

## Review

# Unusual inflammation in gynecologic pathology associated with defective endometrial receptivity

Kotaro Kitaya<sup>1,2</sup>, Tadahiro Yasuo<sup>3</sup>, Yoshihiro Tada<sup>1</sup>, Terumi Hayashi<sup>1</sup>, Yuri Iwaki<sup>1</sup>, Masako Karita<sup>1</sup>, Miyako Funabiki<sup>1</sup>, Sagiri Taguchi<sup>1</sup>, Dustin Spillers<sup>1</sup>, Yoshitaka Nakamura<sup>1</sup> and Hisao Yamada<sup>2</sup>

<sup>1</sup>IVF Center, Oak Clinic, Nishinari-ku, Osaka, <sup>2</sup>Department of Anatomy and Cell Science, Kansai Medical University, Hirakata and

<sup>3</sup>Department of Obstetrics and Gynecology, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto, Japan

**Summary.** Human cycling endometrium displays a series of periodic transitions unique to this mucosal tissue, which includes rapid proliferation, secretory transformation, physiological angiogenesis, interstitial edema, and menstrual shedding. Among these properties of the endometrium are the inflammatory changes that occur dynamically across the menstrual cycle. Immunocompetent cell composition and inflammatory gene expression pattern in the human endometrium drastically fluctuate from the proliferative phase to the secretory phase, particularly at the time of ovulation. These local immune responses are fine-tuned by the direct or indirect action of two representative ovarian steroids, estradiol and progesterone, and are essential for successful blastocyst implantation. Meanwhile, studies have been accumulating the evidence that such physiological endometrial inflammatory status is altered in the presence of certain gynecologic pathologies. Given that blastocysts are semi-allografts for maternal tissue, even subtle alterations in endometrial immunity potentially have a negative impact on implantation process. In this article, we aimed to review and discuss the physiological and pathological mucosal inflammatory conditions that can affect endometrial receptivity.

**Key words:** Embryo implantation, Endometrium, Implantation failure, Immune responses, Inflammation

## Introduction

The remarkable advances in time-lapse imaging in assisted reproductive technology enabled us to visualize the chronological steps of the embryogenesis, i.e. fertilization, cleavage, compaction, and blastocyst formation (Basile et al., 2013). In addition, progress in pre-implantation genetic diagnosis using trophoctoderm biopsy and comparative genomic hybridization/whole genome amplification technique has expanded the possibility for safe and rapid selection of euploid blastocysts (Capalbo et al., 2013). With all this expertise, the practical management system from oocyte pickup to intrauterine embryo/blastocyst transfer improved in an in vitro fertilization-embryo transfer (IVF-ET) program. Nevertheless, blastocyst implantation, the final step of the conception is yet beyond our control.

Successful implantation is an unhindered sequential phenomenon that consists of hatching from zona pellucida, apposition and adhesion to the endometrial epithelium, and deposit and invasion into subepithelial interstitial areas (Fig. 1). Human endometrium turns to receptive phenotype only in the limited period during the mid-to-late secretory phase, typically between days 19 to 23 in the spontaneous menstrual cycle. This mucosal modulation is under the great influence of progesterone secreted by the corpus luteum, a specific endocrine

**Abbreviations.** IL: interleukin, TNF-alpha: tumor necrosis factor-alpha, IVF-ET: in vitro fertilization-embryo transfer, TGF-beta: transforming growth factor-beta, GnRH: gonadotropin-releasing hormone, BCL-2: B-cell lymphoma-2, STAT3: signal transducer and activator of transcription 3, VEGF: vascular endothelial growth factor

organ that is formed in the ovulating ovary (Sharkey and Macklon, 2013).

In this period, endometrial surface epithelial cells undergo membranous transformation for embryo adhesion and invasion, whereas they lose progesterone receptor expression. In parallel, numerous processes (termed pinopodes) and microvilli structures appear on the apical side of the surface epithelium. Endometrial glands gain luminal dilatation and tortuosity, and reduce epithelial cell height and subnuclear vacuoles. Additionally, the volume of the gland secretion peaks at this phase (Ozturk and Demir, 2010).

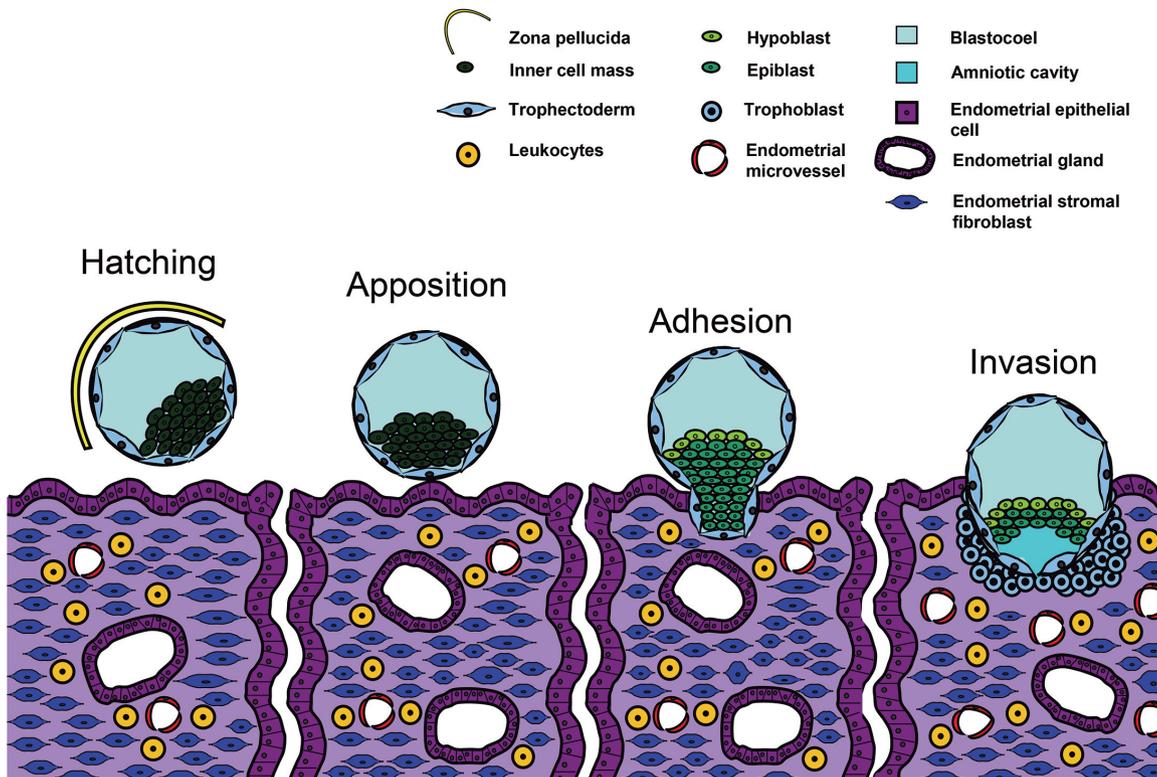
In the endometrial interstitial areas, stromal fibroblasts grow larger and rounder decidualizing cells, whereas extracellular matrices begin to develop around these decidualizing stromal fibroblasts and form meshwork architecture. Spiral arterioles become conspicuous in the subepithelial layer with significant growth and coiling. Moreover, a wide variety of leukocytes amass as single cells or aggregates in the stromal compartments, particularly around the glands and microvessels (Kitaya, 2008).

