Molecular mechanisms of thymus aging: identification of novel target of intervention

PhD Thesis

Dávid Ernszt

Basic Medical Sciences

Basic Immunology

Head of PhD school: Prof. Dr. Júlia Szekeres
Head of PhD program: Prof. Dr. Tímea Berki

Supervisor: Dr. Krisztián Kvell

University of Pécs Medical School

Pécs, 2018.

Introduction

The PPAR (peroxisome proliferator-activated receptor) molecular family is widely studied **(1-3)**. These nuclear receptor proteins possess transcription factor activities and influence multiple cellular events at the molecular level including adipocyte differentiation and metabolism. Among them, PPARgamma is of particular interest being expressed by all adipose tissue subtypes and being indispensable for adipose tissue development and for the homeostasis of physiological metabolism **(4-7)**. As a consequence, in the mouse systemic loss of PPARgamma activity severely impairs glucose and lipid metabolism as characterized by others (**8-10**). In accordance, PPARgamma null mice are only viable if using conditional knockout strategy **(11)**. Similar to the mouse above, in human PPARgamma haplo-insufficiency leads to the development of a rare metabolic condition known as familial partial lipodystrophy, type 3 (FPLD3, ORPHA 79083) also characterized by diabetes and dyslipidemia **(12-15)**.

In mammals systemic PPARgamma activity may be increased at multiple levels. Environmental factors including excessive caloric consumption or corticosteroid exposure increase PPARgamma activity systemically **(16-18)**. Pharmacological systemic activation may be achieved through administration of thiazolidinediones (TZDs) previously used as part of oral anti-diabetic treatment, but currently neglected due to adverse cardiovascular side-effects **(19, 20)**. Genetic engineering-based enhancement of PPARgamma activity in mouse models has also been performed **(21)**. In every case increased PPARgamma activity promotes adipose tissue development at multiple sites of the body.

Thymic aging is observed as adipose involution during which the functional thymus niche that normally supports T-cell production is gradually lost and replaced by adipose tissue **(22)**. The process starts focally in childhood then spreads and accelerates with puberty due to hormonal changes **(23)**. Diminishing T-cell production results in decreased availability of fresh naïve T-cells **(24)**. Consequences include increasing incidence of infection, cancer and autoimmunity observed at senior ages **(25, 26)**. Thymic adipose involution appears to be PPARgamma-dependent: any condition that systemically enhances PPARgamma activity – either environmental, pharmacological or genetic – accelerates thymic senescence or adipose involution with all its immunological consequences **(27-32)**. However, the opposite

phenomenon whether systemically decreased PPARgamma activity can ameliorate long-term functional immune parameters has barely been addressed **(33, 34)**. For this reason we have set out to characterize the effect of systemic genetic PPARgamma loss of function on long-term immune homeostasis in both mouse and human.

Objectives

A major cause of immune pathologies problems in the elderly is premature aging of the thymus and T-cell immunity. As time progresses, the histological structure of the thymus becomes disorganized, the number of epithelial cells decreases and adipoid cells accumulate in the thymus, resulting in a reduced production of naive T cells. The molecular processes underlying thymus aginge are still not fully understood. A key hypothetic player is transcription factor PPARγ, an indispensable organizer of adipose cell development. Earlier studies suggested that overexpression of PPARγ accelerates thymic aging. The opposite, however, whether the lack of PPARγ has an impact on thymus aging is still not known. Therefore, we aimed to study the aging of the thymus in the absence of the PPARγ, in both mouse and human.

Our objectives were:

1. Investigation of age-associated thymus histology in the absence of PPARγ in mouse
2. Evaluation of the effect of PPARγ deficiency on naive T cell production in mouse
3. Examination of T-cell dependent immune functions in the absence of PPARγ in mouse
4. Investigation the human immune relevance of PPARγ deficiency

Methods

Human thymus samples

Formalin-fixed, paraffin-embedded (FFPE) human thymus samples from age groups 30-40 years, 50-60 years and 70-80 years were obtained from the Department of Pathology (Faculty of Medicine, University of Pecs, Hungary.) Experiments involving human thymus samples were performed with the consent of the Regional and Local Ethics Committee of Clinical Centre, University or Pecs (ref. no.: 6331/2016) according to their guidelines. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

Human immunohistochemistry

Human thymus lobes were fixed in paraformaldehyde (4% PFA in PBS) then paraffin embedded. 5µm thick sections were stained using immunohistochemistry **(35)**. First the slides were rinsed in heated xylene and were washed with a descending series of alcohol to remove paraffin. After de-paraffination the slides were rehydrated in distilled water and antigen retrieval was performed by heating the slides in Target Retrieval Solution (pH 6 DAKO) at 97°C for 20-30 minutes. Subsequently slides were washed in dH_2O and endogenous peroxidase activity was blocked with 3% H_2O_2 containing TBS (pH 7.4) for 15 minutes. Then slides were washed three times with TBS containing Tween (0.05%, pH 7.4). Pre-blocking was carried out with 3% BSA in TBS for 20 minutes before overnight incubation with anti-PPARgamma (1:100, rabbit monoclonal antibody clone: C26H12 Cell Signalling Technology) primary antibody at 4°C. Following incubation slides were washed with TBS for three times then incubated with peroxidase conjugated secondary antibody (1:100, Polyclonal Goat Anti-Rabbit IgG, DAKO) for 90 minutes. Antibody labeling was visualized with the help of liquid DAB Substrate Chromogen System (DAKO). For nuclear counterstaining hematoxylin staining was performed. Finally slides were mounted with Faramount Aqueous Mounting Medium (DAKO). Histological evaluation was performed with the help of Panoramic MIDI digital slide scanner (3DHistech). Image analysis was performed using ImageJ software with IHC toolbox plug-in.

