

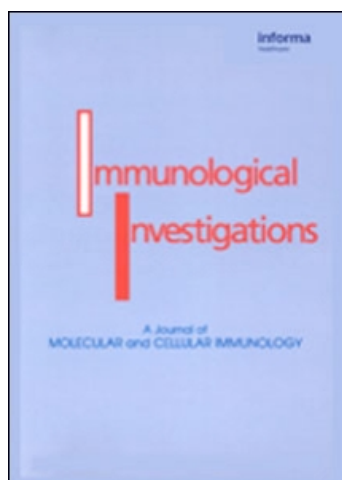
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Activation of Peripheral Th17 Lymphocytes in Patients with Asthma

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A recently identified interleukin (IL)-17-producing T-helper (Th) lymphocyte subset, which comprises Th17 cells producing hallmark cytokines IL-17A, IL-17F and IL-22, is involved in chronic inflammatory diseases. Elevated gene and protein expressions of IL-17 are manifested in allergic asthma. We further characterized the activation of Th17 cells in asthmatic patients. Peripheral blood mononuclear cells (PBMC) were purified from 31 asthmatic patients and 20 sex- and age-matched control subjects. The number of IL-17A secreting cells in peripheral blood was enumerated by enzyme-linked immunosorbent spot assay. Cell surface expression of Th17-related chemokine receptor CCR6, and plasma level of IL-17A, IL-17F and IL-22, and *ex vivo* production of IL-17A and IL-22 were measured by flow cytometry and enzyme-linked immunosorbent assay, respectively. The number of peripheral Th17 lymphocytes, expression of CCR6 on Th cells, and *ex vivo* IL-23, anti-CD3 and anti-CD28 induced production of IL-22 by PBMC were significantly elevated in asthmatic patients compared with control subjects (all $p < 0.01$). This clinical study further confirmed increased number of peripheral Th17 lymphocytes and cell surface expression of CCR6 receptors on Th cells in asthmatic patients. Pro-inflammatory cytokine IL-23 can exacerbate disease severity by activating pathogenic Th17 lymphocytes to release downstream inflammatory cytokine IL-22 in asthma.

Keywords Asthma, CCR6, IL-22, Th17 cells.

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INTRODUCTION

Asthma is a complex and heterogeneous disease that is characterized by intermittent reversible obstruction and chronic inflammation of the airways, bronchial hyperreactivity, and infiltration of lymphocytes and eosinophils into the airway submucosa (Yssel and Groux, 2000). Activation and differentiation of T helper (Th)2 lymphocytes for the secretion of interleukin (IL)-4, IL-5 and IL-13 have been shown to play a central role in mediating the pathogenesis of asthma (Larche et al., 2003). Our previous studies have revealed the elevation of plasma concentration of inflammatory cytokines IL-6, IL-12, IL-17 and IL-18, Th2 chemokines CCL5, CCL17 and CCL22, and the cell surface expression of Th2 related chemokine receptor CCR3 on peripheral blood lymphocytes in asthmatic patients (Wong et al., 2001; Leung et al., 2002, 2003; Lun et al., 2006). In addition, the significantly lower ratio of peripheral Th1/Th2 cells and Th1 transcription factor T-bet expression in asthmatic patients indicated a predominance of Th2-mediated immunity in asthma (Wong et al., 2001; Ko et al., 2007).