In synchrony with these morphological changes, the transcripts encoded by diverse genes are up-regulated in endometrial-component cells. These genes include the

subgroup of cytokines, chemokines, growth factors, transcription factors, adhesion molecules, enzymes, and prostaglandins. These molecules are thought to exert a pivotal role in dynamic remodeling and transformation of the endometrium in the blastocyst implantation process (Nakayama et al., 2003; Daikoku et al., 2004; Kitaya et al., 2004; Yamaguchi et al., 2006; Kitaya and Yasuo, 2009; Yasuo and Kitaya, 2009; Yasuo et al., 2010; Kitaya and Yamada, 2011; Kitaya et al., 2012a; Darlington et al., 2013).

**Inflammation in receptive endometrium in natural cycle**

Human cycling endometrium contains various types of immunocompetent cells under a physiological condition (Fig. 2). The absolute number and relative proportion of endometrial immunocompetent cells significantly fluctuate across the menstrual cycle. The predominant endometrial leukocyte subpopulation in the proliferative phase (from the menstrual period to the ovulatory phase) is a subset of CD8+ T cells. At the time of ovulation (2 days after luteinizing hormone surge), unique CD16<sup>neg</sup> CD56<sup>bright</sup> natural killer cells in this mucosa start to increase in number in the early secretory



**Fig. 1.** Schema of human in-utero multi-step blastocyst implantation model. Developing blastocysts that transferred into the uterine cavity at day 5 or 6 following insemination get out of the zona pellucida opening by enzymatic digestion and mechanical force (hatching). The trophoctoderm, the outer layer of the free-floating polarized blastocysts, faces the endometrial epithelium in close proximity, which is mediated through the action of the chemokines (apposition) (Sela et al., 2013).

Around day 7, the trophoctoderm attaches and encroaches the endometrial epithelial layer utilizing adhesion molecules and proteases (adhesion) and migrates further into the decidualizing endometrial interstitial layer via chemokine-chemokine receptor interaction (invasion). During the course, blastocysts grow and differentiate to form hypoblasts (GATA-4+), epiblasts (nanog+), and the amniotic cavity (Roode et al, 2012). Endometrial stromal fibroblasts, leukocytes, and microvascular endothelial cells understandably play a major role in receiving blastocysts successfully.

phase, accompanied by a smaller delayed rise of macrophages and neutrophils in the mid-to-late secretory phase. When pregnancy occurs, the density of natural killer cells and macrophages further rises in the decidualized endometrium towards the first trimester of pregnancy. Meanwhile, the density of T cell subsets is relatively unchanged throughout the menstrual cycle. The lineage of B cells is a rare subpopulation that resides sparsely within the endometrial basal layer as the core of the lymphoid aggregates surrounded by CD8+ T cells and macrophages (Kitaya et al., 2007). The exact nature of these endometrial leukocytes are not fully determined, but studies have unveiled their potential contribution to both conventional (prevention of infection and carcinogenesis) and unique (involvement in menstruation and blastocyst implantation) mucosal immune responses.

Recent microarray techniques allowed simultaneous wide genomic or proteomic analysis within a tissue or cell. With the assistance of these comprehensive measurement systems, human receptive endometrium was found to display unique immunological features in transcriptome and proteome of pro-inflammatory mediators. Even though there are yet some variances among the reports, the expression of several immune-associated genes such as osteopontin, decay accelerating factor for complement, serine or cysteine proteinase, and interleukin (IL)-15 was confirmed to be up-regulated stably in the mid-to-late secretory phase endometrium throughout the studies (Kitaya et al., 2000, 2005; Horcajadas et al., 2007; Kitaya and Yasuo 2013a). These findings indicate that certain types of local inflammation are required for the human endometrium to establish a

successful conception.

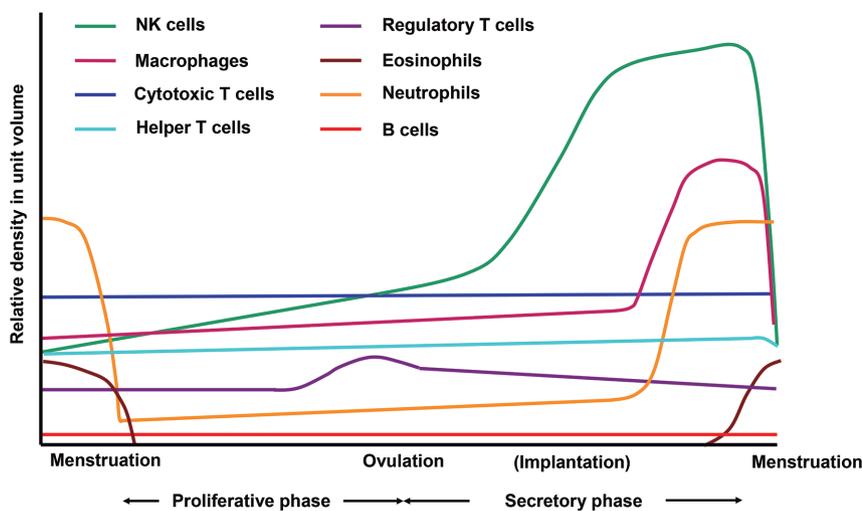
### Inflammation in endometrium with gynecologic pathological conditions

Studies have been accumulating the evidence that such physiological endometrial inflammatory status is altered in the presence of certain gynecologic pathology. As embryos/blastocysts are semi-allografts (or allografts for host surrogate mothers) for maternal endometrial tissue, even subtle alterations in local mucosal immunity may have a negative impact on their implantation process.

### Uterine fibroids

Uterine fibroids (leiomyoma) are common estrogen-dependent benign tumors that arise in the myometrium in premenopausal women. The etiology and pathogenesis underlying the myometrial transformation remain unknown, although the involvement of genetic backgrounds, ovarian steroids, growth factors, and immunomodulators is implicated. Uterine fibroids cause a variety of pelvic and urogenital symptoms, including menorrhagia, menorrhagia, dyspareunia, metrorrhagia, dyschezia, and pollakisuria. The influence of uterine fibroids on embryo implantation has been controversial, but meta-analysis of clinical trials disclosed that it largely depends on the location of tumors (Pritts et al., 2009).

According to the localization of the major part of tumors, uterine fibroids are hysteroscopically categorized into three subtypes; (i) subserosal fibroids



**Fig. 2.** Fluctuation of leukocyte subpopulations in the human cycling endometrium. In the menstrual period, the endometrium is infiltrated by a large number of granulocytes such as neutrophils and eosinophils. These granulocytes are postulated to play a role not only in the destruction and scavenging of the old tissues, but also mucosal regeneration for the next menstrual cycle via synthesis and release of multiple angiogenic and growth factors. Granulocytes leave the tissue around the cycle day 5-6 and mononuclear cell subpopulations infiltrate the stromal compartment in synchrony with endometrial epithelial cell proliferation. The major leukocyte subpopulation in the proliferative phase is a subset of CD8+ cytotoxic T cells. Endometrial leukocyte composition drastically changes at the time of ovulation. Unique CD16neg CD56bright natural killer cells acutely start to increase in number on day 2 after the pituitary luteinizing hormone surge, along with smaller delayed rise of macrophages and neutrophils in the mid-to-late secretory

phase. If blastocyst implantation is successful in the uterine cavity, these leukocyte subpopulations further increase in the decidualizing endometrium in the first trimester pregnancy. By contrast, if implantation does not occur, these leukocytes peak in density during the late secretory phase and are shed with other endometrial cells in the menstruation period. Again, granulocytes accumulate a few days prior to the endometrial shedding.

