Molecular characterization of the mouse involuted thymus: aberrations in expression of transcription regulators in thymocyte and epithelial compartments

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Abstract

Despite playing a critical role in the development of naive T cells, the thymus is involuted with age. Whether a single age-associated defect or multiple aberrations contribute to thymic involution remains controversial. Here, we determined molecular aberrations in the thymocyte and epithelium compartments of the aging thymus. We demonstrated that total thymocyte numbers declined with a stepwise kinetics; clear demarcations occurred at 1.5, 3, 12 and 22 months of age. By quantitative PCR, a 2.4-fold reduction in the copies of signal joint TCR-excised circle (sjTREC)/10⁵ thymocytes was first detected at 3 months; no further reduction observed thereafter. Nevertheless, the combined reductions in thymocyte numbers and sjTREC/10⁵ cells caused a 7-fold decrease in sjTREC/thymus by 3 months, 21-fold by 18 months and 72-fold by 22 months as compared to 1 month. We showed aberration in expression of E2A, a transcription regulator critical for TCRβ rearrangement. While E2A expression declined 3-fold by 3 months and 18-fold by 7 months, expression of LMO2, a negative regulator of E2A activities, increased 5-fold by 18 months. Interestingly, expression of pre-Ta and its transcriptional regulator HEB were not reduced with age. Furthermore, keratin-8 expression, specific for cortical thymic epithelium, declined 3-fold by 7 months and remained stable thereafter. In contrast, Foxn1 expression was reduced 3-fold by 3 months, 16-fold by 12 months and 37-fold by 18 months. IL-7 expression was not reduced until 7 months and reached 15-fold reduction by 22 months. Thus, the data demonstrate that thymic involution results not from a single defect, but culminates from an array of molecular aberrations in both the developing thymocytes and thymic epithelials.

Introduction

The thymus in mammals is a primary site for the development and output of the majority of naive TCRβ T cells that are essential for mounting immune responses against new antigens (1,2). Despite this critical immune function, the thymus undergoes physiological changes associated with aging that is termed thymic involution (3,4). Consequently, thymic involution precedes an array of immune dysfunction that manifests as increased susceptibility to infections, increased incidence of autoimmunity and poor response to vaccination due to loss of newly made naive T cells (2,3,5). Thus, understanding the mechanism that triggers age-associated thymic involution is critical for designing therapeutic approaches to ameliorate thymopoiesis in the elderly.

Using signal joint TCR-excised circle (sjTREC) as a molecular marker for naive T cells, it has been shown that the thymus remains functional through 60 years of age in humans, albeit with a significant reduction in thymic output of naive T cells (6,7). In mice, thymic reconstitution studies have suggested that the aged mouse thymus remains functional (8). Recently, we have also shown that the level of mouse sjTREC/thymus is reduced with age (9). These findings indicate that T cell development continues to exist; however,
age-associated aberrations in the process of T cell development results in decreased naive T cell output (6, 7). While certain TCR transgenic mice show no age-associated thymic involution, other TCR transgenic mice exhibit involution of the thymus with age (10–12). It was suggested that other events such as T cell commitment or some unidentified processes contribute to thymic involution (10). Evidence to support this notion is currently lacking.

It has been demonstrated that aberrations in T cell development, from the early double-negative (DN) to double-positive (DP) stages, reduce the total thymocyte numbers and thymic cellularity (13). The DN thymocyte population can be further divided into four distinct subsets based on the expression of CD44 and CD25 (14). The four DN subsets have been found localized in distinct sub-anatomical regions of the thymic cortex indicating that functional heterogeneity of the cortical thymic epithelial cells (TEC) has differential effects in the development of the DN thymocytes in each subset (15). In aged mice, there is an accumulation of the earliest DN1 (CD44+CD25−) cells, and a concomitant reduction in the number of DN2 (CD44+CD25+) and DN3 (CD44−CD25+) thymocytes (10,11). Because the DN2 and DN3 thymocytes actively proliferate, it was suggested that the decline of DN2 and DN3 cells contributes to the reduction in total thymocyte numbers observed in aged mice (10,11,16). The exact molecular mechanism for the arrest and accumulation of precursors at the DN1 stage is unknown.

