

Original article

Increased production of IL-5 and dominant Th2-type response in airways of Churg–Strauss syndrome patients

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Abstract

Objective. Churg–Strauss syndrome (CSS) is a rare systemic vasculitis associated with eosinophilia and asthma. We assessed the local immune response in airways of CSS patients with different activity of the disease.

Methods. Concentration of IL-5, CCL17, CCL22 and CCL26 (ELISA) together with cell expression of T-helper-related genes (real-time PCR array) were measured in bronchoalveolar lavage fluid (BALF) sampled from 11 patients with active CSS, 11 patients with CSS in remission and 9 control subjects with bronchial asthma.

Results. In active CSS, both BALF and blood eosinophil counts were increased ($P < 0.01$). BALF cells in active disease were characterized by an increased expression of Th2 and regulatory-type transcripts: *STAT6*, *STAT3*, *GATA3*, *IL4*, *IL5* and *IL10* as compared with asthmatics, and *STAT5A*, *CCR4*, *FOXP3*, *IL4*, *IL5* and *IL10* when compared with inactive CSS. There was significant increase in BALF concentration of IL-5 and CCL26 in exacerbation of CSS. CCR4-active chemokines were detected more frequently in active disease. We found a strong positive correlation between clinical parameters of disease activity (BVAS, eosinophilia) and expression of *IL4*, *IL5*, *IL10* and *STAT5A*.

Conclusion. These results indicate that as compared with asthma, active-CSS patients have much stronger local Th2 response in the airways. Airway cells may contribute to lung eosinophilia in CSS by producing IL-5 and eosinophil active chemokines.

Key words: bronchoalveolar lavage fluid, chemokines, Churg–Strauss syndrome, interleukin-5, Th2 lymphocytes.

Introduction

Churg–Strauss syndrome (CSS) is a small-vessel necrotizing vasculitis associated with asthma, high peripheral blood and tissue eosinophilia and granuloma formation [1–3]. The mechanism of eosinophilic accumulation in CSS has not been elucidated, although it is likely a

consequence of enhanced eosinopoiesis and prolonged survival, both depending on IL-5 and other eosinophil-specific mediators that seem to be produced at the site of inflammation [4]. Studies on allergic diseases have revealed that activated Th2-type helper cells are the major source of IL-5 [5]. Thus it is likely that in CSS, a yet unknown antigen may induce a strong Th2-type immune response with subsequent overproduction of eosinophil-active mediators [6]. Indeed, the exacerbation of CSS is associated with increased plasma levels of IL-5 and chemokines associated with Th2-type response [7–9]. The role of T-helper lymphocytes in CSS has only been investigated in peripheral blood. In our previous study we detected both increased numbers of Th17 cells and higher levels of Th2-type chemokines, suggesting that both subsets of

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T cells could be involved in the pathogenesis of CSS [7]. Little is known, however, of whether similar combined responses occur within the affected tissue. Additionally, there are limited data on the local production of mediators acting on eosinophils. Although some investigators detected CCL26 and CCL17 in tissue biopsies from active CSS, the quantitative data are scarce [8, 10]. It is mainly due to difficulty in the collection of standardized samples in larger cohorts of CSS patients with an active disease. Therefore, in the current study, we focused on immune responses in peripheral tissues of CSS patients with disease exacerbation, and chose to study bronchoalveolar lavage fluid (BALF) cells, as they reflect local inflammation in the airways and can be easily sampled.

We hypothesized that pulmonary exacerbation of CSS may be associated with a local increase in Th2-type response and production of eosinophil-active mediators. To test this hypothesis, we analysed differential cell counts, concentrations of cytokines and expression of T-helper signature genes in BALF sampled from patients with different CSS activity and control subjects with bronchial asthma.

