Original Article

Macrophages and Natural Killer Cells Characteristics in Variously Colored Endometriotic Lesions: A Cross-Sectional Analytic Study

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Abstract

Background: Dysregulation of the immune response contribute to a significant role in endometriosis. This research examined macrophages and natural killer (NK) cells numbers in endometriotic lesions and their association with the different lesion colors: red, black, and white. To investigate the amount of the CD68 and CD56 in eutopic endometrium and different type of the endometriotic lesions.

Materials and Methods: A cross-sectional analytic study was conducted. Women suspected endometriosis requiring laparoscopic surgery between July 2016 and January 2017 were recruited. Their lesions were classified as red, black, or white and these lesions were excised by standard laparoscopic surgery. Twenty-four endometriotic lesions from each color group were obtained from 45 women who met the inclusion criteria. One type of lesion was collected from 25 women. Two different lesion types and three-color lesion types were collected from the same women in 13 and 7 subjects, respectively. Immunohistochemistry staining with anti-human mouse cluster of differentiation (CD) 68 monoclonal antibody for macrophages and mouse anti-human CD56 monoclonal antibody for NK cells were performed.

Results: The number of CD68 macrophages in red lesions was higher than in black and white lesions [median (25^{th}-75^{th} percentile); 10 (5-19.4), 0 (0-6.9), 0 (0-2.5) cells per mm^2, respectively, adjusted P=0.001 for red vs. black lesions and red vs. white lesions, and adjusted P=1.000 for black and white lesions]. The number of CD56 NK cells was not significantly different among red, black, and white lesions [median (25^{th}-75^{th} percentile; 5 (2-16.5), 3.8 (0-14.4), 1.3 (0-6.9) respectively, adjusted P=1.000 for red vs. black lesions and black vs. white lesions, and adjusted P=0.617 for red vs. white lesions].

Conclusion: The dynamic changes in the immune cells in ectopic endometrium were specific to the macrophages but not to the NK cells, as demonstrated by the highest number of CD68 macrophages in the red lesion, the earliest established ectopic endometrium. NK cells in endometriosis may have a role in the uterus.

Keywords: Endometriosis, Endometrium, Killer Cells, Macrophages, Natural

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Introduction

Endometriosis is an important gynecologic disease in women of childbearing age; however the pathophysiology of endometriosis is still poorly understood. Retrograde of menstruation into the peritoneal cavity established by Sampson is the most widely believed theory for the origin of the disease. At present, studies of certain genetic, environmental, and immunological factors enhance clinical practice of eradication of ectopic endometrial cells and may or may not allow the implantation of endometriotic lesions onto peritoneal tissue or pelvic organs. Current endometriosis treatments are not satisfactory due to the high rate of recurrence after surgery and the requirement of long-term pharmacological therapy. Treatments tailored to pathophysiology, for example an abnormal immunologic response, might yield a better outcome.

Endometriosis is associated with abnormal immunologic responses to both innate and adaptive mediated-immune cells, including T and B cells. Endometrial cells that retrograde during menstruation from the intrauterine cavity to the peritoneal cavity lyse and release chemokine and hypoxic substances. Macrophages then ingest these endometrial cells. However, these recruited macrophages also release certain cytokines leading to the recruitment of

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more macrophages to the peritoneal cavity, inducing anti-apoptosis of endometrial cells, and stimulating neo-angiogenesis. Thus, exaggerated activities of macrophages contribute to the survival of ectopic endometrial cells and the development of endometriotic lesions (1). The number and proportion of macrophages in the peritoneal fluid are significantly increased in women with endometriosis (2). Angiogenesis caused by vascular endothelial growth factor (VEGF) and induced by hypoxia is partly regulated by macrophages and might contribute to the persistence of ectopic endometrial tissue. Macrophages expression of cluster of differentiation (CD) 68 was significantly increased in eutopic endometrium of endometriosis women during the proliferative phase of the endometrium (3). Therefore, results from previous studies support the role of macrophages in the pathogenesis of the disease.