(which extend 50% or more outside the myometrium without cavity distortions, (ii) intramural fibroids (which extend less than 50% outside the myometrium without any cavity distortions), and (iii) submucosal fibroids (which cause uterine cavity distortions) (Bajekal and Li, 2000). While subserosal fibroids did not affect the reproductive outcome, the presence of intramural or submucosal fibroids significantly reduced the clinical pregnancy and embryo implantation rate, and increased the miscarriage rate. In addition, surgical removal of subserosal or intramural fibroids did not improve the reproductive outcome, whereas hysteroscopic myomectomy of submucosal fibroids appears to increase the clinical pregnancy and embryo implantation rate (Pritts et al., 2009). By contrast, the influence of the size and number of the intramural fibroid on endometrial receptivity remains to be determined, although a retrospective study demonstrated that these parameters in intramural fibroids did not affect embryo implantation rate and pregnancy course following transfer of the embryos/blastocysts obtained in an oocyte donation program (Horcajadas et al., 2008).

The endometrium adjacent to the submucosal or intramural fibroids contains an unusually high density of CD68+ macrophages throughout the menstrual cycle (Miura et al., 2006; Kitaya and Yasuo, 2010a). CCL2, a specific chemoattractant for macrophages, and prostaglandin F2 $\alpha$ , a strong uterine contracting compound, are expressed at higher level in the submucosal and intramural fibroids than in the subserosal fibroids and the intact myometrium. These soluble factors derived from nearby fibroids are likely to be involved in macrophage migration into the local endometrium and induction of uterine contraction and peristalsis that can negatively affect the blastocyst implantation process (Miura et al., 2006). Furthermore, the mid-secretory phase endometrium close to uterine fibroids contains lower density of natural killer cells than those away from fibroid tissues or without uterine fibroids (Kitaya and Yasuo, 2010a), suggesting that the unstructured myometrial microvasculature in the fibroid uterus may impede the selective migration of natural killer cells, the minor lymphocyte subpopulation in peripheral blood, from tissue microcirculation (Aitken et al., 2006; Kitaya et al., 2007).

Meanwhile, in the endometrium lining over submucosal fibroids, the expression of some immunosuppressive molecules such as progesterone-associated endometrial protein, IL-10, and leukemia inhibitory factor are reported to be lower than in the endometrium covering the intact myometrium (Ben-Nagi et al., 2010; Hasegawa et al., 2012). Progesterone-associated endometrial protein is a soluble angiogenic and immunosuppressive glycoprotein that appears in endometrial epithelial cells during the secretory phase in response to progesterone stimulation (Dell et al., 1995). IL-10 is produced mainly by endometrial epithelial cells and plays a protective role for blastocysts by counteracting pro-inflammatory cytokines such as

interferon- $\gamma$  or tumor necrosis factor (TNF)-alpha and inducing human leukocyte antigen-G on extravillous trophoblasts (Chaouat et al., 1995; Kitaya and Yasuo, 2013a). Leukemia inhibitory factor is a glandular epithelium-derived glycoprotein indispensable for endometrial decidualization mediated through signal transducer and activator of transcription 3 (STAT3) pathways that regulates epithelial junctional integrity and stromal proliferation and differentiation (Stewart et al., 1992; Pawar et al., 2013). Indeed, studies showed that an early rise of serum IL-10 concentration following IVF-ET and high endometrial expression of leukemia inhibitory factor in the mid-secretory phase have a significant positive correlation with high clinical pregnancy rate in infertile women (Wu et al., 2001; Mariee et al., 2012). Given the unique properties of these molecules, embryo receptivity seems to be impaired in the endometrium overlying the submucosal fibroids.

It remains unclear how intramural fibroids affect nearby endometrium. It is noteworthy that a large amount of transforming growth factor (TGF)-beta is synthesized and secreted by the fibroid tissue. TGF-beta plays a central role in growth and differentiation of leiomyoma cells as well as tissue fibrosis in an autocrine manner via regulation of multiple genes associated with immunity, angiogenesis, and extracellular matrix turnover (Dou et al., 1996). TGF-beta is also actively involved in endometrial tissue remodeling. While TGF-beta is capable of stimulating trophoblast attachment to the endometrium by enhancing production of fibronectin (Feinberg et al., 1994), it inhibits trophoblast invasion via promotion of cell adhesiveness to extracellular matrices in the decidualizing endometrium (Irving and Lala, 1995). These apparently contradictory findings imply a complicated role of this cytokine in blastocyst implantation. Although its clinical impact is undetermined, local high TGF-beta production by fibroid tissue may affect embryo implantation process.

Meanwhile, a genome-wide analysis employing complementary DNA microarray found the dysregulated expression of 69 genes in the endometrium with various sizes of intramural fibroids. However, only three out of 25 genes associated with endometrial receptivity (progesterone-associated endometrial protein, glutathione peroxidase 3, and aldehyde dehydrogenase 3 family member B2) were downregulated in patients with a single intramural fibroid with 5 cm or greater diameter undergoing IVF-ET using donated oocytes (Horcajadas et al., 2008). Further clinical and molecular studies are required to clarify the influence of the location, size, number, and distance of fibroid tissue on endometrial receptivity and blastocyst implantation.

### **Adenomyosis**

Adenomyosis is defined as ingrowth and invasion of endometrial glands and surrounding stromal compartments in the hypertrophic and hyperplastic myometrium, leading to diffuse uterine wall thickening.

## *Endometrial inflammation in embryo implantation failure*

Similar to uterine fibroids, adenomyosis exhibits estrogen-dependent growth and development in pre- and peri-menopausal women and causes several gynecologic symptoms. Meanwhile, adenomyotic tissues are often scattered in the myometrium with less clear boundaries than uterine fibroids. It is thereby hard to achieve the complete resection of the lesions without impairing uterine anatomy and functions. Long-term hormonal therapies including progestins and gonadotropin-releasing hormone (GnRH) agonists, which are generally used for treatment of adenomyosis in peri-menopausal women, are hard to be introduced to women desiring a pregnancy due to ovulation suppressive effects (Tomassetti et al., 2013). Clinical management of adenomyosis occurring in infertile women is therefore often challenging (Campo et al., 2012).

In baboons, there is a retrospective study supporting the relationship between adenomyosis and infertility (Barrier et al., 2004). These researchers obtained 37 cases with spontaneous adenomyosis and 37 controls with normal uterine histology that were selected randomly from a necropsy record database. The female baboons with spontaneous adenomyosis are strongly associated with lifelong infertility, regardless of concomitance or absence of endometriosis. Despite that there was no matching of the confounding factors between the cases and controls, the strength of this study is that a uniform examination was introduced to all uterine samples.

