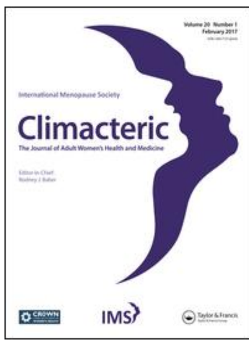

Climacteric

ISSN: 1369-7137 (Print) 1473-0804 (Online) Journal homepage: https://www.tandfonline.com/loi/icmt20

Evaluation of $CD4^+CD25^+FOXP3^+$ regulatory T cells and FOXP3 mRNA in premature ovarian insufficiency

J. Xiong, R. Tan, W. Wang, H. Wang, D. Pu & J. Wu

To cite this article: J. Xiong, R. Tan, W. Wang, H. Wang, D. Pu & J. Wu (2020): Evaluation of $CD4^+CD25^+FOXP3^+$ regulatory T cells and FOXP3 mRNA in premature ovarian insufficiency, Climacteric, DOI: 10.1080/13697137.2019.1703938

To link to this article: https://doi.org/10.1080/13697137.2019.1703938

Published online: 15 Jan 2020.

Submit your article to this journal

Article views: 3

View related articles

View Crossmark data

Full Terms & Conditions of access and use can be found at
https://www.tandfonline.com/action/journalInformation?journalCode=icmt20

CLIMACTERIC
https://doi.org/10.1080/13697137.2019.1703938

Taylor & Francis
Taylor & Francis Group

Check for updates

ORIGINAL ARTICLE

Evaluation of $CD4^{+}CD25^{+}FOXP3^{+}$ regulatory T cells and FOXP3 mRNA in premature ovarian insufficiency

J. Xiong, R. Tan, W. Wang, H. Wang, D. Pu and J. Wu

State Key Laboratory of Reproductive Medicine, Department of Obstetrics and Gynecology, The First Affiliated Hospital of Nanjing Medical University, Nanjing, China

ABSTRACT

Objective: T cell-mediated injury plays an important role in the pathogenesis of autoimmune premature ovarian insufficiency (POI). The purpose of this study was to assess the percentage of $CD4^{+}CD25^{+}FOXP3^{+}$ regulatory T (Treg) cells and the level of forkhead box protein 3 (FOXP3) mRNA expression in POI patients.

Methods: The case–control study compared 30 POI patients with 30 healthy subjects. Peripheral blood mononuclear cells were collected. The percentage of $CD4^{+}CD25^{+}FOXP3^{+}$ Treg cells was measured by flow cytometry using specific monoclonal antibodies recognizing the $CD4^{+}$, $CD25^{+}$, and $FOXP3^{+}$ markers. FOXP3 gene expression was evaluated by real-time polymerase chain reaction. In addition, the levels of transforming growth factor-β1 (TGF-β1), interferon-γ (IFN-γ), and adrenal cortex autoantibody (AAA) were determined by enzyme-linked immunosorbent assay.

Results: The percentage of $CD4^{+}CD25^{+}FOXP3^{+}$ Treg cells and the level of FOXP3 mRNA expression were significantly decreased in the POI patients compared with the control subjects. Moreover, the women with POI showed significantly increased levels of IFN-γ and AAA but reduced levels of TGF-β1.

Conclusions: Our study suggested that POI may be associated with an abrogated function of circulating $CD4^{+}CD25^{+}FOXP3^{+}$ Treg cells and a decreased level of FOXP3 gene expression. However, these results require further investigation.

ARTICLE HISTORY
Received 16 September 2019
Revised 31 October 2019
Accepted 27 November 2019
Published online 15 January 2020

KEYWORDS
Premature ovarian insufficiency; regulatory T cells; FOXP3 gene; transforming growth factor-β1; interferon-γ; adrenal cortex autoantibody

