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Three-Dimensional Visualization of the Mouse Thymus Organization in Health and Immunodeficiency

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Lymphoid organs exhibit complex structures tightly related to their function. Surprisingly, although the thymic medulla constitutes a specialized microenvironment dedicated to the induction of T cell tolerance, its three-dimensional topology remains largely elusive because it has been studied mainly in two dimensions using thymic sections. To overcome this limitation, we have developed an automated method for full organ reconstruction in three dimensions, allowing visualization of intact mouse lymphoid organs from a collection of immunolabeled slices. We validated full organ reconstruction in three dimensions by reconstructing the well-characterized structure of skin-draining lymph nodes, before revisiting the complex and poorly described corticomедullary organization of the thymus. Wild-type thymi contain ~200 small medullae that are connected to or separated from a major medullary compartment. In contrast, thymi of immunodeficient Rag2−/− mice exhibit only ~20 small, unconnected medullary islets. Upon total body irradiation, medullary complexity was partially reduced and then recovered upon bone marrow transplantation. This intricate topology presents fractal properties, resulting in a considerable corticomedullary area. This feature ensures short distances between cortex and medulla, hence efficient thymocyte migration, as assessed by mathematical models. Remarkably, this junction is enriched, particularly in neonates, in medullary thymic epithelial cells expressing the autoimmune regulator. The emergence of a major medullary compartment is induced by CD4+ thymocytes via CD80/86 and lymphotoxin-α. The development of the medulla emphasizes a complex topology favoring efficient interactions between developing T cells and autoimmune regulator–positive medullary thymic epithelial cells, a key process for central tolerance induction. The Journal of Immunology, 2013, 190: 586–596.

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lymphocytes play critical roles in cell-mediated immunity and are generated in the thymus by sequential maturation and selection events. The thymus is divided into a cortex, supporting the earliest events in thymocyte development, and a medulla, in which thymocytes undergo final selection processes (1, 2). The thymus is composed of a highly organized three-dimensional (3D) network of cortical and medullary thymic epithelial cells (cTECs and mTECs, respectively) (3). The cTECs promote positive selection, leading to survival of double-positive (DP) CD4⁺CD8⁺ thymocytes carrying TCRs capable of interacting with self-MHC (4). Positively selected thymocytes become CD8⁺ or CD4⁺ single-positive (SP), depending on their interactions with mTECs class I or II expressed by cTECs. They then migrate into the medulla in a CCR7-dependent manner (5). The medulla promotes negative selection, which consists in eliminating autoreactive SP thymocytes that carry TCRs exhibiting high affinities for self-antigen–MHC complexes (6). The mTECs play a critical role in this process by expressing a wide range of tissue-restricted self-antigens representing peripheral tissues (7). The expression of these Ags is regulated in part by the autoimmune regulator (Aire) (8). Thymic selection events ultimately lead to the emergence of a T cell population carrying a randomly generated TCR repertoire that has been purged of autoreactive specificities. Mature naive T cells are exported to the periphery, where they circulate and migrate into secondary lymphoid organs, such as lymph nodes (LNs). In LNs, T cells are localized in the deep cortex (T-zone), whereas B cells are found in the outer cortex, arranged in follicles (B-zones) (9, 10).

Lymphoid organ structures were originally unraveled by classical histological techniques. Their complex and highly organized pattern was documented using tissue section counterstaining or immunofluorescence staining, with Abs revealing specific cell types. A pioneering study (11) suggested that the 3D geometry of the thymic medulla displays more convoluted structures than previously thought, with irregular foci and extensive branching. Another approach based on serial sections recently described the 3D organization of the bone marrow (12). More recently, advanced imaging techniques, such as two-photon microscopy, allowed a deeper comprehension of the 3D organization of lymphoid organs. Ex vivo LN explants and in vivo surgically exposed LNs and thymic slices were dynamically imaged by two-photon microscopy (13–15). However, because tissue penetration remains limited to ~400 μm, the 3D topology of entire lymphoid organs remains largely elusive, particularly for the thymus.
We have developed a user-friendly method allowing visualization of the 3D architecture of lymphoid organs from fluorescence microscopy of immunolabeled tissue sections with multiple Abs. This technique was first validated for skin-draining LNs and then applied to the thymus, in healthy and immunodeficient conditions, using the Rag2−/− mouse model, which is almost devoid of medulla, and during thymic recovery upon total body irradiation (TBI) and bone marrow transfer (BMT). We provide novel insight into the organization, development, and function of the thymic medulla, which is composed of a large medulla surrounded by a hundred islets. Unlike Rag2−/− thymi, this organization remains preserved upon TBI. This complex topology provides a significant corticomedullary junction (CMJ) that is enriched, particularly in neonates, in Aire+ mTECs, implicated in the induction of T cell tolerance. We show that CD4+ thymocytes constitute the main cellular actor mediating this 3D organization. This effect is mediated by lymphotixin (LT) α signaling, which is upregulated in SP thymocytes by the CD80/86 (20) mice were maintained under specific pathogen–free (SFP) conditions. Unless mentioned, male and female mice were used at the age of 6–12 wk for adults and 10 d for neonates. All experiments were approved and performed in accordance with guidelines from the Swiss veterinary authorities.