On the other hand, the association between adenomyosis and infertility is not conclusive in humans, although some studies explored its clinical impact on endometrial receptivity and blastocyst implantation. Maubon et al. (2010) first described the close relationship between adenomyosis and implantation failure. They found that junctional zone thickening on magnetic resonance imaging (an average thickness more than 7 mm and maximal thickness superior to 10 mm), the cardinal finding of adenomyosis, is significantly associated with a reduction in implantation rate in an IVF-ET program, independently from infertility etiology or patient age. Mijatovic et al. (2010) compared the reproductive outcome following IVF-ET between infertile women suffering from endometriosis and adenomyosis and infertile women with endometriosis and normally appearing uterus. Endometriosis was diagnosed by laparoscopy, whereas adenomyosis was detected on ultrasound. When a GnRH agonist ultralong protocol, one or two fresh cleavage stage embryo transfer, and luteal support with vaginal progesterone suppository was adopted for these women, there were no significant differences in the fertilization, embryo implantation, miscarriage, and ongoing pregnancy rate between the two groups. Martínez-Conejero et al. (2011) also reported the reproductive outcome in infertile women with laparoscopically-diagnosed endometriosis and sonographically or magnetic resonance-detected adenomyosis, infertile women with endometriosis alone, and women with unexplained infertility. In the hormone

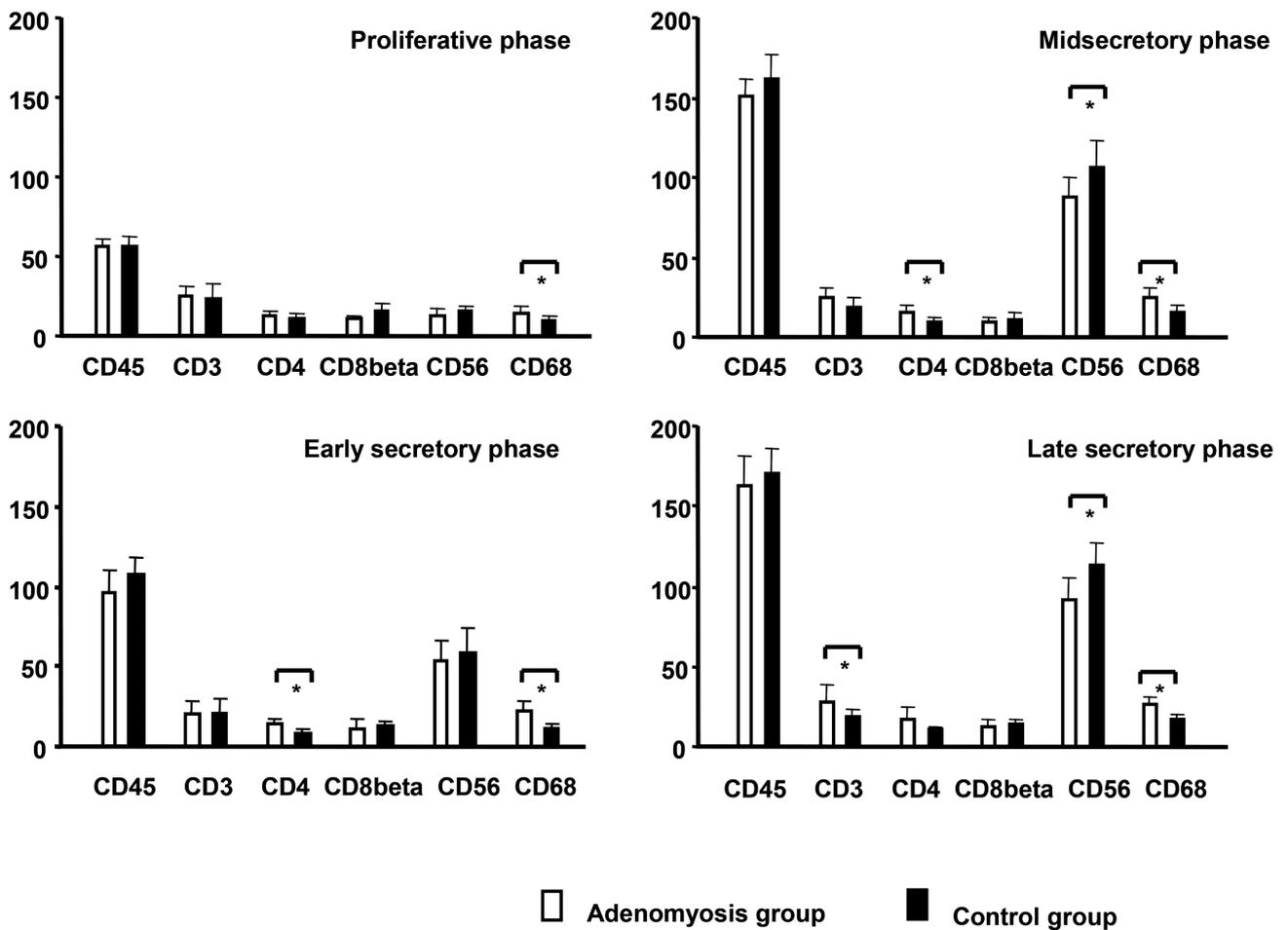
replacement cycles using oral estradiol administration and intramuscular progesterone injection, fresh transfer of one or two day 2/3 cleavage stage embryos or day 4/5 blastocysts were performed using the donated oocytes obtained from healthy volunteers undergoing GnRH agonist long protocol. Although miscarriage rate and live birth rate were lower in the adenomyosis/endometriosis group compared with the other groups, fertilization, embryo implantation, and ongoing pregnancy rate were comparable among the three groups. Thus, the authors concluded that adenomyosis has a potentially negative impact on post-implantation process, but not on implantation itself, although the age of the patients was younger and the number of the past treatment cycles was smaller in the endometriosis alone group than in the other two groups in this study. By contrast, Thalluri and Tremellen (2012) compared the pregnancy outcome following IVF-ET in infertile women with adenomyosis versus intact myometrium using a fixed GnRH antagonist protocol combined with oocyte maturation triggering with GnRH agonist and human chorionic gonadotropin for, day4/5 fresh and/or frozen-thawed blastocyst transfer, and luteal support with oral estradiol and vaginal progesterone supplementation. The clinical pregnancy and embryo implantation rate were significantly lower in infertile women with sonographically-detectable adenomyosis than those without adenomyosis. Taken together, these findings implicate that, to improve the local microenvironment for blastocyst implantation in patients with adenomyosis, GnRH agonist protocol may be superior in an IVF-ET cycle to GnRH antagonist protocol. Larger cohort studies are awaited to validate the accuracy of these findings.

