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# Therapeutically Targeting the Inflammasome Product in a Chimeric Model of Endometriosis-Related Surgical Adhesions

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**Abstract**

Development of adhesions commonly occurs in association with surgery for endometriosis. Even in the absence of surgery, women with endometriosis appear to be at an enhanced risk of developing adhesions. In the current study, we utilized a chimeric mouse model of experimental endometriosis in order to examine the role of inflammasome activation in the development of postsurgical adhesions. Mice were randomized to receive peritoneal injections of human endometrial tissue fragments or endometrial tissue conditioned media (CM) from women with or without endometriosis 16 hours after ovariectomy and placement of an estradiol-releasing silastic capsule. A subset of mice receiving CM was also treated with interleukin (IL) 1 receptor antagonist (IL-1ra). Our studies demonstrate that peritoneal injection of endometrial tissue fragments near the time of surgery resulted in extensive adhesive disease regardless of tissue origin. However, adhesion scores were significantly higher in mice receiving CM from tissues acquired from patients with endometriosis compared to control tissue CM ($P = .0001$). Cytokine bead array analysis of endometrial CM revealed enhanced expression of IL-1$\beta$ from patients with endometriosis compared to controls ($P < .01$). Finally, the ability of human tissue CM to promote adhesive disease was dramatically reduced in mice cotreated with IL-1ra ($P < .0001$). Our data implicate enhanced expression of IL-1$\beta$ in women with endometriosis as a potential causal factor in their increased susceptibility of developing postsurgical adhesions. Thus, targeting inflammasome activation may be an effective strategy for the prevention of surgical adhesions in patients with endometriosis.

**Keywords**

adhesions, inflammasome, interleukin 1, endometriosis, endometrium

## Introduction

Endometriosis is common and debilitating disease of reproductive-age women defined by the presence of endometrial glands and stroma outside the uterine corpus, most often as a consequence of inadequate clearance of displaced tissues following retrograde menstruation.[1] Nevertheless, most physicians and scientists now recognize that endometriosis is a systemic disease reflecting alterations in the function of both the endocrine and the immune systems.[2,3] While the precise etiology of the multiple presentations of the disease remains to be defined, once ectopic endometrial growth is established, chronic inflammation within the peritoneal cavity not only contributes to the progression of ectopic endometrial growth but may also promote the development of adhesive disease. Although adhesions can be a significant medical complication following any peritoneal surgery, women with endometriosis appear to be at an enhanced risk of this comorbidity.[4] Even in the absence of surgery, women with endometriosis have been found to spontaneously develop adhesive disease,[3,5] further suggesting an intrinsic phenotypic difference in systemic inflammatory processes among these patients.

The basic pathophysiology of postsurgical adhesion development is not well understood; however, both mechanical and physiological processes associated with tissue injury and repair likely contribute to this disorder. Importantly, peritoneal inflammation has been implicated in both the early establishment of endometriosis and the development of endometriosis-

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related surgical adhesions.[6,7] In order to address this clinical concern, we previously utilized a chimeric murine model of experimental endometriosis in which human endometrial tissue fragments were injected intraperitoneally into mice within 16 hours of ovariectomy. This study demonstrated that acute inflammation associated with wound healing following surgical trauma within the peritoneal cavity can significantly promote the development of both postsurgical adhesions and ectopic endometrial growth.[2] This study also identified less extensive experimental endometriosis with few or no adhesions in mice receiving endometrial tissue injections into the peritoneal cavity beyond 36 hours of the time of surgery.[2] This additional observation suggested that biologic components of wound healing likely played a mechanistic role in connecting experimental endometriosis to adhesive disease development in our model. Within the innate immune system, macrophages and neutrophils each play important roles in the initiation of inflammatory processes associated with normal wound healing by releasing inflammatory cytokines and proangiogenic factors. Thus, endometriosis-related alterations in immune cell phenotypes could potentially alter immunological responses to surgical injury within the peritoneal cavity such that adhesion formation becomes more likely to occur.[8,9]