Natural killer (NK) cells, a component of innate immunity, release cytotoxic cytokines to kill malignant and infected cells. NK cells react with heat shock protein-70 and human leukocyte antigen (HLA)- G found on endometrial cells and release cytokines in order to kill them during retrograde menstruation (4). Many studies have provided evidence of NK cell dysfunction in women with endometriosis through the decreased cytotoxic activity and increased inhibitory activity of peripheral blood and peritoneal fluid NK cells (5). Dysfunction of NK cells might lead to endometrial cell survival and implantation and likely promotes the development of the endometriotic lesion. However, to the best of our knowledge, the amount and activity of NK cells in different endometriotic lesions of the peritoneum have not been reported yet.

Each color of the lesion (red, black, or white) of superficial peritoneal endometriotic has been studied for their association with different levels of inflammation, angiogenesis, and immunologic response. The red lesion is the most active, earliest lesion, resembling eutopic endometrium. VEGF level, microvessel density score, and surrogate markers of angiogenesis, are significantly higher in the red endometriotic lesions than black or non-opaque lesions in all phases of menstruation (6). Studies have also demonstrated that the inflammatory response, proliferating cell nuclear antigen index, endoglin or CD105 [a marker of transforming growth factor (TGF)-β type 1), interleukin-1 (IL-1) receptor type 1, hepatocyte growth factor (HGF), and HGF receptor] expressions are higher in red lesions than black and white endometriotic lesions (7, 8). Such findings suggest that red lesions are the most active lesion among all colors and confirm the retrograde menstruation theory and the occurrence of the red endometriotic lesion before black lesion.

Most studies have focused on markers of angiogenesis and inflammation, but very few have investigated dysregulated immune cells within endometriotic lesions. It was hypothesized that different types of endometriotic lesions would have differences in the expression of macrophages and NK cells. The present cross-sectional analytic study was conducted to compare the expression of CD68 macrophages and CD56 NK cells in red, black, and white endometriotic lesions.

Materials and Methods

This cross-sectional study was done at the Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynaecology, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok, Thailand. It was approved by the Ethics Committee on Human Rights Research related to Research involving Human Subjects of Ramathibodi Hospital, Mahidol University (MURA 2016/364).

Study subjects

Women between 18 and 45 years old who had symptoms and signs of pelvic pain, dysmenorrhea, and infertility or ovarian cyst compatible with endometriosis were recruited in this study. All subjects provided informed consent before recruitment. These women underwent laparoscopic surgery from July 2016 to January 2017. The present study recruited women who had regular menstrual periods (cycle length within 28 ± 7 days in the 3 months before enrollment), were in the proliferative phase of their menstrual periods on the day of operation and had no history of any hormone therapy at least 3 months before the surgery. Each participant was questioned about parity, history of surgery, any previous pelvic inflammatory disease, pre-operative symptoms, duration of symptoms, and underlying disease as the baseline assessment. All operative data, including pre- and post-operative diagnosis, operation, and the stage of endometriosis, were also recorded. Subjects who had no peritoneal endometriosis, according to laparoscopic finding or histopathology, were excluded (Fig.1).

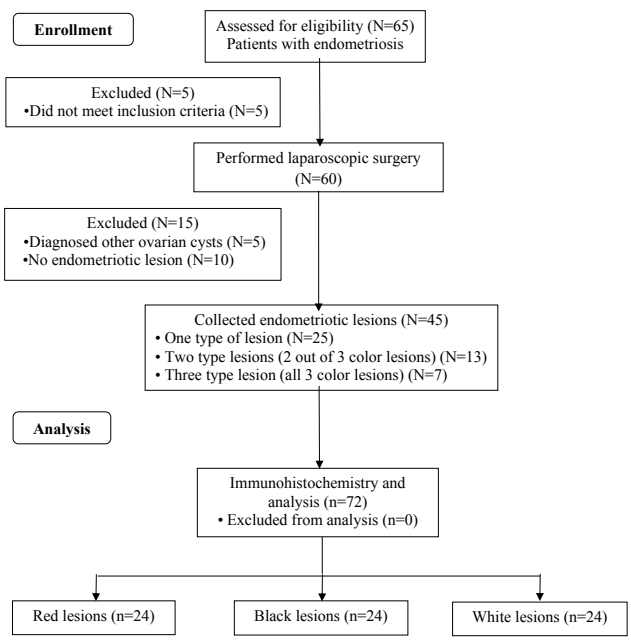

Fig.1: The study flow chart. N; Number of participants and n; Number of endometriotic lesions.

