T-cell mediated immunity in Wegener's granulomatosis
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2008

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CHAPTER 6

Functional defect of circulating regulatory CD4+ T-cells in patients with Wegener’s granulomatosis in remission

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Arthritis & Rheumatism 2007; 56: 2080-91
Abstract

Objective: Accumulating data support the role of regulatory T-cells (T_{Reg} cells), a subset of CD4^{+}T-cells that express CD25^{High} and the transcription factor forkhead box P3 (FoxP3), in controlling and preventing autoimmunity. In Wegener’s granulomatosis (WG), an autoimmune vasculitis, up-regulation of CD25 on circulating CD4^{+}T-cells has been observed, even in patients in remission. The objective of this study was to test whether the frequency and/or function of T_{Reg} cells from WG-patients in remission (R-WG) are disturbed.

Methods: Peripheral blood mononuclear cells (PBMCs) were freshly isolated from 52 R-WG-patients and from 27 age- and sex-matched healthy control (HC) subjects. The proportion of circulating T_{Reg} cells was assessed by flow cytometry using CD4, CD25, FoxP3, and CD45RO markers. Anergy and suppressive function of CD25^{High}CD4^{+}T-cells were determined using polyclonal stimulants and co-culture assay in 10 R-WG-patients and in 10 age- and sex-matched HCs.

Results: In R-WG-patients, a significant increase was observed in the percentage of circulating CD25^{High}CD4^{-} and CD25^{Low}CD4^{+}T-cells, whereas CD25CD4^{+}T-cells were decreased, as compared with HC. Among circulating CD4^{+}T-cells, an expanded percentage of T_{Reg} cells (CD25^{High}FoxP3^{+}) with memory phenotype was present in R-WG-patients. However, when the suppressive function of CD25^{High}CD4^{+}T-cells was tested, CD25^{High}CD4^{+}T-cells from R-WG-patients showed diminished or absent suppression of responder T-cell proliferation. The impaired suppression was not due to responder cell resistance (as shown by criss-cross experiments with T-cells from HC) or altered survival of T_{Reg} cells.

Conclusion: These data indicate that R-WG-patients have an expanded proportion of T_{Reg} cells which are functionally defective. This observation may be relevant in relation to the development and relapsing course of this autoimmune vasculitis.
Introduction

Despite the presence of several mechanisms that sustain T-cell tolerance, such as thymic deletion of auto-reactive T-cells and induction of T-cell anergy to auto-antigens, auto-reactive T-cells can be detected in normal individuals who never clinically develop autoimmune disease\(^1\);\(^2\). Thus, additional regulatory mechanisms must exist to prevent clinically manifest autoimmunity. During the last decade, circulating regulatory lymphocytes (T\(_{\text{Reg}}\) cells), a subset of CD4\(^+\)T-cells that express the interleukin (IL)-2 receptor \(\alpha\)-chain (CD25), have been described in experimental animals and in humans as an additional mechanism that sustains tolerance. Adoptive transfer of T\(_{\text{Reg}}\)-depleted CD4\(^+\)T-cells from wild-type mice into thymectomized nude mice induced multi-organ autoimmune disease, whereas co-transfer of purified T\(_{\text{Reg}}\) cells resulted in disease prevention\(^3\). In humans, decreased frequencies and/or defective function of T\(_{\text{Reg}}\) cells have been documented in several autoimmune diseases, such as multiple sclerosis\(^4\);\(^5\), rheumatoid arthritis (RA)\(^6\), autoimmune polyglandular syndrome type-II\(^7\), Kawasaki disease\(^8\), myasthenia gravis\(^9\), type-I diabetes mellitus\(^10\), systemic lupus erythematosus (SLE)\(^11\), and psoriasis\(^12\). These observations demonstrate that T\(_{\text{Reg}}\) cells are an essential component of peripheral suppressor mechanisms and suggest a role of these cells in the pathogenesis of autoimmune diseases.

T\(_{\text{Reg}}\) cells are characterized by high levels of surface expression of CD25\(^13\) and intracellular expression of the transcription factor forkhead box P3 (FoxP3)\(^14\);\(^15\). FoxP3, a member of the forkhead/winged helix family, is a master gene that controls development and function of T\(_{\text{Reg}}\) cells\(^16\);\(^17\), and is therefore a more specific marker for T\(_{\text{Reg}}\) cells. Further studies have shown that cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) and glucocorticoid-induced tumor necrosis factor receptor (GITR) can also be considered additional markers for these cells\(^18\);\(^19\).

