REPRODUCTION
REVIEW

Is there an immune modulating role for follicular fluid in endometriosis? A narrative review

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Abstract

Follicular fluid (FF) surrounds the granulosa cell–oocyte complex and is one of the mediating factors in the communication between the cells within the follicle. Literature reveals that human FF and its components are key factors to the success of natural fertilization. Among other substances, FF consists of multiple cytokines and immune cells, including interleukin 6 (IL6), IL12, sHLA-G, macrophages, NK cells and lymphocytes. Together, these cells and cytokines might influence the oocyte–granulosa–cell complex. Altered balances of immune content might be involved in changes on folliculogenesis, oocyte maturation, oocyte quality and ovulation. Furthermore, these altered balances are possibly involved in infertility associated with immune-mediated diseases such as endometriosis. The aim of this narrative review is to elaborate on the function and contents of FF and its immunological profile in patients with endometriosis. A comprehensive literature search was performed for the published literature on FF (immune) contents, FF function and FF content alterations in endometriosis patients. In FF of patients with endometriosis, elevated levels of macrophages and several cytokines have been reported. The role of specific immune cells in FF and a clarification of the biological mechanism in healthy women and endometriosis patients remain largely unknown. Future studies in this field will give us more insight in the role of FF immune cells and the effect of altered balances in patients with endometriosis.

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Introduction

Follicular fluid (FF) is an important component in the growth and development of the follicle. Therefore, changes in FF will influence the developing oocyte (Hennet & Combelles 2012). FF consists of many substances, such as hormones, immune cells, cytokines, enzymes, anticoagulants, electrolytes, reactive oxygen species, lipids, cholesterol and antioxidants (Hennet & Combelles 2012, Nagy *et al.* 2015, Basuino & Silveira 2016).

Endometriosis is a disorder characterized by the presence and growth of endometrial tissue outside the uterine cavity (Kennedy *et al.* 2005). The most accepted theory on the origin of endometriosis is Sampson's theory of retrograde menstruation (Sampson 1927, Izumi *et al.* 2018). Research has demonstrated retrograde menstruation in most menstruating women (Ahn *et al.* 2015). However, additional factors are essential to allow implantation and proliferation of endometrial cells and the development of endometriosis since clinical detected endometriosis only affects up to 10% of women in the reproductive age. In short, the clearance of endometrial debris in the peritoneal cavity is decreased in women with endometriosis, due to defective natural killer (NK) cell function, decreased phagocytosis by macrophages and induction of regulatory T cells (Zhang *et al.* 2018). Together, these factors may contribute to the tolerance of endometrial cells in the peritoneal cavity.

The peritoneal fluid (PF) in women with endometriosis is also marked by increased inflammation, including increased concentrations of leukocytes and cytokines secreted by ectopic endometrial cells and infiltrating immune cells. This promotes endometrial cell proliferation, endometrial cell adhesion and angiogenesis (Zhang *et al.* 2018). Changes of the immune response detected in the peritoneal cavity can also be seen in uterine endometrial tissue, peripheral blood and FF (Zhang *et al.* 2018).

The immunological profile of FF in patients with endometriosis could be a reflection of the immunological changes in the systemic circulation or alternatively a reflection of local inflammation due to endometriosis lesions in the ovary or peritoneal cavity (de Barros *et al.* 2017). Furthermore, FF is a contributor to the PF at ovulation (Bahtiyar *et al.* 1998). Interestingly, FF was found to stimulate the proliferation of ectopic endometrial cells *in vitro* and therefore repetitive exposure to FF during ovulation may be involved in the enhancement of peritoneal endometriosis (Bahtiyar *et al.* 1998). Lower fecundity in women with endometriosis is attributed to anatomic alterations such as adhesions leading to disturbed folliculogenesis and ovum pick-up

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mechanisms (Somigliana *et al.* 2017). Also involved in the lower fecundity in endometriosis is the trafficking of leukocyte subsets to the eutopic endometrium due to inflammatory changes. The specific chemokines that direct their migration and the inflammatory changes could have detrimental effects on endometrial receptivity, possibly via progesterone resistance and changes in endometrial gene expression (Lessey *et al.* 2013). Our hypothesis is that the altered systemic and local peritoneal immune profile in endometriosis patients has its effects on the endometrium/decidua in the uterus. And furthermore on FF surrounding the maturing oocyte which both might lead to a reduced fecundity independent of the anatomical changes such as adhesions.

The aim of this narrative review is to summarize literature on the most prominent immune cells in the FF in the general infertile population and in patients with endometriosis. The key points relevant to FF immune cells and cytokines in reproductive outcomes and endometriosis that emerge from this review are listed in Box 1.

