Expansion of a CD26^{low} effector TH subset and reduction of circulating levels of sCD26 in stable allergic asthma in adults

Running title: CD26 levels in allergic asthma in adults

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ABSTRACT

Background: Asthma pathogenesis is dependent on the balance between regulatory and effector T cells, which display differential expression of CD25 and CD26. Therefore, alteration of circulating levels of sCD25 and sCD26 during allergic asthma could be conditioned by leukocyte phenotype changes.

Objectives: To analyse CD25 and CD26 expression on T lymphocytes and their soluble derivatives (sCD25, sCD26) during stable phases of moderate-severe allergic asthma.

Methods: Cross-sectional study with two adult cohorts of allergic asthmatics. Clinical, anthropometric, lung function, haematological and biochemical parameters were measured. Flow cytometry phenotyping was done in both circulating and cultured leukocytes. Dipeptidyl peptidase 4 (DPP4) activity was assayed in culture supernatants.

Results: *In vitro* studies showed an up-regulation of CD26 on human T lymphocytes upon activation, especially by TH17-favouring conditions, and a correlation with soluble DPP4 activity (r_s=0.641; $P < 0.001$). CD26 expression on lymphocytes was higher in asthmatics, while serum sCD26 was lower in women and patients. The last finding could be related with an expanded $CD25^{low}/CD26^{low}/CD127^{low}$ subset of effector $CD4^+$ T cells in allergic asthma, without changes in Treg percentages. However, women showed an increased Teff/Treg ratio, which could explain their higher susceptibility to asthma.

Conclusions: Allergic asthma causes an increment in $CD25^{low}CD26^{low}$ TH cells detected in stable stages. These changes are mirrored in serum and should be considered in the light of the down-modulating role of CD26 on important chemokines related to asthma pathogenesis such as CCL11 (eotaxin), CCL5 (RANTES) or CXCL12a (SDF-1α).

Key words: Asthma biomarkers; CD25; CD26; DPP4; Lymphocytes; T helper cells.

RESUMEN

Introducción: La patogénesis del asma depende del equilibrio entre células T reguladoras y T efectoras, las cuales presentan distintos niveles de CD25 y CD26. Por tanto, la alteración de la concentración de sCD25 y sCD26 durante el asma alérgica podría estar condicionada por cambios en el fenotipo de los leucocitos.

Objetivos: Analizar la expresión de CD25 y CD26 en linfocitos T y sus derivados solubles (sCD25 y sCD26) durante asma alérgica moderado severa y en fases estables.

Métodos: Estudio transversal con dos cohortes de adultos con asma alérgica. Se han medido parámetros clínicos, antropométricos, de función pulmonar, hematológicos y bioquímicos. Se ha hecho el fenotipado de leucocitos circulantes y en cultivo mediante citometría de flujo. Se ha analizado la actividad Dipeptidil peptidasa 4 (DPP4) en sobrenadantes de cultivo.

Resultados: Los estudios *in vitro* mostraron un aumento de expresión de CD26 en linfocitos T humanos tras activación, especialmente en condiciones favorables para TH17, y una correlación con la actividad DPP4 soluble (r_s=0.641; $P < 0.001$). La expresión de CD26 en linfocitos fue mayor en asmáticos, mientras que sCD26 estaba reducido en sueros de mujeres y pacientes. Este último hallazgo podría ser relacionado con la expansión de una subpoblación $CD25^{low}/CD26^{low}/CD127^{low}$ de células T $CD4^{+}$ efectoras en asma alérgica, sin cambios en los porcentajes de Treg. Sin embargo, las mujeres mostraron un incremento del cociente Tef/Treg, lo cual podría explicar su mayor susceptibilidad al asma.

Conclusiones: El asma alérgica causa un incremento de células TH $CD25^{low}CD26^{low}$ durante fases no activas. Estos cambios se reflejan en suero y deberían tenerse en cuenta a la luz de la función inhibidora de CD26 sobre quimioquinas importantes relacionadas con la patogénesis del asma, como CCL11 (eotaxina), CCL5 (RANTES) o CXCL12a (SDF-1α).

Key words: Biomarcadores de asma; CD25; CD26; DPP4; Linfocitos; Células T cooperadoras.

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INTRODUCTION

Asthma is influenced by genetic (e.g., dipeptidyl peptidase 10 (*DPP10*), ADAM metallopeptidase domain 33 (*ADAM33*)) and environmental factors [1], and its management should be based on endotypes [1, 2]. Allergens trigger lung epithelial cells to release cytokines during asthma attacks, which activates innate leukocytes and drives the TH_2 lymphocytes differentiation [1]. These cells release IL-4, IL-5, and IL-13, stimulate IgE production, and favour the activation of eosinophils, mast cells and basophils [1]. This effector role is counteracted by regulatory T cells (Tregs) [3], whose number and/or function may be altered in asthma [4]. Both $CD4^+$ T subsets express differential levels of interleukin receptor 2 (IL-2R). Thus, CD25 (IL-2R-α) is mainly expressed by Tregs [4], while a $CD25^{-/low}$ phenotype is present in effector $CD4^+$ T lymphocytes (Teff). T cell receptor (TCR)-triggered Teff cells release soluble CD25 (sCD25), an activation marker [5] elevated in serum/plasma during asthma exacerbations [6] that positively correlates towards the disease severity of allergic asthma [7]. In addition, Tregs are suggested to be another source of sCD25 [4, 8, 9]. Furthermore, sCD25 is also increased in bronchoalveolar lavage fluid (BALF) from asthma patients [8, 9].

Another interesting protein in asthma pathogenesis is CD26/DPP4, a surface glycoprotein enriched in $CD4^+$ T cells [10]. As dipeptidyl peptidase 4 (DPP4, EC: 3.4.14.5), the enzyme belongs to the serine peptidase subfamily S9B, which includes an asthma susceptibility locus (*DPP10* [11]) as well as dipeptidyl peptidase 8 (*DPP8*), dipeptidyl peptidase 9 (*DPP9*) and fibroblast activation protein alpha (*FAP*) [12-16]. Additionally, dipeptidyl peptidase 2 (*DPP2*, *DPP7*) from serine peptidase subfamily S28 also displays DPP4-like activity at acidic pH. As CD26, the glycoprotein interacts with adenosine deaminase, CD45, caveolin-1 or C-X-C chemokine receptor type 4 (CXCR4), thereby fulfilling either inhibitory or enhancing roles upon association [17]. CD26 is an activation marker known to be upregulated on lymphocytes (especially $CD4^+$) in adult allergic asthma [18]. $CD4^+$ T cells are major actors in the asthma pathogenesis, but contrary to CD25, Treg cells display lower CD26 levels than Teff lymphocytes. Indeed, CD26 is reported as a negative marker of Treg cells and a marker of the remaining TH subsets. Thus, expression of CD26 on TH cells follows the order of TH_{17}>>TH_1>TH_2>Treg [19, 20]. Hence, elevated presence of CD26 on $CD4^+$ T cells in adult allergic asthma suggests an activated status [18, 21] and may point to a specific T cell phenotype. Moreover, a soluble form of CD26 (sCD26/sDPP4) is released from T cells into blood circulation either shedded by metalloproteases [22] or secreted by $CD26^+$ vesicles (http://www.exocarta.org). In plasma/serum sCD26 accounts for >90% of total sDPP4-like activity [23, 24], and the remaining 10% is derived from intracellular peptidases DPP2, DPP8, or DPP9. These DPP4 homologues are also involved in asthma pathogenesis [13] and show a differential expression in leukocytes [14, 15] and eosinophils [25]. Moreover, both DPP8 and

DPP9 enzymes are upregulated in activated macrophages and trimmed for antigen presentation [14, 15]. Besides, DPP2 is necessary for maintaining the quiescence of lymphocytes cells and it is downmodulated upon activation [15].

