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Functional Study of CD4⁺CD25⁺ Regulatory T Cells in Health and Autoimmune Hepatitis

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Regulatory CD4⁺CD25⁺ T cells (Tregs) are defective numerically and functionally in autoimmune hepatitis (AIH). We have investigated and compared the mechanism of action of Tregs in healthy subjects and in AIH patients using Transwell experiments, where Tregs are cultured either in direct contact with or separated from their targets by a semipermeable membrane. We also studied Treg FOXP3 expression and effect on apoptosis. Direct contact is necessary for Tregs to suppress proliferation and IFN-γ production by CD4⁺CD25⁻ and CD8⁺ T cells in patients and controls. Moreover, in both, direct contact of Tregs with their targets leads to increased secretion of regulatory cytokines IL-4, IL-10, and TGF-β, suggesting a mechanism of linked immunosuppression. Tregs/CD4⁺CD25⁺ T cell cocultures lead to similar changes in IFN-γ and IL-10 secretion in patients and controls, whereas increased TGF-β secretion is significantly lower in patients. In contrast, in patients, Tregs/CD8⁺ T cell cocultures lead to a higher increase of IL-4 secretion. In AIH, Treg FOXP3 expression is lower than in normal subjects. Both in patients and controls, FOXP3 expression is present also in CD4⁺CD25⁻ T cells, although at a low level and not associated to suppressive function. Both in patients and controls, addition of Tregs does not influence target cell apoptosis, but in AIH, spontaneous apoptosis of CD4⁺CD25⁺ T cells is reduced. In conclusion, Tregs act through a direct contact with their targets by modifying the cytokine profile and not inducing apoptosis. Deficient CD4⁺CD25⁺ T cell spontaneous apoptosis may contribute to the development of autoimmunity.


Several T cell subpopulations with regulatory activity have been described, such as NKT, Th3, Tr1, CD8⁺CD28⁻, and γδ T cells, although CD4⁺ T cells naturally expressing the IL-2Rα chain (CD25) have emerged as a pivotal regulatory subpopulation (1, 2). These cells, representing 5–10% of the CD4⁺ T lymphocytes in healthy mice and humans (3–5), control the innate and the adaptive immune response by preventing the proliferation and effector function of autoreactive T cells. Their mechanism of action is not fully elucidated, possibly involving a direct contact with the target cells, as indicated by the majority of in vitro data (4, 6), or implicating the release of cytokines with regulatory properties (IL-10 and TGF-β) as suggested by in vivo studies (7–9).

It has been demonstrated that CD4⁺CD25⁺ regulatory T cells (Tregs) represent a highly differentiated T cell subpopulation particularly prone to apoptosis (10), although their effect on the spontaneous apoptosis rate of other T lymphocyte subsets, to our knowledge, has not been investigated.

Apart from CD25, additional markers expressed by Tregs include the glucocorticoid induced TNFR (GITR) (11), CD62L (12, 13), CTLA-4 (11), and the forkhead/winged helix transcription factor (FOXP3) (14, 15). It has been reported that FOXP3 induction in nonregulatory CD4⁺ T cells is associated with the acquisition of regulatory properties typical of the Tregs’ compartment (16), an observation with potential important implications for the therapeutic management of autoimmune disease and transplant acceptance (17, 18).

CD4⁺CD25⁺ Tregs are increasingly being investigated in human autoimmune pathology, including autoimmune hepatitis (AIH), an inflammatory liver disorder characterized by presence of elevated levels of transaminases and circulating autoantibodies, hypergammaglobulinemia, histological evidence of interface hepatitis, and response to immunosuppressive treatment (19, 20). Results from our group have shown that in patients with AIH Tregs are numerically and functionally defective (21, 22). This impairment relates to the stage of disease being more evident at presentation than during drug-induced remission, where an increase in Treg number and ability to suppress proliferation of, and IFN-γ production from, target cells (CD4⁺CD25⁻ or CD8⁺ T cells) is observed.

The aims of this study were to investigate both in healthy individuals and in patients with AIH whether CD4⁺CD25⁺ T cells exert their function by direct cell contact or through the release of soluble factors; to analyze their FOXP3 and cytokine gene expression; and to determine their effect on the apoptosis rate of CD4⁺ and CD8⁺ T lymphocytes.