The Th17 lymphocytes are 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 (Sato et al., 2007; Liang et al., 2006). IL-1 β , 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 ROR γ t (Acosta-Rodriguez et al., 2007; Brustle et al., 2007; Wilson et al., 2007). Both IL-17A and IL-17F can induce the expression of IL-6, granulocyte macrophage colony-stimulating factor, CXCL10 from epithelial and vascular endothelial cells and neutrophil chemokine CXCL8 from fibroblasts and defensin from keratinocytes (Wilson et al., 2007; Kawaguchi et al., 2004). Our recent study also revealed that IL-17A and IL-17F could activate eosinophils to release inflammatory IL-6 and CXC chemokines in allergic inflammation (Cheung et al., 2008). Hyperproduction of IL-17 family cytokines such as IL-17A was found in allergic asthmatic patients (Wong et al., 2001; Hashimoto et al., 2005). Other studies in keratinocytes and colonic myofibroblasts showed that Th17 cytokine IL-22 could induce anti-microbial proteins, defensins, acute-phase proteins, inflammatory cytokines, chemokines, and hyperplasia (Boniface et al., 2005; Wolk et al., 2006). Elevated IL-22 expression has been shown to be clinically associated with Crohn's disease, interstitial lung disease, rheumatoid arthritis and psoriasis (Commings et al., 2008). However, the production mechanisms of IL-22 have not been investigated in asthmatic patients.

Th17 cells but not Th1 and Th2 cells selectively express chemokine receptor CCR6 and CCR6 ligand CCL20 (Acosta-Rodriguez et al., 2007; Annunziato et al., 2007; Hirota et al., 2007). CCR6 mediates homing to skin and mucosal tissues for the recruitment of pathogenic T cells in many IL-17 related inflammatory diseases including psoriasis, inflammatory bowel diseases, allergic

asthma and rheumatoid arthritis (Tesmer et al., 2008). CCL20 is upregulated in stromal cells by IL-17, allowing Th17 cells in inflamed tissues to attract additional Th17 and Th1 cells (Hirota et al., 2007; Chabaud et al., 2001; Kao et al., 2005).

Activation of Th17 cells has been related to the pathogenesis of allergic asthma since CD4⁺ Th cells of atopic asthma patients can produce IL-17 upon restimulation with allergen *Dermatophagoides farinae* extract (Hashimoto et al., 2005). Apart from the circulation, sputum and bronchial alveolar lavage fluid, elevated IL-17 was also detected in airway eosinophils in asthmatic patients (Molet et al., 2001; Tesmer et al., 2004). However, the circulating numbers of Th17 cells and the production mechanisms of Th17 related cytokine have not been fully elucidated in asthmatic patients. In an attempt to further confirm the pathophysiological roles of Th17 cells in asthma, the circulating number of Th17 cells, plasma concentrations of Th17 cytokines, expression of CCR6 on Th cells, and the *ex vivo* production of IL-22 from peripheral blood mononuclear cells (PBMC) upon co-stimulation have been studied in this cross sectional case-control study.

PATIENTS AND METHODS

Asthmatic Patients, Control Subjects and Blood Samples

Thirty-one Chinese adult patients with asthma were recruited from the Asthma Clinic of the Prince of Wales Hospital, Hong Kong. Diagnosis of asthma was based on the guidelines of the American Thoracic Society (1993). Lung function of the subjects was assessed by spirometry (Model S, Vitalograph, Buckingham, UK) according to the American Thoracic Society standards (American Thoracic Society, 1995). Forced expiratory volume in 1 second (FEV₁), forced vital capacity (FVC), and the FEV₁/FVC ratio were measured before and 15 minutes after the inhalation of salbutamol (Glaxo Operations Ltd, Greenford, UK). The results were compared with the local predicted age- and sex-matched values (Lam et al., 1983). The severity of asthma in these patients was expressed as the level of asthma control assessed with symptoms, lung functions and exacerbation (National Heart, Lung and Blood Institute, 2007). All our studied asthmatic patients were on short acting bronchodilator as needed. Four patients were on inhaled steroids such as beclomethasone dipropionate (Becloforte; Glaxo Wellcome, Research Triangle Park, NC, USA) or budesonide (Pulmicort; AstraZeneca, London, UK). All patients and control subjects had no oral intake of steroid or change of asthma medications four weeks before recruitment of study. Twenty sex- and age-matched healthy Chinese volunteers were recruited as control subjects. All subjects were either life-long non-smokers or ex-smokers for at least half a year and free from upper respiratory tract infection for 8 weeks before

recruitment. Nine milliliters of venous peripheral ethylenediaminetetraacetic acid (EDTA) blood were collected. Aliquots of whole blood were processed immediately for peripheral blood mononuclear cell (PBMC) isolation. PBMC were prepared by centrifugation of blood using a density gradient (Ficoll-Paque Plus; GE Healthcare Life Sciences, NJ, USA).

