The role of Foxp3+ regulatory T-cells in endometriosis: a potential controlling mechanism for a complex, chronic immunological condition

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BACKGROUND: Endometriosis is an inflammatory condition, associated with highly dysregulated immune response at both uterine and peritoneal levels. Surprisingly, Foxp3+ regulatory T-cells, which control and suppress a range of immune responses, have not previously been investigated in endometriosis.

METHODS AND RESULTS: Immunohistochemical analysis of Foxp3+ cells in 127 eutopic endometrial samples and 59 ectopic peritoneal lesions revealed that these immune cell populations are highly disturbed in women suffering from endometriosis. We showed that Foxp3+ cells remained highly up-regulated during the secretory phase of the menstrual cycle, while at this time their expression is significantly down-regulated in women without endometriosis (P<0.001). Foxp3+ cells were detected in the stroma of 18 of the 59 peritoneal endometriotic lesions, but not in the surrounding or control peritoneal tissue.

CONCLUSIONS: We propose that in eutopic endometrium in women with endometriosis Foxp3+ cells decrease the ability of newly recruited immune cell populations to effectively recognize and target endometrial antigens shed during menstruation, allowing their survival and ability to implant in ectopic sites. At these ectopic sites, variable expression of Foxp3+ cells within some peritoneal endometriotic lesions is likely to be linked to the characteristics and stage of individual lesion development and be playing key roles in pathogenesis and progression of this unique condition.

Key words: regulatory T-cells / Foxp3 / endometriosis / endometrium / peritoneal lesions

Introduction

Endometriosis is a benign gynaecological condition, characterized by the growth of endometrial-like glands and stroma at sites outside the uterine cavity (ectopic endometriotic lesions). Although the exact aetiology of endometriosis remains poorly understood, it has been postulated that during the process of menstruation, endometrial fragments which are commonly displaced through the fallopian tubes via retrograde flow are more viable in women with endometriosis and are thus more likely to implant at ectopic sites, establishing the disease (Healy et al., 1998; Taylor et al., 2002). Predisposing genetic and environmental factors are likely to play important roles in pathogenesis of endometriosis; however, factors which permit survival of endometrial fragments following the process of menstruation could almost certainly be attributed to defective immune surveillance within the eutopic endometrium of women with this disease.

Although numerous immune factors have been reported to be significantly altered within endometriotic lesions and the surrounding peritoneal fluid (Oosterlynck et al., 1993; Jones et al., 1998; Lebovic et al., 2002), recent evidence suggests that dysregulated immune response in endometriosis is likely to originate within the eutopic endometrium (Ota et al., 1996; Bulmer et al., 1998; Akoun et al., 2006; Al-Jefout et al., 2009; Berbic et al., 2009; Schulke et al., 2009). It is here that, under the normal conditions, sloughed
endometrial cells are likely to be targeted by the immune system, such that even if they did escape via retrograde flow, they would no longer be able to implant and establish the disease.

Our recent studies show that a range of uterine immune cell populations, including macrophages, and immature and mature dendritic cells (DCs), are altered in women with endometriosis (Berbic et al., 2009; Schulke et al., 2009). Of particular interest was the observation that CD83+ mature DCs were significantly down-regulated across all phases of the menstrual cycle in women with endometriosis, suggesting that previously unrecognized components of adaptive immune response and antigen presentation are likely to lead to insufficient or ineffective specific responses against sloughed endometrial fragments in women with endometriosis.

It remains uncertain as to how sloughed endometrial cells signal the immune response and subsequent attack; however, regardless of the mechanisms involved, the immune cells which are likely to play roles in this destruction, including cells such as macrophages, natural killer (NK) and cytotoxic T-cells, must be tightly regulated in order to ensure that the immune response is specific to sloughed endometrial fragments and not the intact uterine tissue. The cells which are almost certainly the key regulators of this response are a distinct population of T-cells, known as regulatory T-cells (Tregs).