Patients and methods

Patients and study design

We studied 11 patients with active CSS (CSS diagnosed according to American College of Rheumatology criteria [11]), 11 patients in disease remission (inactive CSS) and 9 mild to severe asthmatic patients (control group). Sixteen patients (73%) were investigated in our earlier project [7], yet for the current study, they were sampled at different stages of the disease. Activity of the disease was evaluated using BVAS [12]. Disease exacerbation was defined as a BVAS score ≥ 3 and blood eosinophilia

≥ 800 cells/ μ l. Asthma severity was established according to the guidelines of the National Heart, Lung and Blood Institute [13]. Organ symptoms characteristic for CSS, as evidenced by medical records, imaging techniques or tissue biopsies were similarly distributed between the groups: 100% respiratory tract, 59% heart involvement, 64% mono- or polyneuropathy, 73% skin lesions and 23% digestive tract symptoms. Clinical features of the patients are summarized in Table 1. The study was approved by the Jagiellonian University Ethics Committee, and informed consent was obtained from all the participants.

BALF preparation

BALF was sampled during fiberoptic bronchoscopy performed according to British Thoracic Society guidelines [14]. BALF samples were spun down to collect cells. Supernatants were concentrated 8-fold by centrifugation through 3 kDa membranes (Nanosep, Ann Arbor, MI, USA) and frozen for further measurements. After preparation of cytospin slides (Thermo Scientific, Waltham, MA, USA), the remaining cells were collected to extract RNA (Total RNA Isolation Kit, A&A Biotechnology, Gdynia, Poland). In 18 individuals (CSS: $n = 13$, asthma: $n = 5$) with adequate recovery of samples, BALF cells were also cultured *in vitro* ($0.1\text{--}0.5 \times 10^6$ cells per sample) for 18 h in 10% fetal bovine serum-supplemented RPMI-1640 (Sigma-Aldrich, St Louis, MO, USA). Cell lysates and culture supernatants were stored at -80°C for further measurements.

Transcriptome profile of BALF cells

The focused transcriptome profile of BALF cells was analysed in cDNA samples by real-time PCR (7900HT System, Applied Biosystems, Foster City, CA, USA) in eight subjects from each study group using custom-designed TaqMan

TABLE 1 Clinical characteristics of the subjects studied

	Active CSS ($n = 11$)	Inactive CSS ($n = 11$)	Asthma ($n = 9$)
Age, years	41 (33–52)	42 (41–46)	55 (37–60)
Sex, females, n (%)	5 (45)	9 (81)	7 (78)
Disease duration, years	2 (0.5–3)	2 (1–4)	NA
ANCA positive, n (%) ^a	1 (9)	4 (36)	0
Blood eosinophilia (cells/ μ l)	1254 (883–4106)	128 (89–587)**	31 (9–128)**
BVAS (score)	10 (6–24)	0 (0–1)**	NA
Asthma severity, n (%) ^b			
Mild/moderate	7 (64)	7 (64)	2 (44)
Severe	4 (36)	4 (36)	5 (56)
Systemic steroids, n (%)	7 (64)	11 (100)	6 (67)
Systemic steroids, mg/d ^c	4 (0–8)	8 (6–16)	12 (0–16)
Immunosuppressive drugs, n (%)	0	3 (27)	0
Serum IgE, IU/ml	52 (42.4–215)	54.8 (38.6–90.1)	22.1 (18.7–127)
Serum CRP, mg/ml	3.9 (<3.1–6.1)	3.1 (<3.1–3.8)	3.1 (<3.1–3.6)
Serum IL-5, pg/ml	14.8 (9.8–20.7)	6 (5.5–9.5)**	NA

Data are presented as median (IQR) unless indicated otherwise. NA: not applicable. ^aIn all, 23% of patients were ANCA positive. It is within the range reported for European cohorts of CSS patients. ^bAsthma severity established according to the guidelines of NHLBI [13]. ^cSystemic steroid daily dose recounted for methylprednisolone. ** $P < 0.01$ as compared with active CSS.

Low-Density Arrays (Applied Biosystems). In the transcriptome panel, we included genes encoding signature cytokines (*IFNG*, *IL4*, *IL5*, *IL17A*, *IL17F*, *IL10*, *IL22*, *TGFB1*), receptors (*CD4*, *IL23R*, *CCR4*, *CCR6*, *CXCR3*, *CXCR6*) and transcription factors (*STAT3*, *STAT4*, *STAT5A*, *STAT6*, *GATA3*, *TBX21*, *RORC*, *FOXP3*). Data were normalized to *GAPDH* and relative quantities (RQ) of individual transcripts were estimated using the $2^{-\Delta\Delta CT}$ method, as previously described [3]. Expression of chemokine genes (*CCL5*, *CCL17*, *CCL22*, *CCL24*, *CCL26*, *CXCL5*, *CXCL8*, *CXCL10*) was analysed by quantitative real-time PCR using specific primers (TIB Molbiol, Poznan, Poland) and SYBR-Green I (Amresco, Solon, OH, USA). PCR products were visualized with standard imaging techniques (Amersham Pharmacia, Uppsala, Sweden).

