

## Increased Expression of Plasma and CD4+ T Lymphocyte Costimulatory Molecule CD26 in Adult Patients with Allergic Asthma

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CD26, which is a costimulatory molecule and peptidase, is responsible for the degradation of interferon (IFN)- $\gamma$ -induced chemokines. To elucidate the immunopathological role of CD26 in allergic asthma, we investigated plasma soluble CD26 (sCD26) concentration and its cell surface expression on lymphocytes, monocytes, CD4+ T helper, CD8+ T suppressor plus cytotoxic T, invariant natural killer T (iNKT), and CD19+ B lymphocytes in allergic asthmatic patients. Plasma sCD26 was significantly elevated in asthmatic patients regardless of inhaled corticosteroid treatment (all  $P < 0.05$ ). Cell surface expression of CD26 was significantly up-regulated on lymphocytes, especially on CD4+ and iNKT lymphocytes (all  $P < 0.05$ ), but not on other cell types. Significant positive correlations were found between sCD26 and the percentage of eosinophils, Th2-related chemokines CCL5 and CCL22, and costimulatory molecule sCTLA-4 (all  $P < 0.05$ ). In conclusion, the aberrant expression of CD26 may contribute to the inflammatory process and Th2 predominance in the immunopathogenesis of allergic asthma.

**KEY WORDS:** allergy; asthma; costimulatory molecules; CD26; T lymphocytes.

### INTRODUCTION

Allergic asthma is characterized by infiltration into the airway submucosa of leukocytes, which contributes to airway obstruction and bronchial hyperresponsiveness (1).

Its pathogenesis is centrally mediated by the activation, differentiation, and chemotaxis of T lymphocytes, which is regulated by cytokines and chemokines (2). The degradation of such cytokines by peptidases, such as CD13 and CD26, is an important mechanism to modulate peptide-mediated inflammation in asthma (3, 4).

CD26, a membrane-anchored ectoenzyme with dipeptidyl peptidase IV activity in its extracellular domain, is a 110-kDa transmembrane glycoprotein, which belongs to the serine protease family. It acts to selectively remove the N-terminal dipeptide from peptides with proline or alanine in the penultimate position (5). CD26 alone cannot directly degrade intact cytokines such as IL-1 $\beta$ , IL-2, and IL-6 under *in vitro* conditions. It can only degrade synthetic oligopeptides with sequences analogous to the N-terminal part of these natural or recombinant cytokines. For the hydrolysis of these cytokines, a concerted action of both CD26 and another CD26-related enzyme peptidase aminopeptidase N is needed (6). A number of chemokines are also substrates of CD26 including CXCL9 (monokine induced by  $\gamma$ -interferon, MIG) and CXCL10 (interferon (IFN)- $\gamma$  inducible protein-10, IP-10) (5). CD26 is expressed on a variety of tissues including endothelial, epithelial, and T-cells, with a preferential expression on the subset of CD4+ memory T-cells (5). It is up-regulated after T-cell activation and is highly expressed on activated T helper (Th) type 1 lymphocytes (7). CD26 also exists as a secreted isoform, soluble CD26 (sCD26), which lacks the cytoplasmic tail, transmembrane region, and circulates in plasma (8).

The cell surface expression of CD26 has been shown to positively correlate with the production of Th1 cytokines by T-cell clones. Also, its expression is induced by stimuli favoring the development of Th1 response (9–11). The number of CD26+ T lymphocytes is increased in

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the inflamed tissues, such as thyroid and synovial fluids, of patients with autoimmune diseases such as rheumatoid arthritis (12). Despite the substantial evidence on the involvement of CD26 in Th1-related diseases, its role as costimulatory factor and peptidase with regard to the pathogenesis of asthma is not yet fully understood.

