

PPARgamma deficiency counteracts thymic senescence

Ernszt, David; Banfai, Krisztina; Kellermayer, Zoltan; Pap, Attila; Lord, Janet; Pongracz, Judit; Kvell, Krisztian

DOI:

[10.3389/fimmu.2017.01515](https://doi.org/10.3389/fimmu.2017.01515)

License:

Creative Commons: Attribution (CC BY)

Document Version

Peer reviewed version

Citation for published version (Harvard):

Ernszt, D, Banfai, K, Kellermayer, Z, Pap, A, Lord, J, Pongracz, J & Kvell, K 2017, 'PPARgamma deficiency counteracts thymic senescence', *Frontiers in immunology*, vol. 8, 1515.
<https://doi.org/10.3389/fimmu.2017.01515>

[Link to publication on Research at Birmingham portal](#)

Publisher Rights Statement:

This Document is protected by copyright and was first published by Frontiers. All rights reserved. It is reproduced with permission. See the publisher's version: PPARgamma Deficiency Counteracts Thymic Senescence, by David Ernszt, et. al. *Front. Immunol.*, 06 November 2017, <https://doi.org/10.3389/fimmu.2017.01515>

General rights

Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

- Users may freely distribute the URL that is used to identify this publication.
- Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.
- User may use extracts from the document in line with the concept of 'fair dealing' under the Copyright, Designs and Patents Act 1988 (?)
- Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

Take down policy

While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact UBIRA@lists.bham.ac.uk providing details and we will remove access to the work immediately and investigate.

1 **PPARGAMMA DEFICIENCY**
2 **COUNTERACTS THYMIC SENESENCE**

3

4

5David Ernszt^{1,2}, Krisztina Banfai^{1,2}, Zoltan Kellermayer³, Attila Pap⁴, Janet M. Lord⁵, Judit E.

6Pongracz^{1,2}, Krisztian Kvell^{1,2,*}

7

8

9

101: Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, University of Pecs,
11Pecs, Hungary

122: Szentagothai Research Center, University of Pecs, Pecs, Hungary

133: Department of Immunology and Biotechnology, Faculty of Medicine, University of Pecs,
14Pecs, Hungary

154: Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of
16Debrecen, Debrecen, Hungary

175: Institute of Inflammation and Aging, College of Medical and Dental Sciences, University
18of Birmingham, Birmingham, United Kingdom

19

20

21

22

23* Corresponding author:

24

25Krisztian KVELL MD PhD dr. habil.

26

27Address: Department of Pharmaceutical Biotechnology

28Faculty of Pharmacy, University of Pecs

292 Rokus Str, H-7624 Pecs, Hungary

30

31Phone: +36-72-536-000 ext. 20551

32E-mail: kvell.krisztian@pte.hu

33

34

35manuscript text word count: 3,917 words

36

37figure count: 6 figure files, 1 supplementary material file

1 ABSTRACT

2

3 Thymic senescence contributes to increased incidence of infection, cancer and autoimmunity
4 at senior ages. This process manifests as adipose involution. As with other adipose tissues,
5 thymic adipose involution is also controlled by PPARgamma. This is supported by
6 observations reporting that systemic PPARgamma activation accelerates thymic adipose
7 involution. Therefore we hypothesized that decreased PPARgamma activity could prevent
8 thymic adipose involution, although it may trigger metabolic adverse effects.

9 We have confirmed that both human and murine thymic sections show marked staining for
10 PPARgamma at senior ages. We have also tested the thymic lobes of PPARgamma haplo-
11 insufficient and null mice. Supporting our working hypothesis both adult PPARgamma haplo-
12 insufficient and null mice show delayed thymic senescence by thymus histology, thymocyte
13 mTrec qPCR and peripheral blood naïve T-cell ratio by flow-cytometry. Delayed senescence
14 showed dose-response with respect to PPARgamma deficiency. Functional immune
15 parameters were also evaluated at senior ages in PPARgamma haplo-insufficient mice (null
16 mice do not reach senior ages due to metabolic adverse effects). As expected, sustained and
17 elevated T-cell production conferred oral tolerance and enhanced vaccination efficiency in
18 senior PPARgamma haplo-insufficient, but not in senior wild-type littermates according to
19 ELISA IgG measurements.

20 Of note, humans also show increased oral intolerance issues and decreased protection by
21 vaccines at senior ages. Moreover, PPARgamma haplo-insufficiency also exists in human
22 known as a rare disease (FPLD3) causing metabolic adverse effects, similar to the mouse.
23 When compared to age- and metabolic disorder-matched other patient samples (FPLD2 not
24 affecting PPARgamma activity), FPLD3 patients showed increased hTrec values by qPCR
25 (within healthy human range) suggesting delayed thymic senescence, in accordance with
26 mouse results and supporting our working hypothesis.

27 In summary our experiments prove that systemic decrease of PPARgamma activity prevents
28 thymic senescence, albeit with metabolic drawbacks. However, thymic tissue-specific
29 PPARgamma antagonism would likely solve the issue.

