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Peripheral and endometrial dendritic cell populations during the normal cycle and in the presence of endometriosis

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Abstract

Background—Dysfunctional immune response may be implicated in endometriosis pathogenesis, and dendritic cells (DC) may play greater roles in this response than previously recognized. This study set out to evaluate peripheral blood and endometrial DC population changes in the presence and absence of endometriosis pathology.

Methods—Endometrial ($n = 83$) and peripheral blood samples ($n = 30$) were subjected to immunohistochemical techniques and flow cytometry, respectively, to assess DC populations in women with and without endometriosis. Three circulating DC subsets (MDC1, MDC2 and PDC, expressing CD1c, CD303 and CD141), and late-stage mature endometrial DCs (using DC-LAMP antibody) were investigated.

Results—A highly significant reduction in CD1c intensity on MDC1 populations in peripheral blood was observed between normal cycle proliferative and menstrual phases ($p = 0.025$), but not in women with endometriosis, in whom CD1c intensity was markedly increased at the time of menstruation ($p = 0.05$). A significant reduction in peripheral blood MDC2 ($p = 0.016$) and apparent reduction in endometrial DC-LAMP+ DC (trend, $p = 0.062$) were observed in women with endometriosis compared with controls, consistent with our preliminary DC data.

Conclusions—Cyclical variation in endometrial and circulating DC populations appears to be crucial during normal menstrual cycles and in the establishment of pregnancy. In endometriosis, circulating and endometrial DC populations are significantly dysregulated at a number of levels, and are likely to contribute to inefficient immunological targeting of endometrial fragments shed at menstruation, facilitating their survival and establishment of endometriosis.

Keywords

Blood; Dendritic cells; Endometriosis; Endometrium; Immune cells

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INTRODUCTION

Endometriosis is a common gynecological condition defined by the presence of endometrial-like tissue outside the uterine cavity causing symptoms such as pain and infertility (1). Although several theories of pathogenesis have been proposed, endometriosis remains an enigmatic disease (2–5). In recent years, evidence has suggested that dysfunctional immune response may be implicated in the pathogenesis of this disease (6–9). It has been proposed that inefficient immunological targeting via defective antigen capture and presentation may allow for the survival and ectopic establishment of shed endometrial fragments at the time of menstruation. Dendritic cells (DCs), professional antigen-presenting cells (APCs), which play crucial roles in efficient antigen capture and presentation are likely to be implicated in this response (9).

DCs are specialized immune cells, involved in both innate and adaptive T-lymphocyte-mediated immunological responses. DCs originate from both myeloid and lymphoid precursors, which in peripheral circulation give rise to myeloid DCs (MDCs) and plasmacytoid DCs (PDCs), respectively. While MDCs are involved in antigen presentation and response via major histocompatibility complex II (MHC-II), PDCs are thought to be involved in mechanisms of the innate immunity, through secretion of interferons and in induction of tolerance (10–12).

DCs that migrate from circulation into peripheral tissues undergo maturation and acquire the ability to efficiently target foreign antigens. Late-stage mature DCs play a major role in antigen presentation (13–15). We have previously shown that endometrial DCs, like other endometrial immune cell populations, exhibit specific patterns during the normal menstrual cycle. Significantly increased numbers of immature and mature DCs were observed during normal menstruation (16). Furthermore, in endometriosis, despite a sufficient presence of immature DCs, numbers of mature DCs were significantly reduced across all phases of the cycle, suggesting that the numbers and/or function of DCs may be affected in the disease (9).

To better define the roles of DCs in the normal menstrual cycle and in endometriosis, it is important to characterize DCs not only in the endometrium, but also within the peripheral blood. However, to our knowledge no previous study has investigated circulating DCs during the normal menstrual cycle or in the presence of endometriosis. This study aimed for the first time to characterize peripheral blood DC subsets and to quantify late-stage mature endometrial DCs to better understand the potential roles of DCs in the presence and absence of disease.

