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Collagen IV Is Increased in the Cortex of Thymus by Acute Dietary Restriction

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Malnutrition, a global health problem, impairs immune function and increases susceptibility to infection. The thymus, a primary lymphoid organ, is highly sensitive to nutritional status and is known to undergo atrophy under malnutrition. However, the temporal progression of thymic changes during malnutrition remains unclear. In this study, we used a mouse model of short-term starvation, in which dietary intake was completely withheld for 24 or 48 h to model acute fasting. The thymus of 48-h-starved mice, but not that of 24-h-starved mice, exhibited marked atrophy with a significant reduction in absolute weight. Expression levels of Th2-associated cytokines, including *Il5*, *Il6*, and *Il10*, were increased following 48 h of starvation, indicating polarization of Th0 cells toward a Th2-like phenotype. These phenotypic changes were not observed after 24 h of starvation. In addition, the protein level of type IV collagen was elevated in the thymus of 48-h-starved mice, proposing that dietary-restriction-induced remodeling of thymic extracellular matrix represents a novel mechanism underlying acute fasting-induced immunodeficiency.

Key words thymus, malnutrition, collagen, extracellular matrix

INTRODUCTION

Malnutrition is a global challenge, affecting nearly 10% of the world's population.[1] It leads to a wide range of physiological impairments, including immunodeficiency. A vicious cycle between malnutrition and immunodeficiency is well recognized: immunodeficiency exacerbates malnutrition by increasing susceptibility to infections, which elevate energy demands and suppress nutrient intake. Malnutrition also induces functional decline in lymphoid organs such as the thymus, spleen, and lymph node.[2–5] In the thymus, nutritional deficiency causes structural alterations and functional impairment, a phenomenon known as thymic atrophy.[4] Thus, thymic atrophy is widely used as an indicator of malnutrition.[6] Although both acute (two to three days of fasting) and chronic (20–40% reduction in food intake for several weeks) malnutrition result in thymic atrophy, the cellular characteristics of the atrophied thymus differ depending on the type of malnutrition.[7]

The thymus is a primary lymphoid organ essential for T cell development, where thymic progenitor cells differentiate into naïve helper T type 0 (Th0) cells. These cells emigrate from the thymus and subsequently differentiate into helper T type 1 (Th1) and 2 (Th2) cells depending on the exposed immunological stimuli at the periphery.[8] Although Th0 cells remain undifferentiated in the thymus, their phenotypic states are not static. Our previous studies demonstrated that during 48-h starvation-induced thymic atrophy, naïve helper T cells become biased toward a Th2-like phenotype, characterized by increased expression of Th2 cytokines such as *Il5*, *Il6*, and *Il10*.[9,10] These findings suggest that T cell properties are influenced by nutritional deficiency. However, the mechanisms underlying these starvation-induced phenotypic changes remain unknown.

The thymic microenvironment plays a critical role in T cell development. Structurally and functionally, the thymus consists of two distinct regions: the cortex and the medulla. In the cortex, cortical thymic epithelial cells (cTECs) mediate the positive selection of thymocytes expressing T-cell receptors (TCRs) with low-affinity for self-peptide-major histocompatibility complex. In the medulla, medullary thymic epithelial cells (mTECs) facilitate the elimination of autoreactive T cells. Thymic stromal cells, including cTECs and mTECs, produce extracellular matrix (ECM) components–such as collagen–that shape the thymic microenvironment.[11] Collagen not only provides structural support but also transmits biochemical signals that regulate cellular differentiation.

In the present study, we examined the temporal dynamics of the thymus during starvation. Consistent with our previous findings, 48-h-starved mice exhibited reduced thymic weight and increased expression of Th2 cytokine mRNAs, whereas these changes were not observed after 24 h starvation. These results indicate that the thymic atrophy develops between 24 and 48 h after the onset of starvation. Furthermore, we found that starvation selectively upregulated the expression of col-

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lagen type IV at both the mRNA and protein levels, and this increase correlated with thymic atrophy.

