The investigation of immune tolerance mechanism during pregnancy

Ph.D. Thesis

Árpád Ferenc Kovács, MD

Semmelweis University
Doctoral School of Molecular Medicine

Supervisor:
Éva Pállinger, MD, Ph.D.

Official reviewers:
Krisztina Buzás, Ph.D.
Orsolya Dobay, Ph.D.

Head of the Final Examination Committee:
Dóra Szabó, MD, D.Sc.

Members of the Final Examination Committee:
Mihály Józsi, D.Sc.
Henriett Butz, MD, Ph.D.

Budapest
2020

1. Introduction

The birth of a new life depends on the cooperation between two different genomes. The continuous communication between the developing embryo and the maternal immune system has essential importance. One of the most dynamic form of intercellular communication is mediated by extracellular vesicles (EV), which are produced by living cells. EVs play an active role in the maturation of germ cells, in embryo implantation, and probably in the formation and maintenance of immune tolerance during pregnancy. Classification of EVs can be done based on their size or biogenesis. According to their size, three main subtypes are distinguishable: small EVs (sEV), intermediate-sized EVs (iEV) and large EVs (lEV). The structured multimolecular composition (proteins, nucleic acids, lipids and carbohydrates) can influence the function of their target cells.

Almost 40% of decidual cells are immune cells. The immune cell composition of the decidua is tightly regulated, including the ratio and kinetics of the different cell types found here. This is of crucial significance for the establishment and maintenance of a successful pregnancy: (1) the developing immune tolerance ensures a suitable immune environment for the fetus and (2) at the same time the immune system remains immune competitive, ensuring an effective protection against pathogens. Regulatory T cells (T_{reg} cells) play a central role in immune tolerance. T_{reg} cells act as key regulators at the maternofetal interface, influencing the function of all immune cells. Single cell transcriptomics analysis showed, that T_{reg} cells found at the maternofetal interface are differentiated in the decidua.

Although peripheral T_{reg} cell differentiation is not yet fully understood, trophoblast cells may play a defining role in it. The multinucleated syncytiotrophoblast cells are in direct contact with the maternal blood. At the maternofetal interface the syncytiotrophoblast cells are the main source of EVs. The main target cells of trophoblast-derived iEVs are circulating T-cells. The investigation of trophoblast-derived EV induced T-cell functions is one of the main topics of the thesis.

Dysfunction of the maternal immune system can be detected in the background of many pregnancy complications. Preeclampsia is a progressive, systemic pregnancy-specific disorder, affecting around 3-7% of human pregnancies. The inadequate adaptation of the maternal immune and cardiovascular system leads in the second half of the pregnancy to a syndrome of new-onset hypertension, proteinuria, or organ dysfunction during preeclampsia. Although the immunological aspects of preeclampsia have been extensively studied, its pathomechanism is not yet fully discovered. A significantly higher number of M1 macrophages have been found in preeclamptic placentas. M1 macrophages may contribute to the development of an exaggerated inflammation and inhibit the motility of trophoblast cells, negatively impacting trophoblast invasion, and consequently spiral artery remodelling. Trophoblast-derived EVs may influence the function of monocytes, which ultimately contribute to the development of preeclampsia.

2. Objectives

Our objective was the investigation of the effects of EVs on the development of maternal immune tolerance in *in vitro* and *ex vivo* research settings.

Questions to answer, problems to be solved:

Analysis of trophoblast-derived EV induced effects by using an *in vitro* model system, the BeWo trophoblastic cell line:

1. Do BeWo-derived EVs (BeWo EV) influence the function and polarization of $CD4^+$ T cells?
2. Which molecular components of BeWo EVs are responsible for the EV induced effects observed in $CD4^+$ T cells?
3. Does HSPE1 expression of BeWo EVs play a role in the observed EV induced $CD4^+$ T cell function?
4. Does HSPE1 play a role in T_{reg} cell heterogeneity?