Sample size calculation

For the sample size estimation, we performed a pilot study for the expression of macrophages in different colors of endometriotic lesions using the n4Studies software with two independent means (two-tailed test) (9). Since the main aim of the present study was to compare the number of macrophages among three groups-red, black and white lesions-we chose means and standard deviations (SDs) of black and white lesions to calculate. The difference of means between a pair of black and white was less than both pairs of red and black and red and white.

$$n_1 = \frac{(z_{1-\frac{\alpha}{2}} + z_{1-\beta})^2 \left[\sigma_1^2 + \frac{\sigma_2^2}{r}\right]}{\Delta^2}$$
$$r = \frac{n_2}{n_1},\ \Delta = \mu_1 - \mu_2$$

The mean ± SD (σ) value in the black lesion group (μ) was 1.00 ± 0.30. Mean ± SD (σ) in the white lesion group (μ_2) was 0.80 ± 0.10. The ratio (r), alpha (α), Z (0.975), beta (β), and Z (0.800) values were 1.00, 0.05, 1.96, 0.200 and 0.84, respectively. The sample size for each group was 20 subjects. Allowing for a 20% dropout rate, 24 samples were recruited to each color group.

Endometriotic lesions collection

All operations were performed according to the standard laparoscopic surgical technique. Endometriotic lesions were carefully excised by laparoscopic scissors along with attached peritoneum no more than 2 mm from the lesions. Electro-cauterization was avoided to preserve tissue quality. Macroscopic appearances of endometriotic lesions were classified into red, black, or white in accordance with the revised American Society for Reproductive Medicine (rASRM) classification (10) confirmed by a second observer during operation. A red endometriotic lesion has red, red-pink, or clear morphology. The black endometriotic lesion has either a black or blue morphology. In addition, a white endometriotic lesion is a lesion containing a white, yellow-brown morphology, or peritoneal defects. The menstruation phase was confirmed by pathological dating of the eutopic endometrial tissue.

Tissue processing and immunohistochemistry

All lesions were fixed overnight in 10% formalin, followed by 70% alcohol, and embedded in paraffin. The tissues were then cut and prepared in a 3-mm thick slide for subsequent histopathological and immunohistochemical assesments. Every tissue was stained with hematoxylin and eosin (H&E) for histological study and examined by a pathologist to confirm the diagnosis. Tissues that were not diagnosed with endometriosis were discarded. The criterion for the diagnosis of endometriosis was tissues composed of both the endometrial gland and stroma.

An immunohistochemical study was performed with mouse anti-human CD68 monoclonal antibody (514H12,1:100; Novocastra, UK) for macrophages and mouse anti-human CD56 monoclonal antibody (CD56, predilution; Novocastra, UK) for NK cells. CD68 is a transmembrane glycoprotein receptor found in the endosome surface of monocytes and macrophages (lysosomal-associated membrane protein) and has been used as the principal marker of macrophages in most studies (11). CD56 is a cell membrane protein of an unknown function found on human lymphoblastoid cells, including NK cells (12). CD56 has been widely used as a marker of NK cells. The deparaffinization of paraffin-embedded tissue sections was performed in xylene solution. Tissue slide sections were incubated with Bond Dewax Solution (Leica Biosystems, Bannockburn, IL) for 60 minutes at 60°C. The slides were then incubated for 20 minutes at 100°C in Bond Epitope Retrieval Solution. The Bond Polymer Refine Detection kit (Leica Biosystems, Bannockburn, IL) was used for immunohistochemistry analysis (13). Briefly, the tissue slides were incubated with primary antibody for 45 minutes at room temperature. Hydrogen peroxide solution at 3% concentration was added for 5 minutes and then washed with Bond Wash Solution. The tissue slides were incubated for 9 minutes in the post-primary polymer. Polymer poly-HRP IgG was added for 7 minutes and then rinsed with bond wash solution before applying the diaminobenzidine chromogen for 4 minutes. Counterstaining of the slides with hematoxylin solution was done for 5 minutes. A tonsil tissue was used as a positive control slide.

