

Wnt4 signaling is associated with the decrease of proliferation and increase of apoptosis during age-related thymic involution

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Abstract. The thymus is a central lymphoid organ that is responsible for T-lymphocyte development and maturation. Through negative and positive selection, lymphoid progenitor cells, which initiate from the bone marrow, develop into mature T cells in the thymus, and are subsequently involved in peripheral cell immunity. It has been reported that the Wnt signaling pathway exists widely in thymic epithelial cells and T lymphocytes. Wnt signaling affects the shape and function of thymic epithelial cells and has an important role in maintaining pro-T-cells, and in the subsequent T-cell differentiation. Previous studies have demonstrated that the Wnt signaling pathway participates in age-associated thymic involution. In the present study alterations in proliferation and apoptosis were investigated in murine thymic cells during aging. The results of the present study demonstrated that the aged thymus was characterized by markedly decreased cell numbers, as well as decreased proliferation and increased apoptosis. Concurrently, age-associated changes in thymic cell number and function were accompanied by a decrease in the transcription levels of Wnt4, and downregulation of forkhead box N1 and B-cell lymphoma-extra large, which are two target genes of the Wnt4 signaling pathway. *In vitro* studies demonstrated that activation of the Wnt4 signaling pathway promotes mouse thymus epithelial cell 1 (MTEC1) cell proliferation,

and that Wnt4 signaling modulation alleviates dexamethasone-mediated MTEC1 cell apoptosis. These results suggest that normal expression levels of Wnt4 have a critical role in maintaining the balance between cell proliferation and apoptosis. Alterations in the Wnt signaling pathway may disrupt the epithelial network structure of the thymus, eventually leading to microenvironmental damage. Therefore, further studies regarding the effects of the Wnt signaling pathway on thymus development and age-related thymic involution, may be beneficial for improving the health conditions of the elderly.

Introduction

The thymus is essential for a properly functioning immune response. The three-dimensional structure of the thymic meshwork comprises the cortex and medulla, which are predominantly composed of distinct developing T-cell subsets and thymic stromal cells (TSCs). TSCs are a diverse group of cells that includes cortical thymic epithelial cells (cTECs), medullary (m)TECs, mesenchymal cells, fibroblasts, pericytes, endothelial cells, and adipocytes (1). TECs provide essential microenvironments for the maturation of T cells. In addition, cTECs are responsible for the attraction of T-cell precursors, commitment of cells to the T-cell lineage, expansion of immature double-negative (DN) thymocytes, and positive selection of double-positive (DP) thymocytes (2). mTECs consist of a heterogeneous population of epithelial cells, which generate a suitable microenvironment for recently positively selected CD4 and CD8 single-positive thymocytes (3).

It is currently unclear whether thymic involution is initiated early on in life; however, thymic involution has severe negative effects on immune responsiveness with progressive age (4). Age-related thymic involution has been shown to be associated with reduced immune surveillance, increased risk of infection and cancer, vaccination failure, and delayed T-cell reconstitution in patients undergoing hematopoietic stem cell transplantations (5-7). The progressive loss of thymic function results in a decrease in adaptive immunity. The predominant causes of age-related thymic involution include: Reduced numbers of hematopoietic stem cells (HSCs), accompanied with intrinsic defects (8,9); reduced TECs and deterioration of the stromal microenvironment (10,11); and extrinsic circulating factors that affect the aged microenvironment, including alterations in hormones, growth factors and cytokines (12). Microenvironmental alterations in the aged thymus

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Abbreviations: TSCs, thymic stromal cells; TECs, thymic epithelial cells; cTECs, cortical thymic epithelial cells; mTECs, medullary thymic epithelial cells; DN, double negative; DP, double positive; GSK-3, glycogen synthase kinase 3; LEF, lymphoid enhancer factor; TCF, T-cell factors; Dex, dexamethasone; MACS, magnetic cell sorting; PCR, polymerase chain reaction; PI, propidium iodide; CT, threshold cycles; MTEC1, mouse thymus epithelial cell 1; SD, standard deviation; ETP, early thymocyte progenitors; LiCl, lithium chloride

Key words: age-associated thymus involution, thymic epithelial cells, thymocytes, Wnt4, cell proliferation, cell apoptosis

are considered to be the determining cause for defective T-cell development (13,14). During aging of the immune system, the gradual reduction in the number of naïve T cells is associated with a marked destruction of the epithelial network, including reduced TECs, increased fibroblasts, disrupted thymic perivascular space, and the increasing presence of adipocytes (10,15,16). Thymocytes are the major components of the thymic microenvironment in young individuals; however, adipocytes constitute the bulk of the aged thymic cellular space (15,16). As age increases, the thymus undergoes marked fibrotic and fatty alterations, which result in its transformation into adipose tissue.

Despite studies regarding thymic senescence, the molecular mechanism underlying thymic ageing remains to be elucidated. The formation of an appropriate thymic microenvironment is dependent on interactions between developing thymocytes and TECs, which is known as thymic crosstalk (17,18). Understanding the signaling mechanisms that regulate tissue development and maintenance of TECs may result in the identification of the process of thymic involution. Previous studies have demonstrated that maintenance and functional integrity of the thymic stroma requires stimulation via the Notch, bone morphogenetic protein, and Wnt signaling pathways (19-21). Numerous studies have reported the importance of Wnt signaling and Wnt proteins in the maintenance of various stem cell lineages. For example, Wnt3A deficiency has been shown to result in decreased numbers of HSCs in fetal liver, and decreased self-renewal capacity (22). In addition, the Wnt signaling pathway has been implicated in lineage-commitment and cell-fate regulation during development and aging (23,24). The majority of the members of the Wnt glycoprotein family, which currently consists of 19 molecules, have been implicated in the development of the embryonic thymus, and in the maintenance of adult thymic epithelium (25).

