# Distinct proteinase 3-induced cytokine patterns in Wegener's granulomatosis, Churg-Strauss syndrome, and healthy controls

U. Fagin[1], E. Csernok[1], A. Müller[1], S. Pitann[1], J. Fazio[2], K. Krause[1], P. Bremer[1], E. Wipfler Freißmuth[1,3], F. Moosig[1], W.L. Gross[1], P. Lamprecht[1]

*[1]University of Lübeck, Department of Rheumatology, Vasculitis Center UKSH and Clinical Center Bad Bramstedt, Lübeck, Germany; [2]Christian Albrechts University Kiel, Institute of Immunology, Kiel, Germany; [3]Department of Rheumatology, University Hospital for Internal Medicine, Graz, Austria.*

*Ursula Fagin, PhD*
*Elena Csernok, PhD*
*Antje Müller, Eng D*
*Silke Pitann,*
*Juliane Fazio, Dipl Biol*
*Kristina Krause, Dipl Biol*
*Philip Bremer, MD*
*Edith Wipfler Freißmuth, MD*
*Frank Moosig, MD*
*Wolfgang L. Gross, MD*
*Peter Lamprecht, MD*

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*Please address correspondence to:*
*Dr Peter Lamprecht,*
*University of Lübeck,*
*Department of Rheumatology,*
*Vasculitis Centre UKSH and Clinical Center Bad Bramstedt,*
*Ratzeburger Allee 160,*
*23538 Lübeck, Germany.*
*E-mail: peter.lamprecht@uk-sh.de*

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*Competing interests: none declared.*

## ABSTRACT

**Objective:** *To analyse whether a specific cytokine pattern is elicited in response to the autoantigen proteinase 3 (PR3) in active Wegener's granulomatosis (WG).*

**Methods:** *Six-colour flow cytometry was used to analyse cytokine production and surface markers of the total CD4+ T-cell population ex vivo and in PR3-stimulated T-cell lines of patients with active PR3-ANCA-positive WG, PR3-ANCA-negative Churg-Strauss syndrome (CSS), and healthy controls (HC).*

**Results:** *The cytokine response of the total PB CD4+ T cell population was skewed towards distinct pro-inflammatory cytokine patterns in WG (Th1-type) and CSS (Th17, Th1- / Th2-type). Th2-type as well as Th17 cell populations including Th17/Th1, Th17/Th2 and Th22 cells were elicited in response to PR3 stimulation in WG. In contrast, CSS patients displayed a Th2-type dominated response following PR3 stimulation.*

**Conclusion:** *These data suggest that the cytokine response of the total CD4+ T-cell population and PR3-specific cells is influenced by the underlying disorder.*

## Introduction

Wegener's granulomatosis (WG) and Churg-Strauss syndrome (CSS) are two rare diseases characterised by a necrotising systemic vasculitis and a predilection for chronic granulomatous inflammation of the respiratory tract. Both disorders progress from a prodromal stage to a generalised systemic disorder. Respiratory and constitutional symptoms herald early systemic WG, whereas asthma usually precedes CSS. Pulmonary-renal syndrome and other systemic vasculitic features prevail in generalised WG, whereas blood eosinophilia, eosinophilic pulmonary infiltrates and vasculitic manifestations such as polyneuropathy are found in full-blown CSS. Anti-neutrophil cytoplasmic autoantibodies with proteinase 3-specificity (PR3-ANCA) are detected in nearly all Caucasian patients with generalised WG, but rarely in CSS (1-3).

Previous studies reported the predominance of a peripheral blood (PB) Th1-type cytokine response after unspecific stimulation in WG and a mixed Th1/Th2-type cytokine pattern in CSS (4, 5). Interestingly, the autoantigen PR3 elicits T-cell proliferation both in WG patients and in healthy controls (6). PR3 induces a Th2-type and Th17 response in WG patients in clinical remission (3, 6-8). To determine whether a specific cytokine pattern is elicited in active WG, we investigated the *ex vivo* cytokine response of PB CD4+ T-cells following PR3 stimulation. In the present study we show that distinct cytokine patterns were displayed by the total CD4+ T-cell population and PR3-stimulated T-cells in WG and CSS.

