The aged thymus shows normal recruitment of lymphohematopoietic progenitors but has defects in thymic epithelial cells

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Abstract

Aging is associated with reduced numbers of all thymocyte sub-populations, including early T-cell progenitors. However, it is unclear if this is due to inadequate recruitment of lymphohematopoietic progenitor cells (LPCs) to the aged thymus or to abnormal development of T cells within the thymus. We found that LPCs from young mice were recruited equally well to the thymi of young or aged mice and that thymic stromal cells (TSCs) from young and old mice expressed similar levels of P-selectin and CCL25, which are believed to mediate recruitment of LPCs to the adult thymus. However, the number of recruited thymocytes in old thymus was markedly reduced after two weeks, indicating that T-cell development or proliferation is defective in the aged thymus. We also found that LPCs from aged and young mice have similar capacities to seed a fetal thymus that was transplanted under the kidney capsule. Thymic epithelial cells (TECs) in aged mice had lower proliferative capacity and higher rate of apoptosis, compared with findings in young animals. In addition, immunofluorescence staining with antibodies to cortical and medullary TECs revealed that aged thymus had a disorganized thymic stromal architecture, combined with reduced cellularity of the medulla, and apoptosis of thymocyte sub-populations in the medullary microenvironment was increased, compared with that in young mice. We conclude that aging does not impair recruitment of LPCs to the thymus, but is characterized by abnormalities in thymic epithelial architecture, especially medullary TEC function that may provide sub-optimal support for thymic development of LPCs.

Introduction

The thymus, a major T-lymphocyte generator, is mainly composed of T-cell precursors—thymocytes of hematopoietic origin and thymic stromal cells (TSCs) of non-hematopoietic origin, which are primarily thymic epithelial cells (TECs). Development and generation of functional T cells in the thymus depend on the cross-talk between thymocytes and TSCs (1, 2). During fetal thymic organogenesis, interactions between thymocytes and TSCs are essential for differentiation of TSCs from a two-dimensional cortical epithelium to a threedimensional cortical-medullary epithelium (1, 3). In the postnatal adult thymus, the fully developed three-dimensional TEC meshwork consists of two distinct anatomical regions, the cortex and the medulla, each containing specific TECs: cortical TECs or medullary TECs, respectively. These regions provide a unique microenvironment for lymphohematopoietic progenitor cell (LPC) thymic colonization, intra-thymic migration and development (4, 5).

During T-cell development, an important function of TSCs is periodical recruitment of bone marrow (BM)-derived LPCs from the bloodstream (6, 7). Although the mechanism of adult thymic recruitment of LPCs is unclear (8, 9), platelet (P)-selectin, expressed on the endothelium of thymic stroma, and platelet-selectin glycoprotein ligand-1 (PSGL-1), expressed on circulating LPCs (10, 11), are believed to contribute to...
this process. The chemokine, CCL25 (thymus-expressed chemokine), expressed on TECs (12), and its receptor, CCR9, expressed on multipotent progenitors (13), are also believed to regulate thymic colonization by LPCs (10).

The thymus undergoes age-related progressive involution with decreased thymic lymphopoiesis, reduced thymic size and disrupted thymic architecture. Thymocytes in the aged thymus have been shown to have altered proliferation and apoptosis (14). In addition, there are reduced numbers of all thymocyte sub-populations from early T-cell progenitors (ETPs) to double-negative (DN)2, DN3, DN4, double-positive (DP) and CD4 or CD8 single-positive (SP) populations (15–17). However, it is uncertain if some of these defects result from reduced recruitment of LPCs to the aged thymus. In addition, it is controversial if there are age-related changes of TSCs that may adversely affect thymocyte development. For example, the aged cortical epithelial region was generally thought to be shrunken, based on the morphologic observations of sections stained with hematoxylin and eosin (14, 18, 19). In contrast, flow cytometric analysis showed that only the number of medullary, but not cortical, cells declined with age (20). It is important to ascertain if altered TECs result in thymocyte defects, as it will enhance our understanding of the cellular mechanisms of age-related thymic involution and lay the groundwork for strategies to rejuvenate age-related T-cell immunodeficiency.