Mouse breeding and maintenance

For certain experiments we have used wild-type and PPARgamma heterozygous (haplo-insufficient) or PPARgamma null (KO) mice of C57BL/6J genetic background. The mice

were age matched, and both genders were used for the investigation. The design to generate PPARgamma KO mice was described previously **(11)**. Briefly, PPARgamma +/-/Sox2Cre+ male mice were crossed with PPARgamma fl/fl female mice to generate heterozygous PPARgamm afl/-/Sox2Cre- and homozygous PPAR gammaΔfl/-/Sox2Cre+ mice, wherein the floxed allele was recombined resulting a null allele. Mice were housed under minimal disease (MD) conditions in the Laboratory Animal Core Facility of University of Debrecen. Animal rooms were ventilated 15 times / hour with filtered air, mice received autoclaved pellet diet (Altromin VRF1) and tap water ad libitum. The cages contained sterilized bedding. Room lightning was automated with 12 hours light and 12 hours dark periods. The room temperature was 21±2 °C, the relative humidity is between 30-60%. Senescent animals developed and aged normally, without any treatment. Permission to perform the described animal experiments was granted to the relevant utilities of the University of Pecs (ref. no.: BA02/2000-46/2016). Permission to generate PPARgamma GM mice was granted to the relevant utilities of the University of Debrecen (ref. no.: TMF/82-10/2015). Permission to perform experimental procedures with PPARgamma GM mice was granted to the relevant utilities of the University of Pecs (ref. no.: TMF/124-11/2017).

Mouse immunofluorescence

Immunofluorescent staining was performed on 8µm cryo-sections of mouse thymus lobes as described previously **(35)**. Briefly, the slides were fixed in cold acetone, then dried and blocked to prevent non-specific staining using 5% BSA in PBS for 20 min before staining with fluorochrome-conjugated or primary antibodies: anti-EpCAM1-FITC (1:100, rat monoclonal antibody clone: G8.8,), anti-Ly51-PE (1:100, rat monoclonal antibody clone: 6C3, eBioscience), anti-PPARgamma (rabbit monoclonal antibody clone: C26H12 Cell Cell Signaling Technology). For secondary antibody Alexa-555 conjugated a-rabbit goat IgG (1:200, Life Technologies) was used. In certain cases DAPI (Life Technologies) nuclear counterstain was also applied. Sections were analyzed using a Nikon Eclipse Ti-U microscope equipped with a CCD camera (Andor Zyla 5.5) and NIS-Elements software. The medulla/cortex ratio was calculated using ImageJ software.

Mouse flow-cytometry

Thymocyte subsets and T-cell subpopulations in blood were investigated by flow-cytometry as published by others **(36, 37)**. Thymocytes and PBMC were isolated from mice and labeled with fluorophore-conjugated antibodies in PBS-BSA (5% BSA diluted in PBS). In every case

100,000 cells were stained for measurement. Incubation with antibodies was performed at 4°C for 60 minutes followed by a washing step. FACSCanto II flow-cytometer and FACSDiva software (Becton Dickinson) were used for analysis. In every case 10,000 events (parent R1 morphological lymphocyte gate) were recorded by flow-cytometry. For thymocyte subset measurement Alexa-647 conjugated anti-mouse CD4 (clone: YTS 191) and FITC conjugated anti-mouse CD8 (clone: IBL 3/25) antibodies were used (both produced in the Department of Immunology and Biotechnology, University of Pecs, Hungary). For peripheral blood T cell subpopulation analysis, Pacific Blue conjugated anti-mouse CD3 (clone: 17A2), PerCP conjugated anti-mouse CD4 (clone: GK1.5), APC/Cy7 conjugated anti-mouse CD8 (clone: YTS156.7.7), PE conjugated anti-mouse CD44 (clone: IM7), APC conjugated anti-mouse CD62L (clone: MEL-14) (all purchased form BioLegend) and FITC conjugated anti-mouse CD19 (clone: 1D3, produced by the Department of Immunology and Biotechnology, University of Pecs, Hungary) were used.

TREC measurement by digital qPCR in mouse and human

TREC (T-cell recombination excision circle) by-products of gene-rearrangement in fresh naive T-cells were also assessed. We performed mTREC digital qPCR using mouse and hTREC digital qPCR using human samples by adapting methods published by others **(38)**. Briefly, DNA was isolated from mouse thymocytes using the NucleoSpin Tissue kit (Macherey-Nagel) according to the manufacturer's instruction. For human, peripheral-blood samples were processed using the DNA Blood Mini kit (Qiagen) following the manufacturer's guides. Absolute copy numbers were measured by digital PCR on the QuantStudio 3D Digital PCR platform (ThermoFisher) using 30 ng DNA per sample. Taqman primers / probes and digital qPCR reagents were also purchased from ThermoFisher and used as suggested. For age-matched range of healthy human hTrec values please refer to the work of Lynch et al **(38)**. Permission to perform the described animal experiments was granted to the relevant utilities of the University of Pecs (ref. no.: BA02/2000-46/2016). Experiments involving human blood samples were performed with the consent of the Regional and Local Ethics Committee of Clinical Centre, University or Pecs (ref. no.: 6439/2016) according to their guidelines.