MATERIALS AND METHODS

This study was approved by the human ethics committees of the Sydney South West Area Health Service and The University of Sydney.

Patients

Blood samples were collected from 30 women with or without endometriosis, at the time of investigative laparoscopy at Royal Prince Alfred Hospital (RPAH), Sydney. Paraffin-embedded endometrial curetting samples (n = 83) were obtained from the RPAH pathology archives. The presence or absence of endometriosis was visually and histopathologically confirmed in all patients at surgery. The stage of the menstrual cycle was determined by an experienced gynecological histopathologist according to the appearance of the endometrium. Blood samples were from 16 patients with endometriosis (mean age 30.5, range 29–40; proliferative n = 6, secretory n = 7, menstrual n = 3) and 14 control subjects (mean age 35.4, range 29–43; proliferative n = 5, secretory n = 6, menstrual n = 3). Endometrial samples were from 42 women with endometriosis (mean age 35.1, range 20–48; proliferative phase n = 14, secretory phase n = 16, menstrual phase n = 12) and 41 women without endometriosis (mean age 34.7, range 21–49; proliferative n = 17, secretory n = 13, menstrual n = 11).

Comprehensive clinical information was collected for all subjects. Samples were from regularly menstruating women, none of whom had received hormonal treatment within the 3 months prior to surgery. None were known to have an autoimmune condition, and they had not had a recent cold/flu infection. Indication for laparoscopic investigation in control subjects included benign gynecological pathologies such as fibroids, ovarian cysts or tubal blockage.

Flow cytometry

Blood samples collected into Vacutainer Lithium Heparin tubes (BD Biosciences, Franklin Lakes, NJ, USA) were prepared using a Blood Dendritic Cell (BDC) Enumeration kit, Human (Miltenyi Biotec Inc., Auburn, CA, USA). Anti-BDCA cocktail contained monoclonal antibodies conjugated to fluorochromes to identify MDC1, MDC2 and PDC DCs (CD1c-PE, CD141-APC and CD303-FITC, respectively). Dead Cell Discriminator and Red Blood Cell Lysis solutions were utilized. Cells were washed and fixed with formaldehyde in Fix Solution.

Labeled cells were analyzed by flow cytometry using the BD FACSCanto II flow cytometer and BD FACSDIVA software (BD Biosciences, Franklin Lakes, NJ, USA). Approximately 1 million events were collected for each sample. Compensation beads (Miltenyi Biotec Inc., Auburn, CA, USA) coupled to each fluorochrome were utilized to improve data integrity. Gates were established to exclude dead cells, debris, B cells and monocytes. The relative proportion and total numbers of the 3 DC subsets (MDC1, MDC2 and PDC) were calculated, and CD1c, CD141 and CD303 expression assessed within these populations (respectively) (Fig. 1).

Immunohistochemistry

Paraffin-embedded endometrium blocks were cut at 5 μ m and mounted onto glass slides. Following deparaffinization and rehydration, slides were treated in alcohol-ammonia solution for 1 hour to remove formalin pigment. Antigen retrieval was performed on slides for immunohistochemical staining at 95°C–99°C in diluted, preheated pH 9 Target Retrieval Solution (Dako, Glostrup, Denmark). Dual Endogenous Enzyme Block (Dako, Glostrup,

Denmark) was applied followed by monoclonal mouse anti-human DC-LAMP antibody (Dendritics, Lyon, France; 15 µg/mL dilution) to specifically detect late-stage mature DCs. An EnVision+ Dual Link detection system (Dako, Glostrup, Denmark) was used with Liquid Diaminobenzidine+ (DAB+) Substrate Chromogen System (Dako, Glostrup, Denmark) for visualization. The Dako Autostainer Plus Model S3400 (Dako, Glostrup, Denmark) was used to perform all immunostaining. Staining with isotype controls was negative.

Slide analysis was conducted using an Olympus BXS1 microscope (Olympus, Tokyo, Japan) coupled with a DP70 camera (Olympus, Tokyo, Japan). Blinded DC counting was performed on 20 random fields of view captured under $\times 400$ magnification. DCs were identified according to their morphology and staining characteristics. Counts were expressed as density per mm².