Introduction

Premature ovarian insufficiency (POI) is clinically defined as 4–6 months of amenorrhea with elevated gonadotropins and low estrogen levels, and POI affects approximately 1% of women before the age of 40 years. It presents a highly heterogeneous clinical phenotype and etiology. Genetic, iatrogenic, and autoimmune factors are potential causes of POI[1]. Autoimmunity is responsible for approximately 4–30% of POI cases. Accumulative evidence indicates that POI is associated with alterations in cellular immunity and humoral immunity. These alterations may include abnormal T and B lymphocyte activation[2], disruptions in cytokines[3], and the production of various autoantibodies, such as adrenal cortex autoantibody (AAA)[4]. The prevalence of associated clinical autoimmune diseases in POI patients varies between 10 and 55%, with thyroid disorders representing the most common of these diseases, being detected in 12–40% of patients[5]. The histopathological analysis of ovaries from women with POI reveals the infiltration of $CD4^{+}$ and $CD8^{+}$ T lymphocytes and plasma cells into the theca interna and externa layers of the follicle and within the corpus luteum, and, occasionally, the granulosa cell layer is also involved[6]. However, the contribution of autoimmune deregulation to the pathogenesis of ovarian follicle depletion and dysfunction remains elusive.

Studies have elucidated the role of the regulatory T (Treg) cell and transcription factor forkhead box protein 3 (FOXP3) in countless autoimmune diseases, such as autoimmune thyroiditis, type I diabetes mellitus, multiple sclerosis, myasthenia gravis, systemic lupus erythematosus, and X-linked (IPEX) syndrome[7–9]. FOXP3 is an essential molecular marker of Treg cells[10] and is functionally important for Treg cell function because transgenic FOXP3 expression endows T cells with immune suppressive activity[11,12]. In addition, FOXP3 deletion leads to the aberrant activation of immune function and systemic autoimmunity[13].

Little is known about the possible role of Treg cells in POI. Experimental studies in animals have shown that day-3 thymectomized mice develop autoimmune oophoritis characterized by the rapid loss of oocytes with the infiltration of lymphocytes and circulating anti-oocyte antibodies[14], and that polyclonal Treg cells from antigen-positive donors suppress ovarian autoimmune disease[15]. Recently, Kobayashi *et al.*[16] found that the number of $CD4^{+}$ T cells or $CD4^{+}CD69^{+}$ T cells was significantly higher and that the number of effector Treg cells was significantly lower in POI patients. However, the association between Treg cell subpopulations and POI has not been well studied. Moreover, FOXP3 gene expression in POI patients has not been determined. Therefore, the aim of our present study was to

CONTACT Jie Wu wujiemd@126.com State Key Laboratory of Reproductive Medicine, Department of Obstetrics and Gynecology, The First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, China

© 2020 International Menopause Society

examine the levels of $CD4^{+}CD25^{+}FOXP3^{+}$ Treg cells and FOXP3 mRNA in the peripheral blood of patients with POI.

Methods

Subjects

A total of 30 Chinese POI patients and 30 age-matched healthy women were recruited from the First Affiliated Hospital of Nanjing Medical University. Inclusion criteria were the cessation of secondary menses for at least 4 months before the age of 40 years, together with follicle stimulating hormone (FSH) plasma levels >25 IU/ml on two occasions at least 1 month apart. Control individuals were younger than 40 years of age with normal menstrual cycles, proven fertility, and normal FSH levels and without a history of subfertility treatment or autoimmune disease. Women with chromosomal abnormalities or a clinical record of pelvic surgery, radiotherapy, or chemotherapy were excluded. All POI patients were prescribed with hormone replacement therapy (HRT) (2 mg 17β-estradiol daily, sequentially combined in one tablet with 10 mg dydrogesterone for 14 days per 28-day cycle). After 3 months of treatment, the peripheral blood of POI patients was collected on the 2nd to 5th day of bleeding. This study was approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University. All participants signed informed written consent forms.

Flow cytometry analysis of Treg cells

To determine the percentage of $CD4^{+}CD25^{+}FOXP3^{+}$ Treg cell populations in the peripheral blood, flow cytometry was performed using anti-human CD4, CD25, and FOXP3 monoclonal antibodies. Erythrocytes were lysed in ammonium chloride buffer, and the remaining living cells were washed three times and resuspended in phosphate-buffered saline. Cells were incubated with normal rat serum for 30 min at room temperature and then in a mixture of anti-human CD4 fluorescein isothiocyanate and anti-human CD25 allophycocyanin (eBioscience, Thermo Fisher Scientific, USA) at 4 °C for 30 min in the dark. After the cells were permeabilized, anti-human FOXP3 phycoerythrin (eBioscience, Thermo Fisher Scientific, USA) was added to the cell suspension, and the cells were analyzed using BD FACSVerse (BD Biosciences).

