Lower numbers of FoxP3 and CCR4 co-expressing cells in an elevated subpopulation of CD4$^+$CD25^{high} regulatory T cells from Wegener's granulomatosis

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Abbreviations:

WG	Wegener's granulomatosis
Treg cells	regulatory T cells
PB	peripheral blood
(c)ANCA	(cytoplasmic) antineutrophil cytoplasmic antibodies
HC	healthy controls
BVAS	Birmingham vasculitis activity score
FoxP3	transcription factor forkhead box P3
CCR4	chemokine receptor 4
IFNRI	interferon receptor I
(i)PR3	(heat-inactivated) proteinase 3
IFN-α	interferon alpha,
TNF-α	tumour necrosis factor alpha
SLE	systemic lupus erythematosus
CFDA-SE	carboxy fluorescein diacetate succinimidylester

ABSTRACT

Defects in regulatory T (Treg) cells have been implicated in the pathogenesis of chronic inflammatory and autoimmune diseases, such as Wegener's granulomatosis (WG). This study aimed at evaluating numbers, phenotype and suppressive capacity of Treg cells in WG. Peripheral blood (PB) mononuclear cells from 22 WG-patients (17 active, 5 remission) and 22 sex- and age-matched healthy controls (HC) were examined for Treg cells by flow cytometry measuring CD4, CD25, transcription factor forkhead box P3 (FoxP3), chemokine receptor CCR4 and interferon receptor I (IFNRI). Suppressive function of CD4$^+$CD25^{high} Treg cells from 3 WG-patients and 3 HC was analysed using a carboxyfluoresceindiacetate-succinimidylester-based in vitro proliferation assay. Endonasal biopsies of 10 WG- and 5 sinusitis-patients were investigated for CD3$^+$FoxP3$^+$ cells, employing double immunohistochemistry. WG-patients displayed elevated numbers of CD4$^+$CD25^{med} T cells and of CD4$^+$CD25^{high} Treg cells. CD4$^+$ T cells of WG-patients contained higher numbers of CCR4$^+$ cells. However, CD4$^+$CD25^{high} Treg cells of WG-patients exhibited decreased numbers of cells co-expressing FoxP3 and CCR4. A low but significant increase of CD4$^+$CD25^{high}IFNRI$^+$ Treg cells was detected in WG-patients. 9 days following stimulation with interferon (IFN)α + proteinase 3 (PR3), a reduced suppression of proliferation of responder T cells was observed for WG and proliferated CD4$^+$CD25^{high} Treg cells still showed downregulated co-expressions of FoxP3 and CCR4. Wegener's granuloma exhibited increased numbers of CD3$^+$FoxP3$^+$ cells. The results indicate upregulated numbers of Treg cells in PB and nasal mucosa as well as phenotypical and functional alterations of PB Treg cells in WG, some presumably mediated through PR3 and IFN-α.

Introduction

Regulatory T (Treg) cells play a major role in regulating immune responses, through interaction (mainly suppression) of responder T cells and antigen presenting cells (1, 2). Thus, numbers as well as phenotypic and functional features of Treg cells in chronic inflammatory and autoimmune diseases have become one of the primary focuses of current T cell research. Treg cells are mostly described as being CD4$^+$CD25^{high}FoxP3$^+$ (1). However, identification of Treg cells remains problematic, since all the presently-used Treg cell markers represent general T cell activation markers (3). For example, the transcription factor FoxP3 is also expressed by non-regulatory T cells (4) and the same is true for CD25 (3). Despite these difficulties, when applied as CD4$^+$CD25^{high} or CD4$^+$CD25^{high}FoxP3$^+$ template, CD25 and FoxP3 still are the most .accepted/ used marker in studies of human Treg cells (1, 5-7). For instance, a study of

PB $CD4^+CD25^{high}$ Treg cells in active rheumatoid arthritis displayed compromised functions (8). In multiple sclerosis, the number of circulating $CD4^+CD25^{high}$ Treg cells was not different from healthy controls (HC), but a reduced inhibitory effect on antigen-specific T cell proliferation was observed (9). PB $CD4^+CD25^{bright/high}$ Treg cells of patients with systemic lupus erythematosus (SLE) exhibited decreased numbers (10-13). Another study reported an increased number of Treg cells in PB of SLE (14), but this may be due to methodological differences in analysing Treg cells ($CD4^+CD25^+FoxP3^+$ cells *vs*. $CD25^{bright/high}$ population). In active sarcoidosis an increase of PB $CD4^+CD25^{bright/high}$ T cells was reported (15). Moreover, Miyara and colleagues demonstrated an accumulation and proliferation of $CD4^+FoxP3^+$ regulatory T cells in the periphery of sarcoidosis granulomas, but there was no correlation with the extent of granulomatous inflammation (15, 16).

Wegener's granulomatosis (WG) is a rare chronic inflammatory disease of unknown etiology, characterised by disseminated necrotising granulomas, systemic vasculitis predominantly affecting small blood vessels, pauci-immune glomerulonephritis and circulating autoantibodies directed against the serine protease PR3 (17, 18). When examining Treg cells in WG, one has to consider earlier studies describing elevated levels of soluble CD25 as well as increased numbers of $CD4^+CD25^+$ T cells (19-22), which emphasises some of the difficulties that come along with regard to a valid differentiation between Treg cells and activated T cells. As additional phenotypic markers we included the chemokine receptor CCR4, which has been shown as one of the well-known phenotypic features of $CD4^+CD25^{high}$ Treg cells (23) as well as the interferon receptor type I (IFNRI) for reasons mentioned below. The only study of PB Treg cells in WG so far, described an elevated number, which exhibited less suppression of proliferation 6 days after polyclonal stimulation of $CD25^{neg}$ T cells (24). However, this study primarily examined Treg cells from WG in remission, therefore the question remains, if the quantitative imbalance and dysfunction of Treg cells occurs in active WG, too. Furthermore, for examination of the suppressive capacity of Treg cells in an *in vitro* proliferation assay the use of carboxyfluoresceindiacetate-succinimidylester (CFDA-SE) as employed herein, instead of ^{3}H-thymidine (24), permits an analysis of potential phenotypic changes of proliferated cells. Such an approach, *i.e*. using CFDA-SE for proliferation analysis, has also been applied in a recent study, describing abnormalities in the number and function of Treg cells, which was most pronounced in active WG (25). In addition to polyclonal stimulation, we employed IFN-α as a modulatory reagent within the *in vitro* proliferation assay. IFN-α is of interest, because it has been demonstrated that it contributes to the conversion of autoreactivity into disease in an autoimmune diabetes mouse model (26). Moreover, it has been suggested that IFN-α can push the immune system toward an autoimmune state (27). Since it has also been shown that IFN-α induces expression of Wegener's autoantigen PR3 by PB leukocytes (28), in addition, we used IFNα + heat-inactivated (=i)PR3 for stimulation within the *in vitro* proliferation assay. Thus, our study investigated numbers, phenotype and the suppressive capacity of Treg cells in WG in comparison to sex- and age-matched HC or non-WG sinusitis, using flow cytometry (CD4, CD25, FoxP3, CCR4, IFNRI), immunohistochemistry (CD3 + FoxP3) and a CFDA-SE-based *in vitro* proliferation assay.