30

31

32 **Keywords:** PPARgamma, thymus, immunity, senescence, rejuvenation

33

11. INTRODUCTION

2

3The PPAR (peroxisome proliferator-activated receptor) molecular family is widely studied (1-43). These nuclear receptor proteins possess transcription factor activities and influence 5multiple cellular events at the molecular level including adipocyte differentiation and 6metabolism. Among them, PPARgamma is of particular interest being expressed by all 7adipose tissue subtypes and being indispensable for adipose tissue development and for the 8homeostasis of physiological metabolism (4-7). As a consequence, in the mouse systemic loss 9of PPARgamma activity severely impairs glucose and lipid metabolism as characterized by 10others (8-10). In accordance, PPARgamma null mice are only viable if using conditional 11knockout strategy (11). Similar to the mouse above, in human PPARgamma haplo- 12insufficiency leads to the development of a rare metabolic condition known as familial partial 13lipodystrophy, type 3 (FPLD3, ORPHA 79083) also characterized by diabetes and 14dyslipidemia (12-15).

15

16In mammals systemic PPARgamma activity may be increased at multiple levels. 17Environmental factors including excessive caloric consumption or corticosteroid exposure 18increase PPARgamma activity systemically (16-18). Pharmacological systemic activation may 19be achieved through administration of thiazolidinediones (TZDs) previously used as part of 20oral anti-diabetic treatment, but currently neglected due to adverse cardiovascular side-effects 21(19, 20). Genetic engineering-based enhancement of PPARgamma activity in mouse models 22has also been performed (21). In every case increased PPARgamma activity promotes adipose 23tissue development at multiple sites of the body.

24

25Thymic aging is observed as adipose involution during which the functional thymus niche that 26normally supports T-cell production is gradually lost and replaced by adipose tissue (22). The 27process starts focally in childhood then spreads and accelerates with puberty due to hormonal 28changes (23). Diminishing T-cell production results in decreased availability of fresh naïve T- 29cells (24). Consequences include increasing incidence of infection, cancer and autoimmunity 30observed at senior ages (25, 26). Thymic adipose involution appears to be PPARgamma- 31dependent: any condition that systemically enhances PPARgamma activity – either 32environmental, pharmacological or genetic – accelerates thymic senescence or adipose 33involution with all its immunological consequences (27-32). However, the opposite 34phenomenon whether systemically decreased PPARgamma activity can ameliorate long-term 35functional immune parameters has barely been addressed (33, 34). For this reason we have set 36out to characterize the effect of systemic genetic PPARgamma loss of function on long-term 37immune homeostasis in both mouse and human.

38

12. METHODS

2

32.1. Human thymus samples

4Formalin-fixed, paraffin-embedded (FFPE) human thymus samples from age groups 30-40
5years 50-60 and 70-80 years were obtained from the Department of Pathology (Faculty of
6Medicine, University of Pecs, Hungary.) Experiments involving human thymus samples were
7performed with the consent of the Regional and Local Ethics Committee of Clinical Centre,
8University of Pecs (ref. no.: 6331/2016) according to their guidelines. All subjects gave
9written informed consent in accordance with the Declaration of Helsinki.

10

112.2. Human immunohistochemistry

12Human thymus lobes were fixed in paraformaldehyde (4% PFA in PBS) then paraffin
13embedded. 5µm thick sections were stained using immunohistochemistry (35). First the slides
14were rinsed in heated xylene and were washed with a descending series of alcohol to remove
15paraffin. After de-paraffination the slides were rehydrated in distilled water and antigen
16retrieval was performed by heating the slides in Target Retrieval Solution (pH 6 DAKO) at
1797°C for 20-30 minutes. Subsequently slides were washed in dH₂O and endogenous
18peroxidase activity was blocked with 3% H₂O₂ containing TBS (pH 7.4) for 15 minutes. Then
19slides were washed three times with TBS containing Tween (0.05%, pH 7.4). Pre-blocking
20was carried out with 3% BSA in TBS for 20 minutes before overnight incubation with anti-
21PPARgamma (1:100, rabbit monoclonal antibody clone: C26H12 Cell Signalling Technology)
22primary antibody at 4°C. Following incubation slides were washed with TBS for three times
23then incubated with peroxidase conjugated secondary antibody (1:100, Polyclonal Goat Anti-
24Rabbit IgG, DAKO) for 90 minutes. Antibody labeling was visualized with the help of liquid
25DAB Substrate Chromogen System (DAKO). For nuclear counterstaining hematoxylin
26staining was performed. Finally slides were mounted with Faramount Aqueous Mounting
27Medium (DAKO). Histological evaluation was performed with the help of Panoramic MIDI
28digital slide scanner (3DHitech). Image analysis was performed using ImageJ software with
29IHC toolbox plug-in.