The development of DN thymocytes in the thymus is tightly regulated by specific transcription regulators expressed at specific stages in the early developmental process (17–19). E2A and HEB, members of the basic helix-loop-helix (bHLH) transcription factor family, play a critical role in the development of DN thymocytes and the transition from the DN to DP (19). In E2A-deficient mice, early T cell development is arrested at the DN1 stage (20). This early developmental defect likely contributes to the significant decrease in thymocyte number observed in E2A-deficient mice (20). Recent studies have also demonstrated that HEB plays an essential regulatory role in the expression of pre-α (21), and E2A and HEB play a critical role in TCRβ rearrangement (22,23). Conversely, E2A and HEB functions are negatively regulated by the LIM only protein 2 (LMO2) (21,24). LMO2 has been shown to be expressed specifically at the DN1 and DN2 stages, and must be down-modulated and inactivated for continuing progression of the DN thymocytes toward the DP stage (21). This is further supported by the findings that LMO2 transgenic mice develop thymomas with the immature cells arrested at DN1 and DN2 stages (25). Whether perturbations in the expression pattern of the above-mentioned transcription regulators occur in the thymocyte compartment of the involuted thymus have not been investigated.

In addition to alterations in the thymocyte compartment, there is evidence that changes in the functions of the thymic stroma also affect T cell development (8,26). Various cytokine levels are affected in the aged human thymus (27,28). In aged mice, the IL-7 level is reduced; however, it is not clear whether the reduction is due to loss of the IL-7-producing TEC or to a decline in TEC functions (29). The Foxn1 (Whn) transcription factor is expressed by TEC and is required for TEC proliferation and differentiation during thymic organogenesis (30,31). Mutations in the Foxn1 gene in both humans and mice result in an atrophic condition and severe immune deficiency (30–32). Recent studies using chimeric mice generated from nuclear fusion of wild-type and nude mice further demonstrate that expression of Foxn1 is required for differentiation of all TEC subsets from a common thymic epithelial precursors (33). The timely expression of Foxn1 is also essential for the successful colonization of the thymic rudiment by bone marrow-derived precursors (30,31). Although a role of Foxn1 during thymic organogenesis is established (31,33), little is known of Foxn1 functions in the postnatal thymus. Whether perturbation in Foxn1 expression in the adult thymus is associated with thymic involution has not been investigated.

In this study, we determined whether aberrations in the expression of critical transcription regulators occur in the aging thymus. We have developed real-time quantitative RT-PCR assays to measure transcript levels of these factors in both thymocyte and thymic epithelial compartments. Our data demonstrate an array of aberrations in the expression of critical transcription regulators and suggest that thymic involution is not caused by a single age-associated defect but rather culminates from multiple aberrations in both the developing thymocytes and epithelium.

Methods

Mice

Female BALB/c mice, 1–27 months of age, were purchased from the National Institute of Aging (Harlan Sprague-Dawley, Indianapolis, IN). Mice were housed in specific pathogen-free conditions at Loyola University Medical Center animal facility. Mice from different age groups were housed in the same room and were allowed to acclimate for 7–10 days before experimentation. Mice handling and experimental protocols were conducted according to institutional guidelines for animal care and use to minimize environmental stress.

Isolation of genomic DNA and total RNA

Thymocytes were obtained from individual thymi by gentle teasing in RPMI 1640 (CellGro) supplemented with 10% FCS (Life Technology, Gaithersburg, MD) and 10% penicillin/streptomycin (Life Technology, Gaithersburg, MD) and 10% FCS (Life Technology). After depletion of thymocytes, the thymic stroma was agitated, rinsed with media and immediately processed for total RNA isolation. Genomic DNA was isolated from 1×10^6 thymocytes using 0.5 ml DNAzol in the presence of 5 µg of polyacryl carrier according to manufacturer’s instructions (Molecular Research Center, Cincinnati, OH). DNA was dissolved in 40 µl of 8 mM NaOH and neutralized with 1.2 µl of 0.1M HEPES. Total RNA from thymocytes (20×10^6 cells) and thymic stroma was processed immediately for total RNA isolation using Trizol reagent (Life Technology) in the presence of 20 µg/ml of glycogen (Ambion, Austin, TX) to facilitate precipitation and maximize total yields. RNA was dissolved in 25 µl of DEPC-treated H2O. Isolated total RNA was treated with DNA-free (Ambion) to remove contaminating genomic DNA as per the manufacturer’s instruction. Isolated total RNA was stored at −80°C until used.
cDNA synthesis

