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# Possible Contribution of Circulating Blood Cells to Embryo Implantation

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## *Introduction*

It has been widely accepted that the endocrine system regulates endometrial differentiation to prepare for blastocyst implantation. To induce and maintain embryo implantation in the uterus, mammalian mothers utilize progesterone by constructing a new endocrine organ, corpus luteum (CL). Upon progesterone stimulation, the estrogen-primed endometrium is further differentiated, which is suitable for embryo implantation (1). In the uterus, direct cross-talking of the embryo and the maternal endometrium is considered necessary to achieve a subsequent successful implantation of the embryo (2). However, the precise mechanisms of the initial step of human embryo implantation remain unknown. Recently, accumulating evidence suggests that local immune cells at the implantation site actively contribute to embryo implantation (3, 4). In this article, we introduce new mechanisms by which circulating blood immune cells are involved in endometrial differentiation and embryo-maternal cross-talk.

## *Regulation of corpus luteum (CL) of pregnancy*

### Endocrinological regulation

In humans, CL function continues only for 14 days during each menstrual cycle. However, when pregnant, the implanting embryo secretes human chorionic gonadotropin (HCG). This hormone shares a receptor with luteinizing hormone (LH) and stimulates the function of CL of menstrual cycle to induce its transformation into CL of pregnancy and maintain embryo implantation. Accordingly, CL of pregnancy is an essential organ for the embryo implantation and it has been accepted that HCG is a major regulator of human CL of pregnancy (1).

However, several basic studies demonstrated that the level of binding of labeled HCG to the CL of pregnancy was lower than that to the CL of menstrual cycle (5, 6). In addition, there are many lines of clinical evidence to suggest that different mechanisms are also involved in regulation of human CL of pregnancy. For

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example, despite a high HCG level in blood, progesterone production by the CL decreases in patients with ectopic pregnancy or natural abortion. This can be explained as rapid and continuous increase in HCG concentration is necessary to maintain CL of pregnancy (7). However, such a high concentration is not required to activate LH/HCG receptor system. It should be noted that regulatory mechanisms for CL of pregnancy are completely different among mammals and chorionic gonadotropin is only detected in mare and primates (8). Therefore, many researchers have investigated essential mechanisms and/or hormones other than HCG. However, no soluble factor other than HCG has been identified and the precise regulatory mechanisms remain unknown (9).

**Immunological regulation**

To clarify regulatory mechanism(s), we focused on the molecules that are expressed in human CL of pregnancy. We found that several molecules such as HLA-DR and leukocyte functional antigen (LFA)-3/CD58, which mediate interaction with immune cells, were expressed on the luteal cell surface in the human CL of pregnancy (10, 11). In general, immune cells are considered to enhance CL regression (12). However, since these molecules appear during CL formation, it is speculated that interaction with immune cells plays some role in the functional and morphological transition from CL of menstrual cycle to CL of pregnancy.

At the initial step of implantation, the human embryo attaches to endometrial epithelial cells, and then invades the endometrium as a mass through the epithelial layer, becoming buried within endometrial stromal tissue within 8-9 days after ovulation. Thereafter, trophoblast invasion transiently slows down and the lacunar spaces, which will become the intervillous spaces, are formed within the trophectoderm layer. Maternal blood gently flows in these spaces and then returns to the maternal systemic circulation (13). At this stage, HCG produced by the trophectoderm can be transmitted to the ovary through the blood circulation, stimulating corpus luteum to produce progesterone via the HCG receptor. During formation of the lacunar spaces, maternal blood cells including peripheral blood mononuclear cells (PBMC: lymphocytes and monocytes) infiltrate here and these cells also return to the maternal systemic circulation.

Considering that the human CL expresses several molecules that can mediate direct interaction with T lymphocytes, we speculated that not only HCG, but also immune cells contribute to the systemic crosstalk between embryo and mother (ovary) via blood circulation. Accordingly, we hypothesized that signals from the developing embryo in the genital tract are transmitted to the ovary by not only the endocrine system, but also the immune system, in other words, via not only soluble factors, but also circulating cells (11).

To examine this hypothesis, we investigated the effects of PBMC derived from pregnant women in early pregnancy on progesterone production by luteal cells in culture. We found that PBMC derived from women in early pregnancy promoted progesterone production, suggesting that circulating blood immune cells in early pregnancy stimulate CL function (14). Based on these findings, we extended our hypothesis to the further concept that circulating immune cells transmit information about the presence of the developing embryo to various organs throughout the whole body and induce adequate functional change or differentiation in these organs to facilitate embryo implantation (15) (Fig. 1).