MATERIALS AND METHODS

Animals ICR male mice (8–12 weeks old) were housed in a temperature-controlled room in the Animal Facility in Kobe Pharmaceutical University under a 12-h light/dark cycle. All experimental procedures were conducted following the Guidelines for Proper Conduct of Animal Experiments of the Science Council of Japan, and all protocols were approved by the Kobe Pharmaceutical University Committee for Animal Care and Use.

Starvation Mice were housed in individual cages and acclimatized for at least one week. For starvation experiments, mice were transferred to clean cages and deprived of food pellets for 24 or 48 h. Water was accessible throughout the starvation period.

RNA Extraction and RT-quantitative PCR (RT-qPCR) Analyses Mice were anesthetized and perfused transcardially with phosphate-buffered saline (PBS; 137-mM NaCl, 8.1-mM Na_2HPO_4, 1.5-mM KH_2PO_4, and 2.7-mM KCl). Collected thymus samples were stored at −80°C. Tissues were homogenized in ice-cold Sepasol-RNA I super G (Nacalai Tesque Inc., Kyoto, Japan) using a Potter homogenizer, and total RNA was extracted and purified according to the manufacturer's instructions. Purified total RNA was reverse-transcribed into cDNA using ReverTra Ace reagent (Toyobo Co., Ltd., Osaka, Japan) according to the manufacturer's instructions. Prepared cDNA samples were subjected to quantitative PCR analysis using a CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories Inc., Hercules, CA, USA) and SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories). Primer sequences for detecting mRNAs encoding cytokines and prostanoid synthesizing enzymes have been published in the previous manuscript.[10] Primer sequences for detecting collagen mRNAs were as follows: *Col1a1* (F, 5′- *AGCACGTCTGGTTTGGAGAG* -3′; R, 5′- *GACATTAGGCGCAGGAAGGT* -3′); *Col1a2* (F, 5′- *GCTGGGAAACATGGAAACCG* -3′; R, 5′- *TCCTTTGTCACCTCGGATGC* -3′); *Col3a1* (F, 5′- *CTCCTGGTCTGCAAGGGATG* -3′; R, 5′- *TTTCCTGGAACTCCGTCAGC* -3′); *Col4a1* (F, 5′- *TTTGGCTCGCCACCATAGAG* -3′; R, 5′- *GCAGAGGCGAGCATCATAGT* -3′); *Col4a2* (F, 5′- *GAGGGCTCGGTTTTTACGGA* -3′; R, 5′- *ATCCGGGTGGATCGTTGATG* -3′); *Col4a3* (F, 5′- *CTCCAGGACCTATCGGAAAAGG* -3′; R, 5′- *ACCTCTGACACCGGGAAGAC* -3′); *Col4a4* (F, 5′- *AGACAGTGGCTCACCTGGA* -3′; R, 5′- *CCAGGTACACCATCCACACC* -3′); *Col4a5* (F, 5′- *GGCCTCAAGGCCCTCCT* -3′; R, 5′- *TACCTCCATTTCCAGGGGGT* -3′). *Rplp2* was used as the reference gene for normalization.

Preparation of Tissue Sections Mice were anesthetized and perfused transcardially with PBS. Thymus tissues were postfixed in 4% paraformaldehyde (PFA) in PBS at 4°C for 4 h and cryoprotected in 30% sucrose in PBS at 4°C overnight. Samples were embedded in O.C.T. compound (Sakura Finetek Japan Co. Ltd., Tokyo, Japan), and 24-μm sections were prepared using a cryostat (SLEE medical GmbH, Mainz, Germany).