At this juncture, identifying an *endometriosis-specific* link to altered immune cell behavior following surgery may allow the development of a targeted therapy to prevent or reduce adhesive disease in these patients. In this regard, using our chimeric model of experimental endometriosis induced after ovariectomy, we demonstrated a significant reduction in postsurgical adhesions when animals were maintained on a fish oil-enriched diet prior to surgery.[10] The dramatic results achieved with the supplemented diet likely reflect the well-known anti-inflammatory actions of omega-3 fatty acids, which have been demonstrated to reduce inflammasome activation.[11,12] The inflammasome is a multiprotein complex that is expressed in macrophages and other immune cells, where they act as immune system receptors and sensors and regulate the inflammatory response associated with both infectious microbes and damage-associated host proteins. Inflammasome complexes have also been identified in some epithelial cells, in particular, in tissues with mucosal surfaces.[13] Presently, 4 inflammasome complexes have been identified, each having a distinct protein composition formed in a stimulus-dependent manner (reviewed in and Bullon and Navarro[13]). Of these, NLR Family Pyrin Domain Containing 3 (NLRP3) is perhaps the most well studied in a variety of normal and disease states.[14] These protein complexes cleave pro-interleukin (IL) 1β, leading to its activation and promoting an inflammatory cascade. Not surprisingly, activation of inflammasome complexes has been implicated in a host of inflammatory disorders, including endometriosis[13] as well as in association with chronic pain.[15] In the current study, we investigated endometrial expression of the NLRP3 inflammasome complex as well as endometrial tissue production of IL-1α and IL-1β in women with and without endometriosis. We also examined whether therapeutically blocking the action of IL-1β, the primary product of the inflammasome, would reduce the development of postsurgical adhesions in our experimental model.

## Materials and Methods

### Acquisition of Human Endometrium

Approval for use of human tissues was obtained from the Vanderbilt University Institutional Review Board and Committee for the Protection of Human Subjects. For in vitro studies, endometrial tissues from women undergoing hysterectomy for endometriosis (PT; n = 5) were obtained through Vanderbilt University Medical Center's Cooperative Human Tissue Network. These surgical samples were placed on ice for transport to the laboratory, and endometrial tissues were removed from the myometrial layer using a scalpel, aided by the use of a dissecting microscope. Surgical tissues were washed in media and used only for conditioned media (CM) experiments (tissues were discarded after culture).

Additional endometrial samples for in vivo studies were obtained by Pipelle biopsy (Unimar, Inc, Wilton, Connecticut), following written informed consent during the proliferative phase (days 9-12) from women with a confirmed history of endometriosis (PT; n = 4) and normally cycling women with no prior history of adhesions or endometrial disease, including endometriosis (controls [CT]; n = 5). A serum progesterone level of <1.5 ng/mL was used as a cutoff value for the proliferative phase; cycle stage was later confirmed by morphological analysis of fixed tissues. Individuals with a recent (<3 months) history of hormonal therapy (ie, oral contraceptives) or other medications that could impact study results were excluded. Tissues and/or CM were prepared for injection into mice as described subsequently.

Finally, for immunochemistry studies, archived samples of formalin-fixed, paraffin-embedded tissues were obtained from women with (N = 16) or without endometriosis (N = 10) who had previously had a hysterectomy due to endometriosis or leiomyoma, respectively, at Vanderbilt University Medical Center. Archived CT and endometriosis (PT) samples were selected after histologic evaluation to determine cycle phase, resulting in an equal distribution of proliferative and early secretory phase samples. Prior to surgical removal of any organ, all patients were consented for use of discarded tissue for medical research. Although archived tissues were not originally collected specifically for this project, Vanderbilt's Institutional Review Board approved our use of these samples for retrospective analysis.

### Human Tissue Preparation and Culture Conditions

Fresh endometrial specimens from biopsies or surgery were minced into 1 × 1 $mm^3$ cubes and then suspended in tissue culture inserts (Millipore Corp, Bedford, Massachusett) as described previously.[16] Endometrial fragments within each well were maintained overnight under serum-free conditions in 500 μL DME/F-12 media supplemented with 1 nmol/L

estradiol (Sigma-Aldrich, St Louis, Missouri), 1% insulin-transferrin-selenium (Collaborative Biomedical, Bedford, Massachusett), 0.1% Excyte (Miles Scientific, Kankakee, Illinois), and 1% antibiotic/antimyotic (Sigma-Aldrich) and incubated in a 5% $CO_2$ humidified chamber at 37°C. After 24-hour incubation, tissues were removed from media and washed in phosphate-buffered saline (PBS) prior to injection into mice (biopsied tissue) or discarded (surgical specimens). The CM was centrifuged to remove any residual tissue fragments; supernatant was then collected for subsequent injection into mice or analysis by Cytokine bead array (described subsequently).