***Immunological regulation of endometrial differentiation in mice***

To investigate the above concept, we first examined the effects of circulating immune cells on endometrial differentiation and embryo implantation using mouse implantation experiments. When blastocysts were transferred

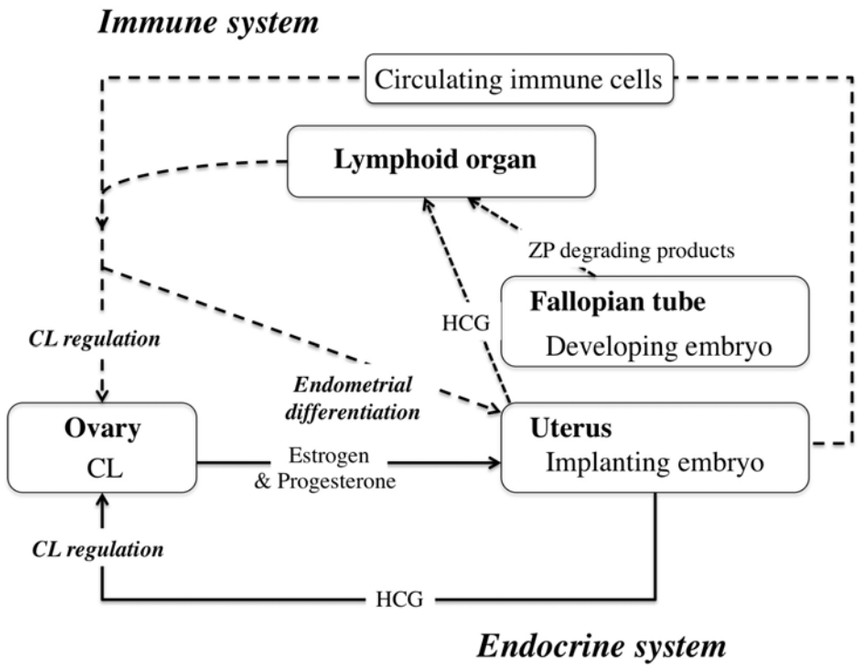


Fig. 1 : Proposed dual control of CL function and endometrial differentiation by the endocrine and immune systems.

The maternal immune system recognizes the presence of developing and implanting embryo in the Fallopian tube and the uterus by embryo- and species-specific signals such as degraded products of zona pellucida glycoprotein and/or HCG. Then, effector immune cells move to the ovary and the endometrium via blood circulation to regulate CL function and induce endometrial differentiation.

into the uterine cavity of pseudopregnant recipient mice that had been mated with vasectomized male mice, successful implantation was only achieved during 3-5 days after ovulation when the endometrium was adequately differentiated. This period is called the “implantation window” (16, 17). However, when spleen cells, stocked circulating immune cells, were obtained from pregnant day 4 mice and were administered to pseudopregnant mice, embryo implantation was induced prior to the implantation window (1-2 days after ovulation) when embryos cannot normally be implanted (18).

In order to examine the direct effects of the splenocytes on endometrial differentiation, we then used a delayed implantation model in which pseudopregnant mice were treated with daily progesterone supplementation following an oophorectomy on post-ovulatory day 3. In this model, in the absence of ovarian estrogen, embryos that are transferred into the uterine cavity remain floating in the luminal spaces, and the exogenous administration of estrogen induces expression of leukemia inhibitory factor (LIF) in the endometrium, promoting a restart of embryo implantation (19). Interestingly, instead of estrogen, intravenous administration of splenocytes derived from early pregnancy restarted embryo implantation along with induction of LIF expression (20). These results indicated that circulating immune cells could induce early endometrial differentiation that was necessary for subsequent embryo implantation. These findings also support a novel concept that endometrial differentiation just prior to

embryo attachment can be achieved by dual control of the endocrine and immune systems.

Notably, it was demonstrated that thymocytes from non-pregnant immature mice, especially CD8-negative population, could promote embryo implantation along with inducing LIF expression in the uterus. This indicates that there is a certain immune cell population that can induce endometrial differentiation and embryo implantation even in non-pregnant mice (21).

