FINAL REPORT

(01. September 2012. – 31. August 2016)

„Role of the Galectin-9/TIM-3 pathway in the maintenance of immune-tolerance during pregnancy”

OTKA-K 104960

PI: Laszlo Szereday MD, PhD., Med. Habil.

1. PHD THESIS DEFENSE

My PhD student, Mátyás Meggyes successfully defended his PhD thesis titled “**Investigation of TIM-3 and Galectin-9 molecules in healthy and pathologic pregnancies and in infertile women**” on the 2nd of July 2015. This PhD thesis was completely supported and funded by this grant.

2. ORAL AND POSTER PRESENTATIONS

All the results obtained during this time period were presented in international (12 oral and poster presentation) and national (14 oral and poster presentation) conferences.

3. PUBLICATIONS WITH "OTKA SUPPORT" IN THE ACKNOWLEDGEMENT

1. Miko E, Meggyes M, Bogar B, Schmitz N, Barakonyi A, Varnagy A, Farkas B, Tamas P, Bodis J, Szekeres-Bartho J, Illes Zs, **Szereday L.** Involvement of Galectin-9/TIM-3 pathway in the systemic inflammatory response in early-onset preeclampsia. Plos One 2013; 8: e71811 **Impact factor: 3,534**

2. Hatzipetros I, Gocze P, Koszegi T, Jaray A, **Szereday L**, Polgar B, Farkas N, Farkas B. Investigating the clinical potential for 14-3-3 zeta protein to serve as a biomarker for epithelial ovarian cancer. J Ovarian Res. 2013 Nov 15;6(1):79. [Epub ahead of print] **Impact factor: 2,03**

3. Barakonyi A, Miko E, **Szereday L**, Polgar PD, Nemeth T, Szekeres-Bartho J, Engels GL. Cell Death Mechanisms and Potentially Cytotoxic Natural Immune Cells in Human Pregnancies Complicated by Preeclampsia. Reprod Sci. 2014 Feb;21(2):155-66. **Impact factor: 2,23**

4. Meggyes M, Miko E, Polgar B, Bogar B, Farkas B, Illes Z, **Szereday L.** Peripheral blood TIM-3 positive NK and CD8+ T cells throughout pregnancy: TIM-3/galectin-9 interaction and its possible role during pregnancy. PLoS One. 2014 Mar 20;9(3):e92371. **Impact factor: 3,234**

5. Horvath G, Reglodi D, Brubel R, Halasz M, Barakonyi A, Tamas A, Fabian E, Opper B, Toth G, Cohen M, **Szereday L.** Investigation of the Possible Functions of PACAP in Human Trophoblast Cells. J Mol Neurosci. 2014 Nov;54(3):320-30. **Impact factor: 2,343**

6. Meggyes M, Lajko A, Palkovics T, Totsimon A, Illes Z, **Szereday L**, Miko E. Feto-maternal immune regulation by TIM-3/galectin-9 pathway and PD-1 molecule in mice at day 14.5 of pregnancy. Placenta. 2015 Oct;36(10):1153-60 **Impact factor: 2,71**

7. Par G, **Szereday L**, Berki T, Palinkas L, Halasz M, Miseta A, Hegedus G, Szekeres-Bartho J, Vincze A, Hunyady B, Par A. Increased baseline proinflammatory cytokine production in chronic hepatitis C patients with rapid virological response to peginterferon plus ribavirin. PLoS One. 2013 Jul 9;8(7):e67770. doi: 10.1371 **Impact factor: 3,534**

8. L. Szereday, M. Meggyes, M. Halasz, J. Szekeres-Bartho, A. Par, G. Par Immunological changes in different patient populations with chronic hepatitis C virus infection World J Gastroenterol. May 28, 2016; 22(20): 4848-4859 **Impact factor: 2,369**

9. A. Lajko, M. Meggyes, B. Polgar, **L. Szereday** The possible role of Galectin-9/TIM-3 pathway in Mifepristone induced medical abortion in mice Submitted to Placenta **Impact factor: 2,972**

10. E. Miko, M. Meggyes, K. Doba, N. Farkas, B. Bogar, A. Barakonyi, **L. Szereday**, J. Szekeres-Bartho, E. Mezosi. Innate immune response in euthyroid and hypothyroid women with thyroid autoimmunity experiencing reproductive failure Submitted to J Autoimmunity **Impact factor: 7,76**

4. BOOK CHAPTER

1. G. Horvath, J. Nemeth, R. Brubel, B. Opper, M. Koppan, A. Tamas, **L. Szereday**, D. Reglodi. Occurrence and Functions of PACAP in the Placenta. In. D. Reglodi, A. Tamas Eds. Pituitary Adenylate Cyclase Activating Polypeptide - PACAP. 2016 Springer

5. RESULTS OF HUMAN EXPERIMENTS

Pregnancy is an ideal condition to study active immunotolerance. During pregnancy the fetus will not be attacked or rejected by the maternal immune system but rather successfully accepted by the mother. Precise immunoregulation of the maternal immune system is critical for normal pregnancy and fetal development.

We had planned to conduct our research in two areas: first investigating the immunological mechanisms in the periphery (human experiments) analyzing peripheral blood mononuclear cells (PBMC) obtained from heparanized venous blood. The other part of the work (animal experiments) focused on the local immunological regulatory mechanisms directly investigated in placental samples (decidua and trophoblast).

