Both Th2 and Th17 responses are involved in the pathogenesis of Churg-Strauss syndrome

B. Jakiela, M. Sanak, W. Szczeklik, B. Sokolowska, H. Plutecka, L. Mastalerz, J. Musial, A. Szczeklik

Department of Medicine, Jagiellonian University Medical College, Krakow, Poland.

Bogdan Jakiela, MD, PhD
Marek Sanak, MD, PhD
Wojciech Szczeklik, MD, PhD
Barbara Sokolowska, MD, PhD
Hanna Plutecka, PhD
Lucyna Mastalerz, MD, PhD
Jacek Musial, MD, PhD
Andrzej Szczeklik, MD, PhD

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Reprints are not available from the authors.

Please address correspondence to:
Andrzej Szczeklik, MD, PhD,
Department of Medicine,
Jagiellonian University Medical College,
ul. Skawinska 8,
31-066 Krakow, Poland.
E-mail: mmszczek@cyf-kr.edu.pl

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ABSTRACT

Objectives. *Churg-Strauss syndrome (CSS) is a rare systemic vasculitis associated with eosinophilia and granuloma formation. The contribution of in-dividual T-helper cell lineages in pathogenesis of CSS is unknown. We hypothesised that in CSS an imbalance of major effector T-cell subpopulations takes place, and is further influenced by the mode of treatment.*

Methods. *We investigated the immunophenotype, cytokine production and transcriptome profile in peripheral blood lymphocytes (PBL) from 19 patients with stable CSS (10 were treated with glucocorticoids alone (CSS/GC), 9 with steroids and other immunosuppressive drugs (CSS/IS)), and 13 healthy controls. Furthermore, serum IL-5 and CCR4-active chemokines (CCL17, CCL22) were measured in six patients with active disease and upon remission.*

Results. *All CSS patients had decreased percentage of FoxP3+ regulatory T cells. In the CSS/GC group we found an increase in the Th17/Treg ratio and up-regulation of both Th2 and Th17 markers as evidenced by (1) over expression of Th2-related genes (GATA3, STAT6) in PBL, (2) elevated concentrations of serum IL-5 and CCL17, and (3) a concomitant increase in the number of Th17 cells, and secretion of IL-17A by stimulated PBL. The level of CCR4-active chemokines was increased in active-CSS, and correlated with blood eosinophilia. The combined treatment with steroids and other immunosuppressive drugs was associated with a significant decrease in both Th2-related chemokines and the number of Th17 cells.*

Conclusion. *Our results indicate that both Th2 and Th17 lineages are involved in the pathogenesis of CSS, while CCR4-active chemokines contribute to eosinophilia in the active disease. These phenomena are down regulated by immunosuppressive therapy.*

Introduction

Churg-Strauss syndrome (CSS) is characterised by asthma, necrotising vasculitis, peripheral blood and tissue eosinophilia, and granuloma formation (1-3). Histological lesions typical for CSS constitute of small-vessel vasculitis with adjacent eosinophil rich infiltrates (4). The mechanism of tissue eosinophil accumulation has not been fully explained, though it is likely a consequence of T-helper (Th)2 mediated release of IL-5 and eosinophil specific chemokines (5, 6). Nonetheless, it does not fully explain the mechanisms leading to vasculitis or autoimmunisation.

The classical view of Th2 and Th1 adaptive immune responses has been recently challenged by the discovery of Th17 cells, which differentiate from naïve T cells upon stimulation with mediators of innate immunity, and produce large amounts of potent proinflammatory cytokines IL-17A, IL-17F and IL-22 (7, 8). This picture is even more complicated, as the function of effector T-helper cells, apart from their reciprocal interactions, is also controlled by regulatory T-lymphocytes (Treg), secreting anti-inflammatory TGF-β and IL-10 (9).

It has been established that sustained activation of effector T-cell responses, while meeting defective suppressory mechanisms, can drive the onset of allergy, autoimmunity or other chronic inflammatory diseases. Similar dysregulation could be responsible for the development of CSS (1, 3). Indeed, a recently published study described the imbalance between Th17 lymphocytes and regulatory T cells during active phase of the disease (10). The contribution of Th17 cells to the eosinophilic inflammation is still not well understood. Furthermore, not all CSS patients express high levels of IL-5 (11). Additionally, a proportion of CSS patients experience disease flares despite steroid treatment, and little is known

whether Th17 cells or other mediators could drive eosinophilia in these cases. Therefore, in the current study, we aimed to analyse functional subsets of CD4+ lymphocytes, expression of T-helper cell genes, and the profile of secreted cytokines in patients with CSS. We also evaluated the impact of immunosuppressive therapy by comparing patients treated only with steroids with those receiving additional immunosuppressive agents.

Methods

Patients and study design

Nineteen patients with confirmed diagnosis of CSS (fulfilling at least 4 criteria of the American College of Rheumatology (2)) were studied. The severity of the disease was evaluated using the Birmingham Vasculitis Activity Score (BVAS) (12). Eleven sex- and age-matched healthy non-atopic volunteers served as controls. The study was approved by the ethics committee of the Jagiellonian University. Informed consent was obtained from all participants.

Isolation of peripheral blood lymphocytes

Peripheral blood mononuclear cells (PBMC) were isolated from EDTA-anticoagulated blood by Histopaque 1.077 (Sigma-Aldrich, St. Louis, MO) gradient centrifugation. PBMC were resuspended in RPMI-1640 (Sigma-Aldrich) with 5% human AB serum (ABS, Lonza, Basel, Switzerland) and incubated for 30 min at 37° C in a plastic flask in order to deplete monocytes. Non-adherent PBL (peripheral blood lymphocytes) were cryopreserved for further experiments up to one month.

Lymphocyte immunophenotyping

PBL were washed in phosphate buffered saline (PBS) and triple stained for 30 minutes with labelled monoclonal antibodies (all from BD Biosciences) to identify subpopulations of peripheral blood lymphocytes: CD45, CD3, CD4, CD8, CD45RA, CD45RO. To identify regulatory T cells, PBL were first labelled with PerCP-CD4 and PE-CD25, and then fixed (FACS Lysing Solution, BD Biosciences), permeabilised (0.2% saponin, Sigma-Aldrich), and intracellulary stained with FITC-anti-FoxP3 (eBiosciences, San Diego, CA). Samples were washed with PBS or Perm/Wash buffer (BD Biosciences) and analysed by flow cytometry (Coulter EPICS XL, Beckman Coulter, Fullerton, CA). Data were presented as the percentage of the marker-specific fraction of lymphocytes in comparison to the relevant isotype controls. Treg cells were recognised as CD4+ cells expressing $CD25^{high}$ and FoxP3+ protein.

Stimulation of peripheral blood lymphocytes and detection of intracellular cytokines

Frozen PBL were thawed, washed in RPMI-1640 (10% ABS) and pre-cultured for 2 hours (2x 10^6 cells per well) before assessment of viability (>98% as verified by flow cytometry) and addition of stimuli. PBL were then left untreated (baseline control) or incubated for 5 hours with PMA (final 50ng/mL; Sigma-Aldrich) and ionomycin (0.7μg/mL; Sigma-Aldrich) in the presence of brefeldin-A (10μg/mL; Sigma-Aldrich). Cells were washed in PBS and labelled with PerCP-CD4 (all antibodies from BD Biosciences, if not otherwise stated). Samples were fixed and permeabilised with Cytofix/Cytoperm Kit (BD Biosciences) and double-stained for 30min with appropriate monoclonal antibodies: FITC-IFN-γ, PE-IL-4, PE-IL-17A (eBiosciences), and PE-IL-5. Samples were washed in Perm/Wash buffer and analysed by flow cytometry. Specificity of cytokine staining was confirmed with relevant isotype controls. Cytokine expressing CD4+ cells were classified as Th1 (producing only IFN-γ), Th2 (producing only IL-4), IL-4+ (all cells producing IL-4), Th17 (producing IL-17A, but not IFN-γ), Th1/17 (producing both IL-17A and IFN-γ), and IL-5+ (all cells producing IL-5). Representative dot plots and gating strategy used in identification of individual phenotypes are presented in Figure 1.

Expression of lymphocyte differentiation genes

The transcriptome profile was analysed in PBL (2.5x10^6 cells per sample) in control conditions or upon stimulation with PMA (50ng/mL) and ionomycine (0.7μg/mL) for 5 or 48 hours. Culture supernatants were frozen for cytokine measurements. Total RNA was isolated from pelleted cells with Total RNA Isolation Kit (A&A Biotechnology, Gdynia, Poland) and reverse transcribed (HC Reverse Transcription KIT, Applied Biosystems, Foster City, CA). Relative expression of mRNA was quantified with real time-PCR using target specific primers (TIB Molbiol, Poznan, Poland; see Supplementary Table S1) and SYBR-Green I (Amresco, Solon, OH), using iCycler System (BioRad, Hercules, CA). Data were normalised to the mean C_T (threshold cycle) value of *GAPDH* and *ACTB*, and relative quantities (RQ) of individual transcripts were estimated using $2^{-\Delta\Delta CT}$ method (13). In volcano graphs, $\log_2$ RQ values (fold change, biological significance) are plotted against $-\log_{10}$ *p*-values (statistical significance) estimated using the *t*-test.