Measurement of cytokine concentrations in BALF samples

Levels of IL-5, CCL17 (thymus and activation-regulated chemokine), CCL22 (macrophage-derived chemokine) and CCL26 (eotaxin-3) were measured by ELISA (Quantikine Human ELISA Kits, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was determined using GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA, USA). All data in the text and tables are shown as median and interquartile range (IQR), if not otherwise stated. Horizontal bars on graphs represent means (S.E.M.). The Mann-Whitney U-test was used in two-group comparison. Differences between three groups were analysed using ANOVA or the Kruskal-Wallis test. Spearman's rank sum test was used to analyse correlation coefficients. $P < 0.05$ was considered significant.

Results

Increase in BALF eosinophilia in active CSS

Total BALF cell count did not differ between CSS patients and asthmatics. Active-CSS patients were characterized by a significant increase in the percentage of BALF eosinophils and a concomitant decrease in the percentage of macrophages as compared with other groups (Table 2).

The percentage of BALF eosinophils positively correlated with blood eosinophilia ($r = 0.75$), serum IL-5 ($r = 0.76$) and disease activity score (BVAS, $r = 0.81$). No such correlation was found for lymphocytes or neutrophils.

Th2 dominant transcriptome profile of BALF cells in active CSS

To determine the profile of adaptive immune responses in CSS airways, we analysed the BALF cell expression of 22 T-helper cell-related genes. Out of eight signature cytokines analysed, mRNA levels of *IL4*, *IL5* and *IL10* were significantly increased in active CSS as compared with disease in remission or asthmatics, whereas *IFNG* tended to be upregulated in CSS independently of disease activity (Fig. 1). Products of *IL17A* and *IL22* genes were present only in three individuals, whereas *IL17F* transcript was below the threshold of detection. Active-CSS patients were also characterized by a significant increase in expression of *STAT3*, *STAT6* and *GATA3* as compared with asthma, and *STAT5A*, *FOXP3* and *CCR4* as compared with inactive CSS (Fig. 2). We found a positive correlation between the percentage of BALF eosinophils and the RQ of *IL4* ($r = 0.6$), *IL5* ($r = 0.69$), *IL10* ($r = 0.7$) and *STAT5A* ($r = 0.62$) transcripts. Expression of these genes significantly correlated with clinical markers of disease activity (BVAS), blood eosinophilia and serum IL-5 ($r > 0.6$ in each comparison). Altogether, these results suggest that Th2-type cytokine transcripts (IL-4, IL-5, IL-10) dominate in BALF cells from active-CSS patients and are accompanied by an up-regulation in expression of other Th2 and regulatory-type genes.

