FINAL REPORT

„Immunology of pre-eclampsia: characterization of the inflammatory stage and therapeutical approaches”

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PUBLICATIONS WITH “OTKA SUPPORT” IN THE ACKNOWLEDGEMENT

1. Meggyes M, Szanto J, Lajko A, Farkas B, Varnagy A, Tamas P, Hantos E, **Miko E**, Szereday L.
   The possible role of CD8+/Vα7.2+/CD161+ T (MAIT) and CD8+/Vα7.2+/CD161^{lo} T (MAIT-like) cells in the pathogenesis of early-onset preeclampsia. Resubmitted in revised form to *Am J Reprod Immunol.* 2017 **Impact factor: 3,013**
2. **Miko E**, Meggyes M, Doba K, Farkas N, Bogar B, Barakonyi A, Szereday L, Szekeres-Bartho J, Mezosi E.
   Characteristics of peripheral blood NK and NKT-like cells in euthyroid and subclinical hypothyroid women with thyroid autoimmunity experiencing reproductive failure. Accepted for publication in *J Reprod Immunol*, 2017 **Impact factor: 2,798**
3. 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: 3,013**
4. 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. doi: 10.1016/j.placenta.2015.07.124. **Impact factor: 2,71**

ORAL AND POSTER PRESENTATIONS WITH “OTKA SUPPORT” IN THE ACKNOWLEDGEMENT

1. Matyas Meggyes, **Eva Miko**, Beata Polgar, Adrienn Lajko, Julia Szekeres-Bartho, Laszlo Szereday. TIM-3/Galectin-9 in normal pregnancy and in early-onset preeclampsia. A Magyar Immunológiai Társaság 43. Vándorgyűlése 2014. október 15-17. Velence

2. Lajko A, Meggyes M, Totsimon A, Szanto J, **Miko E**, Szereday L. Investigating Galectin-9 molecule expression by peripheral and decidual mononuclear cells in pregnant mice. Doktoranduszok Országos Szövetsége TAVASZI SZÉL konferencia. 2015. április. 10-12. Eger

3. Lajko A, Meggyes M, Totsimon A, Szanto J, **Miko E**, Szereday L. The possible role of Galectin/Tim-3 pathway in mifepristone induced medical abortion in mice. A Magyar Immunológiai Társaság 44. Vándorgyűlése 2015. október Velence. Immunológiai Szemle VII évfolyam, 3. szám, 2015 október 14-16. p40

4. Lajko A, Meggyes M, Totsimon A, Szanto J, **Miko E**.Galektin-9 molekula vizsgálata perifériás és deciduális mononukleáris sejteken terhes egérmodellben. Doctoral Workshop 2015 OE-EA 13.

5. Lajko A, Meggyes M, Totsimon A, Szanto J, **Miko E**, Szereday L. The possible role of Galectin-9/TIM-3 pathway in fetomaternal tolerance in pregnant mice. 4th European Congress of Immunology September 6-9. 2015. Vienna, Austria (PC.02.08)

6. Lajko A, Meggyes M, Totsimon A, Szanto J, **Miko E.** Immune regulation by PD-1 molecule in mice at day 14.5 of pregnancy 5th Interdisciplinary Doctoral Conference May 27-29. 2016 Pécs

7. Lajko A, Meggyes M, Totsimon A, Szanto J, **Miko E**, Szereday L**.** PD-1 molekula immunregulációja terhes egérben Magyar Farmakológiai, Anatómus, Mikrocirkulációs és Élettani Társaságok Közös Tudományos Konferenciájára (FAMÉ 2016) 2016. Június 1-4. Pécs

RESULTS OF ANIMAL STUDIES

Investigation of the maternal inflammatory response in the reduced uteroplacental perfusion pressure (RUPP) pregnant rat model – establishing the RUPP rat model

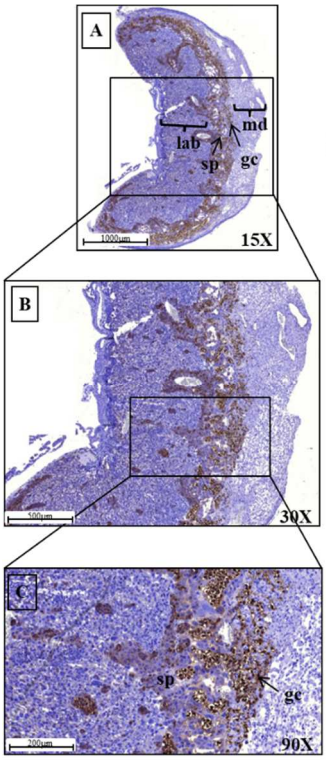
Female and male Sprague-Dawley rats were obtained from the Department of Anatomy. Since we didn't get any technical help and information from researchers who first developed the RUPP model about the source and handling of silver clips used for the surgery, and silver clips for this purpose are not official available we ordered special tiny disposable needles for ligation purposes from the U.S. This procedure took several months, so we started with the establishment of the RUPP pregnancy rat model in spring 2015. The surgical intervention was carried out on 15 animals. Unfortunately, our modified surgical method didn't succeed and the animals did not survive the intervention. Since the RUPP pregnant rat model is the only animal model mimicking placenta derived pre-eclampsia in human we focused on the investigation of the role of co-inhibitory molecules in murine pregnancy.

Feto-maternal immune regulation by TIM-3/Galectin-9 pathway and PD-1 molecule in pregnant mice

Since pregnancy represents a unique model of local immunotolerance, regulatory pathways exerted by these co-inhibitory molecules could have significant impact on maternal immuno-suppression. Therefore, the aim of our study was to investigate the expression pattern of TIM-

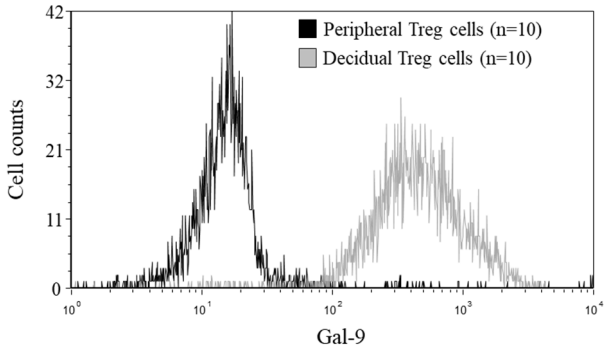
3, PD-1 and Gal-9 on different immune cell subsets in the peripheral blood and at the fetomaternal interface.

