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Research article

The unilateral involution in the thymus of a 96-year-old male leads to the preservation of structural integrity in one thymic lobe, as assessed by the expression of medullar and cortical antigens and the presence of CD3+ cells

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ABSTRACT

The process of thymic involution begins soon after birth and continues through adult life. Although evolutionary conserved in all vertebrates, the thymic involution has no defined kinetics. Little is known about the pace of its regression in humans, except that there is a marked increase of thymic involution after puberty. This report describes the unusual structural findings in the thymus of a 96-year-old male. The morphological parameters of the organ were evaluated using H&E and immunohistochemistry (IHC) techniques. The macroscopic examination showed a typical organ's weight and size, except that the right thymic lobe presented a well-preserved organ and the left lobe was significantly adiposed. The H&E staining of the thymic sections from the left and right lobes confirmed advanced thymic adiposity in the left lobe and preserved thymic epithelial space containing hematoxylin-stained cells in the right lobe. The multiplex immunostaining of the right lobe sections with antibodies specific to cytokeratins -14 and -8, CD3, and CD4 revealed the presence of medullar and cortical epithelium and mix population of CD3+/CD4+ and CD3+/CD4- T cells. The T cells were associated with the medulla but not with the cortex of the thymus. The immunostaining with an antibody to FoxN1 showed that the protein was expressed in the thymic epithelium. Taken together, we provide evidence that the thymus of a 96-year-old man involuted different kinetics in each of the two thymic lobes. Furthermore, the presence of CD3+/CD4+ and CD3+/CD4-cells gives a hand to the hypothesis that a pool of T-cells may associate with this primary lymphatic organ for as long as there is the available thymic epithelium and be a source of lymphocytes aiding adaptive immune responses to old age.

1. Introduction

The thymus is the primary lymphatic organ responsible for T-cell development and the acquisition of immune fitness. The thymic growth and thymopoiesis start in the fetal life and continue into the early postnatal years (Chinn et al., 2012; Goldschneider, 2006). Then, in early adulthood, the thymus begins the process of involution, when the organ decreases the rate of T-cell output (Cowan et al., 2020; Palmer, 2013; Rezzani et al., 2014; Thapa and Farber, 2019; Thome et al., 2016). Interestingly, the thymopoietic activity remains constant until at least the age of fifty (Bertho et al., 1997; Flores et al., 1999; Jamieson et al., 1999), and, in isolated cases, it could be detected in centenarians (Levy et al., 2019).

The structure of the mature thymus is complex and consists of lobular compartments composed of cytokeratin positive and negative cells providing a microenvironment for T-cell development (Hale, 2004). The cytokeratin-positive thymic epithelial cells (TECs) are further specialized into medullar and cortical TECs, referred to as thymic epithelial space (Hale, 2004). The medullar and cortical TECs can be distinguished by expressing complex or simple epithelium cytokeratins (CK) represented by antigens to CK14 and CK8, respectively (Lee et al., 2011). The cytokeratin-negative mesenchymal and non-mesenchymal cells form the thymic capsule, septae, and blood vessels (Rodewald, 2008). The thymus region spanning between the thymic epithelial space and a capsule is called the perivascular space (Rezzani et al., 2014). With aging, the perivascular space becomes more prominent and progressively infiltrated by the adipose cells, whereas epithelial space declines and its primary organization becomes disrupted (Chinn et al., 2012; Hale, 2004). Interestingly, the increasing perivascular compartment enables recirculation of peripheral lymphocytes consistent with cytotoxic or memory T or B cell characteristics (Flores et al., 2001; Haynes and Hale, 1998).

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The forkhead transcription factor (FoxN1) has an essential role in early thymic development (Nowell et al., 2011; Vaidya et al., 2016). Interestingly, the same protein is required to maintain the postnatal thymic environment until the adulthood (Vaidya et al., 2016), and its expression in a murine aging model was associated with restoration of thymic architecture and T cell export (Bredenkamp et al., 2014a; Zook et al., 2011). In addition, the wealth of evidence points to the fact that the declining expression of the FoxN1 contributes to the aging of the human thymus (Chen et al., 2009; Reis et al., 2015; Romano et al., 2012). Remarkably, there are reports of thymic function occurring in the late age (Flores et al., 1999; Jamieson et al., 1999), including the autopsy evidence of thymi with well-defined cortex and medulla in subjects in their 70s (Smith and Ossa-Gomez, 1981) and thymocytes in subjects older than 50 years (Bertho et al., 1997; Levy et al., 2019).

