RHEUMATOLOGY

Original article

Aberrant expression of the negative costimulator PD-1 on T cells in granulomatosis with polyangiitis

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Abstract

Objective. Persistent T-cell activation is frequently observed in granulomatosis with polyangiitis (GPA, formerly known as Wegener's granulomatosis). T-cell activation is usually balanced by negative costimulatory molecules. The negative costimulator programmed death receptor-1 (PD-1) and its relevance to T-cell immunity have not been studied so far in GPA. Thus it is the aim of the study to characterize the role of PD-1 in GPA.

Methods. Thirty-two patients suffering from GPA and 19 age-matched healthy controls (HCs) were enrolled. T-lymphocyte subsets from peripheral blood were analysed by flow cytometry for the expression of PD-1. The frequency of memory T cells and T cells producing pro-inflammatory cytokines was determined. Renal biopsies from GPA patients were stained for CD3 and PD-1.

Results. PD-1 expression was increased on T-helper cells (Th cells) from GPA patients as compared with HCs. In addition, parameters of persistent T-cell activation and production of pro-inflammatory cytokines were positively associated with numbers of PD-1$^+$ Th cells in patients but not in HCs. Latent infection with CMV seemed to enhance PD-1 expression on CD4$^+$ and CD4$^+$CD25$^-$ T cells. Interestingly, expression of PD-1 on CD4$^+$CD25$^+$T cells was inversely correlated with relapse rate. Importantly, lesional T cells were mostly lacking PD-1.

Conclusions. The expression of the negative costimulator PD-1 is altered in GPA and might counterbalance persistent T-cell activation.

Key words: ANCA, vasculitis, T cells, PD-1.

BASIC SCIENCE

Introduction

Granulomatosis with polyangiitis (GPA, formerly known as Wegener's granulomatosis) is an autoimmune vasculitis of small- to medium-sized vessels that is characterized by

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the presence of ANCAs [1–4]. Granulomatous necrotizing inflammation of the upper/lower airways and rapidly progressive GN is usually observed. This disorder leads to acute organ failure and fatal prognosis if left untreated [5]. The adaptive and the innate immune system both contribute to disease mechanisms [6]. ANCA-triggered activation and degranulation of neutrophils causes endothelial injury and vasculitic damage [6]. Along with this, granulomatous necrotizing inflammation is found in affected organs. T cells are usually present in the inflammatory infiltrate [7, 8] and granuloma formation is T-cell dependent [9, 10]. Furthermore, determination of IgG subclasses suggests a T-cell-dependent class switch of B cells [11]. In line with these findings, several authors report abnormalities of the T-cell compartment in GPA. Persistent expansion and activation of T cells is a well-established finding [12–15]. In autoimmunity, breakdown of T-cell tolerance is a prerequisite [16, 17]. Inhibitory pathways

normally restrain T-cell function and maintain peripheral tolerance keeping auto-reactive T cells anergic. The programmed death receptor-1 (PD-1) belongs to the family of negative costimulatory molecules and inhibits T-cell activation. The importance of PD-1 to immune tolerance has been demonstrated in animal models [18–20]. Blockade of the PD-1 pathway leads to enhanced rejection in transplant models and promotes inflammation in animals prone to autoimmunity [21]. In RA, multiple sclerosis (MS) and SLE, alterations of the PD-1 pathway with implications for disease pathogenesis have been shown [22–26]. PD-1 polymorphisms have been investigated in GPA but further studies on the impact of PD-1 on T-cell immunity are lacking [27]. Given the role of T cells in GPA, we explored the expression of PD-1 on T cells in relation to disease pathogenesis.

Methods

Patient cohort

Thirty-two consecutive patients with GPA visiting the outpatient clinic of the Department of Nephrology or Department of Rheumatology and Clinical Immunology were enrolled (Table 1 and supplementary Table S1, available as supplementary data at *Rheumatology* Online). Fifteen of these patients were sampled and assessed twice. Thirty of 32 patients were measured with quiescent disease and 3 of these 30 patients were sampled in addition during relapse of disease. Nineteen age-matched healthy individuals [median age 55 (48–58) years; 9 males and 10 females) with no history of chronic infections, cancer or autoimmune diseases were enrolled as control cohort. For the functional studies on PD-1 ligation, five additional HCs and five consecutive patients with quiescent GPA visiting the outpatient clinic of the Division of Clinical Immunology were recruited. A relapse was defined as new disease manifestation occurring after a period in remission and requiring intensified treatment with steroids, increased dose of maintenance therapy or CYC. Two of 32 patients were sampled once with new-onset disease. The diagnosis of GPA was made in accordance with the criteria of the ACR and Chapel Hill

TABLE 1 Patients' characteristics

Patients' characteristics	
Total, *n*	32
Age, median (IQR), years	62 (46–69)
Gender, female/male, *n*	14/18
PR3/MPO, *n*	29/3
Disease duration, median (IQR), months	71 (23–168)
Localized/systemic disease, *n*	8/24
Active disease, *n*	5
CMV anti-IgG, +/−	11/13

Localized disease was defined as GPA restricted to the upper and lower respiratory tract, whereas systemic disease also involves organs outside the respiratory tract [28].

Consensus [3, 4]. Detailed patient characteristics are given in Table 1 and supplementary Table S1, available as supplementary data at *Rheumatology* Online. Clinical data were obtained retrospectively based on the patient's file records. CMV IgG antibody status was determined using the Enzygnost anti-CMV IgG Assay (Siemens Healthcare Diagnostics, Eschborn, Germany) according to the manufacturer's instructions. Informed consent was obtained from each patient. The local ethical committee of the University Duisburg-Essen and the medical ethical committee of the University Hospital Maastricht approved the study.