Methods

A comprehensive literature search was performed in the PubMed database for published literature on FF immune content, FF functions and FF composition in patients with and without endometriosis. We undertook a search on all literature published between 1990 and March 2019. English was used as language restriction. Relevant articles were identified by searching for (combinations of) search terms such as FF, endometriosis, cytokine, immunology, interleukin, T cell, B cell, lymphocyte, macrophage, antigen-presenting cell and NK cell. Additional studies were identified by analysis of reference lists. Data from studies were analyzed, interpreted and are presented in this review.

Box 1 Key features of FF immune cells and cytokines

- Human FF is an important factor present in every stage of conception
- High levels of $CD56^{bright}CD16^{-}$ NK cells in FF are associated with good reproductive outcomes
- High levels of $CD56^{dim}CD16^{+}$ NK cells in FF are associated with adverse reproductive events
- sHLA-G levels in FF are not a marker for good-quality oocytes
- High levels of IL1B in FF are associated with good fertilization rates
- Elevated levels of IL12 in FF are detrimental to reproductive success in IVF
- In patients with endometriosis, NK cell levels in FF are higher compared to controls
- In patients with endometriosis, $CD14^{+}$ macrophages/monocytes levels in FF are higher compared to controls
- In patients with endometriosis, IL6, IL8 and IL12 levels in FF are higher compared to controls

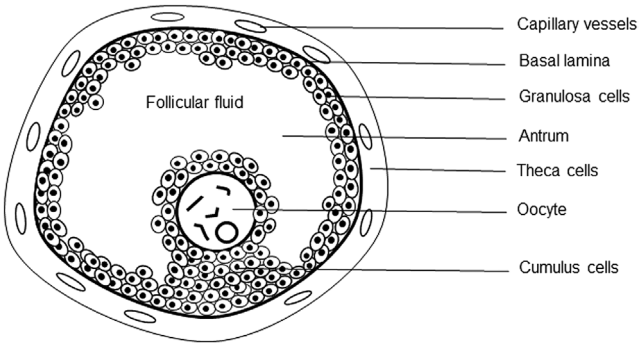

Figure 1 Schematic representation of an antral follicle with follicular fluid.

Results

Human FF

In antral follicles the oocyte undergoes a process of growth and maturation, to reach the optimal maturation stage for fertilization. This process occurs through a dynamic relationship between the oocyte within the antral follicle, consisting of granulosa and theca cells separated by the basement membrane (Fig. 1) (Hennet & Combelles 2012). During follicle growth an antrum is formed and filled with FF. FF is a combined product of those blood plasma constituents that cross the blood follicular barrier, and the granulosa and thecal cells' secretory activity (Gosden *et al.* 1988). The follicular microenvironment in which the oocyte matures is also influenced by the oocyte itself, through gap junctional communication and secreted factors within the oocyte-cumulus complex (Gilcrist *et al.* 2008). FF consists of hormones, immune cells, cytokines, enzymes, anticoagulants, electrolytes, reactive oxygen species, lipids, cholesterol and antioxidants. Likely, immune cells secrete products in the FF as well (Fig. 1) (Hennet & Combelles 2012, Nagy *et al.* 2015, Basuino & Silveira 2016).

The composition of FF varies throughout the phases of follicular development (Baskind *et al.* 2014). It is postulated that during the follicular phase, the basement membrane is intact, thereby preventing exchange from the theca cell compartment to the avascular granulosa cell compartment (Duffy *et al.* 2019). When the luteal surge occurs, the basement membrane ruptures, causing vascular changes within the follicle resulting in invasion of capillaries with influx of various cells into the FF (Baskind *et al.* 2014). The phases of follicular development are therefore relevant when reviewing FF composition and function. However, literature on FF composition does not always report these phases. Furthermore, hyper-stimulation in ART alters FF contents as well. Using immunohistochemical analysis, it has been shown for example that levels of $CD45^{+}$ leucocytes were significantly higher in FF from stimulated IVF compared to FF from natural cycle IVF (Kollmann *et al.* 2017). When using flow cytometry to further characterize

the difference in $CD45^+$ cells, no statistically significant differences were found in percentages of $CD3^+$, $CD4^+$, $CD8^+$ and $CD19^+$ cells, showing that it is not a subset-specific increase (Kollmann *et al.* 2017).