Here, we report that the thymic involution occurred unilaterally. Applying the IHC techniques, we assessed the structural and functional components of the thymus and cells in both thymic lobes. Based on our findings, we hypothesize that preservation of the thymic structural microenvironment may contribute to the prolonged health of the individual to an advanced age.

2. Materials and methods

2.1. Reagents

Hematoxylin and Eosin solutions, Hoechst 33342 nuclear stain, and Histoplast paraffin were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Directly labeled mouse monoclonal antibodies to cytokeratin 14 (AlexaFluor-594), cytokeratin 8 (AlexaFluor-488), CD3 (AlexaFluor-405), CD4 (AlexaFluor-546), FoxN1 (AlexaFluor-488), and isotype control normal mouse IgG1 conjugated to Alexa Fluor 488, Alexa Fluor 594 or Alexa Fluor 405 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Bovine serum albumin, Phosphate Buffered Solution (PBS), Tris Buffered Saline (TBS) were purchased from Sigma-Aldrich, Inc, (St. Louis, MO, USA).

2.2. Tissue harvesting and processing

The cadaver was procured for gross anatomy training from the Anatomical Gift Program (Dayton, OH, USA). As a standard procedure, the cadavers used at the Gross Anatomy course are embalmed within 24 h of death in a formalin-based fixative solution. The thymus was harvested using 4.5″ surgical Sharp-Sharp scissors and thumb forceps (Nasco, Fort Atkinson, WI, USA), and we assessed the organ's weight, length, and thickness of each lobe, and the length of the transverse diameter. Subsequently, three samples from each thymic lobe were fixed and embedded in paraffin employing Histocore Arcadia-H (Leica Biosystems, IL, USA) and standard histology protocols (Gupta, 2011; Kim et al., 2016). Finally, paraffin-embedded tissue was cut into 5μM sections using HistocoreBiocut (Leica Biosystems, IL, USA) and placed on gelatin-coated microscope slides.

2.3. Immunohistochemistry (IHC)

All microscope slides with thymic sections were de-paraffinized in xylene, re-hydrated in a series of descending concentrations of 100%–