Oral tolerance induction in mouse

Induction and evaluation of oral tolerance was performed as described by others **(39, 41, 42)**. Briefly, both wild-type and PPARgamma haplo-insufficient mice received 5mg/ml ovalbumin

(OVA, Sigma-Aldrich) in drinking water for seven days. On day 7 mice were challenged with an intra-peritoneal injection of 5µg ovalbumin in 200µl of 1:1 of PBS:complete Freund adjuvant. On day 14 mice received an intra-peritoneal injection of 5µg ovalbumin in 200 µl of 1:1 of PBS:incomplete Freund adjuvant. Serum was collected on day 21 and anti-OVA IgG antibodies were measured by ELISA. Briefly, 96 well Microtest Plates (Sarstedt) were coated with OVA and blocked with BSA. Then plates were incubated with serial dilutions of mouse serum samples (1:100 - 1:3200). The antibody content was visualized with the help of HRP conjugated a-mouse immunoglobulin antibody (rabbit polyclonal, Dako). Optical density was measured at 492 nm with iEMS Reader MF equipment (Thermo Labsystems).

Influenza vaccination in mouse

The efficiency of influenza vaccination was investigated as described elsewhere **(40)**. Briefly, both wild-type and PPARgamma haplo-insufficient mice were injected intramuscular once with 0.1ml human seasonal influenza vaccine cocktail (3Fluart) to mimic human vaccination at 9 months of age. In order to imitate human exposure pattern serum antibody IgG titer against H1N1 A/California/7/2009 strain (part of 3Fluart) was measured by ELISA three months after initial single vaccination at 12 months of age. For detection ELISA plates were coated with 0.05ug HA protein of influenza strain A (Recombinant subtype H1N1 A/California/7/2009 His Tag, Life Technologies). Then plates were incubated with serial dilutions of mouse serum samples (1:5 - 1: 1600). The antibody content was visualized with the help of HRP conjugated a-mouse immunoglobulin antibody (rabbit polyclonal, Dako). Optical density was measured at 492 nm with iEMS Reader MF equipment (Thermo Labsystems).

Statistical analysis

All experiments were performed at least on three occasions, representative experiments are shown. Measures were obtained in triplicates, data are presented as mean and +SD as error bars. Graphpad Prism software was used for statistical analysis. Two-tailed T-student test was applied. Significant differences are shown by asterisks (ns for not significant, * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$).

Results

PPARgamma distorts the ratio of thymic epithelial compartments with age

Previously reported mouse results showed increasing PPARgamma expression with age in the thymic epithelial compartments, accompanied by thymic adipose involution. We have set out to prove the human relevance of these findings and test whether PPARgamma activity influences the ratio of thymic epithelial compartments.

Human FFPE thymic sections were analyzed for their PPARgamma expression in several adult age groups from young through middle-aged to senior. Our results indicate that PPARgamma expression significantly and progressively increases with age. Immunofluorescent staining of mouse thymic cryo-sections at 15 months of age provides visual support for thymic epithelial to adipose trans-differentiation in harmony with the working hypothesis of cellular trans-differentiation. A portion of stromal cells shows dual staining for epithelial identity and adipose differentiation, a hallmark of thymic adipose involution. This phenomenon is not observed at young adult age.

Mouse thymic cryo-sections were differentially stained for medullary and cortical epithelial compartments at several ages and using various genetic backgrounds. Our results show that in the wild-type setting the medullary epithelial compartment significantly shrinks with age as reported previously **(31)**. This, however, is not observed in PPARgamma deficient settings. PPARgamma deficiency efficiently and significantly prevents the erosion of the medullary epithelial compartment, otherwise prone to shrink with senescence.

PPARgamma affects thymic T-cell production and peripheral blood T-cell distribution with age

We have observed changes in thymus architecture in response to PPARgamma status. Consequently, we were interested in whether morphological changes alter thymus function: naïve T-cell production. Going beyond, we were eager to see if sustained influence of PPARgamma status on thymocyte function is also reflected in the peripheral blood.

Age-related changes in thymocyte levels of mTrec (DNA loop by-product of mouse T-cell receptor gene rearrangement) were evaluated in wild-type and PPARgamma deficient settings using digital qPCR. Our results indicate slight (though not significant) decrease of mTrec and hence fresh-naïve T-cell output with age in thymocytes of wild-type mice. PPARgamma deficiency significantly and progressively counteracts the process, showing increase of

thymocyte mTrec levels. In further analyses the percent distribution of thymocyte subpopulations was assessed using flow-cytometry in wild-type and PPARgamma deficient mice. Thymocyte subpopulations showed near identical distribution patterns. Taken together, PPARgamma deficiency progressively enhances thymocyte development in adult age, without skewing the distribution of thymocyte subpopulations or their differentiation preference.

Peripheral blood T-cell subpopulations were evaluated by flow-cytometry at 12 months of age in wild-type and PPARgamma deficient animals. Our results do not show any difference in the percent distribution of the major T-cell groups of helper T-cells and cytotoxic T-cells. However, the evaluation of naive T-cell and memory T-cell ratio reveals a significant effect of PPARgamma deficiency. There is a significant increase of naïve T-cells in the peripheral blood of PPARgamma deficient animals compared to wild-type animals, inversely and significantly decreasing the memory T-cell pool of T-cells. Deeper analysis of memory T-cell pool reveals that it is the mobile effector memory T-cell subpopulation, which shows significant decrease. Sustained and prolonged naïve T-cell production due to PPARgamma deficiency in the thymus as suggested by mTrec values above apparently affects peripheral blood T-cell subpopulations as shown here.

Functional immunological consequence and human relevance

Having seen the far-reaching influence of PPARgamma status on thymus architecture, thymus function and peripheral blood T-cell composition with age, we have set out to test whether these changes have any functional immunological relevance. If so, it would be also of high interest to test if our comprehensive mouse results have any human relevance.