Although the dual role of Th2 lymphocytes and eosinophils is widely accepted as being critical for the onset and progression of the pathogenesis of allergic asthma, recent studies have suggested a role of costimulatory molecule CD26 in the pathogenesis by enhancing Th2-dependent allergic inflammation (13). Originally described as a T-cell activation molecule, CD26 as a costimulatory molecule have been studied extensively *in vitro* (5). The optimal activation of T-cells require two signals—a primary signal delivered by the antigenic peptide presented by the major histocompatibility complex (MHC) molecules, and a nonspecific signal generated by the interaction of costimulatory molecules such as cytotoxic T-lymphocyte-associated antigen (CTLA)-4 and CD28 (14, 15). It has been suggested that allergen-sensitized CD26+ Th1 lymphocytes produce IFN- $\gamma$  and Th1-polarized dendritic cells, which are later capable of augmenting allergen-induced pulmonary inflammation (13).

Natural killer T (NKT) cells is a subset of T-cells that exhibit both properties of natural killer cells and conventional T-cells. A majority of NKT cells expressing an invariant T-cell receptor, which is restricted to interactions with CD1d, are known as the invariant NKT (iNKT) cells. Upon activation, these cells produce a large amount of cytokines IL-2, tumor necrosis factors (TNF)- $\alpha$ , IL-4, and IFN- $\gamma$  (16). Recent studies have shown activated iNKT cells causes exacerbation of allergic airway inflammation in mouse models, and in addition, the number of these cells are found to be increased in the lungs of patients with chronic asthma (17).

To elucidate the immunopathological role of CD26 in the pathogenesis of allergic asthma, we investigated the plasma concentration of sCD26 and cell surface expression of CD26 on lymphocytes, monocytes, CD4+ T helper, CD8+ T suppressor plus cytotoxic T lymphocytes, CD19+ B lymphocytes, and iNKT lymphocytes in patients with allergic asthma treated or not treated with inhaled corticosteroid treatment (ICS). Both Th2-related chemokine CCL5 (regulated upon activation normal T-cell expressed and secreted (RANTES)) and CCL22 (macrophage-derived chemokine (MDC)) can be truncated by CD26 (18, 19), and these Th2-related chemokines in patients with allergic asthma have been studied by our group. Our previous results indicated a significant elevation of plasma CCL5 and CCL22, and

the elevation showed positive correlation with GINA disease severity score (20, 21). For assessing the role of CD26 in Th2 predominance in allergic asthma, the association between plasma concentration of sCD26 and other costimulatory molecules in plasma such as sCTLA-4 and sCD28 and Th2-related chemokines CCL5, CCL11 (eotaxin), and CCL22 were also investigated in this study cohort.

## MATERIALS AND METHODS

### *Asthmatic Patients, Control Subjects, and Blood Samples*

Fifty-one Chinese adult patients with allergic asthma were recruited from the Asthma Clinic of the Prince of Wales Hospital, Hong Kong. The atopic status of these patients was ascertained by positive serum-specific IgE assays to house dust mite (*Dermatophagoides pteronyssinus* 1, Der p 1), cat, dog, mixed cockroaches, and mixed moulds by fluorescence enzyme immunoassay (AutoCAP analyzer, Pharmacia Diagnostics AB, Uppsala, Sweden) (22). Diagnosis of asthma was based on the guidelines of the American Thoracic Society (23), and lung function of the subjects was assessed by spirometry (Model S, Vitalograph, Buckingham, UK) according to the American Thoracic Society standards (24). Forced expiratory volume in 1 s (FEV<sub>1</sub>), forced vital capacity (FVC), and the FEV<sub>1</sub>/FVC ratio were measured before and 15 min after the inhalation of salbutamol (Glaxo Operations Ltd., Greenford, UK). The results were compared with the local predicted age- and sex-matched values (25). The severity of asthma in these patients was assessed according to the Global Initiative for Asthma (GINA) guidelines (26, 27). All our studied asthmatic patients were on short acting bronchodilator as needed. Some of them were on inhaled corticosteroid 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 for 4 weeks before recruitment of study. Thirty-five sex- and age-matched nonasthmatic healthy Chinese volunteers were recruited as control subjects. All subjects were non-smokers and free from upper respiratory tract infection for 4 weeks before recruitment. Nine milliliters (mL) of venous peripheral ethylenediaminetetraacetic acid (EDTA) blood and 5 mL of clotted blood were collected from each participant. Aliquots of whole blood were processed immediately for peripheral blood mononuclear cell (PBMC) isolation. Plasma and serum samples were preserved at  $-70^{\circ}\text{C}$  for subsequent assays. 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.