## Animal Procedures

For all studies, a total of 105 nude mice (NCr strain) were utilized and purchased at 5 weeks of age from Envigo (Indianapolis, Indiana). Upon completion of a 1-week acclimation period, mice were anesthetized with isoflurane and subjected to standard surgical ovariectomy via a single 5-mm, dorsal/ventral incision between the rib cage and the hind limb as described in detail previously.[7] At the time of surgery, all mice were implanted subcutaneously with a slow-release estradiol capsule prepared in our laboratory as described previously.[17]

For the first series of studies (Table 1), anesthetized mice were subjected to intraperitoneal injection of human endometrial tissues (8-10 pieces/mouse in 400 μL PBS) or CM (400 μL) *from those same tissues* along the ventral midline 16 hours after surgery. A single biopsy was typically adequate for induction of experimental disease in 2 to 3 mice. Each experiment used 1 biopsy from a single donor and included multiple mice per group. Separate experiments (using a single biopsy for each experiment) were replicated with multiple human endometrial biopsies from different women (PT, n = 5; CT, n = 5).

The fresh endometrial tissues obtained from patients undergoing hysterectomy for endometriosis were utilized for a second series of in vitro studies (Table 2), utilizing only the CM obtained after tissue incubation. For these studies, mice were ovariectomized and provided estradiol as described earlier, and 16 hours after surgery, mice received a single injection of 400 μL CM along the ventral midline with or without recombinant human IL-1 receptor antagonist (IL-1ra; 50 ng/mL; Sigma, #SRP3084). Additional animals received injections of 400 μL PBS supplemented with IL-1β (10 or 2 ng/mL; Sigma, #I9401) or IL-1ra (50 ng/mL) in the absence of CM. As in the first series of studies, the second series of studies utilized ovariectomized mice implanted with slow-release estradiol capsules.

At necropsy, 5 to 7 days after intraperitoneal introduction of human tissues or CM, the presence or absence of peritoneal adhesions was determined. Scoring of adhesions was as previously described,[10] which considers the number, strength, and distribution of adhesions. Specifically, 0 = no adhesions; 1 = thin filmy adhesions; 2 = more than 1 thin adhesion; 3 = thick adhesion with focal point; 4 = thick adhesion with planar attachment; and 5 = very thick vascularized adhesions or more than 1 planar adhesion.

## Immunohistochemistry

Immunohistochemical staining of archived human tissues was performed by standard methodology for formalin-fixed, paraffin-embedded tissues. Briefly, tissues were deparafinized with xylene and rehydrated in serial dilutions of ethanol. After heat-activated antigen retrieval, primary antibody was applied (NLRP3: Sigma, HPA 012878, original concentration 0.2 mg/mL, 1:50 dilution) and slides incubated overnight at 4°C in a humid chamber. Primary antibody was removed, slides rinsed in PBS/Tween, and secondary antibody applied (HK 336-9R, Biogenex, Fremont, CA; 50 mL ready-to-use biotinylated anti-immunoglobulin in PBS with carrier protein and 0.09% sodium azide). Slides were incubated in secondary antibody for 1 hour at room temperature, washed in PBS and NLRP3, and visualized using the Dako Envision+ HRP/DAB System (DakoCytomation, Carpenteria, CA) with nickel enhancement followed by counterstaining with Mayer's hematoxylin. After staining, all slides were dehydrated, cleared, and coverslipped for morphological analysis. For the negative control, the primary antibody was omitted. Histopathological assessments were performed using an Olympus BX51 microscope system and images captured using an Olympus BX51 microscope system and Olympus DP71 digital camera (Center Valley, PA).

## Assessment of Staining Intensity of NLRP3

Staining intensity was assessed based on the Histologic score (H-score) method originally developed by Budwit-Novotny and colleagues.[18] For calculation of H-score, 8 to 10 fields were selected at random per group and photographed at 400× magnification. Staining intensity within endometrial glands and stroma was independently assessed by 2 authors (SM and KB-T) and scored as 0, 1, 2, or 3 corresponding to the presence of negative, weak, intermediate, or intense staining, respectively. The average of percent positive cells at each level of intensity was determined and the H-score calculated based on the following formula:

$$\begin{aligned}\text{H-score} = {} & (\%\text{ of cells stained at level 1 intensity} \times 1) \\ & + (\%\text{ of cells stained at level 2 intensity} \times 2) \\ & + (\%\text{ of cells stained at level 3 intensity} \times 3)\end{aligned}$$

An H-score of between 0 and 300 was obtained, where a score of 300 is equivalent to 100% of cells maximally stained. The H-score from each observer (2 observers) per field was averaged; thus, 8 to 10 numbers were plotted for each group. The interobserver variation was less than 15% across all samples.

## Masson's Trichrome Staining

Adhesions excised from mice at necropsy were formalin fixed, paraffin embedded, and subjected to Masson's trichrome staining in order to assess the extent of collagen deposition. Specifically, Masson's trichrome staining results in blue collagen fibers (with intensity of staining dependent on the amount of

collagen), red cytoplasm, and purple nuclei. Histochemical staining was performed by Vanderbilt Translational Pathology Shared Resource Core using standard methods.