Flow cytometry: surface and intracellular staining

Expression levels of the receptors including PD-1 were measured by four-colour surface staining on unstimulated lymphocytes from whole blood. Phycoerythrin (PE), FITC, peridin chlorophyll protein (PerCP) and allophycocyanin (APC)-labelled antibodies with different specificity were used: CD3 (mouse IgG1, PerCP), CD4 (mouse IgG1, PerCP), CD25 (mouse IgG1, FITC), CD45RO (mouse IgG1, FITC), CD127 (mouse IgG1, APC; R&D Systems, Wiesbaden, Germany), PD-1 (mouse IgG1, PE), CD197 (CCR7, rat IgG2a, Alexa Fluor 647) and MultiTest CD3/CD8/CD45/CD4. All antibodies except CD127 were purchased from Becton Dickinson, Heidelberg, Germany. Appropriate isotype controls (Becton Dickinson) were used. Peripheral whole blood was stained with labelled mAbs for 20 min at room temperature followed by red blood cell lysis. Absolute numbers of T cells were determined using the TruCount system (Becton Dickinson). Intracellular cytokine staining was performed on Ficoll-separated peripheral blood mononuclear cells (PBMC) of patients and HCs after mitogen stimulation. A surface staining was performed with anti-CD3, anti-PD-1 or appropriate isotype controls followed by fixation and permeabilization (Cytofix/Cytoperm Kit, Becton Dickinson). Afterwards, intracellular staining with anti-IFN-γ or appropriate isotype controls was done. Analysis was performed with an FACS from Becton Dickinson. Effector memory T cells were defined as CD4+CD45RO+CCR7− (T_{em}), late differentiated effector memory T cells were defined as CD4+CD45RO+CD28− (CD28^{null}) and activated T-helper cells (Th cells) were defined as CD4+CD25+ (T_{act}).

T-cell cytokine and proliferation assays

For intracellular IFN-γ stainings, cells were stimulated in the absence or presence of phorbol-12-myristate-13-acetate (PMA) (5 ng/ml) and Ionomycin (1 μM) (Sigma-Aldrich, Seelze, Germany) for 4 h. Cytokine secretion was inhibited by Brefeldin A (Ebioscience, Frankfurt, Germany). PD-1 ligation studies were performed similar to Bertsias *et al*. [23] and Raptopoulou *et al*. [26]. Briefly, CD4+ T cells were isolated by negative selection (Rosette CD4+ T-cell Enrichment Kit, Stem Cell Technologies, Grenoble, France), stained with 1 μM 5,6-carboxy-fluorescein-succinimidyl ester (Molecular Probes, Invitrogen, Breda, The Netherlands) to track proliferation and stimulated for 72 h with immobilized anti-CD3 (0.25 μg/ml, clone

2G3, kind gift from Biomedical Research Institute, Diepenbeek, Belgium) in the presence or absence of immobilized human PDL-1 IgG chimera (R&D systems). After 72 h, the proliferation of cells was assessed by flow cytometry. Suppression of proliferation was calculated as described in ref. [29].

Immunohistochemistry

Eight renal biopsies with diagnosed necrotizing crescentic GN (NCGN) from GPA patients were available and provided by the Department of Pathology, University Duisburg-Essen. Three biopsies were classified as focal NCGN and the other five as crescentic GN according to the histopathological classification of Berden *et al.* [30]. Moreover, five renal biopsies with minimal change GN (MCGN) were provided by the Department of Clinical and Experimental Immunology, Maastricht University Medical Center, The Netherlands. All specimens were fixed in 10% neutral buffered formalin and paraffin embedded. Sections 4 μm thick were deparaffinized in xylene and rehydrated in ethanol at different concentrations (100, 95, 70 and 50%). EDTA buffer pH 9.0 for heat-induced epitope retrieval was applied, followed by neutralization of endogenous peroxidase with 0.3% H_2O_2. Primary antibodies (CD3 obtained from DCS, Hamburg, Germany and PD-1 obtained from Becton Dickinson) and HRP-conjugated secondary antibodies (Zytomed) were incubated on slides (each for 30 min at room temperature. Tonsils were used as positive controls for CD3 and PD-1 staining [31]. Washing steps with PBS were performed after each incubation step. A DAB Substrate Kit (Zytomed) was used for visualization. Finally, the slides were counterstained with haematoxylin. CD3 and PD-1 expression in biopsies was scored in a similar fashion as described earlier [32]. Analysis was performed semi-quantitatively. A score ranging from 0 (0 = absence of mononuclear cell infiltration; 0.5 = scattered, positive cells present; 1 = few mononuclear cell infiltration; 2 = moderate mononuclear cell infiltration) to 3 (3 = severe mononuclear cell infiltration) was used for assessing the severity of cell infiltration. Serial sections were used to reveal colocalizations.

IF double staining

Tissues were prepared as indicated above. Primary antibodies against CD3 (rabbit IgG1, DCS) and PD-1 (mouse IgG1, Becton Dickinson) were used and incubated for 30 min at room temperature simultaneously. Secondary antibodies conjugated to Cy2 and Cy3 (Dianova, Hamburg, Germany) were applied for 30 min. Finally, the slides were mounted with Immu Mount (Thermo Fisher, Kehl, Germany).

Statistics

All values are expressed as medians, with the lower and upper bounds of the interquartile range (IQR) given in brackets. Significance for the differences between groups was determined using the Mann–Whitney U-test. Spearman's rank correlation was applied for detecting correlations between different study parameters.

Results

Persistent T-cell activation in GPA patients with quiescent disease

Persistent T-cell activation was defined as expansion of CD25+ Th cells (CD4+CD25+) and/or effector memory Th cells (T_{em}, CD4+CD45RO+CCR7−) [14, 33]. GPA patients in remission showed an expanded CD25+ Th-cell population [GPA vs HC: 36 (25–46) vs 21 (16–27)%, P < 0.001, supplementary Table S2, available as supplementary data at *Rheumatology* Online] [34]. Likewise, an expansion of the T_{em} population was found [53 (40–68) vs 36 (32–45)%, P = 0.002]. Furthermore, GPA patients harboured an increased fraction of Th cells lacking CD28 [CD28null 1.9 (0.7–11.4) vs 0.9 (0.4–3.7)%, P = 0.06, supplementary Table S2, available as supplementary data at *Rheumatology* Online]. These results indicate persistent activation of T-cell-mediated immunity in patients in remission.