CD26/DPP4 cleaves X-Pro or X-Ala amino terminal dipeptides from chemokines (e.g., CXCL12a (stromal cell-derived factor-1α, SDF-1α), CCL11 (eotaxin) and CCL5 (regulated on activation, normal T cell expressed and secreted, RANTES), thereby modulating their biological activity and immunological function as recently reviewed [17, 26, 27]. Other substrates include neuropeptides or peptide hormones like incretins [28, 29] whose half-life is prolonged by DPP4-inhibitors currently used as antidiabetic drugs [30]. Vascular substrates of DPP4 may either be cleaved by sDPP4, DPP4 expressed on leucocytes or endothelial DPP4 [31]. Increased DPP4-like activity has been observed in BALF from asthmatic rat lungs, contributed mostly by sDPP4 and to some extent by DPP8, DPP9 or DPP2 [13, 32].

The most important source of sCD26 is $CD4^+$ T lymphocytes, as this subpopulation displays the highest percentage of $CD26^+$ cells [10, 23, 33]. Therefore, this molecule could be used as a “fingerprint" to test either the activation status or differentiation status of $CD4^+$ T cells in asthma. However, only a few studies have been undertaken. Thus, augmented levels of sCD26 were reported in allergic asthma and positively correlated with eosinophils and IgE [18]. In contrast, sCD26 was inversely associated with inflammation in chronic eosinophilic pneumonia, a disease linked to asthma [34], while no changes were observed for sCD26 in children with asthma or atopy [35].

Up to now, there has been few studies monitoring sCD26 in asthma, and none considering the potential roles of CD26 in asthma pathogenesis and bearing in mind that alterations of circulating biomarkers (sCD25 and sCD26) may reflect changes in leukocyte phenotype such as $CD25^{-/low}CD26^+$ Teff and $CD25^{+/high}CD26^{low}$ Treg cells. Therefore, in the present study a comprehensive analysis was carried out aiming to assess the immune biomarkers described above in patients with moderate-severe allergic asthma.

MATERIALS AND METHODS

Subjects

The study was conducted between 2009 and 2012 and included patients from hospital consultations for Pneumology and Allergy in Galicia (Spain). Patients had confirmed diagnosis of asthma and allergy for at least one year according to Global Strategy for Asthma Management and Prevention (GINA 2006,

http://www.seicap.es/documentos/archivos/GINA2006general.pdf) criteria. The research project was approved by the Ethics Committee of Clinical Research of Galicia (2011/001), Spain, and informed consent was obtained from all individual participants included in the study. A validation cohort was also recruited from 2014 to 2016 (Neumology Service, University Hospital of Santiago de Compostela, Spain). All patients were in stable phase for at least 4 weeks before sample collection. Healthy Controls (HC) were selected from patients scheduled in the hospital for minor surgeries such as inguinal hernia or orthopedic surgery; they were non-smokers and systemic diseases or allergies were absent. None of the patients or healthy controls were on DPP4 inhibitors treatment.

Sensitization in allergic asthmatic patients (AAP) was confirmed through a skin prick test or serum IgE specific to frequent allergens. Other variables were also accounted: body mass index (BMI), rural-urban residence, profession, smoking or comorbidities. Biochemical determinations were performed using an ADVIA®1650 analyzer (SIEMENS Healthcare Diagnostics S.L., Berlin, Germany). The nucleated cells number was measured using an ADVIA®2120 hematology counter (SIEMENS Healthcare Diagnostics S.L., Berlin, Germany).

Magnetic purification of $CD4^+$ T cell subsets and *in vitro* culture

Buffy coats from healthy subjects were donated by "Axencia Galega de Sangue, Órganos e Tecidos" (Santiago de Compostela, Spain) and used to isolate peripheral blood mononuclear cells (PBMCs) by Ficoll® density gradients. Teff and Treg cells were prepared from PBMCs by means of the Dynabeads® Regulatory $CD4^+CD25^+$ T cell Kit (Life-Technologies, Spain). Viability was always >90% (trypan blue exclusion).

Teff ($CD4^+CD25^-$) and Treg ($CD4^+CD25^+$) cells were cultured *in vitro* for 4 days in 96-microwell U-bottom plates with ImmunoCult™-XF T Cell Expansion Medium (StemCell, Grenoble, France) supplemented (or not) with soluble tetrameric antibody complexes (ImmunoCult™ Human CD3/CD28 T Cell Activator; StemCell). To promote a partial Teff differentiation, the following cytokines (PeproTech, London, UK) were added: 10 ng/mL IL-12 (TH_1-like), 10 ng/mL IL-4 (TH_2-like), or IL-1β (100 ng/mL), IL-6 (30 ng/ml) and IL-23 (100 ng/mL) (TH_{17}-like). In addition, 800 ng/mL of IL-2 was used for Treg cells maintenance.

Flow cytometry assays

Venous peripheral blood was collected (BD Vacutainer K2E) ir order to examine the proportion of Teff and Treg cells. Leukocytes from 100 µL of whole blood were stained (30 min, room temp.) with mouse IgG1 κ isotype antibodies (BD Biosciences) labelled with FITC, PE-Cy7, AlexaFluor-647 and PE as negative controls. Alternatively, cells were stained with mouse IgG1 κ specific antibodies (BD Biosciences) against CD4 (FITC), CD25 (PE-Cy7) or CD127 (Alexa

Fluor-647) and a mouse IgG2b antibody specific for CD26 (PE; Immunostep); then, red cells were lysed (BD FACS™ Lysing Solution). Purity of Teff/Treg lymphocytes prior (or after) *in vitro* cell culture was also assayed with the same specific (CD4-FITC, CD25-PE-Cy7, CD26-PE) or isotype antibodies (Isotype-FITC, Isotype-PE-Cy7, Isotype-PE). Finally, samples were analysed (BD FACSCalibur and FACSort) and a number of 10,000-200,000 events collected. Data were examined using WinMDI 2.9 software (Joseph Trotter, La Jolla, CA. USA).

DPP4 activity measurement

Total DPP4 enzymatic activity was colourimetrically assayed by means of a flat-bottom 96-well microplate-adapted and end-point protocol. Cell culture supernatants (50 μL) were diluted with 50 μL reaction buffer (0.05 M tris(hydroxymethyl)aminomethane (TRIS)-HCl pH 8.0 buffer) and 100 μL of 2 mM glycyl-prolyl-paranitroanilide (Gly-Pro-pNA). Plates were incubated at 37ºC and absorbance sequentially (30-120 min) recorded at 405 nm (Labsystems Multiscan MS microtiter plate reader). The concentration of pNA was calculated from a standard curve, ranging from 0 – 2000 μM. One international unit (IU) was defined as the amount of enzyme that processes 1 μmol Gly-Pro-pNA (or releases 1 μmol pNA from this substrate) per minute. Assays were performed in duplicate for each sample.