Increased concentration of IL-5 and CCL26 in BALF from active CSS

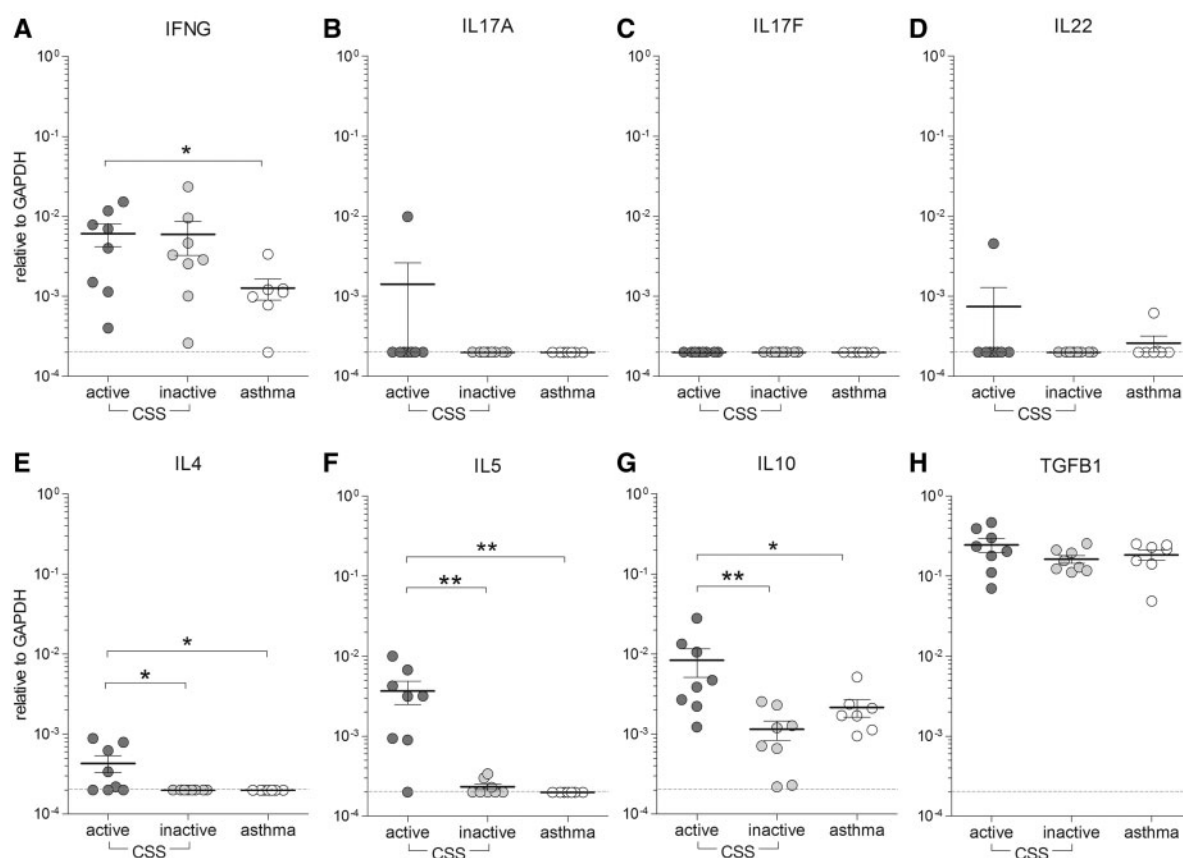
Next we determined BALF concentrations of IL-5 and Th2-type chemokines. The level of IL-5 was increased in active CSS as compared with disease remission ($P < 0.05$) and the asthma group ($P < 0.01$) (Fig. 3). We found a significantly elevated concentration of CCL26 ($P < 0.05$) and a trend ($P = 0.1$) towards increase in the level of CCL22 in BALF from patients with active disease (Fig. 4). The levels of CCL17 were mostly below the detection limit, although positive results were seen in the CSS group only (Fig. 4A). We observed a significant correlation between the concentration of IL-5 in BALF with both blood eosinophilia

TABLE 2 Bronchoalveolar lavage fluid differential cell count

	Active CSS (n = 11)	Inactive CSS (n = 11)	Asthma (n = 9)
Total BALF cells ($\times 10^3/\text{ml}$)	18.6 (10.5–42.9)	15.9 (10.6–29.4)	28.5 (9.6–44.7)
Macrophages, %	77 (50–83)	92 (88–97)*	92 (89–96)**
Lymphocytes, %	6 (2–12.5)	4.5 (1.5–10)	5.5 (3.5–8.5)
Neutrophils, %	1.5 (1–2)	3 (1.5–5)	1 (0.5–2)
Eosinophils, %	16.5 (5–29)	0 (0–1)**	0.5 (0–1)**
Eosinophil count ($\times 10^3/\text{ml}$)	3.06 (0.41–21.5)	0 (0–0.24)**	0.09 (0–0.46)**

Data presented as median (IQR). * $P < 0.05$, ** $P < 0.01$ as compared with active CSS.

Fig. 1 Relative abundance of cytokine transcripts (compared with GAPDH reference gene) in CSS patients and asthmatics.



(A) Interferon- γ (*IFNG*), (B) IL-17A (detected only in one subject), (C) IL-17F (not detected), (D) IL-22 (detected in two subjects), (E) IL-4, (F) IL-5, (G) IL-10 and (H) TGF- β 1 (*TGFB1*). Dashed lines indicate the limit of detection ($\Delta C_t < 12.3$). Horizontal bars on graphs represent means. * $P < 0.05$, ** $P < 0.01$.