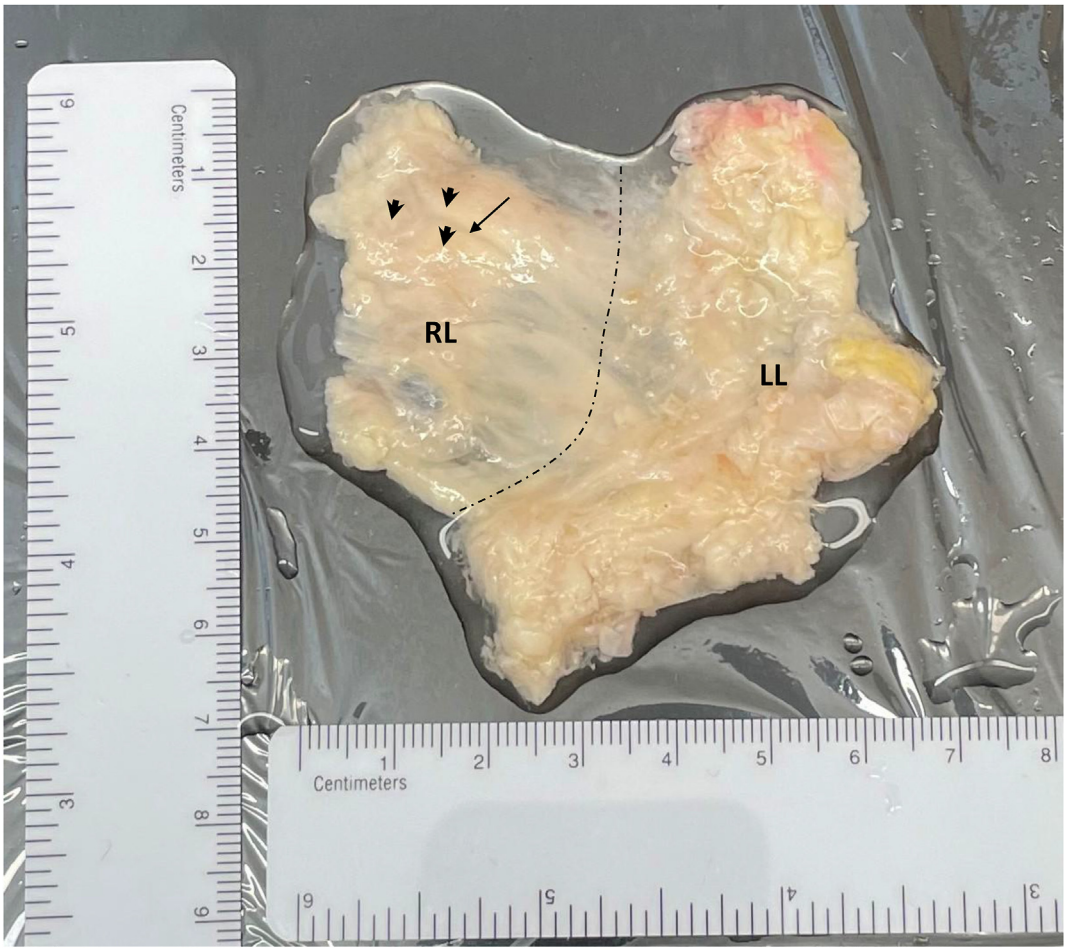

Figure 1. Anatomical evaluation of the thymus from the 96-year old male body donor. The thymus was removed from the donor body and photographed in an anatomical position. The LL and RL depict the left and right thymic lobes, respectively. The dotted line marks the LL and RL boundaries. The arrow shows the thymic septum, and the arrowheads depict individual lobules in the right thymic lobe.

95% ethanol, and rinsed with distilled H_2O. For antigen unmasking, slides were placed in 10mM citrate buffer, pH 6.0, and heated for 10 min in a microwave. Subsequently, slides were washed in 1xTBS. The H&E staining was performed according to the manufacturer's protocols, and tissue sections were observed under a light microscope (Olympus BH-2). The tissue images were captured with the Ep50 microscope digital camera (Olympus).

The multiplex antibody staining was performed as published elsewhere (Bolognesi et al., 2017) with some modifications. Briefly: to increase tissue permeabilization and prevent the non-specific binding, slides were treated for 40 min with the increasing concentration of albumin (1%–5%) in 1xPBS supplemented with 0.4%Triton-100 (PBS-T), and then washed in 1xPBS. The working antibody solutions in 1xPBS supplemented with 1% albumin were applied on the slides and incubated in a dark chamber for 2 h. Each incubation was followed by a wash in 1% albumin in PBS-T buffer for 10 min. After the incubation with the last antibody, slides were washed thoroughly in 1xPBS. The immunofluorescent staining of antigens was evaluated under a confocal laser scanning microscope (Olympus FV/1000).

3. Results

The thymus was isolated from a 96-year-old white male who died from acute toxic metabolic encephalopathy. The length of the left (LL) and right (RL) thymic lobes was 7.0 cm and 5.0 cm, whereas the thickness measured 0.2 cm and 0.1 cm, respectively. The thymic transverse diameter measured 4.0 cm, and the organ's weight was 4.03 g. The macroscopic examination of the harvested organ showed that the left thymic lobe was overgrown with the adipose tissue, while the right lobe presented well preserved thymic lobules separated by septae and no infiltration by adipose tissue (Figure 1). The striking difference in the presentation of both thymic lobes suggested that the process of involution involved different kinetics.

Following the anatomical evaluation, we performed the H&E staining to determine the cellular structures of the organ. The H&E showed that the left lobe consisted of the adipose tissue, and we could not find any remains of the thymic epithelium (Figure 2d). However, the right lobe showed well-preserved TECs filled with hematoxylin-stained cells (Fig. 2a-c), which confirmed the anatomical observations.

