



## Immunology of endometriosis

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Endometriosis is a benign gynecologic disorder characterized by the ectopic growth of misplaced endometrial cells. A unifying hypothesis to explain endometriosis has not been elucidated as yet but numerous investigations have implicated disturbances in the immune response as fundamental to its etiology and pathogenesis. Clearly, the immune system is involved in endometriosis. It is not clear, however, whether and to what extent this involvement is a primary response leading to the initiation, promotion, and progression of the disease or a secondary response to the ectopic endometrial growth in an attempt to restore homeostasis. Thus, although numerous studies have shown alterations in cell-mediated and humoral immunity in subjects with endometriosis, the importance of these changes remains obscure. This review considers the past two decades of investigation of immune function changes in women with endometriosis with the expectation that this information will ultimately provide the basis for developing new approaches to patient management.

**Key words:** apoptosis; chemokines; cytokines; endometriosis; endometrium; immunity.

The histogenesis of endometriosis is not entirely clear and several theories have been proposed. Most of the available evidence supports the concept of retrograde tubal dissemination of the endometrial cells (ECs) and tissue fragments, and their subsequent ectopic implantation. However, retrograde transport seems to operate in all women and there is evidence that misplaced ECs in healthy women do not implant but are eliminated from the ectopic locations (see references 1–8 for a review).

It has been suggested that the immune 'disposal system' removes ectopic EC and prevents their implantation and development into endometriotic lesions in healthy women.<sup>1</sup> This process might be facilitated by EC apoptotic changes, which normally

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increase at the end of the menstrual cycle but are significantly decreased in endometriosis.<sup>9</sup> Thus, in healthy women, ECs disseminated into the ectopic locations might be programmed to die and are easily eliminated by the immune system. A deficiency in cell-mediated immunity and/or a decrease in EC apoptosis might thus lead to the survival and implantation of misplaced cells.

Reports have also indicated that in endometriosis, ectopic ECs differ from eutopic ECs, which in turn differ from ECs in healthy women. In endometriosis, for example, eutopic and ectopic ECs proliferate in response to stimuli that cause apoptosis in normal endometrium. Moreover, ectopic ECs seem to have a survival advantage through their ability to synthesize estrogen and other growth factors, which exert paracrine and autocrine effects.<sup>10,11</sup>

This aberrant function of the ectopic endometrium at the cellular and molecular level might be secondary to genomic alterations. Recent studies detected abnormal chromosomes, loss of heterozygosity, clonal changes, and allelic imbalances in the ectopic EC and in endometriosis-derived cell lines.<sup>12-14</sup> Microarray analysis demonstrated changes in expression of cell cycle and metabolic detoxification enzyme genes in the ectopic endometrium. It has been suggested that women with latent genetic endometrial instability might be predisposed to environmental toxins, which could confer increased survival characteristics to the ectopic EC.<sup>15-17</sup>

Ectopic endometrial growth, infiltration by immune cells, increased production of pro-inflammatory cytokines, growth factors, and angiogenesis factors are all features of the profound inflammatory response that surrounds endometriotic implants. This in turn leads to the mobilization of fibroblasts and proliferation of connective tissue as a homeostatic mechanism to sequester and heal the site of injury. The intensity of this reaction varies, depending on the degree and character of the immune activation. In some women, florid polypoid growth of ectopic endometrium is surrounded by little or no fibrosis; in others there is predominant fibrotic reaction and only occasional endometriotic implants are detected. There is no question that the immune system is mobilized and that there is a profound immune response. It is unclear, however, whether this is a primary or secondary response.

## THE IMMUNE SYSTEM AND ITS FUNCTION IN ENDOMETRIOSIS

It has been hypothesized that a deficiency in cell-mediated immunity prevents clearance of the retrograde menstrual debris from the peritoneal environment and permits the implantation of misplaced EC that results in the development of endometriosis.<sup>18</sup> Alternatively, it has been suggested that endometriosis is a type of autoimmune disease.<sup>19</sup> The results and interpretation of these studies have been criticized because of design, methodological, and conceptual flaws, and because ectopic endometrium as a self-tissue is not expected to elicit immune responses.<sup>20</sup>

### Peripheral blood and peritoneal fluid immune cells, their subsets, and function in endometriosis

#### *Monocytes/macrophages*

Peripheral blood monocytes (PBMs) and macrophages (PM) in the peritoneal fluid (PF) are key cellular constituents of the immune system. In women with endometriosis, circulating PBMs demonstrate increased activation status as indicated by increased

chemiluminescence.<sup>21</sup> Under basal and stimulated conditions, they produce higher levels of tumor necrosis factor (TNF $\alpha$ ), interleukin (IL-6), and IL-8—but not IL-10—than the PBMs of healthy controls.<sup>22</sup> Resident PMs sequestered in the peritoneal cavity (PC) remove red blood cells, damaged tissue fragments, apoptotic cells, and probably ECs that gain access to the PC through the fallopian tubes. In endometriotic PF (EPF), the concentration and numbers of PM are significantly increased. These are large, activated PMs that, under basal and stimulated conditions, produce higher levels of TNF $\alpha$ , IL-6, IL-8, and IL-10 than the PMs of healthy women.<sup>23</sup> They also produce higher levels of smooth-muscle-contracting prostaglandins (PGs) such as PGE2 and PGF2 $\alpha$ .<sup>24</sup>

Endometriotic PMs are also a principal source of other cytokines, growth factors, adhesion molecules, complement components, hydrolytic enzymes, reactive oxygen radicals, and other substances—increased levels of which have been demonstrated in EPF. Autologous PBM/PMs from women with endometriosis, and/or their secretory products, stimulate eutopic and ectopic EC proliferation *in vitro* and decrease EC apoptosis.<sup>25</sup> The opposite effects—increased apoptosis and decreased EC proliferation—are observed when autologous endometrium is co-cultured with PBM/PM from healthy controls. This interesting phenomenon has been attributed to the differential effect of TNF $\alpha$  on EC function in women with and without endometriosis.