Immunological substances in human FF

FF consists of several immune cell subsets and cytokines. Within FF immune cells such as NK cells, T- and B lymphocytes, dendritic cells (DCs) and macrophages have been found (Lachapelle *et al.* 1996, Takaya *et al.* 1997, Fainaru *et al.* 2012). In general, most studies use pre-ovulatory FF collected during IVF cycles with ovarian hyperstimulation for their analyses. The origin of FF leukocytes and cytokines in general is hard to determine, due to (some) contamination of the aspirate with blood during oocyte harvesting, collected after the induced LH surge or hCG injection. One study has examined leukocytes and erythrocyte levels within peripheral blood and follicular aspirate in patients with male factor infertility undergoing controlled ovarian hyperstimulation. This study reported that leukocytes appear independently of erythrocytes in follicular fluid, which indicates that active infiltration into FF takes place. It is unknown whether this infiltration is before or after the hCG administration which induces the final maturation of the oocyte in IVF (Smith *et al.* 2005). In the following sections of this review, we will summarize the literature on immune cells – and the interaction between these cells and cytokines – in FF and in endometriosis-related alterations in FF.

NK cells in human FF

NK cell subsets are distinguished phenotypically and functionally by their CD56 and CD16 surface antigen expression (Fainaru *et al.* 2011). The $CD56^{dim}CD16^+$ and $CD56^{bright}CD16^+$ NK cell subsets are cytotoxic, whereas the $CD56^{bright}CD16^-$ NK cell subset has a more immune regulatory and pro-angiogenic function. One study measured levels of the pro-angiogenic ($CD56^{bright}CD16^-$) and cytotoxic ($CD56^{dim}CD16^+$) NK cells in the FF of antral follicles (<10 mm), collected 38 h after hCG injection in patients with PCOS ($n=8$) and patients with a contraindication for hormonal therapy ($n=2$) (endometrial and breast carcinomas) in the context of *in vitro* maturation (IVM). This was compared with levels of NK cells in the FF of mature follicles (>18 mm), collected 34 h after hCG injection during IVF treatment in patients with an infertility diagnosis (male, unexplained, tubal). The distribution of NK cell subpopulations in FF derived from an antral follicle of IVM patients revealed a predominance of cytotoxic $CD56^{dim}CD16^+$ and $CD56^{bright}CD16^+$ NK cell in the antral follicle compared to mature follicles (Fainaru *et al.* 2011). The infertility diagnoses in each group (IVM versus IVF) were different, which might introduce a difference in the reported distribution of NK cell subpopulations. This could be due to an altered immune profile of the various diagnostic categories of the patients instead of the maturity of the follicles. Krizan and colleagues reported that the relative distribution of $CD56^{bright}CD16^-$ immune regulatory NK cells in FF was significantly higher in patients after a successful IVF treatment resulting in a pregnancy, compared with a negative outcome (Krizan *et al.* 2009). On the other hand, higher levels of the $CD56^{dim}CD16^+$ cytotoxic NK cells in FF from IVF patients with unexplained infertility compared with a control group, consisting of patients with male factor infertility, are associated with diminished fertilization rates. The balance between angiogenic and cytotoxic NK cell subsets in the developing follicle might contribute to maturation of the cumulus-oocyte complex and the final goal of achieving a pregnancy. However, the functional role of these cells was not determined (Lukassen *et al.* 2003).

Macrophages in human FF

The presence of macrophages in FF, obtained after hCG injection during IVF cycles with ovarian hyperstimulation, are described in several studies (Lachapelle *et al.* 1996, Smith *et al.* 2005). Macrophages can be classified into different functional subsets, with specific roles in vascularization, tissue remodeling and inflammation (Wu *et al.* 2004, Sica & Mantovani 2012). However, only total macrophage fractions have been studied and to our knowledge macrophage subsets in FF have not yet been analyzed in humans. Brannstrom *et al.* (1994) found elevated macrophage density in the infiltrate of the thecal layer of the human follicle wall immediately after the LH surge. Takaya *et al.* (1997) analyzed macrophage levels in normal human ovulating cycles and reported lower macrophage infiltration in the stroma and theca cell layer in developing follicles as compared to corpora lutea. Wu *et al.* (2004) proposed that ovarian macrophages stimulate theca and granulosa cell proliferation, follicle growth and suppress follicular apoptosis, by secreting growth factors and cytokines. Ovarian macrophages secrete growth factors like epidermal growth factor (EGF) and insulin-like growth factors (IGFs), granulocyte macrophage-colony stimulating factors (GM-CSF) as well as cytokines such as IL1B, IL1, IL6, IL10, IL12, IFNa and TNFa (Wu *et al.* 2004). Cytokine profiles in FF will be further discussed in 'Cytokines in human FF'. To our knowledge, there are no studies describing the macrophage levels in human FF in the different follicular phases despite their presumed relevance in cytokine production.

Human leukocyte antigen (HLA)-G in human FF

HLA-G is a non-classical HLA-class Ib molecule from the major histocompatibility complex.

