Increased apoptosis induction in CD4+CD25+ Foxp3+ T cells contributes to enhanced disease activity in patients with rheumatoid arthritis through IL-10 regulation

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Abstract. – BACKGROUND: Abnormality in the number and function of regulatory T cells (Tregs) has been linked to initiation and progression in patients with Rheumatoid Arthritis (RA).
AIM: This study aims to demonstrate the apoptosis status of regulatory T cells (Tregs) and its correlation with clinical activity of RA patients and the effect of interleukin-10 (IL-10) on Tregs apoptosis in RA.
MATERIALS AND METHODS: Apoptosis rates and its related molecules including Fas, Bcl-2, Caspase-3 and Caspase-8 were examined using flow cytometry. The correlation between the apoptosis level of Tregs and clinical activity parameters including ESR (erythrocyte sedimentation rate), CPR (C reactive protein), RF (Rheumatoid Factor) and DAS28 (Disease activity score 28) was analysed. PBMCs isolated from RA patients were cultured with IL-10 or anti-IL-10, and the apoptosis frequency of Tregs was then analyzed.
RESULTS: The frequency of Tregs in active RA patients was decreased, and Fas, Caspase-3 and Caspase-8 expression on Tregs was much higher compared with the healthy subjects. The expression of anti-apoptotic protein, Bcl-2 on Tregs did not display significant changes between active RA patients and healthy subjects. There was a significantly positive correlation between the levels of apoptosis rates and Caspase-3 expression in Tregs and DAS28 of active RA patients. The apoptosis rates of Tregs in RA patients decreased or increased, respectively, following treatment with IL-10 or anti-IL-10 antibody in vitro.
CONCLUSIONS: Apoptosis pathways are defective in Treg cells from RA patients with active disease. IL-10 treatment can modulate apoptosis in Tregs via extrinsic (type I) pathway, which may lead to restoration of the Tregs towards that of controlling autoimmune reaction in RA patients.

Introduction

In mice, CD4+CD25+Foxp3+ regulatory T cells (TREGs) play a pivotal role in preventing autoimmunity. Regulatory T cells are also present and functional in healthy humans. Quantitative and/or qualitative (functional) deficiencies of CD4+CD25+Foxp3+ Treg subset have been suggested to contribute to the development of autoimmune diseases. In recent years, Treg cell counts and function have also been examined in patients with rheumatoid arthritis (RA)\(^1\)\(^-\)\(^7\). Several studies have shown that numbers of Treg cells have diminished in the peripheral blood of patients with RA or those with other chronic rheumatic diseases, implying that defect of frequency and function of Tregs is the one of the main reasons of excessive immune reaction in RA patients. However, studies on Tregs have focused on their function and mechanisms of action, few have concentrated on the mechanisms involved in controlling their homeostasis.

Given the critical role of Tregs in suppressing autoimmunity, studying the regulation of Tregs apoptosis is important in understanding the mechanism of RA pathogenesis. The objective of this study was to investigate the frequency of Tregs apoptosis in active RA patients and effects of IL-10 on Tregs apoptosis. Our data showed that the apoptosis of Treg cells was significantly elevated and correlated with RA active index, DAS28. Interestingly, we found that IL-10 could modulate the apoptosis rate of Treg cells in the peripheral blood. Our data describ a novel role for IL-10 in RA Treg survival via downregulation of Treg apoptosis, providing a potential approach to treat patients RA via modulate the serum IL-10 level.
Materials and Methods

Patient Characteristics

Forty-five patients with active RA were recruited. All patients (30 females, 15 males; mean±SD age 46.32±13.31 years; disease duration 70.48±50.12 months) fulfilled the 1987 American College of Rheumatology criteria for RA44. The patients complicated with pregnancy, viral hepatitis, diabetes mellitus, active infections or other autoimmune diseases were excluded.

Complete clinical and laboratory evaluations were conducted for the patients. Age, sex, disease duration, the erythrocyte sedimentation rate (ESR), the C-reactive protein (CRP) and rheumatoid factor (RF) levels, the presence of radiographic bony erosions, and current medications were recorded. The study was approved by the Tongji University Ethics Committee, and written informed consent was given by all patients. Data were collected into a predesigned form.

Preparation of PBMCs

PBMCs were purified from peripheral blood by centrifugation, using a Ficoll-Hypaque gradient (Fresenius Kabi, Norway). PBMCs (peripheral blood mononuclear cells) were adjusted to a final concentration of 10^6/mL in RPMI (Roswell Park Memorial Institute) 1640 medium supplemented with 10% heat-inactivated FCS (Fetal Calf Serum), 1% glutamine/penicillin/streptomycin, and 2% HEPES. A quarter of the PBMCs was detected apoptosis related parameters using flow cytometry directly. The remaining cells were divided into joint counts, including tender joints and swollen joints, were recorded. Thirty age- and sex-matched healthy donors (19 females, 11 males; mean±SD age 42.12±10.15 years) were also enrolled as controls in the study.