Single-strand cDNA was synthesized with 2–5 µg of total RNA (DNA-free) from either thymocytes and thymic stroma and random hexamers in a total volume of 33 µl using the First Strand cDNA synthesis kit (Pharmacia Biotech, Piscataway, NJ) according to manufacturer’s instructions.

Quantitative real-time PCR for sjTREC

Real-time PCR was performed with 80 ng of genomic DNA in a total volume of 20 µl of 1 × Platinum Quantitative PCR Super Mix-UDG (1.5 U Platinum Taq DNA polymerase, 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 3 mM MgCl2, 200 µM dGTP, 200 µM dATP, 200 µM dCTP, 400 µM dUTP and 1 U UDG) (Life Technology), 0.25 µM Rox (Life Technology), 1/40,000 SYBR Green (Molecular Probes, Eugene, OR) and 0.25 µM primers. PCR amplifications were performed in triplicate using a Perkin-Elmer Gene Amp 5700 sequence detector thermal cycler (Applied Biosystems). The thermal profile included 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, followed by 40 cycles of amplification consisting of denaturing at 95°C for 15 s and annealing/elongation at 60°C for 1 min. PCR data was analyzed using SDS software (Applied Bioscience). A PCR product of the rearranged signal joints of the δRec1 3’ sequence and sjα1c5 5’ sequence was cloned into the PCR3.1 plasmid and known concentrations of the plasmid were used to generate a standard curve. The standard curve consisted of the following concentrations: 100,000, 10,000, 1000 and 100 copies/µl. GAPDH levels in each sample were determined and were used to normalize DNA sample inputs. Each sample was performed in triplicate and the data expressed as number of copies of sjTREC/10^6 cells based on the mouse genome of 2.6 × 10^9 bp (i.e. 1 ng of genomic DNA is equal to 350 genomes or 350 cells).

Quantitative real-time RT-PCR

The conditions for quantitative real-time RT-PCR were similar to those described for sjTREC. Standard curves for target genes were performed in each experiment with concentrations as described above. cDNA samples were used at 1:4 or 1:8 dilutions (40–20 ng of total RNA). Each sample was performed in triplicate and the data were expressed as number of transcripts/µg of total RNA used for cDNA synthesis. A triplicate PCR reaction without cDNA or known DNA template was included for each set of primers in every experiment. Expression of GAPDH housekeeping gene was determined in each sample to correct for variations in RNA concentration.

Primers

Specific primer sequences were selected using the Primer Express program (Applied Biosystems) and sequences available from the NCBI database. Primers were synthesized by Life Technology. Primer sequences are presented in Table 1.

Statistical analysis

Mann–Whitney U-test, Tukey–Kramer and Kruskal–Wallis non-parametric ANOVA were performed.