5.1. Possible role of TIM-3/Gal-9 pathway in healthy pregnancy

We investigated the percentage of CD3+ T cells, CD4+ Th, CD8+ Tc cell subpopulations, NK cells, NKT cells and Gal-9+ Th cells in the peripheral blood of normal pregnant women during each trimester of pregnancy and in non-pregnant women.

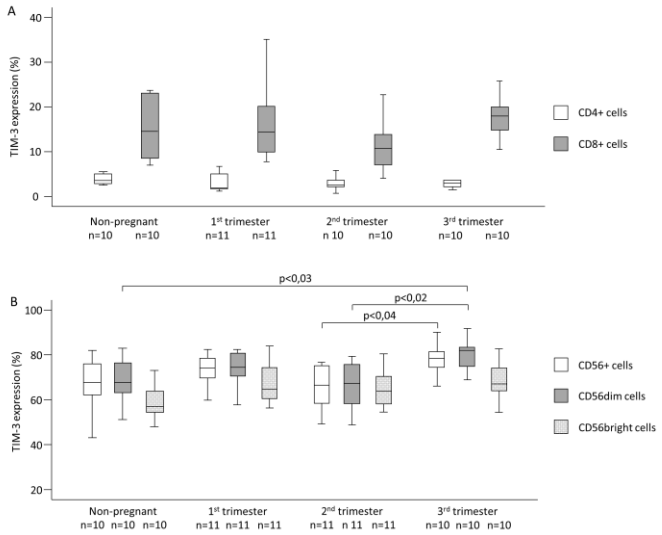
	Non-pregnant	1st trimester	2nd trimester	3rd trimester
No. of patients	30	16	19	22
Age (years)	33 (19-44)	31 (27-35)	32 (26-45)	33 (28-43)
Gestation age at sampling (weeks)	-	12 (11-16)	26 (24-28)	36 (35-38)
Gestation age at birth (weeks)	-	38,8	38,0	39,0
Gravidity	1,5	1,2	1,9	1,7
Parity	1,0	0,4	0,7	0,5

The frequency of NK cells and NK^{dim} cells throughout pregnancy was lower and the frequency of NK^{bright} cells was higher than in non-pregnant women but these results did not reach the level of significance. The frequency of Gal-9+ Th cells were approximately 1% in non-pregnant women and we detected an increased frequency throughout pregnancy reaching 2,39 % in the third trimester. The frequency of Gal-9 Th cells in the third trimester was significantly higher than in non-pregnant women, as well as women in the first and second trimester.

	Non-pregnant	1st trimester	2nd trimester	3rd trimester	P-value
CD3+ T cells	**66,64**±2,23	**65,10**±3,27	**69,43**±3,33	**67,74**±1,34	NS
CD4+ T cells	**44,04**±2,32	**39,92**±2,28	**45,68**±3,47	**39,03**±2,60	NS
CD8+ T cells	**32,02**±1,51	**35,25**±1,53	**28,98**±2,67	**34,31**±2,41	NS
CD8+ TIM-3+ T cells	**5,59**±0,83	**6,0**±0,89	**4,36**±0,88	**5,97**±0,50	NS
CD3- CD56+ cells	**13,65**±1,38	**12,31**±1,85	**10,69**±1,74	**11,47**±1,53	NS
CD3- CD56dim cells	**12,72**±1,31	**10,56**±1,75	**9,29**±1,69	**9,74**±1,39	NS
CD3- CD56bright cells	**1,03**±0,24	**1,80**±0,29	**1,44**±0,30	**1,76**±0,24	NS
CD3- CD56+ TIM-3+ cells	**9,61**±1,41	**9,16**±1,53	**7,34**±1,30	**9,05**±1,30	NS
CD3- CD56dim TIM-3+ cells	**9,02**±1,38	**8,10**±1,42	**6,49**±1,25	**8,07**±1,23	NS
CD3- CD56bright TIM-3+	**0,61**±0,18	**1,11**±0,22	**0,93**±0,24	**1,11**±0,17	NS
CD3+ CD56+ cells	**4,08**±0,75	**6,37**±1,21	**4,70**±0,98	**5,46**±1,26	NS

Gal-9+ Th cells	**0,97**±0,13	**0,66**±0,09	**1,17**±0,3	**2,39**±0,49	**3rd vs. NP p<0,01**