Investigation of the effect of circulating EV pattern on immune system in *in vitro* model system:

1. Detection of preeclampsia associated EVs based on cellular origin.
2. Identification of target cells of preeclampsia associated EVs.
3. *In vitro* analysis of the effects of circulating EVs on monocytes.
4. Complex molecular characterization of the effects of EVs on monocytes.

3. Methods

Biological samples

Peripheral venous blood was collected from 45 healthy non-pregnant donors obtained from the Hungarian National Blood Transfusion Service. Clinical samples: blood samples from both healthy and preeclamptic third trimester pregnant women were obtained from the Department of Obstetrics and Gynaecology at Semmelweis University, Budapest, Hungary. Normotensive patients were age and gestational age matched with no detectable proteinuria or history of preeclampsia. Exclusion criteria were the presence of any chronic disease or acute infection in either group. During the entire investigation period, we followed the guidelines and regulations of the Helsinki Declaration in 1975, and the experiments were approved by the Hungarian Scientific and Research Ethics Committee; all tested individuals signed an informed consent form.

Isolation of mononuclear cells

Mononuclear cells (PBMC) were isolated under sterile conditions with gradient centrifugation (2 500 *g*; 20 minutes). The separated cells were wished with phosphate buffered saline (PBS) and stored at -80 °C degrees in fetal bovine serum containing 10% dimethyl sulfoxide.

Separation of platelet free plasma

To separate the plasma blood samples were centrifuged at 800 *g* for 5 minutes. Platelet poor plasma (PPP) was obtained with centrifugation of plasma samples at 2 500 *g* for 15 minutes at room temperature. With a further centrifugation step (2 500 *g*; 15 minutes)

platelet free plasma (PFP) was isolated and stored at -80 °C degrees until use.

Cell culture and establishment of gene knockout cell line

BeWo cells were cultured in Ham F12/K media containing 2 mM L-glutamin, 10% HyClone® FBS, 5% glucose, 1 mM sodium pyruvate, 1% of Gibco® MEM NEAA, 100 U/mL penicillin és 100 µg/mL streptomycin.

For the development of HSPE1 KO cell line, the *HSPE1* was knocked out in BeWo cells using the CRISPR/Cas9 genome editing system. We used the Synthego CRISPR Design Tool to design sgRNAs targeting the *HSPE1* gene.

For transfection 1.8 x 10^5 BeWo cells, 100 pmol/µL sgRNA, 20 pmol Cas9-NLS, 19.6 µL transfection reagent (82%:18% ration SF media transfection buffer) and 0.4 µg pMaxGFP (1µg/mL) plasmid vector was used. BeWo cell suspension was transferred to the nucleocuvette and cell line optimization protocol was run on Nucleofector 4D-X. Transfection efficiency was verified at DNA level by Sanger sequencing, at RNA level by qPCR and Synthego ICE analysis was performed.

Culturing, sorting, and differentiation towards T_{reg} cells of human lymphocytes

Frozen PBMC samples were thawed and cultured in RPMI 1640 for 24 hours. Cells were centrifuged, resuspended in sorting buffer and stained with anti-human CD4 antibody. $CD4^+$ cells were sorted with SONY SH800S cell sorter. 5 x 10^5 CD4+ cells were cultured and treated with 32 IU/mL recombinant human IL-2 and co-activated with Dynabeads™ Human T-Activator beads. $CD4^+$ T cells were

treated with recombinant human HSPE1 (rHSPE1) protein (1 µg/mL, 2.5 µg/mL, 10 µg/mL, 15 µg/mL), 1 x 10^6 GFP transfected BeWo cell-derived iEVs, or 1 x 10^6 HSPE1 KO BeWo cell-derived iEVs for T_{reg} differentiation. After 72 hours, the activation beads were removed with magnetic columns and cells were washed and resuspended in 100 µL autoMACS buffer and immunophenotyped. To identify T_{reg} cells we applied the following staining combination: anti-human CD25-FITC, anti-human CD3-AF647, anti-human CD127-PE/Cy7. The $CD3^+/CD25^{high+/}CD127^{low+}$ T_{reg} cells were sorted under sterile condition for holomicroscopic analysis.