The phagocytic activity of PMs is thought to be crucial in healthy women for the elimination of menstrual detritus. This activity is mediated through surface scavenger receptors, which are regulated by a variety of cytokines and growth factors. It has also been demonstrated that scavenger receptors play a role in cellular adhesion and that non-adherent PMs do not express type A scavenger receptors. Thus an increase in non-adherent PMs without scavenger receptors might contribute to the pathogenesis of endometriosis.<sup>26</sup> The cytotoxicity of PM to cultured cell lines was decreased in severe as compared to limited endometriosis. This decrease appears to be prostaglandin-dependent because it was reversed *in vitro* by addition of prostaglandin synthesis inhibitors such as indomethacin.<sup>27</sup>

#### *Natural killer (NK) cells*

NK cells are large granular lymphocytes that participate in the destruction of abnormal cells arising from viral infections, malignant changes, or senescence. One of the mechanisms through which NK cells kill their targets is through antibody-dependent cellular cytotoxicity. For this purpose, NK cells have receptors that bind immunoglobulin G (IgG) and then kill IgG-coated target cells. Another mechanism involves the recognition of target cells through characteristic killer-activating receptors (KAR) and killer-inhibitory receptors (KIR). If KAR are occupied, NK cells demonstrate cytotoxic activity; when KIR are occupied, cytotoxic activity is suppressed. The cytotoxic activity of NK cells can be increased by lymphokines such as IL-2, a phenomenon referred to as lymphokine-activated killer (LAK) function.

Several investigators have demonstrated a decrease in NK cell cytotoxic activity against autologous and heterologous EC in women with endometriosis.<sup>4</sup> This decrease was observed with both peripheral and peritoneal NK cells. The decrease in NK cell cytotoxicity against autologous EC might also reflect increased resistance of these cells to NK-mediated cytolysis, as suggested by Oosterlynck et al.<sup>28</sup> This indicates that, in endometriosis, alteration in the immune response coexists with endometrial abnormalities. If NK cell cytotoxicity is one of the components of the immune 'disposal system' of the menstrual detritus, NK cell deficiency might facilitate development of endometriosis. Alterations in NK cell cytotoxicity in endometriosis

appear to be secondary to functional rather than quantitative changes. The percentage of peripheral NK cells is not altered and contradictory reports indicate a decrease, no change, or an increase in peritoneal NK cells.

Several studies have demonstrated increased expression of the family of KIRs on NK cells from women with endometriosis. These molecules interact with major histocompatibility complex (MHC) class I molecules on potential target cells to block NK killing of the cell. Cells with absent or aberrant class I expression, therefore, activate the lytic program of NK cells.<sup>29,30</sup> Studies by Maeda et al<sup>31</sup> demonstrated—in endometriosis—increased proportions of NK cells that expressed the KIR2DL1 phenotype compared to normal controls; this was found for both circulating and peritoneal NK cells. Similarly, Wu et al demonstrated decreased NK cytotoxicity against the reference NK target, K562, in endometriotic peritoneal leukocytes in association with increased expression of the NKBI and EB6 forms of KIRs.<sup>32</sup> The effect was most pronounced in women with extensive disease.

Evidence of NK inhibitory factors in endometriotic EC or PF has also been documented. Somigliana et al demonstrated that conditioned media from stromal EC could inhibit NK-cell-mediated cytotoxicity against 51Cr-labeled EC to a greater extent in endometriosis than in controls.<sup>33</sup> The same group has presented evidence that suggests that the p40 subunit of the NK-activating cytokine, IL-12, which is expressed in increased amounts in the EPF, can block NK-mediated EC lysis.<sup>34</sup> This group subsequently reported that soluble intracellular adhesion molecule 1 (ICAM-1) from stromal EC cultures could also block the lysis of K562 cells<sup>35</sup>, an effect that is consistent with reports by Fukaya et al, who showed that soluble ICAM-1 is increased in the EPF and interferes with NK-mediated killing.<sup>36</sup>

#### *T lymphocytes*

T lymphocytes originate from pluripotent stem cells in the fetal liver and in bone marrow. From these sites they travel to the thymus, where they complete their development into two major subpopulations characterized by the expression of the glycoproteins CD4 and CD8, which function as co-receptors for MHC class II and class I molecules, respectively. The CD4 T cells can be further subdivided into CD4/TH1 and CD4/TH2 cells. TH1 cells enhance the differentiation of the CD8 cells into killer cells and activate PBM/PM to facilitate cell-mediated immunity. TH2 cells enhance B cell differentiation into antibody-secreting cells. TH1 and TH2 cells can be distinguished by their cytokine synthetic characteristics. Fully activated CD8 T lymphocytes can eradicate intracellular pathogens by killing virally infected cells and by activating PBM/PM.

Several investigators have quantitated T lymphocytes and their subsets in PB and PF from women with endometriosis. No changes have been found in total lymphocyte numbers and CD4/CD8 ratio in PB, but in PF, an increase in ratio and concentration of each subset has been reported. In eutopic endometrium, total lymphocyte counts and CD4/CD8 ratios were similar in endometriosis and controls. In the ectopic endometrium, although the number of T lymphocytes increased compared to proliferative and secretory eutopic endometrium, the CD4/CD8 ratio remained unchanged.