Wegener’s granulomatosis (WG) is an autoimmune vasculitis of small- to medium-sized vessels, associated with anti-neutrophil cytoplasm auto-antibodies (ANCAs) with specificity for proteinase-3 (PR3)\(^20\), and characterized by granulomatous inflammation, particularly of the upper and lower airways, pauci-immune vasculitis, and glomerulonephritis\(^21\). Although the etiology of this disease is not yet known, the observation that remission
can be induced in WG-patients by drugs directed at removing T-cells suggests the involvement of T-cells in the pathogenesis of this disorder. In addition, during remission, an increase in memory T-cells with a concomitant decrease in naïve T-cells within the circulating CD4⁺T-cell subset in WG-patients points to persistent T-cell activation, which is associated with vasculitis severity. Since T₅ cells are important inhibitors of the activation and proliferative responses of both naïve and memory T-cells and act on auto-aggressive T-cells and B-cells to inhibit maturation of auto-antibody responses, a malfunction of these cells might sustain recurrence of auto-reactivity and chronic inflammation in WG. Although involvement of T₅ cells in WG has not yet been elucidated, increased expression of CD25 and CTLA-4 (T₅ cell markers) on circulating CD4⁺T-cells has been reported in this disease.

In the present study, we sought to determine the frequency and function of circulating T₅ cells derived from a group of patients with WG in remission, as compared with those from age- and sex-matched healthy individuals. We hypothesize that an altered frequency and/or function of circulating T₅ cells from WG-patients in remission (R-WG) may contribute to loss of self-tolerance and underlie the relapsing course of this disease.

**Materials and Methods**

**Study population**

Fifty-two patients with WG (table 1) and 27 age- and sex-matched healthy control subjects (17 males, 10 females, mean age 50 years, range 22-83 years) were included in this study. The diagnosis of WG was established according to the Chapel Hill Consensus Conference classification criteria for WG. Only patients without clinical signs and symptoms of active vasculitis and considered to be in complete remission, as indicated by a score of 0 on the Birmingham Vasculitis Activity Score (BVAS), were included in the study. Of the 52 patients, 35 were considered to have generalized-WG that included renal involvement, and 17 patients were considered to have localized-WG in which the disease is
Regulatory T-cells in WG-patients in remission

confined to the upper and/or lower respiratory tract. None of the patients or controls experienced an infection at the time of sampling.

Thirty-seven of the 52 patients were receiving maintenance immunosuppressive therapy at the time of blood sampling. Seventeen patients were receiving monotherapy with azathioprine (n=13), cyclosporine (n=1), prednisolone (n=1), or cotrimoxazole (n=2), and 20 patients were receiving prednisolone in combination with either azathioprine, cyclophosphamide, or mycophenolate mofetil.

The main clinical and laboratory data of the patients are summarized in Table 1. All study subjects provided informed consent, and the Local Medical Ethics Committee approved the study.

### Table 1: Clinical and laboratory characteristics of the WG-patients at the time of blood sampling

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>52</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. male / no. female</td>
<td>34 / 18</td>
</tr>
<tr>
<td>Age, mean (range) years</td>
<td>54 (17 – 87)</td>
</tr>
<tr>
<td>No. with localized / generalized WG</td>
<td>17 / 35</td>
</tr>
<tr>
<td>No. positive / negative for ANCA</td>
<td>40 / 12</td>
</tr>
<tr>
<td>No. receiving / not receiving treatment</td>
<td>37 / 15</td>
</tr>
<tr>
<td>Disease duration, mean (range) months</td>
<td>94 (5 – 409)</td>
</tr>
<tr>
<td>No. of relapses, median (range)</td>
<td>2 (0 – 12)</td>
</tr>
</tbody>
</table>

*ANCA, Anti-neutrophil cytoplasmic antibody; WG, Wegener’s granulomatosis.

Measurement of ANCA titres

ANCA titres were measured by indirect immunofluorescence on ethanol-fixed human granulocytes according to the standard procedure, as previously described. ANCA titers lower than 1:20 were considered negative.

Antibodies used in flow cytometry

The following conjugated antibodies were used in flow cytometry: fluorescein isothiocyanate (FITC)-conjugated anti-CD45RO, FITC-conjugated anti-CD8, FITC-conjugated anti-CD56, FITC-conjugated anti-
γ/δTCR, phycoerythrin (PE)-conjugated or allophycocyanin (APC)-conjugated anti-CD25, and APC- or peridinin-chlorophyll protein (PerCP)-conjugated anti-CD4. All antibodies were purchased from Becton & Dickinson (Amsterdam, The Netherlands). PE-conjugated anti-human FoxP3 (clone PCH101) was obtained from eBioscience (San Diego, CA, USA). Isotype matched control antibodies of irrelevant specificity were purchased from Becton & Dickinson and eBioscience.