Extracellular vesicle isolation and characterization

For EV isolation we used two different methods: (1) differential centrifugation, and (2) size exclusion chromatography. After isolation, EVs were characterized according to the recommendations of International Society for Study of Extracellular Vesicles. For BeWo derived EV isolation we used 10 mL of cell supernatant of 2 x 10^6 BeWo cells. The different EV fraction isolation was performed in 4 steps: 1) cell debris removal (800 *g*, 5 minutes), 2) apoptotic body removal (2 000 *g*, 15 minutes), 3) iEV fraction enrichment (12 500 *g*, 20 minutes), 4) sEV fraction enrichment (100 000 *g*, 70 minutes).

EVs were isolated from 1 mL PFP plasma samples, which were diluted with 1.5 mL of 0.2 µm filtered PBS. Isolation of iEV, sEV and EV-free fractions was performed as described for the BeWo-cell supernatant. EV size distribution was assessed by dynamic light scattering and high-resolution flow cytometry. Mass spectrometry

was used for the analysis of EV proteomics, and next generation sequencing was applied for EV miRNA pattern identification.

EV-target cell identification and functional studies

Flow cytometry was used to immunophenotype cells and EVs, to identify the target cells of EVs, to analyse the phagocytosis of EVs and to detect the EV induced cell cycle.

EV induced cell migration was assessed with holomicroscope, EV induced chemotaxis was determined with Neuroprobe assay and cell adhesion was recorded with a real-time impedance-based method.

EV induced cytokine gene expression was evaluated with ABI7900 qPCR.

Single cell transcriptomics analysis

T_{reg} cell, $CD4^+$ T cell and 68K PBMC raw sequencing data was downloaded from 10x Genomics online database. Python based Scanpy 1.3.7 software was used for pre-processing and data analysis. For data filtering and quality control the following parameters were used: cell filter, gene filter and mitochondrial filter. Cells were assigned in clusters with louvain algorythm (resolution = 0.8). For data visualisation Uniform Manifold Approximation and Projection (UMAP) dimension reduction method was used. Cell clusters were annotated manually, by using top 50 highest gene expression in each cluster and known markers from the literature.

Statistics

Two-sided Student's unpaired t-test was used for normally distributed data, and ANOVA analysis followed by Bonferroni correction was applied for multiple parameter analysis. In the cases of non-normal distribution data, Mann–Whitney U or Wilcoxon tests

were used, while multiple parameter analysis was calculated by ANOVA test followed by Kruskal–Wallis test. The level of significance was set at $p < 0.05$.

4. Results

BeWo iEVs and rHSPE1 induce T_{reg} cell differentiation *in vitro*

BeWo iEVs induced the differentiation of $CD4^+$ T cells into T_{reg} cells. To interpret the molecular mechanism underlying T_{reg} cell differentiation mass spectrometry analysis of iEVs was performed. We verified the presence of HSPE1 protein in EVs, which functions in *de novo* protein folding (GO:0006458, p = 0.00072) and has the capacity to activate T cells. We proved the presence of HSPE1 protein, its topology and association with iEVs with flow cytometry. We showed, that HSPE1 is found both in the membrane of iEVs and also in the intravesicular lumen. To elucidate the role of HSPE1 in T_{reg} cell differentiation we first investigated the role of the recombinant human rHSPE1 protein. We demonstrated that recombinant HSPE1 treatment induced T_{reg} differentiation in a dose-dependent manner after 72 h. Holomicroscopic studies confirmed that differentiated $CD25^+/CD127^{lo}$ T_{reg} cells were viable.

