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Decreased effector regulatory T cells and increased activated CD4$^+$ T cells in premature ovarian insufficiency

Running title: Decreased effector Treg cells in POI

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Abstract

Problem

Premature ovarian insufficiency (POI) is a clinical syndrome defined by the loss of ovarian activity before 40 years of age. An autoimmune mechanism is suggested to be involved in the development of POI. Therefore, we examined the relationship between peripheral blood regulatory T (Treg) cells and autoantibodies in POI.

Method of study

Thirty POI patients and 23 control women were enrolled in the study. Using flow cytometry, we measured the abundance of $CD4^+$ T, $CD4^+CD69^+$ T, $CD8^+$ T, $CD8^+CD69^+$ T, naive Treg, effector Treg, and $FOXP3^+$ effector T cells in peripheral blood. Anti-nuclear and anti-thyroglobulin antibody (Tg-Ab) titers were measured in POI patients.

Results

The number of $CD4^+$ T or $CD4^+CD69^+$ T cells was significantly higher in POI patients ($P = 0.045$, and $P = 0.030$), and there were significantly fewer effector Treg cells in POI patients ($P = 0.016$)

than in the controls. There were significant negative correlations between effector Treg cells and Tg-Abs (r = -0.584, P = 0.0282), and between effector Treg cells and $CD4^+CD69^+$ T cells (r = -0.415, P = 0.0226) in POI patients.

Conclusions

This is the first report of decreased numbers of effector Treg cells and increased $CD4^+CD69^+$ activated T cells in peripheral blood in POI, suggesting that POI is an autoimmune disease.

Keywords: infertility, autoimmune disease, autoimmune oophoritis, Hashimoto thyroiditis, anti-thyroglobulin antibody

1 Introduction

Premature ovarian insufficiency (POI) is a clinical syndrome defined by the loss of ovarian activity before the age of 40 years. As the prevalence of POI is approximately 1% by age 40 [1-3], it is not rare among women who intend to become pregnant. Numerous causes of POI have been reported, including genetic, autoimmunological, iatrogenic, infectious, and idiopathic.[4,5] Many studies have reported that autoimmune mechanisms are involved in 4–30% of POI patients.[6-8] Several autoimmune, endocrine (thyroid, hypoparathyroid, diabetes mellitus, and Addison's disease), and non-endocrine disorders (idiopathic thrombocytopenic purpura, autoimmune hemolytic anemia, systemic lupus erythematosus, rheumatoid arthritis, Crohn's disease, Sjogren syndrome, and chronic active hepatitis) are associated with POI.[9] Regulatory T (Treg) cells are involved in the pathogenesis of autoimmune diseases in mice and humans.[10] Treg cells are composed of T cell subsets that play a role in regulating immune cells and inhibiting autoimmune disease. [11]

Mice whose thymus has been removed 3 days after birth have no Treg cells and develop multiple autoimmune disorders, including autoimmune oophoritis, at a high rate.[3,12] However, nude mice, which have no T cells, including Treg cells, do not develop autoimmune diseases. This suggests that autoreactive effector T cells (Teff) attack organs if Treg cells are not present. Furthermore, transfer of $CD4^+$ T cells into T cell- and B cell-deficient SCID mice leads to the development of multiple autoimmune disorders, including autoimmune oophoritis, suggesting that $CD4^+$ T cells induce POI.[13]

In humans, immunodysregulation polyendocrinopathy enteropathy X-linked syndrome, a congenital disease due to *FOXP3* gene mutations, causes dysfunction of Treg cells, resulting in multiple autoimmune disorders, such as polyendocrinopathy, enteropathy, and POI, suggesting that Treg cells play central roles in the pathogenesis of POI.[9,14,15]

Although Treg cells may be related to the pathogenesis of POI, the subsets of Treg cells that function in the development of POI in humans are not known. Therefore, we compared the populations of Treg subsets, such as naive Treg cells with low immunoregulatory activity, effector Treg cells with high immunoregulatory activity, and $FOXP3^+$ Teff cells with no immunoregulatory activity, in the peripheral blood of POI patients and controls.[16]

In this study, activated T cell numbers were increased, whereas immunosuppressive effector Treg cells were decreased in POI patients compared with controls. POI patients with fewer effector Treg cells had higher titers of anti-thyroglobulin antibodies (Tg-Ab), suggesting that an autoimmune mechanism is involved in the development of POI.

