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Dysfunctional Signaling Underlying Endometriosis: Current State of Knowledge

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Abstract

Endometriosis is defined as the presence of endometrial tissue outside the uterine cavity. It affects approximately 5-10% of women of reproductive age. Endometriosis is associated with dysmenorrhea, dyspareunia and, often, severe pelvic pain. In addition to pain, women with endometriosis often experience infertility. Defining the molecular etiology of endometriosis is a significant challenge for improving the quality of women's lives. Unfortunately, the pathophysiology of endometriosis is not well understood. Here, we summarize the potential causative factors of endometriosis in the following three categories: 1) dysregulation of immune cells in the peritoneal fluid and endometriotic lesions; 2) alteration of apoptotic signaling in retrograde menstrual tissue and cytotoxic T cells involved in endometriosis progression; and 3) dysregulation of oxidative stress. Determining the molecular etiology of these dysregulated cellular signaling pathways should provide crucial clues for understanding initiation and progression of endometriosis. Moreover, improved understanding should suggest new molecular therapeutic targets that could improve the specificity of endometriosis treatments and reduce the side effects associated with current approaches.

Introduction

Endometriosis, defined as the presence of endometrial tissue outside the uterine cavity, results in severe pelvic pain and infertility in up to 5–10% of women of reproductive age (Eskenazi and Warner 1997; Giudice 2010). Understanding the molecular etiology of endometriosis is essential to providing better treatment for this disease. There are many unresolved side effects of treatment, including adverse consequences for normal reproductive function, because current systemic estrogen deficiency therapy using gonadotropin-releasing hormone agonists (Descamps and Lansac 1998), oral contraceptives, synthetic progestins and/or aromatase inhibitors prevents pregnancy (Attar and Bulun 2006). To minimize these side effects, new and essential pathological pathways involved in endometriosis and endometriosis-associated dysfunction need to be evaluated.

There are several hypotheses regarding how endometriosis is initiated and progresses (Bulun 2009). The most widely accepted hypothesis involves retrograde menstruation (Sampson's hypothesis), wherein viable endometrial tissue fragments move into the pelvic cavity through the fallopian tubes during menstruation (Sampson 1927). These refluxed endometrial cells subsequently adhere to various tissues (such as the ovary, peritoneum, intestine, and uterus), invade them, and then proliferate until they become endometriotic lesions. Abnormalities of the genital tract, genetic predispositions, hormonal imbalances, altered immune surveillance, inflammatory responses, and abnormal regulation of endometrial cells are potential causative drivers of endometriosis progression (Sourial, et al. 2014). Although numerous studies have sought to determine the causative factors underlying the initiation and progression of endometriosis, the precise pathogenesis of endometriosis remains unknown. To help address this crucial question, we have summarized how the dysregulation of inflammation, apoptosis,

and oxidative stress signaling in immune cells, endometriotic lesions, and peritoneal fluid drives the initiation and progression of endometriosis (Barrier 2010; Gupta, et al. 2006; Taniguchi, et al. 2011). A review of the literature was conducted to identify the most relevant studies reported in the English language. We searched the PubMed MEDLINE electronic database (https://www.ncbi.nlm.nih.gov/pubmed) for articles published between 1996 and 2017. The major keywords used were as follows: “endometriosis and inflammation”, “endometriosis and immune dysregulation”, “endometriosis and apoptosis”, and “endometriosis and oxidative stress”. Here, our goal was to present relevant research related to the pathophysiology of endometriosis, and we considered both in vitro studies using human samples and animal model studies. To specify our purpose, we have included additional keywords as follows: “T cell/B cell dysfunction”, “macrophage”, “natural killer cells”, “cytokine signal” and “inflammation and estrogen receptor” along with endometriosis. Moreover, references in each article were searched to identify studies potentially overlooked in our initial search.

Dysregulation of immune signaling during endometriosis progression

During each menstrual cycle, viable endometrial fragments are transported into the peritoneal area by retrograde menstruation. Several studies have indicated that endometriosis patients have dysregulated immune systems that allow retrograde menstrual tissue to survive. For example, endometriosis patients have elevated levels of activated macrophages, T and B cells, but reduced levels of cytotoxic natural killer (NK) cells compared to healthy women (Jeung, et al. 2016). They also show significant upregulation of stem cell growth factor b (SCGFB), interleukin (IL) 8, human growth factor (HGF), and monocyte chemoattractant protein 1 (MCP1), and downregulation of IL13 (Jorgensen, et al. 2017). These dysregulated

immune cells and their cytokine networks could stimulate the initiation and progression of endometriosis.

A) Alterations of macrophages and their cytokine profiles in endometriosis.

Macrophages, the internal components of the mononuclear phagocyte system, are derived from bone marrow progenitors and enter the bloodstream as monocytes. In peripheral tissues, macrophages mature and are activated in response to various external stimuli (such as lineage-determining growth factors, T helper (Th) cell cytokines, and microbial products) to modulate the immune system (Santanam, et al. 2002).

Are macrophages required for the progression of endometriosis? Significantly increased numbers of macrophages are detected in eutopic endometria in women with endometriosis (Berbic, et al. 2009), raising questions regarding their role during endometriosis progression. A rat endometriosis model showed that macrophage depletion using liposomal alendronate (LA) effectively inhibited the initiation and growth of endometriotic lesions, as determined by reduced implantation rates, adhesion scoring, implant size and weight, and numbers of infiltrating macrophages in implants following LA treatment compared to vehicle treatment (Haber, et al. 2009). Another study revealed that endometrial fragments adhered to and implanted in the peritoneal wall, whereas endometriotic lesions failed to organize and develop in the absence of macrophages because blood vessels failed to reach the inner layers of endometriotic lesions, which subsequently stopped growing (Bacci, et al. 2009). These observations suggest an important role for macrophages in endometriosis progression.

How do macrophages drive endometriosis progression? As macrophages secrete various cytokines to modulate normal cell functions, dysregulated macrophage-secreted cytokines

have been associated with several diseases (Arango Duque and Descoteaux 2014). An abundance of peritoneal neutrophils and macrophages in the peritoneal fluid of endometriosis patients increases the levels of vascular endothelial growth factor (VEGF), which stimulates endometriosis progression (Lin, et al. 2006). Higher levels of macrophages may play a role in endometriosis by increasing the levels of cytokines responsible for amplifying the angiogenic signal. Interleukin 24 (IL24) is a novel tumor suppressor gene active in a broad range of human cancer cells. In decidual stromal cells, IL24 also significantly restricts the stimulatory effects of estrogen (Shao, et al. 2013). Interestingly, macrophages markedly reduce the expression of IL24 in endometrial stromal cells to limit the inhibitory effects of IL24 on cell viability and invasion, as well as on the expression levels of the proliferation-related gene Ki-67, proliferating cell nuclear antigen (PCNA), and cyclooxygenase 2 (COX2) (Shao, et al. 2016). Macrophage-mediated downregulation of IL24 leads to the increased proliferation and invasiveness of endometrial stromal cells and contributes to endometriosis progression.