HSPE1 expression and heterogeneity analysis of T_{reg} cells

To examine the role of HSPE1 in the maintenance of T_{reg} cells, single cell transcriptomics analysis was applied. Analysis of T_{reg} cell sequencing data (10x Genomics repository) revealed 7 T_{reg} cell subtypes based on the global gene expression pattern. HSPE1 expression was detectable in all T_{reg} subgroups, however, the level of expression was found to be different. We have also identified key marker gene panels suitable for T_{reg} cells clustering. Based on global gene expression profile, we could identify 3 T_{reg} cell subtypes in

CD4$^+$ T cell data: naïve, effector-activated and memory T_{reg} cells. From the 3 different cell subtypes only 2 cell clusters were distinguishable, because based on the global gene expression pattern naïve and effector-activated T_{reg} cells showed a significant overlap. The *CAPG* gene showed in CD4$^+$ T cell data the highest expression in T_{reg} cells. In all 3 analysed sequencing data *HSPE1* showed T_{reg} cell subtype dependent expression. Among T cell clusters memory T cells and memory T_{reg} cells showed the highest CAPG expression.

miRNome of BeWo EVs contributes to T_{reg} cell differentiation

miRNA networks can play a significant part in immune tolerance induction, including T_{reg} cell differentiation. We detected immune tolerance associated miRNAs in trophoblast-derived EVs: 1.) hsa-miR-23b miRNA, which inhibits Th17 signalling pathway; 2.) hsa-miR-146a and hsa-miR-155 miRNAs critical for T_{reg} cell differentiation; 3.) high expression of hsa-miR-22 and hsa-miR-221 miRNAs, known as important immune tolerance mediating miRNAs; 4.) every miRNA of the hsa-miR-17~92 polycistronic miRNA cluster, which are key to antigen specific IL-10 producing T_{reg} cell differentiation. Furthermore, every miRNA of the C19MC trophoblast-specific miRNA cluster was expressed both in iEV and sEV fraction.

Identification of circulating preeclamptic EV pattern based on cellular origin

We compared the circulating EV pattern of healthy and preeclamptic pregnant women. Platelet-derived and trophoblast-derived EVs were

detectable at the highest concentration in both groups, however the activated platelet-derived EV number was significantly lower in preeclamptic samples. Opposite of it, significantly higher amounts of tissue factor expressing EVs was detected in preeclamptic samples.

Analysis of EV-target cell interaction

iEVs isolated from healthy and preeclamptic pregnant women's plasma bound to THP-1 monocytic cells. THP-1 cells phagocytosed isolated EVs. Comparing the uptake of EVs isolated from preeclamptic or control plasma samples, we found that phagocytosis of preeclamptic EVs was significantly lower.

Analysis of EV induced cell migration

Both healthy and preeclamptic iEVs are chemoattractant for THP-1 cells. The chemotactic effects of iEVs differed: preeclamptic iEVs induced significantly lower chemotactic activity of THP-1 cells.

Holomicroscopic analysis revealed, that both healthy and preeclamptic iEVs stimulated THP-1 cell migration, and there was no significant difference between their effects. Opposite of it, preeclamptic iEVs induced significantly lower cell motility, compared to iEVs isolated from healthy pregnant.

Investigation of EV induced cytokine gene expression

Both healthy and preeclamptic iEVs induced increased TNF mRNA expression, but the impact of preeclamptic iEVs were significantly higher. The increased TNF production was confirmed at protein level

as well: we showed the intracellular and secreted TNF in the supernatant of THP-1 cells.

To uncover the molecular background of iEV induced cytokine expression and functional effects, we performed mass spectrometry analysis of healthy and preeclamptic EVs. The complex interaction map of healthy iEVs showed a chemoattractant, adhesion and migration related protein pattern. In contrast with this, in preeclamptic iEVs, we found less chemoattractant and cell migration related proteins and more inflammation associated proteins. Comparison of interprotein connections, weaker and fewer protein interactions could be detected in preeclampsia associated EVs.

5. Conclusions

• BeWo iEVs induce *in vitro* T_{reg} cell differentiation.

• BeWo EVs have a protein network that activates immune cells and plays a role in *de novo* protein folding, of which HSPE1 should be highlighted.

