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Effect of endometriosis on white blood cell subpopulations in peripheral blood and peritoneal fluid of baboons*

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In women with endometriosis, changes in peripheral blood and peritoneal fluid white blood cell (WBC) populations have been reported, but it is not known whether these alterations are causally related to or a consequence of endometriosis. The purpose of this study was to test the hypothesis that peripheral blood and peritoneal fluid WBC populations are altered in baboons with spontaneous and induced endometriosis compared to animals without disease. Peripheral blood and peritoneal fluid samples were obtained at laparoscopy from 60 baboons with a normal pelvis (n = 23), spontaneous endometriosis (n = 19) and induced disease (n = 18) during menses (n = 9), follicular phase (n = 12), luteal phase (n = 20), pregnancy or nursing (n = 11) and in non-cycling animals (n = 8). The WBC concentration was analysed with a Coulter counter and fluorescent antibody cell separation (FACS) analysis was used to measure cluster designation (CD)2, CD4, CD8, interleukin (IL)2R and leucine (Leu) M5 subsets. In peripheral blood, the percentage of $CD4^+$ and $IL2R^+$ cells was increased in baboons with stage II–IV spontaneous or induced endometriosis, suggesting that alterations in peripheral blood WBC populations may be an effect of endometriosis. In peritoneal fluid the WBC concentration and percentages of Leu $M5^+$ macrophages and $CD8^+$ lymphocytes were only increased in baboons with spontaneous endometriosis and not in animals with induced disease, suggesting that alterations in peritoneal fluid WBC populations may lead to the development of endometriosis. In summary, the results of this study suggest that peripheral blood and peritoneal fluid immune cell populations are affected in baboons with endometriosis.

Key words: baboon (*Papio*)/endometriosis/menstrual cycle/peritoneal fluid/white blood cells

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Introduction

Endometriosis is an important gynaecological disease associated with pain and subfertility. The pathogenesis of this disease is not well understood. It is generally accepted that endometriosis can be caused by retrograde menstruation and pelvic implantation of endometrial cells (Sampson, 1927). However, it is not clear why not all women develop endometriosis, since retrograde menstruation has been reported in 70–90% of women (Halme *et al.*, 1984; Liu and Hitchcock, 1986). An altered immune response has been proposed to explain this paradox (Dmowski *et al.*, 1981). White blood cell (WBC) populations in both peritoneal fluid and peripheral blood may be important in promoting ectopic endometrial growth and diminishing fertility by their activity (Muscato *et al.*, 1982) and their secretion of growth factors (Halme *et al.*, 1988) and cytokines (Hill, 1992). An increased concentration and total number of macrophages, lymphocytes and their subsets has been reported in both peritoneal fluid and peripheral blood from women with endometriosis when compared to those without the disease (Haney *et al.*, 1981; Halme *et al.*, 1982; Muscato *et al.*, 1982; Haney *et al.*, 1983; Badawy *et al.*, 1987; Hill *et al.*, 1988; Badawy *et al.*, 1989; Odukaya *et al.*, 1995a,b). However, it is not known whether changes in WBC subpopulations in peritoneal fluid and peripheral blood are causally related to endometriosis or a consequence of this disease. This aspect is difficult to study in women, since the temporal relationship between pain, infertility and endometriosis is not known at the time of diagnosis. Endometriosis in non-human primates provides a valuable opportunity to address this issue because endometriosis occurs spontaneously (D'Hooghe *et al.*, 1991) and induced endometriosis has similar appearances to the spontaneous disease (D'Hooghe *et al.*, 1995a). The baboon is an attractive species for the study of WBC populations since spontaneous endometriosis occurs in 25% of animals and peritoneal fluid is present in sufficient amounts to allow analysis (D'Hooghe *et al.*, 1991). The purpose of this study was to test the hypothesis that peripheral blood and peritoneal fluid WBC subpopulations are altered in baboons with spontaneous and induced endometriosis compared to animals without disease.