Masson's Trichrome Staining Masson's trichrome staining was performed using the following protocol. All staining solutions were purchased from Muto Pure Chemical Co. Ltd. (Tokyo, Japan). Tissue sections were washed in tap water, immersed in mordant for 30 min, and stained with Weigert's iron hematoxylin for 20 min. After incubation in a second mordant for 30 seconds and washing in 1% acetic acid, sections were stained with 0.75% orange G for 1 min and Masson's solution B for 30 min. After an additional wash in 1% acetic acid, sections were incubated in phosphomolybdic-phosphotungstic acid solution for 30 min and stained with aniline blue solution for 30 min. Sections were then dehydrated through graded ethanol (75%, 85%, 95%, 100%), cleared in xylene, and mounted with Entellan New (MilliporeSigma, Burlington, MA, USA).

Fluorescent Immunostaining Fluorescent immunostaining was conducted as follows. Tissue sections were fixed in 4% PFA in PBS for 5 min, washed briefly in PBS, and subjected to antigen retrieval in Tris-EDTA buffer (10-mM Tris, 1-mM EDTA, pH 9.0) at 65°C for 40 min. After cooling to room temperature, sections were washed in PBS three times and permeabilized in 0.3% Triton X-100 in PBS for 10 min at room temperature. Following three washes in PBS, sections were blocked in 0.5% fetal bovine serum in PBS for 30 min at room temperature. Sections were incubated with an anti-Collagen IV antibody [EPR22911-127] (ab256353, Abcam, Cambridge, UK) at 4°C overnight. After washing with PBS three times, sections were incubated with an Alexa488-conjugated anti-rabbit IgG antibody (711-545-152, Jackson ImmunoResearch, West Grove, PA, USA) diluted in 0.5% fetal bovine serum in PBS for 2 h at room temperature. After washing with PBS three times, sections were mounted with Fluoromount-G (Thermo Fisher Scientific, Waltham, MA, USA).

Image Acquisition Images were acquired using a BZ-X800 microscope (Keyence Co., Osaka, Japan) with a 20× objective lens for fluorescent immunostaining and a 10× objective lens for Masson's trichrome staining. Images were processed using Fiji, an open-resource software for manipulating images. For quantification of cortical collagen IV signals, fluorescence intensity was measured from four randomly selected ROIs per thymic section, and the mean value was calculated.

Statistical Analysis Results were represented as dot plots with mean values. Statistical significance was assessed using paired t-test (Fig. 1A) and Tukey-Kramer test (Fig. 1B-D, Fig. 2, Fig. 3B and Fig. 3D), as indicated in the figure legends. Statistical analyses were performed using R software. A p-value of < 0.05 was considered statistically significant.

RESULTS

Thymic Changes Were Induced by 48 h, but not 24 h, of Starvation Our previous study demonstrated that 48 h of starvation induces thymic atrophy, accompanied by disorganization of the cortico-medullary boundary and alteration in naïve T cell polarization in mice.[9] To further characterize the temporal changes in the thymus during starvation, we measured thymic weights in food-deprived mice. Body weight was significantly reduced after both 24 and 48 h of starvation (Fig. 1A). The degree of reduction increased in a time-dependent manner, with 24-h and 48-h starvation decreasing body weight by 12.1% and 20.2%, respectively (Fig. 1B). As previously reported, the absolute weight of thymus was significantly decreased after 48 h of starvation (Fig. 1C).[10] In contrast, 24 h of starvation did not significantly affect absolute thym-