Measurement of cytokine concentrations in sera and culture supernatants

Serum samples and culture supernatants were stored at -80° C. Levels of IL-5, IL-17A, C-C motif chemokine 17 (CCL17) and C-C motif chemokine 22 (CCL22) were measured by ELISA (Quantikine Human ELISA Kits, R&D Systems, Minneapolis, MN) according to the manufacturer instruction.

Statistical analysis

The statistical analysis was performed using GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA). All data were presented as medians and IQR (interquartile range). The between-group comparison was determined using the Mann-Whitney U- or the Friedman test. Differences between three groups were analysed with the ANOVA or the Kruskal-Wallis test. Spearman's rank sum test was used to analyse correlation coefficients. For convenience, in correlation statistics real-time PCR data were transformed according to formula: $\Delta C_{T(calc)} = -1 \bullet \Delta C_T$, therefore positive value of (*r*) coefficient indicates the presence of positive correlation between mRNA level and given variable. A *p*-value <0.05 was considered significant.

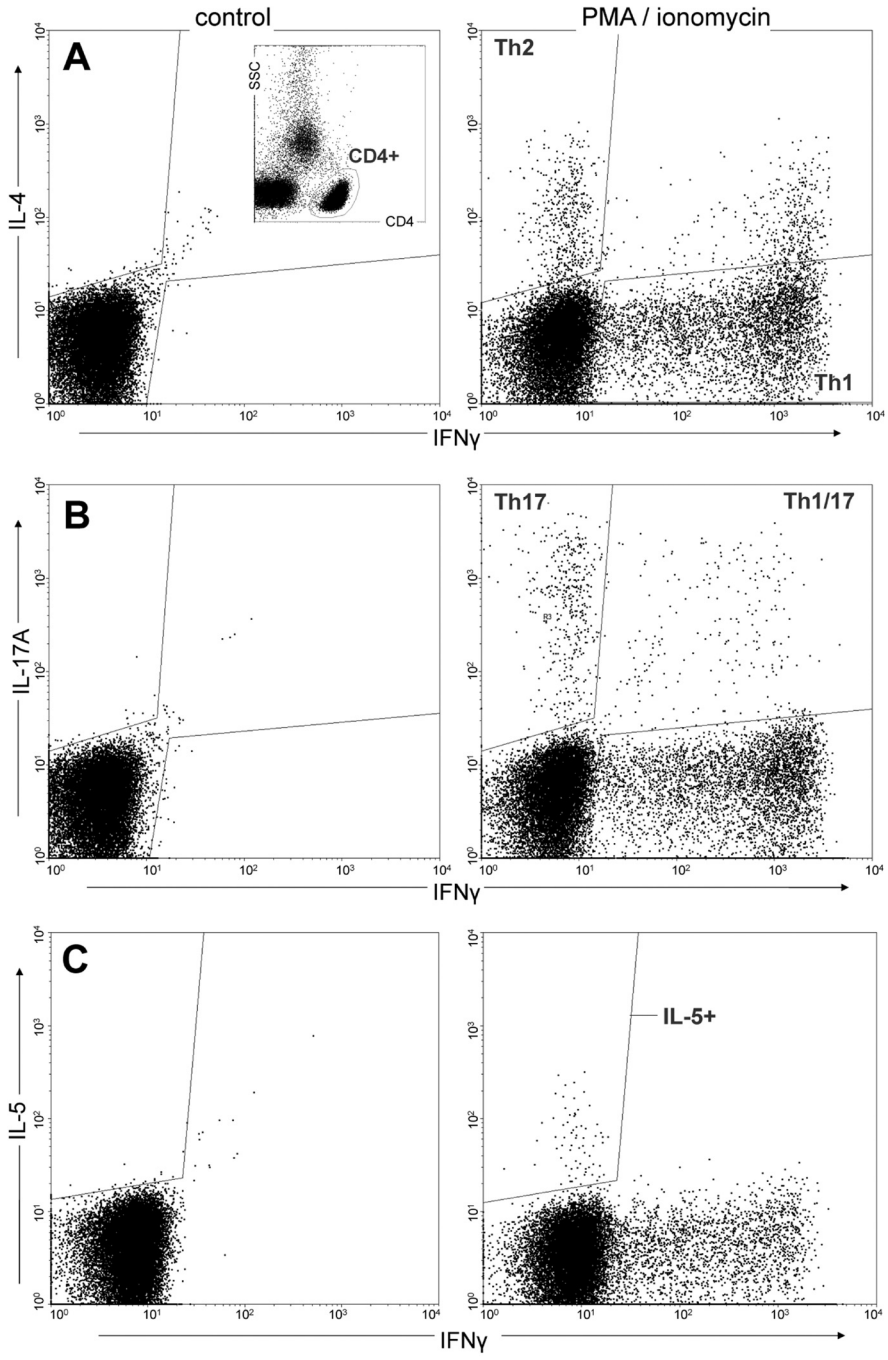

Fig. 1. Representative dot-plots showing the expression of cytokines in control or PMA/ionomycin stimulated CD4+ T cells (CSS patient no.6). **A**. Staining for IL-4 and IFN-γ to distinguish Th1 (only IFN-γ) and Th2 (only IL-4) cells, CD4+ gate is shown in the inset. **B**. Staining for IL-17A and IFN-γ to detect Th17 (only IL17A) and Th1/17 cells (express both cytokines). **C**. Staining for IL-5 and IFN-γ to detect IL-5 producing cells (IL-5+).

Results

Clinical characteristics

All CSS patients gave a history of sinusitis (100%), asthma (100%), and other symptoms typical for CSS (90% pulmonary infiltrates, 63% skin lesions, 58% polyneuropathy, 58% heart involvement, 21% digestive tract symptoms). Blood eosinophilia at the initial diagnosis was markedly elevated in all patients (6795 (3953–9629) cells/μL), but at the study entry both the disease activity scores and eosinophil counts were low (except for patients no. 9 and no. 14), due to the maintenance therapy with glucocorticoids (GC) or combined therapy with steroids and additional immunosuppressive (IS) drugs (azathioprine, cyclosporine A or cyclophosphamide). In the following statistical comparisons, if relevant, CSS patients treated only with GC (CSS/GC-group) were compared with those, who received both steroids and additional immunosuppressive drugs (CSS/IS-group). The two CSS groups did not differ by BVAS score, and basic clinical and laboratory characteristics (Table I).

Decreased percentage of CD25^{high}FoxP3+ regulatory T cells in CSS

CSS patients were characterised by increased percentage of T cells (p<0.05) in comparison to healthy subjects. However, the frequency of CD4+ cells (both naïve or effector/memory) did not differ between the groups. We noticed a trend toward increased number of CD8+ cells and decreased CD4/CD8 ratio in CSS patients treated with immunosuppressive drugs. Detailed characteristics of peripheral blood lymphocytes are summarised in Supplementary Table S2.

CSS patients were characterised by a significant (p<0.05) decrease in the percentage of CD25^{high}FoxP3 regulatory T cells (2.3% (1.7–3.1)) when compared to healthy controls (3.2% (2.6–5.2)). The lower frequency of Treg cells was present in all CSS patients, irrespective of the therapy given (Fig. 2A). Interestingly, the change in Treg fraction was only qualitative, as their absolute counts were not decreased in CSS.

Increased frequency of IL-17 producing cells and elevated Th17/Treg ratio in CSS

To identify lineage committed effector CD4+ T cells, PBL were stimulated *in vitro* with PMA/ionomycin, stained with cytokine specific antibodies and counted by flow cytometry. Analysis of CD4+ subsets revealed increased frequency and absolute counts of Th17 cells (p<0.05), Th1/17 cells (p<0.05) and IL-4 producing CD4+ cells (p=0.06) in all CSS patients as compared to healthy controls. Unexpectedly, the percentage and absolute counts of both Th2 and IL-5 secreting cells did not differ between the groups (Table II).

We next determined the ratios between main effector subpopulations of CD4+

Table I. Clinical characteristics of the CSS patients (regarding the immunosuppressive therapy) and healthy controls.