Determination of sCD25 and sCD26

Serum sCD25 levels were measured through enzyme-linked immunosorbant assay (ELISA) from R&D Systems, MN, USA (Quantikine® Human IL-2Rα Immunoassay), and sCD26 was quantified with ELISAs plates from eBioscience®, Vienna, Austria (Human sCD26 Platinum ELISA). Optical densities were recorded at 450 nm and protein concentration calculated from standard curves.

Statistics

Descriptive data are presented as either median (interquartile range; IQR1-3) or percentages. To assess the changes between AAP and HC in non-normally distributed variables we used Mann–Whitney U two-tailed test, or the Kruskal–Wallis test followed by Dunn's multiple comparison test for more than 2 groups. Spearman´s test was used to measure association between variables. All analyses were conducted using GraphPad Prism 6.0 (GraphPad Software, Inc., San Jose, California, USA). The statistical significance was defined as $P < 0.05$.

RESULTS

Characteristics of the first cohort of allergic asthmatic patients

The characteristics of this first cohort of patients are summarised in table 1. The median (IQR1-3) of forced expiratory volume in the first second (FEV1) (%) was 93 (74.5-102.2), while the FEV1/forced vital capacity (FVC) ratio (%) was 74.2 (65.2-81.9) in AAP. Asthma was mainly moderate-persistent (71.6%) and patients displayed an allergic disease, with skin prick test positive reactions against common allergens. In addition, most patients had their residence in rural areas (72%), but only a small percentage were farmers (Table 1). As expected, a significant group of AAP had peripheral blood eosinophilia, with 45% with > 350 eosinophils/μL, and elevated total IgE (Table 1). There was a positive correlation between eosinophils and IgE, but not between FEV1% and serum IgE in AAP (Table 2). Patients were under different treatments, mostly non-smokers, and displayed a well-controlled asthma (Table 1).

Given the well-known leptin-BMI association, and the assumed correlation between the development/worsening of asthma and BMI, we also studied these parameters. Firstly, BMI in AAP showed a positive correlation with leptin and C-reactive protein (CRP), but a negative association with IgE and both FEV1% and FEV1/FVC (Table 2). Secondly, leptin levels were generally 4- to 6-fold higher in women, though no differences were detected between HC and AAP (Figure S1, Table 1). The latter findings underline the disassociation between overall adiposity and asthma. In contrast, some parameters were more elevated in men, including IgE, basophil counts, and sCD26 (data not shown). The influence of age was also taken into consideration, indicating a positive correlation with BMI and TNF, but a negative interdependence with IgE, FEV1% and FEV1/FVC; no association with age was detected for sCD26 or sCD25 (Table 2).

sDPP4 activity is highly correlated with CD26 expression on $CD4^+$ T lymphocytes *in vitro*

CD26 was mainly expressed by lymphocytes, especially $CD4^+$ T cells. Furthermore, CD26 expression was higher in Teff ($CD4^+CD25^{low}CD127^{high}$) than Treg cells ($CD4^+CD25^{high}CD127^{low}$) (Figure S2). To investigate whether phenotypic differences in $CD4^+$ T cells regarding CD26 expression could be translated into altered sCD26 levels, we performed *in vitro* experiments with Treg cells ($CD4^+CD25^{high}$) or Teff lymphocytes ($CD4^+CD25^-$) cultured in TH_1-, TH_2-, or TH_{17}-skewing conditions. Results from sDPP4 activity determination in cell culture supernatants (highly correlated with sCD26 levels according to the literature) and flow cytometric assays against CD26 showed a high positive correlation between sDPP4 and CD26 (MFI) (Figure 1a), and a direct association between CD26 levels and absolute lymphocyte

counts (Figure 1b). Likewise, there was a positive relationship between sDPP4 and the number of lymphocytes (Figure 1c). To maximize the influence of $CD4^+$ T cell phenotype, we weighted sDPP4 by the lymphocyte count, and we continued to observe a strong correlation between CD26 expression (MFI) and sDPP4 (Figure 1d), supporting the influence of the TH phenotype on the last variable. Moreover, comparable results were obtained for both cell surface CD26 expression (MFI) (Figure 1e) and sDPP4 (Figure 1f) with data segregated according to *in vitro* culture conditions. Indeed, two opposite poles were observed: resting Treg and TH cells cultured under TH_{17}-favouring conditions. Moreover, TH_1-like and TH_2-like cells expressed intermediate levels of CD26 on lymphocytes (Figure 1e) and this was mirrored in sDPP4 levels (Figure 1f). Therefore, at least *in vitro*, there was a positive correlation between the density of CD26 molecules on $CD4^+$ T cells and sDPP4 levels (i.e., sCD26). Furthermore, we found a differential behaviour in Teff and Treg cells, with a high positive correlation between CD26 intensity and CD26 percentage in activated Teff cells and an opposite correlation for Tregs (Figures 1g, h).

Reduction of serum sCD26 and sCD25 and increment of $CD4^+CD127^{low}CD25^{low}CD26^{low}$ T cells in allergic asthma patients

Despite corticosteroids, circulating lymphocytes were increased in AAP (Table 1). Considering that a) *in vitro* experiments showed a positive correlation between CD26 on $CD4^+$ T cells and sDPP4; b) Treg and Teff cells have a reverse phenotype ($CD25^{high}CD26^{low}$ and $CD25^{low}CD26^{high}$, respectively); and c) asthmatic donors were expected to have an effector/regulatory disequilibrium, we evaluated both sCD26 and sCD25. Our results showed a slight positive correlation of sCD26 with basophils, and a negative correlation with neutrophils (Table 2). However, sCD26 levels were not positively associated with BMI and there was a slight negative relationship with leptin (Table 2). As well, sCD25 was directly associated with CRP and neutrophils (%), but negatively with eosinophils (Table 2). Importantly, instead of the previously reported sCD26 upregulation [18], it was found a slight but no significant decrease of the absolute concentration of sCD26 in AAP (Figure 2a), even upon data segregation by gender (data not shown). Moreover, we found a small association of sCD26 with the percentage of lymphocytes (Table 2), but not with the absolute number (as showed in our experiments *in vitro*). Despite this last finding, sCD26 was still divided by the lymphocyte count to maximize the effect of T-cell phenotype, revealing significantly lower concentrations in AAP (Figure 2b). A significant down-modulation in AAP was observed for the absolute (Figure 2c) and normalised (Figure 2d) concentrations of sCD25. Anyhow, there was no correlation between sCD26 and sCD25 levels (Table 2).