## Materials and methods

*Study population*

In this study, consecutive patients with active PR3-ANCA-positive WG (n=16) and PR3-ANCA-negative CSS (n=10) were included. All WG patients were MPO-ANCA-negative; all but one CSS patient were MPO-ANCA-negative (Table I). Patients fulfilled the ACR criteria and the CHC definition for WG and CSS, respectively. Disease activity and stages were defined and recorded according to EULAR recommendations (9). All patients and controls provided informed consent. Ten healthy volunteers served as controls. The study was approved by the ethics committee of the University of Lübeck (no. 07-059).

*Antibodies used for flow cytometry*

The following antibodies were used in different combinations: Pacific Blue (PB)-conjugated anti-CD3, anti-CD4, FITC-conjugated anti-CD3, anti-TNF-α,

**Table I.** Clinical characterisation of WG and CSS patients according to EULAR recommendations (8).

| Patient no. | Age (Yr) /sex | Activity | Organ involvement | BVAS V3.0 | ANCA | Treatment |
|---|---|---|---|---|---|---|
| WG-1 | 57/M | First manifestation | E, L, K, P, S, B | 32 | PR3 | None |
| WG-2 | 55/F | Major relapse | E, L | 12 | PR3 | Pred, AZA |
| WG-3 | 39/M | Refractory disease | E, L, Ey | 10 | PR3 | Pred, AZA |
| WG-4 | 37/M | Low activity | E, L | 6 | PR3 | Pred, MTX |
| WG-5 | 45/F | Refractory disease | E, L, K | 16 | PR3 | Pred, CYC |
| WG-6 | 52/F | Refractory disease | E, L, K, Ey | 22 | PR3 | Pred, CYC |
| WG-7 | 39/F | Major relapse | E, C | 15 | PR3 | Pred, MMF |
| WG-8 | 20/F | Minor relapse | E, A | 5 | PR3 | Pred, MTX |
| WG-9 | 83/M | Refractory disease | E, K, B | 14 | PR3 | Pred, CYC |
| WG-10 | 51/M | Refractory disease | E, L, K, H | 23 | PR3 | Pred, CYC |
| WG-11 | 79/F | First manifestation | E, K, C, Ey, A, B | 20 | PR3 | None |
| WG-12 | 54/M | Low activity | E, L | 7 | PR3 | Pred, LEF |
| WG-13 | 27/M | Low activity | E, L | 7 | PR3 | Pred, MTX |
| WG-14 | 50/M | First manifestation | E, L, K, A, B | 24 | PR3 | None |
| WG-15 | 64/M | First manifestation | E, L, K, Ey, S, B | 23 | PR3 | None |
| WG-16 | 50/M | Low activity | P | 6 | PR3 | Pred, AZA |
| CSS-1 | 56/M | Low activity | A, B | 2 | Ø | Pred MTX |
| CSS-2 | 73/F | Major relapse | L | 6 | Ø | Pred, MTX |
| CSS-3 | 79/M | Low activity | P | 6 | Ø | Pred, AZA |
| CSS-4 | 64/M | First manifestation | E, L, P, H, GI, B | 29 | Ø | None |
| CSS-5 | 57/F | Low activity | A, B | 3 | Ø | Pred, LEF |
| CSS-6 | 64/F | Minor relapse | E | 4 | Ø | Pred, AZA |
| CSS-7 | 59/F | Major relapse | L | 4 | Ø | Pred, MTX |
| CSS-8 | 60/M | Minor relapse | A | 1 | MPO | Pred, MTX |
| CSS-9 | 40/F | Low activity | A, B | 3 | Ø | Pred, MTX |
| CSS-10 | 60/M | First manifestation | E, L | 8 | Ø | None |

F: female; M: male; WG: Wegener's granulomatosis; CSS: Churg-Strauss syndrome; Low activity: minor symptoms, which do not warrant an escalation of therapy; Minor relapse: re-occurrence or new onset of disease, which is neither organ- nor life-threatening; Major relapse: re-occurrence or new onset of organ- or life-threatening disease; Refractory disease: unchanged or increased disease activity in spite of treatment; E: ENT; L: lung; K: kidney; C: central nervous system; P: peripheral nervous system; Ey: eye; H: heart; GI: gastro-intestinal tract; S: skin; A: arthralgia/arthritis; B: constitutional symptoms; BVAS V3.0: Birmingham Vasculitis Acitivity Score, version 3.0; ANCA: antineutrophil cytoplasmic autoantibody; PR3: proteinase 3-specific ANCA, MPO: myeloperoxidase-specific ANCA; Ø: no ANCA; AZA: Azathioprine; CYC: cyclophosphamide; LEF: leflunomide; MMF: mycopholate mofetil; MTX: methotrexate; Pred: prednisolone.