#### *Assays for sCD26, sCTLA-4, sCD28, CCL5, CCL22, and CCL11 in Plasma*

Plasma sCD26, sCTLA-4, sCD28, CCL11, and CCL22 concentration was measured using enzyme-linked immunosorbent assay (ELISA) reagents from Bender Medsystems Diagnostics GmbH, Vienna, Austria and R&D Systems Inc., MN, USA, respectively. CCL5 was assayed by human chemokine cytometric bead array (BD Pharmingen, CA, USA) using flow cytometry (BD Calibur flow cytometer) (20, 28).

#### *Cell Surface Expression of CD26 on CD4+, CD19+, CD8+, and iNKT Cells*

PBMC were prepared by centrifugation of blood using a density gradient (Ficoll-Paque Plus; Amersham Pharmacia Biotech Ltd., Uppsala, Sweden). The cells were incubated with fluorescein isothiocyanate (FITC)-conjugated CD26-specific monoclonal antibody (mAb), followed by phycoerythrin (PE)-conjugated CD4, allophycocyanin (APC)-conjugated CD19 and peridinin-chlorophyll-protein (PerCP)-conjugated CD8 antibodies (Becton Dickinson Biosciences Pharmingen). FITC-conjugated mouse immunoglobulin (Ig) G was used as isotypic control. The typical forward and side scatter gates for lymphocytes were set to exclude contaminating cells from the analysis.

For the study of cell surface expression of CD26 on iNKT cells, PBMC were incubated with purified CD26-specific mAb (BD Pharmingen) followed by APC-conjugated secondary antibody (R&D Systems). These cells were subsequently incubated with PerCP-conjugated CD3 (BD Pharmingen), FITC-conjugated V $\alpha$ 24 and PE-conjugated V $\beta$ 11 antibodies (Beckman Coulter, Fullerton, CA) for the identification of iNKT cells.

The expression of CD26 on CD4+, CD19+, iNKT, and CD8+ cells was then analyzed by flow cytometry (BD FACSCalibur). Altogether, 5000 events were collected in the log mode and were expressed as mean fluorescence intensity (MFI).

#### *Statistical Analysis*

The Mann–Whitney *U* test was used to assess the differences of data between the asthmatic patients and the control subjects. The Kruskal–Wallis test followed,

when significance arose, by Dunn's test for pair-wise comparisons of data between asthmatic patients with or without receiving ICS and the control subjects. Spearman correlation test was used for correlation analysis between parameters. All analyses were performed using statistical software (SPSS for Windows, version 9.0, SPSS Inc., Chicago, IL). Probability (*P*) values of less than 0.05 were considered significant. Unless otherwise specified, results are expressed as the median (interquartile range (IQR)).

## RESULTS

### *Asthma Severity and Atopic Status*

The characteristics of the study groups are summarized in Table I. The mean  $\pm$  SD FEV<sub>1</sub> of all asthmatic patients was 2.5  $\pm$  0.9 L/min (85.9  $\pm$  16.9% of predicted normal values), while the FEV<sub>1</sub>/FVC ratio was 84.1  $\pm$  8.2%. The severity of asthma in these patients according to GINA comprised: intermittent asthma 9 patients (17.7%), mild persistent asthma 6 patients (11.8%), moderate persistent asthma 18 patients (35.3%), and severe persistent asthma 18 patients (35.3%).