Wnt protein expression has previously been detected in thymocytes and TECs, and their receptors and associated regulatory molecules. In addition, TEC cell lines and primary TECs are both capable of responding to Wnt proteins *in vitro* (21,25-27). Canonical and non-canonical signaling pathways have been identified, which are initiated by various combinations of the Wnt/Frizzled complex. The most studied signaling pathway is the canonical Wnt pathway, which results in stabilization of β -catenin via inactivation of the 'destruction complex', which consists of adenomatous polyposis coli, axin, and glycogen synthase kinase 3 (GSK-3) (28). In the absence of Wnt signaling, GSK-3 phosphorylates axin, which culminates in the degradation of β -catenin (28). In the presence of Wnt signaling, GSK-3 activity is inhibited, resulting in the hypophosphorylation of axin, which protects β -catenin from degradation and allows it to accumulate (29). Stabilized β -catenin may then translocate to the nucleus where it targets the lymphoid enhancer factor (LEF)-1, as well as T-cell factors (TCF)-1, TCF-3 and TCF-4. The binding of β -catenin to LEF or TCF initiates the transcription of numerous genes, including forkhead box (FoxN1), B-cell lymphoma-extra large (Bcl-xL), axin, cyclin D1 and c-Myc (30). The thymic degeneration observed in response to transgenic Dickkopf Wnt signaling pathway inhibitor 1 expression is caused by a loss of TEC stem/progenitor cell maintenance or proliferation of an immature TEC subset (3). In addition, loss of Wnt signaling within

TECs results in a decrease in the K5K8DP TEC subset, which is localized at the cortico-medullary junction, and a reduction in the number of cycling TECs primarily within immature subsets (3). Downregulation of Wnt4 has previously been identified as a trigger for epithelial-mesenchymal transition and pre-adipocyte-differentiation during thymic involution (31).

The aim of the present study was to evaluate the role of the specific Wnt protein, Wnt4, in thymic involution. Wnt4 is one of the most abundant Wnt molecules present in the thymus, and it is produced by both thymocytes and TSCs (25). Previous studies have demonstrated that Wnt4 is responsible for the direct upregulation of FoxN1, which is associated with the differentiation of TECs and the subsequent maintenance of thymic epithelial identity (25). FoxN1 is an essential transcription factor that is required for adequate epithelial morphogenesis, and the capacity of TECs to attract lymphoid precursors from the bone marrow (32). Apoptosis inhibiting factor Bcl-xL is highly expressed in normal mouse thymocytes, whereas in Tcf-1 knockout mice Wnt4 signaling is suppressed, which leads to decreased expression of Bcl-xL and increased apoptosis in thymocytes (33). Exposure to glucocorticoids, similar to physiological aging, results in significant thymic senescence (34). Dexamethasone (Dex) treatment has been shown to trigger Wnt4 and FoxN1 downregulation, leading to the increased expression of preadipocyte-type differentiation markers lamina-associated polypeptide 2 and peroxisome proliferator-activated receptor γ (31,34). Furthermore, in a Wnt4-overexpressing TEPI cell line (35), Wnt4 overexpression effectively suppressed Dex-induced increases in adipocyte markers, and protected thymic epithelium-derived cells against Dex-induced senescence at the molecular level (36). Considering the vital role Wnt4 has in Dex-induced senescence, it was hypothesized that Wnt4 may regulate proliferation and apoptosis within the thymus, and contribute to age-related thymic involution. Using *in vitro* and *in vivo* approaches, the present study provided evidence that a decreased expression of Wnt4 in the aging thymus may be one of the molecular triggers underlying the process of age-related thymic senescence.

Materials and methods

Murine model. C57BL/6 mice were purchased from Charles River Laboratories, Inc. (Wilmington, MA, USA). The mice were 1 month old at the time of purchase, and were housed in the Laboratory Center Animal Ministry of Shengjing Hospital (China Medical University, Shenyang, China) until they had reached 5, 10, 15, or 20 months old. All of the mice were maintained in a specific, pathogen-free environment. There were six mice in each group, (male:female, 1:1) and they were maintained at 21~27°C with 40~70% humidity and a 12/12 h light/dark cycle. Animal procedures were performed according to the protocol approved by the Experiment Animal Center of China Medical University, in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (37).

Separation of thymocytes and TSCs. The mice were sacrificed with ether gas anaesthesia, then the pleura was cut open using eye scissors, the thymus was exposed and then it was removed with curved eye tweezers. Thymic lobes were rinsed

in sterilized phosphate-buffered saline (PBS) a number of times. The lobes were enzymatically digested using 1.5 mg/ml collagenase II (cat. no. 17101; Gibco Life Technologies, Grand Island, NY, USA), 1 mg/ml trypsin (cat. no. 25200-056; Gibco Life Technologies) and 0.2 mg/ml DNase I (cat. no. D5025; Sigma-Aldrich, St. Louis, MO, USA), and were incubated at 37°C for 30 min with intermittent agitation. The resulting single cell suspension was resuspended in buffer (2 mM EDTA in PBS with 0.5% bovine serum albumin; Gibco Life Technologies) and passed through a 100 μ m strainer (cat. no. 130-041-407; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) to remove any remaining undigested tissue. The cells were then purified using magnetic cell sorting (MACS) LS separation columns (Miltenyi Biotec GmbH). Cell suspensions were labeled with anti-CD45-fluorescein isothiocyanate (FITC) and washed with MACS buffer (Miltenyi Biotec GmbH), followed by incubation with anti-FITC microbeads (Miltenyi Biotec GmbH). Thymocytes (positively separated) and TSCs (negatively separated) were subsequently used for total RNA isolation, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis, and flow cytometric analysis.

