

# Quantify each Dendritic Cell Subsets along with Normal Thymus Tissues in Different Ages

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## Research

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# Abstract

## Background

Dendritic cells (DCs) in the thymus are involved in central tolerance formation, but they also play other roles in the thymus. In this study, thymuses of different ages were collected for observation after tissue sectioning and staining. The area of cortex and medulla parts of the thymus in the sections and the density of different subsets of DCs in the thymus were also calculated.

## Results

We found that, along with the increasing age, the thymic cortex atrophies faster, leading to a gradual rise in the medullary's surface area ratio to that of the cortex with an increase in age. The medullary's surface area ratio to cortex can be used as an indirect reference to reflect the thymus hyperplasia. The density of DCs in the thymus showed different changes with the increasing age, and the density of plasmacytoid DCs (pDCs) in the thymus gradually increased with aging, suggesting that pDCs may play an essential role in the thymus in addition to central tolerance.

## Conclusions

These findings complement our knowledge about the DCs' subsets in the thymus along with physical ageing and help us understand the full function of DCs in the thymus beyond central tolerance.

# Background

The thymus is the central lymphoid organ in which T lymphocytes develop and mature. In this process, the body goes through a series of elaborate mechanisms to remove the thymocytes that cannot recognize their own human leukocyte antigen (HLA) or autoreactive thymocytes and finally output the naive T cells. This is the formation of T cells' central tolerance, which is a critical component of the body's immune tolerance.[1, 2] The destruction of central tolerance often leads to autoimmune diseases, such as myasthenia gravis, whose pathogenesis is closely related to the thymus.[3–5]

Dendritic cells (DCs) and thymic epithelial cells are the main components for thymocyte screening (central tolerance) in the thymus[2, 6, 7]. Currently, it is believed that there are two origins of DCs in the thymus. Some of the DCs are derived from the precursor cells of DCs or dendritic cells in the blood. And the others are differentiated from the progenitor cells, which migrate from the blood to the thymus.[8–10] Regardless of the origin, DCs in the thymus participate in central tolerance formation and are mainly concentrated in the medulla. DCs in the medulla collect various self-antigens expressed by the thymic epithelial cells. DCs migrated from the blood and can carry peripheral antigens to the thymus to enrich the central tolerance self-antigen library.[7]

Like those in other tissues, DCs in the thymus is divided into plasmacytoid dendritic cells (pDCs) and myeloid dendritic cells (mDCs).[11] And mDCs can be further divided into CD1c positive and CD141

positive mDCs by labeling these two different markers.[12, 13] The function of DCs in the thymus and the details of the process of central tolerance that DCs execute are still not fully understood.

Although the specific function of DCs determines their role in the immune response, the number of DCs in the immune response cannot be ignored.[14] Therefore, it is vital to understand the role of DCs in thymus tissue in detail and define the distribution and quantitative characteristics of the two DC subsets in the thymus. Also, the thymus atrophies gradually with the increase of age, especially after puberty. It is not clear what changes have taken place about the distribution and density of DCs while the thymus atrophies. The study of the distribution of DC subsets in the thymus and their density changes with aging might help us to understand the role of DCs in the thymus.

## Results

### Dendritic cell subsets and morphological characteristics in the thymus

Thymus tissue was digested into single-cell suspension, and dendritic cells in the thymus were analyzed by flow cytometry. Using lineage cocktail antibody to exclude thymocytes, T cells, B lymphocytes, NK cells, and macrophages. Cells with lineage cocktail negative and HLADR positive were selected for further analysis. Plasmacytoid DCs (pDCs) were labeled by CD123 (Fig. 1, A). Another dendritic cell subset, myeloid DCs (mDCs) (Fig. 1, B), were labeled by CD11c and further divided into CD1c positive DC and CD141 positive DC (Fig. 1, B) by CD1c and CD141. Using CD123 and CD11c as markers in immunohistochemistry, the two subsets of DCs in thymus tissue were identified respectively (Fig. 1, C). pDCs were elliptic and granular, while mDCs were typical dendrite-like with irregular shapes and many projections. Though mDCs could be further labeled by CD1c and CD141 in poly colors flow cytometry, these two markers have poor specificity when used in immunohistochemistry (an observed phenomenon). Both pDCs and mDCs were mainly distributed in the thymus medulla (Fig. 1, D), and the density of pDCs in the thymus is higher than that of mDCs (Fig. 1, D).

