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Disruption of Thymic Microenvironment with Age: Remodeling of Blood Vessels and Extracellular Matrix

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Thymic involution is a hallmark of aging characterized by reduced thymic size and function, leading to impaired T lymphocyte development and increased susceptibility to infection and cancer. While previous studies have described histological and cellular changes during thymic aging, the impact on vasculature and extracellular matrix (ECM) organization remains incompletely understood. In this study, we analyzed thymic tissue from young (8–10 weeks) and aged (18–27 weeks) mice to investigate age-associated changes in blood vessels and ECM components. Immunohistochemical analysis revealed that CD34-positive blood vessels in the cortex were abundant, elongated, and oriented toward the medulla in young thymus, but were significantly reduced and structurally disorganized in aged thymus. In contrast, CD31-positive vessels were localized mainly in the medulla and remained largely unaffected. ECM proteins laminin and tenascin-C displayed well-aligned vascular structures in young mice, but became more dispersed and mesh-like in aged thymus. Collagen type I, prominently detected in large blood vessels and some microvessels in young thymus, was nearly absent in aged samples. These structural changes were accompanied by a specific upregulation of *Mmp3* mRNA, encoding matrix metalloproteinase-3, which is known to degrade ECM proteins and disrupt vascular integrity. The remodeling of blood vessels and ECM in the aged thymus may weaken the blood-thymus barrier, impair T lymphocyte development, and contribute to inflammaging. Our findings provide new insights into the microenvironmental deterioration of the thymus with age and identify vascular and ECM remodeling as potential therapeutic targets to mitigate age-related immune decline.

Key words thymus, aging, blood vessel, extracellular matrix, matrix metalloproteinase

INTRODUCTION

The thymus is a primary lymphoid organ essential for the differentiation and maturation of T lymphocytes. It develops earlier than most other organs and reaches its maximum size during infancy. Both its size and function begin to decline as early as 2 to 3 years of age, resulting in reduced T lymphocyte output.[1,2] This age-related reduction in thymic size and function, known as thymic involution, is associated with increased susceptibility to infection and a higher risk of tumorigenesis in older individuals.[3] Therefore, mitigation or delaying thymic involution could be beneficial for maintaining immune health in the elderly.

Thymic involution is accompanied by various histological changes. The thymus is anatomically divided into the outer cortex and inner medulla, where immature T lymphocytes undergo positive and negative selections, respectively. These two regions are separated by cortico-medullary junction, through which developing T cells migrate at specific developmental stages, regulated in part by the CCL21-CCR7 signaling axis.[4] The organization of this boundary is maintained by cortical and medullary thymic epithelial cells, namely cTECs and mTECs, which reside in their respective compartments.[5] Aging disrupts TEC identity, promoting the emergence of atypical TEC populations, which in turn leads to the breakdown of cortico-medullary architecture and impaired T lymphocyte development.[6,7] Additionally, aging induces fibroblast expansion, epithelial-to-mesenchymal transition, and lipid accumulation in the thymus.[2,8] Despite these well-documented structural changes, the molecular characteristics of the involuted thymus remain incompletely understood.

Vascular dysfunction and structural alterations are hallmark features of aging and are implicated in a wide range of age-related diseases. In large arteries, aging increases wall thickness and lumen diameter, leading to stiffness and reduced responsiveness to vasodilatory signals.[9] In contrast, aging affects microvasculature differently depending on tissue type. For example, in the nervous system, age-related microvascular changes impair neuronal plasticity despite the preservation of neuronal and synaptic density.[10] Aged brains also show distorted vascular patterning and compromised blood-brain barrier integrity.[11] While brain vasculature has been extensively

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studies in the context of aging, much less is known about how microvasculature in other organs, such as the thymus, is affected.

In this study, we investigated histological change in thymic vasculature in aged mice to gain insights into the vascular components of age-associated thymic involution.

MATERIALS AND METHODS

Animals Male young mice (8 to 10-week-old) and aged mice (18 to 27-week-old) of ICR were purchased from Japan SLC, Inc (Shizuoka, Japan). They were raised in the animal facility in Kobe Pharmaceutical University and acclimatized in individual housing for at least one week before the experiment. All procedures of animal experiments in this study were conducted following the Guidelines for Proper Conduct of Animal Experiments of the Science Council of Japan. The protocols were approved by the Kobe Pharmaceutical University Committee for Animal Care and Use.