Measurement of CD68 macrophages and CD56 NK cells number of endometriotic lesions

Positive CD68 macrophages and CD56 NK cells will appear as brown chromogenic granules. Two investigators,who were blinded for the type of tissues, counted the number of positive cells under microscopy with ×200 magnification independently. The cells were randomly counted in ten different fields, 200×200 microns each, and reported as (cells per mm^2). Results from two investigators were checked for sample correlation and recounted if differences occurred.

Statistical analysis

All data in the present study were statistically analyzed using an SPSS program version 23.0 (Statistical Package of the Social Security, IBM, Armonk/USA). The baseline demographic data of all women was analyzed as a whole and as a group by the color of the lesion. The number of CD68 macrophages and CD56 NK cells was compared between groups. Data were presented as median with 25^{th}-75^{th} percentile, or number with percentage as appropriate. The normality of the data was assessed by the Shapiro-Wilk test. Categorical variables were assessed with the Chi-square test. Continuous non-normally distributed variables between groups were compared with the Kruskal Wallis test. A $P<0.05$ was used to determine statistical significance. Multiple comparisons were used to assess the number of CD68 macrophages and CD56 NK cells between each group. Adjusted $P<0.05$ was chosen as the cut off for statistical significance.

Results

Sixty women suspected of endometriosis and admitted to Ramathibodi Hospital for laparoscopic surgery were recruited into the study if they met the inclusion criteria. All of them had no underlying medical condition, except two women with a thyroid nodule, one woman with hyperthyroidism, and the other one with hepatitis B carrier. Fifteen women were excluded because the diagnosis during surgery was not an ovarian endometriotic cyst and no endometriotic lesions were present. Twenty-four endometriotic lesions were obtained for every three groups. Twenty-five women had only one type of lesion. Thirteen and seven women had two and three matched different lesions, respectively. All tissues were histologically diagnosed as endometriosis (Fig.1). The demographic data of endometriosis women are shown in Table 1. The median (25^{th}-75^{th} percentile) age of the subjects was 38 (33.3-40.0) years. Their presenting symptoms included chronic pelvic pain (4.4%), dysmenorrhea (57.8%), an ovarian cyst (37.8%), and infertility (35.6%). Forty percent had a history of previous surgery. None had past medical illnesses related to pelvic inflammatory disease. The distribution of the rASRM stage was severe (60%), moderate (13.3%), mild (8.9%), and minimal (17.8%). There was a significant difference in the number of CD68 macrophages in the eutopic endometrial gland, eutopic endometrial stroma, red lesions, black lesions and white lesions (P<0.001, Fig.2). The red endometriotic lesions contained the highest number of CD 68 macrophages when three ectopic endometrial tissues were compared; red vs. black vs. white [median (25^{th}-75^{th} percentile); 10 (5-19.4), 0 (0-6.9), and 0 (0-2.5), respectively]. The eutopic endometrial stroma had higher number of CD 68 macrophages than the eutopic endometrial gland [median (25^{th}-75^{th} percentile); 20 (7.5-35) and 0 (0-2.5), adjusted P<0.001] (Table 2).

Table 1: Baseline demographic data of women with endometriosis

Characteristics	All women (n=45)
Age (Y)	38 (33.3-40.0)
Parity	
0	43 (95.6)
1	2 (4.4)
Previous history of surgery	18 (40.0)
Symptoms	
Chronic pelvic pain	2 (4.4)
Dysmenorrhea	26 (57.8)
Ovarian cyst	17 (37.8)
Infertility	16 (35.6)
Duration of symptoms in months, median (25^{th}-75^{th} percentile)	8 (4-21)
Stage of endometriosis	
I	8 (17.8)
II	4 (8.9)
III	6 (13.3)
IV	27 (60.0)

Data are presented mean ((25^{th}-75^{th} percentile) or n (%). Statistical analysis was performed by SPSS version 23.0.