### *Immunological regulation of human endometrial receptivity*

To examine human endometrial receptivity, we then developed an attachment assay using a human choriocarcinoma-derived BeWo cell mass and a human endometrial epithelial cell monolayer culture. In this assay, high attachment rates were observed in endometrial culture derived from women in the mid-luteal phase, supporting the concept of an implantation window in human endometrial receptivity. Importantly, when these endometrial cells were co-cultured with autologous PBMC, attachment rates significantly increased in the culture derived from women in the late proliferative and early secretory phases, showing that autologous PBMC promote endometrial cell receptivity *in vitro* (22).

As a growing clinical problem in reproductive medicine, increasing attention has been paid to repeated implantation failure in infertile patients who had undergone in vitro fertilization (IVF) therapy. Unfortunately, no effective therapy had been developed. Based on this background, we developed a novel therapy using autologous PBMC. In this therapy, autologous PBMC are administrated into the uterine cavity prior to blastocyst transfer in order to induce endometrial differentiation that facilitates subsequent embryo implantation. We applied this treatment to patients who had experienced implantation failure in IVF therapy in a trial approved by the ethics committee of Kyoto University Hospital. As a result, PBMC treatment effectively improved pregnancy and implantation rates (23).

### *Possible mechanisms for maternal recognition of developing embryos by the immune system*

#### Effects of HCG

In order to transmit pregnancy-related signals to distant organs such as the CL and the endometrium, the immune system must recognize the presence of the embryo in the female genital tract. First, we focused on the pregnancy-specific hormone, HCG, which is secreted from the developing and implanting human embryo. In invasion assays using murine embryo and BeWo cells, PBMC derived from women in early pregnancy promoted murine trophectoderm and BeWo cell invasion more than those obtained from non-pregnant women. Importantly, when PBMC derived from non-pregnant women were incubated with HCG, HCG-treated PBMC promoted invasion more than non-treated PBMC (24, 25). These findings suggest that HCG can change PBMC functions to facilitate embryo implantation.

Several decades ago, HCG crudely purified from urine was reported to suppress immune reactions (26). However, it was later shown that highly purified HCG had no effect on lymphocyte function (27). Accordingly, the effects of HCG on immune cell function have been controversial for a long time. Recently, we found that recombinant-HCG enhanced IL-8 production by human monocytes at relatively high concentrations via activation of NF-κB. HCG shares a receptor with LH to commonly access the LH/HCG receptor. However, the so-called LH/HCG receptor was not detected on the cell surface of monocytes. Therefore, it was speculated that there was a different pathway besides the LH/HCG-R system, which could respond to high HCG concentration. HCG is an evolutionarily recent hormone that is present in primates (28). The

most important difference between LH and HCG is the abundance of sugar chains at the C-terminal of the HCG β-subunit. Notably, HCG-induced IL-8 production was inhibited by an exogenous excess of sugars, suggesting that HCG can regulate PBMC function through sugar chain receptors, which is a primitive regulatory mechanism in the immune system (29). It should also be noted that the sugar chains of purified HCG are largely cleaved before urine production (30).

A high concentration of HCG is produced at the embryo implantation site. It is well known that the initial change around the implantation site is an increase in vascular permeability, leading to recruitment of certain immune cells to the area. Recently, it was reported that human trophoblasts invading the implantation site produce hyperglycosylated HCG, and that the hyperglycosylated HCG up-regulates trophoblast invasion in humans (31). Therefore, it is reasonable to speculate that HCG stimulates endometrial immune cells to produce chemoattractants and vasodilators and that these cytokines in turn induce embryo invasion (15) (Fig. 2). It should also be attended that BeWo cells are derived from human choriocarcinoma. It was reported that particularly invasive choriocarcinoma produces hyperglycosylated HCG β-subunits (28). Considering that HCG-treated PBMC promote BeWo cell invasion, it is possible that hyperglycosylated HCG promotes choriocarcinoma invasion by stimulating adjacent immune cells at both primary and metastatic lesions (25) (Fig. 2).