Histological Analyses Mice were deeply anesthetized and transcardially perfused with phosphate buffered saline (PBS). The collected thymus was fixed in 4% paraformaldehyde in PBS at 4°C for 5 h and cryoprotected in 30% sucrose in PBS at 4°C overnight. The thymus was embedded in O.C.T. compound (Sakura Finetek Japan Co. Ltd., Tokyo, Japan). Then, 30-μm tissue sections were prepared with a cryostat (SLEE medical GmbH, Mainz, Germany).

For hematoxylin-staining, the sections were incubated in Mayer's Hematoxylin Solution (FUJIFILM Wako Pure Chemical Co., Osaka, Japan) for 2 min at room temperature. After washing in distilled water, they were mounted with TE-glycerol solution (10 mM Tris, pH=8.0, 1 mM ethylenediaminetetraacetic acid, 80% glycerol).

Immunohistochemistry was performed as described in our previous manuscript with slight modifications.[12] The tissue sections were fixed in 4% PFA in PBS for 5 min, briefly washed with PBS three times, and incubated in Tris-EDTA buffer (10 mM Tris, pH=9.0, 1 mM ethylenediaminetetraacetic acid) at 75°C for 40 min for antigen retrieval. They were then washed three times in PBS and incubated in 0.3% hydrogen peroxide-methanol solution at room temperature for 15 min. After being washed three times in PBS, they were blocked in 1.5% fetal bovine serum-PBS for 1 h at room temperature. After the blocking step, they were incubated with the following primary antibodies at 4°C overnight: anti-CD31 (AF3628, R&D Systems, Inc., Minneapolis, MN, USA), CD34 (ab81289, Abcam, plc., Cambridge, UK), anti-laminin (NB300-144, Novus Biologicals, LLC, Centennial, CO, USA), anti-collagen I (ab270993, Abcam), anti-tenascin-C (ab108930, Abcam). Following three times washes with PBS, they were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (A16035, Thermo Fisher Scientific, Inc., Wilmington, DE, USA) or anti-goat IgG (A16005, Thermo Fisher Scientific) in 1.5% fetal bovine serum in PBS, at room temperature for 2 h. After being washed three times with PBS, they were developed using ImmPACT DAB substrate kit (Vector laboratories, Inc., Newark, CA, USA) according to the manufacturers' instruction. The sections were dehydrated in a series of 70%, 80%, 95%, 100% ethanol solution and xylene, and mounted with Entellan New mounting medium (Merck KGaA, Darmstadt, Germany).

All images were acquired using an BZ-X810 microscope (KEYENCE, Osaka, Japan) and processed in Fiji and GIMP, open-resource softwares for manipulating images.

RNA Purification and qRT-PCR RNA extraction and qRT-PCR analysis was performed as described in previous manuscript.[13] Mice were deeply anesthetized and transcardially perfused with PBS. The thymus was collected and stored in Sepasol-RNA I super G solution (Nacalai Tesque, Inc., Kyoto, Japan) at -80°C, followed by homogenization and total RNA purification according to the manufacturer's instruction. The concentration of purified RNA was determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). Complementary DNA was synthesized using ReverTra Ace reagent (Toyobo Co. Ltd, Osaka, Japan) according to the manufacturer's instructions. The expression levels of target genes were determined using CFX Connect real-time PCR detection system (Bio-Rad Laboratories Inc., Hercules, CA, USA), where PCR amplification was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories) with the primer pairs listed in Supplementary Table 1. The PCR parameters were as follows: 1 min of initial DNA polymerase activation, DNA denaturation at 95°C, and 40 cycles of denaturation at 95°C for 15 s and primer annealing-fragment extension at 60°C for 30 s. The melting curves of the real-time PCR products were analyzed from 65°C to 90°C. Differences in gene expression, expressed as fold change, were calculated using the ΔΔCt method with Excel software (Microsoft, Co., Redmond, WA, USA). *Rplp2* was used as a reference gene for normalizing the expression. Results were indicated as means ± standard deviation. Statistical significance was determined using Student's t-test.

RESULTS

Blood Vessel Remodeling in the Involuted Thymus of Aged Mice To investigate age-related change in thymic vasculature, we compared the thymus of young (8 to 10-week-old) and aged (18 to 27-week-old) mice. The thymus-to-body weight ratio was significantly lower in aged mice compared to young mice, consistent with thymic involution (Fig. 1A and B). Hematoxylin staining of thymus sections revealed a well-defined cortico-medullary boundary in young mice, whereas this boundary was disrupted or indistinct in aged mice (Fig. 1C), further confirming age-associated thymic degeneration.

