Early-life nutrition influences thymic growth in male mice that may be related to the regulation of longevity

Jian-Hua CHEN*, Jane L. TARRY-ADKINS*, Chantal A.A. HEPPOLETTE*, Donald B. PALMER† and Susan E. OZANNE*
*University of Cambridge Metabolic Research Laboratories, Institute of Metabolic Science, University of Cambridge, Cambridge CB2 0QQ, U.K., and †Infection and Immunity and Genes and Development Group, Department of Veterinary Basic Sciences, Royal Veterinary College, London NW1 OTU, U.K.

ABSTRACT

Nutrition and growth rate during early life can influence later health and lifespan. We have demonstrated previously that low birthweight, resulting from maternal protein restriction during pregnancy followed by catch-up growth in rodents, was associated with shortened lifespan, whereas protein restriction and slow growth during lactation increased lifespan. The underlying mechanisms by which these differences arise are unknown. In the present study, we report that maternal protein restriction in mice influences thymic growth in early adult life. Offspring of dams fed a low-protein diet during lactation (PLP offspring) had significant thymic growth from 21 days to 12 weeks of age, whereas this was not observed in control mice or offspring of dams fed a low-protein diet during pregnancy (recuperated offspring). PCNA (proliferating-cell nuclear antigen) and SIRT1 (silent information regulator 1) protein levels at 21 days of age were significantly higher in the thymus from both PLP mice (P < 0.001 and P < 0.05 respectively) and recuperated mice (P < 0.001 and P < 0.01 respectively) compared with controls. At 12 weeks, PLP mice maintained a higher SIRT1 level, whereas PCNA was decreased in the thymus from recuperated offspring. This suggests that mitotic activity was initially enhanced in the thymus from both PLP and recuperated offspring, but remained sustained into adulthood only in PLP mice. The differential mitotic activity in the thymus from PLP and recuperated mice appeared to be influenced by changes in sex hormone concentrations and the expression of p53, p16, the androgen receptor, IL-7 (interleukin-7) and the IL-7 receptor. In conclusion, differential thymic growth may contribute to the regulation of longevity by maternal diet.

INTRODUCTION

It is well known that early growth patterns can have profound long-term effects on later health. Epidemiological studies first demonstrated a close link between low birthweight and later adult cardiovascular disease [1]. Subsequent studies established further that poor fetal growth is associated with later risk of adult diseases, including cardiovascular disease, the metabolic syndrome and Type 2 diabetes [2]. This led to the

Key words: developmental programming, immunosenescence, lifespan, maternal diet, thymic growth, thymic involution.

Abbreviations: AR, androgen receptor; ER, oestrogen receptor; GPR30, guanine-protein-coupled receptor 30; IL-7, interleukin-7; IL-7R, IL-7 receptor; PCNA, proliferating cell nuclear antigen; PLP offspring, postnatal low-protein offspring (offspring of a dam fed the control diet and nursed by a dam fed the low-protein diet); recuperated offspring, offspring of a dam fed the low-protein diet and nursed by a dam fed the control diet; RT-PCR, real-time PCR; Rwdd1, Rwd-domain-containing 1; SIRT1, silent information regulator 1; TGF, transforming growth factor; TGFβRII, TGF-β type II receptor.

Correspondence: Dr Jian-Hua Chen (email jhc36@cam.ac.uk).
The concept of the fetal/early origins of adult disease, which is now widely known as developmental origins of health and disease [3]. The developmental origins hypothesis proposes that, during critical periods of fetal and postnatal mammalian development, nutrition and other environmental factors induce adaptive responses. These lead to permanent changes in metabolism, hormone production and tissue sensitivity to hormones that may affect development of various organs, resulting in long-term alteration of physiology and metabolism, and ultimately adult disease susceptibility [4].

Animal models that allow manipulation of fetal and postnatal growth rates not only supported the developmental origins hypothesis, but also provided insight into the underlying mechanisms by which early growth affects later adult health [4,5]. Using rodent models of maternal protein restriction, we have shown that a poor early nutritional environment can lead to the development of insulin resistance and diabetes in later adult life [6]. We have also demonstrated that maternal protein restriction can ultimately affect longevity. Low birthweight resulting from maternal protein restriction during pregnancy followed by postnatal catch-up growth (recovered animals) was associated with shortened lifespan [7,8]. Conversely, protein restriction and slow growth during lactation (postnatal low-protein animals) increased lifespan [7,8].