The ratio between the medullary and cortex area in the thymus increases with an increase in age.

Normal thymus with different ages was collected, and HE staining was performed on the tissue slides. Four specific age groups (1 year - infant, 14 years - adolescent, 26 years - early adult, 40 years - middle adult) were selected to represent the characteristics of thymus changes with age (Fig. 2, A). The thymus gradually atrophies with increasing age and rapidly atrophies after puberty. By adulthood, the thymus has been replaced mainly by adipose tissue, but a tiny amount of thymus medulla and cortex remains. Although the thymus gradually atrophies with age increase and is replaced by adipose tissue, the atrophy degree of the thymic cortex and medulla is not uniform (Fig. 2, A). We calculated the area of the medullary and cortex in the stained thymus tissue and calculated the ratio ( $\text{AreaM}/\text{AreaC}$ , M/C) between the medullary area (AreaM) and the cortical area (AreaC) to establish the relationship between this ratio and age. With the increase of age, the ratio of M/C gradually increased (Fig. 2, B), and there was a significant correlation between them. This suggested that with aging, the thymic cortex atrophied more rapidly than the medulla.

The density of mDCs did not change significantly with aging, while pDCs' increased gradually with aging.

Using CD123 and CD11c to mark thymus tissues of different ages, we found the changes of pDCs and mDCs density. With the increase in age, most parts of the thymic medulla and cortex parts had been replaced by fat components in the typical 40 years old thymus, which was not suitable for the staining and density calculation of DCs. Therefore, normal thymus tissue over 40 years old was not included in the analysis. By analyzing the density of the two DC subsets in the thymic medulla and cortex, respectively, we found that the density of DC subsets in the thymic medulla and cortex changed with special tendency. As the age increases, the density of pDCs in the thymic medulla and cortex gradually increased, and the density of pDCs in the medulla was always much higher than that in the cortex. The density of mDCs in the medulla was significantly higher than that in the cortex regardless of age. Still, the density of mDCs in the medulla and cortex showed an opposite trend with age increase, with a gradual decrease in the medulla and a gradual increase in the cortex (Fig. 3, A). Combining the density of the two DC subsets in the medulla and cortex, we found that the density of pDCs still gradually increased with the age growth, thymus atrophy, and medulla proportion increase. Simultaneously, the density of mDCs also increased but did not change significantly (Fig. 3, B).

## Discussion

In 2010, Ziegler-Heitbrock, L et al. declared a consensus on a flow cytometry panel for human peripheral blood DCs labeling. In this consensus, Linage (negative) HLA-DR (positive) CD11c (positive) for mDCs, or Linage (negative) HLA-DR (positive) BDCA2 (positive) for pDCs. [16] There is no uniform labeling method for DCs in different organs, but Linage, HLADR, BDCA2 (or CD123), CD1c, CD141 and CD11c are widely used to mark DCs in human tissues. However, immunohistochemistry cannot identify DCs by multiple staining as flow cytometry does. Currently, CD11c, CD123 and BDCA2 are commonly used to label DCs in immunohistochemistry. In some previous studies, CD86, DC-LAMP and other markers were also used to mark DCs in tissues.[11, 17, 18] Although CD11c and CD123 are not specific markers, these two markers can be used alone to specifically mark two different dendritic cell subsets, as our results showed.

The roles of the thymic cortex and medulla are different in the thymocytes development. [19] Bone marrow hematopoietic stem cells enter the thymic cortex through the blood vessels at the junction of cortex and medulla and gradually develop into CD4CD8 double-positive thymocytes. In this process, thymocytes expressing TCR that can bind to HLA are screened out through positive selection. These double-positive thymocytes enter the medulla undergoing negative selection. Thymocytes expressing TCR that can bind with self HLA with appropriate intensity are screened out and developed into CD4 or CD8 single-positive naive T cells.[20] The thymus epithelial cells in the medulla and DCs are responsible for deleting self-reactive thymocytes, which point to the medulla's indispensable roles in central tolerance. As a result, the medulla may need to keep the whole function during the thymus atrophy, meaning a relatively slowly shrinking, avoiding the release of the self-reactive naive T cells (leading to autoimmune disease) during the process of thymic atrophy. On the other hand, for an economical energy spending

strategy (a relatively complete naive T cell repertoire and can deal with most pathogens in the future natural environment), the thymic cortex shrinks relatively faster.