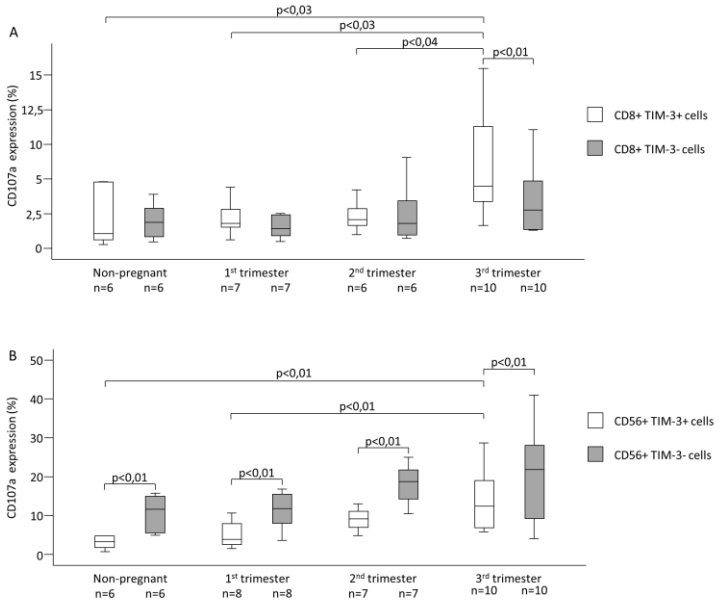
Investigating TIM-3 expression by CD8+ T cells we found a decrease in the 2nd trimester compared to other trimesters and to the samples from the non-pregnant group, however this change did not reach the level of statistical significance. Furthermore TIM-3 expression was significantly increased by NK cells in samples from the 3rd trimester compared to the samples in the 2nd trimester. Analyzing the NK cell subsets, the TIM-3 expression by NK^{dim} cells was significantly increased in samples from the 3rd trimester compared to the samples from the 2nd trimester and from non-pregnant women.



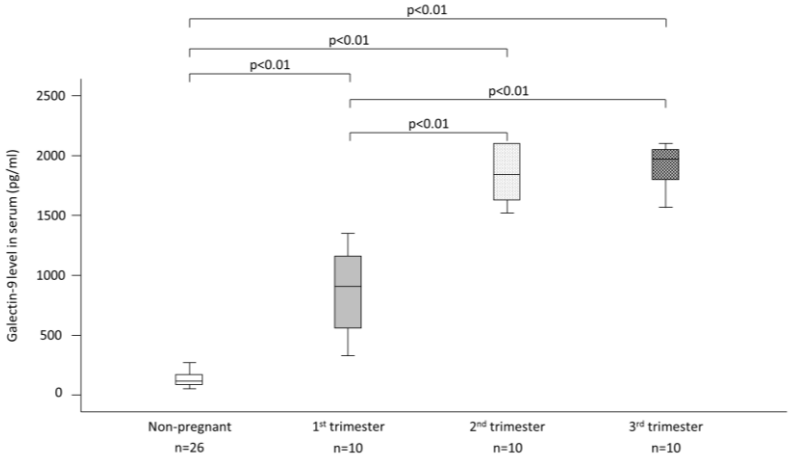
Th1, Th2 and Th17 cytokines were analyzed by CBA system, where IL-4, IL-6 and IL-1 cytokines were under the detectable level. CD8+ Tc cells expressing TIM-3 produced significantly lower level of proinflammatory (IL-2, TNF-α and IFN-Ɣ) and Th17 cytokines compared to TIM-3 negative counterparts in the 1st and 3rd trimester of pregnancy and in healthy non-pregnant controls. IL-2 cytokine production by TIM-3+ NK^{dim} cells was significantly lower compared to TIM-3 negative NK^{dim} cells in the 1st and 2nd trimester of pregnancy. In the 2nd trimester of pregnancy IFN-Ɣ cytokine production by TIM-3+ NK^{bright} cells was significantly higher compared to TIM-3 negative NK^{bright} cells.

pg/ml		**Non-pregnant**			**1st trimester**			**2nd trimester**			**3rd trimester**		
		TIM-3+	TIM-3-	p	TIM-3+	TIM-3-	p	TIM-3+	TIM-3-	p	TIM-3+	TIM-3-	p
CD56 dim cells	IL-2	9,18	117,18	ns	9,53	62,95	**<0,04**	9,25	17,17	**<0,01**	9,23	80,71	ns
	TNF-α	1625,01	2157,81	ns	1820,63	2278,48	ns	104,75**	793,57	ns	439,75**	403,62	ns
	IFN-Ɣ	2010,29	2172,1	ns	3095,85	3516,78	ns	218,03**	1095,87	ns	816,23***	696,77$	ns
	IL-17	<min	<min	ns	<min	<min	ns	<min	<min	ns	<min	<min	ns
CD56 bright cells	IL-2	20,97	114,27	ns	13,25	39,99	ns	14	12,78	ns	12,93	50,89	ns
	TNF-α	1211,37	1786,06	ns	1036,76	1315,13	ns	641,82	461,83	ns	1188,97	1046,67	ns
	IFN-Ɣ	1961,81	3513,19	ns	2656,23	2543,13	ns	1632,29	979,18	**<0,04**	1894,9	2136,18	ns
	IL-17	<min	<min	ns	<min	<min	ns	<min	<min	ns	<min	<min	ns
CD8 T cells	IL-2	3076,06	6400,32	**<0,05**	2463,73	7812,88	**<0,01**	687,87	1276,9*	ns	2587,58	7471,57	**<0,03**
	TNF-α	1385,92	6064,46	**<0,01**	1217,89	6654,4	**<0,01**	356,7	1468,89$	ns	861,61	3427,65	**<0,01**
	IFN-Ɣ	3455,09	5750,34	ns	4223,87	6306,06	**<0,05**	908,5$$$	2093$$	ns	2364,49	4851,96	**<0,04**
	IL-17	17,33	345,2	**<0,04**	16,93	247,9	**<0,05**	9,83	11,49	ns	20,83	257,01	**<0,04**

Investigating the cytotoxic activity of TIM-3+ CD8+ Tc cells during pregnancy, we found that CD107a expression was significantly higher in samples from 3rd trimester compared to 1st and 2nd trimester and non-pregnant women. CD8+ Tc cells expressing TIM-3 in the 3rd trimester of pregnancy showed significantly increased CD107a expression compared to TIM-3 negative CD8+ Tc cells. Analyzing CD107a expression by the NK^{bright} subpopulation showed no significant differences. Interestingly cytotoxic activity of TIM-3+ NK cell and NK^{dim} cell were significantly lower in non-pregnant women and in all trimesters compared to TIM-3 negative counterparts.