- We investigated the Gal-9 protein expression at the murine fetomaternal interface on gestational day 14.5 using immunohistochemistry. As a result, our stainings highlight that we can now effectly detect the Gal-9 molecule in the placentae spongiotrophoblast layer.



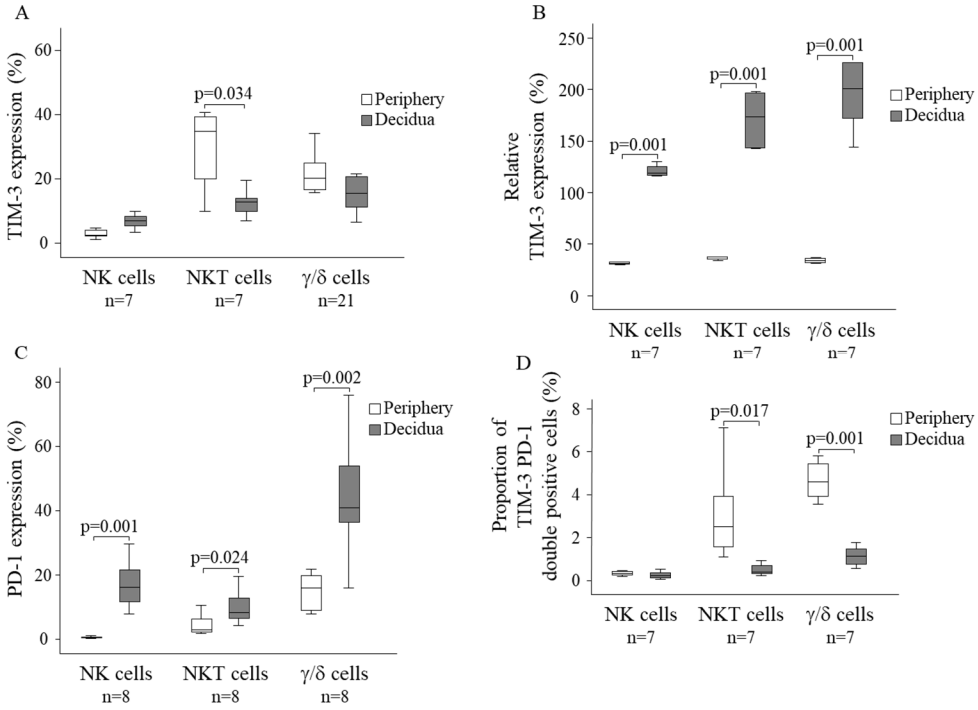
lab: labyrinthine trophoblast
sp: spongiotrophoblast
gc: trophoblast giant cells
md: maternal decidua

- We analysed the surface Gal-9 expression by splenic and decidual regulatory T cells, in pregnant mice. We discovered a significantly increase in Gal-9 expression by the decidual Treg cells when compared to the splenic Treg cells.

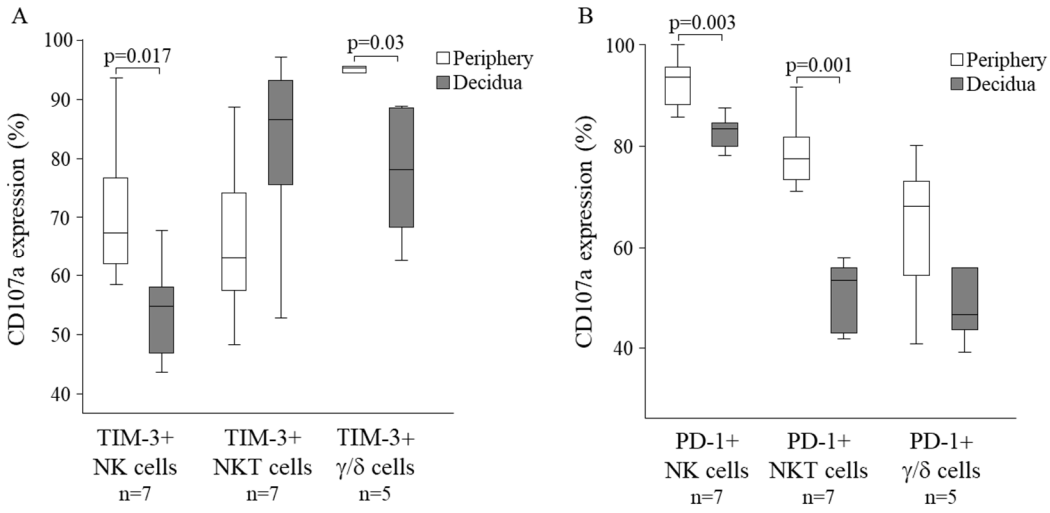


- We measured the surface expression of TIM-3 on different lymphocyte subsets by flow cytometry. We observed a significantly decreased TIM-3 expression by decidual NKT cells compared to the periphery. TIM-3 expression showed no statistical difference between the spleen and the decidua by NK and γ/δ T cells (A). Furthermore, we also noted a significant increase in receptor density within the decidual NK, NKT and γ/δ T cells when compared with the periphery (B). During our investigation of the PD-1

expression by NK cells, γ/δ T and NKT cells in pregnant mice we discovered that all analysed cell populations demonstrated an increase in PD-1 expression within the decidua compared to the periphery (C). As a result of our investigation of the peripheral and decidual TIM-3/PD1 double positive cells we found a significant decrease in decidual NKT and γ/δ T cells proportion compared with the periphery. Lastly, we could not detect TIM-3/PD-1double positive NK cells in the examined tissues (D).



- The cytotoxic activity was evaluated by measuring the CD107a expression by the investigated cell subsets. We analysed 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+ γ/δ T cells compared to the periphery (A). We observed an increasing tendency in the CD107a expression by decidual TIM-3 positive NKT cell which did not attain the level of statistical significance (A). Moreover, we analysed 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 (Fig. 5/B). Additionally, we discovered a decreased tendency in the cytotoxic activity by decidual PD-1 positive γ/δ T cell which did not reach the level of statistical significance (B).