Despite the presence of soluble HLA-G1 (sHLA-G) and sHLA-G5 in FF, the role of intrafollicular sHLA-G

molecules remains largely unknown (Shaikly *et al.* 2008, Jee *et al.* 2011, Ouji-Sageshima *et al.* 2016). Granulosa cells are identified as the primary source of sHLA-G in FF, but sHLA-G could also emanate from peripheral circulation, possibly during oocyte harvesting (Shaikly *et al.* 2008, Rizzo *et al.* 2009). Several studies have investigated HLA-G as a marker for oocyte quality and implantation potential. Studies showed an inverse correlation between sHLA-G levels in FF and oocyte quality, reflected by fertilization rate, but no association with successful pregnancy (Jee *et al.* 2011, Ouji-Sageshima *et al.* 2016).

T and B lymphocytes and subpopulations in human FF

The presence of T and B subsets in FF has been described in several heterogeneous groups of infertile patients with different indications and comparing stimulated IVF with natural cycle IVF using markers as CD3, CD4, CD8, CD14, CD15, CD19, CD20, CD27, CD45 and CD57 (Hill *et al.* 1989, Lachapelle *et al.* 1996, Vujisic *et al.* 2004, Wu *et al.* 2007, Qin *et al.* 2016, Kollmann *et al.* 2017). However, the function of these cells in FF remains largely unknown. As $CD4^+$ T cells can be divided in Th1, Th2, Th17 and Treg subsets (Guerin *et al.* 2009, Wan *et al.* 2010), which all have their specific function, future studies analyzing these subsets in FF in more detail related to the outcome of IVF are needed.

Cytokines in human FF

Cytokines are small-molecular-weight glycoproteins that act as intercellular mediators across various immune effector cells, ovarian somatic cells and the oocyte (Vujisic & Zidovec 2005, Baskind *et al.* 2014). The following cytokines have been found in FF: IL1a and IL1B, IL2, IL6, IL8, IL10, IL11, IL12, IL13, IL17A, IL18, IL23, granulocyte- colony stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), TNFa, vascular endothelial growth factor (VEGF), EGF, platelet-derived growth factor (PDGF), IGF, leukemia inhibitory factor (LIF), fibroblast growth factor (FGF), RANTES (CCL5) and stem cell factor (SCF) (Vujisic & Zidovec 2005, Sabbaghi *et al.* 2014). Many cytokines have been found in FF, in the current review, we have focused on those cytokines which are mainly reported in FF of patients with endometriosis. Below we briefly describe these cytokines.

TNFa is a pro-inflammatory cytokine released by macrophages and its production can be induced by VEGF. It has a role in proliferation of granulosa cells and enhances its IL8 secretion (Kilic *et al.* 2007, Opøien *et al.* 2013). TNFa level is significantly higher in FF associated with poor quality oocytes and embryos (Wunder *et al.* 2006).

VEGF is a mediator that helps folliculogenesis and formation of the corpus luteum by stimulating ovarian angiogenesis, and it can be secreted by granulosa cells. Intrafollicular VEGF concentrations have no predictive value to evaluate IVF treatment outcome (Kilic *et al.* 2007).

IL1B is a pro-inflammatory cytokine that stimulates proliferation and activation of T and B cells, this cytokine is mainly produced by macrophages and monocytes. In the thecal cell layer of the human ovarian follicle, the presence of IL1B has been described, and an increase of intrafollicular IL1B at the time of ovulation is seen (Brannstrom *et al.* 1994). Furthermore, IL1B induces granulosa cell and theca cell IL6 and IL8 production (Baskind *et al.* 2014). In IVF patients, high levels of IL1B in FF were associated with good fertilization rates (Zollner *et al.* 2013). There is no correlation between FF IL1B levels and embryo morphology or pregnancy rates. Also, IL1B levels show no variation in FF concentrations during follicular development.

IL6 is not only produced by macrophages, Th1 cells and B cells, but also by fibroblasts and endothelial cells (Cameron & Kelvin 2003). The expression of IL6 has been described in the granulosa cells of the follicle, the corpus luteum, ovarian theca cells, the endometrium and in the preimplantation embryo (Altun *et al.* 2011). IL6 affects the vascular permeability (Bergqvist *et al.* 2001) and angiogenesis (Kawasaki *et al.* 2003) during follicular growth and development of the oocyte. Moreover, IL6 has effects on most immune cells including B and T cells (Hunter & Jones 2015).

IL6 in FF is significantly increased in the peri-ovulatory phase as compared to the follicular phase Baskind *et al.* (2014). Contradictory results exist on the possible effects of IL6 on IVF outcome. IL6 levels in FF were significantly higher in pregnant compared to non-pregnant IVF treated women (Bedaiwy *et al.* 2007). In contrast to these findings, another study reported detrimental effects of elevated FF IL6 levels on the outcome of IVF. Patients with IL6 levels in FF lower than the median were twice as likely to achieve clinical pregnancy as patients with higher IL6 levels (Altun *et al.* 2011). Both studies included comparable patients, but Bedaiwy *et al.* (2007) only performed univariate analysis, whereas Altun *et al.* (2011) did multivariate analysis including duration of COH, FF IL6 level and the number of embryos transferred, which could explain different results. The latter study used group medians for statistical analysis, but in the final statistical model included a FF IL6 cut-off level of <4.0 pg/mL as the independent variable. IL6 cut-offs could possibly be more reliable.