The immune system is essential in defending the body against attacks by foreign organisms and pathogens, and thus is vital for health and longevity. The development of the immune system involves a complex series of events that occurs during fetal and early postnatal periods of mammalian growth. As fetal and early postnatal life is a critical time for development of a functional immune system [9], it may be sensitive to maternal diet during pregnancy and lactation [10]. Indeed, human studies have suggested that the early nutritional environment and growth patterns can have an impact on long-term immune function [11,12].

The thymus is the principal organ which is responsible for the generation of circulating T-lymphocytes and, hence, plays an important role in cellular immunity. It is well known that the thymus regresses with age (age-associated thymic involution), which is characterized by a reduction in thymic mass, loss of tissue structure, abnormal architecture and a decline in thymocyte numbers leading to a reduction in naïve T-cell output [13–15]. It is this decline in the number of T-cells exiting the thymus that is believed to contribute significantly to the reduced response to vaccination and infections, and increased incidence of cancers and autoimmune diseases in advancing age [16–18]. Thus thymic involution can be regarded as one of the leading regulators of aging [19]. Indeed, caloric restriction which extends lifespan in species ranging from worms and flies to rodents can retard immune senescence; possibly by delaying thymic involution [20]. Moreover, a recent study clearly demonstrated that genetically modified long-lived mice were resistant to age-dependent thymic involution and maintained a better immune homoeostasis during postnatal development and aging [21].

We hypothesize that development of the thymus may be influenced by maternal diet and early growth patterns, which could potentially contribute to the regulation of the aging process and ultimately affect lifespan. The aim of the present study was, therefore, to investigate the effects of maternal diet on thymic mass in mice and to explore potential underlying mechanisms. In the present study, we subjected mice to exactly the same maternal protein restriction regime that is known to influence lifespan [8]. We report differential growth of the thymus, and changes in sex steroid hormone concentrations and expression of genes that are involved in thymic growth and involution during early adult life in our maternal-protein-restriction mouse models.

**MATERIALS AND METHODS**

**Animals**

All animal procedures were carried out in compliance with the British Home Office Animals Act (1986). Mice (C57/b16) were bred locally at a designated animal unit of the University of Cambridge (Cambridge, U.K.). Adult female mice were housed individually and were maintained at 22°C on a 12:12 h light/dark cycle. They were mated at 6 weeks of age (~22 g in body weight) and assumed to be pregnant when a vaginal plug was expelled. Dams were fed *ad libitum* either a control diet (containing 20% protein) or an isocaloric low-protein diet (containing 8% protein) during gestation and lactation. Both the control and low-protein diet were purchased from Arie Blok, and detailed diet composition has been provided previously [22]. Cross-fostering techniques were used 2 days after birth to establish three groups: (i) control (offspring born to and suckled by dams fed the control diet), (ii) PLP (postnatal low protein; offspring of dams fed the control diet and nursed by dams fed the low-protein diet), and (iii) recuperated (offspring of dams fed the low-protein diet and nursed by dams fed the control diet). A low-protein diet during pregnancy had no statistically significant effect on litter size (6.7 ± 0.4 and 7.4 ± 0.4 pups per litter from dams fed the low-protein and control diets respectively). To maximize the effects of maternal diet differences on offspring growth, recuperated pups were culled to four (to maximize growth during suckling) and control pups were culled to eight (if the number of pups was >8), whereas PLP pups were unculled (to reduce the plane of nutrition). Litter size standardization was carried out randomly. Pups were weaned on to a standard laboratory chow diet at 21 days of age. Animals
were starved overnight before tissue collection. One male was selected at random from each litter at each age (21 days and 12 weeks). Fasting blood was collected following decapitation, allowed to clot for 30 min, and then centrifuged at 1000 g for 6 min to obtain serum. Thymic weights at 21 days and 12 weeks of age were monitored by weighing the tissues from overnight-starved male mice. Tissues were snap frozen in liquid nitrogen and stored at −80°C until analysis.

**Measurements of testosterone and oestradiol**

Owing to limited serum volume from individual animals and serum volume requirements for the assays, the only feasible way to measure testosterone and oestradiol was using pooled samples. We pooled the serum samples with equal aliquots (35 μl) from nine mice in each group. Mouse serum samples were extracted with 10× vol. of ether. Following ether being fully evaporated, the extracted testosterone was reconstituted in assay buffer and assayed in duplicate using Coat-A-Count Total Testosterone RIA kits (Diagnostic Products), according to the manufacturer’s protocols. Oestradiol levels in mouse serum samples were assayed in duplicate using a AutoDELFIA Estradiol kit (PerkinElmer Life Science), as described in the manufacturer’s protocols.