Table 2: The number of CD68 macrophages and CD56 natural killer cells in eutopic endometrium and different types of endometriotic lesions

Immune cells	Endometrial gland	Endometrial stroma	Red lesion	Black lesion	White lesion	P value
Macrophages (cells/mm^2)	0 (0-2.5)	20* (7.5-35)	10* (5-19.4)	0**,*** (0- 6.9)	0**,*** (0-2.5)	<0.001
NK cells (cells/mm^2)	5 (0-10)[a]	70* (17.5-220)	5** (2-16.5)	3.8** (0-14.4)	1.3** (0-6.9)	<0.001

[a]; Positive cells were expressed as median (25^{th}-75^{th} percentile), *; Significant difference from endometrial gland, adjusted P<0.001, **; Significant difference from the endometrial stroma, adjusted P=0.001, *; Significant difference from the red lesions, adjusted P=0.001, and NK; Natural killer. Statistical analysis was performed by SPSS version 23.0.**

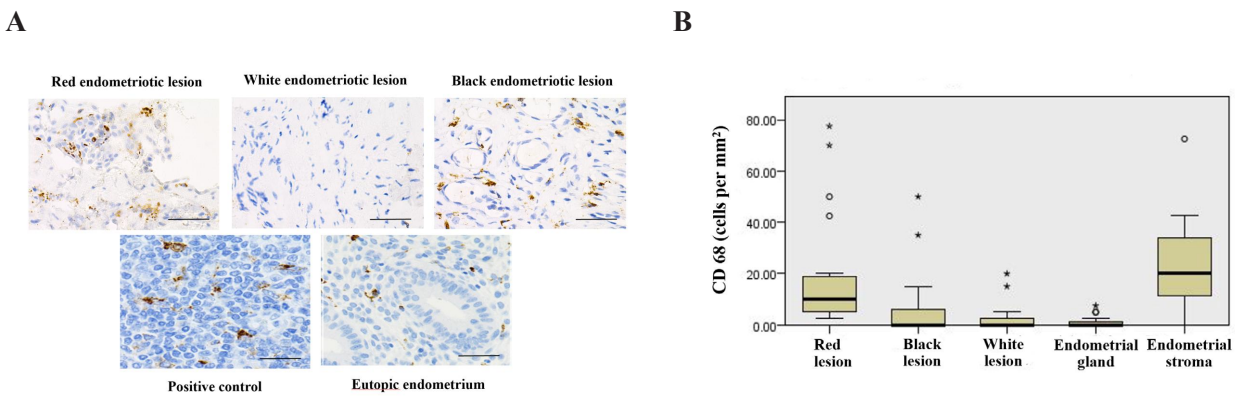

Fig.2: The immunohistochemistry by anti-CD68 to identify macrophages in eutopic endometrium and different types of endometriotic lesions. **A.** The immunohistochemistry images of each type of endometriotic lesions (scale bar: 50 µm), **B.** Boxplot graph of macrophages number of each type of endometriotic lesions.

There was a significant difference in the number of CD56 NK cells in the eutopic endometrial gland, eutopic endometrial stroma, red, black and white lesions (P<0.001, Fig.3). The number of CD56 NK cells was not significantly different among red, black, and white endometriotic lesions [median (25^{th}-75^{th} percentile); 5 (2-16.5), 3.8 (0-14.4), and 1.3 (0-6.9), respectively]. The eutopic endometrial stroma exhibited a higher number of CD56 NK than the eutopic endometrial gland [median (25^{th}-75^{th} percentile); 70 (17.5-220) and 5 (0-10), adjusted P<0.001].

