

Endometrial inflammation and effect on implantation improvement and pregnancy outcome

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Abstract

Implantation failure, which is presently the major barrier in human fertility, is attributed, in many cases, to the failure of the uterus to acquire receptivity. The transition into a receptive uterus includes cellular changes in the endometrium and the modulated expression of different cytokines, growth factors, transcription factors, and prostaglandins. These molecules partake in the generation of an inflammatory response followed by the recruitment of immune cells. These cells have shown to be involved in the maternal immune tolerance toward the implanted embryo as well as in the maternal–fetus interaction during pregnancy. Most of the accumulated evidence indicates that embryo implantation is associated with an active Th1 inflammatory response while a Th2-humoral inflammation is required for pregnancy maintenance. Yet, recent findings suggest that a Th1 inflammatory response is also necessary for the acquisition of uterine receptivity. This notion was originally suggested by reports from our and other clinical centers worldwide that IVF patients with repeated implantation failure subjected to endometrial biopsy exhibit a substantial improvement in their chances to conceive. These findings, followed by the demonstration of an elevated pro-inflammatory cytokine/chemokine expression, as well as an increased abundance of immune cells, in the endometrium of these patients, raised the idea that acquisition of uterine receptivity is closely associated with an inflammatory response. This review summarizes the molecular and biochemical evidence that confirm this notion and proposes a mechanism by which injury-induced inflammation improves uterine receptivity and the subsequent pregnancy outcome.

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Inflammation

Inflammation is a process induced by either viral/microbial infection or mechanical trauma of the tissue, such as injury. Inflammation is characterized by upregulation of cytokines and chemokines followed by the recruitment of immune cells from the blood system to the infected/injured tissue. These cells, in turn, secrete different cytokines that induce remodeling of the tissue by stimulating cell proliferation and differentiation. Based on the cytokine type produced by the inflamed tissue, the immune reaction is functionally divided into the Th1 and Th2 responses. The Th1 response is mediated by pro-inflammatory cytokines such as interleukin 1 (IL1), IL2, IL6, IL12, IL15, IL18, interferon- γ (IFN γ), and tumor necrosis factor- α (TNF α), whereas the Th2 response is characterized by the involvement of regulatory cytokines such as IL4, IL5, IL10, IL13, and granulocyte macrophage colony stimulating factor (GM-CSF) and is therefore known also as anti-inflammatory (reviewed by [Challis *et al.* \(2009\)](#) and [Mor *et al.* \(2011\)](#)). It is the delicate balance between

the activities of Th1/Th2 that determines the nature of the immune reaction.

Implantation

Implantation of the embryo, which is crucial for successful reproduction, takes place in a receptive uterus. In human, the uterus becomes receptive during the mid-secretory phase, between days 19 and 23 of the menstrual cycle. This period of endometrial receptivity is also known as the window of implantation (WOI). The specific cellular changes during the WOI include the transformation of the fibroblast-like endometrial stromal cells into larger and rounded decidual cells ([Dunn *et al.* 2003](#)) and the emergence of large apical protrusions (pinopodes) and microvilli on the luminal epithelium ([Paria *et al.* 2002](#)). In parallel, modulations in the expression of different cytokines, growth factors, transcription factors, prostaglandins, and adhesion molecules take place ([Paria *et al.* 2002](#), [Wang & Dey 2006](#)). The slightest imbalance in each of these protein expressions could result in pathological conditions and subsequent infertility.

Inflammatory events in the endometrium

Although, by definition, the assignment of the immune system is to provide protection from invading organisms, this system appears to be crucial for successful implantation and maintenance of pregnancy (Mor *et al.* 2011). Furthermore, a key role of inflammatory components of the immune system in maintaining uterine homeostasis and regeneration has been established and the involvement of cytokines and immune cells in menstruation has been demonstrated. Menstruation occurs following the withdrawal of progesterone at the end of the menstrual cycle that releases the inhibition of the pro-inflammatory NF- κ B pathway, leading to an increase in the levels of pro-inflammatory cytokines, prostaglandins, and matrix metalloproteinases followed by lysis of the connective tissue and bleeding (reviewed by Kelly *et al.* (2001) and Maybin & Critchley (2011)). Their production by the endometrial cells throughout the cycle is regulated by the sex steroids, estrogen, and progesterone (Dominguez *et al.* 2003, Kitaya *et al.* 2003, Jones *et al.* 2004, Carlino *et al.* 2008). During the proliferative phase, it is estrogen that regulates the endometrium regeneration. Under the influence of this steroid hormone, the endometrium stops bleeding, the luminal lining re-epithelializes, the stromal tissue starts to grow, and vessels are repaired. After ovulation, during the secretory phase, cellular changes in the uterine endometrium that include the transformation and decidualization of the stromal cells and the development of secretory glandules are regulated by progesterone (Paria *et al.* 2002, Dunn *et al.* 2003, King & Critchley 2010).