Tregs are specialized immune cells which function to control and suppress a range of immune responses including; T-cell proliferation (Giatromanolaki et al., 2008) and activation (Nandakumar et al., 2009); macrophage, B-cell, DC and NK-cell function (Thornton, 2005; Sakaguchi et al., 2008); mast-cell degranulation (Nandakumar et al., 2009); cell proliferation and cytokine release (Sakaguchi et al., 2008). The vast majority of Tregs arise naturally from a specific lineage in the thymus; however, evidence suggests that a small population of CD4+, and to an even smaller extent CD8+, T-cells can be induced to become ‘adaptive’ Tregs in the periphery, often as a result of T-cell activation in the absence of optimal antigen exposure (Nandakumar et al., 2009). Naturally occurring Tregs, however, play the most crucial roles in controlling, suppressing and modulating a vast variety of immune responses to infection (Fehervari and Sakaguchi, 2004; Giatromanolaki et al., 2008) and/or to the presence of tumors (Arruvito et al., 2007), maintaining self-tolerance and preventing autoimmunity.

Foxp3 (forkhead—winged helix transcription factor family member; Sakaguchi, 2003; Zheng and Rudensky, 2007) has been reported to be a master controlling gene for the development and function of naturally occurring Tregs (Fehervari and Sakaguchi, 2004) and to be essential for both development and function of Treg populations (Liu and Zheng, 2009). Although flow-cytometric immunophenotyping allows for better characterization of Tregs using a collection of markers such as CD4+, CD25+, Foxp3+; CD127low (Liu et al., 2006), to date Foxp3 remains the best marker for analysis and quantification of Treg populations in fixed, paraffin embedded tissue (Giatromanolaki et al., 2008).

Surprisingly, despite the crucial roles Foxp3+ Tregs play in regulation and suppression of immune response, these immune cell populations have not previously been investigated in the eutopic or the ectopic endometrium in women with endometriosis. Immune regulators are likely to play crucial roles in diseases within which numerous immune factors appear to be highly disturbed. This study has thus aimed to analyse the expression of Foxp3+ cells in the eutopic endometrium and peritoneum of women with and without endometriosis, in order to gain insights as to how and at what stages of the cycle these distinct immune cell populations are likely to play the most crucial roles in regulation and/or suppression of immune responses in endometriosis.

Materials and Methods

This study was approved by the Human Ethics Committees of the Sydney Southwest Area Health Service and the University of Sydney.

Tissue collection

Tissue samples from women with and without endometriosis, collected between 1996 and 2009, were identified from the Royal Prince Alfred Hospital Anatomical Pathology Archives.

Uterine curetting samples from 127 women of reproductive age, with and without endometriosis (as confirmed by laparoscopy), were collected for analysis. There were 35 menstrual phase samples (17 and 18 from women with and without endometriosis, respectively), 46 proliferative samples (23 and 23 from women with and without endometriosis, respectively) and 46 secretory samples (25 and 21 from women with and without endometriosis, respectively).

Sound clinical data were available for all subjects. The mean age for women with and without endometriosis was 32.5 years (range 16–48 years) and 36.8 years (range 24–54 years), respectively. Among endometriosis-positive subjects, 65% had peritoneal endometriosis and 35% had ovarian endometriosis with and without endometriosis at other sites. All women with endometriosis were experiencing pain symptoms; however, none had received medical or surgical therapy for endometriosis in the 3 months prior to endometrial sampling.

All samples were staged as accurately as possible by one experienced gynaecological histopathologist (P.R.) according to histological appearance of endometrium (Noyes et al., 1950; Robboy et al., 2002) as early (Days 1 and 2; n = 13), mid (Days 3 and 4; n = 16) or late (Day 5; n = 6) menstrual; early (Days 6–8; n = 14), mid (Days 9–11; n = 17) or late (Days 12–14; n = 15) proliferative and early (Days 15–18; n = 16), mid (Days 19–23; n = 15) or late (Days 24–28; n = 15) secretory phase of the menstrual cycle.

In addition to this, 59 ectopic peritoneal lesions from the same cohort of women in whom peritoneal endometriosis has been identified were collected for analysis. The excision of the lesion occurred between 1995 and 2009, for clinical management of endometriosis.

