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A Novel Pilot Study of Endometrial Stromal Cells and Immune Cell Populations in Sentinel Uterine-Draining Lymph Nodes During the Menstrual Cycle and in Endometriosis

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Abstract

Recent studies suggest that changes in certain uterine immune cell populations in endometrium of women with endometriosis are likely to precede changes at ectopic sites. This preliminary study is a first look into the function of uterine-draining lymph nodes (LNs) during the menstrual cycle and in the presence of endometriosis. Paraffin-embedded obturator LNs were obtained from women with (n = 7, mean age 44.3) and without (n = 9, mean age 38.4) endometriosis, who had undergone hysterectomy for cervical or ovarian cancer and in whom LN involvement was not detected. Immunohistochemical staining for endometrial stromal cells and a range of immune cell populations was performed. The CD10+ endometrial stromal cells were detected in uterine-draining LNs throughout the menstrual cycle with numbers peaking during menstruation. The inflammatory process of menstruation was also associated with increased numbers of CD3+, CD4+, Foxp3+, DC-Sign+, CD68+, CD20+, CD79+, and plasma cells. In endometriosis, CD10+ endometrial stromal cells were further increased in numbers, but CD3+, CD4+, DC-Lamp+, FoxP3+, and plasma cells were reduced. This study indicates that efficient immunological responses may be required to contain shed endometrial fragments within the draining uterine LNs thus preventing their further dissemination with establishment of ectopic lesions at distant sites.

Keywords

lymph nodes, menstrual cycle, endometriosis, immunology, lymphatic spread

Introduction

Uterine immune cell populations exhibit dynamic alterations throughout the menstrual cycle which are believed to be associated with endometrial tissue destruction, remodeling, and repair.[1-4] Recruitment of neutrophils and macrophages to the endometrium premenstrually is thought to be important for orchestration of normal menstruation.[1,3] In contrast, very little is known about the immunological environment in the lymph nodes (LNs) draining the uterus and the role of the lymphatic system during menstruation.

The LNs are organized collections of lymphoid tissues strategically located to trap foreign antigens and products of tissue damage (eg, cell debris) during injury, infection, or cancer. As a result a combination of innate (nonantigen specific) and specific adaptive immune responses is initiated with the aim of containing and then destroying the original insult. In the case of uterus, the draining lymph primarily enters the obturator LNs,[5] but there is also drainage into ureteral, hypogastric, external and common iliac, periaortic, lateral, and middle sacral, and femoral LNs.[6]

Menstruation is an inflammatory process characterized by tissue edema and leukocyte infiltration, which results in the shedding of the endometrium and potential exposure of host tissues to microorganisms within the uterine cavity.[7,8] It is therefore likely

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that the endometrial tissue shed at menstruation may enter the lymphatic vessels, exposed by tissue breakdown and be channeled via afferent lymphatic vessels into the uterine-draining LNs, where appropriate immunological targeting is likely to occur. On the other hand, if these endometrial fragments were to escape efficient immune surveillance mechanisms, either locally (within the endometrium) or within draining LNs, viable cells would have the potential to implant within the peritoneal cavity (and elsewhere) in the form of endometriotic lesions.

Endometriosis is a benign gynecological condition that affects up to 20% of women during their reproductive years. Like menstruation, it is considered to be an inflammatory condition,[9] and recent evidence suggests that distinct leukocyte subsets, such as macrophages, dendritic cells (DCs) and T cells, are significantly dysregulated within the eutopic endometrium and that these alterations may precede the well-documented changes at ectopic sites.[10-14] Moreover, one may infer that uterine-draining LNs from patients with this condition have failed to contain endometrial fragments. Investigation of the immunological capabilities of those LNs in endometriosis is therefore of particular interest given that it is an invasive condition, characterized by increased angiogenesis, cellular proliferation, and lesion progression[15-17] possibly associated with lymphatic spread as well as retrograde menstrual flow.[18-20]

To our knowledge, no previous study has examined the changes in leukocyte subsets, which occur in sentinel uterine LNs throughout the menstrual cycle or those of women with endometriosis, presumably due to the difficulty in obtaining appropriate samples. Although the current literature contains a number of reports on LN involvement, in isolated cases of deep infiltrating endometriotic lesions,[21,22] the role of uterine-draining LNs in recognizing shed endometrial fragments during menstruation and in the presence of endometriosis has never been investigated previously. The aim of the present pilot study was to rectify this situation by characterizing the leukocyte subsets present in the obturator LNs from individuals with and without endometriosis.

Materials and Methods

This study was approved by the Human Ethics Review Committee of the Sydney South West Area Health Service and The University of Sydney Human Research Ethics Committee.

Tissue Collection

Obturator LNs collected from 16 premenopausal women (mean age 41.2, range 30-48) who had undergone hysterectomy or parametrectomy and LN dissection were obtained from recent pathology archives at Royal Prince Alfred Hospital (Sydney, Australia). Hysterectomy samples were staged by an experienced gynecological histopathologist according to the histological appearance of the endometrium as menstrual (days 1-5; n = 4), proliferative (days 6-14; n = 9), or secretory (days 15-28; n = 3) phase of an idealized 28-day menstrual cycle. Detailed clinical information was collected from medical records and recorded in a standard format. Because the removal of LNs from disease-free women is limited by ethical considerations, all these patients had early-stage ovarian or cervical cancer. All patients were free of nodal metastases and had not been taking hormonal contraceptives or other hormonal treatment at any time within the 3 months prior to surgery. An additional diagnosis of endometriosis had been made in 7 patients (mean age 44.3, range 39-48; menstrual phase n = 1, proliferative phase n = 4, and secretory phase n = 2). In order to investigate the effects of endometriosis on LN cell composition, these patients with endometriosis were compared with the remaining "control" participants (mean age 38.4, range 30-47; menstrual phase n = 3; proliferative phase n = 5; secretory phase n = 1).