The above protocol was approved by the Clinical Research Ethics Committee of The Chinese University of Hong Kong-New Territories East Cluster Hospitals, and informed consent was obtained from all participants according to the Declaration of Helsinki.

Ex vivo Induction of IL-17A and IL-22

The method of Viallard et al. (1999) was adopted with modification. After a maximum storage period of 1 h of collected EDTA blood at room temperature, PBMC were purified using Ficoll-Paque gradient centrifugation (GE Healthcare). PBMC ($1 \times 10^6/\text{mL}$) was then incubated with or without anti-CD3 and CD28 monoclonal antibody (BD Pharmingen Corp, CA, USA) (each $1 \mu\text{g}/\text{mL}$) and IL-23 ($50 \text{ ng}/\text{mL}$) (Peprotech EC Ltd, London, UK) for 24 h at 37°C in a 5% CO_2 atmosphere. After incubation, the cell free supernatant of *ex vivo* culture was harvested and stored at -70°C for subsequent ELISA of IL-17A and IL-22. The percentage increase of the induction of IL-17A and IL-22 was calculated by $[\text{treatment (pg/ml)} - \text{medium control (pg/ml)}]/\text{medium control (pg/ml)} \times 100\%$.

Cell Surface Expression of CCR6

PBMC were prepared by centrifuging EDTA venous blood using a Ficoll-Paque density gradient (GE Healthcare). The viability of PBMC was more than 95% as determined by trypan blue exclusion method. PBMC ($1 \times 10^6/\text{mL}$) were harvested and resuspended in cold phosphate buffered saline (PBS) supplemented with 0.5 % bovine serum albumin (BSA). After blocking with 2% human pooled serum for 20 min at 4°C and washed with PBS supplemented with 0.5% BSA, cells were incubated either with mouse anti-human CCR6 monoclonal antibody or mouse IgG₁ isotype (R & D systems Corp., MN, USA) for 30 min at 4°C in the dark. After washing, cells were stained with secondary FITC-conjugated anti-mouse antibody (Becton Dickinson Biosciences Pharmingen Corp., CA, USA) for 45 min before finally suspending in 1% paraformaldehyde in PBS as fixative. Cell surface expression of CCR6 was then analyzed by flow cytometry (FACSCalibur, Becton Dickinson, CA, USA) with R-phycoerythrin-conjugated CD4+ gating (BD Pharmingen). The typical forward and side scatter gate for lymphocytes was set to exclude contaminating monocytes from the analysis. A total of 5,000 events were collected in the log mode and were expressed as mean fluorescence intensity (MFI) by subtracting the basal fluorescence level of isotypic control.

Assay of Human IL-17A, IL-17F and IL-22

Plasma concentrations of IL-17A, IL-22, and IL-17F were measured by enzyme-linked immunosorbent assay (ELISA) kit from R & D Systems, MN, USA and Peprotec Inc., NJ, USA, respectively.

Assay of Circulating IL-17A Secreting Cells by Enzyme-Linked Immunosorbent Spot (ELISPOT)

Briefly, PBMC was first purified from EDTA blood using Ficoll gradient centrifugation (GE Healthcare). PBMC was then preserved using Cryostar™ freezing medium (U-CyTech Biosciences Corp, Yalelaan, Netherlands) at -80°C until the assay. For assay, the number of IL-17A secreting cells in PBMC (1×10^5) upon 24 h activation by phorbol-myristate acetate (PMA) (50 ng/ml) and ionomycin (1 $\mu\text{g/ml}$) in polyvinylidene difluoride filter 96-well plate (Millipore Corp., MA, USA) was detected using Human IL-17A ELISPOT kit (eBioscience Corp., CA, USA). The number of red spots was counted using a dissection microscope (Wong et al., 2008).