30

312.3. Mouse breeding and maintenance

32For certain experiments we have used wild-type and PPARgamma heterozygous (haplo-
33insufficient) or PPARgamma null (KO) mice of C57BL/6J genetic background. The mice
34were age matched, and both genders were used for the investigation. The design to generate
35PPARgamma KO mice was described previously (11). Briefly, PPARgamma +/-/Sox2Cre+
36male mice were crossed with PPARgamma fl/fl female mice to generate heterozygous
37PPARgamma fl/-/Sox2Cre- and homozygous PPAR gammaΔfl/-/Sox2Cre+ mice, wherein the
38floxed allele was recombined resulting a null allele. Mice were housed under minimal disease
39(MD) conditions in the Laboratory Animal Core Facility of University of Debrecen. Animal
40rooms were ventilated 15 times / hour with filtered air, mice received autoclaved pellet diet
41(Altromin VRF1) and tap water ad libitum. The cages contained sterilized bedding. Room
42lightning was automated with 12 hours light and 12 hours dark periods. The room temperature
43was 21±2 °C, the relative humidity is between 30-60%. Senescent animals developed and
44aged normally, without any treatment. Permission to perform the described animal
45experiments was granted to the relevant utilities of the University of Pecs (ref. no.:
46BA02/2000-46/2016). Permission to generate PPARgamma GM mice was granted to the
47relevant utilities of the University of Debrecen (ref. no.: TMF/82-10/2015). Permission to
48perform experimental procedures with PPARgamma GM mice was granted to the relevant
49utilities of the University of Pecs (ref. no.: TMF/124-11/2017).

50

12.4. Mouse immunofluorescence

2 Immunofluorescent staining was performed on 8µm cryo-sections of mouse thymus lobes as
3 described previously (35). Briefly, the slides were fixed in cold acetone, then dried and
4 blocked to prevent non-specific staining using 5% BSA in PBS for 20 min before staining
5 with fluorochrome-conjugated or primary antibodies: anti-EpCAM1-FITC (1:100, rat
6 monoclonal antibody clone: G8.8.), anti-Ly51-PE (1:100, rat monoclonal antibody clone:
7 76C3, eBioscience), anti-PPARgamma (rabbit monoclonal antibody clone: C26H12 Cell
8 Signaling Technology). For secondary antibody Alexa-555 conjugated a-rabbit goat IgG
9 (1:200, Life Technologies) was used. In certain cases DAPI (Life Technologies) nuclear
10 counterstain was also applied. Sections were analyzed using a Nikon Eclipse Ti-U microscope
11 equipped with a CCD camera (Andor Zyla 5.5) and NIS-Elements software. The
12 medulla/cortex ratio was calculated using ImageJ software.

13

14 2.5. Mouse flow-cytometry

15 Thymocyte subsets and T-cell subpopulations in blood were investigated by flow-cytometry
16 as published by others (36, 37). Thymocytes and PBMC were isolated from mice and labeled
17 with fluorophore-conjugated antibodies in PBS-BSA (5% BSA diluted in PBS). In every case
18 100,000 cells were stained for measurement. Incubation with antibodies was performed at 4°C
19 for 60 minutes followed by a washing step. FACSCanto II flow-cytometer and FACSDiva
20 software (Becton Dickinson) were used for analysis. In every case 10,000 events (parent R1
21 morphological lymphocyte gate) were recorded by flow-cytometry. For thymocyte subset
22 measurement Alexa-647 conjugated anti-mouse CD4 (clone: YTS 191) and FITC conjugated
23 anti-mouse CD8 (clone: IBL 3/25) antibodies were used (both produced in the Department of
24 Immunology and Biotechnology, University of Pecs, Hungary). For peripheral blood T cell
25 subpopulation analysis, Pacific Blue conjugated anti-mouse CD3 (clone: 17A2), PerCP
26 conjugated anti-mouse CD4 (clone: GK1.5), APC/Cy7 conjugated anti-mouse CD8 (clone:
27 YTS156.7.7), PE conjugated anti-mouse CD44 (clone: IM7), APC conjugated anti-mouse
28 CD62L (clone: MEL-14) (all purchased from BioLegend) and FITC conjugated anti-mouse
29 CD19 (clone: 1D3, produced by the Department of Immunology and Biotechnology,
30 University of Pecs, Hungary) were used.

31

32 2.6. TREC measurement by digital qPCR in mouse and human

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

47

48 2.7. Oral tolerance induction in mouse

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

1(OVA, Sigma-Aldrich) in drinking water for seven days. On day 7 mice were challenged with
2an intra-peritoneal injection of 5µg ovalbumin in 200µl of 1:1 of PBS:complete Freund
3adjuvant. On day 14 mice received an intra-peritoneal injection of 5µg ovalbumin in 200 µl of
41:1 of PBS:incomplete Freund adjuvant. Serum was collected on day 21 and anti-OVA IgG
5antibodies were measured by ELISA. Briefly, 96 well Microtest Plates (Sarstedt) were coated
6with OVA and blocked with BSA. Then plates were incubated with serial dilutions of mouse
7serum samples (1:100 - 1:3200). The antibody content was visualized with the help of HRP
8conjugated a-mouse immunoglobulin antibody (rabbit polyclonal, Dako). Optical density was
9measured at 492 nm with iEMS Reader MF equipment (Thermo Labsystems).

10

112.8. Influenza vaccination in mouse

12The efficiency of influenza vaccination was investigated as described elsewhere (40). Briefly,
13both wild-type and PPARgamma haplo-insufficient mice were injected intramuscular once
14with 0.1ml human seasonal influenza vaccine cocktail (3Fluart) to mimic human vaccination
15at 9 months of age. In order to imitate human exposure pattern serum antibody IgG titer
16against H1N1 A/California/7/2009 strain (part of 3Fluart) was measured by ELISA three
17months after initial single vaccination at 12 months of age. For detection ELISA plates were
18coated with 0.05ug HA protein of influenza strain A (Recombinant subtype H1N1
19A/California/7/2009 His Tag, Life Technologies). Then plates were incubated with serial
20dilutions of mouse serum samples (1:5 - 1: 1600). The antibody content was visualized with
21the help of HRP conjugated a-mouse immunoglobulin antibody (rabbit polyclonal, Dako).
22Optical density was measured at 492 nm with iEMS Reader MF equipment (Thermo
23Labsystems).