Proliferation analysis. The mice were intraperitoneally injected with 10 mg/kg 5-ethynyl-2'-deoxyuridine (EdU; Invitrogen Life Technologies) in PBS. EdU is a nucleoside analog to thymidine, which is incorporated into DNA during active DNA synthesis. Detection is based on a click reaction, also known as a copper catalyzed covalent reaction, between an azide and an alkyne. After 24 h, the thymi of the mice were harvested and single-cell suspensions were purified using MACS, as mentioned above. Thymocytes were stained with CD4-allophycocyanin (APC) (cat. no. 100515; BioLegend, San Diego, CA, USA) and CD8-PerCP (cat. no. 100731; BioLegend) antibodies, whereas TSCs were labeled with EpCAM1-APC (clone G8.8) antibodies (cat. no. 118213; BioLegend). The cells were then prepared for EdU detection, according to the manufacturer's instructions (cat. no. C10425; Invitrogen Life Technologies, Carlsbad, CA, USA). The proliferation and apoptosis of the cells was analyzed using a flow cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA).

Apoptosis analysis. As described above, thymocytes and TSCs were separated using MACS. TSCs were labeled with EpCAM1-APC, whereas thymocytes were stained with CD4-APC and CD8-PerCP. For the early thymocyte progenitors (ETP), freshly isolated thymocytes were stained with phycoerythrin (PE)-conjugated lineage markers, as well as CD25-PE (cat. no. 101903) and CD127-PE (cat. no. 135009), CD117-APC (cat. no. 105811) and CD44-FITC (cat. no. 103021) (BioLegend). For the Annexin V apoptosis assay, the cells were stained with Annexin V-FITC (cat. no. KGA107; Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Propidium iodide was used to exclude dead cells from the apoptosis assays.

RT-qPCR analysis. Total RNA was extracted from the separated thymocytes and TSCs using TRIzol[®] reagent (Invitrogen Life Technologies), and reverse transcribed using PrimeScript RT reagent kit (cat. no. RR047A; Takara Bio Inc., Otsu, Japan). For qPCR analysis, ABI 7500 system (Applied Biosystems Life Technologies, Foster City, CA,

USA) and SYBR[®] *Premix Ex Taq*[™] (cat. no. RR820A; Takara Bio Inc.) were used, with 2 μ l cDNA. The threshold cycles (CT) for three replicate reactions were determined using Sequence Detection System software (ABI 7500 software, version 2.3; Applied Biosystems Life Technologies), and relative transcription abundance was calculated following normalization with β -actin. The primer pairs used were: FoxN1, forward 5'-TTCCTCAAGGGCAACCAC-3', reverse 5'-CCCATGTCCACAGGGATC-3'; Wnt4, forward 5'-CTCAAAGGCCTGATCCAGAG-3', reverse 5'-TCACAGCCACACTTCTCCAG-3'; Bcl-xL, forward 5'-GCGTGGAAA GCGTAGACAAGGAGATG-3', reverse 5'-ACTGAAGAG TGAGCCCAGCAGAACC-3'; and β -actin, forward 5'-GCT GTCCTGTATGCCTCT-3', and reverse 5'-ATGTCACGC ACGATTTCC-3' (Takara Bio Inc.). The cycling conditions were as follows: Initial denaturation at 95°C for 30 sec, 45 cycles of 95°C for 5 sec and 60°C for 20 sec.

MTEC1 cell stimulation. The mouse thymus epithelial cell 1 (MTEC1) cell line originated from a mouse thymic medulla cell line established by the Department of Immunology, Peking University Health Science Center (China). The cells were cultured in medium (Gibco Life Technologies) supplemented with 10% fetal bovine serum (Gibco Life Technologies) and plated in 24-well cultures to ensure that total cell density (5×10^6) was uniform between the compared samples. The cell lines were separately treated with 30 mM LiCl (cat. no. 213233; Sigma-Aldrich), 10^{-5} M Dex (cat. no. D4902; Sigma-Aldrich) or 500 ng/ml mouse recombinant Wnt4 protein (cat. no. 475-WN; R&D Systems, Inc., Minneapolis, MN USA). For some experiments, the cells were co-cultured in 10^{-5} M Dex and 500 ng/ml recombinant Wnt4 protein. The control groups of all of the experiments were cultured in common cell medium. After 24 h, the cells underwent total RNA isolation and flow cytometry.

MTEC1 cell investigation. Following the treatments previously described, the MTEC1 cell line was investigated. For proliferation analysis, EdU was added to the cell culture medium at a final concentration of 50 μ M, and incubated for 2 h at 37°C. The cells were then harvested and EdU detection was conducted according to the manufacturer's instructions (cat. no. C10425; Invitrogen Life Technologies). For apoptosis analysis, MTEC1 cells were stained with Annexin V-FITC (cat. no. KGA107; Nanjing KeyGen Biotech Co., Ltd.). Propidium iodide (PI) was used to exclude dead cells from the apoptosis assays. To determine the mRNA expression levels of FoxN1 and Bcl-xL, RT-qPCR was performed as described above.

