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Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed
☐	☒ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒ The statistical test(s) used AND whether they are one- or two-sided Only common tests should be described solely by name; describe more complex techniques in the Methods section.
☐	☒ A description of all covariates tested
☐	☒ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e .g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒ For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted Give P values as exact values whenever suitable.
☒	☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☐	☒ Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	scRNA-seq and V(D)J libraries (scTCR-seq) were generated using the 10X Genomics Chromium Controller instrument, Chromium Single Cell 5' Library & Gel Bead Kit, and V(D)J Enrichment Kit, following the manufacturer's instructions. Spatial sequencing (ST) data were generated using the 10X Visium platform.
Data analysis	**R packages:** Seurat v4.1.0; MASS v7.3; FeatureSelection v1.0.0; CARD v1.0; CellTrek v0.0.94; clusterProfiler v4.2.0; Monocle2 v2.22.0; ComplexHeatmap v2.13.1; CellChat v1.1.3; corrplot v0.92; pheatmap v1.0.12; ggsci v2.9; SingleCellExperiment v1.10.1; SummarizedExperiment v1.24.0; GenomeInfoDb v1.24.2; matrixStats v0.61.0; SeuratWrappers v0.3.0; SeuratData_0.2.1; SeuratDisk_0.0.0.9020; reticulate_1.25; ape_5.5; ggtree v3.2.0; writexlsx v1.4.0; ggplot2 v3.3.5; tidyverse v1.3.1; colorRamps v2.3; caTools v1.18.2; dplyr v1.0.6; SeuratObject v4.0.4 ; CellphoneDB v5.0; DoubletDection v3.0; xgboost v1.7.5.1; quanteda v2.1.2; ROGUE v1.0; harmony v0.1.1; DoubletFinder v2.0.3. **Python packages:** Scanpy v1.8.1; sklearn v1.3.0; CellPhoneDB v5.0; scvelo v0.3.2; Scrublet v0.2.2. **Other:** CellRanger v4.0.0.
Code Availability	All custom code used in this work are available at Github: https://github.com/lihuamei/Thymus. And can also be accessed on Zenodo via DOI: 10.5281/zenodo.12803343.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g . GitHub). See the Nature Portfolio guidelines for submitting code & software for further information .

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

1. The raw sequence data generated in this study have been deposited in the Genome Sequence Archive (GSA-Human) and are publicly accessible via the following links: GSA-Human: HRA007984 (scRNA-seq) at https://ngdc.cncb.ac.cn/gsa-human/s/C87MlGbL, HRA007980 (Spatial transcriptomics data) at https://ngdc.cncb.ac.cn/gsa-human/s/eOQ2yy34, and HRA007988 (scTCR-seq) at https://ngdc.cncb.ac.cn/gsa-human/s/38O9Q5t8. The processed data (Seurat object) corresponding to these datasets can be accessed from Zenodo repository (DOI: 10.5281/zenodo.13207776).
2. Thymus scRNA-seq data from Park et al. is publicly available on the Zenodo repository (DOI: 10.5281/zenodo.3572422).
3. Processed human thymus scRNA-seq data by Cordes et al. can be accessed via GEO: GSE195812.
4. Three thymus Spatial Transcriptomics (ST) samples are accessible from the study by Suo et al. (Science, 2022) (https://developmental.cellatlas.io/fetal-immune).
5. The Bioconductor org.Hs.eg.db annotation package and the ENSEMBL_MART_ENSEMBL BioMart database are available for public use.

Research involving human participants, their data, or biological material

Policy information about studies with human participants or human data . See also policy information about sex, gender (identity/presentation), and sexual orientation and race, ethnicity and racism.

Reporting on sex and gender	1. Four fetal thymus samples for scRNA-seq and scTCR-seq in this study, with unknown gender information. 2. Out of the eight pediatric thymus samples for scRNA-seq, ST-seq and scTCR-seq in this study, four are male, and four are female. 3. Adult (n = 2) and elderly (n = 2) thymus samples for scRNA-seq and scTCR-seq in this study are all male. **Notably, this study did not involve any analyses specific to sex differences. Details are listed from Extended Data Table 1.**
Reporting on race, ethnicity, or other socially relevant groupings	The research was not involved in race, ethnicity or other socially relevant groupings. The relevant information was not taken other socially relevant under consideration.
Population characteristics	The patients involved in this study encompass four distinct developmental stages of the thymus, including the prenatal, pediatric, adult, and geriatric stages. 1.Fetal samples (n = 4) were sourced from miscarriages or stillbirths. 2.Pediatric samples (n = 8) were obtained from patients with congenital heart disease undergoing surgical intervention, necessitating the removal of obstructing thymic tissue. 3.Adult (n = 2) and geriatric (n = 2) samples were procured posthumously from organ donors. All tissue samples were gathered from January 2020 to November 2022 from Nanjing Drum Tower Hospital. And the clinical information is summarized in Extended Data Table 1.
Recruitment	Our study does not involve donor recruitment. Thymus sample information was retrospectively retrieved.
Ethics oversight	The research was carried out in alignment with the guidelines delineated in the Declaration of Helsinki, and ethical clearance was acquired from the Research Ethics Committee of Nanjing Drum Tower Hospital (ID 2020-015-01). Every participant within the study granted informed consent for the retrieval of tissue samples and subsequent analyses.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	**For individual:** No statistical methods were used to establish the sample size in advance. This study included four samples from the fetal stage, eight from pediatric, two from adulthood, two from the geriatric stage. Each sample was provided by an individual patient. **For mouse:** Sample sizes were determined based on mouse cohort availability for adoptive transfered mice. Experiments were repeated to increase sample sizes.
Data exclusions	Exclusions from the scRNA-seq dataset were made based on the following criteria: Cells exhibiting low counts of unique molecular identifiers, low gene counts, and/or a high proportion of mitochondrial reads were presumed to be cells in a deceased or deteriorating state, prompting their removal. Furthermore, cells identified as consensus doublets through hashtag demultiplexing were also eliminated. No samples were excluded from mouse, scRNA-seq, and Spatial-seq datasets.
Replication	**For individual:** Each donor was individually studied without sample repetition, but there were repeated samples across different age stages. **For mouse:** All experiments were replicated at least three biological replicates.
Randomization	This is an observational study, rather than a clinical trial, so no randomization was performed.
Blinding	Blinding was not implemented in the experiments and analyses due to the involvement of a single investigator who conducted sample collection, sample processing, data generation, and data analysis.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☒	☐ Plants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☐	☒ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used