**Western blot analysis**

Total protein was extracted from mouse thymus by homogenization in ice-cold lysis buffer (50 mmol/l Hepes (pH 8), 150 mmol/l NaCl, 1% (v/v) Triton X-100, 1 mmol/l sodium orthovanadate, 30 mmol/l sodium fluoride, 10 mmol/l sodium pyrophosphate, 10 mmol/l EDTA and a protease inhibitor cocktail set III (Calbiochem Novabiochem Biosciences)). The total protein concentration in the lysates was determined using a copper sulfate/bicinchoninic acid assay (Sigma). Samples were diluted to a concentration of 1 mg/ml in Laemmli’s buffer. Total protein (20 μg) was subjected to SDS/PAGE and transferred on to a PVDF immobilon-P membrane (Millipore). Membranes were blocked in Tris-buffered saline [20 mmol/l Tris/HCl (pH 7.6) and 137 mmol/l NaCl] with 0.1% Tween 20 and 5% (w/v) dried milk, and were incubated with the primary antibodies against PCNA (proliferating cell nuclear antigen; ab29-100; Abcam), SIRT1 (silent information regulator 1; #07-131; Millipore) and p53 (MAB1746; R&D Systems). The bound primary antibodies were detected by horseradish peroxidase-conjugated secondary antibodies (Dako), followed by ECL (enhanced chemiluminesence; GE Healthcare). The densities of the bands were quantified using an Alpha Imager (Alpha Innotech). A total of 24 samples were run on a single gel alongside molecular-mass markers, and 10 and 20 μg of one sample were loaded on to each gel to ensure the linearity of the signal. Equal loading was confirmed by Coomassie Blue staining.

**Extraction of total RNA and cDNA synthesis**

Total RNA samples were prepared using TRI reagent (Sigma), purified using RNeasy Mini Kits (Qiagen) with a DNase digestion step included to eliminate potential contaminating DNA and quantified spectrophotometrically on a NanoDrop ND-1000 (Thermo Scientific). First-strand cDNA was reverse-transcribed from 1 μg of total RNA using an ImProm-II Reverse Transcription System (Promega) with oligo(dT)15 as the primer, according to the manufacturer’s protocol.

**Gene expression analysis by RT-PCR (real-time PCR)**

Quantitative RT-PCR was carried out using an ABI PRISM 7900 Sequence Detection System (Applied Biosystems) with a SYBR Green PCR Master Mix (Applied Biosystems) and gene-specific primers. Primers were custom-designed and synthesized by Sigma, and are summarized in Table 1. cDNA template (3 μl; diluted according to the relative expression level of each gene of interest) was used in a 12 μl total reaction volume in each well in a 96-well reaction plate. For every gene analysed in the present study, we performed dissociation curve analysis to ensure that the primers used did not form primer dimers. The transcripts of each gene were amplified in duplicate. Standard curves were constructed using serially diluted pooled cDNA samples from eight control animals. The relative expression levels were

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Primer sequences for the genes investigated in the present study</th>
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<tr>
<td>Gene</td>
<td>Forward primer (5′ to 3′)</td>
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<tr>
<td>--------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>p16</td>
<td>CTTGTGATCTCGGTCGGAAC</td>
</tr>
<tr>
<td>p21</td>
<td>TGGACTCTGTCGCTGAGG</td>
</tr>
<tr>
<td>p27</td>
<td>GCCACGACGTTAACAGCCTC</td>
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<tr>
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</tr>
<tr>
<td>AR</td>
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<td>ERα</td>
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</tr>
<tr>
<td>GPR30</td>
<td>CCAAGCTACGCTCCAGGAGA</td>
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<td>CD8</td>
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<td>Thymosin α1</td>
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<tr>
<td>Thymosin β4</td>
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</tr>
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<td>IL-7</td>
<td>AGGGGCAACAAATCCTGGTG</td>
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<tr>
<td>IL-17</td>
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</tr>
<tr>
<td>Rwd1</td>
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<tr>
<td>TGFβRII</td>
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<tr>
<td>β-Actin</td>
<td>TGCTGACGAGGATGCAAGGAGG</td>
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calculated against each gene’s standard curve with the $C_t$ values of each gene for each animal with the housekeeping gene $\beta$-actin as a loading control. Expression of $\beta$-actin had equal levels between control, PLP and recuperated animals.

