European Review for Medical and Pharmacological Sciences 2014; 18: 78-85

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

N. LI, T. MA, J. HAN, J. ZHOU, J. WANG, J. ZHANG, S. ZHENG#

Department of Rheumatology, Tong Ji University Affiliated Shanghai East Hospital, Shanghai, China
#Division of Rheumatology and Immunology, Department of Medicine, Keck School of Medicine of the University of Southern California, Los Angeles, CA, USA

Ning Li and *Tianrui Ma* are co-first Author

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.

Key Words:
Treg cells, Rheumatoid arthritis, apoptosis, Interleukin 10, Fas, Caspase, Bcl-2.

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. Interstingly, 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 dawnregulation of Treg apoptosis, providing a potential approach to treat patients RA via modulate the serum IL-10 level.

Corresponding Author: Jie Han, MD; e-mail: hjgj85528@sina.com

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 RA[44]. 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. Standardized 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.

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 blank group, IL-10 group and anti-IL-10 group, respectively. After stimulation *in vitro* for 24 hours, the cells were collected again and detected using flow cytometry.

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 12×75 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.

Groups	Cases	Age (year)	Gender (F/M)	Duration (month)
RA	45	46.32 ± 13.31	30/15	70.48 ± 50.12
Controls	30	42.12 ± 10.15	19/11	—

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 amomg 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.

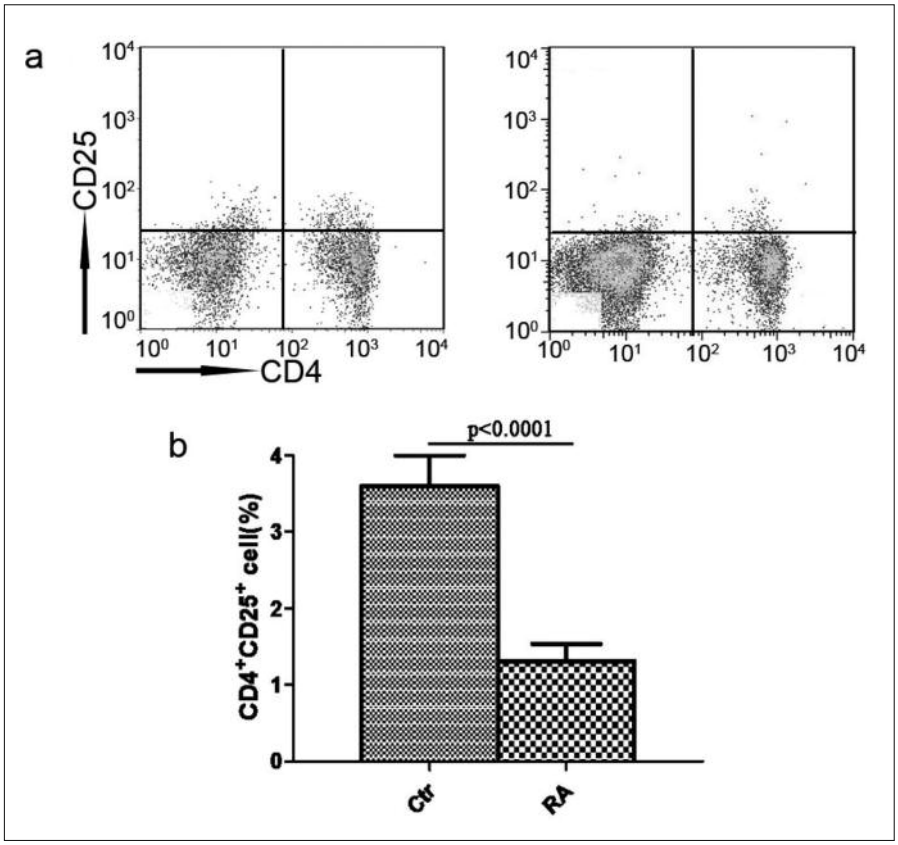

Figure 1. Frequencies of circulating Tregs in patients with RA.