Tissue samples had been previously fixed in formalin–acetic acid and embedded in paraffin wax according to a standardized protocol. Paraffin-embedded tissue blocks were cut at 4 μm and mounted onto glass slides. The sections were cut through the central regions of LNs, ensuring that full-thickness cortex and medulla regions were obtained. Dried slides were deparaffinized through xylene and a series of alcohols into water. Rehydrated slides were treated in alcohol–ammonia solution for 1 hour to remove formalin pigment.

Immunohistochemistry

Deparaffinized slides for immunohistochemical staining underwent antigen retrieval for 20 minutes at 95 to 99°C in preheated Target Retrieval Solution, pH 9 (Dako, Glostrup, Denmark). Dual Endogenous Enzyme Block (Dako) was applied for 10 minutes followed by primary antibodies for 30 minutes as detailed in Table 1. Additionally, slides stained for Foxp3 were then incubated for 15 minutes with EnVision *FLEX*+ Mouse (LINKER; Dako) to amplify the signal.

EnVision+ Dual Link System-horseradish peroxidize detection system was applied to all slides for 30 minutes followed with liquid diaminobenzidine+ (DAB+) substrate chromagen system (Dako) for 10 minutes. As a result of the enzyme–substrate reaction, DAB+ produced a brown end product at the site of the target antigen. A Dako Autostainer Plus Model S3400 (Dako) was used to perform all immunostaining. Following immunostaining, the slides were counterstained with Mayer hematoxylin solution, dehydrated, and cover slipped using ultramount.

As a part of the optimization process, staining with isotype controls matched to the host species and working concentration of the primary antibody (μg/mL) was performed. All staining with isotype controls was negative.

Quantification

Staining intensity (brownness) and positive (brown) and negative (blue) areas were quantified for all the antibodies using an Automated Cellular Imaging System (ACIS; Dako). With standardized tissue processing and immunostaining protocols, as used in this study, ACIS provides sensitive, reproducible, and accurate quantification.[23,24] Positive (light and dark brown) and negative (blue) color thresholds were set for each antibody.

Table 1. Primary Antibodies Used in Lymph Node Immunostaining.

Antibody	Identifies	Dilution	Species	Supplier
CD10	Endometrial stromal cells	Ready to use	Monoclonal mouse (clone 56C6)	Dako, Glostrup, Denmark
T cells and dendritic cells				
CD3	All T cells	1:600	Polyclonal rabbit	Dako, Glostrup, Denmark
CD4	Effector T cells	Ready to use	Monoclonal mouse (clone 4B12)	Dako, Glostrup, Denmark
CD8	Cytotoxic T cells	1:100	Monoclonal mouse (clone C8/144B)	Dako, Glostrup, Denmark
FoxP3	Regulatory T cells	1:100	Monoclonal mouse (clone 236A/E7)	Abcam, Cambridge, UK
DC-Sign	Immature DCs	1:1000	Monoclonal mouse (clone 102E11.06)	Dendritics, Lyon, France
DC-Lamp	Mature DCs	1:200	Monoclonal mouse (clone 1010E1.01)	Dendritics, Lyon, France
B cells				
CD20	All B cells	1:800	Monoclonal mouse (clone L26)	Dako, Glostrup, Denmark
CD79	B cells associated with antigen recognition	1:400	Monoclonal mouse (clone JCB117)	Dako, Glostrup, Denmark
Plasma	Plasma cells	1:400	Monoclonal mouse (clone VS38c)	Dako, Glostrup, Denmark
Macrophages				
CD68	Macrophages	1:600	Monoclonal mouse (clone PG-M1)	Dako, Glostrup, Denmark
NK cells				
CD57	NK cells	1:100	Monoclonal mouse (clone TB01)	Dako, Glostrup, Denmark

Abbreviations: DC, dendritic cell; NK, natural killer.

Free-hand regions were drawn to include representative LN areas. Staining intensity measurements gave the average brownness of all positive pixels within the region, expressed as a number on a continuous scale from 0 to 255 (gray scale). Intensity measurements reflect the level of antigen expression within the tissue and provide an indication for functional status of that particular antigen.[25] The percentage of the total region area that was positively stained was determined from the brown and blue area measurements by the following equation:

$$\%\ \text{Area positively stained} = \frac{\text{Brown area}}{\text{Brown area} + \text{Blue area}} \times 100$$

This area measurement is a quantitative representation of the number of positive cells.[26]

CD10 is an endometrial stromal cell marker which detects the endometrial stromal cells within the uterus and at ectopic locations.[27] In LNs, CD10 stains only the germinal centers and is generally absent in other areas of the LN.[28,29] Thus, regions for CD10 analysis were carefully drawn to exclude germinal centers within lymphoid follicles because some immature T and B precursors, as well as proliferating B cells and mature neutrophils, may express CD10.[30] Remaining CD10+ cells displayed morphology and size consistent with endometrial stromal cells rather than lymphocytes. Positive (brown) color thresholds were set such that weakly stained lymphocytes were not detected (Figure 1).

Statistical Analyses

Statistical analysis was performed using SPSS 17.0 Statistical Analysis Software. Since intensity data were significantly skewed and area data were proportions, nonparametric analyses were used to compare data. Statistical significance was established at P values of less than .05.