Statistical Analysis

Numerical data were expressed as median (interquartile range, IQR) if they were not in Gaussian distribution. Difference between two groups was assessed by Mann–Whitney rank-sum test. Comparison of basal and *ex vivo* culture supernatant concentrations was made with the Wilcoxon paired test. Statistical analysis was performed using the statistical package for the Social Sciences (SPSS) statistical software for Windows, Version 16.0 (SPSS Inc, IL, USA). Probability values (p) < 0.05 were considered as significant.

RESULTS

Asthmatic Patient and Control Subjects

The characteristics of the study groups are summarized in Table 1. The mean $\pm$ SD FEV₁ of all asthmatic patients was 2.16 ± 0.63 L/min (82.0% of predicted normal values), while the FEV₁/FVC ratio was 80.1%. The severity of asthma in these patients in terms of the level of asthma control assessed with symptoms, lung functions and exacerbation was controlled asthma 9 patients (29%), partly controlled asthma 13 patients (42%), and uncontrolled asthma 9 patients (29%).

Elevated Circulating IL-17A Secreting Th17 Cells in Asthmatic Patients

As shown in Figure 1, the number of circulating IL-17A secreting Th17 cells in asthmatic patients was significantly higher than that of control

Table 1: Characteristics of asthmatic patients treated or not treated with inhaled corticosteroid (ICS) and their control subjects.[†]

Parameters	Asthmatic patients (n = 31)	Control subjects (n = 20)
Sex, male/female		
Male	14	9
Female	17	11
Age, yr (range)	45.6 ± 12.3 (22–69)	48.4 ± 12.8 (24–68)
Lung functions		
FEV ₁ , L/min (% predicted)	2.16 ± 0.63 (82.0)	NE
FEV ₁ /FVC ratio, %	80.1	NE
ICS treatment		
Percentage of patients receiving treatment	4/31 (13%)	0 (0%)
Daily dose, µg	855 ± 405	0

[†]Values given as mean ± SD.
NE: not examined

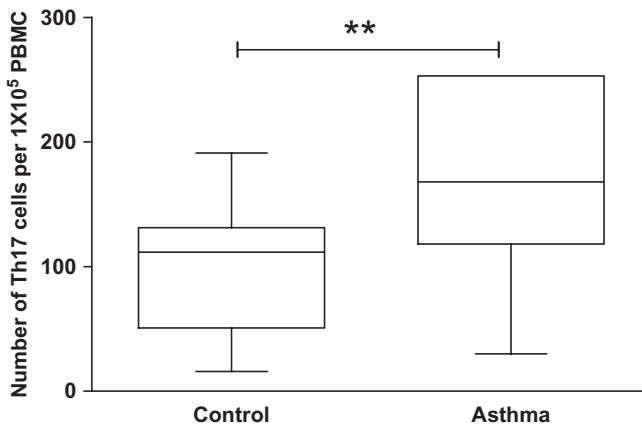


Figure 1: IL-17A secreting Th17 cells in PBMC of asthmatic patients and control subjects. IL-17A secreting Th17 cells upon 24 h activation by PMA (50 ng/ml) and ionomycin (1 µg/ml) in 1 × 10⁵ PBMC was assayed using human IL-17A ELISPOT kit (eBioscience Corp., CA, USA). Results are presented with Box-and-Whisker plot showing the median (interquartile range). Mann-Whitney rank sum test was used to assess the differences of concentration between patients and control subjects. **p < 0.01 compared to control subjects.

subjects [median (interquartile range) asthma: 168 (118 - 253) versus control: 112 (51 - 131) per 1 × 10⁵ PBMC, *p* < 0.01]. For the analysis of the Th17 cells by ELISPOT, PMA and ionomycin were commonly used stimulators to activate the expression of IL-17 in Th17 cells according to previous study (Wong et al., 2001). IL-17A secreting Th17 cells were not detected using ELISPOT without the stimulation by PMA and ionomycin (data not shown).

