Biological and Pharmaceutical Bulletin Advance Publication by J-STAGE
DOI:10.1248/bpb.b22-00842

Advance Publication
December 27, 2022

Biol Pharm Bull: Regular Article

Different Properties of Involuted Thymus upon Nutritional Deficiency in Young and Aged Mice

Kei **Nakayama***,[a] Mari **Kondo**,[a] Tomoko **Okuno**,[a] Nurhanani **Razali**,[a,b] Hiroshi **Hasegawa**[a]

[a] *Laboratory of Hygienic Sciences, Kobe Pharmaceutical University, 4-19-1 Motoyamakita-machi, Higashinada-ku, Kobe 6588558, Japan;* [b] *Current affiliation: Membranology Unit, Okinawa Institute of Science and Technology Graduate University, 1919-1, Tancha, Onna-son, Kunigami-gun, Okinawa 904-0495, Japan*

*Correspondence: knakayam@kobepharma-u.ac.jp

Summary

Immune suppression in elderly individuals is one of the most important hygienic problems in aged societies. The primary immune organ thymus is histologically and functionally reduced by aging, which is known as thymic involution. The thymus is also involuted by nutritional deficiency, which frequently occurs in elderly individuals. However, there is no information on the thymic changes caused by nutritional deficiency with aging. Therefore, this study was conducted to examine the histological and molecular responses of the thymus to nutritional deficiency in young and aged mice. The thymic size was significantly smaller in 16- or 18-week-old aged mice than in 7-week-old young mice. Dietary restriction for 48 h reduced the thymic size in young mice, but not in aged mice. Immunostaining with anti-keratin 5 antibody revealed that the integrity of the corticomedullary boundary was maintained in the aged thymus, whereas dietary restriction induced its disorganization in both young and aged thymus. The numbers of IgG-positive cells were increased upon dietary restriction in aged, but not in young, thymus. Dietary restriction, but not aging, upregulated the mRNA levels of Th2-related *Il5*, *Il6*, and *Il10*, whereas aging increased that of Th1-related *Ifng*. The dietary restriction–induced upregulation of prostanoid-synthesizing enzymes was clearly observed in the young thymus but attenuated in the aged thymus. Thus, nutritional deficiency and aging cause an involuted thymus with different properties. Moreover, the thymus in aged mice does not show further reduction in size by nutritional deficiency but still responds differently compared with that in young mice.

Keywords: thymus, nutritional deficiency, aging, inflammation, thymic epithelial cell

INTRODUCTION

The elderly population experiences numerous health issues, among which a decrease in immune capacity is the most important medical problem as it increases the risks of developing bacterial and viral infections, autoimmune diseases, and cancer progression.[1)] Elderly individuals have chronic mild inflammation, with increased production of proinflammatory cytokines and activation of T lymphocytes and macrophages, due to continuous exposure to external antigens under reduced immune functions.[2)] Although the maintenance of immune capacities is essential for the quality of life in the elderly population, there is no clear information on the mechanisms causing the decline of immune functions by aging.

The thymus is a primary immune organ responsible for the development and maturation of T lymphocytes. It is vulnerable to various biological stresses, including infection, mental stresses, toxic chemicals, malnutrition, and aging, which cause changes in its size and functions, a process known as thymic involution or thymic atrophy. In humans, the thymus reaches its maximum size in juvenile stages. After puberty, it begins to shrink and the substances of the thymus are replaced with fat by the middle age. The development and maturation of T cells occur prominently in younger stages. Studies have reported that the removal of the thymus in newborns causes a complete depletion of T cells in the blood and lymph tissues, whereas its removal in adults causes no apparent defect.[3,4)] However, the thymus continues to supply naïve T cells also in adult stages.[5,6)] Thymic involution is related to increased risks for infection, autoimmune disease, and cancer in adults.[7)] Immune reconstitution after recovery from human immunodeficiency virus infection depends on the level of thymic function in human patients.[8)] Therefore, the maintenance of thymic function throughout life contributes to the quality of life.

During nutritional deficiency–induced thymic involution, the characteristics of naïve helper T cells are modulated.[9)] Helper T cells develop and mature, but not finally differentiate, in the thymus and reside at the stage known as helper T cell type 0 (Th0). The final differentiation of Th0 cells to each Th subtype requires exposure to immunological stimulation in the periphery. Hence, Th0

cells remain undifferentiated in the thymus, but not completely neutral to subtype-specific characteristics, and they transiently exhibit some characteristics of differentiated T cell subtypes. This plasticity of Th0 cells is termed as polarization. Our previous study demonstrated that the balance of Th0 cell polarization is affected by the nutritional state, wherein dietary restriction for 48 h suppressed the Th1-like phenotype and biased it to the Th2-like phenotype.[9] This unbalanced Th0 cell polarization involves increased production of prostanoids and could be a cause of reduced cellular immunity upon nutritional deficiency.[9]

Thus, both nutritional deficiency and aging cause thymic involution. Because aging is often accompanied with reductions of food intake and nutritional absorption in the digestive organs, several elderly people are under a condition of nutritional deficiency. This nutritional deficiency with aging results in increased risks of the onset and progression of several age-associated diseases. However, there are no data on the combinatory effects of nutritional deficiency and aging on the thymus. Therefore, in this study, we compared the effects of dietary restriction, mimicking nutritional deficiency, on the thymus of young and aged mice.

MATERIALS AND METHODS

Animals

Male ICR mice were purchased from Japan SLC, Inc (Shizuoka, Japan) and raised in the animal facility at Kobe Pharmaceutical University till they reached 7, 16, and 18 weeks of age. They were individually housed and acclimatized for at least 1 week before the experiment. All the animal experimental procedures in this study were conducted according to the Guidelines for Proper Conduct of Animal Experiments of the Science Council of Japan. All protocols were approved by the Kobe Pharmaceutical University Committee for Animal Care and Use.

Dietary restriction was accomplished as described previously.[9] Briefly, the mice were placed in new cages with (normal feeding) or without (dietary restriction) food pellets and housed for 48 h. Water was freely accessible to the mice during dietary restriction. All the tested mice were weighed

before and after treatment.

Histological Analyses

Mice were deeply anesthetized with isoflurane, sacrificed by cervical dislocation, and dissected to obtain the thymus. The thymus was fixed in 4% paraformaldehyde in PBS at 4°C for 6 h. Immunohistochemistry was performed as described in our previous study with minor modifications.[10] The paraformaldehyde-fixed thymus tissues were cryoprotected in 30% sucrose in PBS at 4°C overnight. Furthermore, 30-μm-thick sections were prepared using a cryostat (SLEE medical GmbH, Mainz, Germany). The tissue sections were fixed in 4% paraformaldehyde in PBS at room temperature for 5 min, briefly washed with PBS, and incubated in 10 mM citrate buffer (pH 6.0) at 65°C for 40 min for antigen retrieval. The sections were then cooled to room temperature, washed three times in PBS, and blocked with 1.5% fetal bovine serum in PBS for 1 h. For the detection of immunoglobulin G (IgG) and immunoglobulin M (IgM), the sections were incubated with Alexa 568- or Cy3-conjugated antibodies against mouse IgG (A11019; Thermo Fisher Scientific, Wilmington, DE, USA), IgM (715-165-140; Jackson ImmunoResearch Inc., West Grove, PA, USA), or rabbit IgG as a control (A10042; Thermo Fisher Scientific).