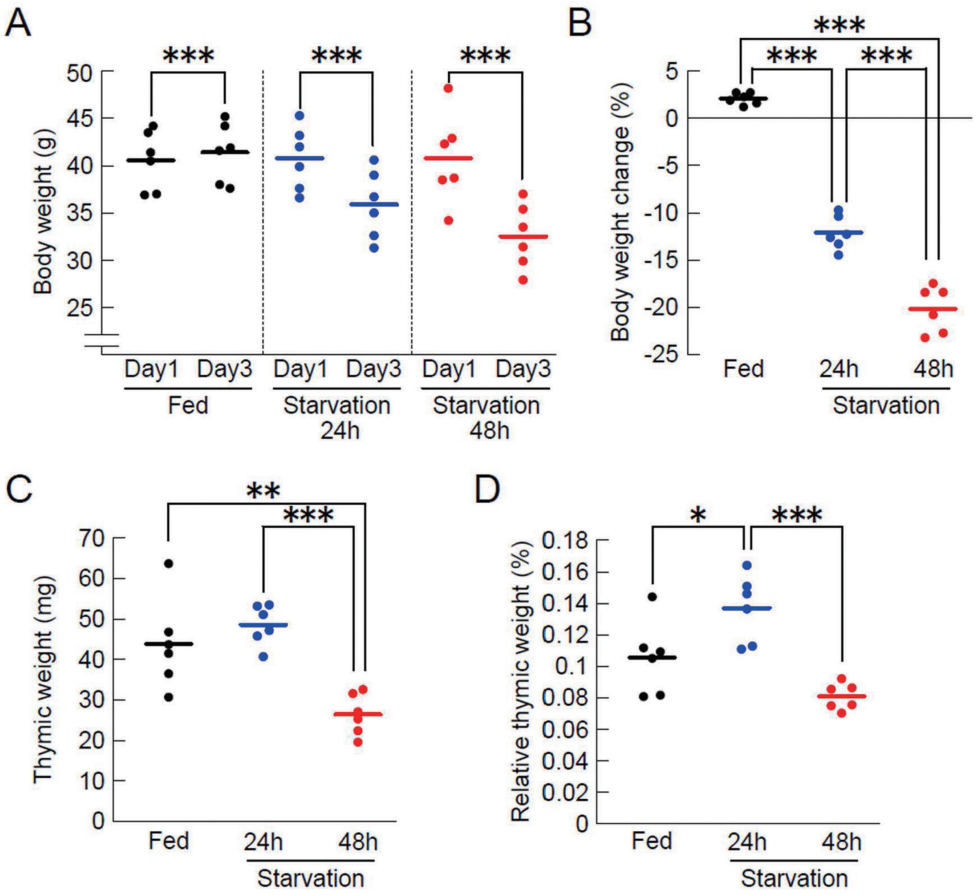

Fig. 1. Changes in the Thymic Weight by the Starvation.

A. Body weights of fed and starved mice at indicated time points. n = 6. ***$p < 0.005$ using Student's t-test. B. Body weight changes of fed and starved mice. n = 6. C. Absolute thymic weights of indicated mice. n = 6. D. Relative thymic weights of indicated mice. n = 6. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ using Tukey-Kramer test in (B) – (D).

ic weight (Fig. 1C). Consequently, thymic weight relative to body weight was significantly increased 24 h of starvation (Fig. 1D).

Our previous study also showed that 48-h starvation increases thymic expression of *Il5*, *Il6*, and *Il10* mRNAs–cytokines preferentially expressed by Th2 cells.[9] To evaluate the temporal dynamics of Th2 cytokine expression, we examined mRNA levels by RT-qPCR (Fig. 2A). Expression of *Il5*, *Il6*, and *Il10* mRNAs was significantly elevated after 48 h of starvation, consistent with previous results.[7] In contrast, the 24-h starvation did not significantly alter the expression of Th2 cytokines. The expression of interferon-γ (*Ifng*) mRNA, predominantly expressed by Th1 cells, was not significantly affected at either 24 or 48 h. This differs from our previous observations, potentially due to seasonal or environmental variations. These results indicate that 24 h of starvation is insufficient to induce polarization of thymic Th0 cells toward a Th2-like phenotype.

Because starvation-induced polarization of Th0 cells involves increased prostanoid synthesis,[9] we also examined temporal changes in the mRNA expression of prostanoid-synthesizing enzymes (Fig. 2B). mRNA expression levels of *Cox1*, *Cox2*, *Ptgds*, *Hpgds*, *Ptges1*, and *Tbxas1* were significantly increased after 48 h of starvation but were unchanged after 24 h. Together, these findings indicate that starvation-induced thymic alterations emerge between 24 and 48 h after the onset of dietary restriction.