IL8 is a pro-inflammatory cytokine secreted by granulosa cells and involved in the recruitment and activation of neutrophils as well as cell proliferation and angiogenesis, and with that likely important in folliculogenesis, ovulation and repair of the ruptured

follicle (Vujisic & Zidovec 2005). In FF, significantly higher levels of IL8 were found in the pre-ovulatory phase and LH surge, as compared to the follicular phase, both in natural cycles and stimulated cycles (Baskind *et al.* 2014). Therefore, it is thought that granulosa cell and theca cell IL8 secretion is associated with follicle growth, oocyte maturation and ovulation, rather than being solely the result of hormonal stimulation (Runesson *et al.* 2000). IL8 seems to vary inversely with IL10, IL12 and TNFa, indicating that immune cells may interact with granulosa cell function (Opøien *et al.* 2013). IL8 levels in FF are not associated with fertilization and implantation rates or IVF outcome (Gazvani *et al.* 2000).

IL12 is a pro-inflammatory cytokine that regulates the immune system as an inductor of Th1 response and regulator of biological activities of T cells and NK cells (Gazvani *et al.* 2000). IL12 levels are significantly higher in FF of women who failed to become pregnant with IVF (Gazvani *et al.* 2000, Bedaiwy *et al.* 2007, Altun *et al.* 2011). High levels of IL12 may represent an increased inflammatory state of the maternal immune system and with that lower pregnancy success.

RANTES (CCL5) is a chemokine, widely expressed in tissue and a potent monocyte, macrophage, T lymphocyte and eosinophil attractant. The regulation of RANTES can reflect local modulation of the immune system together with MCP-1 (Xu *et al.* 2006).

Many studies have focused on the analysis of the individual number of cytokines, despite the fact that these mediators are known to operate in networks rather than in isolation, exhibiting features such as pleiotropism, synergy, antagonism and functional redundancy. It follows that the effect of any given cytokine is therefore not just dependent upon its absolute concentration, but rather on a balance between its own effects and those induced by other local mediators, including other cytokines (Field *et al.* 2014).

Dendritic cells in human FF

Dendritic cells (DC) initiate and coordinate innate and adaptive immune responses. After maturation, DCs migrate into lymph nodes, where interaction with antigen-specific T cells takes place to evoke an immune response (Fainaru *et al.* 2012). Following activation, DCs produce cytokines such as IL6, IL23 and TNFa. DCs function as a bridge between pathogen recognition and effector cells of both the innate and adaptive immunity (Sallusto 2013). DCs are a significant part of the bone marrow-derived leukocytes in the FF of the maturing oocyte immediately before ovulation, making up to 15% of the FF content of these cells (Fainaru *et al.* 2012). In patients undergoing IVF treatment DCs are present in pre-ovulatory FF and are characterized by expression of CD11c and HLA-DR. DC maturation correlates positively with the ovarian response to gonadotropins, but correlations between immune parameters in FF and oocyte quality, pregnancy outcomes are not reported (Fainaru *et al.* 2012, Zhang *et al.* 2017*a*).

Alterations of immune cells in patients with endometriosis

Our hypothesis is that the altered systemic and peritoneal immune profile in endometriosis patients has its effects on the endometrium/decidua in the uterus, as well as on FF surrounding the maturing oocyte. Both could possibly lead to a reduced fecundity due to an imbalanced immune profile, where a balanced profile is required for the maturing cumulus-oocyte complex in the follicles (Fig. 2).

NK cells in FF of patients with endometriosis

In patients with superficial peritoneal endometriosis (stage I/II) undergoing IVF treatment, higher levels of NK cells have been reported in FF compared to patients without endometriosis. Approximately 20% of $CD56^+$ NK cells expressed CD16, which indicates that the increase in NK cells was caused predominantly by the pro-angiogenic NK subset ($CD16^-$) (Lachapelle *et al.* 1996). Endometriosis is an angiogenesis dependent condition; thus, it is not surprising that there is an abundance of pro-angiogenic NK cells. In light of these results, modifications in the distribution of immune cell populations in the FF of patients with endometriosis likely influence the follicular environment and could subsequently influence folliculogenesis and cumulus-oocyte maturation. The cellular immune dysregulation in human ovaries could