Thymus hyperplasia is a typical feature of patients with general myasthenia gravis (MG). [3, 21] It is believed that hyperplasia of the thymus was the initial pathogenesis of myasthenia gravis. [5, 22] In current clinical work, the method of evaluating thymus hyperplasia is relatively complex, requiring sectioning analysis of the whole thymus. By analyzing sections of thymus tissue with different ages, we found that while aging, although undergoing obvious atrophy, cortex parts atrophied relatively faster. To confirm this phenomenon, we calculated the area ratio of medulla and cortex in tissue sections to figure this trend. Finally, we found that the ratio gradually increased with age growth. Thus, we wondered if the area ratio between medulla and cortex in the thymus was a predictor of thymic hyperplasia, and we did find that the area ratio of the medulla and cortex in the thymus of MG patient was significantly higher than that in the normal thymus of the similar age (results have not been published).

The two DC subsets are functional differences in some parts. [8, 23, 24] Those differences may also occur in the thymus, but their roles were not as important as those in central tolerance. In this study, we analyzed the changes of density of different DC subsets in the thymic medulla and cortex in aging. The density changes of mDCs and pDCs in the thymus are inconsistent and suggest that the two DC subsets' functions in the thymus may be quite different. MDCs, as a critical cellular component for central tolerance formation, have lost their importance as thymus could not keep their role in immunity and gradually became fat tissue, thus decreasing its density. However, the density of pDCs increases slowly with an increase in age, suggesting that pDCs may play other vital roles even the thymus was experiencing atrophy. This role may be beyond the thymic function, but the specific role needs to be further studied.

## Conclusions

The relative proportion of cortical and medulla structure in thymus tissue changes with age, and the relative retention of medulla acknowledges the importance of central tolerance. The density of different dendritic cell subsets in the thymus is not fixed during one person's birth, development and growing old, which may indicate that the function of dendritic cells in the thymus changes with physical ageing. The density of plasmacytoid dendritic cells increases with ageing, suggesting that some of the functions they perform in the thymus become relatively more important during ageing process.

## Methods

### Samples collection and processing

Thymus samples were collected from patients undergoing corrective cardiology surgery from the Cardiac Surgery Department of the First Affiliated Hospital of Sun Yat-sen University and Guangdong Provincial People's Hospital from June to November 2014. Samples were taken from patients aged 4 months to 62

years (n = 23). The surgically removed specimens were immediately transferred to the specimen transfer box for laboratory processing. All thymus specimens were collected, transferred, and treated per the Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University's regulations and under its supervision.

### Flow cytometry

The thymus samples were cut into approximately 1mm<sup>3</sup> pieces and digested in RPMI medium (with 10%FBS) containing Collagenase D (1mg/mL), Collagenase IV (1.5mg/mL) and DNase I (0.05mg/mL). The digestion medium was put into an incubator containing 5%CO<sub>2</sub> for 45 min, and the tissue blocks were suspended by shaking every 15 min. After digestion, the tissue blocks were blown to dissociate by pipette, and the resulting digestion medium was filtered through a 70um filter to form a single cell suspension for flow cytometry staining. Antibodies Linage cocktail (anti-CD3, CD14, CD16, CD19, CD20, and CD56 antibodies, 348701, Biolegend), HLA-DR (327013, Biolegend), CD11c (301641, Biolegend) and CD123 (306015, Biolegend) were added into the single-cell suspension incubated for 30 minutes at 4°C for staining. Please refer to the previous article [15] for specific staining methods. After staining, the single cells of thymus samples were analyzed by flow cytometry. Flow cytometry data were collected on a CytoFLEX Flow Cytometer (Beckman Coulter) and analyzed using Flowjo Vx software (TreeStar software).

