

Role of immunologic and inflammatory factors in the development of endometriosis: indications for treatment strategies

Attila Mihalyi,
Kyama C Mutinda,
Peter Simsa,
Sophie Debrock,
Jason M Mwenda &
Thomas M D'Hooghe[†]

[†]Author for correspondence
Leuven University Fertility
Center, Department of
Obstetrics & Gynecology,
University Hospital
Gasthuisberg, Herestraat 49
B-3000 Leuven, Belgium
Tel.: +32 16 343 624
Fax: +32 16 343 607
thomas.dhooghe@uz.kuleuven.ac.be

This article presents an overview of immunologic/inflammatory factors related to endometriosis with special attention to their potential relevance to future treatment strategies. The development of endometriosis appears to involve several immunologic and inflammatory factors. Increased inflammatory, proteolytic and angiogenic activity of the peritoneum and the peritoneal fluid, and poor immune surveillance and clearance of endometrial cells from the pelvic cavity all appear to contribute to the development of endometriosis. The presently available pharmacotherapeutic treatments are characterized by high recurrence rates, severe side effects and limited duration of application. These and the consequences of endometriosis on the quality of life of affected women, their environment and the society, clearly indicate the need for the development of new drugs, abolishing endometriosis and its symptoms, without interfering with the hormonal homeostasis. Substances potentially capable of modulating immunologic/inflammatory mechanisms involved in the onset and/or progression of the disease could be targets for future research in endometriosis.

Endometriosis is a gynecologic disease characterized by the presence of endometrial glands and stroma at ectopic sites. It is mostly found at visceral and peritoneal locations within the pelvis and is associated with pelvic pain, adhesion formation and infertility. Endometriosis occurs in 13 to 33% of women with infertility [1] and is progressive in 40 to 50% of patients [2].

Despite great efforts in recent decades, the pathomechanism of endometriosis remains unclear. Several theories attempt to explain the development of endometriosis; however, none can explain all cases. The theories that have drawn much attention, include the retrograde menstruation [3] and the metaplasia theory [4]. Although both theories have supportive evidence, Sampson's theory of implantation of retrogradely shed endometrial cells at ectopic sites, has gained the most supportive evidence.

Retrograde menstruation occurs in at least 76 to 90% of women undergoing peritoneal dialysis and laparoscopy [5,6]; however, the much lower prevalence of endometriosis (6.2–8.2%) [7,8] suggests that other factors must determine susceptibility to the disease. There is growing evidence that immunologic abnormalities may be major contributing factors in the development of endometriosis. Studies demonstrated increased inflammatory parameters in both humans and nonhuman primates when compared with controls [9,10]. Immunologic and inflammatory

mediators are postulated to be significantly involved in the development of endometriosis [11–13]. This review focuses on the role of these factors and their potential relevance to the development of novel treatment strategies for endometriosis.

Medical treatment

Endometriosis is a benign, estrogen-dependent disease. Medical treatments are usually based on hormone preparations and are aimed at generating a low-estrogen milieu. Therapies have included combined oral contraceptives, progestins, antiprogestins, androgenic derivatives, and gonadotropin-releasing hormone agonists (GnRHa) and antagonists [14–18]. Combined oral contraceptives alleviate cyclic pain by suppressing the cyclic growth of endometriotic implants. However, success seems to be limited to younger patients with milder disease [16]. Progestins applied alone produce a hypoestrogenic, acyclic hormonal environment by suppressing gonadotropins, inhibiting ovulation and producing amenorrhea [19].

Danazol, an isoxazol derivative of 17 α -ethinyl-testosterone, causes anovulation by attenuating the midcycle peak of luteinizing hormone secretion, inhibiting enzymes in the steroidogenic pathway and increasing free testosterone concentrations [17]. However, recently danazol has been reported to substantially increase the risk of ovarian cancer in

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patients with endometriosis [20,21]. GnRHa diminish the secretion of follicle stimulating hormone and luteinizing hormone, resulting in hypogonadotropic hypogonadism. The eventual hypoestrogenic state results in endometrial atrophy and amenorrhea [17].

Attempts have been made to introduce other drugs in the management of endometriosis; however, these either have excessive side effects, have not been properly evaluated or have not been proven effective. To a certain extent, all of the mentioned drugs mitigate endometriosis-related pain and are more or less effective in the regression of endometriotic lesions or the prevention of further progression of the disease. However, all of these medical treatments interfere with the function of the pituitary–gonadal axis, and have an explicit influence, not only on the endometrium and the endometriotic lesions, but several other hormonally controlled, fine-tuned mechanisms. This nonspecific targeting leads to a large number of side effects ranging from dizziness to increased risk of deep-vein thrombosis and loss of bone density [17,22]. Another important and sometimes underestimated problem of these treatment strategies is the large recurrence rate after discontinuation of the therapy [23]. Furthermore, they all suppress ovulation, therefore they have no benefit to endometriosis-associated infertility [24].

The classic nonsteroidal anti-inflammatory drugs (NSAIDs) are the first-line agents in the treatment of endometriosis-related pain [17]. These drugs are of low cost and can effectively alleviate the painful symptoms of endometriosis; however, their long-term application may lead to gastrointestinal side effects. Although pain relief undoubtedly increases the quality of life of the patient, care must be taken as the symptom-free condition may induce a negligent behavior towards the disease, which may eventually facilitate the silent progression of endometriosis.