Materials and methods

Study population

Peripheral blood was obtained after informed consent from 22 consecutive adult WG patients (16 women and 6 men; mean age: 44.2±2.6 yr; range: 20-69 yr), who fulfilled the American College of Rheumatology classification criteria (29) and the Chapel Hill Consensus Conference definition (30). At the time blood was drawn, 20 patients were treated. Disease activity was described employing the Birmingham Vasculitis Activity Score (BVAS) according to the EULAR recommendations (31). Twenty-two age- and sex-matched healthy volunteers (16 women and 6 men; mean age 44.2±2.9 yr; range: 22-63 yr) served as controls and provided peripheral blood samples following informed consent. Formalin-fixed, paraffin-embedded nasal biopsies from active WG (n=10) and from non-WG sinusitis (n=5) patients were taken after informed consent and employed for the tissue analysis. Clinical and laboratory data are reported in Table I. The study design has been approved by the local ethics committee (no. 07-045, no. 07-058).

Flow cytometry

Briefly, mononuclear cells were isolated from peripheral blood by density gradient centrifugation on Biocoll (Biochrom, Berlin, Germany). Cells recovered from the interface were washed twice in phosphate buffered saline (PBS, Biochrom) and resuspended in RPMI-1640 medium (Biochrom). Thereafter, cells (1×10^6) were stained with appropriate concentrations of anti-CD4-PacificBlue®/APC (clone: RPA-T4), anti-CD8-PerCP (SK1), anti-CD25-FITC/PE-Cy7 (M-A251), anti-CD28-PE-Cy7 (CD28.2), anti-CD103-PE (Ber-ACT8), anti-CD-152-PE (BNI3), anti-CCR4-PE (1G1) (all from BD, Heidelberg, Germany), anti-PD-1-APC (MIH4, eBioscience/NatuTec, Frankfurt/M., Germany) and anti-IFNR1 (85228; R&D Systems, Wiesbaden, Germany) or corresponding isotype controls for 30 min at 4°C in the dark. Intracellular detection of FoxP3 with anti-FoxP3-PE (PCH 101; eBioscience) was performed using a FoxP3 staining kit (eBioscience) according to the manufacturer's instructions. The stained cells were analysed by flow cytometry on a FACSCalibur and FACSCanto II (BD). The analysis was made using FACSDiva version 6.1.1.

Proliferation assay, suppression experiment and cytokine detection

Blood from three patients with active WG as well as from three age- and sex-matched HC was used. The WG patients were treated with low-dose corticoids only and had no cyclophosphamide at present and in the history, because of the inhibiting influence on Treg cells

Table I. Clinical characteristics of the Wegener's granulomatosis patients.

n.	sex	age (years)	disease activity	cANCA (titer)	CRP (mg/dl)	BVAS	DEI	Therapy
1	F	38	A	1024	11.5	15	7	MTX/Pr
2	F	44	A	512	1.74	6	6	C/Pr
3	F	35	A	1024	0.3	8	4	MMF/Pr
4	F	65	R	512	0.3	0	0	MTX/Pr
5	F	51	R	2048	0.3	0	0	MTX/Pr
6	F	35	A	0	0.3	3	1	L/Pr
7	M	58	A	1024	0.3	6	2	C/Pr
8	M	58	R	32	0.3	0	0	none
9	F	69	R	256	0.64	0	0	MTX/Pr
10	F	39	A	1024	11.8	10	8	none
11	M	37	A	20	0.3	9	4	C/Pr
12	F	61	A	2560	6.8	11	11	MTX/Pr
13	F	38	A	40	0.7	2	3	MTX/Pr
14	F	44	A	1280	5.8	17	13	AZA/Pr
15	M	60	A	80	0.4	6	4	MTX/Pr/L
16	M	26	R	0	0.7	0	4	L/Pr
17	F	54	A	160	0.2	6	2	AZA/Pr
18	F	45	A	1024	0.4	6	10	MTX/Pr
19	F	39	A	40	0.2	4	2	C/Pr
20*	F	20	A	1280	10.4	28	10	Pr
21*	F	30	A	2560	0.2	12	2	Pr
22*	M	26	A	320	15.9	5	4	Pr
T - 1	M	43	A	1280	13.5	6	5	C/Pr
T - 2	F	75	A	64	0.3	4	2	none
T - 3	F	28	A	0	0.3	4	2	none
T - 4	M	62	A	n.d.	n.d.	n.d.	n.d.	n.d.
T - 5	M	78	A	256	0.3	4	4	C/Pr
T - 6	F	65	A	256	17.8	4	6	Pr
T - 7	F	52	A	n.d.	n.d.	n.d.	n.d.	n.d.
T - 8	M	21	A	512	11.4	9	6	MTX
T - 9	M	85	A	0	0	13	4	C/Pr
T - 10	M	78	A	0	25.7	4	4	C/Pr

A: active; R: complete remission; cANCA: cytoplasmic anti-neutrophil cytoplasmic antibodies; CRP: C-reactive protein; BVAS: Birmingham Vasculitis Activity Score; DEI: disease extent index (50); AZA: azathioprine; L: leflunomide; M: mycophenolate mofetil; Pr: prednisolone; C: cyclophosphamide; MTX: methotrexate; *patients, whose cells were taken for proliferation experiments; T – WG patients, whose biopsies were used for double immunohistochemistry; n.d. - not determined.

