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Endometrial leukocyte subpopulations associated with *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and *Trichomonas vaginalis* genital tract infections

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Abstract

Objective—Characterize endometrial inflammation associated with common genital tract pathogens.

Study Design—Immunohistochemical characterization of endometrial leukocyte subpopulations from 37 controls and 45 women infected with *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, or *Trichomonas vaginalis*.

Results—Compared to uninfected women, endocervical infection with *C. trachomatis*, *N. gonorrhoeae*, or *T. vaginalis* was associated with significant increases in endometrial T cells, B cells, plasma cells, and polymorphonuclear leukocytes. Even more substantial increases in T cell, B cell, and plasma cell numbers were detected among women infected endocervically and endometrially with *C. trachomatis*.

Conclusions—As lower genital tract *C. trachomatis*, *N. gonorrhoeae*, or *T. vaginalis* infections were associated with comparable increases in the same endometrial leukocyte subpopulations, our results suggest underappreciated involvement of *T. vaginalis* in upper genital tract inflammatory processes. The more robust inflammatory infiltrate associated with *C. trachomatis* endometrial ascension may offer insight into host inflammatory responses associated with pelvic inflammatory disease development.

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Condensation: *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and *Trichomonas vaginalis* genital tract infections are associated with significant increases in endometrial T cell, B cell, and plasma cell numbers.

Keywords

endometritis; *Chlamydia trachomatis*; *Neisseria gonorrhoeae*; pelvic inflammatory disease; *Trichomonas vaginalis*

Introduction

Endometritis is a histopathological diagnosis formed by identification of abnormal endometrial inflammatory cell infiltrates and disturbances in normal endometrial growth [1]. The leukocyte subpopulations most firmly established with diagnosis of histologic endometritis are polymorphonuclear leukocytes (PMNs) and plasma cells (2). Although not necessarily indicative of disease duration, inflammatory cell infiltrates are also used to stratify endometritis into acute and chronic forms of disease [3]. Acute endometritis is identified by the presence of PMNs in the absence of menstrual endometrium [4], while chronic endometritis relies upon plasma cell identification in the absence of neutrophilic infiltration [5, 6]. Although offending pathogens are not always found, migration of *Chlamydia trachomatis* or *Neisseria gonorrhoeae* from the lower to upper genital tract is the most common cause of chronic (also called plasma cell) endometritis [7, 8].

Because diagnosis of chronic endometritis has been associated with development of pelvic inflammatory disease (PID), chronic endometritis may represent an intermediary step between lower genital tract infection and upper genital tract damage [9]. Accurate identification of histologic endometritis, however, is problematic as many leukocyte subpopulations are also present in normal endometrial tissue [10, 11]. Adding to this diagnostic complexity, the frequency of some endometrial leukocyte subpopulations vary with stages of the menstrual cycle [12]. In proliferative and early secretory phases, the predominant leukocyte subpopulations are macrophages, natural killer (NK) cells, and T cells (accounting for approximately 10% of endometrial stromal cells), while in the late secretory stage these leukocyte subpopulations account for 20% of the stromal cells [13]. This increase is primarily the result of an increase in NK cell numbers as T cell numbers remain relatively constant throughout the menstrual cycle [14]. Unlike NK and T cells, plasma cells are infrequently detected in normal endometrial tissue [4], while B cells are only sporadically identified (comprising < 2% of all endometrial leukocytes) [15].

While prior immunohistochemistry-based studies comparing leukocyte subpopulations among non-pregnant women with normal endometrium and women with chronic endometritis found no differences in the number of endometrial macrophages and NK cells [1, 3], large increases in endometrial B cells [16], and more modest increases in endometrial T cells, [17] have been associated with diagnosis of chronic endometritis. Less well described, however, is the endometrial inflammatory infiltrate associated with the specific genital tract pathogens causing chronic endometritis. In the present study, we performed immunohistochemical analyses of endometrial tissue using markers specific for NK cells, T cells, B cells, plasma cells, and macrophages to characterize endometrial inflammatory responses associated with genital tract infection with *C. trachomatis*, *N. gonorrhoeae*, and a putative PID pathogen, *Trichomonas vaginalis*.