The limitations of the therapeutic methods presently available indicate that they are obviously not optimal solutions to the problem. The ideal endometriosis drug should:

- Prevent the development of endometriosis
- Permanently extinguish the disease
- Prevent recurrence after cessation of the treatment
- Not interfere with the menstrual cycle
- Have no or negligible side effects
- Provide the possibility of pregnancy even during the treatment

Novel endometriosis drugs will have to aim at disease-specific targets in order to suite these demands. The immunologic and inflammatory factors associated with endometriosis may be potential targets for future drug development.

Immune cells

Macrophages

Macrophages represent a continuously changing population of monocyte-derived cells that are involved in immunologic and inflammatory responses. They play a key role in host defense against intracellular microorganisms as well as against tumors. Macrophages produce over a hundred different substances, which include modulators of both immune and nonimmune cells; thus, macrophages and their products have been investigated intensively in the pathogenesis of endometriosis.

In women with endometriosis, total number of peritoneal macrophages is increased, concentration and activation status [25–28]. In parallel, there is an increased concentration of their products, such as growth factors and cytokines, which can affect the survival and growth of ectopic endometrial cells [13]. Under *in vitro* conditions, autologous mononuclear phagocytes from women with endometriosis, and/or their secretory products, stimulate eutopic and ectopic endometrial cell proliferation and decrease their apoptosis [29]. In contrast, increased apoptosis and decreased endometrial cell proliferation were observed when autologous endometrium was cocultured with macrophages from healthy controls [29], suggesting that macrophage activity towards endometrial cells is markedly different in diseased women and controls.

Scavenger function of the macrophages is vital when encountering foreign material, cellular debris or apoptotic cells. A variety of surface receptors are involved in this activity, and they are regulated by several factors, including cytokines and growth factors [30]. As these cytokines and hormones are present in abnormal levels in the peritoneal fluid (PF) of women with endometriosis, they may cause insufficient scavenger receptor function of the macrophages that could facilitate the growth of ectopic endometrial cells [31]. When macrophages are not attached to the extracellular matrix in tissues, they do not express A-type scavenger receptors [32] and may not be competent scavengers despite their differentiated status. Therefore, the increased number of nonadherent

macrophages found in the PF of women with endometriosis [33] could result in insufficient scavenger function.

CD36 – a type-B scavenger receptor – is involved in the uptake and degradation of apoptotic cells and other debris [34]. Retinoic acid receptor agonists and peroxisome proliferator activated receptor (PPAR)- γ agonists are able to increase CD36 expression on THP-1 cells, a human monocyte cell line. Furthermore, the two agonists showed a synergistic effect, when applied together [35]. In addition, retinoic acid treatment increased CD36 expression on both protein and mRNA levels, and the upregulation also occurs in the absence of cellular adhesion [31]. These data support the notion that disturbed macrophage function may be an important factor in the pathogenesis of endometriosis. Restoring the secretory activity of macrophages to physiologic levels and/or stimulating their scavenger function by selectively modulating the regulatory receptors, could serve as targets for the development of novel treatment strategies.

Natural killer cells

Natural killer (NK) cells are large, granular lymphocytes capable of lysing a variety of autologous and allogenic target cells without previous sensitization. They are involved in antitumor surveillance, defense against viral, bacterial and parasitic infections and rejection of transplants. Several studies have tried to assess the changes in NK cell quantities in patients with and without endometriosis; however, the results are inconsistent. Some studies found decreased, others increased, while still others unchanged numbers or percentages of NK cells in affected women [36–38]. In some cases, these controversies may be explained by the low numbers of samples or poorly defined study populations. Nevertheless, other cofounders (age, parity, oral contraception, smoking or recent acute infection) that can strongly influence the proportions of leukocyte subsets could also contribute to the discrepancies [39].

NK cells are important in the clearance of endometrial cells from the peritoneal cavity. NK cells from endometriosis patients show decreased cytotoxicity to both autologous and heterologous endometrium [38,40]. Additionally, the PF of women with endometriosis exhibits significantly greater NK cell suppressive activity than that of fertile controls [41]. Similarly, the serum of diseased women shows increased NK cell suppressive activity than that of

controls [42]. Thus, it is postulated that the decreased NK cell cytotoxicity towards retrogradely shed endometrial tissue may allow the establishment of endometriosis within the peritoneal cavity.

One of the mechanisms used by NK cells to kill their targets involves the killer-activating receptors (KAR) and the killer-inhibitory receptors (KIR). If KAR are stimulated, NK cells exert cytotoxicity; whereas KIR stimulation suppresses cytotoxic activity [43]. Increased KIR expression has been reported on the PF and peripheral blood NK cells from women with endometriosis, which represents a likely cause of decreased peritoneal NK cell activity [44,45] and suggests both a local and a systemic decrease in NK cell function. Furthermore, overexpression of these KIR receptors on NK cells in endometriosis patients remains present even after laparoscopic surgery and GnRHa treatment [46]. Interestingly, publications have reported increased NK cell number and activity following GnRHa treatment [36,47]. A recent *in vitro* study suggests that the increased NK cell activity is not a direct effect of the GnRHa, but of the decreased estradiol levels induced by the GnRHa treatment [48].