Statistical analysis. Data are presented as the mean \pm standard deviation. The differences between various experimental groups were compared using a Student's t-test with GraphPad Prism software, version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Significant downregulation of Wnt4 signaling is accompanied by age-related thymic involution. Given that the early stages

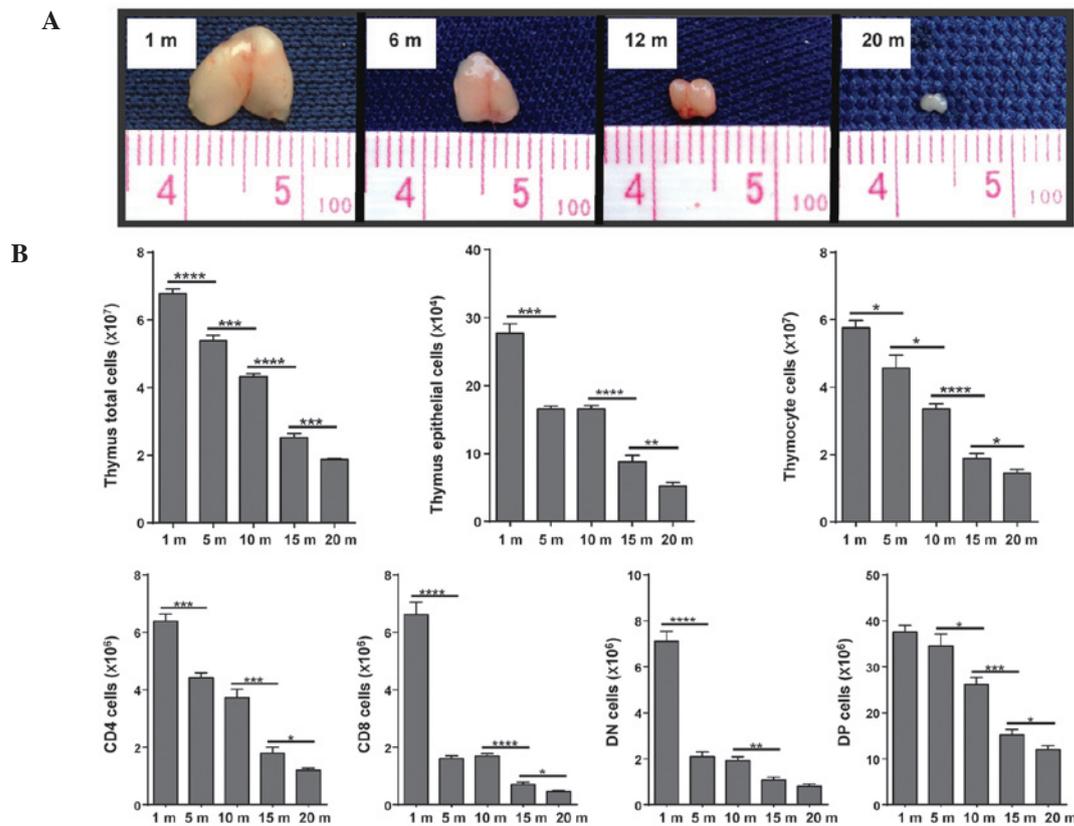


Figure 1. Changes in mouse thymic size and absolute numbers of thymic cells. (A) Comparison of C57BL/6 mouse thymic size at 1, 6, 12 and 20 months. (B) Absolute numbers of thymic total cells, thymic epithelial cells, thymocytes and CD4, CD8, double-negative (DN) and double-positive (DP) cells at 1, 5, 10, 15 and 20 months. Data are presented as the mean \pm standard deviation. Results are representative of six independent experiments (n=6). *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

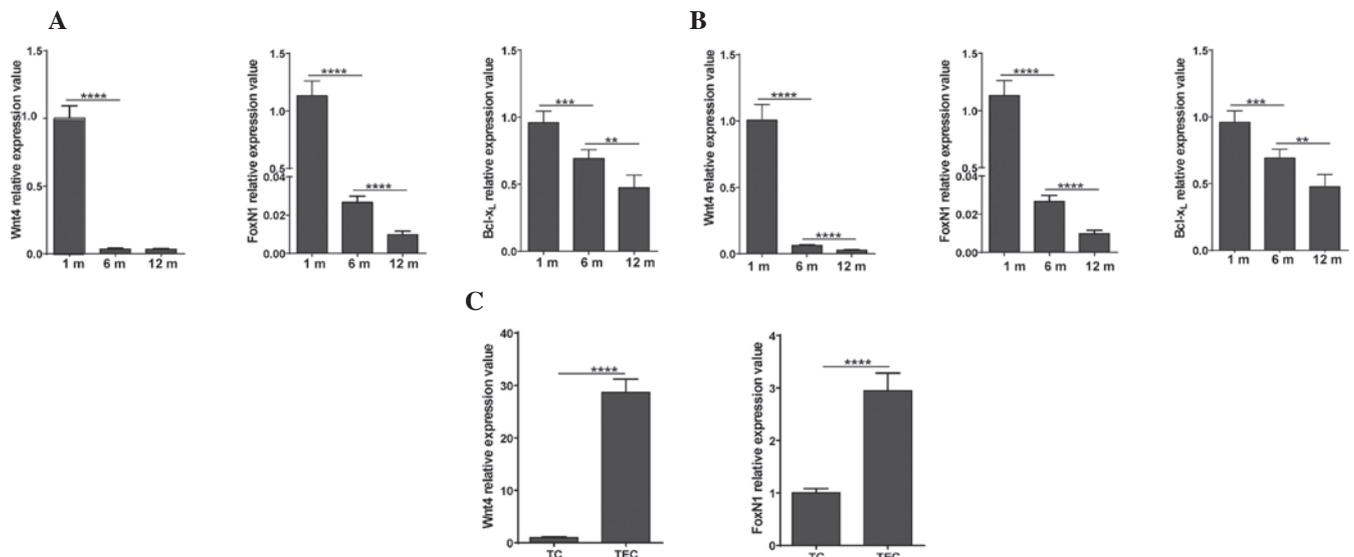


Figure 2. Age-related gene expression alterations in the thymus. Relative mRNA expression levels of Wnt4, forkhead box (Fox)N1 and B-cell lymphoma-extra large (Bcl-xL) in (A) thymic epithelial cells (TEC) and (B) thymocytes (TC) at various ages (1, 6 and 12 months), expressed as the mean $2^{-\Delta\Delta CT}$ standardized to the endogenous control β -actin. (C) Relative mRNA expression levels of Wnt4, FoxN1 compared between the TEC and TC of 1-month old mice. Data are expressed as the mean $2^{-\Delta\Delta CT}$ standardized to the endogenous control β -actin. Data are presented as the mean \pm standard deviation. Results are representative of six independent experiments (n=6). **P<0.01; ***P<0.001; ****P<0.0001.