No	sex	age (years)	BVAS (score)	oral GC (mg/d)	IS drug (mg/d)	organs involved in the history	pANCA (titer)	WBC (10^3/μL)	Blood eosinophilia (cells/μL)	IgE (IU/mL)	CRP (mg/L)
CSS/GC (n=10)											
1	F	45	2	16	–	2 (NS)	1:10	7.7	770	38.6	<3.08
2	M	38	0	8	–	2 (LS)	–	11.8	328	65.7	<3.08
3	M	48	0	4	–	4 (LSHD)	1:10	5.8	125	69.9	3.85
4	F	41	0	4	–	5 (LNSHD)	–	9.3	28	90.1	3.81
5	F	33	0	6	–	2 (NS)	–	10.7	420	65.7	4.7
6	F	37	4	8	–	2 (LH)	–	8.4	649	502	<3.08
7	F	57	0	10	–	2 (LH)	–	3.8	254	17.6	<3.08
8	F	57	0	6	–	4 (LNSH)	–	6.6	652	61.7	<3.08
9	F	41	8	0	–	2 (LH)	–	7.7	1320	389	9.9
10	F	33	0	16	–	2 (LN)	–	7.2	454	52	<3.08
Median (IQR)	F 80%	41 (36–50.3)	0 (0–2.5)	7 (4–11.5)	NA	2 (2–4)	n=2 20%	7.7 (6.4–9.7)	437 (223–683)*	65.7 (48.7–165)*	3.08 (3.08–4.06)
CSS/IS (n=9)											
11	M	51	0	16	CTX (50)	5 (LNSHD)	1:10	6.5	65	157	<3.08
12	M	53	0	8	CSA (25)	2 (LH)	–	7.2	432	106	3.16
13	F	31	2	8	CSA (25)	4 (LNSH)	–	12	646	58.4	<3.08
14	M	31	6	4	AZA (75)	2 (LS)	–	10.3	1197	527	4.76
15	F	48	0	8	AZA (50)	4 (LNSH)	1:160	5.7	28	18.5	<3.08
16	M	55	0	16	CTX (50)	3 (LND)	–	4.8	307	17.6	<3.08
17	M	79	0	4	AZA (100)	2 (LH)	1:40	7.8	505	216	12.6
18	M	57	0	8	CTX (50)	3 (LNS)	1:80	11.4	94	24.8	4.81
19	M	42	0	8	AZA (100)	3 (LNH)	–	7.9	587	48.9	3.16
Median (IQR)	F 22%	51 (36.5–56)	0 (0–1)	8 (6–12)		3 (2–4)	n=4 44%	7.8 (6–10.9)	432 (79–617)	58.4 (21.7–187)	3.16 (3.08–4.8)
Healthy subjects (n=11)											
median (IQR)	F 55%	36 (29–41)	NA	NA	NA	NA	–	6.8 (5.4–7.7)	211 (115–237)	21 (17.2–60.4)	<3.08 in all subjects

Oral glucocorticoid daily dose is recounted for methylprednisolone. Statistics: Results are presented as medians (IQR). Significant differences: CSS/GC *vs*. healthy controls: **p*<0.05. AZA: azathioprine; BVAS: Birmingham Vasculitis Activity Score; CSA: cyclosporine-A; CSS: Churg-Strauss Syndrome; CRP: C-reactive protein; CTX: cyclophosphamide; GC: glucocorticoids, IS: immunosuppressive drugs; pANCA: perinuclear-staining antineutrophil cytoplasmic antibodies; WBC:white blood cells; NA: non-applicable. Abbreviations in 'organs involved' column: L: infiltrates in lungs; N: mono- or polyneuropathy; S: skin lesions; H: heart involvement; D: digestive tract involvement.

cells. There was no difference in the proportion of Th1, Th2 and Th17 cells between the studied groups. We noticed, however, a considerably higher ratio of cytokine producing effector T cells to regulatory T cells in CSS when compared to healthy controls (Table II). Particularly, the Th17/Treg ratio was markedly elevated in CSS patients (p<0.01), and positively correlated with blood eosinophilia (r=0.41, p<0.05) and serum IL-5 (r=0.62, p<0.01) (Fig. S1 in Supplementary materials).

We further determined the frequencies of T-helper cell subpopulations in CSS patients stratified by the mode of maintenance therapy. Patients with CSS treated only with systemic steroids had increased frequency of Th1, Th1/17 and Th17 cells, when compared to CSS treated with steroids and immunosuppressive drugs (Fig. 2B-E), whilst the number of IL-4 producing cells (including Th2) was similar in both groups.

Increased secretion of IL-17A by in vitro stimulated lymphocytes in CSS

We further investigated the ability of lymphocytes from CSS to produce IL-17 family cytokines. In resting conditions, transcripts of *IL17A* and *IL17F* genes in PBL were not detected, and the concentration of IL-17A in culture media was just above the limit of detection. Stimulation with PMA/ionomycin resulted in the induction of IL-17A and IL-17F mRNA and subsequent increase in the level of secreted IL-17A, though no difference was observed at 5 hours between CSS patients and controls (Fig. S2 in Supplementary materials). After 48 hours of stimulation, the concentration of IL-17A in supernatants was 3 to 4-fold higher (p<0.01) in CSS patients (1217 (1028–5226)pg/mL) as compared to controls (305 (146–775)pg/mL). The level of secreted IL-17A was comparable in CSS/GC and CSS/IS patients. We also noted a significant correlation of IL-17A concentration with both the level of IL-17A mRNA and the absolute count of circulating Th17 cells (data not shown).

The production of IL-5 in vitro is not increased in CSS

We next determined the capacity of lymphocytes in CSS to produce IL-5. The flow cytometric analysis of PMA/ionomycin stimulated lymphocytes revealed, that only small fraction of CD4+ (<0.5%) expressed IL-5. The frequencies and absolute counts of IL-5+ cells

Table II. Characteristics of peripheral blood CD4+ lymphocytes.

	Unit	Healthy subjects (n=11)	CSS (n=19)
Lymphocytes	cells / μL	2060 (1512–2448)	2509 (1800–2690)
CD4+	cells / μL	755 (518–1009)	905 (553–1352)
Subpopulations of CD4+ lymphocytes (% of CD4+)			
Treg	% of CD4+	3.2 (2.6–5.2)	2.4 (1.7–3.1) **
Th1	% of CD4+	18.2 (14.6–19.8)	22.9 (13.8–26.7)
Th2	% of CD4+	2.9 (2.1–3.3)	3.1 (2.3–4.5)
Th17	% of CD4+	0.54 (0.34–0.72)	0.74 (0.47–1.22) *
Th1/17	% of CD4+	0.04 (0.02–0.1)	0.08 (0.05–0.29) *
IL-4+	% of CD4+	3.4 (2.7–4.2)	4.5 (3.1–5.4)
IL-5+	% of CD4+	0.22 (0.18–0.36)	0.3 (0.23–0.5)
Subpopulations of CD4+ lymphocytes (absolute counts)			
Treg	cells / μL	26.6 (19.6–32.2)	15.6 (13.1–36.7)
Th1	cells / μL	153 (80–190)	188 (86–381)
Th2	cells / μL	18.0 (13.5–32.3)	23 (18–50)
Th17	cells / μL	3.6 (2.2–5.8)	6.1 (3.9–10.3) §
Th1/17	cells / μL	0.26 (0.19–0.66)	0.97 (0.29–2.35) *
IL-4+	cells / μL	25.8 (16.2–40.4)	39.3 (23.8–68.8) §§
IL-5+	cells / μL	1.7 (1.2–3.6)	3.5 (1.3–5.1)
Ratio of particular CD4+ subpopulations			
Th1/Th2	ratio	6.3 (5.1–8.5)	5.7 (4.4–10.7)
Th1/Th17	ratio	32.7 (22.6–53)	31.7 (18.6–44.7)
Th2/Th17	ratio	5.1 (3.6–5.8)	3.5 (1.9–6.9)
Th1/Treg	ratio	5.7 (3.6–7.5)	8.6 (5.2–13.3) *
Th2/Treg	ratio	0.8 (0.6–1.2)	1.3 (0.9–2.2) *
T17/Treg	ratio	0.19 (0.11–0.21)	0.33 (0.2–0.57) **

CSS: Churg-Strauss syndrome; Treg: regulatory T cells; IL-4+ - CD4+ cells producing interleukin-4, IL-5+ - CD4+ cells producing interleukin-5. Statistics: Results are presented as medians (IQR). *P*-values: $^{**}p<0.01$, $^{*}p<0.05$ ($^{\S}p=0.05$, $^{\S\S}p=0.06$) in comparison to helthy controls.