Materials and Methods

Patients

Twenty-five patients with anincular Ab- and/or smooth muscle Ab-positive autoimmune hepatitis (AIH type 1 (AIH-1)) were investigated. A liver biopsy performed at diagnosis showed histological features of interface hepatitis in all patients. A retrograde cholangiopancreatography, performed
whether immunomagnetic purification yields an enriched CD4 T cell population, the purified CD4^CD25^ T cells were compared phenotypically and functionally to CD4^CD25^ T cells sorted from PBMCs using a cell sorter (model BD Vantage SE/DiVa; Immunocytochemistry Systems; BD Biosciences) (Fig. 1). Immunomagnetically purified cells colocalized (Fig. 1B) with the CD4^CD25^ population sorted from PBMCs (Fig. 1A) and both immunomagnetically purified CD4^CD25^ T cells and sorted CD4^CD25^ inhibited CD4^CD25^ T cell proliferation by 50%. CD4^CD25^ and CD8 T cells, to be used as targets, were obtained by negative selection using magnetic beads coated with an Fc-specific human IgG4 Ab (Dynal Biotech). The purity of the three-cell subpopulations, assessed by flow cytometry using FITC-conjugated anti-CD4, CD19, CD14, PE-conjugated CD25, and CyChrome (Cy)-conjugated anti-CD8 and CD56 mAbs, exceeded 95% for the CD4^CD25^ Treg subset and was consistently higher than 90% (range, 92–97%) for the CD4^CD25^ and CD8^T cells subsets, the 3–8% contaminating cells being double positive CD4^CD8^ T cells (30–35%), CD56^+^ cells (20–25%), CD19^+^ cells (10–15%), and dead cells (20–25%).

**Transwell experiments and proliferation assay**

To assess whether CD4^CD25^ T cells exert their regulatory function through direct cell contact or through release of soluble factors, we performed a series of Transwell experiments. Once purified, CD4^CD25^ T cells were added at a ratio of 1:8 to autologous CD4^CD25^ or CD8^T cells seeded at 5 × 10^5\textsuperscript{+}/well in the lower chamber of a 24-well plate. The 1:8 ratio was selected because it is capable of exerting a detectable regulatory function in preliminary experiments, where ratios of 1:16, 1:8, and 1:4 were compared (21). CD4^CD25^ T cells were either cultured in the lower chambers directly in contact with the target cells or in the upper chambers separated from the target cells by a 0.4-μm pore membrane (BD Biosciences Discovery Labware), which allows diffusion of small molecules, such as cytokines, but not of cells. Control cultures using CD4^CD25^ T cells instead of Tregs and CD4^CD25^ and CD8^T cells cultured on their own were performed under identical conditions. All the experiments were performed in duplicate. Cells were cultured for 5 days in RPMI 1640, supplemented with 2 mM L-glutamine, 25 mM HEPEs, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2.5 μg/ml amphotericin B, and 10% inactivated FCS at 37°C and 5% CO\textsubscript{2}, in the presence of a T cell expander capable of preserving the original T cell function (CD3/CD28 Dynabeads; Dynal Biotech) (25). rIL-2 (Chiron) was added at a concentration of 30 U/ml on day 3. On the last day of culture, cells from the lower chambers were collected, transferred after centrifugation in 96-well plates (2 × 10^5 cells/well), and their proliferative response tested by addition of 0.25 μCi/well of [\textsuperscript{3}H]thymidine/well, followed by harvesting 18 h later. The amount of incorporated [\textsuperscript{3}H]thymidine was determined by liquid scintillation spectroscopy (beta counter; Canberra Packard). Percentage inhibition was calculated using the formula: 1 – (cpm in the presence of CD4^CD25^ T cells/cpm in the absence of CD4^CD25^ T cells) × 100.

**Cytokine measurement by ELISA**

On the last day of culture, 200 μl of supernatant was collected from each well, and the levels of IFN-γ, IL-4, IL-10, and TGF-β were measured by ELISA (26). Changes between cytokine levels at baseline and after Treg addition were calculated in each individual as fold decrease/increase, i.e., the ratio between the levels of the cytokine secreted after Treg addition over the baseline levels. Comparison between the mean fold decrease/increase in the two groups of subjects was then performed.

**Neutralization assay**

To investigate whether the suppressor function of Tregs is related to the release of regulatory cytokines, anti-human-IL-4 (clone 34019.111; R&D Systems), anti-human-IL-10 (clone 23738, R&D Systems), and anti-human-TGF-β (clone 9016; R&D Systems) were added to cocultures of CD4^CD25^ or CD8^T cells with Tregs at a final concentration of 10 μg/ml, chosen on the basis of previous titration studies (27). Cells were cultured under the same conditions as described above in Transwell experiments and proliferation assay. Controls consisted of CD4^CD25^ and CD8^T cells cultured on their own using the same culture medium and reagents described for Transwell experiments. Following a 5-day culture, cells were tested for their proliferative response.