The presence of soluble NK cell inhibitory factors produced by ectopic endometrial cells has also been suggested by a study, in which conditioned media from endometrial cells showed increased inhibition of NK cell-mediated cytotoxicity of endometrial cells in endometriosis patients compared with controls [49]. However, such a factor has not yet been identified. Nevertheless, the p40 subunit of interleukin (IL)-12 – an NK cell-activating cytokine – is able to block NK cell-mediated lysis of endometrial cells. Both IL-12 and its free p40 subunit are present in the PF of women with and without the disease. However, the level of free p40 subunit seems to be elevated in the PF of women with endometriosis [50]. This could explain – at least in part – the decreased NK cell activity in diseased women. The literature suggests that decreased NK cytotoxicity in endometriosis is generally accepted. Synthesis of substances capable of increasing NK cytotoxicity could lead to new approaches in the management of endometriosis. Inhibition of the expression or the signaling pathways of KIR receptors on NK cells, decreasing the concentration of the free p40 subunit of IL-12 in the peritoneal cavity or NK cell responsiveness to estrogens could potentially improve NK cytotoxicity.

T-lymphocytes

T-lymphocytes are key figures of cell-mediated immune responses. Following their maturation processes they can be classified into two major subpopulations by their expression of CD4 and CD8 glycoproteins. CD4 serves as a coreceptor for major histocompatibility complex (MHC) II, while CD8 has the same function towards MHC I. It has been demonstrated that peripheral blood lymphocytes in endometriosis show decreased proliferation in response to recognition of endometrial antigens and cells [51]. Lymphocyte subpopulations in women with endometriosis have been extensively analyzed; however, the results are rather inconsistent [52–56].

In a recent study involving 90 women (60 endometriosis patients and 30 controls) the investigators found an increase in the CD4/CD8 ratio in the peripheral blood of endometriosis patients compared with control patients [57]. In contrast, in the PF of endometriosis patients, the CD8/CD4 ratio was higher compared with healthy controls. Moreover, the number of activated CD8⁺ and CD4⁺ cells in the PF was significantly lower in the former than in the latter group [58]. Additionally, activated T-cells from women with endometriosis may adhere to components of the extracellular matrix more intensively than T-cells from control patients; therefore, they might facilitate destruction of peritoneal epithelium integrity and promote ectopic implantation [59]. In general, it is difficult to define the changes in T-lymphocyte function and distribution and their possible role in endometriosis. However, the existing data seem to delineate an imbalance in the CD4/CD8 ratio, which may facilitate the survival and implantation of endometrial cells at ectopic sites.

B-lymphocytes

B-cells are precursors of plasma cells, the antibody-producing cells of the immune system. Knowledge available on their possible role in endometriosis is limited. Some reports suggest no significant alterations regarding B-lymphocytes in the ectopic [54,60] or eutopic endometrium of diseased patients [61]. Conversely, B-lymphocytes are increased in peripheral blood and the PF [53,56,62]. Activated B-cells are known to produce soluble CD23 protein (sCD23). The concentration of sCD23 is higher in the PF of endometriosis patients when compared with controls, suggesting increased B-cell activation in diseased patients [63]. Uchiide and colleagues hypothesized that a possible reason for not detecting increased levels of

B-cells in ectopic endometrium is that the cells in these lesions have already advanced to plasma cells [64]. The data available on plasma cells in endometriosis are rather poor; however, this hypothesis needs to be investigated experimentally. Taken together, the function or malfunction of B-cells in endometriosis needs to be investigated in more detail in order to determine whether it could provide any benefit in endometriosis treatment.

Cytokines & growth factors

Interleukin-1

IL-1 is a proinflammatory cytokine secreted mainly by activated monocytes and macrophages, although T-cells, B-cells and NK cells also produce it [65]. It affects the activation of T-cells and the differentiation of B-cells. There are two distinct, functionally similar isoforms of IL-1 (IL-1 α and -1 β) derived from two different genes.

Interleukin-1 α

IL-1 α is a major stimulatory cytokine for matrix metalloproteinase (MMP)-1. IL-1 α protein levels were found to be elevated, together with MMP-1, in endometriotic lesions when compared with their matched eutopic counterparts. The increased expression of both MMP-1 and IL-1 α in endometriotic lesions suggests their involvement in local invasion and tissue destruction [66].

Interleukin-1 β

IL-1 β levels were found to be elevated in the PF of women with endometriosis, although some studies reported conflicting results [67–69]. IL-1 β promotes angiogenesis in endometriotic lesions, but not in normal endometrial stromal cells, by upregulating several angiogenic factors [70,71]. This is supportive to results from clinical studies that have demonstrated increased angiogenic activity associated with IL-8 levels in the PF of women with endometriosis [72]. IL-1 β can also induce regulated upon activation, normal T-cell expressed and secreted (RANTES) gene expression in endometrial stromal cells [73,74], and thus could contribute to the maintenance of the local inflammatory environment. IL-1 β has also been associated with increased shedding of soluble (s) intercellular adhesion molecule (ICAM)-1 from endometrial cells, which may interfere with peritoneal immune surveillance [75].

Furthermore, IL-1 β can increase cyclooxygenase (COX)-2 expression and concomitant prostaglandin (PG)E₂ production in both

eutopic and ectopic endometrial stromal cells, suggesting that increased sensitivity of IL-1-dependent COX-2 expression in these cells may play an important role in the development of endometriosis [76]. The effects of IL-1 are mediated by the IL-1 receptor Type I (IL-1RI), whereas IL-1 receptor Type II (IL-1RII) acts as decoy receptor for IL-1, buffering its effects. The membrane-bound IL-1RII can be cleaved from the cell surface and released in soluble form. Both soluble and membrane-bound forms of the receptor are able to bind IL-1 and to neutralize its effects. IL-1RII expression and release of soluble (s)IL-1RII are decreased in eutopic endometrial cells of women with endometriosis compared with healthy controls [77,78]. These decreases may enhance IL-1-mediated activation of endometrial cells and contribute to the local inflammatory conditions.