## Increased PD-1 expression on Th cells in quiescent GPA is linked to persistent T-cell activation

Negative costimulation by PD-1 is thought to balance T-cell activation and maintain immune tolerance. Therefore PD-1 expression was analysed on circulating T cells from GPA patients with quiescent disease (Fig. 1 and supplementary Table S2, available as supplementary data at *Rheumatology* Online). Interestingly, the proportion of PD-1 expressing CD4+ T cells was increased as compared with HCs [9.8 (6.7–17.7) vs 5.6 (3.4–8.7)%, P < 0.001, Fig. 1a].

As GPA patients showed a reduction in absolute CD4+ T-cell count, the absolute number of CD4+PD-1+ T cells was comparable in GPA patients and HCs [48 (31–76) vs 45 (16–73) cells/μl, P > 0.05]. Disease extent had an impact on PD-1 expression on CD4+ Th cells; in systemic GPA, PD-1 expression tended to be higher as compared with localized GPA [12.8 (7.5–21.5) vs 7.5 (6.2–8.6)%, P = 0.06]. There was a strong association between the relative number of PD-1+ Th cells and the presence of CD28null memory Th cells as well as T_{em} (%CD28null Th cells and %PD-1+ Th cells: n = 29, r = 0.71, P < 0.0001; %T_{em} and %PD-1+ Th cells: n = 29, r = 0.67, P = 0.0002). No significant association of PD-1+ Th cells and CD28null memory T cells or T_{em} numbers was detected in HCs (%CD28null Th cells and %PD-1+ Th cells: n = 19, r = 0.12, P = 0.7; %T_{em} and %PD-1+ Th cells: n = 19, r = 0.44, P = 0.07). In GPA, the relative number of PD-1+ Th cells was also strongly correlated with T cells producing IFN-γ (%IFN-γ+ T cells and %PD-1+CD4+ T cells, GPA: n = 25, r = 0.72, P = <0.0001; HCs: n = 19, r = 0.46, P = 0.09). Interestingly, PD-1 expression was increased on IFN-γ+ T cells from GPA patients as compared with HCs [35 (31–38) vs 22 (16–24)%, P = 0.02, n = 5 and n = 4, Fig. 1b]. Taken together, persistent T-cell activation in GPA seems to be linked to PD-1 expression.

Effector Th cells show enhanced expression of PD-1

Expansion of activated CD25+ Th cells is a specific feature of GPA [33, 34]. Therefore the presence of the negative

Fig. 1 Expression of PD-1 on circulating Th cells in GPA patients and HCs. (a) Expression of PD-1 on peripheral CD4$^+$ T cells was enhanced in patients with quiescent GPA as compared with HCs. (b) The fraction of IFN-γ^+ T cells expressing PD-1 is increased in quiescent GPA as compared with HCs. (c) Expression of PD-1 on peripheral CD4$^+$CD25$^+$ T cells was increased in patients with quiescent GPA as compared with HCs. (d) Expression of PD-1 on peripheral CD4$^+$CD25$^+$ in patients with active GPA and HCs. The illustrated data are shown as medians (IQRs). Significant differences by the Mann–Whitney U-test are indicated: $^*P < 0.05$; $^{**}P < 0.001$.
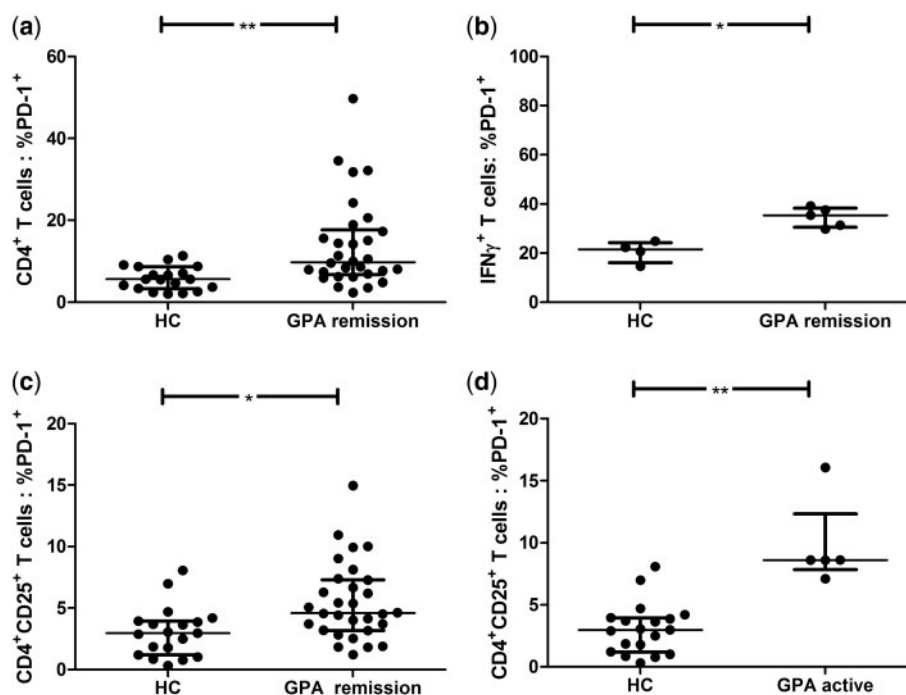
costimulator PD-1 was determined on the CD25$^+$ and the CD25$^-$ Th-cell subset. PD-1 expression on CD25$^+$ Th cells was enhanced in GPA patients as compared with HCs [4.6 (3.2–7.3) vs 3.0 (1.2–3.9)%, $P = 0.002$, Fig. 1c], whereas the absolute cell count did not differ [8 (3–14) vs 7 (2–9) cells/μl]. Disease extent did not influence expression of PD-1 on CD25$^+$ Th cells, as it was comparable in localized disease and systemic disease (data not shown). Further analysis revealed that PD-1$^+$CD25$^+$CD127^{low} T cells (PD-1$^+$ Tregs) were not different in GPA patients vs HCs, whereas PD-1$^+$CD25$^+$CD127^{high} T$_{eff}$ cells were significantly increased [percentage of CD25$^+$ Th cells: PD-1$^+$ Tregs 1.74 (1.04–5.59) vs 1.32 (0.81–1.83)%, $P > 0.05$ and PD-1$^+$ T$_{eff}$ 3.05 (1.97–4.36) vs 1.81 (0.85–2.41)%, $P = 0.02$]. PD-1 expression on CD25$^+$ Th cells remained stable over time in quiescent disease (see supplementary Fig. S1, available as supplementary data at *Rheumatology* Online).