($r = 0.64$) and BALF eosinophilia ($r = 0.5$). As expected, the concentration of IL-5 in BALF supernatants correlated strongly both with serum levels of IL-5 ($r = 0.68$) and the expression of *IL5* gene in BALF cells ($r = 0.72$).

BALF cells of CSS patients express only low amounts of Th2-type chemokines

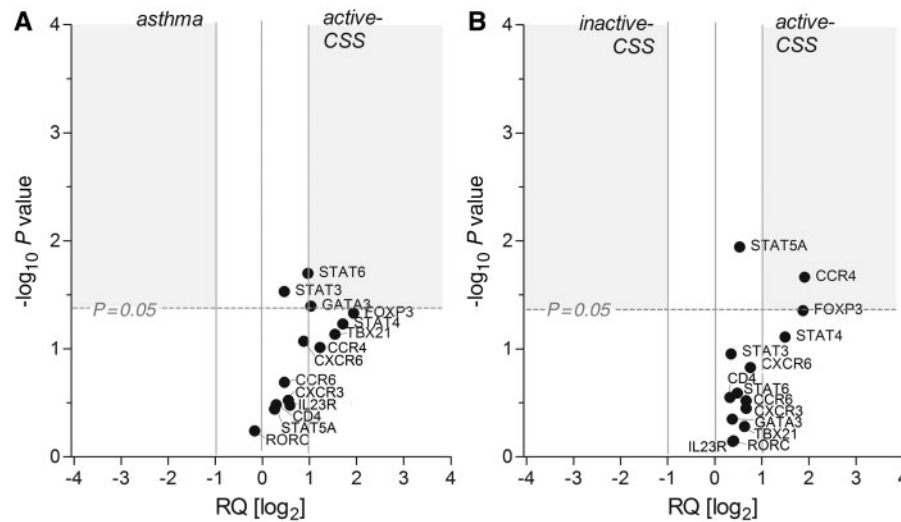
Finally, we investigated whether the Th2-type chemokines found to be elevated in CSS could be produced by inflammatory cells present in BALF. Expression of mRNA of selected chemokine genes was analysed in BALF cells. Transcripts of CCR4-active chemokines (CCL17 and CCL22) were detected at a very low level in three CSS subjects and two asthmatics, whereas CCL26 was found in only one asthmatic (supplementary data Fig. 1, available as supplementary data at *Rheumatology* Online). This observation was corroborated by ELISA; as in the majority of cell culture supernatants, Th2-type chemokine levels were below the detection limit of the assay (data not shown). In contrast, CCL5, CXCL5 and CXCL8, which are

produced by a variety of innate immunity cells present in BALF (e.g. macrophages), were expressed in large quantities (supplementary data Fig. 1, available at *Rheumatology* Online).

Discussion

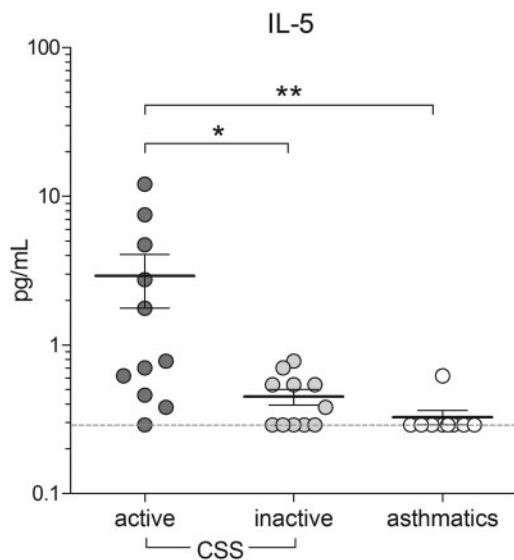
The presence of high blood and tissue eosinophilia allows CSS to be distinguished from other vasculitic disorders, and suggests the existence of a specific molecular mechanism responsible for their selective recruitment. Among numerous mediators regulating eosinophil biology, IL-5 is unique because it is crucial for both eosinophilic lineage differentiation, their activation and sustained viability in the periphery. Therefore an increase in serum IL-5, which occurs in active CSS, usually correlates with blood eosinophilia and decreases rapidly following the administration of immunosuppressive therapy [7, 15–17]. However, peripheral blood lymphocytes from CSS patients do not secrete higher amounts of IL-5 [18, 19]. Rather, during the active phase of the disease, the production of IL-5 could be

Fig. 2 Volcano plots showing the differences in BALF cell expression of T-helper-related genes between active-CSS patients and other groups.