It is well documented that IL-1 – like other cytokines – has a rather complex function. This includes activation of lymphocytes, chemotaxis of immunocytes, facilitation of angiogenesis in response to hormonal effects and decreasing immune surveillance. These different effects, and the observation that most of them are indirect, mediated by other factors, indicates that clear understanding of the function of this cytokine still demands great efforts. Nevertheless, future experiments aiming at restoring sIL-1RII levels, inhibiting proteolysis of membrane-bound IL-1RII or inhibiting IL-1 activity by specific antagonists may reveal potential target sites for novel endometriosis medications.

Interleukin-6

IL-6 is a cytokine mainly produced by T-cells, but also by macrophages, fibroblasts and endothelial cells. It stimulates B-cell activity, enhances T-cell differentiation and modulates secretion of other cytokines. IL-6 also regulates ovarian steroid production, folliculogenesis and embryo implantation and acts as a growth regulator for various human cells.

IL-6 can inhibit the proliferation of endometrial stromal cells from the secretory phase, but shows no such effect on cells from the proliferative phase. In contrast, stromal cells of endometriotic tissues were resistant to the inhibitory effects of IL-6 [79], suggesting different responses of ectopic endometrium and the eutopic counterpart. Both serum and PF IL-6 levels are significantly higher in endometriosis patients compared with controls and the PF levels positively correlate with the severity of endometriosis [69,80,81]. Its elevated

concentration in endometriosis is also accompanied by decreased membrane-bound IL-6 receptor (IL-6R) and soluble IL-6R expression (sIL-6R)[81]. Since both IL-6R and sIL-6R are necessary for appropriate IL-6 effects [82], it seems possible that deficient expression of these receptors leads to resistance to the regulatory effects of IL-6. Nevertheless, high PF concentration of IL-6 is associated with increased embryotoxicity [83], suggesting a contribution to endometriosis-related infertility.

These findings indicate that IL-6, and perhaps more importantly its decreased inhibitory effect on the growth of ectopic endometrial cells, may largely contribute to the progression of endometriosis and at the same time could also be involved in endometriosis-related infertility. Future studies should aim at restoring the sensitivity of ectopic endometrium to IL-6 by stimulating IL-6R and/or sIL-6R expression. It could be hypothesized that the increased levels of IL-6 is, at least in part, induced by inappropriate tissue response. Thus, restoring IL-6 sensitivity of ectopic endometrium could lead to a decrease in IL-6 levels, which in turn could improve the fertility parameters of the patients. However, this speculation requires verification.

Interleukin-8

IL-8 is a chemokine with chemotactic and activating effects on neutrophils and T-cells, and is a potent angiogenic factor. Peritoneal mesothelial cells, macrophages and endometrial cells are its potential sources [84,85]. CXC-chemokine receptor (CXCR)1 and 2 are the receptors for IL-8. IL-8 levels are increased in the PF of women with endometriosis, compared with controls, and were found to correlate with the severity of the disease [85–87]. However, there was no difference detected in the PF IL-8 levels between patients with endometriosis or with idiopathic infertility [69]. Interestingly, in the circulation, IL-8 levels were reported to be highest in women with limited disease [88].

Cytoplasmic IL-8 levels in endothelial cells of ectopic endometrium are also elevated when compared with eutopic endometrium of women either with or without endometriosis. However, IL-8 immunostaining of endothelial cells of eutopic endometrium from women with endometriosis did not show a significant difference compared with that of women without the disease [89]. In addition, in both eutopic and ectopic endometrium of the diseased women, epithelial and stromal IL-8 receptor expression were increased throughout the menstrual cycle, when

compared with controls [90]. IL-8 significantly stimulates cell proliferation of the stromal cells in both eutopic and ectopic endometrium [87,91] and increases the adhesion of endometrial cells to fibronectin [92]. The adherence of endometrial cells induces further IL-8 expression [93], suggesting the establishment of a positive-feedback loop.

IL-8 is also known for its potent angiogenic properties [94]. This might be a key feature in terms of establishing new vascular structures for the implanted ectopic endometrium. Moreover, IL-8 upregulates the FasL protein in human endometrial cells, suggesting that increased PF levels of IL-8, by stimulating FasL-induced apoptosis in activated T-lymphocytes, may contribute to the survival of endometriosis implants [95]. Overall, it appears that IL-8 is involved in the pathogenesis of endometriosis at several levels. It promotes ectopic adhesion and proliferation of endometrial cells, facilitates angiogenesis, and subserves apoptosis of T-cells. In addition, both IL-8 and its receptors are present at increased levels in endometriosis patients. Since the structure of IL-8 and its receptors are available, synthesis and experimental testing of specific IL-8 inhibitors could reveal essential information regarding possible new endometriosis treatments.

Interleukin-12

IL-12 is a heterodimer composed of two disulfide-linked subunits of 35 kDa (p35) and 40 kDa (p40), encoded by separate genes. Simultaneous expression of the two genes is required for the production of the biologically active IL-12 heterodimer. Although the p35 gene is constitutively expressed in most cell types, the presence of p40 gene transcripts is restricted to those cells able to produce IL-12 [96,97]. IL-12 is produced by macrophages, monocytes and B-cells and the biologic effects are directed at T-cells and NK cells. IL-12 is involved in the stimulation and maintenance of T-helper cell 1 responses and proliferation and cytotoxicity of NK cells. IL-12 is present in the PF of women both with and without endometriosis and its most likely sources are macrophages [98].