Fifteen peritoneal biopsies were collected from women undergoing laparotomy for conditions completely unrelated to endometriosis to act as a control group. Informed consents were available for all subjects.

Samples had all been fixed in 10% neutral buffered formalin for ~18–24 h, processed and embedded in paraffin using standard techniques.

Immunohistochemistry

The paraffin embedded tissue blocks were cut at 5 μm and mounted onto glass slides (SuperFrost Ultra Plus, Menzel Glaser, 100 Deckglaser 22 × 50 mm). Prior to immunostaining, the tissue was deparaffinized and rehydrated. Heat-induced antigen retrieval techniques were applied (1: 10 dilution of Target Retrieval-pH 9, Dako Cytomation, Australia). Sections were subjected to DEEB (dual endogenous enzyme block, Dako Cytomation) for 10 min, following which they were immunostained using Foxp3 (236A/E7 clone; 1:100 dilution) monoclonal mouse, antihuman antibody (Sapphire Bioscience—Abcam distributor for Australia) for 30 min.

EnVision+ Dual Link System-HRP (Dako Cytomation) visualization system was used in conjunction with EnVision FLEX+ Mouse (LINKER) for 15 min to amplify the staining signal. Application of liquid
Diaminobenzidine-Substrate Chromogen System (Dako Cytomation) led to formation of brown end-product at the site of target antigen. A sample of tonsil tissue, known to contain FoxP3+ cells, was used as positive control. An additional endometrial tissue specimen was classed as negative control and was only subjected to the immunoglobulin G1 isotope control (matched to the concentration of Foxp3 antibody). All immunostaining was performed using Dako Cytomation Autostainer—Universal Staining System (S3400, Dako Cytomation Inc., Carpinteria, CA, USA). The quality of sections produced by the Autostainer is far superior to manual staining, as it ensures that every section is treated in the same manner, reducing the chances of human error and allows for much better standardization between sections (Hasui et al., 2002). Following staining, the slides were counterstained using Mayer’s haematoxylin solution, dried and cover-slipped using ultramount.

Quantification
Slide analysis was performed using the Olympus BX51 microscope (Olympus, Tokyo, Japan) under ×400 magnification. Tregs were characterized by brown intracellular staining of Foxp3 antibody (Fig. 1A and B). Where possible up to 20 fields of view were analysed for each sample, however, poor quality of some menstrual samples permitted smaller numbers of fields of view to be analysed. In the menstrual group, the slides that showed at least five fields of view of the tissue were included for further analysis and quantification.

The fields of view were captured using Olympus DP70 digital camera (Olympus) and Image Pro Plus Discovery Software, Media Cybernetics (Olympus). The counting was performed on random fields on these images, and the investigator was blinded to the presence or the absence of endometriosis. The counting was also carried out blind with respect to the subphase of the menstrual cycle. All fields of view were quantified by two separate blinded investigators, and the correlation between the two investigators was analysed. The third investigator, experienced gynaecological pathologist, who was also blinded to the presence or the absence of endometriosis, classed the samples as early, mid and late proliferative, menstrual or secretory. In total, 980 and 1025 fields of view were analysed for women with and without endometriosis, respectively.

Statistical analysis
Statistical analysis was performed using the Statistical Package for the Social Sciences 16.0 Statistical Analysis Software. Correlation between two blinded investigators was conducted using interclass correlation (interclass correlation coefficient = 0.958, \( P < 0.001 \)). Within the eutopic endometrium, Foxp3+ cell counts were analysed with respect to the presence and the absence of peritoneal or other endometriosis throughout menstrual, proliferative and secretory phases and were analysed separately according to early, mid or late subgroups. Owing to variation in the number of fields of view between subjects, the data analysis was based on the fields of view. The distribution of Tregs was found to be significantly skewed, thus non-parametric statistical analysis, using Mann–Whitney \( U \)-test (denoted by W \( U \) \( z \)), was performed. The phases and subgroups were analysed using the descriptive statistics, which showed the minimum, maximum, mean and SD values. Statistical analysis using Kruskal–Wallis \( \chi^2 \) (denoted by \( H \) ) was also conducted to investigate the density of Foxp3+ cells within the positive ectopic lesions, across all phases of the menstrual cycle. The differences between the comparison groups were considered to be statistically significant at \( P \)-value of \( < 0.05 \) and highly significant at \( P \)-value of \( < 0.001 \).