We investigated the potential role of co-inhibitory molecules PD-1 and TIM-3 in maternal immune responses, since it has been shown their ligands are present at the fetomaternal interface. Our research concludes PD-1 and TIM-3 expressing decidual lymphocytes were found to be more dominant than in the periphery. While PD-1 positive lymphocytes show reduced cytotoxicity, lytic activity of TIM-3 expressing cells varies with cell type suggesting opposite roles of TIM-3 on different lymphocyte subsets.

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.

RESULTS OF HUMAN EXPERIMENTS

Involvement of the PD-1/PD-L1 co-inhibitory pathway in the pathogenesis of the inflammatory stage of early onset pre-eclampsia

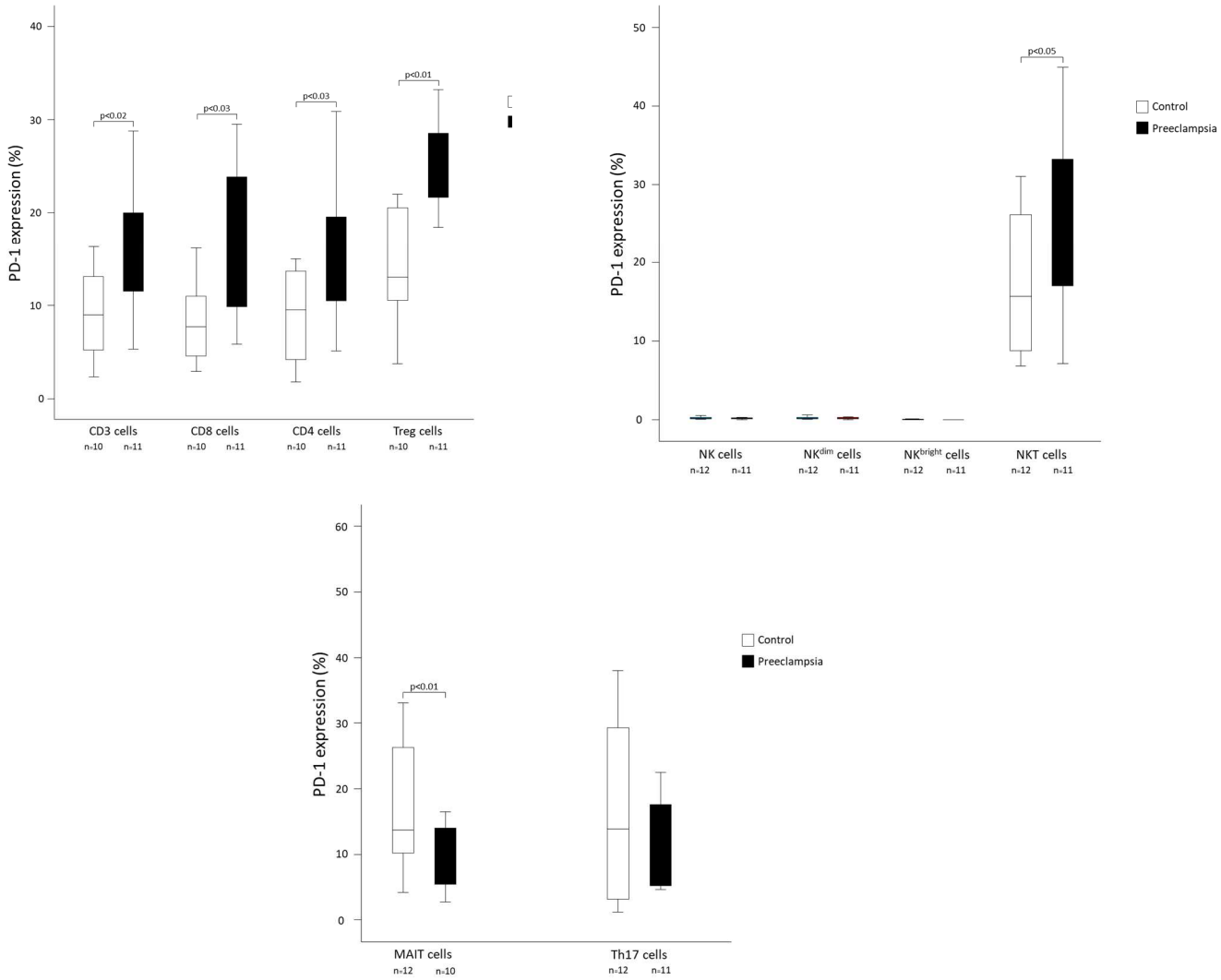
T-cell costimulatory pathways are central in modifying the delicate balance between protective immunity and tolerance. The new molecular and costimulatory pathway, consisting of a programmed death-1 (PD-1) receptor and its ligand PD-L1, has been reported to deliver inhibitory signals that regulate the balance among T-cell activation, peripheral tolerance, and immune-mediated tissue damage. In this study we investigated the possible involvement of this pathway in the pathogenesis of the clinical phase of early onset pre-eclampsia analysing phenotype and functional characteristics of peripheral blood lymphocytes.

- We determined the ratio of different lymphocyte subpopulations in healthy pregnant and pre-eclamptic women. Regulatory T cells and Mucosal-Associated Invariant T (MAIT) cells were found to be significantly reduced in pre-eclampsia compared to healthy pregnancy.