of T-cell development are highly dependent on the availability of stromal niches (38), and since the number of TECs has been suggested to be the limiting factor in determining

thymic size (39), the present study measured the thymic size and the absolute cell numbers of the C57BL/6 mice at various ages (Fig. 1). To detect gene expression alterations, thymocytes

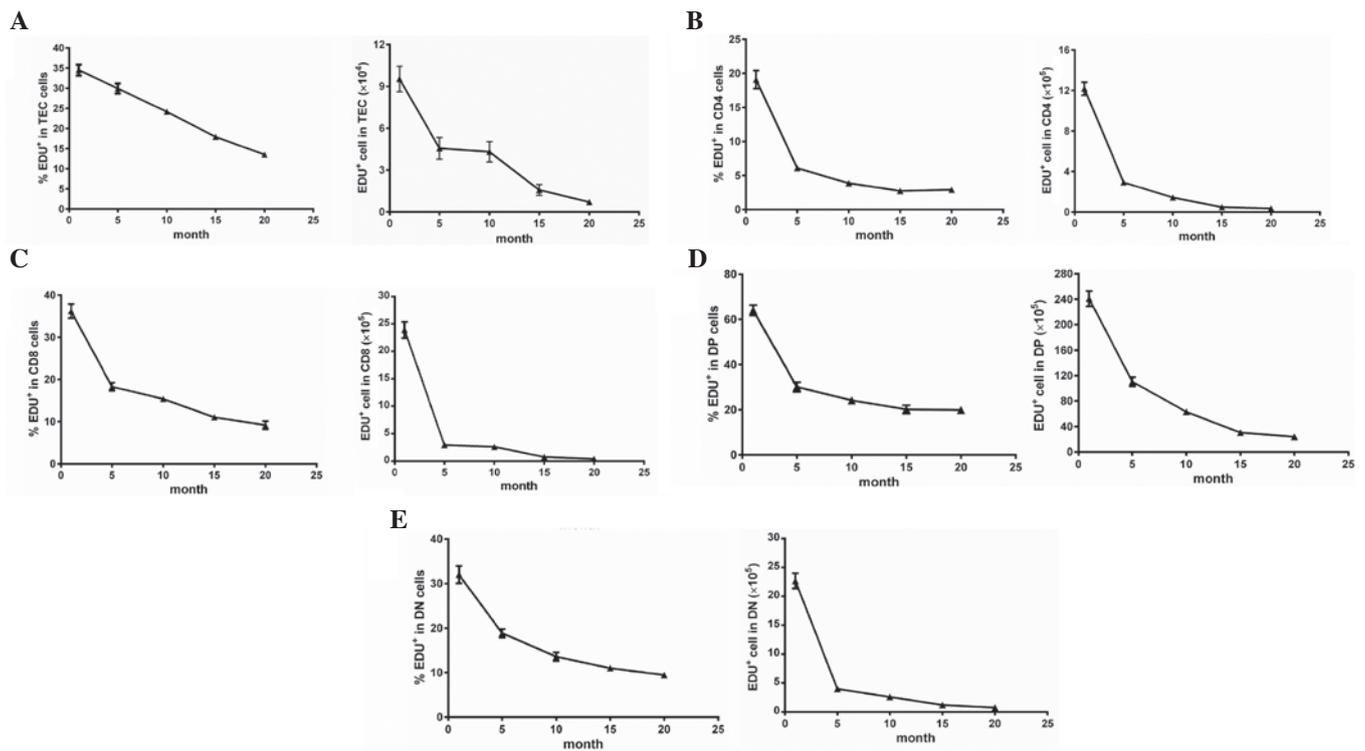


Figure 3. Percentage of proliferation in the thymus at various ages. 5-ethynyl-2'-deoxyuridine (EdU) flow cytometric analysis of proliferation and total number of various thymic cells. (A) Thymic epithelial cells (TECs), (B) CD4, (C) CD8, (D) double-positive (DP), and (E) double-negative (DN) cells. Results are representative of six independent experiments (n=6).

and TECs were purified from 1 month, 6 month and 1-year old C57BL/6 mice based on CD45 expression, and RT-qPCR analysis was performed. *Wnt4* signal-related genes, including *Wnt4*, *FoxN1* and *Bcl-xL* were detected. In addition, *Wnt4* and *FoxN1* mRNA expression was compared by RT-qPCR between the thymocytes and TECs of 1-month old C57BL/6 mice (Fig. 2).

Thymic size (Fig. 1A) and the absolute numbers of thymocytes and TECs (Fig. 1B) were significantly decreased as the age of the mice increased. Concurrently, the mRNA expression levels of *Wnt4*, *FoxN1* and *Bcl-xL* decreased in the same manner (Fig. 2A and B). Highly decreased levels of *FoxN1* may be caused by the strong downregulation of *Wnt4*, which occurred by the time the mice reached 1 year of age. Furthermore, *Wnt4* expression was almost 30 times higher in the TECs, as compared with in the thymocytes. Concordantly, the mRNA expression levels of *FoxN1* were also more abundant in TECs, as compared with in thymocytes. These results indicate that *Wnt4* is predominantly expressed in TECs, and changes in thymic size and absolute numbers of thymic cells are accompanied by a decrease in the transcriptional levels of *Wnt4*, as well as the expression levels of *FoxN1* and *Bcl-xL*, which are two target genes of the *Wnt* signaling pathway.

