CD4\(^+\) CD25\(^+\) FOXP3\(^+\) regulatory T cells in peripheral blood and peritoneal fluid of patients with endometriosis

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**STUDY QUESTION:** Is endometriosis associated with changes in CD4\(^+\) CD25\(^+\) FOXP3\(^+\) regulatory T cells (Treg cells)?

**SUMMARY ANSWER:** Endometriosis is associated with disturbed compartmentalization of CD25\(^{\text{high}}\) FOXP3\(^+\) Treg cells.

**WHAT IS KNOWN ALREADY:** Endometriosis is associated with an abrogated immune response and displays some features of an autoimmune disorder. Treg cells play a part in the development of autoimmune reactions; however, their role in pathogenesis of endometriosis is still poorly recognized.

**STUDY DESIGN, SIZE AND DURATION:** Case–control study comparing 17 women with laparoscopically and histopathologically confirmed ovarian endometriosis with 15 control women without visible endometriosis foci, pelvic inflammation or related pathology who were subjected to laparoscopic surgery between 2010 and 2011.

**PARTICIPANTS/MATERIALS, SETTING AND METHODS:** Peripheral blood and peritoneal fluid were collected during laparoscopy and T cell subpopulations were analysed by flow cytometry using specific monoclonal antibodies recognizing CD4\(^+\), CD25\(^+\) and FOXP3\(^+\) markers.

**MAIN RESULTS:** The percentage of CD25\(^{\text{high}}\) FOXP3\(^+\) Treg cells was significantly decreased in the peripheral blood of women with ovarian endometriosis compared with control women. On the other hand, the proportion of these cells was significantly increased in the peritoneal fluid of women with endometriosis.

**LIMITATIONS, REASONS FOR CAUTION:** The present study is limited to patients with ovarian endometrioma and further investigations are needed, including patients with lower grade of endometriosis.

**WIDER IMPLICATIONS OF THE FINDINGS:** The present results suggest that Treg cells may play a part in immunopathogenesis of endometriosis being responsible for abrogated local cellular immune responses and facilitation and development of autoimmune reactions. Treg cells may be thus a potential target in the treatment of endometriosis.

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\(^{†}\)The authors consider that the first two authors should be regarded as joint First Authors.

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Introduction

Endometriosis is a common, benign disorder characterized by the presence of endometrial tissue outside the uterine cavity, mainly on the pelvic peritoneum and/or ovaries. It is associated with chronic pelvic pain, pelvic inflammatory reactions and infertility (Giudice and Kao, 2004).

Endometriosis appears to be a complex disorder. Its aetiopathogenesis may involve a variety of environmental, immune and genetic factors. However, the role of which still remains to be elucidated (Giudice and Kao, 2004).

There is a growing evidence that endometriosis is associated with disturbed local and systemic immune responses. These may include increased levels of activated peritoneal macrophages and various proinflammatory and angioregulatory cytokines, abnormal T and B lymphocyte activation, reduced natural killer (NK) cell activity and the production of various autoantibodies such as anti-endometrial, anti-ovarian, anti-nuclear and anti-phospholipid antibodies (Vinatier et al., 1996; Matarrese et al., 2003; Giudice and Kao, 2004; Berbic and Fraser, 2011). Moreover, some polymorphisms of autoimmune-related genes were recently postulated to be associated with the development of endometriosis (Bianco et al., 2012). Therefore, one may argue for endometriosis to be regarded as an autoimmune disease (Nothnick, 2001; Matarrese et al., 2003; Giudice and Kao, 2004). The mechanisms of these endometriosis-associated immune deviations are, however, still poorly understood.

Autoimmune disorders might be a consequence of disturbance in the function of T regulatory cells. These include CD4⁺ CD25⁺ FOXP3⁺ regulatory T cells (Treg cells) which appear to play a key role in control and suppression of many types of effector cells such as macrophages, NK cells, dendritic cells, cytotoxic T cells (Sakaguchi et al., 2008; Berbic and Fraser, 2011). The role of Treg anomaly in aetiopathogenesis of several autoimmune disorders such as autoimmune gastritis, thyroiditis, type I diabetes mellitus, inflammatory bowel disease, multiple sclerosis, myasthenia gravis, systemic lupus erythematosus, rheumatoid arthritis, and psoriasis as well as immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome has already been well documented (Sakaguchi et al., 2006; Rouse, 2007; Kondělková et al., 2010).