To visualize blood vessels, thymus sections were immunostained with antibodies against CD31 and CD34. CD31 and CD34 label distinct endothelial populations in several immune organs.[14-16] CD31 is generally regarded as a marker of endothelial cells with high angiogenic potential, whereas CD34-positive endothelial cells show lower proliferative activity and secret IL-33 and angiopoietin-2.[17,18] In young mice, CD31-positive signals were detected in a limited number of vessel-like structures, primarily within the medulla, with minimal staining in the cortex (Fig. 2A). The distribution pattern of CD31-positive structures appeared similar between young and aged mice showing no apparent age-related difference (Fig. 2A). In contrast, CD34 immunostaining revealed clear blood vessel-like structures in both cortex and medulla regions of the young thymus (Fig. 2A). In the cortex, numerous CD34-positive vessels exhibited strong intensity and elongated longitudinally from the thymic surface toward the medulla. However, in the aged thymus, these cortical CD34-positive structures were markedly altered: the elongated pattern was largely lost, and

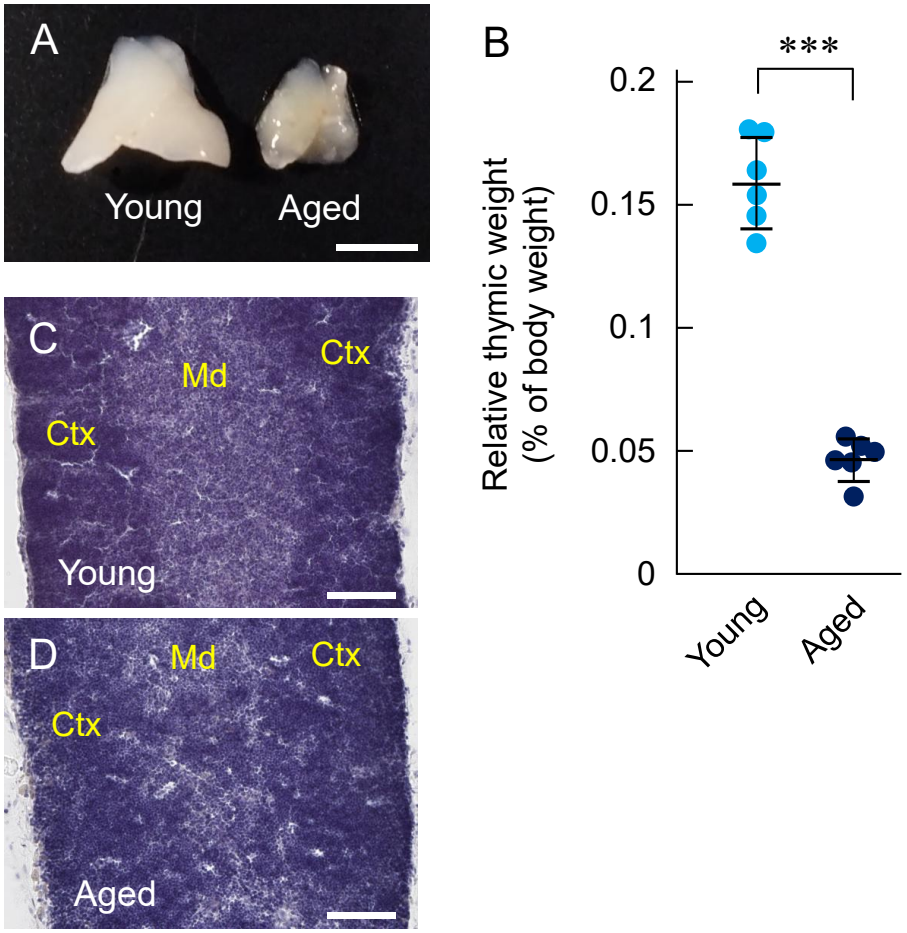

Fig. 1. Age-Related Thymic Involution

A: Whole view of the thymus of young and aged mice. B: The thymic weight relative to body weight. Light and dark blue circles indicate individual animal of young and aged mice, respectively. ***$p < 0.001$. C and D: Hematoxylin-staining of the thymus sections. Ctx, cortex; Md, medulla. Scale bars: 10 mm (A), 100 μm (for C and D).