According to our *in vitro* assays, the slight reductions of sCD26 (and sCD25) in AAP were suggesting the expansion of a small $CD26^{low}$ subset of $CD4^+$ T cells. To test this

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hypothesis, another independent set of samples with similar characteristics (validation cohort) was obtained from intermittent-mild (n=53) and moderate-severe (n=50) allergic asthma, and HC (n=32). Firstly, Teff ($CD26^{high}$) and Treg ($CD26^{low}$) cells within circulating $CD4^+$ T lymphocytes were detected based on CD25 and CD127 (Figure 3a-c), but an additional $CD25^{low}CD127^{low}$ subset within Teff cells was also found in intermittent-mild (Figure 3b) and moderate-severe (Figure 3c) asthma. Besides, this small subset displayed the lowest levels of CD26 ($CD26^{-/low}$) amongst $CD4^+$ T cells (Figure 3d). We detected in intermittent-mild and moderate-severe AAP a slight (but not significant) reduction in the percentage of Teff lymphocytes ($CD25^{low}CD127^{high}CD26^{high}$) (Figure 3e), which is accompanied by a significant increased proportion of "triple low" $CD4^+$ T cells ($CD25^{low}CD127^{low}CD26^{low}$) (Figure 3f). In contrast, the percentage of Treg lymphocytes ($CD25^{high}CD127^{low}CD26^{low}$) remained unchanged (Figure 3g). In line with the augmented proportion of "triple low" $CD4^+$ T cells, our data revealed significantly reduced absolute levels of sCD26 in serum samples from AAP (especially in moderate-severe asthma) compared to HC (Figure 3h), which was in line with results in the first cohort. Moreover, in this second cohort it was unnecessary to weight sCD26 levels by the lymphocyte count to maximize the effect of $CD4^+$ T cell phenotype. Downmodulation of sCD26 in AAP was mainly associated to men, due to their higher levels in the reference population (HC) compared to women. In contrast, CD26 expression in total lymphocytes was higher in women than men (data not shown), and also in AAP than in HC (Figure 3i). Besides, despite we did not perceived alterations in both Teff ($CD26^{high}$) and Treg ($CD26^{low}$) percentages between HC and AAP that could explain the reduction in sCD26 levels, women had increased and reduced proportions, respectively, of Teff and Treg subsets compared to men (Figure 4).

DISCUSSION

In the current study, *in vitro* experiments have given evidence to a direct correlation of lymphocyte surface CD26 with sDPP4 activity and a differential expression/secretion of CD26 related to the TH phenotype. Although we expected an elevation of sCD26 in patients (in line with the enhanced expression of CD26 in lymphocytes), we found lower levels of sCD26 than in healthy donors, which can be partially explained through the expansion of a $CD25^{low}CD127^{low}$ TH subset with the lowest levels of CD26 amongst $CD4^+$ T lymphocytes ("triple low"). This subpopulation could have an important pathogenic role in asthma (Figure 5).

CD26 is a multifunctional molecule with both stimulatory and inhibitory roles [17] and remarkable expression differences between Treg and Teff cells [19, 20], like CD25 or CD127 [36]. IL-12 leads to TH_1 polarization [37] and potentiates the TCR-mediated up-regulation of

CD26 on $CD4^+$ T cells [38]. On the other hand, in 2012 Bengsch *et al.* used flow cytometry and cell sorting assays with human peripheral blood and tissue samples to show that human TH_{17} cells express the highest levels of CD26, followed by TH_1, TH_2 and Treg lymphocytes [20]. In our *in vitro* assays, we show a similar pattern where Teff cells submitted to TH_{17}-polarizing conditions (IL-1β, IL-6, IL-23) also acquire the greatest levels of CD26 and sDPP4 activity (TH_{17}>TH_1≥TH_2>Treg). Although 90% of soluble DPP4 activity is derived from sCD26 [23, 24], we must also consider the likely contribution to the total DPP activity in cell culture supernatants of other DPP enzymes expressed by lymphocytes, such as DPP8, DPP9 or DPP2. Nevertheless, these enzymes are intracellular and only minute amounts of them reach the extracellular compartment because of cell turnover. Besides, the most likely candidate, DPP2, is a structurally unrelated aminopeptidase expressed in quiescent lymphocytes, downregulated upon activation, and active at both acidic and neutral pH, but not at the alkaline pH used in our DPP4 enzyme activity assays. Therefore, sDPP4 activity is a bona fide indicator of the actual sCD26 levels in cell culture supernatants.

Stimulation by IL-4 results only in moderate upregulation of CD26 followed by $CD4^+$ T cell activation. Thus, TH_2-differentiation cells in allergic asthma should lead to cells with slightly higher levels of CD26 (although lower than TH_1 or TH_{17} lymphocytes) as well as enhanced concentrations of sCD26 in serum. Accordingly, Lun *et al.* found an up-regulation of CD26 in $CD4^+$ T cells from adult allergic asthma patients [18] that we have confirmed in the second cohort. Moreover, they also described an elevation of sCD26 in allergic asthmatics [18]. However, sCD26 has more frequently been described in literature to have decreased [23] rather than increased concentrations [39] under pathological conditions. Indeed, we found a trend towards sCD26 down-modulation in allergic asthma. A similar sCD26 down-modulation has been reported in chronic eosinophilic pneumonia [34], while Remes *et al.* detected no differences in serum sCD26 levels in children with asthma, and no association with atopy [35]. Therefore, it is difficult to ascertain the reasons for these different results as most of them (including our study) used the same ELISA kit (Bender MedSystems) for the determination of CD26 concentration [18, 35], but at least in the study of Lun *et al.* the results might be partially explained by a higher proportion of males in patients or perhaps a more active disease status.

Contrary to *in vitro* studies, circulating sCD26 values in serum samples are not correlated with the number of lymphocytes, probably due to different processes: a) shedding of sCD26 [22] or release of $CD26^+$ vesicles [http://exocarta.org/gene_summary?gene_id=1803]; b) changes in proportions of $CD26^{high}$ and $CD26^{low}$ subsets; c) release of sCD26 by adipose tissue or hepatocytes [40, 41]. However, the latter possibility is not supported by the present study due to unaltered levels of CRP. Furthermore, lack of variation in overall adiposity and no differences in leptin levels between AAP and HC as noticed in the present study, indicates that

adipose tissue is not a major source of sDPP4/sCD26. It has been also detected a negative correlation of sCD26 (higher in men [42, 43]) with leptin (higher in woman [44]) in patients, and no correlation with BMI. Besides, results from the CD26-specific ELISA reveal that sCD26 is found down-modulated rather than augmented in asthma patients, contrary to what should be expected in obesity-related asthma.

sCD25 is considered a T cell activation marker as it has been found augmented during asthma flare-ups [5-8]. To our surprise, we detected a significant reduction of sCD25 in the first asthmatic cohort, and no changes in the second group. These different results are difficult to interpret, but one explanation is the presence of a "stable" disease in our cohort compared to previous works. In addition, these results show a similar behaviour of sCD26 and sCD25. Indeed, both molecules could share the same cell origin despite both parameters are not correlated. Thus, they are carried by vesicles or released by matrix metalloproteinase-9 (MMP-9)-mediated cleavage [22]. Moreover, our data support that CD26 is mainly expressed by peripheral blood $CD4^+$ T cells and primarily secreted by Teff cells upon TCR-triggering *in vitro*. Similarly, Teff cells up-regulate and release CD25 following activation [45]. Therefore, the slight reduction of sCD26 and sCD25 in asthmatics might be attributed to the expansion of a $CD25^{low}CD26^{low}$ subset of $CD4^+$ T cells.