Figure 1 Expression of Foxp3+ regulatory T cells (Tregs) in endometriosis. Foxp3+ Tregs are characterized by brown diaminobenzidine chromogen spots in the eutopic endometrium under ×400 (A) and ×1000 (B) magnification, and within ectopic peritoneal lesion under ×400 magnification (C), in a subject with endometriosis.
Results

The counts of Foxp3+ Tregs were expressed as the mean numbers (±SD) of Foxp3+ cells per square millimetre in eutopic endometrium of women with and without endometriosis, across menstrual, proliferative and secretory phases of the menstrual cycle. Figure 1 shows the expression of Foxp3+ cells in the eutopic endometrium for an endometriosis positive subject under ×400 (A) and ×1000 (B) magnifications.

Expression of Foxp3+ cells in the eutopic endometrium of women with and without endometriosis

Across all phases of the menstrual cycle, the mean density ±SD, of Foxp3+ cells was found to be 9.3 ± 27.9 and 9.6 ± 19.7 for women without and with endometriosis, respectively (W U z = −2.75; P = 0.010). Figure 2 compares the mean density of Foxp3+ cells in women with and without endometriosis, across menstrual, proliferative and secretory phases of the cycle.

Secretory-phase Foxp3 expression

Highly significant increase in Foxp3+ cell density was observed in women with endometriosis (mean ± SD = 10.3 ± 26.3; range = 0–339.1), compared with those women who did not have this disease (mean ± SD = 4.4 ± 8.5; range = 0–52.2), during the secretory phase of the menstrual cycle (W U z = −5.36; P < 0.001). This significant increase was also evident throughout all subgroups of the secretory phase in women with endometriosis, including: early (W U z = −3.34; P = 0.001), mid (W U z = −3.65; P < 0.001) and late (W U z = −1.92; P = 0.055) (Fig. 3C).

Menstrual-phase Foxp3 expression

During the menstrual phase, an observed trend suggested that the density of uterine Foxp3+ cells was generally higher across the first two parts of the phase in women without endometriosis (mean ± SD = 10.3 ± 44.1; range = 0–643.5) than those with endometriosis (mean ± SD = 5.8 ± 9.3; range = 0–43.5); however, this difference was not statistically significant (W U z = −1.33; P = 0.185). Significant difference in the density of Foxp3+ cells in women with and without endometriosis was most evident during the early menstrual phase, with significantly lower levels in endometriosis (W U z = −3.48; P = 0.001); however, significant differences between the two groups were not demonstrated during the mid (W U z = −0.64; P = 0.521) and late (W U z = −0.07; P = 0.946) stages of the menstrual phase. The histogram (Fig. 3A) shows Foxp3+ cell densities per square millimetre within the functional layer of eutopic endometrium in women with and without endometriosis during the early, mid and late stages of the menstrual cycle phase.

Proliferative-phase Foxp3 expression

Although an overall significant difference was not detected between women with (mean ± SD = 11.2 ± 15.1; range = 0–121.7) and without (mean ± SD = 12.9 ± 21.6; range = 0–208.7) endometriosis during the proliferative phase (W U z = −0.28; P = 0.779), a highly significant increase in Foxp3+ cell density was observed during the early proliferative phase in women with endometriosis (W U z = −5.61; P < 0.001). Although the density of Foxp3+ cells rose linearly from early to late proliferative phase in women without endometriosis, the density of Foxp3+ cells in women with endometriosis did not follow the same pattern (Fig. 3B). Interestingly, a trend suggested a much lower density of Foxp3+ cells was present in women with endometriosis during the mid (W U z = −1.970; P = 0.049) and late (W U z = −1.72; P = 0.086) stages of the proliferative phase, in comparison to controls.