Tumor Growth Factor (TGF)β levels are also elevated in endometriotic lesions and macrophages in women with endometriosis compared to healthy women (Omwandho, et al. 2010). TGFβ-mediated autocrine and paracrine signaling in peritoneal macrophages plays an essential role in endometriosis progression by stimulating macrophage DNA synthesis, macrophage cell-cell interactions and the expression of macrophage cell surface adhesion molecules, such as integrin-α/β (Dou, et al. 1997).

Is there any difference in the macrophage populations between the normal endometrium and endometriotic lesions? Macrophages are activated into classic (M1) or alternative (M2) phenotypes depending on the type of stimulation (Martinez and Gordon 2014). Lipopolysaccharides (LPS), interferon-γ (IFN-γ), and granulocyte-macrophage colony

stimulating factor (GM-CSF) induce macrophages towards the M1 phenotype. M1 macrophages produce significant levels of pro-inflammatory cytokines, such as IL1β, tumor necrosis factor (TNF), IL12, IL18, and IL23 (Wang, et al. 2014a). These help drive antigen-specific Th1 and Th17 cell inflammatory responses that suppress tumor cell growth (Roberts, et al. 2015). In addition to pro-inflammatory cytokines, M1 macrophages upregulate the expression of intracellular protein suppressor of cytokine signaling 3 (SOCS3) and activate inducible nitric oxide synthase (NOS2 or iNOS) to produce NO from L-arginine and inhibit tumor growth (Arnold, et al. 2014). Macrophages are guided towards the M2 type by fungal cells, immune complexes, helminth infections, complement components, apoptotic cells, macrophage colony-stimulating factor (MCSF), IL4, IL13, IL10, and transforming growth factor (TGF)-β (Martinez and Gordon 2014). Activated M2 macrophages secrete high levels of IL10, IL1, IL1ra, and IL6 to stimulate tumor growth (Arango Duque and Descoteaux 2014).

A rhesus macaque model of endometriosis revealed that, compared to controls, the activation state of macrophages in endometriosis tissues in nonhuman primates was skewed towards the M2 phenotype (Smith, et al. 2012). Large peritoneal macrophages (LPMs) and small peritoneal macrophages (SPMs) have been found to polarize towards either M1 or M2 cells, respectively, in a murine model. Accordingly, the proportion of SPMs increased immediately after peritoneal injection of endometrial tissue, whereas LPMs exhibited the opposite trend (Yuan, et al. 2017). Thus, it is possible that retrograde menstrual tissues could stimulate peritoneal macrophage polarization to the M2 type. In human endometriosis patients, there is high M2 macrophage polarization, and *in vitro* co-culture analyses have shown that M2 macrophages significantly upregulate proliferation of

147 endometrial stromal cells by activating signal transducer and activator of transcription 3
148 (STAT3) signaling (Itoh, et al. 2013). STAT3 signaling is aberrantly activated in epithelial
149 and endometrial stromal cells in human endometriotic lesions (Kim, et al. 2015). Therefore,
150 endometriosis-associated M2 macrophages may stimulate STAT3 signaling in endometriotic
151 lesions and thereby stimulate endometriosis.

152 *What causative factors drive M2 macrophage polarization in endometriotic cells?* M2
153 macrophage polarization is regulated by the endometrium. Abnormal expression of
154 indoleamine 2,3-dioxygenase-1 (IDO1) in endometrial stromal cells promotes an
155 inflammatory response that subsequently initiates M2 macrophage polarization, which may
156 facilitate the survival of retrograde menstrual tissues (Mei, et al. 2017). Fractalkine (FKN),
157 which is secreted by eutopic endometrial stroma cells, also stimulates M2 macrophage
158 polarization and enhances endometriosis progression (Wang, et al. 2014b). FKN
159 induces M2 macrophage polarization by decreasing CD86 expression. In addition, FKN
160 increases the expression of matrix metalloproteinase 9 (MMP9) by decreasing the expression
161 of tissue inhibitor of MMP1 and 2. This promotes the invasiveness of endometrial stromal
162 cells by activating p38 mitogen-activated protein kinases (MAPKs) and the integrin β1
163 signaling pathway to stimulate endometriosis progression (Collette, et al. 2006; Wang et al.
164 2014b).

165 Exposure to endocrine disrupting chemicals interferes with the endocrine system, causing
166 cancerous tumors, birth defects and other developmental disorders, resulting in the
167 progression of several human diseases (Mallozzi, et al. 2017; Ribeiro, et al. 2017). For
168 example, exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) compounds stimulate
169 endometriosis progression (Smarr, et al. 2016). To induce endometriosis, TCDD alters

patterns of macrophage activation. Combining 17β-estradiol with TCDD has a synergistic effect on the induction of M2 macrophage activation when macrophages are co-cultured with endometrial stromal cells, because it activates STAT3 and p38 MAPK signaling pathways (Wang, et al. 2015). In addition to *in vitro* assays, the combination of TCDD and high levels of local 17β-estradiol in endometriotic lesions has been shown to synergistically induce M2 macrophage polarization and stimulate endometriosis in humans (Delvoux, et al. 2009).

Annexin A2 is involved in various cellular processes, such as cell motility, cytoskeletal regulation, and endocytosis. Levels of annexin A2 are markedly reduced in peritoneal macrophages from women with endometriosis compared to controls, and downregulation of annexin A2 inhibits the phagocytic capacity of macrophages (Wu, et al. 2013). The level of annexin A2 mRNA in macrophages is reduced by prostaglandin E2 (PGE2) via the EP2/EP4 receptor-dependent signaling pathway. Indeed, elevated levels of PGE2 have been detected in endometriotic lesions (Rakhila, et al. 2015), where they may reduce the ratio of M1/M2 peritoneal macrophages and stimulate the progression of endometriosis.

Endometriotic lesions exhibit high levels of the C-C chemokine regulated on activation, normal T cell expressed and secreted (RANTES). During osteogenesis, RANTES stimulates the transition of M1 to M2 macrophages in osteoprogenitors (Cordova, et al. 2017). Elevated RANTES levels has been linked to endometriosis progression (Hornung, et al. 2001; Wang, et al. 2010) and is likely involved in M2 peritoneal macrophage polarization in endometriosis patients. TCDD promote RANTES expression, and a combination of 17β-estradiol and TCDD significantly enhanced RANTES secretion in an endometriosis-associated human endometrial cell co-culture system, recruiting greater numbers of macrophages (Wang et al.

2010). RANTES could be a molecular therapeutic target for endometriosis, as suggested by the action of shikonin, an anti-inflammatory phytocompound derived from *Lithospermum erythrorhizon*, that mediates the inhibition of RANTES secretion and reduces endometriosis progression (Yuan, et al. 2014).

The activation of TGFβ signaling in endometriosis also induces M2 macrophage polarization to stimulate inflammatory signaling and tissue repair (Gong, et al. 2012).

B) Dysregulation of T cell-mediated cytokine profiling in endometriosis.