Materials and methods

Animals

All animals in this study were of proven fertility in the wild and were housed in single or group cages at the Institute of Primate Research, Nairobi, Kenya, as described previously (D'Hooghe *et al.*, 1991). Perineal staging was used to determine the menstrual cycle phase when peritoneal fluid and/or peripheral blood samples were

obtained. The perineal inflation and deflation phases approximate the follicular and luteal phases of the menstrual cycle respectively Ovulation occurs ~3 days before the onset of perineal deflation (Hendrickx and Kraemer, 1971) Peripheral blood and peritoneal fluid were obtained during laparoscopy, as described previously (D'Hooghe *et al.*, 1991). Peritoneal fluid was aspirated with a laparoscopic needle from the posterior cul-de-sac, vesicouterine fold and pararectal gutters prior to any organ manipulation at the beginning of the laparoscopy In three cases, the peritoneal fluid was excluded from the study when bleeding from the puncture site was noticed and peritoneal fluid was stained red, because of probable contamination with peripheral blood The peritoneal fluid was collected in sterile heparinized tubes, kept at 4°C and processed within 2 h Primates with endometriosis had either recent (acquired within the last 2 years before the study) or long-term (present for >2 years at the moment of the study) spontaneous, or experimentally-induced endometriosis. Endometriosis had been induced by retro- or i.p. injection of endometrium, as described previously (D'Hooghe *et al.*, 1995a). All animals with endometriosis had histologically proven disease (presence of both endometrial glands and stroma) In baboons without adhesions caused by previous hysterotomies, endometriosis could be classified according to the revised classification system of the American Fertility Society (AFS, 1985), modified for the smaller size of the baboon.

Initially, the peripheral blood of 10 female baboons (five with a normal pelvis and five with spontaneous endometriosis stage I or II), six of which were in the luteal phase and four in the follicular phase, was analysed to determine whether mouse antihuman monoclonal antibodies used to measure lymphocyte subsets were cross-reactive with baboons

Subsequently, the distribution of WBC subsets was analysed in peripheral blood from 60 female baboons (23 controls, eight with recent spontaneous endometriosis, 11 with spontaneous long-term endometriosis, 18 with induced disease) during menses (n = 9), follicular phase (n = 12), luteal phase (n = 20), pregnancy or while nursing (n = 11) and in animals that were not cycling for unknown reasons (n = 8). In this group, 33 primates that could be classified according to the revised AFS system had endometriosis stage I (n = 10, eight with recent spontaneous disease, two with long-term spontaneous disease), endometriosis stage II (n = 10, two with spontaneous and eight with induced disease) and endometriosis stages III–IV (n = 13, five with spontaneous and eight with induced disease)

Finally, the distribution of WBC subsets was analysed in peritoneal fluid from 57 female baboons (21 controls, nine with recent spontaneous endometriosis, 11 with spontaneous long-term endometriosis, 16 with induced disease) during menses (n = 8), follicular phase (n = 13), luteal phase (n = 18), pregnancy or while nursing (n = 10) and in animals that were not cycling for unknown reasons (n = 8). In this group, 31 primates that could be classified according to the revised AFS system had endometriosis stage I (n = 11, nine with recent spontaneous disease, two with long-term spontaneous disease), endometriosis stage II (n = 9, two with spontaneous and seven with induced disease) and endometriosis stages III–IV (n = 11, five with spontaneous and six with induced disease)

Analysis of peripheral blood and peritoneal fluid

A Coulter counter was used to measure the total WBC concentration A FACStar Plus (Becton-Dickinson, Mountain View, CA, USA) was used to determine WBC subsets in peritoneal fluid and peripheral blood. Flow cytometry with light scattering was used to determine proportions of lymphocytes, monocytes/macrophages and granulocytes. Phycoerythrin (PE) or fluorescein isothiocyanate (FITC)-conjugated mouse anti-human monoclonal antibodies (Becton-Dickinson, Mountain View, CA, USA) were used to measure WBC subsets.