**Apoptosis staining**

To assess whether the presence of CD4^CD25^ T cells affects the spontaneous rate of apoptosis of target subpopulations, CD4^CD25^ T cells were added to autologous CD4^CD25^ and CD8^T cells either directly or separated by the semipermeable membrane. The apoptosis rate of CD4^CD25^ and CD8^T cells was assessed by staining the cells with Annexin VFITC (BD Biosciences), an early marker of apoptosis, and with

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**FIGURE 1.** Characterization of CD25\textsuperscript{high}. Dot plots of anti-CD4-FITC fluorescence (x-axis; log scale) vs anti-CD25-PE fluorescence (y-axis; log scale). A, CD4^CD25^; B, CD4^CD25^ and CD8^T (ordinate) and CD4^CD25^ (abscissa) within PBMCs. R, An immunomagnetically purified CD4^CD25^ T cell population, which colocalizes with the CD4^CD25^ population, occupying the same quadrant region. Data shown are from a representative patient with AIH.
propidium iodide (BD Biosciences), which allows the exclusion of necrotic cells. The apoptosis rate of CD4+ CD25+ Tregs, cultured on their own, was also assessed.

Staining was performed at days 1 and 5 of culture. The incubation period of 5 days was chosen because a consistently higher percentage of apoptotic cells at day 5 was seen when compared with shorter incubation periods (1, 2, 3, and 4 days) in preliminary experiments.

A total of 1 × 10^6 cells was washed once with PBS/1% FCS and resuspended in 1× binding buffer containing 0.1 M HEPES/NaOH, 1.4 M NaCl, and 25 mM CaCl_2. After addition of 5 μl of Annexin V-FLICA and propidium iodide, cells were incubated at room temperature for 15 min in the dark and resuspended in 400 μl of 1× binding buffer. Analysis was performed within 1 h on a BD Biosciences FACS and CellQuest software was used for analysis (Immunocytochemistry Systems; BD Biosciences).

Quantification of gene expression by real-time PCR

To quantify FOXP3, IFN-γ, IL-4, IL-10, and TGF-β gene expression, 1 × 10^6 purified CD4+ CD25+ T cells were lysed with TRIzol reagent (Invitrogen Life Technologies) at a concentration of 0.1 ml/1 × 10^6 cells, and total RNA was extracted. mRNA was reverse transcribed using oligo(dT)12-18 primer (Invitrogen Life Technologies) and Omniscript Reverse Transcriptase (Qiagen). GAPDH was used as control gene. FOXP3 and cytokine gene transcripts were quantified by real-time PCR using gene-specific probes and TaqMan Master Mix (Applied Biosystems). PCR amplification conditions were as follows: 50°C for 2 min, 95°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min. Samples were run in triplicate using a real-time PCR thermocycler (ABI PRISM 7000 Sequence Detection System; Applied Biosystems), and results were analyzed by matched software. Relative expression of FOXP3 and cytokine genes was determined by normalizing to GAPDH expression in each set of samples according to the manufacturer’s instructions.

Statistical analysis

The normality of variable distribution was assessed by the Kolmogorov-Smirnov goodness of fit test and, once the hypothesis of normality was accepted (p > 0.05), comparison was performed by paired and unpaired Student’s t tests as appropriate. If the hypothesis of normality was rejected, analysis was performed by Wilcoxon’s rank-sum test. Correlation between variables was determined by Pearson’s or Spearman’s correlation coefficient. A value of p < 0.05 was considered significant.

Results

Transwell experiments and proliferation assay

The ability of CD4+ CD25+ Tregs to suppress the proliferation of target cells (CD4+ CD25− and CD8+ T cells) was investigated in 8 normal controls and in 11 patients with AIH-1. In normal controls, CD4+ CD25− T cells, stimulated with CD3/CD28 T cell expander, gave a mean cpm value of 16,966 ± 3,042, which decreased to 9,487 ± 2,034 (44% reduction, p = 0.01) when cultured in direct contact with Tregs (Fig. 2A). A reduced CD4+ CD25− proliferation was also observed when these cells were cocultured with Tregs separated by a semipermeable membrane (13,685 ± 2,272, 19% reduction), although this reduction did not reach statistical significance (p = 0.2). Proliferation of CD4+ CD25− T cells was lower when cultured in direct contact with Tregs than when cultured in the presence of the semipermeable membrane (p = 0.028). In patients, the mean cpm value of stimulated CD4+ CD25− T cells in the absence of Tregs (10,509 ± 1,783) was lower than in normal controls (p = 0.05) and decreased to 8,223 ± 1,474 (23% reduction, p = 0.037) when cultured in direct contact with Tregs (Fig. 2B). No reduction was observed when Tregs were separated from CD4+ CD25− T cells by the semipermeable membrane (10,741 ± 1,649). Proliferation of CD4+ CD25− T cells was lower when cultured in direct contact with Tregs than when cultured in the presence of the semipermeable membrane (p = 0.033).