**Isolation of peripheral blood mononuclear cells (PBMCs) and flow cytometry**

PBMCs were prepared from heparinized venous blood by density-gradient centrifugation on Lymphoprep (Axis-Shield PoC AS, Oslo, Norway) immediately after blood was drawn. Cells recovered from the gradient interface were washed twice in phosphate buffered saline (PBS), pH 7.2, and adjusted to 1 x 10^7 cells/mL in RPMI 1640 (Cambrex Bio Science, Verviers, Belgium) supplemented with 5% human pool serum and 50μg/ml of gentamycin (Gibco, Paisley, UK). Foxp3 staining was performed using a Foxp3 staining set (eBioscience/ITK Diagnostic BV, Uithoorn, The Netherlands) according to the manufacturer’s instructions. Briefly, freshly isolated PBMCs (0.5 x 10^6 cells in 100μL) were immediately incubated with appropriate concentrations of FITC-conjugated anti-CD45RO, PerCP-conjugated anti-CD4, and APC-conjugated anti-CD25, for 30 minutes at 4 °C in the dark. Cells were then washed with cold PBS, followed by fixation and permeabilization in Fix/Perm buffer (eBioscience) for 45 minutes at 4 °C. Subsequently, cells were washed twice with cold 1X Permeabilization buffer (eBioscience). To block non-specific binding, normal rat serum was added for 10 minutes, followed by the addition of PE-conjugated rat anti-human-FoxP3. After incubation for 30 minutes at 4 °C, the cell suspension was washed twice with cold 1X Permeabilization buffer, and four-color staining was immediately analyzed on FACS-Calibur (Becton & Dickinson).

For all flow cytometry analyses, data for 10^5 cells were collected. Lymphocytes were gated by forward and side scatter patterns, and plotted using the Win-List software package (Verity Software House Inc, ME, USA).
Positively and negatively stained populations were calculated by quadrant dot plot analysis, as determined by the appropriate isotype controls.

**Purification of CD4^+CD25^{&Low} and CD4^+CD25^{High} T-cells**

PBMCs from 10 of the WG-patients in remission and 10 of the age- and sex-matched healthy controls were immediately isolated from freshly drawn blood and incubated for 45 minutes at 4 °C with appropriate concentrations of APC-conjugated anti-CD4, PE-conjugated anti-CD25, and a cocktail of anti-CD8, anti-CD56, and anti-γ/δTCR, all of which were labeled with FITC. Cells were washed and sorted on a FACS MoFlo (Becton & Dickinson) according to their forward and side scatter properties. Subsequently, FITC-positive cells were excluded, and the CD4^+T-cell fraction was sorted into a CD4^+CD25^{&Low} population as responder T-cells (TResp) and a CD4^+CD25^{High} population as regulatory T-cells (TReg cells) (Figure 3A). The purity of the sorted TResp cells and TReg cells, as determined by flow-cytometric reanalysis, was >95% and >90%, respectively (Figure 3A).

**Proliferation assays and suppression experiments**

Freshly sorted TResp cells (1x10^4) and TReg cells (1x10^4) were cultured in triplicate, either separately or in co-culture (1:1 ratio), in round-bottomed 96-well plates in 200µL of complete RPMI1640 medium (containing 10% heat-inactivated human pool serum, 50µg/ml gentamycin, 50µM 2-mercaptoethanol, and 25mM HEPES with 2,1 mM L-glutamine). Cells were incubated in the absence or presence of 10% soluble anti-CD3 (supernatant of clone WT-32) and 10% soluble anti-CD28 (supernatant of clone 20–4996) (both from CLB, Amsterdam, The Netherlands) for 6 days at 37°C in 5% CO2. For the last 18 hours of culture, 100µL of the supernatant was removed, and 1 μCi/well [³H]-thymidine (ICN Biomedical Inc., Costa Mesa, CA) was added. Cells were then harvested onto glass-fiber filters using a Skatron Combi Cell Harvester (Skatron Inc., Oslo, Norway), and sealed in plastic bags containing 4,5 mL of Betaplate Scint cocktail (Wallac Inc., Turku, Finland).
[^H]-thymidine incorporation was counted on a 1450 Microbeta Trilux β-scintillation counter (Wallac) using Wallac 1450 Microbeta Trilux & Micobeta Jet software (version 4.01). Results were expressed as counts per minute (cpm). The percentage suppression of proliferation was calculated as follows:

\[\%\text{ Suppression} = \left[1 - \left(\frac{\text{mean cpm of co-culture}}{\text{mean cpm of responder cells alone}}\right)\right] \times 100\%\]

Quantification of Apoptotic T\text{Reg} cells

Freshly sorted T\text{Reg} cells (3 x10⁴ cells) were cultured in 600µL of complete RPMI1640 medium in the absence or presence of soluble anti-CD3 and anti-CD28 for 6 days at 37°C in 5% CO₂. Thereafter, cell death was assessed using the Annexin V-FITC Apoptosis Detection kit (Becton & Dickinson) according to the manufacturer’s instructions. Briefly, cells were harvested and washed with annexin V-bending buffer and stained with FITC-conjugated annexin V for 15 minutes in the dark. Cells were washed and resuspended in 1μg/mL of propidium iodide (PI), followed by flow cytometry analysis. In this assay, viable cells (annexin V - PI -) can be distinguished from early apoptotic (annexin V + PI -), late apoptotic (annexin V + PI +Low), and necrotic (PI +High) cells.