Materials and Methods

We selected 82 paraffin embedded endometrial specimens from a prior investigation of the risk factors associated with PID [18]. Both the parent and current investigation were approved by the University of Pittsburgh's Institutional Review Board. In the parent study, endocervical swab specimens had been collected for culture-based detection of *N.*

gonorrhoeae and *T. vaginalis* and PCR-based detection of *C. trachomatis*. Trans-cervical endometrial biopsy specimens had also been obtained, and a tissue portion had undergone identical diagnostic testing for *C. trachomatis*, *N. gonorrhoeae* and *T. vaginalis*, while another portion had been paraffin embedded. For the current study, we selected paraffin embedded endometrial samples from 37 women that had no evidence of *C. trachomatis*, *N. gonorrhoeae*, or *T. vaginalis* infection. Among the other selected samples, 27, 7, and 11, respectively, had been collected from women with endocervical *C. trachomatis*, *N. gonorrhoeae*, or *T. vaginalis* infection. None of the cases selected were from women co-infected with any 2 or 3 of these genital tract pathogens, while 10 of the 27 women with endocervical *C. trachomatis* infection had also been diagnosed with endometrial *C. trachomatis* infection. Endometrial specimens were excluded from further analysis if hematoxylin and eosin (H&E) stained slides demonstrated menstrual cycle stage consistent with late secretory or menses as increased interstitial hemorrhage, tissue necrosis, and neutrophilic infiltration makes interpretation of endometrial inflammatory changes more problematic [19]. H&E stained slides were also used to grade PMN infiltrate in 10 high power (400X) fields of endometrial surface epithelium using a 0-2 semi-quantitative scale: grade 0 = no PMNs detected; grade 1 = mean number of 1- 4 PMNs per field; grade 2 = mean number ≥ 5 PMNs per field.

The monoclonal antibodies used in this investigation (and leukocyte subpopulations they identify) were CD3 (T cells), CD8 (CD8⁺ T cells), CD20 (B cells), CD56 (NK cells), CD68 (macrophages), CD138 (plasma cells), and the class II major histocompatibility complex (class II MHC) molecule (Table 1). MHC class II molecule staining was performed without antigen retrieval, determination of CD8, CD56, and CD138 expression utilized microwave antigen retrieval in citrate buffer and indirect immunolabeling, and CD3, CD20, and CD68 immunostaining was completed using Ventana pre-diluted antibodies (Ventana, Tucson, AZ). Positive control slides were included with each stain to confirm antibody activity, while absence of reactivity among negative controls confirmed adequacy of blocking procedures and specificity of positive results. Two surgical pathologists blinded to all infectious disease diagnoses independently assessed each slide. Positive cells for each marker were graded using a 0-4 semi-quantitative scale: grade 0 = no positive cells; grade 1 = single scattered positive cells; grade 2 = loosely scattered cells or occasional small aggregates; grade 3 = densely scattered cells; grade 4 = dense collections of positive cells throughout entire specimen. Because the vast majority of CD3⁺ T cells in the endometrium are either CD8 or CD4 positive, immunohistochemical identification of CD3⁺ and CD8⁺ positive cells also allowed determination of endometrial CD4⁺ T cell numbers.

Statistical analyses were completed using GraphPad Prism® 5 software (San Diego, California). Calculation of medians and percentile distributions were used to determine semi-quantitative differences in each of the measured leukocyte subpopulations between: 1) women with no genital tract infection or women diagnosed with lower genital tract *C. trachomatis*, *N. gonorrhoeae*, or *T. vaginalis* infection and 2) women with no genital tract infection, women diagnosed with endocervical *C. trachomatis* infection, or women diagnosed with endocervical and endometrial *C. trachomatis* infection. Comparisons of scores between groups were performed using Kruskal-Wallis test on ranks and Dunn's multiple comparison post hoc test (p values ≤ 0.05 were considered statistically significant).

Results

We first compared PMN numbers and the number of CD3, CD8, CD20, CD56, CD68, CD138, and MHC class II positive cells in the endometria of women with no genital tract infection to the number of these cells seen in the endometrial tissue of women diagnosed with *C. trachomatis* or *N. gonorrhoeae* endocervical infection; the two most firmly

established infectious causes of upper genital tract inflammation [20]. Similar to the results from prior comparisons of endometrial biopsies from healthy women and women diagnosed with chronic endometritis [1], we found no significant differences in the numbers of endometrial macrophages and NK cells among uninfected women and women with endocervical *C. trachomatis* or *N. gonorrhoeae* infection (Figure 1). On the other hand, women diagnosed with *C. trachomatis* or *N. gonorrhoeae* endocervical infection demonstrated statistically significant increases in endometrial PMN, CD3⁺ T cell, B cell, and plasma cell numbers compared to the numbers seen among uninfected controls (Figure 1). As the number of CD3⁺, but not CD8⁺, cells was increased, we were also able to conclude CD4⁺ T cells were the T cell population increased among women with endocervical *C. trachomatis* or *N. gonorrhoeae* infection.