Quantitative RT-PCR analysis of FOXP3 mRNA

Total RNA was extracted from cultured cells using TRIzol reagent (Takara, Shiga, Japan) according to the manufacturer's instructions. Complementary DNA was synthesized from 2 mg of total RNA using random primers and Superscript II Reverse Transcriptase (Takara) in 20-ml reaction volumes. Quantitative real-time polymerase chain reaction (PCR) was performed with an Applied Biosystems Step One real-time PCR system using QuantiTect SYBR Green PCR kits (Takara). Expression levels were normalized against U6 or glyceraldehyde 3-phosphate dehydrogenase expression. The $2^{-\Delta\Delta Ct}$ method was used to analyze relative mRNA expression. RNA samples were prepared in three independent experiments.

Evaluation of TGF-β1, IFN-γ, and AAA

Cytokines transforming growth factor-β1 (TGF-β1), interferon-γ (IFN-γ), and AAA in the serum were evaluated using specific enzyme-linked immunosorbent assay kits (Proteintech Group, Inc., USA) according to the manufacturer's instructions. Briefly, sera were diluted properly with the provided sample buffer, and 100-μl aliquots were assayed in a 96-well plate format. After incubation with diluted goat anti-human IgG (heavy and light chain)–horseradish peroxidase conjugate (1×), the antibodies were detected by the addition of chromogenic substrate (3,3′,5,5′-tetramentylbenzidine). The absorbance at 450 nm was measured using an automatic microplate reader (BioTek Synergy2, BioTek, USA). The measurement of each sample was repeated two times to obtain an average value. The intra-assay and inter-assay coefficients of variation were less than 10%.

Statistical analysis

Statistical analysis was performed using SPSS version 22.0 (IBM Corp., USA). The mean ± standard error and the median (interquartile range) were used to describe normal and non-normal distribution, respectively. The Shapiro–Wilk test was used to check the normal distribution of quantitative variables. The differences between the groups were determined by Student's t test or the Mann–Whitney test, where applicable. All results were considered statistically significant at $p < 0.05$.

Results

Clinical characteristics

Clinical characteristics of POI patients and control women are presented in Table 1. There were no statistical differences in age and body mass index between the two groups. The levels of serum FSH and luteinizing hormone in POI patients were significantly elevated (both $p < 0.01$). On the other hand, the level of 17β-estradiol did not significantly differ between POI patients and controls. In the present study, 20% of POI patients were positive for anti-thyroperoxidase antibodies (anti-TPO) and/or anti-thyroglobulin antibodies

Table 1. Baseline characteristics of POI patients and control subjects.

Variable	*POI* (N = 30)	*Control* (N = 30)	p-*Value*
Age (years)	26.50 ± 1.02	27.07 ± 1.04	0.70
BMI (kg/m^2)	21.17 ± 0.29	21.60 ± 0.26	0.28
FSH (IU/l)	71.26 ± 5.40	7.08 ± 0.10	0.00
LH (IU/l)	23.75 ± 1.80	4.71 ± 0.08	0.00
E2 (pmol/l)	195.48 ± 6.71	206.01 ± 6.62	0.27
TPOAb and/or TgAb	6 (20%)	0 (0%)	NA

Data are given as mean ± standard error or n (%). BMI, body mass index; E2, 17β-estradiol; FSH, follicle stimulating hormone; LH, luteinizing hormone; NA, not applicable; POI, premature ovarian insufficiency; TPOAb, anti-thyroperoxidase antibody; TgAb, anti-thyroglobulin antibody.

(anti-TG), while none of the controls were with positive anti-TPO and/or anti-TG.

Analysis of $CD4^+CD25^+FOXP3^+$ Treg cells

The gating strategies for the Treg cell subsets are shown in Figure 1(A–C). The positive staining of the CD4, CD25, and FOXP3 Treg cell subpopulations corresponded to the effector Treg levels. The median percentage (interquartile range) of effector Treg cells in $CD4^+$ cells in the peripheral blood of the POI patients and control women was 1.52 (0.96–2.06) and 3.91 (2.85–4.40), respectively, and this difference was statistically significant ($p < 0.001$, Figure 1(D)).

Analysis of the FOXP3 mRNA level

FOXP3 gene expression is closely related to the development and function of most Treg cells. Figure 2 shows that the expression of FOXP3 mRNA was significantly decreased in the POI patients compared with the controls ($p < 0.001$). The median percentage (interquartile range) was 0.45 (0.31–0.72) and 0.99 (0.66–1.32), respectively.