very few vessels extended toward the medulla. In the medulla of young mice, CD34-positive vessels typically ran parallel to the thymic surface, whereas in aged mice, these structures appeared more fragmented and disorganized (Fig. 2A). Quantitative analysis showed that the number of CD34-positive vessels in the medulla remained relatively unchanged with age. In contrast, the number of cortical CD34-positive vessels–particularly the longer ones–was significantly reduced in aged mice (Fig. 2B). Additionally, the diameter of CD34-positive vascular structures was significantly smaller in the aged thymus compared to the young thymus (Fig. 2C). It is worth noting that CD34 is a marker for proliferating progenitor cells.[19] In the young thymus, CD34-positive progenitor-like cells were readily detected, whereas their presence was markedly diminished in the aged thymus (Fig. 2A). These findings suggest that aging induces significant remodeling of thymic vasculature, particularly within the cortical region, and is associated with structural alterations and reduced vascular integrity.

Extracellular Matrix Remodeling in the Involuted Thymus of Aged Mice The extracellular matrix (ECM) plays a critical role in maintaining vascular structure and function, and its remodeling is closely linked to the development of various pathologies.[20,21] To investigate ECM changes associated with thymic aging, we examined the localization of major ECM proteins in young and aged thymus tissue by immunohistochemistry. Immunostaining with an anti-collagen type I antibody revealed strong signals in large blood vessel-like structures located at the cortico-medullary boundary in young mice (Fig. 3A, blue arrows), as well as in a subset of microvessel-like structures near the thymic surface (Fig. 3A, orange arrows). Fragmented collagen I-positive structures were also observed throughout both the cortex and medulla. In contrast, collagen I staining was markedly reduced in the aged thymus. Only a few thick blood vessel-like structures showed weak collagen I signals, indicating a substantial loss of collagen I with aging. An anti-laminin antibody stained vascular structures in both cortex and medulla of young thymus, showing a distribution pattern similar to that of CD34. In addition, reticulated laminin-positive networks were visible in the medulla, which were not apparent with CD34 staining. In the aged thymus, laminin-positive signals remain detectable; however, their diameter was notably increased, particularly in areas proximal to the medulla (Fig. 3d, yellow arrow), suggesting structural remodeling. Staining with an anti-tenascin-C antibody also highlighted blood vessel-like structures in both cortex and medulla of young thymus, again resembling the distribution of