24

252.9. Statistical analysis

26All experiments were performed at least on three occasions, representative experiments are
27shown. Measures were obtained in triplicates, data are presented as mean and +SD as error
28bars. Graphpad Prism software was used for statistical analysis. Two-tailed T-student test was
29applied. Significant differences are shown by asterisks (ns for not significant, * for $p \leq 0.05$,
30** for $p \leq 0.01$, *** for $p \leq 0.001$).

31

13. RESULTS

2

33.1. PPARgamma distorts the ratio of thymic epithelial compartments with age

4Previously reported mouse results showed increasing PPARgamma expression with age in the
5thymic epithelial compartments, accompanied by thymic adipose involution. We have set out
6to prove human relevance of previous mouse findings and test whether PPARgamma activity
7influences the ratio of thymic epithelial compartments.

8

93.1.1. PPARgamma expression increases in the adult thymus with age

10Human FFPE thymic sections were analyzed for their PPARgamma expression in several
11adult age groups from young through middle-aged to senior (**Figure 1A-D**). Our results
12indicate that PPARgamma expression significantly and progressively increases with age
13(**Figure 1A-C**). Of note, total cellular areas shrink at senior ages in both human (**Figure 1C**)
14and mouse (**Figure 1F**). As a result the ratio of PPARgamma-expressing cellular areas shows
15relative increase with age (**Figure 1D**). Immunofluorescent staining of mouse thymic cryo-
16sections at 15 months of age (**Figure 1F**) provides visual support for thymic epithelial to
17adipose trans-differentiation in harmony with the working hypothesis of cellular trans-
18differentiation. A portion of stromal cells shows dual staining for epithelial identity and
19adipose differentiation, a hallmark of thymic adipose involution. This phenomenon is not
20observed at young adult age (**Figure 1E**).

21

223.1.2. PPARgamma skews the ratio of epithelial compartments with age

23Mouse thymic cryo-sections were differentially stained for medullary and cortical epithelial
24compartments at several ages and using various genetic backgrounds (**Figure 2A-D**). Our
25results show that in the wild-type setting the medullary epithelial compartment significantly
26shrinks with age as reported previously (**31**). This, however, is not observed in PPARgamma
27deficient settings. Loss of PPARgamma activity shows protection in a progressive manner
28presenting dose-response (**Figure 2E**). PPARgamma deficiency efficiently and significantly
29prevents the erosion of the medullary epithelial compartment, otherwise prone to shrink with
30senescence.

31

32

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

35We have observed changes in thymus architecture in response to PPARgamma status.
36Consequently, we were interested in whether morphological changes alter thymus function:
37naïve T-cell production. Going beyond, we were eager to see if sustained influence of
38PPARgamma status on thymocyte function is also reflected in the peripheral blood.

39

403.2.1. PPARgamma disturbs thymic T-cell output with age

41Age-related changes in thymocyte levels of mTrec (DNA loop by-product of mouse T-cell
42receptor gene rearrangement) were evaluated in wild-type and PPARgamma deficient settings
43using digital qPCR (**Figure 3A**). Our results indicate slight (though not significant) decrease
44of mTrec and hence fresh-naïve T-cell output with age in thymocytes of wild-type mice.
45PPARgamma deficiency significantly and progressively counteracts the process also showing
46dose-responsive increase of thymocyte mTrec levels. In further analyses the percent
47distribution of thymocyte subpopulations was assessed using flow-cytometry in wild-type and
48PPARgamma deficient mice (**Figure 3B**). All thymocyte subpopulations showed near
49identical distribution pattern with all genetic backgrounds. Taken together, PPARgamma

1 deficiency progressively enhances thymocyte development in adult age, but without skewing
2 the distribution of thymocyte subpopulations or their differentiation preference.

3

4 3.2.2. PPARgamma influences T-cell subpopulation distribution in adult peripheral blood

5 Peripheral blood T-cell subpopulations were evaluated by flow-cytometry at 12 months of age
6 in wild-type and PPARgamma deficient animals. Our results do not show differences in the
7 percent distribution of the major T-cell groups of helper T-cells and cytotoxic T-cells (**Figure**
8 **4A**) within the CD3-gate of T-cells. However, the evaluation of naïve T-cell and memory T-
9 cell ratio reveals significant effect of PPARgamma deficiency (**Figure 4B**). There is
10 significant increase of naïve T-cells in the peripheral blood of PPARgamma deficient animals
11 compared to wild-type animals, conversely and significantly decreasing the memory T-cell
12 pool within the CD3-gate of T-cells. Deeper analysis of the memory T-cell pool reveals it is
13 the mobile effector memory T-cell subpopulation that shows significant decrease and not
14 central memory T-cells (**Figure 4C**) within the CD3-gate of T-cells. Sustained and prolonged
15 naïve T-cell production due to PPARgamma deficiency in the thymus as suggested by mTrec
16 values above apparently affects peripheral blood T-cell subpopulations as shown here.