Statistical analysis

Data are presented as the mean ± SD. Comparison of mean values between WG-patients and healthy controls was assessed using nonparametric Mann-Whitney U-test, and differences were considered statistically significant at two-sided \(P\)-values of less than 0.05.
Results

Increased percentage of circulating CD25\(^{\text{High}}\) and CD25\(^{\text{Low}}\) memory CD4\(^+\)T-cells in WG-patients in remission compared with healthy donors

First, we compared the proportion of circulating CD25\(^{\text{High}}\), CD25\(^{\text{Low}}\), and CD25\(^{-}\) cells within the CD4\(^+\)T-cell population in WG-patients and healthy donors. A representative flow cytometric analysis for one patient and one age- and sex-matched healthy control subject is shown in figure 1A.

Figure 1: Surface expression of CD25 on circulating CD4\(^+\)T-cells in Wegener’s granulomatosis (WG)-patients in remission and in age- and sex-matched healthy controls (HC).

(A) Representative flow-cytometric analysis of CD4 and CD25 expression on gated lymphocytes in freshly isolated peripheral blood mononuclear cells obtained from one WG-patient, and one HC. The gate strategy for CD25, CD25\(^{\text{High}}\), and CD25\(^{\text{Low}}\) populations is shown. The same gates were used for data analysis of each patient and HC. Values in each gate are the percentage of positive cells. Data were plotted using the Win-List software package, and quadrants were established using isotype controls. (B) Percentage expression of CD25\(^{\text{High}}\), CD25\(^{\text{Low}}\), and CD25\(^{-}\) cells among CD4\(^+\)T-cells in peripheral blood from WG-patients and HC. The percentages of CD25\(^{\text{High}}\) and CD25\(^{\text{Low}}\) populations of CD4\(^+\)T-cells are increased, whereas the percentages of CD25\(^{-}\)CD4\(^+\)T-cells are decreased, in WG-patients as compared with HC. (C) Percentage expression of CD25\(^{\text{High}}\), CD25\(^{\text{Low}}\), and CD25\(^{-}\) on memory (CD45RO\(^{+}\)) and naïve (CD45RO\(^{-}\)) T-cells among the circulating CD4\(^+\)T-cells from WG-patients and HC. Horizontal bars in B and C show the mean. P-values were determined by nonparametric Mann-Whitney U-test.
WG-patients in remission had a significantly higher percentage of CD25\textsuperscript{High} and CD25\textsuperscript{Low} cells, and a significantly lower percentage of CD25\textsuperscript{-} cells among their CD4\textsuperscript{+}T-cells as compared with the control group (Figure 1B). Next, we investigated whether the relatively increased CD25\textsuperscript{High} and CD25\textsuperscript{Low} populations were confined to the naïve (CD45RO\textsuperscript{-}) or memory (CD45RO\textsuperscript{+}) CD4\textsuperscript{+}T-cells. Within the CD4\textsuperscript{+}T-cells from WG-patients, a significant increase was observed in the percentage of CD25\textsuperscript{High} naïve and CD25\textsuperscript{High} memory T-cells and in the percentage of CD25\textsuperscript{Low} memory T-cells as compared to healthy controls. The percentage of CD25\textsuperscript{Low} naïve CD4\textsuperscript{+}T-cells did not differ between the 2 groups (Figure 1C). In addition, the percentage of CD25 naïve cells among the CD4\textsuperscript{+}T-cells was significantly lower in WG-patients, whereas no differences were seen in the percentage of CD25 memory T-cells between patients and healthy individuals. These results demonstrate a shift from CD25 naïve CD4\textsuperscript{+}T-cells in WG-patients toward CD25\textsuperscript{High} and CD25\textsuperscript{Low} memory CD4\textsuperscript{+}T-cells.

No differences were found in the percentages of CD25\textsuperscript{High}, CD25\textsuperscript{Low}, and CD25 naïve and memory CD4\textsuperscript{+}T-cells between patients with localized and generalized disease, or between patients who were ANCA-positive and those who were ANCA-negative at the moment of sampling (data not shown).

**Increased proportion of T\textsubscript{Reg} cells in peripheral blood of WG-patients in remission**

Since CD25\textsuperscript{High} is not a unique marker for T\textsubscript{Reg} cells, it was appropriate to establish the identity of circulating CD25\textsuperscript{High}CD4\textsuperscript{+}T-cells through expression of the FoxP3 marker, which is a master regulator and a more specific marker of T\textsubscript{Reg} cells\textsuperscript{16,17}. The expression level of FoxP3 was measured using FACS technique. To determine whether the expression of this transcription factor was specific for CD25\textsuperscript{High}CD4\textsuperscript{+}T-cells, we compared FoxP3 expression in the CD25\textsuperscript{High}, CD25\textsuperscript{Low}, and CD25 naïve populations of CD4\textsuperscript{+}T-cells (Figure 2A). The majority of CD25\textsuperscript{High}CD4\textsuperscript{+}T-cells expressed FoxP3 (>79%) and <20% of the CD25\textsuperscript{Low}CD4\textsuperscript{+}T-cells expressed FoxP3, whereas only 1.74% of the CD25 CD4\textsuperscript{+}T-cells expressed FoxP3.
Figure 2: Frequency of circulating Treg cells in Wegener’s granulomatosis (WG)-patients in remission and in age- and sex-matched healthy controls (HC).