anti-CD69, anti-CD45RA, APC- PE-conjugated anti-CCR6, and Alexa-Fluor-647-conjugated anti-CCR7 were purchased from BD Biosciences, Heidelberg, Germany. PE-conjugated anti-IL-17A was obtained from eBioscience (Frankfurt, Germany), PE-conjugated anti-TGF-β from IQProducts (Hannover, Germany), APC-conjugated anti-CD4 and PE-conjugated anti-IL-10 from Miltenyi (Bergisch Gladbach, Germany) and APC-conjugated anti-IL-22 from R&D systems (Wiesbaden, Germany), respectively. PE-Cy7-conjugated anti-IL-4 and Cy7-conjugated anti-IFN-γ were a kind gift of S. Dimitrov, Lübeck. Appropriate isotype controls were included.

*Surface marker and intracellular cytokine staining*

Cell surface and intracellular stainings were performed as described previously (10). Briefly, lithium heparinised whole blood was stimulated with PMA (Sigma, Munich, Germany) (10ng/ml) and ionomycin (Sigma) (1μg/ml) for 4h at 37°C before the addition of Brefeldin (1μg/ml) (Sigma). After lysing cells were subsequently stained with previously determined optimal concentrations of fluorochrome-conjugated monoclonal antibodies for cell surface antigens and anti-cytokine antibodies or appropriate negative (isotype) controls. Thereafter, cells were immediately analysed on a FACS CantoII cytometer (BD) using FACSDiva software (BD).

*Flow cytometry*

Six-colour flow cytometric analysis was performed on a FACS CantoII cytometer (BD) using FACSDiva software (BD). Lymphocytes were gated for analysis based on light-scattering properties and on CD3 and CD4. For all flow cytometry analysis, $5x10^4$ CD3+CD4+ cells were collected. Unstimulated control samples were used to discriminate between negative and positive populations. Additionally appropriate isotype controls were included in each experiment to determine background staining. CD69 expression was used to exclude T cell anergy due to immunosuppressive therapy. Frequencies of cell subsets were calculated using FACSDiva™ Software (BD).

*In vitro cell stimulation with PR3*

Short-term PR3-stimulated T-cell lines were generated as described earlier (5). Cells were incubated with 10μg/ml heat-inactivated PR3 (Athens Research & Technology, GA, USA) and cultured for 18 days at 37°C in the presence of 20U/ml IL-2. Cytokine production and phenotypic characterisation of cells was determined by intracellular staining and flow cytometry as described above. Staining with 7-aminoactinomycin D