Serum Gal-9 levels differ significantly between non-pregnant and healthy pregnant women in each trimester. Analyzing Gal-9 levels throughout pregnancy we found an increasing tendency with a significant elevation of serum Gal-9 concentration in the 2nd and 3rd trimester compared to the 1st trimester.



To conclude, our results indicate that **TIM-3/Galectin-9 pathway**, especially Galectin-9 expressing regulatory T cells, TIM-3+ cytotoxic T cells and NK cells could **play an important role in the maintenance of healthy pregnancy**.

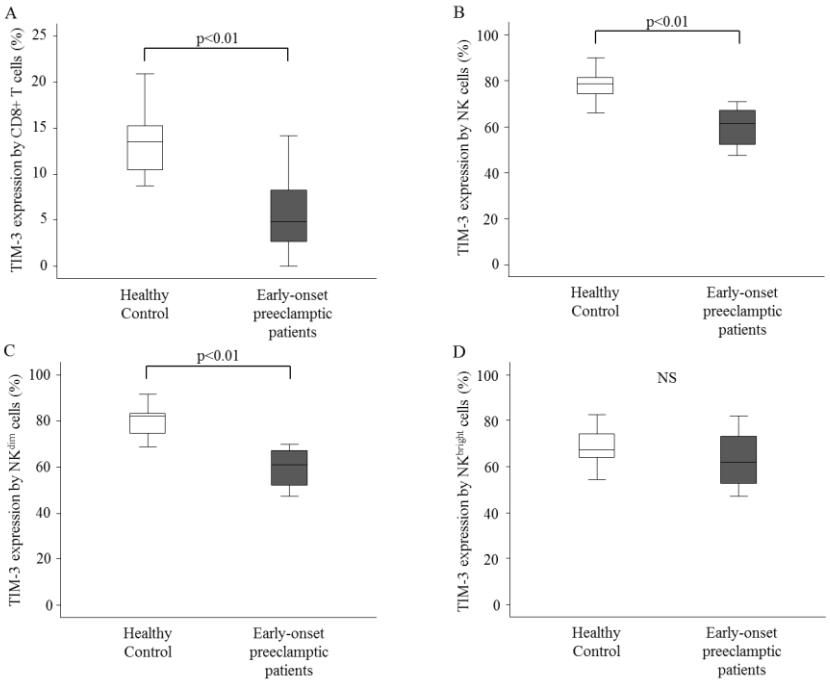
5.2. Possible role of TIM-3/Gal-9 pathway in early-onset preeclampsia

	Healthy pregnant women	**Early-onset preeclamptic women**	P-value
No. of patients	25	27	
Age (years) (mean)	32,6±1,03	29,1±1,53	NS
Gestational age at sampling (mean)	35,64±0,27	33,74±0,48	NS
Gestational age at birth (mean)	38,9±0,27	34,1±0,64	P<0,05
Birth weight (mean)	3420±126,84	1881±148,28	P<0,05
Previous live birth/ patients	058±0,15	088±0,31	NS

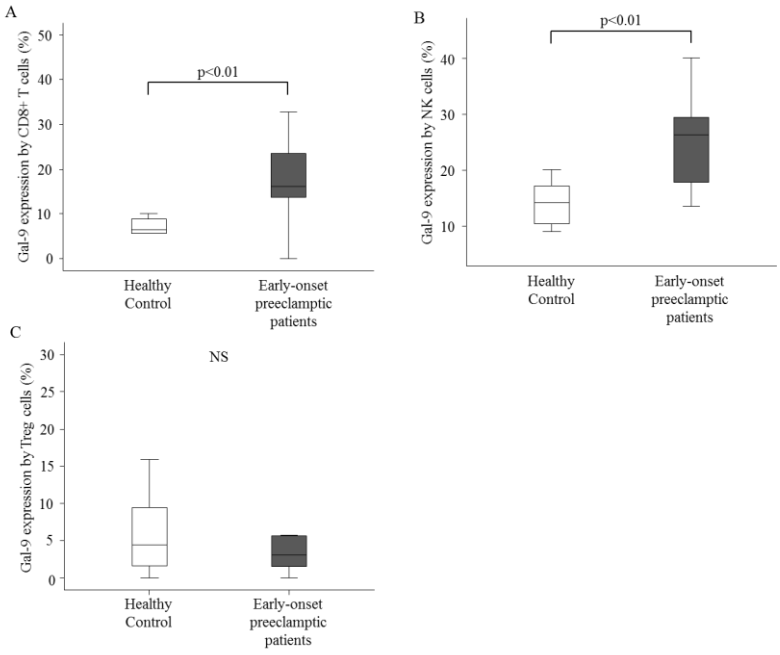
We compared the frequency of CD3+ T cells, helper and cytotoxic T cell subpopulations, regulatory T cells, NK cells, NK^{dim} cells, NK^{bright} cells, and NKT cells among peripheral blood mononuclear cells in women with early-onset preeclampsia and in healthy pregnant women. Compared to healthy pregnant controls, in the peripheral blood of early-onset preeclamptic women there is a significant decrease in the frequency of regulatory T cells and in the frequency of NK^{bright} cells.