17

18

19 **3.3. Functional immunological consequence and human relevance**

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

24

25 3.3.1. PPARgamma modulates immune regulation and immune response

26 We have tested the capacity to mount oral tolerance to the foreign protein OVA in wild-type
27 and PPARgamma deficient aged adult mice by measuring OVA-specific IgG titers following
28 oral and / or intra-peritoneal OVA challenge (**Figure 5A**). As reported by others, age impairs
29 oral tolerance in wild-type animals (**41, 42**). As a consequence, there is only moderate,
30 insufficient decrease of OVA-specific IgG titers in case of parallel oral OVA administration
31 and i.p. OVA-injection in senior animals. However, PPARgamma deficiency rescues oral
32 tolerance in the same experimental setting despite of age, profoundly and significantly
33 decreasing OVA-specific IgG titers (**Figure 5A**). Consequently, naïve T-cell dependent
34 immune regulation (oral tolerance) remains efficient in PPARgamma heterozygous animals
35 despite their age.

36 The capacity to mount immune reaction to foreign influenza antigens was also tested as
37 human seasonal influenza vaccine was injected into aged adult wild-type and PPARgamma
38 deficient animals. Subsequent analysis of serum IgG titers specific to a vaccine component
39 showed elevated protective antibody production (maximal ELISA OD values) in PPARgamma
40 deficient animals, but not in their wild-type littermates (**Figure 5B**). This tendency is not
41 significant because of individual variation observed due to the applied human vaccination
42 protocol being inferior to standard mouse immunization protocol. Nevertheless, naïve T-cell
43 dependent immune response proves to be efficient in aged, PPARgamma heterozygous
44 animals.

45

46 3.3.2. Human evidence of PPARgamma deficiency preventing thymic senescence

47 Genetic PPARgamma deficiency is a rare, but existing condition in human called FPLD3 (**15**).
48 It leads to a metabolic phenotype called lipodystrophy, similar to the mouse (**11-15**). Other
49 rare human conditions not affecting PPARgamma can also lead to lipodystrophy (**12-15**). In
50 case of FPLD2 lamin mutations trigger similar metabolic changes (**14**). Peripheral blood

1hTrec (DNA loop by-product of human T-cell receptor gene rearrangement) levels were
2measured using digital qPCR in age-matched patients with FPLD2 condition and FPLD3
3condition (**Figure 6**). As expected and in perfect harmony with previous mouse thymocyte
4results elevated mean hTrec levels were detected in FPLD3 samples compared to FPLD2
5samples. The tendency is not significant due to individual variation within the patient groups.
6Unfortunately, current patient sample numbers cannot be increased due to the extremely rare
7nature of these conditions (FPLD2 or ORPHA 2348 has prevalence of $\leq 1/1,000,000$ and
8FPLD3 or ORPHA 79083 also has prevalence of $\leq 1/1,000,000$) (**14, 15**). For age-matched
9range of healthy human hTrec values please refer to the work of Lynch et al (**38**). Lower limit
10of healthy human hTrec threshold (approx. 200 copies / μg DNA) is not reached by FPLD2
11(lamin) patient samples, but this is rescued in FPLD3 (PPARgamma) patients despite being
12age- and disease-matched.

13

14. DISCUSSION

2

34.1. PPARgamma drives thymic epithelial to adipose trans-differentiation with age

4It has been previously suggested based on direct fate-mapping experiments that with
5senescence thymic adipose tissue develops from the thymic stromal or epithelial compartment
6(28). Based on indirect evidence others have also supported this concept (29). In further
7support, we here present visual evidence of epithelial to adipose trans-differentiation in the
8mouse. This is indicated by the presence by EpCAM-1 / PPARgamma double-positive cells
9shown by histology (Figure 1D). These cells still express cell surface markers of their fading
10thymic epithelial identity (EpCAM-1), but already show early signs of the novel adipocyte
11differentiation program in their nuclei (PPARgamma). The fact that such double positive cells
12show rather scattered and not uniform staining pattern at a given time point may provide
13explanation for gradual thymic adipose involution observed during senescence.