Similar to menstruation, implantation is also characterized by elevated levels of endometrial cytokines, prostaglandins, and leukocytes (Kelly *et al.* 2001). A gradient of chemokines and cytokines, produced by the endometrial cells, guides the blastocyst to the site of implantation allowing its interaction with the uterine lining. During invasion, trophoblast cells break through the epithelial and stromal cells. The endometrial tissue is then repaired and remodeled by the growing placenta. This local 'wound healing-like' process is characterized by a strong Th1, pro-inflammatory response in which high levels of pro-inflammatory cytokines such as IL6, LIF, IL8, and TNF α are involved (Dominguez *et al.* 2005, van Mourik *et al.* 2009). Among their other activities, these cytokines recruit immune cells to the decidua. Both, in human and in mouse, infiltration of large populations of decidual leukocytes to the implantation site has been observed. Of these cells, 65–70% are uterine-specific natural killer (uNK) cells and 10–20% are antigen-presenting cells (APC) such as macrophages and dendritic cells (DCs; Kämmerer 2005, Hanna *et al.* 2006). Unlike NK cells, macrophages and DCs stay in the decidua throughout pregnancy and have a pivotal role at the maternal–fetal interface (Gardner & Moffett 2003, Fest *et al.* 2007, Plaks *et al.* 2008, Renaud &

Graham 2008). Specifically, these cells form the initial contact with external antigens, mediating the antigen-specific adaptive immune response. Several lines of evidence suggest that APCs play a pivotal role in shaping the cytokine profile in the maternal–fetal interface (Laskarin *et al.* 2007, Mor 2008). Macrophages and DCs have the ability to secrete an array of anti-inflammatory cytokines/chemokines (IL4, IL10, and IL13) and enzymes that are involved in tissue remodeling and angiogenesis (Goetzl *et al.* 1996, David Dong *et al.* 2009). In addition, macrophages have been suggested to regulate trophoblast invasion and may play a key role in cleaning up the debris, resulting from apoptosis of the trophoblast in the various stages of pregnancy (Abrahams *et al.* 2004, van Mourik *et al.* 2009). Accumulation of DCs in the maternal tissue surrounding the implanting embryo has been reported (Blois *et al.* 2004a, 2004b, Plaks *et al.* 2008). Their involvement in the establishment of tolerance toward the semi-allograft fetus has been extensively investigated (Steinbrink *et al.* 1997, Blois *et al.* 2007). These cells are responsible for changing the Th1, pro-inflammatory, into the Th2, anti-inflammatory environment also during later stages of pregnancy (Miyazaki *et al.* 2003, Nagamatsu & Schust 2010). Toward the end of pregnancy, the anti-inflammatory environment changes again into a pro-inflammatory one, inducing the contractions of the uterus that lead to parturition (Romero *et al.* 2006a, 2006b).

Taken together, the above-mentioned observations describe the pivotal role of inflammation starting at implantation and throughout pregnancy. However, evidence for the role of inflammatory processes before implantation is scarce.

Local injury to endometrium increases its receptivity

The capacity of the female reproductive tract to remodel after every menstruation presents features that are similar to that of injured tissue repair, involving balanced activity of inflammation, associated with vascular, connective tissue, and epithelial cell remodeling (Ruszczak & Schwartz 2000). Morphological studies demonstrated similarities between endometrial regeneration and the recovery from a mechanical trauma, supporting this notion. Mechanical manipulation has been first shown to be associated with decidual formation in guinea pig in 1907 (Loeb 1907). This study demonstrated that scratching the uterus during the progestational phase of the estrous cycle provoked a rapid growth of decidual cells. Later experiments showed that decidual formation in pseudopregnant rodents could be induced by other forms of local injury, such as suturing the uterine horn (Turner & Bagnara 1976) and i.u. injection of oil (Finn & Martin 1972). This phenomenon may go along with the recent intriguing findings of a positive association between uterine mechanical manipulation and pregnancy outcome in