• Recombinant HSPE1 and iEV-associated HSPE1 induce *in vitro* T_{reg} cell differentiation from human naïve $CD4^+$ T cells.

• Based on single cells transcriptomics data, T_{reg} cell subtypes can be characterized by distinct expression of HSPE1.

• Single cell transcriptomics data reveal *CAPG* gene to be highly expressed in memory T_{reg} cells, which could be used as a marker for memory T_{reg} cells.

• The miRNA pattern of BeWo EVs favours the development of immune tolerance, mainly by inducing T_{reg} cell differentiation.

• A different circulating EV pattern can be detected in preeclamptic patient compared to healthy pregnant women.

• Both preeclamptic and healthy pregnant plasma-derived iEVs bind to monocytes.

• Binding of pregnancy associated iEVs to monocytes induces phagocytosis, however, preeclampsia associated iEVs are less phagocytosed by THP-1 cells.

• Preeclampsia associated iEVs decrease THP-1 cell motility and increase their adhesion compared to healthy pregnant iEVs.

• Preeclampsia associated iEVs induce TNF and IL-6 production in THP-1 cells.

• Preeclampsia associated iEVs contain altered cell migration, adhesion and inflammatory protein network.

6. Bibliography of own publications

Publications related to the current Ph.D. thesis:

1. **<u>Kovács ÁF</u>**, Fekete N, Turiák L, Ács A, Kőhidai L; Buzás EI, Pállinger É (2019) Unravelling the Role of Trophoblastic-Derived Extracellular Vesicles in Regulatory T Cell Differentiation. **Int. J. Mol. Sci.**, 20: 3457.

2. **<u>Kovács ÁF</u>**, Láng O, Turiák L, Ács A, Kőhidai L, Fekete N, Alasztics B, Mészáros T, Buzás EI, Rigó J Jr, Pállinger É. (2018) The impact of circulating preeclampsia-associated extracellular vesicles on the migratory activity and phenotype of THP-1 monocytic cells. **Sci. Rep.**, 8(1): 5426.

3. Théry C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, Antoniou A, Arab T, Archer F, Atkin-Smith GK, Ayre DC, Bach JM, Bachurski D, Baharvand H, Balaj L, Baldacchino S, Bauer NN, Baxter AA, Bebawy M, Beckham C, Bedina Zavec A, Benmoussa A, Berardi AC, Bergese P, Bielska E, Blenkiron C, Bobis-Wozowicz S, Boilard E, Boireau W, Bongiovanni A, Borràs FE, Bosch S, Boulanger CM, Breakefield X, Breglio AM, Brennan MÁ, Brigstock DR, Brisson A, Broekman ML, Bromberg JF, Bryl-Górecka P, Buch S, Buck AH, Burger D, Busatto S, Buschmann D, Bussolati B, Buzás EI, Byrd JB, Camussi G, Carter DR, Caruso S, Chamley LW, Chang YT, Chen C, Chen S, Cheng L, Chin AR, Clayton A, Clerici SP, Cocks A, Cocucci E, Coffey RJ, Cordeiro-da-Silva A, Couch Y, Coumans FA, Coyle B,