CD4$^+$CD25$^-$ T cells from GPA patients showed increased expression of PD-1 when compared with HCs [13.3 (8.1–13.8) vs 5.9 (3.8–10.2)%, $P < 0.002$]; absolute numbers did not differ significantly (supplementary Table S2, available as supplementary data at *Rheumatology* Online). In systemic GPA, PD-1 expression on CD4$^+$CD25$^-$ T cells tended to be enhanced as compared with localized GPA [16.1 (9.0–24.5) vs 9.0 (6.8–10.2)%, $P = 0.05$].

Increased PD-1 expression on CD25$^+$ and CD25$^-$ Th cells is maintained during active disease

Patients with active GPA ($n = 5$, Table 1 and supplementary Table S1, available as supplementary data at *Rheumatology* Online) were assessed to elucidate if increased PD-1 expression is maintained during the acute phase of the disease. Patients with active GPA showed enhanced PD-1 expression on CD25$^+$ Th cells as compared with HCs and was even higher in comparison with quiescent GPA [8.6 (7.8–12.3) vs 3.0 (1.2–3.9)%, $P < 0.001$, Fig. 1d and 8.6 (7.8–12.3) vs 4.6 (3.2–7.3)%, $P = 0.02$]. PD-1 expression on CD25$^-$ T cells did not differ between active GPA and quiescent GPA (data not shown).

Latent CMV infection in GPA associates with increased PD-1 expression on CD4$^+$CD25$^-$ but not CD4$^+$CD25$^+$ T cells

As latent infection with CMV is described to drive expansion of T$_{em}$ and also impacts PD-1 expression on T cells, the patient cohort was stratified according to CMV serostatus (Table 1) [35]. Interestingly, PD-1 expression on CD4$^+$ and CD4$^+$CD25$^-$ T cells was higher in CMV$^+$ patients as compared with CMV$^-$ patients [17.3 (14.2–49.7) vs 7.4 (5.4–8.3)%, $P < 0.001$ and 20.8 (15.1–36.8) vs 8.4 (6.4–10.2)% $P = 0.002$, Fig. 2a and b]. CMV$^+$ and CMV$^-$ patients did not differ regarding PD-1

F $_{IG.}$ 2 PD-1 expression on circulating Th cells of CMV$^+$ and CMV$^-$ GPA patients. (**a**) PD-1 expression on CD4$^+$ and (**b**) CD4$^+$CD25$^-$ T cells was higher in CMV$^+$ patients as compared with CMV$^-$ patients. (**c**) CMV$^+$ and CMV$^-$ patients did not differ regarding PD-1 expression on CD4$^+$CD25$^+$ T cells. The illustrated data are shown as medians (IQR). Significant differences by the Mann–Whitney U-test are indicated: *$P < 0.05$.
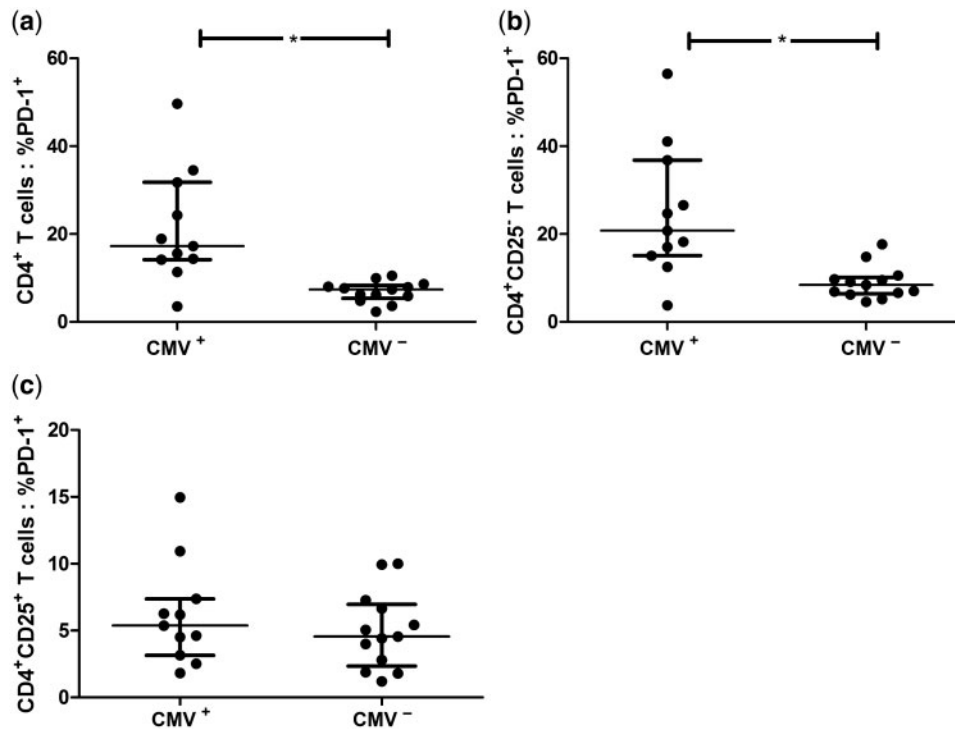
expression on CD4$^+$CD25$^+$ T cells [5.4 (3.2–7.4) *vs* 4.6 (2.4–7.0)%, $P = 0.45$, Fig. 2c).

Relapse rate in GPA correlates with PD-1$^+$CD25$^+$ Th cells

Interestingly, PD-1$^+$CD25$^+$—but not PD-1$^+$CD25$^-$ Th cells—showed a strong negative association with relapse rate (relapses per month of disease duration, PD-1$^+$CD25$^+$ T cells and relapse rate: $r = -0.43$, $P = 0.03$, $n = 26$, Fig. 3). Separate correlation analysis for PD-1$^+$ Treg and PD-1$^+$ T$_{eff}$ showed similar results ($r = -0.5$, $P = 0.03$ and $r = -0.48$, $P = 0.03$).