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 no 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 acivity-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-

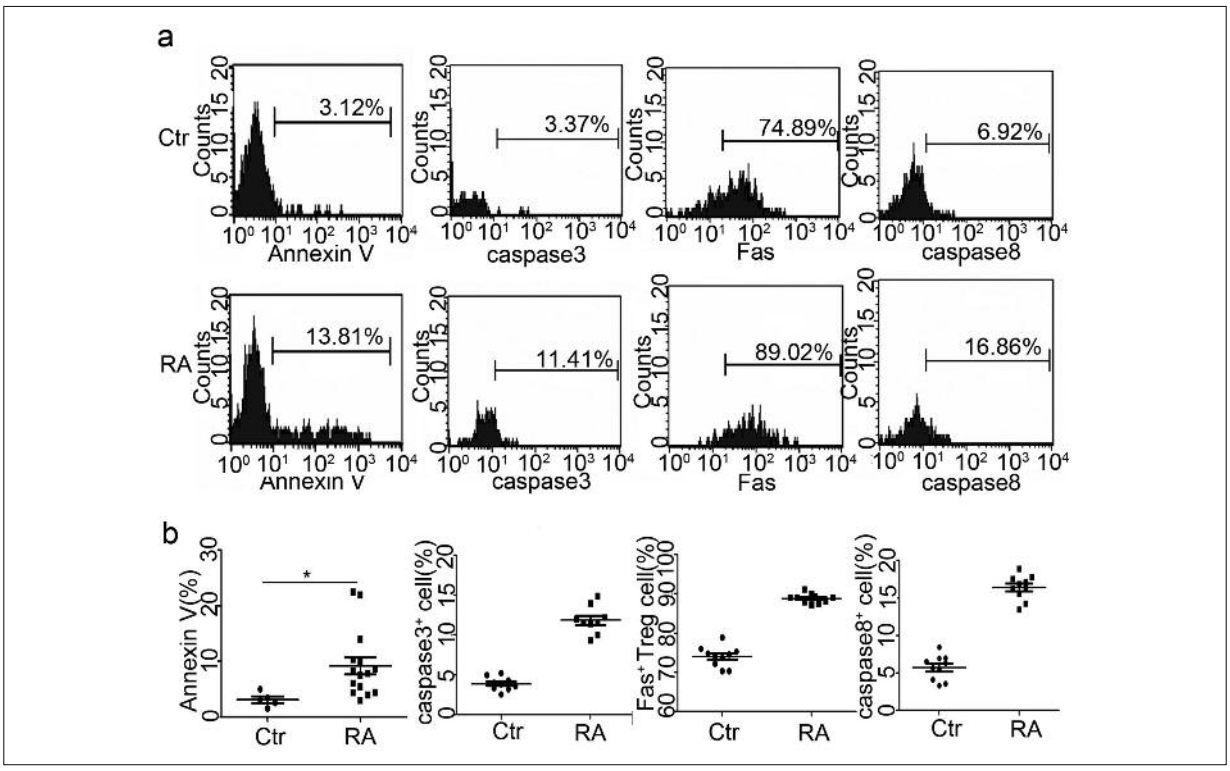

Figure 2. Increased expression of caspase 3, Fas, and caspase 8 molecules on CD4+CD25+Foxp3+ Treg cells from the peripheral blood (PB) obtained from (RA) patients with rheumatoid arthritis.