We also observed similar increases in the number of endometrial PMNs, CD3⁺ T cells, B cells, and plasma cells among women diagnosed with *T. vaginalis* infection, a much less firmly established cause of upper genital tract inflammation (Figure 1). Combined, these findings demonstrate that all 3 genital tract pathogens, even in the absence of documented upper genital tract infection, were associated with similar increases in endometrial CD4⁺ T cell, B cell, and plasma cell numbers. In other words, upper genital tract inflammation evoked by all 3 genital tract pathogens appeared to be indistinct, at least as related to the infiltration of endometrial leukocytes. Although a significant increase in the number of endometrial plasma cells was observed with all 3 genital tract infections (Figure 1), we found that endocervical *C. trachomatis* infection was the least consistently associated with detection of endometrial plasma cells. While endometrial biopsy sections from all women infected with *N. gonorrhoeae* or *T. vaginalis* had a detectable plasma cell population, only 70.4% (19/27) of women in our study with endocervical *C. trachomatis* infection had a demonstrable endometrial plasma cell infiltrate (data not shown).

We next compared the numbers of PMNs and CD3, CD8, CD20, CD56, CD68, CD138, and MHC class II positive cells present in the endometrial tissue of uninfected controls, women with endocervical *C. trachomatis*, and women with both endocervical and endometrial *C. trachomatis* infection (representative results for CD3, CD20, and CD138 immunohistochemical staining are displayed in Figure 2). We observed that women with endocervical *C. trachomatis* infection and women with endocervical and endometrial *C. trachomatis* infection had significantly higher numbers of endometrial PMNs compared to the numbers seen among uninfected controls. We also observed that women with both endocervical and endometrial *C. trachomatis* infection demonstrated substantially higher numbers of CD3⁺ T cells, B cells, and plasma cells compared to the numbers seen among uninfected women or women diagnosed with isolated endocervical *C. trachomatis* infection (Figure 3). Because CD3⁺, but not CD8⁺ cell numbers were increased, these results were consistent with the fact that CD4⁺ T cells were the endometrial T cell subset preferentially increased among women with upper genital tract *C. trachomatis* infection. The more substantial increases in CD4⁺ T cell, B cell, and plasma cell numbers seen in the latter group also suggested that more dramatic host inflammatory responses were elicited by migration of *C. trachomatis* into the upper genital tract as opposed to when detection of this pathogen was restricted to the lower genital tract. Furthermore, because PID is caused primarily by ascension of pathogenic microorganisms from vagina and cervix into the upper genital tract [2], our findings may serve to delineate endometrial inflammatory responses associated with an increased risk for PID development.

Comment

Our study demonstrates increased numbers of PMNs, T cells, B cells, and plasma cells in the endometrial tissue of women with lower genital tract *C. trachomatis*, *N. gonorrhoeae*, and *T.*

vaginalis infections. As endometrial mucosa is site to immune responses capable of eradicating pathogens from the upper genital tract, these results may provide insight into the inflammatory responses associated with sterilizing immunity against these genital pathogens. On the other hand, the increased inflammatory infiltrate associated with these infections may increase the likelihood for immunopathologic upper genital tract damage. Any conclusion as to which of these alternative scenarios is more likely, however, is prevented by the cross-sectional design of our investigation. We further demonstrate CD4⁺ cells are the T-cell subset preferentially increased in endometria of women with lower genital tract *C. trachomatis*, *N. gonorrhoeae*, and *T. vaginalis* infections. As the majority of CD3⁺ cells in healthy, uninfected endometrial tissue are CD8⁺ [21], our findings also suggest the increased endometrial CD4:CD8 T-cell ratio observed in our investigation occurred in response to the presence of a lower genital tract infection. These changes mirror those seen in the endometrial tissue of women with unexplained infertility where increased numbers of CD4⁺ T-cells have also been detected [22, 23]. Our findings, therefore, raise the interesting possibility that endometrial CD4⁺ T-cell numbers may also be increased among women with PID-associated infertility. Further investigation will be needed to determine the validity of this hypothesis and to delineate possible etiologic roles for CD4⁺ T cells in PID pathogenesis.

Because endometrial inflammatory changes elicited by *C. trachomatis*, *N. gonorrhoeae*, and *T. vaginalis* infections appear indistinguishable in our investigation, our findings suggest an underappreciated contribution by the latter pathogen in upper genital tract inflammatory processes. As culture-based diagnosis of *T. vaginalis* infection, as performed in the parent investigation, is less sensitive than PCR (24), it is also possible that undetected *T. vaginalis* infections made additional contribution to the increased endometrial inflammation seen among women with *C. trachomatis* and *N. gonorrhoeae* infection. Although causal connections between *T. vaginalis* and the pathogenesis of endometritis and PID are not well delineated, prior reports suggest the possibility of a linkage. For example, *T. vaginalis* is capable of ascension into the endometrial cavity and fallopian tubes [25], and women in a nested case control study that were co-infected with *T. vaginalis* and *C. trachomatis* exhibited increased risk for PID development [26]. Risk of infertility due to tubal adhesions or occlusions was also twice as high among women with self-reported history of vaginal trichomoniasis [27]. As our cross-sectional design cannot address causality, further investigation is now needed to determine if *T. vaginalis* alone can elicit endometrial inflammation or if this motile organism acts as a carrier to allow other bacterial pathogens improved access to the upper genital tract.

