ORIGINAL ARTICLE

Expansion of a CD26^{low} Effector T_H Subset and Reduction in Circulating Levels of sCD26 in Stable Allergic Asthma in Adults

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Abstract

Background: The pathogenesis of asthma 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 changes in leukocyte phenotype.
Objectives: To analyze expression of CD25 and CD26 on T lymphocytes and their soluble derivatives (sCD25, sCD26) during stable phases of moderate-severe allergic asthma.
Methods: Cross-sectional study with 2 adult cohorts of allergic asthmatics. Clinical, anthropometric, pulmonary, hematological, and biochemical parameters were measured. Phenotyping was performed with flow cytometry in both circulating and cultured leukocytes. Dipeptidyl peptidase 4 (DPP4) activity was assayed in culture supernatants.
Results: In vitro studies revealed upregulation of CD26 on human T lymphocytes upon activation, especially under T_H17-favoring conditions, and a correlation with soluble DPP4 activity (r_s=0.641; P<.001). CD26 expression on lymphocytes was higher in asthmatics, while serum sCD26 was lower in women and patients. The latter finding could be associated with an expanded CD25^{low}/CD26^{low}/CD127^{low} subset of effector CD4$^+$ T cells in allergic asthma, with no changes in Treg percentages. However, women showed an increased Teff/Treg ratio, which could explain their greater susceptibility to asthma.
Conclusions: Allergic asthma causes an increment in CD25^{low}CD26^{low} helper T cells detected in stable stages. These changes are mirrored in serum and should be considered in the light of the downmodulating role of CD26 in major chemokines related to the pathogenesis of asthma such as CCL11 (eotaxin), CCL5 (RANTES), and CXCL12a (SDF-1α).

Key words: Asthma biomarkers. CD25. CD26. DPP4. Lymphocytes. Helper T 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 moderada-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 T_H17, 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

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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).

Palabras clave: Biomarcadores de asma. CD25. CD26. DPP4. Linfocitos. Células T cooperadoras.

Introduction

Asthma is influenced by genetic factors (eg, dipeptidyl peptidase 10 [*DPP10*] and ADAM metallopeptidase domain 33 [*ADAM33*]) and environmental factors [1]. Its management should be based on endotypes [1,2]. During asthma attacks, allergens trigger lung epithelial cells to release cytokines, which in turn activate innate leukocytes and drive type 2 helper T cell (T_H2) lymphocyte differentiation [1]. These cells release interleukins (IL-4, IL-5, and IL-13), stimulate IgE production, and favor 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-2Rα) 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] that is elevated in serum/plasma during asthma exacerbations [6] and that correlates positively with the severity of allergic asthma [7]. In addition, Tregs are thought to be another source of sCD25 [4,8,9]. sCD25 is also increased in bronchoalveolar lavage fluid from asthma patients [8,9].

Another interesting protein in the pathogenesis of asthma is CD26/DPP4, a surface glycoprotein enriched in CD4^{+} T cells [10]. In the form of 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]), dipeptidyl peptidase 8 (*DPP8*), dipeptidyl peptidase 9 (*DPP9*), and fibroblast activation protein alpha (FAP) [12-16]. 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, and 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 adults with allergic asthma [18]. CD4^{+} T cells are major actors in the pathogenesis of asthma; however, unlike CD25, Treg cells display lower CD26 levels than Teff lymphocytes. Indeed, CD26 is a negative marker of Treg cells and a marker of the remaining TH subsets. Thus, expression of CD26 on T_H follows the order of $T_H17 >> T_H1 > T_H2 >$ Treg [19,20]. Hence, an 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 the bloodstream, either shed 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 the intracellular peptidases DPP2, DPP8, and DPP9. These DPP4 homologs are also involved in the pathogenesis of asthma [13] and show 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 and is downmodulated upon activation [15].

CD26/DPP4 cleaves X-Pro or X-Ala amino terminal dipeptides from chemokines (eg, 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 and peptide hormones such as incretins [28,29], whose half-life is prolonged by the DPP4 inhibitors currently used as antidiabetic drugs [30]. Vascular substrates of DPP4 may be cleaved by sDPP4, DPP4 expressed on leukocytes, and endothelial DPP4 [31]. Increased DPP4-like activity has been observed in bronchoalveolar lavage fluid from asthmatic rat lungs and is due mostly to sDPP4 and, to some extent, DPP8, DPP9, and DPP2 [13,32].

CD4^{+} T lymphocytes are the main source of sCD26, as this subpopulation displays the highest percentage of CD26^{+} cells [10,23,33]. Therefore, this molecule could be used as a "fingerprint" to test the activation status or differentiation status of CD4^{+} T cells in asthma. However, the few studies that have been undertaken show augmented levels of sCD26 in allergic asthma that were 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].

To date, few studies have monitored sCD26 in asthma, and none consider the potential roles of CD26 in the pathogenesis of asthma or take into account the possibility that abnormalities of circulating biomarkers (sCD25 and sCD26) may reflect changes in leukocyte phenotype such as CD25$^{-/low}$CD26^{+} Teff cells and CD25$^{+/high}$CD26^{low} Treg cells. Therefore, in the present study a comprehensive analysis was carried out to assess the aforementioned immune biomarkers in patients with moderate-severe allergic asthma.

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Material and Methods

Patients

The study was conducted between 2009 and 2012 and included patients from respiratory medicine and allergy departments in Galicia, Spain. Patients had been diagnosed with asthma and allergy at least 1 year earlier according to the criteria of the Global Strategy for Asthma Management and Prevention guidelines (GINA 2006, http://www.seicap.es/documentos/archivos/GINA2006general.pdf). The research project was approved by the Clinical Research Ethics Committee 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 (Respiratory Medicine Department, University Hospital of Santiago de Compostela, Spain). All patients were in a stable phase for at least 4 weeks before sample collection. Healthy controls (HCs) were selected from patients scheduled for minor surgeries such as inguinal hernia or orthopedic surgery. They were nonsmokers and had no systemic diseases or allergies. None of the patients or HCs were receiving DPP4 inhibitors.

Sensitization in allergic asthmatic patients (AAPs) was confirmed through a skin prick test or serum specific IgE to frequent allergens. Other variables included body mass index (BMI), area of residence (rural-urban), profession, smoking history, and comorbidities. Biochemical determinations were performed using an ADVIA1650 analyzer (SIEMENS Healthcare Diagnostics SL). The nucleated cell number was measured using an ADVIA2120 hematology counter (SIEMENS Healthcare Diagnostics SL).