Decreased proliferation and increased apoptosis of thymic cells with age. To analyze the cause of the reduction in thymic cell number, the present study evaluated the proliferation and apoptosis of thymic cells at various ages. TSC composition, as well as organization, is severely disrupted with advancing age. In addition, naïve T-cell production is highest in young individuals and is reduced as thymic involution progresses with age. These

processes lead to a significant decrease in the absolute numbers of thymocytes and TECs. With advancing age, the thymus undergoes marked fibrotic and fatty changes, which culminate in its transformation into adipose tissue (40). A balance between cell proliferation and apoptosis can maintain a normal number of cells; therefore, the present study aimed to examine thymic cell proliferation and apoptosis at various ages.

Thymocytes and TSCs were separated from 1-, 5-, 10-, 15- and 20-month old C57BL/6 mice, based on CD45 expression. Based on the expression of CD4 and CD8, the proliferation of distinct developing T-cell subsets was determined by EdU-flow cytometric analysis. The proliferation of thymocytes and TECs continuously declined with advancing age (Fig. 3). In particular, the percentage of proliferation was markedly decreased between months 1 and 5, which may provide evidence suggesting that thymic involution is initiated early on in life. Furthermore, Annexin V analysis demonstrated that the apoptosis of TECs and TCs, including CD4, CD8, DP, DN and ETPs, was significantly increased in the aged thymi (Fig. 4).

Wnt4 signaling modulation alleviates Dex-induced MTEC1 cell apoptosis. Glucocorticoids are particularly effective immune suppressants, as they induce rapid peripheral T cell and thymocyte apoptosis, resulting in impaired T cell-dependent immune responses. Primary TECs express glucocorticoid receptors, and high-dosage Dex induces degeneration of the thymic epithelium within 24 h of treatment (37). The present study aimed to determine how exogenous glucocorticoids affect the thymic epithelial network, and if *Wnt4* has an important role in thymic senescence. The MTEC1 cells were treated with 10^{-5} M Dex, or co-cultured in 10^{-5} M Dex and

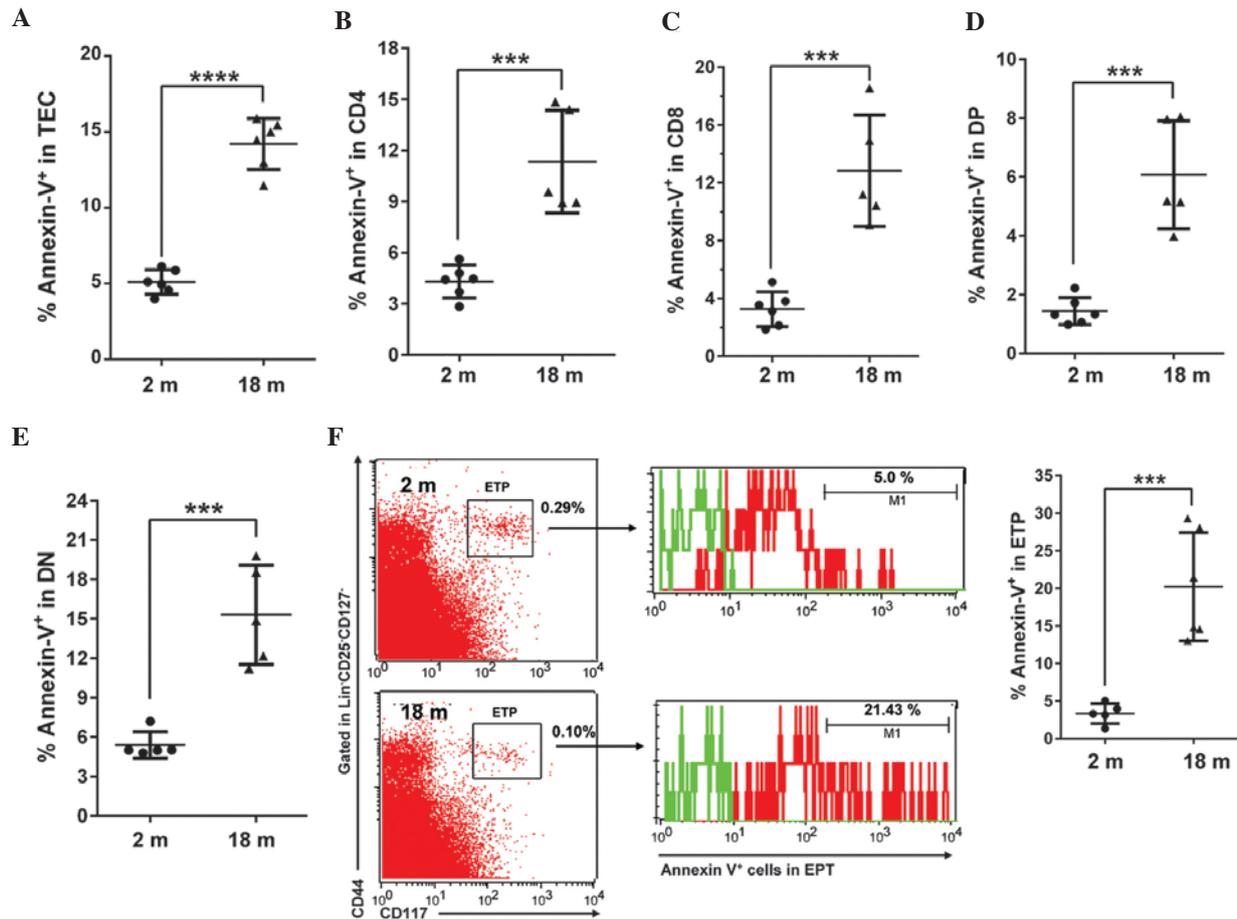


Figure 4. Percentage of apoptotic thymic cells at various ages. Flow cytometric analysis of thymic cells from 2- and 18-month old mice. Apoptotic cells were detected by annexin V and propidium iodide staining (A) Thymic epithelial cells (TECs), (B) CD4, (C) CD8, (D) double-positive (DP), (E) double-negative (DN), and (F) early thymocyte progenitors (ETP). Results are representative of six independent experiments (n=6). ***P<0.001; ****P<0.0001.