Remodeling of Thymic ECM Was Induced by 48 h, but Not 24 h, of Starvation Substantial changes in tissue structure are often accompanied by remodeling of the ECM. Previous studies have shown that collagen, a major ECM protein family, plays important roles in T cell behavior and function.[12] This prompted us to examine changes in collagen expression and localization in the atrophied thymus. To assess starvation-induced collagen alterations, thymic sections were subjected to Masson's trichrome staining (Fig. 3A). In the thymus of normally fed mice and 24-h-starved mice, blue collagen signals were predominantly located in the medulla. In contrast, 48 h of starvation resulted in prominent collagen deposition in both the cortex and medulla, indicating that starvation notably increased cortical collagen contents.

Type I, III, and IV collagens are known to be major ECM components in the thymus.[11,13] To identify which collagen subtypes were altered in the cortex during starvation, we examined the mRNA expression levels of *Col1a1, Col1a2, Col3a1, Col4a1, Col4a2, Col4a3, Col4a4,* and *Col4a5* using RT-qPCR (Fig. 3B). Expression of *Col1a1, Col1a2,* and *Col3a1* was slightly reduced after 24 h of starvation, but no significant differences were observed between normally fed and 48-h-starved mice. In contrast, the expression levels of *Col4a1, Col4a2,* and *Col4a5* mRNAs were significantly increased after 48 h of starvation. *Col4a3 and Col4a4* mRNAs were not detected.