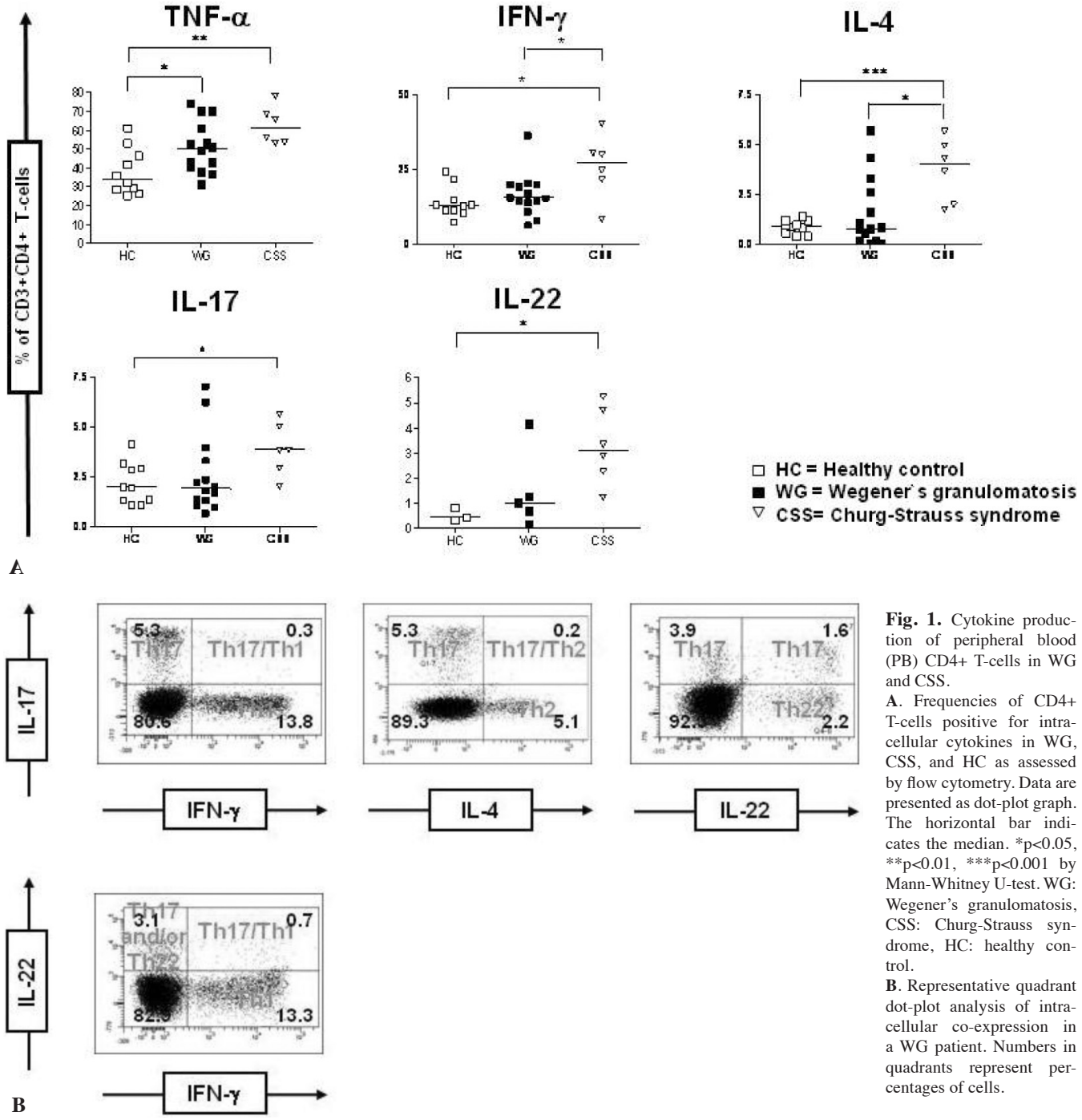


**Fig. 1.** Cytokine production of peripheral blood (PB) CD4+ T-cells in WG and CSS.
**A**. Frequencies of CD4+ T-cells positive for intracellular cytokines in WG, CSS, and HC as assessed by flow cytometry. Data are presented as dot-plot graph. The horizontal bar indicates the median. *p<0.05, **p<0.01, ***p<0.001 by Mann-Whitney U-test. WG: Wegener's granulomatosis, CSS: Churg-Strauss syndrome, HC: healthy control.
**B**. Representative quadrant dot-plot analysis of intracellular co-expression in a WG patient. Numbers in quadrants represent percentages of cells.

(7-AAD, BD) was included to discriminate between live and dead cells.

*Statistical analysis*

Statistics were performed using Prism 4.0 (GraphPad Software, San Diego, CA, USA). Comparisons between patients and control subjects were analysed by non-parametric Mann-Whitney U-test. *P*-values equal to or less than 0.05 were considered to be statistically significant.

## Results

*PB CD4+ T-cells display differences in cytokine production in WG and CSS*

Using flow cytometry we analysed intracellular cytokine expression of PB CD4+ T-cells in active WG (n=16), CSS (n=6), and healthy controls (n=10). WG patients displayed an intracellular TNF-α expression in a significantly higher percentage of CD4+ T-cells compared to healthy individuals. In CSS significantly higher percentages of TNF-α, IFN-γ, IL-4, IL-17, and IL-22 expressing CD4+ T-cells were found compared to healthy controls. Moreover, the percentages of TNF-α and IL-4 expressing CD4+ T-cells were higher in CSS as compared to WG (Fig. 1a). There were no significant differences in the percentages of IL-10+ and TGF-β+ CD4+ T-cells between WG, CSS, and healthy controls (data not shown).