### Cytokine Bead Array Analysis

Conditioned medium was assayed simultaneously for IL-1α and IL-1β using a multiplex magnetic bead array (Cat # HCYTOMAG-60K; Milliplex, Millipore, Temecula, California) on the Luminex platform. All samples were run in duplicate alongside a standard curve and unconditioned media.

### Statistical Analysis

Analyses were performed with GraphPad Prism5 (version 5.00 for Windows, GraphPad Software, La Jolla California USA, http://www.graphpad.com) software and presented as mean ± standard error of the mean. The statistical difference between samples was determined using an unpaired 2-tailed *t* test. $P < .05$ was considered significant.

## Results

### Enhanced Risk of Adhesive Disease Associated With Endometriosis

Using a chimeric model of experimental endometriosis, we previously demonstrated an increased risk of adhesion development following introduction of viable human endometrial fragments into the peritoneum of nude mice within 16 hours of abdominal surgery.[7] However, our previously published work utilized endometrial samples obtained from disease-free women only. Herein, organ cultures were established using endometrial tissues from patients with surgically confirmed endometriosis and from control women without endometriosis. As shown in Table 1, current studies reveal that there is only a nonsignificant difference between development of adhesive disease in response to peritoneal injection of human endometrial tissues, washed and resuspended in PBS, from women with or without disease. However, injection of the CM obtained from the same tissues into mice resulted in a significant increase in the risk of adhesive disease associated with CM from PT tissues ($PT_{CM}$) compared to CM from women without endometriosis ($CT_{CM}$). The marked difference in the adhesion score following injection of $PT_{CM}$ versus $CT_{CM}$ suggests differential production of inflammatory cytokines by endometrial tissues from women with endometriosis.

**Table 1.** Enhanced Risk of Adhesions Associated With Tissues From Women With Endometriosis.[a]

| Mouse Treatment[b] | N (Mice) | % With Adhesions[c] | Adhesion Score (Mice With Adhesions) | *P*[d] |
|---|---|---|---|---|
| $CT_{CM}$ (N = 5) | 15 | 67 | 1.1 | |
| $CT_{endometrial\ tissue}$ (N = 5) | 16 | 81 | 3.2 | .001 |
| $PT_{CM}$ (N = 4) | 12 | 100 | 3.6 | <.0001 |
| $PT_{endometrial\ tissue}$ (N = 4) | 12 | 100 | 3.9 | <.0001 |

Abbreviations: CT, controls; CM, conditioned media; PT, patients.
[a]Tissue and CM samples from women undergoing volunteer endometrial biopsy.
[b]Treatments conducted 16 hours postsurgery.
[c]Mice euthanized 5 to 7 days postsurgery.
[d]Compared to mice receiving control conditioned media.

**Table 2.** Influence of Peritoneal Interleukin 1β on Risk of Postsurgical Adhesive Disease.[a]

| Mouse Treatment[b] | n | % With Adhesions[c] | Adhesion Score (Mice With Adhesions) | *P*[d] |
|---|---|---|---|---|
| IL-1β (10 ng/mL) | 10 | 100 | 4.4 | |
| IL-1β (2 ng/mL) | 9 | 100 | 1.8 | .0002 |
| $PT_{CM}$ (N = 5) | 13 | 100 | 3.7 | .0327 |
| $PT_{CM}$ + IL-1ra (N = 5) | 14 | 71 | 1.6 | .0001 |
| IL-1ra only (50 ng/mL) | 4 | 0 | 0 | .0039 |

Abbreviations: IL-1β, interleukin-1 β; PT, patients; CM, conditioned media; IL-1ra, IL-1 receptor antagonist.
[a]Patient samples from women undergoing hysterectomy due to endometriosis.
[b]Treatments conducted 16 hours postsurgery.
[c]Mice euthanized 6 to 8 days postsurgery.
[d]Compared to IL-1β (10 ng).

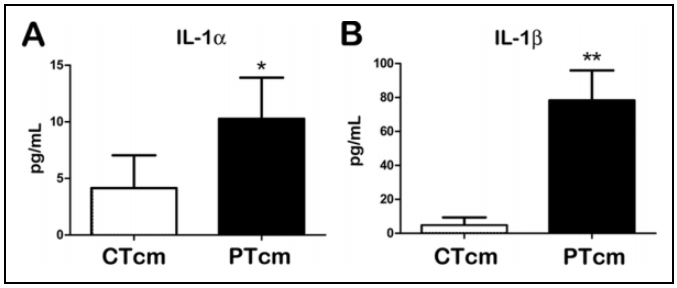


**Figure 1.** Cytokine bead array analysis of IL-1α and IL-1β in conditioned media samples. Endometrial organ cultures were established from proliferative phase samples obtained from women with (N = 5) and without (N = 5) endometriosis. Following 24 hours in overnight culture, conditioned media was collected and subjected to cytokine bead array analysis, revealing a significant increase in both IL-1α (A; $P = .0468$) and IL-1β (B; $P = .0089$) compared to control samples. Note the difference in scale between panels A and B, demonstrating a greater increase in expression of IL-1β compared to IL-1α among patients with endometriosis. IL-1 indicates interleukin-1.