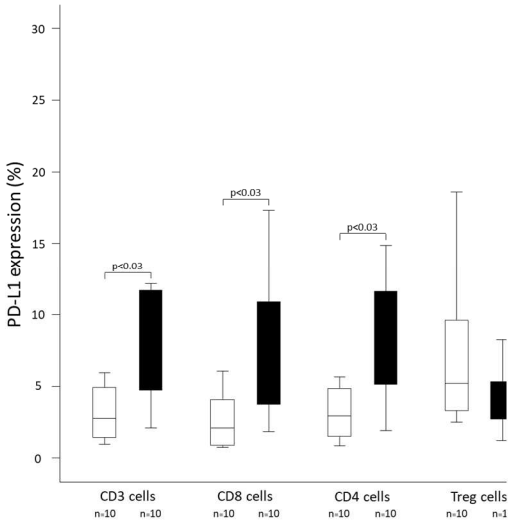
Distribution of lymphocyte subpopulations

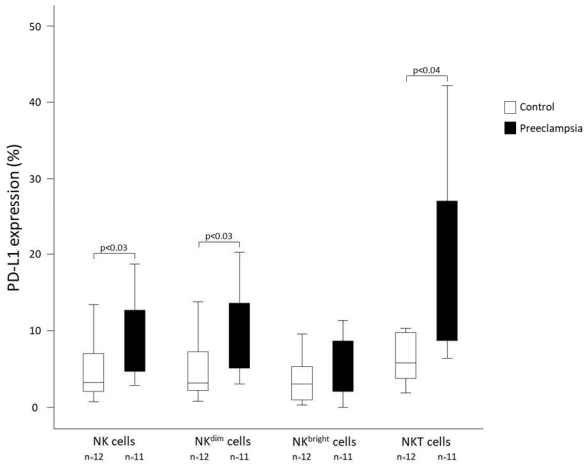
	3rd trimester	Preeclampsia	p-value
CD3+ T cells	70,11	66,87	ns
CD4+ Th cells	42,15	37,46	ns
CD8+ Tc cells	23,21	23,58	ns
Treg cells	**2,66**	**1,55**	**0,04**
NK cells	12,92	13,31	ns
NK^{dim} cells	11,54	11,48	ns
NK^{bright} cells	1,43	1,84	ns
NKT cells	2,52	2,97	ns
MAIT cells	**0,71**	**1,47**	**0,05**

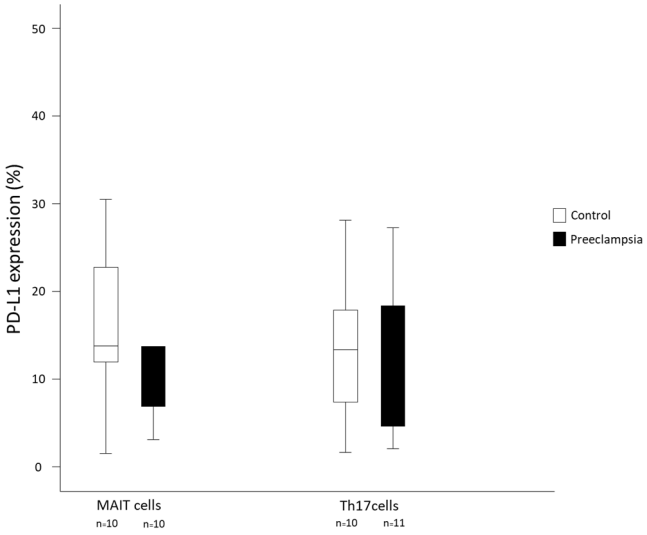
- Analysing the PD-1 expression on the different lymphocyte subsets, CD3+ T cells, CD8+ T cells, CD4+ T cells, regulatory T cells, NKT cells showed an upregulation of the PD-1 checkpoint molecule in the case of pre-eclampsia. In contrast to that, MAIT cells express a significantly reduced amount of PD-1 on their cell surface in pre-eclamptic patients. NK cells express a negligible amount of PD-1



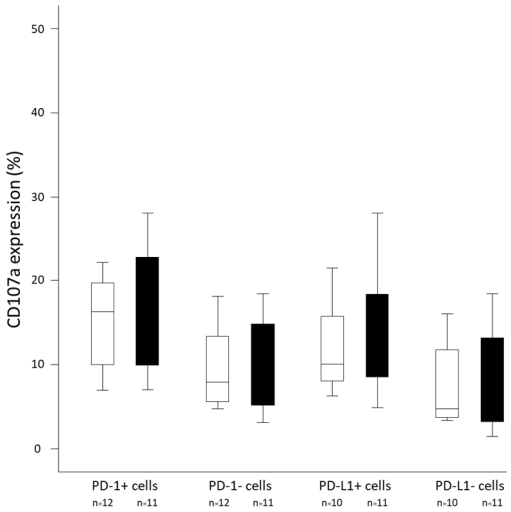
- We determined the surface expression of the ligand PD-L1 on different lymphocyte subsets. We found significantly elevated levels of PD-L1 on CD3+ T cells, CD8+ T cells, CD4+ T cells, NK and NKT cells in pre-eclamptic patients.

