY11-7082, SB: SB203580, SP: SP600125

Multiple comparisons were done between control and treated samples by one-way ANOVA with Bonferroni post hoc test. *p<0.05, **p<0.01, ***p<0.001 when compared between groups denoted by horizontal lines.
**Figure 6.** Effect of signaling molecule inhibitors on the cell surface expression of ICAM-1 on BEAS-2B cells in the co-culture. KU821 cells (5 x 10⁵ cells) and confluent BEAS-2B cells (1 x 10⁵ cells), either cultured separately or together, were pretreated with AG490 (5 µM), BAY11-7082 (1 µM), LY294002 (5 µM), SB203580 (20 µM), SP600125 (5 µM) or U0126 (10 µM) for 1 h, followed by incubation with inhibitors for further 24 h. Surface expression of ICAM-1 on 10,000 cells was analyzed by flow cytometry as MFI. Results have been normalized by subtracting appropriate isotypic control and are expressed as the arithmetic mean plus SD of three independent experiments. DMSO (0.1%) was used as the vehicle control.
Multiple comparisons were done between control and treated samples by one-way ANOVA with Bonferroni post hoc test. ***p<0.01 when compared between groups denoted by horizontal lines B: BEAS-2B cells only, K: KU812 cells only, AG: AG490, BAY: BAY11-7082, LY: LY294002, SB: SB203580, SP: SP600125

Fig.6