Little is known about a possible role of Treg in endometriosis. Recently, Budiu et al. (2009), Berbic et al. (2010) and Basta et al. (2010) have reported the presence of Treg in eutopic and ectopic endometrial tissue of patients with endometriosis. Increased levels of mRNA of Treg-specific FOXP3 transcriptional factor were also detected in human endometriotic lesions (Budiu et al., 2009). Moreover, endometriosis was reported to be associated with polymorphisms of FOXP3 gene (André et al., 2011). However, Treg cells in the peripheral blood and peritoneal fluid of endometriosis patients have not been analysed, so far. Therefore, the aim of our present study was to investigate the levels of CD4⁺ CD25⁺ FOXP3⁺ Treg cells in the peripheral blood and peritoneal fluid of patients with endometriosis.

Materials and Methods

Patients

The study included 17 women (mean age 31 years, range 19–39) with laparoscopically and histopathologically confirmed endometriosis. All patients had ovarian endometriotic cysts and were classified as stage III/IV of endometriosis according to the revised criteria of the American Fertility Society (rAFS) (American Fertility Society, 1985). The control group consisted of 15 women (mean age 34 years, range 18–46) without visible endometriosis foci, pelvic inflammation or related pathology who underwent laparoscopic excision of ovarian dermoid cysts or diagnostic laparoscopy.

Material from all patients and controls was collected on day 5–10 of the menstrual cycle. None of the patients and control subjects suffered from any other chronic inflammatory or autoimmune disorder and was not subjected to pharmacological treatment which would affect immune response for at least 3 months prior to the study. All patients gave an informed consent to the study, and the investigations were approved and conducted according to the strict guidance of the local Ethics Committee at the Medical University of Warsaw.

Collection and preparation of blood and peritoneal fluid

Peritoneal fluid was aspirated from the cul de sac at the beginning of the standard laparoscopic procedure under general anaesthesia. Samples of peritoneal fluid contaminated with blood were excluded from the study. Mononuclear cells (MNCs) were isolated from heparinized blood and peritoneal fluid of patients by Gradisol L (Aqua Medica, Łódź, Poland) density-gradient centrifugation at 2900 rpm for 15 min at 4°C. The cells were collected from the interphase, washed in ice-cold PBS and suspended to an antibody staining buffer with a desired concentration for further flow cytometry analysis.

Evaluation of anti-nuclear antibodies (ANA)

ANA in sera and peritoneal fluids were evaluated by indirect immunofluorescence on HEP-2 cells using Fluorescent ANA Test System supplied by Immunon Concepts N. A. Ltd., Sacramento, CA, USA, as described in detail elsewhere (Ploski et al., 2009).

Flow cytometry analysis of Treg cells

For identification and evaluation of Treg cells, MNCs were stained with peridinin chlorophyll protein-conjugated anti-CD4 and allophycocyanin-conjugated anti-CD25 monoclonal antibodies (all from BD Biosciences, San Jose, CA, USA) as described previously (Baecher-Allan et al., 2001; Bocian et al., 2010). This was followed by intracellular staining of FOXP3 using the fluorescein isothiocyanate (FITC) Anti-Human Foxp3 Staining Set (eBioscience Inc., San Diego, CA, USA) according to the manufacturer’s instructions. IgG1 antibodies conjugated with the respective fluorochromes served as isotype controls.

Flow cytometry analysis was carried out on an FACS-Calibur using Cell Quest software (BD Biosciences). The cells were specifically analysed by selective gating as shown in Fig. 1. The results were based on analysis of at least 100 000 cells and were shown as the percentage of positively labelled cells.
Statistical analysis

The differences between the groups were calculated by Wilcoxon’s matched-pair test or Mann–Whitney U-test, where applicable. They were considered significant at least at $P \leq 0.05$.

Results

General characteristics of patients’ immune status

Evaluation of ANA in sera and peritoneal fluid of women enrolled in the study has revealed the positive reactivity in four (23.5%) women with endometriosis and one (6.7%) woman from the control group. In all positive cases, ANA titres did not exceed 1:80.

The volume of the peritoneal fluid and the numbers of peritoneal fluid leucocytes appeared to be higher in the endometriosis group when compared with the control women, but these differences were not statistically significant (Table I).

Table I The volume of peritoneal fluid collected from control subjects and women with endometriosis and the concentration of leucocytes in it [mean (range)].

<table>
<thead>
<tr>
<th>Peritoneal fluid</th>
<th>Control (n = 15)</th>
<th>Endometriosis (n = 17)</th>
<th>$P^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (ml)</td>
<td>6.2 (1.5–11.0)</td>
<td>9.2 (0.9–20.0)</td>
<td>0.104</td>
</tr>
<tr>
<td>Leucocyte concentration (10^6/ml)</td>
<td>0.82 (0.1–1.6)</td>
<td>1.26 (0.2–5.3)</td>
<td>0.868</td>
</tr>
</tbody>
</table>

*P-value was judged by Mann–Whitney U or Student’s t-test.

