THE MECHANISM OF EMBRYO IMPLANTATION: THE ROLE OF PIBF, IDENTIFYING THE COMPETENT EMBRYO

Doctoral (Ph.D.) dissertation theses

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INTRODUCTION

The incidence of infertility is increasing in developed countries, partly due to the postponement of the first pregnancies. Higher maternal age negatively affects both the condition of the endometrium and the quality of embryos [1]. The first step in achieving pregnancy is the implantation of the blastocyst into a receptive endometrium, during which the embryo adheres to the endometrial epithelium, implants, and the trophoblast invades the decidualized endometrial tissue [2]. Progesterone plays a crucial role in this process, mediated by progesterone receptors (PR-A and PR-B) [3]. PR-A is essential for decidual transformation of the endometrium and endometrial receptivity, while PR-B is involved in mammary gland development [4].

Implantation failure may be due to inadequate endometrial development, or to poor implantation capacity of the embryo.

Our research aims to investigate the immunological mechanisms influencing embryo implantation and to identify the embryo with good implantation capacity.

During a normal pregnancy, the maternal immune system recognizes but does not attack paternal antigens expressed by the foetus [5]. PIBF is present in various pregnancy-associated tissues (decidua, placenta, amnion) and in the serum, with serum concentrations increasing throughout pregnancy [6]. The phylogenetically conserved PIBF gene, produces 16 different mRNAs during transcription. The longest of these, contains 18 exons and codes for a 90 kDa protein [7]. Due to alternative splicing several smaller MW isoforms are produced. The full length PIBF is centrosome-associated [8], while the smaller isoforms are localized in the cytoplasm and secreted [9]. The small isoforms affect the immune response, by promoting Th2 cytokine dominant cytokine production and inhibiting NK cell activity [10].

Extracellular vesicles (EVs)

Communication between the embryo and the maternal organism is facilitated by extracellular vesicles (EVs). These are "transport vehicles" surrounded by a double phospholipid membrane and carry various cargoes, e.g.: DNA, RNA, proteins (MHC molecules, cytokines), Based on their size EVs are classified into three groups: exosomes (diameter below 100 nm), microvesicles (100-800 nm), and apoptotic bodies (diameter over 1000 nm). EVs [11].

EVs play a role in fertilization, as EVs in seminal fluid contain enzymes necessary for sperm survival and motility [12, 13] and carry proteins that facilitate the communication between the embryo and the maternal immune system. The role of EVs in the implantation process is less well understood, but it is assumed that EVs produced by the embryo might influence the maternal immune response. EVs produced by extravillous trophoblasts, for example, are positive for the human leukocyte antigen G (HLA-G) molecule which acts as

a ligand for the inhibitory receptor on decidual NK cells and might play a role in establishing maternal immune tolerance [14- 17].

This research aims to investigate:

- the role of PIBF in implantation,
- how embryo-derived EVs affect the maternal immune response,
- and whether there is a marker in the EVs produced by cultured human embryos that can identify embryos with the highest implantation potential.

MATERIALS AND METHODS

Investigating the expression of molecules involved in implantation by cultured mouse embryos

Two-cell stage embryos were isolated from the fallopian tubes of 1.5-day pregnant CD1 mice and cultured in KSOM medium at 37°C, 5% CO2, and 100% humidity until they reached the 4, 8 cell, morula, or blastocyst stage. The embryos were fixed, in 4% paraformaldehyde (PFA) solution for 25 minutes at room temperature, then washed in PBS and permeabilized with 0.5% Triton X-100.

Endogenous peroxidase activity was blocked with 3% hydrogen peroxide, and non-specific binding sites were blocked with 1% bovine serum albumin (BSA). The embryos were incubated with primary antibodies (VEGF, IGF2, PIBF, LIF, PLGF, TNFα, PAI1, MIF) for 1 hour, followed by treatment with horseradish peroxidase (HRP) conjugated secondary antibodies. The reaction was visualized with diaminobenzidine (DAB) staining, and the results were examined under a light microscope.

Examining the role of PIBF in implantation in a mouse model

8–12-week-old female CD1 mice were mated with male CD1 mice. On days 1.5 and 4.5 of pregnancy, the mice were treated intraperitoneally with monoclonal anti-PIBF antibody to neutralize the biological effect of PIBF. Control mice were injected with PBS instead of anti-PIBF antibody.

On day 10.5 of pregnancy, the mice were sacrificed by cervical dislocation, when the number of implantation sites and the ratio of resorbed embryos were determined.

Identification of PIBF+ NK cells and B cells in the decidua of PIBF-deficient mice

Implantation sites were fixed in 6% buffered formalin, embedded in paraffin. and 5 μm sections were prepared. Immunohistochemical reaction was performed with biotinylated monoclonal anti-PIBF antibody and HRP-conjugated streptavidin to identify PIBF+ NK cells.

Alexa647 fluorochrome-conjugated B220 antibody and fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-PIBF were used to identify B cells.