Antibodies and Flow Cytometry

Antibodies to the following markers were employed in these studies: FITC (fluorescein isothiocyanate)-conjugated anti-Foxp3 and AnnexinV (all from BD Biosciences, San Jose, CA, USA), PE (phycoerythrin)-conjugated anti-CD25, CaspGLOW Red Caspase-3,8 and CD95 (Fas), Bcl2 (all from Invitrogen, Carlsbad, CA, USA); PerCP-Cy5.5-conjugated anti-CD4 (BD Biosciences), and appropriate fluorochrome-labeled control IgG from BD Biosciences. For whole blood staining, a mixture of antibodies against surface markers was placed into 12x75 mm polystyrene tubes (BD Biosciences). 50 µl of whole blood were then added and the mixture was incubated for 15 min at room temperature. 1-2 ml of FACSlyse (BD Biosciences) was added and lysis allowed to proceed for 10 min at room temperature. The tubes were filled to the top with calcium- and magnesium-free phosphate buffered saline (PBS) containing 2% FBS (fetal bovine serum) (stain buffer) and centrifuged to collect the cells. Following centrifugation, if necessary, intracellular staining was performed according to instructions provided by the manufacturer of the antibody. Purified PBMC were stained according to a similar protocol, except that the antibody mixture was added to cells suspended in 50 µl of stain buffer. Data were collected on a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA) and analyzed using CellQuest software.

Stimulation of PBMCs with IL-10 or anti-IL-10

1×10^6/mL PBMCs were added in 24-well, U bottom plates and treated with IL-10 as 0 ng/ml (blank), 20 ng/mL, 40 ng/mL and 80 ng/mL, or anti-IL-10 as 0 µg/mL (blank), 20 µg/mL, 40 µg/mL and 80 µg/mL, respectively. The cells were cultured for 6h, 12h, 18h and 24h, respectively, then were collected at each time and stained as described above. The experiments were repeated three times, and the average was calculated. Then we got the optimal treatment time and concentration of IL-10 was 24h and 20 ng/mL, those of anti-IL-10 were 24h and 20 µg/mL, respectively, which were used in the following experiments.

1×10^6/mL PBMCs were added to the wells of a 24-well plate alone (blank) or together with IL-10 (20 ng/mL, IL-10 group) or anti-IL-10 (20 µg/mL, anti-IL-10 group) (Peprotech Inc., Rocky, IL, USA). Plates were placed in the incubator for 24h before harvesting, staining, and analysis using the FACSCalibur and CellQuest software as described above.

Statistical Analysis

The results for the different groups were analyzed by using one-way analysis of variance (ANOVA), followed by the Bonferroni multiple-comparison test. To compare the effects of the different treatments in each group, we used the ANOVA test for repeated measures or a paired
Table I. The characteristics of RA patients enrolled in the study.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cases</th>
<th>Age (year)</th>
<th>Gender (F/M)</th>
<th>Duration (month)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA</td>
<td>45</td>
<td>46.32 ± 13.31</td>
<td>30/15</td>
<td>70.48 ± 50.12</td>
</tr>
<tr>
<td>Controls</td>
<td>30</td>
<td>42.12 ± 10.15</td>
<td>19/11</td>
<td>—</td>
</tr>
</tbody>
</table>

Student’s t test. To calculate the correlation between apoptosis items and RA activity, we used the Pearson test. A \( p \) value of 0.05 was considered statistically significant and the \( p \) value is two-sided. All statistical analyses were carried out with SPSS17.0 (SPSS Inc., Chicago, IL, USA). This study is a cross sectional study. The apoptosis rate in our study is showed by the rate of Annexin V⁺ cell to all gated cells (Annexin V⁺/Total cells). \( p < 0.05 \) was considered statistically significant.

Results

Clinical and Demographic Characteristics

The characteristics of RA patients enrolled in the study are shown in Table I. All patients were Han people and had active RA at study entry, with the mean DAS-28 (Disease Activity Score in 28 joints) 5.39 (5.39± 0.78, range 3.89-8.21).