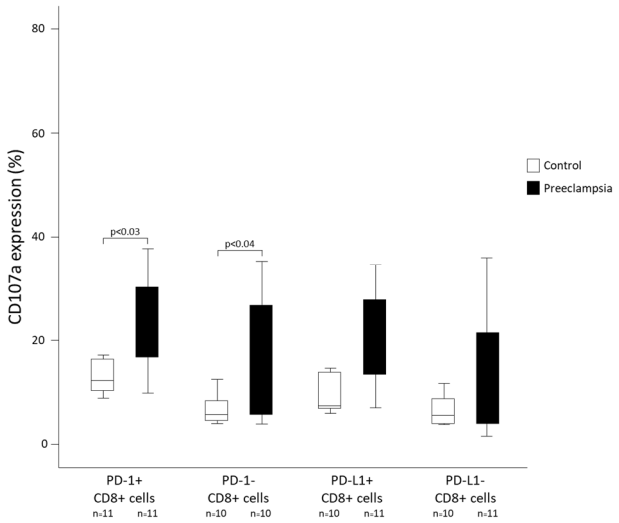


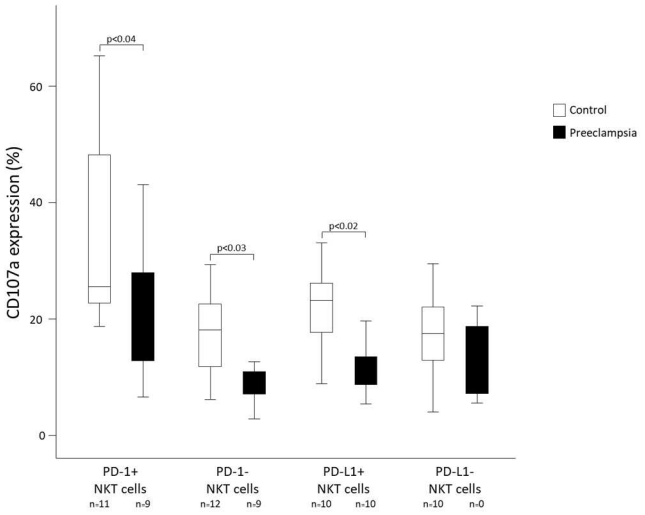




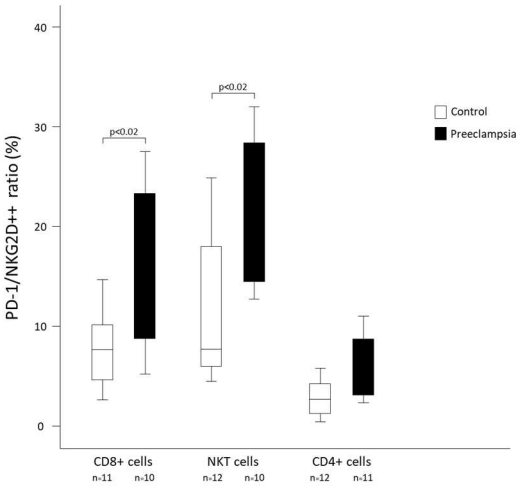
- Cytotoxic activity of PD1 or PD-L1 expressing lymphocytes was determined with the CD107a degranulation assay. While cytotoxicity of PD1+ CD8+ T cells was found to be elevated in pre-eclampsia, PD-1 and PD-L1 expressing NKT cells showed reduced lytic activity compared to healthy pregnancy.

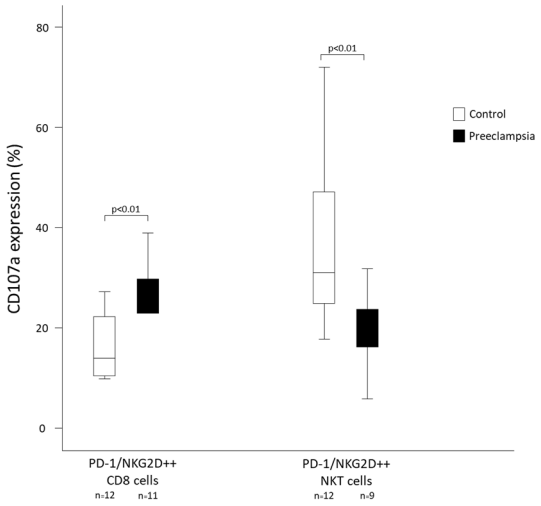




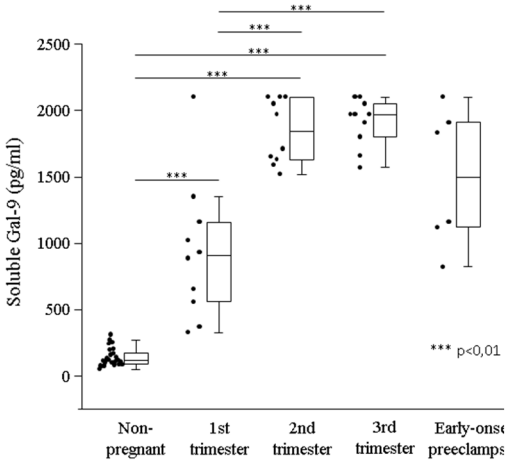


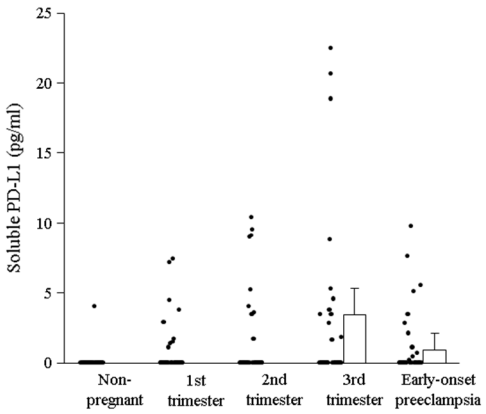
- Co-expression of PD-1 with the NK cell activating receptor NKG2D was determined on the different lymphocyte subpopulations. The percentage of PD-1/NKG2D++ CD8+ T cells and NKT cells was found to be significantly increased in pre-eclamptic women. Regarding cytotoxicity, PD-1/NKG2D++ CD8+ T cells displayed enhanced cytolytic activity while PD-1/NKG2D++ NKT cells showed reduced cytotoxicity in the case of pre-eclampsia.





- Soluble forms of the ligands galectin-9 and PD-L1 for the immune checkpoint receptors TIM-3 and PD-1 were analysed in the serum of healthy pregnant women and in women suffering from early-onset pre-eclampsia. We could detect a significant increase of Gal-9 serum concentrations throughout healthy pregnancy, while concentrations of sPD-1 were stable in all trimesters. In pre-eclampsia, there are no changes of sGal-9 and sPD-1 levels compared to third trimester concentrations.





According to our results, the expression of the immune checkpoint molecule PD-1 and its ligand PD-L1 is significantly altered in several lymphocyte subsets in the peripheral blood of pre-eclamptic women compared to healthy pregnant controls. Moreover, these changes result in a difference of cytotoxicity of the lymphocytes. However, our findings indicate distinctive role of the PD-1/PD-L1 pathway on different lymphocytes subsets.

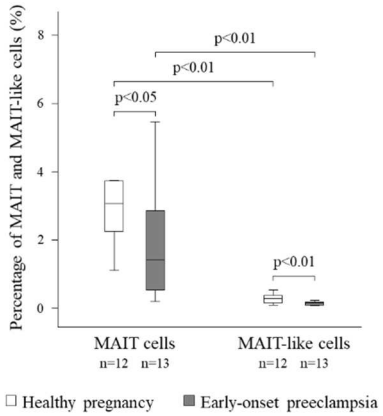
Manuscript in preparation.

The possible role of Mucosal-Associated Invariant T (MAIT) cells and MAIT-like cells in the pathogenesis of early-onset preeclampsia.