Cytotoxicity tests:

The cytotoxic activity of decidual and peripheral NK cells was determined using PKH67 vital dye-stained YAC-1 target cells. Target cell propidium iodide (PI) staining was analysed by flow cytometry, and cytotoxicity was expressed as the percentage of PI+ cells within PKH67+ population.

Lymphocyte activation and Th1 and Th2 cell differentiation

A prime PCR array from Bio-Rad was used for determining the markers for T cell activation and Th1 or Th2 differentiation in separated CD4+ and CD8+ spleen cells from anti-PIBF-treated and control pregnant mice. CD4+ and CD8+ T cells were separated from splenic single-cell suspension by magnetic separation with the Mini-MACS system. The collected cells were washed, and the cell count was adjusted to 5×10^6. For the stabilization of RNA, separated CD4+ or CD8+ splenocytes were stored frozen in RNAlater®-ICE solution. Total RNA was extracted from the cells with the Qiagen RNeasy Mini Kit according to the supplier's protocol. The expression of 41 genes was determined using a Bio-Rad prime PCR array (Immune response-Th1 and Th2 cell differentiation M384; Bio-Rad Laboratories, Inc., California, USA). Quantitative PCR reactions were carried out on an ABI 7900 real-time PCR instrument according to the manufacturer's instructions.

Electron microscopy of EVs at the feto-maternal interface in day 5 murine pregnancy

To confirm the presence of extracellular vesicles (EVs) at the feto-maternal interface in day 5 murine pregnancy, transmission electron microscopy was used. Uterine tissues (1 mm^3) from day 5 murine pregnancy were washed in PBS and fixed with 2.5% glutaraldehyde in phosphate buffer (0.1 M, pH 7.4) overnight at 4°C. After PBS washing, tissues were post-fixed in 1% osmium tetroxide in 0.1 M PBS for 1 hour at 4°C, then dehydrated in ethanol. Completely dehydrated blocks were treated with propylene oxide twice for 4 minutes, then immersed in a propylene oxide and Durcupan resin mixture for 30 minutes. The blocks were placed in Durcupan-filled aluminium-foil boats overnight and embedded in gelatine capsules with Durcupan resin. After polymerizing at 56°C for 72 hours, 700 nm semi-thin sections were cut, stained with toluidine blue, and examined under a light microscope. Subsequently, 65 nm ultrathin sections were cut, mounted on copper grids, contrasted with uranyl acetate and lead citrate, and examined using a JEOL 1200EX-II electron microscope.

Detecting PIBF in murine embryo-derived Evs by immuno-electron microscopy

In vitro cultured morula stage mouse embryos were stained in droplet. The embryos were fixed in 4% formaldehyde buffered in PBS for 20 minutes at room temperature. Following fixation, blocking of endogenous peroxidase was achieved by immersing the embryos in 1% hydrogen peroxide for 15 minutes, non-specific binding sites were blocked with 3% of bovine serum albumin for 40 minutes. Embryos were then reacted with 1:50 diluted rabbit anti-PIBF primary antibody [6] for 2 hours at room temperature. Binding of the primary antibody was detected with horseradish peroxidase conjugated anti-rabbit secondary antibody (1:100) at room temperature for 1 hour. Peroxidase reaction was visualized using diaminobenzidine as chromogen. Following the immunoreaction, embryos were mixed in 3% agar solution. After agar was hardened, small blocks of approximately 1 mm^3 were cut and fixed with 2.5% glutaraldehyde diluted in PB overnight at 4 °C. Post-fixation with 1% osmium tetroxide, embedding and cutting of the blocks were performed as described above.

The effect of PIBF+ EVs on the cytokine production of CD8+ T cells'

Two-cell stage embryos from pregnant CD1 mice were cultured in KSOM medium. The culture medium was collected and stored at -80°C. Evs in the culture medium were identified with PE-labelled annexin V, which labels the phosphatidylserine in the membrane.

Some samples were treated with detergent to dissolve the membrane, which eliminates the signals.

EV binding to mouse spleen cells:

Spleen cells of 16 weeks old female CD1 mice were prepared by mechanical separation Multicolour staining method was used for the detection of CD4+ and CD8+ T cells.

For visualization the phosphatidylserine receptor (PSR) on CD4+ and CD8+ cells, the cells were reacted with anti-phosphatidylserine receptor antibody for 20 min at room temperature, and with FITC-labelled secondary antibody for 30 min at room temperature. FITC-labelled secondary antibody was used as negative control to distinguish non-specific background signal from specific antibody signal.

PKH–labelled EVs were incubated with 5×10^4 anti-CD4 and anti-CD8 labelled splenocytes. Diluted PKH dye solution without EVs (mock-stained control) was used to assess non-specific (EV-independent) staining. At the end of the incubation period, cells were washed with PBS and analysed by flow cytometry.