Statistical analysis

The distribution of all variables (peripheral blood DC populations MDC1, MDC2 and PDC and DC-LAMP+ endometrial DCs) was examined using the 1-sample Kolmogorov-Smirnov test, and all were found to be significantly skewed. Comparisons were made between the 2 groups (women with and without endometriosis), and then these group comparisons were repeated within each of the 3 menstrual cycle phases separately. These 2 group comparisons were made with the nonparametric Mann-Whitney U z test (denoted by U z). The data were also split by group, and phase effects explored within the women with and without endometriosis separately with the Mann-Whitney U z test (denoted by U z). SPSS Statistical Analysis Software (Version 17.0) was used to perform all statistical analyses. Statistical significance was established at p values of less than 0.05.

RESULTS

Peripheral blood DCs

Variations in MDC1, MDC2 and PDC numbers and intensity of antigen expression were observed in the peripheral blood during the normal menstrual cycle, as shown in Figure 2.

MDC1

A significant increase in CD1c staining intensity in peripheral blood occurred during the proliferative phase of the normal cycle ($p = 0.025$, $U z = -2.236$; Fig. 2B). While in the normal cycle, CD1c intensity gradually reduced from the proliferative phase to menstruation, this decrease was not seen in women with endometriosis (Fig. 2B). Hence, CD1c intensity was greatly reduced during normal menstruation; however, a strong trend indicated that in women with endometriosis, CD1c expression was greatest during menstruation ($p = 0.050$, $U z = -1.960$; Fig. 2B).

Numbers of peripheral blood CD1c+ MDC1 appeared to be higher in women with endometriosis compared with controls during both the proliferative and secretory phases of the cycle; however, these differences were not statistically significant (Fig. 2A).

MDC2

A highly significant reduction in MDC2 numbers in peripheral blood occurred between the secretory and menstrual phases of the cycle in women with endometriosis ($p = 0.016$, $U z = -2.400$; Fig. 2C). In women without endometriosis, however, there were no statistically significant changes in MDC2 density between phases of the menstrual cycle. CD141 expression did not significantly vary during the normal menstrual cycle or in women with endometriosis (Fig. 2D).

PDC

In women both with and without endometriosis, numbers of circulating PDCs increased between the proliferative and secretory phases, before decreasing again in menstruation (Fig. 2E). In women without endometriosis, the intensity of CD303 expression on PDCs appeared to decrease from the beginning to end of the menstrual cycle. In contrast, in the endometriosis group, intensity of CD303 expression tended to increase during the cycle (Fig. 2F).

Endometrial DCs

DC-LAMP+ DCs were demonstrated in the endometrium (Fig. 3) from endometriosis and control subjects, with changes in density apparent during the menstrual cycle (Fig. 4). A highly significant increase in the numbers of endometrial DC-LAMP+ DCs was observed between secretory and menstrual phases of the normal cycle in women without endometriosis ($p = 0.013$, $U z = -2.495$). In these women without endometriosis, endometrial DC-LAMP+ DC density then decreased between menstruation and the proliferative phase ($p = 0.060$, $U z = -1.883$). In contrast, in women with endometriosis, these cyclical variations were not observed. Overall, DC-LAMP+ DC density appeared to be reduced in women with endometriosis ($p = 0.062$, $U z = -1.870$) compared with controls.

DISCUSSION

DC populations appear to be playing important roles during the normal menstrual cycle and are altered at both local and systemic levels in women with endometriosis. Like other immune cell populations that are recruited into the endometrium during the normal cycle, DCs are likely to facilitate the clearance of the endometrial cavity following menstruation. Failure of DCs to do this efficiently may facilitate survival of shed endometrial fragments and their consequent establishment in the form of ectopic lesions in women with endometriosis. While it is not yet clear whether DCs are recruited into the endometrium from the systemic circulation, or undergo clonal expansion locally, it is plausible that cyclical fluctuations in peripheral blood DC pools may at least to some degree be related to the changes in DC density and function within the endometrium.