PD-1-mediated suppression of T-cell proliferation is less efficient in GPA

In HCs, T-cell proliferation was suppressed in a PDL-1 dose-dependent fashion with maximal suppression at 5 μg/ml PDL-1. In four of five GPA patients, PD-1 ligation also resulted in suppression of T-cell proliferation at 5 μg/ml PDL-1, whereas in one patient no effect was seen. However, suppression was significantly more efficient in HCs when compared with GPA patients [43.3 (25.3–58.0) *vs* 13.4 (4.3–34.3)%, $P = 0.03$].

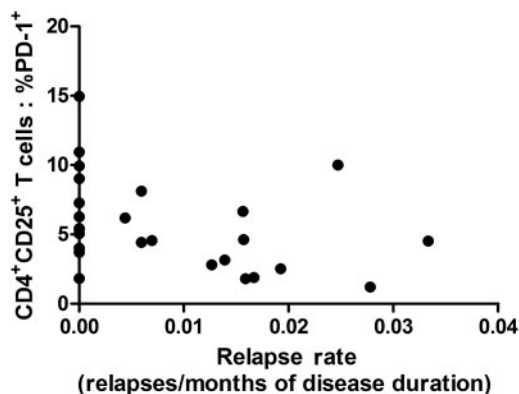### Lesional T cells in GPA mostly lack PD-1 expression

The PD-1/PDL-1 pathway has a special role in maintaining tolerance and attenuation of the immune response. At active sites of inflammation, engagement of PD-1 via

PDL-1 dampens T-cell activation and prevents tissue damage. To address the question if this pathway is also operative in GPA, eight renal biopsies of patients with GPA and NCGN were stained for the presence of PD-1$^+$ cells and CD3$^+$ T cells (Fig. 4 and supplementary Fig. S2, available as supplementary data at *Rheumatology* Online). Tonsil sections were used as positive control (supplementary Fig. S3, available as supplementary data at *Rheumatology* Online). PD-1 expression in NCGN was scarce and rare. Only single cells were found in one out of eight NCGN biopsies. The average PD-1 expression was scored with 0.03 (0.08). No expression was found in glomeruli. T-cell infiltration was found in all eight biopsies and was scored with an average of 0.85 (0.3). Serial sections revealed a lack of PD-1 expression in areas where T-cell infiltrates are present (Fig. 4). MCGN served as non-inflammatory kidney control tissue. Five different biopsies with MCGN were assessed and were completely lacking CD3$^+$ T cells and PD-1 expression (Fig. 4). Given the scoring and the findings revealed by serial sections, it seemed that the majority of T cells infiltrating the kidney in NCGN lacked PD-1 expression.

Discussion

Our results demonstrate increased PD-1 expression on circulating CD4$^+$ Th cells in GPA patients. PD-1 expression was associated with persistent activation of T cells

Fig. 3 Correlation between relapse rate and expression of PD-1 by CD4+CD25+ T cells ($n = 26$, relapse rate: $r = -0.43$, $P = 0.03$). The percentage of PD-1+CD25+ Th cells measured at Visit 1 was correlated with the relapse rate (number of relapses per month of disease duration) retrieved by retrospective file search. The median BVAS was 10 (9–12). The correlation was calculated by means of Spearman's rank test.

and latent CMV infection. Importantly, PD-1 expression on CD4+CD25+ T cells seemed to protect from relapses. However, PD-1-mediated inhibition of T-cell proliferation was less potent in GPA patients as compared with HCs. Lesional T cells in NCGN mostly lacked PD-1.

T-cell activation is inhibited by negative costimulatory molecules like cytotoxic T-lymphocyte antigen 4 (CTLA-4) and PD-1, which is an important mechanism of tolerance [17, 36]. More in detail, ligation of PD-1 inhibits the proximal signalling cascade pivotal for T-cell activation by attenuating phosphatidylinositol-3-kinase activation, which finally results in decreased cytokine production, decreased proliferation and decreased survival. As shown in animal models and in humans, breakdown of PD-1-mediated immune modulation leads to autoimmunity and uncontrolled T-cell responses [17, 36].