human patients. Specifically, it has been demonstrated by us and others that endometrial biopsies taken during the spontaneous cycle that preceded the IVF treatment more than doubled the rates of implantation, clinical pregnancies, and live birth (Barash *et al.* 2003, Raziel *et al.* 2007, Zhou *et al.* 2008, Karimzadeh *et al.* 2009, Narvekar *et al.* 2010, Tiboni *et al.* 2011). Moreover, as reported by Raziel *et al.* (2007), this treatment improved IVF outcome in patients with a mean of seven previous implantation failures resulting in 30 vs 12% clinical pregnancy rate and 11 vs 4% implantation rate in the treated and untreated patients, respectively. Recent meta-analysis of all of the above-mentioned studies further demonstrated the beneficial effect of local endometrial injury on IVF success rate (El-Toukhy *et al.* 2012). Still, there is no consensus protocol regarding the optimal number and timing of the biopsies that are needed for achieving the improvement in implantation and a large-scale prospective randomized trial is required in order to elucidate this issue. Along this line, other forms of mechanical manipulation in human, such as curettage and hysteroscopy (Friedler *et al.* 1993, Mooney & Milki 2003, Rama Raju *et al.* 2006, El-Toukhy *et al.* 2009, Bosteels *et al.* 2010), also generate a favorable effect on the pregnancy outcome in IVF patients. Taken together, these findings suggest that the success of implantation in all these cases is apparently attributed to an injury-induced inflammatory reaction that leads to the development of an adequate decidua supporting implantation.

Local injury-induced increase in endometrial receptivity is mediated by inflammation

The hypothesis that the mechanism by which local injury increases endometrial receptivity involves an inflammatory response was recently confirmed by us. Specifically, endometrial samples were collected from two groups of IVF patients, on day 21 of their spontaneous menstrual cycle that represents the WOI. Patients in the experimental group underwent a previous biopsy during the proliferative phase of that same cycle. Comparison of the day 21 samples of the two groups revealed elevated levels of different pro-inflammatory cytokines such as TNF α , growth-regulated oncogene α (GRO α), IL15, macrophage inflammatory protein 1B (MIP-1B), and osteopontin (OPN) in the endometrium of the biopsy-treated patients. An increased abundance of the specific immune cells, DCs, and macrophages was also observed in endometrial samples of the treated patients (Gnainsky *et al.* 2010). The suggested mediatory role of inflammation in the injury-induced transition of a nonreceptive uterus into its receptive stage is supported by previous findings that show expression of the pro-inflammatory cytokine TNF α (Haider & Knöfler 2009) and MIP-1B (Kitaya *et al.* 2003) in human endometrium during

the WOI. These results are compatible with our findings that show that the expression of these two cytokines in the endometrium of the biopsy-treated patients during the WOI positively correlated with pregnancy outcome (Gnainsky *et al.* 2010). Such correlation was also demonstrated by Boomsma *et al.* (2009), showing that cytokine levels in endometrial secretions, including TNF α , in ovarian stimulated IVF cycles, are associated with the likelihood of conceiving.

As TNF α is essential for the initiation of an inflammatory response (Haider & Knöfler 2009), this cytokine could possibly be involved in the injury-induced inflammation by stimulating endometrial cells to produce other cytokines and chemokines. Analysis of the direct effect of TNF α on endometrial stromal and epithelial cells *in vitro* indeed demonstrated that MIP-1B, GRO α , and IL15, which were upregulated following biopsy treatment *in vivo*, were also increased by TNF α *in vitro* (Gnainsky *et al.* 2010). However, the specific role of these cytokines in facilitating endometrial receptivity and allowing implantation is still unclear. MIP-1B is one of the characteristic progesterone-regulated endometrial chemokines, the expression of which is elevated during the mid-secretory phase. It is expressed by epithelial and decidualized stromal cells in the endometrium during the WOI (Kitaya *et al.* 2003, Jones *et al.* 2004). This cytokine may have a direct favorable effect on implantation due to its additional effect on trophoblast migration (Hannan *et al.* 2006). GRO α was also shown to be expressed in the decidualized endometrial cells (Nasu *et al.* 2001). Both these cytokines bear the ability to attract monocytes *in vitro* (Menten *et al.* 2002, Traves *et al.* 2004). Moreover, previous studies demonstrated that MIP-1B, like other cytokines, that is released by injured tissue attracts macrophages and DCs. These specific immune cells play a crucial role in the process of wound healing (Sozzani *et al.* 1997, Luster 1998). This may suggest that the macrophages and DCs, the levels of which were elevated in the endometrium following biopsy treatment (Gnainsky *et al.* 2010), are either recruited by MIP-1B and GRO α or are differentiated from monocytes that were stimulated to migrate to the site of injury by these cytokines.