Lymphocyte subpopulations in endometriotic lesions are markedly different from those in normal endometrial tissue. Specifically, endometriotic lesions display increased numbers of CD4 and CD8 cells and activated T cells compared to normal endometrial tissue (Witz, et al. 1994). Additionally, T cell subtypes are also differentially regulated. The proportion of Th1 lymphocytes is significantly lower, whereas the Th17 lymphocyte fraction is significantly elevated in endometriotic lesions (Takamura, et al. 2015). One recent study has shown that IL-10^{+}Th17 cell papulation are significantly elevated in the peritoneal fluid of endometriosis patients as compared to the women without endometriosis (Chang, et al. 2017). Interestingly, elevation of IL-10^{+}Th17 cell papulation is associated with increased levels of IL-27, IL-6 and TGF-β. Especially, TGF-β stimulates IL-10 production in Th17 cells in vitro and in vivo in human endometrial stromal cells to stimulate the proliferation and implantation of ectopic lesions and accelerate the progression of endometriosis (Chang, et al. 2017). Although these patterns are not fully understood, this differential T lymphocyte activation appears to clearly be involved in the pathophysiology of endometriosis.

Altered ratios of Th1/Th2 cells in endometriotic lesions: CD4+ T lymphocytes, or Th cells, can be further subdivided into Th1 and Th2 cells, and the cytokines they produce are referred

to as Th1-type and Th2-type, respectively (Berger 2000). Th1-type cytokines tend to generate pro-inflammatory responses, whereas Th2-type cytokines, such as IL4, IL5, IL10, and IL13, tend to elicit anti-inflammatory responses. A well-balanced Th1 and Th2 response is important for various immune challenges (Berger 2000). In endometriotic lesions, the levels of IFN-γ, IL10, and the ratios of IL4/IFN-γ, IL4/IL2, IL10/IFN-γ, and IL10/IL2 are significantly elevated in the peritoneal fluid of endometriosis patients compared to healthy controls (Podgaec, et al. 2007), which reflects a shift towards the Th2 immune response. Endometriosis progression may be associated with a reduced Th1/Th2 ratio among T cells in the peritoneal fluid.

Role and determinants of Th2 cytokine production during endometriosis progression: In humans, cytokines secreted from Th2 cells stimulate endometriosis progression. For example, IL4, a typical Th2 cytokine, has been shown to increase the mRNA expression of 3β-hydroxysteroid dehydrogenase (HSD3B2) in a dose-dependent manner (Urata, et al. 2013). HSD3B2 is a pivotal enzyme for estrogen production. IL4 increases local estrogen levels to stimulate endometriosis progression. In addition, IL4 increases the proliferation of endometriotic stromal cells by activating p38 MAPK, stress-activated protein kinase/c-Jun kinase, and p42/44 MAPK to stimulate endometriosis progression (OuYang, et al. 2008b). Similar changes have been observed in mouse models. The weights and areas of endometriotic lesions have been found to be significantly reduced following treatment with INF-γ and IL2 (Th1 cytokines) compared to treatment with IL4 and IL10 (Th2 cytokines) or saline solution (controls) (Mier-Cabrera, et al. 2013). Th1 cytokine milieus suppress the progression of endometriosis in a murine endometriosis model.

Eutopic endometrial tissues from patients with endometriosis have higher mRNA levels of

GATA binding protein 3 (GATA3) compared to normal endometrial tissue (Chen, et al. 2012). Expression of GATA3 is regulated by estrogen, and their synergistic action regulates Th2 cytokine (e.g., IL6, IL8, and IL10) expression in eutopic endometrial cells (Chen, et al. 2016). Therefore, GATA3 integrates estrogen signaling to induce Th2 cytokine expression in endometriotic lesions, thereby promoting endometriosis progression.

IL6 levels are also elevated in endometrial stromal cells isolated from women with endometriosis compared to healthy controls (Tsudo, et al. 2000). IL6 expression in endometriotic cells is induced by IL1β and TNF-α (Akoum, et al. 1996). IL6 promotes CD4+ Th2 differentiation and inhibits Th1 differentiation via two independent molecular mechanisms (Diehl, et al. 2000). Elevated IL6 levels promote Th2 differentiation by activating transcription mediated by nuclear factor of activated T cells (NFAT) (Diehl and Rincon 2002). Additionally, IL6 inhibits Th1 differentiation by interfering with IFN-γ signaling and the expression of suppressor of cytokine signaling 1 (SOCS1). These findings may support a role for IL6 in Th2 differentiation and Th2 cytokine production in endometriotic lesions.

Alteration of Treg cells in endometriosis: In addition to Th1 and Th2 cells, naïve T cells can differentiate into regulatory T (Treg) cells (Josefowicz, et al. 2012). Treg cells suppress a range of immune responses, such as T-cell proliferation and activation (Giatromanolaki, et al. 2008), as well as macrophage, B-cell, dendritic cell, and NK-cell function (Thornton 2005). Because of its immuno-suppressive function, the infiltration of large numbers of Treg cells into tumor tissues is associated with a poor prognosis (Enokida and Nishikawa 2017). Consistent with tumor progression, a higher concentration of Treg cell phenotypes and/or expression markers has been detected in peritoneal fluid and endometriotic lesions but

not in samples from healthy control women (Bellelis, et al. 2013; de Barros, et al. 2017; Slabe, et al. 2013). To initiate and establish endometriosis, retrograde menstrual tissues in the peritoneal region must escape the host immune surveillance system. To achieve this, the large numbers of Treg cells in the T cell population and endometriotic lesions decrease the recruitment of immune cells to prevent the recognition and targeting of retrograde menstrual tissues, thus allowing their survival and implantation into ectopic sites.

Th17 cells and IL23 levels in endometriosis: In addition to Th2 cytokines, the levels of IL23 and the Th17 cytokine IL17 are highly elevated in the peritoneal fluid of women with minimal or mild endometriosis (Andreoli, et al. 2011). Th17 cells are involved in the pathogenesis of several autoimmune diseases, and endometriosis is associated with a higher risk (20–60%) of autoimmune disease, such as multiple sclerosis, systemic lupus erythematosus, and Sjögren syndrome (Nielsen, et al. 2011; Ouyang, et al. 2008a). *In vitro* stimulation of endometrial epithelial carcinoma cells, Ishikawa cells, and HUVECs with IL17A revealed that IL17A treatment significantly increased angiogenic (VEGF and IL8), pro-inflammatory (IL6 and IL1β), and chemotactic cytokine levels (G-CSF, CXCL12, CXCL1, and CX3CL1) (Ahn, et al. 2015). The levels of IL23 were significantly higher in the peritoneal fluid of women with endometriosis compared to normal controls (Andreoli et al. 2011). Activated naïve T cells produce IL23, which then increases the levels of IL10 and IL17, both of which are required for endometriosis progression (Vanden Eijnden, et al. 2005). Dysregulation of IL23 is also involved in several endometriosis-associated endometrial dysfunctions, such as infertility (Andreoli et al. 2011; Frazer, et al. 2013).