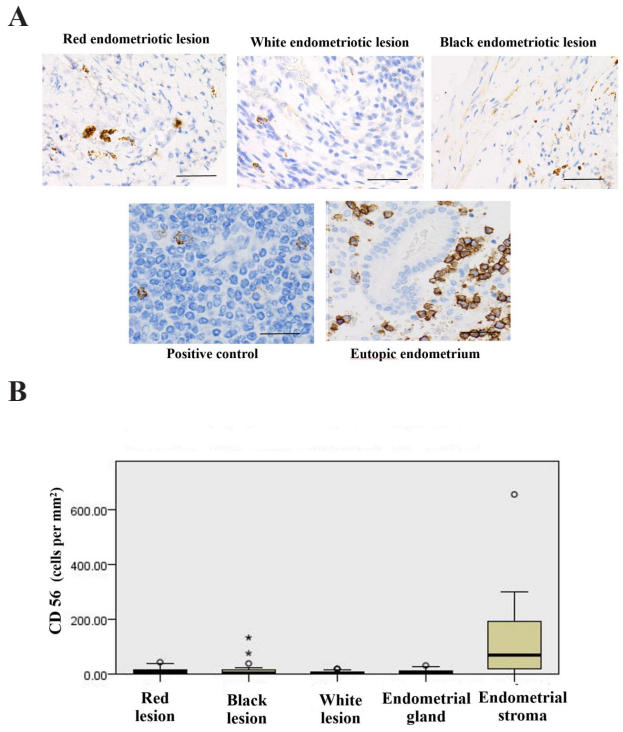

Fig.3: The immunohistochemistry by anti-CD56 to identify natural killer cells in eutopic endometrium and different types of endometriotic lesions. **A.** The immunohistochemistry images of each type of endometriotic lesions (scale bar: 50 µm), **B.** Boxplot graph of macrophages number of each type of endometriotic lesions.

Discussion

The study investigated macrophages and NK cells in peritoneal endometriosis by comparing the number of CD68 and CD56 positive cells according to the different colors of endometriotic lesions; black, red, and white. The number of CD68 macrophages in red endometriotic lesions was significantly higher than in the black and white endometriotic lesions. The expression of CD68 macrophages in red lesions was similar to the stroma of the eutopic endometrium. CD56 NK cell abundance was not significantly different among all color types of endometriotic lesions. Nevertheless, CD56 NK cells were more abundant in the endometrial stroma than in all endometriotic lesions.

The dynamic features and metabolic activities of peritoneal endometriosis have been studied in both animal models and humans. Multiple studies have reported that red lesions show increased vascularization (14), increased expression of VEGF (15), increased epithelial mitotic and proliferation activity (16), higher incidence of complex glands, and increased matrix metalloproteinase (MMP)-1 (17) and MMP-2 (18) when compared to white and black lesions. The study of dynamic change of lesions investigated in the monkey model of endometriosis demonstrated that the red lesion had a higher proliferation index, endothelial cells numbers, and vascularity compared with the black and white lesions, but similar to the endometrium (19). These data support the hypothesis that red endometriotic lesions are the initial stage of peritoneal lesions and consist of more active metabolic function than the other types of lesions. Black and white lesions are assumed to be advanced endometriosis and healed endometriosis or quiescent lesions, respectively (16). Many studies have reported that red lesions consist of a more pro-inflammatory process and immune cells than the other lesion types. Increased a pro-inflammatory transcription factor nuclear factor-kappa B (NF-kB) (20), IL-1 receptor type 1 (7), a receptor for a macrophage-derived pro-inflammatory IL-1, and a pro-inflammatory macrophage migration inhibitory factor (MIF) were found in red lesions (21). MIF was believed to function to retain macrophages in the lesions. Therefore, the inflammatory process, specifically macrophage and macrophage-related cytokines, plays an important mechanism involving the pathogenesis of early endometriosis development.

Macrophages in endometriosis play multiple dynamic roles (or phenotypes), for example, growth of lesion, neurogenesis and angiogenesis in endometriosis. Women with endometriosis have an increased number of CD68 macrophages in eutopic endometrium (3), an increased number of macrophages and a higher level of pro-inflammatory in peritoneal fluid (22) compared to healthy controls. The present study found that the number of CD68 positive cells, a marker for macrophages, is significantly higher in red lesions than in black or white lesions and similarly to eutopic endometrium. The results from the present study are consistent with those of Khan et al. (23). Endometrial macrophages derived from women with endometriosis contributed lower expression of CD163, a marker of wound healing, than those from women with no disease (24). Macrophages may increase the deposition of the retrograded endometrial tissue in the peritoneal cavity since the expression of MMP-9, reflecting tissue remodeling, was demonstrated to co-localized with CD 68 macrophages in the endometrium of women with endometriosis (25). A decrease in the phagocytotic activity of macrophages enhanced the growth of endometriotic lesions, as demonstrated *in vitro* by Shao et al. (26). Macrophages contribute to neurogenesis in endometriosis. Macrophages were found densely in the high nerve fiber density area of the endometriotic lesions, and the *in vitro* study demonstrated that the outgrowth of a nerve fiber by chemokines secreted by macrophages was estrogen-dependent (27). The mouse endometriosis model demonstrated that deletion of endothelial growth factor receptor 1 (*VEGFR1*) gene decreased

endometriotic lesion and vascularity. VEGFR1 positive cells in endometriotic lesions derived from macrophages in bone marrow (28). Previous data demonstrated the progressive change of the macrophage phenotype over time in endometriosis. Initially, macrophages expressed pro-inflammatory cytokine inducible nitric oxide synthase but they then switched to tissue modeling markers, that is, CD204 and arginase, after one to two weeks of induction of the endometriotic lesions (29). In addition, the present study showed the same direction of the dynamic change in terms of the macrophage numbers which were found more in the active red lesions than less active, black and white lesions.