We have tested the capacity to mount oral tolerance to the foreign protein OVA in wild-type and PPARgamma deficient aged adult mice by measuring OVA-specific IgG titers following oral and / or intra-peritoneal OVA challenge. As reported by others, age impairs oral tolerance in wild-type animals **(41, 42)**. As a consequence, there is only moderate, insufficient decrease of OVA-specific IgG titers in case of parallel oral OVA administration and i.p. OVA-injection in senior animals. Our results show that PPARgamma deficiency rescues oral tolerance at senior age, profoundly and significantly decreasing OVA-specific IgG titers. This suggests that naïve T-cell dependent immune regulation (oral tolerance) remains efficient in PPARgamma heterozygous animals despite their age.

The capacity to mount immune reaction to foreign influenza antigens was also tested as human seasonal influenza vaccine was injected (single i.m. shot) into aged adult wild-type and PPARgamma deficient animals. Subsequent analysis of serum IgG titers specific to a

vaccine component showed elevated protective antibody production in PPARgamma deficient animals, but not in their wild-type littermates. This tendency was not significant due to individual variation. Nevertheless, naïve T-cell dependent immune responses prove to be efficient in aged, PPARgamma heterozygous animals.

Genetic PPARgamma deficiency is a rare, but existing condition in human patients called FPLD3 **(15)**. It leads to the development of a metabolic disease called lipodystrophy, similar to the mouse **(11-15)**. Other rare human conditions not affecting PPARgamma can also lead to the development of lipodystrophy **(12-15)**. In case of FPLD2, lamin mutations trigger similar metabolic changes **(14)**. Peripheral blood hTrec (DNA loop by-product of human T-cell receptor gene rearrangement) levels were measured using digital qPCR in age-matched patients with FPLD2 condition and FPLD3 conditions. As expected, and in harmony with previous mouse thymocyte results elevated mean hTrec levels were detected in FPLD3 samples compared to FPLD2 samples. The tendency is not significant due to individual variation. Unfortunately, current patient sample numbers cannot be increased due to the extremely rare nature of these conditions (both FPLD2 and FPLD3 have prevalence of $\leq$ 1/1,000,000) **(14, 15)**. Lower limit of healthy human hTrec threshold (approx. 200 copies / μg DNA) is not reached by FPLD2 (lamin) patient samples, but this is rescued in FPLD3 (PPARgamma) patients despite being age- and disease-matched.

Discussion

PPARgamma drives thymic epithelial to adipose trans-differentiation with age

It has been previously suggested based on direct fate-mapping experiments that with senescence thymic adipose tissue develops from the thymic stromal or epithelial compartment **(28)**. Based on indirect evidence others have also supported this concept **(29)**. In harmony, we here present visual evidence of epithelial to adipose trans-differentiation in the mouse. This is indicated by the presence by EpCAM-1 / PPARgamma double-positive cells. These cells still express cell surface markers of their fading thymic epithelial identity (EpCAM-1), but already show early signs of the novel adipocyte differentiation program in their nuclei (PPARgamma). The fact that such double positive cells show rather scattered and not uniform staining pattern at a given time point may provide explanation for gradual thymic adipose involution observed during senescence.

PPARgamma impairs naïve T-cell production with age

Thymus histology data show that the medullary compartment is rescued from age-related involution in case of PPARgamma deficiency. Extended survival of this stromal niche ensures permissive environment for sustained thymus function: naïve T-cell production. This is indicated by elevated mTrec values showing direct correlation with PPARgamma deficiency. Of note and highlighting human relevance, peripheral blood leukocyte hTrec values from adult FPLD3 patients (with genetic PPARgamma deficiency) also exceed adult FPLD2 patient values (with unrelated genetic background) despite being age-matched and disease-matched (lipodystrophy, diabetes). Unlike lower than physiological hTrec values measured in FPLD2 (lamin) patients, those measured in FPLD3 (PPARgamma) patients are within healthy human physiological range. Since both mTrec and hTrec DNA loops originate from gene rearrangement during thymocte development this is direct evidence of sustained T-cell development indicating intact thymic niche in PPARgamma deficient animal models and human patients **(38)**. Of note, the distribution of thymocyte subpopulations shows identical pattern irrespective of PPARgamma status proving that sustained, enhanced thymocyte development does not skew differentiation preference, but rather enhances fresh, naive T-cell production of all thymocyte subtypes uniformly. Finally, since sustained thymic naïve T-cell production is a continuous trend, peripheral blood naïve T-cell populations are rescued from age-driven shrinking, against the effector memory T-cell pool.

PPARgamma hampers T-dependent immune regulation and immunity with age

Oral consumption of foreign T-depended antigen normally initiates immune tolerance inhibiting any eliminative immune response (e.g. serum IgG), despite parallel immunization in young adult individuals with appropriate naïve T-cell supply. Unfortunately, the phenomenon is disrupted at senior age due to the lacking naïve T-cell pool in the Peyer's patches of the gut **(41, 42, 45)** This loss of oral tolerance (impaired immune regulation) is a possible link to increasing food intolerance prevalence observed in the aging adult population **(46-49)**. However, the phenomenon is rescued by PPARgamma deficiency providing evidence that sustained T-cell production is necessary for efficient oral (immune) tolerance.

Senescence-triggered decrease of naïve T-cell output also impairs T-dependent immunity. An example in the senior human population is decreased protection from seasonal flu strains despite annual vaccination campaigns **(50-52)**. This is caused by low levels of neutralizing antibody titers due to lacking naïve T-cells necessary during T-B cooperation to mount adequate innate immune response against T-dependent antigens of the vaccine. The phenomenon has well established mouse models **(53-55).** This, however, is not the case with PPARgamma deficient mice. Single intramuscular vaccination against seasonal flu (mimicking human vaccination campaign) resulted in higher maximal antibody production three months later (a typical delay in human exposure). This confirms that the cause of decreased vaccination efficiency in the senior population is impaired T-dependent immunity due to thymic senescence.