Magnetic Purification of $CD4^+$ T-Cell Subsets and In Vitro Culture

Buffy coats from healthy participants were donated by Axencia Galega de Sangue, Órganos e Tecidos, Santiago de Compostela, Spain and used to isolate peripheral blood mononuclear cells by Ficoll density gradients. Teff and Treg cells were prepared from peripheral blood mononuclear cells by means of the Dynabeads Regulatory $CD4^+CD25^+$ T cell Kit (Life-Technologies). 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 partial Teff differentiation, the following cytokines (PeproTech) were added: 10 ng/mL IL-12 (T_H1-like), 10 ng/mL IL-4 (T_H2-like), or IL-1β (100 ng/mL), IL-6 (30 ng/mL), and IL-23 (100 ng/mL) (T_H17-like). In addition, 800 ng/mL of IL-2 was used for maintenance of Treg cells.

Flow Cytometry Assays

Venous peripheral blood was collected (BD Vacutainer K2E) in order to examine the proportion of Teff and Treg cells. Leukocytes from 100 µL of whole blood were stained (30 minutes, room temperature) 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), and CD127 (Alexa Fluor-647), and a mouse IgG2b antibody specific for CD26 (PE; Immunostep). Red cells were then lysed (BD FACS Lysing Solution). The purity of Teff/Treg lymphocytes before (or after) in vitro cell culture was also assayed with the same specific antibodies (CD4-FITC, CD25-PE-Cy7, CD26-PE) or isotype antibodies (Isotype-FITC, Isotype-PE-Cy7, Isotype-PE). Finally, samples were analyzed (BD FACSCalibur and FACSort), and 10 000-200 000 events were collected. Data were examined using WinMDI 2.9 software (Joseph Trotter).

DPP4 Activity Measurement

Total DPP4 enzymatic activity was colorimetrically assayed using a flat-bottom 96-well microplate–adapted and endpoint protocol. Cell culture supernatants (50 µL) were diluted with 50 µL of 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 recorded sequentially (30-120 minutes) at 405 nm (Labsystems Multiscan MS microtiter plate reader). The concentration of pNA was calculated from a standard curve, ranging from 0 µM to 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 using ELISA (Quantikine Human IL-2Rα Immunoassay, R&D Systems), and sCD26 was quantified using ELISA plates (eBioscience, Human sCD26 Platinum ELISA). Optical densities were recorded at 450 nm, and the protein concentration was calculated from standard curves.

Statistics

Descriptive data are presented as median (IQR) or percentages. To assess changes between AAPs and HCs in nonnormally distributed variables, we used the 2-tailed Mann-Whitney test or the Kruskal-Wallis test followed by the Dunn multiple comparison test for more than 2 groups. The Spearman test was used to measure associations between variables. All analyses were conducted using GraphPad Prism 6.0 (GraphPad Software, Inc). Statistical significance was set at $P<.05$.

Results

Characteristics of the First Cohort of Allergic Asthmatic Patients

The characteristics of this first cohort of patients are summarized in Table 1. In AAPs, the median forced expiratory volume in the first second (FEV_1) (%) was 93 (74.5-102.2), while the FEV_1/forced vital capacity (FVC) ratio (%) was 74.2 (65.2-81.9). Asthma was mainly moderate-persistent (71.6%),

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and patients had an allergic disease, with positive skin prick test reactions against common allergens. In addition, most patients lived in rural areas (72%), although only a small percentage of them were farmers (Table 1). As expected, a significant group of AAPs had peripheral blood eosinophilia (45% had >350/μL) and elevated total IgE (Table 1). There was a positive correlation between eosinophils and IgE, but not between FEV_1 and serum IgE in AAPs (Table 2). Patients were under different treatments, were mostly nonsmokers, and had well-controlled asthma (Table 1).

Table 1. Characteristics of the Study Population[a]

	Allergic Asthmatic Patients[b]			Healthy Controls		
	Male	Female	All	Male	Female	All
No. (%)	33 (40.7)	48 (59.3)	81 (100)	36 (37.1)	61 (62.9)	97 (100)
Age	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	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	-	-	-
FEV_1 (%)	93 (74-101)	91.8 (73-102)	93 (74-102)	-	-	-
FEV_1/FVC (%)	74.5 (64-83)	73.5 (65-81)	74.2 (65-82)	-	-	-
Lymphocytes, cells/μL	2480 (1935-2835)	2150 (1770-2680)	2310 (1848-2765)[d]	1941.5 (1615-2372)	1892.3 (1585-1892)	1912.7 (1594-2362)[d]
Eosinophils, cells/μL	327 (154-502)[d]	326 (182-492)[d]	327 (175-494)[d]	173 (110-268)[d]	133 (97-198)[d]	152 (103-218)[d]
Monocytes, cells/μL	501 (414-658)	420 (306-518)[d]	444 (349-575)[d]	425 (350-528)	318.8 (231-399)[d]	361 (271-451)[d]
Neutrophils, cells/μL	4458 (3202-5617)	3851 (2814-4859)	4045 (3003-5232)	3286 (2645-5685)	3399 (2812-4472)	3395 (2776-4809)
IgE, IU/mL	355 (87-707)[d]	204 (89-680)[d]	241 (90-682)[d]	78 (19-195)[d]	22 (7-55)[d]	34 (10-95)[d]
CRP, mg/dL	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	9.4 (6.6-12.0)	9. (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	2.9 (1.3-7.2)[e]	18.1 (11.2-27.9)[e]	11.2 (3.1-23.9)	2.9 (1.0-8.0)[e]	13.0 (5.35-26.9)[e]	8.1 (3.3-20.0)

Abbreviations: BMI, body mass index; CRP, C-reactive protein; FEV_1, forced expiratory volume in the first second; FVC, forced vital capacity; TNF, tumor necrosis factor.
[a]Values are expressed as median (IQR), unless otherwise specified.
[b]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%).
[c]Apart from the nonregistered (8.6%), former smoker (11.1%) and current smoker (29.6%) patients, they were mostly nonsmokers (50.6%).
[d]Differences between AAPs and HCs (Mann-Whitney, $P<.05$).
[e]Differences between male and female (Mann-Whitney, $P<.05$).