Evaluations of cytokines TGF-β1, IFN-γ, and AAA

The level of TGF-β1 was significantly decreased (15.03 ± 1.13 vs. 18.49 ± 1.00, $p < 0.05$), and the values for IFN-γ (23.41 [17.97–32.79] vs. 16.39 [9.85–23.71], $p < 0.001$) and AAA (62.68 ± 1.44 vs. 45.69 ± 1.93, $p < 0.001$) were significantly increased in the serum of the POI patients compared to the serum of the control subjects (Figure 3).

Discussion

The results of the present study showed a significant decrease in the frequency of $CD4^+CD25^+FOXP3^+$ Treg cells in the POI patients as well as reduced expression of FOXP3 mRNA, which may be associated with the insufficiency of the $CD4^+CD25^+$ Treg cell immune suppressive function in women with POI. These findings strongly suggest that POI is associated with the disruption of circulating Treg cells.

POI is a complex disorder that is associated with a highly heterogeneous clinical phenotype and etiology. Autoimmunity may be the pathogenic mechanism in 4–30% of POI cases, as evidenced by the presence of anti-ovarian autoantibodies, autoimmune lymphocytic oophoritis, or any other associated autoimmune disorders[17–19].

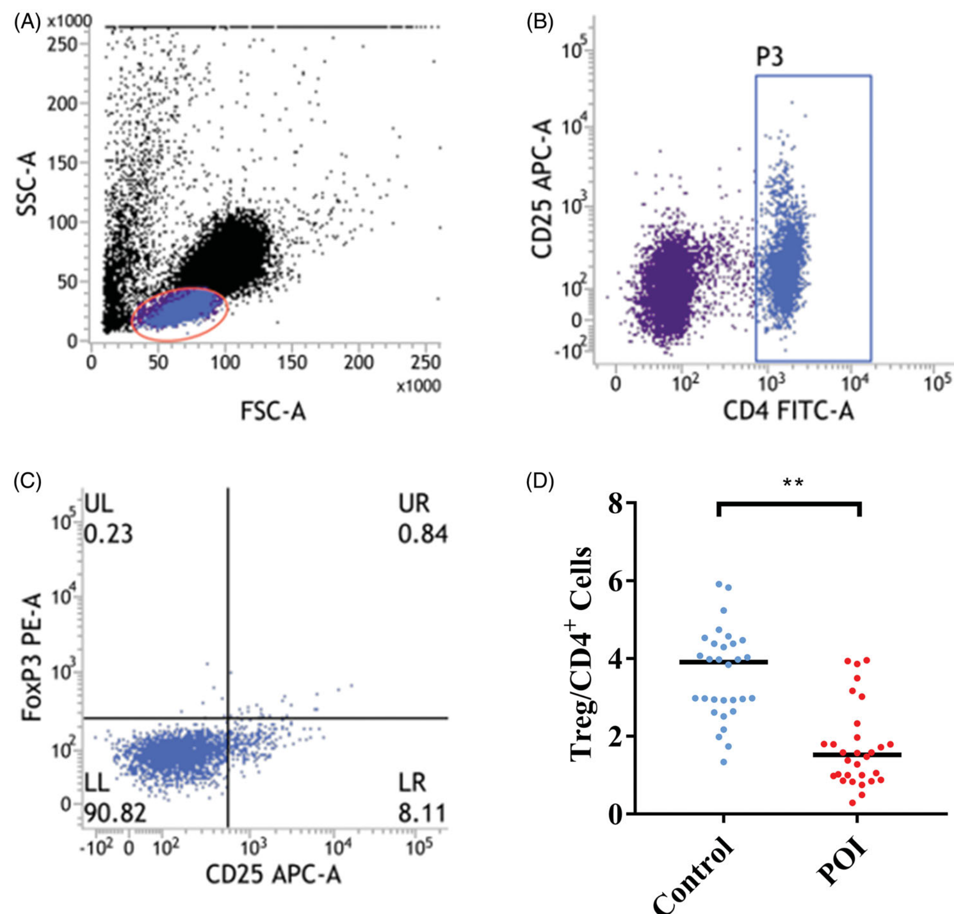

Figure 1. Gating strategies for T cell and regulatory T (Treg) cell subsets: (A) lymphocytes in the peripheral blood were gated according to forward-scatter (FSC) and side-scatter (SSC) parameters; (B) the $CD4^+$ cell subpopulation among the lymphocyte population was chosen; and (C) $CD4^+CD25^+FOXP3^+$ Treg cells were gated. APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin. (D) Analysis of the proportion of $CD4^+CD25^+FOXP3^+$ Treg cells in the peripheral blood of premature ovarian insufficiency (POI) patients and control subjects. Compared to that of the controls, the percentage of $CD25^+FOXP3^+$ Treg cells in the $CD4^+$ cell subpopulation was significantly decreased in the POI patients. The horizontal bars represent medians. $^{**}p < 0.001$.

Bats *et al.*[2] reported the observation of a dense infiltration of activated T lymphocytes in POI patients, which was very similar to findings in murine models of autoimmune oophoritis[14]. Some studies have been shown that POI patients have a low number of $CD8^+CD57^+$ T cells (cytotoxic T lymphocytes), an increased $CD4^+$ T cell count, and an increased $CD4^+/CD8^+$ ratio[20,21]. However, other