The eutopic endometrium in women with adenomyosis shares various immunologic characteristics with those with uterine fibroids. For example, hyperactivated CD163+ macrophages invade the stromal areas of the eutopic endometrium in women suffering from repeated implantation failure and severe diffuse adenomyosis (Tremellen and Russell, 2012). Additionally, an increase in eutopic endometrial gamma-delta T cell density was reported in infertile women with adenomyosis (Ota et al., 1996). However, the comprehensive studies detailing eutopic endometrial lymphoid subsets were deficient about infertile women with adenomyosis. Using immunohistochemistry and morphometry, we measured the density of mononuclear cells in the unit areas of the eutopic endometrial biopsy samples in women suffering from adenomyosis and repeated implantation failure, but not from endometriosis and/or uterine fibroids (Fig. 3). Consistent with previous studies, the density of CD68+ macrophages was significantly higher throughout the menstrual cycle in the adenomyosis group. Moreover, the density of endometrial CD4+ T cells was significantly higher in the early-to-mid secretory phase compared with the control group. On the contrary, the density of CD56+ natural killer cells in the mid-to-late

secretory phase endometrium was significantly lower in the adenomyosis group, suggesting that a decrease in local natural killer cells may provide the microenvironment that facilitates ingrowth and invasion of eutopic endometrial cells into the myometrium.

A genomic study using complementary DNA microarray (Martínez-Conejero et al., 2011) found 34 dysregulated genes in the endometrium of infertile women with adenomyosis, but these did not include any of endometrial receptivity-associated genes. On the

contrary, the protein expression level of the implantation markers such as leukemia inhibitory factor and its specific receptor CD118, IL-11, and homeobox protein HOXA10 was reported to be significantly lower in the midsecretory phase endometrium and/or uterine cavity flushing fluid in infertile women with adenomyosis than in those with fertile women with intact myometrium (Yen et al., 2006; Xiao et al., 2010; Fischer et al., 2011). Like leukemia inhibitory factor, IL-11 is a cytokine that utilizes STAT3 signaling pathways and plays a critical



**Fig. 3.** Endometrial mononuclear cell density in infertile women with adenomyosis and a history of repeated implantation failure (open bars) and fertile age- and body mass index-matched control women (closed bars). The samples were obtained by endometrial biopsy from 27 infertile women suffering from adenomyosis and a history of more than three cycles of repeated implantation failure, as well as 26 fertile women undergoing hysterectomy for benign ovarian tumors (n=22) or uterine prolapse (n = 4) under informed consent. Adenomyosis had been diagnosed on ultrasound and/or magnetic resonance. The cases with known endometriosis and uterine fibroids were excluded from the analysis. Paraformaldehyde-fixed paraffin-embedded endometrium was cut into sections and subjected to conventional hematoxylin and eosin staining for endometrial dating and immunohistochemistry for mononuclear cell subpopulations, including CD45 (pan-leukocytes), CD3 (pan-T cells), CD4 (helper T cell subset), CD8beta (cytotoxic T cell subset), CD20 (B cells), CD56 (natural killer cells), and CD68 (macrophages). According to the histologic dating criteria, the endometrium was subclassified into seven proliferative phase, five early secretory phase, seven mid-secretory phase, and eight late secretory phase in the adenomyosis/repeated implantation failure group, and seven proliferative phase, six early secretory phase, seven mid-secretory phase, six late secretory phase in the control group. The immunoreactive cells were counted in 10 nonoverlapping stromal areas in triplicate under a light microscope (x400 magnifications) by two independent observers. The data following Gaussian distribution were compared using two-tailed Student's t test and shown as mean (bars) and SD (whiskers). The density of CD20+ cells not following Gaussian distribution (data not shown, less than five cells in majority of the samples) was compared using nonparametric Mann-Whitney U-test.

role in induction of decidualization in mice (Robb et al., 1998). Meanwhile, the lack in HOXA10 gene causes female mice infertility via uterine malfunction (Satokata et al., 1995). Taken together, these results indicate the impaired endometrial inflammatory condition during the implantation period in the presence of adenomyosis.

On the contrary, the expression of IL-10 in eutopic endometrial epithelial cells with adenomyosis was found to be significantly higher than in those overlying the intact myometrium, which was in contrast to low IL-10 expression in the endometrium adjacent to uterine fibroids (Wang et al., 2009). TGF-beta exhibits an inhibitory effect on IL-10 secretion by endometrial natural killer cells (Eriksson et al., 2004). The discrepancy in endometrial epithelial IL-10 expression pattern between adenomyosis and uterine fibroids may be attributable to high TGF-beta production by fibroids, but not by adenomyosis lesions. Considering the immunosuppressive effects of IL-10 on tumor growth, IL-10 may contribute to the development of adenomyotic tissues in the myometrium. In addition, the expression level of the extracellular matrix degrading enzymes (matrix metalloproteinase-2 and -9), IL-6, IL-8 receptors (CXCR1 and CXCR2), and vascular endothelial growth factor (VEGF) are elevated in the endometrium with adenomyosis compared with intact endometrium (Li et al., 2006; Ulukus et al., 2006; Yang et al., 2006). Recently, it was underscored that a combination of antibiotics and glucocorticoid therapy reduced matrix metalloproteinase-2 and -9 activities in uterine cavity flushing fluid and improved reproductive outcome in patients suffering from repeated implantation failure following two or more cycles of IVF-ET treatment (Yoshii et al., 2013), implying a deleterious role of these enzymes on endometrial receptivity. Moreover, VEGF concentration in the uterine cavity flushing fluid obtained during the mid-secretory phase was reported to be significantly lower in women with unexplained infertility compared with fertile women. Additionally, solubilized endometrial VEGF or recombinant VEGF are capable of enhancing blastocyst outgrowth and increasing endometrial epithelial cell adhesive properties in an animal model (Hannan et al., 2011). These findings suggest that VEGF release into the uterine cavity by endometrial epithelial cells during the implantation period may play some essential role in embryo implantation process.

### Chronic endometritis

Chronic endometritis is an unusual inflammatory condition localized to the endometrium. Chronic endometritis is histopathologically characterized by edema in the superficial layer, increased stromal cell density, dissociated maturation between the epithelium and stroma. The finding unique to chronic endometritis is, however, unusual infiltration of plasmacytes and B cells (Kitaya and Yasuo, 2011). These antibody-producing lymphocytes not only amass the endometrial