Cytotoxicity of NK cells is a response of NK cells using activating and inhibitory receptors on its cell surfaces to target cells. The balance between both activating and inhibitory receptors affects the action of NK cells on the target cells. NK cell attacks the target cell when it binds to the NK cell's activating receptor. However, the NK cell does not act on the target cell when it binds to the NK cell's inhibitory receptor. An important function of NK cells in the peritoneal cavity is to get rid of the refluxed eutopic endometrium bearing a non-classic HLA-G during menstruation. NK cells use the killer immunoglobulin-like receptor (KIR) 2DL4 (CD158d) to bind to HLA-G on endometrial cells and destroy endometrial cells (30). Many studies have reported that the numbers of cytotoxic NK cells were reduced in the peritoneal fluid and circulation of patients with endometriosis association with an overall decrease in NK cell activity (12, 31, 32), while another study reported that the number of NK cells in blood circulation was increased (33). The expression of inhibitory receptors on the cell surface of NK cells, such as killer immunoglobulin-like receptor 2DL1 and immunoreceptor tyrosine-based activation motif-killer immunoglobulin-like receptor (ITAM-KIR), were up-regulated in women with endometriosis compared to healthy women (34-36). The dysfunctional NK cell cytotoxicity might allow reflux endometrium to survive in the peritoneal cavity.

No dynamic changes in the number of NK cells in the ectopic endometrium of women with endometriosis were demonstrated from the present study. This indicates that NK cells might not play a differential role in the dynamic progression of endometriosis lesions. However, the expression of NK cells was more prominent in the eutopic endometrium than in peritoneal ectopic lesions. The results of the present study were comparable to Drury's study. They collected the eutopic endometrium from women with and without endometriosis, 30 subjects for each group, and ectopic endometrium from 22 women with endometriosis, having matched eutopic endometrium for seven women. They reported strikingly low NK cells numbers in ectopic lesions when compared to uterine NK (uNK) cells. Dynamic change of uNK cells across the menstrual phase cycle was also demonstrated (37).

The role of uterine NK (uNK) cells is not well understood (12). The number of uNK cells is low during the proliferative phase of the endometrium, but the number of uNK cells is increased in the secretory phase (38). Moreover, uNK cells proliferation was increased during early pregnancy and associates with good pregnancy outcomes (39). However, a recent retrospective study did not demonstrate the association between endometriosis and an increased number of uNK cells (40). The present study compared NK cell abundance between different peritoneal lesions and eutopic endometrium, but no tissues from women without endometriosis were compared side by side. Future work should compare the uNK cell abundance of women with and without endometriosis and study the functional role of NK cells in the uterus.

Knowledge from the present study could be useful for designing the potential therapeutic intervention, for example, a drug for blocking macrophages or blocking recruitment of macrophages, and the appropriate timing for prescribing these medications. The limitation of the present study was the sample of all colors could not be simultaneously collected from the same subject because one endometriosis woman could have more than one color of lesions. Moreover, only the number but not the activity of both macrophages and NK cells were studied.

Conclusion

The dynamic changes in the immune cells in ectopic endometrium are specific to the macrophages but not to the NK cells, as shown by the highest number of CD68 macrophages in the red lesion, the earliest established lesion of peritoneal endometriosis. NK cells in endometriosis may contribute their role to the uterus, and they have been reported to be a significantly low number in ectopic endometrium in women with endometriosis.

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Authors' Contributions

A.S., N.A., Y.T.; Participated in study design, data collection, drafting and statistical analysis. M.S., S.S., W.W., K.D., T.C., A.J.; Conducted laboratory results. A.S., Y.T.; Participated in the finalization of the manuscript. All authors read and approved the final manuscript.

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