In the current study, we found that recruitment of LPCs from the bloodstream into the aged atrophic thymus was not impaired and that expression of P-selectin and CCL25, which are believed to mediate thymic LPC recruitment, was not reduced. Meanwhile, we also found that LPCs from aged mice can normally seed the young thymus in a system of kidney capsule transplantation. However, the aged thymus was characterized by decreased expansion of LPCs, altered thymic epithelial architecture, as well as decreased proliferation and increased apoptosis of TECs, suggesting that a dys-functional microenvironment may contribute to abnormal development of LPCs.

Methods

Mice

Aged (16–20 months old) C57BL/6 (B6) mice were purchased from the National Institute on Aging (Bethesda, MD, USA). Middle-aged (10–12 months old) and young (1.5–2 months old) mice were originally purchased from Jackson Laboratory (Bar Harbor, ME, USA) and bred in the University of Texas Health Center at Tyler (UTHCT) animal facility or were directly purchased from the National Institute of Cancer (Bethesda, MD, USA). We used B6 congenic mice expressing CD45.1 or CD45.2 on the cell surface. RAG-1−/−CD45.1 mice were generated from mating of RAG1−/−CD45.2 with CD45.1 wild-type mice (both were purchased from Jackson Laboratory) for at least three generations, and the RAG-1−/−CD45.1 mice were screened out by FACS assay. All animal experiments were done according to the protocols approved by the UTHCT Animal Research Committee in accordance with guidelines set forth by the National Institutes of Health.

Analysis of thymic recruitment of LPCs

BM cells from a pool of four to five CD45.1+ young mice were harvested by flushing the femur and tibia. RBCs in the BM were depleted with ACK lysing buffer (pH 7.2, 0.15 M NH4Cl/1.0 mM KHCO3/0.1 mM Na2EDTA) followed by a reaction with a biotin-conjugated cocktail of lineage antibodies, and then streptavidin beads (kit from BD Pharmingen, San Diego, CA, USA). The bead-labeled cells were removed by two rounds of negative selection through LS columns (Miltenyi Biotec, Auburn, CA, USA), and the efficiency of enrichment was checked by staining with CD117, followed by flow cytometric analysis. The lineage negative-enriched BM cells (∼1 × 10^5 per mouse) were intravenously (i.v.) injected through the retro-orbital route into sub-lethally irradiated (500 rads) CD45.2+ young and aged mice. Three, 7 and 10 days after injection, the recipient thymocytes were analyzed by staining with fluorescence-conjugated antibodies to CD45.1, a cocktail of lineage markers including CD3ε, CD8α, CD19, CD11b, B220, TCRβ, TCRγδ, Gr-1, NK1.1 and TER-119 (7, 21). CD44 and CD25. All antibodies were purchased from BD Pharmingen or BioLegend (San Diego, CA, USA).

Enrichment of adult TECs

The thymic sub-capulse was torn gently with forceps and shaken several times in cold PBS to remove thymocytes. The thymic sub-capsule was torn gently with forceps and then streptavidin beads (kit from BD PharMingen, San Diego, CA, USA). The bead-labeled cells were removed by two rounds of negative selection through LS columns (Miltenyi Biotec, Auburn, CA, USA). The bead-labeled cells were subjected to negative selection through LS magnetic columns to obtain TEC-enriched cells, which were used for flow cytometric assays of proliferation and apoptosis.

Semi-quantitative RT-PCR assay for expression of P-selectin

Total RNA (~0.35 μg per lane) from the TEC-enriched thymic tissue was isolated by Trizol (Invitrogen, Carlsbad, CA, USA). RT was done using the SuperScript III RT kit (Invitrogen). Semi-quantitative PCR was done using Taq DNA polymerase (Promega, Madison, WI, USA). The primers for P-selectin were (forward) 5’-GTGCAGAGCGGTCAAATGC-3’ and (reverse) 5’-CTGAGAGCTTCTTACGAGGC-3’ (22). The product length was 303 bp. PCRs were performed for 31 or 35 cycles at 94°C for 45 s, 58°C for 45 s and 72°C for 1 min. PCR products were resolved by 5% PAGE with TBE buffer and the densities of the PCR bands were measured with the VersaDoc imaging system and Quantity One software (Bio-Rad, Hercules, CA, USA). The ratios of the density of P-selectin bands to that of the control GAPDH bands from the same sample were compared.
Thymic lobes from RAG-kidney and a small hole was made in the kidney capsule. A small dorsolateral incision was made to expose the young (2 months old) and aged (17–18 months old) wild-type host mice. A small dorsolateral incision was made to expose the