For the detection of keratin 5, the blocked sections were incubated with rabbit antibody against keratin 5 (905504, Biolegend, Inc., San Diego, CA, USA) at 4°C overnight. The sections were then washed with PBS and incubated with Alexa 488-conjugated anti-rabbit IgG (715-545-152, Jackson ImmunoResearch) diluted in PBS containing 0.5% fetal bovine serum at room temperature for 3 h. Next, they were washed in PBS and mounted with Fluoromount-G (SouthernBiotech, Birmingham, AL, USA).

All images were captured using an Axio Scope A1 microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) and processed in the Affinity Designer (Serif, Ltd., Nottingham, England) and GNU Image Manipulation Program, an open resource software program for manipulating images. The quantification was performed using the ImageJ software.

RNA Purification and Quantitative Reverse Transcription Polymerase Chain Reaction

RNA extraction and quantitative reverse transcription polymerase chain reaction (RT-qPCR) were performed as described previously.[11] Briefly, mice were deeply anesthetized with isoflurane and transcardially perfused with PBS. The thymus was dissected out and frozen in Sepasol-RNA I Super G solution (Nacalai Tesque, Inc., Kyoto, Japan). Tissues were homogenized, and total RNA was purified according to the manufacturer's instructions. The concentrations of purified RNA were determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). Complementary DNA was synthesized using the ReverTra Ace reagent (Toyobo Co. Ltd, Osaka, Japan) according to the manufacturer's instructions. The expression levels of target genes were determined using a CFX Connect real-time PCR detection system (Bio-Rad Laboratories Inc., Hercules, CA, USA). PCR amplification was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories). The primer sequences used in this study have been described in our previous study, except those for *Il6* and *Actb* mRNA.[9] The sequences for *Il6* were 5′-GGAGCCCACCAAGAACGATA-3′ and 5′-GTCACCAGCATCAGTCCCAA-3′, and those for *Actb* were 5′-TATTGGCAACGAGCGG-3′ and 5′-CGGATGTCAACGTCAC-3′. PCR was performed under the following conditions: 1 min of initial DNA polymerase activation and DNA denaturation at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s, and primer annealing and fragment extension at 60°C for 30 s. The melting curves of the real-time PCR products were analyzed from 65°C to 95°C. Differences in gene expression, expressed as fold change, were calculated using the standard curve method. *Actb* was used as a reference gene for normalizing the expression.

Statistical Analyses

Results were expressed as mean ± standard deviation. Data were statistically analyzed using the R software. When a significant interaction was observed in the two-way factorial one-way analysis

of variance (ANOVA), the Tukey–Kramer post-hoc test was performed to compare the mean values among four groups.

RESULTS

Unresponsiveness of Thymus Size to Dietary Restriction in Aged Mice

Dietary restriction for 48 h reduced the body weight of both young (7-week-old) and aged (16- to 18-week-old) mice (Fig. 1A, B), a finding that was supported by the significant effect of the nutritional condition on the change in body weight (Table 1). The extent of reduction was smaller in aged mice than in young mice (Fig. 1C), probably because of the reduction of basal metabolism and/or decreased physical activity.[12] Dietary restriction reduced the absolute weight of the thymus by 43.1% in young mice (Fig. 1D). Aging itself induced thymic involution and reduced the weight of the thymus relative to body weight by 61.4% under normally fed conditions. In aged mice, dietary restriction exerted no significant effect on the thymic weight (Fig. 1D). Because the body weights of young and aged mice are largely different, we evaluated the reduction of thymic weight by dividing with body weight (Fig. 1E). The relative thymic weight was smaller by 24.0% in young mice subjected to dietary restriction compared to that in normally fed young mice. In contrast, the relative thymic weight remained unchanged by dietary restriction in aged mice; the relative thymic weight of aged mice subjected to dietary restriction was 91.6% of that of normally fed aged mice. Hence, both nutritional deficiency and aging result in thymic size reduction, but there is no additive effect between these two stresses.

The liver weight relative to body weight was reduced by both dietary restriction and aging (Fig. 2A, Table 1). The weight of the kidney and heart relative to body weight was significantly reduced by aging, but not by dietary restriction (Fig. 2B, C, Table 1). The testicular weight relative to body weight showed no statistically significant difference with both aging and dietary restriction (Fig. 2D). The epididymal fat in aged mice was heavier than that in young mice (Fig. 2E, Table 1). Interestingly, dietary restriction did not reduce the content of epididymal fat, although the

subcutaneous fat content apparently reduced after dietary restriction (Fig. 2E and data not shown).

Disorganization of Corticomedullary Boundary in the Thymus of Dietary-Restricted Mice

Our previous study indicated that the 48-h dietary restriction in 8-week-old mice induced a disorganization of the corticomedullary boundary in the thymus.[9] This disorganization is a major feature of the involuted thymus. As this disruption of segmentation in the thymus would be a mechanism responsible for reduced cellular immunity, we explored whether a similar change in the corticomedullary boundary occurs in aged mice by immunostaining of the thymus sections of young and aged mice with anti-keratin 5 antibody. In normally fed young mice, the corticomedullary boundary in the thymus was clearly formed (Fig. 3A), but it was disorganized after dietary restriction (Fig. 3B). In normally fed aged mice, the corticomedullary boundary was jaggy and not straight as in normally fed young mice, but it was still apparent (Fig. 3C). Dietary restriction resulted in disorganization of the boundary in aged mice, comparable to the condition in young mice (Fig. 3D). Thus, the disorganization of the corticomedullary boundary was induced by nutritional deficiency in both young and aged mice. Aging is not directly related to the integrity of the corticomedullary boundary, but it affects the shape of the thymic cortex and medulla.

Accumulation of IgG-Positive Cells in the Thymus of Dietary-Restricted Aged Mice

Our previous studies demonstrated that the 48-h dietary restriction induced the accumulation of IgM-positive cells, but not IgG-positive cells, in the spleen, whereas it reduced IgG immunoreactivity in the lymph nodes.[13,14] Hence, the response of immune capacities upon nutritional deficiency differs among organs. To examine the change of immune capacity in the thymus by nutritional deficiency, the thymus sections were stained for detecting IgG, which revealed only a very few IgG-positive cells in normally fed young mice (Fig. 4A). Dietary restriction did not affect the number of IgG-positive cells in young mice (Fig. 4B). In aged mice, the number of IgG-positive cells in the thymus under the normally fed condition was comparable to that in normally

fed young mice (Fig. 4C). In aged mice, dietary restriction significantly increased the number of IgG-positive cells, which were distributed in both the cortex and medulla (Fig. 4D, K, Table 2). These results indicate that nutritional deficiency induces inflammatory response in an age-dependent manner. The background signal for IgG, which might be of IgG molecules secreted from thymic and/or peripheral B lymphocytes and deposited on the extracellular space, increased in the aged thymus (Table 2). Dietary restriction increased the IgG intensity, although no statistical significance was obtained (Fig. 4L, Table 2). The number of IgM-positive cells slightly increased in the aged thymus (Fig. 4G, M, Table 2). Dietary restriction did not affect the number of thymic IgM-positive cells in both young and aged mice (Fig. 4F, H, M, Table 2). The IgM intensity in the extracellular space remained unchanged by dietary restriction and aging (Fig. 4E–H, N, Table 2). No immunoreactivity was observed with antibody against rabbit IgG (Fig. 4K and L), confirming the specificity of antibodies against mouse IgG and IgM.