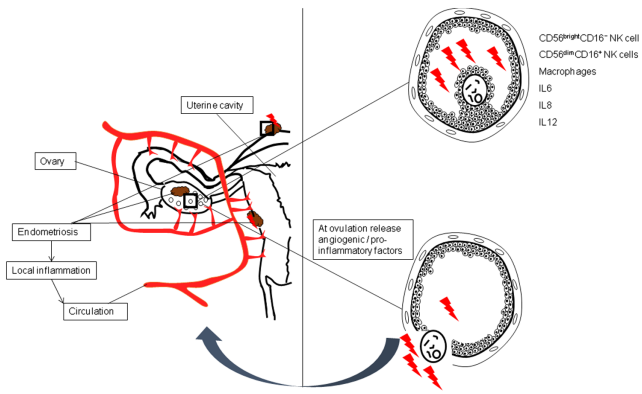

Figure 2 Schematic representation of immunological follicular fluid (FF) alterations in endometriosis patients and suggested role of FF in endometriosis and its associated infertility. A tolerogenic immune balance together with invasiveness of endometrial cells causes endometriosis lesions after retrograde menstruation. The endometriosis lesions cause local inflammation. Release of inflammatory factors through the circulation into the ovary causes alterations in follicular fluid contents. At ovulation, these factors are released into the peritoneal cavity. The pro-angiogenic factors stimulate endometriosis lesions. Furthermore, these factors are involved in the infertility associated with endometriosis. FF, follicular fluid; IL, interleukin; NK, natural killer.

be a factor altering fertility. The presence of $CD56^{+}CD16^{-}$ pro-angiogenic NK cells has been associated with successful implantation, favorable IVF outcome, spiral artery remodeling and placental maturation (Fainaru *et al.* 2010). However, higher levels of these pro-angiogenic NK cells in FF of endometriosis patients are likely related to endometriosis itself and any positive effects on placentation will be too small to compensate for the higher inflammatory state in the endometrium. The pro-angiogenic NK cells in FF released during ovulation could create a certain peritoneal environment stimulating the development of peritoneal endometriosis, which in turn creates an even more inflammatory environment and with that compromised fecundity.

Macrophages in FF of patients with endometriosis

Macrophages are among the first immune cells to be recruited when endometrial fragments enter the peritoneal cavity. Compared to women without endometriosis, in women with endometriosis, the number of peritoneal macrophages, the activation status and the concentration of inflammatory cytokines (i.e. IL1B, IL6, TNFa) secreted by these macrophages are elevated (Wunder *et al.* 2006). By secreting these pro-inflammatory cytokines, macrophages may promote the establishment of an inflammatory environment favorable for endometriosis development.

In patients with endometriosis (stage I/II), compared with groups of idiopathic infertility and tubal factor, flow cytometric evaluation in FF revealed higher levels of $CD14^{+}$ macrophages/monocytes which could lead to altered cytokine levels, leading to dysregulation of folliculogenesis and thereby impacting fertility (Lachapelle *et al.* 1996). These $CD14^{+}$ cells were not further characterized into macrophage or monocyte subsets.

Many macrophage-derived cytokines, such as IL1B and IL6, are increased in the FF of patients with endometriosis, indicating strong macrophage activity in FF, which may contribute to the pathogenesis of endometriosis (Pellicer *et al.* 1998, Tsudo *et al.* 2000, Wu *et al.* 2004).

Although higher levels of $CD14^{+}$ macrophages/monocytes in FF of endometriosis patients have been found, to our knowledge, these levels have not been further characterized into specific subsets. Therefore, it is difficult to interpret the exact role of these higher FF levels of macrophages in endometriosis and its associated fertility problems. More research is needed to determine the differences in peritoneal and FF macrophage subsets and the effects on folliculogenesis, maturation of the oocyte or oocyte quality and the involvement in the pathophysiology of endometriosis.

HLA-G in FF of patients with endometriosis

sHLA-G has been demonstrated to induce apoptosis of activated $CD8^{+}$ T cells and to modulate the NK cell and cytotoxic T lymphocyte responses, whereas membrane-bound HLA-G proteins have been shown to inhibit both natural killer cell and T cell-mediated cytolysis, to suppress proliferation of $CD4^{+}$ T lymphocytes and to induce a Th2 cytokine profile (Eidukaite & Tamosiunas 2008). In endometriosis, HLA-G was found in ectopic endometrial cells in the peritoneal cavity in women which is postulated to aid in their escape from immunosurveillance, due to their role in the protection against NK cell-mediated lysis (Barrier *et al.* 2006, Kawashima *et al.* 2009, Rached *et al.* 2019).