(A) Flow cytometric analysis of forkhead box P3 (FoxP3) expression in CD25^{High} (left), CD25^{Low} (middle), and CD25^{-} (right) CD4^{+} T-cells. Values at the top of each plot are the percentage of FoxP3 positive cells. (B) Representative example of FACS-plots and gating of CD25^{High}FoxP3^{+} cells among CD4^{+} T-cells (left), and the expression of CD45RO in CD25^{High}FoxP3^{+} gated cells (right), in a WG-patient and HC. Values in each gate are the percentage of positive cells. (C) The percentage of total Treg cells (FoxP3^{+}CD25^{High}), memory Treg cells (FoxP3^{+}CD45RO^{+}CD25^{High}), and naïve Treg cells (FoxP3^{+}CD45RO^{−}CD25^{High}) among CD4^{+} T-cells from the peripheral blood of WG-patients and HC. Flow cytometry was performed as described in Materials and Methods. Horizontal bars show the mean. P-values were determined by nonparametric Mann-Whitney U-test.
Next, we examined the percentages of $CD25^{\text{High}}FoxP3^{+}$ cells among the $CD4^{+}$T-cell population in both healthy controls and WG-patients (Figure 2B and C). Unexpectedly, a significantly increased percentage of $CD25^{\text{High}}FoxP3^{+}$ cells was observed in the $CD4^{+}$T-cell population in WG-patients as compared with healthy controls. Since circulating T$_{\text{Reg}}$ cells are found in both memory and naïve $CD4^{+}$T-cell populations, we determined the percentages of the memory and naïve $FoxP3^{+}CD25^{\text{High}}$ T-cell populations. As shown in figure 2C, the increased percentages of T$_{\text{Reg}}$ cells ($CD25^{\text{High}}FoxP3^{+}$) in WG-patients were confined to memory T-cells, whereas percentages of naïve T$_{\text{Reg}}$ cells did not differ between WG-patients and healthy controls. In addition, no difference was seen between patients with localized and those with generalized WG, nor between patients who were ANCA-positive and those who were ANCA-negative patients with respect to the percentage of $FoxP3^{+}CD25^{\text{High}}$ $CD4^{+}$T-cells, in both the naïve and memory populations (data not shown).

Thus, based on phenotype analysis, these results imply that WG-patients whose disease is in remission have an elevated percentage of circulating $CD45RO^{+}$T$_{\text{Reg}}$ cells as compared with normal individuals.

**No involvement of therapy in the relative increase in T$_{\text{Reg}}$ cells in WG-patients**

It has been reported that treatment may alter the frequency of T$_{\text{Reg}}$ cells. To further rule out the possibility that the increased proportions of T$_{\text{Reg}}$ cells in WG-patients were the result of current treatment, the WG-patient group was separated into treated ($n=37$) and untreated ($n=15$) patients, and the percentages of $FoxP3^{+}CD25^{\text{High}}$ cells in naïve and memory $CD4^{+}$T-cell population were compared. As shown in table 2, no significant differences in these percentages were observed between treated and untreated patients. Since patients with generalized-WG previously may have received more aggressive treatment in the past than patients with localized-WG, which might have influenced the distribution of lymphocyte subsets, we compared the percentages of naïve and memory T$_{\text{Reg}}$ cells between currently untreated patients with generalized and localized WG. No differences were found between these patient groups (data not shown).
Table 2: Percentages of circulating T<sub>reg</sub> cells (naïve and memory) among the CD<sup>4</sup><sup>+</sup>T-cell subset in untreated and treated patients with WG in remission.

<table>
<thead>
<tr>
<th>T&lt;sub&gt;reg&lt;/sub&gt; cell subset</th>
<th>Untreated (n=15)</th>
<th>Treated (n=37)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>FoxP3&lt;sup&gt;+&lt;/sup&gt;CD&lt;sub&gt;25&lt;/sub&gt;&lt;sup&gt;High&lt;/sup&gt;</td>
<td>5.9 ± 0.5</td>
<td>6.8 ± 0.5</td>
<td>NS</td>
</tr>
<tr>
<td>FoxP3&lt;sup&gt;+&lt;/sup&gt;CD&lt;sub&gt;45RO&lt;/sub&gt;&lt;sup&gt;+&lt;/sup&gt;CD&lt;sub&gt;25&lt;/sub&gt;&lt;sup&gt;High&lt;/sup&gt;</td>
<td>5.2 ± 0.5</td>
<td>5.9 ± 0.5</td>
<td>NS</td>
</tr>
<tr>
<td>FoxP3&lt;sup&gt;+&lt;/sup&gt;CD&lt;sub&gt;45RO&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;CD&lt;sub&gt;25&lt;/sub&gt;&lt;sup&gt;High&lt;/sup&gt;</td>
<td>0.8 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Thirteen of the 37 treated patients with Wegener’s granulomatosis (WG) received azathioprine, 2 received cotrimoxazole, 1 received cyclosporine, 1 received prednisolone, and 20 received prednisolone with a low-dose of either azathioprine, cyclophosphamide, or mycophenolate mofetil. Values are the mean ± SD percentages of positive cells in the CD<sup>4</sup><sup>+</sup>T-cell subset. P-values for untreated versus treated WG were not significant (NS).*