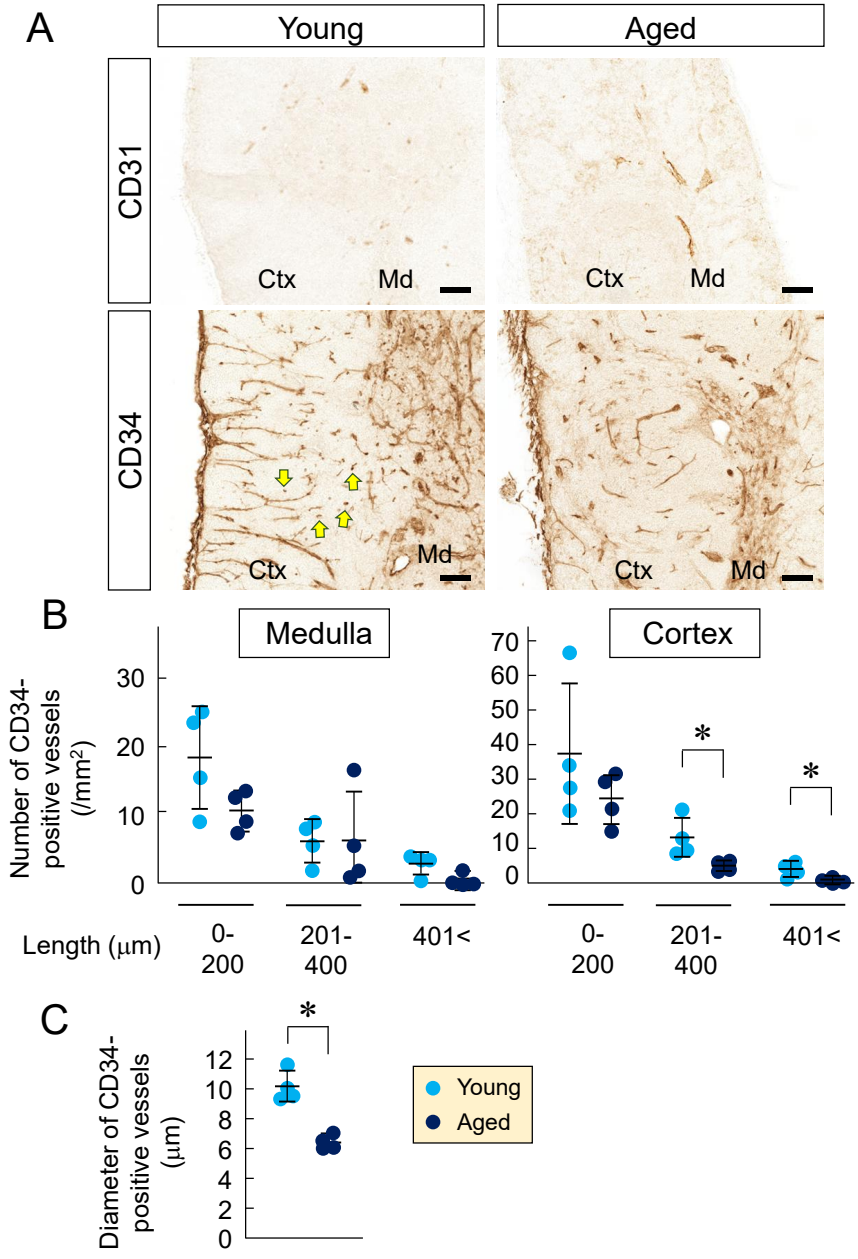

Fig. 2. Blood Vessel Remodeling in the Thymus of Aged Mice

A: The thymus sections from young and aged mice were immunostained with an anti-CD31 and CD34 antibodies. Ctx, cortex; Md, medulla. Yellow arrows indicate CD34-positive progenitor cell-like signals. Scale bars: 100 μm. B: Quantification of the number of CD34-positive vessels in the cortex and medulla. C: Average diameter of CD34-positive vessels. Light and dark blue circles indicate individual animal of young and aged mice, respectively. *$p < 0.05$.

CD34 and laminin. In the aged thymus, tenascin-C staining in the cortex appeared as mesh-like, more diffuse structures, distinct from the more organized laminin pattern. These mesh-like tenascin-C structures were largely absent in young thymus. Tenascin-C-positive signals were markedly increased in the medulla of the aged thymus, compared to the young thymus. Collectively, these findings indicate that the ECM architecture in the thymus undergoes substantial remodeling during age-associated involution, particularly affecting the composition and organization of key structural proteins such as collagen I, laminin, and tenascin-C.

Age-Associated Change in Matrix Metalloproteinase Expression in the Thymus Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases responsible for degrading ECM components and are central to tissue remodeling processes.[22,23] While MMPs have been implicated in age-related skin change,[24,25] their roles in age-associated changes in other organs, including the thymus, remain poorly understood. To explore the potential contribution of MMPs to ECM remodeling in aged thymus, we analyzed the expression of multiple MMP family genes using qRT-PCR. Among the genes tested, *Mmp3* mRNA was specifically upregulated in the aged thymus compared to young con-