Crescitelli R, Criado MF, D'Souza-Schorey C, Das S, Datta Chaudhuri A, de Candia P, De Santana EF, De Wever O, Del Portillo HA, Demaret T, Deville S, Devitt A, Dhondt B, Di Vizio D, Dieterich LC, Dolo V, Dominguez Rubio AP, Dominici M, Dourado MR, Driedonks TA, Duarte FV, Duncan HM, Eichenberger RM, Ekström K, El Andaloussi S, Elie-Caille C, Erdbrügger U, Falcón-Pérez JM, Fatima F, Fish JE, Flores-Bellver M, Försönits A, Frelet-Barrand A, Fricke F, Fuhrmann G, Gabrielsson S, Gámez-Valero A, Gardiner C, Gärtner K, Gaudin R, Gho YS, Giebel B, Gilbert C, Gimona M, Giusti I, Goberdhan DC, Görgens A, Gorski SM, Greening DW, Gross JC, Gualerzi A, Gupta GN, Gustafson D, Handberg A, Haraszti RA, Harrison P, Hegyesi H, Hendrix A, Hill AF, Hochberg FH, Hoffmann KF, Holder B, Holthofer H, Hosseinkhani B, Hu G, Huang Y, Huber V, Hunt S, Ibrahim AG, Ikezu T, Inal JM, Isin M, Ivanova A, Jackson HK, Jacobsen S, Jay SM, Jayachandran M, Jenster G, Jiang L, Johnson SM, Jones JC, Jong A, Jovanovic-Talisman T, Jung S, Kalluri R, Kano SI, Kaur S, Kawamura Y, Keller ET, Khamari D, Khomyakova E, Khvorova A, Kierulf P, Kim KP, Kislinger T, Klingeborn M, Klinke DJ 2nd, Kornek M, Kosanović MM, **Kovács ÁF**, Krämer-Albers EM, Krasemann S, Krause M, Kurochkin IV, Kusuma GD, Kuypers S, Laitinen S, Langevin SM, Languino LR, Lannigan J, Lässer C, Laurent LC, Lavieu G, Lázaro-Ibáñez E, Le Lay S, Lee MS, Lee YXF, Lemos DS, Lenassi M, Leszczynska A, Li IT, Liao K, Libregts SF, Ligeti E, Lim R, Lim SK, Linē A, Linnemannstöns K, Llorente A, Lombard CA, Lorenowicz MJ, Lörincz ÁM, Lötvall J, Lovett J, Lowry MC, Loyer X, Lu Q, Lukomska B, Lunavat TR, Maas SL,

Malhi H, Marcilla A, Mariani J, Mariscal J, Martens-Uzunova ES, Martin-Jaular L, Martinez MC, Martins VR, Mathieu M, Mathivanan S, Maugeri M, McGinnis LK, McVey MJ, Meckes DG Jr, Meehan KL, Mertens I, Minciacchi VR, Möller A, Møller Jørgensen M, Morales-Kastresana A, Morhayim J, Mullier F, Muraca M, Musante L, Mussack V, Muth DC, Myburgh KH, Najrana T, Nawaz M, Nazarenko I, Nejsum P, Neri C, Neri T, Nieuwland R, Nimrichter L, Nolan JP, Nolte-'t Hoen EN, Noren Hooten N, O'Driscoll L, O'Grady T, O'Loghlen A, Ochiya T, Olivier M, Ortiz A, Ortiz LA, Osteikoetxea X, Østergaard O, Ostrowski M, Park J, Pegtel DM, Peinado H, Perut F, Pfaffl MW, Phinney DG, Pieters BC, Pink RC, Pisetsky DS, Pogge von Strandmann E, Polakovicova I, Poon IK, Powell BH, Prada I, Pulliam L, Quesenberry P, Radeghieri A, Raffai RL, Raimondo S, Rak J, Ramirez MI, Raposo G, Rayyan MS, Regev-Rudzki N, Ricklefs FL, Robbins PD, Roberts DD, Rodrigues SC, Rohde E, Rome S, Rouschop KM, Rughetti A, Russell AE, Saá P, Sahoo S, Salas-Huenuleo E, Sánchez C, Saugstad JA, Saul MJ, Schiffelers RM, Schneider R, Schøyen TH, Scott A, Shahaj E, Sharma S, Shatnyeva O, Shekari F, Shelke GV, Shetty AK, Shiba K, Siljander PR, Silva AM, Skowronek A, Snyder OL 2nd, Soares RP, Sódar BW, Soekmadji C, Sotillo J, Stahl PD, Stoorvogel W, Stott SL, Strasser EF, Swift S, Tahara H, Tewari M, Timms K, Tiwari S, Tixeira R, Tkach M, Toh WS, Tomasini R, Torrecilhas AC, Tosar JP, Toxavidis V, Urbanelli L, Vader P, van Balkom BW, van der Grein SG, Van Deun J, van Herwijnen MJ, Van Keuren-Jensen K, van Niel G, van Royen ME, van Wijnen AJ, Vasconcelos MH, Vechetti IJ Jr, Veit TD, Vella LJ, Velot É, Verweij FJ, Vestad B,