Treg cells are $CD25^{high}CD26^{low}$ lymphocytes [19] that tend to maintain the expression of CD25 upon activation but turn into $CD25^{low}$ lymphocytes when their suppressive function is abrogated [45]. Indeed, deficient function and reduced frequency of Tregs has been described in allergic asthma [4, 46, 47]. However, we fail to detect a numerical alteration of Tregs, which discards a link between this subset and alteration of sCD26/sCD25 levels in asthma. Furthermore, we notice augmented percentages of Teff cells and reduction of Tregs in women, which can explain their higher susceptibility to asthma.

The nature of "triple low" lymphocytes is unknown, but they are probably $CD25^-$ $CD127^{high}CD26^{high}$ Teff cells that have reduced these three surface markers and whose expansion causes the down-modulation of sCD26 and sCD25 in AAP. Reduction of CD26/sCD26 could be important for TH cells to abrogate the inhibitory role of this molecule and burst cell proliferation, potentiate the activity of TH_2-chemokines (e.g., CCL11/eotaxin), or favour the pro-inflammatory effect of adenosine or substance P [17] (Figure 5). Thus, reduced DPP4 activity in cells or plasma could enhance the bioavailability of TH_2 (e.g., IL-4) and myeloproliferative cytokines (G-CSF, GM-CSF) [17]. Besides, CD26 down-modulation in Teff lymphocytes could be parallel to the loss of caveolin-1 (a CD26 ligand) in bronchial epithelial cells and monocytes from asthmatics [48].

DPP4 activity reversible competitive inhibitors or "gliptins" are a novel class of small molecules used orally. These include the peptidomimetic and less specific vildagliptin (Galvus; EU, 2007) and saxagliptin (Onglyza; US FDA, 2010), and the non-peptidomimetic, long-lasting, and more specific/potent sitagliptin (Januvia; US FDA, 2006), linagliptin (Tradjenta; US FDA and EU, 2011), and alogliptin (Takeda Pharmaceutical Company Limited; US FDA, 2013). These drugs, with an excellent tolerability profile, have been introduced into clinical practice as a second-line therapy in type 2 diabetes mellitus (T2DM) to reduce the inactivation of incretins. However, several mild adverse drug reactions (ADRs) have been reported, such as skin-related disorders, infections (e.g., nasopharyngitis and upper respiratory tract infections), and respiratory disorders (e.g., dyspnea, coughing, wheezing) [VigiBase®, WHO Programme for International Drug Monitoring; 49]. Some studies fail to detect significant differences in the incidence of upper respiratory tract infections or nasopharyngitis [50, 51]. However, the mere likelihood of enhanced risk of viral infections in the upper respiratory tract deserves a careful assessment of the safety profile of gliptins, particularly in patients susceptible to airway inflammation or with an already established (sometimes unnoticed) disease (e.g., allergic asthma with reduced extracellular sCD26 levels, as we have described). Indeed, viral upper respiratory infections trigger or worsen asthma symptoms (https://www.nhlbi.nih.gov/health/health-topics/topics/asthma/signs). For example, respiratory viral infections (e.g., rhinovirus) or viral-derived dsRNA sensed by pattern-recognition receptors lead to asthma exacerbations in murine models of asthma induced by ovalbumin (OVA) [52] or house dust mite (HDM) [53], but also in children [54].

Apart from CD26/DPP4, DPP4 activity and/or structure homologue (DASH) proteins include FAP, DPP8, DPP9, DPP-like protein 1 (DPPL1) and two proteins linked to asthma: the soluble DPP2 (an enzyme important in lymphocyte quiescence) [15], and the catalytically-inactive and bronchi/trachea-associated DPPL2/DPP10 [15]. Some ADRs could be linked to off-target effects on some of these DASH proteins [55], but the presence of similar ADRs with different gliptins and the "moonlighting" nature of DPP4, with a great number of substrates related with the immune system, indicate that these drugs could be enhancing T_{H2}-mediated responses and the asthma-dependent down-modulation of sCD26 levels. Thus, gliptins have been proposed for the treatment of the autoimmune disease diabetes type 1 by, amongst other mechanisms, up-regulating TH_2 cells [56], while the highly specific and potent inhibitor sitagliptin reduces sDPP4 activity without altering CD26 levels on cells and causes a temporary proinflammatory state in T2DM patients through the reduction of Treg cells [57]. In the same sense, gliptins also favour the development of allergic rhinitis [49] and a non-inflammatory variant of bullous pemphigoid, two diseases where eotaxins and eosinophils play important roles [58, 59]. In clear contrast, sitagliptin seems to play a beneficial role in a chronic murine

model of asthma induced by OVA [60]. Therefore, additional research is needed to ascertain the impact of gliptins in allergic asthma.

Taken together, our findings evidence the expansion of a $CD25^{low}CD127^{low}CD26^{low}$ subpopulation of $CD4^{+}$ T cells in allergic asthmatic patients, coincident with a decrease in the levels of sCD26 and sCD25 in these patients. Reduction of sCD26 and CD26 levels on effector TH lymphocytes might be important to improve their migratory or proliferative capabilities. Moreover, this downmodulation of CD26/sCD26 could be emulated upon the administration of certain drugs, which should be considered in the light of the clinical usage of DPP4 inhibitors [17] and humanised anti-CD26 antibodies [61] in patients with a predisposition toward developing certain allergic hypersensitivity reactions (atopy).