Intracellular IL-10 staining of splenocytes

Anti-CD4 and anti-CD8 labelled splenocytes stimulated with embryo-derived EVs for 4 hours were incubated with phycoerythrin-conjugated anti-mouse IL-10 monoclonal antibody for 15–30 min, at room temperature. The cells were washed twice in 2 ml of PBS, centrifuged for 5 min at 300 g, and fixed with 300 µl of 2% paraformaldehyde. Stained cells were stored at 4 °C in dark before analysis. Tests were carried out by measuring, 5×10^4 cells/tube, on the day of the staining. All the FACS data were analysed with CellQuest Pro software.

Determining the number of nucleic acid -containing extracellular vesicles in day 5 culture medium of human embryos

Embryos from women undergoing IVF treatment were individually cultured in G-1 medium for three days, when the medium was replaced by G-2 medium and the embryos were cultured for two more days. On day 5, the culture medium was collected and stored at -80°C.

Flow cytometry:

The absolute number of EVs was determined using internal standard beads. The nucleic acid content of EVs was detected with propidium iodide (PI) staining and measured by BD FACSCalibur flow cytometer. Megamix-Plus SSC beads were used for setup and gating.

RESULTS

Cultured mouse embryos express markers involved in implantation

The expression of eight molecules implicated in implantation was tested in 2, 4, 8 cell, morula, and blastocyst stage mouse embryos. The embryos strongly expressed VEGF, IGF2, MIF, PAI1, LIF, and PIBF at all developmental stages. In contrast, PLGF and TNFα were not detectable.

Anti-PIBF treatment in the peri-implantation period results in impaired implantation and increased resorption rates in later pregnancy

The mice were treated with anti-PIBF antibody at days 1.5 and 4.5 of pregnancy to render them functionally PIBF deficient during the implantation window. The mice were sacrificed at day 10.5. This enabled us to record not only the implantation sites but also the resorption rate among the implanted embryos. While the average number of implanted embryos was 6.5 in the controls, in females treated with anti- PIBF antibody in the peri-implantation period, the mean implantation sites decreased to four. The unusually low 2% resorption rate in the control group increased to 40% in the functionally PIBF-deficient mice.

PIBF+ large, granulated cells are depleted from the deciduae of anti-PIBF-treated mice

In an earlier study, we demonstrated a high number of large, granulated cells—with a strong PIBF reactivity in the cytoplasmic granules—in the deciduae of day 12.5 pregnant mice. These cells were identified as members of the PAS+ DBA+ uterine NK cell population. PIBF co-localized with perforin in the cytoplasmic granules of the cells [18].

In the present study, we found a significantly decreased number of PIBF+ NK cells in the day 10.5 deciduae of resorbed embryos from anti-PIBF-treated mice compared to normal deciduae from untreated mice.

Functionally PIBF deficient mice exert increased NK cell activity

Anti-PIBF treatment significantly increased the cytotoxic activity of both peripheral and decidual NK cells.

B cells are depleted from the deciduae of anti-PIBF-treated embryos

The endometria of the control animals contained decidual NK cells and a discrete layer of B cells at the choriodecidual interface. While the NK cells were still present, the B cells disappeared from the deciduae of the resorbed embryos of anti-PIBF-treated mice. A recent study showed that the IL-33-induced expression of PIBF1 by decidual B cells protects against preterm labor both in humans and in mice [19]. B cells constitute a minor population among decidual lymphocytes, yet they might be important for the immunological balance of the decidua.

Functional PIBF deficiency in the peri-implantation period results in impaired CD4+ T cell activation and Th1 type differentiation

Peripheral Th cell subsets from anti-PIBF-treated and control mice were tested for the differential expression of 48 genes using a prime PCR array. Twelve of these showed a significantly higher or lower expression in the lymphocytes of anti-PIBF-treated mice compared to the controls. When analysing the results, the differentially expressed genes were assigned to the following groups: (1) genes involved in T cell activation, (2) those involved in Th1 differentiation, and (3) those involved in Th2 differentiation.

The genes implicated in T cell activation, e.g., members of the CD3 complex (CD 247, CS3D, CS3E, CS3G, and IL2RG), were significantly downregulated in the CD4+ spleen cells of anti-PIBF-treated mice but significantly increased in the CD8+ cells of the same animals. In the anti-PIBF-treated mice, the beta chain of the IL2R was downregulated in the CD4+ population, while in the CD8 population the alpha and the gamma chain of the IL2R and the IL2 increased. The genes of the co-stimulatory molecules were altered in a similar fashion. Upon anti-PIBF treatment, the genes for CD4, CD28, CD40L, and CD86 were downregulated in the CD4 and upregulated in the CD8 population. These data suggest that the absence of PIBF inhibits the activation of CD4+ cells and facilitates that of CD8+ T cells.