Functional changes in peripheral lymphocytes in women with endometriosis and adenomyosis were first suggested by Startseva in 1980.<sup>37</sup> A year later, our group reported decreased *in vivo* reactivity to intradermal injection of autologous endometrial antigens (measured as the intensity of perivascular lymphocytic infiltration)

in Rhesus monkeys with spontaneous endometriosis as compared to healthy controls.<sup>18</sup> Similarly, lymphocyte proliferation in response to the same autologous endometrial antigens was reduced in monkeys with endometriosis. In women with endometriosis, cytotoxicity assays using peripheral blood lymphocytes and autologous <sup>51</sup>Cr-labeled endometrial target cells demonstrated decreased target cell lysis when comparisons were made between patients and controls and between patients with moderate and severe endometriosis.<sup>38</sup> Nonspecific immune function was comparable for patients and controls, suggesting that both monkeys and women with endometriosis were otherwise immunologically competent.

### **Humoral immunity in endometriosis**

#### *B lymphocytes*

B cells are precursors of plasma cells, the antibody-producing cells of the immune system. It has been suggested that CD5 + B cells, which represent 10–20% of the B-cell population, are responsible for the production of autoantibodies. CD5 + B cells are elevated in patients with autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis. No changes in peripheral B cell populations have been identified in women with endometriosis. In the uterine endometrium, B lymphocytes are present almost exclusively in lymphocyte aggregations, and this pattern is seen in women with and without endometriosis throughout the cycle.

#### *Autoantibodies*

An increase in B-cell reactivity in women with adenomyosis and endometriosis was first suggested in 1980.<sup>37</sup> The same year, another group reported C3 and IgG deposits in the uterine endometrium of women with endometriosis, and a reduction in total serum complement levels suggestive of the intra-endometrial antigen–antibody reaction.<sup>39</sup> Subsequent studies by Mathur et al<sup>40</sup> identified IgG and IgA autoantibodies against endometrial and ovarian tissues in the sera and in cervical and vaginal secretions of women with endometriosis. However, another report by the same group indicated that both fertile controls and women with endometriosis had low levels of circulating and PF antibodies to endometrial antigens of varying molecular weights.<sup>41</sup> They suggested that this might be a mechanism for clearing the reproductive tract of the menstrual debris. Autoantibodies to endometrial antigens with molecular weights of 26 and 34 kDa were identified only in endometriosis. A high frequency of anti-endometrial antibodies in the sera, PF, and endometrial tissues of women with endometriosis has been confirmed by other investigators using different techniques (see references 6,41–44 for reviews). Antibodies to endometrial transferrin, alpha 2 Heremans–Schmidt glycoprotein, and 2 carbonic anhydrase have also been reported in women with endometriosis.<sup>41</sup> Recent reports indicate that a common carbohydrate epitope—the Thomsen–Friedenreich glyco (T) antigen—found on many of these molecules might be involved in the autoantibody reaction and could indicate an underlying genetic defect.<sup>45</sup>

Other investigators demonstrated circulating autoantibodies against subcellular elements (i.e. antinuclear antibodies) or against chemical substances integral to the cell structure (i.e. anti-DNA or antiphospholipid antibodies). A high frequency of these autoantibodies has also been reported in women with autoimmune diseases and various forms of reproductive failure, such as unexplained infertility and recurrent abortions. Gleicher and associates reported that among 31 women with endometriosis, 65% had IgG and 45% had IgM autoantibodies to at least 1 of 16 antigens investigated.<sup>19</sup>

Those detected most frequently were autoantibodies to phospholipids (particularly phosphatidylserine), histones, and nucleotides. We have also reported that circulating autoantibodies to phospholipids, histones, and/or polynucleotides are associated with lower pregnancy rates from in vitro fertilization procedures and that the mechanism of this effect most likely involves interference with embryo implantation.<sup>46</sup> In addition to autoantibodies against specific antigens, women with endometriosis seem to have a higher frequency of anti-tissue autoantibodies (anti-endometrial and anti-endothelial autoantibodies) and anti-organ autoantibodies (anti-ovarian and anti-thyroid autoantibodies). The presence of multiple autoantibodies indicates polyclonal B cell activation and, together with other characteristics, suggests that endometriosis might be a form of autoimmune disease.<sup>19,41,42,47</sup>

If endometriosis is an autoimmune disease, it remains to be determined whether autoantibodies represent a loss of self-tolerance or an acquired sensitization to antigenic determinants not normally expressed. Loss of a cell's tolerance could be secondary to the loss of suppressor T cells or exposure to hyperstimulatory B cell activators. New antigenic determinants might represent 'altered-self' or foreign antigens that are similar to endometrial antigens. In either case, exposure to immunotoxins might facilitate these events. Furthermore, it has been demonstrated that a decrease in apoptosis leads to the development of autoimmunity. Apoptosis is decreased in the eutopic endometrium in women with endometriosis and further decreased in the ectopic endometrium.

### **Cytokines, growth factors, and other soluble proteins with autocrine and paracrine effects**

An increase in the amount of PF is a characteristic finding in endometriosis, as is the increased presence of various free-floating cells such as macrophages, lymphocytes, eosinophils, NK cells, mast cells, mesothelial cells, and EC, as well as a wide range of soluble substances including autoantibodies, cytokines, growth factors, adhesion molecules, enzymes, hormones, prostaglandins, and reactive oxygen species.<sup>41,48-55</sup> Early reports from the 1980s demonstrate toxic effects of EPF on different steps in reproductive function, such as ovum pickup by the fimbria, sperm mobility and survival, sperm-oocyte interaction, embryo development, and implantation. Some of these effects were reversed during hormonal treatment of endometriosis and were attributed to the presence of the secretory products of the endometriotic and/or immune cells. Subsequent reports identified some of these substances.