As discussed, levels of the free p40 subunit – an inhibitor of IL-12-induced NK activity, and also decreases IL-12 receptors on NK cells – are higher in the PF of women with endometriosis than in healthy women. Heterodimeric IL-12 greatly enhances NK-cell-mediated cytotoxicity towards endometrial targets from endometriosis patients [50]. Furthermore, both IL-12 level and

(IL-12 + free p40)/IL-12 ratio are elevated in the PF of women with endometriosis [50], suggesting elevated free p40 subunit concentration that could diminish the NK cell activating effect of IL-12. Collectively, it seems probable that the immunostimulatory capacity of IL-12 might be exploited as a novel therapeutic strategy to control the cytolytic arm of the initial immune surveillance to ectopic endometrial antigens. In this regard, controlling the balance between the free p40 subunit and IL-12 might be of great significance.

RANTES

RANTES is an 8-kDa protein with chemoattractant actions on monocytes, NK cells, T-cells and eosinophils [99,100]. It has been shown that the concentration of RANTES is increased in the PF of women with endometriosis. Its distribution in endometriotic lesions shows localization primarily in the stromal compartment, as in the normal endometrium [101]. It has been postulated that secretion of RANTES may result in recruitment of peritoneal macrophages and T-lymphocytes after T-cell activation in the PF [102]. In the PF from endometriosis patients, RANTES has been shown to be responsible for 70% of monocyte migration [103].

When stimulated, PPAR- γ decreases endometrial stromal cell expression of RANTES *in vitro* by inhibiting transcription of the mRNA [104,105], suggesting that the abnormal inflammatory response in endometriosis could be decreased by PPAR- γ activation. Long-term treatment with progestins is also able to decrease RANTES gene expression, suggesting that this type of therapy could have beneficial effects on pelvic pain by suppressing inflammation within endometrial implants. The effect is progesterone receptor (PR) dependent, and the PR-B isoform appears to be the most effective [106]. However, PR-B is decreased or absent in endometriotic tissues [107], which may limit efficacy of such therapy. Although evaluation of RANTES in endometriosis is not complete, it seems possible that selective interference with its regulation could lead to the development of safer treatments to blunt the inflammatory response in diseased women by suppressing the chemoattraction of immunocompetent cells.

Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF), a 30 to 40 kDa glycoprotein, is a potent stimulator of angiogenesis. It has six known isoforms:

VEGF-A, -B, -C, -D, -E and placental growth factor (PlGF), which bind to three receptors: VEGFR-1, -2 and -3 [108]. VEGF is produced by various tissues, such as endometrium, ovary, placenta [109], and macrophages [110]. It is a potent inducer of vascular permeability and is a survival factor for newly formed blood vessels.

There is growing evidence that VEGF is involved in both the onset and maintenance of peritoneal endometriosis. Circulating VEGF levels were found to be higher in infertile women with endometriosis compared with controls and this increase was stage dependent [111]. These findings suggest that detection of VEGF could serve as an additional diagnostic tool in endometriosis. In parallel, the PF concentrations of VEGF in endometriosis are significantly higher in moderate-to-severe cases than minimal-to-mild cases [110]. In eutopic endometrium, VEGF is localized predominantly in endometrial glands, and estradiol increases its gene expression [112,113]. Besides estradiol, hypoxia, IL-1, platelet-derived growth factor, transforming growth factor- β , epidermal growth factor, and PGE2 are other upregulating factors of VEGF [70,114,115]. The expression of VEGF by endometriotic implants is involved in neovascularization, which is commonly observed around lesions [116], suggesting an active role for VEGF in disease development. A recent study tested two antagonists of VEGF-A in a murine model of endometriosis. The antagonists – a truncated inhibitory receptor, soluble fms-like tyrosine kinase-1, and a VEGF antibody – were initially shown to have antiangiogenic properties *in vivo* [117].

They inhibited the growth of human endometrial tissue transplanted into mice. In most cases explants were undetectable in the treated animals. Where they were, the numbers of blood vessels were limited. In contrast, explants maintained in animals receiving estrogen and demonstrated numerous blood vessels [117]. The study also showed that a large number of the blood vessels supplying endometrial explants in women are immature. Thus, anti-VEGF agents have the potential to disrupt the vasculature of endometriotic lesions [117].

The importance of angiogenesis in the survival of endometriotic implants is evident and VEGF seems to have a crucial role in it. As demonstrated, several factors participate in the regulation of VEGF. Modifying these regulatory factors or direct inhibition of VEGF appears to

be a possible tool to develop medications that could prevent vascularization of the endometriotic lesions and promote the regression of the ectopic implants

Tumor necrosis factor- α

Tumor necrosis factor (TNF)- α is a proinflammatory cytokine able to initiate inflammatory cascades. It is secreted by neutrophils, activated lymphocytes, macrophages, NK cells and several other cells. TNF- α is suggested to play a major role in the pathogenesis of endometriosis [118,119]. TNF- α concentrations are increased in the PF of women with endometriosis, and its levels correlate with the stage of the disease [69].

TNF- α has been shown to increase the adherence of cultured stromal cells to mesothelial cells [119], suggesting that the presence of TNF- α in the PF may promote adherence of ectopic endometrial tissue to the peritoneum. As mentioned previously, TNF- α promotes the proliferation of endometriotic stromal cells by inducing IL-8 expression [120]. Furthermore, estrogen-induced IL-8 upregulation is also mediated by TNF- α [121].

The expressions of matrix metalloproteinase (MMP)-1 and -3 (regulators of the extracellular matrix) are also induced by TNF- α [122], suggesting a role in matrix degradation and implantation of ectopic endometrium. TNF- α also possesses significant embryotoxic effects, which may contribute to endometriosis-related infertility [123]. These results suggest that TNF- α has multiple functions in endometriosis; thus, it seems to be a possible target for drug development.