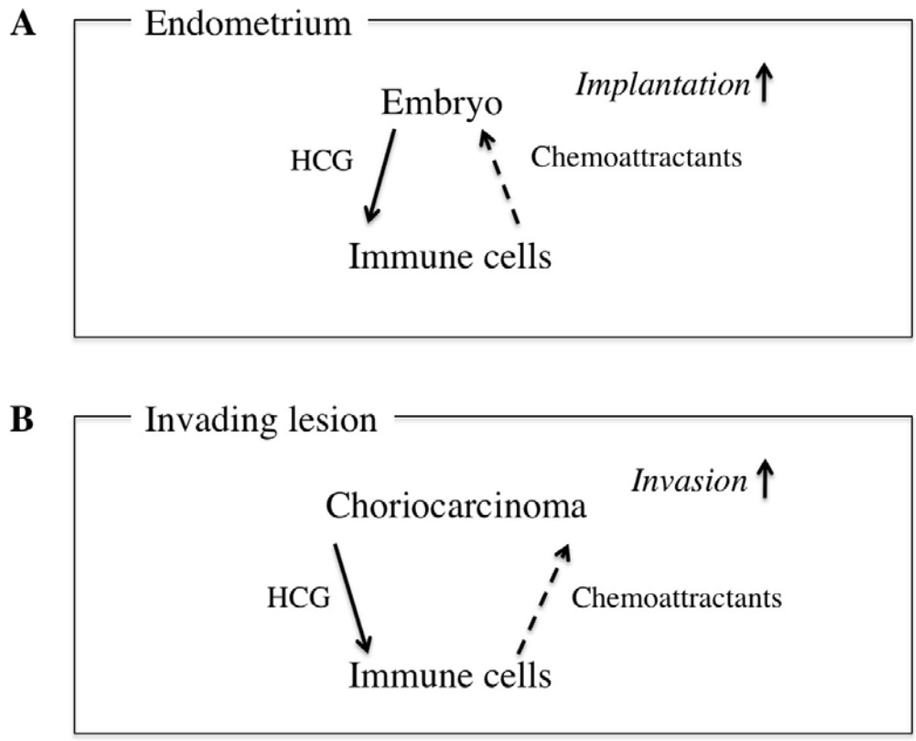


Fig. 2 : The invasion-inducing effects of HCG on the human embryo and choriocarcinoma via adjacent immune cells. (A) At the implantation site, HCG secreted from the implanting embryo stimulates endometrial immune cells to produce chemoattractants, and these cytokines then induce embryo invasion. (B) At the invading lesion, hyperglycosylated HCG secreted by choriocarcinoma promotes its own invasion by stimulating adjacent immune cells to produce chemoattractants.

### *Maternal recognition of the developing embryo in the Fallopian tube by the immune system*

The findings from mouse implantation experiments suggest that functional changes in the immune system have already occurred in the early stage of pregnancy when the developing embryo passes through the Fallopian tube. In order to accurately identify the embryo, the maternal immune system must distinguish non-self tissues belonging to the same species from those of other organisms. The immune system must also discriminate between developing embryos and unfertilized eggs. However, in the Fallopian tube, immune cells cannot directly interact with the embryo because the embryo is surrounded by the zona pellucida. Therefore, it is deduced that the developing embryo actively releases species-specific and embryo-specific factors into the Fallopian tube. However, it appears difficult for the embryo to produce a sufficient amount of such soluble factors to successfully activate the maternal immune system at such an early stage in its development.

Since it was shown that the sugar chains of the HCG effectively activate immune cells, we then paid attention to the zona pellucida that contains abundant glycoproteins. It is well known that the zona pellucida is composed of glycoproteins that mediate species-specific interaction between spermatozoa and oocytes (32). Accordingly, the zona pellucida can be considered an abundant store of species- and oocyte-specific glycoproteins. It should be noted that one of the most evident differences between the developing embryo and unfertilized oocytes is the active degradation of the zona pellucida. The zona pellucida of fertilized oocytes can be a target for acrosomal enzymes of sperm and cortical granules of oocytes (33, 34). In addition, developing embryos further degrade the zona pellucida in order to achieve hatching. Thus, in contrast to unfertilized eggs, degradation products of zona pellucida glycoproteins may be released from fertilized oocytes/developing embryos into the Fallopian tube. Accordingly, we speculate that mammals have developed the ability to utilize these glycoproteins, especially their sugar moieties, to transmit information about the presence of the developing embryo to the immune system in the female genital tract (35).

Taken together, we propose that degraded products of zona pellucida glycoprotein and HCG are important candidates for embryo- and species-specific signals for maternal recognition by the immune system (Fig. 1).