Transcriptional Characterization of the Thymus of Dietary-Restricted and Aged Mice

The thymic expression of inflammatory cytokines, which are believed to be transcriptional markers for Th1 and Th2 cells, was examined by RT-qPCR (Fig. 5A). Dietary restriction increased the expression of *Il5*, *Il6*, and *Il10* mRNAs encoding interleukin (IL)-5, IL-6, and IL-10, respectively, although no statistically significant differences were obtained for *Il10* (the *p* value for nutritional condition was 0.0741 by the two-way factorial ANOVA) (Fig. 5A, Table 2). These ILs are preferentially expressed by Th2 cells. In contrast, the expression of interferon-γ (*Ifng*) mRNA, which is primarily secreted from the Th1 subtype, was higher in the aged thymus than in the young thymus (Fig. 5A, Table 2). Dietary restriction did not affect the expression of *Ifng* (Table 2). These results are consistent with our previous results and indicate that nutritional deficiency regulates the polarization of Th0 cells toward the Th2 phenotype.[9] The levels of expressional changes of *Il5*, *Il6*, and *Il10* were more apparent in the young thymus than in the aged thymus (Fig. 5A). Hence, the

response of Th0 cells to nutritional deficiency observed in the young thymus disappeared in the aged thymus.

The change of Th0 cell polarization by nutritional deficiency involves increased prostanoid synthesis.[9] Therefore, we investigated the expression of mRNAs encoding prostanoid-synthesizing enzymes (Fig. 5B). The results of the two-way factorial ANOVA indicated an interaction between dietary restriction and aging for the expression of *Cox1*, *Hpgds, Ptges1*, and *Tbxas1* (Table 2). The expression of these mRNAs significantly increased in the young thymus by dietary restriction, but such dietary restriction–induced upregulation was abolished in the aged thymus (Fig. 5B). The expressions of *Cox2*, *Ptgds*, and *Akr1b7* were significantly affected by dietary restriction, but not by aging (Fig. 5B, Table 2). The mRNA expression levels of *Ptges2*, *Ptges3*, *Akr1b3*, and *Ptgis* were almost similar between young and aged mice and not affected by dietary restriction (Fig. 5B, data not shown). Therefore, the patterns of mRNA induction of prostanoid-synthesizing enzymes by nutritional deficiency are different in the young and aged thymus.

DISCUSSION

There are two primary findings of our analyses, as follows: (1) the thymus undergoes involution in response to both nutritional deficiency and aging. Both these stimuli effectively induce the reduction in size, but the characteristics of the involuted thymus are different; (2) nutritional deficiency causes a size reduction of the thymus in young mice, but not in aged mice. However, the thymus in aged mice is also susceptible to nutritional deficiency and changes its properties.

Different Properties of Nutritional Deficient and Aged Thymus

Our results showed that nutritional deficiency resulted in common and different responses in young and aged mice. Dietary restriction significantly reduced the thymic size in young mice, but not in aged mice (Fig. 1). The integrity of the corticomedullary boundary of the thymus was maintained in normally fed aged mice, and it was disorganized by dietary restriction in both young and aged

mice (Fig. 3). Dietary restriction resulted in the accumulation of IgG-positive cells and deposition of IgG molecules on the extracellular space in the thymus of aged, but not young, mice (Fig. 4). The expression of prostanoid-synthesizing enzymes strongly increased in the young thymus, but mostly not in the aged thymus (Fig. 5B). Hence, young and aged thymuses respond differently to nutritional deficiency.

The thymic cortex and medulla have different functional roles and characteristics. Therefore, an appropriate segregation of these compartments is functionally important for the thymus. The 48-h dietary restriction resulted in disorganization of the corticomedullary boundary, which was not apparent in the aged thymus in our analysis (Fig. 3). However, Gui *et al.* showed that the corticomedullary boundary was disorganized in the thymus of 12-month-old mice.[15] Because the size reduction was apparent as early as 16 weeks of age (Fig. 1), the disorganization of the corticomedullary boundary may not be directly related to the reduction of thymic size. The corticomedullary boundary is maintained by cortical and medullary thymic epithelial cells (cTECs and mTECs, respectively) through Eph signaling.[16] In the thymus of 24-month-old mice with disorganization of the corticomedullary boundary, the numbers of cTECs and mTECs are reduced and the cTEC:mTEC ratio is disturbed.[17] Although we did not observe an apparent reduction of keratin 5 immunoreactivity by acute nutritional deficiency and week-level aging (Fig. 3), the numbers and ratio of cTECs and mTECs after these stresses must be investigated in future study.

In contrast to the effect on the corticomedullary boundary, the dietary restriction–induced accumulation of IgG-positive signals depended on aging (Fig. 4). Aging is associated with chronic mild inflammation.[2] In the human aged thymus, the levels of some inflammatory mediators, such as leukemia inhibitory factor, oncostatin M, stem cell factor, macrophage colony-stimulating factor, and IL-6, are elevated.[18] Our study showed a higher *Ifng* mRNA expression in the thymus of normally fed aged mice than in the thymus of normally fed young mice (Fig. 5, Table 2). The elevated expression of interferon-γ by aging may trigger the basal activation and/or infiltration of IgG- and IgM-positive B lymphocytes under static conditions (Fig. 4). Although dietary restriction

exerted no effect on the expression level of *Ifng* by itself in the aged thymus, the combination of higher *Ifng* expression with some histochemical changes in thymic properties caused by dietary restriction, such as the disorganization of the corticomedullary boundary, may result in the accumulation of IgG-positive cells and deposition of IgG molecules. Further studies are required to reveal the molecular mechanisms underlying age-dependent IgG-positive cell activation/recruitment.

Differential Regulation of Prostanoid Synthesis and Their Roles

Our previous study indicated that prostaglandin D_2 (PGD_2) and thromboxane A_2 (TxA_2) were effectively induced by the 48-h dietary restriction.[9] The results of mRNA expression in the present study also support this finding, as the mRNA expressions of *Ptgds*, *Hpgds*, *Ptges1*, *Akr1b7*, and *Tbxas1* were upregulated after dietary restriction (Fig. 5B). The upregulation of the expressions of *Hpgds*, *Ptges1*, and *Tbxas1* was attenuated in the aged thymus. In contrast, dietary restriction resulted in the accumulation of IgG-positive cells and enhanced the secretion and deposition of IgG molecules in the aged thymus (Fig. 4). These findings suggest that prostanoid production after nutritional deficiency plays different roles than the induction of mild inflammation. The synthesis of prostanoids by aging was implicated in cell culture experiments. The induction of prostaglandin E_2, prostaglandin $F_2\alpha$, prostacyclin (PGI_2), and TxA_2 in response to angiotensin, thrombin, or bradykinin decreased upon senescence in human embryo lung fibroblasts.[19] In contrast, prostanoid production by epinephrine enhanced in the aged muscles of rhesus monkeys. Hence, the changes in prostanoid responses are different among tissues. Interestingly, another study also reported the upregulation of the mRNAs of prostanoid-synthesizing enzymes in the ovine thymus during early pregnancy.[20] As the thymus is involuted during pregnancy, the functional significance of prostanoids may be common to the thymic involution by various biological stresses, which is unrelated to the induction of thymic inflammation.