Foxp3+ cells in ectopic peritoneal lesions in women with endometriosis and within the normal peritoneum in women without the disease

Analysis of Tregs within peritoneal endometriotic lesions revealed that only 18 out of 59 lesions were positive for Foxp3, whereas the remaining 41 lesions did not demonstrate any Foxp3+ cells. Within these positive lesions, Foxp3 expression was observed exclusively within the lesion tissue and was never seen in the adjacent or more distant peritoneum. In addition to this, no Foxp3 staining was identified within 15 peritoneal biopsies of women without endometriosis. Within the positive lesions, the density of Tregs ranged from 3.0 to 51.6 positive cells per square millimetre, (mean ± SD = 17.2 ± 15.1), the mean being greater than in any of the eutopic endometrium.
groups. Significant differences could not be observed in the ectopic expression of Foxp3 during the time eutopic endometrium was at menstrual, proliferative and secretory phases (\(H = 0.77, df = 2, P = 0.697\)); however, the pattern of Foxp3 expression within the positive lesions correlated broadly with the expression of Foxp3 within the eutopic endometrium of women with endometriosis (Fig. 4). At present, no clinical or other correlates could be identified, which would obviously explain why some lesions were negative, yet others were positive.

**Discussion**

This study has demonstrated that significant changes in the density of endometrial Foxp3+ cells occur across the menstrual cycle in women with and without endometriosis. Foxp3+ naturally occurring Tregs play crucial roles in modulating immune response (Arruvito et al., 2007) and function to maintain immune tolerance, which allows the immune system to differentiate between self and non-self antigens (Roncador et al., 2005). In a disease in which viable
endometrial ‘self-antigens’ almost certainly escape immune surveillance (Matarese et al., 2003; Kibangou Bondza et al., 2006) prior to their subsequent ectopic establishment and growth, alterations in Foxp3+ Treg populations are likely to be linked to pathogenesis and progression. Our novel findings are likely to influence the way we view and better understand dysregulated immune response in endometriosis.

Strikingly, our results showed that secretory phase endometrial Foxp3+ cell populations in particular are significantly altered in endometriosis. A significant decrease, which was observed between late proliferative and early secretory phases in women without endometriosis (P < 0.001), was not seen in women with the disease. Previous evidence suggests that during the normal menstrual cycle, peripheral blood Foxp3+ cells are dramatically down-regulated in the luteal phase compared with the follicular phase (Arruvito et al., 2007). Our data showed that in endometriosis, the density of uterine Foxp3+ cells remained relatively constant in women with endometriosis. However, the density of Foxp3+ cells in the controls did not follow this pattern. This may be because elevated expression of Foxp3+ cells during the previous phase in women with endometriosis interferes with the ability of newly recruited immune cell populations to effectively target endometrial antigens. When menstruation does occur in women with endometriosis, Foxp3 levels are reduced, as there is no further need to suppress already reduced immunological responses. Instead, in women without endometriosis Foxp3 cell density was elevated during the menstrual phase, and this difference was particularly evident during the early menstrual phase (P = 0.001). It is likely that during this time, under the normal conditions, increased Foxp3 expression functions to tightly regulate the immunological reactions within the endometrium, and in doing so, ensuring that the ability of immune cells to exert their response against autologous endometrial tissue is now suppressed and controlled. However, immune cell populations did show great variability among individual subjects throughout the menstrual phase. These interactions appear extremely complex and thus it is very difficult to speculate as to what these differences in Foxp3 cell expression, among women with and without endometriosis are likely to be attributed to.