Bone marrow chimeric mice

C57BL/6 mice were lethally irradiated (900 rad), sacrificed after 3 d or injected i.v. with 10^7 bone marrow cells obtained from wild-type (WT) donors.

**Materials and Methods**

**Mice**

C57BL/6 mice (Charles River) were maintained in a conventional animal facility. Rag2−/− (16), β2m−/− (17), H2-Aα−/− (18), LTα−/− (19), and CD80/86−/− (20) mice were maintained under specific pathogen–free conditions. Unless mentioned, male and female mice were used at the age of 6–12 wk for adults and 10 d for neonates. All experiments were approved and performed in accordance with guidelines from the Swiss veterinary authorities.

Bone marrow chimeric mice

C57BL/6 mice were lethally irradiated (900 rad), sacrificed after 3 d or injected i.v. with 10^7 bone marrow cells obtained from wild-type (WT) donors.

**TEC isolation and flow cytometry**

Individual thymi were digested with collagenase D (1 mg/ml)/DNase I (2 μg/ml; Roche). Cells were passed through a 70-μm mesh to remove debris. Hematopoietic cells were depleted with anti-CD45 magnetic beads (Miltenyi Biotec) by AutoMACS. CD45+ cells were incubated at 4°C with Fc-block (anti-CD16/CD32; Pharmingen), costained with ant-EpCAM (Miltenyi Biotec) by AutoMACS. CD45bris. Hematopoietic cells were depleted with anti-CD45 magnetic beads (Invitrogen). Sections were counterstained with 1 μm conjugated streptavidin (Invitrogen), or Alexa Fluor 546 anti-hamster for 30 min with Cy3-conjugated anti-rabbit (Invitrogen), Alexa Fluor 488–conjugated streptavidin (Invitrogen), or Alexa Fluor 546 anti-hamster (Invitrogen). Sections were counterstained with 1 μl/ml DAPI and mounted with Mowiol (Calbiochem).

**Immunofluorescence staining**

Organs were dissected, and fat tissue was removed to avoid interference with 3D reconstruction. Frozen tissues embedded in O.C.T. (Sakura Finetek) were cut into 20-μm-thick sections. Sections were fixed with 2% paraformaldehyde. Abs were diluted in hybridization buffer (1% BSA; 0.01% Triton; and 0.1 M Tris buffer, pH 7.4). LN sections were costained for 30 min with Alexa Fluor 647–conjugated anti-IgD (clone 11-26; eBioscience) and Alexa Fluor 488–conjugated anti-CD3 (clone 500A2; Invitrogen). Thymic sections were costained for 30 min with rabbit anti-keratin 14 (clone AF648; Covance Research) and either biotin-conjugated rat anti-keratin 8 (clone C51; ProGene) or Alexa Fluor 488–conjugated anti-Aire (clone 5H12; eBioscience) or hamster anti-LTo (clone AFB3; Pharmingen). Thymic slices were then incubated for 10 min in blocking buffer (3% BSA in 0.01% Triton and 0.1 M Tris buffer) before incubation for 30 min with Cy3-conjugated anti-rabbit (Invitrogen), Alexa Fluor 488–conjugated streptavidin (Invitrogen), or Alexa Fluor 546 anti-hamster (Invitrogen). Sections were counterstained with 1 μl/ml DAPI and mounted with Mowiol (Calbiochem).