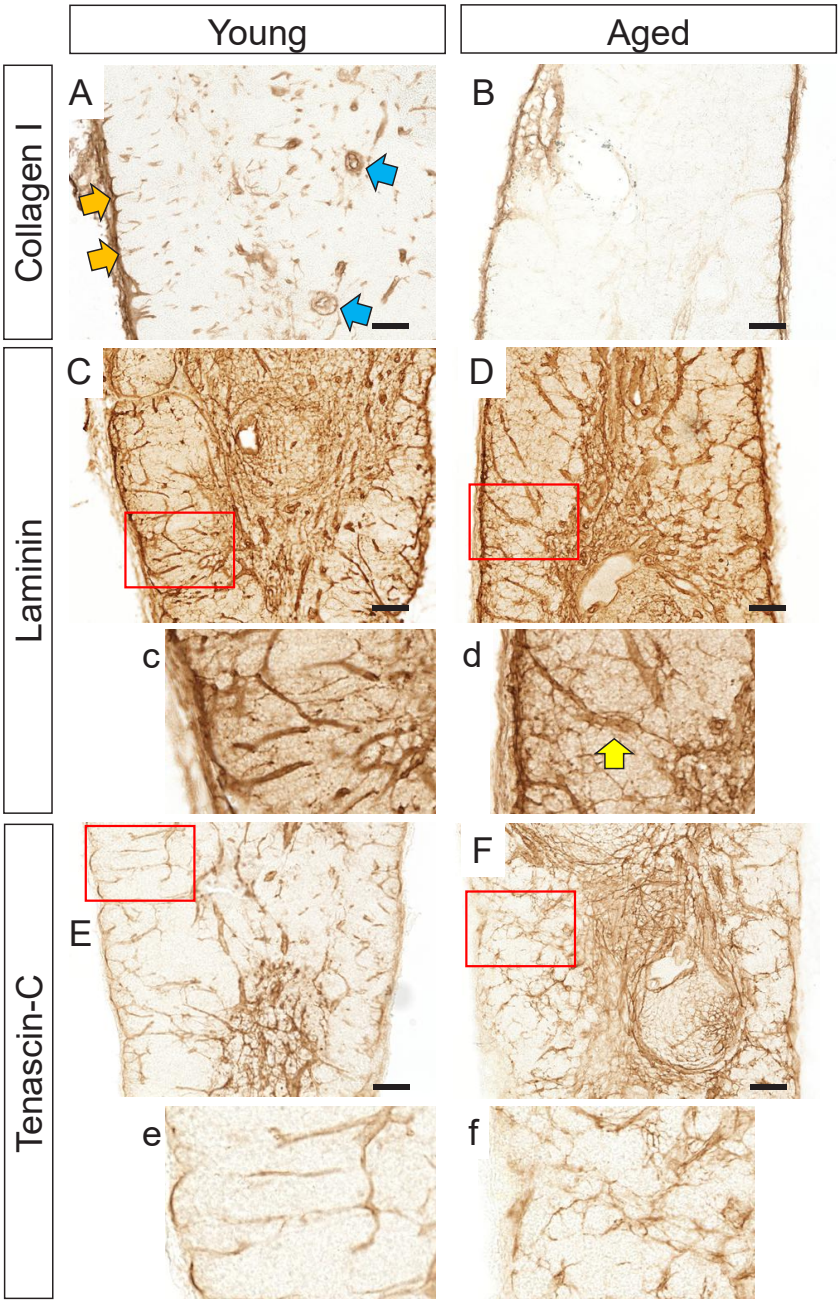

Fig. 3. Localization of ECM Proteins in the Thymus of Young and Aged Mice

The thymus sections from young (A, C, c, E, e) and aged (B, D, d, F, f) mice were stained with anti-collagen I (A, B), laminin (C, c, D, d), or tenascin-C (E, e, F, f) antibodies. Blue and orange arrows in panel A indicate the large blood vessels at the cortico-medullary boundary and cortical microvessels, respectively. Red square regions in panels C, D, E, and F, are enlarged in panels c, d, e, f, respectively. Yellow arrow in panel d indicates the dispersed signals of laminin in the cortex of aged thymus. Scale bars: 100 μm.

trols (Fig. 4; Table 1). This result suggests that MMP-3 may play a central role in ECM remodeling during thymic involution. In contrast, *Mmp7* mRNA expression was significantly downregulated in the aged thymus. Although expression levels of some other MMPs tended to decrease with age, inter-individual variability was high, preventing definitive conclusions. Nonetheless, the selective upregulation of *Mmp3* mRNA highlights it as a candidate mediator of age-related ECM remodeling in the thymus.

DISCUSSION

The Function of Thymic Blood Vessels and Potential Consequences of Vascular Remodeling during Aging

Thymic blood vessels play essential roles in T lymphocyte development. Hematopoietic progenitor cells originating from the bone marrow enter the thymus via the vasculature. Following differentiation in the cortical and medullary regions, immature T lymphocytes exit the thymus and enter the periphery circulation through blood vessels. Thus, blood vessels serve as both the entry and exit points for thymocyte development. Importantly, the thymic vasculature includes the blood-

Table 1. Expression of mRNAs of MMPs and TIMPs in the Thymus of Young and Aged Mice