Encouraged by this result, we evaluated further the structural components of the thymic epithelial cell (TEC) using monoclonal antibodies to cytokeratin 14 and 8 specific to the medullar and cortex epithelium (Klug et al., 1998). As shown in Fig. 3a-c, the TEC expressed CK14 and CK8 antigens, suggesting the presence of the medullar and cortical regions in the right thymic lobe. Interestingly, we determined that most of the thymic epithelial cells were CK14+ (Figure 3b). The CK8+ cells were in the minority and intercalated with the CK14 + cells, likely indicating the remnants of the cortico-medullary junction (Fig. 3a & c). This organization of epithelial cells was observed in several sections proving that the right thymic lobe had a well-preserved medulla and a rudimentary cortex epithelium (Fig. 3a & c).

There is a wealth of knowledge showing that FoxN1 serves as a mediator of TEC differentiation and maintenance (Chen et al., 2009; Javan et al., 2020; Nowell et al., 2011; Vaidya et al., 2016), and its expression in thymus continues into the adulthood (Reis et al., 2015; Vaidya et al., 2016). As such, the expression of this protein in TEC

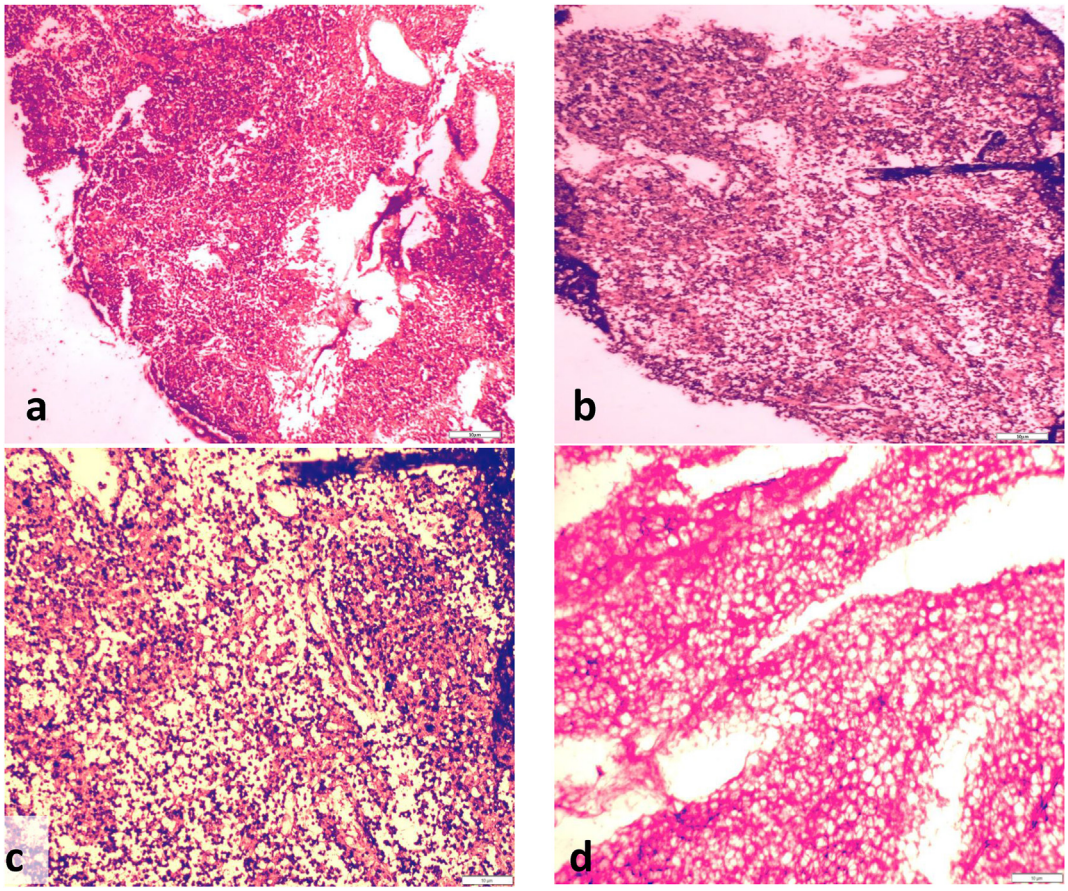

Figure 2. Histologic evaluation of the thymus in the 96-year-old male body donor. The H&E staining of the right (a–c) and left (d) thymic lobes was evaluated in the light microscope under the scanning (a-b & d) and 10x (c) magnifications. The left lobe (c) shows adipose tissue, whereas the right lobe (a–c) shows thymic epithelium (pink) filled with T-cells (blue). The (a & b) photographs show slides from two different tissue blocks prepared from the right lobe; (c) - 10x magnification of the exposure taken in (b). The scale bar: 10 μm.