Results

Production of naive T cells in the thymus is reduced with age

We first determined the kinetics of the decline in total thymocyte numbers in aging thymus. The total number of thymocytes was obtained from thymi of 110 mice ranging from 1 to 27 months of age. The data were collected from four or five experiments, each comprised of mice from several age groups. Although it is known that the total number of thymocytes declines with age, the kinetics of this reduction has not been thoroughly investigated. Rather than gradually declining with age, the loss of thymic cellularity appears to occur in a stepwise fashion (Fig. 1). There were significant differences in thymocyte numbers between 1 versus 1.5 months (P < 0.0001), 1.5 versus 3 months (P = 0.0009), 3 versus 12 months (P = 0.0001), 12 versus 18 months (P = 0.0080) and 18 versus 22 months (P = 0.0047). In contrast, there were no significant differences in thymocyte numbers among mice between the 3–7, 12–16 and 22–27 months age groups (Fig. 1). Next, we determined whether production of naive T cells changes as dramatically. We have developed a real-time quantitative PCR for the mouse sjTREC to measure thymic output of naive T cells. The results were expressed as copies of sjTREC/10^6 thymocytes and total sjTREC/thymus based on the total number of thymocytes obtained from each animal. In contrast to the multiple stepwise reduction seen with the total thymocyte numbers, a significant reduction in the levels of sjTREC/10^6 cells was observed only between 1 versus 3 months (P = 0.0357); the level of sjTREC/10^6 cells was not significantly different between 3 months and any other age groups up to 22 months of age (Fig. 2A). The total number of sjTREC/thymus was calculated to determine the relative levels of naive T cell output per thymus. The total numbers of sjTREC/thymus were different between 1 versus 3 months (P = 0.0179), 12 versus 18 months (P = 0.0286) and 18 versus 22 months (P = 0.0143) (Fig. 2B). We did not detect significant reductions in the total number of sjTREC in mice between 3, 7 and 12 months of age. Taken together, the data demonstrate (i) an aberration in TCR rearrangement, and (ii) that the reduction in the levels of sjTREC/10^6 cells and total thymocyte numbers are not directly correlated. This indicates that alteration in TCR rearrangement is not solely responsible for the reduced thymocyte numbers observed in aging mice. Conversely, the patterns of perturbation in total thymocyte numbers and sjTREC/10^6 cells suggest two independent events that contribute to the overall decline in naive T cell output in the aging thymus.

Changing profiles of the expression of pre-α, HEB, E2A and LMO2 in aging thymocytes

Because the reduction in sjTREC/10^6 thymocytes suggests aberrations in either TCRα and/or TCRβ rearrangement, we determined whether expression of pre-α and transcription regulators critical for TCR rearrangement is perturbed in thymocytes from the aging thymus. We first determined whether expression of pre-α is altered in aging thymocytes because it is required for TCRβ selection that subsequently leads to rearrangement of the TCRα genes (13,34). Surprisingly, the expression of pre-α was not reduced at
3 months, the age at which a significant reduction in the levels of sjTREC/105 cells was observed (Figs 2A and 3A). Furthermore, the pre-Tα expression remained constant from 1 to 23 months of age (Fig. 3A). We did observe fluctuations from 12 to 23 months; however, the differences were not significant. Expression of the bHLH transcription factor HEB, a transcription regulator of pre-Tα expression, corroborated the pre-Tα results because HEB expression displayed a similar fluctuation pattern (Fig. 3B).

We next determined expression of E2A, a transcription regulator that has been shown to play a critical role in TCRβ rearrangement (19). In contrast to HEB, expression of E2A was significantly reduced at 3 (P = 0.0179) and 7 (P = 0.0179) months (Fig. 4A). The levels of E2A transcript in the 7-, 12- and 18-month-old thymocytes remained low until 23 months, when we observed a reversed trend in the expression of E2A (P = 0.0087). In normal T cell development, expression of E2A increases as T cell differentiation proceeds from the early DN to DP stages, while the expression of LMO2 is concomitantly down-modulated (21). We determined that expression of LMO2 was not changed between 1 and 12 months mice; however, a 5-fold increase was observed at 18 months of age (P = 0.0052) (Fig. 4B). We also observed an increasing trend between 18 and 23 months of age (Fig. 4B). Thus, expression