The increased leukocyte numbers found in EPF are attributable to the synthesis of various chemokines, which increase leukocyte trafficking and activation. These molecules can be synthesized by resident or inflammatory peritoneal leukocytes and endothelial cells as well as by misplaced EC.<sup>51,52</sup> Recent studies have also demonstrated stimulation of chemokine synthesis by peritoneal mesothelium co-cultured with epithelial EC.<sup>56</sup> Finding multiple sources for chemokines in the uterus, pelvis, and PC is not surprising, given their role in normal physiology and reproduction.<sup>51,52,57</sup> In endometriosis, however, increased levels of chemokines in PB and PF have been reported.

Both  $\alpha$ -chemokines (typified by IL-8) and  $\beta$ -chemokines (typified by monocyte chemoattractant protein; MCP-1) are increased in endometriosis. With respect to neutrophils, the  $\alpha$ -chemokines, IL-8, and growth-regulated oncogene-alpha (GRO- $\alpha$ ) are increased in EPF and EPB,<sup>22,23,51-53,58,59</sup> apparently as a consequence of increased

synthesis by PM and PBM.<sup>22,23</sup> The level of IL-8 in the EPF correlates with increasing severity of the disease,<sup>52,53,59</sup> whereas, in the circulation, IL-8 levels were reported to be highest in women with limited disease.<sup>59</sup> By contrast, no correlation between disease severity and GRO- $\alpha$  level was found in EPF by Szamatowicz et al.<sup>58</sup>

Levels of the  $\beta$ -chemokine MCP-1 are increased in EPF and EPB.<sup>51,59</sup> Increased biosynthesis by both EC<sup>60</sup> and mesothelial cells<sup>56</sup> has been documented. The lymphocyte chemokine RANTES (regulated on activation normal T-expressed and secreted), which is a member of the  $\beta$ -chemokine family and which is also chemotactic for monocytes, is increased in EPFs and correlates with disease severity.<sup>61</sup> Evidence for RANTES gene expression in the stroma of endometriotic implants in proximity to macrophages has been reported,<sup>62</sup> with suggestions that macrophage-derived TNF $\alpha$  was responsible for upregulation of RANTES mRNA synthesis. Enhancement of RANTES mRNA and protein expression by endometriotic cells has also been observed following treatment with IL-1 $\beta$ , with further stimulation evident in cells pretreated with estradiol.<sup>63</sup>

Chemokine-mediated mobilization, recruitment, and activation of leukocytes within PC in endometriosis is amplified by a variety of other cytokines.<sup>53</sup> These molecules are secreted primarily by the activated leukocytes within PC and circulation, but are also synthesized by endometriotic cells, resident cells, and mesothelium.<sup>52,53,56</sup> It is important to appreciate that the immunologic economy does not allow the synthesis of an individual cytokine without eliciting a cascade of cytokine biosynthesis. Recent conventions have delineated cytokines into those that mediate cellular immunity (termed type 1 or TH1 cytokines) from those that facilitate humoral immunity (termed type 2 or TH2 cytokines). Nevertheless, most cytokines have more than one target tissue and, thus, more than one biologic effect. Therefore, although there are numerous examples of activated cytokine biosynthesis in endometriosis, the role that any individual cytokine might play in the etiology or pathogenesis of this condition has been much more difficult to elucidate.

A recent report by Nasu et al.<sup>64</sup> illustrates this point. In this study, the effect of the type 2 cytokine IL-13 and the type 1 cytokine TNF $\beta$  on the capacity of highly purified stromal EC to synthesize a variety of cytokines and chemokines was investigated. The results demonstrated that both IL-13 and TNF $\beta$  increased IL-6, IL-8, MCP-1, and eotaxin synthesis by stromal cell cultures in a dose-dependent manner. By contrast, IL-13 suppressed the synthesis of IL-11 and leukemia inhibitory factor (LIF), whereas TNF $\beta$  enhanced this. TNF $\beta$  also increased the synthesis of the chemokines GRO- $\alpha$  and RANTES by stromal EC cultures whereas IL-13 had no effect on the synthesis of these molecules. Thus, it is easy to appreciate that the multiplicity of effects that can be exerted by a single cytokine against a single target tissue can be greatly amplified in a physiologic condition in which multiple cell types and cytokines participate in the event. The complexity of this situation should caution against oversimplification of the role played by cytokines in endometriosis.

The majority of studies to date have shown increased synthesis of cytokines by immune cells and EC in endometriosis when compared to normal controls.<sup>53</sup> This has been found for both type 1 and type 2 cytokines. Depending on the cytokine in question, there are numerous examples of correlation between cytokine level and disease severity, although some interesting exceptions and some surprising relationships have been documented. But the consequences of this increased biosynthetic activity are rarely elucidated by these studies.

Perhaps a more meaningful issue to consider is how the increase in cytokine biosynthesis influences specific aspects of etiology and/or pathogenesis in endometriosis. Studies of this kind have demonstrated clearly that endometriotic ECs

appear to have the capacity to utilize cytokines to facilitate critical disease processes such as attachment, invasion, angiogenesis, and growth. Historically, the most frequently studied cytokines in endometriosis are IL-1 $\beta$ , TNF $\alpha$ , and IL-6—potent inflammatory mediators that also contribute to the establishment of the disease.