(32). PB mononuclear cells (PBMC) were prepared as described before. Removal of $CD25^+$ T cells, including the $CD25^{high}$ T cells, was done using the CD25 MicroBeads II kit according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). The positive selection resulted in >95% purity of the $CD25^+$ T cell fraction and <1% $CD25^+$ T cells in the $CD4^+CD25^{neg}$ fraction (data not shown). Thereafter, 5 different cellular compositions were generated to use in the *in vitro* proliferation assay, *i.e.* PBMC, $CD25^{high}$ depleted cells (=$CD25^{neg}$), $CD25^+$ T cells, containing the $CD25^{high}$ T cells, and two co-cultures of $CD25^{neg}$ cells with $CD25^+$ T cells in ratios of 1:1 and 2:1. It has been described that a proportion of $CD4^+CD25^{high}FoxP3^+$ Treg cells is generated by proliferation (33). In addition, it has been reported that Treg cells do not need other cell types to suppress proliferation of $CD4^+CD25^{neg}$ responder T cells (34). Thus, all five samples/co-cultures were resuspended in sterile PBS and CFDA-SE (Invitrogen, Karlsruhe, Germany) was added in the dark to a final concentration of 0.5 μM (35). The cells were incubated at 37°C for 10 minutes, then put on ice and washed twice with cold PBS. Thereafter, PBMC (1 x 10^6/ml) and T cell mixtures (3 x 10^5/ml) were incubated in a 48-well plate (BD) with either RPMI-1640 medium (Biochrom) supplemented with 10% heat-inactivated human pooled serum (PAA, Coelbe, Germany), 1% L-glutamine and 1% penicillin-streptomycin (Biochrom) alone or with the addition of soluble anti-CD3 antibodies (0.5 μg/ml and 1.0 μg/ml, Sanquin, Amsterdam, Netherlands), IFN-α (universal type I interferon, 500 units/ml, PBL Biomedical Laboratories, Piscataway, NJ, USA) and with IFNα + heat-inactivated Proteinase 3 (iPR3, 5 μg/ml, Athens Research and Technology, Athens, GA, USA) at 37°C and 5% CO_2 for nine days. Following nine days the cells were collected and analysed by flow cytometry. Dead cells were stained and excluded using 7-aminoactinomycin D (7-AAD, BD). Proliferated cells were defined as $CFDA\text{-}SE^{low}$ cells as described by Venken *et al*. (35). Suppression (%) of proliferation was calculated as reported (24, 36, 37) and as follows:

% suppression = 100 – (100 x [% of proliferated cells of co-culture [[1:1 or 1:2]] / % of proliferated responder T cells alone])

On day one after stimulation, 100 μl of supernatant were taken and stored at -80°C for cytokine detection. TNF-α was measured using a cytokine reagent kit (Microbionix, München, Germany) following the manufacturer's instructions.

Double immunohistochemistry

Briefly, 2-3 μm thick paraffin-embedded tissue sections on superfrost+ slides (Menzel, Braunschweig, Germany) were dewaxed using xylene, rehydrated and then submitted to antigen retrieval by heating in target retrieval solution, pH 9.0 (Dako, Hamburg, Germany) in a microwave oven. After cooling, the slides were washed with Tris-buffered saline (TBS). Endogenous peroxidase was blocked by means of a commercial peroxidase-blocking reagent (Dako) for 5 min in a humidified chamber at room temperature. Slides were rinsed with TBS and then incubated for 60 min with mouse anti-human Foxp3 antibody (236A/E7, eBioscience). Thereafter, the slides were washed and immunodetection was performed with a polymer HRP-labelled anti-mouse secondary antibody (Dako) for 30 min and the reaction was developed for 5 min using diaminobenzidine (Dako) as chromogenic substrate. Slides were then rinsed with distilled water. Endogenous alka-

line phosphatase was blocked employing a commercial double stain blocking reagent (Dako). Double labelling was carried out incubating the slides with a polyclonal rabbit anti-human CD3 antibody (Dako) for 30 min, followed again by washing steps and another incubation with a polymer-labelled AP anti-rabbit secondary antibody (Dako) for 30 min. The reaction was developed for 5 min using fast red (Dako) as chromogenic substrate. Finally, sections were counterstained with hematoxylin and mounted with aquatex (Merck, Darmstadt, Germany). All sections were stained in parallel with appropriate isotype controls (Dako) for the primary antibodies to provide negative controls.

Statistical analysis

Statistics were perfomed using Prism v4.0 (GraphPad Software, San Diego, CA, USA). Comparisons between patients and control subjects were done employing either the unpaired *t*-test or the Mann-Whitney U-test. The correlations between the number of circulating $CD4^+CD25^{high}$ Tregs and the cANCA titer as well as between $CD4^+CD25^{neg}CCR4^+$ T cells and WG disease activity (BVAS) were calculated using the Spearman's correlation coefficient. Unless otherwise indicated, data are shown as mean ± standard error of the mean (SEM). *P*-values equal to or less than 0.05 were considered to be statistically significant.