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

Apoptosis parameters (%)	ESR		CRP		RF		DAS28	
	r	p	r	p	r	p	r	p
AnnexinV/Treg	0.58	0.17	0.34	0.46	0.03	0.95	0.82	0.00[b]
Caspase3/Treg	0.65	0.11	0.69	0.09	0.12	0.80	0.79	0.03[a]
Caspase8/Treg	0.62	0.91	0.67	0.15	0.12	0.83	0.84	0.06
Fas/Treg	0.65	0.17	0.10	0.86	0.80	0.06	0.16	0.76
Bcl-2/Treg	0.20	0.71	0.48	0.33	–0.18	0.74	–0.19	0.71

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

plored 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 24h at 37°C with 5% CO_2. 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 experssion of the interleukin-2 (IL-2) α-chain receptor (CD25) and of the transcription factor X-linked forkhead/winged helix (Foxp3) has received much attention[6-8] and will be our focus. The potent inhibitory activity of Tregs has been observed in various disease models including RA[9]. However, the relative numbers of $CD4^+CD25^+$ T cells in PB of patients with RA remain controversial. Some experments 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

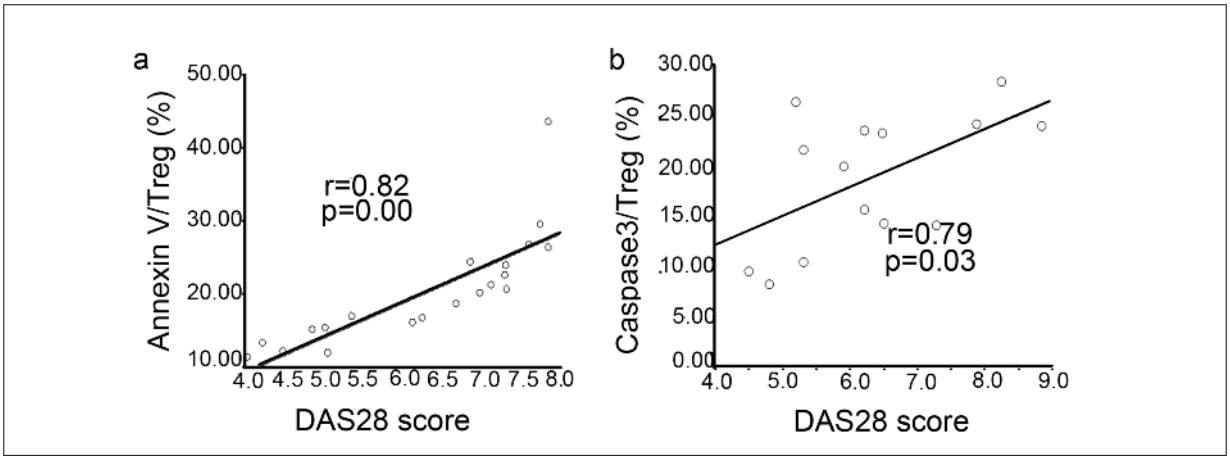

Figure 3. Correlation of Annexin V+ Tregs and caspase3+ Tregs in peripheral blood with DAS28 score. In patients with RA.