For example, TNF $\alpha$  and IL $\beta$  were both found to increase the adhesion of endometriotic EC to laminin and fibronectin, whereas TNF $\alpha$  also increased adherence to collagen. By contrast, these cytokines increased adherence of EC from normal women to fibronectin only.<sup>65</sup> Similarly, the release of the extracellular matrix degrading enzyme matrix metalloproteinase 3 (MMP3) from cultured uterine EC from endometriosis patients was up-regulated by IL-1 $\beta$  and TNF $\alpha$  in studies by Sillem et al<sup>66</sup> Differential effects of IL-1 $\beta$  were shown when EC from endometriosis patients and controls was compared, whereas TNF $\alpha$  stimulated MMP3 release in both groups of cells. However, these actions of TNF $\alpha$  and IL-1 $\beta$  might be opposed by TGF $\beta$ —another cytokine that is increased in the EPF. This was suggested in studies by Bruner-Tran et al who assessed MMP3, MMP7 mRNA, and protein expression in endometriotic eutopic and ectopic endometrium<sup>67</sup> and found that progesterone exposure failed to inhibit MMP expression normally unless the cells were also treated with retinoic acid and TGF $\beta$ . Such treatments also restored progesterone control of disease establishment in an *in vivo* experimental model.

Neovascularization of endometriotic lesions can coincide with MMP expression and this function also appears subject to regulation by cytokines. This is not surprising given the capacity of activated endometriotic PM to elaborate both MMPs and vascular endothelial growth factor (VEGF) directly.<sup>68,69</sup> In studies by Lebovic et al<sup>70</sup> IL-1 $\beta$  treatment increased expression of VEGF and IL-6 mRNA expression in stromal EC from endometriotic lesions but not from control endometrium. IL-1 $\beta$  has increased expression of IL-8 (a cytokine with known angiogenic promoting activity) in estradiol-treated EC.<sup>71</sup> This is in keeping with clinical studies that have demonstrated increased angiogenic activity associated with IL-8 levels in EPF<sup>72</sup>, although other factors in the fluid also appeared to modulate angiogenesis.

A study by Maas et al suggests that one such factor might be TNF $\alpha$ .<sup>73</sup> In this study, angiogenic activity measured in the chick embryo chorioallantoic membrane assay was detected in 85% of EPFs. The angiogenic activity was positively correlated with TNF $\alpha$  levels, but not with levels of IL-1 $\beta$  or IL-8 in the fluids. Another factor that might contribute to angiogenesis of endometriotic lesions is IL-15, a newly described cytokine with immunomodulatory and angiogenic effects that was found to be increased in the EPF by Arici et al<sup>74</sup> Interestingly, the levels of IL-15 were inversely correlated with the extent of the disease and depth of invasion, which led the authors to suggest that the role of IL-15 might be most important in facilitating establishment of early endometriosis. This contrasts with the positive association found between angiogenic activity and MMP production by endometriotic implants, and depth of invasion of the disease found in the study by Ria et al.<sup>69</sup>

Yet another candidate cytokine present in increased amounts in the EPF is the macrophage migration inhibition factor (MIF). Kats et al<sup>75</sup> measured MIF concentrations and angiogenic activity in EPF and control PF. The results demonstrated a significant increase in MIF levels in endometriosis (that was most pronounced in women who also had infertility) and the ability of an anti-MIF antibody to block the angiogenic activity of EPF. This is one of the few studies that have attempted to demonstrate a causative relationship between a specific cytokine and the function of interest. The majority of studies have relied more on correlative data to infer the importance of a particular cytokine in the function.

IL-6 is a cytokine involved in the regulation of cell growth and differentiation of immunocompetent cells and in acute phase inflammatory responses. It also regulates ovarian steroid production, folliculogenesis, and embryo implantation. A principal function of IL-6 is to promote the differentiation of B cells into antibody-producing cells. However, in certain autoimmune diseases, IL-6 has been shown to be responsible for polyclonal B cell stimulation. IL-6 also has been implicated in the production of immune complexes in different inflammatory sites. IL-6 is one of the key mediators in the cytokine cascade in endometriosis and has been shown to be elevated systemically and locally and to correlate with disease activity.<sup>50,53</sup> Serum IL-6 concentrations were significantly higher in women with endometriomata and were suppressed by gonadotropin-releasing hormone (GnRH) agonist treatment.<sup>76</sup> There seems to be no consistent effect of endometriosis, pelvic adhesions, or chronic pelvic pain on PF concentrations of IL-6. High concentrations, however, have been reported in women with active, red endometriotic implants. Increased IL-6 secretion has been demonstrated by endometriotic PM and by ectopic as well as eutopic stromal EC. IL-6 production by the EC *in vitro* is significantly enhanced by IL-1 $\beta$ , TNF $\alpha$ , and IFN $\alpha$ . Progesterone and danazol displayed inhibitory effects on both spontaneous and cytokine-induced IL-6 secretion.

IL-6 inhibits proliferation of normal stromal EC and might be a growth-inhibiting factor in the human endometrium. However, ectopic EC were resistant to growth inhibitory effects of IL-6. Basal synthesis of IL-6 by endometriotic PBM was significantly greater than by PBM of fertile controls. Laparoscopic surgery and treatment with GnRH agonist or danazol significantly reduced serum IL-6 levels. IL-6 might contribute to the development of autoantibodies in endometriosis. Normalization of increased IL-6 levels by danazol could be the mechanism of autoantibody suppression by this drug. IL-6 is embryotoxic and inhibits blastocyst implantation, which might be the mechanism through which it contributes to endometriosis-associated infertility.