Analysis of total CD4$^+$ and CD4$^+$ CD25$^+$ Treg cells

FACS analysis demonstrated that the median prevalence of total CD4$^+$ T lymphocytes (Th cells) in the peritoneal fluid of women with endometriosis and control subjects was 4.1 and 6.0%, respectively, and this difference was not statistically significant ($P = 0.07$).
were also no significant differences between the tested groups in the percentage of total peripheral blood CD4+ T cells (data not shown). Similarly, there were no differences between endometriosis and control women in respect of expression of CD25 within CD4+ T cell population (data not shown).

**Analysis of CD25+ FOXP3+ cells**

Analysis of CD25+ Th cells in respect of expression of FOXP3 showed that there were no significant differences between patients with endometriosis and control women in the percentage of CD25+ FOXP3− activated Th cells, both in the peripheral blood and in the peritoneal fluid (data not shown).

Comparison of total CD25+ FOXP3+ cells within CD4+ cells in the peripheral blood and peritoneal fluid of control group and women with endometriosis is shown in Fig. 2. The median percentage (interquartile range) of these cells in the peripheral blood of the control group and the women with endometriosis group was 5.2 (4.1–5.71) and 4.4 (3.11–5.5), respectively, and this difference was not statistically significant (P = 0.4). On the other hand, the respective median percentage of these cells in the peritoneal fluid of control women and endometriosis subjects was 3.4 (3.2–4.3) and 6.1 (5.1–8.6), respectively, and this difference reached high significance (P = 0.003).

Further analysis of peripheral blood and peritoneal fluid leucocytes revealed two different subpopulations of FOXP3+ cells displaying low (CD25low) and high (CD25high) expression of CD25 marker (Fig. 1). A majority (70–90%) of FOXP3+ cells were CD25high, whereas only 10–20% of these cells were CD25low. The CD25high FOXP3+ subpopulation corresponds to Treg cells (Baecher-Allan et al., 2001, 2004; Askenasy et al., 2008).

Figure 3A shows the percentages of CD25low FOXP3+ cells within the CD4+ population. The median percentage (interquartile range) of these cells in the peripheral blood of the control and endometriosis groups was 3.7 (2.4–4.5) and 3.3 (2.1–4.9), respectively, and was not significantly different (P = 0.95). In the peritoneal fluid, the median percentage of these cells in the control group was 1.2 (0.7–2.4), whereas in the women with endometriosis group this value was 2.7 (2.3–3.8) and was significantly increased (P = 0.015).
The percentage of CD25high FOXP3+ cells within CD4+ population (Treg cells) is shown in Fig. 3B. The respective median percentage (interquartile range) of these cells in the peripheral blood of control women and women with endometriosis was 1.6 (1.0–2.5) and 0.8 (0.5–1.2), respectively, and this decrease was statistically significant ($P = 0.021$). On the contrary, the percentage of these cells in the peritoneal fluid of endometriosis patients was significantly increased ($P = 0.014$): the median percentage (interquartile range) of Treg cells in control women was 2.0 (1.5–2.7) whereas in the women with endometriosis this value was 3.1 (2.2–4.4).

Discussion

The results of the present study show for the first time that the frequency of CD25high FOXP3+ cells in CD4+ population (Treg cells) in the peritoneal fluid is significantly increased in women with ovarian endometriosis compared with control women. On the other hand, the proportion of these cells was significantly lower in the peripheral blood of endometriosis patients. This strongly suggests that endometriosis is associated with the disturbance of circulating Treg cells.

Although, to our knowledge, Treg cells in the peripheral blood and peritoneal fluid in patients with endometriosis have not been studied so far, their prevalence has been recently studied in eutopic endometrium and ectopic endometrioid tissue (Basta et al., 2010; Berbic et al., 2010). Basta et al. (2010) reported that the percentage of CD25+ FOXP3+ cells within CD4+ population from ovarian endometrioma was significantly higher than in ectopic decidua from ectopic pregnancies. They, however, did not reveal any difference in the numbers of these cells between endometriotic lesions and normal endometrium from healthy women. On the other hand, Berbic et al. (2010) showed that the numbers of Treg cells in normal eutopic endometrium from healthy women were significantly decreased during secretory phase, whereas similar change was not observed in eutopic endometrium from endometriosis patients.