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Table 2. Spearman Correlation Matrix of the Study Population

Variables	BMI	IgE	Leptin	sCD25	sCD26	Age
Eosinophil count	–0.004P	0.420[a]	–0.009	–0.306[b]C	0.063	–0.144
Eosinophils, %	–0.010P	0.379[a]	0.005	–0.358[b]C	0.051	–0.103
Basophil count	0.011P	0.025	–0.234[b]	–0.073	0.261[b]	–0.157
Basophils, %	–0.005P	–0.048	–0.263[a]	–0.108	0.265[b]	–0.125
Lymphocyte count	0.021P	0.178[c]	0.024	–0.327[b]C	0.091	–0.098
Lymphocytes, %	–0.043P	–0.015	–0.090	–0.341[b]C	0.201[c]	–0.032
Monocyte count	–0.004P	0.266[a]	–0.138	–0.018	0.111	–0.103
Monocytes, %	–0.015P	0.100	–0.138	–0.037	0.190[c]	–0.038
Neutrophil count	0.041P	0.101	0.057	–0.091	–0.174[c]	–0.062
Neutrophils, %	0.075P	–0.094	0.136	0.346[b]C	–0.249[b]	0.095
FEV_1,%	–0.277cP	–0.059P	–0.243[c]P	–0.035P	0.019P	–0.240[c]P
FEV_1/FVC	–0.252cP	–0.046P	–0.174P	–0.102P	–0.013P	–0.428[a]P
CRP	0.403aP	–0.016	0.272[a]	0.214[b]	–0.057	0.072
BMI		–0.260[c]P	0.282[c]P	0.071P	–0.072P	0.504[a]P
TNF		–0.299[b]C	0.221[b]	0.170[c]	0.040	0.252[c]C
IgE			–0.230[c]C	–0.123	–0.066	–0.275[c]P
Leptin				0.102	–0.280[c]P	0.147
sCD25					0.001	0.015
sCD26						0.003

Abbreviations: BMI, body mass index; C, control population; CRP, C-reactive protein; FEV_1, forced expiratory volume in the first second; FVC, forced vital capacity; P, patient population; TNF, tumor necrosis factor.
[a]$P<.001$
[b]$P<.01$
[c]$P<.05$

Given the well-known association between leptin and body mass index (BMI) and the assumed correlation between the development/worsening of asthma and BMI, we also studied these parameters. First, BMI in AAPs was positively correlated with leptin and C-reactive protein (CRP), but negatively associated with IgE and both FEV_1% and FEV_1/FVC (Table 2). Second, leptin levels were generally 4 to 6–fold higher in women, although no differences were detected between HCs and AAPs (Supplementary Figure 1, Table 1). These findings underline the lack of association between BMI 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 between BMI and TNF and a negative interdependence with IgE, FEV_1%, and FEV_1/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 ($CD4^+CD25^{high}CD127^{low}$) (Supplementary Figure 2). To investigate whether phenotypic differences in $CD4^+$ T cells with respect to CD26 expression could be translated into altered sCD26 levels, we performed in vitro experiments with Treg cells ($CD4^+CD25^{high}$) and Teff lymphocytes ($CD4^+CD25^-$) cultured under T_H1-, T_H2-, or T_H17-skewing conditions. Results from determination of sDPP4 activity 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 lymphocyte count (Figure 1C). To maximize the influence of $CD4^+$ T-cell phenotype, we weighted sDPP4 for the lymphocyte count and continued to observe a strong correlation between CD26 expression (MFI) and sDPP4 (Figure 1D), thus supporting the influence of the T_H phenotype on the latter 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, 2 opposite poles were observed: resting Treg and T_H cells cultured under T_H17-favoring conditions. Moreover, T_H1-like and T_H2-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

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positive correlation between the density of CD26 molecules on CD4+ T cells and sDPP4 levels (ie, sCD26). Furthermore, we found differential behavior in Teff and Treg cells, with a high positive correlation between the intensity and percentage of CD26 in activated Teff cells and an opposite correlation for Tregs (Figures 1G, H).

Reduction of Serum sCD26 and sCD25 and Increment of CD4+CD127lowCD25lowCD26low T Cells in Allergic Asthma Patients

Despite therapy with corticosteroids, circulating lymphocytes were increased in AAPs (Table 1). Given that