	Healthy pregnant women	**Early-onset preeclamptic women**	P-value
CD3+ T cells	67,51±1,5	66,1±4,39	NS
CD4+ T cells	39,98±2,93	37,53±2,98	NS
CD8+ T cells	31,29±2,52	29,92±2,52	NS
Regulatory T cells	0,92±0,12	0,55±0,08	P<0,05
CD3-CD56+ cells	9,91±0,94	8,12±1,25	NS
$CD3^{-}CD56^{dim}$cells	8,53±0,92	7,54±1,15	NS
$CD3^{-}CD56^{bright}$ cells	1,41±0,12	0,61±0,13	P<0,01
CD3+CD56+ cells	5,11±1,18	3,35±1,11	NS

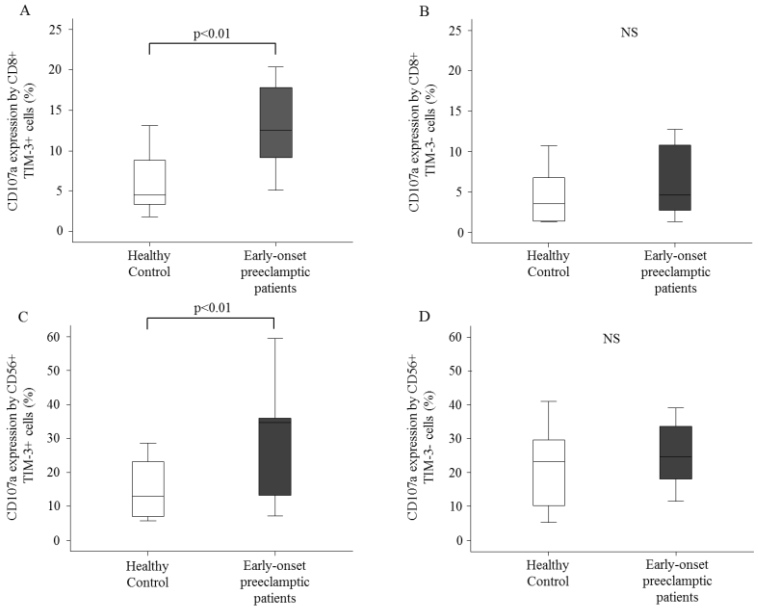
Investigating peripheral blood mononuclear cells of women with early-onset preeclampsia, our results showed a decreased TIM-3 expression by CD8+ Tc cells, NK cells and NK^{dim} cells compared to healthy pregnant women.



Analyzing the Gal-9 expression of peripheral lymphocytes we found a notably increased frequency of Gal-9 positive CD8+ Tc, NK and NK^{bright} cells in the case of early-onset preeclamptic patients when compared to healthy pregnant controls while the frequency of Gal-9+ Treg cells did not changed.



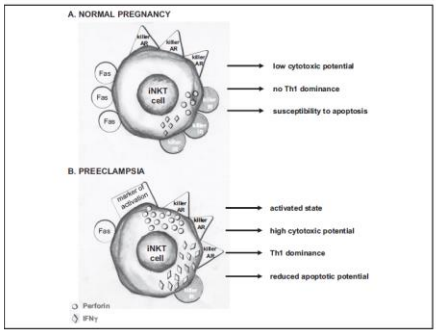
Investigating the cytotoxic activity of CD8+ Tc and NK cells, we found that only TIM-3 positive CD8+ Tc and NK cells showed increased cytotoxicity in women with early-onset preeclampsia compared to healthy pregnant women. Interestingly, TIM-3 positive NK^{dim} cells from women with early-onset preeclampsia showed significantly increased CD107a expression compared to healthy pregnant women and this difference was not observed in the case of NK^{bright} cells.



To conclude, our data suggest that Gal-9/TIM-3 pathway could play an important role in the immune regulation during pregnancy and the **altered Galectin-9 and TIM-3 expression could result an enhanced systemic inflammatory response** including the activation of Th1 lymphocytes **in preeclampsia**.

5.3. Cell death mechanisms and potentially cytotoxic natural immune cells in human pregnancies complicated by preeclampsia

Recent studies confirm that preeclampsia is the extreme end of a normal inflammatory reaction, which also occurs in healthy pregnancies. **Our review focuses on maternal immune changes during preeclampsia leading to altered cytotoxic responses.** The potential role of Perforin/Granzyme-, Fas/FasL-, TNF-α- or TRAIL-mediated apoptotic mechanisms in the pathomechanism is analyzed. Effector cytotoxic cells of natural immunity itself such as NK-, NKT- and gamma/delta T-cells are also changed in the frequency and function both in the periphery and locally in the uterus influencing the outcome of pregnancy. Here, authors conclude that beside exaggerated inflammatory responses apoptotic and killing mechanisms seem to be also implicated in the pathogenesis of preeclampsia.



