FINAL REPORT

(01. October 2016. – 31. March 2021)

„ The role of immune checkpoint molecules in the maintenance of feto-maternal tolerance during pregnancy and in pathologic pregnancies”

NKFI-6 K-119529

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1. PH.D. THESIS DEFENSE

My Ph.D. student, Adrienn Lajkó, successfully defended her Ph.D. thesis titled “**Feto-maternal immune regulation by TIM-3/GAL-9 and PD-1/PD-L1 pathways in healthy, mifepristone treated and PACAP-deficient pregnant mice**” on the 5th of March 2021. This Ph.D. thesis was completely supported and funded by this grant.

2. ORAL AND POSTER PRESENTATIONS

All the results obtained during this time period were presented at international (oral and poster presentation) and national (oral and poster presentation) conferences.

3. PUBLICATIONS WITH "NKFIH SUPPORT" IN THE ACKNOWLEDGEMENT

2017

1. Meggyes M, **Szereday L**, Jakso P, Bogar B, Bogdan A, Nörenberg J, Miko E, Barakonyi A. Expansion of CD4 phenotype among CD160 receptor-expressing lymphocytes in murine pregnancy. Am J Reprod Immunol. 2017 Sep 16. doi: 10.1111/aji.12745. (Impact factor: **2.745**)

2. Brubel R, Bokor A, Pohl A, Schilli GK, **Szereday L**, Bacher-Szamuel R, Rigo J, Polgar B. Serum galectin-9 as a noninvasive biomarker for the detection of endometriosis and pelvic pain or infertility-related gynecologic disorders. Fertility and Sterility 2017 Dec;108(6):1016-1025.e2 (Impact factor: **4.803**)

2018

3. Meggyes M, Szanto J, Lajko A, Farkas B, Varnagy A, Tamas P, Hantosi E, Miko E, **Szereday L.** The possible role of CD8+/Vα7.2+/CD161++ T (MAIT) and CD8+/Vα7.2+/CD161lo T (MAIT-like) cells in the pathogenesis of early-onset preeclampsia. Am. J. Reprod Immunol. 2018 Feb;79(2). doi: 10.1111/aji.12805. (Impact factor: **3.091**)

4. Lajko A, Meggyes M, Polgar B, **Szereday L.** The immunological effect of Galectin-9/TIM-3 pathway after low dose Mifepristone treatment in mice at 14.5 day of pregnancy. PLoS One. 2018 Mar 22;13(3):e0194870. doi: 10.1371/journal.pone.0194870. (Impact factor: **2.776**)

5. Lajko A, Meggyes M, Fulop BD, Gede N, Reglodi D, **Szereday L.** Comparative analysis of decidual and peripheral immune cells and immune-checkpoint molecules during pregnancy in wild-type and PACAP-deficient mice. Am J Reprod Immunol. 2018 Oct;80(4):e13035. doi: 10.1111/aji.13035. (Impact factor: **3.091**)

2019

6. Meggyes M, Miko E, Lajko A, Csiszar B, Sandor B, Matrai P, Tamas P, **Szereday L.** Involvement of the PD-1/PD-L1 Co-Inhibitory Pathway in the Pathogenesis of the Inflammatory Stage of Early-Onset Preeclampsia. Int J Mol Sci. 2019 Jan 29;20(3). (Impact factor: **4.556**)

7. Meggyes M, Miko E, Szigeti B, Farkas N, **Szereday L.** The importance of the PD-1/PD-L1 pathway at the maternal-fetal interface. BMC Pregnancy Childbirth. 2019 Feb 19;19(1):74. (Impact factor: **2.239**)

8. Miko E, Meggyes M, Doba K, Barakonyi A, **Szereday L.** Immune Checkpoint Molecules in Reproductive Immunology. Front Immunol. 2019 Apr 18;10:846. (Impact factor: **5.085**)

2020

9. Meggyes M, Nagy DU, **Szereday L.** Investigation of the PD-1 and PD-L1 Immune Checkpoint Molecules Throughout Healthy Human Pregnancy and in Nonpregnant Women. J Clin Med. 2020 Aug 6;9(8):2536. (Impact factor: **3.303**)

10. Meggyes M, Nagy DU, Szigeti B, Csiszar B, Sandor B, Tamas P, **Szereday L.** Investigation of mucosal-associated invariant T (MAIT) cells expressing immune checkpoint receptors (TIGIT and

CD226) in early-onset preeclampsia. Eur J Obstet Gynecol Reprod Biol. 2020 Sep;252:373-381. (Impact factor: **1.868**)

11. Meggyes M, Lajko A, Fulop BD, Reglodi D, **Szereday L.** Phenotypic characterization of testicular immune cells expressing immune checkpoint molecules in wild-type and pituitary adenylate cyclase-activating polypeptide-deficient mice. Am J Reprod Immunol. 2020 Mar;83(3):e13212. (Impact factor: **2.739**)