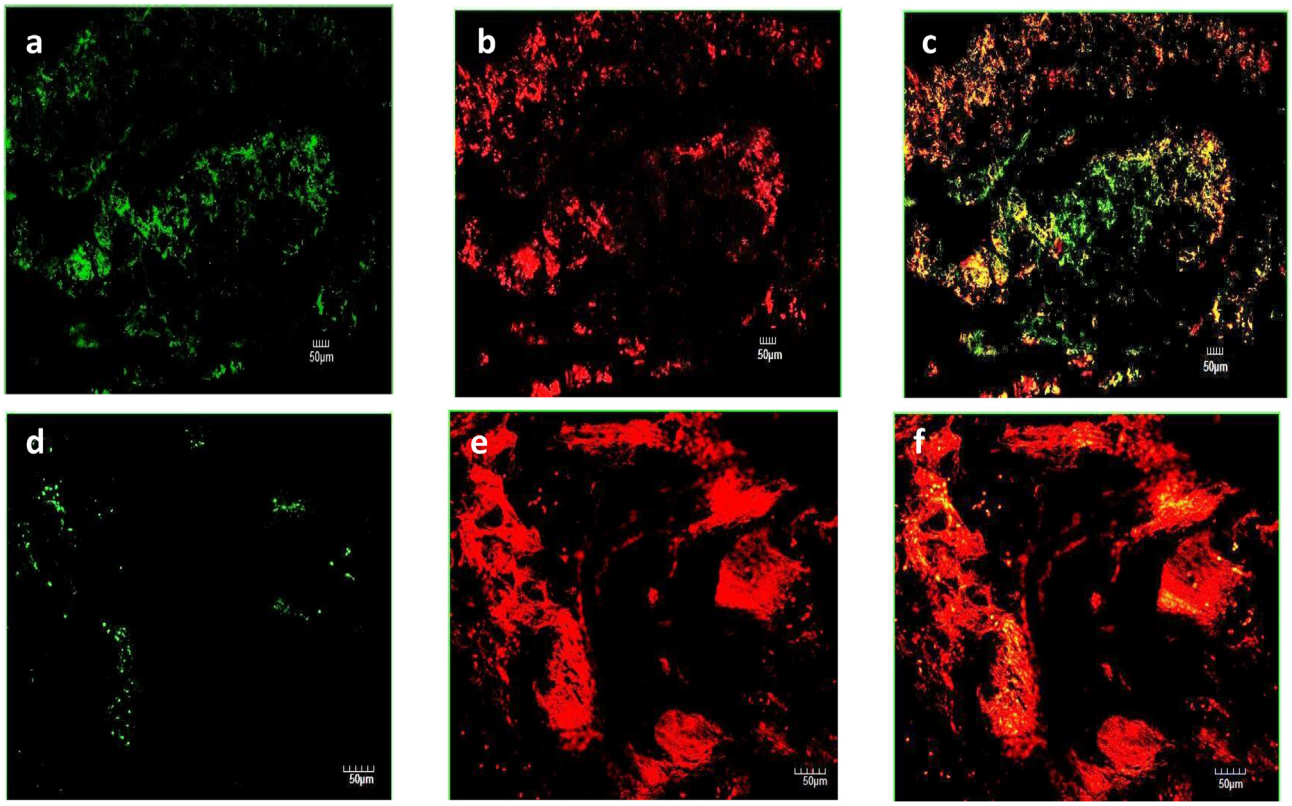

Figure 3. The multiplex immunostaining of the right lobe for the presence of cortex and medullar markers and FoxN1 protein expression in the thymus of a 96-year-old male body donor. (a & b) shows the immunostaining of the thymic sections with antibodies to CK8 (a) and CK-14 (b) representing cortical and medullar epithelial cells at the cortico-medullary junction; an overlay of (a & b) is shown in (c). (d & e) show the immunostaining with antibodies to FoxN1 (d) and CK14 (e); an overlay is shown in (f). The isotype control normal mouse IgG1 conjugated to Alexa Fluor 488 and Alexa Fluor 594 is shown in Figure 4 k & i. All images were obtained under 10x magnification and represent one of three slides taken from two tissue blocks. The scale bar: 50 μm.