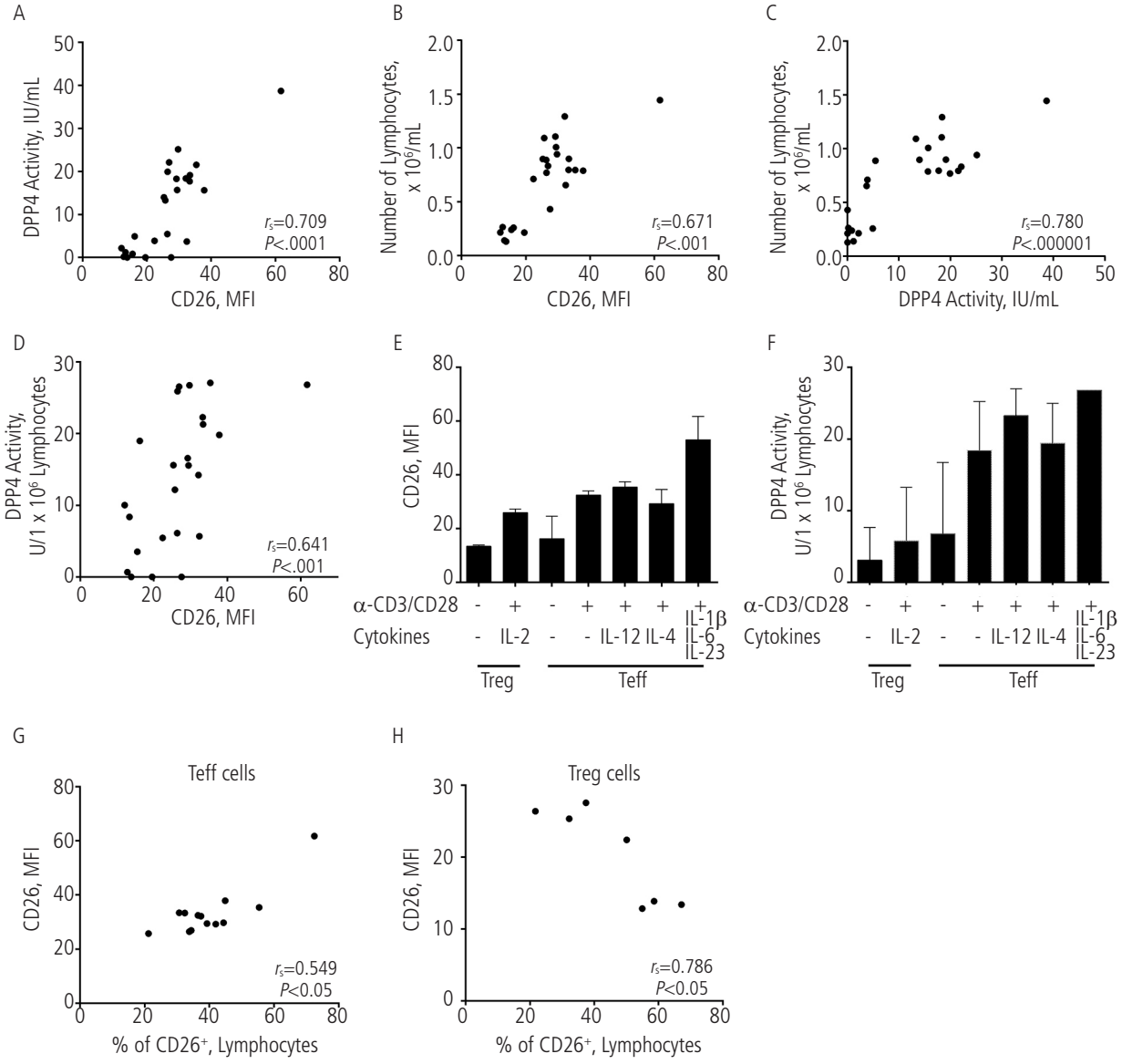

Figure 1. CD26 surface expression and DPP4 activity in culture supernatants of magnetically isolated lymphocytes. Spearman rank correlation test showing the positive relationship between the following: A, CD26 expression (MFI) in resting/activated CD4+ T lymphocytes and soluble DPP4 activity (sDPP4; 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 lymphocyte 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/L × 106 lymphocytes) of sDPP4 in culture supernatants (n=24). Potential outliers in A-C were not eliminated, because their removal did not significantly affect rs 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), and activated effector T cells expanded with activation beads in the absence (Teff + B) or the presence of the cytokines required for differentiation of effector T_H subsets differentiation: T_H1 (Teff+ B + IL-12), T_H2 (Teff + B + IL-4)- and T_H17 (Teff + B + IL-1β + IL-6 + IL-23) (n=4). Figures (G) and (H) show, respectively, the positive and negative association (Spearman rank 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.

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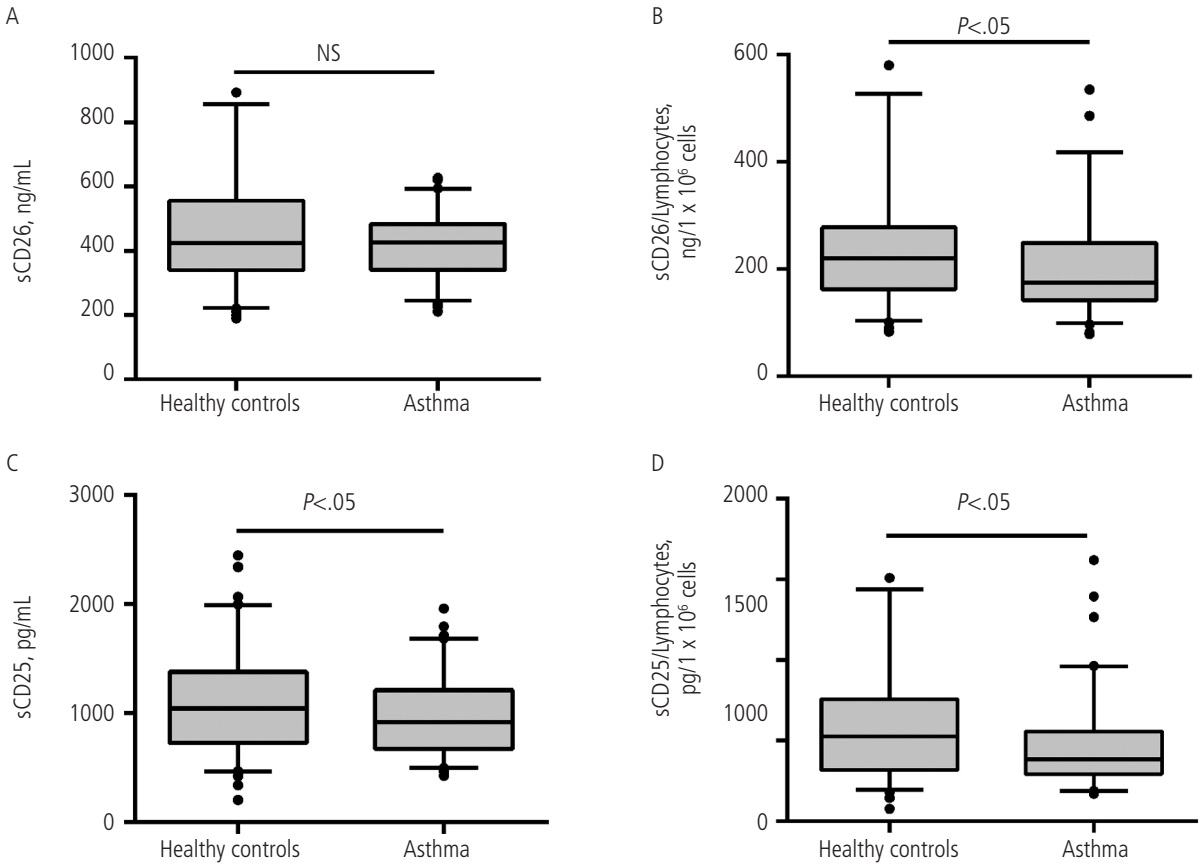

Figure 2. Concentration of sCD26 and sCD25 in serum samples from healthy and allergic asthmatic patients. Data are presented in box and whisker plots where median (IQR), 5-95 percentiles (error bars), and anomalous values are shown. Data refer to levels of sCD26 (A and B; healthy individuals, n=89; asthma patients, n=72) or sCD25 (C and D, healthy individuals, n=96; asthma patients, n=81), which were normalized (B and D) or not (A and C) for the absolute lymphocyte count in peripheral blood. The numbers on the graph represent *P* values (Mann-Whitney). NS, not significant.

in vitro experiments showed a positive correlation between CD26 on $CD4^+$ T cells and sDPP4, Treg and Teff cells have a reverse phenotype ($CD25^{high}CD26^{low}$ and $CD25^{low}CD26^{high}$, respectively) and asthmatic donors were expected to have effector/regulatory disequilibrium, we evaluated both sCD26 and sCD25. Our results showed a slight positive correlation between sCD26 and basophils and a negative correlation between sCD26 and neutrophils (Table 2). However, sCD26 levels were not positively associated with BMI, and there was a slight negative relationship with leptin (Table 2). In addition, sCD25 was directly associated with CRP and neutrophils (%), although negatively with eosinophils (Table 2). Importantly, instead of the previously reported sCD26 upregulation [18], a slight but nonsignificant decrease was observed in the absolute concentration of sCD26 in AAPs (Figure 2A), even after classification of data by gender (data not shown). Moreover, we found a small association between sCD26 and the percentage of lymphocytes (Table 2), but not between sCD26 and the absolute number (as shown in our experiments in vitro). Despite the latter finding, sCD26 was still divided by the lymphocyte count to maximize the effect of T-cell phenotype, revealing significantly lower concentrations in AAPs (Figure 2B). Significant downmodulation in AAPs was observed for the absolute concentrations of sCD25 (Figure 2C) and for the normalized concentrations (Figure 2D). In any case, there was no correlation between sCD26 and sCD25 levels (Table 2).