Effects of Nutritional Deficiency in the Aged Thymus

Our results indicated that nutritional deficiency still induced histological changes in the aged thymus, although the size change caused by nutritional deficiency was not apparent due to its smaller size upon aging. We found two major histological changes in the nutritional deficient aged thymus, viz., the disruption of the corticomedullary boundary and the accumulation of IgG-positive cells. The corticomedullary boundary is essential for the segregation of cortical and medullary compartments with different developmental stages of T lymphocytes. Its disruption causes the failure of central tolerance and the development of autoimmune phenomena.[21,22)]

IgG is normally synthesized by mature B lymphocytes. The thymus contains a unique population of B lymphocytes, thymic B cells, which have a distinct origin and phenotype from those of peripheral B cells.[23)] They reside at the corticomedullary boundary and function as antigen-presenting cells.[23)] In contrast, peripheral B lymphocytes can be recruited into the thymus upon pathological situation.[24)] Future studies would clarify which population of B lymphocytes appear in the nutritional deficient aged thymus. The physiological consequences of the increased appearance of IgG-positive cells and accumulation of IgG molecules in the thymus still remain unclear. The thymus continuously secretes thymic humoral factors that are important for the maintenance of peripheral T lymphocytes, whose numbers could decrease upon IgG-involved inflammation.[25)] Interestingly, it has been proposed that the thymic humoral factors affect the biological aging of the entire body through the regulation of self-destructive and nonprotective thymic activities.[26)] Thus, an enhanced IgG-related inflammatory state in the aged thymus after nutritional deficiency could affect the quality of life. It is also important to determine the mechanisms that are responsible for the recruitment of IgG-positive cells specifically in the aged thymus. Prostanoids might not be critical for this process (Fig. 5). Studies have indicated that aging is related to increased expression of some CC and CXC chemokines,[27-29)] which should be examined in future studies.

Consequently, the combination of the disruption of the corticomedullary boundary and enhanced IgG-related inflammation in the aged thymus revealed in this study may be a cause of the severe

reduction of immune capacity in elderly individuals in an aged society.

ACKNOWLEDGMENTS

We thank Dr. Koji Teramoto, Laboratory of Hygienic Sciences, Kobe Pharmaceutical University, for his kind help for animal maintenance. This work was supported by the Kobe Pharmaceutical University President's Discretionary Expenses.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. Bueno V, San'Anna OA, Lord JM. Ageing and myeloid-derived suppressor cells: possible involvement in immunosenescence and age-related disease. *Age*, **2014**, *36*, 9729. https://doi.org/10.1007/s11357-014-9729-x.

2. Campos C, Pera A, Lopez-Fernandez I, Alonso C, Tarazona R, Solana R. Proinflammatory status influences NK cells subsets in the elderly. *Immunol. Lett.*, **2014**, *162*, 298-302. https://doi.org/10.1016/j.imlet.2014.06.015.

3. Sempowski G, Thomasch J, Gooding M, Hale L, Edwards L, Ciafaloni E, Sanders D, Massey J, Douek D, Koup R, Haynes B. Effect of thymectomy on human peripheral blood T cell pools in myasthenia gravis. *J. Immunol.*, **2001**, *166*, 2808-2017. https://doi.org/10.4049/jimmunol.166.4.2808.

4. van Gent R, Schadenberg AWL, Otto SA, Nievelstein RAJ, Sieswerda GT, Haas F, Miedema F, Tesselaar K, Jansen NJG, Borghans JAM. Long-term restoration of the human T-cell compartment after thymectomy during infancy: a role for thymic regeneration? *Blood*, **2011**, *118*, 627–634. https://doi.org/10.1182/blood-2011-03-341396.

5. Douek DC, Vescio RA, Betts MR, Brenchley JM, Hill BJ, Zhang L, Berenson JR, Collins RH, Koup RA. Assessment of thymic output in adults after haematopoietic stem-cell transplantation and prediction of T-cell reconstitution. *Lancet*, **2000**, *355*, 1875–1881. https://doi.org/10.1016/S0140-6736(00)02293-5.

6. Poulin JF, Viswanathan MN, Harris JM, Komanduri KV, Wieder E, Ringuette N, Jenkins M, McCune JM, Sékaly RP. Direct evidence for thymic function in adult humans. *J. Exp. Med.,* **1999**, *190*, 479–486. https://doi.org/10.1084/jem.190.4.479.

7. Lynch HE, Goldberg GL, Chidgey A, Van den Brink MRM, Boyd R, Sempowski GD. Thymic involution and immune reconstitution. *Trends Immunol.*, **2009**, *30*, 366-373. https://doi.org/10.1016/j.it.2009.04.003.

8. Ye P, Kirschner DE, Kourtis AP. The thymus during HIV disease: role in pathogenesis and in immune recovery. *Curr. HIV Res.,* **2004**, *2*, 177–183. https://doi.org/10.2174/1570162043484898.

9. Razali N, Hohjoh H, Inazumi T, Maharjan BD, Nakagawa K, Konishi M, Sugimoto Y, Hasegawa H. Induced prostanoid synthesis regulates the balance between Th1- and Th2-producing inflammatory cytokines in the thymus of diet-restricted mice. *Biol. Pharm. Bull.*, **2020**, *43*, 649-662.

https://doi.org/10.1248/bpb.b19-00838.

10. Kondo M, Okazaki H, Nakayama K, Hohjoh H, Nakagawa K, Segi-Nishida E, Hasegawa H. Characterization of astrocytes in the minocycline-administered mouse photothrombotic ischemic stroke model. *Neurochem. Res.*, **2022**, *47*, 2839-2855. https://doi.org/10.1007/s11064-022-03703-z.

11. Razali N, Horikawa I, Hohjoh H, Yoshikawa C, Hasegawa H. Prostaglandin-modulated interaction of thymic progenitor cells with blood vessels during estradiol-induced thymic involution. *BPB Rep.*, **2019**, *2*, 39-47. https://doi.org/10.1248/bpbreports.2.4_39.

12. Houtkooper RH, Argmann C, Houten SM, Cantó C, Jeninga EH, Andreux PA, Thomas C, Doenlen R, Schoonjans K, Auwerx J. The metabolic footprint of aging in mice. *Sci. Rep.*, **2011**, *1*, 134. https://doi.org/10.1038/srep00134.

13. Nakayama K, Yoshida T, Nakayama Y, Iguchi N, Namba Y, Konishi M, Hasegawa H. Activation of macrophages mediates dietary restriction-induced splenic involution. *Life Sci.*, **2022**, *310*, 121068. https://doi.org/10.1016/j.lfs.2022.121068.

14. Hasegawa H, Nakayama K. Malnutrition-induced involution of lymph nodes in mice. *BPB Rep.*, **2022**, *5*, 133-139. https://doi.org/10.1248/bpbreports.5.6_133.

15. Gui J, Zhu X, Dohkan J, Cheng L, Barnes PF, Su D.-M. The aged thymus shows normal recruitment of lymphohematopoietic progenitors but has defects in thymic epithelial cells. *Int. Immunol.*, **2007**, *19*, 1201–1211. https://doi.org/10.1093/intimm/dxm095.

16. García-Ceca J, Jiménez E, Alfaro D, Cejalvo T, Chumley MJ, Henkemeyer M, Muñoz J-J, Zapata AG. On the role of Eph signalling in thymus histogenesis; EphB2/B3 and the organizing of the thymic epithelial network. *Int. J. Dev. Biol.*, **2009**, *53*, 971-982. https://doi.org/10.1387/ijdb.082702jg.

17. Chinn IK, Blackburn CC, Manley NR, Sempowski GD. Changes in primary lymphoid organs with aging. *Semin. Immunol.*, **2012**, *24*, 309-320. https://doi.org/10.1016/j.smim.2012.04.005.

18. Sempowski GD, Hale LP, Sundy JS, Massey JM, Koup RA, Douek DC, Patel DD, Haynes BF. Leukemia inhibitory factor, oncostatin M, IL-6, and stem cell factor mRNA expression in human thymus increases with age and is associated with thymic atrophy. *J. Immunol.*, **2000**, *164*, 2180-

2187.
https://doi.org/10.4049/jimmunol.164.4.2180.

19. Taylor L, Schneider E, Smith J, Polgar P. Prostaglandin production and cellular aging. *Mech. Ageing Dev.*, **1981**, *16*, 311-317.
https://doi.org/10.1016/0047-6374(81)90015-4.