5.4. The possible role of PACAP in healthy pregnancy.

This investigation was done in collaboration with Prof. Reglodi at the Dept. of Anatomy and these preliminary result let us have the opportunity to continue this project in our next founded NKFIH project.

Pituitary adenylate cyclase activating polypeptide (PACAP) is an endogenous neuropeptide having a widespread distribution both in the nervous system and peripheral organs including the female reproductive system. The aim of the study was to investigate the effects of PACAP on invasion, proliferation, cell survival and angiogenesis of trophoblast cells.

In summary, **PACAP seems to play various roles in human trophoblast cells**, depending on the cell type and microenvironmental influences.

6. RESULTS OF ANIMAL STUDIES

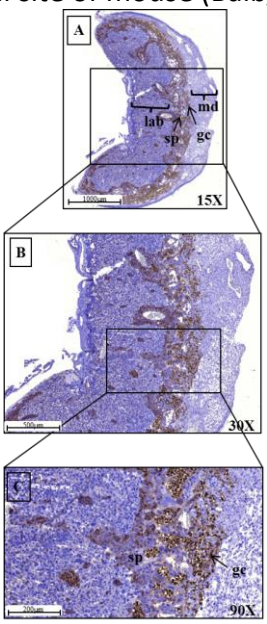
Animal models are important, as they allow for controlled experiments and analysis of multiple time-points during pregnancy.

Since pregnancy represents a unique model of local immunotolerance, regulatory pathways exerted by these co-inhibitory molecules like TIM-3 and PD-1 could have significant impact on maternal immuno-suppression.

Therefore, the aim of our study was to investigate the expression pattern of TIM-3, PD-1 and Gal-9 on different immune cell subsets in the peripheral blood and at the feto-maternal interface.

6.1. TIM-3/Galectin-9 pathway and PD-1 molecule in mice at day 14.5 of pregnancy

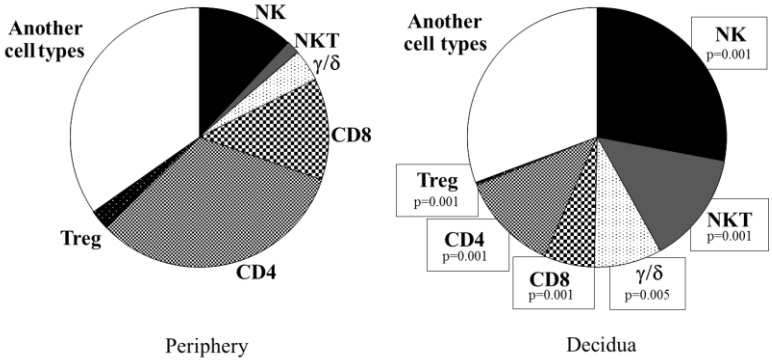
Galectin-9 (the ligand for TIM-3) expression by spongiotrophoblast was successfully detected at the feto-maternal site of mouse (Balb/c mice) placenta by immuno-histochemistry.



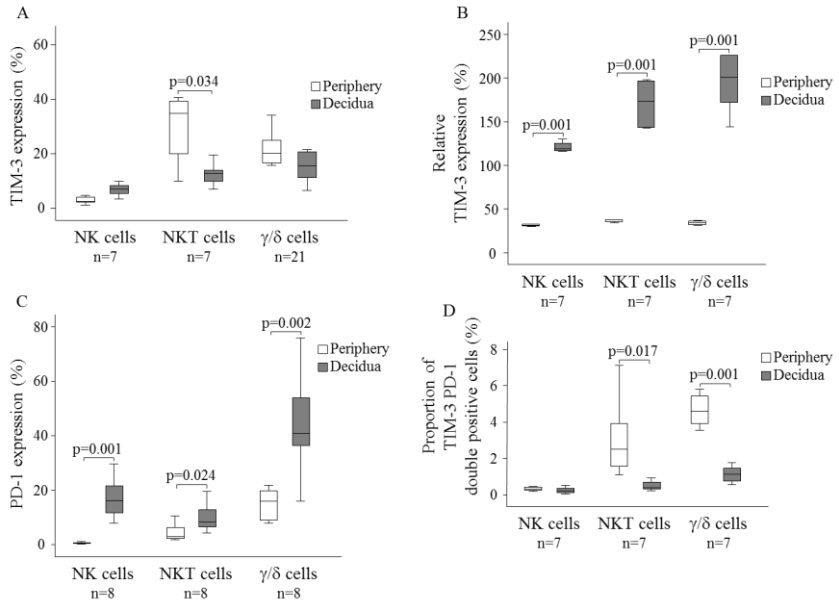
lab: labyrinthine trophoblast
sp: spongiotrophoblast
gc: trophoblast giant cells
md: maternal decidua

We discovered a significantly increase in Gal-9 expression by the decidual Treg cells when compared to the splenic Treg cells.