Kidney capsule transplantation

Survival surgery was performed under sterile conditions after intra-peritoneal administration of the anesthetics, ketamine (100 mg kg⁻¹) and xylazine (10 mg kg⁻¹) to CD45.2 young (2 months old) and aged (17–18 months old) wild-type host mice. A small dorsolateral incision was made to expose the kidney and a small hole was made in the kidney capsule. Thymic lobes from RAG⁻/⁻/CD45.1⁺ donor mice at day 14 of embryonic development [embryonic day 14 (E14), the morning on which the vaginal plug was found was defined as day 0] were placed under the kidney capsule, and the incision was closed with sterile sutures. The grafted thymus was analyzed for CD45.2 (derived from recipients) and CD45.1⁺ (carried over from grafted thymus) cells on 4, 7 and 14 days after the transplantation.

Immunohistology and immunofluorescence staining

Serial 4-μm sections from paraffin embedded thymic tissue were cut and hematoxylin–eosin staining was performed (23). For immunofluorescence staining of frozen sections (24), serial 8-μm sections from optimal cutting temperature medium (OCT)-embedded frozen thymic tissue were fixed in acetone and incubated with optimal dilutions of anti-keratin-8 antibody (Developmental Studies Hybridoma Bank, Iowa City, IA, USA), which stains the cortex and anti-keratin-5 antibody (Developmental Studies Hybridoma Bank, Iowa City, IA, USA), which stains the medulla. Immunoreactivity was detected by fluorochrome-Cy3-conjugated (Jackson ImmunoResearch Laboratories) or AlexaFluor-488-conjugated antibody (Molecular Probes, Inc. product from Invitrogen) secondary antibodies.

Measurement of proliferation by 5-bromo-2-deoxyuridine incorporation and Ki67 staining

Mice were injected intra-peritoneally twice with the nucleotide analog 5-bromo-2-deoxyuridine (BrdU) (0.1 mg g⁻¹ body weight) in 0.2 ml PBS, with an interval of 1.5 h between injections; 1.5 h after the second injection, the thymus was collected and processed to enrich for TECs, as outlined above. The TEC-enriched preparation was treated with 2.4G2 hybridoma supernatant (from American Type Culture Collection) to block Fc receptors, and then labeled with antibodies to various cell-surface determinants, fixed and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate, pH 7.2. For the BrdU assay (25), the cells were treated with DNase I at 37°C for 15 min. A negative control (without DNase I treatment) was set up for each sample. The cells were then stained with FITC-conjugated anti-BrdU. For the Ki67 assay, after fixation and permeabilization, cells were stained with FITC-conjugated antibody to Ki67 and the negative control was stained with FITC–anti-mouse IgG1. The BrdU and Ki67 antibodies were obtained from BD PharMingen.

Analysis of apoptosis with the TUNEL assay for TECs and by Annexin V staining for thymocyte sub-populations

Methods for the TUNEL assay of TEC apoptosis are detailed in our previous paper (23). Briefly, TEC-enriched cells were stained with APC–anti-CD45 and PE–anti-MHC Class II, and then fixed and permeabilized as outlined above. Terminal deoxynucleotidyl transferase reactions were conducted with the in situ Cell Death Detection Kit (Roche, Boehringer Mannheim, Indianapolis, IN, USA). A negative control without the terminal deoxynucleotidyl transferase enzyme was included with each sample. For the Annexin V assay of thymocyte apoptosis, cells were labeled with antibodies to CD4 and CD8, or lineage markers, CD44 and CD25 (23), and then stained with AlexaFluor-488-conjugated antibody to Annexin V (Molecular Probes, Inc.).