14

154.2. PPARgamma impairs naïve T-cell production with age

16Thymus histology data show that the medullary compartment is rescued from age-related
17shrinking in case of PPARgamma deficiency (Figure 2A-D). Extended survival of this
18stromal niche ensures permissive environment for sustained thymus function: naïve T-cell
19production. This is indicated by elevated mTrec values showing direct correlation with
20PPARgamma deficiency (Figure 3A). Of extreme importance and highlighting human
21relevance, peripheral blood leukocyte hTrec values from adult FPLD3 patients (with genetic
22PPARgamma deficiency) also exceed adult FPLD2 patient values (with unrelated genetic
23background) despite being age-matched and disease-matched (lipodystrophy, diabetes)
24(Figure 6). Of note, such metabolic disorders are known to impair thymus function indicated
25by decreased hTrec values as reported by others (43, 44). For exactly this reason have we used
26disease-matched controls (FPLD2 vs FPLD3) to show enhanced thymus function with
27PPARgamma deficiency despite metabolic disorders. Unlike lower than physiological hTrec
28values measured in FPLD2 (lamin) patients, those measured in FPLD3 (PPARgamma)
29patients are within healthy human physiological range (Figure 6). Since both mTrec and
30hTrec DNA loops originate from gene rearrangement during thymocyte development this is
31direct evidence of sustained T-cell development indicating intact thymic niche in
32PPARgamma deficient animal models and human patients (38). Of note, the distribution of
33thymocyte subpopulations shows identical pattern irrespective of PPARgamma status proving
34that sustained, enhanced thymocyte development does not skew differentiation preference, but
35rather enhances fresh, naïve T-cell production of all thymocyte subtypes uniformly (Figure
363B). Finally, since sustained thymic naïve T-cell production is not restricted to a given time-
37point, but rather represents a continuous trend, the peripheral blood naïve T-cell population
38shows cumulative differences as it is rescued from age-driven shrinking, against the memory
39T-cell population – more specifically against the effector memory T-cell pool (Figure 4B-C).

40

414.3. PPARgamma hampers T-dependent immune regulation and immunity with age

42Oral consumption of foreign T-dependent antigen normally initiates immune tolerance
43inhibiting any eliminative immune response (e.g. serum IgG), despite parallel immunization
44in young adult individuals with appropriate naïve T-cell supply. Unfortunately, the
45phenomenon is disrupted at senior age due to the lacking naïve T-cell pool in the Peyer's
46patches of the gut (41, 42, 45) This loss of oral tolerance (impaired immune regulation) is a
47possible link to increasing food intolerance prevalence observed in the aging adult population
48(46-49). However, the phenomenon may be rescued by PPARgamma deficiency despite of
49age providing evidence that sustained T-cell production is necessary for efficient oral
50(immune) tolerance (Figure 5A).

1 Senescence-triggered decrease of naïve T-cell output also impairs T-dependent immunity. An
2 example in the senior human population is decreased protection from seasonal flu strains
3 despite annual vaccination campaigns (50-52). The phenomenon has well established animal
4 models (53-55). This is caused by low levels of neutralizing antibody titers due to lacking
5 naïve T-cells necessary during T-B cooperation to mount adequate innate immune response
6 against T-dependent antigens of the vaccine. This, however, is not the case with PPARgamma
7 deficiency (Figure 5B). Single intramuscular vaccination against seasonal flu (mimicking
8 human vaccination campaign) resulted in higher maximal antibody production three months
9 later (a typical delay in human exposure). This confirms that the cause of decreased
10 vaccination efficiency in the senior population is impaired T-dependent immunity due to
11 thymic senescence.

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

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

22

23 4.5. Limitations and perspectives

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

37

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

44

1ACKNOWLEDGEMENTS

2The authors wish to thank the PPARgamma^{+/-} and PPARgamma^{fl/fl} mice that were obtained
3from Yaacov Barak PhD (Salk Institute, La Jolla, California, USA) and also the Sox2Cre mice
4obtained from Beatrice Desvergne MD, PhD (University of Lausanne, Switzerland). We are
5grateful for Gregory D. Sempowski MD PhD (Duke Human Vaccine Institute, Duke
6University, Durham, USA) for providing the Trec primer and probe sequences along with
7protocols for TaqMan qPCR, and also Peter Balogh MD PhD (Department of Immunology
8and Biotechnology, University of Pecs, Hungary) for providing test antibodies for mouse
9CD3, CD4, CD8. The authors wish to thank David B. Savage MD PhD (Metabolic Research
10Laboratories, School of Clinical Medicine, University of Cambridge, Cambridge, United
11Kingdom) for providing peripheral blood DNA samples from genetically verified FPLD2 and
12FPLD3 rare disease patients.

13

14CONFLICTS OF INTEREST

15The authors declare that they have no conflicts of interest with the contents of this article. The
16research was conducted in the absence of any commercial or financial relationship that could
17be construed as a potential conflict of interest.

18

19FUNDING

20Scientific research support was provided by the Hungarian National Science Foundation (No.
2178310) and PTE AOK KA-2016-16 to KK. The project was also supported by the University
22of Pecs in the frame of Pharmaceutical Talent Center program and the Viral Pathogenesis
23Talent Center program via KK. The Janos Bolyai Scholarship of the Hungarian Academy of
24Sciences also supported KK. JEP was supported by the European Union and he State of
25Hungary, co-financed by the European Social Fund in the framework of GINOP 2.3.2-15-
262016-00022 TAMOP-4.2.2. A-11/1/KON-2012-0024, TAMOP-4.2.4.A/2-11/1-2012-0001
27'National Excellence Program', PTE AOK-KA-2013/22 and EFOP-3.6.1-16-2016-00004. The
28present scientific contribution is also dedicated to the 650th anniversary of the foundation of
29the University of Pecs, Hungary.