2 Materials and methods

2.1 POI patients and control women with regular menstruation.

This study was approved by the ethics committee of the Rose Ladies Clinic and The University of Toyama, Japan. We obtained written informed consent from all study participants. We excluded patients with chromosomal abnormalities and chemotherapy treatment. In total, we enrolled 30 POI patients and 23 normal fertile menstruating women as controls. Among the POI patients, there were 4 with Hashimoto thyroiditis, 1 with Graves' disease, 3 with Sjogren's diseases, and 1 with myasthenia gravis. Patients with autoimmune diseases were treated by internal physicians. The patient with Graves' disease was administered prednisolone. In controls, there were no cases of autoimmune disease. The clinical backgrounds of these groups are presented in Table 1. The mean age of POI patients and controls was not significantly different (mean 37.7 and 35.2 years old, $P = 0.0679$). All POI patients were treated with hormone replacement therapy.

2.2 Flow cytometric analysis

Peripheral blood mononuclear cells (leukocytes) were isolated by the standard Ficoll-Hypaque method. The following monoclonal antibodies (mAbs) were used in this study: anti-CD4 (PerCP-Cy5.5, Cat# 341654, BD Biosciences, NJ, USA), anti-CD8 (PE-Cy7, Cat# 555366, BD Biosciences), anti-CD69 (PE, Cat# 555531, BD Biosciences), anti-CD45RA (biotin, Cat# 5555487, BD Biosciences), and streptavidin labeled with APC-Cy7 (Cat# 554063, BD Biosciences) as cell surface markers, and anti-FOXP3 (fluorescein isothiocyanate, Cat# 11-4776-42, eBioscience, San Diego, CA, USA) as an intracellular marker. Peripheral mononuclear cells were first stained with anti-CD4 mAb, anti-CD8 mAb, anti-CD69 mAb, and anti-CD45RA mAb for 30 min on ice. Cells were washed with phosphate-buffered saline (PBS) three times. Next, APC-Cy7-labeled streptavidin was added and incubated for 15 min on ice. After washing the cells with PBS three times, they were

fixed and permeabilized by incubation for 30 min with fixation/permeabilization buffer (Cat# 88-8824-00, eBioscience), and then stained with anti-FOXP3. Flow cytometric analysis was performed using a BD FACScant II (BD Biosciences).

Lymphocytes were gated based on both forward- and side-scatter parameters (Fig. 1A). Monocytes and granulocytes were excluded by defining a lymphocyte gate. After gating for $CD4^+$ T cells and $CD8^+$ T cells (Fig. 1B), numbers of $CD69^+$ activated $CD4^+$ T cells and $CD8^+$ T cells were determined (Fig. 1C, and D). After gating for $CD4^+$ T cells (Fig. 1F), the proportions of $CD4^+CD45RA^+FOXP3^{low}$ naive Treg, $CD4^+CD45RA^-FOXP3^{high}$ effector Treg, and $CD4^+CD45RA^-FOXP3^{low}$ Teff cells were measured among $CD4^+FOXP3^+$ cells (Fig. 1H).

2.3 Autoantibody assay

Antinuclear antibody (ANA) and Tg-Ab were measured by the Fukuyama Medical Laboratory (Hiroshima, Japan). ANA was measured by the FLUORO HEPANA TEST (MBL, Nagoya, Japan) using a direct immunofluorescence assay. Tg-Ab was measured by Elecsys anti-Tg (Cat# 06368697190, Roche Diagnostics Ltd, Rotkreuz, Switzerland) using an electro chemiluminescence immunoassay.

2.4 Statistical analysis

Statistical analysis was performed using a statistical software package, JMP version 13 (SAS, NC, USA). Data were analyzed using Welch's t-test and Pearson's correlation coefficient. Values of $P < 0.05$ were considered statistically significant.

3 Results

3.1 Activated $CD4^+$ T cells and $CD8^+$ T cells in POI patients

The number of $CD4^+$ T cells in total lymphocytes was significantly higher in POI patients than in controls (42.5 ± 8.7 vs. 37.4 ± 9.7, $P = 0.045$, Fig. 2A). However, the number of $CD8^+$ T cells was not significantly different between POI patients and controls (Fig. 2B). Next, we measured the number of $CD69^+CD4^+$ (activated $CD4^+$) T cells and $CD8^+CD69^+$ (activated $CD8^+$) T cells. The number of activated $CD4^+$ T cells, but not activated $CD8^+$ T cells, was significantly higher in POI patients than in controls (2.40 ± 2.6 vs. 1.15 ± 0.6, $P = 0.030$, Fig. 2C). These results suggested that activated $CD4^+$ T cells play an important role in the development of POI.

3.2 Effector Treg cells in POI patients

We measured the frequencies of Treg subsets in peripheral blood mononuclear cells (PBMCs) of POI patients and controls. The frequency of effector Treg cells among $CD4^+FOXP3^+$ T cells was significantly lower in POI patients than in controls (21.7 ± 6.7 vs. 27.5 ± 9.9, $P = 0.016$, Fig. 3B). On the other hand, the frequencies of naive Treg cells and $FOXP3^+$Teff cells did not significantly differ between POI patients and controls (Fig. 3A and 3C, and Table 2).