Frequencies of Circulating Treg cells in Patients with RA and Health Controls

PBMCs were separated from PB (peripheral blood) of active RA patients and healthy controls, and then the surface marker of CD4⁺CD25⁺Foxp3⁺ cells (Treg cells) were detected using flow cytometry. Frequency of Tregs was expressed as percentages of Tregs among PBMCs. Although there were controversial results about Tregs frequency in RA patients, our study showed that the frequency of Tregs in PB was significant lower in active RA group than in healthy controls (\( p < 0.05 \)). Figure 1 showed one of 3 representative results of flow cytometry analysis and the mean of all experiments.
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Changes in Mediators of Apoptosis in Treg cells of Active RA Patients

There are two possibilities to explain the reduction of Tregs in PB. One is the Tregs have increased apoptosis, and another is Tregs migrate to joints or other lymph organs from PB. To address these possibilities, we first studied the apoptosis of Tregs in active RA patients. Apoptosis- and survival-related molecules expressed by Tregs have been detected by flow cytometry. As shown in Figure 2, the expressions of Annexin-V, caspase3, caspase8 and Fas in Tregs, which are all pro-apoptotic molecules, were markedly elevated in active RA patients than in healthy subjects \((p < 0.05)\). However, the expression of Bcl-2, an anti-apoptotic molecule, was not significantly different between the two groups (data not shown).

Increased Apoptosis of Tregs was Positively Correlated with Disease Activity

We further determined whether increased apoptosis of Tregs may correlate with the disease status of RA. To address this problem, we have analyzed the expression of apoptosis parameters in Tregs and the levels of serum CRP, ESR or RF. As shown in Table II, there was no correlation between the apoptosis of Tregs and CRP, ESR or RF \((p > 0.05)\). To our interest, there was a statistically positive correlation between Annexin-V and caspase3 expression of Tregs with the marker of disease activity-DAS28 \((r = 0.82, p < 0.01\) and \(r = 0.79, p < 0.05\), respectively; Figure 3), indicating that apoptosis status of Tregs is likely associated with disease activity in RA patients. However, there were no significant correlations of DAS28 with caspase8, Fas and Bcl-2 expression levels in Tregs \((p > 0.05)\).

Role of IL-10 in the Apoptosis of Tregs from Patients with Active RA

As demonstrated above, the apoptosis of Treg from RA patients with active disease is increased and positively associated with disease activity. To investigate whether the decreased frequency of Tregs was induced by suppressive cytokines such as IL-10, we examined the level of serum IL-10 in peripheral blood of RA patients and health control. As shown in Figure 4a, the level of serum IL-10 was significantly higher in active RA group than in healthy controls. Next, we ex-

![Figure 2](image-url). Increased expression of caspase 3, Fas, and caspase 8 molecules on CD4+CD25+Foxp3+ Treg cells from the peripheral blood (PB) obtained from (RA) patients with rheumatoid arthritis.
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explored the effect of IL-10 on Treg apoptosis using exogenous IL-10. PBMCs separated from active RA patients or healthy controls were cultured with 20 ng/mL, 40 ng/mL and 80 ng/mL of IL-10 and 1640 medium *in vitro* for 24 h at 37°C with 5% CO₂. As shown in Figure 4b, the addition of exogenous IL-10 (20 ng/mL) inhibited Tregs apoptosis in active RA patients by approximately 10%. In contrast, the addition of 40 ng/mL of IL-10 was able to reverse the Tregs survival (Figure 4b).

Next we examined the effects of IL-10 on the apoptosis-related molecules. As seen in Figure 4c, the addition of exogenous IL-10 (20 ng/mL) elevated the frequency of Tregs and reduced the expression of Annexin V, caspase 3 and caspase 8. But, the addition of 40 ng/mL and 80 ng/mL IL-10 reverse the effect of 20 ng/mL IL-10 (data not shown). There was no statistical significance in terms of Fas and Bcl-2 expression after treatment of medium and IL-10 group (*p > 0.05*) (data not shown).

### Discussion

Tregs are considered an important therapeutic target for a large range of human immune mediated disease, and ongoing clinical trials attempt to modulate the population of Tregs, and, thereby, restore immune balance. Among the different types of CD4⁺ Treg cells that have been defined, one particular subset characterized by high expression of the interleukin-2 (IL-2) α-chain receptor (CD25) and of the transcription factor X-linked forhead/winged helix (Foxp3) has received much attention⁶-⁸ and will be our focus. The potent inhibitory activity of Tregs has been observed in various disease models including RA⁹. However, the relative numbers of CD4⁺CD25⁺ T cells in PB of patients with RA remain controversial. Some experiments have confirmed that the number of Tregs was reduced in RA patients and could be one of the reasons for excessive autoimmunologic reactions in RA. Decrease of peripheral Treg cells have been found