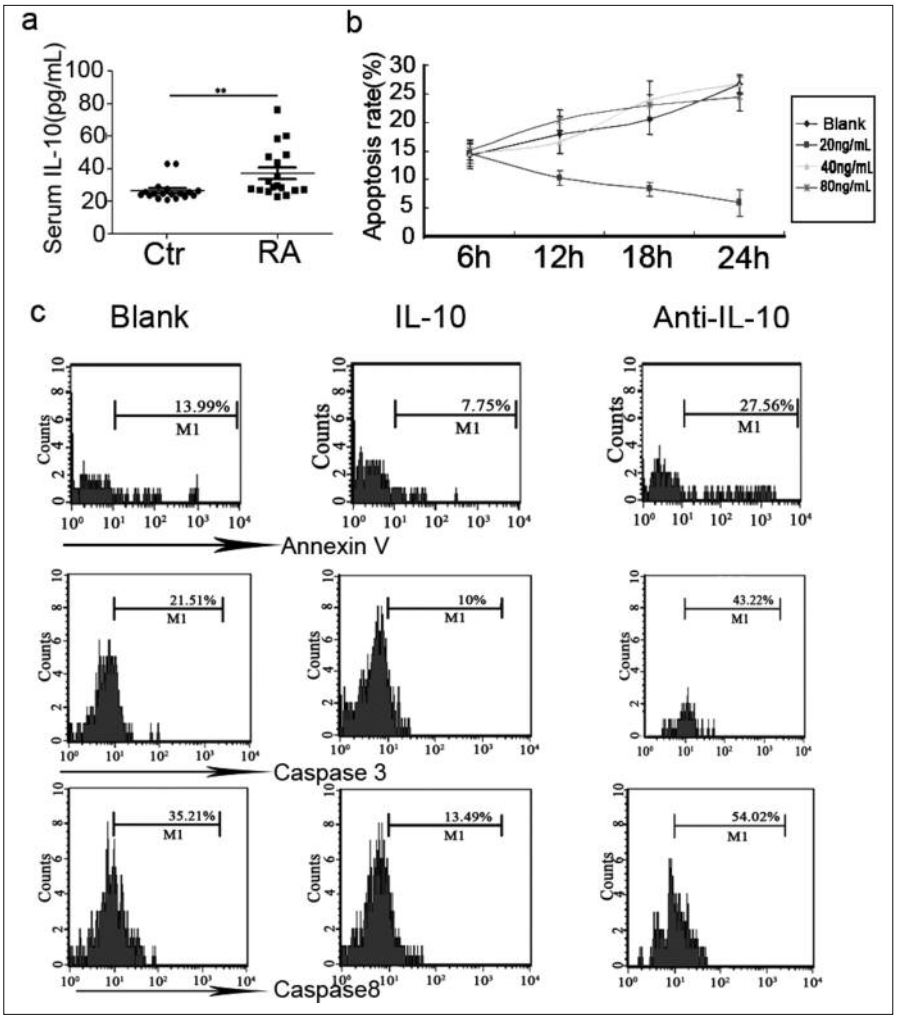

Figure 4. Regulation of IL-10 to the apoptosis of Treg cells in RA patients.

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[10].

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[11,12]. 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[13-16]. Several caspases are key effector molecules in apoptosis, forming the apoptosome, with caspase 3 functioning as an executioner caspase[17], 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 wether the apoptosis pathways are active in a particular cell[18].

As reported, IL-10 can function as a potent immunoregulating cytokine to regulate inflammatory and autoimmune responses[19]. Several studies have shown that IL-10 can enhance the function of $CD4^+$ Treg cells[20,21] and suppress organ-specific autoimmune diseases[22].

IL-10 have different regulation to different cell apoptosis since it can induce mononuclear cell and hepatic stellate cell apoptosis[23,24] 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[25]. 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 regulated 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 mighe be a balance between promote or inhibit the survival of Tregs and affect the disease.

Acknowledgements

This work was partly funded by Research Grant for Health Science and Technology development and innovation of Pudong New Area of Shanghai (Grant No. PKJ2011-Y06).

Conflict of Interest

The Authors declare that there are no conflicts of interest.