20. Yang L, Lv W, Liu Y, Chen K, Xue J, Wang Q, Wang B, Zhang L. Effect of early pregnancy on the expression of prostaglandin synthases in the ovine thymus. *Theriogenology*, **2019**, *136*, 166-171.
https://doi.org/10.1016/j.theriogenology.2019.06.040.

21. Ueno T, Saito F, Gray DH, Kuse S, Hieshima K, Nakano H, Kakiuchi T, Lipp M, Boyd RL, Takahama Y. CCR7 signals are essential for cortex-medulla migration of developing thymocytes. *J. Exp. Med.*, **2004**, *200*, 493–505.
https://doi.org/10.1084/jem.20040643.

22. Kurobe H, Liu C, Ueno T, Saito F, Ohigashi I, Seach N, Arakaki R, Hayashi Y, Kitagawa T, Lipp M, Boyd RL, Takahama Y. CCR7-dependent cortex-to-medulla migration of positively selected thymocytes is essential for establishing central tolerance. *Immunity*, **2006**, *24*, 165–177.
https://doi.org/10.1016/j.immuni.2005.12.011.

23. Perera1 J, Huang H. The development and function of thymic B cells. *Cell. Mol. Life Sci.*, **2015**, *72*, 2657–2663.
https://doi.org/10.1007/s00018-015-1895-1.

24. Hodge DL, Reynolds D, Cerbán FM, Correa SG, Baez NS, Young HA, Rodriguez-Galan MC. MCP-1/CCR2 interactions direct migration of peripheral B and T lymphocytes to the thymus during acute infectious/inflammatory processes. *Eur. J. Immunol.*, **2012**, *42*, 2644-2654.
https://doi.org/10.1002/eji.201242408.

25. Palmer DB. The effect of age on thymic function. *Front. Immunol.*, **2013**, *4*, 316.
https://doi.org/10.3389/fimmu.2013.00316.

26. Csaba G. The immunoendocrine thymus as a pacemaker of lifespan. *Acta Microbiol. Immunol. Hung.*, **2016**, *63*, 139-158.
https://doi.org/10.1556/030.63.2016.2.1.

27. Anthony D, Dempster R, Fearn S, Clements J, Wells G, Perry VH, Walker K. CXC chemokines generate age-related increases in neutrophil-mediated brain inflammation and blood–brain barrier breakdown. *Curr. Biol.*, **1998**, *8*, 923–926.
https://doi.org/10.1016/S0960-9822(07)00373-9.

28. Felzien LK, McDonald JT, Gleason SM, Berman NEJ, Klein RM. Increased chemokine gene

expression during aging in the murine brain. *Brain Res.*, **2001**, *890*, 137-146. https://doi.org/10.1016/S0006-8993(00)03090-0.

29. Chen J, Mo R, Lescure PA, Misek DE, Hanash S, Rochford R, Hobbs M, Yung RL. Aging is associated with increased T-cell chemokine expression in C57Bl/6 mice. *J. Gerontol. A: Biol. Sci. Med. Sci.*, **2003**, *58*, B975–B983. https://doi.org/10.1093/gerona/58.11.B975.

Table1. Results of two-way factorial ANOVA, related to Figure 1 and 2

	$p_{nutritional\ condition}$	p_{aging}	$p_{nutritional\ condition \times aging}$
Change in body weight (Figure 1C)	0.000433 ($F_{1,43}$=14.542)	0.241180 ($F_{1,43}$=1.412)	0.428285 ($F_{1,43}$=0.640)
Thymic weight (Figure 1D)	0.000141 ($F_{1,43}$=17.458)	1.42×10^{-13} ($F_{1,43}$=112.417)	0.003629 ($F_{1,43}$=9.469)
Relative thymic weight (Figure 1E)	0.0056 ($F_{1,43}$= 8.508)	8.51×10^{-16} ($F_{1,43}$= 153.83)	0.0219 ($F_{1,43}$= 5.653)
Relative liver weight (Figure 2A)	3.26×10^{-7} ($F_{1,19}$= 58.472)	7.77×10^{-7} ($F_{1,19}$= 51.793)	0.386 ($F_{1,19}$= 0.786)
Relative kidney weight (Figure 2B)	0.082778 ($F_{1,19}$= 3.354)	0.000266 ($F_{1,19}$= 19.921)	0.113077 ($F_{1,19}$= 2.760)
Relative heart weight (Figure 2C)	0.55095 ($F_{1,19}$= 0.369)	0.00137 ($F_{1,19}$= 14.019)	0.39031 ($F_{1,19}$= 0.773)
Relative testis weight (Figure 2D)	0.102 ($F_{1,19}$= 2.954)	0.114 ($F_{1,19}$= 2.739)	0.837 ($F_{1,19}$= 0.044)
Relative epididymal fat weight (Figure 2E)	0.53897 ($F_{1,19}$= 0.391)	0.00048 ($F_{1,19}$= 17.680)	0.15659 ($F_{1,19}$= 2.176)

Table2. Results of two-way factorial ANOVA, related to Figure 4 and 5

	$p_{nutritional\ condition}$	p_{aging}	$p_{nutritional\ condition \times aging}$
Density of IgG^+ cells (Figure 4K)	0.03326 ($F_{1,12}$=5.781)	0.00362 ($F_{1,12}$=12.991)	0.01744 ($F_{1,12}$=7.591)
Relative intensity of IgG (Figure 4L)	0.1157 ($F_{1,12}$=2.875)	0.0486 ($F_{1,12}$=4.818)	0.1093 ($F_{1,12}$=2.991)
Density of IgM^+ cells (Figure 4M)	0.7806 ($F_{1,12}$=0.081)	0.0281 ($F_{1,12}$=6.232)	0.9108 ($F_{1,12}$=0.013)
Relative intensity of IgM (Figure 4N)	0.623 ($F_{1,12}$=0.254)	0.211 ($F_{1,12}$=1.747)	0.987 ($F_{1,12}$=0.000)
Il5 expression (Figure 5A)	0.016 ($F_{1,20}$=6.919)	0.533 ($F_{1,20}$=0.402)	0.382 ($F_{1,20}$=0.800)
Il6 expression (Figure 5A)	0.00676 ($F_{1,20}$=9.124)	0.85473 ($F_{1,20}$=0.034)	0.16723 ($F_{1,20}$=2.054)
Il10 expression (Figure 5A)	0.0741 ($F_{1,20}$=3.553)	0.3800 ($F_{1,20}$=0.806)	0.2349 ($F_{1,20}$=1.500)
Ifng expression (Figure 5A)	0.319142 ($F_{1,20}$=1.044)	0.000919 ($F_{1,20}$=15.098)	0.954738 ($F_{1,20}$=0.003)
Cox1 expression (Figure 5B)	0.00764 ($F_{1,20}$=8.796)	0.14163 ($F_{1,20}$=2.342)	0.00542 ($F_{1,20}$=9.721)
Cox2 expression (Figure 5B)	0.0152 ($F_{1,20}$=7.045)	0.1040 ($F_{1,20}$=2.900)	0.5800 ($F_{1,20}$=0.316)
Ptgds expression (Figure 5B)	0.0123 ($F_{1,20}$=7.574)	0.7196 ($F_{1,20}$=0.133)	0.1505 ($F_{1,20}$=2.235)
Hpgds expression (Figure 5B)	0.00389 ($F_{1,20}$=10.649)	0.07683 ($F_{1,20}$=3.481)	0.00489 ($F_{1,20}$=10.005)
Ptges1 expression (Figure 5B)	0.00343 ($F_{1,20}$=11.008)	0.61432 ($F_{1,20}$=0.262)	0.03838 ($F_{1,20}$=4.915)
Akr1b7 expression (Figure 5B)	0.000346 ($F_{1,20}$=18.521)	0.156299 ($F_{1,20}$=2.170)	0.155322 ($F_{1,20}$=2.181)
Ptgis expression (Figure 5B)	0.6538 ($F_{1,20}$=0.207)	0.0783 ($F_{1,20}$=3.444)	0.6960 ($F_{1,20}$=0.157)
Tbxas1 expression (Figure 5B)	0.000287 ($F_{1,20}$=19.212)	0.007995 ($F_{1,20}$=8.677)	0.001137 ($F_{1,20}$=14.399)