A study which investigated the expression of Foxp3+ cells in the peripheral blood of healthy, non-pregnant women suggested that peripheral Treg populations undergo profound changes during the menstrual cycle (Arruvito et al., 2007). This study showed that the density of peripheral Foxp3+ cells increases during the follicular phase, reaching its peak during the late proliferative phase, at which time serum estradiol levels are also elevated (Arruvito et al., 2007). It has been proposed that under normal conditions a pre-ovulatory rise in Foxp3+ cells may be required for the induction of immune tolerance required to facilitate successful embryo implantation, should it occur (Arruvito et al., 2007). Our results suggest that endometrial Foxp3+ cell densities in women without endometriosis also follow this pattern, with Foxp3+ cells increasing linearly from early to late proliferative phase. In women with endometriosis, however, Foxp3+ cells appear to behave differently during the proliferative phase: the density of Foxp3+ cells was significantly higher during the early proliferative phase in women with endometriosis, in comparison to controls (P < 0.001). Although the density in the controls rose gradually throughout the proliferative phase, in endometriosis a substantial decrease in Foxp3+ cell density was observed between early and mid proliferative phase and was maintained throughout the late proliferative phase. Down-regulation of pre-ovulatory Foxp3 expression is likely to contribute to reduced fertility rates in women with endometriosis (Arruvito et al., 2007).

There appears to be a positive association between estrogen and Foxp3+ cell expression. Estrogen has been shown to modulate Tregs by inducing cell proliferation and subsequent immune response suppression in endometrium (Arruvito et al., 2007). Endometriosis is an estrogen-dependent disease (Zeitoun et al., 1998) and increased local tissue aromatase activity in women with endometriosis has been shown to enhance estrogen biosynthesis and growth of endometriotic tissue (Bulun et al., 2001).

During the normal menstrual cycle, the major influx of immune cell populations into the endometrium occurs during the secretory phase (Salamonsen and Lathbury, 2000). It is during this time, that in endometriosis, up-regulated Foxp3 expression is likely to exert immunosuppressive effects (Jasper et al., 2006) on newly recruited immune

Figure 4 Density of Foxp3+ cells in ectopic peritoneal lesions in women with endometriosis, during the times when eutopic endometrium was in menstrual, proliferative or secretory phase of the cycle. Although no significant differences were observed between various stages of the menstrual cycle, the pattern of expression of Foxp3+ cells broadly followed that observed in eutopic endometrium in women with endometriosis, and densities of Foxp3 cells tended to be higher than in eutopic endometrium. Data are represented as mean ± SD, n = number of subjects.
cell populations, decreasing their cytotoxicity, phagocytic capacities and their general ability to effectively target endometrial antigens. Subsequently, these antigens may escape immune surveillance and implant at ectopic sites, establishing the classic lesions which we call endometriosis. In addition to this, convincing evidence suggests that Tregs-induced down-regulation of CD80 and CD86 molecules on mature DCs decreases their ability to effectively present antigens (Fehervari and Sakaguchi, 2004). CD80 and CD86 play important roles in the antigen presentation process by co-stimulating major histocompatibility complex, T-cell receptor interaction, required for T cell activation and antigen-specific response (Melichar et al., 2000).

Our previous study has shown that another marker of DC maturation, CD83, is down-regulated across all phases of the menstrual cycle in the eutopic endometrium in women with endometriosis (Schulke et al., 2009). Whether or not Tregs are solely responsible for altering the function of DCs in endometriosis is difficult to say, however, it is highly likely that they are implicated in this response.

Other factors are also likely to be influencing the expression of Treg and DC populations in endometriosis. Vascular endothelial growth factor, an angiogenic factor which is highly up-regulated in both the eutopic and DC populations in endometriosis. Vascular endothelial growth factor, an angiogenic factor which is highly up-regulated in both the eutopic and DC populations in endometriosis. In conclusion, we believe that previously unrecognized components of the adaptive immune response, particularly DC development, antigen capture and presentation, as well as Treg-mediated immune regulation, are almost certainly playing crucial roles at various stages during the establishment and progression of endometriosis. A better understanding and defining of the role of specific immune responses in endometriosis, with respect to study characteristics, lesion types and associated symptoms is a matter of priority, in order to better understand how the immune system truly fights endometriosis throughout its progression, and why and at what stages this response fails. Such understanding has considerable potential to change the way we view other chronic diseases that have a substantial immunological component, how we detect them and may better manage them in the future.

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