Antibodies used for Western blot (Catalogue, supplier, dilution):
Anti-Actin (Cat： MAB501, Sigma, 1:10,000), anti- ELOVL4 (Car: 55023-1-AP, Proteintech, 1:1000), horseradish peroxidase-conjugated donkey anti-mouse IgG (Cat:715-035-151, Jackson ImmunoResearch, 1:3000), horseradish peroxidase-conjugated donkey anti-Rabbit IgG (Cat:711-035-152, Jackson ImmunoResearch, 1:3000).

The functional-grade antibodies utilized in this study included CD3 (Cat: 14-0031-82, clone 145-2C11,1:1000) and CD28 (Cat: 16-0281-82, clone 37.51, 1:1000), both sourced from eBioscience. Fluorescence-labeled antibodies for CD3 (Cat: 11-0032-82, 17A2,1:200), CD4 (Cat: MCD0428, RM4-5, 1:200), CD8 (Cat: A15385, 53-6.7, 1:200), CD25 (Cat: 17-0251-82, PC61.5, 1:200), FOXP3 (Cat: 25-5773-82, FJK-16s, 1:200), CD44 (Cat: 17-0441-82, IM7, 1:200), CD62L (Cat: 12-0621-82, MEL-14, 1:200) and CD99 (Cat: 12-0997-42, 3B2/TA8, 1:200) were from eBioscience. Mouse CD99 PE-conjugated Antibody were from R&D (Cat: FAB3905P,1:200). The antibody targeting mouse ELOVL4 (Cat： 55023-1-AP ,clone 55023-1-AP， 1:1000) was procured from Proteintech. Anti-Actin (Cat: MAB1501, C-4, 1:10,000) was from Sigma.

Validation

All commercial antibodies were validated by the manufactures as indicated on their web sites. Antibodies used for Western blot were further validated by molecular weights of the target proteins. The validation statements of all antibodies are accessible to the public on the manufacturer's website.

Animals and other organisms

Mice

Rag1-KO (C57BL/6, Strain NO. T004753) mice were purchased from Gempharmatech Co., Ltd. ROSA26-Cas9 mice (C57BL/6, Cat. NO. NM-KI-00120) were purchased from Shanghai Model Organisms Center, Inc. Cas9-expressing bone marrow cells isolated from ROSA26-Cas9 mice were spin transduced with lentiviral constructs on a Retronectin-coated plate and adoptively transferred into irradiated (950rad on an X-Rad irradiator) Rag1-KO mice. After 8 weeks, the chimeric mice were euthanized using CO_2 asphyxiation followed by cervical dislocation for analysis of thymus T cell development and peripheral T cell activation. All animal experiments were conducted in accordance with the guidelines set forth by the institutional and national committees. Explicit permission to perform animal experiments was granted by the Institutional Animal Care and Use Committee (IACUC) at Nanjing Medical University, under the protocol number 2308051. The animals were housed in a specific pathogen-free (SPF) facility. For each experiment, sex-matched and age-matched (6–8 weeks old) mice were used, with experimental and control animals co-housed to ensure consistent environmental conditions. The facility maintains strict protocols to prevent pathogen contamination and to ensure the well-being of the animals.

Eukaryotic cell lines

Cell line source(s)

HEK293T cells were originally purchased from ATCC (catalogue no. CRL-3216)

Authentication

None of the cell line have been authenticated.

Mycoplasma contamination

All cell lines were tested negative for mycoplasma contamination.

Commonly misidentified lines(See ICLAC register)

All cell lines were tested negative for mycoplasma contamination.

Flow Cytometry

Plots

Confirm that:

☒ The axis labels state the marker and fluorochrome used (e.g. CD4- FITC).

☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☒ All plots are contour plots with outliers or pseudocolor plots.

☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation	Thymus and spleen sample were gently ground under nylon mesh using the flat end of syringes. Red blood cells were removed by ACK lysing buffer. Following by washing cell with isolation buffer. Cell were then filtered, pelleted and staining for FACS or soring. Cells were blocked with Fc blocker(CD16/32), and stained for specific surface markers.
Instrument	Flow cytometry data were collected by FACS Celesta and FACS Aria.
Software	Flow cytometry data were analyzed by FlowJo software (TreeStar, Ashland, OR).
Cell population abundance	The purities of the sorted T cells were more than 99%.
Gating strategy	For immune cells, first we gated the lymphocytes based on the FSC-A and SSC-A. Singlet cells were gated according to the pattern of FSC-H vs FSC-A. The detailed gating strategies are shown in the Supplementary Figure 6a,b. Further gates identifying cell subpopulation basing on specific markers are shown in the manuscript and described in the Figure legends.

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.