### *Plasma sCD26*

As shown in Fig. 1, sCD26 was present in plasma. Its concentration was significantly higher in allergic asthmatic patients than control subjects [526 (390–658) versus 398 (316–504) ng/mL; *P* < 0.001] (Fig. 1A). It was significantly higher in both ICS-treated [515 (390–687) versus 398 (316–504) ng/mL; *P* < 0.01] and non-ICS-treated asthmatic patients than that of control subjects [542 (390–637) versus 398 (316–504) ng/mL; *P* < 0.05] (Fig. 1B).

Plasma concentration of sCD26 and sCTLA-4 were found to have a strongly significant and positive correlation (*r* = 0.47, *P* < 0.01) (Table II). However, there were no significant correlations between sCD26 and sCD28 (*P* > 0.05). We previously found that plasma concentrations of both sCTLA-4 and sCD28 were elevated in asthmatic patients (28). We also observed that there was a significant and positive correlation between plasma concentration of sCD26 and the percentage of circulating eosinophils in white blood cells (*r* = 0.43, *P* < 0.01) (Table II).

There were significant and positive correlations between plasma sCD26 and Th2 chemoattractants CCL5 and CCL22 (CCL5: *r* = 0.25, *P* < 0.05; CCL22: *r* = 0.56, *P* < 0.05) (Table II). We have shown the elevated plasma concentration of CCL5 and CCL22 in asthmatic patients than control subjects (20, 21). Although the concentration

**Table I.** Characteristics of Allergic Asthmatic Patients With or Without Receiving Inhaled Corticosteroid (ICS) and Their Control Subjects<sup>a</sup>

Parameters	ICS-treated asthmatic patients (n = 28)	Non-ICS-treated asthmatic patients (n = 23)	Control subjects (n = 35)
Sex, male/female			
Male	15	9	11
Female	13	14	24
Age, year	43.5 ± 15.7	37.1 ± 14.0	40.9 ± 11.9
Range	(21–69)	(20–63)	(22–66)
Lung functions			
FEV <sub>1</sub> , L/min (% predicted)	2.4 ± 0.8 (82.5 ± 14.9)	2.6 ± 0.9 (89.9 ± 18.5)	NE
FEV <sub>1</sub> /FVC ratio (%)	82.0 ± 7.1	86.5 ± 8.9	NE
ICS treatment			
Daily dose (μg)	1110.7 ± 631.5	0	0

<sup>a</sup>Values given as mean ± SD.  
NE: not examined.

of plasma CCL11 was significantly elevated in allergic asthmatic patients compared with control subjects [67.45 (51.39–87.28) versus 50.92 (43.19–65.95) pg/mL,  $P < 0.01$ ], no significant correlation was found between plasma CCL11 and sCD26 ( $r = 0.096$ ,  $P > 0.05$ ).

In addition, we observed an increase in plasma sCD26 concentration along with the progression of disease severity. Asthmatic patients with GINA severity score of 1, in which 6% of these patients were treated with inhaled corticosteroids, showed a median sCD26 concentration of 423 pg/mL; while those patients with higher GINA severity scores of 2, 3, or 4, in which 33, 73, and 92% of these patients were treated with inhaled corticosteroids, showed median sCD26 concentrations of 576, 544, and 540 pg/mL, respectively, which were higher than that of patients with GINA severity score of 1.

#### *Expression of CD26 on Cell Surface of Lymphocytes, Monocytes, CD4+, CD19+, CD8+, and iNKT Lymphocytes*

As shown in Fig. 2, there were no significant differences in cell surface expression of CD26 on monocytes and CD8+ and CD19+ lymphocytes between allergic asthmatic patients and control subjects ( $P > 0.05$ ). However, there was a significant up-regulation in cell surface expression of CD26 on total lymphocytes of asthmatic patients than that of control subjects [7.71 (6.51–8.79) versus 5.12 (3.90–6.19) MFI,  $P < 0.01$ ]. Using immunophenotyping of CD4 T lymphocytes by flow cytometry, we observed that the CD4+ lymphocytes of allergic asthmatic patients showed significant up-regulation in cell surface expression of CD26 than that of control subjects [12.97 (11.80–16.45) versus 8.81 (6.81–9.81) MFI,  $P < 0.01$ ]. Results, therefore, indicate that CD26 expression is significantly