Lines indicate thresholds of statistical significance ($P < 0.05$) and a 2-fold change in the relative quantity of a given transcript ($RQ < -1$ and > 1). **(A)** Active CSS vs asthmatics. Genes significantly upregulated in active CSS: *GATA3*, *STAT3* and *STAT6*. **(B)** Active CSS vs inactive CSS: genes significantly upregulated in active CSS: *FOXP3*, *CCR4* and *STAT5A*.

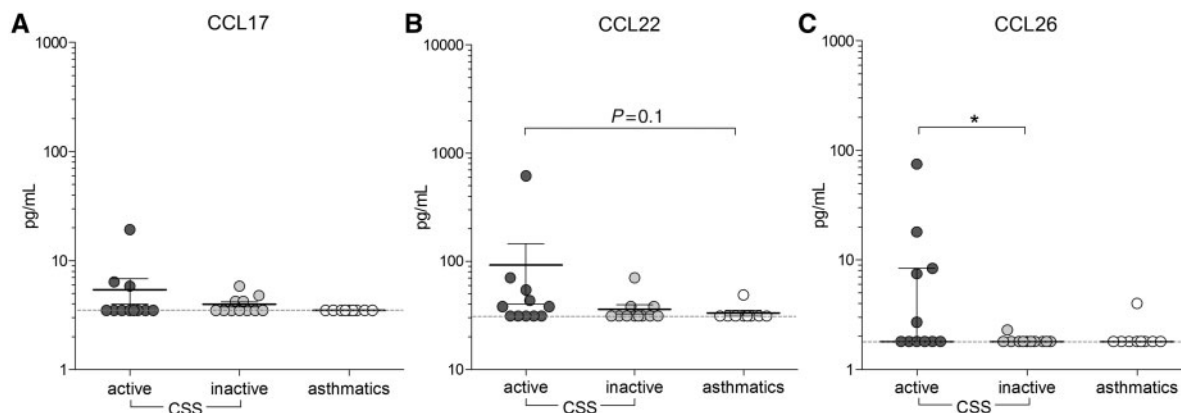
Fig. 3 Concentration of IL-5 in BALF supernatants of CSS patients and asthmatics.



The dashed line indicates the limit of detection. Horizontal bars on graphs represent means. * $P < 0.05$, ** $P < 0.01$.

restricted to the affected tissue. But apart from one small study, where IL-5 was detected in BALF samples from two active-CSS patients [16], there is limited evidence on the secretion of IL-5 at the site of CSS-related lesions. This is of special importance in a context of recent reports indicating a beneficial role of anti-IL-5 therapy in CSS [20].

In the current study we evaluated the production of IL-5 at both the mRNA and protein level in BALF samples isolated from CSS patients. We observed a marked increase in expression of the *IL5* gene and the concentration of IL-5 in BALF from active CSS, as compared with inactive disease and asthma. Additionally, products of the *IL5* gene correlated well with clinical markers of disease activity, and more importantly, with BALF eosinophilia. Our results indicate that expression and release of IL-5 is elevated in the airways of active-CSS patients. In the broader analysis of BALF cell transcriptome, active CSS was characterized by increased expression of transcription factors (*STAT3*, *STAT6*, *FOXP3* and *GATA3*) and two other cytokines (*IL10*, *IL4*) in comparison with asthmatics. Most of these genes are required for Th2 function and are engaged in the development of allergic inflammation. This is in line with previously reported data showing upregulation of *STAT6* and *GATA3* in peripheral blood lymphocytes from CSS patients [7]. Interestingly, when data from active-CSS and inactive-CSS patients were compared, we found upregulation of not only Th2-type cytokines, but also *FOXP3*, *STAT5A* and *CCR4* in the active phase of the disease. The increase in local expression of *FOXP3* could be a consequence of its upregulation in recently activated T cells or Treg reprogramming, a phenomenon observed in experimental models of autoimmune disorders [21]. Transcription factor *STAT5A*, apart from regulating both Th2 and Treg development, is also engaged in IL-5 signalling and contributes to eosinophil differentiation and tissue homing [22, 23]. In line with this, our results show a significant positive correlation of *STAT5A* expression with BALF eosinophilia. Summarizing, our data

Fig. 4 Expression of Th2-type chemokines in BALF samples.