### Alterations in IL-1 Production in Endometriosis

The CM obtained following 24-hour incubation with endometrial tissues from women with or without endometriosis were subjected to cytokine bead array analysis for IL-1α and IL-1β. As shown in Figure 1, we observed significant increases in both IL-1α ($P < .05$) and IL-1β ($P < .01$) in $PT_{CM}$ samples compared to $CT_{CM}$ samples; however, the most dramatic change was the increase in IL-1β ($P < .01$). Since IL-1β is the primary

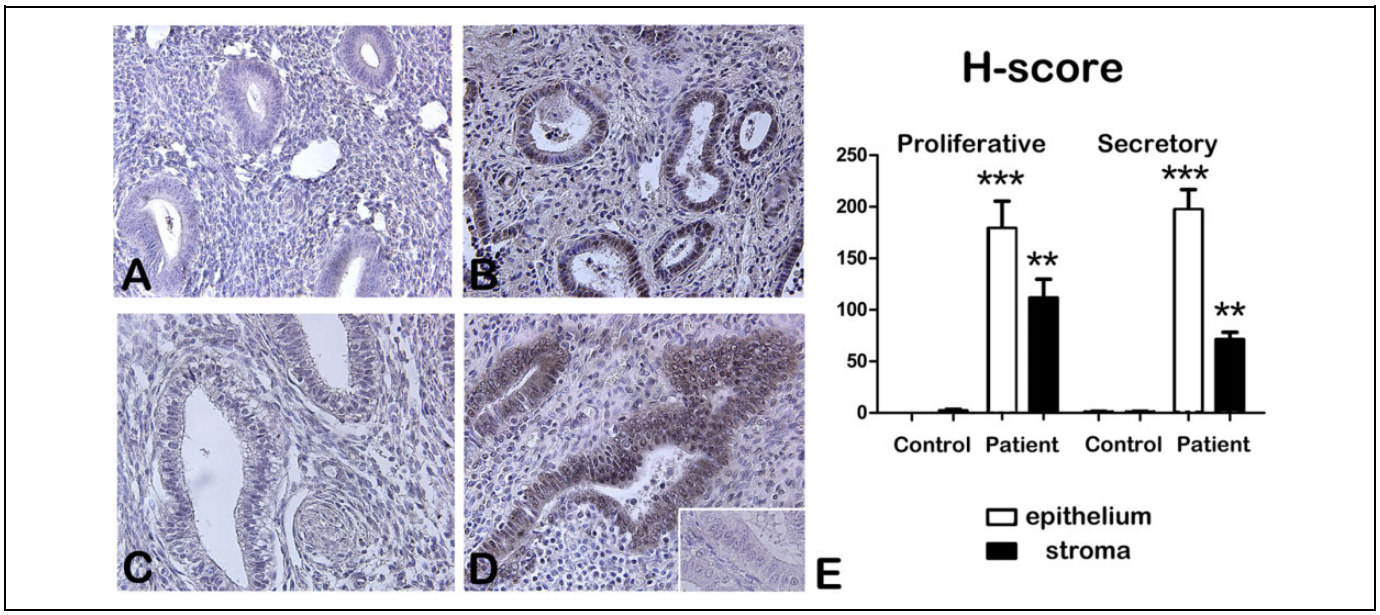


**Figure 2.** Immunohistochemical localization of NLR Family Pyrin Domain Containing 3 (NLRP3) protein in endometrial tissues from women with and without endometriosis. Archived samples of hysterectomy specimens were obtained from women with (N = 16) and without (N = 10) endometriosis, equally distributed between proliferative phase (A and B) and the early secretory phase (C and D). Immunohistochemical localization of NLRP3 revealed minimal expression in tissues from women without endometriosis (A and C) but robust expression of this protein in tissues from women with this disease (B and D), regardless of cycle phase. E, Semiquantitative analysis of NLRP3 as determined by H-score. Compared to control samples, $P \leq .001$ for all patients with endometriosis. Representative photomicrographs are shown (original magnification, ×400). Inset: omission of primary antibody. $^{**}P = .001$; $^{***}P = .0001$. H-score indicates Histologic score.

product of the inflammasome, these data suggested a role for dysregulated peritoneal inflammation involving inflammasome as a potential trigger for endometriosis-associated adhesive disease.