**Automated image acquisition**

Slices were scanned using either an LSM 510 confocal or the automated epifluorescence microscope Mirax (Zeiss) (Fig. 1A; Supplemental Video 1A). The Mirax system uses a Plan-Apochromat 20×0.8 objective, a Zeiss AxioScan MRm camera, and shift-free filter-sets: DAPI [excitation (ex.) BPBlue/550–emission (em.) BP555/50], Alexa Fluor 488 (ex. BP470/40–em. BP530/50), Cy3 (ex. BP565/30–em. BP620/60), and Alexa Fluor 647 (ex. BP640/30–em. BP690/50). A picture of each section was saved with a Panoramic Viewer (3DHistech). The highest possible magnifications, 8× for LN sections, 2× for entire thymic sections, and 10× for high resolution for visualizing Aire+ cells, led to pixel widths of 2.6 μm, 6.5 μm, and 1.3 μm, respectively.

**Computational analysis**

Full organ reconstruction in three dimensions (FOR3D) is automatically performed through the following steps.

**Section registration**

Sections were first registered, using ImageJ (National Institutes of Health) and the dedicated plugin StackReg (21) (Fig. 1B, Supplemental Video 1B).

**Intensity equalization**

This and the following steps are performed automatically by starting the rendering 3D function in Matlab (Mathworks). Labeling variations may result in artifactual disparities in fluorescence intensity between sections, as denoted within the collection of uncorrected sections. With the assumption of a nearly parabolic profile over depth, as generated by a pseudospheroidal organ, intensities could be corrected for possible abrupt variations arising between images (see dashed boxes in image collections before and after equalization, Fig. 1B, 1C, respectively; Supplemental Video 1C). We performed a least-square fit of the mean intensity profile I_{mean} with a second-order equation (Supplemental Fig. 1B). Frame intensities were then corrected by normalizing according to the fit results I_{eq}: I_{eq}(x,y,z) = I(x,y,z) \left( \frac{I_{fit}(Z)}{I_{mean}(Z)} \right)^\alpha.

The exponent \alpha allows tuning this normalization. Because organs are not totally spherical, \alpha was set empirically to 0.2, according to the degree of homogeneity among slices. This setting resulted in homogeneous variations of intensity over depth.

**Data filtering and subsampling**

Images were smoothed by median filtering (Fig. 1D, Supplemental Video 1D) to better reveal macromolecular structures. Voxel size is set by section thickness and microscope settings, typically generating an axial resolution 2× larger than the lateral resolution. Hence, thymic images with an initial pixel width of 6.5 μm were subsampled by 2-fold, leading to a final pixel width of 13 μm. Filtered images were combined into a single 3D array. Gaussian filtering over 3D neighbors further reduced frame-to-frame discrepancies (Fig. 1E, Supplemental Video 1E). To visualize Aire+ cells (Fig. 5F), FOR3D was performed at higher resolution, using images captured at 10× magnification, with a pixel size of 1.3 μm. Aire+ cell detection was improved by background subtraction.

**Rendering in 3D**

Thresholds were set for each channel according to fluorescence intensity, using the Matlab functions graythresh and otsu (Fig. 1F, Supplemental Video 1F), with particular attention to the K14 staining, used to discriminate medulla from cortex. Users may also set thresholds. Surfaces were determined using the Matlab function isosurface (Fig. 1G), with partial transparency to allow visualizing inside organ structures. A 3D rotation video was obtained by viewing the organ from various orientations (Fig. 1H, Supplemental Video 2).

**Individual volume computation**

We quantified 3D structures by identifying individual objects within the organ. Matlab allowed definition of an isosurface, which was often split into several distinct objects. These regions were measured individually using Imaris (Bitplane) (Fig. 1I, Supplemental Video 3).

**Results**

Reconstructing lymphoid organs by automated FOR3D analysis

Fig. 1 and Supplemental Video 1 depict the steps used by FOR3D. Serial sections from WT mouse thymi were immunostained and images acquired over the entire sections. Images were first aligned...
relative to each other, and fluorescence intensity was equalized;
images were smoothed, combined as a 3D volume, and thresh-
olded to define an isosurface enclosing each labeled structure. The
fully automated FOR3D process uses the homemade Matlab
function rendering_3D, which first displays a graphical user in-
terface listing default parameters (Supplemental Fig. 1A). Starting
analysis is hence straightforward and user friendly, provides 3D
movies, and allows volume quantification of specific structures.