Results

Sentinel Uterine-Draining LNs Throughout the Menstrual Cycle

Endometrial stromal cells. CD10+ endometrial stromal cells were present in all LN samples, with significant changes in the CD10+ area within LNs throughout the menstrual cycle. CD10+ endometrial stromal cells were primarily observed within vessels in trabecular sinuses of the nodes. A peak in the area occupied by CD10+ cells at the time of menstruation (median [interquartile range] = 1.6 [0.8-2.7]) was followed by a significant decrease in the proliferative phase (0.5 [0.4-0.7]; $P = .014$; Mann-Whitney $Uz = -2.469$; Figure 2).

Leukocyte populations. Significant changes in the area (cell numbers) and the intensity (antigen expression) of various immunological markers were noted within the uterine-draining LNs throughout the menstrual cycle (Figure 3). CD8+ T cells and CD57+ natural killer (NK) cells were not significantly altered (data not shown). The number of CD3+, CD4+, Foxp3+, DC-Sign+, CD68+, CD20+, CD79+, and plasma cells peaked during menstruation; however, due to a small sample size, trends within some populations did not reach significance (Figure 4).

A significant increase in CD3+ antigen expression was evident between the secretory (intensity median [interquartile range] = 102.2 [102.2-106.3] grayscale units) and menstrual (115.2 [113.0-122.8]) phases of the cycle ($P = .034$; Mann-Whitney $Uz = -2.121$). Significant decreases in Foxp3+ cell area and intensity were observed within LNs between the menstrual and the proliferative phases of the cycle (area: 4.1 [2.5-10.4] vs 0.7 [0.3-2.7], respectively; $P = .031$; Mann-Whitney $Uz = -2.160$; intensity 86.9 [83.6-88.5] vs 78.4 [73.9-82.5], respectively; $P = .014$; Mann-Whitney $Uz = -2.469$).

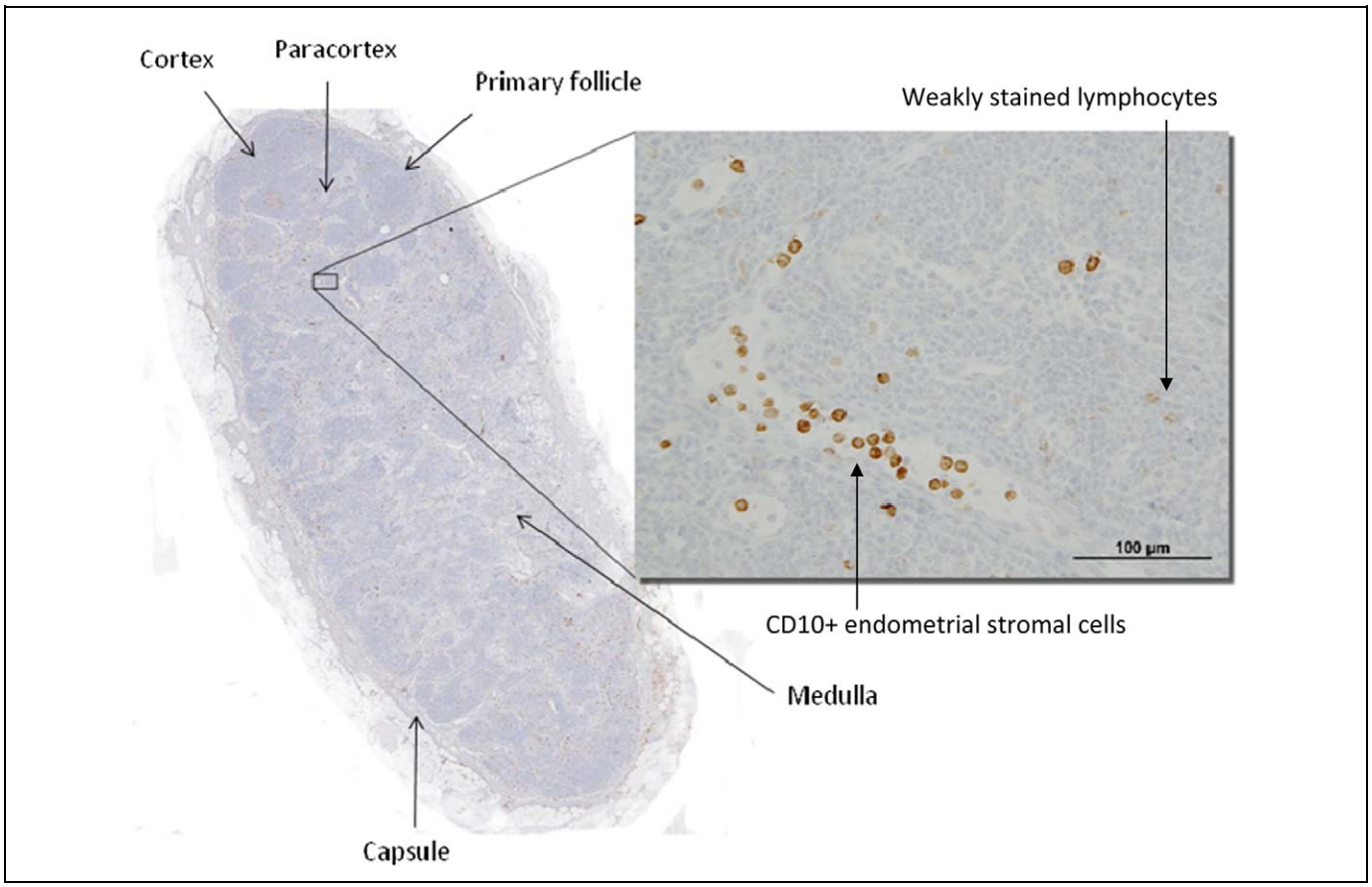

Figure 1. Microstructural regions and CD10+ cells in obturator lymph node vessels. Low-power magnification showing the microstructures of the lymph node, including the cortex, paracortex, medulla, primary follicle, and capsule. CD10+ endometrial cells (primarily observed within vessels in trabecular sinuses of the nodes) stained brown with DAB+ chromagen in an obturator lymph node during the menstrual phase (×400 magnification). Although certain lymphocytes weakly expressed CD10 antigen, intense staining of CD10+ endometrial stromal cells was observed within the lymphatic and larger blood vessels (veins) of the lymph node. DAB+ indicates diaminobenzidine+.