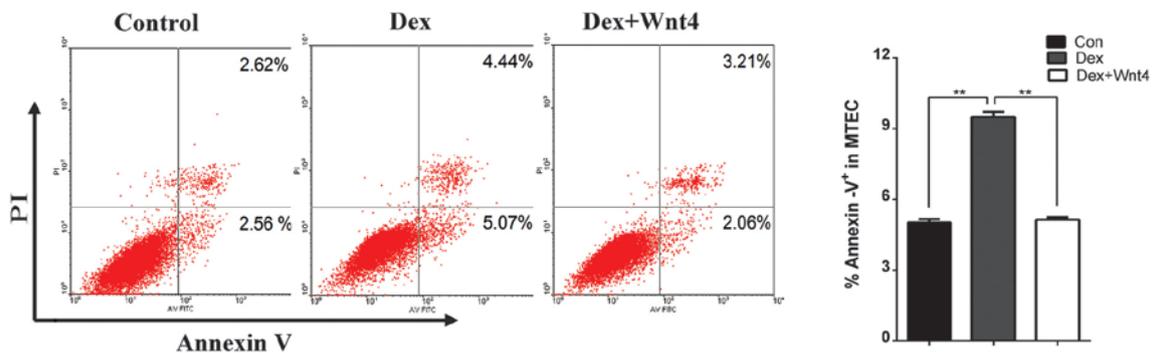


Figure 5. Effects of Wnt4 signaling on dexamethasone (Dex)-mediated mouse thymus epithelial cell 1 (MTEC1) cell apoptosis. The MTEC1 cells were treated with 10^{-5} M Dex, or co-cultured in 10^{-5} M Dex and 500 ng/ml recombinant Wnt4. After 48h apoptotic cells were detected by annexin V and propidium iodide (PI) staining. Numbers indicate the percentage of cells in each quadrant. Data are presented as the mean \pm standard deviation. Data shown are representative of at least three independent experiments. **P<0.01. Con, control.

500 ng/ml recombinant Wnt4. After 48 h, it was determined whether high glucose altered the rate of apoptosis of MTEC1 cells. As compared with the control group, Dex significantly promoted cell apoptosis, whereas recombinant Wnt4 attenuated Dex-induced cell apoptosis (Fig. 5).

Activation of the Wnt4 signaling pathway promotes MTEC1 cell proliferation. The biological role of Wnt4 in

Dex-mediated MTEC1 cell apoptosis was detected in the present study. To determine whether activation of the Wnt4 signaling pathway has a role in cell proliferation, the effects of LiCl and exogenous Wnt4 were determined. LiCl is an inhibitor of GSK-3 β (41), and thus prevents phosphorylation and degradation of β -catenin. MTEC1 cells were cultured in medium containing 30 mM LiCl for 24 h, cell proliferation was then examined using EdU, and the mRNA expression