Viñas JL, Visnovitz T, Vukman KV, Wahlgren J, Watson DC, Wauben MH, Weaver A, Webber JP, Weber V, Wehman AM, Weiss DJ, Welsh JA, Wendt S, Wheelock AM, Wiener Z, Witte L, Wolfram J, Xagorari A, Xander P, Xu J, Yan X, Yáñez-Mó M, Yin H, Yuana Y, Zappulli V, Zarubova J, Žėkas V, Zhang JY, Zhao Z, Zheng L, Zheutlin AR, Zickler AM, Zimmermann P, Zivkovic AM, Zocco D, Zuba-Surma EK. (2018) Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. **J. Extracell. Vesicles**, 7(1): 1535750.

Publications not related to the current Ph.D. thesis:

1. Gézsi, A., **Kovács, Á.,** Visnovitz, T., & Buzás, E. I. (2019). Systems biology approaches to investigating the roles of extracellular vesicles in human diseases. **Exp. Mol. Med.**, 51(3): 33.

2. Csabai T, Pallinger E, **Kovacs AF**, Miko E, Bognar Z and Szekeres-Bartho J (2020) Altered Immune Response and Implantation Failure in Progesterone-Induced Blocking Factor-Deficient Mice. **Front. Immunol**., 11: 349.

3. Melicher D, Illés A, Pállinger É, **Kovács ÁF**, Littvay L, Tárnoki ÁD, Tárnoki DL, Bikov A, Molnár MJ, Buzás EI, Falus A. (2017) Tight co-twin similarity of monozygotic twins for hTERT protein level of T cell subsets, for telomere length and mitochondrial DNA copy number, but not for telomerase activity. **Cell. Mol. Life. Sci**., 75(13): 2447-2456.

4. Sódar BW, **Kovács ÁF,** Visnovitz T, Pállinger É, Vékey K, Pocsfalvi G, Turiák L, Buzás E (2017) Best practice of identification

and proteomic analysis of extracellular vesicles in human health and disease. **Expert Rev. Proteomics**, 26: 1-18.

5. Balogh A, Toth E, Romero R, Parej K, Csala D, Szenasi NL, Hajdu I, Juhasz K, **Kovacs AF**, Meiri H, Hupuczi P, Tarca AL, Hassan SS, Erez O, Zavodszky P, Matko J, Papp Z, Rossi SW, Hahn S, Pallinger E, Than NG. (2019) Placental Galectins Are Key Players in Regulating the Maternal Adaptive Immune Response. **Front. Immunol.**, 10: 1240.

6. Hegyesi, H; Sándor, N; Sáfrány, G; Lovas, Virág; **Kovács, Á**; Takács, A; Kőhidai, L; Turiák, L; Kittel, Á; Pálóczi, K; Bertók L; Buzás E. (2019) Radio-detoxified LPS alters bone marrow-derived extracellular vesicles and endothelial progenitor cells. **Stem Cell Res. Ther.**,10(1): 313.

Scientific dissemination articles:

1. **Kovács ÁF** (2015) Életre kész alkotás. **Élet és Tudomány**, 46 (70): 1454-1455.

2. **Kovács ÁF** (2019) A szabályozó T-sejteket meghatározó HSPE1, azaz a várandósság sorsdöntő génje – A terhesség titkos sorsának felderítése. **Természet Világa**, 150(8): 343-346

Book chapters:

1. **Árpád Ferenc Kovács**. Chapter 2. Current Trends on Exploring the Multifaceted Role of Extracellular Vesicles in Human Pregnancy. In: Lafon (ed.) **Extracellular Vesicles: Mechanisms and Role in Health and Disease. Nova Science Publishers Inc.,** New York, 2019: 37-60.

Cumulative impact factor: **48,499**