The area and the intensity of DC-Sign+ cells increased between secretory (5.8 [0.0-5.8] and 77.2 [64.0-77.2], respectively) and menstrual phases (24.9 [22.9-33.0] and 98.2 [94.4-103.6], respectively; both $P = .034$ Mann-Whitney $Uz = -2.121$). These increases were followed by significant decreases during the proliferative phase (area 13.2 [7.2-23.0], $P = .009$, Mann-Whitney $Uz = -2.623$; intensity 82.6 [75.6-90.5], $P = .014$, Mann-Whitney $Uz = -2.469$).

Furthermore, the area occupied by CD79+ B cells significantly increased between the secretory (44.5 [23.6-44.5]) and menstrual (52.8 [48.3-68.9]), phases of the cycle ($P = .034$; Mann-Whitney $Uz = -2.121$). Significant decreases in the area of CD79+-activated B cells and plasma cells were noted between the menstrual and the proliferative phases (CD79 52.8 [48.3-68.9] vs 37.0 [29.8-47.3], $P = .045$, Mann-Whitney $Uz = -2.006$; plasma cell 1.6 [0.9-4.5] vs 0.7 [0.2-1.2], $P = .021$, Mann-Whitney $Uz = -2.315$).

Sentinel LNs in Women With Endometriosis

Endometrial stromal cells and immune cell populations were also compared in LNs from women with and without endometriosis. The most striking difference observed between endometriotic and control LNs was the significant increase in the area occupied by CD10+ cells in women with endometriosis during the proliferative phase of the cycle ($P = .014$, $Z = -2.449$; Figure 5).

Due to small numbers within endometriosis and control groups in the secretory and menstrual phases, only the proliferative phase (endometriosis n = 4, control n = 5) showed statistical significance when menstrual cycle phases were considered separately.

Overall, significant reduction in CD3+ T-cell area ($P = .05$, $Z = -1.958$) was noted in LNs from patients with endometriosis (phases combined). The numbers of CD4+, DC-LAMP+, FoxP3+, and plasma cells were also reduced in women with endometriosis, compared to controls; however, due to small sample size, these differences were not found to be statistically significant (Figure 6).

Discussion

This preliminary study has demonstrated a range of statistically significant changes occurring within uterine-draining

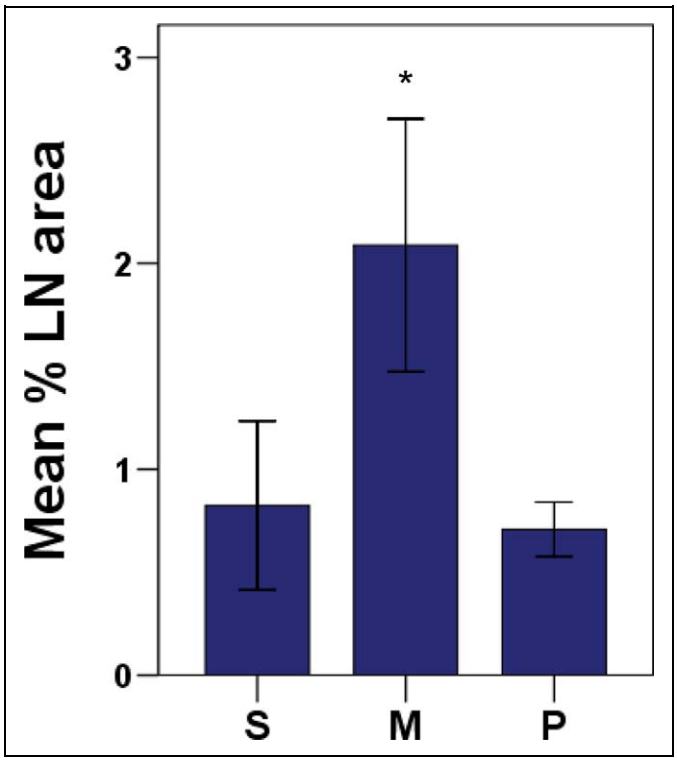

Figure 2. Median percentage of the lymph node area occupied by CD10+ cells throughout the menstrual cycle. Box plot showing the median percentage of the lymph node area occupied by CD10+ cells during the secretory (S, n = 3), menstrual (M, n = 4), and proliferative (P, n = 9) phases of the menstrual cycle. The CD10+ area peaks during the time of menstruation, *$P = .014$. The solid line inside the box represents the median value, the length of the box denotes the interquartile range, and the whiskers the minimum and maximum values excluding outliers (represented by circles).

LNs throughout the menstrual cycle and in women with endometriosis. Specifically, changes in immune and endometrial cell numbers (mean percentage positive area) and levels of antigen expression (staining intensity) were recorded.