EPF concentrations of IL-8 are also increased, especially in active and advanced disease. This is relevant because, as an angiogenic agent, IL-8 might contribute to the induction of a new blood supply to the ectopic endometrium. IL-8 might also facilitate the initial attachment of the EC to the peritoneal surfaces because it stimulates adhesion of endometrial stromal cells to fibronectin and metalloproteinase activity.<sup>52</sup>

Endometriotic PMs produce several times higher levels of IL-8 under basal and stimulated conditions than PMs from fertile controls.<sup>23</sup> Similarly, elevated IL-8 production by PBM from endometriosis patients has been reported.<sup>22</sup> Ectopic ECs express high concentrations of IL-8 *in vivo* regardless of the menstrual phase and IL-1 induces IL-8 secretion by the endometriotic cells, which is further enhanced by estradiol.<sup>71</sup> IL-8 is expressed in the healthy uterine endometrium, with concentrations highest in the late-secretory and early-proliferative phases. IL-8 is also expressed in the cultured mesothelial cells and its expression is up-regulated by IL-1 and TNF $\alpha$ , indicating that, when stimulated by pro-inflammatory cytokines, mesothelium can also be a source of IL-8.<sup>77</sup>

Cytokines that are present in increased amounts in the EPF also can enhance proliferation of eutopic and ectopic EC. This was shown for IL-8 and TNF $\alpha$  in studies by Iwabe et al. and Harada et al.<sup>53,78</sup> The results suggest that TNF $\alpha$  and IL-8 collaborate in the proliferation of stromal cells from ectopic EC and endometriomata. A study by Braun et al also implicated TNF $\alpha$  as the factor in PF from women with endometriosis that promotes proliferation of eutopic and ectopic EC.<sup>25</sup> In this study, PF or recombinant TNF $\alpha$  stimulated proliferation of endometriotic EC but failed to do so, or even inhibited, the proliferation of EC from normal controls. EPF, which enhanced proliferation of autologous EC, also enhanced proliferation of heterologous

endometriotic EC but failed to stimulate the proliferation of heterologous EC from controls. This illustrates the differential growth capacities of EC from women with and without the disease. TNF receptor protein inhibited the proliferation-enhancing activity of EPF.

### IMMUNOLOGICAL RESISTANCE OF EC IN ENDOMETRIOSIS

Increased chemokine and cytokine levels within PC in endometriosis might be expected to eliminate any ectopic EC or tissue fragments. The survival and implantation of these fragments leading to development of endometriosis, its persistence, and subsequent clinical sequelae, testifies to a remarkable capacity of these cells to resist and exploit an otherwise overwhelming homeostatic response. It should be appreciated that this resistance can reflect either or both inherent resistance of the endometriotic EC and/or impaired capacity of the immune cells to mediate EC killing. Both mechanisms appear to be operative in endometriosis.

Perhaps the clearest evidence that ectopic ECs in endometriosis are resistant to the cytolytic actions of lymphocytes and macrophages comes from histological studies demonstrating the presence of leukocytes in viable endometriotic lesions. The majority of these studies have shown increased levels of T cells and macrophages with decreased levels of NK cells in ectopic lesions compared to eutopic endometrium.<sup>79-82</sup> Similarly, increased numbers of T cells and macrophages compared to normal are found in the EPF, although a relative reduction in TH1 T cells has been reported in this compartment.<sup>83</sup> The content of lymphocytes in normal eutopic endometrium, which fluctuates with the menstrual cycle, does not seem to be substantially altered in endometriosis.<sup>84</sup> By contrast, macrophage infiltration appears to be diminished compared to normal during the early-proliferative and late-secretory phases of the menstrual cycle in eutopic endometrium of women with endometriosis<sup>85</sup> and is positively correlated with reduced apoptosis.

Activation of T cells and macrophages in endometriosis does not appear to be diminished, with most studies reporting hyperactivation of these cells. However, the results are not consistent. For example, Chiang and Hill measured the expression of gamma interferon ( $\gamma$ -IFN) and the class II molecule human leukocyte antigen (HLA)-DR on T cells localized to eutopic and ectopic endometrium from women with and without endometriosis.<sup>86</sup> The results showed increased numbers of T cells and increased expression of  $\gamma$ -IFN and HLA-DR in the ectopic compared to the eutopic tissues. This result is consistent with activation of type I, T cell-mediated immunity against the ectopic disease. By contrast, studies by Ho et al<sup>87</sup> demonstrated impaired TH1 cytokine synthesis in peritoneal lymphocytes from women with endometriosis as evidenced by inhibition of  $\gamma$ -IFN and IL-2 synthesis following stimulation with mitogens.

With respect to macrophages, the capacity of endometriotic PM to mediate cytolytic activity against a reference tumor cell line was substantially increased compared to normal PM in studies by Braun et al.<sup>88</sup> Although there was a reduction in the level of cytolytic activity when comparing extensive to mild endometriosis, both groups were significantly greater than normal. This is consistent with numerous studies showing increased cytokine synthesis by endometriotic PM. Once again, however, there are conflicting studies that suggest some level of impaired activation in macrophages from these patients. Thus, Maeda et al used flow cytometry to demonstrate reduced expression of the cellular adhesion molecule ICAM-1 on endometriotic PM compared to controls.<sup>89</sup> Comparable results were obtained by Izumiya et al<sup>90</sup>, who showed

depressed expression of HLA-DR, ICAM-1, and the CD14 molecule on endometriotic PM compared to normal.