12. Meggyes M, **Szereday L,** Bohonyi N, Koppan M, Szegedi S, Marics-Kutas A, Marton M, Totsimon A, Polgar B. Different Expression Pattern of TIM-3 and Galectin-9 Molecules by Peripheral and Peritoneal Lymphocytes in Women with and without Endometriosis. Int J Mol Sci. 2020 Mar 28;21(7):2343. (Impact factor: **4.556**)

13. Szereday L, Meggyes M, Berki T, Miseta A, Farkas N, Gervain J, Par A, Par G. Direct-acting antiviral treatment downregulates immune checkpoint inhibitor expression in patients with chronic hepatitis C. Clin Exp Med. 2020 May;20(2):219-230. (Impact factor: **2.642**)

2021

14. Peterfalvi A, Meggyes M, Makszin L, Farkas N, Miko E, Miseta A, **Szereday L.** Forest Bathing Always Makes Sense: Blood Pressure-Lowering and Immune System-Balancing Effects in Late Spring and Winter in Central Europe. Environ Res Public Health. 2021 Feb 20;18(4):2067. (Impact factor: **2.849**)

5. RESULTS OF HUMAN EXPERIMENTS

5.1. The possible role of immune checkpoint pathways in healthy pregnancy

5.1.1. At the maternal-fetal interface

Investigation of the immune-checkpoint molecules at the maternal-fetal interface is crucial to clarify the exact immunological relationship between the mother and the fetus. This knowledge could help us to understand the background of pathological pregnancy conditions such as preeclampsia and intrauterine growth restriction. One of the biggest challenges in reproductive immunology is finding a proper biomarker or biomarker panel for the early diagnosis of preeclampsia. We hope that our results supplemented with previous findings, will contribute to understanding the background of this pathological condition.

The aim of this present study was to clarify the contribution of the PD-1/PD-L1 immune-checkpoint pathway in the induction of maternal tolerance during a healthy pregnancy.

The results of this present study demonstrated the complexity of the activating and inhibitory mechanisms at the maternal-fetal interface. Our findings suggest that PD-1/PD-L1 immune-checkpoint pathway could play a novel role in the maintenance of this sensitive immunological balance between the mother and the fetus.

5.1.1. At the periphery

This study focused on the involvement of the PD-1/PD/L1 mediated immunoregulation at the periphery throughout pregnancy.

Our results about the peripheral expression of PD-1, PD-L1, and the connection with NKG2D activatory receptor, primarily in the case of CD8+T and NKT-like cells, could contribute to the changes of the Th1/Th2/Th1 predominance and can help to maintain maternal immunotolerance in the periphery too.

5.2. The possible role of immune checkpoint pathways in early-onset preeclampsia

5.2.1 Immune checkpoint expression by MAIT, MAIT-like cells in patients with early-onset preeclampsia

During this work, we examined the possible contribution of Mucosal associated invariant T cells (MAIT) cells in the pathogenesis of the clinical phase of early-onset preeclampsia and how this could be influenced by TIGIT and CD226 immune checkpoint molecules.

Considering that we could not detect a notable difference between early-onset preeclampsia and healthy pregnancy, we hypothesize that peripheral MAIT cells expressing TIGIT and CD226 have a marginal role in the pathogenesis of early-onset preeclampsia.

5.2.2 The role of PD-1/PD-L1 co-stimulatory immune checkpoint pathway in the pathogenesis of early-onset preeclampsia

Since little is known about the involvement of PD-1 mediated immunoregulation in pregnancy and pregnancy-related disorders. In this study, we investigated the possible role of the PD-1/PD-L1 co-stimulatory immune checkpoint pathway in the pathogenesis of the clinical phase of early-onset preeclampsia, analyzing phenotypic and functional characteristics of peripheral blood lymphocytes.

Our data indicate a disturbed balance between immunotolerance and immunoactivation, suggesting the failure of immune regulation allowing exaggeration of inflammatory responses in early-onset preeclampsia.

Our findings indicate an enhanced presence of the PD-1/PD-L1 immune checkpoint pathway in early-onset preeclampsia, theoretically creating a more likely condition for interacting and damping inflammation as observed in a healthy pregnancy.

5.3. The possible role of immune checkpoint pathways in endometriosis

5.3.1 Serum Galectin-9 as a non-invasive biomarker for the detection of endometriosis

Because our previous studies showed increased Gal-9 staining in ectopic endometriosis lesions, our main goal was to examine the diagnostic potential of serum soluble Gal-9 measurement in the non-invasive diagnosis of endometriosis.

We found a notable elevation of soluble Gal-9 level in the serum of I.-II. stages of endometriosis in comparison to healthy controls strongly suggest its potential usefulness in the sensitive, early laboratory diagnosis.

We conclude that we identified Gal-9 as a new, potential biomarker for the non-invasive, laboratory diagnosis endometriosis that has a better diagnostic performance than that of the other endometriosis biomarkers like CA-125, IL-6, Hs-CRP, TNF-α or VEGF. We suggest that Gal-9 might be potentially used for the non-invasive monitoring of endometriosis.

5.3.2 The involvement of the TIM-3/Gal-9 pathway in the development of endometriosis-associated immunological abnormalities

We demonstrated that the distribution and the cell surface expression of the TIM-3 receptor and the Gal-9 ligand are significantly altered on different peripheral and peritoneal T and NK cell subsets of endometriosis-affected patients.