### Tissue section staining and Immunohistochemistry

The collected thymus samples were cut into 15X15X5mm pieces and fixed in 4% paraformaldehyde (Sigma-Aldrich) for 24 hours at room temperature. The selected tissue samples were trimmed for dehydration and paraffin embedding. After embedding, 4um thin slices of tissue were sectioned and fixed on slides. After dewaxing and hydration, the tissue sections were stained with hematoxylin and eosin (HE) (BASO) is a typical procedure. For Immunohistochemistry (IHC), Antigen retrieval was performed on rehydrated tissue by boiling sections in antigen retrieval Citra Solution (Gene tech). Sections were blocked for 30 min at room temperature using 3% H<sub>2</sub>O<sub>2</sub> solution (Gene tech), followed by incubation with anti-CD11c (ab52632, Abcam) or CD123 (GT2136, Gene tech) primary antibody overnight at 4°C. Staining with biotinylated secondary antibody was performed for one hour at room temperature. Slides were developed using an ABC kit (Gene tech) and DAB kit (Gene tech) and counterstained with hematoxylin (BASO).

### The calculation of thymic medullary and cortex area and DCs' density

Nikon ECLIPSE Ni-U series microscope was used to collect the staining images of HE and IHC in thymus tissue sections with good staining quality, complete tissue sections and attached were selected. The whole slide's tissue images were collected through the image synthesis software (NIS-Elements D) to form an entire tissue section image. HE staining slides were used for the area of the medullary cortex of thymus tissue calculation. The cortical and medullary parts of thymus tissue were circled, and the actual size was calculated by the software (NIS-Elements D). IHC section slides were used to calculate the

density of dendritic cells. Using the software (NIS-Elements D), the specific number of dendritic cells in the cortex and medulla was counted and divided by the area of the cortex or medulla ( $\text{mm}^2$ ) to obtain the average density of dendritic cells in the cortex or medulla (per  $\text{mm}^2$ ).

## Statistical analysis

The unpaired, parametric, two-tailed Student's t-test was used for statistical significance calculation between two groups. The correlation between age and the area ratio of medullary to cortex was fitted using a logarithmic regression, while correlation analysis of age and density of dendritic cells in thymus and cortex, using linear regression fitting.

## Abbreviations

BDCA

blood dendritic cell antigen; DCs:dendritic cells; HLA:human leukocyte antigen;

IHC:immunohistochemistry; mDCs:myeloid dendritic cells; MG:myasthenia gravis; pDCs:plasmacytoid DCs

## Declarations

### Conflict of interest

The authors declare that they have no competing interests

### Authors' contributions

Li Y, Chen P, Liu WB and Ran H contributed to the study concept and design. Li Y, Chen P, Huang H and Feng HY acquired and analyzed the data. Li Y, Chen P, Liu WB and Ran H interpreted the data. Li Y and Chen P drafted the manuscript. Liu WB and Ran H revised the manuscript. All authors read and approved the final manuscript.

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### **Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### **Ethics approval and consent to participate**

All thymus specimens were collected, transferred, and treated per the Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University's regulations and under its supervision. Not consent applicable.

### **Consent for publication**

Not applicable.