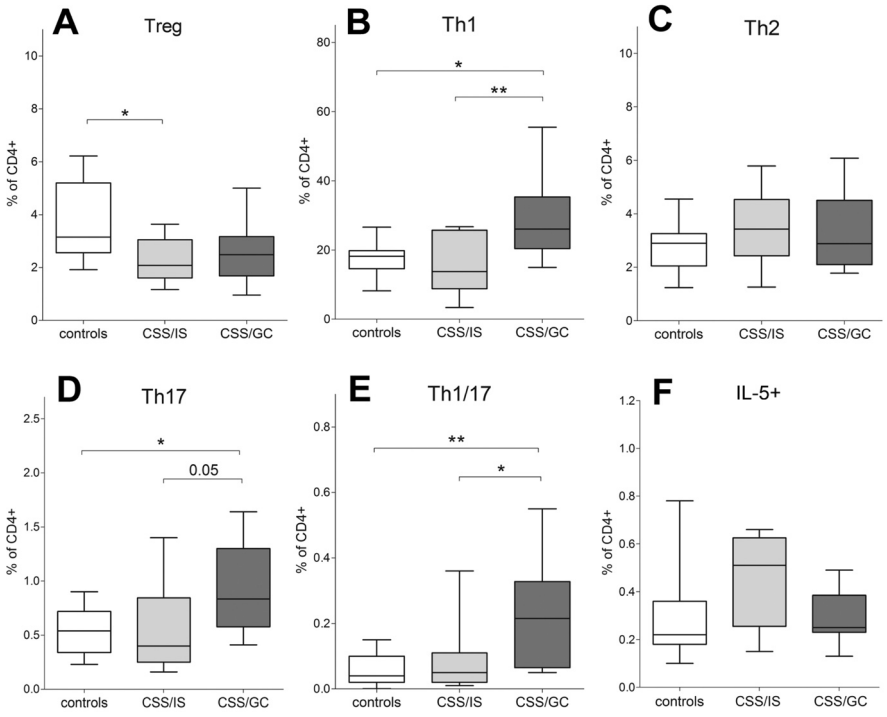

Fig. 2. Graphs showing the frequency of indicated CD4+ subpopulations in CSS patients (stratified by the mode of treatment) and the control group: Treg (**A**), Th1 (**B**), Th2 (**C**), Th17 (**D**), Th1/17 (**E**) and IL-5+ cells (**F**). Data presented as medians with IQR ('min to max' whiskers), $^{*}p<0.05$, $^{**}p<0.01$.

did not differ between CSS patients and healthy controls (Fig. 2F). These results were further confirmed by *in vitro* assay, as both the abundance of IL-5 mRNA, and the level of IL-5 in culture supernatants, did not differ between the groups (Fig. S3 in Supplementary materials).

Transcriptome profile of lymphocytes in CSS is skewed towards Th2

Transcriptome profiles were analysed to distinguish whether lymphocytes from CSS differ in the expression of T-helper related genes. In the transcriptome panel we included genes encoding signature cytokines (*IFNG, IL4, IL5, IL10, IL17A, IL17F, TGFB1*), cytokine receptors (*IL4R, IL12RB1, IL12RB2, IL23R*), and transcription factors (*STAT3, STAT4, STAT6, TBX21, GATA3, RORC, FOXP3*). At resting conditions, PBL expressed the majority of transcripts included in the mRNA panel, except for *IL5*, *IL4*, *IL17A* and *IL17F*. Resting lymphocytes from CSS patients were characterised by a significant increase in expression of only two, Th2-related genes, *STAT6* and *GATA3*, as compared to healthy subjects (Fig. 3A). When CSS/GC patients were analysed separately, we noted a significant increase in expression of *IL12RB2*, *STAT4*, *STAT6* and *GATA3* (Fig. S4 in Supplementary materials).

As expected, stimulation with PMA/ionomycin resulted in the induction of *IL5*, *IL4*, *IL17A* and *IL17F*, and led to a tremendous increase in expression of *IFNG* (1000- to 4000-fold), and *IL10* (200- to 500-fold). Other genes were up-regulated 2- to 10-fold (*e.g. TBX21*, *IL23R*, *RORC*, *IL12RB2*), or down-regulated (*CD4*, *IL12RB1*) in all subjects (Fig. S5 in Supplementary materials). Interestingly, among all transcripts analysed, only *TGFB1* was down-regulated in stimulated lymphocytes of CSS patients when compared to control group (Fig. 3B, left panel). No differences were detected in comparison between CSS/GC and CSS/IS groups (data not shown). After 48-hours of stimulation a majority of transcripts were down-regulated, and we no longer observed any differences between the groups (Fig. 3B, right panel).

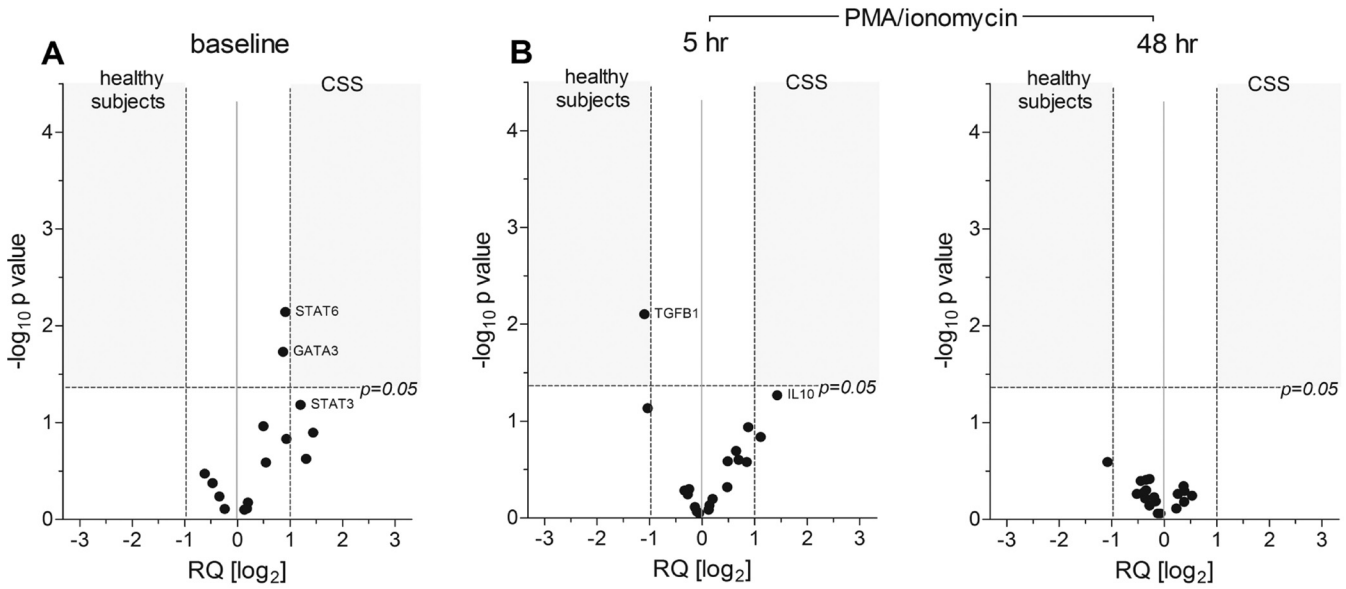

Fig. 3. Volcano graphs showing the difference in the expression of T-helper cell differentiation genes (in PBL) between CSS patients and healthy controls. Grey areas are delineated by thresholds of both biological (>2-fold change in expression when log_2RQ <-1 or >1), and statistical (p<0.05) significance. **A**. At baseline *STAT6* and *GATA3* were significantly up-regulated in PBL of CSS patients, **B**. PBL stimulated with PMA/ionomycin. 5 hr: the expression of *TGFB1* was significantly lower in CSS group. 48 hr: no difference between the groups.

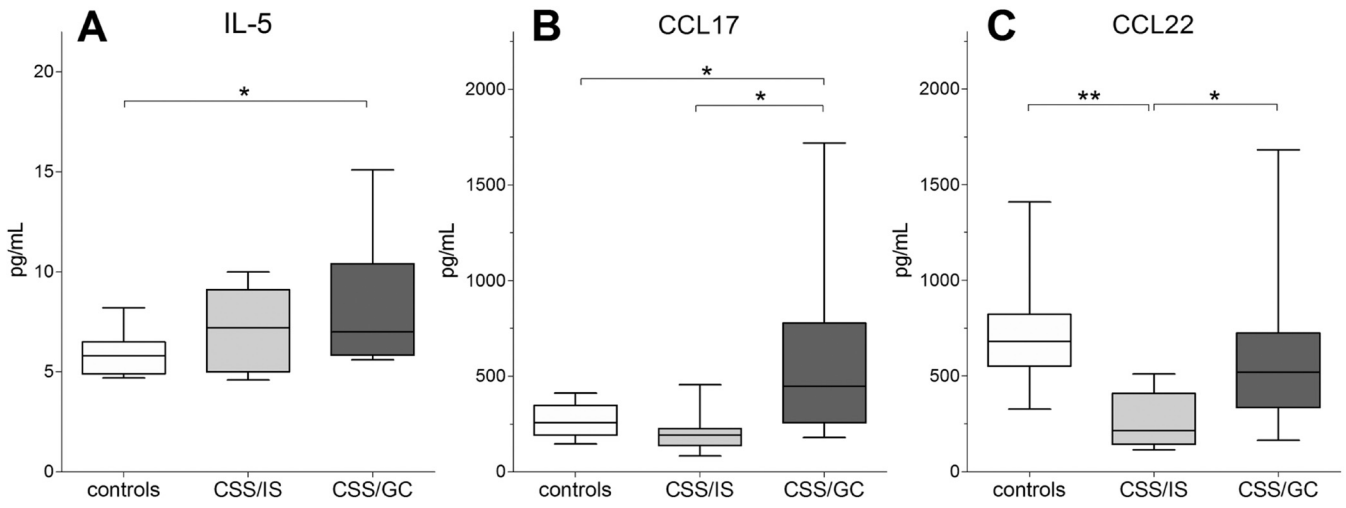

Fig. 4. Serum concentration of IL-5 and CCR4-active chemokines. **A**. Serum levels of IL-5 in healthy controls and CSS patients. **B**. Serum levels of CCL17. **C**. Serum levels of CCL22. Data are presented as medians with IQR ('min to max' whiskers). *p<0.05, **p<0.01.