**Table 1. Primer sequences for real-time quantitative PCR**

<table>
<thead>
<tr>
<th>Target genes</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. δRec-1</td>
<td>CAGGGAGAGATGGGCCCTCTCT</td>
<td>GAAGGCATAAACCGACACGAA</td>
</tr>
<tr>
<td>2. φα1</td>
<td>TCCTCCCCCAACAGTACGCT</td>
<td>GCCAACACCCAGCATTGGA</td>
</tr>
<tr>
<td>3. Pre-Tα</td>
<td>GGGCCAGCTTTGGCATACCCGA</td>
<td>CCTCGCTCATACCCATTGC</td>
</tr>
<tr>
<td>4. E2A</td>
<td>CATCGCCCAATCTCGGATGAT</td>
<td>GGCTGCTTGCTCGCCAGGCA</td>
</tr>
<tr>
<td>5. HEB</td>
<td>CTGAGGAAACCCGTGGATGAG</td>
<td>GCCACCCACAGAGTCACA</td>
</tr>
<tr>
<td>6. LMO2</td>
<td>GACCTTGGAGACTGACCGAT</td>
<td>TGCCCTTTTCGCGCACAA</td>
</tr>
<tr>
<td>7. Foxn1</td>
<td>TCTGCTGACGGACATCATC</td>
<td>GGCATAGTGAATCTCTCAGTATTC</td>
</tr>
<tr>
<td>8. IL-7</td>
<td>CTCCGGCAGATCCATGAAAG</td>
<td>GCTCGGCTGGCAATTG</td>
</tr>
<tr>
<td>9. Keratin-8</td>
<td>GTGAGGCCTGCTGAGTAT</td>
<td>TCATGAGCCCTCCACAATG</td>
</tr>
<tr>
<td>10. GAPDH</td>
<td>GTGAGGCCTGCTGAGTAT</td>
<td>GAAGGCATAAACCGACACGAA</td>
</tr>
</tbody>
</table>
of E2A and LMO2 is not only perturbed, but shows a reversed pattern of expression in the aging thymocytes. Figure 5 shows a summary of the perturbations in the expression patterns of pre-Ta, HEB, E2A and LOM2 in the aging thymocytes.

Changing profiles of the expression of keratin-8, IL-7 and transcription factor Foxn1 in the epithelial component of the aging thymus

The thymic epithelial component plays a crucial role in the development of thymocytes and aberrations in TEC functions are thought to contribute to the overall functional deterioration of the involuted thymus (27,35). After thorough depletion of thymocytes, thymic stroma was examined for expression of keratin-8, IL-7 and Foxn1. It has been shown that keratin-8 is associated predominantly with cortical TEC, thus its expression can be utilized to indirectly determine the proportion of cortical TEC in the thymic stroma (36,37). We demonstrated that there are non-coordinate effects of age on the expression of different genes by cortical TEC. Expression of keratin-8 gradually declined from 1–1.5 to 7 months of age with a significant reduction (3-fold) first detected by 7 months ($P = 0.0014$; 1 versus 7 months) (Figs 6 and 9). Beyond 7 months of age, keratin-8 expression remained relatively stable over most of the adult life (Figs 6 and 9). Nevertheless, there was a 6-fold reduction in keratin-8 expression by 22 months as compared to 1-month-old thymus (Figs 6 and 9). In contrast, IL-7 expression was reduced 1.5-fold at 7 months, 3-fold at 12 months and 15-fold at 22 months of age (Figs 7 and 9). Because the decline in IL-7 expression is parallel with the reduction in keratin-8 expression from 3 to 12 months, the reduced IL-7 expression could be due to a decline in the relative proportion of cortical TEC in thymic stroma, assuming that expression of keratin-8 is not altered with age. However, the subsequent 15-fold reduction in IL-7 expression observed at 22 months was likely due to a specific aberration in the regulation of IL-7 expression in aging TEC because expression of keratin-8 remained unchanged after 7 months of age (Figs 7 and 9).
Finally, we examined expression of Foxn1, a transcription factor that is required for the functional development of cortical TEC during thymic organogenesis (30,31). The level of Foxn1 expression was drastically reduced with age, and showed distinct stepwise declines between 1±1.5 and 3 months ($P = 0.0159$), 7 and 12 months ($P = 0.004$) and 12 and 18 months ($P = 0.0159$) (Fig. 8). We determined that there was a 3-fold reduction at 3 months, a 16-fold reduction at 12 months and a 37-fold reduction at 18 months (Figs 8 and 9). In contrast to IL-7, the first significant reduction (3-fold) in Foxn1 expression was observed at 3 months when expression of keratin-8 was not altered (Fig. 9). Similar to IL-7, the decline in Foxn1 expression between 3 and 7 months was parallel with keratin-8 expression (Fig. 9). The stable expression of keratin-8 after 7 months of age indicates there were no alterations in the composition of thymic stroma past 7 months of age. Thus, perturbations in Foxn1 expression observed at 12, 18 and 22 months were not due to the reduced numbers of cortical TEC as judged indirectly by keratin-8 expression or changes in the composition of thymic stroma at these ages, but rather reflected alterations in transcription of Foxn1 with age (Fig. 9).