Table I. Reactivity percentage (SD) of mouse anti-human monoclonal antibodies with leukocyte subsets in peripheral blood from baboons (n = 10)

Marker	Predominant reactivity	Baboons	Humans[a]
CD3/Leu4	T cells	0.5 (0 7)	73 (6)
CD2/Leu5b	T cells	72 5 (0–7)	
CD45RA	Naive T cells	40.5 (10.2)	
HLA-DR	Activated T/B cells	23 (6 7)	
CD4/Leu3a	T helper/inducer cells	37 7 (9 6)	43 (7)
CD4/HLA-DR$^+$	Activated	7 3 (3 8)	
CD4/CD45RA$^+$	T suppressor/inducer	6 8 (2 9)	
CD8/Leu2a	T cytotoxic/suppressor	55 4 (19 5)	33 (7)
CD8/CD3-		60 2 (8 4)	
CD8/HLA-DR$^+$	Activated	8 6 (5 1)	
CD8/CD11B$^+$	T suppressor	18 1 (8 8)	
CD19/Leu12	B cells	0 5 (0 8)	14 (4)
CD20/Leul6	B cells	11 (2 6)	
CD16	NK cells	2 2 (1 4)	14 (6)
CD56	NK cells	2 1 (2 2)	14 (6)
CD57	NK cells	1 0 (0 8)	
CD57/CD11B$^+$	NK cells	0 4 (0–5)	
CD57/CD3$^+$	pre-NK cell	0	
CD57/CD8$^+$	Active killer cell	1 1 (0 4)	
CDllB/Leul5	NK/T suppressor cell	17 8 (9 6)	
CD15/LeuM1	Macrophages/monocytes	1 8 (0 4)	
CD11c/LeuM5	Macrophages/monocytes	5 4 (2 8)	

[a]Lymphocyte subset reference ranges in adult male and female Caucasians (Reichert *et al*, 1991)

Peritoneal fluid and peripheral blood were analysed without Ficoll–Paque separation, except for the initial study. FITC- and/or PE-conjugated antibody (20 µl) was added to 200 µl of peripheral blood or peritoneal fluid, in a 1/2 dilution with phosphate-buffered saline, and staining was performed at 4°C for 20 min. Subsequently, red blood cells were lysed with 2 ml NH_4Cl (9 3 g/l) for 5 min. Cells were centrifuged, fixed with 1 ml of 0.5–1% paraformaldehyde and analysed by flow cytometry.

Ethics

The study protocol had been reviewed and approved by the Institution Scientific Resources Evaluation and Research Committee, which assesses the need for primate studies, the health care of the baboons involved and the experimental design and methods of proposed studies.

Statistics

Parametrically-distributed data were analysed using Student's t-test and one-way analysis of variance (ANOVA) with subsequent Newman–Keuls comparison and/or Scheffé post-hoc analysis to determine whether the differences between pairs of means were significant after a significant ANOVA If the data were non-parametrically distributed, the Kruskal–Wallis test was used with subsequent Dunn's multiple comparisons test to determine whether any pairs of categorized data were significantly different following a significant Kruskal–Wallis test. The Pearson correlation test was used to determine the correlation between two continuous variables.

Results

Percentage of WBC subsets in peripheral blood of baboons

In peripheral blood, WBC subsets could be determined by the following monoclonal antibodies (Table I): cluster designation (CD)2 (T cells), CD45RA (naive T cells), CD4 (T helper cells), CD8 (T cytotoxic/suppressor cells), CD20 (B cell

Table II. Effect of cycle stage in white blood cell (WBC) concentration and subpopulations (mean ± SD) in peripheral blood and peritoneal fluid of baboons with endometriosis

	Menses	Follicular phase	Luteal phase	Pregnant/nursing	Non-cycling	*P*
Peripheral blood						
WBC (× 10^3/μl)	8.5 (2.7)	7.0 (1 8)	11.3 (4 3)	18 4 (1 3)	7.8 (1.6	0 008 (AN)[a]
$CD4^+$ cells	28 4 (7 5)	39 2 (7 4)	30.2 (4 9)	28 5 (4 9)	34 8 (4 6)	0 02 (AN)[b]
Peritoneal fluid						
WBC (× 10^3/μl)	5.6 (5.6)	1.6 (0.5)	2 8 (1 2)	2 8 (0.3)	1 0 (0.5)	<0 0151 (KW)[c]
No WBC (× 10^6)	11 5 (10 9)	4.4 (2 4)	6.3 (2 4)	10 4 (3 7)	3 8 (1 7)	<0 05 (KW)[d]
M5 (%)	70 (10 8)	54 (17 8)	55 (27)	43 (12 2)	58 (14 7)	0.2 (KW)

AN = analysis of variance, KW = Kruskal–Wallis test
[a]Significant difference between pregnant/nursing and other phases (Scheffé post-hoc test)
[b]Significant difference between follicular phase and other phases (Scheffé post-hoc test)
[c]Significant difference between luteal phase and non-cycling (Dunn's multiple comparison test)
[d]No significant difference between subgroups (Dunn's multiple comparison test)

marker), CD11B (NK/T suppressor), CD8/CD11B (T suppressor) CD11C/leucine (Leu) M5 (macrophage marker). The CD4/CD8 ratio in peripheral blood was 0 67 in baboons. Monoclonal antibodies for the measurement of natural killer (NK) cell populations (CD16, CD56, CD57) and macrophages (Leu M1) in humans reacted poorly with baboon WBCs.