References

1) Askenasy N, Kaminitz A, Yarkoni S. Mechanisms of T regulatory cell function. Autoimmun Rev 2008; 7: 370-375.
2) Zheng SG, Wang J, Horwitz DA. Cutting edge: Foxp3+CD4+CD25+ regulatory T cells induced by IL-2 and TGF-beta are resistant to Th17 conversion by IL-6. J Immunol 2008; 180: 7112-7116.
3) Wang CR, Liu MF. Regulation of CCR5 expression and MIP-1alpha production in CD4+ T cells from patients with rheumatoid arthritis. Clin Exp Immunol 2003; 132: 371-378.
4) Furuzawa-Carballeda J, Vargas-Rojas MI, Cabral AR. Autoimmune inflammation from the Th17 perspective. Autoimmun Rev 2007; 6: 169-175.
5) Shahrara S, Huang Q, Mandelin AM, Pope RM. TH-17 cells in rheumatoid arthritis. Arthritis Res Ther 2008; 10: R93.
6) Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003; 299: 1057-1061.
7) Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol 2003; 4: 330-336.
8) Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol 1995; 155: 1151-1164.
9) Hu G, Liu Z, Zheng C, Zheng SG. Antigen-non-specific regulation centered on CD25+Foxp3+ Treg cells. Cell Mol Immunol 2010; 7: 414-418.
10) Ehrenstein MR, Evans JG, Singh A, Moore S, Warnes G, Isenberg DA, Mauri C. Compromized function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFalpha therapy. J. Exp. Med. 2004; 200: 277-285.
11) Krammer PH, Arnold R, Lavrik IN. Life and death in peripheral T cells. Nat. Rev. Immunol 2007; 7: 532-542.
12) Strasser A, Pellegrini M. T-lymphocyte death during shutdown of an immune response. Trends Immunol 2004; 25: 610-615.
13) Banz A, Pontoux C, Papiernik M. Modulation of Fas-dependent apoptosis: a dynamic process controlling both the persistence and death of CD4 regulatory T cells and effector T cells. J Immunol 2002; 169: 750-757.
14) Fisson S, Darrasse-Jeze G, Litvinova E, Septier F, Klatzmann D, Liblau R, Salomon BL. Continuous activation of autoreactive CD4+ CD25+ regulatory T cells in the steady state. J Exp Med 2003; 198: 737-746.
15) Fritzsching B, Oberle N, Eberhardt N, Quick S, Haas J, Wildemann B, Krammer PH, Suri-Payer E. In

contrast to effector T cells, CD4+CD25+FoxP3+ regulatory T cells are highly susceptible to CD95 ligand- but not to TCR-mediated cell death. J Immunol 2005; 175: 32-36.

16) Fritzsching B, Oberle N, Pauly E, Geffers R, Buer J, Pschl J, Krammer P, Linderkamp O, Suri-Payer E. Naive regulatory T cells: a novel subpopulation defined by resistance toward CD95L-mediated cell death. Blood 2006; 108: 3371-3378.

17) Riedl SJ, Salvesen GS. The apoptosome: signalling platform of cell death. Nat Rev Mol Cell Biol 2007; 8: 405-413.

18) Smith MD, Walker JG. Apoptosis a relevant therapeutic target in rheumatoid arthritis? Rheumatology (Oxford) 2004; 43: 405-407.

19) Moore KW, de Waal Malefyt R, Coffman RL, O'-Garra A. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol 2001; 19: 683-765.

20) Annacker O, Pimenta-Araujo R, Burlen-Defranoux O, Barbosa TC, Cumano A, Bandeira A. CD25+ CD4+ T cells regulate the expansion of peripheral CD4 T cells through the production of IL-10. J Immunol 2001; 166: 3008-3018.

21) Hara M, Kingsley CI, Niimi M, Read S, Turvey SE, Bushell AR, Morris PJ, Powrie F, Wood KJ. IL-10 is required for regulatory T cells to mediate tolerance to alloantigens in vivo. J Immunol 2001; 166: 3789-3796.

22) von Herrath MG, Harrison LC. Antigen-induced regulatory T cells in autoimmunity. Nat Rev Immunol 2003; 3: 223-232.

23) Hosaka N, Oyaizu N, Kaplan MH, Yagita H, Pahwa S. Membrane and soluble forms of Fas (CD95) and Fas ligand in peripheral blood mononuclear cells and in plasma from human immunodeficiency virus-infected persons. J Infect Dis 1998; 178: 1030-1039.

24) Wang XZ, Zhang SJ, Chen YX, Chen ZX, Huang YH, Zhang LJ. Effects of platelet-derived growth factor and interleukin-10 on Fas/Fas-ligand and Bcl-2/Bax mRNA expression in rat hepatic stellate cells in vitro. World J Gastroenterol 2004; 10: 2706-2710.

25) Bachis A, Colangelo AM, Vicini S, Doe PP, De Bernardi MA, Brooker G, Mocchetti I. Interleukin-10 prevents glutamate-mediated cerebellar granule cell death by blocking caspase-3-like activity. J Neurosci 2001; 21: 3104-3112.