30

31AUTHOR CONTRIBUTIONS

32DE performed most histological, molecular biology and statistics work in the project and was
33involved in manuscript preparation. KB performed all human IHC work. ZK performed oral
34immune tolerance experiments. AP was in charge for the breeding, metabolic and genetic
35characterization of PPARgamma haplo-insufficient and null mice. JML was in charge for
36planning human experiments, involved in manuscript preparation as well as local supervision
37of respective department. PJE was involved in planning mouse experiments, involved in
38manuscript preparation as well as local supervision of respective department. KK was
39involved in histological, molecular biology and statistics work, also in planning experiments
40and manuscript preparation, and supervised the project.

41

1 FIGURE LEGENDS

2

3 **Figure 1.**

4 **PPARgamma expression in the adult thymus**

5 Human FFPE thymic sections were analyzed for PPARgamma expression by
6 immunohistochemistry in age groups of 20-30 years called young adult (Figure 1A), 50-60
7 years called middle-aged (Figure 1B) and 70-80 years called senior (Figure 1C). Brown color
8 reaction (DAB) shows PPARgamma expression. Blue color (hematoxylin) shows nuclear
9 counter-stain and defines total cellular areas. The ratio of PPARgamma-expressing cellular
10 areas and total cellular areas is also shown for the different age groups (Figure 1D).
11 Immunofluorescent staining is also shown for mouse at 1 month of age called young adult and
12 at 15 months of age called senior (Figure 1E-F). Green color shows epithelial cells (anti-
13 EpCAM1-FITC), red color shows pre-adipocytes (anti-PPARgamma primary AB with Alexa-
14 555 secondary AB) and blue color defines nuclei (DAPI counter-stain). Please note
15 arrowheads pointing at double-staining (EpCAM-1⁺ / PPARgamma⁺) cells (Figure 1F). Both
16 stainings show expected patterns: EpCAM-1 staining presents cell surface markers, while
17 PPARgamma-staining shows nuclear localization (observed in magenta color due to overlap
18 with DAPI nuclear counter-stain on Figure 1F). For exact numerical data please refer to
19 Supplementary material. Significant differences are shown by asterisks (ns for not significant,
20 * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$).

21

22 **Figure 2.**

23 **Ratio of epithelial compartments in the adult thymus**

24 Mouse thymic cryo-sections were stained differentially for medullary (anti-EpCAM1-FITC⁺⁺,
25 anti-Ly51-PE⁻) and cortical (anti-Ly51-PE⁺⁺, anti-EpCAM1-FITC⁺) epithelial compartments.
26 Wild-type thymus is shown at 1 month (Figure 2A) and 8 months of age (Figure 2B).
27 PPARgamma heterozygous (Figure 2C) and PPARgamma KO (Figure 2D) animals are shown
28 at 8 months of age. The ratio of medullary and cortical epithelial compartment is also shown
29 (Figure 2E) for both ages and genetic backgrounds. For exact numerical data please refer to
30 Supplementary material. Significant differences are shown by asterisks (ns for not significant,
31 * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$).

32

33 **Figure 3.**

34 **Thymocyte development in the adult thymus**

35 Changes in level of mouse T-cell recombination excision circles (mTrec) was evaluated by
36 Taqman digital qPCR in wild-type, PPARgamma heterozygous and PPARgamma KO
37 thymocytes (Figure 3A). The columns represent mTrec values measured at 8 months divided
38 by those measured at 1 month for every strain. The ratio of thymocyte subpopulations was
39 assessed by flow-cytometry at 8 months of age in wild-type, PPARgamma heterozygous and
40 PPARgamma KO animals (Figure 3B). Double negative (CD4⁻, CD8⁻), double positive (CD4⁺,
41 CD8⁺) and single positive (CD4⁺ or CD8⁺) subpopulations are shown. For the measurement of
42 every sample 100,000 cells were stained and 10,000 events (parent R1 morphological
43 lymphocyte gate) were recorded by flow-cytometry. For exact cell numbers please refer to
44 Supplementary material. Significant differences are shown by asterisks (ns for not significant,
45 * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$).

46

47 **Figure 4.**

48 **T-cell subpopulations in adult peripheral blood**

49 Peripheral blood T-cell subpopulations were evaluated by flow-cytometry at 12 months of age
50 in wild-type and PPARgamma heterozygous animals (KO animals decrease by this age).

1 Percent distribution of T-cells (CD3⁺), helper T-cells (CD3⁺, CD4⁺) and cytotoxic T-cells
2 (CD3⁺, CD8⁺) is shown by Figure 4A. Also, the percent distribution of naive T-cells (CD3⁺,
3 CD44⁻, CD62L⁺) and memory T-cells (CD3⁺, CD44⁺, CD62L^{+/-}) was evaluated within the
4 CD3-gate of T-cells (Figure 4B). Further analysis of memory T-cell subpopulation shows
5 percent distribution of effector memory T-cells (CD3⁺, CD44⁺, CD62L⁻) and central memory
6 T-cells (CD3⁺, CD44⁺, CD62L⁺) within the CD3-gate of T-cells (Figure 4C). For the
7 measurement of every sample 100,000 cells were stained and 10,000 events (parent R1
8 morphological lymphocyte gate) were recorded by flow-cytometry. For exact cell numbers
9 please refer to Supplementary material. Significant differences are shown by asterisks (ns for
10 not significant, * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$).