Discussion

An essential role of the thymus is to produce TCR$\alpha$$\beta$ naive T cells for the peripheral T cell pool; however, the thymus has
been shown to deteriorate with age. Although a great deal of work has characterized the aged thymic architecture, the mechanism of thymic involution remains elusive, and little information on the molecular aberrations in the developing thymocytes and thymic stroma in the aged thymus is known.

The declining pattern of total thymocytes that we observed with age reveals a more complex process than previously appreciated. When we examined a large number of mice over a broad spectrum of ages from 1 through 27 months (110 mice), a stepwise decline was observed with distinct reductions at 1.5, 3–7, 12–16, 18 and 22–27 months (Figs 1 and 10). The kinetics reveal a much more complex process and suggest that the reduction in thymocyte numbers is caused not by a single defect, but rather culminates from an accumulation of multiple aberrations. It is less likely that the reduced thymocyte numbers were due to environmental factors rather than age differences for the following reasons. First, the data were collected from multiple experiments (four or five experiments), each comprised of mice from various age groups. Second, all mice were purchased from the same vendor. Third, all mice were housed in the same room for at least 7 days before tissues were collected for analysis. We have found that the acclimation period of 7 days shows no stress effect on the early B lineage cells and stem cells (P. L. White, unpublished observation). Furthermore, the small standard deviations of each age group indicate that there were no vicissitudes in the thymocyte number due to environmental stress in mice from different age groups.

The sjTREC levels have been shown to reflect thymic output of naive T cell in humans and mice (6, 9). In normal T cell development, the presence of sjTREC directly shows that a T cell has successfully rearranged TCR\(\beta\) genes, and indicates that the same T cell has rearranged TCR\(\beta\) gene and passed the \(\beta\) checkpoint (13). Thus, the levels of sjTREC on the per cell basis indicate the number of T cells with rearranged TCR\(\beta\). We detected a completely different declining profile in the levels of sjTREC/10\(^5\) thymocytes as compared to the reduction in thymocyte numbers. While there was a 2.4-fold reduction at 3 months, the levels of sjTREC/10\(^5\) thymocytes were stabilized between 3 and 22 months, indicating that after the initial reduction at 3 months, the levels of TCR\(\beta\) rearrangement were not further reduced with age (Fig. 10). In contrast, the total thymocyte numbers were significantly reduced at 3 months, and were further reduced at 12–18 and 22 months. The results demonstrate that the subsequent reduction in the number of thymocytes, particularly from 12 months onward, is not directly caused by perturbations in TCR\(\beta\) and \(\alpha\) rearrangements. However, the reduced levels of sjTREC/10\(^5\) cells together with the decline in total thymocyte numbers cause a 21- and 72-fold reduction in the levels of total sjTREC/thymus, or the relative number of naive T cells, at 18 and 22 months respectively (Fig. 10). Currently, we cannot rule out the possibility that the reduction in sjTREC/10\(^5\) cells together with some unknown defects contribute to the overall reduction in total thymocyte numbers. We caution that because the sjTREC assay was designed using the \(V_\beta\) pseudogene \(\delta\)Rec1, the dominant rearranged gene among three \(\delta\)Rec genes in mice (38), the calculated values could be underestimated. Whether the continuing presence of sjTREC in the aged thymocytes is associated with productive rearrangement of TCR \(V_\beta\) genes has not been investigated.