Effect of PIBF+ EVs on cytokine production of CD8+ T cells

With immuno-electron microscopy, we confirmed that embryo-derived EVs carry PIBF. In the presence of embryo-derived EVs IL-10 production by CD8+ T cells increased, and this effect was counteracted by pre-treatment of the EVs with anti-PIBF antibody. These findings suggest, that at the early stages of pregnancy, signals from the embryo might alter the maternal immune response, in order to establish a tolerant environment for the developing foetus.

The number of nucleic acid -containing EVs produced by cultured embryos indicates implantation potential

88 women undergoing IVF were included in the study. More than 1 embryos were transferred to most patients. In 58 women, the transfer resulted in clinical pregnancy, whereas in 30 women in implantation failure. In 112 culture media of embryos from the "clinical pregnancy" group, the number of PI+ EVs was significantly lower than in those of 49 embryos, from the "implantation failure" group. In 14 women, transfer of a single embryo resulted in a singleton pregnancy, or transfer of two embryos in twin pregnancy. The culture media of 19 out of the 20 "confirmed competent" embryos contained a lower level of PI+ EVs than the cut off level, suggesting that the competent embryo can indeed be identified by low PI+ EV counts. We developed a non-invasive, simple, inexpensive, quick test, which identifies the embryos that are most likely to implant.

The research results indicate that PIBF and EVs play crucial roles in embryo implantation and regulation of maternal immune response. Neutralization of PIBF negatively affects implantation and increases embryo resorption rates, while the presence and content of EVs can predict successful embryo implantation. These findings improve infertility treatment and the efficiency of IVF procedures.

DISCUSSION

Earlier findings, suggest that PIBF might play an active role in implantation [20]. To confirm this hypothesis, we neutralized the biological activity of PIBF during the peri-implantation period in mice and investigated the consequences of functional PIBF deficiency at several levels.

Anti-PIBF treatment of pregnant mice in the peri-implantation period resulted in a significantly reduced number of implantation sites, and the implantations that took place nevertheless must have been compromised as shown by the high resorption rates.

We further investigated the underlying mechanisms of implantation failure and pregnancy loss in functionally PIBF-deficient mice.

Failed pregnancies are characterized by high peripheral NK activity, both in humans and in mice [20- 28]. Decidual NK cells constitute 60% of decidual lymphocytes [30]. Is spite of the availability of perforin and granzyme in their cytotoxic granules [30], these cells have a very moderate cytotoxic potential [31, 32] but secrete angiogenic factors and cytokines [30].

The low cytotoxic activity of decidual NK cells might be due to the presence of PIBF in their cytoplasmic granules [18]. PIBF blocks the upregulation of perforin expression in activated decidual lymphocytes and inhibits NK cell cytotoxicity by blocking granule exocytosis [33, 34]. Bogdan et al. demonstrated a high number of PIBF+ NK cells in the day 12.5 decidua of pregnant mice [18].

Here we show that anti-PIBF treatment during the peri-implantation period results in the reduced presence of PIBF+ NK cells in the day 10.5 decidua, together with significantly increased decidual and peripheral NK activity, compared to the controls.

Anti-PIBF treatment of mid-pregnant mice has been shown to boost both the peripheral NK activity and the resorption rates. Increased resorption rates in anti-PIBF-treated mice were corrected by simultaneous treatment of the mice with anti-NK antibodies [35], suggesting that PIBF prevents pregnancy loss in mice—at least partly—by blocking NK activity. Increased decidual NK activity owing to the loss of PIBF+ decidual NK cells could be one of the reasons for the increased resorption rates in the anti-PIBF treated mice.

B cells constitute a minor population among decidual lymphocytes, yet they might be important for the immunological balance of the decidua. A recent study showed that the IL-33-induced expression of PIBF1 by decidual B cells protects against preterm labor both in humans and in mice [19].

In the present study, we detected a distinct layer of B cells at the choriodecidual interface of control pregnant mice on day 10.5 of pregnancy. These cells were completely absent from the deciduae of mice that had been treated with anti-PIBF during the peri-implantation period.

Taken together, it is conceivable that anti-PIBF treatment—by depleting decidual B cells—will, at a later stage, put pregnancy at risk due to the lack of PIBF-positive B cells [19].

Finally, we performed a gene array on the spleen cells of anti-PIBF-treated and control mice in order to investigate whether the absence of functional PIBF influenced T cell activation and differentiation.

The T cell receptor is a complex of T cell receptor alpha and beta chains and the CD3 proteins. Activation of CD4+ T cells occur through the simultaneous engagement of the T cell receptor and a co-stimulatory molecule on the T cell by the MHCII peptide and the co-stimulatory molecules on the APC. In the absence of co-stimulation, T cell receptor signalling results in anergy.

In addition to TCR alpha/beta, a whole set of cell surface receptors are also engaged by their ligands on APCs, which regulate Th differentiation. CD4 acts as a cellular adhesion molecule that binds MHC class II and stabilizes the interaction of T cells and APCs [36, 37]. CD28 is a costimulatory receptor on T cells, which binds CD80 and CD86 on activated APCs [38]. The TCR alpha/beta/CD3 complex provides the first signal and CD28 the second signal for T cell activation. Both signals are required for IL-2 production and T cell proliferation. CD40 ligand, expressed by activated T cells, binds to CD40 on APCs, initiating a T cell-mediated immune response [39].