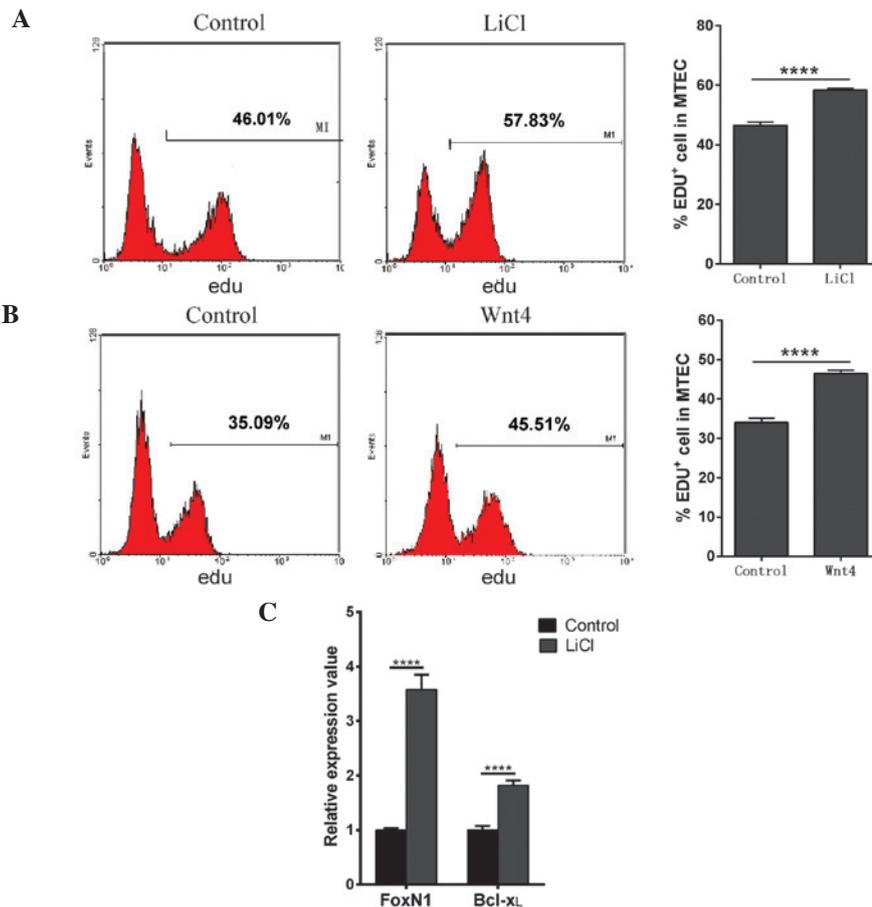


Figure 6. Effects of Wnt4 signaling activation on mouse thymus epithelial cell 1 (MTEC1) cell proliferation. (A) Percentage of cell proliferation after 24 h incubation in LiCl-supplemented medium, cell proliferation was detected using 5-ethynyl-2'-deoxyuridine (EdU). (B) Percentage of proliferation was quantified following treatment with 500 ng/ml exogenous Wnt4 protein. (C) Forkhead box (Fox)N1 and B-cell lymphoma-extra large (Bcl-xL) mRNA expression levels were detected using reverse transcription-quantitative polymerase chain reaction following cell culture in LiCl-supplemented medium. Data are presented as the mean \pm standard deviation. Data shown are representative of at least three independent experiments. **** $P < 0.0001$.

levels of Bcl-xL and FoxN1 were determined by RT-qPCR. Concurrently, to determine whether exogenous Wnt4 resulted in increased cell proliferation, MTEC1 cells were examined following incubation with recombinant Wnt4-supplemented media for 16 h. MTEC1 cells cultured in LiCl and exogenous Wnt4 exhibited $\sim 10\%$ increased proliferation, as compared with the control group (Fig. 6A and B). As expected, LiCl treatment alone resulted in an increase in FoxN1 and Bcl-xL mRNA expression levels (Fig. 6C). Notably, cell culture with medium supplemented with LiCl also resulted in increased proliferation, suggesting that activation of Wnt signaling is a critical step during MTEC1 proliferation, and is not solely limited to the expression of Wnt4.

Discussion

It has been demonstrated that high-dosage Dex induces degeneration of thymic epithelium within 24 h of treatment. In addition, the overexpression of Wnt4 can prevent the upregulation of adipose differentiation-related aging markers, thus suggesting an important role of Wnt4 in thymic senescence (37). The present study hypothesized that during age-associated thymic involution Wnt4 signaling is associated with thymic proliferation and apoptosis. The results of the

present study suggested that enhancing the canonical Wnt4 signal transduction pathway alleviates Dex-mediated MTEC1 cell apoptosis and promotes MTEC1 cell proliferation.

Thymic involution is believed to be initiated early on in life. In the present study, a progressive reduction in thymic size occurred from month 6, and almost complete thymic involution had occurred by month 20. TECs provide growth factors and signals that promote the migration and T lineage commitment of developing lymphocytes. To maintain normal function, the key thymic microenvironment is dependent on proficient cell numbers and normal cell function. By contrast, an imbalance between cell proliferation and apoptosis results in a marked reduction of thymic cell numbers. The present study demonstrated that the aged thymus is characterized by markedly decreased TEC numbers, as well as decreased levels of proliferation, and increased apoptosis of TECs. Notably, the proliferation rate of TECs plummeted from month 5, suggesting that a dysfunctional microenvironment contributes to abnormal thymic function. The lack of a normal thymic microenvironment also results in defective thymic lymphopoiesis in aged thymi. As compared with normal thymic lymphopoiesis in the young thymus, the number of thymocyte sub-populations, especially CD8 and DN, in the elderly mice was markedly reduced after 5 months, alongside decreased

proliferation and increased apoptosis. These results indicated that T-cell development or proliferation is defective in the aged thymus. With advancing age, normal functional thymocytes and TECs in the thymus are reduced, and adipocytes constitute the bulk of the aged thymic cellular space; the thymus undergoes striking fibrotic and fatty alterations that culminate in its transformation into adipose tissue.

It has been suggested that the absence of Wnt4 may suppress fetal and early postnatal thymic expansion, resulting in decreased TEC numbers, an alteration of the medullary-to-cortical TEC ratio, and a disproportionate loss of immature thymic precursors (42). The present study demonstrated that changes in thymic cell numbers and function are accompanied by a decrease in the transcription levels of Wnt4, as well as the downregulation of FoxN1 and Bcl-xL, which are two target genes of the Wnt signaling pathway. In addition, Wnt4 and FoxN1 were shown to be more abundant in TECs, as compared with thymocytes. A marked decrease of FoxN1 mRNA expression levels was detected in the aged thymus. FOXN1 is a key transcription factor that is responsible for the differentiation of TECs, and its decreased expression may be one reason underlying the reduced proliferation of TECs in elderly mice. Stabilization of β -catenin, which is a critical coactivator of TCF, enhances DP thymocyte survival via the upregulation of Bcl-xL. Spontaneous or glucocorticoid-induced thymocyte apoptosis has previously been shown to be associated with reduced levels of β -catenin and Bcl-xL (43). The results of the present study suggested that downregulation of Bcl-xL may result in increased apoptosis of thymocytes and TECs in elderly mice. Concurrently, activation of Wnt4 signaling was shown to result in increased FoxN1 and Bcl-xL mRNA expression levels. In order to further study the role of Wnt4 signaling in cell proliferation and apoptosis, *in vitro* studies were conducted. The results indicated that activation of the Wnt4 signaling pathway may promote MTEC1 cell proliferation, and alleviate Dex-mediated MTEC1 cell apoptosis. These findings suggested that normal expression level of Wnt4 have a critical role in maintaining the balance between cell proliferation and apoptosis.

In conclusion, based on the present study, it may be suggested that continuously decreasing proliferation and increasing apoptosis occurs during age-related thymic involution. In addition, an age-associated downregulation of Wnt4 may be responsible for the disruption between the balance of cell proliferation and apoptosis. Wnt4 and its downstream signaling pathways may therefore represent interesting candidates to improve thymic function in patients with thymic atrophy.

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