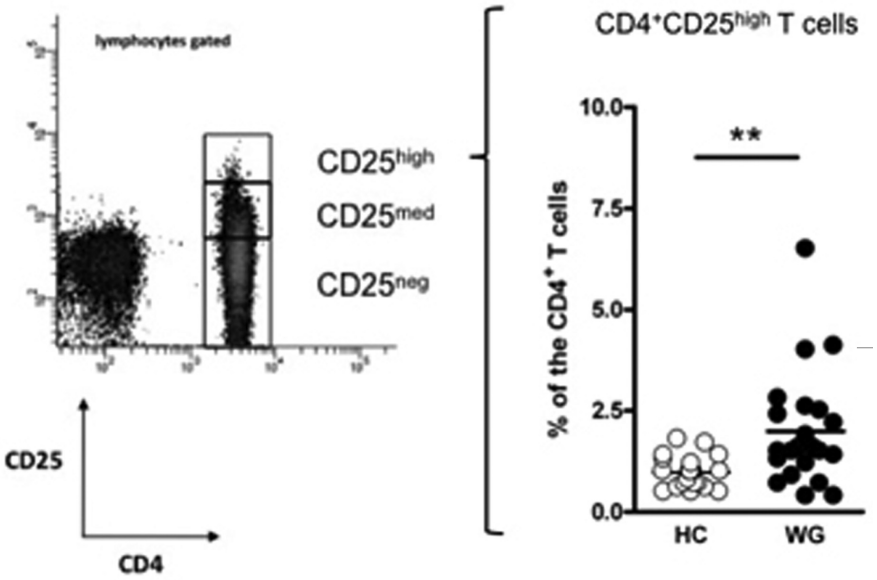

Fig. 1. Flow cytometric analysis of the phenotype and proportion of $CD4^+CD25^{high}$ T cells of HC and WG. Representative dot plot demonstrating the gating of $CD4^+CD25^{neg}$, $CD4^+CD25^{med}$ and $CD4^+CD25^{high}$ T cells (left panel). $CD4^+CD25^{high}$ T cells of WG exhibited an elevated number (%), when compared to HC ($p<0.005$), (right panel).

Results

Elevated numbers of peripheral blood $CD4^+CD25^{high}$ T cells in WG

No significant differences between WG and HC in terms of absolute numbers of $CD4^+$ and $CD8^+$ lymphocytes as well as of percentages were observed. This is in agreement with a previous study (38). Regarding the co-expression of CD25 on both, $CD4^+$ and $CD8^+$ T cells, elevated numbers were found for WG compared to HC ($CD4^+CD25^{med}$: WG *vs*. HC: 15.09±1.31% *vs*. 8.04±0.70%, $p<0.0001$; $CD8^+CD25^+$: WG *vs*. HC: 3.16±0.78% *vs*. 0.70±0.15%, $p<0.005$, data not shown). Moreover, the percentage of $CD25^{high}$ cells among $CD4^+$ T cells was also about 2fold higher for WG compared to HC (WG *vs*. HC: 1.99±0.3% *vs*. 0.97±0.08%, $p<0.005$, gating exemplarily shown in Fig. 1, left panel, Fig. 1 right panel). On the other side, the $CD4^+CD25^{neg}$ T cell population was decreased in WG, when compared to HC (WG *vs*. HC: 84.91±1.3% *vs*. 91.46±0.89%, $p<0.001$, data not shown).

Decreased numbers of $FoxP3^+$ cells within the $CD4^+CD25^{high}$ T cell compartment in WG

Using FoxP3 as a defining marker for Treg cells no differences were found in the total $CD4^+$ T cell population between WG and HC (WG *vs*. HC: 3.38±1.03% *vs*. 1.43±0.35%, *p*=not significant=ns, data not shown). When evaluating the CD25 subpopulations, our data revealed that about 56% of the $CD4^+CD25^{high}$ cells from HC co-expressed FoxP3 (Fig. 2A and 2B, upper panels). Other studies demonstrated higher values of about 85% of $CD4^+CD25^{high}FoxP3^+$ Treg cells in HC, however, this was based on either sorted $CD4^+CD25^{high}$ T cells or PCR analysis (11, 15). Unexpectedly, we observed that the number of $FoxP3^+$ cells within the $CD4^+CD25^{high}$ T cell subpopulation of WG was substantially reduced (Fig. 2A and 2B, upper panels, WG *vs*. HC: 27.55±8.31% *vs*. 55.60±6.62%, $p<0.05$). At first, such a result seems somewhat contradictory, because strictly speaking Treg cells are defined as $FoxP3^+CD4^+CD25^{high}$ T cells. However, similar findings of a reduced FoxP3 expression have been described for active SLE (11, 12). There was no difference in the number of $FoxP3^+$ cells within the $CD4^+CD25^{med}$ population (Fig. 2B, middle panel, WG *vs*. HC: 15.65±3.34% *vs*. 14.97±2.86%, *p*=ns). A small number of FoxP3-expressing cells were also found within the $CD4^+CD25^{neg}$ population and higher numbers of $FoxP3^+$ cells were detected in WG, when compared to HC (Fig. 2B, lower panel, WG *vs*. HC: 3.40±1.06% *vs*. 0.92±0.19%, $p<0.05$).

When examining the number of $CD4^+CD25^{high}$ T cells from PB of 50 consecutive WG patients (adding another 28 to the 22 patients described herein), we observed a positive correlation with the cANCA titer ($r^2=0.35$, $p=0.002$, data not shown).

Diminished numbers of $CCR4^+$ cells within the $CD4^+CD25^{high}$ T cell compartment in WG

More $CD4^+$ T cells from WG co-expressed the chemokine receptor CCR4, when compared to HC (WG *vs*. HC: 26.85±2.44% *vs*. 20.22±1.44%, $p<0.05$,