In this study, we found that members of the T cell receptor CD3 complex were significantly downregulated on CD4+ T cells of anti-PIBF-treated mice, while CD3D and IL2R B and G were upregulated in CD8+ cells, suggesting that Th cell activation is severely inhibited in the anti-PIBF-treated pregnant mice.

IL-4 is the main cytokine driving Th2 cell differentiation. IL-4 is produced by various cell types, including mast cells, basophils, eosinophils, NK cells, activated CD4+ T cells, and differentiated Th2 cells [40]. **Here we found that the gene for IL-4 was significantly downregulated in CD4+ cells, while that of IL-12A was upregulated in CD8+ cells of the anti-PIBF-treated mice. The above data show that PIBF is indispensable for creating a Th2 dominant cytokine balance.**

This poses the question, how does the maternal immune system receive the information, that pregnancy has been established.

Embryo-derived extracellular vesicles are detectable in the embryo culture medium as well as at the implantation site.

The PIBF content of embryo-derived EVs has been verified with immune electron microscopy, and the former induced increased IL-10 production by murine spleen cells. These data suggest that the implanted embryo lets its presence known by sending PIBF+ EVs to the mother, resulting in a Th2 shifted immune response.

There is now ample evidence that the recognition of paternal antigens by the maternal immune system is not simply harmless but absolutely necessary for the setting in of the mechanisms that adapt the immune response to tolerate the foetus [41]. Following recognition of foetal antigens, the immune system becomes activated, and this will result in the establishment of regulatory mechanisms, e.g., a Th2 dominant immune response [42, 43].

Neutralizing PIBF in the peri-implantation period abolishes this mechanism right at the start. CD4+ T cell activation is disturbed, T cells differentiate in the Th1 direction, and as a result, implantation as well as ongoing pregnancies is compromised.

Multiple pregnancy is a risk for preterm birth and prematurity. The goal of assisted reproduction is to achieve a single pregnancy, by transferring a single embryo. This requires improved methods to identify the competent embryo. **Here, we describe such a test, based on flow cytometric determination of the nucleic acid (PI+) containing extracellular vesicle (EV) count in day 5 embryo culture media. The test is non-invasive, simple, inexpensive, quick, which identifies the embryos that are most likely to implant.**

REFERENCES

[1] A. Chemerinski, J. Garcia de Paredes, K. Blackledge, N. C. Douglas, and S. S. Morelli, "Mechanisms of endometrial aging: lessons from natural conceptions and assisted reproductive technology cycles," *Front Physiol*, vol. 15, p. 1332946, Feb. 2024, doi: 10.3389/FPHYS.2024.1332946/BIBTEX.

[2] S. Pawar, A. M. Hantak, I. C. Bagchi, and M. K. Bagchi, "Minireview: Steroid-Regulated Paracrine Mechanisms Controlling Implantation," *Molecular Endocrinology*, vol. 28, no. 9, pp. 1408–1422, Sep. 2014, doi: 10.1210/ME.2014-1074.

[3] R. Fernandez-Valdivia *et al.*, "Revealing progesterone's role in uterine and mammary gland biology: insights from the mouse," *Semin Reprod Med*, vol. 23, no. 1, pp. 22–37, Feb. 2005, doi: 10.1055/S-2005-864031.

[4] B. Mulac-Jericevic, R. A. Mullinax, F. J. DeMayo, J. P. Lydon, and O. M. Conneely, "Subgroup of reproductive functions of progesterone mediated by progesterone receptor-B isoform," *Science (1979)*, vol. 289, no. 5485, pp. 1751–1754, Sep. 2000, doi: 10.1126/SCIENCE.289.5485.1751/SUPPL_FILE/1051453S2_THUMB.GIF.

[5] W. D. Billington, "2 The normal fetomaternal immune relationship," *Baillieres Clin Obstet Gynaecol*, vol. 6, no. 3, pp. 417–438, Sep. 1992, doi: 10.1016/S0950-3552(05)80004-5.

[6] B. Polgár, E. Nagy, E. ´ Va Mikó, P. Varga, and J. Lia Szekeres-Barthó, "Urinary Progesterone-Induced Blocking Factor Concentration Is Related to Pregnancy Outcome 1," *Biol Reprod*, vol. 71, pp. 1699–1705, 2004, doi: 10.1095/biolreprod.104.030437.

[7] B. Polgar *et al.*, "Molecular Cloning and Immunologic Characterization of a Novel cDNA Coding for Progesterone-Induced Blocking Factor," *The Journal of Immunology*, vol. 171, no. 11, pp. 5956–5963, Dec. 2003, doi: 10.4049/JIMMUNOL.171.11.5956.