Previous findings that showed that macrophages and DCs that are present in the endometrium during the menstrual cycle increase in their abundance at the WOI (Rieger *et al.* 2004, Kämmerer 2005, Laskarin *et al.* 2007) together with our findings that demonstrated a higher abundance of these cells in the endometrium of IVF patients following biopsy treatment was associated with increased pregnancy rate indicate that these cells play an important role in acquiring endometrial receptivity. However, their specific role in preparation of the uterus for implantation remains unknown. It is important to mention that the immune cells accumulated in the endometrium following local injury were

characterized by combined expression of HLA-DR⁺ and CD11c⁺. Among these, CD14⁺ (DCs) as well as CD14⁺ (macrophages) cells were detected. Although previous studies found that macrophages comprise 20–25% the total decidual leukocytes whereas DCs comprise only 2%, it is quite difficult to distinct between these two immune cell subpopulations. Human endometrial macrophages exhibit a high phenotypic plasticity that is characterized by expressing specific DCs intercellular adhesion molecules (DC-SIGN, a C type lectin receptor) and ability to differentiate in certain inflammatory conditions into CD83⁺ DCs (Nagamatsu & Schust 2010).

A strong evidence for the indispensability of DCs in decidualization and implantation was provided using the elegant mouse model, the DCs of which express the diphtheria toxin (DTx) receptor, thus allowing their conditional ablation by DTx administration. Depletion of uterine DCs caused impaired decidualization that was characterized by a reduced proliferation, differentiation, and delayed angiogenesis. Embryos neither attached nor invaded the uterine epithelium (Plaks *et al.* 2008). Another study in the mouse showed that therapy by DCs administration significantly decreases the rate of spontaneous resorption of embryos in the uterus (Blois *et al.* 2004a, 2004b). Taken together, these results suggest that in addition to their induction of tolerogenic microenvironment at the maternal–fetal interface (Laskarin *et al.* 2007), uterine DCs have a specific and direct role in decidual development and embryo implantation.

In vitro studies suggest that DCs in cooperation with uNK cells exert a synergistic effect on uterine cell proliferation (Blois *et al.* 2011). Although the role of NK cells in decidualization has been thoroughly investigated, their direct effect on endometrial cells is largely elusive. Accumulating evidence suggest that uNK cells affect the profile of human endometrial gene expression and that their main contribution is attributed to their positive effect on vascularization, thus providing an adequate blood flow to the tissue (Blois *et al.* 2011). Furthermore, uNK cells have been shown to play a crucial role in the regulation of stromal cell differentiation. In addition, NK cells have a role in regulating trophoblast invasion by the production of IL8 and interferon-inducible protein-10 chemokines (Croy *et al.* 2003, Hanna *et al.* 2006). Interestingly, their involvement in the development of a receptive endometrium in human is crucial whereas in the mouse, mature NK cells do not appear in the uterus before implantation (King 2000). Further studies demonstrated that uNK cell differentiation is regulated by DCs via IL15 and IL12 (Blois *et al.* 2011). The increase in IL15 in the endometrium following local injury (Gnainsky *et al.* 2010) is probably attributed, at least in part, to the increase in the abundance of DCs. IL15 has been shown to be present in mice fetomaternal interface (Zourbas *et al.* 2001) and human endometrial glandular cells and

decidualized stromal cells (Kitaya *et al.* 2000). Although IL15 knockout mice were fertile, they display impaired decidual integrity, unmodified spiral arteries, and lack of uNK at the implantation sites (Ashkar *et al.* 2003). This phenomenon was reported in human endometrium as well. Low *IL15* mRNA levels were correlated with sub-endometrial vascular flow and with low CD56⁺ (NK) cell number in IVF patients with implantation failure (Ledee *et al.* 2008). Furthermore, it has been demonstrated that the endometrial levels of IL15 in IVF patients with implantation failure were lower than in the control, suggesting that this cytokine may serve as a potential pre-conception immune biomarkers for clinical practice (Lédée *et al.* 2011). In agreement with these findings, another recently published study demonstrated a correlation between stromal IL15 and the number of uNK cells. However, in disagreement with these studies, it has been reported that women with repeated implantation failure have higher IL15 in the stroma compared with controls (Mariee *et al.* 2012).