In fact, among all potential targets in endometriosis, TNF- α inhibition is the most advanced in terms of evaluation. Recombinant human TNF-binding protein (TBP)-1, a soluble form of TNF receptor Type I, was reported to reduce the size of endometriotic-like foci in a rat model by up to 64% [124]. These findings were supported by a study of D'Hooghe and colleagues using the baboon model [125]. They demonstrated that, following endometriosis induction, neutralization of TNF- α with TBP-1 partially inhibited the development of endometriotic lesions and reduced the establishment of endometriosis-related adhesions. It is important to note that the TBP-1 treatment did not interfere with the menstrual cycle in the baboons, which is a major advantage compared with the presently available medical treatments for endometriosis. Additionally, in baboons with induced endometriosis, anti-

TNF- α treatment also effectively reduced the extent of established endometriosis, mainly due to a reduction in both number and area of red lesions [126]. Furthermore, another study conducted on baboons with spontaneous endometriosis showed that 8 weeks of treatment with etanercept – an anti-TNF- α currently used to treat disorders such as rheumatoid arthritis, juvenile rheumatoid arthritis and psoriatic arthritis – diminished the amount of spontaneously occurring active endometriosis [127].

One of the mechanisms by which anti-TNF- α substances may exert their action is the inhibition of MMP transcription, which could result in diminished implantation at the ectopic sites. Another possible mechanism is the suppression of IL-8 expression, which may lead to a subsequent decrease in proliferation, adhesion and angiogenesis. The results from the studies using TNF- α blockers provided potential prospects for clinical application. Considering that anti-TNF- α substances (i.e., etanercept and infliximab) with other indications are already available in the clinical practice, it seems possible that clinical evaluations of this therapeutic approach may take place in the near future.

Other potential targets in immunologic, inflammatory pathways

Intercellular adhesion molecule/soluble intercellular adhesion molecule -1

ICAM-1 is a member of the immunoglobulin family of adhesion molecules, and has been found in several cell types, including endometrial cells from both eutopic and ectopic endometrium [128]. It affects inflammatory and immune responses, has been associated with migration of both tumoral and normal cells [129–131]. ICAM-1 is particularly important in antigen presentation by macrophages as a costimulatory molecule that functions together with MHC and T-cell receptor [132–134]. The shedding of the surface molecule generates sICAM-1. There seems to be no difference in PF sICAM-1 concentrations between endometriosis patients and controls [135,136]. However, other investigators reported increased concentrations of sICAM-1 in the PF of diseased women [137,138].

A recent study involving 67 women with and 19 without endometriosis investigated the PF concentration of sICAM-1 in endometriosis and could not demonstrate a significant difference compared with controls [139].

There was also no significant difference in serum levels of sICAM-1 in women with endometriosis compared with control patients [140–142]. However, in other studies either an increase [143,144] or a decrease [145] was observed in endometriosis patients when compared with controls. A significant correlation between sICAM-1 concentration and the inhibition of NK-cell-mediated lysis was described in culture systems [135,137], suggesting that increased sICAM-1 levels may be involved in decreased pelvic clearance of endometrial fragments. Furthermore, ICAM-1 expression by macrophages in the PF of the diseased women was found to be decreased, which may also contribute to local immunotolerance [146]. Endometrial ICAM-1 expression in endometriosis patients is reduced during the luteal phase, which could further contribute to the decreased recognition and killing of endometrial cells in the peritoneal cavity [147]. In conclusion, ICAM-1 and sICAM-1 seem to have a key role in the recognition and elimination of endometrial cells from the peritoneal cavity. Exact determination of the changes in ICAM-1 expression in endometriosis could provide deeper understanding of how ectopic endometrial cells can escape immunosurveillance and thus could reveal novel drug targets.

Peroxisome proliferator activated receptor- γ

PPARs are ligand-activated transcription factors that have an effect on physiologic processes such as adipocyte differentiation, lipid metabolism, monocyte/macrophage activation and cytokine expression [148–150]. There are three types of PPAR, α , β (or δ) and γ . Although all three PPARs are widely expressed, their relative levels differ greatly between tissues, reflecting their distinct biologic functions.

All PPARs are present in cells of the vascular wall and the immune system. PPARs are activated by natural ligands such as fatty acids, eicosanoids and oxidized fatty acids. Pharmacologic compounds, such as the antihyperlipidemic fibrates and the antidiabetic thiazolidinediones, are also ligands for PPAR- α and γ , respectively [151–153]. PPARs have been shown to mediate anti-inflammatory processes [149,154,155]. Activated PPAR- α inhibits the production of inflammatory markers, such as ICAM-1, vascular cell adhesion molecule (VCAM)-1, MMP-9 and TNF- α [156]. PPAR- γ ligands inhibit TNF- α , IL-6 and β expression in monocytes [155], MMP-9 expression in macrophages [157] and increase the production of the IL-1 receptor

antagonist [158]. Evidence suggests that PPAR- γ ligands, such as thiazolidinediones, are potent inhibitors of cell growth and inducers of apoptosis [159–161]. They also have antiangiogenic effects that are mediated by decreased production of VEGF [162]. Human endometrial epithelial and stromal cells also contain PPAR- γ [163,164]. In *in vitro* models, thiazolidinediones inhibit monocyte migration [165] and the accumulation of inflammatory peritoneal cells [166].