In normal subjects, CD8+ T cells, stimulated with CD3/CD28 T cell expander, gave a mean cpm value of 3346 ± 562, which decreased to 2375 ± 534 (29% reduction, p = 0.03) when cultured in direct contact with Tregs (Fig. 2C). No change was observed when CD8+ T cells were cultured separated from Tregs by the membrane (3398 ± 528). Proliferation of CD8 T cells was lower...
when cultured in direct contact with Tregs than when separated from them by the semipermeable membrane \( (p = 0.045) \). In patients with AIH-1, the mean cpm count of CD8\(^+\) T cells in the absence of Tregs \( (5829 \pm 616) \) was higher than in normal subjects \( (3346 \pm 562; p = 0.01) \) and decreased to 4365 \( \pm 495 \) (22\% reduction, \( p = 0.04 \)) when cultured in direct contact with Tregs (Fig. 2D). No significant reduction was observed when Tregs were separated from CD8\(^+\) T cells by the semipermeable membrane \( (5741 \pm 937; 1.5\% \) reduction). Proliferation of CD8 T cells was lower when cultured in direct contact with Tregs than when separated from them by the semipermeable membrane \( (p = 0.08) \). Control experiments in which CD4\(^+\)CD25\(^-\) T cells were used instead of Tregs had no detectable effect on proliferation of either CD4\(^+\)CD25\(^-\) or CD8\(^+\) T cells both in patients and normal controls, both in the absence or presence of the semipermeable membrane.

**Cytokine measurement in culture supernatants**

The amount of IFN-\(\gamma\), IL-4, IL-10 and TGF-\(\beta\) released in the culture supernatant was observed; how-

lower when cultured in direct contact with Tregs than when sep-

arated from CD8\(^+\) T cells by the semipermeable membrane (3346 \( \pm 562; p = 0.01 \)) and decreased to 4365 \( \pm 495 \) (22\% reduction, \( p = 0.04 \)) when cultured in direct contact with Tregs (Fig. 2D). No significant reduction was observed when Tregs were separated from CD8\(^+\) T cells by the semipermeable membrane \( (5741 \pm 937; 1.5\% \) reduction). Proliferation of CD8 T cells was lower when cultured in direct contact with Tregs than when separated from them by the semipermeable membrane \( (p = 0.08) \). Control experiments in which CD4\(^+\)CD25\(^-\) T cells were used instead of Tregs had no detectable effect on proliferation of either CD4\(^+\)CD25\(^-\) or CD8\(^+\) T cells both in patients and normal controls, both in the absence or presence of the semipermeable membrane.

No correlation between the baseline levels of IFN-\(\gamma\), IL-4, IL-

10, and TGF-\(\beta\) secreted by both CD4\(^+\)CD25\(^-\) and CD8\(^+\) T cells and duration of immunosuppressive treatment was observed; how-

ever, the highest levels of the regulatory cytokines IL-4, IL-10, and TGF-\(\beta\) secreted by CD4\(^+\)CD25\(^-\) T cells were noted in patients treated for longer than 3 years.

Both in normal controls and patients with AIH, the direct addition of CD4\(^+\)CD25\(^-\) Tregs to CD4\(^+\)CD25\(^-\) T cells resulted in a decrease of IFN-\(\gamma\) and an increase of IL-4, IL-10, and TGF-\(\beta\) in the supernatant. Although the changes in IFN-\(\gamma\) and IL-4 were similar in patients and controls, the increase in TGF-\(\beta\) levels was significantly higher in controls (359-fold) than in patients (2.9-

fold; \( p = 0.008 \)). There was also a more pronounced increase in IL-10 levels in controls (6-fold) compared with patients (4-fold), although the difference was not significant. When CD8\(^+\) T cells were used as targets, in normal controls the addition of Tregs induced a decrease in the levels of IFN-\(\gamma\) and an increase in those of IL-10 but not of IL-4 and TGF-\(\beta\), whereas in AIH-1 patients, a decrease in the level of IFN-\(\gamma\) and an increase in the level of IL-4, IL-10, but not of TGF-\(\beta\) was observed. The changes in secretion of IFN-\(\gamma\), IL-10, and TGF-\(\beta\) were similar in patients and controls, while that of IL-4 was significantly lower in controls (1-fold) than in patients (80-fold; \( p = 0.01 \)). CD4\(^+\)CD25\(^-\) Tregs cultured on their own did not release detectable amount of IFN-\(\gamma\), IL-4, and TGF-\(\beta\), except in one patient in whom an IL-10 level of 436 pg/ml was detected.