Lack of correlation between clinical features of WG and increased percentages CD45RO<sup>+</sup>T<sub>reg</sub> cells

Since CD4 memory T<sub>reg</sub> cells were relatively increased in WG-patients, we addressed the question whether this increase was correlated with the clinical findings in WG-patients. The percentage of CD45RO<sup>+</sup>T<sub>reg</sub> cells showed no correlation with disease duration, age, total number of relapses, or ANCA-titre in patients with localized-WG or in patients with generalized WG (data not shown).

Defective suppressor function of circulating T<sub>reg</sub> cells in WG-patients in remission

In order to evaluate the immunosuppressive properties of T<sub>reg</sub> cells purified from WG-patients whose disease was in remission and from age- and sex-matched normal donors, proliferation and co-incubation studies were performed (Figure 3). Since cyclophosphamide inhibits the function of T<sub>reg</sub> cells<sup>33</sup>, patients receiving cyclophosphamide were excluded. Of the 10 patients evaluated, 4 were untreated, 1 was receiving prednisolone plus mycophenolate mofetil, and 5 patients were receiving monotherapy with azathioprine (n=3), cyclosporine (n=1), or prednisolone (n=1).

Upon induction by soluble anti-CD3 and soluble anti-CD28 antibodies, sorted T<sub>resp</sub> cells from both patients and controls showed a high
level of proliferation. In contrast, sorted T\textsubscript{Reg} cells from neither the patients nor the controls proliferated in response to these polyclonal stimuli. Next, we measured the ability of T\textsubscript{Reg} cells to inhibit the proliferation of autologous T\textsubscript{Resp} cells (at a 1:1 ratio) induced by polyclonal stimuli. In the healthy control group, the proliferation of T\textsubscript{Resp} cells was strongly suppressed by T\textsubscript{Reg} cells (mean 77%), whereas T\textsubscript{Reg} cells from WG-patients induced a significantly lower level of suppression (mean 10%) (Figure 3C). Importantly, T\textsubscript{Reg} cells from some patients induced increased proliferation of T\textsubscript{Resp} cells instead of suppression. The results suggest an impaired suppressive function of circulating T\textsubscript{Reg} cells in WG-patients.

Figure 3: Isolation and functional characterization of T\textsubscript{reg} cells in Wegener’s granulomatosis (WG)-patients in remission and in age- and sex-matched healthy controls (HC). (A) CD4 T-cells freshly isolated from peripheral blood mononuclear cells were stained as described in Patients and Methods and separated into T\textsubscript{Resp} (CD25\textsuperscript{Low}) and T\textsubscript{Reg} (CD25\textsuperscript{High}) cells by FACS-sorting to a purity >95% and >90%, respectively. Values in each gate are the percentage of positive cells. (B) Proliferation of sorted T\textsubscript{Resp} cells, T\textsubscript{Reg} cells, or of both populations in a 1:1 ratio from a WG-patient and a HC in response to polyclonal stimuli with anti-CD3 plus anti-CD28. Cells were incubated for 6 days, and proliferation was measured by \textsuperscript{3}H-thymidine uptake. T\textsubscript{Reg} cells from the WG-patient are less potent inhibitors of T\textsubscript{Resp} cell proliferation than those from the control. Values are the mean and SEM of triplicate results. (C) Percentage suppression of T\textsubscript{Resp} cell proliferation by autologous T\textsubscript{Reg} cells (at a 1:1 ratio) from WG-patients (n=10) and HCs (n=10). Values were calculated from the proliferation assay in response to polyclonal stimulation (see Patients and Methods). Defective suppressor activity of T\textsubscript{Reg} cells from WG-patients is observed compared with T\textsubscript{Reg} cells from HCs. Horizontal bars show the mean. P-values were determined by nonparametric Mann-Whitney U-test.
Impaired suppression of $T_{Reg}$ cells from WG-patient is unrelated to $T_{Resp}$ cell resistance

Next, we analyzed whether the lack of suppression of WG-patients' cells in co-culture assay was attributable to functional impairment of the $T_{Reg}$ cells or to resistance of the $T_{Resp}$ cells to suppression. To test both possibilities, we performed criss-cross experiments in which polyclonally stimulated $T_{Resp}$ cells from WG-patients were co-cultured with autologous $T_{Reg}$ cells or with $T_{Reg}$ cells from a healthy donor and vice versa. As illustrated in figure 4 (A-C), $T_{Reg}$ cells from HCs inhibited the proliferation of both autologous $T_{Resp}$ cells (70% and 86%; Figures 4A and B, respectively) and $T_{Resp}$ cells (56% and 77%; Figure 4A and B, respectively) from WG-patients, whereas $T_{Reg}$ cells from WG-patients failed to suppress the proliferation of either autologous $T_{Resp}$ cells (-62% and 19%; Figures 4A and B, respectively) or $T_{Resp}$ cells (-45% and 17%; Figure 4A and B) from HCs. Collectively, these findings indicate that the lack of suppression of $T_{Reg}$ cells in WG-patients is related to a functional impairment of these cells, rather than to a resistance of $T_{Resp}$ cells to suppression.