In a recent study, 1-month's treatment with ciglitazone, a thiazolidinedione-type activator of PPAR- γ , resulted in significant regression of the endometriotic implants in the treated rats when compared with controls. Furthermore, the ciglitazone dose used for the effective treatment did not seem to influence estrous cycling or folliculogenesis of the rats [167]. As discussed earlier, stimulation of PPAR- γ decreases endometrial expression of RANTES. Although knowledge regarding the role of PPARs in endometriosis is incomplete, it seems probable that this receptor type could provide targets for the development of novel therapeutic tools. Naturally, the promising results observed in rodents need to be verified by primate investigation. Together it could give a firm basis to plan clinical trials with the commercially available thiazolidinediones.

Matrix metalloproteinases

There are approximately 28 known types of MMPs, which can be synthesized by macrophages, fibroblasts, neutrophils, epithelial and endothelial cells. MMPs initiate tissue remodeling by degradation of extracellular matrix molecules. Inhibitor molecules of MMPs known as tissue inhibitors of metalloproteinases (TIMPs) exist in four distinct forms (TIMP-1, -2, -3 and -4). MMPs are upregulated by TNF- α and IL-1, and TNF- α may also contribute to the decreased expression of TIMPs, which could partially explain the increased invasiveness of endometrial fragments in women with endometriosis [168,169]. In the peritoneal cavity, the survival and implantation of endometrial cells is largely dependent on abnormal MMP and TIMP expression [170]. Ectopic endometrium implants express elevated levels of MMP-1, -2, -3, -7 and -9 [169,171–174] and reduced levels of TIMP-1 and -2 [169].

Suppression of MMPs prevents the development of ectopic lesions in nude mice and in the chicken chorioallantoic membrane model for endometriosis [175,176], suggesting that inhibition

of MMP activity could provide a tool in the prevention of endometriosis, by inhibiting implantation of the endometrial fragments.

Cyclooxygenase-2

COX is the rate-limiting enzyme for the production of prostaglandins. There are three known isoforms of the enzyme: COX-1, -2 and -3, which is a splice variant of COX-1 [177,178]. COX-1 is expressed constitutively in most tissues and is responsible for producing prostaglandins for primary housekeeping functions [177,179]. By contrast, the COX-2 enzyme is expressed at low or undetectable levels but is upregulated by inflammatory, mitogenic and physical stimuli. COX-2 staining was found to be more frequent and denser in the ectopic endometriosis implants than in eutopic endometrium and COX-2 mRNA levels in the lesions was increased [180]. COX-2 overexpression is also present in human cancer types and is linked to angiogenic activity [181]. Thus, it seems possible that selective COX-2 inhibition may suppress endometriotic implants. This was supported by a study, whereas treatment with a selective COX-2 inhibitor, rofecoxib, caused regression of endometriotic explants in the rats [182]. However, a similar study using a mouse model and nimesulide as the COX-2 inhibitor did not confirm those results [183].

Another study investigated the effect of seven NSAIDs – including selective and non-selective COX-2 inhibitors – on endometriosis, using a mouse model. The results indicated that six out of the seven NSAIDs significantly decreased lesion size in the animals. Additionally, two of the tested substances – celecoxib and indomethacin – could significantly reduce the percentage of established endometriotic lesions. Nevertheless, the results also revealed that even celecoxib – the molecule found to be the most efficient in prevention of lesion formation – could not induce the regression of established lesions [184].

Increased activation of COX-2 by estrogen may also be implicated. It has been shown that estradiol induces COX-2 activity, which increases PGE₂ production. PGE₂ is the most potent known stimulator of aromatase in endometriotic stromal cells [185]. In this manner, a positive-feedback cycle is established, in which local production of estrogen and PGE₂ is enhanced, favoring the proliferative and inflammatory characteristics of endometriosis [186]. The inhibition of COX-2 could decrease

angiogenic activity and local estrogen production by inhibiting PGE2-induced aromatase stimulation. Theoretically, both of these mechanisms could be beneficial in treating endometriosis. However, because of the inconsistent results, this theory should be evaluated in primate models. Furthermore, long-term use of COX-2 inhibitors has been associated with an increased risk of cardiovascular side effects, which can be a serious limiting factor [187].

Immunomodulators for endometriosis treatment

Loxoribine

Guanine ribonucleosides substituted at the 8-position of the guanine ring are a unique class of immunomodulators, the lead compound of which is 7-allyl-8-oxoguanosine; loxoribine. In the rat model, intraperitoneal loxoribine administration enhanced cytokine activity and resulted in regression of endometrial explants [188]. Unfortunately, no additional studies have been published on loxoribine in endometriosis.

Pentoxifylline

Pentoxifylline is a methylxanthine derivative that shows immunomodulating activity. It inhibits phagocytosis and the generation of toxic oxygen species and proteolytic enzymes by macrophages and granulocytes, decreases TNF- α production, and reduces inflammatory action of TNF- α and IL-1 on granulocytes [189,190]. Thus, pentoxifylline influences both the production of inflammatory mediators and the responses to inflammatory stimuli. Furthermore, an experimental study showed that pentoxifylline can modulate rat endometriotic implant growth and production of implant-specific proteins [191]. As it is not an inhibitor of ovulation, administration of pentoxifylline could be feasible throughout the time period of attempting conception.

This was supported by a rodent study, where pentoxifylline reversed the inhibition of fertilization by surgically induced endometriosis [190]. However, these findings were not confirmed by human investigations. A placebo-controlled, randomized clinical trial showed no significant difference in the pregnancy rates between pentoxifylline- and placebo-treated patients [192]. Although the difference was not statistically significant, the authors emphasize that increasing the sample population and excluding additional infertility factors could help to evaluate the real value of this drug.