The levels of cytokines in the culture supernatant did not change when Tregs were cultured separated from the targets by the semi-

Table I. **Cytokine levels in culture supernatant before and after Treg addition**

<table>
<thead>
<tr>
<th></th>
<th>IFN-(\gamma) (pg/ml)</th>
<th>IL-4 (pg/ml)</th>
<th>IL-10 (pg/ml)</th>
<th>TGF-(\beta) (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal subjects</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4(^+)CD25(^+) alone</td>
<td>Not detectable</td>
<td>Not detectable</td>
<td>Not detectable</td>
<td>Not detectable</td>
</tr>
<tr>
<td>CD4(^+)CD25(^-) alone</td>
<td>507 ( \pm ) 155</td>
<td>77.2 ( \pm ) 22.5</td>
<td>47 ( \pm ) 13</td>
<td>4 ( \pm ) 0.4</td>
</tr>
<tr>
<td>CD4(^+)CD25(^+) and Tregs</td>
<td>99.6 ( \pm ) 75*</td>
<td>158.6 ( \pm ) 36.3**</td>
<td>769 ( \pm ) 234*</td>
<td>2157 ( \pm ) 1234**</td>
</tr>
<tr>
<td>CD8(^+) alone</td>
<td>813 ( \pm ) 212</td>
<td>50 ( \pm ) 4.6</td>
<td>126 ( \pm ) 15</td>
<td>277 ( \pm ) 110</td>
</tr>
<tr>
<td>CD8(^+) and Tregs</td>
<td>213 ( \pm ) 146**</td>
<td>95 ( \pm ) 33</td>
<td>172 ( \pm ) 6.6*</td>
<td>452 ( \pm ) 282</td>
</tr>
<tr>
<td>Patients with AIH-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4(^+)CD25(^+) alone</td>
<td>Not detectable</td>
<td>Not detectable</td>
<td>436(^6)</td>
<td>Not detectable</td>
</tr>
<tr>
<td>CD4(^+)CD25(^-) alone</td>
<td>2829 ( \pm ) 940*</td>
<td>71 ( \pm ) 30.8</td>
<td>267 ( \pm ) 139*</td>
<td>53 ( \pm ) 20.9(^9)</td>
</tr>
<tr>
<td>CD4(^+)CD25(^-) and Tregs</td>
<td>361.5 ( \pm ) 186.6**</td>
<td>155 ( \pm ) 40.6*</td>
<td>1851 ( \pm ) 585(^4)</td>
<td>285 ( \pm ) 93.7*</td>
</tr>
<tr>
<td>CD8(^+) alone</td>
<td>3801 ( \pm ) 1447**</td>
<td>140 ( \pm ) 76</td>
<td>88 ( \pm ) 28</td>
<td>42.9 ( \pm ) 20.3(^7)</td>
</tr>
<tr>
<td>CD8(^+) and Tregs</td>
<td>1672 ( \pm ) 919**</td>
<td>322 ( \pm ) 71*</td>
<td>148 ( \pm ) 17**</td>
<td>80.3 ( \pm ) 26</td>
</tr>
</tbody>
</table>

\(^a\) Levels of cytokines were measured by ELISA. Results are expressed as mean \( \pm \) SEM. Values of \( p \) comparing the baseline levels of cytokines in patients and normal controls: \( ^* p \) = 0.05; \( ^\text{**} p \) = 0.002; \( ^\text{***} p \) = 0.004; \( ^\text{****} p \) = 0.003; \( ^\text{*****} p \) = 0.04. Values of \( p \) comparing the level of cytokine in culture supernatant before and after Treg addition: \( ^* p < 0.05; ^\text{**} p < 0.01; ^\text{***} p < 0.005; ^\text{****} p < 0.001; ^\text{*****} p < 0.0005 \). The value refers to a single patient.
The experiments were performed in triplicate; data are expressed as mean ± SEM. A, The relative FOX3 mRNA levels in Tregs, CD4+CD25+, and CD8+ T cells in normal controls are shown; B, the relative FOX3 mRNA levels in Tregs, CD4+CD25+, and CD8+ T cells in patients with AIH are shown.

p = NS in both comparisons) (Fig. 3B). To exclude that the expression of FOX3 in CD4+CD25+ and CD8+ T cells was due to a contamination with CD4+CD25+ Tregs, CD4+CD25−, and CD8+ T cells were reassessed for their purity by flow cytometry and were found to contain a negligible percentage (<0.05%) of CD4+CD25+ T cells in both normal controls and patients.