To better understand the role of DCs in the presence of endometriosis, it is important that we first understand their role in the absence of pathology. Previous studies have elucidated a link between peripheral blood DC populations and endometrial function. It has been shown that tight regulation of the numbers and function of blood myeloid and lymphoid DCs may be crucial for establishment of maternal tolerance to allogeneic fetus during a successful

pregnancy (17). While it is less certain what potential roles DC populations may be playing during the normal menstrual cycle, fluctuations in the numbers of peripheral DC pools during various phases of the cycle are likely to be reflective of a normal physiological process.

This study observed variations in peripheral blood DC populations during the normal menstrual cycle. Both myeloid (MDC1 and MDC2) and lymphoid (PDC) populations were most numerous just prior to the onset of menstruation, during the secretory phase, although this increase was not found to be statistically significant. An expansion of circulatory DC pools from the proliferative into the secretory phase was followed by a decline in DC numbers during menstruation, providing some evidence to suggest that during this time, DCs are actively recruited from the systemic circulation into the endometrium. It has previously been shown that during the normal cycle, a range of immune cells are recruited into the endometrium in preparation for menstruation, where early inflammatory changes facilitate endometrial clearance, regeneration and repair following menstrual breakdown (18). Unlike the cells of the innate immune system, APCs are likely to require additional time to mount specialized responses. This may explain why it is not until the menstrual phase that we observe a marked increase in the numbers of endometrial DC populations.

Both immature (CD1a+) and mature (CD83+) DC populations have previously been shown to be most numerous at the time of menstruation (9). The present study demonstrated significant phase effects in the numbers of endometrial DC-LAMP+ cells, with the highest numbers of these mature cell subsets also present during the menstrual phase. DC-LAMP+ cells are believed to function in the presentation of endogenous antigen to T cells (13, 19). This provides additional data concerning the role of mature DCs during menstruation in women with and without endometriosis compared with our earlier study (9). Within the endometrium, DCs are likely to be exerting a number of functions, particularly around the activation of targeted immunological responses, thus facilitating clearance of the endometrial cavity of menstrual debris and in preparation for pregnancy.

While there are clear variations in the numbers of peripheral and endometrial DCs during the phases of the normal cycle, it remains difficult to interpret the exact roles individual DC subset populations may be playing during this time. Alterations in DC function during different stages of the menstrual cycle are suggested by variations in the intensity of antigen expression throughout the normal cycle. The intensity of CD1c antigen expression on MDC1 populations significantly decreased from the proliferative to menstrual phase during the normal cycle. CD1c is known to play a role in antigen recognition and presentation of microbial and altered lipid antigens (20, 21). With recruitment of MDCs into the endometrium, the highest CD1c expression during the proliferative phase under normal conditions is likely to contribute to recognition and efficient clearing of shed fragments following menstruation.

The findings from the current study indicate that CD1c expression on circulating MDC1 populations is significantly reduced from the proliferative to menstrual phase of the normal cycle ($p = 0.025$). CD1c is 1 of 4 CD1 functional isoforms expressed on APCs (22). Alongside CD1a, b and d, CD1c is capable of presenting a wide variety of self and foreign

antigens to T cells (23). Evidence suggests that CD1c expression is up-regulated when DCs are activated (23). Unlike CD1d, which may down-regulate APC function, CD1c can “override” a CD1-induced inhibitory effect, thus potentiating the DC function and their antigen-presenting capacity (22). A reduction in CD1c expression from the proliferative to menstrual phase during the normal cycle is thus likely to directly reflect changes in MDC function during the cycle.