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FIGURES

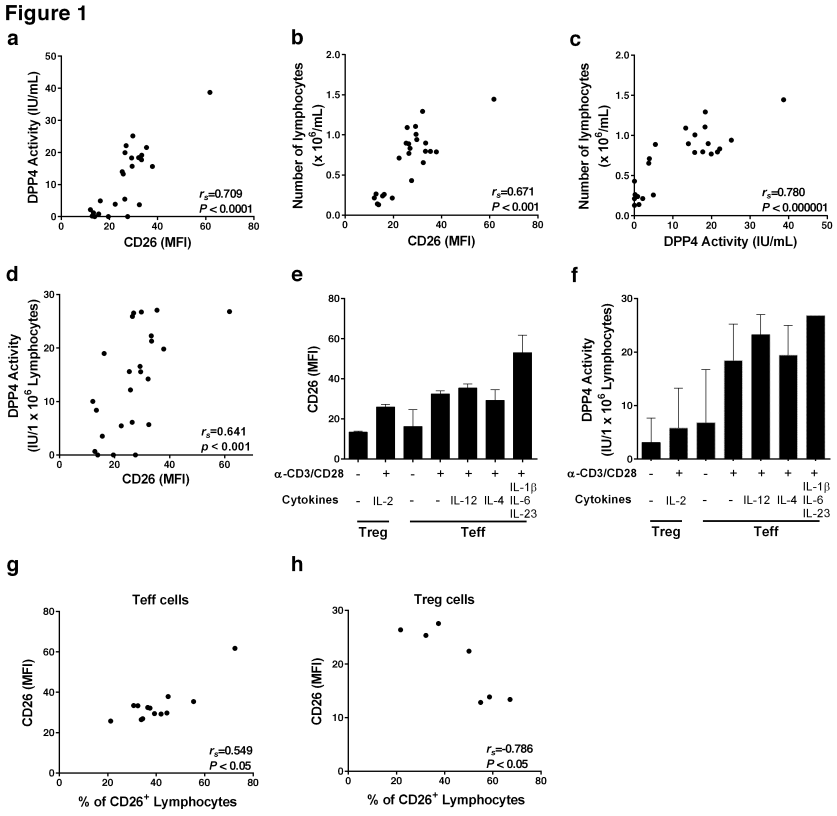

Figure 1. CD26 surface expression and DPP4 activity in culture supernatants of magnetically isolated lymphocytes. Spearman correlation test showing the positive relationship between: (a) CD26 expression (MFI) in resting/activated $CD4^+$ T lymphocytes and soluble DPP4 activity (sDPP4; IU/mL) in cell culture derived supernatants (n=24); (b) CD26 expression (MFI) in resting/activated $CD4^+$ T cells and the lymphocyte count (lymphocytes/mL) at the end of the culture period (n=24); (c) $CD4^+$ T lymphocytes count (lymphocytes/mL) and sDPP4 activity (IU/mL) in culture supernatants (n=24); and (d) CD26 expression (MFI) in the membrane of $CD4^+$ T cells and the relative activity (IU/1 x 10^6 lymphocytes) of sDPP4 in culture supernatants (n=24). Potential outliers in a-c were not eliminated because their removal did not significantly affect r_s values. (e-f) Histogram plots (median ± IQR) showing the alteration in membrane CD26 (MFI) in TH cells (e) and the corresponding sDPP4 activity in cell culture supernatants (f) from resting regulatory T cells (Treg), regulatory T cells expanded with activation beads and IL-2 (Treg + B + IL-2), resting effector T cells (Teff), or activated effector T cells expanded with activation beads in the absence (Teff + B) or the presence of different cytokines required for effector TH-subsets differentiation: TH_1 (Teff+ B + IL-12), TH_2 (Teff + B + IL-4)- and TH_{17} (Teff + B + IL-1β + IL-6 + IL-23) (n=4). Figures (g) and (h) show, respectively, the positive and negative association (Spearman correlation test) between the fluorescence intensity (geometric mean; CD26 MFI) and the percentage of $CD26^+$ lymphocytes in activated Teff (n=13) and resting/activated Treg cells (n=7). Results with resting Teff cells were not included in figure g for clarity, since resting Teff and Treg cells are more alike than resting and activated Teff cells.

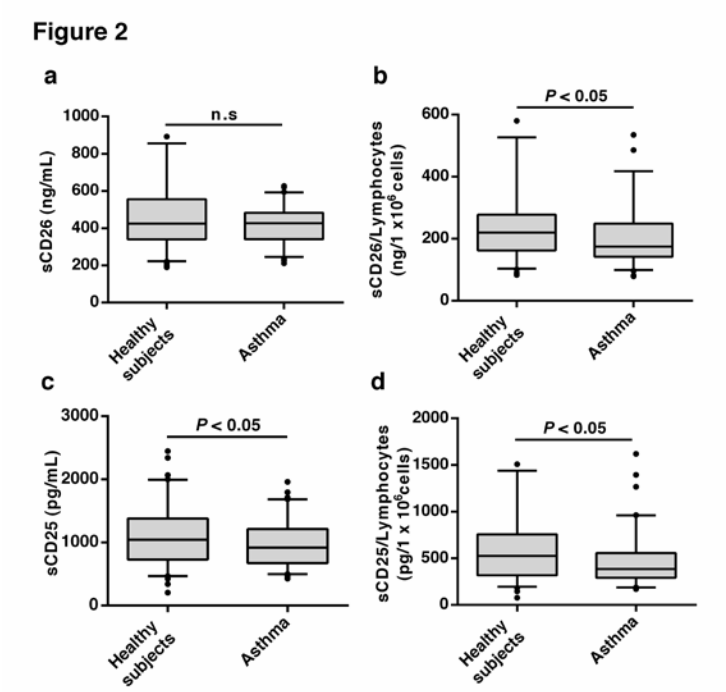

Figure 2. Concentration of sCD26 and sCD25 in serum samples from healthy and allergic asthmatic subjects. Data are presented in box and whisker plots where median (IQR), 5-95 percentiles (error bars) and anomalous values are shown. Data refer to sCD26 (a-b; healthy subjects, n=89; asthma patients, n=72) or sCD25 (c-d; healthy subjects, n=96; asthma patients, n=81) levels normalised (b, d) or not (a, c) for the absolute number of lymphocyte counts in peripheral blood. Numbers on the graph represent *P*-values (Mann-Whitney U Statistic). n.s., no significant.