We addressed the possibility that reduction in sjTREC/10\(^5\) thymocytes at 3 months is due to an aberration in the rearrangement of TCR\(\alpha\) genes. Signaling through the pre-TCR that is comprised of TCR\(\beta\), CD3\(\varepsilon\) and pre-T\(\alpha\) is required for the induction of TCR\(\alpha\) gene rearrangements (34). We showed that there were no significant changes in the levels of pre-T\(\alpha\) transcripts in aging thymocytes. This is further supported by the finding that there was no reduction in the transcript levels of HEB, a transcription regulator that controls pre-T\(\alpha\) expression (21) (Fig. 10). Thus, it is unlikely alteration in pre-T\(\alpha\) expression is responsible for the reduction in levels of sjTREC/10\(^5\) cells or TCR\(\alpha\) rearrangements. However, we can not exclude the possibilities that aberrations in signaling via
the pre-TCR complex in the TCRβ selection process may contribute to the decline in TCRα rearrangements or that TCRα rearrangement in aged thymocytes is independent of the β-selection process.

Alternatively, the reduced number of sTREC/10^5 cells could result from the failure of thymocytes at the DN3 (CD44^+CD25^+) stage to rearrange TCRβ genes. It has been shown that E2A–HEB heterodimers are required for V(D)J rearrangement of the TCRβ gene locus (23). Furthermore, TCRβ rearrangement is dependent on two E-boxes present in the TCRβ gene enhancer (39) and expression of RAG-1 is induced by E2A gene products in a pre-T cell line (40,41). Our observation that the transcript levels of E2A are reduced at 3 months provides a potential mechanism to explain the reduction in the levels of sTREC/10^5 cells at this age. We suggest that the reduction in E2A expression could result in a decline in the rearrangement of TCRβ genes that, subsequently, could lead to the low levels of sTREC/10^5 cells and total sTREC/thymus observed in aged mice.

Previous work has shown that DN1 cells are accumulated in the aged thymus (10,11). DN1 thymocytes have been shown to express LMO2 and termination of its expression is critical for E2A gene products to function in the progression to the subsequent DN subsets (21). We found that expression of LMO2 gradually increased with age; 5- and 10-fold increases were observed at 18 and 22 months respectively (Figs 4B, 5 and 10). The increase in LMO2 levels could reflect the accumulation of DN1 thymocytes in the aged thymus. Alternatively, aberration in LMO2 expression could affect the transition from the DN1 to DN2 stage leading to the observed accumulation of DN1 cells. Lacorazza et al. have shown a 2.5-fold increase in the percentage of DN1 cells in 24-month-old mice (10); we observed a 10-fold increase in 22-month-old mice, suggesting that the increased LMO2 levels could result not only from the accumulation of DN1 cells but also from alteration in LOM2 expression in these aged DN1 thymocytes.

It is conceivable that alterations in the expression of E2A and LMO2 were due to the changes in the composition of thymocyte subsets in the aged thymus. However, previous work has shown that changes in the percentages of the DN, DP and single positive (SP) are negligible, indicating that the absolute number of thymocytes in each subset are proportionally reduced with age (11). Thus, changes in the expression of E2A and LMO2 were not likely due to depletion of a specific thymocyte subset. In addition, the genes that we have chosen to study are expressed exclusively in DN1 and DN2 cells (LMO2) or DN3 and DN4 cells (pre-α); E2A and HEB while are strongly expressed in DN thymocytes, particularly DN3 and DN4, are weakly expressed in the DP and SP thymocytes (21,42). Furthermore, because we did not detect significant changes in the expression of pre-α that is expressed exclusively in DN3 and DN4 thymocytes, we suggest that the changes in E2A expression are due to alterations in transcription of this gene in the aged DN thymocytes.

Earlier work showed dramatic alterations in thymic architecture and cellularity not only in the thymocyte compartment, but also in thymic stroma of the involuted thymus (8,35). Production of IL-7, an essential cytokine produced by cortical TEC, was found to be reduced in the aged thymus (43). However, it was not clear whether this reduction reflects reduced TEC numbers in the involuted thymus or a specific decline in TEC functions. Keratin-8 is predominantly expressed by cortical TEC; thus, the level of keratin-8 expression can be used as a measurement of the relative proportion of cortical TEC in thymic stroma (36,37). Although it is not known whether expression of keratin-8 is affected by aging, previous work has shown that its expression in the adult thymus is not sensitive to sex steroids (44). By comparing the changing patterns of keratin-8 and IL-7 expression in aging thymic stroma, it appeared that the degree of reduction of IL-7 from 7 to 18 months correlated with the reduction in keratin-8 expression (~3-fold reduction for both) (Fig. 9). Therefore, if the expression of keratin-8 reflects the relative proportion of cortical TEC at these ages, then the reduction in IL-7 expression is likely due to the reduced number of cortical TEC. However, while keratin-8 expression was only reduced 6-fold, the level of IL-7 transcript was reduced 15-fold by 22 months of age (Figs 6, 7 and 9). The delay in the reduction of IL-7 expression suggests that IL-7 is unlikely to be responsible for the first wave of the decline in total thymocyte numbers and aberration in TCRβ rearrangement at 3 months of age (Fig. 10). Conversely, the decline in IL-7 expression likely contributes to the decline in thymic cellularity at 22 months of age.