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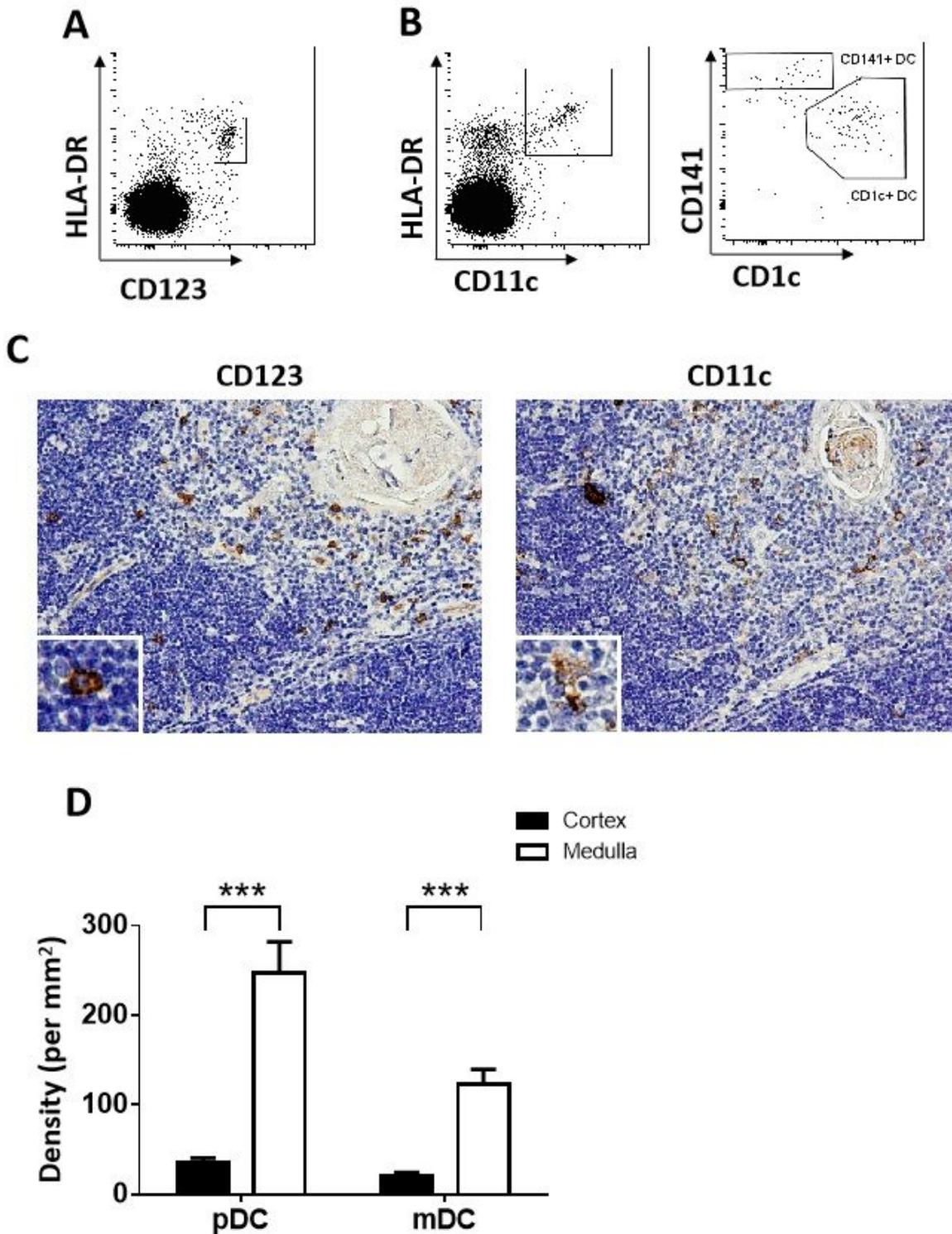
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