In our experiments we have focused on the decline of T-dependent immunity since the thymus shows early and dramatic signs of senescence during adipose involution. This, however, is not the case for the B-cell compartment for which aging has been reported to occur later and in a more gradual fashion, lacking such profound histological changes **(56)**.

PPARgamma is an enigmatic transcription factor showing unique expression pattern in both time and space throughout the body **(57)**. PPARgamma affects both hemopoietic and stromal compartments during development and aging. Further dissection would require e.g. bone-marrow transplantation experiments between control and PPARgamma deficient animals. However, PPARgamma KO animals develop severe metabolic disorders that hamper such experiments, especially at senior ages.

Limitations and perspectives

We here present the long-term thymus- and T-dependent immunity-preserving effect of systemic (genetic) loss of PPARgamma function as observed in PPARgamma deficient mouse models and in a human rare disease (FPLD3 with PPARgamma deficiency). In both cases, there are severe metabolic drawbacks (diabetes, dyslipidemia etc.) due to systemically lacking PPARgamma activity. However, alternative, thymus tissue-restricted suppression of PPARgamma activity would likely solve the issue. Of note, as reported previously, over-expression of Wnt4 glycolipoproteins by thymic epithelial cells can efficiently counteract the effect of PPARgamma **(31)**. Also, Wnt4 was described to travel in extracellular vesicles including exosomes and affect thymocyte differentiation **(58, 59)**. Hence, it is conceivable that thymic epithelium-derived, enriched exosomes would efficiently home to the thymus and deliver their Wnt4 cargo locally even when administered systemically. This would, in theory, allow for tissue-specific, protein-mediated maintenance of thymic epithelial identity and prevent thymic senescence from developing.

Although tissue senescence is ultimately inevitable, there are conditions that accelerate thymic senescence including certain viral infections, intoxications, irradiation, chemotherapy etc. Outcomes include increased incidence of infection, cancer and autoimmune disorder. In any case the identification of novel molecular level targets for potential intervention is highly desired. Therefore, molecular level insight into immune senescence has medical, economical and personal relevance.

REFERENCES

1. Ammazzalorso A, De Filippis B, Giampietro L, Amoroso R. Blocking the peroxisome proliferator-activated receptor (PPAR): an overview. *ChemMedChem.* (2013) Oct;8(10):1609-16. doi: 10.1002/cmdc.201300250.
2. Montagner A, Rando G, Degueurce G, Leuenberger N, Michalik L, Wahli W. New insights into the role of PPARs. *Prostaglandins Leukot Essent Fatty Acids.* (2011) Nov;85(5):235-43. doi: 10.1016/j.plefa.2011.04.016.
3. Christodoulides C, Vidal-Puig A. PPARs and adipocyte function. *Mol Cell Endocrinol.* (2010) Apr 29;318(1-2):61-8. doi: 10.1016/j.mce.2009.09.014.
4. Janani C, Ranjitha Kumari BD. PPAR gamma gene--a review. *Diabetes Metab Syndr.* (2015) Jan-Mar;9(1):46-50. doi: 10.1016/j.dsx.2014.09.015.
5. Lefterova MI, Haakonsson AK, Lazar MA, Mandrup S. PPARγ and the global map of adipogenesis and beyond. *Trends Endocrinol Metab.* (2014) Jun;25(6):293-302. doi: 10.1016/j.tem.2014.04.001.
6. Siersbaek R, Nielsen R, Mandrup S. PPARgamma in adipocyte differentiation and metabolism--novel insights from genome-wide studies. *FEBS Lett.* (2010) Aug 4;584(15):3242-9. doi: 10.1016/j.febslet.2010.06.010.
7. Tontonoz P, Spiegelman BM. Fat and beyond: the diverse biology of PPARgamma. *Annu Rev Biochem.* (2008);77:289-312. doi: 10.1146/annurev.biochem.77.061307.091829.
8. Duan SZ, Ivashchenko CY, Whitesall SE, D'Alecy LG, Duquaine DC, Brosius FC 3rd, et al. Hypotension, lipodystrophy, and insulin resistance in generalized PPARgamma-deficient mice rescued from embryonic lethality. *J Clin Invest.* (2007) Mar;117(3):812-22.
9. Duan SZ, Usher MG, Foley EL 4th, Milstone DS, Brosius FC 3rd, Mortensen RM. Sex dimorphic actions of rosiglitazone in generalised peroxisome proliferator-activated receptor-gamma (PPAR-gamma)-deficient mice. *Diabetologia*. (2010) Jul;53(7):1493-505. doi: 10.1007/s00125-010-1748-2.
10. O'Donnell PE, Ye XZ, DeChellis MA, Davis VM, Duan SZ, Mortensen RM, et al. Lipodystrophy, Diabetes and Normal Serum Insulin in PPARγ-Deficient Neonatal Mice. *PLoS One.* (2016) Aug 9;11(8):e0160636. doi: 10.1371/journal.pone.0160636.
11. Nadra K, Quignodon L, Sardella C, Joye E, Mucciolo A, Chrast R, et al. PPARgamma in placental angiogenesis. *Endocrinology*. (2010) Oct;151(10):4969-81. doi: 10.1210/en.2010-0131.
12. Hegele RA, Joy TR, Al-Attar SA, Rutt BK. Thematic review series: Adipocyte Biology. Lipodystrophies: windows on adipose biology and metabolism. *J Lipid Res.* (2007) Jul;48(7):1433-44.
13. Hegele RA. Familial partial lipodystrophy: a monogenic form of the insulin resistance syndrome. *Mol Genet Metab.* (2000) Dec;71(4):539-44.
14. ORPHANET FPLD2 web resource: http://www.orpha.net/consor/cgi-bin/OC_Exp.php?Expert=2348
15. ORPHANET FPLD3 web resource: http://www.orpha.net/consor/cgi-bin/OC_Exp.php?Lng=GB&Expert=79083
16. Argmann CA, Cock TA, Auwerx J. Peroxisome proliferator-activated receptor gamma: the more the merrier? *Eur J Clin Invest.* (2005) Feb;35(2):82-92
17. Corton JC, Brown-Borg HM. Peroxisome proliferator-activated receptor gamma coactivator 1 in caloric restriction and other models of longevity. *J Gerontol A Biol Sci Med Sci.* (2005) Dec;60(12):1494-509.
18. Luconi M, Cantini G, Serio M. Peroxisome proliferator-activated receptor gamma (PPARgamma): Is the genomic activity the only answer? *Steroids.* (2010) Aug-Sep;75(8-9):585-94. doi: 10.1016/j.steroids.2009.10.012.
19. Choi SS, Park J, Choi JH. Revisiting PPARγ as a target for the treatment of metabolic disorders. *BMB Rep.* (2014) Nov;47(11):599-608.
20. Ahmadian M, Suh JM, Hah N, Liddle C, Atkins AR, Downes M, et al. PPARγ signaling and metabolism: the good, the bad and the future. *Nat Med.* (2013) May;19(5):557-66. doi: 10.1038/nm.3159.
21. Youm YH, Yang H, Amin R, Smith SR, Leff T, Dixit VD. Thiazolidinedione treatment and constitutive-PPARgamma activation induces ectopic adipogenesis and promotes age-related thymic involution. *Aging Cell*. (2010) Aug;9(4):478-89. doi: 10.1111/j.1474-9726.2010.00574.x.
22. Steinmann GG. Changes in the human thymus during aging. *Curr Top Pathol.* (1986);75:43-88.6.
23. Palmer DB. The effect of age on thymic function. *Front Immunol.* (2013) Oct 7;4:316. doi: 10.3389/fimmu.2013.0031
24. Bertho JM, Demarquay C, Moulian N, Van Der Meeren A, Berrih-Aknin S, Gourmelon P. Phenotypic and immunohistological analyses of the human adult thymus: evidence for an active thymus during adult life. *Cell Immunol.* (1997) Jul 10;179(1):30-40.