Altered T cell activation and autoimmune properties of endometriosis: Endometriosis is not itself an autoimmune disease; however, women with endometriosis may have been reported

to have a higher risk of developing several autoimmune diseases, such as systemic lupus erythematosus, Sjögren's syndrome, multiple sclerosis, and rheumatoid arthritis (Haga, et al. 2005; Harris, et al. 2016). This is somewhat controversial however, as another study reported no correlation between them (Nielsen et al. 2011). In many autoimmune diseases, altered activation of $CD4^+$ T cells plays a critical role in activating B cells to stimulate the production of autoantibodies (Palmer and Weaver 2010). Consistent with autoimmune disease, the elevated levels of autoantibodies against the endometrium and ovary are highly elevated in endometriosis patient (Mathur, et al. 1982). Therefore, altered activation of $CD4^+$ T cells, as described above, might be involved in the elevation of autoimmune disease properties in endometriotic lesions.

C) Dysfunction of NK cells in endometriosis patients.

NK cells secrete lytic granules containing granzyme, perforin, and cytotoxins (such as IFN-γ) to destroy other cells (Topham and Hewitt 2009). Cytotoxic NK cells therefore play a critical role in innate immunity to activate the host immune surveillance system following exposure to pathogens. Because of the crucial role of NK cells in innate immunity, dysregulation of NK cells causes immune-related disease progression (Mandal and Viswanathan 2015; Smyth, et al. 2005). The levels of molecular markers of cytotoxic NK cells, such as markers of activation (granzyme B, perforin, TRAIL, CD107a, and CD69) and cell surface markers (NKp46, NKp44, NKG2D, and CD16), are significantly reduced, but the proportion of immature NK cells (CD272CD11b2+) in the NK cell population (CD32CD56+) is elevated in the peritoneal fluid of endometriosis patients compared to normal women (Jeung, et al. 2016; Oosterlynck, et al. 1991).

How are cytotoxic NK cells downregulated in endometriotic lesions compared to normal

endometrial tissue? Cytokines with inhibitory effects on cytotoxic NK cells, such as inflammatory cytokines (IL6, IL8, IL1β, IFN-γ, and TNF-α) and non-inflammatory cytokines (CXCL3, CCL2, CCL5), are significantly elevated in the peritoneal fluid of endometriosis patients compared to controls (Malutan, et al. 2015). Moreover, peritoneal fluid from endometriosis patients also shows elevated levels of antigens (HLA-G and HLA-E), immunoreceptor tyrosine-based inhibitory motif killer cell inhibitory receptors (ITIM-KIRs), inhibitory NK cell receptors containing Ig domains (KIR2DL1, KIR3DL1), EB6, and soluble intracellular adhesion molecule-1 (I-CAM), which also suppress cytotoxic NK cells (Jeung et al. 2016). In addition, HLA-G expression is detected in eutopic endometrial tissue of endometriosis patients during the menstrual phase (Thiruchelvam, et al. 2015). Retrograde menstrual tissues show elevated levels of HLA-G in the peritoneal cavity, where they can interact with the immune surveillance system and counteract the cytotoxicity of NK cells. This would allow retrograde menstrual tissues to survive and implant, eventually developing into endometriotic lesions. Therefore, increased levels of inflammatory cytokines, antigens, and inhibitory receptors in the peritoneal fluid and endometrium downregulate cytotoxic NK activity during the progression of endometriosis.

D) Activation of B cells in endometriosis.

B cells underlie humoral immune responses by producing antibodies against antigens. Increased numbers of B cells are found in the blood and peritoneal fluids of endometriosis patients compared to healthy women (Osuga, et al. 2011). Interestingly, transcriptional factors regulating B cell function are differentially expressed in endometriosis patients compared with healthy women. For example, B lymphocyte inducer of maturation program (Blimp)-1, which is a crucial regulator of plasma cell differentiation, is significantly

increased; the levels of B-cell leukemia lymphoma (Bcl)-6, its antagonist, are significantly reduced in the peritoneal cavities of endometriosis patients (Yeol, et al. 2015). In addition to transcription factors, endometriotic lesions also have higher levels of cytokines that activate B cells, such as B lymphocyte stimulator (BLys) (Hever, et al. 2007). BLys plays an important role in the normal development of B cells and their differentiation into plasma cells (Schiemann, et al. 2001). Therefore, these factors can stimulate B cell function in endometriosis patients.

These hyperactivated B lymphocytes appear to contribute to the pathogenesis of endometriosis
by producing autoantibodies against the endometrium, DNA, and phospholipids, as well as antinuclear antibodies (Osuga et al. 2011). A similar elevation of autoantibodies has also been observed in autoimmune diseases (Eggert, et al. 2010). Because of the many similarities between endometriosis and autoimmune diseases, endometriosis may be treatable as an autoimmune disease (Nothnick 2001).

E) Alteration of cytokine profiling in endometriotic lesions.

In addition to immune cells, endometriotic lesions are themselves a source of secreted cytokines that stimulate endometriosis progression. For example, endometriotic epithelial cells have increased levels of TNF-α compared to normal endometrial tissue during endometriosis progression. Epithelial TNF-α activates the phosphoinositide 3-kinase (PI3K), MAPK, c-Jun N-terminal kinase (JNK), p38, and IκB kinase signaling pathways via autocrine responses to stimulate inflammation and invasion of endometriotic epithelial cells,

thus favoring their proliferation (Grund, et al. 2008). Endometriotic epithelial TNF-α also induces IL6 and IL8 expression in endometriotic stromal cells via nuclear factor kappa-B (NF-κB) and activator protein (AP)1 through paracrine responses to stimulate proliferation of endometriotic stromal cells (Sakamoto, et al. 2003; Yamauchi, et al. 2004). These dysregulated auto- or paracrine cytokine signaling networks in endometriotic lesions are also involved in endometriosis progression.

In addition to immune cells, endometriotic lesions are a source of various cytokines, such as ENA78, RANTES, TNFα, IL6 and IL8 (Akoum, et al. 2001; Bertschi, et al. 2013). IL6 plays a significant role in CD4+ T cell differentiation (Dienz and Rincon 2009), and IL8 induces T lymphocyte infiltration in target tissues (Taub, et al. 1996). Therefore, IL6 and IL8 in endometriotic lesions might generate T cell milieus specific for endometriotic lesions to enhance their survival.