Our results suggest a persistent activation and disturbed TIM-3/Gal-9-dependent regulatory function in endometriosis, which may be involved in the impaired immune surveillance mechanisms, promotes the survival of ectopic lesions and aids the evolution of reproductive failures in endometriosis.

6. RESULTS OF ANIMAL STUDIES

6.1 The role of CD4 T cells among CD160 receptor-expressing lymphocytes in murine pregnancy

Understanding the function and role of CD160 receptor-positive cells is a great challenge for scientists. Available data are hardly adaptable for such a complex immunological situation as pregnancy; therefore, the precise role of CD160 receptor during a healthy pregnancy is still unknown. In addition, due to differing literary data about the role of CD160 receptor on T cells, specific functional investigations for each concrete immunological situation, including pregnancy, are needed in order to understand the impact of CD160 in each scenario.

The increase of the CD4 phenotype among CD160+ cells in murine pregnancy is a novel observation we made in our study, which has potential significance. What factors determine this CD4+ T-cell expansion among CD160+ lymphocytes and what is their real function in the feto-maternal unit remains an open question.

6.2 The possible role of TIM-3/Galectin-9 pathway in Mifepristone induced medical abortion

Since its approval in France in 1988, the abortifacient Mifepristone (RU486) has proven to be a safe, effective, acceptable option for millions of women seeking an abortion during the first several weeks of pregnancy. The exact mechanism of action of Mifepristone is not well investigated and has to be fully elucidated; therefore the development of an animal model that captures the effects of Mifepristone-induced immunological changes during pregnancy may help to expand our understanding of the abortion process.

We examined the alterations of the Gal-9/TIM-3 pathway that might play an important role in the immunological changes caused by Mifepristone treatment using a syngeneic pregnant mouse model.

Our data indicate that even a low dose Mifepristone treatment was effective enough to abrogate Gal-9 production of the placenta. The observed, increased Gal-9 expression by decidual Treg and CD4+Th cells suggest that local immunosuppressive mechanisms are also triggered 24 hours after the treatment, possibly to sustain impaired placental function. In addition, we suppose that the accumulation of the Gal-9 secreting Gal-9+ Th cells might also be involved to partly compensate the decreased placental Gal-9 expression at the feto-maternal interface. These mechanisms might inhibit the pro-inflammatory cytokine production of Th1 and Th17 cell by a Gal-9/TIM-3 dependent fashion and aid the maintenance of the whole embryo-placenta unit. Although low dose RU486 treatment did not cause considerable change in the phenotypic distribution of decidual and splenic immune cells, it altered the Gal-9 and TIM-3 expression by

different NK and T cell subsets. RU486 can induce an immunological alteration in a Gal-9/TIM-3 independent way. In addition, the treatment significantly decreased the CD107a expression (cytotoxicity) by decidual TIM-3+ NK cells but increased its expression by decidual NKT cell compared to the splenic counterparts. These findings suggest that low dose Mifepristone administration might induce immune alterations in both progesterone-dependent and independent ways.

6.3 TIM-3/Galectin-9 pathway and PD-1 molecule in PACAP-deficient pregnant mice

This investigation was done in collaboration with Prof. Reglodi in the Department of Anatomy at the University of Pécs.

PACAP has several regulatory functions in reproduction. PACAP-deficient mice display several abnormalities in several physiological and pathological processes, including reproductive functions. It has long been known that PACAP deficient mice have decreased the reproductive rate, and several mechanisms have been suggested. PACAP is a known modulator of the innate and adaptive immune response. However, it is not known whether PACAP plays a role in the maternal immune tolerance towards a fetus.

In conclusion, despite the found alterations in the peripheral number and function of immune cells, we could not find any remarkable alteration either in the distribution or in the cytotoxicity of the investigated decidual immune cells, which could elucidate any reproductive alterations in pregnant PACAP-deficient mice. The only remarkable finding is the recruitment of Gal-9+ Th cells to the decidua promoting local immune homeostasis in PACAP KO mice, which nevertheless cannot explain the reduced fertility observed in these mice.

6.4 Testicular immune cells expressing immune checkpoint molecules in PACAP-deficient mice

PACAP-deficient male mice have several morphological, biochemical, behavioral defects and show disturbed signaling in spermatogenesis affecting fertility in PACAP KO mice. Reproductive functions such as fertility, mating, and maternal behaviors have been widely investigated, but no immune analyses are available regarding the testicular immune-privileged environment in male PACAP-deficient mice.

In this study, we found that the increased number of testicular CD8+ T cells together with the decrease of Treg cell number could be a key player behind the immunological and fertility alteration documented in male PACAP KO mice. The expression of PD-1 was significantly decreased by these immune cells suggesting an impaired PD-1/PD-L1 pathway. We speculate that these local changes may result in an immune activation with disturbed testicular immunoregulation in PACAP KO mice; however, determining the exact function requires further investigations. Our data further support the view that besides a systemic immune tolerance, localized active immunosuppression is involved in the regulation of testicular immune privilege.