## *Heightened Expression of NLRP3 Protein in Endometriosis*

Numerous studies have demonstrated phenotypic differences between endometrial tissues from women with and without endometriosis (eg, [19-21]), including our previous studies demonstrating a loss of the normal anti-inflammatory effects of progesterone in tissues from women with this disease.[22-25] Herein, using immunohistochemistry, we examined expression of NLRP3 protein in endometrial tissues from women with and without endometriosis as a potential marker of an inflammatory tissue phenotype. As shown in Figure 2, expression of NLRP3 protein is virtually absent in endometrial tissues acquired from women without endometriosis (n = 10), but the majority (14 of 16) of samples acquired from women with endometriosis exhibited robust expression of this complex, regardless of cycle phase (Figure 2B and D). Interestingly, NLRP3 immunolocalized predominantly within the epithelium, with more moderate expression observed within the stroma.

## *Role of IL-1 in Adhesion Development*

In order to directly examine the influence of the inflammasome activation product, IL-1β, on postsurgical adhesion development in our mice, we next conducted intraperitoneal injection of this cytokine versus CM treated with or without IL-1ra. As mentioned earlier, all peritoneal injections occurred 16 hours after ovariectomy and estradiol capsule placement; all animals were euthanized 5 to 7 days after cytokine of CM injections and examined for adhesive disease. As shown in Table 2, gross examination of mice at necropsy revealed a dose-dependent increase in the adhesion score of mice following injection of 2 or 10 ng/mL IL-1β 16 hours after ovariectomy. Furthermore, mice receiving $PT_{CM}$ exhibited adhesion scores similar to those treated with the highest does of IL-1β. In contrast, we observed a significant reduction of adhesive disease in mice treated with $PT_{CM}$ + IL-1ra, compared to either $PT_{CM}$ or 10 mg/mL IL-1β ($P < .0001$). As expected, adhesions were not observed in mice treated only with IL-1ra (Table 2).

## *Collagen Deposition Within Adhesions*

Gross photomicrographs taken at necropsy illustrate the complexity of adhesions developing in mice receiving $PT_{CM}$ or 10 ng/mL IL-1β compared to the minimal disease observed in mice treated with $PT_{CM}$ + IL-1ra (Figure 3A-C). As shown in Figure 3A, mice receiving $PT_{CM}$ exhibit extensive, multipoint adhesions involving several tissues that were similar to adhesions observed in mice receiving the highest dose of IL-1β (Figure 3B). In contrast, $PT_{CM}$ + IL-1ra was associated with significantly fewer adhesions, while adhesions that did develop in the presence of IL-1ra were less complex and thinner, suggesting the structural integrity of these adhesions was reduced