Persistent activation of T cells is consistently reported in GPA [13, 33, 34]. We observed increased PD-1 expression on T cells in GPA. PD-1 expression was positively correlated with expansion of memory T cells, CD28null T cells and T-cell activation and was enhanced on proinflammatory IFN-γ+ T cells in our GPA cohort but not in HCs. Thus persistent immune activation probably resulted in increased PD-1 expression to counteract T-cell activation [36, 37]. Patients with active disease and consequently highly activated immune system showed even more enhanced expression of PD-1+ Th cells as compared with patients in remission. This might reflect an attempt to terminate the ongoing immune response. Next to a balancing effect, chronic challenge with autoantigen or viral antigen might have caused up-regulation of PD-1 in the context of T-cell exhaustion [38]. Indeed, patients with latent CMV infection showed enhanced PD-1 expression on CD25− T cells, suggesting that chronic challenge with viral antigen is related to up-regulation of PD-1 in GPA.
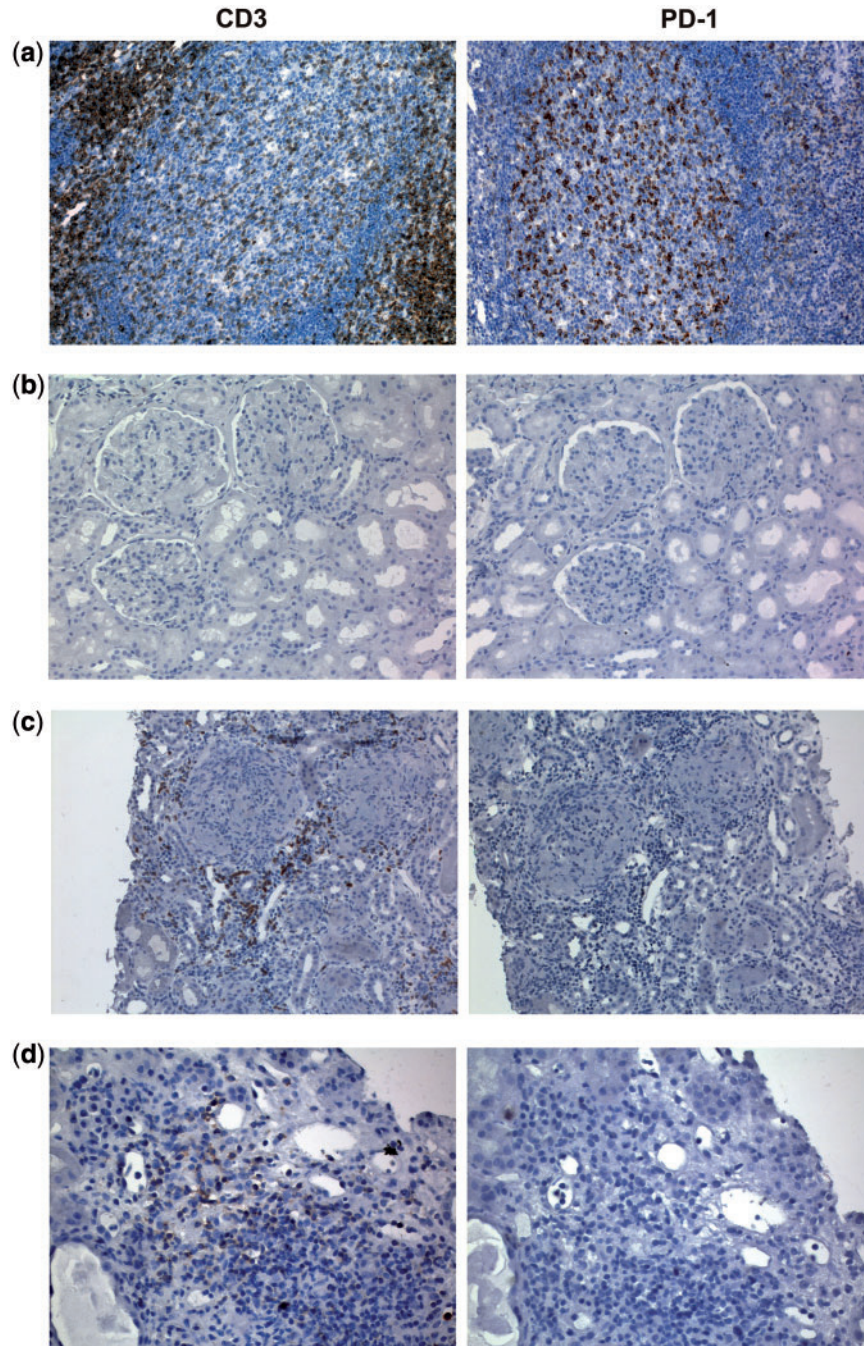
Moreover, CMV infection has been associated with memory T-cell expansion in GPA [35]; this might explain the correlation between PD-1 expression and memory T cell/ CD28null T-cell expansion observed in our study.

In line with our findings, increased expression of a different inhibitory costimulator (CTLA-4 on T cells) was demonstrated in GPA previously by Steiner et al. [39]. In this study, GPA patients showed less strong up-regulation of this molecule in vitro upon mitogenic T-cell stimulation as compared with HCs. This might indicate insufficient CTLA-4-dependent balancing of immune activation in GPA. In our study, PD-1 expression was positively correlated with IFN-γ production and T-cell activation, pointing at a balancing effect. However, it has to be considered that the extent of PD-1 up-regulation in GPA is apparently not sufficient to limit and dampen immune responses. This is supported by our finding that PD-1-mediated suppression of T cells was less potent in GPA patients than in HCs. The cause for diminished PD-1-mediated suppression is not clear. The cytokine environment has a critical role on the outcome of PD-1-mediated inhibition, and IL-2 can counteract negative costimulation [40, 41]. Thus alterations of T-cell polarization and disturbances in cytokine levels reported in GPA might hamper the functionality of the PD-1/PDL-1 axis [6, 40]. Alternatively, it can be hypothesized that PD-1-mediated inhibition is impaired by enhanced responsiveness to specific cytokines (like IL-2) that are known to counteract PD-1/PDL-1 functionality. In line with this, the IL-2Rα chain is overexpressed in GPA patients as compared with HCs [6].

In contrast, decreased expression of PD-1 on circulating T cells is reported in other autoimmune diseases, such as MS and SLE [24, 25]. The pathogenesis of SLE or MS seems to be different from GPA, where T-cell-driven granuloma formation is a hallmark. Different genetic backgrounds might provide an explanation why PD-1 expression is low in SLE/MS and high in GPA. Some polymorphisms of the gene coding for PD-1 [programmed cell death-1 (PDCD1)] seem to be more common and specific for SLE or MS, whereas this has not been found in GPA. No differences between GPA patients and HCs have been detected regarding the distribution of PD1.3 and PD1.5 polymorphisms [23, 25, 27, 42, 43]. Accordingly, the PD1.3A polymorphism, which interferes negatively with transcription of the PDCD1 gene and seems to reduce its protein expression, is more frequently detected in SLE patients than in HCs [23].

Interestingly, CD25+PD-1+ Th cells were negatively associated with relapse rate. PD-1 ligation on activated Teff has been hypothesized to result in contra-conversion, meaning the conversion of Teff into anti-inflammatory T cells like Tregs [38]. Additionally, the PD-1/PDL-1 ligand interaction seems to impact the stability of T cell/dendritic cell (DC) interaction [44]. Moreover, ligation of PD-1 via PDL-1 expressed by endothelial cells is hypothesized to restrict extravasation of T cells [45]. Thus enhanced PD-1 expression on CD25+ effector T cells is likely to increase the threshold for immune activation. It might explain why patients with the lowest relapse rate have the highest

PD-1 baseline expression on CD25$^+$ Th cells. Unexpectedly, also patients with active GPA showed enhanced PD-1 expression on CD25$^+$ T cells. As the cytokine milieu influences the efficacy of PD-1-mediated inhibition, negative costimulation by PD-1 can be overcome under inflammatory conditions [40, 41]. Furthermore, the ligands for the negative costimulator PD-1 but also for the positive costimulatory molecule CD28 are up-regulated on antigen-presenting cells under pro-inflammatory conditions [46]. It has also been demonstrated that positive costimulation via CD28 can override PD-1-mediated negative costimulation [41]. Therefore negative costimulation might be less potent during active disease due to competing positive costimulation.