indicates a functional thymus. Knowing that the T cells observed in the H&E staining had structural thymic support (Fig. 3a-c), we posed the question of whether the thymic epithelium preserved its function to maintain the homeostasis of the TEC and tested the expression of this factor in the right lobe's medulla applying antibodies to CK14 and FoxN1. As shown in Figure 3 d-f, the CK14 + cells representing the medullar epithelium (Figure 3e) also expressed the FoxN1 protein (Figure 3 d & f). Thus, based on the evidence shown in Figure 3, the complex epithelial microenvironment in the right lobe was preserved to support the T cells sufficiently.

We then tested the identity of cells detected in Fig. 2a-c by employing antibodies to the CD3 and CD4 components of the T-cell receptor. As shown in Figure 4a, the cells observed initially on H&E staining (Fig. 2a-c) were CD3-positive and associated with the medulla (Figure 4c) but not the cortex of the thymic tissue (not shown). The immunostaining of the T cells with antibodies to CD3 and CD4 (Fig. 4d-i) showed that the right lobe maintained a mixed population of CD3+ T cells that were either CD4+ or CD4- (Figure 4f). Analysis of the CD4+ cells revealed that the expression of this molecule coincided with the expression of the CD3 (Fig. 4g-i). Together, our data provide evidence that one thymic lobe in the 96-year-old individual maintained the cellular structure and function usually observed in much younger individuals. Thus, the evidence of two thymic lobes involuting such different kinetics adds to our understanding of the aging of the immune system.

4. Discussion

The involution of the human thymus is an unavoidable biological process (Bertho et al., 1997; Chinn et al., 2012; Haynes et al., 2000; Palmer, 2013). However, in an evolutionary view, the human thymus has not been essential for survival far beyond the host's puberty, as the average life span was approximately 40 years until the mid-1800s, and people lived their lives in the same environment (George and Ritter, 1996). According to recent data published by the United Nations (UN, 2019), the current life expectancy at birth doubled to about 80 years, and there is no assumption of living one's life in one geographical locale. Thus, the individuals may be exposed to more diversified antigens and environmental factors than our predecessors, and the biological demand for a thymic function extending to the older age may be more evident.

As part of more extensive studies aiming at the systematic evaluation of thymic involution in the elderly human population, we recorded an unusual process of thymic involution that occurred in one thymic lobe. In contrast, the second lobe displayed a relatively normal epithelial space containing a mixture of CD3+/CD4+ and CD3+/CD4-cells. The anatomical parameters, including variations of the left and right lobe size, were comparable to data reported by others (Araki et al., 2016; Francis et al., 1985). Based on these reports, the length of the left thymic lobe was always more prominent than that of the right lobe, but the left to right lobe proportions varied with gender and age. That difference tends to increase with age in females, while it oscillates between 30-40% in males regardless of age group. Thus, the proportions of the thymic lobes in our study subject were within these established values (Araki et al., 2016). Still, the thymic epithelial cellular structure was preserved only in one lobe, thus indicating that the thymus underwent a unilateral involution. There was a noticeable difference in the thickness of both thymic lobes in our study compared to others (Araki et al., 2016), which could be explained by the de-hydration process to preserve the cadaveric tissue. Interestingly, the thymic transverse diameter recorded in our study was comparable to data published by Araki et al. (2016), but it aligned with the results they observed in the younger cohorts.