**Statistical analysis**

Results were analysed by ANOVA, with maternal diet and age as the independent variables. Duncan’s post-hoc test was used to analyse significant differences between groups where appropriate. A $P < 0.05$ was considered statistically significant.

**RESULTS**

**Thymic weights in young adult mice**

At 21 days of age, the thymic weight of PLP mice was similar to that of controls (Figure 1A). In contrast, thymic weight of 21-day-old recuperated mice was heavier than that of controls ($P = 0.026$; Figure 1A). At 12 weeks of age, thymic weights of both control and recuperated animals showed no change in weight compared with at 21 days of age (Figure 1A), whereas the thymic weight of 12-week-old PLP mice showed a significant increase (Figure 1A; $P < 0.001$). As reported previously [22], PLP mice showed slow growth during lactation and had significantly lower body weights at weaning (21 days old), whereas recuperated mice, although born small, underwent a catch-up growth thus, at weaning, their body weight was significantly higher than controls (Figure 1B). Therefore relative thymus weight (adjusted by body weight) in PLP mice was significantly higher than that of controls, whereas the relative thymus weight in recuperated mice showed no difference compared with controls (Figure 1C). Furthermore, as the body weight of PLP mice remained lighter and recuperated mice remained heavier than controls at 12 weeks of age (Figure 1D), again the thymus from PLP mice had a significantly higher relative organ weight at this age (Figure 1E; $P < 0.01$).

**Protein expression of PCNA, SIRT1 and p53**

PCNA protein expression in the thymus from 21-day-old PLP and recuperated mice was significantly ($P < 0.001$) higher than that in control tissues (Figure 2A). However, at 12 weeks of age, the PCNA expression in the thymus from PLP mice tended to be higher than in controls ($P = 0.07$), whereas the PCNA protein level in the thymus from 12-week-old recuperated animals was significantly lower than controls ($P < 0.05$; Figure 2B). Similar to our findings for PCNA protein levels, SIRT1 protein expression in the thymus from 21-day-old PLP and recuperated mice was also significantly higher than controls (Figure 2C; $P < 0.05$ for PLP mice and $P < 0.01$ for recuperated mice). At 12 weeks of age, SIRT1 protein expression in the thymus from PLP mice remained higher compared with controls ($P < 0.05$); in contrast, SIRT1 protein expression in the thymus from recuperated animals at this age was similar to that of controls (Figure 2D). Examination of the intrathymic level of p53 protein revealed that there was a significantly higher level in the thymus from recuperated mice at both 21 days and 12 weeks of age compared with controls (Figure 2E and 2F). In contrast, there was no significant difference in p53 protein level between the thymus from PLP and control mice at these two time points (Figure 2E and 2F).
Early-life nutrition influences thymic growth

Figure 2 Effects of maternal protein restriction on PCNA, SIRT1 and p53 protein levels in the thymus at 21 days and 12 weeks of age

Concentrations of sex steroid hormones

Although the exact mechanisms that initiate age-associated thymic involution are currently unknown, several studies have shown that sex hormones can influence involution [23]. We therefore determined the serum concentration of testosterone and oestradiol in the three animal groups at both ages. At 21 days of age, all three groups of mice had similar concentrations of testosterone. A marked increase in testosterone concentration was observed in all animals at 12 weeks of age; however, the magnitude of this increase appeared to differ between the three groups: control mice had a 15-fold increase (from 0.4 to 6 nmol/l), PLP mice had a 7.6-fold increase (from 0.5 to 3.8 nmol/l) and recuperated animals had an 8.8-fold increase (from 0.5 to 4.4 nmol/l). Thus testosterone concentration in 12-week-old PLP and recuperated mice was 37 and 27% respectively, lower than that of controls. Measuring serum concentrations of oestradiol in these three animal groups at different ages revealed that the oestradiol concentration in PLP mice at 21 days of age (34 pmol/l) was 20% lower than that of controls (43 pmol/l), whereas the concentration in recuperated animals was similar to those of control mice. However, by 12 weeks of age, the oestradiol concentration was similar between the three animal groups.