Analysis of peripheral blood

In peripheral blood the mean concentration of WBC was 9.5 ± 3.7 (× 10^3/μl) and the percentages for lymphocytes, macrophages and neutrophils were 38 ± 14%, 5 ± 2% and 57 ± 14% respectively. The proportion of WBC subsets was 6 ± 3% (Leu M5+ cells), 72 ± 11% ($CD2^+$ cells), 33 ± 7% ($CD4^+$ cells), 58 ± 8% ($CD8^+$ cells), 17 ± 7% ($CD20^+$ cells) and 14 ± 7% ($IL2R^+$ cells). No differences were found in WBC subsets between baboons with spontaneous endometriosis and those with induced disease. To determine the effect of more advanced degrees of endometriosis on peripheral blood WBC subsets, baboons with long-term spontaneous or induced endometriosis were compared to those with either a normal pelvis or spontaneous and recent minimal endometriosis. An increased percentage of $CD4^+$ (35 ± 7%, P <0.03) and $IL2R^+$ (17 ± 8%, P <0.03) cells were found in baboons with long-term spontaneous and induced endometriosis versus animals with a normal pelvis and recently-developed minimal disease ($CD4^+$ cells: 31 ± 7%; $IL2R^+$ cells: 13 ± 5%). When only cycling animals (n = 41) were analysed, these differences remained significant (P = 0.04 for CD4, P = 0.05 for IL2R), and the proportion of CD2 positive lymphocytes was higher (83.8%; P = 0.02) in baboons with long-term spontaneous endometriosis than in the other subgroups. Analysis of WBC subsets in baboons with a normal pelvis and animals with revised AFS staged endometriosis revealed significantly higher proportions of CD4 (P = 0.03) and IL2R (P = 0.02) cells in primates with stages II, III and IV disease than in animals with stage I endometriosis or a normal pelvis.

Subsequently, analysis of peripheral blood according to cycle stage revealed no differences in baboons with a normal pelvis, but a significantly higher (P = 0.008) concentration of WBC during pregnancy or while nursing and a higher percentage of $CD4^+$ cells (P = 0.02) in the follicular phase (Table II) when compared with the other phases of the cycle by Scheffé post-hoc analysis.

Analysis of peritoneal fluid

The WBC concentration in peritoneal fluid was elevated in the subgroup with recent endometriosis (P = 0.01) when compared with the other subgroups with a normal pelvis, long-term endometriosis or induced disease (Table III). Flow cytometry analysis of peritoneal fluid cells revealed a significant and positive correlation (r = 0.6, P <0.001) between the macrophage population determined by light scattering (61.4 ± 16.8 %) and by staining with Leu M5 monoclonal antibody (57 ± 17%). The peritoneal fluid concentration of macrophages, as determined by Leu M5, was higher (P = 0.03) in baboons with recent and long-term spontaneous endometriosis (65.7 ± 15.3) when compared with animals with a normal pelvis and those with induced disease (51 ± 15, Table III). In peritoneal fluid no differences were found in the other WBC subsets between baboons with spontaneous endometriosis and those with induced disease. The percentage of $CD8^+$ cells was higher (P = 0.01) in the group with long-term endometriosis group than in the other subgroups (Table III). Analysis of revised AFS subgroups revealed a significantly higher (P = 0.04) percentage of Leu M5+ macrophages in baboons with stage I endometriosis (72 ± 10.5%) than in animals with a normal pelvis (53 ± 18%), stage II (49 ± 8%) or stage III–IV disease (49 ± 15).