In women with endometriosis, DC populations appear to be significantly dysregulated, at a number of levels, in comparison with control subjects. Unlike what is seen during the normal menstrual cycle, cyclical variations in CD1c expression on peripheral blood MDC1 cells are not observed in women with endometriosis. Persistent CD1c expression in endometriosis may result from inefficient targeting of viable shed endometrial fragments and is likely to contribute to the highly proinflammatory profile that is characteristic of this disease state. Furthermore, a failure of the immune system to down-regulate the Th1 response and initiate a switch towards Th2 activity in endometriosis is likely to contribute to infertility in these women (24).

In addition to altered DC activity, a highly significant reduction in the numbers of peripheral blood MDC2 populations was observed between the secretory and menstrual phases in endometriosis. MDC2 are an extremely rare subset of DCs in peripheral blood, constituting only 0.02% of all leukocytes (25). Due to their scarcity, their exact function remains uncertain; however, like other MDC populations, MDC2 are thought to contribute to efficient processing, uptake and presentation of foreign antigens (26). It has previously been speculated that, in women with endometriosis, the ability of DCs to effectively capture antigens may be compromised (9). It may be hypothesized that the inability of the immune system to successfully clear the endometrial cavity in the presence of this disease may contribute to the survival of cellular fragments and subsequent establishment of endometriotic lesions. A significant reduction in the numbers of mature endometrial DC populations across all phases of the menstrual cycle in endometriosis has previously been described (9). The reduction in the numbers of DC populations in women with the disease provides evidence for dysregulated recruitment and/or maturation of DCs in the presence of endometriosis. The results from the current study indicate that the numbers of DC-LAMP+ cells are not significantly reduced in women with endometriosis, suggesting that late-stage mature DC populations expressing DC-LAMP are likely not to be significantly disturbed in endometriosis.

The mechanisms by which peripheral DCs are recruited into the endometrium are yet to be elucidated; however, this study suggests that both circulating blood and endometrial DC populations are likely to play important roles during the normal menstrual cycle. While it remains uncertain if alterations in DC subsets are primary, (preceding dysregulation of DC populations within the eutopic endometrium in endometriosis) or secondary (occurring in response to abnormal eutopic endometrium) to the disease state, the current study suggests that distinct DC subsets are significantly altered in women with endometriosis. It is important to acknowledge that one of the main limitations of this preliminary study are the low sample numbers, particularly during the menstrual phase of the cycle. Obtaining fresh, well-characterized human samples is a real challenge, particularly during this time in a

clinical setting. Further functional studies are needed to elucidate the mechanisms by which DCs are recruited into the endometrium in the presence and absence of pathology and how they may participate in the establishment and maintenance of lesions in women with the disease.

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Fig. 1.

A diagram showing gating hierarchy in the flow cytometric analysis of CD1c+ MDC1, CD141+ MDC2 and CD303+ plasmacytoid dendritic cells (PDCs) in peripheral blood.

Fig. 2.

Boxplots showing the numbers and intensity of antigen expression for CD1c+ MDC1, CD141+ MDC2 and CD303+ PDCs in peripheral blood from women with and without endometriosis throughout the menstrual cycle. **A)** CD1c+ MDC1 density, **B)** CD1c intensity on MDC1, **C)** CD141+ MDC2 density, **D)** CD141 intensity on MDC2, **E)** CD303+ PDC density and **F)** CD303 intensity on PDCs. The middle lines in the boxes represent the median. The lower and upper parts of the boxes represent 25th and 75th percentiles of data distribution. The length of the boxes represents the interquartile range (IQR), and the whiskers (lines above and below the box) represent the range of values that fall within 1.5 x IQR.

Fig. 3.
Characteristic DC-LAMP+ dendritic cell morphology in proliferative phase endometrium stained brown with DAB+ Chromogen (x400 magnification).

Fig. 4.

Boxplot showing the density of endometrial DC-LAMP+ DCs (per mm²) in women with and without endometriosis during the menstrual cycle. The middle line in each box represents the median DC-LAMP+ DC density. The lower and upper parts of the boxes represent the 25th and 75th percentiles of data distribution. The length of the boxes represents the interquartile range (IQR), and the whiskers (lines above and below the box) represent the range of values that fall within 1.5 x IQR.