25. Falci C, Gianesin K, Sergi G, Giunco S, De Ronch I, Valpione S, et al. Immune senescence and cancer in elderly patients: results from an exploratory study. *Exp Gerontol.* (2013) Dec;48(12):1436-42. Doi: 10.1016/j.exger.2013.09.011.
26. Fletcher AL, Calder A, Hince MN, Boyd RL, Chidgey AP. The contribution of thymic stromal abnormalities to autoimmune disease. *Crit Rev Immunol.* (2011) ;31(3):171-87.
27. Yang H, Youm YH, Sun Y, Rim JS, Galbán CJ, Vandanmagsar B, et al. Axin expression in thymic stromal cells contributes to an age-related increase in thymic adiposity and is associated with reduced thymopoiesis independently of ghrelin signaling. *J Leukoc Biol.* (2009) Jun;85(6):928-38. doi: 10.1189/jlb.1008621.
28. Youm YH, Yang H, Sun Y, Smith RG, Manley NR, Vandanmagsar B, et al. Deficient ghrelin receptor-mediated signaling compromises thymic stromal cell microenvironment by accelerating thymic adiposity. *J Biol Chem.* (2009) Mar 1;284(11):7068-77. doi: 10.1074/jbc.M808302200
29. Kvell K, Varecza Z, Bartis D, Hesse S, Parnell S, Anderson G, et al. Wnt4 and LAP2alpha as pacemakers of thymic epithelial senescence. *PLoS One.* (2010) May 18;5(5):e10701. doi: 10.1371/journal.pone.0010701.
30. Varecza Z, Kvell K, Talabér G, Miskei G, Csongei V, Bartis D, et al. Multiple suppression pathways of canonical Wnt signalling control thymic epithelial senescence. Mech Ageing Dev. 2011 May;132(5):249-56. Doi: 10.1016/j.mad.2011.04.007.
31. Kvell K, Fejes AV, Parnell SM, Pongracz JE. Active Wnt/beta-catenin signaling is required for embryonic thymic epithelial development and functionality ex vivo. *Immunobiology*. (2014) Aug;219(8):644-52. doi: 10.1016/j.imbio.2014.03.017.
32. Kvell K, Pongracz JE. Central Immune Senescence, Reversal Potentials. In: Nagata T, editor. Senescence [Internet]. Rijeka (HR): *InTech*; (2012) Feb 29. Chapter 31.
33. Yang H, Youm YH, Dixit VD. Inhibition of thymic adipogenesis by caloric restriction is coupled with reduction in age-related thymic involution. *J Immunol.* (2009) Sep 1;183(5):3040-52. doi: 10.4049/jimmunol.0900562.
34. Talaber G, Kvell K, Varecza Z, Boldizsar F, Parnell SM, Jenkinson EJ, et al. Wnt-4 protects thymic epithelial cells against dexamethasone-induced senescence. Rejuvenation Res. 2011 Jun;14(3):241-8. doi: 10.1089/rej.2010.1110.
35. Meggyes M, Lajko A, Palkovics T, Totsimon A, Illes Z, Szereday L, et al. 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.
36. Solti I, Kvell K, Talaber G, Veto S, Acs P, Gallyas F Jr, et al. Thymic Atrophy and Apoptosis of CD4+CD8+ Thymocytes in the Cuprizone Model of Multiple Sclerosis. *PLoS One.* (2015) Jun 8;10(6):e0129217. doi: 10.1371/journal.pone.0129217.
37. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu Rev Immunol.* (2004);22:745-63.
38. Lynch HE, Goldberg GL, Chidgey A, Van den Brink MR, Boyd R, Sempowski GD. Thymic involution and immune reconstitution. *Trends Immunol.* (2009) Jul;30(7):366-73. doi: 10.1016/j.it.2009.04.003.
39. Simioni PU, Fernandes LG, Gabriel DL, Tamashiro WM. Induction of systemic tolerance in normal but not in transgenic mice through continuous feeding of ovalbumin. *Scand J Immunol.* (2004) Sep;60(3):257-66.
40. Ramirez A, Co M, Mathew A. CpG Improves Influenza Vaccine Efficacy in Young Adult but Not Aged Mice. *PLoS One*. (2016) Mar 2;11(3):e0150425. doi: 10.1371/journal.pone.0150425.
41. de Faria AM, Ficker SM, Speziali E, Menezes JS, Stransky B, Silva Rodrigues V, et al. Aging affects oral tolerance induction but not its maintenance in mice. *Mech Ageing Dev.* (1998) May 1;102(1):67-80.
42. Kato H, Fujihashi K, Kato R, Dohi T, Fujihashi K, Hagiwara Y, et al. Lack of oral tolerance in aging is due to sequential loss of Peyer's patch cell interactions. *Int Immunol.* (2003) Feb;15(2):145-58.
43. Hofer J, Hofer S, Zlamy M, Jeller V, Koppelstaetter C, Brandstätter A, et al. Elevated proportions of recent thymic emigrants in children and adolescents with type 1 diabetes. *Rejuvenation Res.* (2009) Oct;12(5):311-20. doi: 10.1089/rej.2009.0863.
44. Iskakova S, Urazayev O, Bekmukhambetov Y, Dworacki G, Dworacka M. [THE DIFFERENCES OF TREC (T-CELL RECEPTOR EXCISION CIRCLES) CONCENTRATION IN TYPE 2 DIABETIC PATIENTS]. *Georgian Med News. (*2015) Jul-Aug;(244-245):29-36.
45. Song F, Guan Z, Gienapp IE, Shawler T, Benson J, Whitacre CC. The thymus plays a role in oral tolerance in experimental autoimmune encephalomyelitis. *J Immunol.* (2006) Aug 1;177(3):1500-9.
46. Galipeau HJ, Verdu EF. Gut microbes and adverse food reactions: Focus on gluten related disorders. *Gut Microbes.* (2014) ;5(5):594-605. doi: 10.4161/19490976.2014.969635.
47. Marietta EV, Murray JA. Animal models to study gluten sensitivity. *Semin Immunopathol.* (2012) Jul;34(4):497-511. doi: 10.1007/s00281-012-0315-y.