studies have reported that the $CD4^+/CD8^+$ ratio in POI patients is unchanged[22] or decreased[23]. Although these findings are contradictory, the data support the important role of T cell-mediated injury in the pathogenesis of autoimmune POI. Treg cells are a specialized subset of T cells that induce the suppression of the immune response to pathogenic agents as well as non-infectious targets. In patients with IPEX syndrome, Treg dysfunction causes several autoimmune disorders, including POI[24]. The high expression of CD25 and CD4 surface markers has classically been used for the identification of Treg cells. A reduced number of $CD4^+CD25^{+high}$ Treg cells in the peripheral blood of POI patients has been revealed[25]. A recent study showed that the frequency of effector Treg cells among $CD4^+FOXP3^+$ T cells, but not the frequencies of naive Treg cells or $FOXP3^+$ effector T cells, was significantly lower in POI patients than in control subjects[16]. In agreement with the aforementioned studies, the present study confirmed a significant decrease in the percentage of $CD4^+CD25^+FOXP3^+$ Treg cells in POI patients.

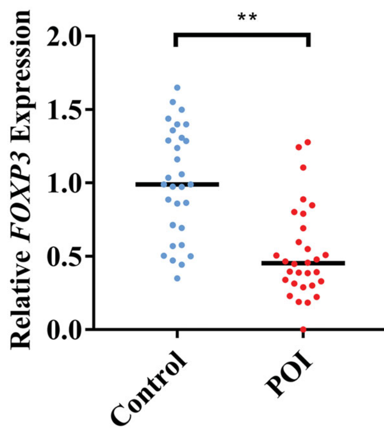

Figure 2. Analysis of FOXP3 mRNA levels in the peripheral blood of premature ovarian insufficiency (POI) patients and control subjects. The expression of FOXP3 mRNA significantly decreased in the POI patients compared with the controls. The horizontal bars represent medians. $^{**}p < 0.001$.

The FOXP3 gene is primarily expressed in $CD4^+CD25^+$ Treg cells in normal physiological conditions; FOXP3 regulates the activation of Treg cells and is an analytical biomarker of autoimmune activity. The double role of FOXP3 as a transcriptional repressor and activator has been demonstrated[10]. The literature indicates that polymorphisms in the FOXP3 gene may change FOXP3 functionally or quantitatively, therefore leading to the lack of functional $CD4^+CD25^+$ Treg cells, resulting in various autoimmune diseases[9], including recurrent idiopathic abortion[26] and endometriosis[27]. The results of the present study showed for the first time that the mRNA levels of the FOXP3 transcriptional factor are significantly decreased in the peripheral blood of POI patients, which may result in the insufficiency of the $CD4^+CD25^+$ Treg cell immune suppressive function.