Increased serum levels of IL-5 and CCL17 in CSS

The concentration of IL-5 in sera was significantly (p<0.05) increased in CSS patients (7.2 (5.7–9.2)pg/mL), when compared to healthy controls (5.8 (4.9–6.5)). The highest level of IL-5 was observed in CSS/GC group (Fig. 4A). We found increased levels of CCL17 in sera of CSS/GC patients (448 (257–778) pg/mL, p<0.05) as compared to both CSS/IS patients (194 (139–228)) and control group (258 (193–358)), Figure 4B. The level of CCL22 was comparable in healthy subjects and CSS/GC patients, but it was significantly decreased in CSS patients treated with additional immunosuppressive drugs (Fig. 4C).

The concentration of IL-5 in sera positively correlated with blood eosinophilia (r=0.79, p<0.001) and the level of CCL17 (r=0.64, p<0.1). Additionally, CCL17 weakly correlated with blood eosinophilia (r=0.45, p<0.05). Interestingly, there was no correlation between the concentration of serum IL-5, and the production of IL-5 by *in vitro* stimulated lymphocytes.

We also attempted to analyse the serum concentration of IL-17A, however due to the low sensitivity of the assay, nearly all measurements were below the detection limit, except for two CSS patients (no. 9:31.8 pg/mL, No. 10:34.4 pg/mL).

CCR4-active chemokines are elevated in active CSS

In the final part of the study we compared the concentration of IL-5, CCL17 and CCL22 in sera from six patients presently in remission, to the samples collected during the disease exacerbation in the past. During exacerbation, their BVAS scores (16 (8.5–29) *vs*. 0

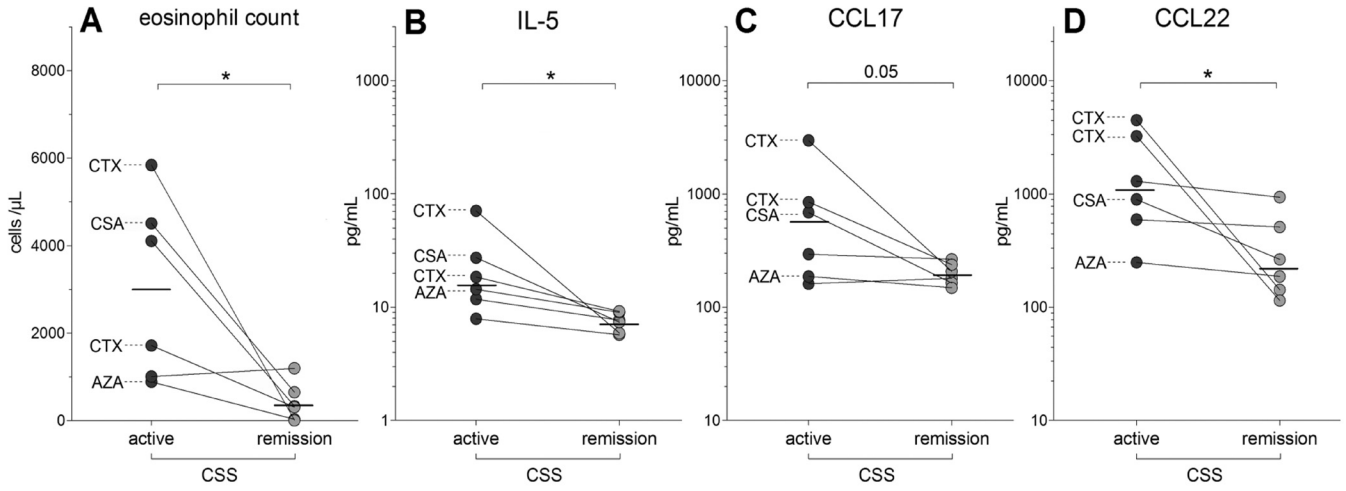

Fig. 5. A. Change in blood eosinophilia, **B**. serum IL-5, **C** CCL17, and **D** CCL22 concentration in six CSS patients, measured in samples collected during disease exacerbation (active), or in inactive CSS (remission). The remission was induced and sustained with glucocorticoids and immunosuppressive agents (labels: CTX: cyclophosphamide; CSA: cyclosporine-A; AZA: azathioprine) or glucocorticoids alone (unlabelled data sets). Horizontal bars on graphs represent medians, *p<0.05.

(0–2) in remission) and blood eosinophilia (Fig. 5A) were markedly elevated. Both IL-5 and CCR4-active chemokines were significantly elevated during the active phase of the disease. Additionally, the decrease in concentration of IL-5, CCL17 and CCL22 was most prominent in those patients who were treated with cyclophosphamide or cyclosporine A in addition to GCs (Fig. 5B-D).

Discussion

In the current study we used complementary methods of T-cell functional phenotyping, focused transcriptome analysis, and measurements of serum chemokines to investigate the individual contribution of effector T-helper cell lineages in the pathogenesis of Churg-Strauss syndrome, and the influence of the mode of immunosuppressive therapy.

We found elevated numbers of Th17 cells, increased secretion of IL-17A *in vitro*, and decreased frequency of Treg cells in CSS patients, as compared to healthy controls. Interestingly, the numbers of Th2 cells (producing exclusively IL-4) did not differ between the studied groups. Alterations in effector T-cell populations were most evident in CSS patients treated with steroids only, where increased percentage and absolute counts of not only Th17 and Th1/17 but also of Th1 cells were observed. In consequence, Th1/Treg and Th17/Treg ratios were elevated, indicating an imbalance between major effector and suppressory T cells in CSS. Additionally, Th17/Treg ratio correlated well with markers of disease activity. This was in line with the results of Tsurikisawa *et al*. (14) who originally found significantly lower numbers of circulating Treg cells in CSS when compared to patients with asthma or chronic eosinophilic pneumonia. Our results also correspond well with recently published data, indicating a marked increase in the fraction of IL-17 producing T cells in active CSS (9). Collectively, these results may suggest that increased activity of Th17 cells, when not properly suppressed by regulatory T cells, could be responsible for maintaining inflammatory reaction in CSS. However, other data indicate that Th17 cells are unlikely to be the sole players in the pathogenesis of CSS. First of all, we found upregulation of Th2-related genes *GATA3* and *STAT6* in circulating lymphocytes isolated from CSS patients. Second, the concentration of IL-5 was elevated in sera of CSS patients and correlated well with blood eosinophilia. Finally, also earlier studies pointed to Th2 cells as a major effector population involved in the pathogenesis of the disease (5, 6), and increased serum levels of IL-5 were reported in active CSS (15, 16). To further address this controversy we decided to analyse other factors that could be involved in the development of Th2 response.

Th2-mediated immune response is associated with two main groups of chemokines: eotaxins (CCL11, CCL24 and CCL26), and CCR4-active chemokines (CCL17 and CCL22) (17). Eotaxins are potent eosinophil chemoattractants, for they bind to CCR3 receptor highly expressed on eosinophils (17). Serum CCL26 (eotaxin-3) was found to be elevated in active CSS and strongly correlated with markers of disease activity (18). On the other hand, both eosinophils and Th2 cells express CCR4 and respond to CCL17 and CCL22 (19, 20). We analysed the levels of CCR4-active chemokines in sera of CSS patients, and observed increase in concentration of CCL17, but only in patients who were treated with glucocorticoids alone. Levels of CCL17 in CSS/GC group were comparable to those reported earlier in patients with bronchial asthma (21), or atopic dermatitis (22), but were several-fold lower than in patients with eosinophilia associated with clonal expansion of T cells (23, 24).

To further elucidate the actual role of CCR4-binding chemokines in active disease, we measured their serum concentration in six patients sampled during overt disease, and upon remission. Levels of CCL17 and CCL22 were elevated in active CSS, and correlated with markers of disease activity, well-fitting previous reports on increased secretion

of these chemokines in exacerbations of Th2-mediated diseases (25, 26). Increase in CCR4-active chemokines could facilitate the recruitment of Th2 effector cells to the sites of inflammation, and may partially explain lower numbers of circulating IL-5+ cells, and the moderate secretion of IL-5 *in vitro*, as seen in this study and reported previously by Kiene *et al*. (6). Unexpectedly low numbers of circulating IL-5+ cells in CSS could be also a consequence of steroid therapy, for glucocorticoids inhibit the production of IL-5 (16).