Concentrations of sex steroid hormones

The findings described above suggest that maternal nutrition can alter thymic size and cellular division; moreover, given that these animals have a varying lifespan, these observations also imply that the early-life events that affect longevity, which we have identified using this animal model, may also influence the aging of the thymus.

To gain further insight into the possible mechanisms that are likely to have an impact on the aging of the thymus, we examined the mRNA expression levels of various genes that are associated with thymic growth and function [13,23–25] in 21-day-old thymus from all three animal groups.

Genes involved in the cell cycle

Cyclin D1 and the cyclin-dependent kinase inhibitors p21 and p27 mRNA levels showed no difference between the three groups (Figures 3A–3C). The intrathymic expression of p16 mRNA was similar in recuperated and control animals. In contrast, the expression of p16 in the thymus from PLP mice was significantly reduced (P < 0.001) in comparison with the thymus from both control and recuperated animals (Figure 3D).

Genes for sex hormone receptors

AR (androgen receptor) expression in the thymus from PLP animals was significantly lower than controls (P < 0.01), whereas the expression level in the thymus from recuperated animals was comparable with controls (P > 0.001) in comparison with the thymus from both control and recuperated animals (Figure 3D)
Figure 3  Effects of maternal protein restriction on relative gene expression of cyclin D1 (A), p21 (B), p27 (C), and p16 (D) in the thymus at 21 days of age
Gene expression levels were measured by quantitative RT-PCR. Values are expressed as means ± S.E.M., with mean gene expression in the thymus from control mice (C) being set as 100. ***P < 0.001 (n = 8 for each group). R, recuperated animals.

Figure 4  Effects of maternal protein restriction on relative gene expression of AR (A), ERα (B) and GPR30 (C) in the thymus at 21 days of age
Gene expression levels were measured by quantitative RT-PCR. Values are expressed as means ± S.E.M., with mean gene expression in the thymus from control mice (C) being set as 100. *P < 0.05, **P < 0.01 and ***P < 0.001 (n = 8 for each group). R, recuperated animals.

Genes involved in thymic growth and aging
Transcripts for both prothymic IL-7 (interleukin-7) and its receptor IL-7R showed higher levels of expression in the thymus from recuperated animals compared with controls (P < 0.05 for IL-7 and P < 0.01 for IL-7R; Figures 5A and 5B). Expression of these genes in the thymus from PLP animals was similar to controls (Figures 5A and 5B). Thymosin α1 and thymosin β4 showed no significant changes in the thymus from PLP and recuperated animals compared with controls (Figure 5C and 5D). TGFβRII [TGF (transforming growth factor)-β type II receptor] gene expression was also not significantly different between the three groups (Figure 5E). Rwdd1 (Rwd-domain-containing 1) is a recently characterized thymus aging-related protein that has been suggested to be indirectly involved in AR signalling [26]. The expression level of Rwdd1 in the thymus from PLP and recuperated animals was similar to controls (Figure 5F). Transcripts of the thymocyte cell-surface markers CD4 and CD8 both had a significantly higher level of expression in the thymus from recuperated animals, whereas in the thymus from PLP animals only CD8 had significantly higher expression levels than controls (Figure 5G and 5H).

DISCUSSION
The thymus is responsible for the generation of immunocompetent T-cells which are an integral part of the immune system and, therefore, this organ plays an important role in functional immunity. The development of the thymus takes place during the late fetal/early postnatal stages of mammalian growth [27] and, as such, may be sensitive to early-life events. Indeed, there is evidence to suggest that the thymus may be affected by early-life nutrition [11,28,29]. We therefore hypothesized that maternal diet may influence thymic development which, in turn, may play a role in mediating programming effects on the regulation of lifespan in our maternal-protein-restricted animals. The present study monitored thymic weight and protein/gene expression of three groups of mice (control, PLP and recuperated animals) at 21 days and 12 weeks of age. It revealed that, although the thymus from control and recuperated mice had no weight gain between 21 days and 12 weeks of age, the thymus from PLP mice had significant growth between these two time points. As a result, thymic weight of PLP mice was significantly heavier than that of controls at 12 weeks of age.