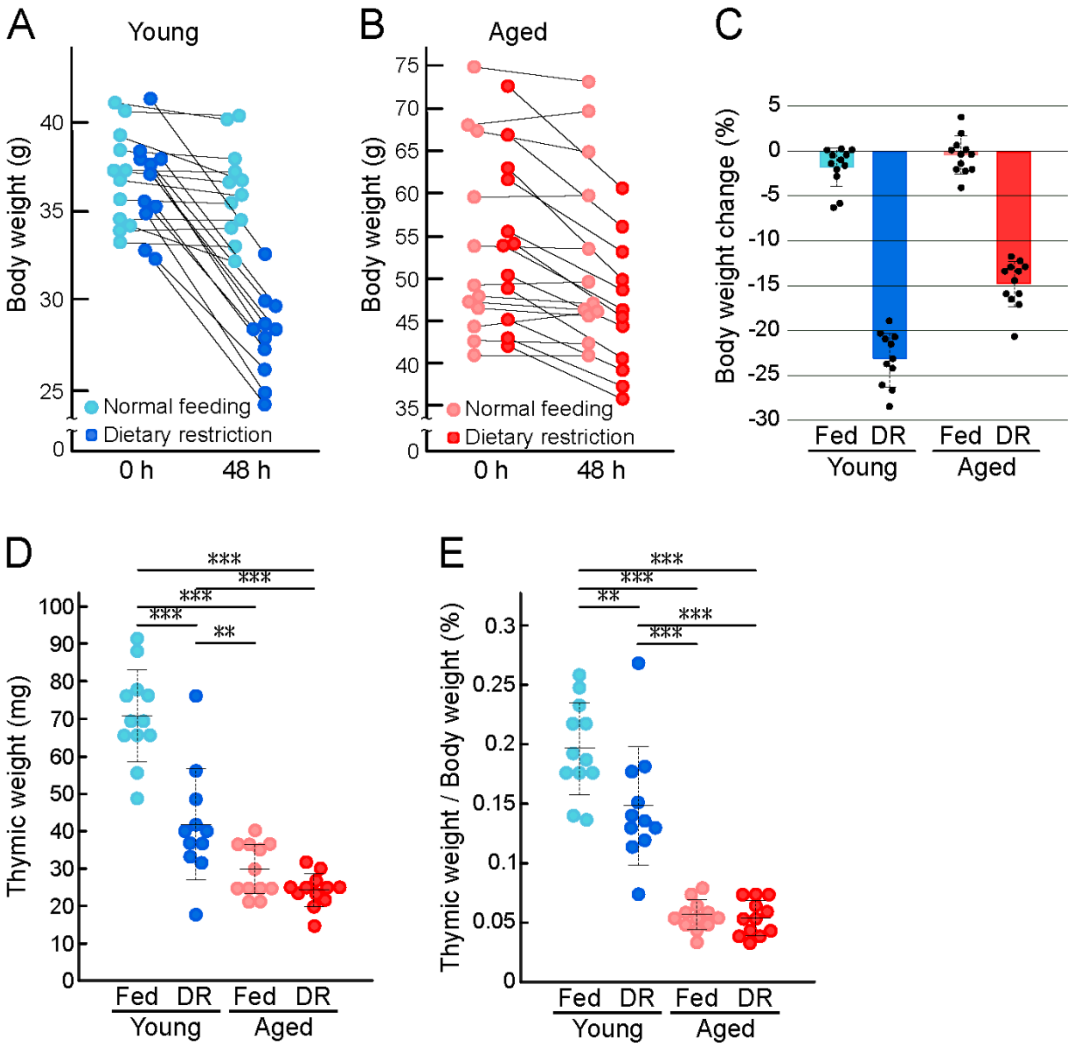
A
Young
Body weight (g)
40
35
30
25
0
Normal feeding
Dietary restriction
0 h
48 h
B
Aged
Body weight (g)
75
70
65
60
55
50
45
40
35
0
Normal feeding
Dietary restriction
0 h
48 h
C
Body weight change (%)
5
0
-5
-10
-15
-20
-25
-30
Fed DR
Young
Fed DR
Aged
D
Thymic weight (mg)
100
90
80
70
60
50
40
30
20
10
0
***
***
***
***
**
Fed DR
Young
Fed DR
Aged
E
Thymic weight / Body weight (%)
0.3
0.25
0.2
0.15
0.1
0.05
0
***
***
**
***
***
Fed DR
Young
Fed DR
Aged

Figure 1. Changes in body and thymic weights by dietary restriction in young and aged mice

A–C: Body weights of young (A, blue) and aged (B, red) mice were examined before and after the 48-h dietary restriction. The change in body weight by dietary restriction is expressed as the percentage of the initial body weight (C). **D, E**: The change in thymic weight. The absolute weight of the thymus (D) and the relative weight to body weight (E) are presented. Fed, normally fed; DR, dietary-restricted. Error bars indicate standard deviation. Each circle indicates an individual animal. The statistical significance of average values among groups was calculated as described in MATERIALS and METHODS. $^{**}p < 0.01$, $^{***}p < 0.001$.

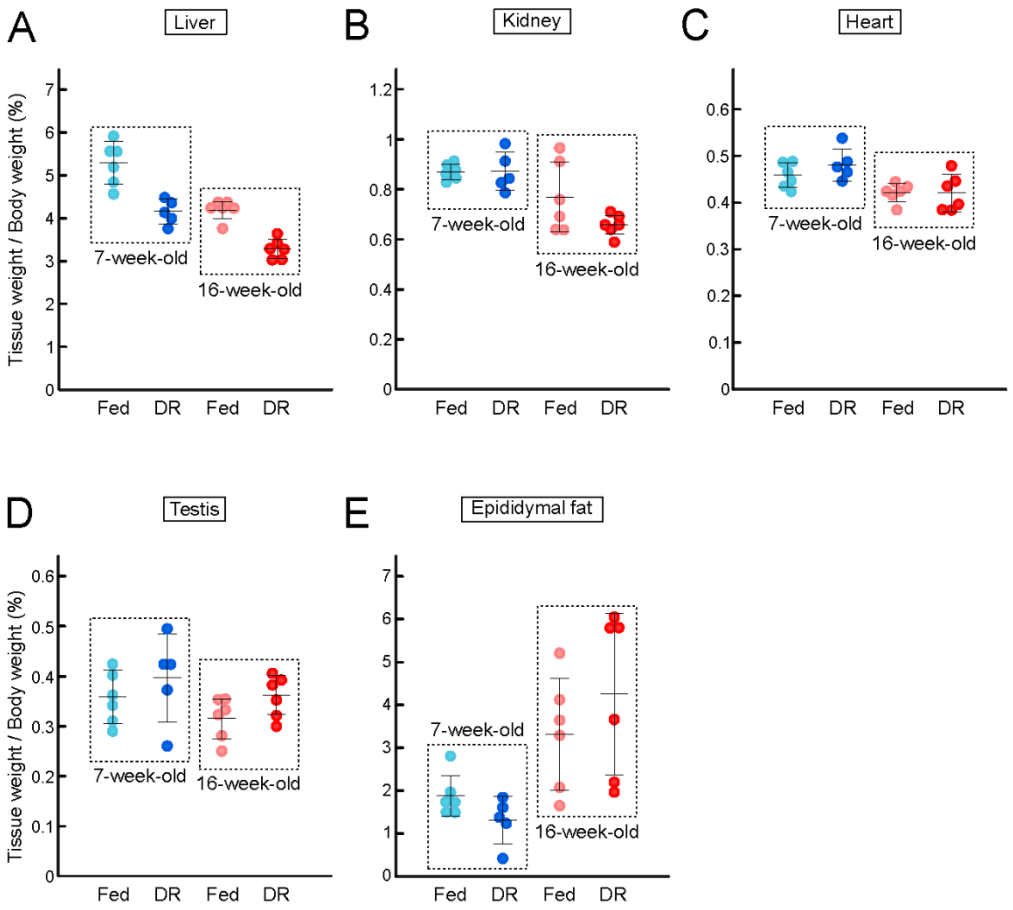
A
Liver
Tissue weight / Body weight (%)
7-week-old
16-week-old
Fed
DR
Fed
DR
B
Kidney
7-week-old
16-week-old
C
Heart
7-week-old
16-week-old
D
Testis
E
Epididymal fat