48. MacDonald TT. Evidence for cell-mediated hypersensitivity as an important pathogenetic mechanism in food intolerance. *Clin Exp Allergy.* (1995) Jul;25 Suppl 1:10-3.
49. Ferguson A, Ziegler K, Strobel S. Gluten intolerance (coeliac disease). *Ann Allergy.* (1984) Dec;53(6 Pt 2):637-42.
50. Cromer D, van Hoek AJ, Jit M, Edmunds WJ, Fleming D, Miller E. The burden of influenza in England by age and clinical risk group: a statistical analysis to inform vaccine policy. *J Infect.* (2014) Apr;68(4):363-71. doi: 10.1016/j.jinf.2013.11.013.
51. Ang LW, Lim C, Lee VJ, Ma S, Tiong WW, Ooi PL, et al. Influenza-associated hospitalizations, Singapore, 2004-2008 and 2010-2012. *Emerg Infect Dis.* (2014) Oct;20(10):1652-60. doi: 10.3201/eid2010.131768.
52. Haq K, McElhaney JE. Immunosenescence: Influenza vaccination and the elderly. *Curr Opin Immunol.* (2014) Aug;29:38-42. doi: 10.1016/j.coi.2014.03.008.
53. Thangavel RR, Bouvier NM. Animal models for influenza virus pathogenesis, transmission, and immunology. *J Immunol Methods.* (2014) Aug;410:60-79. doi: 10.1016/j.jim.2014.03.023.
54. Kim JI, Park S, Lee S, Lee I, Heo J, Hwang MW, et al. DBA/2 mouse as an animal model for anti-influenza drug efficacy evaluation. *J Microbiol.* (2013) Dec;51(6):866-71. doi: 10.1007/s12275-013-3428-7.
55. Kamal RP, Katz JM, York IA. Molecular determinants of influenza virus pathogenesis in mice. *Curr Top Microbiol Immunol.* (2014);385:243-74. doi: 10.1007/82_2014_388.
56. Immunology of Aging, Ahmad Massoud and Nima Rezaei *Springer* Verlag Berlin Heidelberg (2014) doi: 10.1007/978-3-642-39495-9
57. Greene ME, Pitts J, McCarville MA, Wang XS, Newport JA, Edelstein C, et al. PPARgamma: observations in the hematopoietic system. *Prostaglandins Other Lipid Mediat.* (2000) Jun;62(1):45-73.
58. Gross JC, Boutros M. Secretion and extracellular space travel of Wnt proteins. *Curr Opin Genet Dev.* (2013) Aug;23(4):385-90. doi: 10.1016/j.gde.2013.02.017.
59. Lundberg V, Berglund M, Skogberg G, Lindgren S, Lundqvist C, Gudmundsdottir J, et al. Thymic exosomes promote the final maturation of thymocytes. *Sci Rep.* (2016) Nov 8;6:36479. doi: 10.1038/srep36479.