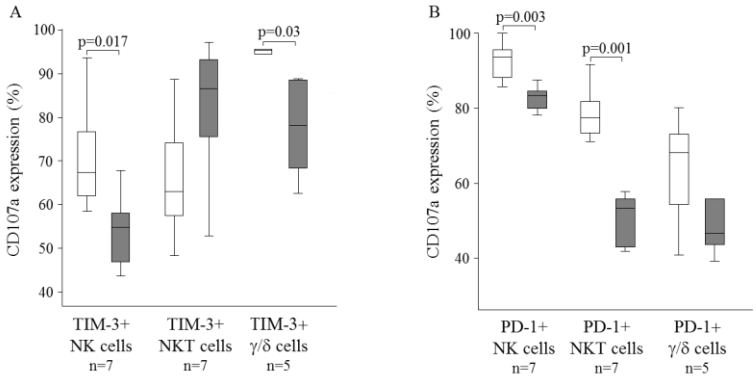
We observed a significant increase in decidual ϒ/δ T, NK and NKT cells frequency compared to the periphery while the decidual CD4+, CD8+ T and Treg cells frequency significantly decreased when compared to the periphery.



We observed a significantly decreased TIM-3 expression by decidual NKT cells compared to the periphery. TIM-3 expression showed no difference between the spleen and the decidua by NK and ϒ/δ T cells. During our investigation of the PD-1 expression by NK cells, ϒ/δ T and NKT cells in pregnant mice we discovered that all analyzed cell populations demonstrated an increase in PD-1 expression within the decidua compared to the periphery.



We analyzed the CD107a expression by TIM-3+ cell subsets and our results demonstrated a significantly decreased CD107a expression by the decidual TIM-3+ NK and TIM-3+ ϒ/δ T cells compared to the periphery. Moreover, we analyzed the CD107a expression by the PD-1+ cell populations. Decidual PD-1 positive NK and NKT cells showed significantly decreased cytotoxicity compared to the periphery.



Since we could detect Gal-9 expression by healthy mouse placentae, we analyzed the gene expression of Gal-9 by real-time RT-PCR during healthy pregnancy investigating healthy placentae and fetal resorptions. In fetal resorptions examined during mid-pregnancy, Gal-9 expression is 4-times lower compared to healthy mouse placentae, suggesting the role of TIM-3/galectin-9 pathway in the pathomechanism of pathologic pregnancies.

To conclude, our investigations revealed a particularly **complex, tissue and cell type specific immunoregulatory mechanism** by the investigated co-inhibitory receptors (TIM-3 and PD-1) **at the feto-maternal interface**.

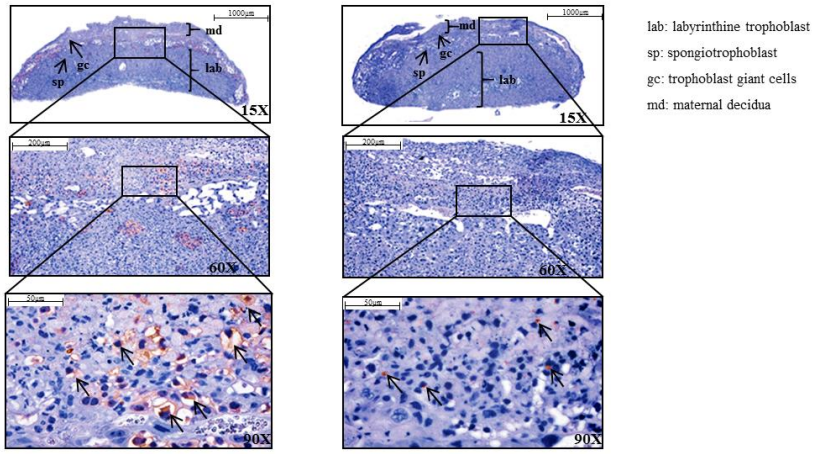
6.2. TIM-3/Galectin-9 pathway in Mifepristone induced medical abortion in mice

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 abortion during the first several weeks of pregnancy.

TIM-3/Gal-9 interaction could play an important role in the regulation of maternal immune tolerance toward the fetus and may be a potent regulator of the innate and adaptive immune response. Therefore, exploring the relationship between Gal-9/TIM-3 pathway and Mifepristone induced medical abortion may provide a better understanding of the pathogenesis of immunological changes during immune-mediated abortions and offer an effective target for therapy.

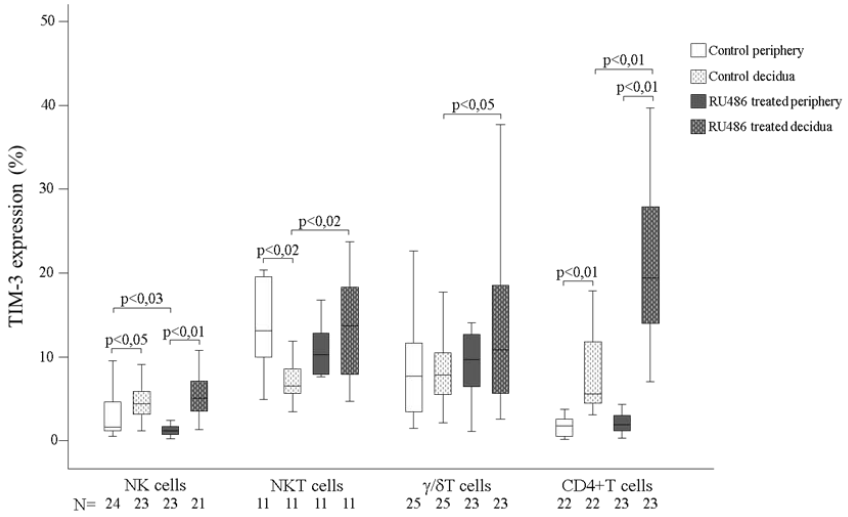
The aim of our study was to determine the immune status of the Mifepristone induced medical abortion mouse model at the feto-maternal interface and at the systemic level simultaneously.