We demonstrated for the first time that expression of a transcription factor critical for TEC proliferation and differentiation is significantly reduced in the aged thymus. The epithelium-specific transcription factor Foxn1 is essential for the functional development of cortical TEC during thymic organogenesis (30,31). In nude mice, mutation in the Foxn1 gene prevents normal development of the thymic anlage past day E11, leading to unsuccessful colonization of the thymic rudiment by bone marrow-derived precursors (30,31). Using chimeric mice generated from nuclear fusion of wild-type and nude mice, it was further shown that expression of Foxn1 is essential for proliferation and differentiation of all TEC subsets from thymic epithelial precursors (33). Although the mechanism by which Foxn1 regulates epithelial cell proliferation and differentiation is not known, the decline in Foxn1 expression may affect the proliferation and differentiation of epithelial precursors in the postnatal thymus. It was demonstrated that thymic epithelial precursors exist in the adult thymus (37), and enforced expression of cyclin D1 in the thymic epithelial precursors expands the epithelial precursor pool and increases proportionally the mature cortical TEC subset and thymopoiesis (36).

In addition to having direct effects on TEC proliferation and differentiation, aberrations in Foxn1 expression may affect TEC functions important for development of the DN and DP thymocytes. First, Lind et al. (15) have reported that differentiation of the DN thymocytes is achieved by the precise sequential migration of each DN subset to a specific anatomical region within the thymic cortex. It was suggested that this precise migration pattern is mediated by chemokines and is an integral component regulating DN differentiation (15,45). In nude mice, the chemokines SDF-1 and TECK are not detectable in the fetal thymic anlage, suggesting that Foxn1-driven differentiation of TEC is required for the expression of SDF-1 and TECK in the embryonic thymus (46). Consequently, it is
possible that reduced expression of Foxn1 in the postnatal thymus leads to down-modulation of SDF-1 and TECK expression which manifests as perturbations in the migrating patterns and differentiation of the DN thymocytes during the early phase of T cell development. Second, PD-1, a type I transmembrane protein of the Ig family with an immunoreceptor tyrosine-based inhibitory motif, has been shown to modulate positive selection of DP thymocytes in the thymus (47). Furthermore, PD-1-deficient mice spontaneously develop lupus-like autoimmune diseases as they age (48). Recently, PD-L1, a ligand for PD-1 (49), has been identified in the involuted thymus, and provide new insights into functions contribute to age-associated thymic involution. PD-1, a type I transmembrane protein of the Ig family with an immunoreceptor tyrosine-based inhibitory motif, has been shown to modulate positive selection of DP thymocytes in the thymus (47). Furthermore, PD-1-deficient mice spontaneously develop lupus-like autoimmune diseases as they age (48). Recently, PD-L1, a ligand for PD-1 (49), has been identified in the involuted thymus, and provide new insights into functions contribute to age-associated thymic involution.

In summary, the data present an array of aberrations in the expression of critical transcription regulators that control early T cell development and TEC functions in this process. The new findings, while underscoring the complexity of thymic involution, help to identify crucial factors and regulatory stages affected in the involuted thymus, and provide new insights into the aging mechanism of this organ.

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Abbreviations

bHLH basic helix-loop-helix
DN double negative
DP double positive
LMO2 LIM only protein 2
sJTREC signal joint TCR-excised circle
SP single positive
TEC thymic epithelial cells

References