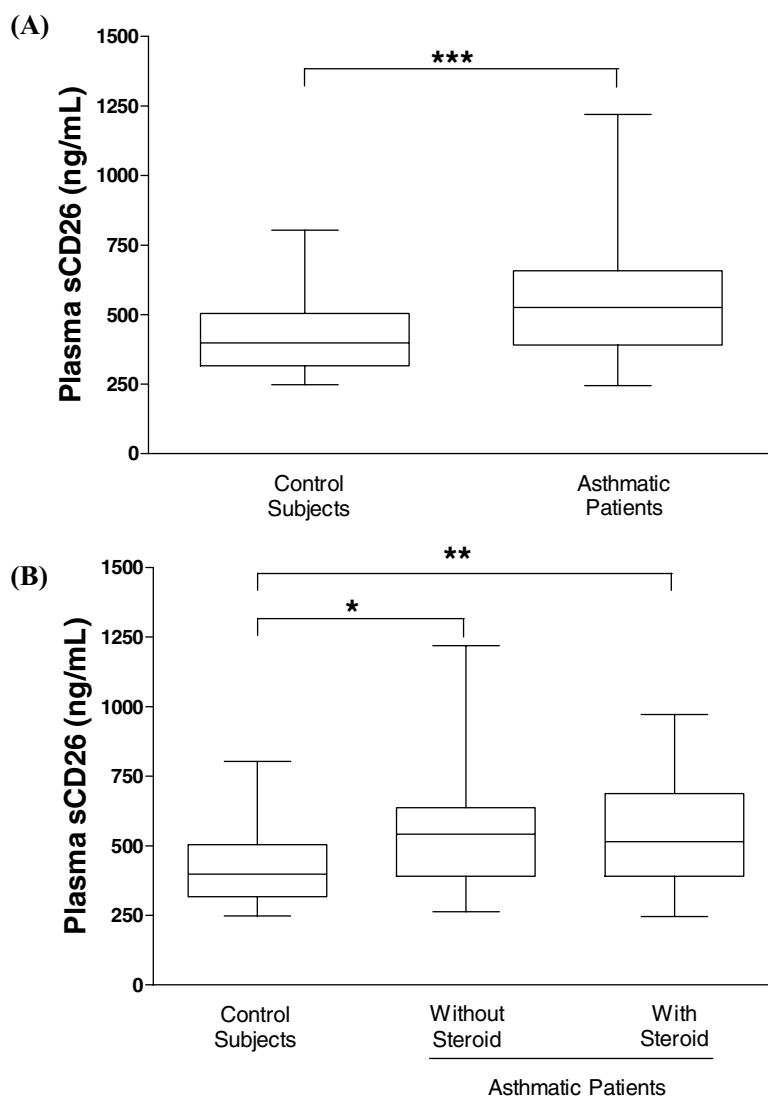
elevated on CD4+ lymphocytes of allergic asthmatic patients. The cell surface expression of CD26 on iNKT lymphocytes was also significantly higher in asthmatic patients than that of control subjects [26.49 (2.18–37.11) versus 2.03 (1.13–3.87) MFI,  $P < 0.05$ ].

The cell surface expression of CD26 was analyzed by flow cytometry as MFI on 10,000 viable cells, which included both the significant increase of CD26 expression on individual cell and the percentage of cells expressing the CD26 (both  $P < 0.05$ ).

## DISCUSSION

This study demonstrated an up-regulation of plasma concentration and cell surface expression of CD26 in patients with allergic asthma. In addition, plasma sCD26 showed positive correlation with the percentage of circulating eosinophils, plasma sCTLA-4, CCL5, and CCL22.