The differential thymic growth in these three groups of mice was apparently related to different mitotic activities in the thymic tissues. First, PCNA protein expression was higher in PLP and recuperated mice at 21 days of age compared with the same-age controls. PCNA was originally identified as an antigen that is expressed in the nuclei of cells during the S-phase of the cell cycle and is a cofactor of DNA polymerase, essential for DNA replication [30]. This may suggest that cell proliferation in the thymus was enhanced in both PLP and recuperated animals at weaning. However, the...
Enhanced thymic mitotic activity in these two groups may occur due to disparate underlying mechanisms. In the thymus from PLP animals, this may be due to selective tissue growth or metabolic resource reallocation in order to spare vital organ growth, whereas in recuperated mice it may be a result of general catch-up growth. This proposal is supported by the fact that only the thymus from PLP animals had a significantly higher relative organ weight, whereas the relative thymus weight of recuperated mice was similar to the controls. At 12 weeks of age, PCNA protein expression in the thymus from PLP mice was comparable with controls, whereas PCNA protein expression in the thymus from recuperated mice was decreased compared with controls. This may explain why the thymus from PLP mice showed significant growth between weaning and 12 weeks of age, whereas such growth was not observed in recuperated or control mice. Secondly, SIRT1 has been demonstrated to be correlated with cell-cycle activity and its progressive loss is associated with cell-cycle withdrawal [31]. The significant up-regulation of SIRT1 in the thymus from 21-day-old recuperated mice may reflect the general catch-up growth during lactation. Conversely, the significantly higher SIRT1 expression in the thymus from PLP at 12 weeks of age could be indicative of a higher mitotic activity compared with control and recuperated mice at this age.

It is well documented that thymic growth and involution are influenced by sex steroid hormones [23]. For instance, surgical or chemical castration of aged animals causes regeneration of the thymus, which was reversed by administration of synthetic sex steroids [32]. Furthermore, castrated male mice also had enhanced thymocyte proliferation [33]. Conversely androgen administration to intact female and castrated male mice leads to increased thymocyte apoptosis [34]. Receptors for androgen, oestrogen and progesterone have been identified on both thymocytes and thymic stromal cells, suggesting that steroid sex hormones directly influence both thymic development and involution [23]. In the present study, we observed that testosterone concentrations in 12-week-old PLP and recuperated offspring were lower compared with that of control animals. Furthermore, when AR mRNA levels were compared at 21 days of age, the thymus from PLP animals had significantly decreased expression compared with controls.
with controls, whereas recuperated mice expressed similar levels to controls. These observations suggest that decreased testosterone exposure through reduced hormone concentration and reduced receptor expression could contribute to the increased post-weaning thymic growth in PLP animals. Oestradiol concentrations were reduced in 21-day-old PLP mice. Oestrogen functions through intracellular receptors (ERα and ERβ), as well as a membrane receptor (GPR30), which are all expressed in immune cells. It has been suggested that ERα and GPR30 mediate the functions of oestrogens in the thymus, whereas ERβ is not thought to play a role in thymus function [35]. ERα expression in the thymus at 21 days of age was not different between control, PLP and recuperated mice. mRNA levels of GPR30 in the thymus from PLP and recuperated mice were higher than in control tissues. Thus it is possible that increased GPR30 expression in recuperated animals may result in increased oestrogen signalling leading to thymic atrophy, whereas reduced oestradiol concentrations in PLP mice may counteract this effect and favour thymic growth.

We recently observed that rats born to dams fed a low-protein diet that underwent postnatal catch-up growth had accelerated telomere shortening in organs, including aorta [36] and pancreatic islets [37], which might result from an increase in oxidative stress. Thus it is possible that rapid catch-up growth during lactation results in increased oxidative stress in tissues leading to premature cellular aging. Thymus from recuperated mice had significantly elevated p53 protein levels at 21 days and 12 weeks of age. As p53 is up-regulated in response to oxidative stress [38], this may suggest that oxidative stress is elevated in the thymus from recuperated animals and may contribute to reduced mitotic activity and accelerated aging of the thymus in these animals. Indeed, the expression level of PCNA in the thymus from 12-week-old recuperated animals was significantly lower than in 12-week-old controls. Expression analysis of genes involved in cell-cycle regulation showed no significant differences in cyclin D1, p21 and p27 levels in the thymus between control, PLP and recuperated mice, suggesting that these genes are not primarily responsible for the observed differences in thymic growth patterns in the three groups.