F) Inflammatory and Estrogen Receptor (ESR) signaling in endometriotic lesions and macrophages

Peritoneal macrophages are activated by exposure to 17β-estradiol (Hong and Zhu 2004). Because a higher activity of the 17β-estradiol axis stimulates endometriosis-associated macrophage activation to synergistically induce endometriosis, endometriosis has been considered an estrogen-dependent inflammatory disease. In addition to higher local estradiol concentrations, ESR levels are also differentially regulated in endometriotic lesions in response to increased estradiol signaling. Accordingly, elevated levels of ESR2 but not ESR1 have been detected in endometriotic tissues compared to normal endometrial tissues. Elevated ESR2 stimulates prostaglandin production in endometriotic tissues through COX2 to promote

endometriosis progression (Bulun, et al. 2012; Wu, et al. 2010). Increased prostaglandin levels suppress the immune system, allowing retrograde menstrual tissues to escape the immune surveillance system and develop into endometriotic lesions. In addition, ESR2 interacts with components of the cytoplasmic inflammasome to increase IL1β in endometriotic lesions, stimulating their adhesion and proliferation properties (Han, et al. 2015). Therefore, increases in ESR2 function modulate the immune response to retrograde menstrual tissues, which can subsequently develop into endometriotic lesions. Hypomethylation of the ESR2 gene promoter region might contribute to higher ESR2 levels in endometriotic lesions (Xue, et al. 2007), but detailed molecular mechanisms underlying ESR2 function in endometriosis progression remain unclear.

Peritoneal macrophages are activated upon 17β-estradiol treatment to stimulate endometriosis progression (Hong and Zhu 2004), and expression levels of ESR2 are significantly increased in peritoneal macrophages of women with endometriosis (Montagna, et al. 2008). Pretreatment of peritoneal macrophages with ERB-041, a selective ESR2 agonist, results in significant inhibition of LPS-induced iNOS expression by suppressing NF-κB activation and endometriosis progression (Harris, et al. 2005; Xiu-li, et al. 2009). Collectively, the alteration of the ESR2-estradiol axis in macrophages is another driver of endometriosis progression.

G) Communication between immune cells and endometriotic lesions drives endometriosis progression.

We have discussed dysregulated immune signaling in both immune cells and endometriotic lesions. Interestingly, altered inflammatory signaling in immune cells induces endometriotic lesions to enhance endometriosis progression (Fig. 1). During the initiation of endometriosis,

altered immune cells release pro-inflammatory cytokines (IL1, IL6, IL8, IL10, IL12, IL13, TNF-α, VEGF, and platelet-derived growth factor, PDGF) by activating the STAT, p38, extracellular signal-regulated kinase (ERK), and JNK signaling pathways. These cytokines bind to their receptors in endometriotic lesions and mediate further downstream signaling via NF-kB to initiate and establish endometriosis progression. For example, mRNA expression levels of steroidogenic acute regulatory protein (StAR), COX2, MMP9, and other pro-inflammatory cytokines is increased in endometriotic lesions as a result of NF-kB-mediated pro-inflammatory cytokines (Tsai, et al. 2001). Elevated StAR expression is involved in estradiol production in endometriotic lesions, further promoting endometriosis progression. Moreover, increased local E2 levels directly induce COX2 expression to promote PGE2 production and activate inflammasomes via ESR2 to induce IL1β, thus enhancing the adhesion and proliferation of endometriotic lesions and endometriosis progression.

Dysregulated apoptosis signaling in endometriotic lesions

Impaired apoptosis in retrograde menstrual tissues and abnormal apoptosis in immune cells are associated with endometriosis progression (Taniguchi et al. 2011). Understanding the molecular mechanisms governing the dysregulation of apoptosis in endometriotic tissues and immune cells is crucial for determining the molecular etiology of endometriosis and providing new molecular therapeutic treatments. Here, we discuss how dysregulated apoptosis is involved in the progression of endometriosis.

A) Reduced apoptosis in endometriotic lesions.

Compared to healthy women, apoptosis is significantly reduced in eutopic endometrial tissue in patients with endometriosis (Gebel, et al. 1998), as are the levels of apoptotic marker genes in endometriotic lesions. Specifically, endometriotic lesions show higher BCL2 (anti-

apoptotic signaling) staining than normal endometrial tissue (Harada, et al. 2004), as well as increased expression of c-myc (a cell-cycle regulator) and TGF-β; in contrast, reduced levels of the pro-apoptotic BCL2-associated X protein (BAX) are found (Meresman, et al. 2000; Vetvicka, et al. 2016; Yu, et al. 2017). Collectively, the reduction of apoptosis in endometriotic lesions represents a concerted effort by retrograde menstrual tissues to evade immune surveillance and develop into endometriotic lesions.

B) Dysregulation of intrinsic apoptosis signaling in endometriosis. Apoptotic signaling occurs via two different pathways: intrinsic (or mitochondrial) and extrinsic (or death receptor-mediated) (Schleich and Lavrik 2013). Suppression of the intrinsic apoptotic pathway has been detected in endometriotic lesions. The ratio of anti- to pro-apoptotic molecules, such as BCL2/BAX, is higher in mitochondria of eutopic endometrial tissues (Meresman et al. 2000) and in macrophages from endometriotic lesions. The BCL2 family of proteins constitutes a critical intracellular checkpoint of the intrinsic apoptotic pathway; increased BCL2 but decreased BAX expression levels are found in the proliferative phase of eutopic endometrial tissues from patients with endometriosis compared with normal endometrial tissue. Women with endometriosis have a large BCL2-positive macrophage population in the peritoneal fluid, whereas women without endometriosis have a peritoneal macrophage population that has elevated levels of BAX (McLaren, et al. 1997). Interestingly, the expression profile of apoptosis-related proteins in endometriotic lesions is regulated in a location-dependent manner. For example, p53 and p21 are higher in ovarian endometriosis, whereas BCL2 expression is higher in peritoneal and colorectal endometriosis (Dufournet, et al. 2006). A different mechanism of suppression of the intrinsic apoptotic pathway might be involved in the development of each type of endometriotic lesion, and targeting specific anti-

apoptotic pathways may be useful as a component of endometriosis treatment for specific endometriotic lesions.

C) Alteration of extrinsic apoptosis signaling in endometriosis.

1) Fas/FasL: The Fas/FasL axis is the traditional extrinsic apoptosis signaling cascade (Curtin and Cotter 2003). Fas (DR2/CD95/Apo-1) is a type I cell membrane protein (mFas), with an extracellular domain that binds FasL (CD95L/CD178/Apo-1L) and a cytoplasmic domain that transduces the death signal (Peter, et al. 2007; Strasser, et al. 2009). Cell death signaling mediated by the Fas/FasL interaction plays an essential role in the immune system and in maintaining immune-privileged sites in the body. For example, Fas/FasL-mediated apoptosis kills cytotoxic T cells (Waring and Mullbacher 1999). FasL is expressed in normal human endometrial cells, where it is stimulated by macrophage cytokines, such as PDGF and TGF-β1 (Garcia-Velasco, et al. 1999). Higher levels of IL8 in the peritoneal fluid of endometriosis patients cause an increase in FasL expression in endometrial cells (Selam, et al. 2002) and endometrial stromal cells. However, increased FasL does not induce apoptosis in endometrial stromal cells (Selam, et al. 2006a). Ectopic epithelial cells of endometriotic lesions have simultaneously increased FasL expression and reduced Fas expression, irrespective of the menstrual cycle phase (Sbracia, et al. 2016). Collectively, induction of FasL in endometrial cells may induce apoptosis in cytotoxic T cells expressing the Fas receptor, thus allowing them to evade immune surveillance and develop into endometriotic lesions.