An additional cytokine, the expression of which was upregulated by the biopsy treatment, is OPN (Gnainsky *et al.* 2010). This pro-inflammatory cytokine, which was previously shown to be secreted by the endometrium and by immune cells (Johnson *et al.* 2003, White *et al.* 2006), is also known for its capacity to recruit and activate macrophages and DCs (Giachelli *et al.* 1998, Renkl *et al.* 2005). The effect of this cytokine was recently shown to be mediated by MIP-1B (Zheng *et al.* 2009). This is compatible with the significant increase in MIP-1B, OPN, as well as in macrophages and DCs observed in the endometrium following local injury (Gnainsky *et al.* 2010). It seems that in addition to MIP-1B and GRO α , OPN is also involved in the recruitment of immune cells to the injured endometrium and is probably induced by TNF α . In addition to its activity as a cytokine, OPN serves as an adhesion molecule that mediates the interaction between the integrins on the luminal uterine surface and that on the trophoblast, thus enabling the attachment of the blastocyst to the uterine lining (Apparao *et al.* 2001, Johnson *et al.* 2003). The increase in the level of OPN following biopsy treatment in IVF patients probably contributed to the injury-induced improvement of the implantation rate. This idea is supported by the positive correlation of endometrial OPN levels and pregnancy outcome (Gnainsky *et al.* 2010). It was previously shown that OPN in the mouse uterus is secreted by endometrial cells as well as by macrophages (White *et al.* 2006). It was further suggested that production of different cytokines by the endometrial immune cells may directly affect the luminal epithelium (van Mourik *et al.* 2009), thus contributing to acquisition of endometrial receptivity. Further, *in vitro* studies are needed in order to elucidate the specific effect of the biopsy-recruited immune cells on the differentiation of each of the endometrial compartments, stromal and epithelial cells.

Inflammation-induced regeneration of endometrial tissue

It is important to mention that the favorable effect of biopsy treatment on the endometrial receptivity is manifested during the IVF treatment performed in the following cycle (Barash *et al.* 2003). This phenomenon apparently relies on the fact that monocytes, the precursors of macrophages and DCs, that are known to be recruited to injured sites are long lived and reside in some tissues even for months, during which time they can differentiate into tissue-resident macrophages and DCs (Chomarat *et al.* 2003, Luster *et al.* 2005, McIntire *et al.* 2008). Facilitation of implantation manifested at the following cycle of treatment is possibly contributed by this 'tissue memory'. In this context, it should be noted that during menstruation, the endometrial thickness is reduced due to the loss of fluid and shrinkage of the spongy layer whereas most of the stroma and apparently the embedded immune cells stay intact. It is the residuum of the functional rather than that of the basal layer that contributes to endometrial regeneration (Brenner & Slayden 1994).

Regeneration of the endometrial tissue may result from proliferation of stem cells that were shown to be present in the endometrium (Gargett & Masuda 2010). Three main populations of these cells were identified:

endothelial cells, endometrial mesenchymal stem cells, and stromal fibroblast cells. Endothelial cells are localized in blood vessels suggesting their involvement in angiogenesis whereas the two other populations are located in the stroma. No stem cells were observed in the luminal surface (Spitzer *et al.* 2012). Along this line, it was shown that re-epithelialization initiates around the remaining glandular stumps (Ludwig & Metzger 1976, Ludwig & Spornitz 1991). The contribution of the stem cells to endometrial regeneration following menses is still unclear. Stromal cells have been shown to be quiescent *in vivo*. It is only after their isolation and culturing *in vitro* for 15 passages that clonogenic endometrial stromal cells differentiate into fibroblast-like elongated cells forming a monolayer (Dimitrov *et al.* 2008). However, these cells were shown to be stimulated *in vivo* when tissue is damaged (Ramalho-Santos *et al.* 2002, Venezia *et al.* 2004), suggesting that endometrial regeneration may be the result of the tissue breakdown caused during menses. It was shown that endometrial stem cells express genes that are involved in the response to inflammation (Spitzer *et al.* 2012) and migrate toward extracellular matrix proteolytic digests, formed in the injured site (Crisan *et al.* 2008). Based on these observations, we postulate that the injury-induced inflammatory reaction stimulate endometrial stem cell proliferation, migration, and differentiation that may be