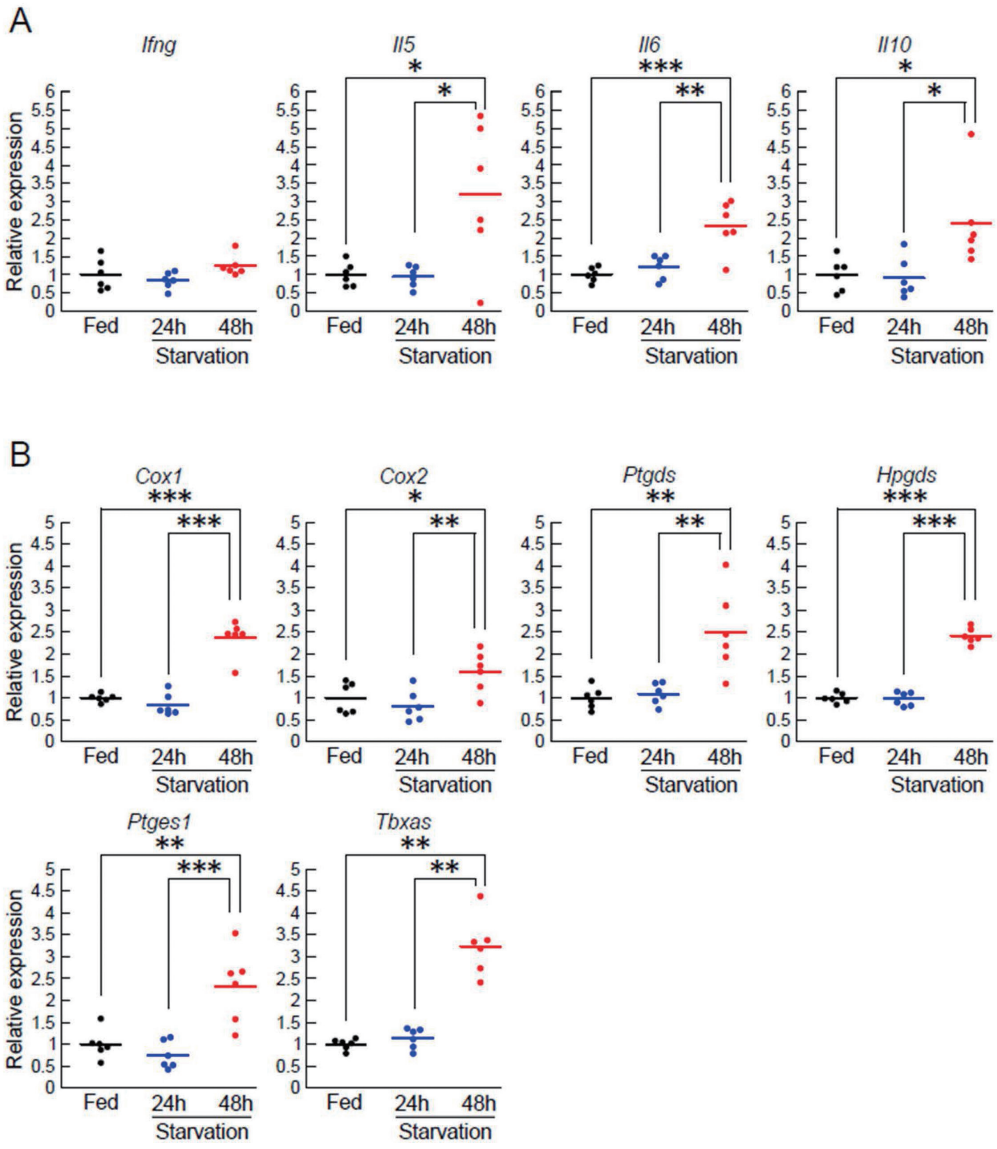

Fig. 2. Changes in Expression Levels of Cytokines and Prostanoid Synthesizing Enzymes in the Thymus by the Starvation.

A. The relative expression of mRNAs encoding cytokines, interferon-γ (*Ifng*), IL-5 (*Il5*), IL-6 (*Il6*), and IL-10 (*Il10*) in the thymus of indicated mice. B. The relative expression of mRNAs encoding prostanoid synthesizing enzymes, COX1 (*Cox1*), COX2 (*Cox2*), prostaglandin D synthases (*Ptgds*, *Hpgds*), prostaglandin E synthase (*Ptges1*), and thromboxane synthase (*Tbxas1*). n = 6. Each circle and bar indicate an individual animal and the average value of the group, respectively. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ using Tukey-Kramer test.

Given the selective upregulation of collagen type IV mRNAs by the starvation, the protein localization of type IV collagen was examined by fluorescent immunostaining of thymic sections using an anti-type IV collagen antibody. In the cortical region, type IV collagen signals were observed in perivascular areas, consistent with its localization in basement membranes (Fig. 3C). Fluorescence images showed a clear increase in type IV collagen protein following 48 h of starvation (Fig. 3C and D). Together, these findings indicate that starvation-induced remodeling of the thymic ECM, particularly the increase in type IV collagen–progresses between 24 and 48 h after the onset of food deprivation.

DISCUSSION

Acute fasting is well known to induce thymic atrophy. Our previous work demonstrated that 48-h starvation causes severe thymic shrinkage; however, it has remains unclear whether this reduction progresses in a linear or nonlinear manner. The present study shows that 24-h starvation does not elicit substantial thymic alterations–such as reduction in tissue weight, polarization of Th0 cells, or remodeling of the thymic ECM–whereas these changes become prominent after 48 h of starvation. These findings suggest that food deprivation-induced thymic atrophy progresses in a nonlinear fashion.