Figure 3

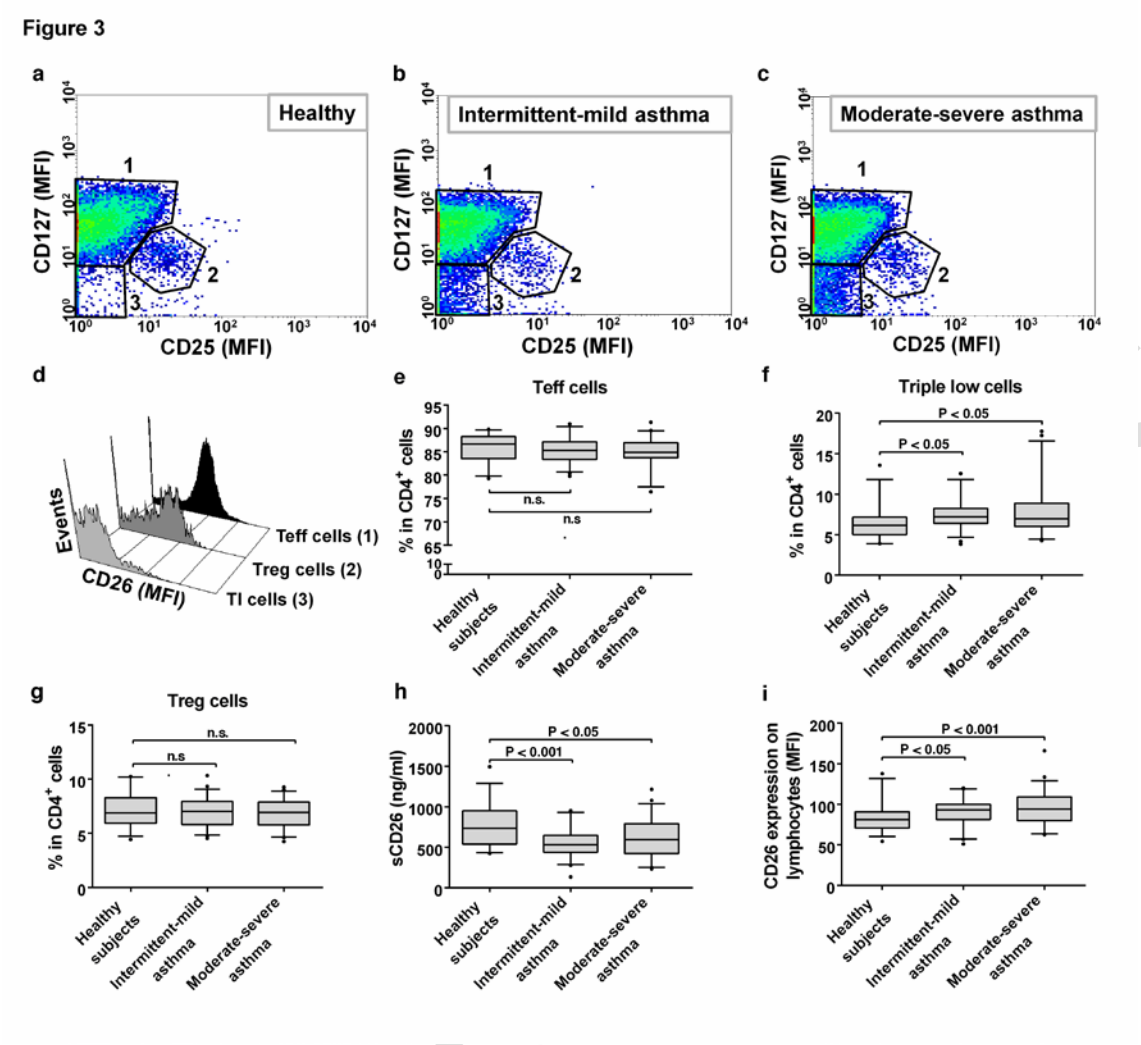

Figure 3. Four-colour flow cytometry analysis of different peripheral blood CD4$^+$ T subsets and evaluation of sCD26 levels in serum samples from healthy and intermittent-mild or moderate-severe allergic asthmatics. (a-c) Representative dot-plots (CD25 vs CD127) of CD4-gated lymphocytes showing the three major cell subsets detected in peripheral blood from healthy donors (a), intermittent-mild asthmatics (b) and moderate-severe asthma patients (c) (validation cohort): Teff (region 1), Treg (region 2) and "triple low" (region 3). (d) CD26 expression levels (MFI) in the different CD4$^+$ T subsets observed in A-C (Teff > Treg >>"triple low" cells). (e-g) Percentage of Teff (e), "triple low" (f) and Treg (g) subsets amongst CD4$^+$ T lymphocytes in intermittent-mild asthma, moderate-severe asthma and healthy subjects. (h) Serum sCD26 levels (ng/mL) in asthmatic and healthy subjects. (i) CD26 expression (MFI) in lymphocytes from healthy subjects and asthmatic patients. Kruskal-Wallis One Way Analysis of Variance on Ranks, $P < 0.05$; All pairwise multiple comparison procedure, Dunn's Method, $P < 0.05$, n.s., no significant.

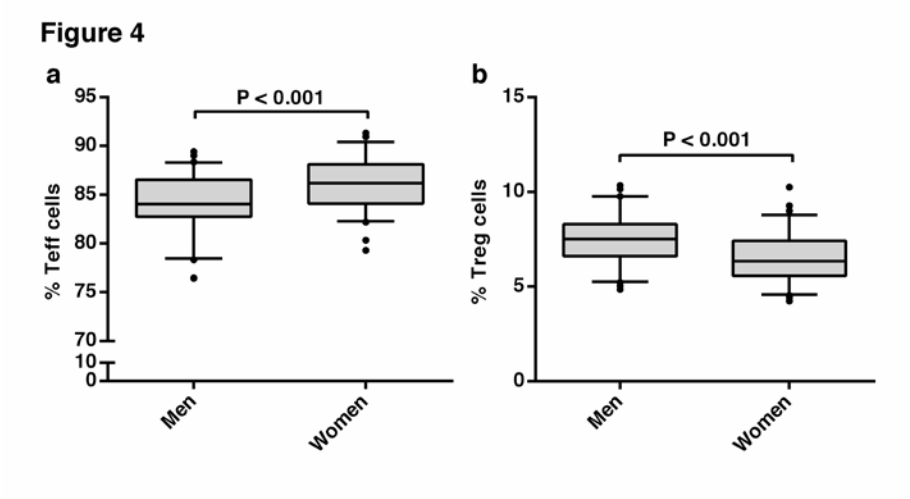

Figure 4. Percentage of Teff and Treg cells in the whole population according to gender. (a) Percentage of Teff cells between men and women. (b) Sex differences in the percentage of Treg cells. Mann-Witney U statistic, $P < 0.001$.