Menstruation has a significant inflammatory component, a key feature of which is infiltration of the endometrium by a range of leukocyte subsets. Recruitment of these cells to this site premenstrually results not only in endometrial tissue damage during menstruation but also in remodeling and repair during the ensuing proliferative phase of the cycle.[2,4,31] When the endometrium breaks down and is shed, endometrial lymphatics are exposed to the uterine cavity, thereby providing the opportunity for endometrial products (cells and fragments) as well as any leukocytes or possible microorganisms present at the time to be channeled into the regional lymphoid tissue for destruction. Although the inflammatory reaction within the endometrium has been extensively analyzed,[7,8] there is essentially no information about the cellular events that occur within the draining LNs during the menstrual cycle including the fate of endometrial products. This is of vital importance given the efficiency of repair of the endometrium postmenstruation, and the fact that the indirect evidence exists for lymphatic spread of endometriosis.[18,21,32]

Initially, we elected to study the cell populations (a mix of resident and recruited cells) present in obturator-draining LNs during the 3 phases of the menstrual cycle as a basis for determining what happens in patients with endometriosis. Of particular interest was our detection for the first time of CD10+ endometrial stromal cells within uterine-draining LNs; as expected their numbers reached a peak during the time of menstruation when the uterine lymphatic vessels are patent. Based on the kinetics of endometrial cell traffic with a number of immune cell populations (including macrophages, NK cells, and immature DCs) recruited to the endometrium premenstrually, it would be predicted on a priori grounds that cells from the innate and adaptive immune systems would also peak within uterine-draining LNs during the menstrual phase or shortly thereafter. A clear increase in LN number of CD3+ T cells, immature DCs, and CD79 +B cells was observed, with similar trends occurring for CD20+ B cells and plasma cells, CD4+ and Foxp3+ T cells, and macrophages.

Increased numbers of certain LN immune cell populations during menstruation are likely to occur not only as a result of transit of cells from the endometrium via the lymphatic circulation but also due to the recruitment of cells in response to increased exposure to endometrial products and antigens during this time of the cycle. Efficient immune response in uterine-draining LNs is required during menstruation to clear displaced endometrial tissue components. In particular, increased numbers of immature DCs, which play roles in antigen internalization and T-cell activation,[33] may be important to recognize shed endometrial cells and initiate appropriate immunological response. Additionally, increased numbers of T cells are likely to facilitate specific, targeted immunological responses, while increased numbers of B cells expressing CD79 following antigen recognition[34] may occur in response to the presence of increased displaced endometrial tissue cell antigens.

These findings raise the question of what are the targets for the immune response within the draining LNs? Clearly, the endometrial stromal cells constitute one overt target, which is removed under normal circumstances, although there may be other subcellular targets like cell debris, apoptotic nuclei, and even microorganisms from the endometrium. As endometrial cells constitute ''self tissue,'' it is likely that following their transit into the lymphatic circulation, they encounter a new environment in which they no longer receive specific growth factors and nutrients required for survival. As such they begin to apoptose, their nuclear envelope breaks down, and their DNA becomes exposed.[35] Consequently, the dying cells become targets for neutrophils and macrophages, which nonspecifically engulf and clear endometrial fragments.

In endometriosis, endometrial cells exhibit a range of inherent characteristics that facilitate their survival, attachment, establishment, and progression at ectopic sites. For example, apoptosis is known to be decreased in the eutopic endometrium in endometriosis.[36-38] This implies that shed endometrial