Our results further imply that starvation-induced thymic changes arise once nutritional deficiency reaches a critical threshold. Starvation elevates circulating corticosterone levels, and adrenalectomy ameliorates starvation-induced thymic hypocellularity and impaired T cell immunity, supporting a central role for corticosterone in this process.[4,14] In addition, dietary restriction promotes ketoacidosis through the accumulation of ketone bodies such as acetone, acetoacetate, and ß-hydroxybutyrate.[15] Whether these metabolic changes contribute to starvation-induced thymic atrophy warrants further investigation. In our model, starvation reduced body weight

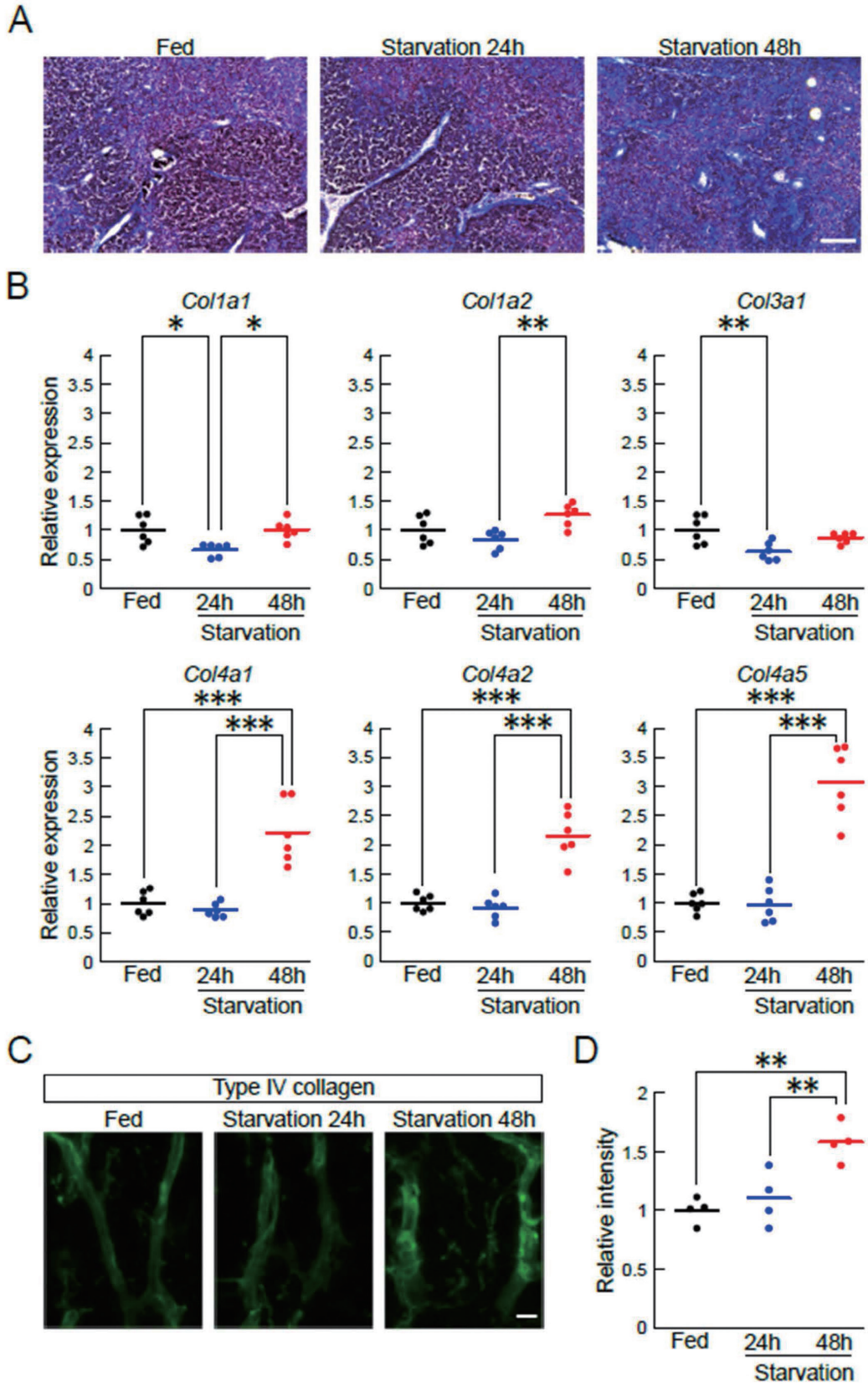

Fig. 3. Changes in the Thymic ECM by the Starvation.