Figure 2. The weight of organs other than the thymus

The weight of the liver (A), kidney (B), heart (C), testis (D), and epididymal fat (E) relative to the body weight of young and aged mice before and after the 48-h dietary restriction is presented. Each circle indicates an individual animal. Error bars indicate standard deviation. The statistical significance of average values among groups was calculated as described in MATERIALS and METHODS.

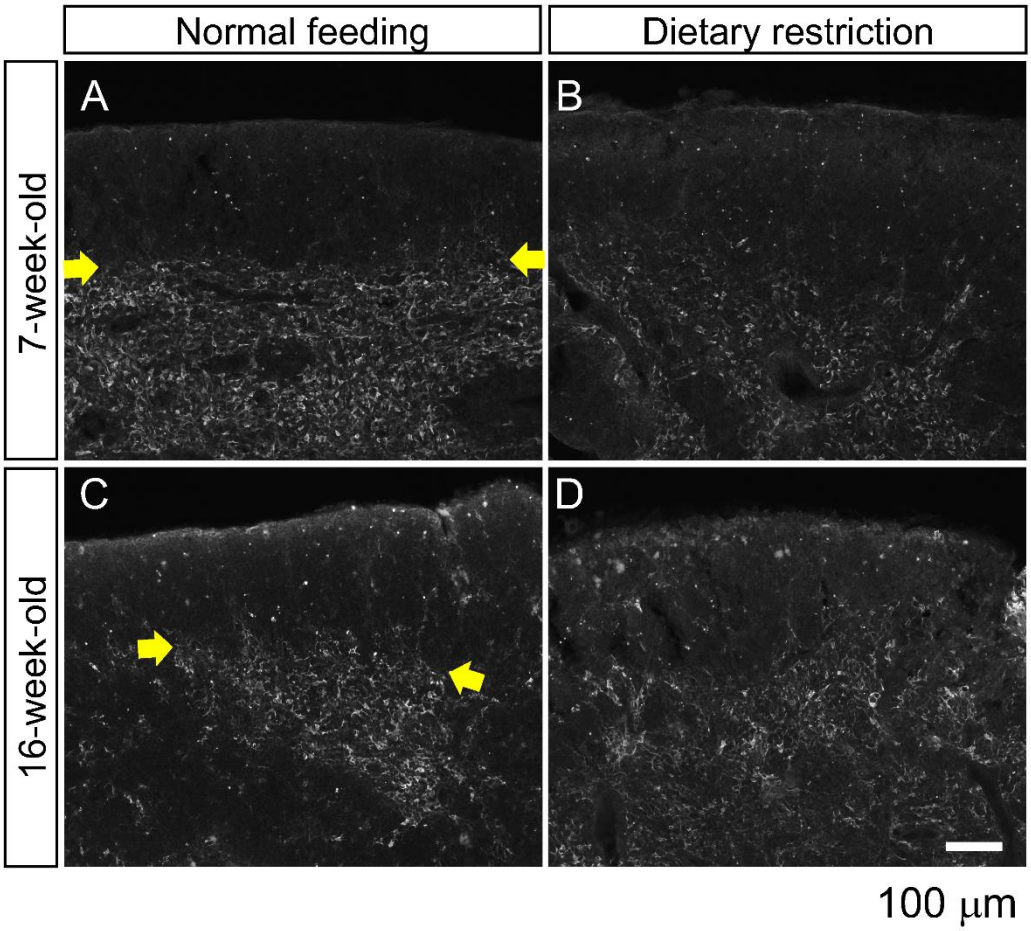
Normal feeding
Dietary restriction
7-week-old
16-week-old
A
B
C
D
100 μm

Figure 3. Disorganization of the corticomedullary boundary by dietary restriction

The thymus sections of young (A, B) and aged (C, D) mice with normal feeding (A, C) and 48-h dietary restriction (B, D) were immunostained with anti-keratin 5 antibody. The yellow arrows indicate the boundary between the cortex and medulla. Scale bar: 100 μm.

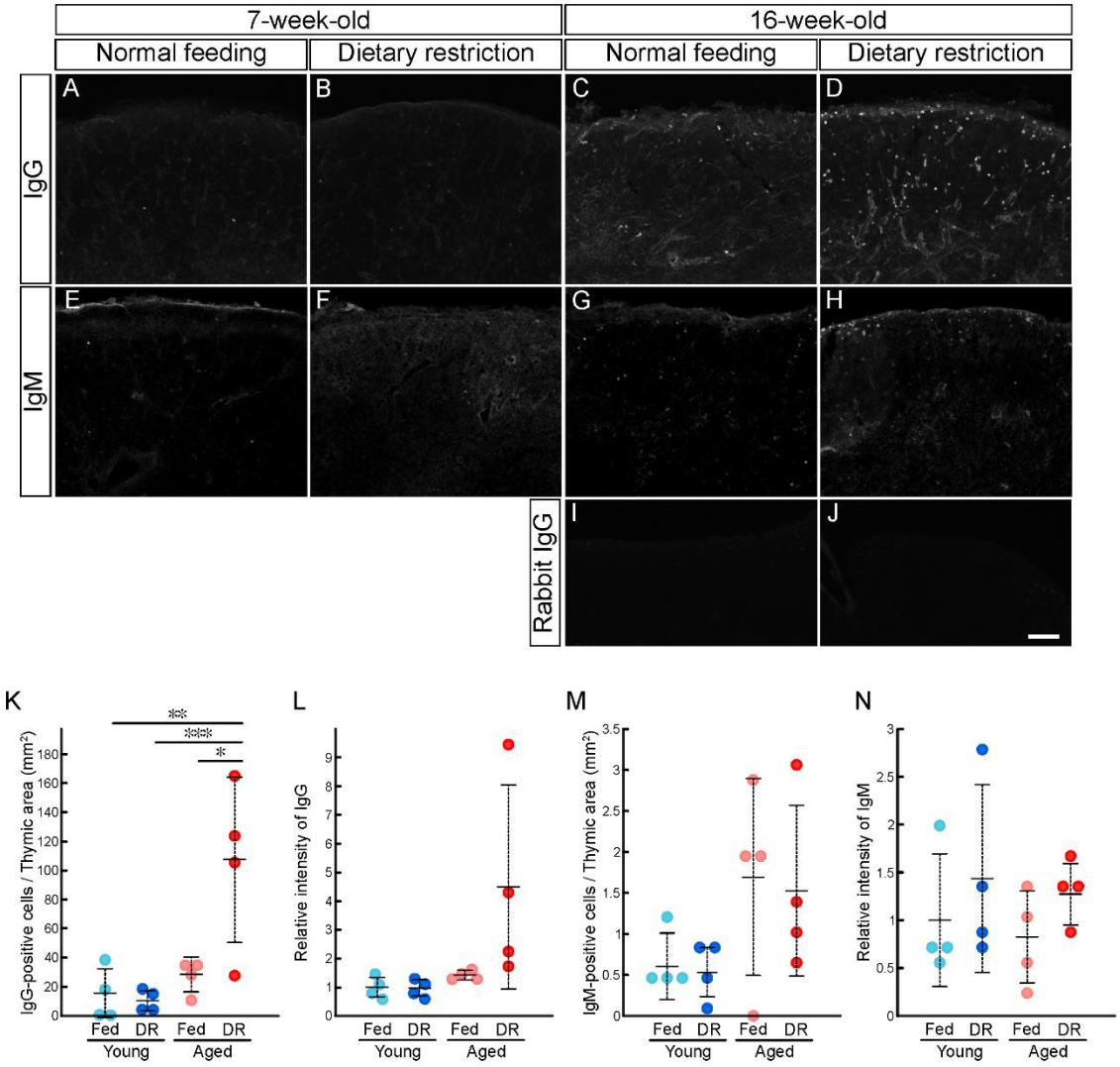
7-week-old
16-week-old
Normal feeding
Dietary restriction
Normal feeding
Dietary restriction
IgG
IgM
Rabbit IgG
A
B
C
D
E
F
G
H
I
J
K
L
M
N
IgG-positive cells / Thymic area (mm²)
Relative intensity of IgG
IgM-positive cells / Thymic area (mm²)
Relative intensity of IgM
Fed
DR
Fed
DR
Young
Aged