[8] K. Kim, K. Lee, and K. Rhee, "CEP90 Is Required for the Assembly and Centrosomal Accumulation of Centriolar Satellites, Which Is Essential for Primary Cilia Formation," *PLoS One*, vol. 7, no. 10, p. 48196, Oct. 2012, doi: 10.1371/JOURNAL.PONE.0048196.

[9] M. Lachmann *et al.*, "PIBF (progesterone induced blocking factor) is overexpressed in highly proliferating cells and associated with the centrosome," *Int J Cancer*, vol. 112, no. 1, pp. 51–60, Oct. 2004, doi: 10.1002/IJC.20326.

[10] N. Kozma *et al.*, "Progesterone-Induced Blocking Factor Activates STAT6 via Binding to a Novel IL-4 Receptor," *The Journal of Immunology*, vol. 176, no. 2, pp. 819–826, Jan. 2006, doi: 10.4049/JIMMUNOL.176.2.819.

[11] M. Yáñez-Mó *et al.*, "Biological properties of extracellular vesicles and their physiological functions," *J Extracell Vesicles*, vol. 4, no. 1, p. 27066, Jan. 2015, doi: 10.3402/JEV.V4.27066.

[12] G. Frenette, M. Thabet, and R. Sullivan, "Polyol pathway in human epididymis and semen," *J Androl*, vol. 27, no. 2, pp. 233–239, Mar. 2006, doi: 10.2164/JANDROL.05108.

[13] J. S. Oh, C. Han, and C. Cho, "ADAM7 is associated with epididymosomes and integrated into sperm plasma membrane," *Mol Cells*, vol. 28, no. 5, pp. 441–446, Nov. 2009, doi: 10.1007/S10059-009-0140-X/METRICS.

[14] L. A. Burnett, M. M. Light, P. Mehrotra, and R. A. Nowak, "Stimulation of GPR30 Increases Release of EMMPRIN-Containing Microvesicles in Human Uterine Epithelial Cells," *J Clin Endocrinol Metab*, vol. 97, no. 12, p. 4613, 2012, doi: 10.1210/JC.2012-2098.

[15] Y. H. Ng *et al.*, "Endometrial Exosomes/Microvesicles in the Uterine Microenvironment: A New Paradigm for Embryo-Endometrial Cross Talk at Implantation," *PLoS One*, vol. 8, no. 3, p. e58502, Mar. 2013, doi: 10.1371/JOURNAL.PONE.0058502.

[16] C. Gercel-Taylor, S. M. O'Connor, G. K. Lam, and D. D. Taylor, "Shed membrane fragment modulation of CD3-zeta during pregnancy: link with induction of apoptosis," *J Reprod Immunol*, vol. 56, no. 1–2, pp. 29–44, Jul. 2002, doi: 10.1016/S0165-0378(02)00025-6.

[17] E. Pallinger, A. Kiss, Pap E, S. Tóth, and A. Falus, "BeWo-derived microvesicles modulate T cell differentiation by the downregulation of IL-6Ralpha expression on CD4 + T lymphocytes," *J Extracell Vesicles*, vol. 1, Jan. 2012, Accessed: Dec. 29, 2022. [Online]. Available: https://onlinelibrary.wiley.com/doi/epdf/10.3402/jev.v1i0.18182

[18] A. Bogdan, G. Berta, and J. Szekeres-Bartho, "PIBF positive uterine NK cells in the mouse decidua," *J Reprod Immunol*, vol. 119, pp. 38–43, Feb. 2017, doi: 10.1016/J.JRI.2016.12.001.

[19] B. Huang *et al.*, "Interleukin-33-induced expression of PIBF1 by decidual B cells protects against preterm labor," *Nat Med*, vol. 23, no. 1, pp. 128–135, Jan. 2017, doi: 10.1038/NM.4244.

[20] B. Mulac-Jeričević, S. Šućurović, T. Gulic, and J. Szekeres-Bartho, "The involvement of the progesterone receptor in PIBF and Gal-1 expression in the mouse endometrium," *Am J Reprod Immunol*, vol. 81, no. 5, May 2019, doi: 10.1111/AJI.13104.

[21] A. R. de Fougerolles and M. G. Baines, "Modulation of the natural killer cell activity in pregnant mice alters the spontaneous abortion rate," *J Reprod Immunol*, vol. 11, no. 2, pp. 147–153, Jun. 1987, doi: 10.1016/0165-0378(87)90018-0.

[22] R. KINSKY, G. DELAGE, N. ROSIN, M. N. THANG, M. HOFFMANN, and G. CHAOUAT, “A Murine Model of NK Cell Mediated Resorption,” *American Journal of Reproductive Immunology*, vol. 23, no. 3, pp. 73–77, Jul. 1990, doi: 10.1111/J.1600-0897.1990.TB00675.X.

[23] K. Aoki *et al.*, “Preconceptional natural-killer-cell activity as a predictor of miscarriage,” *Lancet*, vol. 345, no. 8961, pp. 1340–1342, May 1995, doi: 10.1016/S0140-6736(95)92539-2.