The resistance of endometriotic EC to lymphocyte-mediated cytotoxicity has also been investigated. In the study by Semino et al<sup>91</sup>, ectopic ECs were found to modulate expression of their HLA class I molecules to increase expression of the HLA-B7 allele, which inhibits the lytic activity of NK-like T cells. A number of studies have also demonstrated that endometriotic ECs increase their expression of the lytic molecule FAS ligand. Target cells (which express the receptor FAS) will undergo apoptosis when attacked by cells that express FAS ligand. Both T cells and NK cells express FAS ligand; however, they also express FAS. Thus, attack by a T cell or NK cell can be subverted and lead to inhibition of cell-mediated killing by a cell that expresses increased amounts of FAS ligand. Normal epithelial, but not stromal, ECs express FAS ligand.<sup>92</sup> Moreover, the expression of FAS ligand can be increased by factors produced by activated macrophages and present in increased concentrations in the EPF. Examples that have been demonstrated include increased FAS ligand expression following treatment with both TGF $\beta$  and platelet-derived growth factor (PDGF).<sup>92</sup> Similarly, Selam et al<sup>93</sup> demonstrated that IL-8 treatment of EC increased their expression of FAS ligand and rendered the cells less sensitive and in fact more toxic to the Jurkat T cell line. The same group subsequently demonstrated up-regulation of FAS ligand by stromal EC following attachment to the extracellular matrix proteins, fibronectin, laminin, and collagen factor IV.<sup>94</sup> It was also found that the levels of FAS ligand were greatest with stromal cells obtained from the eutopic endometrium of women with endometriosis compared to normal controls. Finally, evidence for increased levels of soluble FAS ligand in the EPBs and EPFs was provided by Garcia-Velasco et al.<sup>95</sup> This effect was most pronounced in women with moderate-to-severe disease with serum and PF levels being comparable in those patients.

#### **EFFECT OF IMMUNOSUPPRESSION ON THE DEVELOPMENT OF ENDOMETRIOSIS**

The ability of the immune system to protect subjects with endometriosis from foreign antigens appears to be within the normal range. No alterations in the general immune defense mechanisms have been observed in Rhesus monkeys or women with endometriosis. However, recent reports suggest that the frequency of allergic and autoimmune disorders and various types of neoplasia is increased in women with endometriosis.<sup>14,96,97</sup> There are no data on the frequency of endometriosis in immunosuppressed women. However, the incidence of endometriosis in Rhesus monkeys was significantly increased after exposure to radiation or treatment with immunotoxicants. Wood and associates reported that, 7 to 10 years after systemic exposure to a single dose of proton irradiation, Rhesus monkeys had advanced endometriosis twice as often as controls.<sup>98</sup> In another study, Campbell et al<sup>99</sup> reported that monkeys treated with polychlorinated biphenyls (PCBs) frequently develop an aggressive form of endometriosis that results in intestinal obstruction and death. Aggressive endometriosis involving the bowel and leading to intestinal obstruction and death was also noted in a colony of Rhesus monkeys exposed several years before to two different dosages of dioxin in a controlled toxicological study.<sup>100</sup> Both the severity and the incidence of endometriosis diagnosed laparoscopically were higher in treated animals, and were dosage related.

PCBs and dioxin are organochlorides, which are common contaminants in the environment; both are also immunotoxicants. However, studies of immune function in irradiated PCB-treated or dioxin-treated animals were limited or not performed, and it is unclear if and to what degree the affected animals were immunosuppressed. One is tempted to speculate that suppression of the immune system by systemic irradiation, PCBs, or dioxin in some way facilitated implantation of the EC or tissue fragments in ectopic locations, leading to the development of endometriosis. Interesting in this respect are reports indicating that the concentrations of PCBs in the peripheral blood are higher in women with endometriosis.<sup>101</sup> In *Cynomolgus* monkeys with surgically induced endometriosis, exposure to dioxin for 1 year increased survival of endometriotic implants.<sup>102</sup> Similarly, treatment with dioxin resulted in a dose-dependent increase in the size of endometriotic lesions in laboratory rodents.<sup>103</sup> The mechanism through which environmental immunotoxicants might stimulate development of endometriosis is unclear. It has been demonstrated that dioxin increases TNF $\alpha$  production by several types of mammalian cells and that anti-TNF $\alpha$  antibody was able to reverse the toxic effects of dioxin (reviewed in 17). Increased TNF $\alpha$  production by immune and ECs is a characteristic finding in endometriosis and an abnormal response of the endometriotic endometrium to TNF $\alpha$  has also been noted.<sup>22,23,25</sup> It is therefore possible that dioxin dysregulates TNF $\alpha$ , and perhaps other pro-inflammatory cytokine production, leading to the development of the disease. It has also been suggested that dioxin might promote the establishment of endometriosis through an increase in MMP expression or an increase in the expression of cytochrome P-450 isoenzymes, which participate in the formation of EC growth-stimulating catechol estrogens.<sup>17,104</sup> Also interesting are reports demonstrating genetic polymorphism in genes encoding metabolic detoxification enzymes in association with the increased risk of endometriosis.<sup>15</sup> These suggest that women who exhibit reduced activity of metabolic detoxification enzymes might have an increased sensitivity to dioxin and other environmental toxins.

## PROSPECTS FOR FUTURE THERAPY/MANAGEMENT

With an appreciation for the inherent resistance of endometriotic ECs to apoptosis and to immune-mediated destruction, and their ability to exploit peritoneal factors to their growth advantage, it is reasonable to suggest that targeted therapies might be designed to attack these parameters. With respect to normalizing the apoptotic and immunologic sensitivity of ectopic endometrium, studies should first identify the mechanism(s) responsible for this resistance. Although some studies have demonstrated an increase in the cell survival proteins, BCL-2 and survivin in endometriotic EC,<sup>82,105-107</sup> a more concerted effort needs to be made to identify inherent genomic and functional differences in the apoptotic pathways of normal and endometriotic endometrium. How those pathways operate to attenuate spontaneous apoptosis and to increase immunologic resistance is critical information that is needed to provide a rationale and guidance to develop such approaches. In this regard, the demonstration that GnRH agonists induce apoptosis in cultured EC<sup>108</sup> and in eutopic endometrium of patients on GnRH agonist therapy<sup>109</sup> is of interest, as are the results showing that danazol treatment, *in vivo* and *in vitro*, attenuates PBM-mediated stimulation of EC proliferation.<sup>110</sup>

These reports are provocative because they suggest that treatments that target these functions could prove synergistic with standard therapies.