Figure 5

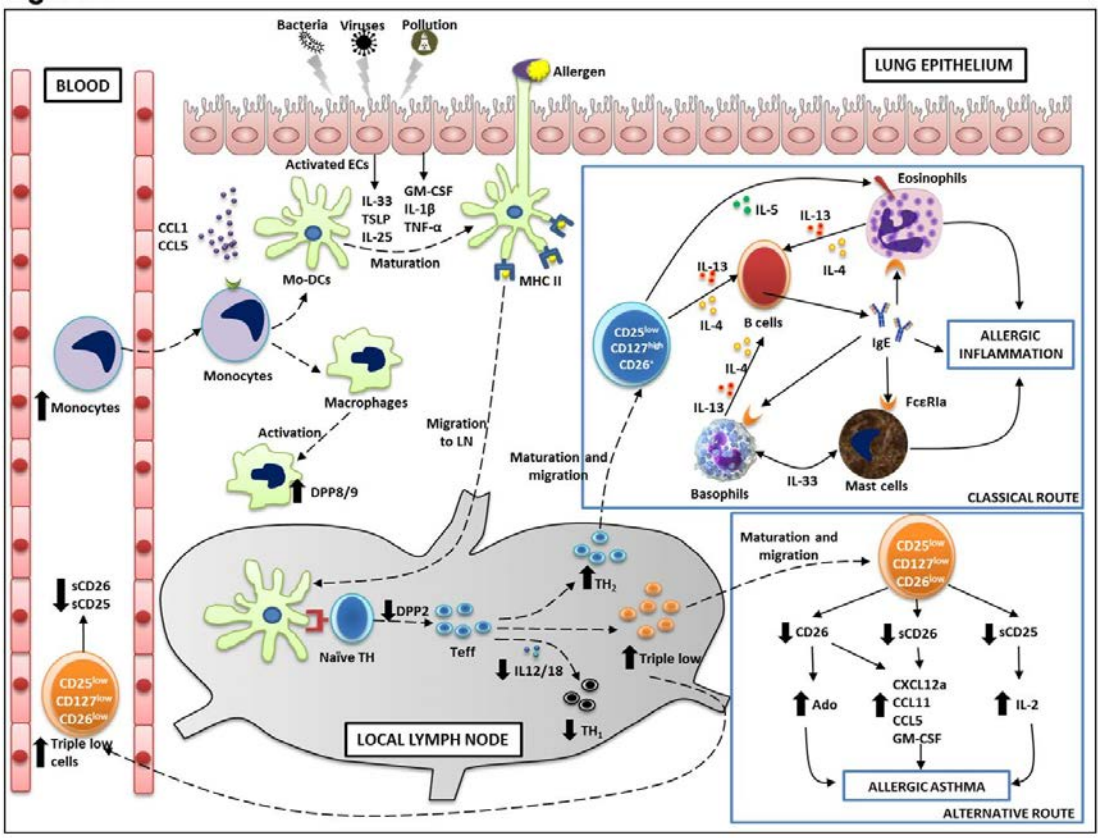

Figure 5. Major immunological pathways in the pathogenesis of allergic asthma. Epithelium damaged by pollution or infection leads to chemokine secretion by airway epithelial cells, enhancing the trafficking of innate cells like monocytes from blood to lung epithelium. Monocytes differentiate into macrophages, which increment the expression of DPP8/9 upon activation, or into monocyte-derived dendritic cells (Mo-DCs), the last subset migrating to lymph nodes (LN) upon maturation. Within the LN, Mo-DCs present antigens to naïve TH cells, which lost DPP2 and differentiate into $CD25^{low}CD127^{high}CD26^{+}$ TH_2 cells (classical route). On the other hand, our results also evidence the expansion of an unconventional $CD25^{low}CD127^{low}CD26^{low}$ TH subset (Triple low) in asthma patients. Triple low cells could have an important role in allergic asthma, as their lower levels of CD26 on plasma membrane or their diminished ability to release sCD26 could promote higher local amounts of chemokines (e.g., CXCL12a/SDF-1α, CCL11/Eotaxin or CCL5/RANTES) and adenosine (Ado) and exacerbate asthma. CCL = Chemokine (C-C motif) ligand; TSLP = Thymic Stromal Lymphopoietin; TH = T-helper cell. GM-CSF = Granulocyte-Macrophage Colony-Stimulating Factor; FcεRI = High-Affinity IgE Receptor.

Table 1. Characteristics of study population.

	AAP[a]			HC		
	MALE	**FEMALE**	**ALL**	**MALE**	**FEMALE**	**ALL**
N (%)	33 (40.7)	48 (59.3)	81 (100)	36 (37.1)	61 (62.9)	97 (100)
Age[d]	33 (21-45)	36 (28-48)	35 (26-47)	35 (27-49)	37 (29-52)	35 (29-51)
Smokers (%)[c]	45.5	18.75	29.6	0	0	0
BMI (Kg/m^2)[c]	26.4 (23.8-29)	26.7 (22.6-28)	26 (23.1-28.4)	-	-	-
Asthma Severity:						
Mild	2	4	6	-	-	-
Moderate/High	31	44	75	-	-	-
Control:						
Good	23	37	60	-	-	-
Bad	10	11	21	-	-	-
Treatment:						
No	1	2	3	-	-	-
Inhaled Corticosteroids	32	46	78	-	-	-
Oral Corticosteroids	5	3	8	-	-	-
Antileukotrienes	6	19	25	-	-	-
Omalizumab	0	1	1	-	-	-
FEV1 (%) [c]	93 (74-101)	91.8 (73-102)	93 (74-102)	-	-	-
FEV1/FVC (%) [c]	74.5 (64-83)	73.5 (65-81)	74.2 (65-82)	-	-	-
Lymphocytes (cells/µL)[c]	2480 (1935-2835)	2150 (1770-2680)	2310 (1848-2765)[*]	1941.5 (1615-2372)	1892.3 (1585-1892)	1912.7 (1594-2362)[*]
Eosinophils (cells/µL)[c]	327 (154-502)[*]	326 (182-492)[*]	327 (175-494)[*]	173 (110-268)[*]	133 (97-198)[*]	152 (103-218)[*]
Monocytes (cells/µL)[c]	501 (414-658)	420 (306-518)[*]	444 (349-575)[*]	425 (350-528)	318.8 (231-399)[*]	361 (271-451)[*]
Neutrophils (cells/µL)[c]	4458 (3202-5617)	3851 (2814-4859)	4045 (3003-5232)	3286 (2645-5685)	3399 (2812-4472)	3395 (2776-4809)
IgE (IU/mL)[c]	355 (87-707)[*]	204 (89-680)[*]	241 (90-682)[*]	78 (19-195)[*]	22 (7-55)[*]	34 (10-95)[*]
CRP (mg/dL)[c]	0.09 (0.07-0.42)	0.16 (0.07-0.32)	0.15 (0.07-0.34)	0.19 (0.1-0.69)	0.16 (0.05-0.39)	0.17 (0.07-0.57)
TNF (pg/mL)[c]	9.4 (6.6-12.0)	9.5 (7.6-12.9)	9.4 (7.2-12.3)	8.6 (7.3-10.4)	10.5 (7.6-13.2)	9.7 (7.4-12.9)
Leptin (ng/mL)[c]	2.9 (1.3-7.2)[#]	18.1 (11.2-27.9)[#]	11.2 (3.1-23.9)	2.9 (1.0-8.0)[#]	13.0 (5.35-26.9)[#]	8.1 (3.3-20.0)

[a]We recorded the professional activity of ~70% of patients, distributed according to the following order: students (14.8%), construction professionals (9.9%), housewives (8.6%), administrative officers (7.4%), cleaning service (6.2%), health professionals (6.2%), waiters (4.9%), educators (4.9%), farmers (2.5%), salespersons (2.5%) and textile workers (2.5%).

[b]Apart from the non-registered (8.6%), formerly smoker (11.1%) and currently smoker (29.6%) patients, they were mostly non-smoking (50.6%).

[*] Differences between AAP and HC: Mann-Witney U Statistic, $P < 0.05$.

[#] Differences between male and female: Mann-Witney U Statistic, $P < 0.05$.

[c] Median value (IQR1-3)

Table 2. Spearman correlation matrix of the study population.

VARIABLES	BMI	IgE	Leptin	sCD25	sCD26	Age
Eosinophil count	-0.004P	**0.420*****	-0.009	**-0.306**C**	0.063	-0.144
Eosinophil (%)	-0.010P	**0.379*****	0.005	**-0.358**C**	0.051	-0.103
Basophil count	0.011P	0.025	**-0.234****	-0.073	**0.261****	-0.157
Basophil (%)	-0.005P	-0.048	**-0.263*****	-0.108	**0.265****	-0.125
Lymphocyte count	0.021P	**0.178***	0.024	**-0.327**C**	0.091	-0.098
Lymphocyte (%)	-0.043P	-0.015	-0.090	**-0.341**C**	**0.201***	-0.032
Monocyte count	-0.004P	**0.266*****	-0.138	-0.018	0.111	-0.103
Monocyte (%)	-0.015P	0.100	-0.138	-0.037	**0.190***	-0.038
Neutrophil count	0.041P	0.101	0.057	-0.091	**-0.174***	-0.062
Neutrophil (%)	0.075P	-0.094	0.136	**0.346**C**	**-0.249****	0.095
FEV1%	**-0.277*P**	-0.059P	**-0.243*P**	-0.035P	0.019P	**-0.240*P**
FEV1/FVC	**-0.252*P**	-0.046P	-0.174P	-0.102P	-0.013P	**-0.428***P**
CRP	**0.403***P**	-0.016	**0.272*****	**0.214****	-0.057	0.072
BMI		**-0.260*P**	**0.282*P**	0.071P	-0.072P	**0.504***P**
TNF		**-0.299**C**	**0.221****	**0.170***	0.040	**0.252*C**
IgE			**-0.230*C**	-0.123	-0.066	**-0.275*P**
Leptin				0.102	**-0.280*P**	0.147
sCD25					0.001	0.015
sCD26						0.003

C, control population; P, patient population. *P<0.05, **P<0.01, ***P<0.001.