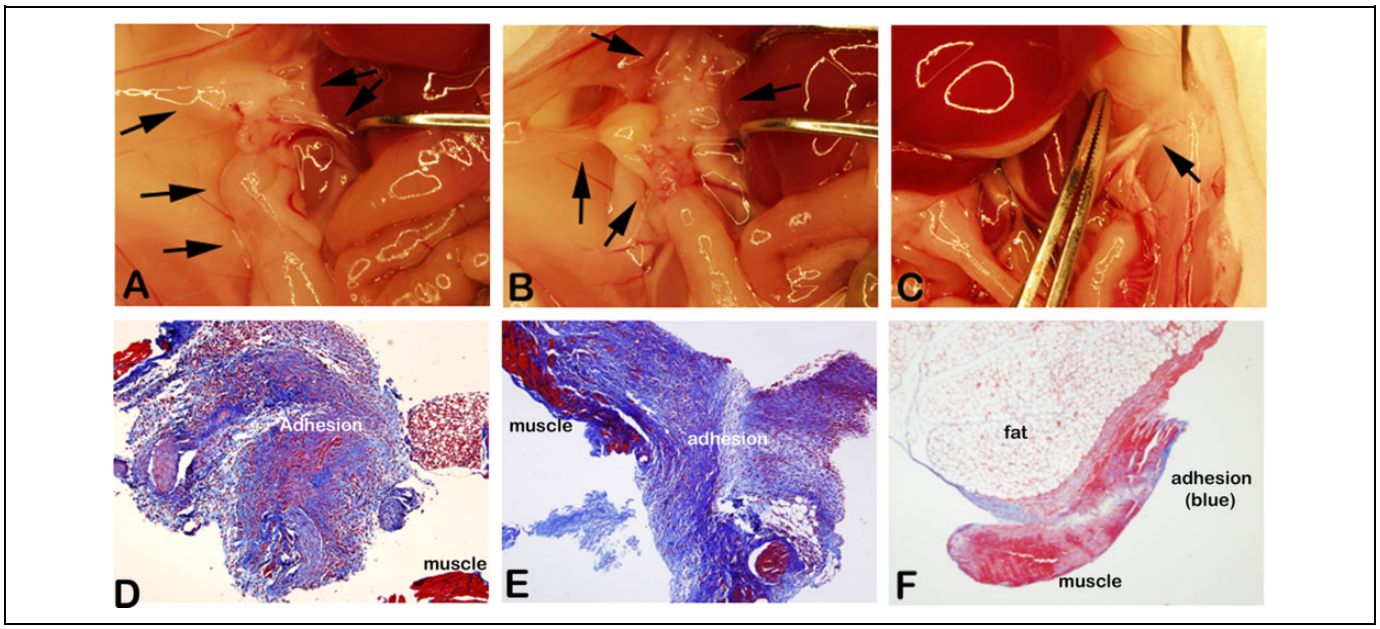


**Figure 3.** Gross photomicrographs of adhesions developing in mice alongside Masson trichrome staining of the same tissues after excision and fixation. Representative images of adhesions developing in mice treated with $PT_{CM}$ (N = 13; A), 10 ng IL-1β (N = 10; B), and $PT_{CM}$ plus IL-1ra (N = 14; C) illustrate the extensive adhesions in association with either $PT_{CM}$ or IL-1β while adhesive disease was dramatically reduced in mice co-treated with IL-1ra. To assess the integrity of the adhesions, tissues shown in (A-C) were excised, formalin fixed, and subjected to Masson trichrome staining (D-F). Masson trichrome results in a distinctive staining pattern in which connective tissue (ie, collagen) is stained blue, nuclei are stained dark red/purple, and cytoplasm is stained red/pink. Collagen deposition was abundant in adhesions forming following either $PT_{CM}$ or IL-1β treatment of mice, while only a limited amount of collagen was observed in adhesions from mice treated with $PT_{CM}$ plus IL-1ra (F). The reduction in collagen within adhesions arising in the presence of $PT_{CM}$ plus IL-1ra suggests these adhesions are weaker, consistent with our observations at necropsy. PT indicates patients; CM, conditioned media; IL-1β, interleukin-1β; IL-1ra, IL-1 receptor antagonist.

compared to those forming in other groups. Therefore, we next examined the collagen content of adhesions excised from each treatment group utilizing Masson's trichrome staining. As shown in Figure 3D-F, although extensive collagen deposition was observed in adhesions associated with $PT_{CM}$ (Figure 3D) or 10 ng/mL IL-1β (Figure 3E), only minimal collagen deposition was observed in mice receiving $PT_{CM}$ + IL-1ra (Figure 3F), which is consistent with the gross observations (Figure 3C).

## Discussion

Postsurgical adhesive disease has been estimated to occur in up to 60% of women undergoing laparoscopy for gynecologic disorders.[26] Compared to disease-free women, patients with endometriosis are at an enhanced risk of developing not only surgery-associated adhesive disease but also spontaneous adhesions occurring in the absence of surgery.[3,5] Thus, a better understanding of whether differences in wound healing processes between women with and without endometriosis contribute to their adhesive disease is essential in order to develop effective preventive therapeutic regimens.

Our group was among the first to demonstrate that women with endometriosis exhibit reduced endometrial responsiveness to ovarian progesterone during the secretory phase of the menstrual cycle.[22] Since the anti-inflammatory action of progesterone is required for normal immune cell migration and function across the menstrual cycle,[8] loss of normal progesterone action may contribute to changes in the immune physiology of both the uterus and the peritoneal cavity as noted among patients with endometriosis.[7] In particular, the presence of refluxed menstrual tissue induces a peritoneal inflammatory response from the innate immune system which in most women aids in the clearance of displaced menstrual debris. However, damaged cell products released by endometrial cell debris are also capable of activating the inflammasome via damage receptors, such as toll-like receptor 4. Therefore, an inappropriate innate immune response to menstrual debris could contribute to disease development as a consequence of reduced phagocytosis and/or excessive production of proinflammatory mediators. Surgical injury can also activate serum-based components of the complement system, thereby stimulating inflammasome activation. Although activated NLRP3 triggers an immune response and inflammatory cascade during normal wound repair, a number of autoimmune diseases have been linked to mutations in the NLRP3 gene (reviewed by Abderrazak et al[27]). Equally significant, Wieser et al[4] reported a significant increase in an IL-1ra polymorphism, which leads to reduced gene and protein expression in women with abdominal adhesions compared to surgical patients who did not develop this disease, implicating dysregulation of the inflammasome system in association with adhesive disease.