The anti-inflammatory cytokine TGF-β induces the proliferation of Treg cells and the differentiation of initial T cells into Treg cells and maintains the expression level and immunosuppressive activity of FOXP3. TGF-β sustains regulatory networks through the modulation of FOXP3 expression and development of ectopic Treg cells[28]. In the case of an immunological abnormality, more pro-inflammatory cytokine IFN-γ molecules are secreted by type 1 T helper cells; however, Treg cells inhibit the proliferation of type 1 T helper cells, thereby inhibiting the production of IFN-γ and maintaining immune tolerance[29]. In the present study, the POI patients had lower levels of TGF-β1 than the controls and increased IFN-γ levels compared to those of the controls, which may be attributable to the decreased frequency of $CD4^+CD25^+FOXP3^+$ Treg cells and FOXP3 gene expression in the peripheral blood of women with POI. Thyroid autoimmunity is the most commonly associated endocrine autoimmune abnormality reported in POI patients (25–60%)[30]. Moreover, 20% of women with POI were positive for anti-TPO and/or anti-TG in the present study. The ovary is a common target of autoimmune attack in organ-specific and systemic autoimmune diseases, and positivity for circulating autoantibodies against ovarian tissue has been demonstrated

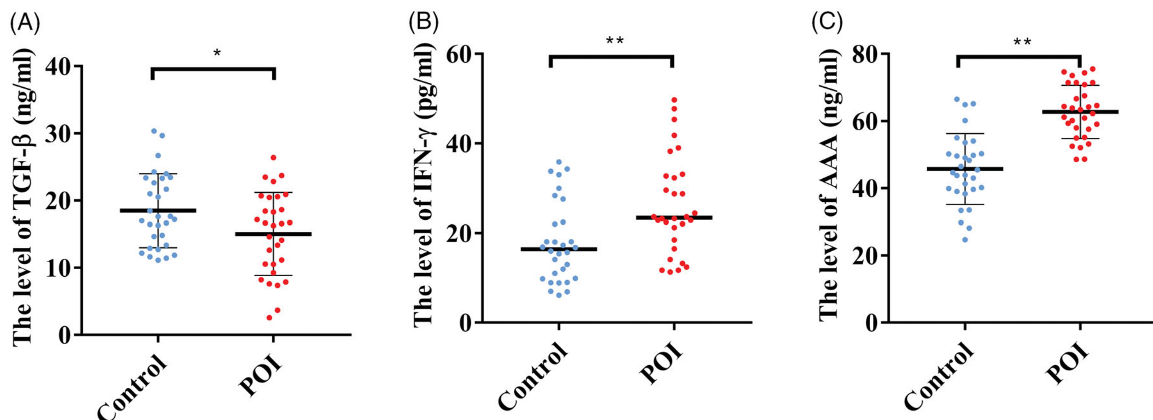

Figure 3. Evaluations of cytokines transforming growth factor-β1 (TGF-β1), interferon-γ (IFN-γ), and adrenal cortex autoantibody (AAA). The levels of (A) TGF-β1, (B) IFN-γ, and (C) AAA in the peripheral blood of the premature ovarian insufficiency (POI) patients and controls. The level of TGF-β1 was significantly decreased, whereas the values of IFN-γ and AAA were significantly increased in the serum of POI patients compared to the serum of the control subjects. The horizontal bars represent means (A and C) and medians (B), respectively. $^{*}p < 0.05$, $^{**}p < 0.001$.

in women with POI, at an incidence of 30–67% of patients[17]. Moreover, it has been reported that all women identified with histological evidence of autoimmune oophoritis by ovarian biopsy are positive for AAA[31]. Therefore, AAA is considered the best marker of POI associated with steroid cell autoimmunity[4]. Consistent with the findings of previous reports, the levels of AAA were significantly increased in the POI patients compared with the controls. Thus, while a specific non-invasive reliable diagnostic test for ovarian autoimmunity is still unavailable, patients should be tested for thyroid, adrenal, and ovarian autoantibodies.

The function and frequency of Treg cells may be regulated by progesterone and 17β-estradiol[32], and these cells may be influenced by hormonal changes during the course of the menstrual cycle[33]. In the present study, all POI patients were treated with the same HRT to keep their hormonal levels steady, and peripheral blood was collected exclusively on the 2nd to 5th day of bleeding. Therefore, the hormonal effects on Treg cells, the expression of FOXP3, and the levels of cytokines were minimized in the two groups.