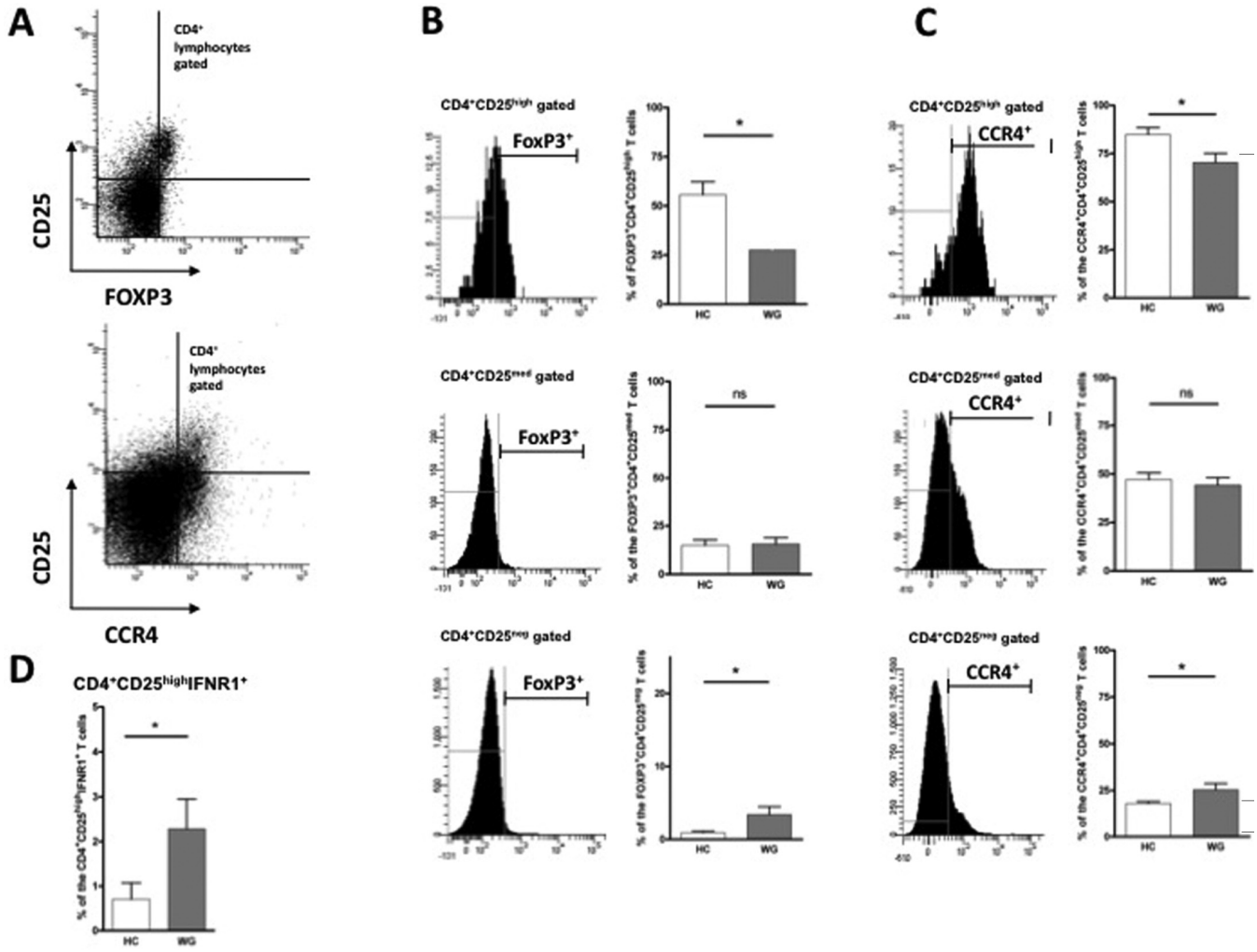

Fig. 2. A: Representative dot plots of the expression of FoxP3 and CCR4 gated on $CD4^+$ T cells. **B**: Expression of intracellular FoxP3 in $CD4^+CD25^{high}$, $CD4^+CD25^{med}$ and $CD4^+CD25^{neg}$ T cells (histograms), arbitrarily (according to (5)) divided on the basis of the CD25 expression (see also Fig. 1, left panel). WG showed a decreased number (%) of FoxP3 expressing cells in the $CD4^+CD25^{high}$ T cell subpopulation, when compared to HC (bar graphs, $p<0.05$). In contrast, the $CD4^+CD25^{neg}$ T cells subpopulation displayed an increased number (%) of FoxP3 expressing cells, when compared to HC (bar graphs, $p<0.05$). **C**: Expression of CCR4 on $CD4^+CD25^{high}$, $CD4^+CD25^{med}$ and $CD4^+CD25^{neg}$ T cells (histograms), arbitrarily (according to (5)) divided on the basis of the CD25 expression (see also Fig. 1, left panel). WG showed a decreased number (%) of CCR4 expressing cells in the $CD4^+CD25^{high}$ T cell subpopulation, when compared to HC (bar graphs, $p<0.05$). Similar to FoxP3, the $CD4^+CD25^{neg}$ T cells subpopulation displayed an increased number (%) of CCR4 expressing cells, when compared to HC (bar graphs, $p<0.05$). **D**: WG demonstrated a low, but increased number (%) of $CD4^+CD25^{high}IFNRI^+$ T cells, when compared to HC (bar graphs, $p\leq0.05$).

data not shown). There was no difference in CCR4 co-expression of $CD8^+$ T cells between WG and HC (WG *vs*. HC: 3.42±0.42% *vs*. 4.56±0.67%, p=ns). Interestingly, a high number of $CD4^+CD25^{high}$ T cells (*i.e*. more than two thirds) from WG and HC exhibited a co-expression of CCR4. Nonetheless and in contrast to the overall $CD4^+$ T cell population, we observed a diminished expression of CCR4 on $CD4^+CD25^{high}$ T cells of WG, when compared to HC (Fig. 2A, lower panel and 2C, upper panel, WG *vs*. HC: 70.29±4.79% *vs*. 84.85±3.56%, $p<0.05$). No differences of the CCR4 co-expression between WG and HC were detected in the $CD4^+CD25^{med}$ compartment (Fig. 2A, lower panel and 2C, middle panel, WG *vs*. HC: 44.25±3.82% *vs*. 47.03±3.46%, p=ns). In line with the total $CD4^+$ T cell population, $CD4^+CD25^{neg}$ T cells of WG showed increased numbers of cells co-expressing CCR4, when compared to HC (Fig. 2A, lower panel and 2C, lower panel, WG *vs*. HC: 25.33±3.25% *vs*. 17.97±1.064%, $p<0.05$). When examining the number of $CD4^+CD25^{neg}CCR4^+$ T cells from PB of 50 consecutive WG patients (adding another 28 to the 22 patients described herein), we observed a negative correlation with the disease activity score BVAS (r^2 =-0.397, $p<0.0001$, data not shown).