A. Masson's trichrome staining images of thymic slices in indicated mice. Scale bar, 200 μm. B. The relative expression of mRNAs encoding type I collagen (*Col1a1, Col1a2*), type III collagen (*Col3a1*), and type IV collagen (*Col4a1, Col4a2, Col4a5*) in the thymus of indicated mice. n = 6. C. Fluorescence images of thymic slices from indicated mice which were immunostained for type IV collagen. Scale bar, 10 μm. D. Relative fluorescence intensity of type IV collagen. n = 4. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ using Tukey-Kramer test in (B) and (D).

by 20% within 48 h (Fig. 1B). In contrast, dietary restriction over two weeks decreases body weight by approximately 10%, after which it stabilizes.[5,7] Thus, dietary restriction model represents a model of chronic malnutrition, rather than acute fasting. Both starvation and chronic dietary restriction induces lymphoid organ atrophy and immunodeficiency.[5] T cell development is arrested at DN2 and DN4 stage due to the starvation and dietary restriction, respectively.[7] Chronic malnutrition, as well as resulting stunting, is associated with elevated blood cortisol levels,[16] raising the possibility that differential glucocorticoid responses underlie the distinct effects of acute versus chronic malnutrition on T cell development. Comparative analyses of corticosterone dynamics in these models will be important for testing this hypothesis.

This study also demonstrates that starvation-induced thymic atrophy is accompanied by remodeling of type IV collagen, whose expression was upregulated at both the mRNA and protein levels (Fig. 3). The expression of type IV collagen is regulated by diverse physiological cues. For example, aging reduces microRNA miR-29a, which in turn upregulates collagen IV.[17] Because aging leads to thymic involution, it will be informative to determine whether miR-29a expression is altered in malnutrition-induced thymic atrophy. Collagen IV protein abundance is also shaped by proteolytic degradation driven by matrix metalloproteinases (MMPs) and ADAM proteinases.[18] Given that MMPs are key mediators of tissue remodeling and closely associated with energy metabolism,[19] MMP-dependent turnover of collagen IV may contribute to malnutrition-induced thymic atrophy. Moreover, MMPs and ADAM proteinases participate in inflammatory responses,[20,21] and several inflammatory cytokines were elevated in the thymus of starved mice (Fig. 2). Thus, both transcriptional upregulation and altered proteolytic degradation may underlie the observed remodeling of type IV collagen during malnutrition.

Type IV collagen is a major structural component of the basement membrane and activates integrin-mediated signaling pathways that regulate T cell development, including modulation of TCR sensitivity and trafficking from the cortex to the medulla.[22,23] Therefore, the starvation-induced increase in type IV collagen may contribute to polarization of Th0 cells through integrin signaling. Although the causal relationship between type IV collagen upregulation and altered polarization of Th0 cells requires future validation, our findings highlight thymic ECM remodeling as a potential mechanism contributing to malnutrition-induced immunodeficiency. Beyond direct effects on developing T cells, type IV collagen may influence thymic function indirectly by modulating vascular integrity. Type IV collagen is essential for vascular homeostasis,[24] and thymic endothelial cells regulate the homing of hematopoietic progenitor cells.[25] Thus, upregulation of type IV collagen during malnutrition may also affect T cell development through vascular-dependent mechanisms. Because our study did not directly test the functional role of collagen IV in starvation-induced thymic atrophy and the current evidence is correlative, the causal contribution of ECM remodeling remains unresolved. Future studies should determine whether ECM remodeling, especially alteration in collagen IV, represents a common pathway driving thymic atrophy and involution triggered by a wide range of nutritional and physiological stresses.

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Conflict of interest The authors declare no conflict of interest.

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