2) TNFα-mediated apoptosis: Changes in TNF-α-mediated cell death signaling are also involved in endometriosis progression (Iwabe, et al. 2000). During retrograde menstruation, the influx of retrograde menstrual tissues into the peritoneal cavity activates macrophages to

secrete cytotoxic cytokines, such as TNF-α, inducing apoptosis signaling in extra-uterine endometrial fragments that need to be removed (Leavy 2015). In endometriosis patients, however, the molecular properties of retrograde menstrual tissues are altered in a way that allows escape from TNF-α-mediated apoptosis. As endometriosis is an estrogen-dependent disease, Nuclear Receptor Coactivator (NCOA)s may play an important role in endometriosis progression. Interestingly, endometriotic lesions have an elevated level of the NCOA-1 isoform, but not full-length NCOA-1 (Han, et al. 2012). The NCOA-1 isoform is proteolytically generated from full-length NCOA-1 by MMP9 in endometriotic lesions. There, the NCOA-1 isoform, but not full-length NCOA-1, interacts with caspase 8 to prevent TNF-α-mediated apoptosis by disrupting apoptosis complex II formation. Endometriotic lesions also express high levels of ESR2 (Hudelist, et al. 2005), which then interacts with caspase 8 or components of the cell death machinery in endometriotic cells to block TNF-α-induced apoptosis (Han et al. 2015). Specifically, high ESR2 induces the formation of apoptosis signal-regulating kinase 1 (ASK1), serine/threonine kinase receptor-associated protein, and the 14-3-3 protein complex to inhibit ASK1 activity required for TNF-α-mediated apoptosis. Moreover, ESR2 disrupts apoptosome formation by interacting with and preventing the activation of caspase 9 in endometriotic lesions. Taken together, induction of the endometriosis-specific NCOA-1 isoform/ESR2 axis actively prevents TNF-α-induced apoptosis signaling in endometriotic lesions by interacting with the apoptotic machinery (Fig. 2).

D) Targeting the dysregulation of apoptosis signaling in endometriotic tissues.

In addition to endometriosis progression, the sophisticated regulation of apoptosis also plays an important role in embryonic development via the appropriate formation of various organs

and structures (Haanen and Vermes 1996). Therefore, defective apoptosis signaling during embryogenesis may cause developmental abnormalities (Haanen and Vermes 1996). Dysregulation of apoptosis is a key driver of many human diseases, and may serve as an effective molecular therapeutic target for the treatment of many human diseases.

PGE2 levels are elevated in endometriosis patients; PGE2 promotes the survival of human endometriotic lesions through EP2 and EP4 receptors and activation of the ERK1/2, AKT, NF-κB, and β-catenin signaling pathways (Banu, et al. 2009). Selective inhibitors of EP2 (AH6809) and EP4 (AH23848) suppress these cell survival pathways and enhance interactions between anti-apoptotic and pro-apoptotic proteins, thereby activating the intrinsic apoptotic pathways in human endometriotic cells.

Pro-inflammatory cytokines also regulate apoptotic signaling in various cells to modulate their cellular function (Grunnet, et al. 2009). In endometriosis, dysregulated cytokines prevent apoptosis and promote the survival of endometriotic lesions. For example, secretion of CXCL8 is significantly higher in eutopic endometrial stromal cells of women with endometriosis compared to normal endometrial tissues, and elevated CXCL8 reduces apoptosis by upregulating BCL2 expression in these cells in an autocrine manner (Li, et al. 2012). Anti-human CXCL8-neutralizing antibodies suppress endometriosis progression by inducing apoptosis in endometriotic lesions. RANTES and IL8 attenuate apoptosis in endometriotic lesions (Selam, et al. 2006b); shikonin-mediated inhibition of RANTES secretion reduces endometriosis progression (Yuan et al. 2014). Treatment with an IL8-neutralizing antibody also suppresses endometriosis progression by inhibiting the attachment of retrograde menstrual tissues and reactivating apoptosis in these cells (Arici 2002). Collectively, molecules that induce anti-apoptotic pathways in endometriotic lesions could be

514 molecular therapeutic targets for alternative endometriosis treatments.

515 **Dysregulation of oxidative stress in endometriosis**

516 Healthy women exhibit balanced levels of reactive oxygen species (ROS) and antioxidants.
517 An overabundance of ROS induces oxidative stress, impacting women throughout their
518 reproductive lifespan, including in the initiation of endometriosis (Carvalho, et al. 2012).
519 Oxidative stress results in damage to cellular lipids, proteins, and DNA, changing their
520 molecular properties and possibly leading to disease. Importantly, ROS overproduction
521 impairs cellular functions by inducing redox-sensitive transcription factor (such as NF-κB)-
522 mediated expression of genes required for the initiation and progression of endometriosis (Fig.
523 3) (Defrere, et al. 2011).

524 Erythrocytes, apoptotic endometrial tissue, and cell debris transplanted into the peritoneal
525 cavity by menstrual reflux, as well as macrophages, have all been cited as potential inducers
526 of oxidative stress. Iron overload has been detected in the cells and peritoneal fluid of women
527 with endometriosis compared to normal endometrial tissues (Carvalho et al. 2012; Van
528 Langendonckt, et al. 2002). Excessive iron induces deleterious ROS in the peritoneal
529 environment, which enhances the attachment and growth of retrograde menstrual tissues
530 (Alizadeh, et al. 2015; Donnez, et al. 2016). In a murine model, iron overload has been shown
531 to further expand endometriosis by promoting epithelial cell proliferation at lesion sites
532 (Defrère et al., 2006). Additionally, excessive iron levels may favor nitric oxide production,
533 resulting in the impaired clearance of endometrial cells by macrophages (Pirdel and Pirdel
534 2014). At present, it remains unclear why iron-mediated oxidative stress is maintained at high
535 levels in endometriosis patients compared to healthy women. One possibility is that it is
536 associated with alterations in ROS detoxification pathways and reductions in catalase levels,

as observed in cancer patients (Ngo, et al. 2009). Retrograde menstruation-mediated hyperactivated oxidative stress leads to stimulation of the ERK and PI3K/AKT/mTOR signaling pathways (Fig. 3), thus promoting adhesion, angiogenesis, and proliferation of endometriotic lesions and subsequent endometriosis progression (McKinnon, et al. 2016).

Development of alternative endometriosis treatments based on drugs targeting the dysregulated immune system, apoptosis, and oxidative stress

The goal of endometriosis treatment is to relieve pain and/or achieve successful pregnancies in infertile patients. Most current medical treatments induce systemic estrogen depletion, because estrogen signaling is an essential driver of endometriosis. However, many current clinical endometriosis treatments are not sufficiently effective and have unacceptable side effects, because the specific molecular etiology of endometriosis has not yet been elucidated. Here, we have discussed endometriosis-associated processes, including dysregulation of inflammation, anti-apoptosis, and oxidative stress in endometriosis patients. Therefore, these dysregulated cellular pathways provide important clues to understanding the molecular etiology of endometriosis and could offer new molecular therapeutic targets to improve the specificity of endometriosis therapy and reduce side effects of current treatments. Based on these findings, several drugs targeting endometriosis-specific inflammation, anti-apoptosis, and oxidative stress pathways, as well as alternative hormonal agents, have been developed and examined using *in vitro* and *in vivo* endometriosis models. The most recently studied drugs are summarized in Table 1.