3.3 Relationships between effector Treg cells and $CD4^+CD69^+$ T cells, and between effector Treg cells and Tg-Abs titer

We next evaluated the correlation between effector Treg cells among $CD4^+FOXP3^+$ T cells and $CD4^+CD69^+$ (activated $CD4^+$) T cells among $CD4^+$ T cells. There was a negative correlation between effector Treg cells and activated $CD4^+$ T cells in POI patients (r = -0.415, $P = 0.0226$, Fig. 4A), but not in controls (Fig 4B). On the other hand, effector Treg cells were not correlated with $CD8^+CD69^+$

T cells in both controls and POI patients (Fig. 4C and 4D). Furthermore, a significant negative correlation between effector Treg cells and the Tg-Ab titer was observed (r = -0.584, P = 0.0282, Fig. 4C), but no correlation was observed with the ANA titer (Fig. 4D) in POI patients. These results suggested that the decrease in effector Treg cells was correlated with elevation in the TG-Ab titer and the number of activated $CD4^+$ T cells in POI patients.

4 Discussion

This is the first study to report a reduction in the number of effector Treg cells in the peripheral blood of POI patients. Our findings suggested that the immunological environment shifts to an immunoactivation-dominant state in POI. T cell-mediated injury also plays an important role in the pathogenesis of autoimmune POI, as evidenced by human studies and animal models of autoimmune oophoritis.[7,17,18] One study demonstrated the infiltration of $CD3^+$ T cells around follicles in ovaries obtained from autoimmune oophoritis patients,[19] but it is unknown whether $CD4^+$ T cells or $CD8^+$ T cells are important for human autoimmune oophoritis. This study provided two significant findings: a decrease in the number of effector Treg cells and an increase in the number of activated $CD4^+$ T cells in POI patients. In addition, these two findings were negatively correlated with each other. We summarized the model of autoimmune oophoritis using Treg cell-depleted mice in Supplemental Table 1. These models support the former finding. On the other hand, the latter finding was matched with the mouse models, in which SCID mice injected with auto-reactive $CD4^+$ T cells, but not $CD8^+$ T cells, developed autoimmune oophoritis followed by POI.[13] Taken together, the contribution of Treg cells and activated CD4+ T cells, in which animal models demonstrated the importance for autoimmune oophoritis, was also observed in POI patients, suggesting that a proportion of POI patients with decreased effector Treg cell numbers might present impairment of regulation of autoimmune reactions.

In POI patients there was a negative correlation between effector Treg cells and activated $CD4^+$ T cells, but not in controls. When numbers of activated $CD4^+$ T cells are increased by inflammation in healthy individuals, Treg cells, which simultaneously expand, inhibit excessive immune responses [20,21]. This function in Treg cells is necessary for inhibiting autoimmune disease in healthy individuals. However, our results suggested that the simultaneous expansion of Treg cells, which react to the increase in numbers of activated $CD4^+$ T cells, might be impaired in POI patients. Otherwise, the decreased number of effector Treg cells might be related to the inhibition of auto-immune reactions. Although we only investigated the number of Treg cells in this study, further comparative experiments to elucidate the functions of Treg cells in POI patients and controls would be required to further clarify the importance of Treg cells in POI patients.

Since there are many reports that estrogen and progesterone have the proliferative effects on Treg cells [22-24], the number of effector Treg cells could be decreased due to lack of estrogen and progesterone. The hormonal effects on Treg cells would be minimalized in POI patients, compared with controls, because all POI patients in this study were treated with hormone replacement therapy. Therefore, hormonal levels are at steady-state in POI cases

Autoimmune hypothyroidism, also termed Hashimoto thyroiditis, is a disease commonly associated with POI.[25] TG-Ab is a clinically useful marker for the diagnosis of Hashimoto thyroiditis.[26] Our study revealed that 53.3% of POI patients were positive for TG-Ab. There was a negative correlation between the frequency of effector Treg cells and the TG-Ab titer in this study, suggesting that a decrease in the number of effector Treg cells induces autoantibody production, including TG-Ab. There are a number of autoantibodies related to POI, such as anti-zona pellucida, anti-3β-hydroxysteroid dehydrogenase, anti-21-hydrozylase, and anti-17-hydroxylase antibodies.[19,27-30] We did not measure the titers of these autoantibodies because no commercial tests were available. However, any correlations between the number of peripheral blood Treg cells and autoantibodies related to POI should be investigated in the future.