### Table II. The analysis of the correlation between apoptosis of Treg and RA activity.

<table>
<thead>
<tr>
<th>Apoptosis parameters (%)</th>
<th>ESR</th>
<th>CRP</th>
<th>RF</th>
<th>DAS28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annexin V/Treg</td>
<td>0.58</td>
<td>0.34</td>
<td>0.03</td>
<td>0.82</td>
</tr>
<tr>
<td>Caspase3/Treg</td>
<td>0.65</td>
<td>0.69</td>
<td>0.12</td>
<td>0.79</td>
</tr>
<tr>
<td>Caspase8/Treg</td>
<td>0.62</td>
<td>0.67</td>
<td>0.12</td>
<td>0.84</td>
</tr>
<tr>
<td>Fas/Treg</td>
<td>0.65</td>
<td>0.10</td>
<td>0.80</td>
<td>0.16</td>
</tr>
<tr>
<td>Bcl-2/Treg</td>
<td>0.20</td>
<td>0.48</td>
<td>−0.18</td>
<td>0.19</td>
</tr>
</tbody>
</table>

*ᵣ = correlation coefficient of apoptosis parameters (Caspase3, Caspase8, Fas, Bcl-2) in Treg cells to RA activity items (ESR, CRP, RF, DAS28); *ᵣ < 0.05; ᵣ < 0.01.

Figure 3. Correlation of Annexin V⁺ Tregs and caspase3⁺ Tregs in peripheral blood with DAS28 score. In patients with RA.
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Associated with active RA and shown to correlate inversely with disease activity. Data showing that Treg cells from patients with RA are functionally defective, and that after infliximab therapy this defect is reversed, have emphasized the potential benefits of biological therapy. A hypothesis for Tregs reduction is abnormal apoptosis. Apoptosis is a tightly regulated process that plays an important role in T cell homeostasis and immune response. During activation, T cell undergo apoptosis triggered via T cell receptor-mediated activation of the Fas/Fas ligand death pathway. Fas ligand (Fas-L) and TNF-a are classic initiators of the Type I pathway, resulting in the activation of caspase 8, which then activates the effector caspase 3 and 7. Several reviews have proposed that Fas-mediated apoptosis of RA synoviocytes is also important for an accurate understanding of the clinical course and pathogenesis of RA.

Conflicting data on the sensitivity of naive CD4+CD25+ Treg cells to apoptosis have been reported, ranging from resistance to Fas signaling to extreme sensitivity to FasL-triggered death. Several caspases are key effector molecules in apoptosis, forming the apoptosome, with caspase 3 functioning as an executioner caspase, and caspase 8 is the principal initiator caspase in the TNF family receptor apoptosis pathway while caspase 9 is the main initiator caspase in the intrinsic mitochondrial pathway. There are a considerable number of activating and inhibitory proteins that ultimately determine the activation status of caspases and determine whether the apoptosis pathways are active in a particular cell. As reported, IL-10 can function as a potent immunoregulating cytokine to regulate inflammatory and autoimmune responses. Several studies have shown that IL-10 can enhance the function of CD4+ Treg cells and suppress organ-specific autoimmune diseases.
IL-10 have different regulation to different cell apoptosis since it can induce mononuclear cell and hepatic stellate cell apoptosis by up-regulating the Fas/Fas-L and down-regulating Bcl-2/Bax mRNA expression but prevents cerebellar granule cell death by blocking caspase 3 like activity. However, few researches have been reported about IL-10 contribution to Tregs apoptosis and the mechanisms by which IL-10 promotes apoptosis have not been clearly identified.

IL-10 is a crucial cytokine involved in T cell survival and development but its signalling in human T cells, particularly in Treg cells, is poorly documented. Our study have investigated the function of IL-10 in peripheral Tregs apoptosis. We found that the frequency of peripheral CD4+CD25+Foxp3+ cells in patients with active RA is significantly lower compared with healthy individuals. At the same time, the expression of annexin V, caspase 3 and caspase 8 were elevated in Tregs from active RA patients than that from healthy controls. In addition, the annexin V and caspase 3 had positive correlation with DAS 28. As reported, IL-10 could regulate the apoptosis of Tregs in mice, so examined the level of serum IL-10 in peripheral blood with RA. We found the level of serum IL-10 was significantly higher in active RA group than in healthy controls. But the addition of exogenous IL-10 (20 ng/mL) inhibited Tregs apoptosis in active RA patients by approximately 10% in vitro. In contrast, the addition of 40 ng/mL of IL-10 was able to reverse the Tregs survival (Figure 4b).

**Conclusions**

Our findings suggest that one plausible explanation for the function of IL-10 in Tregs survival in the patients with RA might be a balance between promote or inhibit the survival of Tregs and affect the disease.

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**Conflict of Interest**

The Authors declare that there are no conflicts of interest.

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