According to our in vitro assays, the slight reductions in sCD26 (and sCD25) in AAPs point to expansion of a small $CD26^{low}$ subset of $CD4^+$ T cells. To test this hypothesis, another independent set of samples with similar characteristics (validation cohort) was obtained from patients with intermittent-mild allergic asthma (n=53) and moderate-severe allergic asthma (n=50) and HCs (n=32). First, Teff cells ($CD26^{high}$) and Treg cells ($CD26^{low}$) 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 asthma (Figure 3B) and moderate-severe asthma (Figure 3C). Besides, this small subset displayed the lowest levels of CD26 ($CD26^{-/low}$) among $CD4^+$ T cells (Figure 3D). In intermittent-mild and moderate-severe AAPs, we detected a slight (but not significant) reduction in the percentage of Teff lymphocytes ($CD25^{low}CD127^{high}CD26^{high}$) (Figure 3E), which was accompanied by a significantly increased proportion of triple-low $CD4^+$ T cells ($CD25^{low}CD127^{low}CD26^{low}$)

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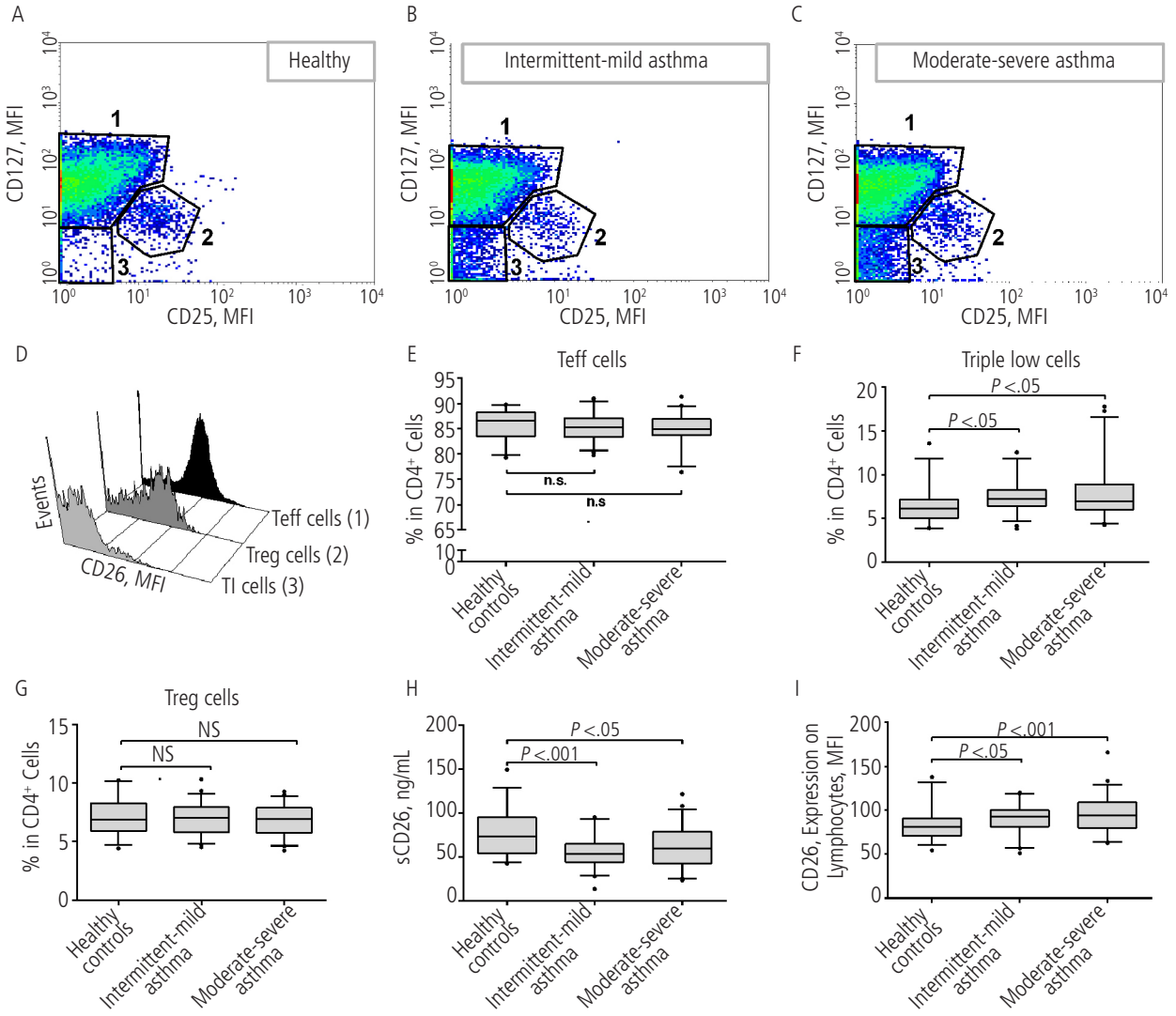

Figure 3. Four-color flow cytometry analysis of peripheral blood CD4+ T subsets and evaluation of sCD26 levels in serum samples from healthy and intermittent-mild or moderate-severe allergic asthmatics. A-C, Representative dot-plots (CD25 vs CD127) of CD4-gated lymphocytes showing the 3 major cell subsets 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 among CD4+ T lymphocytes in intermittent-mild asthmatics, moderate-severe asthmatics, and healthy individuals. H, Serum sCD26 levels (ng/mL) in asthmatic patients and healthy individuals. I, CD26 expression (MFI) in lymphocytes from healthy individuals and asthmatic patients. Kruskal-Wallis 1-way analysis of variance on ranks, *P*<.05. All pairwise multiple comparison procedure, Dunn method, P<.05, NS, non-significant.

(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 lower absolute levels of sCD26 in serum samples from AAPs (especially in moderate-severe asthma) than in HCs (Figure 3H). This finding was in line with the results from the first cohort. Moreover, in the second cohort, it was unnecessary to weight sCD26 levels by lymphocyte count to maximize the effect of CD4+ T-cell phenotype. Downmodulation of sCD26 in AAPs was mainly associated with men, whose levels were higher in the reference population (HCs) than those of women. In contrast, CD26 expression in total lymphocytes was higher in women than in men (data not shown), and also in AAPs than in HCs (Figure 3I). Furthermore, even though we did not perceive abnormalities in Teff ($CD26^{high}$) or Treg ($CD26^{low}$) percentages between HCs and AAPs that could explain the reduction in sCD26 levels, women had higher and lower proportions, respectively, of Teff and Treg subsets than men (Figure 4).