Figure 4. Infiltration of IgG-positive cells in an age-dependent manner

A–F: The thymus sections of young (A, B, E, F) and aged (C, D, G, H, I, J) mice with normal feeding (A, C, E, G, I) and 48-h dietary restriction (B, D, F, H, J) were immunostained with anti-mouse IgG antibody (A–D), anti-mouse IgM (E–H), or anti-rabbit IgG (I, J). The number of IgG-(K) or IgM-(M) positive cells per area was counted. The relative background intensity of IgG (L) and IgM (N) was quantified using ImageJ. Each circle indicates an individual animal. Error bars indicate standard deviation. The statistical significance of average values among groups was calculated as described in MATERIALS and METHODS. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Scale bar: 100 μm.

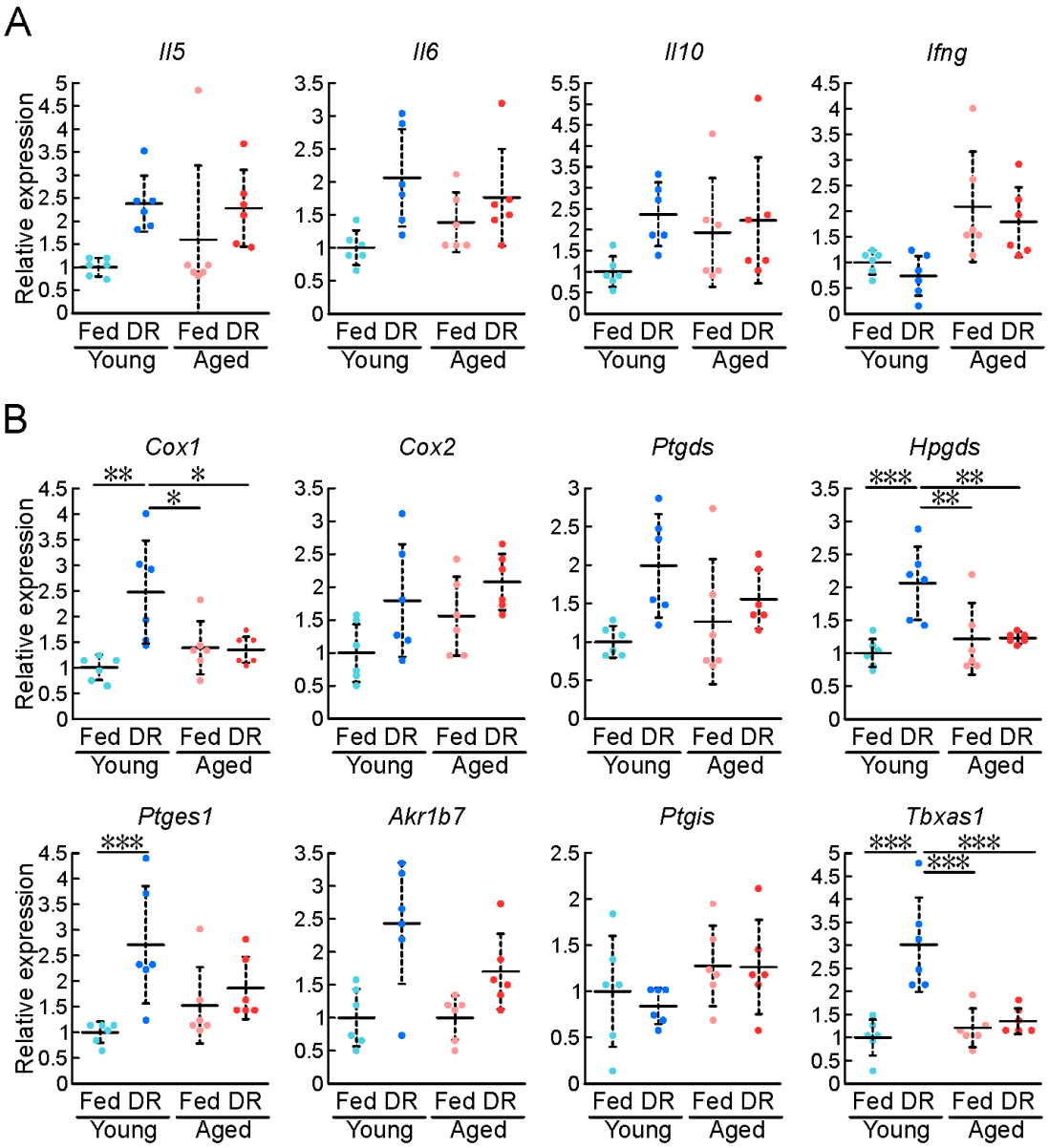
A
Il5
Il6
Il10
Ifng
Relative expression
Fed DR
Young
Aged
B
Cox1
Cox2
Ptgds
Hpgds
Ptges1
Akr1b7
Ptgis
Tbxas1
*
**
***

Figure 5. Characterization of the involuted thymus by mRNA expression

A: The relative expression of mRNAs encoding Th subtype marker cytokines, IL-5 (*Il5*), IL-6 (*Il6*), IL-10 (*Il10*), and interferon-γ (*Ifng*), (A) or prostanoid-synthesizing enzymes, COX1 (*Cox1*), COX2 (*Cox2*), prostaglandin D synthases (*Ptdgs*, *Hpgds*), prostaglandin E synthase (*Ptges1*), prostaglandin F synthase (*Akr1b7*), prostacyclin synthase (*Ptgis*), and thromboxane synthase (*Tbxas1*), (B) in the thymus of young and aged mice with normal feeding or 48-h dietary restriction. Each circle indicates an individual animal. Error bars indicate standard deviation. The statistical significance of average values among groups was calculated as described in MATERIALS and METHODS. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.