	Young	Aged
Mmp2	1.000 ± 0.659	0.907 ± 0.956
Mmp3	1.000 ± 0.440	2.856 ± 1.534 *
Mmp7	1.000 ± 0.323	0.452 ± 0.243 *
Mmp8	1.000 ± 0.675	0.226 ± 0.192
Mmp9	1.000 ± 0.397	0.611 ± 0.507
Mmp10	1.000 ± 0.670	0.355 ± 0.274
Mmp11	1.000 ± 0.234	0.751 ± 0.625
Mmp12	1.000 ± 0.618	0.539 ± 0.502
Mmp13	1.000 ± 0.670	0.721 ± 0.632
Mmp14	1.000 ± 0.282	1.304 ± 1.505
Mmp15	1.000 ± 0.300	0.571 ± 0.520
Mmp16	1.000 ± 0.357	1.242 ± 1.269
Mmp17	1.000 ± 0.168	1.163 ± 1.155
Mmp19	1.000 ± 0.183	0.878 ± 0.794
Mmp20	1.000 ± 0.614	0.810 ± 0.560
Mmp23	1.000 ± 0.309	1.080 ± 0.748
Mmp25	1.000 ± 0.564	0.669 ± 0.569
Mmp27	1.000 ± 0.582	1.848 ± 1.796
Mmp28	1.000 ± 0.307	1.062 ± 0.792
Timp1	1.000 ± 0.174	0.781 ± 0.166
Timp2	1.000 ± 0.262	0.857 ± 0.414
Timp3	1.000 ± 0.139	0.510 ± 0.408
Timp4	1.000 ± 0.397	0.844 ± 0.561

qRT-PCR was performed to assess the expression of matrix metalloproteinases and tissue inhibitor of metalloproteinases using total RNAs isolated from the thymus of young and aged mice. Expression levels were normalized to the average values from the young thymus. Statistical analysis was conducted using Student's *t*-test. *$p < 0.05$.

thymus barrier, a specialized structure that functionally separates developing naïve thymocytes from peripheral mature T lymphocytes.[26] Disruption of this barrier has been shown to impair thymic integrity and T lymphocyte maturation. For example, cadmium exposure in chick compromises the blood-thymus barrier, leading to increased pyroptosis in thymic tissue.[27] These findings highlight the importance of vascular integrity for maintaining thymic architecture and ensuring proper lymphocyte development. Our results demonstrate that CD34-positive blood vessels in the thymus undergo significant remodeling with age (Fig. 2). Notably, antibodies against CD31 and CD34 stained thymic vasculature with different patterns. While most cortical and medullar vessels were positive for CD34, CD31-positive structures were localized only in the medulla. This differential staining pattern is consistent with previous reports in other organs, including the spleen, liver, saphenous vein, and tumors.[14-16] Given the high angiogenic potential of CD31-positive endothelial cells, the presence of CD31-positive vessels specifically in the medulla raises the possibility that these structures may contribute to localized vascular remodeling or regeneration.[15,28,29] Further investigation is needed to clarify the functional significance of CD31-positive endothelial cells in the thymic medulla.

In addition to physiological aging, the thymus is also susceptible to size and functional decline in response to various life style-related biological stresses. This process, referred to as thymic atrophy, can be triggered by infection, malnutrition, exposure to toxic chemicals, psychological stresses, etc.[30,31] Elevated blood estradiol levels are also known to induce thymic atrophy.[32,33] Estradiol administration in rats has been shown to disrupt the blood-thymus barrier, increase vascular permeability, and impair the proper migration of developing T lymphocytes.[34] Thus, disorganization of the blood-