Eidukaite and Tamosiunas compared PF sHLA-G concentrations between endometriosis patients and controls. sHLA-G was found in all groups investigated without significant differences in concentrations (Eidukaite & Tamosiunas 2008). Rached *et al*. assessed sHLA-G levels in serum and PF in women with and without endometriosis. Higher concentration of sHLA-G in serum but not in the PF were observed in women with advanced endometriosis compared to the control group (Rached *et al.* 2019). Binding of the sHLA-G to inhibitory receptors on immune cells might explain the non-significant lower concentrations in sHLA-G available in PF between women with and without endometriosis (Rached *et al.* 2019). Mach *et al*. compared the blood sera among patients with ovarian cancer, ovarian endometrioma and deep endometriosis. The HLA-G concentration levels in the patients with deep endometriosis were comparable to those found in the patients with ovarian cancer, in contrast to significantly lower levels found in patients with ovarian endometrioma (Mach *et al.* 2010). HLA-G was furthermore found in ectopic endometrial cells in the peritoneal cavity in women which is postulated to aid in their escape from immunosurveillance due to their role in the protection against NK cell-mediated lysis (Barrier *et al.* 2006, Kawashima *et al.* 2009, Rached *et al.* 2019). To our knowledge, no studies have been published yet on sHLA-G levels in FF of patients with endometriosis.

T and B cells in FF of patients with endometriosis

Changes in the adaptive immune responses (T and B lymphocytes) have been described in the endometrium, PF and peripheral blood of patients with endometriosis, compared to women without this disorder (Podgaec *et al.* 2012, Olkowska-Truchanowicz *et al.* 2013, Ahn *et al.* 2015). Studies showed a balance toward Th2 cytokines in peripheral blood and PF of women with endometriosis (Hsu *et al.* 1997). This shift toward a Th2 immune response was confirmed by Podgaec *et al*., who found elevated IL4, IL10 and IFN-gamma levels in the PF of patients with endometriosis compared to those without endometriosis (Podgaec *et al.* 2007).

A subset of cells which is involved in the immune processes in endometriosis are regulatory T cells (Tregs). These are specialized T lymphocytes with

immunosuppressive properties (Guerin *et al.* 2009, de Barros *et al.* 2017). Several studies have found Treg cell concentrations to be higher in the peripheral blood, PF and eutopic endometrium in patients with endometriosis, compared to patients without endometriosis (Podgaec *et al.* 2012, Olkowska-Truchanowicz *et al.* 2013). Although it seems logical that the higher levels of Treg cells are involved in the immune tolerance of the endometriotic lesions, this has not been fully elucidated yet.

Similar to other specific cell subsets of T and B cells, there is not much known about Treg cells in FF. One study showed that patients with endometriosis have higher levels of B cells, and comparable levels of T cells in FF compared to groups with other infertility causes (Lachapelle *et al.* 1996). However, to our knowledge, no further analysis of T and B cell subsets in FF have been done in patients with endometriosis. Therefore, no conclusions can be made yet about possible correlations between T and B cells in FF, endometriosis and the effect on folliculogenesis, oocyte maturation and infertility.

Cytokines in FF of patients with endometriosis

Singh *et al.* (2016) reported an increase in the levels of pro-inflammatory (IL1B, TNFa, IL2, IL8, IL12) and anti-inflammatory (IL4, IL6 and IL10) cytokines in FF of patients with endometriosis (grade III/IV) undergoing IVF as compared with patients with tubal factor infertility (Box 2). The percentage of mature oocyte and good-quality embryos was significantly lower in endometriosis as compared with controls. Part of these findings were confirmed by Choi *et al.* (2015) who also reported higher levels of IL6, IL8, TNFa and IL1B in FF of endometriosis patients (Choi *et al.* 2015). Whereas Falconer *et al.* (2009) found lower levels of IL10 in FF of endometriosis patients (Falconer *et al.* 2009). Pellicer *et al.* (1998) reported significantly higher IL6 and higher, but not statistically significant, IL1B levels in FF of patients with endometriosis (grade III/IV) undergoing IVF as compared with patients with tubal factor infertility. Zhang *et al*. also analyzed cytokines in women with severe endometriosis (grade III/IV), although they did not find a difference in these cytokines between the control and grade III/IV endometriosis patients, IL23 levels in FF were significantly higher in patients with stage III/IV endometriosis compared to stage I/II (Zhang *et al.* 2017*b*).

The results of the study by Pellicer *et al*. were confirmed in the study of Wu *et al.* (2017) in natural cycle IVF in women with moderate-to-severe endometriosis. On the other hand, Opøien *et al.* (2013) found no difference in cytokine levels (IL1B, IL6, IL8, IL10, IL12 and TNFa) in the leading follicle adjacent to endometriomas. These results suggest that intra-ovarian endometriosis itself is not associated with local inflammatory reaction.