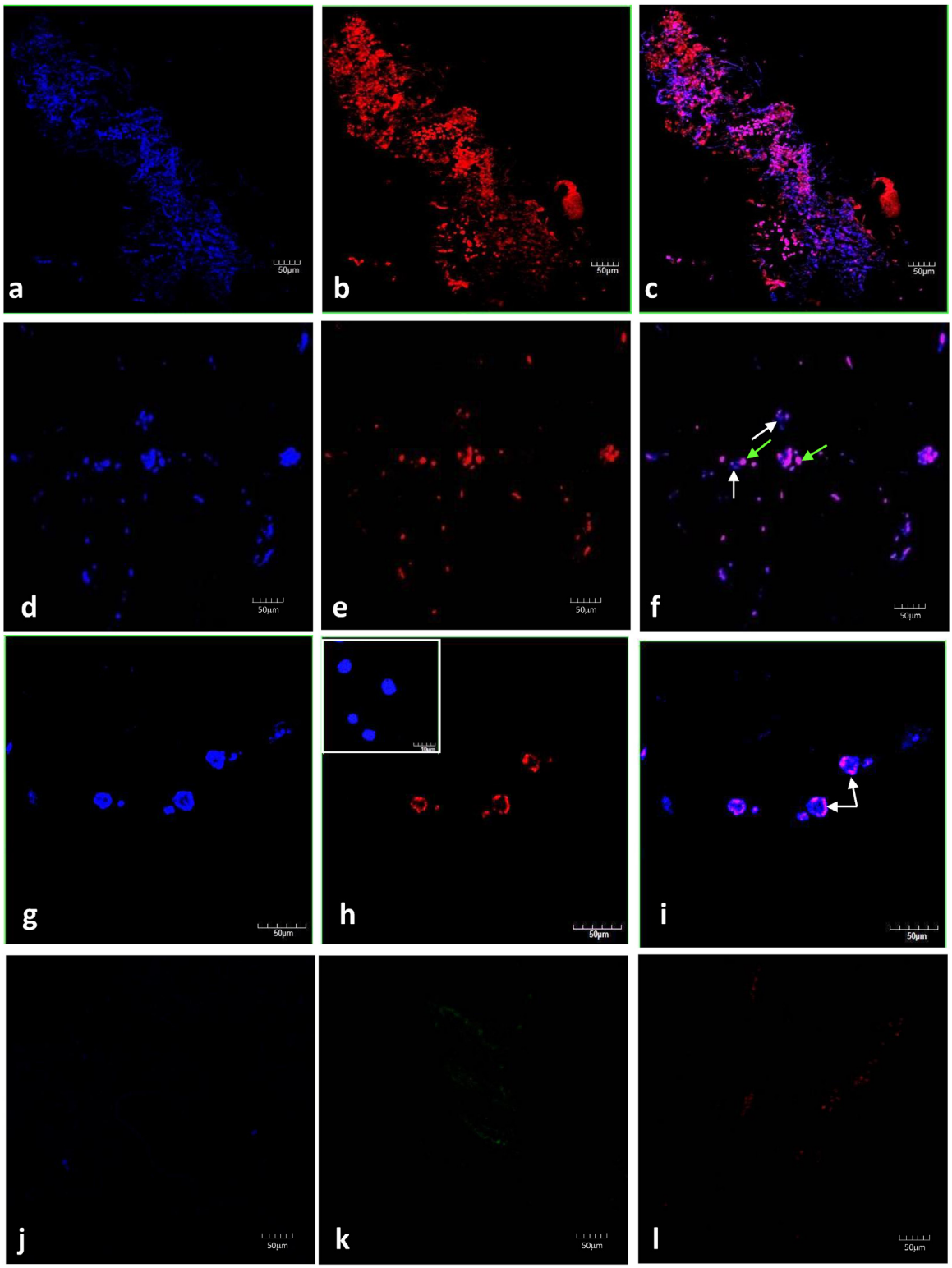

Figure 4. The mixed population of CD3+/CD4-and CD3+/CD4+ T cells associate with the right lobe medullar thymic epithelium in the thymus of a 96-year-old male body donor. (a & b) show the immunostaining of the thymic sections with antibodies to CD3 (a) and CK-14 (b) antigens; an overlay of (a & b) is shown in (c). (d–i) show the immunostaining of the thymic sections with antibodies to CD3 (d & g) and CD4 (e & h) antigens. The overlay of (d & e) is shown in (f); the overlay of (g & h) is shown in (i). The white and green arrows in (f) show CD3+/CD4-and CD3+/CD4+ T cells, respectively; the arrows in (i) show the co-localization of CD3 and CD4 antigens in CD3+/CD4+ cells. The inset in (h) shows the nuclear stain with Hoechst 33342. Images in (a–f) and (g–i) were obtained under 10x and 40x magnification, consecutively, and represent a tissue section from the same block as shown in Figure 3. (j-l) show respectively the isotype controls for monoclonal antibodies used in all experiments discussed in the manuscript. (j) normal mouse IgG1 conjugated to Alexa Fluor 405 represents a control to immunostaining in Fig. 4a, d and g. (k) normal mouse IgG1 conjugated to Alexa Fluor 488 represents a control to immunostaining in Fig. 3a & d. (l) normal mouse IgG1 conjugated to Alexa Fluor 594 represents a control to immunostaining shown in Figures 3b & e and 4 b, e & h. The scale bar: 50 µm.