Subsequently, analysis of peritoneal fluid subsets according to cycle stage demonstrated no differences in baboons with a normal pelvis. In animals with endometriosis the concentration of WBC was higher during the luteal phase than in non-cycling primates (Kruskal–Wallis with Dunn's multiple comparison test, P <0 015, Table II). The WBC concentration, peritoneal fluid macrophage concentration (determined by Leu M5) and the percentages of lymphocyte subsets were comparable during menses, follicular and luteal phase in baboons with and without endometriosis.

Discussion

In this study, the percentages of WBC subsets, determined by mouse anti-human monoclonal antibodies CD2, CD4, CD8,

Table III. Analysis of variance (ANOVA) of white blood cell (WBC) populations (mean ± SD) in peritoneal fluid from 57 baboons with normal pelvis, recent spontaneous endometriosis (n = 9), long-term spontaneous endometriosis (n = 11) and induced disease (n = 16)

	Total	Normal pelvis (n = 21)	Recent spontaneous endometriosis (n = 9)	Long-term spontaneous endometriosis (n = 11)	Induced disease (n = 16)	P
WBC ($\times 10^3$/μl)	2 2 (2)	2.2 (1.7)	4 5 (3 7)	1 3 (0.5)	2 (1 3)	0 01[a]
No WBC ($\times 10^6$)	6 (4 2)	6.4 (3 9)	8 8 (7 6)	4 1 (1.8)	5 7 (3.5)	0 17
FACS	27 5 (15)	26 7 (10)	24 7 (13.3)	25 2 (19 7)	31 6 (17)	0 6
% lymphocytes						
% macrophage	61 4 (16 8)	62 3 (15)	61 9 (13 8)	66 (22.3)	56 4 (15 7)	0 5
% neutrophils	11 (13 4)	10 9 (12)	11.5 (13 8)	9 9 (11 5)	10 7 (12 3)	0.9
M5d	57 (17)	53.2 (18)	70 9 (11 1)	62 1 (17.3)	48 7 (12 6)	0 06[b]
No M5 (%)	3 4 (3)	3.4 (2 6)	6 (5.5)	2 (1 2)	2 7 (1 9)	0 17
CD2	84.3 (7 7)	83 8 (7.5)	79 6 (12 2)	85 8 (5.8)	86 3 (6.2)	0 28
CD4	26.6 (9.3)	28 8 (8.7)	24.4 (9 3)	25 (6 1)	26 1 (12)	0 6
CD8	66 4 (12.9)	67 1 (8 3)	54.8 (21 9)	74.3 (6)	65 8 (8)	0.01[c]
CD20	4 2 (2 5)	4 (1 4)	4 6 (2)	3 4 (2 1)	4 8 (3 7)	0.5
IL2R	12 (8 8)	13 (8.2)	12.3 (3 4)	14 4 (12.8)	8 7 (7 9)	0 4
CD56	5 6 (3)	4 7 (2 8)	5 9 (2 9)	6 6 (3 8)	5 6 (2 8)	0 5

[a]Significant elevation in group with recent spontaneous endometriosis when compared with other groups (Scheffé post-hoc test)
[b]P = 0.03 (ANOVA) when baboons with recent or long-term spontaneous endometriosis (mean 65.7 ± 15 3) are compared with animals with induced disease and primates with a normal pelvis (mean 51 ± 15) P = 0 01 (t-test) comparing baboons with spontaneous endometriosis and those with induced disease
[c]Significant elevation in group with long-term spontaneous endometriosis when compared with other groups (Scheffé post-hoc test)
[d]Leu M5 macrophage marker.

CD11B, CD20 and Leu M5, were comparable in the peripheral blood of baboons to those reported in humans (Table III; Reichert *et al.*, 1991). This observation confirms and extends the results of others (Rappocciolo *et al.*, 1992), is supported by our observation that these immune cells have a similar distribution in immune organs of both baboons and humans (D'Hooghe *et al.*, 1995) and indicates that WBC subsets in baboons can be analysed with commercially-available monoclonal antibodies. However, in contrast to a previous report (Rappocciolo *et al.*, 1992), antibodies for CD3 and CD16 were not cross-reactive and a selective marker for NK cells was not demonstrable. It is also important to note that both this study and a previous report (Rappocciolo *et al.*, 1992) demonstrated a CD4/CD8 ratio of <1 in baboons, rhesus monkeys and African green monkeys, whereas in humans the ratio is 1.4 (Reichert *et al.*, 1991). This 'inverse' ratio is not an indication of immunosuppression since all of the baboons in our study were healthy and normal proliferative responses of their WBC to phytohaemagglutinin, concanavalin A and pokeweed mitogen have been reported (Rappocciolo *et al.*, 1992). The results of our study do not exclude the possibility that WBC subsets in baboons, recognized by anti-human monoclonal antibodies, may be functionally different from WBC subsets in humans.