Important to the current study, our previously published studies using the same chimeric human/mouse model

demonstrated that a peritoneal surgery coinciding with the presence of menstrual debris promotes not only the development of experimental endometriosis but also adhesive disease as a comorbidity.[2] Therefore, we hypothesized that IL-1 β, the primary inflammatory cytokine produced following inflammasome activation, may play a role in development of endometriosis-related adhesive disease. Herein, we demonstrated that the presence of viable endometrial tissue fragments acquired from women with or without endometriosis were equally capable of promoting adhesive disease (perhaps due to the concomitant induction of ectopic lesions and therefore the resulting peritoneal inflammation). However, in similar experiments, involving only the peritoneal injection of the CM from CT versus PT with endometriosis endometrial tissues, we observed a dramatically different result (Table 1). Specifically, significant adhesive disease was noted upon peritoneal injection of CM from patients with endometriosis tissue ($PT_{CM}$), while CM injections from control donor tissues ($CT_{CM}$) resulted in significantly fewer adhesions that were present in only a subset of mice. Since these findings indicated the potential presence of inflammatory activators in the $PT_{CM}$ compared to $CT_{CM}$, we next subjected CM samples to cytokine bead array analysis, specific for human IL-1α and IL-1β. As shown in Figure 1, although both cytokines were increased in patient samples compared to controls, expression of IL-1β predominated, supporting our hypothesis that activation of the inflammasome may precede the development of adhesive disease in our model.

The inflammasome is a multiprotein complex that regulates the inflammatory response associated with both infectious microbes and damaged-associated host proteins. Although expression of this complex is generally attributed to macrophages, NLRP3 and other members of this family have also been detected in epithelial cells, in particular, those associated with muscosal tissues such as the intestine.[13] The NLRP complexes cleave pro-IL-1β, leading to its activation, thereby promoting an inflammatory cascade. Recently, this complex has been identified in the endometrium of women with recurrent pregnancy loss,[28] which the authors suggest leads to "chronic endometrial inflammation." Similarly, inflammasome activation has also been postulated to promote development of endometriosis.[29] Therefore, herein, we examined expression of NLRP3 protein in endometrial tissues from women with and without endometriosis. Our studies revealed that expression of NLRP3 protein is virtually absent in endometrial tissues from women without endometriosis ($n = 10$); however, significant expression of this protein was observed in the majority (14 of 16) of samples from women with endometriosis (Figure 2).

The enhanced expression of NLRP3 in the eutopic endometrium of women with endometriosis as well as the abundance of IL-1β in CM by these tissues supported our hypothesis and provided additional evidence that activation of the inflammasome may promote adhesive disease. Therefore, in order to more directly address this question, we next examined the impact of intraperitoneal injection of IL-1β on development of adhesions in our mice with a recent history of surgery. As shown in Table 2, we identified a dose-dependent increase in adhesion score in mice receiving intraperitoneal injections of IL-1β. At the higher dose (10 ng/mL), adhesive disease was markedly similar to the extent of adhesions developing in response to $PT_{CM}$. Importantly, blocking the action of IL-1β with IL-1ra ($PT_{CM}$ + IL-1ra) resulted in a significant reduction in adhesions (Table 2); moreover, adhesions that were able to form were less complex and contained minimal collagen (Figure 3).

Our previous studies using this chimeric human/mouse model revealed the significant combinatorial impact of surgical inflammation and endometrial fragments within the peritoneal cavity on postsurgical adhesive disease.[2,10] These studies also revealed a significant beneficial effect of anti-inflammatory agents on the development of adhesions. Specifically, we demonstrated that pioglitazone, a member of the thiazolidinediones class of pharmacologic agents which activate the peroxisome proliferator-activated receptor-γ receptor family, dramatically reduced the development of surgical adhesions associated with experimental endometriosis. In a second study, mice were provided an anti-inflammatory diet containing fish oil prior to surgery. As with pioglitazone treatment, the fish oil regimen dramatically reduced the postsurgical development of adhesions.[10] Since both pioglitazone and fish oil fatty acids have been found to reduce inflammasome activation,[30,31] the ability of these agents to block adhesive disease, further supports our hypothesis that inflammasome activation may be critical to development of endometriosis-related adhesions in our model. These previous preclinical studies, taken together with our current study which directly implicates IL-1β in postsurgical adhesion development, strongly suggests that therapeutically targeting the inflammasome may be useful for the prevention of adhesive disease in patients with endometriosis.

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