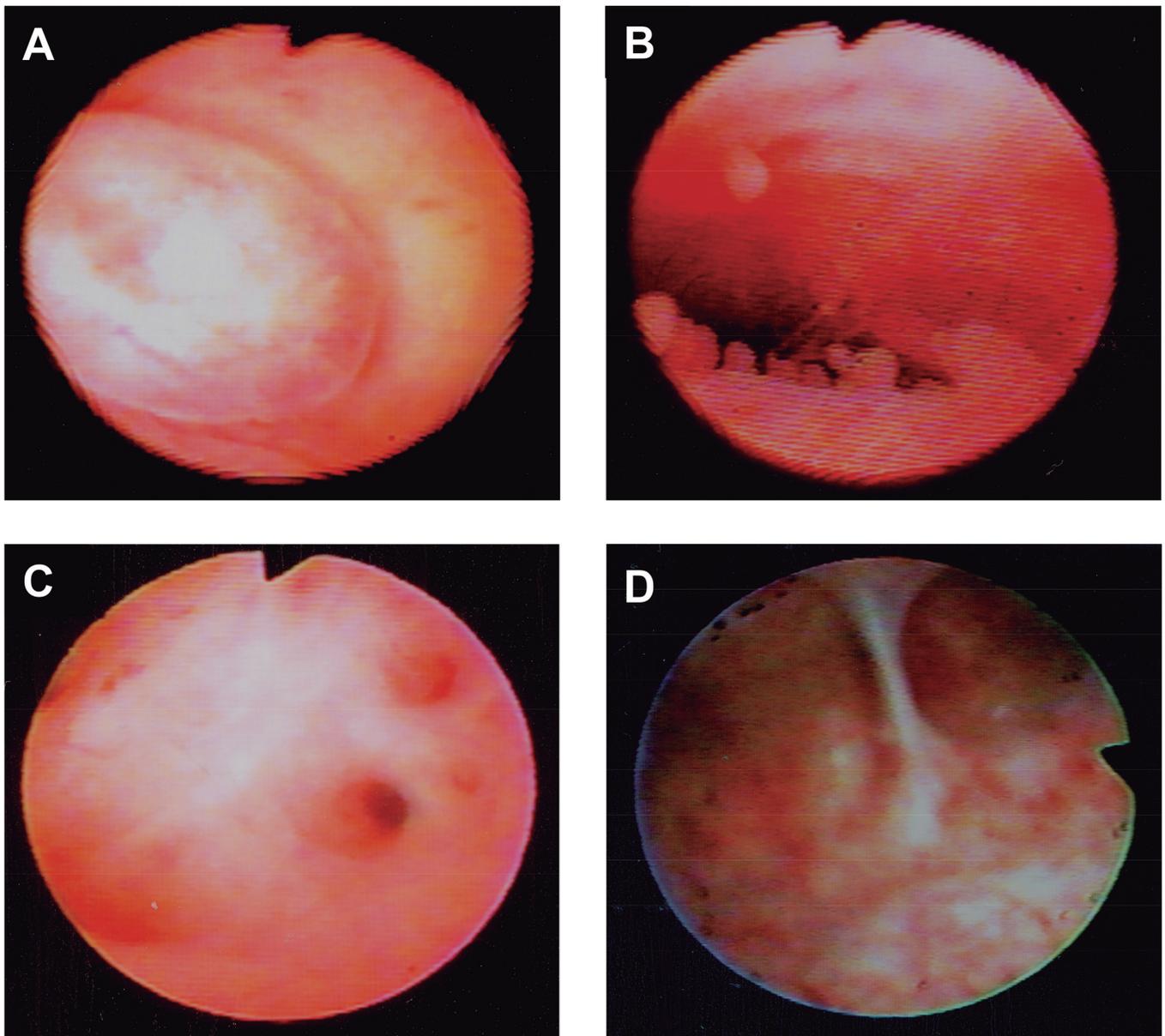
stromal areas, but occasionally invade the gaps among epithelial cells and further advance into the glandular lumina (Kitaya and Yasuo, 2010b). The major cause of chronic endometritis is microbial infections by common bacteria (*Streptococci*, *Escherichia coli*, and *Enterococcus faecalis*) and mycoplasma species (*Mycoplasma genitalium* and *Ureaplasma urealyticum*), which are frequently detectable in the endometrium with chronic endometritis. This microbial infection theory is reinforced by the fact that the antibiotic therapies targeting these microorganisms such as doxycycline are successful to eradicate endometrial stromal plasmacytes in chronic endometritis. Instead, the antibiotics against *Chlamydia Trachomatis* and/or *Neisseria gonorrhoeae* alone are insufficient to treat chronic endometritis (Kitaya et al., 2012b; Wiesenfeld et al., 2012).

Subtle manifestations, time-consuming histopathologic examinations, and nonmalignant pathology hindered the progress of basic and clinical researches on chronic endometritis. An advance in immunohistochemical approach for chronic endometritis, however, facilitated the detection of endometrial stromal plasmacytes in this pathology. CD138 (also known as syndecan-1) is a transmembrane heparan sulphate proteoglycan which is expressed in endometrial stromal plasmacytes but not in other endometrial stromal cell types. Several clones of anti-CD138 monoclonal antibodies were found to be available to diagnose chronic endometritis in formalin-fixed paraffin-embedded endometrial tissue specimens (Bayer-Garner et al., 2004; Kitaya and Yasuo, 2013b). Using immunostaining for CD138, recent studies have disclosed that chronic endometritis is a common pathologic entity frequently seen in the endometrium of women suffering from infertility with unexplained etiology, repeated implantation failure following IVF-ET, and recurrent miscarriages (Johnston-MacAnanny et al., 2010; Kitaya, 2011; Kitaya et al., 2012b). In addition, women with latent chronic endometritis are reported to be at a high risk for infertility in the future (Wiesenfeld et al., 2012).

In contrast to massive accumulation of plasmacytes and B cells in the chronic endometritis lesions, the density of T cells, macrophages, and neutrophils was at a similar level between infertile women with chronic endometritis versus without this mucosal inflammation (Disep et al., 2004; Kitaya and Yasuo, 2010b), indicating the involvement of the B cell lineage-specific pro-inflammatory molecules in the formation and development of the lesions. Interestingly, while the natural killer cell density in the secretory phase endometrium was comparable between chronic endometritis and non-chronic endometritis cases, that in the proliferative phase endometrium was significantly higher in chronic endometritis cases, particularly in the co-existence of endometrial micropolyps, than in non-chronic endometritis cases (Kitaya and Yasuo, 2010b; Kitaya et al., 2012b), although the significance of these findings remains to be elucidated.

We recently found that endometrial microvascular endothelial cells in patients with chronic endometritis unusually express selectin E, which is an adhesion molecule that plays a central role in initial contact between circulating B cells and endothelial cells. In addition, the chemokines CXCL13 (which specifically activates B cell migration) and CXCL1 (which is involved in B cell migration) are aberrantly localized in the microvascular endothelium and glandular epithelium, respectively (Kitaya and Yasuo, 2010b). Lipopoly-

saccharide derived from anaerobic Gram-negative rods was capable of inducing abnormal expression or secretion of these molecules by human uterine microvascular cells and epithelial cells in an in vitro culture system. These findings are consistent with the idea that microbial infection triggers local inflammatory responses which allow the extravasation of circulating B cells into the endometrial stromal areas and attract these extravasated B cells further to the glandular epithelial areas. Moreover, these B cells may potentially invade



**Fig. 4.** Representative hysteroscopic images of intrauterine lesions in infertile women. Endometrial macropolyps (A) are often predictable with other infertility screening examinations, including transvaginal ultrasound, sonohysterogram, and hysterosalpingography, whereas endometrial micropolyps (B) are small protrusions 1 to 2 mm in diameter which are detectable only with hysteroscopy. In Asherman's syndrome following uterine surgery (C), white fibrous connective tissues occupy and obliterate the uterine cavity. In milder cases, synechiae (D) is formed across the cavity.

the basement membrane of the epithelial layer or differentiate in situ into plasmacytes within the endometrial interstitial areas (Kitaya et al., 2013). In chronic endometritis, lipopolysaccharide is likely to be one of the key factors that affect the endometrial receptivity negatively, as poor pregnancy outcome in an IVF-ET cycle was demonstrated in infertile women with a high level of lipopolysaccharide ( $> 200$  pg/mL) in menstrual effluent (Kamiyama et al., 2004).