Increased numbers of $IFNR1^+$ cells within the $CD4^+CD25^{high}$ T cell compartment in WG

The overall number of T lymphocytes expressing the IFN receptor 1 (IFNR1) was quite low compared to for instance CCR4. The differences between WG and HC looking at the expression of IFNR1 within the total $CD4^+$ and the total $CD8^+$ T cell populations were marginal ($CD4^+$: WG *vs*. HC: 0.37±0.12% *vs*. 0.25±0.06%, p=ns; $CD8^+$: WG *vs*. HC: 0.66±0.26% *vs*. 0.17±0.04%,

p=ns). Interestingly, the number of $CD4^+CD25^{high}$ T cells co-expressing the IFNR1 was slightly increased for WG compared to HC (Fig. 2D, WG *vs*. HC: 2.28±0.66% *vs*. 0.71±0.36%, $p \leq 0.05$). Expression of IFNR1 within both, the $CD4^+CD25^{med}$ and the $CD4^+CD25^{neg}$ T cell subpopulation was below detection level. In addition, we found no differences regarding the expression of CD103, CD152 (CTLA4) and PD-1 on $CD4^+CD25^{high}$ T cells between WG and HC (data not shown).

Reduced suppression of in vitro proliferation of responder T cells and impaired phenotype of proliferated $CD4^+CD25^{high}$ T cells in WG, following IFNα and PR3 stimulation

In order to determine a potential influence of $CD25^{high}$ Treg cells on the proliferation of $CD25^{neg}$ cells (=responder T cells), $CD25^+$ and $CD25^{neg}$ cellular subsets were isolated from PBMC of 3 WG patients and of 3 HC employing magnetic beads and cultured with CFDA-SE in co-culture experiments using four different ratios and PBMC as a whole (*i.e*. 100% $CD25^{neg}$ cells, 67% $CD25^{neg}$ cells + 33% $CD25^+$ cells, 50% $CD25^{neg}$ cells + 50% $CD25^+$ cells, 100% $CD25^+$ cells and PBMC). The data showed that 9 days after stimulation with iPR3 + IFNα both co-culture experiments of 50% $CD25^{neg}$ + 50% $CD25^+$ cells (1:1) and of 67% $CD25^{neg}$ + 33% $CD25^+$ cells (2:1) exhibited a reduced suppression of the proliferation of $CD25^{neg}$ responder T cells from WG, when compared to HC ($p<0.05$). In the case of 9 days following stimulation with IFN-α a reduced suppression of proliferation of $CD25^{neg}$ responder T cells from WG was observed for a ratio of 67% $CD25^{neg}$ + 33% $CD25^+$ cells, when compared to HC ($p<0.05$), (Fig. 3). When PBMC were stimulated with iPR3 + IFN-α and with IFN-α, after 9 days we observed phenotypic alterations of proliferated $CD4^+CD25^{high}$ T cells in WG, when compared to HC. Especially following IFNα stimulation proliferated $CD4^+CD25^{high}$ T cells of WG displayed virtually no co-expression of FoxP3 or CCR4, when compared to HC (CCR4: $p<0.05$), (Fig. 4, middle and right panel).

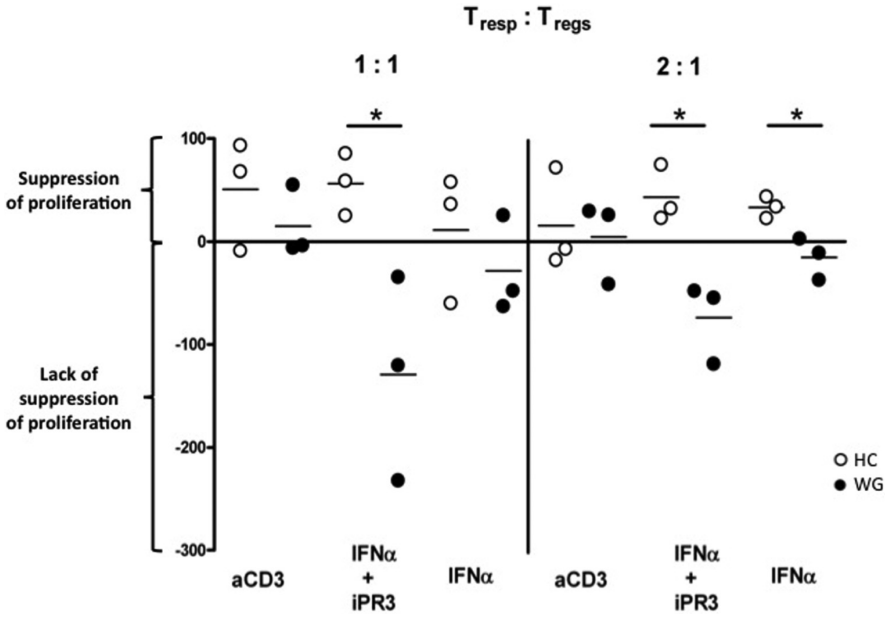

Fig. 3. *In vitro* proliferation and suppression assay of Treg cells from WG and HC. Suppression (%) of the $CD25^{neg}$ responder T (=Tresp) cell proliferation by $CD25^+$ Treg cells (Tregs) at ratios of 1:1 and 2:1. Reduced suppressive capacity of Treg cells from WG, when compared to HC 9 days after stimulation with IFNα + heat-inactivated (=i)PR3 (both ratios, $p<0.05$) and IFNα (ratio 2:1, $p<0.05$). As a control, data 9 days following stimulation with anti-CD3 are also shown.

Following 24h of *in vitro* stimulation with 0.5 μg/ml anti-CD3 we found an elevated release of TNF-α by PBMC from WG compared to HC ($p \leq 0.05$, data not shown).

$CD3^+FoxP3^+$ cells accumulate in the periphery of Wegener's granuloma

Further, we analysed the presence of $FoxP3^+$ T cells in nasal biopsies from 10 WG patients. Compared with sinusitis tissue of 5 non-WG patients, the granulomatous lesions of WG showed increased numbers of $CD3^+FoxP3^+$ T cells in relation to $CD3^+$ T cells (Fig. 5, WG *vs*. non-WG sinusitis: 9.7±1.1%, n=10, *vs*. 2.8±1.36%, n=5, $p \leq 0.005$).