Discussion

In the present study, in vitro experiments provided evidence of a direct correlation between lymphocyte surface CD26 and sDPP4 activity and differential expression/secretion of CD26

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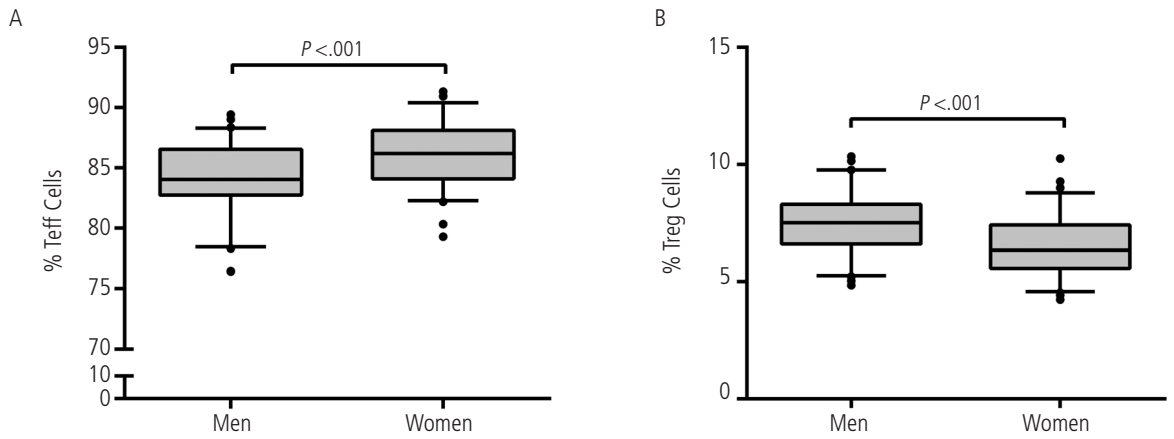

Figure 4. Percentage of Teff and Treg cells in the whole population according to gender. A, Percentage of Teff cells between men and women. B, Sex differences in the percentage of Treg cells. Mann-Whitney, *P*<.001.

related to the T_H phenotype. Although we expected increased sCD26 in patients (in line with the enhanced expression of CD26 in lymphocytes), we found lower levels of sCD26 than in healthy donors. This finding can be partially explained by 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 differences in expression between Treg and Teff cells [19,20], as with CD25 and CD127 [36]. IL-12 leads to T_H1 polarization [37] and potentiates the TCR-mediated upregulation of CD26 on $CD4^+$ T cells [38]. On the other hand, in 2012, Bengsch et al [20] used flow cytometry and cell sorting assays with human peripheral blood and tissue samples to show that human T_H17 cells express the highest levels of CD26, followed by T_H1, T_H2, and Treg lymphocytes [20]. Our in vitro assays reveal a similar pattern, where Teff cells undergoing T_H17-polarizing conditions (IL-1β, IL-6, IL-23) also acquire the highest levels of CD26 and sDPP4 activity ($T_H17>T_H1\geq T_H2>$Treg). Although 90% of soluble DPP4 activity is derived from sCD26 [23,24], we must also consider the likely contribution to total DPP activity in cell culture supernatants of other DPP enzymes expressed by lymphocytes, such as DPP8, DPP9, and 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 that is expressed in quiescent lymphocytes, is downregulated upon activation, and is 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 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, T_H2 cell differentiation in allergic asthma should lead to cells with slightly higher levels of CD26 (although lower than T_H1 or T_H17 lymphocytes), as well as enhanced concentrations of sCD26 in serum. Accordingly, Lun et al [18] found upregulation of CD26 in $CD4^+$ T cells from adult patients with allergic asthma, which we confirmed in the second cohort. Moreover, the authors also reported increased sCD26 in allergic asthmatics [18]. However, under pathological conditions, sCD26 has more frequently been reported to have decreased concentrations [23] than increased concentrations [39]. Indeed, we found a trend towards sCD26 downmodulation in allergic asthma. Similar sCD26 downmodulation has been reported in chronic eosinophilic pneumonia [34], while Remes et al [35] detected no differences in serum sCD26 levels in children with asthma and no association with atopy [35]. Therefore, although it is difficult to ascertain the reasons for these differences, since most studies (including ours) used the same ELISA kit (Bender MedSystems) for determination of CD26 concentration [18,35], at least in the study of Lun et al the results might be partially explained by a higher proportion of males or perhaps a more active disease status.

Contrary to findings from in vitro studies, values for circulating sCD26 in serum samples are not correlated with the number of lymphocytes, probably owing to a series of 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; and (*c*) release of sCD26 by adipose tissue or hepatocytes [40,41]. However, the third possibility is not supported by the present study owing to unaltered levels of CRP. Furthermore, lack of variation in overall adiposity and absence of differences in leptin levels between AAPs and HCs as found in the present study indicate that adipose tissue is not a major source of sDPP4/sCD26. Besides, a negative correlation has also been reported between sCD26 (higher in men [42,43]) and leptin (higher in women [44]), while no correlation was observed with BMI. Finally, CD26-specific ELISA revealed that sCD26 is downmodulated rather than augmented in asthma patients, contrary to what is expected in obesity-related asthma.

sCD25 is considered a T-cell activation marker, as it is increased during asthma exacerbations [5-8]. Surprisingly, we detected a significant reduction in sCD25 in the first asthmatic cohort and no changes in the second group. These differences are difficult to interpret, although one explanation could