No relationship between lack of suppression and altered survival of $T_{Reg}$ cells from WG-patients

It has been reported that $T_{Reg}$ cells from patients are more susceptible to apoptosis than $T_{Reg}$ cells from normal donors. Therefore, it is possible that $T_{Reg}$ cells from WG-patients fail to suppress the proliferation of $T_{Resp}$ cells because they go into apoptosis. To exclude this possibility, we determined the rate of apoptosis of freshly isolated $T_{Reg}$ cells from WG-patients and controls after 6 days of culture in the presence and absence of polyclonal stimuli. Neither unstimulated $T_{Reg}$ cells nor stimulated $T_{Reg}$ cells from either WG-patients or HCs showed an increase of cell death (Figure 4E). Furthermore, incubation of $T_{Reg}$ cells derived from patients and HCs with 10% soluble anti-CD3 and IL-2 (100 units/mL) for 6 days induced a high degree of proliferation as compared with the proliferative response to anti-CD3 and anti-CD28 (Figure 4D). These results clearly suggest that $T_{Reg}$ cells from WG-patients do not display enhanced susceptibility to apoptosis,
and therefore, failure of suppression was not due to altered survival of T_{Reg} cells.

Figure 4: No link between defective suppressor function of T_{Reg} cells in Wegener's granulomatosis (WG)-patients in remission and either T_{Resp} cell resistance or altered survival of T_{Reg} cells. (A and B) In 2 independent criss-cross in vitro proliferation assays, T_{Reg} cells from 2 WG-patients failed to inhibit the proliferation of either autologous T_{Resp} cells (solid bars) or T_{Resp} cells obtained from 2 age- and sex-matched healthy controls (HCs) (cross-hatched bars). T_{Reg} cells from 1 WG-patient (A) even increased the proliferation, instead of the suppression, of both autologous and control T_{Resp} cells. In contrast, T_{Reg} cells from HCs suppressed the proliferation of autologous T_{Resp} cells (open bars) and T_{Resp} cells isolated from WG-patients (diagonally-hatched bars). Values are the mean SEM of triplicate results. (C) Results from the proliferation assays in A and B are presented as percentages of suppression. Horizontal bars show the mean. (D) T_{Reg} cells derived from 2 WG-patients and 2 HCs exhibited a high degree of proliferation in response to anti-CD3 and interleukin-2 (IL-2) as compared with their proliferation in response to anti-CD3 and anti-CD28. Values are the mean and SEM of triplicate results. (E) Percentage of viable (annexin V⁻ PI⁻), early apoptotic (annexin V⁺ PI⁻), late apoptotic (annexin V⁺ PI⁺Low), and necrotic (PI⁺High) T_{Reg} cells in 2 WG-patients and 2 HCs after 6 days of culture in medium alone versus medium supplemented with anti-CD3 and anti-CD28. T_{Resp} cells from WG-patients and HCs exhibit comparable levels of spontaneous apoptosis, and cells from neither WG-patients nor controls show a significant decrease in percentages of viable T_{Reg} cells after 6 days of culture. Values in each plot are the percentage of positive cells.
Discussion

A reduction in number or impaired function of T<sub>Reg</sub> cells has been observed in several autoimmune diseases<sup>4-12</sup>. In the present study, we examined whether a similar abnormality in T<sub>Reg</sub> cells may occur in patients with WG whose disease was in remission. We demonstrated an increase in the percentage of circulating CD25<sup>+</sup>CD4<sup>+</sup>T-cells in WG-patients in remission, in accordance with a previous report<sup>27</sup>; however, in contrast to data from another study<sup>37</sup>, we found that circulating T<sub>Reg</sub> cells (FoxP3<sup>+</sup>CD25<sup>High</sup>) exhibiting a memory phenotype were significantly increased in WG-patients as compared with age- and sex-matched healthy donors. Despite the increased proportion of circulating T<sub>Reg</sub> cells in WG-patients, the in vitro suppressive property of sorted CD25<sup>High</sup>CD4<sup>+</sup>T-cells was significantly decreased. To the best of our knowledge, these data represent the first demonstration of a functional defect in T<sub>Reg</sub> cells in patients with WG.