Furthermore, Kilic *et al.* (2007) compared VEGF and TNFa concentrations in FF of patients with endometriosis and unexplained infertility and reported a significant higher concentration VEGF in the endometriosis group but no difference in TNFa concentration between the two groups. The levels of RANTES and MCP-1 (both chemokines) have also been analyzed in FF of endometriosis patients, Xu *et al.* (2006) have shown higher levels of RANTES and lower levels of MCP-1 in FF of women with endometriosis compared with tubal infertility (Xu *et al.* 2006).

These altered levels of cytokines, including chemokines, could very well be a result of the increased inflammatory state in endometriosis. Moreover, these levels in turn could attract more immune cells into the follicle or into the peritoneal cavity after ovulation. Differences in patient characteristics and methods used can explain the sometimes contradicting results. Although both pro- and anti-inflammatory cytokines have been found higher in FF of endometriosis patients, the balance of these elevated cytokines indicated a pro-inflammatory environment, which confirms the inflammatory character of endometriosis.

Altered cytokine profiles in the FF of patients with endometriosis may play a significant role in an altered folliculogenesis, oocyte maturation, oocyte quality, ovulation and fecundity associated with endometriosis. Possibly, the higher levels of FF cytokines are produced by the granulosa cells or the FF immune cells which might be more activated in endometriosis. However, the activity of these FF immune cells is yet to be analyzed in endometriosis patients (Wu *et al.* 2017).

Box 2 Key cytokines found in FF in endometriosis

Cytokines	Origin	Function	Reference(s)
IL1B	Theca cells Macrophages and monocytes	Proliferation and activation of T and B cells Induces IL6 and IL8 production by granulosa cells	Brannstrom *et al.* 1994Baskind *et al.* 2014
IL6	Macrophages, T cells, B cells fibroblasts, endothelial cells	Effect on vascular permeability Angiogenesis T cell differentiation, proliferation and survival B cell maturation	Bergqvist *et al.* 2001Kawasaki *et al.* 2003
IL8	Granulosa cells Theca cells	Recruitment and activation of neutrophils, cell proliferation and angiogenesis	Vujisic & Zidovec 2005
IL12	B cells, phagocytic cells and other antigen-presenting cells	Inductor of Th1 response and regulator of biological activities on T cells and NK cells	Gazvani *et al.* 2000

Dendritic cells in FF of patients with endometriosis

In mouse models DCs have been shown to be important in the development of endometriosis. In humans the role of DCs in endometriosis is largely unknown (Pencovich *et al.* 2014, Stanic *et al.* 2014). Fainaru *et al.* (2012) described in one case an unilateral endometrioma which was associated with complete absence of DCs, contrary to a follicle of the contralateral ovary, where an abundance of DCs was seen. Another study reported lower levels of HLA-DR$^+$, CD11c$^+$CD123$^+$ DCs in PF of patients with endometriosis compared to controls with no endometriosis; however, this did not reach statistical significance (Tariverdian *et al.* 2009). Further research is necessary on DCs presence and function in the FF of endometrsiosis patients to determine the effect on folliculogenesis, oocyte maturation, quality and fertility potential.

Conclusion

We hypothesized that the altered immune profile in endometriosis patients has its effects not only on the endometrium/decidua in the uterus, but also on FF surrounding the maturing oocyte both possibly leading to a reduced fecundity (Fig. 2). FF represents the microenvironment for the oocyte to develop optimally. FF is composed of many substances including immune cells and cytokines, all of which are likely to play a crucial role in maturing of the oocyte and implantation of the resulting embryo. The physiological role of the immune cells and cytokines in FF in the normal follicle is not yet determined. Therefore, the exact role and differences of FF in women with immune-mediated diseases such as endometriosis remain unknown.

In the PF, peripheral blood and endometrium of patients with endometriosis, multiple alterations have been found that possibly contribute to the establishment and development of the disease, such as impaired NK cell activity, reduced phagocytosis capacity of macrophages and elevated Treg concentrations. Increased quantities of peritoneal and follicular macrophages are held accountable for the secretion of many cytokines, which in turn might alter FF, thereby possibly influencing follicle maturation, oocyte development and quality. Although many studies have addressed FF macrophage, NK cell and cytokine quantity, activity and alterations in patients with endometriosis and compared to patients without endometriosis, the results are not conclusive. The effect of follicle fluid, released at ovulation, to the peritoneal cavity as a synergistic factor in the development of endometriosis also demand further exploration. While decidual T cells seem to play an important role in achieving a normal pregnancy, with an important role for Tregs, no studies so far have been undertaken to analyze these T cells in FF of women with endometriosis. Future studies in this field might give us more insight in the role of immune cells and cytokines in the mechanism of and the effects on the folliculogenesis, oocyte maturation, oocyte quality, ovulation and fecundity in patients with endometriosis. This knowledge might have a great impact in reducing infertility in endometriosis, by interfering with the immune cell and cytokine balance in FF.

Declaration of interest

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

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