The opportunities presented due to the recent findings of the multifaceted roles played by TNF $\alpha$  in women with endometriosis should also be explored. Based on the findings reviewed above, it is reasonable to hypothesize that the control of the effects of TNF $\alpha$  on chemokine synthesis, MMP activity, angiogenesis, and stimulation of EC proliferation in these patients with available TNF inhibitors should be investigated clinically. The capacity of these agents to affect the disease course in selected groups of patients during the evolution of the disease and its management are well within the capabilities of the field today. The ability of a soluble TNF-receptor-1 protein to inhibit the development of endometriosis from human uterine explants in a rat model supports this idea.<sup>111</sup> Similar opportunities exist for IL-1 $\beta$ , IL-6, and IL-8.

Another possibility that can be addressed today in clinical trials is the use of COX-2 (cyclooxygenase-2) inhibitors in women with endometriosis. Both COX-1 and COX-2 (the rate limiting enzymes for the production of pro-inflammatory prostaglandins) are increased in endometriosis.<sup>112-114</sup> With respect to COX-2, studies in patients have shown that levels are increased in both eutopic and ectopic tissues.<sup>112,113</sup> The importance of this with respect to direct modulation of EC growth is based on the capacity of PGE2 to activate the aromatase P450 enzyme involved in biosynthesis of estrogens and expressed by endometrial stromal cells.<sup>115</sup> Thus, COX-2 inhibitors in combination with aromatase inhibitors are expected to reduce local production of estrogens by pathways that circumvent ovarian secretion. Moreover, specific immunologic functions in endometriosis patients are expected to be favorably modulated by these agents based on the capacity of PGs to modulate the activation and function of most cells of the immune response.<sup>116</sup> The recent demonstration of increased COX-1 and COX-2 synthesis in endometriotic PM, especially in advanced disease, is in keeping with this possibility.<sup>114</sup> Indeed, this might explain why the broadly specific cyclooxygenase inhibitor, indomethacin, was found to increase PM-mediated cytolysis of target cells in patients with extensive endometriosis.<sup>27</sup>

## CONCLUSIONS

It is quite likely that, in healthy women, multiple and redundant intracellular and extracellular controls prevent implantation of misplaced EC and that dysregulation in that system results in initiation, promotion, and progression of endometriosis. Abnormal genetically polymorphic ECs, perhaps modified by the environmental toxicants, might respond to the local endometrial signals with proliferation rather than apoptosis. These cells, when misplaced into PC, might not only escape peritoneal destruction but might also exploit the peritoneal environment to their advantage and continue to proliferate in a clonal fashion while normal cells in the same individual are eliminated. Secretory products of these cells offer additional advantages for cell invasion and progression of the disease, and induce a local inflammatory response. This brings about chemotaxis of the leukocytes into PC, their activation, and secretion of pluripotent cytokines. However, these mobilized immune cells might have a decreased ability to eliminate misplaced cells, and their secreted products might stimulate rather than inhibit proliferation of the ectopic cells and progression of the disease. Functional abnormalities in the immune cells

might result from exposure to the same environmental toxins producing concomitant alterations in B cell, NK cell, and T cell functions reported in women with endometriosis.

The immune response, therefore, plays a paradoxical role in endometriosis. Although many of its effects might be expected to facilitate control of the disease, the weight of evidence suggests that the opposite is true. In this respect, endometriosis is comparable to other conditions characterized by chronic, cyclical inflammatory reactions. And, like those other conditions, the substantial increase in knowledge regarding the cellular and molecular mechanisms involved should lead to the development of new forms of treatment.

## SUMMARY

Currently available evidence indicates that endometrial cells misplaced during menses into the peritoneal cavity of healthy women are characterized by increased apoptosis and are eliminated readily by the cells of the immune system. In women with endometriosis, misplaced endometrial cells implant and proliferate in the ectopic locations, an action that is associated with mobilization of the immune cells into the peritoneal cavity and a profound local and systemic immune response. This response, although aimed at the elimination of misplaced cells, is paradoxically exploited by these cells to their own growth advantage. It remains unclear whether the immune response in endometriosis contributes to the etiology/pathogenesis of the disease or is a secondary response in an attempt to restore homeostasis. Both eutopic and ectopic endometrial cells in women with endometriosis demonstrate abnormal proliferative response to the immune cell-produced substances and are capable of the synthesis of growth stimulatory factors that act in autocrine and paracrine fashions. This aberrant proliferative response might be secondary to genomic alterations, which have been demonstrated in the endometriotic cells and endometriosis-derived cell lines. Abnormal expression both of genes encoding cell cycle regulation and of metabolic detoxification enzymes, which was demonstrated in the ectopic endometrium, might predispose affected women to the proliferation-stimulatory effects of the environment toxins. Better understanding of the mechanisms interfering with the immune-mediated destruction of the ectopic endometrial cells, and their ability to exploit peritoneal factors to their growth advantage, should lead to the development of new and more effective methods of treatment.

### Practice points

- women with endometriosis are at increased risk for the development of allergic autoimmune and neoplastic diseases
- hyperactive immune response and accumulation in the peritoneal cavity of immune- and endometrial-cell-secretory products contributes to infertility associated with endometriosis
- medical treatment of endometriosis with danazol or GnRH agonists restores normal immune response, which might improve fertility
- future treatment of endometriosis and/or endometriosis-associated infertility might include immunomodulatory substances

### Research agenda

- clinical testing of COX-2 inhibitors
- clinical testing of TNF $\alpha$  inhibitors (e.g. Remicade) or blocking agents (e.g. Enbrel)
- targeted modulation of pro-inflammatory cytokines/growth factors
- genomic testing of uterine endometrium

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