Although low percentages of naïve CD4<sup>+</sup>T-cells were found in our WG-patients in remission, which is consistent with previous studies<sup>23,37</sup>, we observed a significant increase in the percentage of CD25<sup>High</sup> naive CD4<sup>+</sup>T-cells. Interestingly, the majority of these naïve CD4<sup>+</sup>T-cells from WG-patients were positive for FoxP3; however, no difference was observed in FoxP3<sup>+</sup>CD25<sup>High</sup> naïve CD4<sup>+</sup>T-cells between patients and controls. In comparison with healthy controls, a clear increase of FoxP3-expressing CD25<sup>High</sup>CD4<sup>+</sup>T-cells was found in the memory cell population of patients with WG. Since the WG-patients had received or were still receiving immunosuppressive therapy, it is possible that treatment could have increased the percentage of T<sub>Reg</sub> cells<sup>34,35</sup>. Based on our data, it is unlikely that the proportional increase in T<sub>Reg</sub> cells in WG-patients was due to exposure to immunosuppressive medication, since we did not find a relationship between the percentages of T<sub>Reg</sub> cells and either current or previous immunosuppressive treatment. Likewise, treatment in patients with juvenile idiopathic arthritis or RA has been reported not to alter the percentage of circulating CD25<sup>+</sup>CD4<sup>+</sup>T-cells<sup>6,38</sup>.

Despite the increase in circulating T<sub>Reg</sub> cells, an impaired suppressive function of CD25<sup>High</sup>CD4<sup>+</sup>T-cells was observed in WG-patients. As shown by criss-cross experiments with T-cells from healthy controls, the
failure of T_{Reg} cells to suppress T_{Resp} cells was related to a functional impairment of the T_{Reg} cells rather to a resistance of the T_{Resp} cells to suppression or to an altered survival of T_{Reg} cells. Functional defects in T_{Reg} cells have recently been reported in patients with autoimmune polyglandular syndrome type II (APS-II)\textsuperscript{7}. The percentage suppression induced by T_{Reg} cells from healthy controls in that study was comparable to the percentage suppression found in the present study. Interestingly, consistent with the data from the APS-II patients, T_{Reg} cells from some WG-patients not only failed to suppress, but in some cases even increased the proliferation of autologous and HC T_{Resp} cells, which indicates a complete defect in the suppressive capacity of T_{Reg} cells.

The inconsistency between the increased percentage of circulating T_{Reg} cells and the defective function of this population in WG-patients needs to be explained. The normal expression of FoxP3 in functionally impaired T_{Reg} cells in other autoimmune diseases\textsuperscript{7,12} together with the finding that human CD25^{+}CD4^{+}T-cells upregulate FoxP3 expression following stimulation imply that not all FoxP3-positive cells can be considered regulatory T-cells\textsuperscript{39-41}. It is possible that in WG-patients, the response of CD4^{+}T-cells to an as-yet-unknown antigenic stimulus may induce the expression of FoxP3 in combination with the expression of CD25. Indeed, higher frequencies of CD25^{High}CD4^{+}T-cells and FoxP3-expression have been found in patients with tuberculosis\textsuperscript{42}. Therefore, persistent T-cell stimulation is probably a better explanation for the increase in the percentage of FoxP3^{+}CD25^{High}CD4^{+}T-cells in our WG-patients in remission.

The biological basis for the defective function of T_{Reg} cells in WG remains undefined. Expression of a loss-of-function mutant FoxP3 in T_{Reg} cells from WG-patients could be a possible reason for the defective T_{Reg} cell function. Furthermore, it has recently been reported that human T_{Reg} cells co-express 2 isoforms of FoxP3 (FoxP3 and FoxP3\Delta2), and the suppressive activity of T_{Reg} cells was present only when both FoxP3 isoforms were simultaneously expressed\textsuperscript{43}. It is, therefore, possible that some of the circulating T_{Reg} cells from WG-patients lack the FoxP3\Delta2 isoform, which may be one of several explanations for the inconsistency between the increased percentage of T_{Reg} cells \textit{in vivo} and the defective function of sorted CD25^{High}CD4^{+}T-cells \textit{in vitro}. It should be stressed that although animal models of FoxP3 deficiency result in autoimmune disease, the
human inherited FoxP3 deficiency disease known as immune dysregulation, polyendocrinopathy, and enteropathy, X-linked syndrome (IPEX) does not result in SLE, RA, or WG. Therefore, other factors such as PTPN22 polymorphism, which has been described as a risk factor for WG44, might be important as well in the development of autoimmunity in WG.

Further studies are necessary to establish the exact causes for dysfunction of T\textsubscript{Reg} cells in WG-patients. Nevertheless, imbalances in homeostasis of CD4\textsuperscript{+} T-cell subpopulations\textsuperscript{23}, skewing in Th1/Th2 cytokines\textsuperscript{45,46}, and the production of auto-antibodies (ANCAs) in WG-patients might be related to uncontrolled auto-reactive T-cell activation and proliferation due to a dysfunction of T\textsubscript{Reg} cells in this disease.

In conclusion, this study is the first to demonstrate an expanded proportion and functional deficit of circulating regulatory T-cells in WG-patients in remission, which may underlie loss of self-tolerance in this disease and may contribute to its pathogenesis.

Acknowledgments

We thank Geert Mesander and Henk Moes for their help with the FACS-sorting experiments, and we are grateful to the patients and healthy donors for their co-operation.
REFERENCES