Conclusion

559 Retrograde menstruation occurs in all women of reproductive age. For reasons that remain
560 unknown, retrograde menstrual tissues develop into endometriotic lesions in 5–10% of cases.
561 Here, we have discussed how dysregulation of the immune system, apoptosis, and oxidative
562 stress are closely associated with endometriosis progression. The dysregulated status of these
563 signaling pathways may predispose women to developing endometriosis, although it remains
564 to be determined what causes such dysregulation in the endometrial tissues to develop into
565 endometriotic lesions. Epigenetic changes caused by nutrition and environmental variables or
566 genetic changes might be potential factors that can initiate endometriosis (Borghese, et al.
567 2017). Moreover, further studies on functional correlation between the dysregulated signals
568 and the severity of endometriosis are clearly needed but, taken together, the dysregulated
569 signals herein we have reviewed may also be connected to disease severity. Future studies
570 must determine how these potential endometriosis initiation factors dysregulate the immune
571 system, apoptosis, and oxidative stress pathways, leading to the initiation and progression of
572 endometriosis.

573

574 **Declaration of interest**

575 The authors declare no potential conflicts of interest.

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994

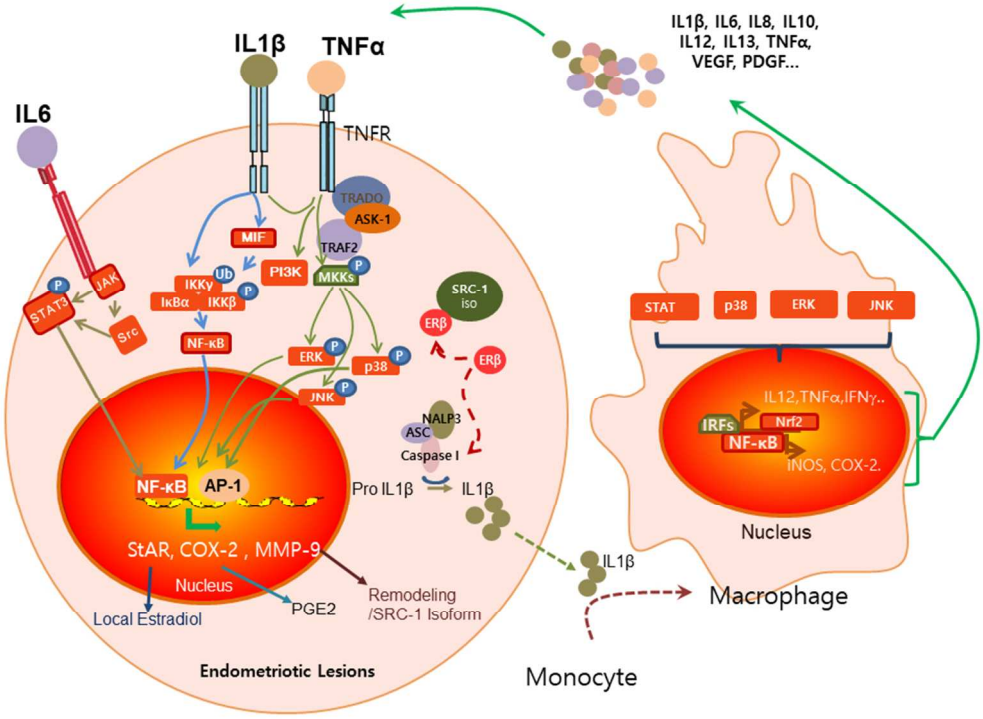
Figure Legends

Figure 1. **Cytokine signaling networks involving endometriotic lesions and peritoneal macrophages**. Activated peritoneal macrophages express inducible nitric oxide synthase (iNOS) and COX2 through interferon regulatory factors (IRFs), NF-κB and nuclear factor (Nrf)2 through activation of STAT, p38, ERK and JNK signaling cascades. Activated macrophages then release cytokines (including IL1, IL6, IL8, IL10, IL12, IL13, and TNFα), growth factors, and angiogenic factors (VEGF and platelet-derived growth factor [PDGF]). The secreted TNFα, IL1β and IL6 bind their membrane receptors in endometriotic lesions. The cytokine/cytokine receptor complex then activates PI3K, MKK, JNK, p38 and IKK pathways to induce the expression of inflammation and invasion mediators, such as StAR, COX2 and MMP9, through NF-κB and AP1 transcription factors to stimulate local estradiol formation, PEG2 formation and tissue remodeling and NCOA-1 isoform generation, which enhances the growth of endometriotic lesions. The estradiol/ESR2/NCOA-1 complex interacts with the cytoplasmic inflammasome to increase IL1β levels to induce monocyte differentiation into macrophages (Schenk, et al. 2014). Therefore, cytokine crosstalk between endometriotic cells and macrophages is the main driver for the initiation, maintenance, and progression of endometriosis.

Figure 2. **Dysregulation of apoptotic signaling in endometriosis.** The decreased apoptosis of endometriotic cells and increased apoptosis of immune cells leads to immune privilege. TNFα, elevated by retrograde menstruation, binds to *tumor necrosis factor receptor (TNFR) to induce caspase 8- and caspase 9-mediated apoptosis in* retrograde menstrual tissues*. In endometriosis patients, however, elevated NCOA-1 isoform/ESR2 complex binds to ASK1 (apoptosis complex I), caspase 8 (apoptosis complex II) and caspase 9 (apoptosome) and suppresses extrinsic apoptosis signaling in* retrograde menstrual tissues. *The elevation of*

PGE2 in endometriosis patients increases the ratio of BCL2/BAX in mitochondria to inhibit intrinsic apoptosis signaling. The endometriotic lesions also exhibit elevated levels of FasL, which binds to Fas in cytotoxic T cells, causing cell death in cytotoxic T cells. This represents a critical defense mechanism of endometriotic lesions against destruction by cytotoxic T cells during retrograde menstruation.

Figure 3. **Alterations of oxidative stress pathways in endometriosis.** An overload of erythrocytes, apoptotic endometrial tissue, and cell debris in the peritoneal cavity stimulate the generation of ROS in mitochondria. The hyperactivated ROS stimulate ERK and PI3K/AKT/mTOR signaling pathways in endometriotic lesions to enhance adhesion, angiogenesis, and proliferation. Overproduction of ROS also impairs cellular function by altering gene expression profiles through the NF-κB signaling cascade to increase inflammatory cytokine production in endometriotic lesions, which enhances endometriosis progression.