Moreover, studies demonstrated the biased gene expression profiles in the endometrium with chronic endometritis (Di Pietro et al., 2013). For instance, the gene transcript expression of several immunomodulators associated with secretory change and decidualization of the endometrium such as IL-11, CCL4, insulin-like growth factor 1, and caspase 8 is reduced in the chronic endometritis lesions (Robb et al., 1998; Kitaya et al., 2003). Meanwhile, the genes involved in cell proliferation and survival such as apoptosis-inhibiting proto-oncogenes B-cell lymphoma-2 (BCL-2) and Bcl-2-associated X protein are up-regulated in the endometrium with chronic endometritis, which corresponds with the findings that the secretory phase endometrium with chronic endometritis occasionally exhibits the morphological appearance of the proliferative phase with delay of two or more days by endometrial histological dating (Kitaya and Yasuo, 2010b), implicating the presence of a time lag between implanting blastocysts and endometrium with chronic endometritis. Of note, chronic endometritis is frequently concomitant with endometrial micropolyps, which are small lesions detectable only by hysteroscopy and often present the morphological appearance of the proliferative phenotype (Fig. 4A) (Cicinelli et al., 2005; Kitaya et al., 2012b). These findings support the possibility that chronic endometritis interferes with the implantation process with the stagnated differentiation of the endometrium.

In search of a more convenient diagnostic tool for chronic endometritis, a more recent study found significantly higher concentration of IL-6, IL-1beta, and TNF-alpha in the menstrual blood of women with chronic endometritis than in those without this pathology (Tortorella et al., 2014). According to logistic regression analysis, they proposed that serum IL-6/TNF-alpha concentration ratio may serve as a potential predictor of chronic endometritis, although further investigations are required to confirm these findings.

### **Endometrial polyps**

Endometrial polyps are pathological protrusions in the uterine cavity that are diagnosed with hysteroscopic and histopathologic examinations (Fig. 4B). Endometrial polyps occurring in women of reproductive age basically possess a benign nature, but confirmed and potential malignant transformation is identifiable in 1.7% of them. Symptomatic vaginal bleeding is notably associated with an increased risk of malignant endometrial polyps (Lee

et al., 2010).

In the literature, there have been some variances in the prevalence of endometrial polyps in infertile patients. While some studies identified endometrial polyps in 6% of infertile women who proceed to an IVF-ET program in the subsequent cycle (Fatemi et al., 2010), others reported that endometrial polyps were found in around 25% of infertile women with unexplained etiology (Hatasaka, 2011). Although the cause-effect relationship between endometrial polyps and infertility has not been confirmed, a systematic review demonstrated the beneficial effects of hysteroscopic removal of endometrial polyps on subfertile women. Endoscopic surgery significantly improved the clinical pregnancy rate in the subsequent intrauterine insemination cycle compared with treatment with a combination of diagnostic hysteroscopy and polyp biopsy (Bosteels et al., 2013).

Endometrial polyps are thought to arise and develop under the influence of an estrogen-dominant environment. This idea is supported by the findings that glandular epithelial cells in the polypoid endometrium express a higher level of estrogen receptor- $\beta$  and aromatase, the enzyme that converts testosterone to estradiol (an active-form estrogen), than those in the naturally cycling endometrium (Maia et al., 2006; Lopes et al., 2007). In addition, glandular epithelial cells and stromal fibroblasts in the polypoid endometrium express a higher level of BCL-2 than in those in the autologous nonpolypoid counterpart, suggesting that endometrial polypoid cells acquire the genotype that can survive shedding during menstruation (Taylor et al., 2003).

Cellular immunity in endometrial polypoid lesions is summarized as intensive infiltration of mast cells in the polypoid lesions, along with regulatory T cell invasion (El-Hamarneh et al., 2013). The density of mast cell subsets is higher in the endometrium with endometrial polyps than in the autologous nonpolypoid endometrium and heterogeneic control endometrium. In addition, a majority of mast cells infiltrating endometrial polyps display degranulated morphological appearance, suggesting the release of the cytoplasmic contents such as histamine, heparin, and tryptase. Furthermore, not only mast cells in the polypoid lesions, but also those infiltrating the surrounding endometrium express CD117, a tyrosine-protein kinase receptor for stem cell factor, a cytokine that is capable of stimulating mast cell migration, aggregation, proliferation, and viability. Experimental animal studies found that pharmacological depletion of mast cells from the polypoid lesions leads to remission of existing polyps, indicating a central role of mast cells in the formation of polyps (Gounaris et al., 2007). These findings explain the possibility that the repetitive recurrence of endometrial polyps following surgical resection is attributable to residual CD117+ mast cells in the surrounding endometrium, promoting regeneration and reorganization of local protrusive lesions.

Regulatory T cells are CD3+, CD4+, CD25+, and

Foxp3<sup>+</sup>-lymphocyte subpopulation involved in immunomodulation and self-antigen tolerance. In the normally cycling endometrium, the density of regulatory T cell subsets increases in the proliferative phase and markedly falls in the secretory phase (Berbic et al., 2010). By contrast, regulatory T cells prevail in the secretory phase endometrium with polyps, suggesting the possible negative effects of these lymphocytes on blastocyst implantation (El-Hamarneh et al., 2013). It should be clarified whether regulatory T cells infiltrating endometrial polyps cooperate with mast cells in the growth and development of the local lesions or exert immunosuppressive effects to compensate the unfavorable inflammatory microenvironment.

Endometrial polyps also highly express IL-1 $\beta$ , interferon- $\gamma$ , TGF- $\beta$ , VEGF, and matrix metalloproteinase-2 and -9 in the lesions (Inagaki et al., 2003; Mollo et al., 2011). These mediators are also the contributory factors to endometrial breakthrough bleeding by degrading the components of the vascular basement lamina. Of note, interferon- $\gamma$  is one of the type 1 helper T cytokines that potentially hampers the implantation process via modulation of metalloproteinase activity and inhibition of embryo outgrowth in mice (Fontana et al., 2010). By contrast, the expression level of the embryo implantation-associated genes, such as TNF- $\alpha$ , osteopontin, and insulin-like growth factor-binding protein-1 is lower in endometrial polyps than in the intact endometrium (Salamonsen and Woolley, 1996; von Wolff et al., 2001; Gnainsky et al., 2010). Such aberrant immunologic properties seem to underlie the association of endometrial polyps with subfertility and miscarriage in premenopausal women, as endometrial polypectomy was shown to improve these abnormal local cytokine profiles and pregnancy outcome (Ben-Nagi et al., 2009b).