In peripheral blood, a higher percentage of $CD4^+$ and $IL2R^+$ cells was found in baboons with long-term spontaneous endometriosis or induced disease (stage II–IV) when compared to those with recent spontaneous endometriosis (stage I) or a normal pelvis. These data suggest that the immune system is activated in the peripheral blood of baboons with stage II–IV endometriosis and that this activation is a consequence of endometriosis since it was also found in animals with induced disease that had had a normal pelvis before induction. Our findings are in agreement with two reports in women (Badawy *et al.*, 1987, 1989) demonstrating an increased number of T cells, B cells and an increased T-helper/T-cytotoxic ratio in peripheral blood from infertile patients with endometriosis in comparison with infertile women without the disease. However, most investigators (Gleicher *et al.*, 1984; Hill *et al.*, 1988; Garzetti *et al.*, 1993; Melioli *et al.*, 1993) have reported that the concentration of peripheral blood macrophages, lymphocytes and their subsets (T cells, B cells, T-helper cells, T-cytotoxic cells, NK cells) are comparable in all women regardless of fertility status or the presence of endometriosis.

Several findings in this study support the hypothesis that alterations in peritoneal fluid WBC cell population may be causally related to endometriosis. Firstly, increased peritoneal fluid cell populations were only found in baboons with spontaneous endometriosis: an increased WBC concentration, an increased percentage of Leu $M5^+$ macrophages and an enhanced proportion of $CD8^+$ cells was demonstrated in peritoneal fluid of baboons with recent endometriosis, animals with recent or long-term disease and primates with long-term endometriosis respectively. Secondly, the peritoneal fluid cell population was comparable in baboons with induced endometriosis and animals with a normal pelvis. Similarly in rabbits (Johnson *et al.*, 1991), induction of endometriosis did not result in an increased total number of peritoneal fluid macrophages. Thirdly, the Leu $M5^+$ macrophage population was higher in baboons with stage I endometriosis than in those with stage II, III or IV disease. Similarly in women, an increased macrophage concentration has been reported in infertile patients with minimal and mild endometriosis, but not in those with moderate or severe disease (Hill *et al.*, 1988). Other studies in women have indicated that the total number of peritoneal fluid macrophages either is not (Haney *et al.*, 1983) or is negatively (Haney *et al.*, 1991) correlated with the extent of endometriosis, further supporting the hypothesis that the presence of endometriosis is not necessarily causally related to the increase in peritoneal fluid macrophage number.

The endocrinological status of the animals affected the WBC population in peripheral blood and peritoneal fluid only in baboons with endometriosis, possibly due to a larger sample size. In peripheral blood, the WBC concentration in baboons with endometriosis was increased during pregnancy or while nursing as is known to occur in women. The increased percentage of $CD4^+$ cells during the follicular phase in baboons with endometriosis can be explained by the fact that nine out of 11 baboons studied in the follicular phase had stage II–IV disease. It can be postulated that the lower concentration of WBC in peritoneal fluid of non-cycling animals was caused by the lack of cyclic peritoneal stimulation (no ovulation, no retrograde menstruation) with reduced activation of the immune system in these primates.

In conclusion, this study demonstrated that mouse anti-human monoclonal antibodies CD2, CD4, CD8, CD20 and Leu M5 can be used in the baboon. The percentage of $CD4^+$ and $IL2R^+$ cells was increased in peripheral blood of baboons with stage II–IV spontaneous or induced endometriosis, suggesting that alterations in peripheral blood WBC populations may be an effect of endometriosis. In peritoneal fluid the WBC concentration and percentage of Leu $M5^+$ macrophages and $CD8^+$ lymphocytes was only increased in baboons with spontaneous endometriosis and not in animals with induced disease, suggesting that alterations in peritoneal fluid WBC populations may lead to the development of endometriosis.

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