Elevated Expression of Cell Surface CCR6

In view of the increase of circulating number of IL-17A secreting Th17 cells, we further investigate the cell surface expression of CCR6 on Th cells using flow cytometry because Th17 cells selectively express chemokine receptor CCR6 (Acosta-Rodriguez et al., 2007; Annunziato et al., 2007; Hirota et al., 2007). Cell surface expression of CCR6 was significantly increased on CD4 + Th cells in the asthmatic group than control group [asthma: 0.81 (0.64–1.23) versus control: 0.37 (0.26 – 0.42) MFI; $p < 0.001$, Figure 2).

Plasma Concentrations of IL-17A, IL-17F and IL-22

There was no significant difference for the plasma concentrations of IL-17A, IL-17F and IL-22 between patient and control groups in this study cohort (all $p > 0.05$). For IL-17A and IL-17F, most of the samples were found to be undetectable for IL-17A and IL-17F (below the detection limit of ELISA kit), while plasma IL-22 concentration was found to be no significant difference between asthmatic and control group [asthma: 2.8 (1.8–5.5) versus control: 4.1 (2.3–7.0) pg/ml, $p > 0.05$].

Elevated *ex vivo* Production of IL-17A and IL-22

We first investigated the *ex vivo* production of IL-17A and IL-22 from anti-CD3 and anti-CD28 co-stimulated PBMC upon IL-23 activation in the patient and control cohorts. IL-23 could significantly induce and augment the release of IL-17A and IL-22 from anti-CD3 and anti-CD28 activated PBMC in both asthmatic and control subjects (all $p < 0.001$, Figures 3A and C). Based on the

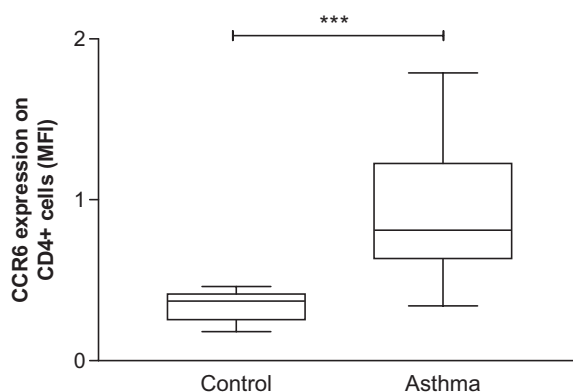


Figure 2: Expression of CCR6 on CD4 + Th cells in asthmatic patients and control subjects. The expression of CCR6 is shown as MFI subtracting corresponding isotypic controls with Box-and-Whisker plot showing the median (interquartile range). Mann-Whitney rank sum test was used to assess the differences of concentration between patients and control subjects. *** $p < 0.001$.