11

12 **Figure 5.**

13 **Functional immunological experiments in adult hosts**

14 Oral tolerance induction capacity to ovalbumin (OVA) was assayed in wild-type and
15 PPAR γ heterozygous animals at 12 months of age. Animals received OVA by either
16 drinking water, i.p. injection, both or neither. OVA-specific IgG titers were evaluated 3 weeks
17 later by ELISA method (Figure 5A). The presented figure was obtained using 1:400 dilution
18 of serum. Mean ELISA OD values are shown for each study group. Human seasonal influenza
19 vaccine (3Fluart) was injected (0.1ml, 1x, i.m.) into wild-type and PPAR γ heterozygous
20 animals at 9 months of age. Serum IgG titers specific to a vaccine component (H1N1
21 A/California/7/2009 strain) were tested 3 months later by ELISA method (Figure 5B). The
22 presented figure was obtained using 1:50 dilution of serum. Maximal ELISA OD values are
23 shown for each study group. For exact numerical data please refer to Supplementary material.
24 Significant differences are shown by asterisks (ns for not significant, * for $p \leq 0.05$, ** for $p \leq$
25 0.01, *** for $p \leq 0.001$).

26

27 **Figure 6.**

28 **Thymus function in adult FPLD patients**

29 Level of human T-cell recombination excision circle (hTrec) was measured by Taqman digital
30 qPCR in peripheral blood leukocytes of age-matched and disease-matched rare disease
31 patients with FPLD2 condition (lipodystrophy due to LMNA-deficiency) and FPLD3
32 condition (lipodystrophy due to PPAR γ -deficiency) (Figure 6). Patient sample numbers
33 were n=3 for FPLD2 and n=5 for FPLD3. For exact numerical data please refer to
34 Supplementary material. For age-matched (approx. 50 years of age) range of healthy human
35 hTrec values please refer to the work of Lynch et al (38). Accordingly, the lower limit of
36 healthy human hTrec threshold (approx. 200 copies / μ g DNA) is represented by dotted line.

37

1 REFERENCES

- 2
3
41. Ammazalorso 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.
72. 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.
103. 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.
124. 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.
145. Lefterova MI, Haakonsson AK, Lazar MA, Mandrup S. PPAR γ 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.
176. 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.
207. 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.
238. 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.
279. 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.
3110. O'Donnell PE, Ye XZ, DeChellis MA, Davis VM, Duan SZ, Mortensen RM, et al. Lipodystrophy, Diabetes and Normal Serum Insulin in PPAR γ -Deficient Neonatal Mice. *PLoS One*. (2016) Aug 9;11(8):e0160636. doi: 10.1371/journal.pone.0160636.
3411. 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.
3712. 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.
4013. Hegele RA. Familial partial lipodystrophy: a monogenic form of the insulin resistance syndrome. *Mol Genet Metab*. (2000) Dec;71(4):539-44.
4214. ORPHANET FPLD2 web resource:
43 http://www.orpha.net/consor/cgi-bin/OC_Exp.php?Expert=2348
4415. ORPHANET FPLD3 web resource:
45 http://www.orpha.net/consor/cgi-bin/OC_Exp.php?Lng=GB&Expert=79083
4616. 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
4817. 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.

118. 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.
419. Choi SS, Park J, Choi JH. Revisiting PPAR γ as a target for the treatment of metabolic disorders. *BMB Rep*. (2014) Nov;47(11):599-608.
620. Ahmadian M, Suh JM, Hah N, Liddle C, Atkins AR, Downes M, et al. PPAR γ signaling and metabolism: the good, the bad and the future. *Nat Med*. (2013) May;19(5):557-66. doi: 10.1038/nm.3159.
921. 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.
1322. Steinmann GG. Changes in the human thymus during aging. *Curr Top Pathol*. (1986);75:43-88.6.
1523. Palmer DB. The effect of age on thymic function. *Front Immunol*. (2013) Oct 7;4:316. doi: 10.3389/fimmu.2013.0031
1724. 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.
2125. 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.
2426. 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.
2727. 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.
3128. 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
3529. 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.
3830. 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.
4231. 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.
4532. Kvell K, Pongracz JE. Central Immune Senescence, Reversal Potentials. In: Nagata T, editor. *Senescence [Internet]*. Rijeka (HR): *InTech*; (2012) Feb 29. Chapter 31.
4733. 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.

134. 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.
435. 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.
836. 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.
1137. 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.
1338. 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.
1639. 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.
1940. 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.
2241. 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.
2542. 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.
- 28
- 29
- 30
3143. 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.
3444. 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.
3845. 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.
4146. 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.
4447. 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.
4648. MacDonald TT. Evidence for cell-mediated hypersensitivity as an important pathogenic mechanism in food intolerance. *Clin Exp Allergy.* (1995) Jul;25 Suppl 1:10-3.
4849. Ferguson A, Ziegler K, Strobel S. Gluten intolerance (coeliac disease). *Ann Allergy.* (1984) Dec;53(6 Pt 2):637-42.
- 49

150. 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.
451. 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.
752. 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.
953. 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.
1254. 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.
1555. 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.
1756. Immunology of Aging, Ahmad Massoud and Nima Rezaei Springer Verlag Berlin Heidelberg (2014) doi: 10.1007/978-3-642-39495-9
1957. 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.
2258. 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.
2459. 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.
- 27
- 28
- 29
- 30