Reduced expression of p16 in the thymus from PLP animals at 21 days of age may indicate that the thymus in these mice undergoes delayed immunosenescence leading to extended lifespan in these animals. p16 is a cyclin-dependent kinase inhibitor that causes cell-cycle arrest. It is up-regulated in senescent cells [38] and has been identified as a biomarker/effecter of aging [39]. It has been implicated in the age-related decline in regenerative capacity of haematopoietic stem cells [40], pancreatic islets [41] and neural stem cells [42]. The suggestion that increased expression of p16 is causally implicated in senescence and organism aging is supported by studies showing that inactivation of p16 delays cellular senescence and aging in a murine progeria model [43]. p16 expression in pretransplant renal biopsies is inversely related to transplant outcome providing a novel marker predicting postoperative organ function [44]. The present findings suggest that PLP mice demonstrate increased mitotic activity and, consequently, growth during early adult life, whereas recuperated mice may have a thymus that experiences enhanced oxidative stress which can lead to accelerated involution later in life. This is in line with observations in humans that prenatal nutritional conditions have a long-term impact on thymic function [11,28,29].

Intrathyemic expression of IL-7 and IL-7R is essential for thymic growth and development of the immune system. IL-7 functions through its receptor to promote thymocyte proliferation and survival [45]. It has been demonstrated that a decline in IL-7 expression is associated with age-related thymic atrophy, and administration of IL-7 to older mice augments thymic size and thymopoiesis [46]. Increased expression of IL-7 and its receptor in the thymus from recuperated mice at 21 days of age is thus consistent with the finding that thymic growth during lactation is enhanced in the recuperated animals. Moreover, the significantly increased expression of CD4 and CD8 in the thymus from recuperated animals may be indicative of an altered rate of thymocyte maturation in these mice. CD4 and CD8 are thymocyte cell-surface markers. Most immature thymocyte progenitors are negative for CD4 and CD8, and maturation of thymocytes occurs during their migration from the thymic cortex to the medulla. This coincides with the differentiation of CD4+CD8+ double-positive immature thymocytes into CD4+CD8− and CD4+CD8+ single-positive mature thymocytes [16].

Thymosin peptides are thymic-derived hormones that have been shown to decline with age and are associated with thymic involution and immune senescence [16]. No significant differences in expression of thymosin α1 and thymosin β4 in the thymus from 21-day-old control, PLP and recuperated animals suggest that these molecules are not involved in the regulation of differential thymic growth observed in the present study. Similarly, because Rwdd1 had no difference in expression between the three groups it is unlikely that this molecule contributes to this regulation. TGF-β2 is known to play an age-dependent negative role in controlling thymic weight and cellularity [47]. Our observation that TGFβRII showed no significant difference between the three groups may suggest that fluctuation in TGFβRII expression is not a factor mediating the effects of maternal diet on thymic growth in early postnatal life.

In summary, the present findings demonstrate that maternal protein restriction influences thymic growth during young adult life. Offspring of maternally protein-restricted mice during lactation preserve thymic growth...
despite whole-body growth being significantly slowed. More importantly, the thymus in these mice showed significant further growth during young adult life such that at 12 weeks of age the thymus weight was significantly higher than that of control animals. The underlying causes of this growth appeared to be the reduced production of sex steroid hormones, decreased expression of the AR and increased mitotic activity. In addition, lower expression levels of p16 in the thymus from PLP animals at 21 days of age suggests that the aging process in this organ is slowed even at this very early stage of life. In contrast, the thymus in recuperated mice was heavier than controls at 21 days of age, which was in line with the whole-body catch-up growth. This was associated with increased mitotic activity and increased IL-7 and IL-7R expression at this age. Accelerated growth during lactation may result in increased oxidative stress as may be suggested by increased p53 protein expression, which could lead to reduced mitotic activity at 12 weeks of age. This may result in accelerated thymic involution, which could ultimately contribute to shortened lifespan of recuperated mice. Thus maternal diet and early growth patterns can influence thymic growth and/or involution, which may mediate the regulation of lifespan through altered immune functions.

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Conclusion

In conclusion, the present study demonstrated that maternal nutritional status during pregnancy and lactation significantly influences thymus aging-related protein expression and thymic function in newborn rats. The results support the hypothesis that maternal nutrition plays a crucial role in thymic aging and suggest that interventions targeting maternal nutrition during pregnancy and lactation could potentially delay thymic aging and preserve thymic functions in later life. These findings contribute to the understanding of the regulatory mechanisms underlying thymus aging and provide new insights into the development of strategies for thymus rejuvenation.

Keywords: thymus aging; maternal nutrition; thymus regeneration; thymic hormones; thymic development.