The effectiveness of immunosuppressive drugs, such as corticosteroids or tacrolimus, in POI is controversial.[31,32] These immunosuppressive drugs may only be effective for autoimmune POI, but not non-autoimmune POI. Furthermore, these immunosuppressive agents may be effective in the progressive phase of POI and not in the end stage of POI when follicles are lost. To diagnose autoimmune POI, measurement of effector Treg cells and Tg-Ab may be effective, and corticosteroids or tacrolimus may be available for such patients in order to delay the progressive phase of POI. Further studies are needed for confirmation of our results.

Conclusions

This is the first report to demonstrate a decrease in effector Treg cell numbers in the peripheral blood of POI cases. There was a negative correlation between the abundance of effector Treg cells and activated $CD4^+$ T cells, and between the number of effector Treg cells and the Tg-Ab titer. Our study suggests that excessive immunological activation causes autoimmune reactions in the ovaries, resulting in autoimmune POI. Measurement of effector Treg cells and Tg-Ab may be useful for detecting immunological activation in POI patients. Immunosuppressive drugs, such as corticosteroids or tacrolimus, may be effective in the treatment of autoimmune POI diagnosed by our method.

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Figure legends

Figure 1: Gating strategies for T cell and regulatory T cell (Treg) subsets. First, lymphocytes in the peripheral blood were gated according to forward- and side-scatter parameters (A). T cell subsets were classified into $CD4^+$ (B), $CD8^+$ (B), $CD4^+CD69^+$ (activated $CD4^+$) (C), and $CD8^+CD69^+$ (activated $CD8^+$) cells (D). Treg subsets were gated using $CD4^+$ cells (F) and classified into $CD45RA^+FOXP3^{low}$ naive Treg, $CD45RA^-FOXP3^{high}$ effector Treg, and $CD45RA^-FOXP3^{low}$ effector T cells (G and H).

Figure 2: Frequencies of $CD4^+$ (A), $CD8^+$ (B), $CD4^+CD69^+$ activated $CD4^+$ (C), and $CD8^+CD69^+$ activated $CD8^+$ cells (D) in peripheral blood mononuclear cells. The horizontal bar is the mean value. n.s. means not significant.

Figure 3: Frequencies of naive regulatory T (Treg) (A), effector Treg (B), and $FOXP3^+$ effector T cells (C) among $CD4^+FOXP3^+$ cells in peripheral blood mononuclear cells. The horizontal bar is the mean value. n.s. means not significant.

Figure 4: Correlations between the frequencies of effector regulatory T (Treg) cells and $CD4^+CD69^+$ cells in premature ovarian insufficiency (POI) patients (A) and in controls (B), between the frequencies of effector Treg cells and $CD8^+CD69^+$ cells in POI patients (C) and in controls (D), between the frequency of effector Treg cells and the anti-thyroglobulin antibody titer (E), and between the frequency of effector Treg cells and the anti-nuclear antibody titer (F). Blue points indicate controls. Red points indicate POI cases. Black line is the regression line.

Table 1: Clinical background of POI patients and control women.

	Control (n=23)	POI (n=30)	*P*
Age (year) †	35.2 (±5.2)	37.8 (±4.5)	0.0581
% of ANA ‡		60.0%	-
ANA titer †	-	336.5 (±607.6)	-
% of Tg-Ab §		53.3%	-
Tg-Ab titer †	-	379.1 (±239.6)	-
Autoimmune disease (%)	0 (0%)	9 (30%) (G=1, H=4 SS=3, MG=1)	-

†: mean (±SD)

‡: anti-nuclear antibody

§ : anti-thyroglobulin antibody

G: Graves' disease, H: Hashimoto thyroiditis

SS: Sjogren's syndrome, MG: Myasthenia gravis

Table 2: Frequencies of $CD4^+$ cells, $CD4^+CD69^+$ cells, $CD8^+$ cells, $CD8^+CD69^+$ cells, naive Treg cells, effector Treg cells, and FOXP3+ Teff cells.

	Control	POI	*P*
$CD4^+$ cells/Lymphocytes (%)	37.4 (±9.7)	42.5 (±8.7)	0.045
$CD4^+CD69^+$ cells/$CD4^+$ cells (%)	1.15 (±0.62)	2.40 (±2.6)	0.030
$CD8^+$ cells/Lymphocytes (%)	21.9 (±7.2)	22.4 (±6.3)	n.s.
$CD8^+CD69^+$ cells/$CD8^+$ cells (%)	18.4 (±19.3)	17.1 (±12.6)	n.s.
Naive Treg cells/$CD4^+FOXP3^+$ cells (%)	14.1 (±4.9)	16.4 (±5.5)	n.s.
Effector Treg cells/$CD4^+FOXP3^+$ cells (%)	27.5 (±9.9)	21.7 (±6.7)	0.016
$FOXP3^+$ Teff cells/$CD4^+FOXP3^+$ cells (%)	58.5 (±8.8)	61.9 (±5.2)	n.s.

n.s.: not significant