Discussion

Our results indicate an elevated number of PB $CD4^+CD25^{high}$ Treg cells in WG, irrespective of disease activity (*i.e*. active or remission), with lower numbers of FoxP3 and CCR4 co-expressing Treg cells and an impaired suppressive function *in vitro*. Thus, the findings confirm and extend the observations for $CD4^+$ Treg cells of WG by two previous studies (24, 25). In aggreement with a recent study (25), we observed a (50%) reduction in the number of $FoxP3^+$ cells within the $CD4^+CD25^{high}$ T cell subpopulation of WG (Fig. 2B, upper panel). Since CD25 is also expressed by activated T cells of WG (20-22), the question arises, if these $CD4^+CD25^{high}FoxP3^{neg}$ cells represent indeed Treg cells or rather activated T cells. In order to solve this problem, expression of CD25 on $CD4^+$ T cells was arbitrarily divided into $CD25^{neg}$, $CD25^{med}$ and $CD25^{high}$ and only the latter have been designated as Treg cells (Fig. 1, left panel; according to previously published criteria, (5)). However, we did not find a correlation between the number of $CD4^+CD25^{high}$ Treg cells and disease activity (BVAS). On the other hand, there was a moderate positive correlation between the number of $CD4^+CD25^{high}$ Treg cells and the cANCA titer, which could be taken as a hint for a potential association of the Treg cells with a disease/diagnostic marker, but this needs to be confirmed. Nonetheless, activated T cells should rather be represented by the $CD4^+CD25^{med}$ T cell subpopulation than by $CD4^+CD25^{high}$ T cells and, in agreement with previous studies of WG (20-22), the number of the $CD4^+CD25^{med}$ T cells was upregulated herein as well. Moreover, similar findings of a reduction of FoxP3 expression levels in $CD4^+CD25^{high}$ Treg

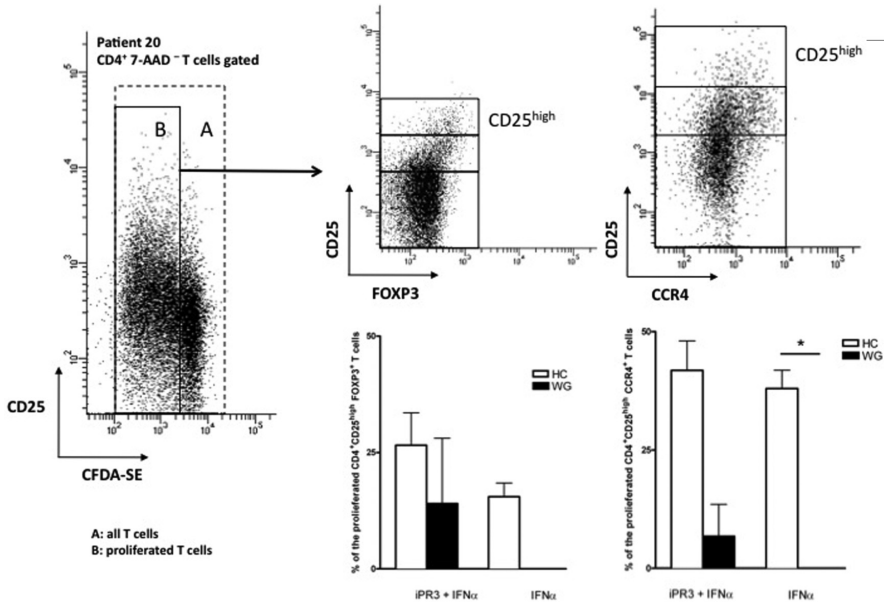

Fig. 4. Representative gating of proliferated T cells defined as CFDA-SE^{low} T cells gated on CD4$^+$7-AAD$^-$ living T cells (dot plot, left panel). Lack of expression of intracellular FoxP3 and surface CCR4 in/on proliferated CD4$^+$CD25^{high} T cells from WG 9 days following stimulation with iPR3 + IFNα and IFNα, when compared to HC (CCR4: $p<0.05$), (middle and right panel).

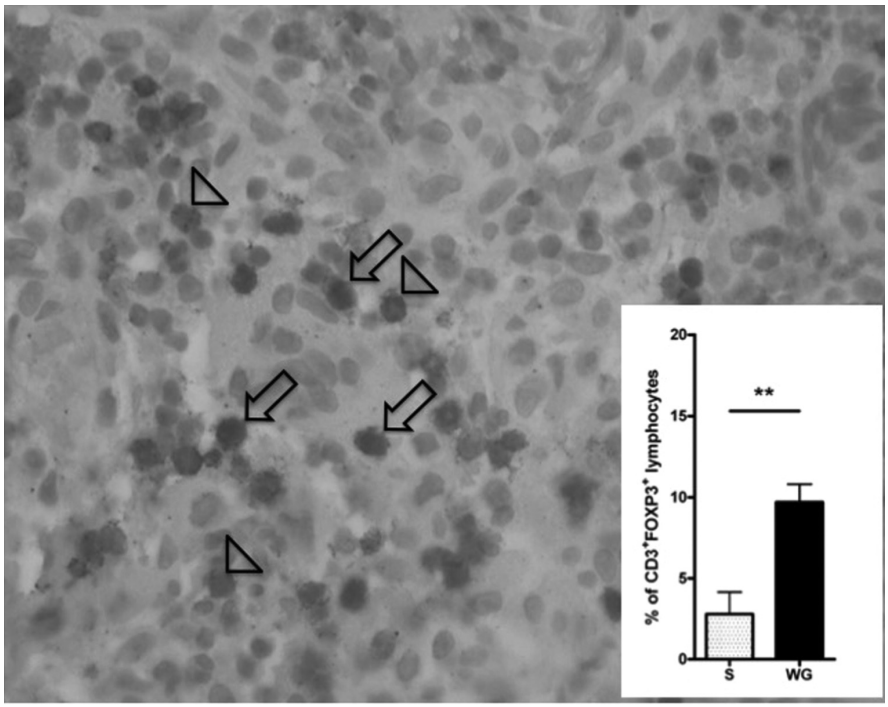

Fig. 5. Accumulation of CD3$^+$FoxP3$^+$ T cells within Wegener's granuloma. FoxP3$^+$ cells (brown nuclear staining) are also CD3$^+$ (red membrane staining, arrow), in contrast to CD3$^+$FoxP3$^-$ cells (red membrane staining, triangle), (original magnification x400). Inset: Overall, nasal tissues of WG demonstrated an increased diffuse accumulation of FoxP3$^+$ T cells in the granulomatous lesion, when compared with non-WG sinusitis tissues ($p<0.005$).