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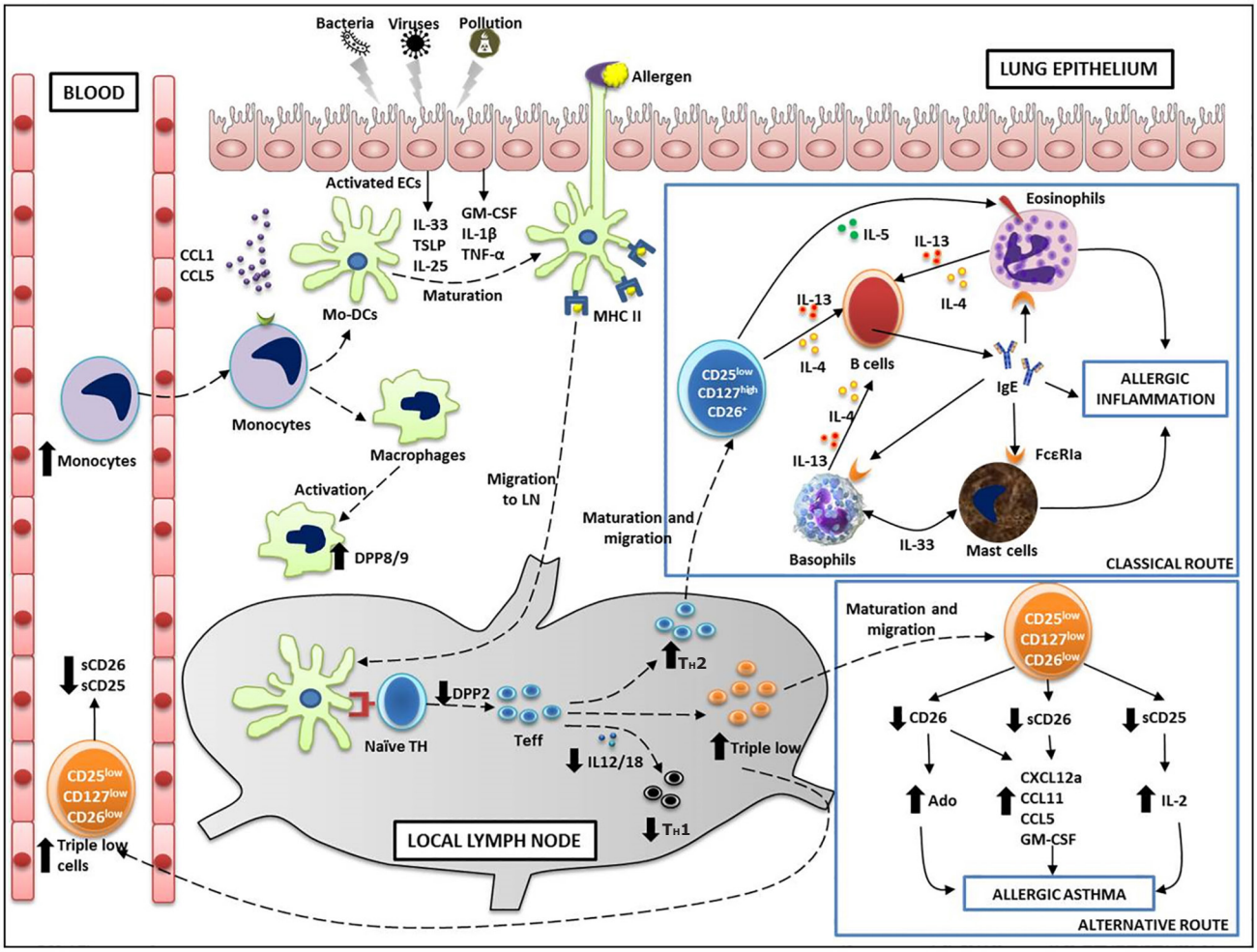

Figure 5. Major immunological pathways in the pathogenesis of allergic asthma. Epithelium damaged by pollution or infection leads to chemokine secretion by airway epithelial cells, enhancing trafficking of innate cells such as monocytes from blood to lung epithelium. Monocytes differentiate into macrophages, which increment expression of DPP8/9 upon activation, or into monocyte-derived dendritic cells (Mo-DCs), the last subset migrating to lymph nodes (LNs) upon maturation. Within the LNs, Mo-DCs present antigens to naïve T_H cells, which lost DPP2 and differentiated into $CD25^{low}CD127^{high}CD26^{+}$ T_H2 cells (classic route). On the other hand, our results also demonstrate the expansion of an unconventional $CD25^{low}CD127^{low}CD26^{low}$ T_H 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 (eg, CXCL12a/SDF-1α, CCL11/Eotaxin, or CCL5/RANTES) and adenosine (Ado) and exacerbate asthma. CCL indicates chemokine (C-C motif) ligand; TSLP, thymic stromal lymphopoietin; TH, helper T cell; GM-CSF, granulocyte-macrophage colony-stimulating factor; FcεRI, high-affinity IgE receptor.

be the presence of "stable" disease in our cohort compared with previous studies. In addition, these results show that the behaviour of sCD26 and sCD25 is similar. Indeed, both molecules could share the same cell origin even though they are not correlated. Thus, they are carried by vesicles or released by matrix metalloproteinase-9 (MMP-9)–mediated cleavage [22]. Moreover, our data support the possibility 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 upregulate and release CD25 following activation [45]. Therefore, the slight reduction in 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 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 have been described in allergic asthma [4,46,47]. However, we failed to detect a numerical alteration of Tregs, thus ruling out a link between this subset and alteration of sCD26/sCD25 levels in asthma. We also recorded increased percentages of Teff cells and reduced percentages of Tregs in women, thus explaining 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 3 surface markers and whose expansion causes the downmodulation of sCD26 and sCD25 in AAPs. Reduction of CD26/sCD26 could be important for T_H cells in abrogation of the inhibitory role of this molecule and the burst in cell

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proliferation, potentiating the activity of T_H2-chemokines (eg, CCL11/eotaxin), or favoring the proinflammatory effect of adenosine or substance P [17] (Figure 5). Thus, reduced DPP4 activity in cells or plasma could enhance the bioavailability of T_H2 (eg, 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].

Reversible competitive DPP4 activity inhibitors or "gliptins" are a novel class of small molecules for oral administration. They include the peptidomimetic and less specific vildagliptin (Galvus; EU, 2007) and saxagliptin (Onglyza; US FDA, 2010), and the nonpeptidomimetic, 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 have an excellent tolerability profile and have been introduced into clinical practice as a second-line therapy in type 2 diabetes mellitus to reduce inactivation of incretins. However, several mild adverse drug reactions (ADRs) have been reported, such as skin-related disorders, infections (eg, nasopharyngitis and upper respiratory tract infections), and respiratory disorders (eg, 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 (eg, allergic asthma with reduced extracellular sCD26 levels [see above]). 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 (eg, rhinovirus) or viral-derived dsRNA sensed by pattern-recognition receptors lead to asthma exacerbations in murine models of asthma induced by ovalbumin [52] or house dust mite [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 2 proteins linked to asthma: the soluble DPP2 (an enzyme that plays an important role 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 large number of substrates associated with the immune system, indicate that these drugs could be enhancing T_H2-mediated responses and the asthma-dependent downmodulation of sCD26 levels. Thus, gliptins have been proposed for the treatment of the autoimmune disease type 1 diabetes by, amongst other mechanisms, upregulating T_H2 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 patients with type 2 diabetes mellitus through the reduction in Treg cell percentages [57]. In the same sense, gliptins also favor the development of allergic rhinitis [49] and a noninflammatory variant of bullous pemphigoid, diseases where eotaxins and eosinophils play important roles [58,59]. In clear contrast, sitagliptin seemed to play a beneficial role in a chronic murine model of asthma induced by ovalbumin [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 that is coincident with a decrease in the levels of sCD26 and sCD25 in these patients. Reduction of sCD26 and CD26 levels on effector T_H lymphocytes might play an important role in improving 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 humanized anti-CD26 antibodies [61] in patients with a predisposition toward developing certain allergic hypersensitivity reactions (atopy).