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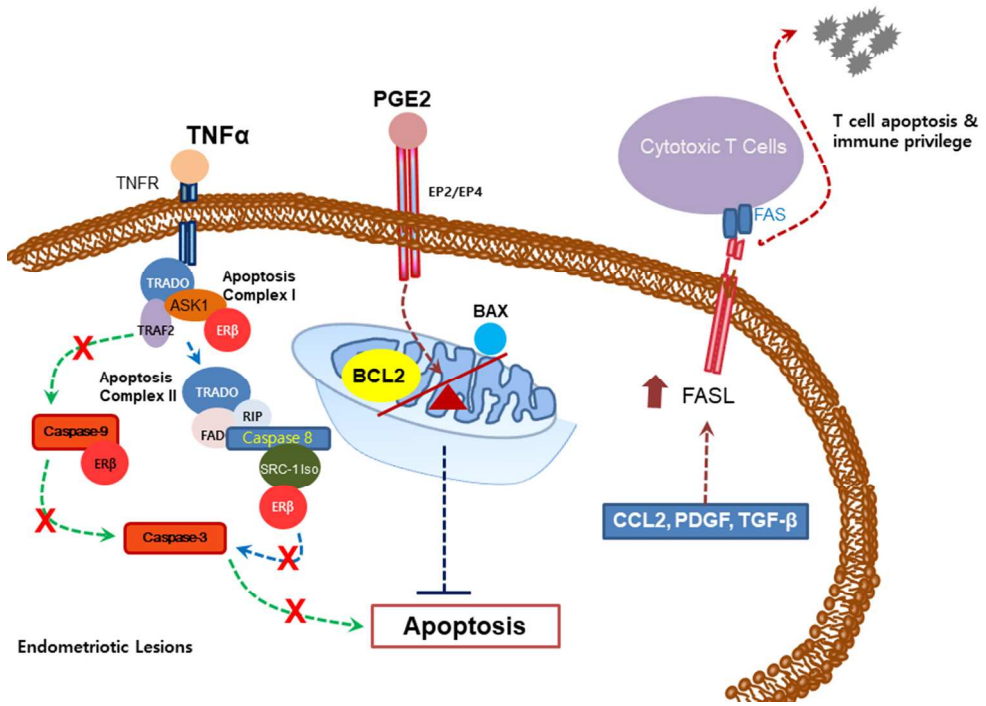

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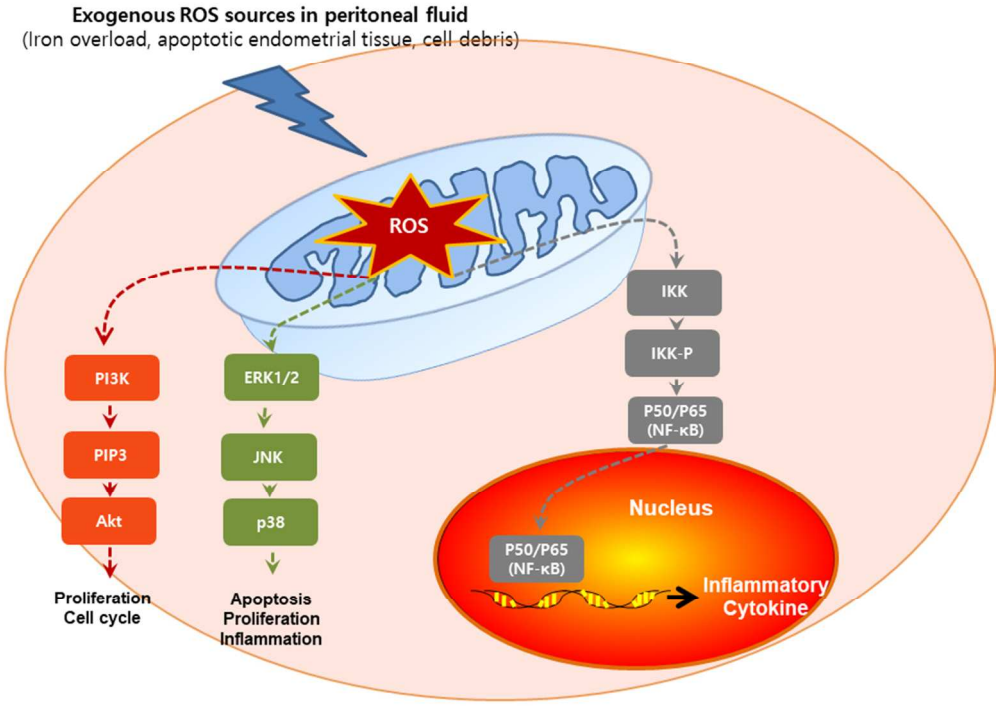

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	Target site	Drug name	Results in Human study	Main Effect
Hormonal agents				
Aromatase inhibitor	Block androstenedione to estrone	Letrozole	Retrospecitve analysis (Abushahin, et al. 2011)	Reduce pain with GnRh agonist
GnRH antagonist	Direct pituitary gonadotropin suppression	Elagolix	RCT(Taylor, et al. 2017)	Reduce pain
SERMs (Selective estrogen receptor modulators)	Nonsteroid selectiveagonist or antagonist effects in different estrogentarget tissues.	Raloxifene	RCT(Stratton, et al. 2008)	Early termination
		Bazedoxifene	None	
		ERB-041	None	
SPRMs (Selective progesterone receptor modulators)	Progesterone receptor antagonist/agonist	Asoprisnil	RCT (Chwalisz, et al. 2005)	Reduce pain and dysmenorrhea
Non-hormonal agents				
Antiangiogenic agents	Anti-VEGF antibody	Avastatin	None	
	3-Hydroxy-3-Methyl Glutaryl Coenzyme A inhibitor	Simvastatin	RCT (Almassinokiani*, et al.* 2013)	Reduce pain
	Dopamine receptor 2 agonist	Quinagolide	Observational study(Gomez, et al. 2011)	Reduce lesion size
	COX-2 inhibitors	Celecoxib	Case control study (Cobellis, et al. 2004)(Cobellis*, et al.* 2004)	Reduce pain

	Epigallocatechin-3-gallate		None	
Antioxidant agents	Melatonin	Melatonin	RCT(Schwertner, et al. 2013)	Reduce pain and dysmenorrhea
	Pentoxifylline	Pentoxifylline	RCT (Alborzi, et al. 2007)	No effect on pain or recurrence
TNF-α blockers	Anti-TNF-α antibody	Infliximab	RCT(Koninckx, et al. 2008)	No effect
Immunomodulators	mTOR inhibitor	Rapamycin	None	
	Endogenous eicosanoid, inhibit MMP-9	LXA4	None	
Apoptotic agent	Natural polyphenolic compound, induce p53 mediated apoptosis	Curcumin	None	
Metformin	Insulin sensitizer from the familyof the biguanides.	Metformin	None	
Matrix metalloproteinase inhibitor		Doxycycline	None	
		ONO-4817	None	

Table 1.Emerging medical therapies in endometriosis with human data: alternative hormonal agents as well as agents targeting endometriosis-specific inflammation, anti-apoptosis and oxidative stress.