No differences in the frequencies of

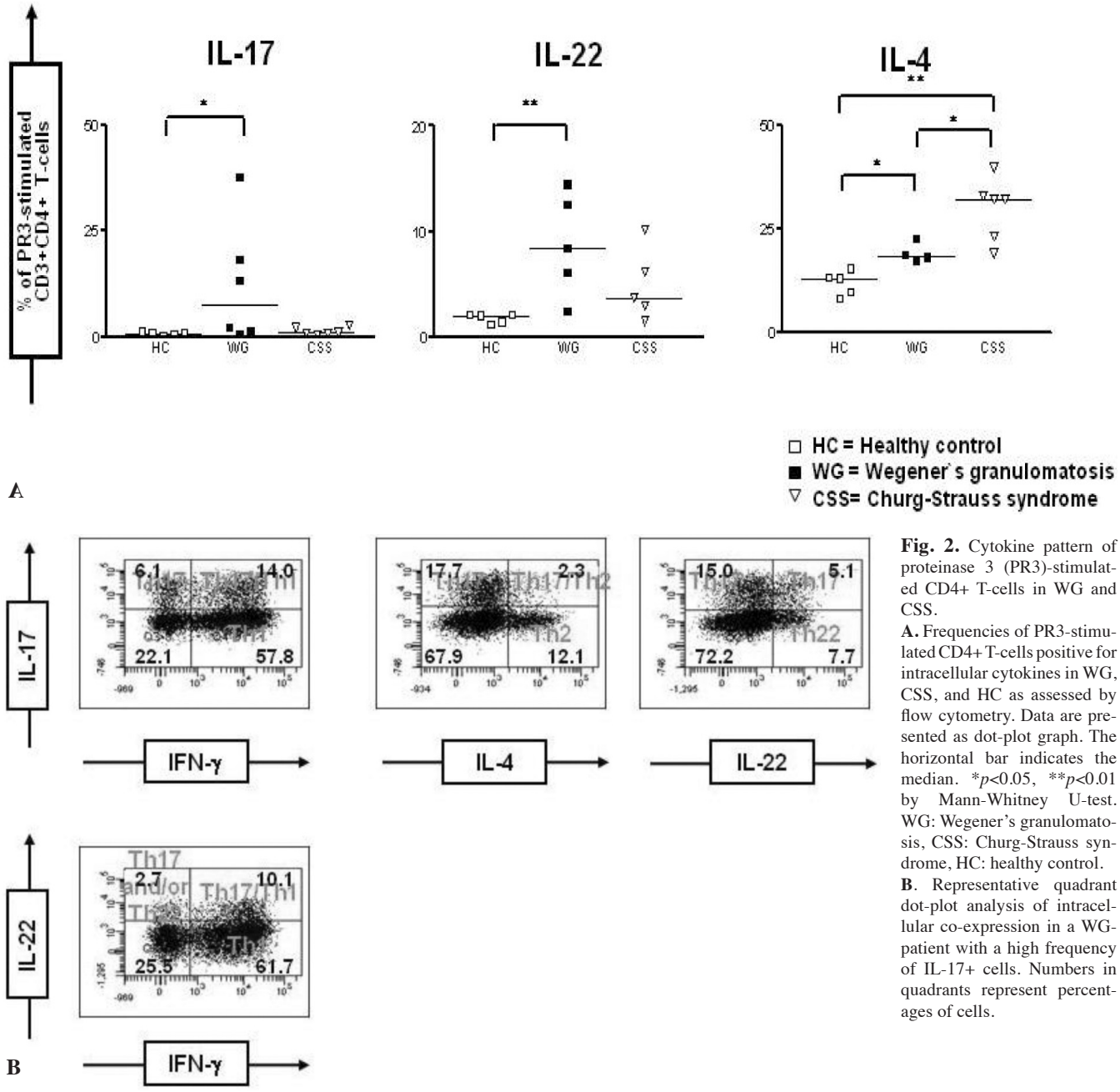


**Fig. 2.** Cytokine pattern of proteinase 3 (PR3)-stimulated CD4+ T-cells in WG and CSS.
**A.** Frequencies of PR3-stimulated CD4+ T-cells positive for intracellular cytokines in WG, CSS, and HC as assessed by flow cytometry. Data are presented as dot-plot graph. The horizontal bar indicates the median. *$p$<0.05, **$p$<0.01 by Mann-Whitney U-test. WG: Wegener's granulomatosis, CSS: Churg-Strauss syndrome, HC: healthy control.
**B**. Representative quadrant dot-plot analysis of intracellular co-expression in a WG-patient with a high frequency of IL-17+ cells. Numbers in quadrants represent percentages of cells.

total PB CD4+ cytokine double-positive staining cells were observed between WG, CSS, and healthy controls. Analysis of intracellular cytokine co-expression showed that only few PB IL-17+ T-cells also produced IFN-γ (Th17/Th1 cells). Virtually no Th17/Th2 cells and few IL-22+ T-cells lacking IL-17 co-expression (Th22 cells) were detected (Fig. 1b).