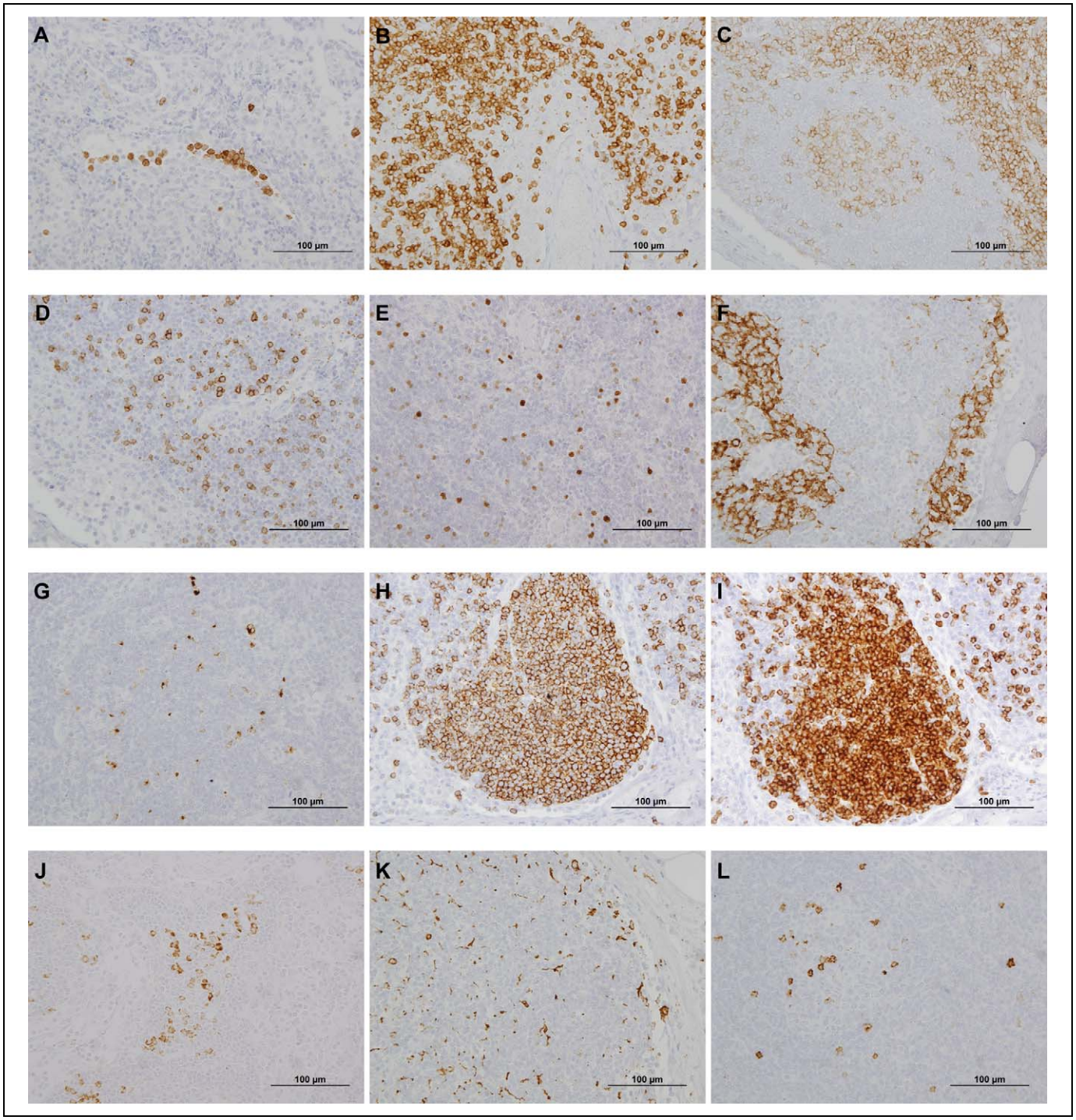

Figure 3. Endometrial stromal and immune cells in obturator lymph nodes during menstruation. Endometrial stromal (A) and immune cell populations in sentinel uterine-draining lymph nodes stained brown with DAB+ chromagen (×400 magnification). CD3+ (B), CD4+ (C), CD8+ (D), and Foxp3+ (E) T cells; DC-Sign+ (F) and DC-Lamp+ (G) dendritic cells; CD20+ (H), CD79+ (I), and plasma+ (J) B cells; CD68+ (K) macrophages, and CD57+ (L) natural killer cells were observed within uterine-draining lymph nodes during menstruation. DAB+ indicates diaminobenzidine+.

fragments in women with endometriosis may be more resistant to apoptosis and consequently remain viable in the LN environment for longer periods of time. Consistent with this interpretation, we have demonstrated a significantly larger number of CD10+ cells in LNs of women with endometriosis compared to controls during the proliferative phase of the menstrual cycle. Furthermore, the increased endometrial lymphatic microvessel density seen in endometriosis during the proliferative phase, as demonstrated by our recent study,[39] is likely to enhance migration of endometrial cells into draining LNs.