### *Clinical application*

Based on these findings, our protocol of PBMC treatment starts with the pre-incubation of autologous PBMC with HCG. Briefly, PBMC are isolated from patients and incubated for 2 days with HCG in order to activate PBMC. Thereafter, activated PBMC are administered into the uterine cavity to induce adequate endometrial differentiation. Three days later, blastocysts are transferred into the uterine cavity (Fig. 3). As described above, we applied this treatment to patients with 4 or more repeated failures in IVF therapy and found that PBMC treatment effectively improved pregnancy and implantation rates (23). Several mechanisms relevant to this procedure should be discussed. 1) PBMC may induce endometrial differentiation that facilitates embryo attachment. 2) Although PBMC are autologous cells from the patient, the induction of PBMC by themselves is expected to evoke favorable inflammatory reactions in the uterine cavity *in vivo*. 3) PBMC can secrete proteases that may effectively change the function or structure of surface molecules expressed on endometrial luminal epithelial cells. 4) PBMC can move from the uterine cavity toward the endometrial stromal tissue, creating a leading pathway for subsequent embryo attachment and invasion (23, 36). In accordance with these clinical

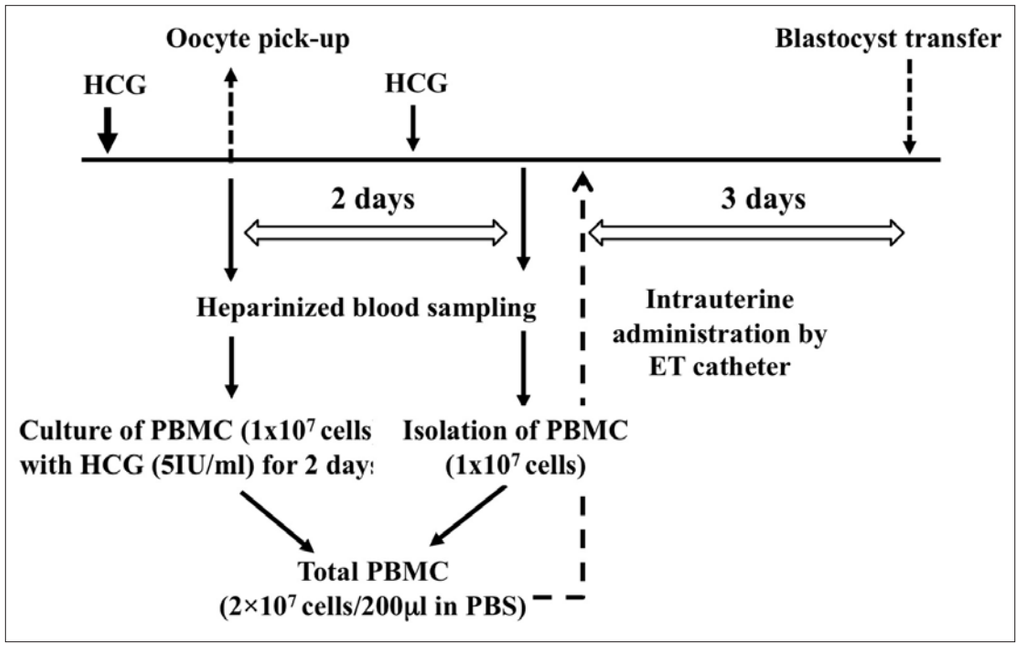


Fig. 3 : Protocol of autologous PBMC therapy for infertile patients receiving IVF-ET.
PBMC isolated from patients are incubated with HCG for 2 days in order to activate PBMC. Thereafter, activated PBMC and freshly isolated PBMC are combined and are administered into the uterine cavity to induce adequate endometrial differentiation. Three days later, blastocysts are transferred into the uterine cavity.

findings, we recently found that autologous PBMC treatment also increases pregnancy rates in cows (37). These findings suggest that the application of autologous PBMC is an effective therapy for infertile patients suffering from repeated implantation failures.

### *Conclusion*

In conclusion, we here introduced a novel mechanism for systemic crosstalk between mother and embryo by circulating immune cells. Through this mechanism, corpus luteum function, endometrial differentiation, and embryo invasion may be controlled. We also highlighted the possibility of a new cross-talk mechanism between endocrine and immune systems, from PBMC to CL or from HCG to PBMC. Importantly, when the endocrine mechanism does not adequately operate, alternative mechanisms involving the immune system can be utilized for infertility therapy. By clarifying the precise mechanisms for maternal recognition of the developing embryo by the immune system, we hope that more effective therapies using autologous PBMC can be developed in the future along with further improvement in the breeding of domestic animals.

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