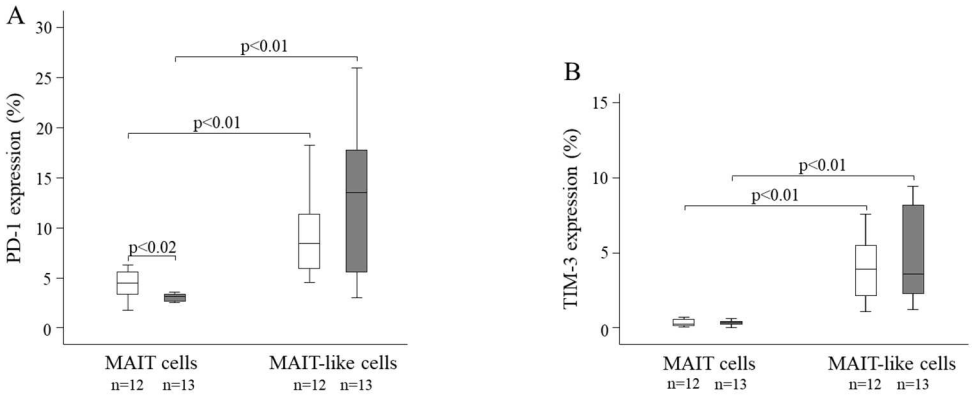
Since pregnancy represents a physiological condition of immune tolerance, we hypothesized whether MAIT cells and their immune checkpoint molecules can play a distinctive role in

maternal immune responses. In this study, we examined the characteristics of MAIT cells in the peripheral blood of healthy pregnant women and in women with early-onset pre-eclampsia.

- In the comparison of MAIT and MAIT cell populations, we detected a significantly increase in the number of MAIT cells compared to MAIT-like cells, both in healthy and in early-onset pre-eclamptic women.

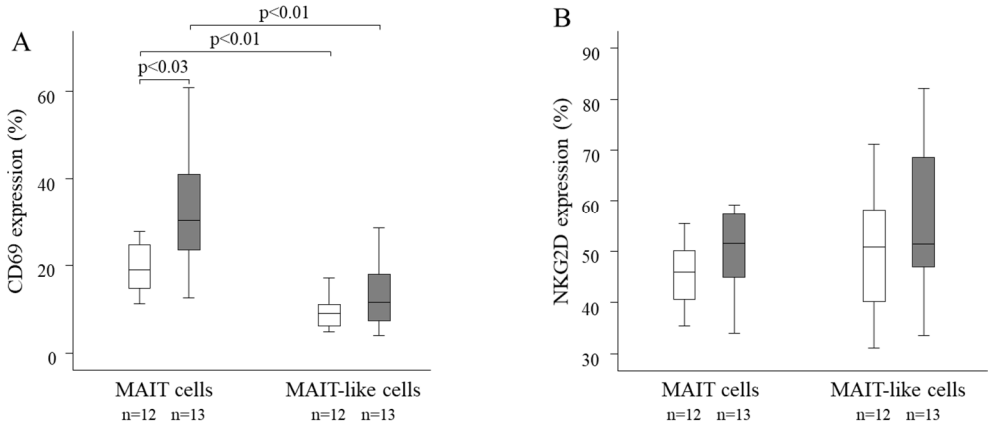


- We measured the surface expression of PD-1 and TIM-3 by MAIT and MAIT-like cells with flow cytometry. In the analysis of the presence of PD-1, we found a significantly lower receptor expression by MAIT cells in women with early-onset preeclampsia compared to healthy pregnant women while MAIT-like cells did not show any significant difference, however, PD-1 expression by MAIT-like cells was significantly elevated compared to MAIT cells in pathological conditions (A). Based on our results, we hereby declare peripheral blood MAIT cells during pregnancy do not really express the TIM-3 receptor, however, we could detect the presence of a TIM-3 molecule on the surface of MAIT-like cells, yet, the expression level does not differ significantly in the two investigated groups (B)

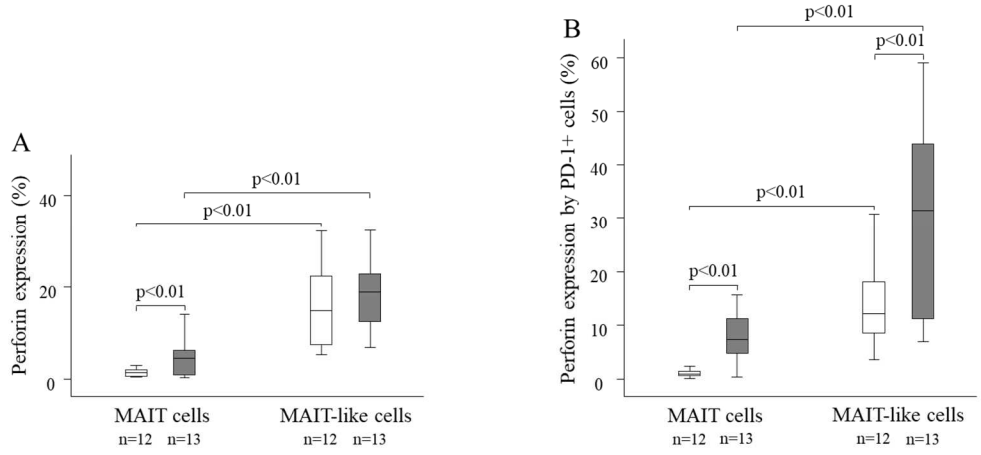


- During our investigation of an early activation marker, we observed in which CD69 expression by MAIT cells was significantly elevated in early-onset pre-eclamptic patients compared to healthy pregnant women (A). CD69 expression by MAIT-like cells show no significant difference between the investigated groups, however, the expression level of CD69 by MAIT cells exhibit a significantly elevated value compared to MAIT-like cells in early-onset pre-eclamptic women (A). In the analysis of NKG2D, which is one of the best characterized activating receptors expressed by NK and T cells,

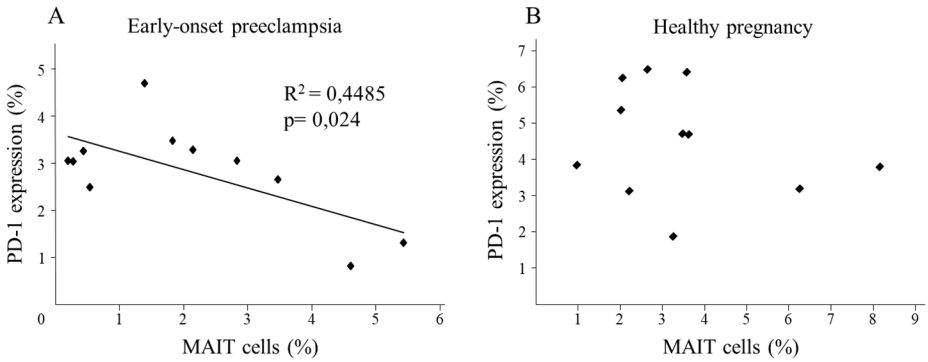
we successfully detected the presence of a NKG2D receptor on the surface of MAIT and MAIT-like cells, but we could not observe any statistical difference between the investigated groups (B).