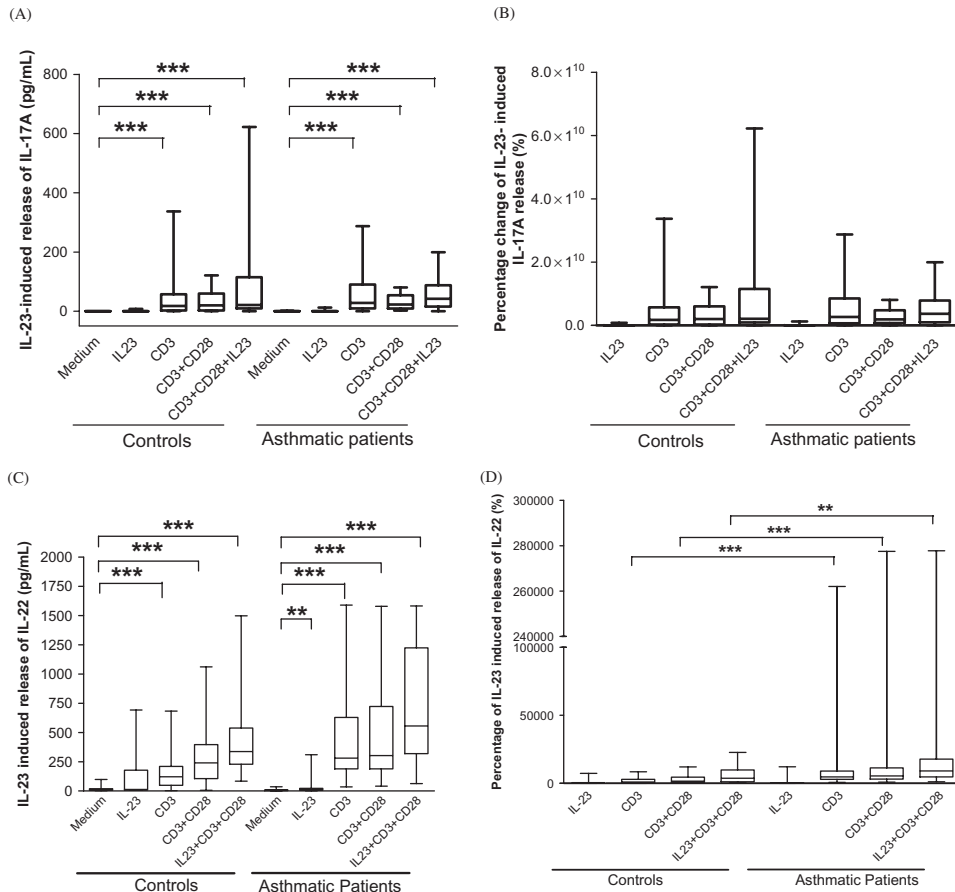


Figure 3: Ex vivo production and percentage increase of IL-17A and IL-22 from anti-CD3, anti-CD28 and IL-23 activated-PBMC of all asthmatic patients and control subjects. The culture supernatant was derived from PBMC cultured with medium with or without anti-CD3 antibody, anti-CD28 antibody (1 μ g/mL each) and IL-23 (50 ng/mL) for 24 h. Results are presented with Box-and-Whisker plots showing median (interquartile range). **p < 0.01, ***p < 0.001 compared to corresponding treatment groups. CD3: anti-CD3 antibody; CD28: anti-CD28 antibody.

above results of IL-23 activated Th17 cells, we further investigated the % increase of IL-17A and IL-22 from anti-CD3 and anti-CD28 co-stimulated PBMC upon IL-23 activation by normalizing with the basal release of IL-17A and IL-22 of medium control.

As shown in Figures 3B and D, the % increase of IL-22 but not IL-17A upon anti-CD3 and anti-CD28 antibody activation with or without IL-23 from PBMC was significantly higher in asthmatic patients than that in control group (all p < 0.001) because asthmatic patients had lower basal release of IL-22 (medium control).

DISCUSSION

Accumulating evidence has suggested the crucial role of the activation of Th17 cells in the pathogenesis of asthma (Molet et al., 2001; Hashimoto et al., 2005; Tesmer et al., 2008). Experimental models suggest that Th17 cells are important for neutrophilic infiltration and inflammation in acute airway inflammation during acute asthma attacks, since IL-17 can activate human bronchial fibroblast and smooth muscle cells to produce IL-6, CXCL8 and CXCL1 for neutrophil migration and activation (Hoshino et al., 2000; Molet et al., 2001; Dragon et al., 2007; Schmidt-Weber et al., 2007).

In addition, IL-17 together with IL-6 can induce the expression of the mucin genes, MUC5B and MUC5AC, by human bronchial epithelial cells (Chen et al., 2003). However, the asthma related Th2 cytokines IL-4, IL-9, and IL-13 did not have the same effect on human bronchial epithelial cells (Chen et al., 2003). In a recent animal study, IL-23 and Th17 cells also up-regulated Th2-cell-mediated eosinophilic airway inflammation (Wakashin et al., 2008). Nevertheless, most of previous clinical studies of asthmatic patients used ELISA or RT-PCR for the analysis of IL-17 level. Since quantitative ELISPOT assay can detect the IL-17A secreting cells at the single cell level, it could be considered as a more superior test, offering a higher sensitivity and specificity than conventional ELISA measurement of circulating IL-17 or mRNA expression by RT-PCR (Wong et al., 2001; Hashimoto et al., 2005; Bullens et al., 2006). Our present study therefore confirmed the elevated number of Th17 cells in asthma.