Unlike reports on lesional PD-1 expression in RA, MS, SLE and SS [24–26, 47, 48], PD-1 was only rarely detected in renal biopsies of patients with GPA-associated NCGN. T cells were present in all of these NCGN biopsies but were mostly lacking PD-1. Therefore the PD-1 pathway might fail to regulate the T-cell response on tissue level sufficiently. The cognate ligand PDL-1 is usually expressed in renal tissue by tubular epithelial cells [49]. PDL-1 is considered to be important for regulating renal inflammation [49]. It has been demonstrated that T$_{em}$ migrate to the kidney in NCGN [50]. Hypothetically, only PD-1^{neg} effector memory T cells are capable of infiltrating renal tissue in GPA. Ligation of PD-1 by PDL-1 expressing endothelial cells might limit T-cell extravasation [45]. Alternatively, PD-1 might be down-regulated on T cells after migration.

In summary, we demonstrated increased PD-1 expression on circulating T cells in GPA, whereas T cells in renal lesions mostly lacked PD-1 expression. Increased PD-1 expression on circulating T cells might counterbalance persistent T-cell activation in GPA. However, PD-1-mediated inhibition seemed less potent in GPA patients than in HCs.

Rheumatology key messages

- Increased expression of the negative costimulator PD-1 on circulating T cells in GPA balances immune activation.
- The majority of lesional T cells lacks PD-1 expression.

Supplementary data

Supplementary data are available at *Rheumatology* Online.

Acknowledgements

References

1. Falk RJ, Gross WL, Guillevin L *et al*. Granulomatosis with polyangiitis (Wegener's): an alternative name for Wegener's granulomatosis. Arthritis Rheum 2011;63: 863–4.

2. Tervaert JW, van der Woude FJ, Fauci AS *et al*. Association between active Wegener's granulomatosis and anticytoplasmic antibodies. Arch Intern Med 1989; 149:2461–5.

3. Leavitt RY, Fauci AS, Bloch DA *et al*. The American College of Rheumatology 1990 criteria for the classification of Wegener's granulomatosis. Arthritis Rheum 1990; 33:1101–7.

4. Jennette JC, Falk RJ, Andrassy K *et al*. Nomenclature of systemic vasculitides. Proposal of an international consensus conference. Arthritis Rheum 1994;37:187–92.

5. Wilde B, Van Paassen P, Witzke O, Cohen Tervaert JW. New pathophysiological insights and treatment of ANCA-associated vasculitis. Kidney Int 2011;79:599–612.

6. Wilde B, Thewissen M, Damoiseaux J *et al*. T-cells in ANCA-associated vasculitis: what can we learn from lesional versus circulating T-cells? Arthritis Res Ther 2010; 12:204.

7. Gephardt GN, Ahmad M, Tubbs RR. Pulmonary vasculitis (Wegener's granulomatosis). Immunohistochemical study of T and B cell markers. Am J Med 1983;74:700–4.

8. Bolton WK, Innes DJ Jr, Sturgill BC, Kaiser DL. T-cells and macrophages in rapidly progressive glomerulonephritis: clinicopathologic correlations. Kidney Int 1987;32:869–76.

9. Saunders BM, Frank AA, Orme IM, Cooper AM. CD4 is required for the development of a protective granulomatous response to pulmonary tuberculosis. Cell Immunol 2002;216:65–72.

10. Co DO, Hogan LH, Il-Kim S, Sandor M. T cell contributions to the different phases of granuloma formation. Immunol Lett 2004;92:135–42.

11. Brouwer E, Tervaert JW, Horst G *et al*. Predominance of IgG1 and IgG4 subclasses of anti-neutrophil cytoplasmic autoantibodies (ANCA) in patients with Wegener's granulomatosis and clinically related disorders. Clin Exp Immunol 1991;83:379–86.

12. Stegeman CA, Tervaert JW, Huitema MG, Kallenberg CG. Serum markers of T cell activation in relapses of Wegener's granulomatosis. Clin Exp Immunol 1993;91: 415–20.

13. Popa ER, Stegeman CA, Bos NA, Kallenberg CGM, Tervaert JWC. Differential B- and T-cell activation in Wegener's granulomatosis. J Allergy Clin Immunol 1999; 103:885–94.

14. Abdulahad WH, van der Geld YM, Stegeman CA, Kallenberg CG. Persistent expansion of CD4+ effector

memory T cells in Wegener's granulomatosis. Kidney Int 2006;70:938–47.

15 Wilde B, Dolff S, Cai X *et al*. CD4+CD25+ T-cell populations expressing CD134 and GITR are associated with disease activity in patients with Wegener's granulomatosis. Nephrol Dial Transplant 2009;24:161–71.

16 Brusko TM, Putnam AL, Bluestone JA. Human regulatory T cells: role in autoimmune disease and therapeutic opportunities. Immunol Rev 2008;223:371–90.

17 Fife BT, Bluestone JA. Control of peripheral T-cell tolerance and autoimmunity via the CTLA-4 and PD-1 pathways. Immunol Rev 2008;224:166–82.

18 Ansari MJ, Salama AD, Chitnis T *et al*. The programmed death-1 (PD-1) pathway regulates autoimmune diabetes in nonobese diabetic (NOD) mice. J Exp Med 2003;198: 63–9.

19 Salama AD, Chitnis T, Imitola J *et al*. Critical role of the programmed death-1 (PD-1) pathway in regulation of experimental autoimmune encephalomyelitis. J Exp Med 2003;198:71–8.

20 Tanaka K, Albin MJ, Yuan X *et al*. PDL1 is required for peripheral transplantation tolerance and protection from chronic allograft rejection. J Immunol 2007;179:5204–10.

21 Wolchok JD, Yang AS, Weber JS. Immune regulatory antibodies: are they the next advance? Cancer J 2010;16: 311–7.

22 Wan B, Nie H, Liu A *et al*. Aberrant regulation of synovial T cell activation by soluble costimulatory molecules in rheumatoid arthritis. J Immunol 2006;177:8844–50.