[24] K. Fukui, I. Yoshimoto, K. Matsubara, R. Hori, H. Ochi, and M. Ito, “Leukocyte function-associated antigen-1 expression on decidual natural killer cells in patients with early pregnancy loss,” *Mol Hum Reprod*, vol. 5, no. 11, pp. 1083–1088, Nov. 1999, doi: 10.1093/MOLEHR/5.11.1083.

[25] E. I. Ntrivalas *et al.*, “Status of peripheral blood natural killer cells in women with recurrent spontaneous abortions and infertility of unknown aetiology,” *Hum Reprod*, vol. 16, no. 5, pp. 855–861, 2001, doi: 10.1093/HUMREP/16.5.855.

[26] E. I. Ntrivalas, C. R. Bowser, J. Kwak-Kim, K. D. Beaman, and A. Gilman-Sachs, “Expression of killer immunoglobulin-like receptors on peripheral blood NK cell subsets of women with recurrent spontaneous abortions or implantation failures,” *Am J Reprod Immunol*, vol. 53, no. 5, pp. 215–221, 2005, doi: 10.1111/J.1600-0897.2005.00268.X.

[27] L. Putowski, D. Darmochwal-Kolarz, J. Rolinski, J. Oleszczuk, and J. Jakowicki, “The immunological profile of infertile women after repeated IVF failure (Preliminary study),” *European Journal of Obstetrics and Gynecology and Reproductive Biology*, vol. 112, no. 2, pp. 192–196, Feb. 2004, doi: 10.1016/j.ejogrb.2003.06.012.

[28] K. Shakhar, S. Ben-Eliyahu, R. Loewenthal, E. Rosenne, and H. Carp, “Differences in number and activity of peripheral natural killer cells in primary versus secondary recurrent miscarriage,” *Fertil Steril*, vol. 80, no. 2, pp. 368–375, Aug. 2003, doi: 10.1016/S0015-0282(03)00611-3.

[29] H. Yamada *et al.*, “High NK cell activity in early pregnancy correlates with subsequent abortion with normal chromosomes in women with recurrent abortion,” *Am J Reprod Immunol*, vol. 46, no. 2, pp. 132–136, 2001, doi: 10.1111/J.8755-8920.2001.460203.X.

[30] L. A. Koopman *et al.*, “Human decidual natural killer cells are a unique NK cell subset with immunomodulatory potential,” *J Exp Med*, vol. 198, no. 8, pp. 1201–1212, Oct. 2003, doi: 10.1084/JEM.20030305.

[31] D. RUKAVINA, G. RUBEŠA, L. GUDELJ, H. HALLER, and E. R. PODACK, “Characteristics of perforin expressing lymphocytes within the first trimester decidua of human pregnancy,” *Am J Reprod Immunol*, vol. 33, no. 5, pp. 394–404, 1995, doi: 10.1111/J.1600-0897.1995.TB00908.X.

[32] T. B. Crncic *et al.*, “Early pregnancy decidual lymphocytes beside perforin use Fas ligand (FasL) mediated cytotoxicity,” *J Reprod Immunol*, vol. 73, no. 2, pp. 108–117, Apr. 2007, doi: 10.1016/J.JRI.2006.07.001.

[33] Zs. Faust, G. Laškarin, and D. Rukavina, “Progesterone-Induced Blocking Factor Inhibits Degranulation of Natural Killer Cells,” *American Journal of Reproductive Immunology*, vol. 42, no. 2, pp. 71–75, Aug. 1999, doi: 10.1111/J.1600-0897.1999.TB00468.X.

[34] G. Laškarin *et al.*, “Progesterone directly and indirectly affects perforin expression in cytolytic cells,” *Am J Reprod Immunol*, vol. 42, no. 5, pp. 312–320, 1999, doi: 10.1111/J.1600-0897.1999.TB00107.X.

[35] J. Szekeres-Bartho, G. Par, G. Dombay, Y. C. Smart, and Z. Volgyi, “The antiabortive effect of progesterone-induced blocking factor in mice is manifested by modulating NK activity,” *Cell Immunol*, vol. 177, no. 2, pp. 194–199, May 1997, doi: 10.1006/CIMM.1997.1090.

[36] D. Leitenberg, Y. Boutin, S. Constant, and K. Bottomly, “CD4 Regulation of TCR Signaling and T Cell Differentiation Following Stimulation with Peptides of Different Affinities for the TCR,” *The Journal of Immunology*, vol. 161, no. 3, pp. 1194–1203, Aug. 1998, doi: 10.4049/JIMMUNOL.161.3.1194.

[37] M. F. Krummel, M. D. Sjaastad, C. W. Wulfing, and M. M. Davis, “Differential clustering of CD4 and CD3zeta during T cell recognition,” *Science*, vol. 289, no. 5483, pp. 1349–1352, Aug. 2000, doi: 10.1126/SCIENCE.289.5483.1349.