(A–C) The concentration of CCL17 (A), CCL22 (B) and CCL26 (C) in BALF supernatants of CSS patients and asthmatics. Dashed lines indicate the limit of detection. Horizontal bars on graphs represent means. $*P < 0.05$.

suggest a strong shift towards Th2 response in the airways of active-CSS patients, and emphasize the role of STAT5A signalling in regulation of lung eosinophilia.

Selective recruitment of eosinophils to the site of inflammation is regulated by Th2-type mediators that activate resident tissue cells to express specific adhesion molecules and chemokines. Eotaxins are CCL-type chemokines regulating eosinophil migration into mucosal sites [24]. Of three known human eotaxins, only CCL26 (eotaxin-3) was found to be specifically elevated in sera from active-CSS patients and correlated with the number of blood eosinophils and inflammatory markers [9, 10]. CCL26 was also detected in peripheral nerve and mucosal biopsies at the onset of CSS [10]. In our study we demonstrated significantly higher levels of CCL26 in BALF samples from active CSS. Binding of CCL26 to CCR3 receptor expressed on resting eosinophils can drive their recruitment into airway tissues. Additionally, it has been found that eotaxin-3 is the only CCR3 ligand significantly induced by Th2-type cytokines in airway epithelial cells [25]. On release, CCL26 can bind to their surface and thus facilitates transepithelial migration of eosinophils. Nevertheless, CCR3 is not present on Th2 cells, and eotaxins do not seem to participate in their recruitment [26].

In search for mediators regulating Th2 influx, we focused on another two chemokines, CCL17 and CCL22, which act on CCR4 receptor expressed primarily by effector Th2-helper cells and activated eosinophils [27]. We speculated that CCR4-acting chemokines could not only drive Th2-helper cell recruitment but may also facilitate eosinophilic infiltration. In our study, BALF levels of CCL17 and CCL22 were usually below the detection limit of the assay; however, a few positive results were observed, mostly in active CSS. Additionally, mRNA transcripts of CCL17, CCL22 and also CCL26 were virtually absent in BALF cells. These observations suggest that Th2-type chemokines could be produced by airway wall resident cells (e.g. epithelial cells) and are released primarily to the surrounding tissues rather than the airway compartment. This is in line with *in vitro* studies showing that airway epithelial

cells produce increased amounts of CCL17 and CCL26, particularly when stimulated with proinflammatory cytokines [25, 28]. Therefore both CCR4-type chemokines and CCL26 levels are usually increased in sera from active-CSS patients [7, 8, 10]. Lower levels of Th2-type chemokines in BALF may also result in part from the dilution of airway secretions during the sampling procedure. Altogether, these data suggest that airway epithelial cells could represent an important source of Th2-type chemokines during CSS-related eosinophilic inflammation in the airways. Nevertheless, further experiments using an epithelial cell culture model are required to verify such a hypothesis.

In conclusion, we observed a strong shift towards Th2 immune response in BALF samples of active CSS patients, with significantly higher expression of Th2-related genes and increased levels of locally produced IL-5. Our results suggest that Th2-type chemokines (eotaxin-3) may facilitate the recruitment of effector cells to the airways, thus amplifying the inflammatory response in CSS. These results confirm earlier reports on a potential use of IL-5 and CCL26 as biomarkers of CSS-related eosinophilic inflammation. Additionally, our results suggest that anti-IL-5 or chemokine receptor targeted therapies may have a beneficial effect in refractory or relapsing CSS.

Rheumatology key messages

- Airway inflammation in CSS patients is characterized by a strong shift towards a Th2 immune response.
- Expression of IL-5 is increased in the airways of patients with active CSS.

Supplementary data

Supplementary data are available at *Rheumatology* Online.

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