The analysis of IFN-γ, IL-4, IL-10, and TGF-β gene expression (Fig. 4) showed that in both patients and controls IFN-γ was not expressed by the CD4+CD25− subset while it was present in CD4+CD25− (18.6 ± 4.9 in patients and 14.8 ± 3 in normal controls; p = 0.03) and CD8+ T cells (5.1 ± 2.4 in patients and 1.3 ± 0.3 in normal controls; p = 0.01); IL-4 was detected at a low level in the CD4+CD25+ subset only, and its level of expression did not differ significantly between patients and normal subjects (2.35 ± 0.3 vs 2.37 ± 0.1); TGF-β was present in all the three cell subpopulations; within each cell subset, its level of expression did not differ significantly between patients and normal subjects (CD4+CD25+, 13.2 ± 1.3 vs 12.4 ± 2.4; CD4+CD25−, 9.8 ± 2 vs 12.9 ± 3.2; and CD8+, 6.8 ± 3.1 vs 5.7 ± 1.5). TGF-β expression levels in CD4+CD25+ T cells did not differ from those in CD4+CD25− T cells, but they were significantly higher than in CD8+ T cells both in patients (p = 0.03) and normal controls (p = 0.03); IL-10 was not detected in CD4+CD25+, in CD4+CD25−, and CD8+ T cells both in patients and normal subjects.

**Effect of Tregs on apoptosis**

The effect of CD4+CD25+ Tregs on apoptosis of CD4+CD25− and CD8+ T cells was assessed in eight patients with AIH-1 and in eight normal controls by determining the percentage of annexin V-positive cells at days 1 and 5 of culture with or without Tregs, added either directly to the target cells or kept separated from them by the semipermeable membrane (Table II). In normal controls, when CD4+CD25+ T cells were cultured on their own without CD4+CD25+ Tregs, the percentage of annexin V-positive cells increased significantly after 5 days (from 29.2 ± 6.2% on day 1 to 79.7 ± 3.8%; p = 0.0001). Addition of Tregs directly to the target did not change the percentage of apoptotic CD4+CD25− T cells both at day 1 and day 5 of culture. In AIH-1 patients in the absence of Tregs, the percentage of annexin V-positive CD4+CD25− T cells also increased after 5 days (from 12.4 ± 2.6% on day 1 to 25.8 ± 7.4%; p = 0.043) but was significantly lower than that observed in normal controls both at day 1 (p = 0.03) and day 5 of culture (p = 0.0006). When Tregs were added directly to the target, the apoptosis rate of CD4+CD25− T cells did not change significantly at both time points.
In normal controls, when CD8\(^+\) T cells were cultured on their own without CD4\(^+\)CD25\(^+\) Tregs, the percentage of annexin V-positive cells increased significantly after 5 days (from 40.2 ± 5.5% on day 1 to 86.3 ± 3.3%; \(p < 0.0001\)). Addition of Tregs directly to the target cells did not change the percentage of apoptotic CD8\(^+\) T cells both at day 1 and day 5 of culture. In AIH-1 patients in the absence of Tregs, the percentage of annexin V-positive CD8\(^+\) T cells also increased significantly after 5 days of culture (from 22.6 ± 4% on day 1 to 42.2%; \(p = 0.02\)) but was significantly lower than that observed in normal subjects both at day 1 (\(p = 0.03\)) and at day 5 (\(p = 0.0001\)) and in patients with AIH (\(p = 0.02\); \(p = 0.0006\)); p values comparing the percentage of annexin V-positive CD8\(^+\) T cells between days 1 and 5 in normal subjects (\(p = 0.001\)) and in patients with AIH (\(p = 0.02\)); p values obtained comparing the percentage of annexin V-positive CD8\(^+\) T cells between normal controls and AIH patients at day 1 (\(p = 0.0002\)) and at day 5 (\(p = 0.03\)).

Similarly, in experiments where Tregs were separated from their targets by the Transwell membranes, no change in the percentage of apoptotic CD4\(^+\)CD25\(^+\) and CD8\(^+\) T cells was observed both in controls and patients (data not shown).

No correlation was found between the rate of apoptosis in CD4\(^+\)CD25\(^-\) and CD8\(^+\) T cells both at baseline and day 5 and the duration of immunosuppressive treatment.

The percentage of annexin V-positive CD4\(^+\)CD25\(^+\) Tregs, cultured on their own, increased after 5 days, from 23 to 48% in three normal controls and from 26 to 36% in three AIH patients, in whom sufficient numbers of cells were available for testing.