The bioavailability of cytokines for mediating inflammation depends on their production, stability, and degradation. Therefore, the presence of peptidases plays a role in determining the availability of cytokines at the site of inflammation. This study is the first to show sCD26 in plasma is significantly elevated in patients with allergic asthma regardless of ICS treatment. Both the membrane-bound and soluble forms of CD26 can rapidly truncate Th1-related chemokine CXCL10; the resulting CXCL10 isoform contains no inflammatory activity (4). Truncation of chemokines may not affect receptor binding, but influencing their ability to induce signaling, can result in antagonist function (29). It is well recorded that leukocyte infiltration in the pathogenesis of allergic asthma is regulated by chemoattractants of eosinophils and Th2 lymphocytes



**Fig. 1.** Concentration of sCD26 in plasma. *Box and whisker plots* showing the comparison of sCD26 between control subjects and (A) all asthmatic patients, with differences determined by Mann–Whitney *U* Test, and (B) asthmatic patients receiving ICS and those not receiving therapy, with differences determined by Kruskal–Wallis test followed by Dunn’s test. Differences were considered significant when  $P < 0.05$ . \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

such as eotaxin and CCL5 (30). It is also widely known that chemoattractants of Th1 and Th2 lymphocytes such as Th1-related CXCL9, CXCL10, and Th2-related CCL17 (thymus and activation regulated chemokine (TARC)), CCL22 counteract each other (20). Thus, the elevation of sCD26 suggests an enhanced truncation of the Th1 chemoattractants, which contributes to a diminished antagonizing effect on Th2 chemoattractants, and hence, the pathological process of Th2-related leukocyte infiltration. This suggestion is in concordance with the hypothesis that CD26 decreases Th1 chemokine receptor CXCR3 bind-

ing by processing the IFN- $\gamma$ -induced chemokines such as CXCL9 and CXCL10, thus, abolishing the chemotaxis of Th1 cells (4). This is further supported by our previous findings that these IFN- $\gamma$ -induced chemokines are diminished in expression in patients with allergic asthma (20). We found no significant correlation between plasma concentrations of sCD26 and Th1-related chemokine CXCL10 ( $r = 0.045$ ,  $P = 0.699$ ) and CXCL9 ( $r = 0.163$ ,  $P = 0.16$ ) (data not shown). Moreover, there was also no significant correlation between plasma concentrations of sCD26 and neutrophil-related chemokines

**Table II.** Correlations of sCD26 with the Percentage of Circulating Eosinophils in White Blood Cells, Other Plasma Costimulatory Molecules and Th2-Related Chemokines in Allergic Asthmatic Patients<sup>a</sup>

	sCD26
Percentage of eosinophils	$r = 0.434$ $P = 0.008^{**}$
sCTLA-4	$r = 0.474$ $P = 0.009^{**}$
sCD28	$r = 0.264$ $P = 0.166$
CCL5	$r = 0.246$ $P = 0.033^*$
CCL11	$r = 0.096$ $P = 0.566$
CCL22	$r = 0.556$ $P = 0.025^*$

<sup>a</sup>Values calculated by Spearman correlation. Differences were considered significant when  $P < 0.05$ .

\* $P < 0.05$ .

\*\* $P < 0.01$ .

CXCL8 ( $r = 0.138$ ,  $P = 0.240$ ) and CXCL5 ( $r = 0.204$ ,  $P = 0.317$ ) (data not shown). In addition, the association between plasma sCD26 and the Th2 chemoattractants CCL5 and CCL22 in this study further suggest a role of sCD26 in the Th2-mediated pathogenesis of asthma. Together with our previous results of the positive correlation of CCL5 and CCL22 with disease severity (20, 21), we, therefore, suggested that these two Th2 chemokines may serve as potential surrogate disease marker of allergic asthma. Together with the elevated cell surface CD26 expression on CD4+ T lymphocytes, it is likely that CD26 exert a role in regulating these chemokines in asthma.