[38] L. L. Lanier *et al.*, “CD80 (B7) and CD86 (B70) provide similar costimulatory signals for T cell proliferation, cytokine production, and generation of CTL.,” *J Immunol*, vol. 154, no. 1, pp. 97–105, Jan. 1995.

[39] C. Dong and R. A. Flavell, “Cell fate decision: T-helper 1 and 2 subsets in immune responses,” *Arthritis Res*, vol. 2, no. 3, p. 179, 2000, doi: 10.1186/AR85.

[40] K. J. Rautajoki, M. K. Kyläniemi, S. K. Raghav, K. Rao, and R. Lahesmaa, “An insight into molecular mechanisms of human T helper cell differentiation,” *Ann Med*, vol. 40, no. 5, pp. 322–335, 2008, doi: 10.1080/07853890802068582.

[41] C. Ober, T. Hyslop, S. Elias, L. R. Weitkamp, and W. W. Hauck, “Human leukocyte antigen matching and fetal loss: results of a 10 year prospective study.,” *Human Reproduction*, vol. 13, no. 1, pp. 33–38, Jan. 1998, doi: 10.1093/HUMREP/13.1.33.

[42] T. G. Wegmann, H. Lin, L. Guilbert, and T. R. Mosmann, “Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon?” *Immunol Today*, vol. 14, no. 7, pp. 353–356, Jul. 1993, doi: 10.1016/0167-5699(93)90235-D.

[43] R. Raghupathy, “Th1-type immunity is incompatible with successful pregnancy,” *Immunol Today*, vol. 18, no. 10, pp. 478–482, 1997, doi: 10.1016/S0167-5699(97)01127-4.

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Pallinger, E., Bognar, Z., Bogdan, A., **Csabai, T.**, Abraham, H., & Szekeres-Barthó, J. **(2018)**. PIBF+ extracellular vesicles from mouse embryos affect IL-10 production by CD8+ cells. Scientific reports, 8(1), 4662. https://doi.org/10.1038/s41598-018-23112-z
Impact factor: 4,011; Classification: Q1

Csabai, T., Pallinger, E., Kovacs, A. F., Miko, E., Bognar, Z., & Szekeres-Barthó, J. **(2020)**. Altered Immune Response and Implantation Failure in Progesterone-Induced Blocking Factor-Deficient Mice. Frontiers in immunology, 11, 349. https://doi.org/10.3389/fimmu.2020.00349
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Other publications:

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Impact factor: 5,296; Classification: Q1

Bognár Zoltán, Szekeres-Barthó Júlia, **Csabai Tímea**, Pállinger Éva, Gödöny Krisztina, Bódis József **(2017)**. Az implantáció eredményességének javítását célzó humán kutatások állatmodellje. MAGYAR NŐORVOSOK LAPJA 80: 4 pp. 166-174., 9 p.
Impact factor: 0; Classification: none

Bognar, Z., **Csabai, T. J.**, Pallinger, E., Balassa, T., Farkas, N., Schmidt, J., Görgey, E., Berta, G., Szekeres-Barthó, J., & Bodis, J. **(2019)**. The effect of light exposure on the cleavage rate and implantation capacity of preimplantation murine embryos. Journal of reproductive immunology, 132, 21–28. https://doi.org/10.1016/j.jri.2019.02.003
Impact factor: 4,018; Classification: Q1

Szekeres-Barthó, J., **Csabai, T.**, & Gorgey, E. **(2021)**. Biologia futura: embryo-maternal communication via progesterone-induced blocking factor (PIBF) positive embryo-derived extracellular vesicles. Their role in maternal immunomodulation. Biologia futura, 72(1), 69–74. https://doi.org/10.1007/s42977-020-00060-2
Impact factor: 1,653; **Classification: Q3**

Ahmadi, H., **Csabai, T.**, Gorgey, E., Rashidiani, S., Parhizkar, F., & Aghebati-Maleki, L. **(2022)**. Composition and effects of seminal plasma in the female reproductive tracts on implantation of human embryos. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie, 151, 113065. https://doi.org/10.1016/j.biopha.2022.113065
Impact factor: 7,419; Classification: Q1

Bognár, Z., **Csabai-Tanics, T. J.**, Görgey, É., Mikó, É., Horváth-Szalai, Z., & Szekeres-Barthó, J. **(2023)**. The effect of calcitriol on the development and implantation capacity of embryos from hyper-stimulated mice. Frontiers in immunology, 14, 1200704. https://doi.org/10.3389/fimmu.2023.1200704
Impact factor: 7,3; Classification: Q1

Ahmadi, H., Aghebati-Maleki, L., Rashidiani, S., **Csabai, T.**, Nnaemeka, O. B., & Szekeres-Barthó, J. **(2023)**. Long-Term Effects of ART on the Health of the Offspring. International journal of molecular sciences, 24(17), 13564. https://doi.org/10.3390/ijms241713564
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