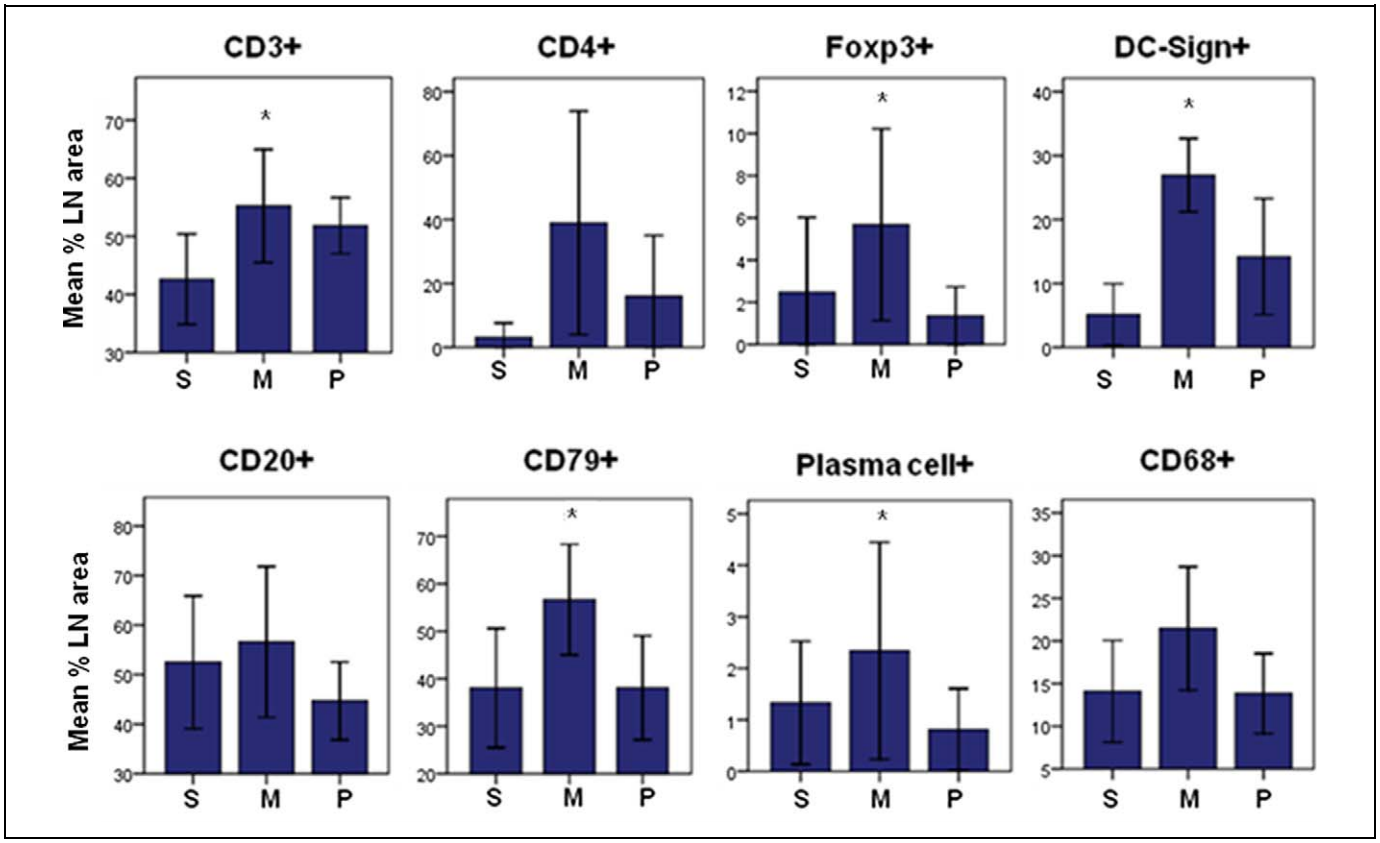

Figure 4. Median percentage area occupied by lymph node immune cell populations throughout the menstrual cycle. The above box plots represent the median percentage area (cell numbers) of immune cell populations during the secretory (S, n = 3), menstrual (M, n = 4), and proliferative (P, n = 9) phases of the cycle. Significant peaks in CD3+, Foxp3+, DC-Sign+, CD79+, and plasma cell numbers were observed during menstruation. Trends also indicate that other immune cell populations peak during this time of the cycle. *$P < .05$.

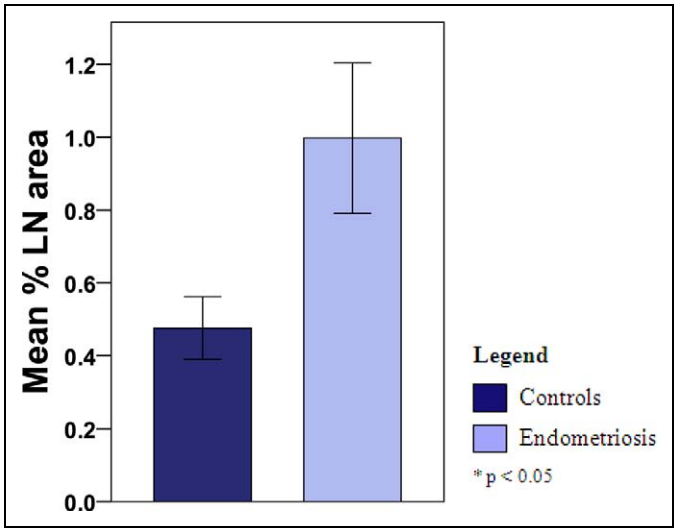

Figure 5. Median percentage area occupied by CD10+ cell populations in endometriosis. The above box plot compares the median percentage area (cell numbers) of CD10+ cell populations between obturator lymph nodes from women with and without endometriosis during the proliferative phase of the menstrual cycle (control n = 5, endometriosis n = 4). A significant increase in CD10+ cell numbers was observed in women with endometriosis, compared to women without the disease (*$P = .014$).

The most commonly accepted theory of endometriosis pathogenesis states that fragments of endometrial tissue shed at menstruation can implant at ectopic locations and persist as endometriotic lesions.[40,41] It is hypothesized that shed endometrial fragments are disseminated not only by the fallopian tubes (retrograde menstruation) but also in some cases via the lymphatic circulation.[19,40] The observations of this study support the theory of lymphatic spread of endometriosis for which only indirect evidence existed previously based on the reports of pelvic LN endometriosis,[18,32] the occurrence of endometriotic lesions at other uncommon locations,[19,42] and the presence of ectopic decidua in pelvic nodes of pregnant women.[43] The main criticisms of the theory of lymphatic spread have concerned how endometrial cells can return from the lymphatics to the peritoneal cavity and why lesions are only seldom found at distant sites. Efferent lymphatic drainage channels leaving sentinel uterine-draining LNs traverse the pelvic region, including the pelvic sidewall.[44-46] Additionally, normal subperitoneal tissue contains numerous small lymphatic vessels,[47,48] indicating communication between the lymphatic drainage channels of the uterus and the peritoneal environment. The lymphatic system functions to mount an immune response against and clear antigenic material, preventing dissemination. However, in some circumstances, such as cancer, metastasis