Statistics

All data were analyzed with Prism software, using a two-tailed Student’s t-test.

Results

Kinetics of newly recruited lymphohematopoietic progenitors in young and aged thymi

To determine if recruitment of LPCs to the aged thymus is normal, we analyzed the kinetics of LPC seeding the thymi of young (6 weeks old) and aged (18 months old) mice. We i.v. injected equal numbers of lineage negative-enriched BM cells from young CD45.1⁺ mice into sub-lethally irradiated young and aged CD45.2⁺ congenic mice, and measured the absolute number of CD45.1⁺ cells in both thymi at 3, 7 and 10 days (Fig. 1A). The absolute numbers of donor cells were similar in young and aged host thymi 3 days after the injection. However, the number of donor cells in young thymi increased much more rapidly than in old thymi after 7 and 10 days (Fig. 1B, left panel). Two to three weeks after the injection, the number of donor cells in the young thymus was >47-fold of those in the old thymus (P < 0.05, Fig. 1B, right panel). These findings suggest that aging does not substantially impair recruitment of LPCs, but that T-cell development or proliferation in the aged atrophic thymus is defective (14).

Profiles of donor DN1 thymocytes in young and aged host thymi

To specifically evaluate the recruitment of LPCs in young and aged host thymi, we measured the percentages and...
number of donor DN1 thymocytes in young and aged host thymi at 3 and 7 days after injection of lineage negative-enriched BM cells from young CD45.1+ mice into sub-lethally irradiated young and aged CD45.2+ congenic mice. DN1 thymocytes are the earliest stage of newly recruited progenitors from blood. The percentage and absolute number of donor DN1 cells (CD45.1+, Lin-/C0 CD44$^{25}$/C0) in young and aged host thymi were similar at both time points (Fig. 2). We did not measure the number of DN1 cells at 10 days because they are likely to have divided by this point, and the numbers may not accurately reflect cell recruitment.

Expression of P-selectin and CCL25

As an alternative means to evaluate the capacity of the aged thymus to recruit LPCs, we analyzed the expression of molecules that mediate LPC recruitment to the thymus, P-selectin and CCL25. Because these molecules are not expressed on thymocytes, we used TEC-enriched thymic preparations, prepared as outlined in the Methods. Because antibodies to P-selectin are not suitable for western blotting of thymic cells, we measured mRNA expression. Semi-quantitative RT-PCR showed that mRNA expression of P-selectin was similar in young, middle-aged and old mice (Fig. 3A and B). CCL25 protein levels measured by western blot were higher in middle-aged and old mice, compared with young, newborn and fetal animals, but the differences were not statistically significant based on four independent experiments (Fig. 3C and D).

Kinetics of LPCs from aged and young mice seeding a fetal thymus

The data in Figs 1 and 2 indicate that there is normal recruitment of young LPCs to the aged thymus. To determine whether LPCs from aged mice have a defect in their capacity to seed the thymus, we evaluated the kinetics of LPCs from aged mice seeding a fetal thymus. We transplanted the fetal thymus from E14 of RAG$^{-/-}$CD45.1+ mice into young and aged CD45.2+ wild-type mice under the kidney capsule (two lobes per kidney). RAG$^{-/-}$ mice were used because they supply an intrinsic normal thymic stroma but have intrinsically defective hematopoietic cells, allowing LPCs from the wild-type donor to colonize and develop in the thymus. The absolute number per lobe and the proportion of CD45.2+ cells seeded from hosts to the grafted thymus were measured by a hemacytometer and FACS staining, 4, 7 and 14 days after the transplantation. LPCs from old and young mice showed similar capacity to colonize the fetal grafts at every time point, as measured by the
percentages (Fig. 4A) or absolute cell numbers (Fig. 4C). Although at the 14 days after the transplantation, the host-derived cell number in the grafts even higher in the aged hosts than in the young hosts (Fig. 4C, right panel), they became closer, 4–5 weeks after the transplantation (37). The gross appearance of the graft was also similar in young and old recipients (Fig. 4B). The results suggest that there is no intrinsic defect in the capacity of LPCs from aged mice to seed a young thymus.