*Distinct cytokine patterns of CD4+ T-cells following PR3 stimulation in WG and CSS*

Cytokine production following PR3 stimulation was analysed in active WG (n=6), CSS (n=5), and healthy controls (n=5). There was no significant difference in disease activity (BVAS 3.0) between the group of patients in which the cytokine response of the total CD4+ T-cell population was determined and those in which the response to PR3 was not determined. Following PR3 stimulation there was no difference in Th1-type T-cell cytokine production between WG, CSS, and healthy controls. However, the percentages of IL-17, IL-22, and IL-4-producing CD4+ T-cells following PR3 stimulation were significantly higher in WG compared to healthy individuals. Interestingly, the 2 WG patients displaying the highest frequencies of IL-17+ cells suffered from a concomitant bacterial infection (*Pseudomonas aeruginosa* and *Staphylococcus aureus*, respectively) at the time of sampling. In contrast, CSS patients showed a Th2-type (IL-4) dominated response following PR3 stimulation. The intracellular IL-4 production in response to PR3 in CSS patients significantly exceeded that displayed by WG patients (Fig. 2a).

Analysis of cellular cytokine co-ex-

pression showed that in those WG patients displaying an IL-17 response to PR3 a substantial proportion of the IL-17+ cells showed a co-expression of IL-22 (Th17 cells) and IFN-γ (Th17/Th1cells). In WG – and to a lesser extent in CSS – a notable fraction of the IL-22+ cell population lacked IL-17 co-expression (Th22 cells). Of note, a small subset of T-cells producing both IL-17 and IL-4 (Th17/Th2 cells) was detected in WG (Fig. 2b).

*Phenotype of PR3-stimulated T-cells in WG and CSS and CCR6 expression of Th17 cells*

The majority of PR3-stimulated CD4+ T-cells lacked CCR7 and CD45RA expression in WG, *i.e.* they were CCR7-CD45RA- effector memory T-cells ($T_{EM}$). In contrast, PR3-stimulated CD4+ T-cells displayed a more heterogeneous phenotype including CCR7+CD45RA- central memory T-cells ($T_{CM}$) in CSS. Th17 cells invariably expressed the Th17-associated chemokine receptor CCR6 (data not shown).

**Discussion**

Earlier reports described an unbalanced Th1-type cytokine production of the total PB CD4+ T-cell population in WG and a Th1/Th2-type cytokine pattern in CSS (3-5, 10). More recently, an increased frequency of PB Th17 cells has been reported in CSS (11). In line with these reports we found an increased percentage of PB CD4+TNF-α Th1-type cells in active WG and CSS and increased frequencies of IFN-γ (Th1-type), IL-4 (Th2-type), and IL-17 (Th17) expressing CD4+ T-cells in CSS. Th17 cells produce IL-17A and IL-17F and, to a lesser extent, IL-22 (12). In this study we show an increased percentage of CD4+IL-22+ T-cells in CSS for the first time. Interestingly, we found no significant differences in the percentages of IL-10 and TGF-β expressing PB CD4+T-cells between WG, CSS, and healthy controls. Induced regulatory CD4+IL-10+ and CD4+TGF-β+ T-cells have been shown to previously ameliorate inflammation in experimental colitis (13). Therefore, the cytokine production of the total PB CD4+ T-cell population differs between both diseases (Th1-type cells in WG and Th17, Th1- and Th2-type CSS). Moreover, the increase in the frequency of circulating pro-inflammatory cytokine producing T-cells is not matched by a corresponding increase in the frequency of induced regulatory T-cells in WG and CSS.