Earlier we demonstrated that healthy pregnant mouse placentae with anti-Gal-9 antibody demonstrated the presence and localization of Gal-9 within the spongiotrophoblast layer and in giant cells. Placentae of RU486 treated pregnant mice showed dramatically reduced Gal-9 expression in the same localization.

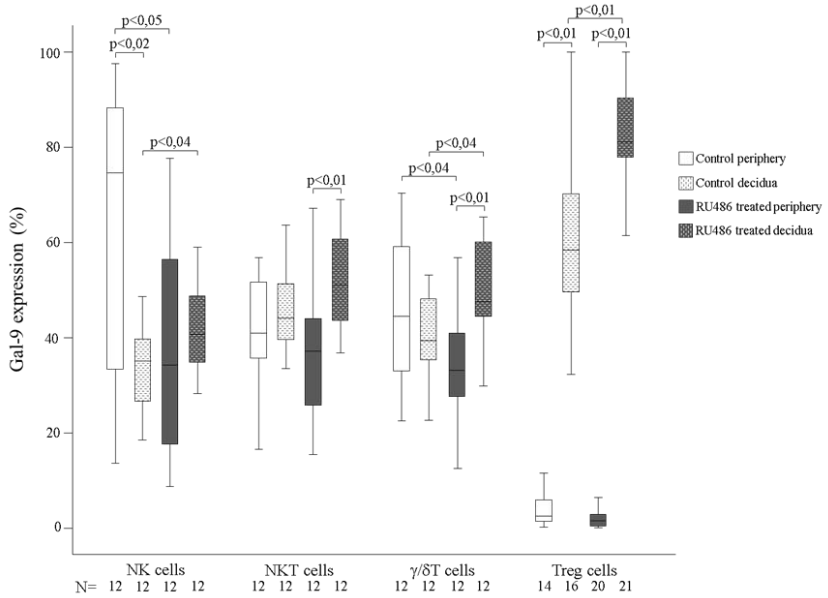


RU486 treatment did not cause much significant change in the phenotype distribution of immune cells both in the decidua and in the periphery when compared to control animals. The only significant difference was the elevation of Treg population in the decidua of RU486 treated animals compared to the controls.

Both in RU486 treated and in untreated control mice TIM-3 expression by NK and CD4+T cells was significantly increased by decidual cells obtained from the feto-maternal site compared to splenocytes obtained from the periphery. In control mice TIM-3 expression by decidual NKT (dNKT) cells showed a significant decrease compared to the periphery. Following RU486 administration TIM-3 receptor expression by dNKT cells, γ/δT cells and CD4+T cells was significantly increased at the feto-maternal interface compared to untreated control mice.

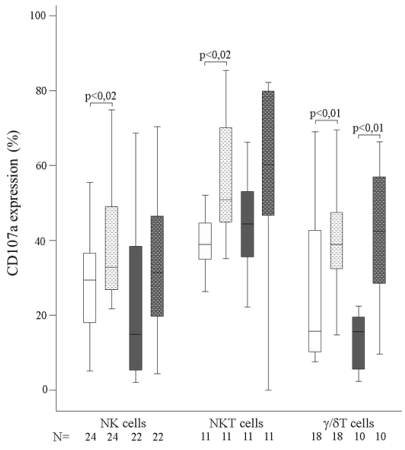


In RU486 treated mice Gal-9 expression was significantly increased by almost all investigated decidual subpopulations (NKT, γ/δ T and Treg cells) compared to the periphery except NK cells. Following RU486 administration Gal-9 receptor expression was significantly increased by NK cells, γ/δ T cells and Treg cells in the decidua while Gal-9 expression was significantly decreased by NK cells and γ/δ T cells in the periphery compared to untreated control mice.



We observed a significant increase in the decidual Gal-9+ Th cell frequency of RU486 treated mice compared to either the periphery of treated mice or control untreated decidua.

Investigating the cytotoxic activity of NK, NKT and γ/δ T cells obtained from the feto-maternal interface we found a significantly higher CD107a expression by all decidual subpopulations compared to splenocytes obtained from the periphery in untreated control mice. In RU486 treated mice decidual γ/δ T cells shows significantly higher cytotoxic potential than peripheral counterparts.



These findings suggest that in the medical abortion mouse model, RU486 can induce immunological chances in both TIM-3 dependent and independent way altering the immune responses that may be involved in the complex process of fetal rejection.

To conclude, these findings suggest that in the medical abortion mouse model, **RU486 can induce immunological chances in both TIM-3 dependent and independent** way altering the immune responses that may be involved in the complex process of fetal rejection.