Altogether, these results indicate that multiple subsets of effector T-lymphocytes seem to be involved in the pathogenesis of CSS. It is tempting to speculate that Th2 subset drives the onset of asthma and chronic tissue eosinophilia, whereas Th17 cells might be responsible for vasculitis symptoms, granuloma formation and inducing disease exacerbations. Nevertheless, such a simplistic model remains controversial, as the role of Th17 cells in the background of Th2-mediated inflammation is still poorly understood. For example, in human asthma, IL-17 family cytokines have been shown to facilitate the neutrophilic inflammation rather than eosinophilia (7, 27). Schnyder-Candrian *et al*. (28) found that IL-17A was required to develop asthma in mice, but in an established disease IL-17A reduced eosinophil recruitment. In animal models, IL-23 and Th17 cells, apart from the effect on neutrophil recruitment, could also enhance Th2-mediated eosinophilic inflammation (29). Such discrepancies could be explained in part by the dual role of IL-23 that not only facilitates the expansion of Th17 cells, but can also enhance the differentiation of Th2 cells (30). Our data suggest that in CSS patients Th17 response develops in parallel to Th2, probably as a result of a particular pattern of innate immunity mediators released at the site of inflammation.

In the current study, we analysed a large cohort of convalescent CSS patients in whom the disease remission was sustained by oral glucocorticoids or combined treatment with steroids and immunosuppressive drugs. Our results indicate, that in patients treated only with steroids, who were clinically in disease remission, we could still observe some signs of ongoing immune activation with involvement of multiple T-cell lineages. Particularly, in these patients we found both elevated levels of Th2-acting cytokines, as well as expanded fraction of circulating Th17 cells, indicating that both Th2 and Th17 lineages might be involved in the pathogenesis of the disease. Introduction of combined immunosuppressive therapy led to a further down-regulation of these pathways, for example, to a strong inhibition in release of both CCL17 and CCL22. These results suggest that such combined therapeutic approach could be effective in CSS. Moreover, alongside IL-5 (16) and other mediators associated with vasculitis (3, 18, 31), measurement of CCR4-active chemokines might constitute a novel biomarker of CSS activity. Finally, this study indicates, that mediators of innate immunity which are capable of inducing both Th2 and Th17 response, could represent an interesting therapeutic target in refractory CSS.

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Supplementary material: Tables S1-S2; Figures S1-S5

Table S1. Sequences of primers used in the study.

no	Gene name	Protein name	Sense primer	Antisense primer	product size (bp)
1	*GAPDH*	Glyceraldehyde-3-phosphate dehydrogenase	5'-AgA ACA TCA TCC CTg CCT CTA C-3'	5'-CTg TTg AAg TCA gAg gAg ACC A-3'	253
2	*ACTB*	Actin. cytoplasmic 1	5'-AGC GGG AAA TCG TGC GTG-3'	5'-GGG TAC ATG GTG GTG CCG-3'	307
3	*CD4*	T-cell surface glycoprotein CD4	5'-CTC Agg TCC CTA CTg gCT CA-3'	5'- AgT CAg CgC gAT CAT TCA g-3'	292
4	*STAT3*	Signal transducer and activator of transcription 3	5'-AgC AgT TTC TTC AgA gCA ggT-3'	5'-gCA TgT CTC CTT gAC TCT TgA-3'	297
5	*STAT4*	Signal transducer and activator of transcription 4	5'-CAT ggC AAC gAT TCT TCT TC-3'	5'-gTg CTC CAC ATT CCT CTg TC-3'	283
6	*STAT6*	Signal transducer and activator of transcription 6	5'-ACA ATg CCA AAg CCA CTA TCC-3'	5'-CCA ggA CAC CAT CAA ACC AC-3'	308
7	*IL4R*	Interleukin-4 receptor alpha chain	5'-gTg gCA AgC TCT ggg AAC AT-3'	5'-AgC CCA CAg gTC CAg TgT AT-3'	258
8	*IL12RB1*	Interleukin-12 receptor beta1 chain	5'-CCA ggA ACC AgA CAg AgA Ag-3'	5'-CCA CTT gCT CCA ggA ACT T-3'	320
9	*IL12RB2*	Interleukin-12 receptor beta2 chain	5'-CAC AAA CAC CAg AAg AAg AgC-3'	5'-TAA TAC gAg Tgg gCA gAg AAC-3'	294
10	*IL23R*	Interleukin-23 receptor	5'-TCC TgA TgA AgT AAC CTg TgT C-3'	5'-TTA Tgg TCT Tgg gCA CTg TAg- 3'	338
11	*TBX21*	T-box transcription factor TBX21	5'-ACT ACA ggA TgT TTg Tgg ACg-3'	5'-gTT CAC CTC AAC gAT ATg CAg-3'	284
12	*GATA3*	Trans-acting T-cell-specific transcription factor GATA-3	5'-gAg ATg gCA Cgg gAC ACT AC-3'	5'-TTC ggT TTC Tgg TCT ggA Tg-3'	273
13	*RORC*	Nuclear receptor ROR-gamma	5'-AgT ggT gCT ggT TAg gAT gTg-3'	5'-TTg CAg AgA TgA TgA TgA AAg g-5'	291
14	*FOXP3*	Forkhead box protein P3	5'-AAg gCT TCA TCT gTg gCA TC-3'	5'-ATT gAg TgT CCg CTg CTT CT-3'	282
15	*IL4*	Interleukin-4	5'-TCT CAC CTC CCA ACT gCT TC-3'	5'-CTg CTT gTg CCT gTg gAA CT-3'	253/ 301
16	*IL5*	Interleukin-5	5'-gCT TCT gCA TTT gAg TTT gCT-3'	5'-TCT CCg TCT TTC TTC TCC ACA-3'	325
17	*IL10*	Interleukin-10	5'-gAg gAC TTT AAg ggT TAC CTg g-3'	5'-CTC ACT CAT ggC TTT gTA gAT g-3'	267
18	*IL17A*	Interleukin-17A	5'-AgA CCT CAT Tgg TgT CAC TgC-3'	5'-CTT TgC CTC CCA gAT CAC Ag-3'	266
19	*IL17F*	Interleukin-17F	5'-AAgAgC TTC CTC CAC AAA gTA Ag-3'	5'-ggT CCC AAg TgA CAg TgT AAT TC-3'	364
20	*IFNG*	Interferon gamma	5'-ATT ggA AAg Agg AgA gTg ACA-3'	5'-ACC TCg AAA CAg CAT CTg AC-3'	311
21	*TGFB1*	Transforming growth factor beta-1	5'-TgA CAg CAg ggA TAA CAC ACT-3'	5'-gTC CAg gCT CCA AAT gTA gg-3'	325

Table S2. Characteristics of peripheral blood lymphocytes

	unit	control	CSS/IS	CSS/GC
WBC	$x10^3/\mu L$	6.8 (5.4–7.7)	7.8 (6.1–10.9)	7.7 (6.4–9.7)
Lymphocytes	cells /μL	2060 (1512–2448)	1881 (1244–3408)	2509 (1800–2690)
Lymphocyte subpopulations (%)		control	CSS/IS	CSS/GC
T cells (CD3+)	%	75.7 (64.5–80.9)	88.4 (77.5–90.7)*	83.8 (80–88.9)*
T-helper (CD4+)	%	39.4 (31.6–43.6)	42.5 (35.2–49.5)	44.1 (36–55.9)
Th naïve (CD45RA+)	% of CD4+	36.7 (29–49.1)	44.1 (25.5–61.1)	34.5 (25.2–53.4)
Th effector/memory (CD45RO+)	% of CD4+	58.2 (42.5–66.8)	49.2 (34.8–69.7)	59.6 (42.9–69.9)
T-cytotoxic (CD8+)	%	27.5 (21.4–34.1)	36.4 (34.6–44.8)	31.2 (23.1–38.3)
CD4/CD8	ratio	1.45 (1.01–1.97)	0.93 (0.87–1.39)†	1.42 (1.01–2.17)
Lymphocyte subpopulations (cells in 1 μL)		control	CSS/IS	CSS/GC
T cells (CD3+)	cells/μL	1630 (1217–1806)	1530 (1091–2716)	1971 (1568–2295)
T-helper (CD4+)	cells/μL	755 (518–1009)	732 (545–1247)	1092 (744–1387)
Th naïve (CD45RA+)	cells/μL	320 (151–476)	365 (136–557)	426 (224–529)
Th effector/memory (CD45RO+)	cells/μL	459 (317–517)	403 (217–671)	444 (333–824)
T-cytotoxic (CD8+)	cells/μL	511 (457–850)	691 (422–1387)	710 (393–886)
Subpopulations of CD4+ lymphocytes (% of CD4+)		control	CSS/IS	CSS/GC
Treg ($CD25^{high}$FoxP3+)	% of CD4+	3.2 (2.6–5.2)	2.1 (1.6–3.1)*	2.5 (1.7–3.2)§
Th1	% of CD4+	18.2 (14.6–19.8)	13.8 (8.8–25.7)$^{\#\#}$	26.0 (20.4–35.3)*
Th2	% of CD4+	2.9 (2.1–3.3)	3.4 (2.4–4.5)	2.9 (2.1–4.5)
Th17	% of CD4+	0.54 (0.34–0.72)	0.40 (0.25–0.85) ††	0.84 (0.58–1.3)**
Th1/17	% of CD4+	0.04 (0.02–0.1)	0.05 (0.02–0.11)$^{\#}$	0.22 (0.07–0.33)**
IL-4+	% of CD4+	3.4 (2.7–4.2)	4.3 (2.6–5.4)	5.2 (3.3–5.5)*
IL-5+	% of CD4+	0.22 (0.18–0.36)	0.51 (0.26–0.63)	0.25 (0.13–0.39)
Subpopulations of CD4+ lymphocytes (counts)		control	CSS/IS	CSS/GC
Treg ($CD25^{high}$FoxP3+)	cells/μL	26.6 (19.6–32.2)	13.4 (11.6–32.7)	20.3 (14.1–38.1)
Th1	cells/μL	153 (80–190)	118 (59–187)$^{\#}$	278 (193–400)**
Th2	cells/μL	18.0 (13.5–32.3)	26.1 (12.3–45.0)	23.0 (18.3–56.4)
Th17	cells/μL	3.6 (2.2–5.8)	3.9 (1.9–5.5)$^{\#}$	8.7 (5.9–13.1)**
Th1/17	cells/μL	0.26 (0.19–0.66)	0.32 (0.19–0.76)$^{\#}$	1.96 (0.89–2.86)**
IL-4+	cells/μL	25.8 (16.2–40.4)	27.3 (14.2–65.4)	42.3 (29.8–71.2)*
IL-5+	cells/μL	1.7 (1.2–3.6)	4.8 (2.5–8.3)	3.1 (1.2–3.9)
T–helper cell functional phenotypes (ratio)		control	CSS/IS	CSS/GC
Th1/Th2	ratio	6.3 (5.1–8.5)	4.4 (2.7–6.4)$^{\#}$	9.6 (5.4–13)
Th1/Th17	ratio	32.7 (22.6–53)	28.8 (17.8–42.4)	31.8 (23.4–45.1)
Th2/Th17	ratio	5.1 (3.6–5.8)	6.9 (3.0–10.9)$^{\#}$	3.2 (1.4–5.9)
Th1/Treg	ratio	5.7 (3.6–7.5)	7.3 (4.1–9.3)$^{\#}$	11.8 (7.4–18.2)**
Th2/Treg	ratio	0.8 (0.6–1.2)	1.4 (1.0–2.2)*	1.2 (0.7–2.1)
Th17/Treg	ratio	0.19 (0.11–0.21)	0.27 (0.16–0.38)$^{\#}$	0.53 (0.3–0.72)**