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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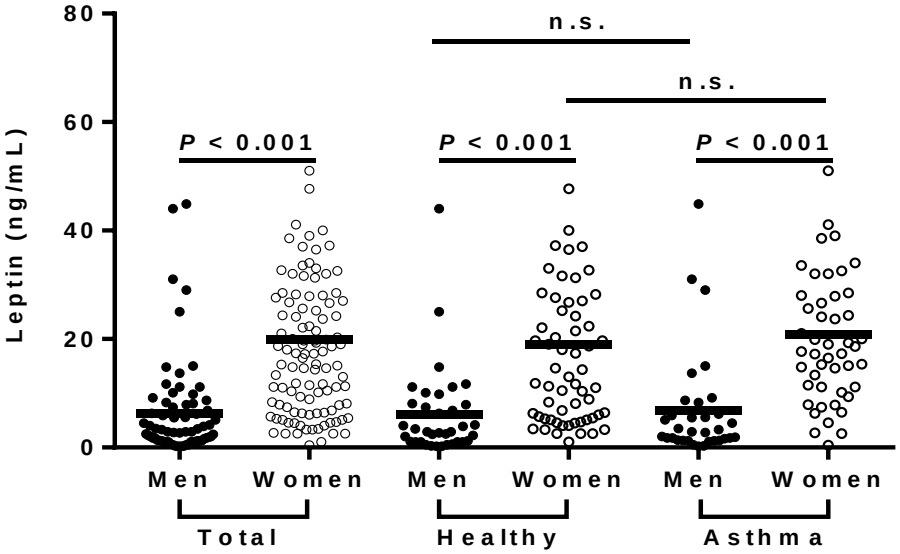

Figure S1. Serum leptin levels in allergic asthma according to gender. Leptin levels (ng/mL) according to gender in both healthy (n=97) and asthmatic (n=81) populations. Mann-Witney U statistic, numbers on the graph represent P-value.

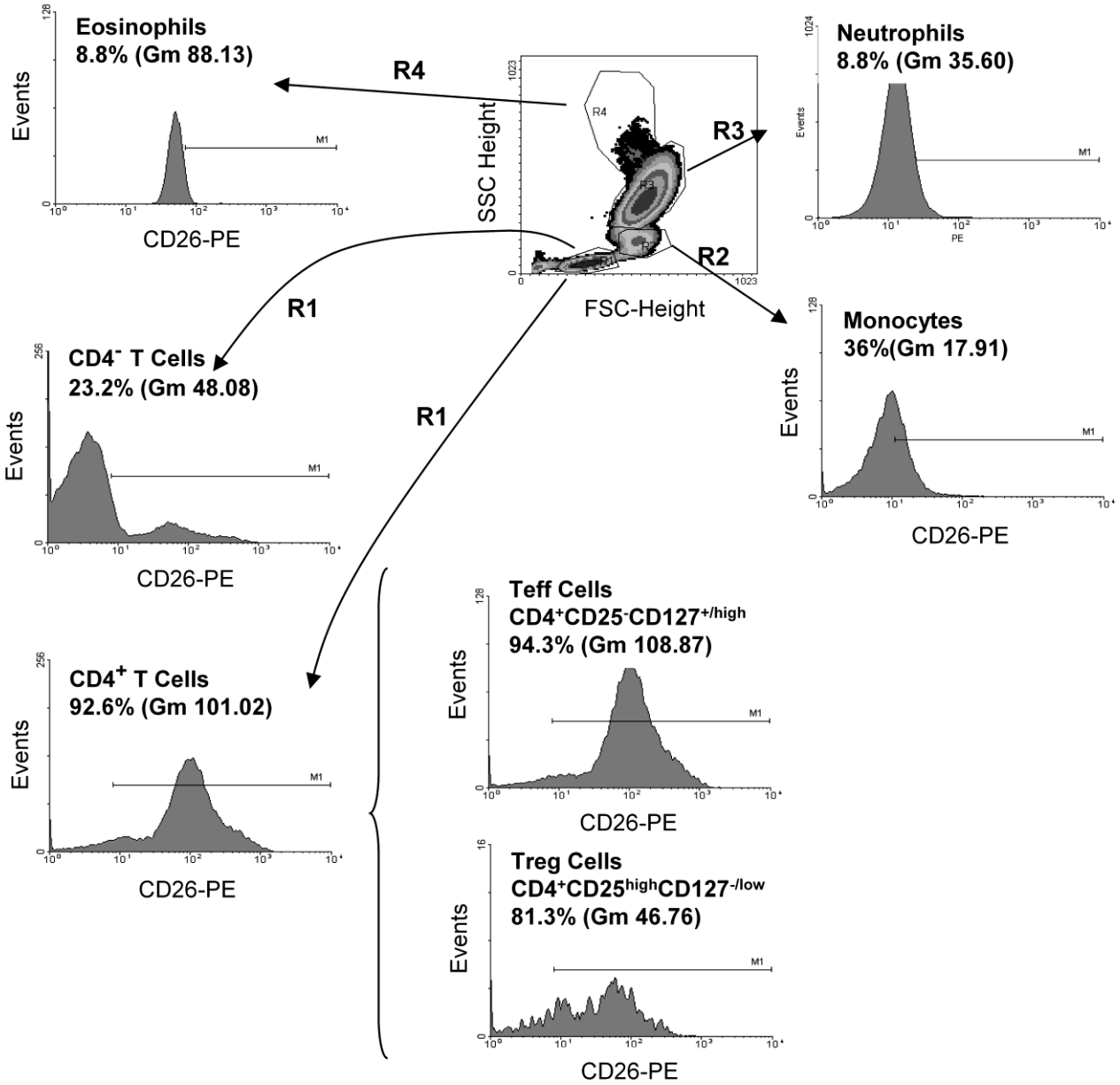

Figure S2. CD26 expression in major peripheral blood leukocyte subpopulations. Leukocytes was marked with CD4-FITC, CD25-PE-Cy7, CD127-AlexaFluor-647, CD26-PE or with isotype antibodies (IgG1κ-FITC, IgG1κ-PE-Cy7, IgGκ-Alexa 647 y IgGκ-PE). Dates are shown as dot plots "forward scatter" (FSC) versus "side scatter" (SSC), where different leukocyte subpopulations are represented (lymphocytes/R1, monocytes/R2, neutrophils/R3 and eosinophils/R4), and histograms, where mean fluorescence intensity (x-axis) is represented versus number of cells (y-axis) of every subpopulation (R1-R4). A representative result is shown.