23 Bertsias GK, Nakou M, Choulaki C *et al*. Genetic, immunologic, and immunohistochemical analysis of the programmed death 1/programmed death ligand 1 pathway in human systemic lupus erythematosus. Arthritis Rheum 2009;60:207–18.

24 Trabattoni D, Saresella M, Pacei M *et al*. Costimulatory pathways in multiple sclerosis: distinctive expression of PD-1 and Pd-L1 in patients with different patterns of disease. J Immunol 2009;183:4984–93.

25 Kristjansdottir H, Steinsson K, Gunnarsson I *et al*. Lower expression levels of the programmed death 1 receptor on CD4+CD25+ T cells and correlation with the PD-1.3A genotype in patients with systemic lupus erythematosus. Arthritis Rheum 2010;62:1702–11.

26 Raptopoulou AP, Bertsias G, Makrygiannakis D *et al*. The programmed death 1/programmed death ligand 1 inhibitory pathway is up-regulated in rheumatoid synovium and regulates peripheral T cell responses in human and murine arthritis. Arthritis Rheum 2010;62:1870–80.

27 Slot MC, Sokolowska MG, Savelkouls KG *et al*. Immunoregulatory gene polymorphisms are associated with ANCA-related vasculitis. Clin Immunol 2008;128: 39–45.

28 Hellmich B, Flossmann O, Gross WL *et al*. EULAR recommendations for conducting clinical studies and/or clinical trials in systemic vasculitis: focus on anti-neutrophil cytoplasm antibody-associated vasculitis. Ann Rheum Dis 2007;66:605–17.

29 Venken K, Hellings N, Broekmans T *et al*. Natural naive CD4+CD25+CD127low regulatory T cell (Treg) development and function are disturbed in multiple sclerosis

30 Berden AE, Ferrario F, Hagen EC *et al*. Histopathologic classification of ANCA-associated glomerulonephritis. J Am Soc Nephrol 2010;21:1628–36.

31 Iwai Y, Okazaki T, Nishimura H *et al*. Microanatomical localization of PD-1 in human tonsils. Immunol Lett 2002;83: 215–20.

32 Wilde B, van Paassen P, Damoiseaux J *et al*. Dendritic cells in renal biopsies of patients with ANCA-associated vasculitis. Nephrol Dial Transplant 2009;24:2151–6.

33 Marinaki S, Kalsch AI, Grimminger P *et al*. Persistent T-cell activation and clinical correlations in patients with ANCA-associated systemic vasculitis. Nephrol Dial Transplant 2006;21:1825–32.

34 Marinaki S, Neumann I, Kalsch AI *et al*. Abnormalities of CD4 T cell subpopulations in ANCA-associated vasculitis. Clin Exp Immunol 2005;140:181–91.

35 Morgan MD, Pachnio A, Begum J *et al*. CD4+CD28− T cell expansion in granulomatosis with polyangiitis (Wegener's) is driven by latent cytomegalovirus infection and is associated with an increased risk of infection and mortality. Arthritis Rheum 2011;63:2127–37.

36 Nishimura H, Honjo T. PD-1: an inhibitory immunoreceptor involved in peripheral tolerance. Trends Immunol 2001;22: 265–8.

37 Freeman GJ, Long AJ, Iwai Y. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J Exp Med 2000;192:1027–34.

38 Francisco LM, Sage PT, Sharpe AH. The PD-1 pathway in tolerance and autoimmunity. Immunol Rev 2010;236: 219–42.

39 Steiner K, Moosig F, Csernok E *et al*. Increased expression of CTLA-4 (CD152) by T and B lymphocytes in Wegener's granulomatosis. Clin Exp Immunol 2001;126:143–50.

40 Carter LL, Fouser LA, Jussif J *et al*. PD-1:PD-L inhibitory pathway affects both CD4+ and CD8+ T cells and is overcome by IL-2. Eur J Immunol 2002;32:634–43.

41 Bennett F, Luxenberg D, Ling V *et al*. Program death-1 engagement upon TCR activation has distinct effects on costimulation and cytokine-driven proliferation: attenuation of ICOS, IL-4, and IL-21, but not CD28, IL-7, and IL-15 responses. J Immunol 2003;170:711–8.

42 Prokunina L, Castillejo-Lopez C, Oberg F *et al*. A regulatory polymorphism in PDCD1 is associated with susceptibility to systemic lupus erythematosus in humans. Nat Genet 2002;32:666–9.

43 Kroner A, Mehling M, Hemmer B *et al*. A PD-1 polymorphism is associated with disease progression in multiple sclerosis. Ann Neurol 2005;58:50–7.

44 Fife BT, Pauken KE, Eagar TN *et al*. Interactions between PD-1 and PD-L1 promote tolerance by blocking the TCR-induced stop signal. Nat Immunol 2009;10:1185–92.

45 Sharpe AH, Wherry EJ, Ahmed R, Freeman GJ. The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. Nat Immunol 2007; 8:239–45.

46 Greenwald RJ, Freeman GJ, Sharpe AH. The B7 family revisited. Annu Rev Immunol 2004;23:515–48.

patients: recovery of memory Treg homeostasis during disease progression. J Immunol 2008;180:6411–20.

47 Kasagi S, Kawano S, Okazaki T *et al*. Anti-programmed cell death 1 antibody reduces CD4+PD-1+ T cells and relieves the lupus-like nephritis of NZB/W F1 mice. J Immunol 2006;184:2337–47.

48 Kobayashi M, Kawano S, Hatachi S *et al*. Enhanced expression of programmed death-1 (PD-1)/PD-L1 in salivary glands of patients with Sjögren's syndrome. J Rheumatol 2005;32:2156–63.

49 Starke A, Lindenmeyer MT, Segerer S *et al*. Renal tubular PD-L1 (CD274) suppresses alloreactive human T-cell responses. Kidney Int 2010;78:38–47.

50 Abdulahad WH, Kallenberg CG, Limburg PC, Stegeman CA. Urinary CD4+ effector memory T cells reflect renal disease activity in antineutrophil cytoplasmic antibody-associated vasculitis. Arthritis Rheum 2009;60: 2830–8.