CSS: Churg-Strauss syndrome; CSS/GC: CSS patients treated only with glucocorticoids (GC); CSS/IS: CSS patients treated with steroids and immunosuppressive agents (IS); WBC: white blood count; Th:T-helper cells; Treg: regulatory T cells; IL-4+:CD4+ cells producing interleukin-4; IL-5+:CD4+ cells producing interleukin-5. Statistics: Results are presented as medians (IQR). *P*-values: $^{**}p<0.01$; $^{*}p<0.05$ ($^{\S}p=0.06$) in comparison to healthy controls; $^{\#\#}p<0.01$; $^{\#}p<0.05$ ($^{\dagger\dagger}p=0.05$,$^{\dagger}p=0.1$) in comparison to CSS/GC group.

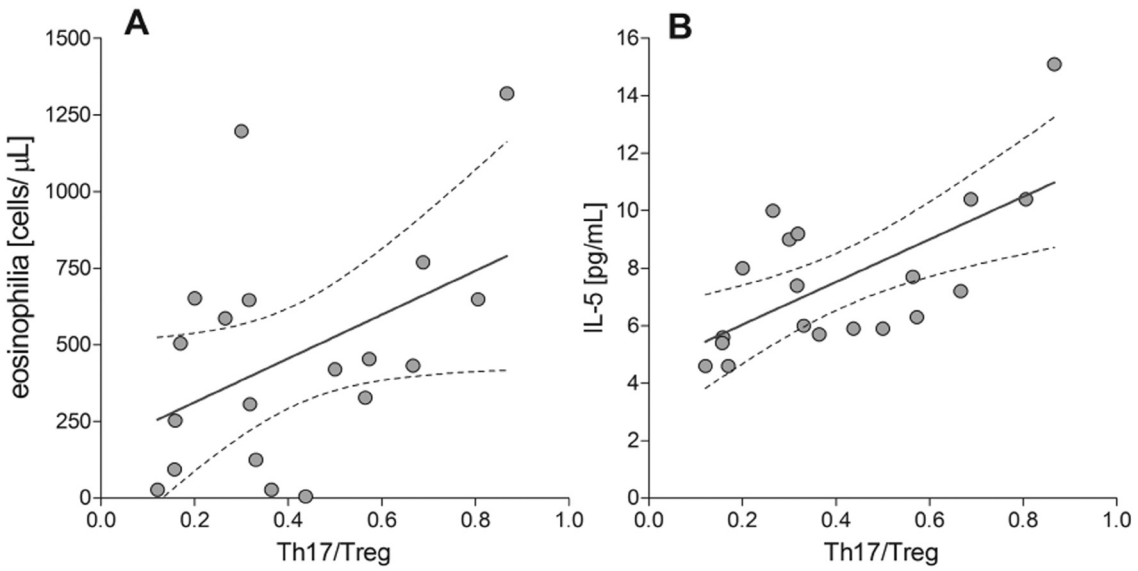

Fig. S1.
Correlation of Th17/Treg ratio with laboratory markers of CSS activity. A. Correlation of Th17/Treg and blood eosinophilia (r=0.41, p<0.05). B. Correlation of Th17/Treg and serum IL-5 (r=0.62, p<0.01).

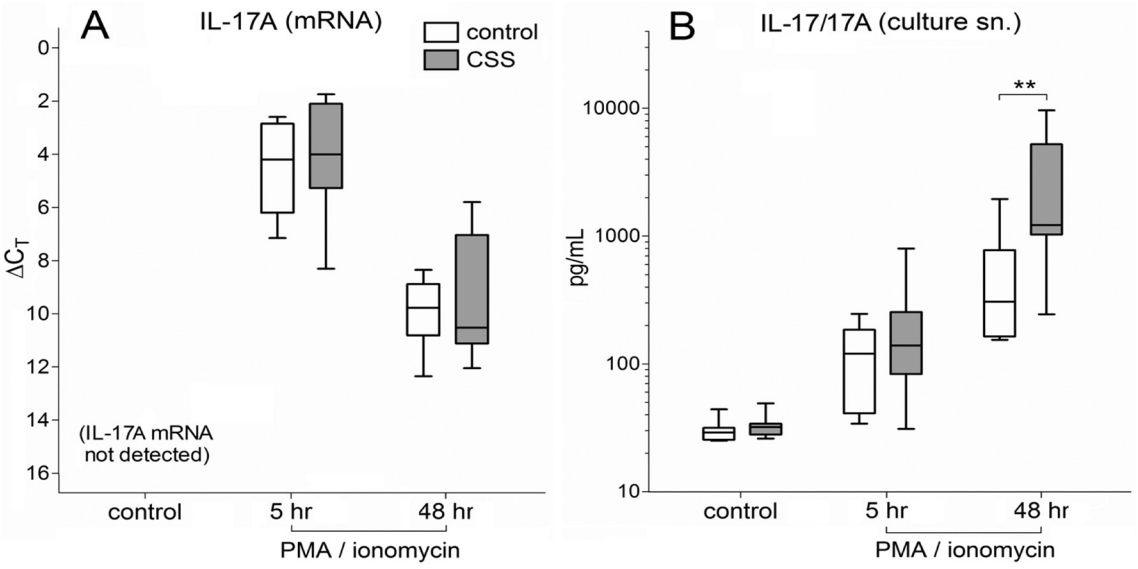

Fig. S2.
Production of IL-17A by *in vitro* stimulated (PMA/ionomycin) lymphocytes. A. The level of IL-17A mRNA (expressed as ΔC_T values) in PBL after 5 and 48 hours of stimulation. B. The concentration of IL-17A in culture supernatants. Data are presented as medians (IQR) with 'min-to-max' whiskers. **p<0.01.