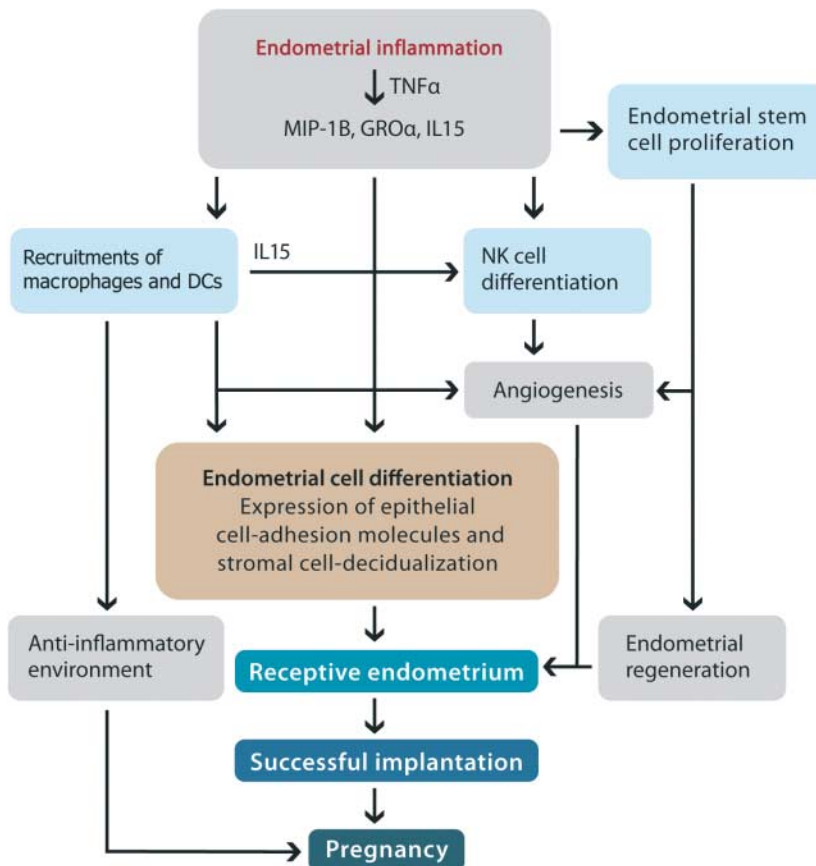


Figure 1 Suggested model for the favorable effect of injury-induced inflammation on implantation. TNF α , tumor necrosis factor- α ; GRO α , growth-regulated oncogene- α ; IL15, interleukin-15; MIP-1B, macrophage inflammatory protein 1B.

involved in acquisition of endometrial receptivity. Stem cell proliferation and differentiation are also controlled by epigenetic changes, such as DNA methylation and chromatin modifications (Tollervey & Lunyak 2012). Alterations in the endometrial global histone acetylation occur during different phases of menstrual cycle, suggesting the idea of involvement of epigenetic regulation in endometrial regeneration (Munro *et al.* 2010).

Conclusive remarks

The above-mentioned information agrees with the idea that local injury generated by endometrial biopsy may increase uterine receptivity by provoking inflammation. Specifically, these findings suggest that endometrial biopsy provokes an inflammatory response that is probably mediated by TNF α . The pro-inflammatory TNF α enhances the expression of other cytokines/chemokines that recruit, in turn, macrophages and DCs to the site of injury. These immune cells secrete different factors that on the one hand may affect uNK cell differentiation and on the other stimulate the luminal endometrial cells to produce adhesion molecules enabling the attachment of the embryo to the uterine lining, facilitating implantation (Fig. 1). The injury-induced inflammation also stimulates stem cell-dependent endometrial regeneration. All these events are essential for the transition of the endometrium from nonreceptive to its receptive stage, which probably do not take place in IVF patients with recurrent implantation failure, in the absence of endometrial biopsy treatment.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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