### **Hydrosalpinx**

Hydrosalpinx is recognized as a unilateral or bilateral fallopian tube(s) being distally blocked and filled with serous fluid, blood, and/or pus. The fallopian tube containing blood component is referred to as hematosalpinx, whereas the tube filled with pus is referred to as pyosalpinx. As the fluid volume increases and the tubal walls are distended, it appears as “sausage” shapes on diagnostic imaging such as ultrasound or magnetic resonance (Kim et al., 2009). Hydrosalpinx mostly originates from pelvic inflammatory diseases, although it also arises potentially from endometriosis, peritubal adhesions, tubal cancer, and tubal pregnancy. Under physiological conditions, tubal fluid is transported to the fimbria by the regular movement of the endosalpinx, the group of cilia lining the inner wall of the fallopian tube, and emptied into the abdominal cavity to undergo peritoneal clearance. When local inflammatory responses reach into and around the endosalpinx and induce adhesion and occlusion of the fimbrial ends and the fluid occupation in the tubal cavity,

the dilated tubal walls eventually lose their integrity and form thin and translucent fibrous scar (Eytan et al., 2001). A prospective randomized controlled trial has proved the close relationship between hydrosalpinx and poor IVF-ET outcome (Strandell et al., 1999). Treatment of hydrosalpinx with salpingectomy, ultrasound-guided sclerotherapy, and proximal tubal blockade (Essure device, Conceptus Inc, Mountain View, CA, USA) improves the pregnancy outcome following IVF-ET (Surrey and Schoolcraft, 2001; Jiang et al., 2010).

It is still controversial whether the congested fluid in hydrosalpinx poses harm to the embryos that are cleaving and passing through the fallopian tube. The chemical composition analysis disclosed that bicarbonate concentration and sodium/potassium ratio were significantly higher in the tubal fluid of hydrosalpinx than in the synthetic human tubal fluid IVF media, whereas the concentrations of potassium, calcium, glucose, and lactate were significantly lower. In regard to pro-inflammatory molecules, the concentrations of interferon- $\gamma$  and epithelial growth factor, were similar between the hydrosalpinx fluid and culture media (Chen et al., 2002). Meanwhile, the expression level of IL-2, IL-8, IL-12, IL-1 $\alpha$ , TNF- $\alpha$ , TGF- $\beta$ 2, and granulocyte macrophage colony-stimulating factor was significantly higher both in the endometrium and fallopian tube with hydrosalpinx than in those without this pathology (Copperman et al., 2006), implicating the potential involvement of multiple cytokines in formation of local inflammation seen in hydrosalpinx (Strandell et al., 2004).

The endometrial expression pattern of several implantation-associated cytokines and adhesion molecules such as leukemia inhibitory factor, antimicrobial elastase inhibitor elafin, selectin L ligands, integrin  $\alpha$ -v  $\beta$ -3, and HOXA10 in patients with hydrosalpinx is very different from those without it (Daftary et al., 2007; Zhong et al., 2012). Furthermore, the endometrial glands and surface epithelium of infertile women with hydrosalpinx were found to be surrounded by unusual numerous lymphoid aggregates, mainly consisting of CD8<sup>+</sup> T cells expressing CD56 and granzyme B as well as CD20<sup>+</sup> B cells in the stromal compartment (Ito et al., 2004). Salpingectomy normalized the expression of some of these endometrial proteins and the density of the endometrial lymphoid aggregates gradually over three to five menstrual cycles (Ito et al., 2004, Neto et al., 2014). These findings support the idea that the negative effect of hydrosalpinx on blastocyst implantation is, at least in part, mediated through disruption of endometrial receptivity via the spread of unfavorable immune responses from the adjacent tubal lesions and tubal fluid influx.

### **Scarred endometrium following uterine surgery**

With a worldwide increase in cesarean section delivery, scar tissue formation in the endometrium at the incision sites on the uterine anterior wall is emerging as

a risk factor of secondary infertility (Hemminki, 1986). It was recently shown that a past history of cesarean section delivery potentially affects the location of the implantation sites in the subsequent pregnancy (Naji et al., 2013). While the gestational sack is mostly visualized at the uterine fundal part during the first trimester of gestation in multiparous pregnant women undergoing vaginal delivery, it was detected most frequently at the uterine posterior wall in pregnant women undergoing previous cesarean section delivery.

Asherman's syndrome is the advanced form of the traumatically scarred endometrium, causing the adhesion and obliteration of the uterine cavity and/or cervical canal (Fig. 4C). Asherman's syndrome occurs not only following surgical intervention such as curettage, myomectomy, endometrial ablation, and cesarean section, but also following intrauterine device insertion and irradiation therapy. In this pathology, histological boundaries between the functional and basal layer of the endometrium become unclear. The functional layer is often covered by an inactive epithelial cell monolayer that does not respond to ovarian steroids. Endometrial glands are also lined by hormone-unresponsive cuboidal-columnar epithelium and appear sparse and thin. The stromal compartment is broadly replaced by fibrous avascular areas that are occasionally accompanied by calcification and ossification (Yu et al., 2008). Intracavitary partial adhesive structures, also referred to as synechiae, are frequently formed across the uterine cavity (Fig. 4D). It was reported that 43% (802 out of 2151 cases) of patients with Asherman's syndrome suffer from infertility (Schenker and Margalioth, 1982). The adhesive occlusion and obstruction of the tubal ostia, uterine cavity, and/or cervical canal are the main reasons that interrupt sperm mobilization, as well as blastocyst implantation.

As aforementioned, the density of natural killer cells, macrophages, and neutrophils in the normally-cycling endometrium markedly increase during the secretory phase. On the contrary, this postovulatory rise of leukocytes is lacking in the scarred endometrium around the incision site of cesarean section (Ben-Nagi et al., 2009b) and in Asherman's syndrome (unpublished observation). The mechanisms underlying the failed post-ovulatory leukocyte increase in the endometrium with cesarean scar remains unknown. Other characteristics of the scarred endometrium following caesarean section include impaired microvascular organization, defective endometrial re-epithelization, and delayed morphological change in the secretory phase (Jauniaux and Jurkovic, 2012). Given that multiple angiogenic factors are produced by endometrial natural killer cells and neutrophils (Mueller et al., 2000; Li et al., 2001), the deficit in these mucosal leukocytes may be attributed to the poor local vasculature and tissue reconstruction seen in the scarred endometrium. These anatomical and histological alterations may result in compromised decidualization and impaired junctional zone contractility associated with secondary infertility.

In addition, endometrial natural killer cells are likely to play a pivotal role in the regulation and monitoring of the trophoblast invasion in the implantation process. Studies suggest that the deficiency in endometrial natural killer cells allows uncontrolled trophoblast migration at the fetal-maternal interface, which potentially leads to abnormal placentation and obstetrical complications in the mid-to-late pregnancy (van Beekhuizen et al., 2010).

## Conclusions

In spite of accumulating evidence supporting the relevance of the immunologic factors to infertility, the diagnostic and therapeutic strategies remain unestablished. Several immunotherapies, including glucocorticoids, aspirin, heparin, paternal leukocyte injection, and intravenous immunoglobulin administration have been introduced empirically to prevent subsequent implantation failure in an IVF-ET program. Nevertheless, randomized controlled trials have failed so far to demonstrate the effectiveness of these immunotherapies on the improvement of reproductive outcome in infertile patients suffering from repeated implantation failure (Stephenson and Fluker, 2000; Urman et al., 2000; Stern et al., 2003; Polanski et al., 2014). Further well-designed studies warrant the feasibility of these therapies for a subgroup of infertile women with repeated implantation failure. Moreover, development of safe new treatment options is yet awaited for these patients.

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*Acknowledgements.* This study is supported by the Grant-in-Aid for Scientific Research (16790964 and 22591840) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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Accepted April 25, 2014