CSS resembles WG to the effect that it is also characterised by granulomatous inflammation and vasculitis. However, WG and CSS display profound differences in immunopathological features such as the predominance of distinct cytokine profiles of PB CD4+ T-cells, as shown above, and neutrophil- *versus* eosinophil-rich inflammation of the tissue (1-8). PR3-ANCA are detected in almost all Caucasian patients with generalised WG, but rarely in CSS patients. PR3-ANCA are associated with vasculitis in WG (1, 14). Therefore, we wondered whether a specific cytokine pattern to PR3 was elicited in WG. In the present study increased frequencies of IL-17, IL-22, and IL-4-producing PB CD4+ T-cells were found in patients with active PR3-ANCA-positive WG following PR3 stimulation. Moreover, PR3-induced cytokine production could be ascribed to Th2-type and Th17 cells including hitherto unreported Th17/Th1, Th17/Th2 and Th22 (solely IL-22 producing) PR3-induced cell populations in WG. In contrast, a Th2-type-dominated cytokine response was detected in PR3-ANCA-negative CSS after PR3 stimulation. Therefore, the cytokine patterns of CD4+ T-cells elicited in response to PR3 stimulation differ between active WG and CSS with a specific Th17 cell component being present in WG.

Previous studies showed that the cytokine response to an antigen may be influenced by the underlying disease state and disorder. Patients with localised tuberculoid leprosy produce IFN-γ in response to *Mycobacterium leprae* antigens, whereas patients with disseminated lepromatous leprosy produce IL-4 (15). The T-cells of patients with autoimmune type 1 diabetes secrete IFN-γ following islet cell autoantigen stimulation, whereas T-cells of healthy individuals secrete IL-10 potentially suppressing the autoimmune response (16). In the present study we show that distinct cytokine responses are also elicited to the autoantigen PR3 depending on the underlying disorder, *i.e.* WG and CSS. Of note, an increased frequency of PR3-specific Th2-type and Th17 cells has been recently reported in WG in clinical remission (6-8). It was argued that Th17 cells migrate to inflamed sites and, thus, become undetectable during active disease (7). However, our data show that PR3-induced PB Th2-type, Th17 and Th22 cell populations remain detectable during active disease. Interestingly, the 2 WG patients with the highest frequencies of IL-17+ cells suffered from a concomitant bacterial infection. Nasal *S. aureus* colonisation as well as other infections may trigger disease activity in WG (17). Th17 cells induce neutrophil-recruitment in experimental pneumonia (18). The precise role of PR3-induced Th17 cells and the mode of Th17 memory cell imprinting remain to be defined in WG. It has been speculated that IL-17 producing cells could sustain inflammation and PR3-ANCA production (7). Th17 cells including Th17/Th1, Th17/Th2 subsets as well as Th22 cells have been demonstrated in inflammatory lesions in different chronic inflammatory diseases more recently. Further, it has been demonstrated that Th17 cells induce inflammation, enhanced germinal centre formation and autoantibody production in animal models (19-23).

The present study focused on the cytokine response to PR3 because WG and CSS differ with respect to the presence of PR3-ANCA. Myeloperoxidase (MPO)-specific ANCA are found in about 10–40% of CSS patients, but rarely in WG (13, 14). In the present study only 1 CSS patient was MPO-ANCA-positive frustrating a comparative analysis of MPO-induced T-cell responses in this group of patients.

Although most patients included were on immunosuppression, disease activity was seen in all of the patients. In line with earlier studies we did not find an apparent difference in the cytokine responses elicited by untreated patients, relapsing and refractory patients (5, 10). Another point of concern refers to CD4 down-regulation, which may follow

stimulation with phorbol esters. In line with previous reports, cytokine producing CD4+ T-cells were detected without problems within a time limit of 4 hours in the present study (10, 21). Some authors suggest to gate on CD3+CD8- cells to represent the CD4+ T-cells when using stimulation with phorbol esters. However, gating on CD3+CD8- cells is biased by the inclusion of NK cells and "double-negative" (CD3+CD4-CD8-) T-cells in that subset resulting in an overestimation of IFN-γ and IL-17 producing cells (24).

Further studies are needed to elucidate in as much and how immune responses to an antigen are additionally determined by disease rather than antigen alone (25). In this context, the reported alteration of the phenotype of cytomegalovirus (CMV)-specific T-cells in WG patients may also find its explanation (26). In the present study we found differences in total PB and PR3-induced CD4+ T-cell cytokine production between WG and CSS. Our data suggest that the response of the total CD4+ T-cell population and PR3-specific cells is influenced by the underlying disease.

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