Thymic architecture in young and aged thymi

Our current findings indicate that aged thymi have no defect in recruitment of LPCs, and aging LPCs have no defect in seeding young thymus. However, it is well known that aged...

![Graph showing percentage of CD45.1+ cells in DN1 population](image1)

![Graph showing total CD45.1+ DN1 cells per thymus](image2)

**Fig. 2.** Donor-derived DN1 cells in young and aged host thymi. CD45.1+ lineage negative-enriched BM cells were injected into five to six sub-lethally irradiated CD45.2+ young (squares with solid line) and old (triangles with broken line) mice. DN1 cells were identified by immunolabeling as LinCD44+CD25− cells. The percentage and number of donor-derived (CD45.1+) DN1 cells were measured in young and aged host thymi, 3 and 7 days after injection. Results shown are the mean ± SEM from five to six mice. The top panel shows the percentage of CD45.1+ cells in the DN1 population, expressed as a percentage of total DN1 cells. The bottom panel shows the absolute number of CD45.1+ DN1 cells per thymus. The percentages and number of the DN1 cells in young and aged mice were similar at both time points (*P* > 0.05).

**Fig. 3.** Expressions of P-selectin and CCL25 in TEC-enriched thymi. (A) A representative result of semi-quantitative RT-PCR results for P-selectin expression in TEC-enriched thymi from young, middle-aged and old mice. The PCR product from 0.35 μg of total RNA was loaded per lane. (B) The density of bands representing PCR product for P-selectin and GAPDH from five young, middle-aged and old mice were measured, using Bio-Rad Gel-Doc and Quantity One software. The means ± SEMs are shown. Differences between age groups are not statistically significant. (C) A representative western blot measuring CCL25 expression (50 μg of total protein per lane) in TEC-enriched thymic cells. (D) The density of bands representing CCL25 and GAPDH from four fetal (gestation of 18 days), newborn (NB), young, middle-aged and old mice were measured, using Bio-Rad Gel-Doc and Quantity One software. The means ± SEMs are shown. Differences between age groups are not statistically significant.
Fig. 4. Kinetics of LPCs from aged or young mice seeding a RAG−/− fetal thymus grafted under the kidney capsule. (A) Thymocytes from the grafted thymi under kidney capsules of aged (left panels) or young (right panels) mice, 4, 7 and 14 days after the graft. The dot plots show representative results. The numbers in each dot plot show the % mean ± SD of CD45.2+ cells from the numbers of animals in each group. (B) Gross appearance of the fetal thymus under the kidney capsules of old and young hosts, 4, 7 and 14 days after the transplantation. (C) Absolute number of CD45.2+ host cells (× 10^7 per lobe) in grafted thymi. These were measured 4 and 7 days (left panel) and 14 days (right panel) after transplantation of CD45.1+ E14 fetal thymic lobes (two lobes in each host) into young and aged hosts. Differences between age groups at all time points are not statistically significant.
thymi do not support normal T-cell development (14, 26). To begin to understand this dichotomy, we analyzed the architecture of young and aged thymi. Compared with thymi from young mice, staining with hematoxylin and eosin revealed that aged thymi were characterized by reduced size, an increased number of large cysts (Fig. 5A, red arrows) in the cortico-medullary junction, more irregular blood vessels (Fig. 5A, blue arrowheads) and decreased cellular density in the medulla. We next stained thymic frozen sections with antibodies to keratin 5, a marker of medullary TECs, and to keratin 8, a marker of cortical TECs. The aged thymus showed disorganized architecture, with a poorly defined boundary between the keratin 5+ medulla and keratin 8+ cortex (Fig. 5B, two right panels), markedly reduced numbers of keratin 5+ cells (Fig. 5B, middle left panels) and keratin 5+, keratin-8+ co-stained TECs which should be in the cortico-medullary junction (Fig. 5B, two right panels). The reduction in cellularity of the medullary region in the aged mouse was more marked in sections stained with the TEC-specific keratin 5 antibody than in those stained with hematoxylin and eosin (Fig. 5A and B, middle left panels).