- During our investigation of the intracellular perforin content, we discovered a significant increase in MAIT cells in early-onset pre-eclamptic patients, compared to healthy women (Fig. 4A). Perforin expression by MAIT-like cells demonstrate no significant difference between the two investigated groups, however, the expression is significantly elevated when compared with MAIT cells in women with early-onset preeclampsia and in healthy pregnant women (A). During our investigation of the PD-1 expressing subsets, we detected a significantly increase in perforin expression by PD-1+ MAIT and MAIT-like cells in patients with early-onset preeclampsia compared to healthy controls (B).



- Based on our regression analyses, the ratio of MAIT cells inversely correlates with their PD-1 expression in women with early onset preeclampsia (A), while we could not detect any correlation in healthy pregnant women (B).



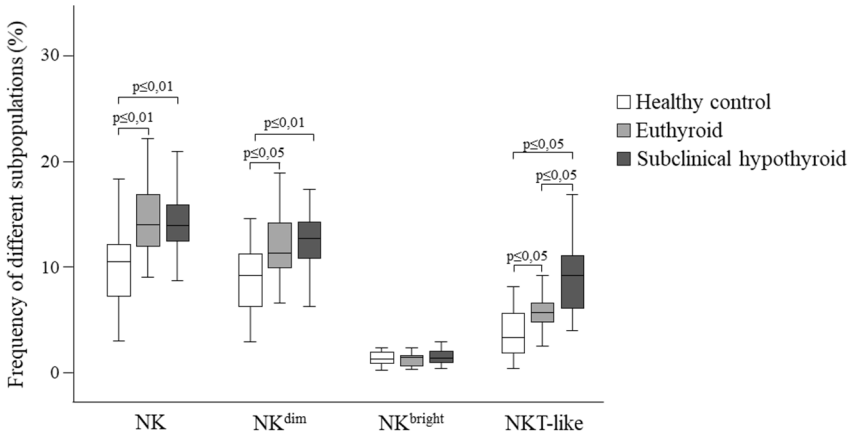
According to our findings, the involvement of MAIT and MAIT-like cells in the systemic inflammatory response in pre-eclampsia is still controversial. Although reduced in number in early-onset pre-eclamptic patients, the remaining circulating cell populations are activated and a higher ratio expresses perforin. The absence of TIM-3 and the reduced expression of PD-1 of pre-eclamptic MAIT cells contraindicate the possibility of T cells exhaustion. Since PD-L1 binds to membrane-bound PD-1, creating a negative signal, which inhibits the activation and proliferation of T cells, these activated MAIT cells escape from this immune inhibition by merely down regulation of their surface PD-1 molecule. On the other hand, MAIT-like cells do express PD-1 and TIM-3, but without changes in early-onset pre-eclampsia.

Meggyes M, Szanto J, Lajko A, Farkas B, Varnagy A, Tamas P, Hantos E, Miko E, Szereday L.The possible role of CD8+/Vα7.2+/CD161+ T (MAIT) and CD8+/Vα7.2+/CD161^{lo} T (MAIT-like) cells in the pathogenesis of early-onset preeclampsia. Resubmitted in revised form to Am J Reprod Immunol. 2017

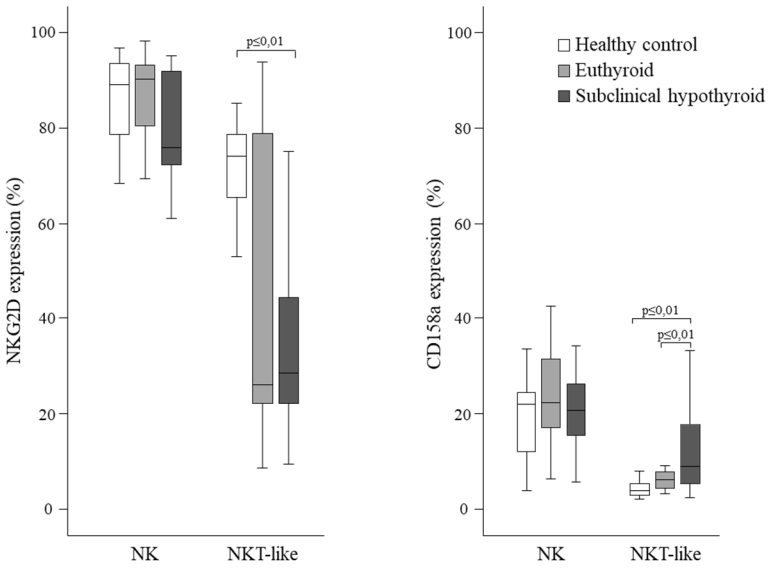
Innate immune response in euthyroid and subclinical hypothyroid women with thyroid autoimmunity experiencing reproductive failure

During the collection of human samples of pre-eclamptic and healthy pregnant women our research group was contacted by the endocrinologist Prof. Emese Mezosi, whit the proposal to start a cooperative research with her patients who were tested positive for thyroid autoantibodies and suffered from pregnancy failure. She suggested us to investigate the immune status of these women. Due to free capacity, beside the research in the field of pre-eclampsia, we started to investigate the immunological background of thyroid autoimmunity (TAI) and infertility associated with it. TAI appears to play a crucial role in female infertility, recurrent pregnancy loss and IVF failure. Thyroid autoantibodies against thyroid peroxidase and thyroglobulin have been shown to represent an independent risk factor for infertility and miscarriage. The aim of this study was to investigate the phenotypic and functional characteristics of peripheral NK and NKT-like cells in euthyroid and subclinical hypothyroid women with TAI compared with healthy female controls.