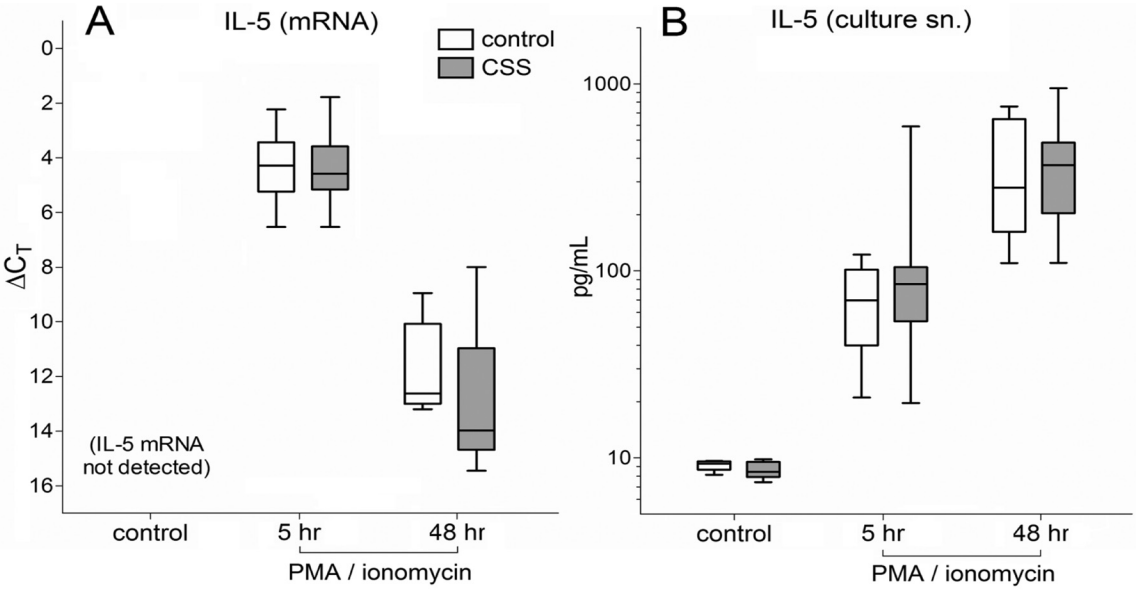

Fig. S3.
Production of IL-5 by in vitro stimulated (PMA/ionomycin) lymphocytes. A. The level of IL-5mRNA (the lower ΔC_T value, the higher amount of transcript is present in the sample) in PBL after 5 and 48 hours of stimulation. B. The concentration of IL-5 in culture supernatants. Data are presented as medians (IQR) with 'min-to-max' whiskers.

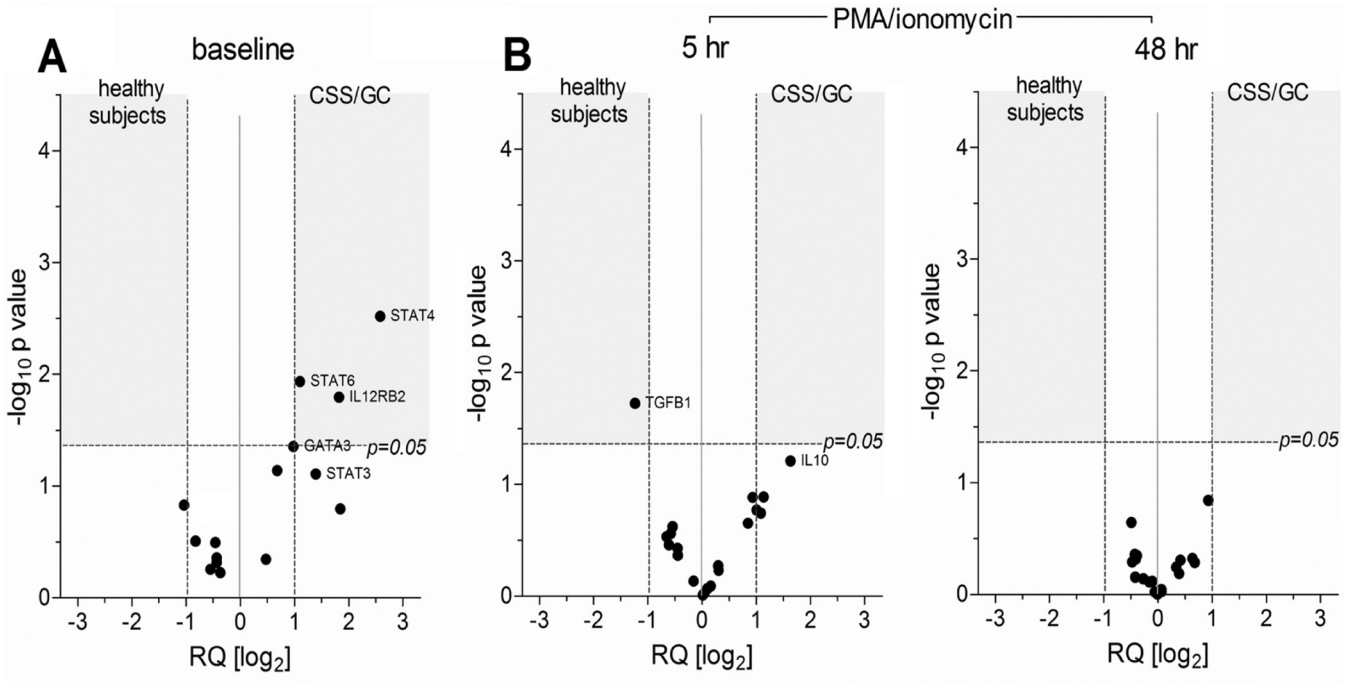

Fig. S4. Volcano graphs showing the difference in the expression of T-helper cell differentiation genes (in PBL) between CSS/GC patients and healthy controls. Grey areas are delineated by thresholds of both biological (>2-fold change in expression when log_2RQ <-1 or >1), and statistical (p<0.05) significance. **A**. At baseline STAT4, STAT6, GATA3 and IL12RB2 were significantly up-regulated in PBL of CSS/GC patients, **B**. PBL stimulated with PMA/ionomycin. 5hr: the expression of TGFB1 was significantly lower in CSS/GC group. 48hr: no difference between the groups.

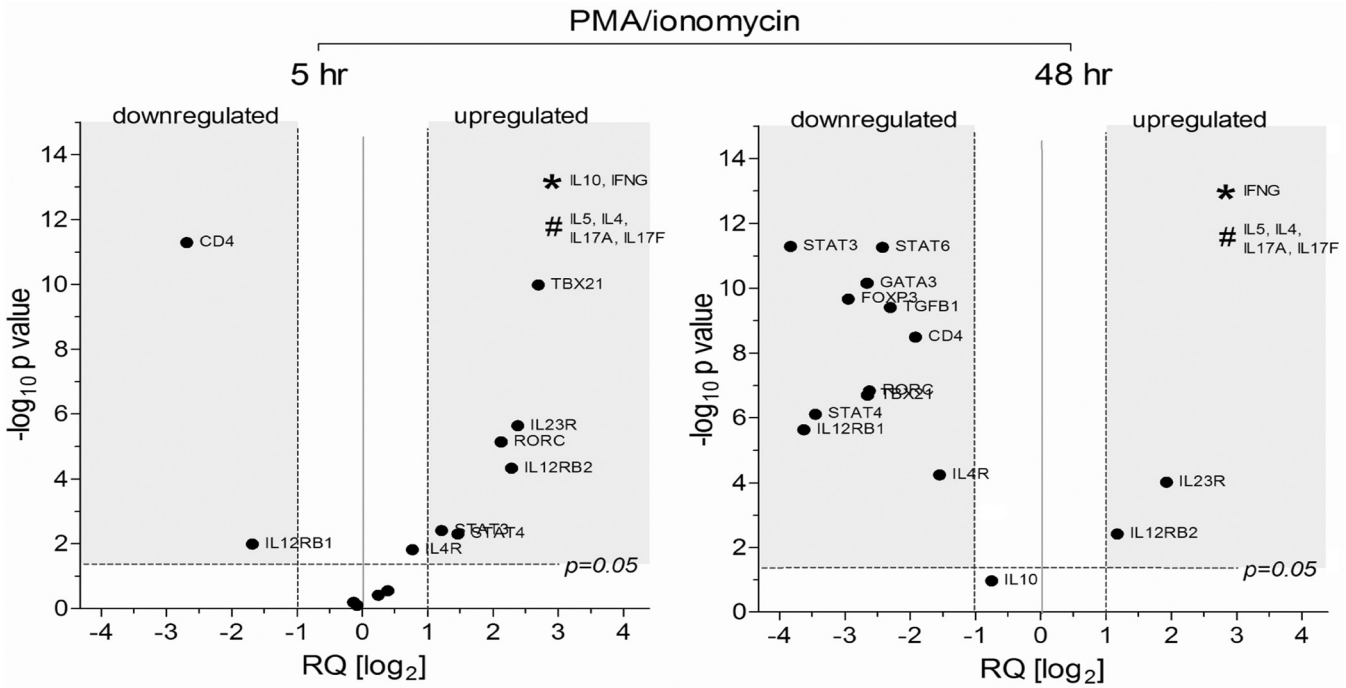

Fig. S5. Volcano plots showing the activation dependent differences in expression of lymphocyte differentiation genes (pooled data from both CSS and controls). The differences between baseline and PMA/ionomycin stimulated levels of individual transcripts are presented separately for 5hr (on the left) and 48hr (on the right) timepoint. Grey areas are delineated by thresholds of both biological (>2-fold change in the level of mRNA (log_2RQ <-1 or >1), when compared to control samples) and statistical (p<0.05) significance. The panel included 19 genes listed in 'results' section. Symbols: # indicate transcripts with highly significant, >100-fold (log_2RQ >6.7) increase in expression; # symbol indicate transcripts, that were not present in control samples but were induced to a high level upon PMA/ionomycin stimulation.