The small sample size was the primary limitation in our study. Kobayashi *et al.*[16] revealed that more than half of the POI patients were positive for thyroglobulin antibody, and the frequency of effector Treg cells negatively correlated with the thyroglobulin antibody titer. However, the percentage of Treg cells and the expression of FOXP3 mRNA were not significantly different between POI patients with and without positive anti-TPO and/or anti-TG in the present study (data not shown), possibly because only six POI patients with positive anti-TPO and/or anti-TG were recruited in our study. Additionally, studies have demonstrated that HRT partially reverses the deleterious effects of aging on the immune system[34], and HRT increased the percentage of Treg cells compared to non-HRT users[35]. However, little is known about the immune status of POI women receiving HRT. We will perform some work to study the mechanisms of Treg cells, including FOXP3, and POI pathogenesis. Importantly, the presented results require further studies on a larger sample size for validation.

Conclusions

Our results revealed decreases in the proportions of $CD4^{+}CD25^{+}FOXP3^{+}$ Treg cells in the peripheral blood of POI patients, as well as the reduced expression of FOXP3 mRNA. These observations suggested that POI is associated with activated cellular immune responses. Further studies are warranted to elucidate changes during the progression of POI and the underlying mechanisms of autoimmune POI.

Acknowledgements

The authors thank all of the subjects for their participation in this research, as well as the clinical co-workers from the Department of Women Health Care.

Potential conflict of interest The authors report no conflicts of interest with respect to the authorship and/or publication of this article.

Source of funding The work was supported by National Natural Science Foundation of China [Grant number: 81771540]; Natural Science Foundation of Jiangsu Province [Grant number: BK20171504]; Jiangsu Women and Children Healthcare Project [Grant number: FXK201701]; Jiangsu Innovation Team Project [Grant number: CXTDA2017004]; a project funded by PAPD of Jiangsu High Education Institutions.

References

1. European Society for Human Reproduction and Embryology (ESHRE) Guideline Group on POI, Webber L, Davies M, Anderson R, *et al.* ESHRE guideline: management of women with premature ovarian insufficiency. *Hum Reprod* 2016;31:926–37
2. Bats AS, Barbarino PM, Bene MC, Faure GC, Forges T. Local lymphocytic and epithelial activation in a case of autoimmune oophoritis. *Fertil Steril* 2008;90:849.e5–8
3. Sun S, Chen H, Zheng X, Ma C, Yue R. Analysis on the level of IL-6, IL-21, AMH in patients with auto-immunity premature ovarian failure and study of correlation. *Exp Ther Med* 2018;16:3395–8
4. Gao J, Jiao X, Dang Y, *et al.* Identification of patients with primary ovarian insufficiency caused by autoimmunity. *Reprod Biomed Online* 2017;35:475–9
5. Kirshenbaum M, Orvieto R. Premature ovarian insufficiency (POI) and autoimmunity-an update appraisal. *J Assist Reprod Genet* 2019;36:2207–15
6. Welt C, Falorni A, Taylor A, Martin K, Hall J. Selective theca cell dysfunction in autoimmune oophoritis results in multifollicular development, decreased estradiol, and elevated inhibin B levels. *J Clin Endocrinol Metab* 2005; 90:3069–76
7. Sakaguchi S, Ono M, Setoguchi R, *et al.* $Foxp3^{+}CD25^{+}CD4^{+}$ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol Rev* 2006;212:8–27
8. Rouse BT. Regulatory T cells in health and disease. *J Intern Med* 2007;262:78–95
9. Bennett CL, Christie J, Ramsdell F, *et al.* The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet* 2001;27:20–1
10. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003;299: 1057–61
11. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of $CD4^{+}CD25^{+}$ regulatory T cells. *Nat Immunol* 2003;4:330–6
12. Sakaguchi S. The origin of FOXP3-expressing $CD4^{+}$ regulatory T cells: thymus or periphery. *J Clin Invest* 2003;112:1310–12
13. Wan YY, Flavell RA. Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression. *Nature* 2007; 445:766–70
14. Taguchi O, Nishizuka Y. Autoimmune oophoritis in thymectomized mice: T cell requirement in adoptive cell transfer. *Clin Exp Immunol* 1980;42:324–31
15. Nishizuka Y, Sakakura T. Thymus and reproduction: sex-linked dysgenesia of the gonad after neonatal thymectomy in mice. *Science* 1969;166:753–5
16. Kobayashi M, Nakashima A, Yoshino O, *et al.* Decreased effector regulatory T cells and increased activated $CD4^{+}$ T cells in premature ovarian insufficiency. *Am J Reprod Immunol* 2019;81:e13125
17. Forges T, Monnier-Barbarino P, Faure GC, Béné MC. Autoimmunity and antigenic targets in ovarian pathology. *Hum Reprod Update* 2004;10:163–75
18. Silva CA, Yamakami LY, Aikawa NE, Araujo DB, Carvalho JF, Bonfá E. Autoimmune primary ovarian insufficiency. *Autoimmun Rev* 2014;13:427–30
19. Hoek A, Schoemaker J, Drexhage HA. Premature ovarian failure and ovarian autoimmunity. *Endocr Rev* 1997;18:107–34
20. Chernyshov VP, Radysh TV, Gura IV, Tatarshuk TP, Khominskaya ZB. Immune disorders in women with premature ovarian failure. *Am J Reprod Immunol* 2001;46:220–5