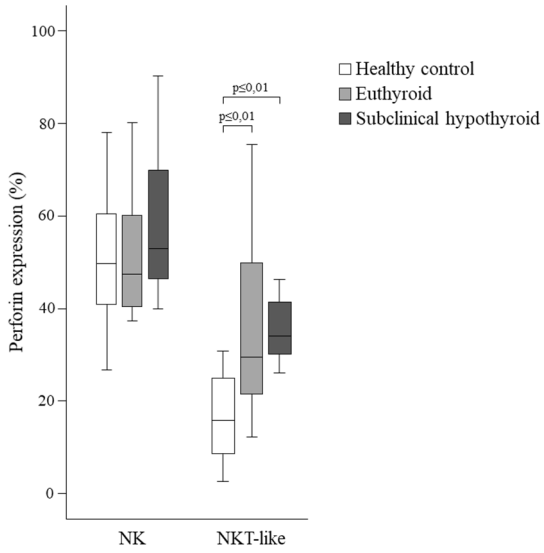
- We investigated the percentage of NK cells, NK cell subpopulations and NKT-like cells in the peripheral blood of euthyroid and subclinical hypothyroid women with thyroid autoimmunity and in healthy female controls. The frequency of NK, NKdim and NKT-like cells was significantly higher in the peripheral blood of euthyroid and subclinical hypothyroid women with TAI compared to healthy controls. NK^{bright} cells showed no significant difference between any investigated groups. In the case of NKT-like cells, their frequency showed a significant increase in subclinical hypothyroid women compared to euthyroid counterparts.



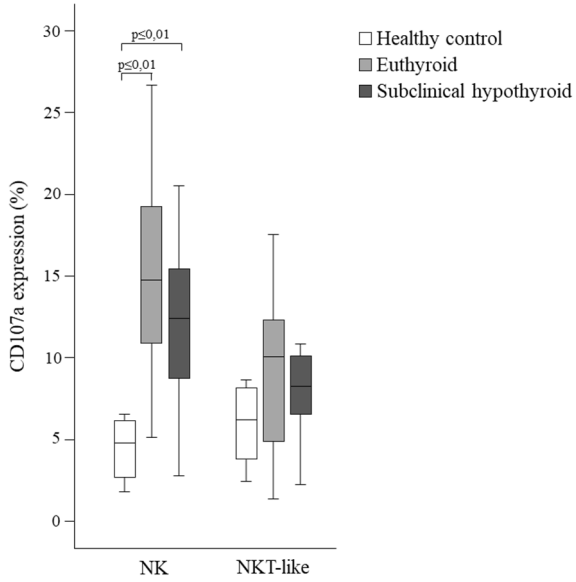
- Since the balance of activating and inhibitory signals through the killer activating and the inhibitory receptors control cell activity, we investigated the activating and inhibitory receptor expression by NK and NKT-like cells in euthyroid and subclinical hypothyroid women and in healthy controls. In the subclinical hypothyroid group, NKT-like cells expressed a significantly decreased level of activating NKG2D receptors. Significantly higher percentage of CD158a inhibitory receptor expression by NKT-like cells were found in euthyroid and subclinical hypothyroid women with TAI compared to controls and to the euthyroid group. Analysing CD158a expression by NK cells we found no significant differences between the investigated groups. In the subclinical hypothyroid group, NKT-like cells expressed a significantly decreased level of activating NKG2D receptors.



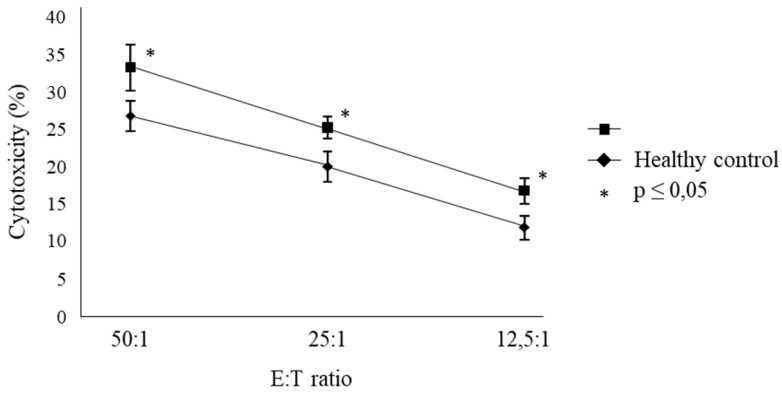
- We measured the intracellular perforin expression by NK and NKT-like cells by flow cytometry. Perforin expression by NK cells showed no significant difference between any investigated groups. Investigating perforin expression by NKT-like cells we found a significant increase in euthyroid and subclinical hypothyroid women compared to healthy controls.



- Investigating the degranulation of NK and NKT-like cells following cytotoxic action, we found that CD107a expression of NK cells was significantly higher in the peripheral blood of euthyroid and subclinical hypothyroid women with TAI compared to healthy female individuals. Analysing CD107a expression by NKT-like cells showed no significant differences between the investigated groups.



- One of the most important function of innate immune cells is the cytotoxic activity, killing target cells without MHC restriction. We determined cytotoxicity of peripheral lymphocytes of the investigated groups against the target cell line K562. Cytotoxicity of PBMC of women with thyroid autoimmunity was significantly increased in all effector:target ratios (50:1, 25:1, 12,5:1) compared to healthy controls.



Our study reveals important and novel information about the immune profile of women with autoimmune thyroid disease. It has been demonstrated that innate immunity of women with TAI shows Th1 oriented changes, similar to that observed in idiopathic reproductive failures, which could have a negative impact on pregnancy success rates. It is important to notice that these unfavourable immune alterations are already established in the euthyroid phase of autoimmune thyroiditis before endocrine dysfunction develops and only the presence of thyroid autoantibodies indicate TAI condition. Women with subclinical autoimmune thyroiditis remain often undiagnosed and are considered otherwise healthy but according to our observations, their reproductive success might be affected.

Miko E, Meggyes M, Doba K, Farkas N, Bogar B, Barakonyi A, Szereday L, Szekeres-Bartho J, Mezosi E. Characteristics of peripheral blood NK and NKT-like cells in euthyroid and subclinical hypothyroid women with thyroid autoimmunity experiencing reproductive failure. Accepted for publication in J Reprod Immunol, 2017

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