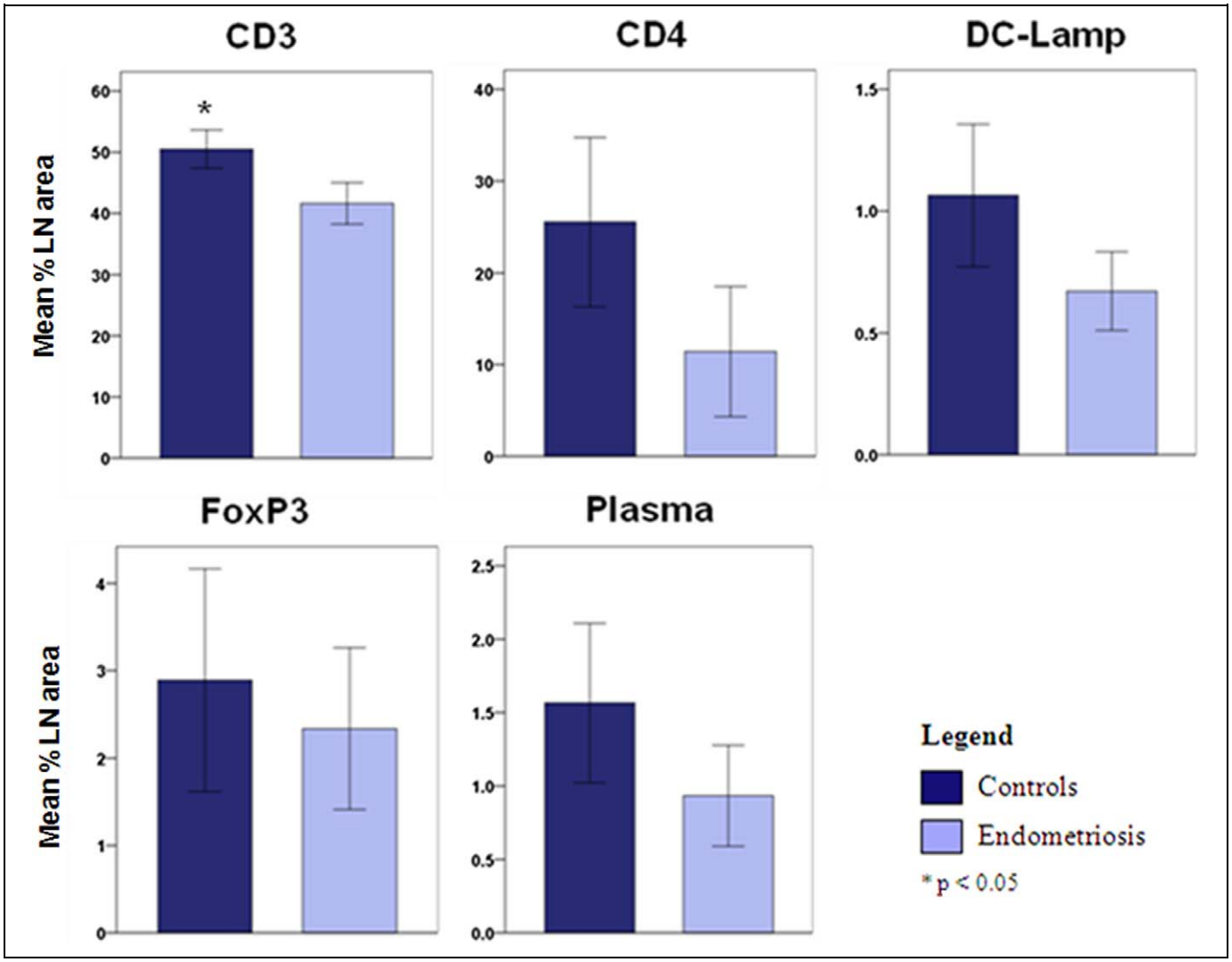

Figure 6. Median percentage area occupied by lymph node immune cell populations in women with and without endometriosis. The above box plots represent the median percentage area (cell numbers) of immune cell populations between women with (n = 7) and without endometriosis (n = 9). A significant decrease in CD3+ cell numbers was observed in women with endometriosis. CD4+, DC-Lamp+, Foxp3+, and plasma cell numbers were also reduced in endometriosis; however, these differences did not reach the level of significance.

may occur through lymphatic circulation due to inefficient containment. Similarly, in most instances, endometrial cells that enter lymphatic circulation would be cleared, but in rare situations may remain viable to reach and implant at distant locations.

Analysis of LN cell composition revealed some subtle but statistically significant changes in women with endometriosis, specifically significant decrease in the number of CD3+ T was observed in LNS from women with endometriosis. Combined with increased numbers of endometrial stromal cells in endometriosis, decreased number of CD3+ T cells may play a role in allowing the survival of endometrial cells and establishment of endometriotic lesions at sites outside the uterine cavity. The numbers of other immune cell populations, including CD4, DC-Lamp, FoxP3, and plasma, also tended to be reduced in women with endometriosis; however, due to small sample size the differences were not statistically significant. The decrease in DC-Lamp+ cells mirrors our previous observation that the density of DCs identified by CD83 as mature cells is significantly reduced in the eutopic endometrium in patients with endometriosis (Schulke et al[10]). The different cell patterns associated with endometriosis are intriguing and raise the question of the mechanism(s) responsible for the decline in leukocyte numbers in endometriotic LNs. Recruitment to ectopic endometriotic sites is one possible explanation, and indeed it is well documented that a number of immune cell populations are recruited to ectopic lesions.[10,14,49-52]

Changes in the level of antigen expression during the menstrual cycle were relatively small in magnitude. Consequently, it is not feasible at this stage to comment in detail on the relationship between these changes in antigenic markers and cell function, which will need to await direct in vitro studies of cell activity and signaling. As a result of this pilot study, we hope to establish collaborations that will enable us to carry out further

studies with larger number of samples and provide more definite conclusions. Animal models of endometriosis, such as the non-human primate (baboon) model, may be particularly useful for increasing our understanding of the complex changes that occur within the uterine-draining LNs, in the presence of this disease. Such knowledge has considerable potential for advancing our current understanding of this complex disease and its pathophysiological mechanisms, which remain poorly understood.

This study represents a novel approach aimed at improving our understanding of the process of menstruation and the role of the immune system in endometriosis. It is however important to acknowledge the limitations associated with this preliminary work. Sample numbers were relatively small, reflecting the difficulty in obtaining such tissues for analysis. Furthermore, although sound clinical data were available for most patients, exact information regarding the stage of endometriosis, the characteristics of endometriotic lesions, and associated symptoms was not always available. It should also be noted that while patients had low-grade cervical or ovarian cancer without evidence of lymphatic involvement on pathological investigation, the possibility that malignant processes may have had an impact on the parameters investigated in the current study cannot be completely excluded. However, we believe this to be unlikely because all cancers were early staged. In addition, the obturator LNs are not considered to be sentinel nodes for cervical or ovarian tumors since these tissues normally drain into ureteral and periaortic LNs, respectively.[6] The LN excision is performed only when malignancy is suspected,[53] thus obtaining truly normal LNs for this kind of study is not feasible. Despite these possible limitations, we do believe that the findings reported here have opened up new avenues for exploring how the innate and adaptive immune systems interact within the endometrium and draining lymphoid tissue both during the normal menstrual cycle and in patients with endometriosis.

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Declaration of Conflicting Interests

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