Soluble CD26 may originate from various sources such as endothelial, epithelial cells from liver or kidney in addition to circulating leukocytes (5). Although, in this study, the origin of sCD26 in plasma is not completely elucidated, a previous study has shown that, by measuring the electrophoretic mobility of the enzymatic activity of CD26, the serum sCD26 coincides with that of lymphocyte CD26 in healthy subjects (5). However, it is still uncertain whether the activity of this enzyme will be changed in pathological conditions.

CD86, produced from antigen-presenting cells (APC), is one of the ligands for CTLA-4 and CD28, which has been shown to be involved in the exacerbation of allergic asthma (28). In this study, the expression of CD26 on cell surface was found to be specifically elevated on CD4+ T lymphocytes. Previous findings have shown that the expression of cell surface CD26 was increased after T-cell stimulation (31). The elevated expression of cell surface CD26 in our present study suggests an enhanced

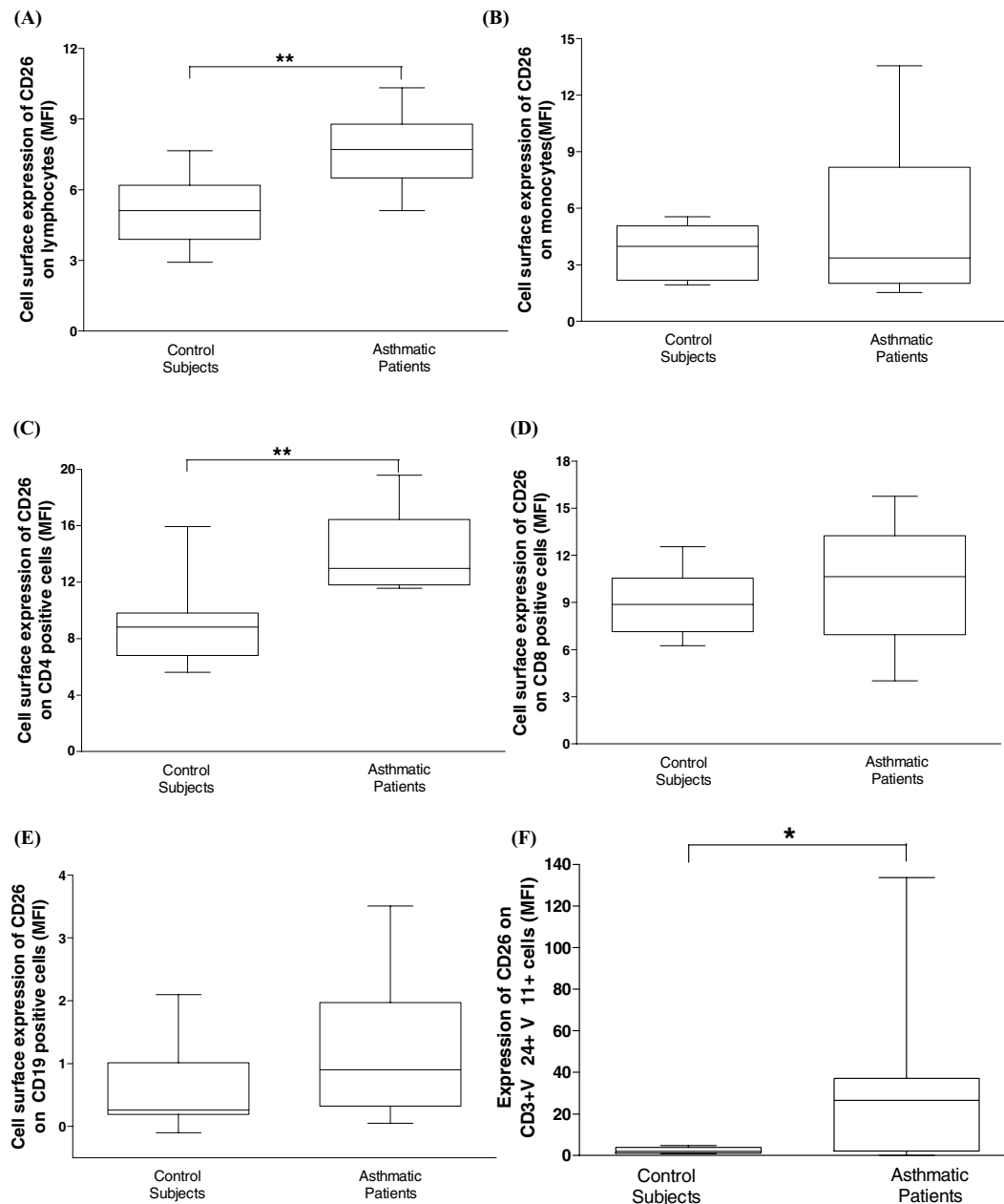
activation of CD4+ lymphocytes in patients with allergic asthma. In addition, previous studies have demonstrated an up-regulation of CD86 expression on APC by CD26 activation (32). This provides greater APC-T lymphocyte interaction, which can enhance T lymphocyte proliferation. The elevated expression of CD26 is in concordance with our previous study demonstrating an enhanced expression of CD86 in patients with allergic asthma (28). Other studies have suggested a role of CTLA-4 in Th2 activation in asthmatic patients (28, 33). Interestingly, sCD26 showed positive correlation with sCTLA-4 in this study, which suggests a role of CD26 in the Th2-mediated pathogenesis of asthma.