cells were described in studies of patients with active SLE, eventually associated with deficiencies of the Treg cell functions (12, 13). Two previous studies of vasculitis and WG, respectively, reported no differences in (i) the levels of FoxP3 mRNA of CD4$^+$ cells between vasculitis and HC and (ii) the numbers of CD4$^+$FoxP3$^+$ T_{EM} cells between active WG and WG in remission (22, 39). These findings might be somewhat reflected by our data showing no difference in the number of cells co-expressing FoxP3 within the CD4$^+$CD25^{med} T cell population, containing the activated T cells, between WG and HC. Interestingly, we observed elevated numbers of CD4$^+$CD25^{neg}FoxP3$^+$ T cells for WG, when compared to HC. Recently, Bonnelli and colleagues (40) suggested that in SLE such cells resemble conventional Treg cells to a certain extent. When interpreting the findings of an increased number of Treg cells in WG (as shown before and herein), one also should consider that an increased number of CD4$^+$CD25^{high} Treg cells might reflect an influence of medication, since it has been shown that treatment with corticosteroids or anti-TNF-α led to increased numbers of CD4$^+$CD25^{high} Treg cells (8, 12). In addition, we and others demonstrated an expanded proportion of effector memory T cells in WG, providing evidence for a dysbalance between effectors and regulators within the T cell population (41-43). The chemokine receptor CCR4 is not solely expressed on Treg cells, but also on the majority of circulating CD4$^+$ memory T cells (44, 45), which is reflected by our findings showing that all subpopulations of CD4$^+$ T cells from HC as well as from WG contained substantial numbers of CCR4$^+$ cells. Surprisingly, Treg cells from WG exhibited a lower number of cells co-expressing CCR4 in our study (Fig. 2C, upper panel). A similar result was again described for SLE, *i.e.* not only a significantly reduced number of Treg cells, but also of CCR4$^+$ Treg cells, when compared to HC (13). In addition, because of the negative correlation between the BVAS and the number of circulating CD4$^+$CD25^{neg}CCR4$^+$ T cells one could speculate about an increased migration of these cells into inflamed tissues of more severe WG. Furthermore, we observed a low, but significant upregulation of the number of IFNRI expressing Treg cells in WG (Fig. 2D). In summary, our results of decreased numbers of FoxP3$^+$ and CCR4$^+$ Treg cells together with an increased number of IFNRI$^+$ Treg cells in WG suggest an altered phenotype, which could be relevant for their functions, *i.e.* suppression

of proliferation. In order to test this assumption, we examined the suppressive capacity of the Treg cells in an *in vitro* proliferation assay and used the ligand of the IFNRI, IFN-α, alone and in combination with Wegener's autoantigen PR3 for stimulation of the cells. Herein, we observed that IFNα-, but especially IFNα+iPR3-treated Treg cells from WG were less effective in suppressing the proliferation of responder T cells (Fig. 3), maybe partially due to an increased interaction between IFNRI and IFN-α. Our results also showed that the altered phenotype of the Treg cells from WG (decreased numbers of FoxP3$^+$ and CCR4$^+$ cells) seemed to be sustained through the *in vitro* assay, especially following IFN-α-treatment (Fig. 4, middle and right panel). Of note, an earlier study showed elevated serum levels of IFN-α in WG and other primary systemic vasculitides (46). A study of SLE implicated in this context that IFN-α-producing antigen-presenting cells are at least partially responsible for a blockade of Treg function (14). Further, in the case of rheumatoid arthritis, it has been demonstrated that TNF-α downmodulates the function of Treg cells (47). Since TNF-α levels were upregulated in the supernatant of PBMC from WG, albeit only following anti-CD3 stimulation, this might be taken as an additional hint, why the Treg cells from WG showed less suppressive capacity than HC within the *in vitro* assay. Thus, for all the methodological limitations of the functional *in vitro* assay, *i.e.* the use of anti-CD25-coated magnetic beads instead of cell sorting (2) and the low experimental number, our data indicate a potential dysfunction of the PB-derived Treg cells in WG, presumably promoted by the autoantigen PR3 in conjunction with IFN-α. Apart from in-, decreased or unchanged numbers of PB Treg cells in autoimmune and inflammatory disorders, some studies described an accumulation of Treg cells in inflamed tissues of humans and in animal models of autoimmunity (15, 16, 48), based on the assumption that FoxP3 detection on fixed tissues mainly identifies FoxP3^{bright} cells corresponding to memory phenotype Treg cells (16). Another study even suggested that CD4$^+$CD25$^+$CD27$^+$ T cells are Treg cells, which participate in the ongoing immune response within lymphoid aggregates of synovial tissue from juvenile idiopathic arthritis (49). In some accordance with the sarcoidosis studies (15, 16), we observed an increased number of FoxP3$^+$ T(reg) cells in granulomatous lesions of WG, when compared to non-WG sinusitis (Fig. 5). Nonetheless, it remains to be determined, if such a potential Treg cell accumulation is of help in suppressing the local *in situ* inflammation in WG. For sarcoidosis granulomas it has been shown that granuloma Treg cells proliferate, but might represent "exhausted" or intrinsically defective memory phenotype Treg cells, which accumulate, but are not able to suppress *in vitro* granuloma formation in contrast to Treg cells from HC (16).

Altogether, our major results indicate upregulated numbers of PB CD4$^+$CD25^{high} Treg cells with a downregulated co-expression of FoxP3 and CCR4 in WG as well as increased numbers of FoxP3$^+$ T(reg) cells in the nasal mucosa of WG. However, we are well aware that these data have to be interpreted with great caution, because as pointed out above, none of the markers used herein, is exclusively present on Treg cells and WG is an autoimmune disease characterised by ongoing chronic inflammation, including persistent T cell activation (21). In order to further test the findings of elevated numbers of Treg cells in blood and tissue of WG, potentially with alterations of phenotype and function, some of them possibly mediated via Wegener's autoantigen PR3 in conjunction with IFN-α, further experimental work and evidence is needed. For instance, it remains to be determined, if IFN-α is present with Wegener's granuloma and if so, subsequently, together with PR3, able to exert similar effects on Treg cells *in situ* as described herein.

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