Expert commentary

Endometriosis is a debilitating condition associated with pain and infertility. Although treatment of this disease with currently available drugs can improve symptoms, the success of the therapy is limited in terms of duration of drug application, side effects and recurrence of endometriosis after cessation of therapy. Furthermore, these pharmacotherapeutic methods do not improve the chances of pregnancy, and as the treatment is hormonally based, it will delay conception even further due to hormonal imbalances introduced to the body. Obviously, there is need for drugs that can abolish endometriosis together with its symptoms and allow conception, even during treatment.

This review has aimed to summarize the immunologic and inflammatory factors that appear to be involved in the pathogenesis of endometriosis. However, at this stage it is difficult to tell whether the changes in quantity and function of these factors cause endometriosis, or are merely consequences of it. The discussed results have shown that anti-inflammatory, antiangiogenic, antiproteolytic substances and agents able to selectively modify specific immune functions may be potential candidates for future endometriosis treatments. These works demonstrate that there are several promising targets, through which it seems possible to interfere pharmacologically with these immunologic and inflammatory functions. Nevertheless, clarification of the controversial results is of great importance in terms of future endometriosis studies and drug developments.

In parallel, results obtained from rodent models of endometriosis should be interpreted carefully. It is well established that these models are valuable tools for first-step evaluations in endometriosis research due to its similarities with human endometriosis, and its favorable financial aspects. On the other hand, there are indeed several very important limitations of this method. Firstly, rodents lack menstrual cycles and spontaneous endometriosis occurs exclusively in menstruating species. Furthermore, in the rodent models, induction is performed via autotransplantation of endometrial fragments or uterine squares, which is not a physiologic event, damages the uterus, and causes adhesions that may interfere with fertility [193]. In addition, evaluating the rat model showed that, despite many similarities between the rat model and human endometriosis, the proliferation of the

lesions declined at 14 days after autotransplantation [194]. This may question the reliability of results obtained from rodent models after the mentioned period of 14 days.

Primates, especially the baboon, has emerged as an ideal animal model for endometriosis. They are phylogenetically very close to humans, experience spontaneous endometriosis of differing stages, and the disease is often progressive. Both induced and spontaneous endometriosis in baboons cause macroscopic lesions similar to those observed in humans. Moreover, baboons in general are bigger and stronger primates, which allows repetitive blood sampling and demanding surgical interventions [195,196].

These features make the baboon an optimal model for testing new endometriosis drugs *in vivo*.

In conclusion, the available results on endometriosis indicate that there is a great need for further basic and clinical research to elucidate the apparently very complex network of mechanisms behind endometriosis. Synthesis of molecules that are able to specifically and selectively modify the function of the immunologic and inflammatory factors are of surpassing importance to these future studies and through this the development of new, more efficient drugs for the management of endometriosis.

Highlights

- Endometriosis is a debilitating disease with an unclarified pathogenesis, affecting millions of women worldwide.
- Available medical treatments do not provide satisfactory relief and there are severe limitations in terms of their application.
- Growing evidence indicates the significant role of immunologic and inflammatory factors in the development of endometriosis.
- The observed alterations of these factors offer potential targets to develop new diagnostic and therapeutic strategies.
- Candidate substances aiming at such targets are already at certain stages of evaluation.
- Due to the peculiarity of the disease, some classical experimental methods – such as rodent models – are of limited value.
- Primate models are indispensable in the appropriate preclinical evaluation of potential new endometriosis drugs.
- Some candidate molecules that are already used in therapeutic practice have shown promising results in several preclinical studies.
- Novel endometriosis drugs may emerge in the near future.

Outlook

Realization the severe consequences of endometriosis on the life of the affected women, as well as on society, has accelerated endometriosis research in the last decade. These still increasing efforts will presumably result in radical innovations in the management of this disease. The overall thinking will increasingly turn from single potential pathogenic factors to the interactions of different molecules – such as cytokines, growth factors and enzymes – on both genetic and proteomic levels. Together with the primate model of endometriosis, such an approach could enable scientists to investigate the early onset of the disease, not only its established form. In turn, the more detailed understanding of the underlying pathomechanism could lead to the development of novel non-invasive diagnostic tools and safer and more efficient medications.

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Affiliations

Attila Mihalyi

Leuven University Fertility Centre,
Department of Obstetrics & Gynecology,
University Hospital Gasthuisberg,
Herestraat 49

B-3000 Leuven, Belgium

Kyama C Mutinda

Leuven University Fertility Centre,
Department of Obstetrics & Gynecology,
University Hospital Gasthuisberg,
Herestraat 49

B-3000 Leuven, Belgium

Peter Simsa

Leuven University Fertility Centre,
Department of Obstetrics & Gynecology,
University Hospital Gasthuisberg,
Herestraat 49

B-3000 Leuven, Belgium

Sophie Debrock

Leuven University Fertility Centre,
Department of Obstetrics & Gynecology,
University Hospital Gasthuisberg,
Herestraat 49

B-3000 Leuven, Belgium

Jason M Mwenda

Institute of Primate Research,
Division of Reproductive Biology,
Karen, Nairobi, Kenya

Thomas M D'Hooghe, MD, PhD

Leuven University Fertility Centre,
Department of Obstetrics & Gynecology,
University Hospital Gasthuisberg,
Herestraat 49

B-3000 Leuven, Belgium

Tel.: +32 16 343 624

Fax: +32 16 343 607

thomas.dhooghe@uz.kuleuven.ac.be