### Discussion

This study provides novel information on Tregs function both in health and autoimmune hepatitis. We describe for the first time that, in an ex vivo condition, CD4\(^+\)CD25\(^+\) Tregs skew the immunological environment toward a Th2-like phenotype through a direct contact with the target cells and that this function is impaired in patients with AIH. This impairment may derive from a defective induction of TGF-\(\beta\) secretion.

Transwell experiments indicate that, both in normal controls and, to a lesser extent, in AIH patients, Tregs inhibit the proliferation of CD4\(^+\)CD25\(^+\) and CD8\(^+\) T cells mainly through direct contact, confirming the findings previously reported for both cell types in healthy subjects (10, 23). The fact that anti-IL-4, anti-IL-10, and anti-TGF-\(\beta\) were unable to restore proliferation of CD4\(^+\) and CD8\(^+\) T cells cultured in direct contact with Tregs suggests that Treg suppressor function is not delivered through the action of these regulatory cytokines. On the other hand, Tregs, which do not secrete cytokines when cultured on their own, do influence the production of cytokines once in direct contact with CD4\(^+\)CD25\(^+\) and CD8\(^+\) T cells. This cell-to-cell contact leads to a decrease in the release of IFN-\(\gamma\), a cytokine with proinflammatory activity, and to an increase in the levels of the regulatory cytokines IL-4, IL-10, and TGF-\(\beta\). This phenomenon was observed both when both CD4\(^+\)CD25\(^+\) and CD8\(^+\) T cells were used as targets, although at a lower degree for the latter. However, in the current experimental setting, it is not possible to determine which cell subset (i.e., Tregs or target cells) is primarily responsible for this cytokine production/release. That Tregs can bias their targets toward a “regulatory” phenotype has been proposed on the basis of in vivo studies in BALB/c/Rag-2\(^{-/-}\) mice, where spleen depletion of CD4\(^+\)CD25\(^+\) Tregs prevented the development of Th2 cells and increased that of Th1 cells (27). Whether Tregs create a regulatory milieu by stimulating preprogrammed cell populations or by acting at a single cell level, skewing the cytokine production/release of a cell toward a Th2 phenotype has to be elucidated. Evidence that Tregs can modulate the cytokine secretion of a single cell has been recently provided in a study conducted at a clonal level by Dieckmann et al. (28), who reported that the addition of Tregs to Ag-specific CD4\(^+\) but not CD8\(^+\) T cell clones decreases the secretion of IFN-\(\gamma\) and enhances that of IL-10 and TGF-\(\beta\). However, this phenomenon was observed only when Tregs were preactivated for 24 h with plate-bound anti-CD3 and soluble anti-CD28 and not when they were used immediately after ex vivo isolation.

Although Treg suppressor function is largely delivered via a cell-to-cell contact mechanism, a cell-contact independent mechanism through soluble regulatory factors cannot be excluded because a reduction in cpm count of ~20% was observed when CD4\(^+\)CD25\(^+\) T cells from normal subjects were cocultured with Tregs in the presence of the semipermeable membrane. In contrast, Tregs from patients appear to act exclusively through a direct cell-contact mechanism. Whether this phenomenon is peculiar to AIH and to what extent is influenced by genetic or environmental factors remains to be determined.

Our data, obtained in a non-Ag-restricted system and closely reproducing the in vivo setting, show that the creation of a regulatory environment is mainly a consequence of the direct contact of Tregs with both CD4\(^+\)CD25\(^+\) and CD8\(^+\) T cells in healthy subjects and AIH patients, extending the concept of “linked immunosuppression” (29) to the interaction between these T cell populations. However, although an overall increase in regulatory cytokines was observed in both normal controls and patients with AIH when Tregs were cultured with CD4\(^+\)CD25\(^-\) or CD8\(^+\) T cells, the magnitude of TGF-\(\beta\) and IL-4 increase differed between the two groups of subjects. The increase in TGF-\(\beta\) levels, observed after direct contact of Tregs with CD4\(^+\)CD25\(^-\) T cells, was higher in normal controls than in patients, suggesting a defect in the up-regulation of this cytokine in AIH. Similarly, we have observed a trend for the regulatory cytokine IL-10 to increase more in normal controls than in patients following Tregs addition. In contrast, following the addition of Tregs to CD8\(^+\) T cells, the increase in IL-4 levels was higher in patients than in controls. Whether IL-4 is secreted to compensate for the defect in TGF-\(\beta\) production and whether such Treg-induced increase in IL-4 secretion during treatment is peculiar to AIH or is present in other autoimmune conditions remain to be elucidated.