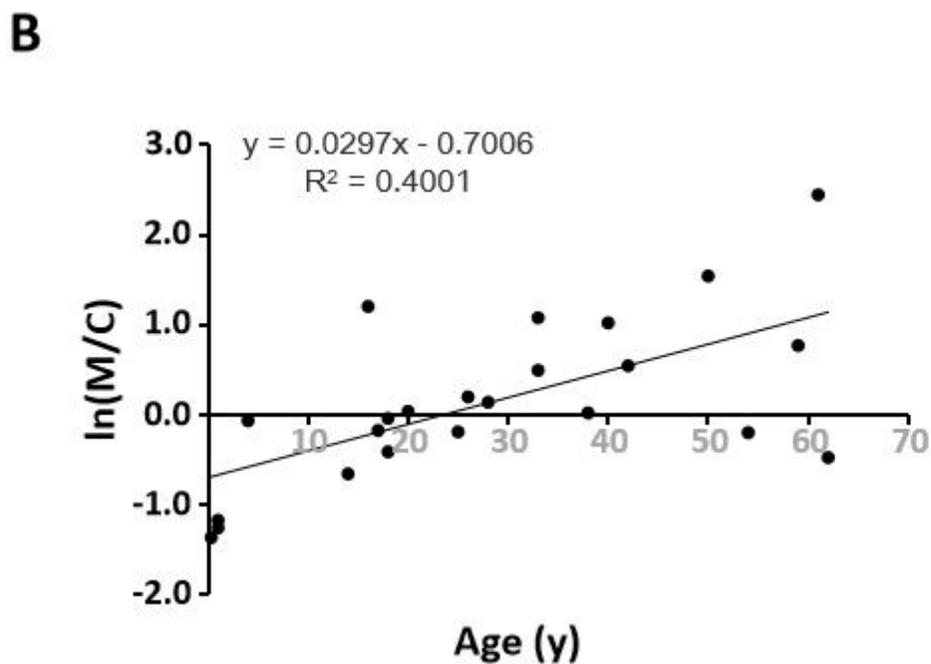
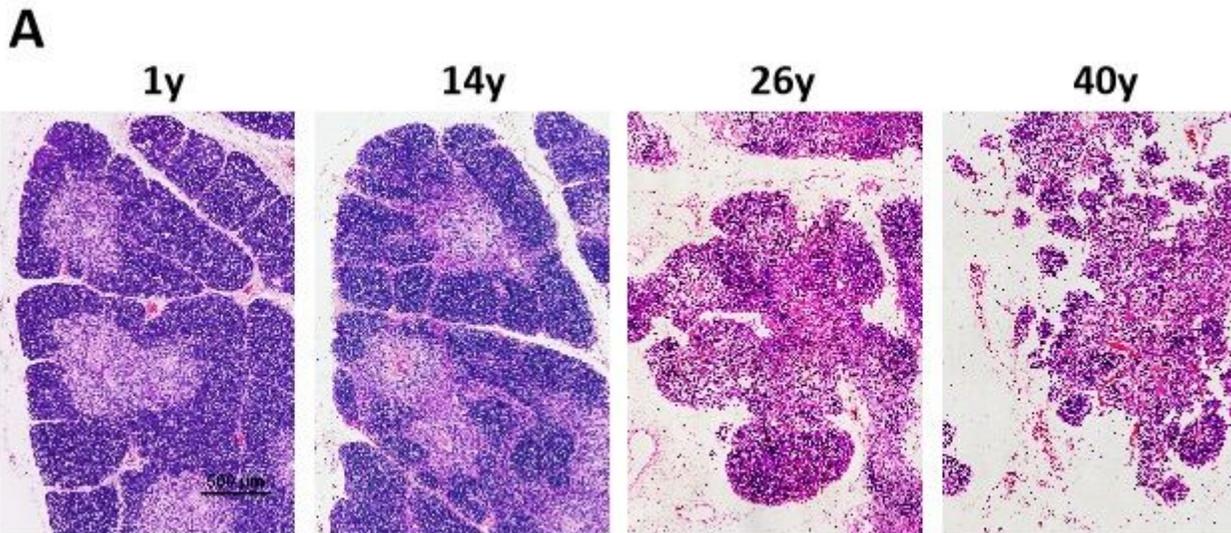
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## Figures