Bibliography

Article related to the thesis

Ernszt D, Banfai K, Kellermayer Z, Pap A, Lord JM, Pongracz JE, Kvell K. PPARγamma Deficiency Counteracts Thymic Senescence. Front Immunol. 2017 Nov 6;8:1515. doi: 10.3389/fimmu.2017.01515. eCollection 2017.

Impact factor: **5.551**

Articles not related to the thesis

Barabás K, Godó S, Ernszt D, Pál J, Lengyel F, Abraham I. Rapid non-classical effects of steroids on the membrane receptor dynamics and downstream signaling in neurons, Hormones and Behavior. 2018. IF: 4.418

Bodó K, Ernszt D, Németh P, Engelmann P. Distinct immune- and defense-related molecular fingerprints in separated coelomocyte subsets in Eisenia andrei earthworms. Invertebrate Survival Journal 15 pp. 338-345., 2018 IF: 0.806

Ugor E, Prenek L, Pap R, Berta G, Ernszt D, Najbauer J, Németh P, Boldizsár F, Berki T. Glucocorticoid hormone treatment enhances the cytokine production of regulatory T cells by upregulation of Foxp3 expression. Immunobiology. 2018 Apr - May;223(4-5):422-431. doi: 10.1016/j.imbio.2017.10.010. Epub 2017 Oct 6. IF: 2.873

Lipták N, Hoffmann OI, Kerekes A, Iski G, Ernszt D, Kvell K, Hiripi L, Bősze Z. Monitoring of Venus transgenic cell migration during pregnancy in non-transgenic rabbits. Transgenic Res. 2017 Apr;26(2):291-299. doi: 10.1007/s11248-016-9994-9. Epub 2016 Nov 10. IF: 2.197

Payrits M, Sághy É, Szolcsányi J, Pohóczky K, Csekő K, Bölcskei K, Barabás K, Ernszt D, Ábrahám I, Helyes Zs, Szőke É: Estradiol sensitises the Transient Receptor Potential Vanilloid 1 channel in pain responses. Endocrinology. 2017. IF: 3.961

Dülk M, Kudlik G, Fekete A, Ernszt D, Kvell K, Pongrácz JE, Merő BL, Szeder B, Radnai L, Geiszt M, Csécsy DE, Kovács T, Uher F, Lányi Á, Vas V, Buday L. The scaffold protein Tks4 is required for the differentiation of mesenchymal stromal cells (MSCs) into adipogenic and osteogenic lineages. Sci Rep. 2016 Oct 6;6:34280. doi: 10.1038/srep34280. IF: 4.259

Engelmann P, Hayashi Y, Bodó K, Ernszt D, Somogyi I, Steib A, Orbán J, Pollák E, Nyitrai M, Németh P, Molnár L. Phenotypic and functional characterization of earthworm coelomocyte subsets: Linking light scatter-based cell typing and imaging of the sorted populations. Dev Comp Immunol. 2016 Dec;65:41-52. doi: 10.1016/j.dci.2016.06.017. Epub 2016 Jun 24. IF: 3.218

Vojkovics D, Kellermayer Z, Heidt D, Mihalj M, Kajtár B, Ernszt D, Kovács T, Németh P, Balogh P. Isolation and Characterization of a Murine Spontaneous High-Grade Follicular Lymphoma with Restricted In Vivo Spreading--a Model for Lymphatic Metastasis Via the Mesentery. Pathol Oncol Res. 2016 Apr;22(2):421-30. doi: 10.1007/s12253-015-0025-6. Epub 2015 Nov 19. IF: 1.736

Kovacs T, Csongei V, Feller D, Ernszt D, Smuk G, Sarosi V, Jakab L, Kvell K, Bartis D, Pongracz JE. Alteration in the Wnt microenvironment directly regulates molecular events leading to pulmonary senescence. Aging Cell. 2014 Oct;13(5):838-49. doi: 10.1111/acel.12240. Epub 2014 Jul 1. IF: 6,340

Cumulative impact factor: **29.808**

Acknowledgments

I would like to express my gratitude to all those who have supported me in the preparation of my thesis.

First of all, I thank my supervisor, Dr. Krisztián Kvell. I am grateful that he has been supporting me throughout the past years with his professional advice and guidance. I would like to say thanks to Prof. Dr. Judit Pongrácz for providing me the conditions of research and supporting my doctoral studies.

I am grateful to the staff of the Department of Pharmaceutical Biotechnology for their help in my work and for a positive atmosphere. Special thanks go to Krisztina Bánfai for helping me with the experiments.

I would like to thank Prof. Dr. Péter Balogh and Dr. Zoltán Kellermayer for their professional support and friendly advice over the years. Also, I would like to thank all the staff of the Department of Immunology and Biotechnology who, with their professional experience, have supported me during my studies.

I would like to thank all the staff of the Department of Pharmacology and Pharmacotherapy for their help and advice.

Special thanks go to my friends Dr. Tamás Kovács, Dr. Dániel Szappanos, Máté Kiss and Diána Feller for supporting and helping me in difficult moments. And because they also contributed to the completion of my thesis with their excellent professional help.

I thank my parents, grandparents and sister, for the support, family atmosphere and love that made my studies possible. Without them, this thesis could not have been born.

I can't say with words how grateful I am to my wife, Maja for the years of help, encouragement, perseverance, and patience she has given me. I thank for her infinite love that supported me during my work. And finally, I thank my son, Miró, for giving me strength in the past few months and encouraging me to write this thesis.