FOR3D was first assessed by 3D reconstruction of skin-draining
LNs, highly compartmentalized structures involved in the initiation
and regulation of adaptive immune responses. Whole inguinal LNs
from adult WT mice were sectioned into 20-μm sections, yielding
∼60 per LN. Sections were stained with Abs against the B- and T-
zeone markers IgD and CD3. Nuclei were counterstained with DAPI
(Fig. 2A). We observed that inguinal LNs exhibit well-defined B-
and T-zones. In agreement with previous studies (10), B cells were
arranged in follicles within the outer cortex. In contrast, T cells
were homogenously distributed in the deep cortex. Using FOR3D,
we reconstructed inguinal LNs in 3D (Fig. 2B, Supplemental Video
4). This procedure permitted quantification of B- and T-zone vol-
umes and numbers. The percentages of volume occupied by the B-
and T-zones in the LNs were 18 ± 6% and 71 ± 25%, respec-
tively (mean LN volume: 2.4 ± 0.7 mm³, n = 4) (Fig. 2C). We
observed an average number of 12.5 ± 3 B-zones per LN. Col-
lectively, these data confirm the highly structured 3D organization
of B- and T-zones of mouse inguinal LNs. Performing FOR3D on
LNs allowed us to validate this process, demonstrating its useful-
ness for reconstructing lymphoid organs in 3D.

FOR3D of WT and Rag2−/− immunodeficient thymi:
visualization of the corticomedullary organization

Cortical and medullary regions of the thymus play critical roles in
early events of thymocyte development and self-tolerance induc-
tion, respectively. To gain better insight into the 3D topological
organization of WT mouse thymus, whole lobes were sectioned for FOR3D analysis. To delineate the structure of the fully developed organ, we focused on the adult thymus, yielding ∼100 sections. Sections were stained with Abs against the cTEC-specific marker keratin 8 (K8) and the mTEC-specific marker keratin 14 (K14) to detect the cortex and medulla, respectively. Nuclei, labeled with DAPI, are particularly dense in the subcapsular region (blue), the cortical compartment (green), and the medullary compartment (red). Arrowhead and arrow respectively indicate medullary islets connected or not connected to the main medulla. (F) Medulla volumes were measured by FOR3D. The dashed circle denotes the largest medulla of each lobe. (G) Representative image of a Rag2<sup>−/−</sup> thymic section, stained as described for WT thymi (D). Scale bar, 1 mm. (H) FOR3D of a Rag2<sup>−/−</sup> thymic lobe depicting the subcapsular region (blue), the cortical compartment (green), and the medullary compartment (red). (I) Individual medulla volumes were measured by FOR3D. In (B), (E), and (H), all axes are graduated in millimeters. In (C), (F), and (I), horizontal lines represent the geometric mean and SD, derived from 4 LNs and 3 WT or 3 Rag2<sup>−/−</sup> thymic lobes, respectively, as indicated by different colors. Statistical significance, from unpaired Student t test p value between WT and Rag2<sup>−/−</sup> medullary volumes: ***p < 0.001. B, B-zones; C, cortex; M, medulla; SC, subcapsule; T, T-zone.
Fig. 2C), emphasizing its complexity. In contrast, the cortex did not exhibit the signature of fractal objects \((D = 3, \text{as for a perfect sphere})\). FOR3D also allowed volume quantification of medullary compartments as well as of total thymic lobes. The percentage of medullary volume was \(9.7 \pm 3.7\%\) (mean lobe volume: \(25.9 \pm 8.8 \text{ mm}^3, n = 3\)). Furthermore, as performed for B-zones in LNs, this procedure permitted the quantification of individual medullary islets (Fig. 2F). We observed, on average, \(171.7 \pm 32.5 \text{ medullary islets per thymic lobe}\). Individual medullary volumes were distributed logarithmically over several orders of magnitude, with a geometric mean volume of \(1.6 \times 10^{-3} \text{ mm}^3\) (geometric SD 9.97). This variability was intrinsic to each analyzed lobe.