Proliferation and apoptosis of TECs from young and aged mice
To begin to study functional aspects of TECs in the aged thymus, we evaluated proliferation and apoptosis. Thymic cells from BrdU-labeled young and old mice were enriched for TECs, as outlined in the Methods. We then gated on CD45+ MHC-II+ cells (TECs) and measured BrdU-positive cells. The percentage of BrdU+ TECs in the thymi of aged mice was reduced by approximately one-third, compared with those in their young counterparts (Fig. 6A, P < 0.01). Similar results were obtained when proliferation was evaluated by nuclear staining with Ki67 (Fig. 6B). In contrast to the reduced proliferation, the percentage of apoptotic TECs was increased approximately 3-fold in aged versus young mice, as measured by TUNEL+ cells (Fig. 6C, P < 0.01). These results suggest that thymic aging is associated with functional defects in TECs, which may reduce the capacity of the thymic microenvironment to support T-cell development.

Apoptotic rates of thymocyte sub-populations in cortex or medulla
Since the development and characteristics of thymocytes serve as good indicators of the function of epithelial compartments in the thymus (1, 27), we evaluated apoptotic rates in thymocyte sub-populations which are present in different TEC compartments. DN1 and DN2 cells are predominant in the cortex (28) and DN3 and DP cells migrate from the cortex to the medulla, while CD4+ and CD8+ SP cells predominate in the medulla (8). We found that the apoptotic rates in DN1 and DN2 cells from aged thymi did not show any differences compared with their young counterparts (Fig. 7, left panel). In contrast, the apoptotic rates in DN3, DP, CD4+SP and CD8+SP cells from aged thymi significantly increased compared with their young counterparts (Fig. 7). The results imply that the aged thymic medulla provides sub-optimal survival signals to thymocytes compared with their young counterpart, suggesting that aging is associated with a more severe stromal defect in the medulla than in the cortex.

Discussion
Because the thymus does not contain self-renewing stem cells, the post-natal thymus recruits BM-derived T-lymphocyte progenitors from the blood. Aging is associated with markedly decreased thymic lymphopoiesis, with reduced number of all thymocyte sub-populations, including ETPs (15–17). Although it is well known that defective development of T cells takes place within the aged thymus, it is unclear if there is also inadequate recruitment of LPCs to the aged thymus. Seeding of the thymus with LPCs is a periodic rather than continuous process that is believed to depend on the availability of a limited number of thymic stromal niches (6). In the young adult thymus, these niches are estimated to contain as few as 200 cells (29, 30) and to accommodate only ~100 new progenitors per day (31). Although thymi of old animals are small and atrophic, they can certainly accommodate this small number of cells. We found that LPCs were recruited equally well to the thymi of young and old mice (Fig. 1) and development of LPCs to DN1 thymocytes was similar in young and old mice (Fig. 2). This was not due to administration of inadequate numbers of LPCs, as we injected ~10⁶ Lin−-enriched BM cells into each mouse. Our findings indicate that aged thymi have adequate TSC niches to accommodate newly repopulating progenitors and that aging is not associated with a defect in recruitment of LPCs.

Chemokines play an important role in mediating LPC colonization in the fetal thymus (5, 9, 32, 33), but the mechanisms that govern this process in adults are uncertain. One study showed that recruitment of LPCs to the adult thymus depended on the interaction of P-selectin in the endothelium of thymic stroma and PSGL-1 ligand in lymphoid progenitors (11). A more recent report found that LPC expression of the chemokine receptor, CCR9, also contributed to this process (13). Therefore, the ligand of CCR9, CCL25, which is expressed on TECs (12), especially in the medulla (34), may also facilitate thymic recruitment (10). We found that expression of P-selectin and CCL25 was not reduced in the thymus of old mice (Fig. 3), providing additional evidence for the normal capacity of the aged thymus to recruit LPCs. Furthermore, when we transplanted a fetal thymus into the kidney capsule of old mice, LPCs from aged mice colonized the fetal thymus as well as their young counterparts (Fig. 4). These data agree with those of others (35) and indicate that aging LPCs have normal capacity to colonize the young thymus.