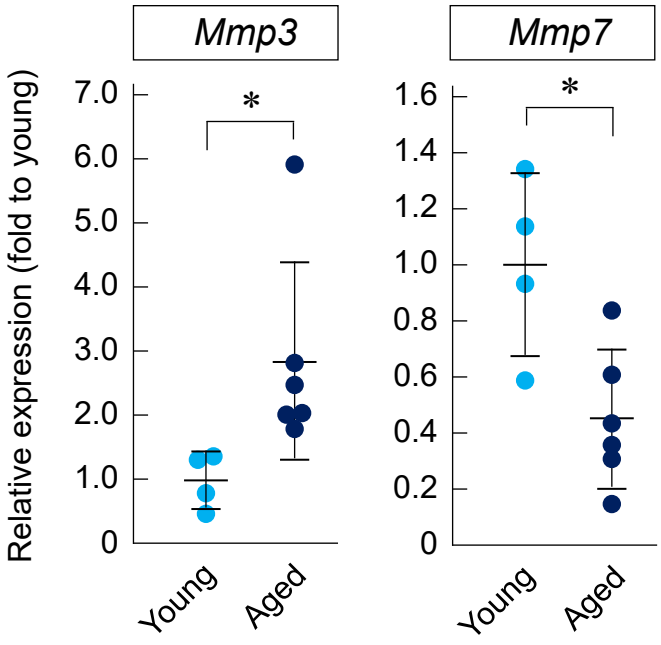

Fig. 4. mRNA Expression of *Mmp3* and *Mmp7* in the Young and Aged Thymus

Relative expression of mRNAs for *Mmp3* and *Mmp7*. Light and dark blue circles indicate individual animal of young and aged mice, respectively. *$p < 0.05$.

thymus barrier may be a common pathological feature of both thymic involution and atrophy. Despite its importance, the precise molecular mechanisms regulating the blood-thymus barrier largely remain poorly understood. Further studies should aim to elucidate the structural and molecular changes underlying barrier disruption during thymic aging and atrophy and examine their impact on systemic immune competence.

The Functional Consequences of ECM Remodeling in the Aged Thymus Our results indicate that aging leads to remodeling of the ECM in the thymus. In young mice, both laminin- and tenascin-C-positive signals detected on well-aligned, vessel-like tubular structures in the cortical and medullary regions (Fig. 3). However, in aged mice, these signals appeared more dispersed and disorganized. Given the concurrent loss of CD34-positive vascular signals in both the cortex and medulla, it is likely that many of these ECM protein-positive structures in aged thymus are no longer associated with functional blood vessels and may represent remnants of regressed vasculature.

Laminin is a major ECM component of the endothelial basement membrane and plays a structural role in supporting blood vessels.[35] Laminin can persist as tubular structures even after blood vessel regression, at least transiently.[36] Beyond its structural role for blood vessels, laminin also influences thymocyte biology directly interacting with the α6β4 integrin to regulate T lymphocyte proliferation, migration, and differentiation.[37,38] Therefore, the residual laminin in aged thymus may retain functional capacity to support T lymphocyte development.

Tenascin-C is another ECM protein observed along vessel-like structures in both cortex and medulla in young thymus. In the aged thymus, tenascin-C appeared more diffusely accumulated, particularly in the medulla, forming mesh-like structures that were more disorganized than laminin signals (Fig. 3E and F). Tenascin-C is implicated in various age-related vascular pathologies, including cerebral vasospasm, intimal hyperplasia, and aortic aneurysm.[39-41] Its elevated presence in the medulla of aged thymus may signal increased vascular vulnerability. Further investigations are needed to reveal whether the laminin- and tenascin-C-positive structures can still support blood flow and nutrient exchange in the aged thymic microenvironment.

Collagens play important roles in vascular integrity and stability. Collagen type I, the most abundant interstitial collagen in the vascular intima, media, and adventitia, is essential for providing stiffness and mechanical resistance blood vessels.[42,43] In the young thymus, collagen I staining was prominent around large vessels at the cortico-medullary boundary, as well as in select cortical and medullary microvessels (Fig. 3). The relatively lower abundance of collagen I-positive microvessels compared to laminin- and tenascin-C-positive ones suggests heterogeneity among the thymic microvasculature. In aged mice, collagen I staining was almost completely absent (Fig. 3B), which, together with the change in laminin and tenascin-C distribution, suggests structural fragility and loss of vascular integrity in the involuted thymus.

Our qRT-PCR screening revealed that *Mmp3* mRNA was selectively upregulated in the aged thymus, while most other *Mmp* genes were downregulated or unchanged (Table 1). MMP-3, also referred to as stromelysin-1, has broad substrate specificity and can degrade various ECM components, including collagen, fibronectin, laminin, and elastin.[44] MMP-3 is known regulator of blood vessel remodeling, facilitating endothelial cell invasion by degrading basement membrane components and disrupting endothelial junctions.[45] In *Mmp3*-knockout mice, reduced extravasation of systematically administered dyes suggests a role for MMP-3 in increasing vascular permeability.[46] Therefore, the upregulation of *Mmp3* mRNA in aged thymus may contribute to ECM degradation, vascular leakage, and potentially promote inflammaging, a chronic low-grade inflammation commonly observed in aging tissues.[47] The precise effects of aging on vascular integrity should be investigated in future studies.

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Conflict of interest The authors declare no conflict of interest.

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