Our study also offers new insight into the nature of the leukocytic infiltrate associated with endometrial *C. trachomatis* infection. PID is caused by pathogen migration into the upper genital tract, so the substantial increases in endometrial T cells, B cells, and plasma cells associated with *C. trachomatis* ascension in our investigation may serve to define the inflammatory responses more likely to cause ectopic pregnancy, tubal infertility, and other PID-associated sequelae. The dramatic increases in plasma cell numbers elicited by endocervical and endometrial *C. trachomatis* infection also imply that prior studies that reported a lack of an association between endometritis (which had been diagnosed upon detection of as few as 2 endometrial plasma cells) and infertility may need reexamined [28, 29]. As endometrial *C. trachomatis* infection appears to be associated with much more substantial increases in endometrial plasma cells, conclusions from these studies may have been limited by their use of diagnostic criteria too non-specific to uncover associations between upper genital tract inflammation and adverse reproductive sequelae. New longitudinal studies will be needed to delineate the actual relationships between genital pathogens, endometrial inflammation, and PID development.

In conclusion, increased numbers of endometrial T cells, B cells, and plasma cells are seen among women with *C. trachomatis*, *N. gonorrhoeae*, and *T. vaginalis* lower genital tract infection, while even more substantial increases in these leukocyte subpopulations are detected upon *C. trachomatis* migration into endometrial space. Additional work is necessary to determine if the increased endometrial inflammation is also associated with increased risk for adverse reproductive outcomes. Compared to the current reliance on plasma cell detection alone for diagnosis of histologic endometritis, it seems possible that increased endometrial CD4⁺ T cell, B cell, and plasma cell numbers may also provide more specific criteria for identification of pathogen-mediated endometrial inflammation. In future PID epidemiologic research, composite characterization of endometrial CD4⁺ T cells, B cells, and plasma cells may also help clarify the relevance of lower genital tract infection with *Mycoplasma genitalium* and other putative PID pathogens, and delineate specific antimicrobial therapies that are best associated with resolution of upper genital tract inflammation and improved reproductive outcomes. Our study, therefore, provides new insight into the improved understanding of PID epidemiology, pathogenesis, and treatment that can be achieved upon a more comprehensive characterization of the endometrial inflammatory changes elicited by specific genital tract pathogens.

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Figure 1. Quantification of endometrial leukocyte subpopulations among women with *C. trachomatis*, *N. gonorrhoeae* or *T. vaginalis* infection of the lower genital tract demonstrates significant increases in the number of endometrial T cells, B cells, and plasma cells

Endometrial biopsy sections from women with no identified genital tract infection or *C. trachomatis*, *N. gonorrhoeae* or *T. vaginalis* lower genital tract infection were H&E stained for PMN detection and immunostained for detection of CD3, CD8, CD20, CD138, CD56, CD68 and MHC II positive cells. Stained sections were scored using semi-quantitative scoring system described in text, and groups compared using Kruskal-Wallis and Dunn's multiple comparison tests (* $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$) (box and whiskers indicate median $\pm$ 10-90 percentile).

Figure 2. Dual endocervical and endometrial *C. trachomatis* infection elicits an intense inflammatory infiltrate

Representative endometrial biopsy specimen from a woman with dual endocervical and endometrial *C. trachomatis* infection shows dramatic A) CD3 (T cell specific), B) CD20 (B cell specific), and C) CD138 (plasma cell specific) immunostaining of endometrial stromal cellular aggregates (all samples developed with brown colorimetric detection systems described in text) (original magnifications X200).

Figure 3. Dual endocervical and endometrial *C. trachomatis* infection is associated with the most substantial increases in endometrial T cell, B cell, and plasma cell numbers

Analyses identical to those described in the Figure 1 legend were completed among women with no genital tract infection, endocervical *C. trachomatis* infection, or dual endocervical and endometrial *C. trachomatis* infection. Groups were compared using Kruskal-Wallis and Dunn's multiple comparison tests (* $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$) (box and whiskers indicate median $\pm$ 10-90 percentile).

Table 1

Primary monoclonal antibodies used to characterize endometrial leukocyte subpopulations

Antibody	Cluster of differentiation	Cellular reactivity	Source
2GV6	CD3	CD3 ⁺ lymphocytes	Ventana
C8/144B	CD8	CD8 ⁺ lymphocytes	Dako
L26	CD20	B lymphocytes	Ventana
123C3.D5	CD56	Natural killer cells	Neomarkers
KP-1	CD68	Macrophages	Ventana
B-B4	CD138	Plasma cells	Serotec
CR3/43	HLA-DP, DQ, DR	MHC II	Dako