21. Mignot MH, Drexhage HA, Kleingeld M, Van de Plassche-Boers EM, Rao BR, Schoemaker J. Premature ovarian failure. II: considerations of cellular immunity defects. *Eur J Obstet Gynecol Reprod Biol* 1989;30:67–72
22. Rabinowe SL, Ravnikar VA, Dib SA, George KL, Dluhy RG. Premature menopause: monoclonal antibody defined T lymphocyte abnormalities and antiovarian antibodies. *Fertil Steril* 1989;51: 450–4
23. Ho PC, Tang GW, Lawton JW. Lymphocyte subsets and serum immunoglobulins in patients with premature ovarian failure before and after oestrogen replacement. *Hum Reprod* 1993;8: 714–16
24. Myers AK, Perroni L, Costigan C, Reardon W. Clinical and molecular findings in IPEX syndrome. *Arch Dis Child* 2005;91:63–4
25. Košir Pogačnik R, Meden Vrtovec H, Vizjak A, Uršula Levičnik A, Slabe N, Ihan A. Possible role of autoimmunity in patients with premature ovarian insufficiency. *Int J Fertil Steril* 2014;7:281–90
26. Saxena D, Misra MK, Parveen F, Phadke SR, Agrawal S. The transcription factor Forkhead Box P3 gene variants affect idiopathic recurrent pregnancy loss. *Placenta* 2015;36:226–31
27. Andre GM, Barbosa CP, Teles JS, Vilarino FL, Christofolini DM, Bianco B. Analysis of FOXP3 polymorphisms in infertile women with and without endometriosis. *Fertil Steril* 2011;95:2223–7
28. Zheng SG, Wang JH, Gray JD, Soucier H, Horwitz DA. Natural and induced $CD4^+CD25^+$ cells educate $CD4^+$CD25-cells to develop suppressive activity: the role of IL-2, TGF-β, and IL-10. *J Immunol* 2004;172:5213–21
29. Nie J, Li YY, Zheng SG, Tsun A, Li B. FOXP3(+) Treg cells and gender bias in autoimmune diseases. *Front Immunol* 2015;28:493
30. Dragojević-Dikić S, Marisavljević D, Mitrović A, Dikić S, Jovanović T, Janković-Ražnatović S. An immunological insight into premature ovarian failure (POF). *Autoimmun Rev* 2010;9:771–4
31. Bakalov VK1, Anasti JN, Calis KA, *et al.* Autoimmune oophoritis as a mechanism of follicular dysfunction in women with 46,XX spontaneous premature ovarian failure. *Fertil Steril* 2005;84:958–65
32. Mjösberg J, Svensson J, Johansson E, *et al.* Systemic reduction of functionally suppressive $CD4^{dim}CD25^{high}Foxp3^+$ T_{regs} in human second trimester pregnancy is induced by progesterone and 17beta-estradiol. *J Immunol* 2009;183:759–69
33. Berbic M, Hey-Cunningham AJ, Ng C, *et al.* The role of $Foxp3^+$ regulatory T-cells in endometriosis: a potential controlling mechanism for a complex, chronic immunological condition. *Hum Reprod* 2010;25:900–7
34. Ghosh M, Rodriguez-Garcia M, Wira CR. The immune system in menopause: pros and cons of hormone therapy. *J Steroid Biochem Mol Biol* 2014;142:171–5
35. Hampras SS, Nesline M, Wallace PK, *et al.* Predictors of immunosuppressive regulatory T lymphocytes in healthy women. *J Cancer Epidemiol* 2012;2012:1