Function and frequency of Treg cells may be regulated by progesterone and 17β-estradiol (Mösb erg et al., 2009) and these cells may be influenced by hormonal changes in the course of the menstrual cycle (Berbic et al., 2010). However, our present clinical material from both groups was collected exclusively during the mid-follicular phase (day 5–10) and, therefore, the differences in the frequency of CD25+ FOXP3+ Treg cells between endometriosis and control group cannot be attributed to differences related to the phase of the menstrual cycle. It is thus possible that they reflect specific endometriosis-associated abrogation of both systemic and local immune systems. The mechanism of this abrogation remains to be elucidated; however, it is tempting to speculate that the differential pattern of peripheral blood and peritoneal fluid CD25high FOXP3+ Treg cells in endometriosis may reflect their active translocation from the peripheral blood to the peritoneal cavity.

Endometriosis is associated with increased peritoneal fluid concentrations of a variety of cytokines including various chemotactic/activatory factors (Harada et al., 2001). These include RANTES and MCP-1 (Harada et al., 2001; Gazvani and Templeton, 2002) which are potent chemotactants for Treg cells (Imai et al., 1997; Ishida and Ueda, 2011). It is, therefore, tempting to speculate that accumulation of Treg cells in the peritoneal cavity of women with endometriosis is a secondary phenomenon related to their chemotaxis due to persistence of local inflammatory reaction.

Increased inflammatory reaction and increased concentrations of peritoneal cytokines are generally associated with the severity of endometriosis (Harada et al., 2001; Gazvani and Templeton, 2002). Thus, the question arises whether changes in the concentration of peritoneal Treg cells may depend on the grade of the disease. Our present study is limited to cases with ovarian endometriosis which classify into the highest moderate/severe grade of the disease. Therefore, further studies are needed to reveal a possible relationship between frequency of Treg cells, severity of endometriosis and local concentration of specific cytokines.

It is also plausible that, in addition to active translocation to the peritoneal cavity, increased numbers of Treg cells may be a result of their local induction. Induction of Treg cells manifested by acquired FOXP3 expression cells may be mediated by some cytokines such as TGF-β and IL-10 (Lu et al., 2010; Okamoto et al., 2011). Increased amounts of TGF-β and IL-10 have been repeatedly reported in the peritoneal fluid of patients with endometriosis (Punnonen et al., 1996; Zhang et al., 2007; Omwandho et al., 2010). Thus, the possibility of local peritoneal induction of Treg cells in women with endometriosis cannot be ruled out. This assumption might be partially supported by our observation of increased frequency of CD25low FOXP3+ cells in the peritoneal cavity of women with endometriosis. Although origin and function of these cells in humans still remain obscure, it is tempting to speculate that they may represent Treg cells in course of their induction from naive T cells (Walker et al., 2003).

Active trafficking of Treg cells to the peritoneum of endometriosis patients and their local induction may be considered as a compensatory anti-inflammatory mechanism. Accordingly, increased numbers of Treg cells might, at least partially, account for previously described local endometriosis-associated immune deviations such as abnormal T and B lymphocyte activation and reduced NK cell activity (Nothnick, 2001; Matarrese et al., 2003). It should be stressed, however, that these immune deviations may be also related to activity of some other regulatory cells such as Th3 and Tr1, which, to our knowledge, have not been studied in endometriosis so far.

Endometriosis is frequently associated with various autoimmune phenomena including production of many different autoantibodies and shows some similarities with autoimmune disorders (Nothnick, 2001). The mechanisms of these autoimmune phenomena in the course of endometriosis are still a matter of discussion. It has been postulated that Treg cells are actively involved in the maintenance of self-tolerance and their abrogation may result in development of autoimmunity (Sakaguchi et al., 2001). Although we did not find any clear association between Treg cells and prevalence of ANA in the present endometriosis patients, it is plausible that some endometriosis-associated autoimmune phenomena are, at least partially, related to an abrogated function of circulating CD25high FOXP3+ cells. This, however, needs further investigation.

Conclusion

The results of our present study suggest that endometriosis is associated with disturbed compartmentalization of CD25high FOXP3+ Treg cells. Increased percentage of Treg cells in the peritoneal fluid may account for abrogated local cellular immune responses, whereas their decreased frequency in the peripheral blood might be
responsible for facilitation and development of autoimmune reactions. It must be stressed, however, that our present study is limited to patients with ovarian endometrioma and further investigations are needed to reveal whether similar changes are also associated with lower grade peritoneal disease.

**Authors’ roles**

J.O.-T. and K.B. were involved in study design, material preparation and flow cytometry measurements, data collection, analysis and interpretation, manuscript drafting and critical discussion. R.B.M., D.W. and W.B. were involved in clinical material collection and analysis. A.B. was involved in material preparation and flow cytometry measurements. J.Z. performed evaluation of ANA. G.K.-K. was involved in study conception and design, data analysis, drafting the article and its final edition. All authors participated in critical reading of the article.

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**Conflict of interest**

None declared.

**References**