**Figure 1**

A, other kinds of cells in the thymus were excluded, pDCs (HL-ADR (positive) CD123 (positive), box) were labeled with HLADR and CD123. B, to exclude other types of cells in the thymus, mDCs were labeled with HLADR and CD11c (HLA-DR (positive) CD11c (positive), in the box). MDCs could be further divided into CD141 (positive) DCs and CD1c (positive) DC by CD1c and CD141. C, in immunohistochemical staining, CD123 was used to mark pDCs (brown, granular). CD11c was used to mark mDCs (brown, dendritic) in the thymus. The two subsets were mainly distributed in the thymus medulla. D, pDCs, and mDCs densities in the thymic medulla and cortex were calculated and compared. \*\*\*,  $P < 0.001$ .



**Figure 2**

A, HE staining of thymus tissue slides with different ages showed medulla, cortex, and gradually increasing adipose tissue in the thymus. B, the ratio of thymic medulla area to cortical area (M/C) and the

relationship between M/C and ages, the variable x in the formula is  $\ln(M/C)$ ,  $n=23$ .

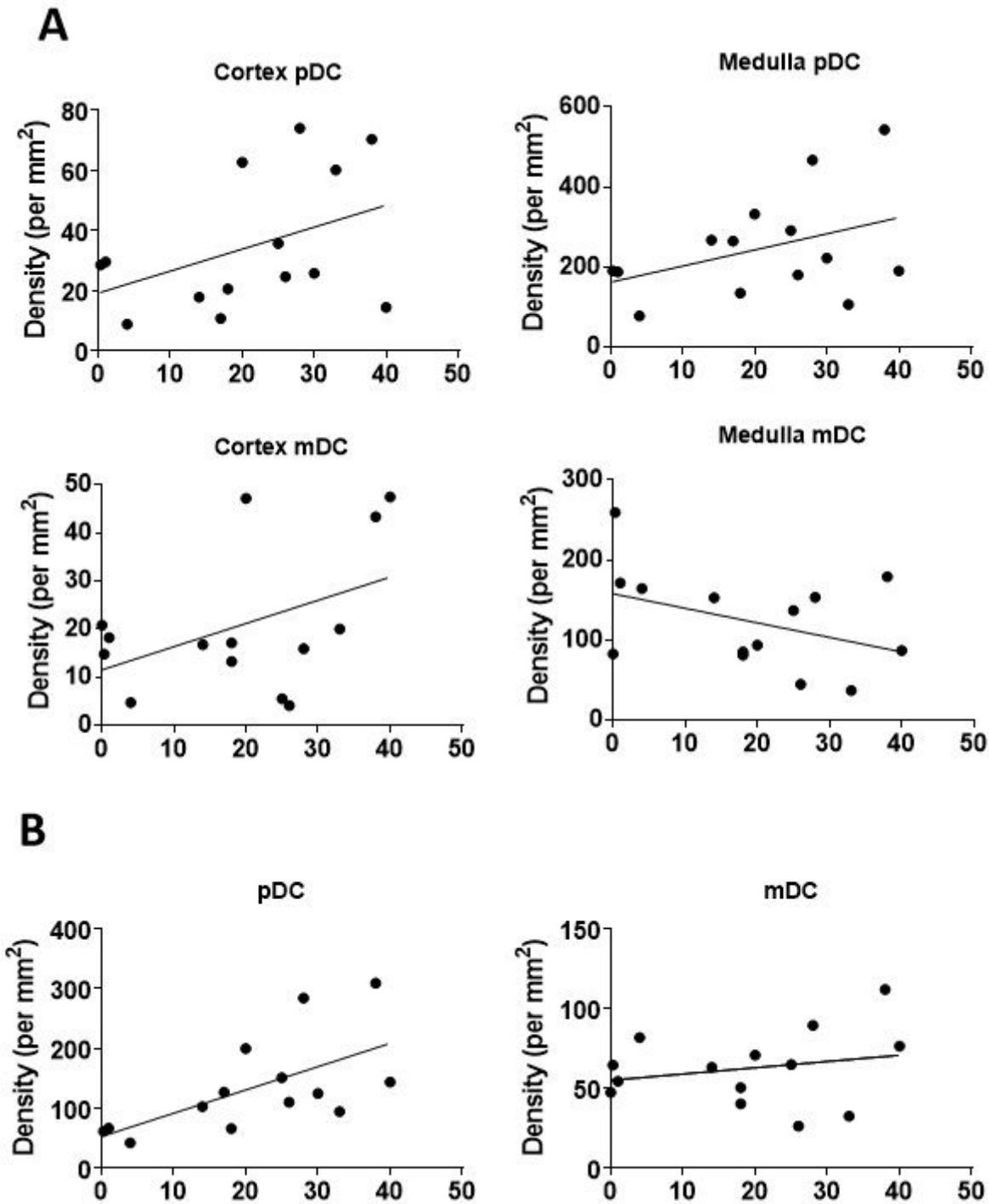


Figure 3

A, the densities of pDCs and mDCs in medulla and cortex were calculated respectively to show their changes with increasing age. B, the density of pDCs and mDCs in the medulla and cortex showed a changing trend with aging, (pDC group,  $n=14$ ; mDC group,  $n=14$ ).