iNKT cells, recently identified to play an essential role in the development of allergen-induced airway hyperactivity in mouse models of allergic asthma, are found to be enriched in the lungs of asthmatic patients (17). The elevated expression of cell surface CD26 on iNKT cells suggested an enhanced activation of these cells, probably leading to an induction of Th2 cytokines, in patients with allergic asthma.

Moreover, it has been shown that CD26-deficient rats showed lower T-cell counts and serum-specific IgE levels (34), which is in line with our previous findings that patients with allergic asthma exhibited significantly higher serum IgE levels (20). In our present study, the significant positive correlation between sCD26 and the percentage of circulating eosinophils indicates that sCD26 maybe related to the exacerbation of allergic inflammation. This suggests that therapeutic drugs that target on sCD26 may alleviate the allergic inflammation. The aberrant expression of CD26 has been shown to be clinically relevant for various diseases such as the differentiated thyroid carcinoma, chronic hepatitis C virus infection, rheumatoid arthritis, diabetes, depression, and atopic dermatitis (5).

CD26 in BAL has been studied in rat asthma model, suggesting the role of CD26 in the pathogenesis of asthma via Th2-cell-dependent processes (34). Elevation of the expression of two CD26-related peptidases, amino-peptidase N and dipeptidyl peptidase IV has also been shown in human bronchus using immunohistochemical enzyme histochemical staining (3). Together with our present results using peripheral blood collected by noninvasive method, we could conclude that CD26 can contribute to the inflammatory process and Th2 predominance in the immunopathogenesis of allergic inflammation.

In conclusion, we have demonstrated that the expression of CD26 is elevated in allergic asthmatic patients and is associated with allergic inflammation. However, the exact pathophysiological function of CD26 requires further investigation.



**Fig. 2.** Box and whisker plots showing the cell surface expression of CD26 on lymphocytes, monocytes, CD4+ , CD8+ , and CD19+ cells. Comparisons were made between the control subjects and asthmatic patients of CD26 on (A) total lymphocytes, (B) total monocytes, (C) CD4+ T helper, (D) CD8+ T suppressor plus cytotoxic, (E) CD19+ B, and (F) iNKT lymphocyte populations, with differences determined by Mann–Whitney *U* Test. Differences were considered significant when  $P < 0.05$ . \*\* $P < 0.01$ .

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