A collateral but interesting finding is that CD8\(^+\) T cells from patients with AIH when cultured on their own secrete much higher levels of IFN-\(\gamma\) than normal subjects and have a high proliferation rate, while their baseline TGF-\(\beta\) secretion is low; findings that...
would suggest a type 1 polarization. CD4+CD25− T cells from patients, on the other hand, not only secrete a higher baseline level of IFN-γ but also of IL-10 and TGF-β in comparison to controls. This CD4+CD25− regulatory profile may be the result of immunosuppressive treatment since prednisolone and azathioprine, the drugs used in our patients, have been reported to favor a regulatory cytokine pattern in renal transplantation (30, 31). In this context, it is of interest that those patients with AIH who had been treated for longer had the highest baseline level of regulatory cytokines secreted by CD4+CD25− cells.

The analysis of cytokine gene expression shows different profiles in the three T cell populations studied. Although TGF-β was present in Tregs, CD4+CD25−, and CD8+ T cells, IL-10 was absent in all three subpopulations, in agreement with murine studies where the expression of IL-10 in Tregs, CD4+CD25−, and CD8+ T cells was either undetectable or very low, although inducible (32–34). IFN-γ was confined to CD4+CD25− and CD8+ T cells, whereas IL-4 transcripts were present only in Tregs, indicating that Tregs constitutively express the immunoregulatory cytokine IL-4, which is expressed by CD4+CD25− T cells only after stimulation (35).

The FOXP3 gene was expressed at a higher level in Tregs from normal subjects than in those obtained from AIH patients, despite the fact that the latter were studied during drug-induced remission, when Treg function, severely impaired at diagnosis (21, 22), is partially restored. This decreased expression may be an original defect predisposing to the development of AIH. In this context, it is of interest that mutations in the FOXP3 gene have been implicated in the development of a fatal human autoimmune disorder, namely immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), although whether these mutations lead to a lower FOXP3 expression and Treg functional impairment is unknown (36–38). Studies of possible mutations within the FOXP3 gene sequence in AIH will clarify whether they account for the decreased gene expression. FOXP3 transcripts were also detected in CD4+CD25− and CD8+ T cells both in patients and normal controls, suggesting that FOXP3 expression is not universally linked to immunoregulatory function, at least in the CD4+CD25− T cell subset, since in the current study these cells were unable to suppress the proliferation of CD4 and CD8 target cells. The presence of FOXP3 transcripts in cells, which do not exhibit regulatory activity, may suggest that regulatory activity is exerted only above a given “threshold” of FOXP3 expression. In agreement with this, a recent report by Morgan et al. (39) shows that the expression of FOXP3 mRNA in humans is not limited to the CD4+CD25− Treg compartment but can also be detected in T cell lines and clones with no suppressor activity, the expression of this gene being possibly a consequence of an activation status. Conversely, Vieira et al. (40) have shown that while expression of FOXP3 is critical for the inhibitory activity of CD4+CD25− Tregs, an IL-10-secreting CD4+CD25+ Treg population can exert suppressor function without its expression.

In view of the fact that a lack of apoptosis of peripheral autoreactive T cells is characteristic of autoimmune conditions, as documented in the lupus-prone mouse strain, MRL/lpr/lpr, where an accelerated disease progression is associated to a deficient expression of Fas protein in T lymphocytes (41), we have investigated, first, whether impaired T cell apoptosis is present in AIH and, second, whether Treg addition influences the apoptosis rate of CD4+CD25− and CD8+ T cells. Although Tregs appear to have a similar apoptotic rate in health and in AIH, we find that both in patients and normal controls they do not increase the apoptotic rate of the target cells. Of interest, the percentage of CD4+CD25− and CD8+ T cells spontaneously undergoing apoptosis was significantly lower in AIH than in normal subjects, suggesting that a reduced apoptosis of T effector lymphocytes may be involved in facilitating the establishment of autoreactive T cells in autoimmune hepatitis. Thus, apoptosis-resistant autoreactive T cells have been described in autoimmune disease and may derive from a variety of mechanisms, such as expression of the anti-apoptotic bcl-2 in CD4+CD25− T cells as shown in rheumatoid arthritis (42), possibly as a consequence of chronic Ag stimulation (43), or functionally relevant mutations within the cell death receptor Fas (CD95) (44) and its ligand, FasL (CD95L) (45), as shown in murine models of lupus.

In summary, we provide evidence that in an ex vivo setting Tregs from both healthy subjects and AIH patients act mainly through a cell-to-cell contact by decreasing the production of the Th1 cytokine IFN-γ and by enhancing that of the Th2/Th3 regulatory cytokines. However, in AIH, Tregs not only are numerically defective, as shown previously (21, 22), but are unable to enhance TGF-β production by CD4+ T cells to the same extent observed in health.

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References