In contrast to the normal recruitment of LPCs to the aged thymus, the number of recruited thymocytes in old animals was markedly reduced after 2 weeks (Fig. 1), indicating that T-cell development or proliferation is defective in the aged thymus. In our experimental system, which involved injection of BM precursors from young mice, this is unlikely to have been due to an intrinsic defect in the T-cell progenitors. An alternative possibility is that the thymic microenvironment in old mice does not provide optimal support for development...
Fig. 5. Micro-architecture of cortex, medulla and cortico-medullary junction in young and aged thymi. (A) Two representative results of staining young and aged thymi with hematoxylin and eosin. Red arrows indicate large cysts and blue arrowheads show irregular blood vessels. (B) Two representative results of immunofluorescence staining with anti-keratin 8 (green) and anti-keratin 5 (red). The far left and middle left panels show representative staining results of the cortex and medulla, respectively, in young, middle-aged and aged (14 and 18 months old) mice, at ×20 magnification. The middle right and far right panels show staining with both antibodies of the cortico-medullary junction, at ×20 and ×40 magnification, respectively. Each experiment was repeated at least three times with similar results.
of LPCs, which normally enter the thymus at the cortico-medullary junction, then migrate to the outer layer of cortex and then to the medulla, where negative selection and TCR rearrangement occurs (28). We found that TECs in aged mice had reduced proliferative capacity and higher rate of apoptosis (Fig. 6). In addition, CD4\(^+\) and CD8\(^+\) SP thymocytes that predominate in the medulla showed higher rates of apoptosis in the thymi of aged mice than in those of young mice (Fig. 7), suggesting that aging is associated with reduced capacity of the medulla to support thymocyte survival. Furthermore, immunofluorescence staining with antibodies to cortical and medullary TECs revealed that aging was associated with a disorganized thymic stromal architecture, with a poorly defined boundary between the medulla and cortex, combined with markedly reduced cellularity of the medulla (Fig. 5). These findings support previous quantitative cytologic analysis with specific antibodies that identified reductions in medullary but not cortical TECs in aged mice (20). These abnormalities may disrupt normal LPC migration and thymocyte development within the aged thymus. We speculate that the age-related reduced medullary size and reduced functional capacity of medullary TECs may impair the quality of the negative selection that deletes self-reactive T cells, perhaps contributing to the increased susceptibility of the elderly to autoimmune diseases. For example, in animals lacking CCR7 or its ligand, thymocytes do not migrate to the medulla; there is an increased likelihood of developing autoimmune dacryoadenitis and sialadenitis (36).

In summary, we found that aging does not impair thymic recruitment of young LPCs, nor does aging LPC seeding young thymus. However, aging was associated with a disrupted thymic architecture, reduced medullary size and abnormalities in apoptosis and proliferation of TECs and
increased apoptosis of thymocyte sub-populations residing in medulla. These changes may provide sub-optimal support for normal thymic development of LPCs and may contribute to some of the immunologic abnormalities associated with aging.

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**Abbreviations**

BM  bone marrow  BrdU  5-bromo-2-deoxyuridine  B6  C57BL/6  DN  double negative  DP  double positive  ETP  early T-cell progenitor  E14  embryonic day 14  i.v.  intravenously  LPC  lymphohematopoietic progenitor cell  P  platelet  PSGL-1  platelet-select glycoprotein ligand-1  RT  reverse transcription  SP  single positive  TEC  thymic epithelial cell  TSC  thymic stromal cell  UTHCT  University of Texas Health Center at Tyler

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**Fig. 7.** Apoptotic rates of thymocyte subsets in the cortical or medullary microenvironment from young and aged mice. Freshly isolated thymocytes from young (~2 months old) and aged (~18 months old) mice were stained with cell-surface markers (PE-lineage cocktail antibodies, PE/Cy5-CD44 and APC–CD25 for the left panel; FITC–CD8 and APC–CD4 for the right panel) and AlexaFluor-488 Annexin V antibody. The results were analyzed in a FACS Calibur by electronic gating different sub-populations, and obtained in three independent experiments using a total of six young and six aged mice. The open bars represent % Annexin V-positive cells from young mice and the filled bars represent % Annexin V-positive cells from aged mice. The percent means ± SEMs are shown. NS = not significant.
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