Thymic epithelial cells have an essential role in all stages of T cell development (Anderson et al., 1993, 1997; Nehls et al., 1994; Oosterwegel et al., 1997), and the aging disrupts the structure of this epithelial microenvironment, including downregulation of keratin (Gray et al., 2006), but it does not disrupt the thymic volume (Steinmann, 1986). We found that the right lobe maintained the relatively complex structure of the thymic epithelial microenvironment. Allying this observation with histological thymic patterns reported by Hale (2004), the structure of the right but not left thymic lobe resembled a tissue of the 11-25-year-old person. The medullar epithelium in this lobe was largely intact, whereas cortical TECs remained present in the outer regions of the medulla. This age-mediated gradual disorganization of thymic compartments and ratio of medullar to cortical TECs was described previously by Chinn et al. (2012).

The thymic epithelium found in the right lobe of our study subject supported structurally the microenvironment for T cells. Several research groups reported on the importance of the FoxN1 transcriptional regulator in the maintenance of the adult thymus (Ki et al., 2014; Nehls et al., 1996; Nowell et al., 2011; O'Neill et al., 2016; Rode et al., 2015), and the down-regulation of this factor was linked to the thymic involution (Chen et al., 2009; O'Neill et al., 2016). Specifically, the FoxN1 protein induces differentiation of functional TEC (Bredenkamp et al., 2014b), which can interact with the T cells. We found that the right thymic lobe of the 96-year-old individual had a rudimentary cortex and a well-preserved medulla expressing the FoxN1 protein and T cells that were associated with the medulla. This evidence indicates that the right lobe provided the structural support to T cells and likely, through the expression of FoxN1 protein, enabled the differentiation of functional TECs (Bredenkamp et al., 2014a, 2014b; Su et al., 2003).

Considering the donor's age, we did not expect thymopoiesis and assumed that the CD3+/CD4+ and CD3+/CD4-cells could be the remaining pool of the initial thymic T-cells aiding central tolerance (Cosway et al., 2017). However, it has been reported that the aging thymus loses its isolation from the peripheral cell migration, and the increasing perivascular compartment enables recirculation of peripheral lymphocytes consistent with cytotoxic or memory T or B cell characteristics (Flores et al., 2001; Haynes and Hale, 1998). The same authors also suggested that while the new lymphocytes are still being produced in the thymic epithelial compartment, the perivascular space participates in the recirculation of peripheral lymphocytes in a fashion similar to the function of a lymph node (Haynes and Hale, 1998). Based on published evidence (Flores et al., 2001; Haynes and Hale, 1998; Sprent and Surh, 2009) and our results, we assume that the medullar CD3+/CD4+ and CD3+/CD4-cells represent the initial T cell pool more likely as opposed to the new T cell thymic immigrants from the periphery. In support of that thought, the research published by Nasi et al. (2006) showed the presence of TREC (T-cell receptor rearrangement excision circles)-positive cells in about 26% of subjects who were more than 100 years old, thus indicating a possibility of active thymopoiesis in these individuals (Levy et al., 2019). Therefore, based on mounting evidence, one cannot exclude that the lymphocytes detected in the medulla of our subject could be a source of the thymocyte pool aiding the immune responses in this advanced age, and detailed functional studies are planned to resolve this question.

Lessons learned from the ongoing COVID-19 pandemic showed that the elderly population was particularly affected by the virus due to the reduced output of naïve T cells (Diao et al., 2020). Our work shows that the thymus may involute unilaterally, thus indicating a biological scenario that may preserve the thymic structure supporting the output of naïve T cells until a late age.

Declarations

Author contribution statement

Pranuthi Kanneganti: Performed the experiments.

Joseph Lyle: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Julia H. Smith; Heather McGuire; Richaela Denlinger: Contributed reagents, materials, analysis tools or data.

Malgorzata Simm, Ph.D: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2022.e11734.

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