A previous study has shown that all IL-17 producing CD4⁺ T cells expressed CCR6 which are not expressed by Th1 and Th2 cells (Singh et al., 2008). Compared with CD4⁺ CD45RO⁺ CCR6⁻ memory T cells, CD4⁺ CD45RO⁺ CCR6⁺ cells expressed more than 100 fold IL-17A, particularly of tissue-infiltrating highly differentiated Th17 cells (Singh et al., 2008; Pene et al., 2008). Our results also indicated that asthmatic patients expressed much more CCR6 on CD4⁺ Th cells than that of control subjects (Figure 2). Since CCR6 is associated with the chemotactic migration of pathogenic T cells, B cells and dendritic cells to epithelial cells, our results of CCR6 expression suggest the mechanisms of the infiltration of Th17 into the inflammatory sites such as bronchial airways in asthma.

We observed that the ex vivo production of IL-22 and IL-17A upon anti-CD3 and anti-CD28 antibody co-stimulation from PBMC was significantly enhanced by the presence of IL-23 (Fig. 3A). More importantly, the percentage increases in IL-22 production were significantly higher in asthmatic patients (Fig. 3B, all $p < 0.05$). These findings indicate that the expressions of Th17 inflammatory cytokine IL-22, and activation of Th17 cells are in part influenced by proinflammatory cytokine IL-23 present in the local environment of the cells at the time of stimulation. Actually, IL-22 can be induced from IL-23

activated naïve T cells (Korn et al., 2009). Previous report revealed that mitogenic stimulation of PBMCs using anti-CD3 and anti-CD28 antibodies induced equivalent levels of IL-17 from control subjects, non-atopic asthmatics, and allergic asthmatics, only the asthmatic patients produced significant higher IL-17 upon house dust mite protein extract-mediated activation (Hashimoto et al., 2005).

Pro-inflammatory cytokine IL-22, a IL-10 superfamily cytokine, has been shown to involve in acute-phase response and innate immunity (Commins et al, 2008). The pattern of IL-22 receptor complex consisting of IL-22R1 and IL-10R2 chains suggests that the cellular targets of IL-22 are within respiratory, skin, kidney and digestive system (Aggarwal et al., 2001). Although there was no significant elevation of plasma IL-22 concentration in asthmatic patients, IL-22 could be highly increased in local inflammatory sites such as bronchial airways in asthmatic patients. In view of the target cells of IL-22 located at the respiratory system; elevated local production of IL-22 can be one of the underlying mechanisms for the inflammation in asthma.

There were no significant differences of Th17 cell numbers, CCR6 + Th cells and *ex vivo* production of IL-17A and IL-22 among asthmatic patients grouped based on the disease severity (all $p > 0.05$). It may be due to the multi-factorial effects including immunological factors, environmental factors and drug effects for the determination of the disease severity of asthma.

In summary, this clinical study further confirms the increased number of peripheral Th17 cells and CCR6 expression on Th cells in asthmatic patients. Inflammatory cytokine IL-23 can promote the disease severity by activating pathogenic Th17 cells and the induction of downstream inflammatory cytokine IL-22 in asthma. Our results should provide new insights for their potential pathological roles in asthma, and facilitate the development of asthma disease surrogate markers. In view of the possibility that interfering with the activity of Th17 cells or IL-17 could be an effective treatment modality (Linden, 2003), our present results may therefore provide a biochemical basis for the novel therapeutic agents for antagonizing the over-activation of Th17-mediate inflammation in asthma.

Declaration of Interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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