We further deciphered the corticomedullary organization of the thymus in immunodeficient conditions. We applied our method to Rag2\(^{-/-}\) mice (16), which exhibit an early block in thymocyte development at the DN3 stage and are consequently devoid of SP thymocytes. Formation of the medulla is controlled by the presence of SP thymocytes (24, 25). Accordingly, Rag2\(^{-/-}\) mice have defective medulla formation (26, 27). A representative image of a Rag2\(^{-/-}\) thymic section stained for K14 and K8 and counterstained with DAPI is shown in Fig. 2G. Using FOR3D, we reconstructed Rag2\(^{-/-}\) thymic lobes in 3D (Fig. 2H, Supplemental Video 6). Rag2\(^{-/-}\) thymi are 10-fold smaller than WT thymi and contain, on average, only \(20.7 \pm 9.3 \text{ small, isolated medullary islets, occupying 0.21 \pm 0.13\% of the thymic volume (n = 3) without any large central medulla}\). Moreover, no fractal feature was observed, denoting a simple topology. Individual medullary volumes were reduced by 2.5-fold, compared with those in WT thymi (Fig. 3A–G, 2 wk after TBI). A geometric mean volume of \(3.25 \times 10^{-3} \text{ mm}^3\) (geometric SD 3.7) was observed, denoting a simple topology. Individual medullary volumes were distributed logarithmically over several orders of magnitude, with a geometric mean volume of \(1.6 \times 10^{-4} \text{ mm}^3\) (geometric SD 9.97). This variability was intrinsic to each analyzed lobe.

3D medullary topology upon thymic recovery after TBI and BMT

Although lymphoablative treatments such as chemotherapy and TBI severely affect thymic cellular composition and architecture (28, 29), their impact on the 3D corticomedullary organization remains unknown. We used FOR3D to investigate how the thymic topology evolves after TBI and subsequent BMT. In WT mice, 3 d after a lethal dose of TBI, DP thymocytes were massively reduced by 2.2-fold, compared with those in WT thymi (Fig. 3A, 3B). FOR3D revealed that the increase in thymic size after 2 wk concerned mainly the cortex (Fig. 3E, Supplemental Videos 8–10), occurring consistently with the reappearance of DP thymocytes. This increase in cortical volume continues 2 wk after BMT because of the accumulation of DP thymocytes (Fig. 3B, 3E). The central medullary compartment (Fig. 3G, dashed circles) and islets started to increase in size only after 2 wk, concomitantly with the reappearance of SP thymocytes. This change was associated with a decrease in the medullary fractal dimension to values similar to those in WT conditions \((D_f \approx 2.2, \text{Supplemental Fig. 2C})\), relating the complexity of the medullary shape directly to the development of SP thymocytes. At 3 wk after BMT, the thymic volume became comparable to what it was before TBI, whereas the CMJ area progressively increased (Fig. 3E). Because FOR3D allowed us to segment the thymus into two compartments, cortex and medulla, we computed the migration distance required for thymocytes located anywhere within the cortex to reach the CMJ. This distance was reduced upon TBI, consistently with an extensively affected cortex, but recovered to a basal value after BMT (Fig. 3E).

The complex medullary topology favors the encounter of SP thymocytes with the CMJ

We investigated the impact of a fractal medulla by comparing experimental measures obtained from WT (Fig. 4A) and immunodeficient thymi (Rag2\(^{-/-}\) and upon TBI) (Fig. 4B, 4C, respectively) with two mathematical models. In one model, the medulla was represented by a single sphere (Fig. 4D), whereas in the other, the medullary compartment was modeled by a set of randomly located spheres (Fig. 4E). Computing migration distances from cortical locations to the CMJ led to a rather narrow distribution for WT thymi, with an average distance of \(114 \pm 42 \text{ mm}^2 (n = 3, \text{Fig. 4A})\). Distances were overall increased in Rag2\(^{-/-}\) thymi (Fig. 4B), consistently with a noticeably reduced medulla, and in contrast decreased in thymi after TBI (Fig. 4C), harboring a reduced cortex. The spherical medulla model (Fig. 4D) led to a broad distance distribution. In contrast, the random pattern, composed of several objects (Fig. 4E), led to a distribution similar to that obtained experimentally. This comparison illustrates that the complex medullary topology allows shorter distances between the cortex and the CMJ than that afforded by a simple spherical medulla. FOR3D also allowed direct measurement of the CMJ area. Rag2\(^{-/-}\) thymi, being almost devoid of medulla, afford a negligible CMJ area \((0.42 \pm 0.23 \text{ mm}^2)\), compared with the WT CMJ area \((40.5 \pm 10.6 \text{ mm}^2)\), whereas thymi after TBI display an intermediate area \((22.3 \pm 8.8 \text{ mm}^2)\). Compared with the WT medullary volume, a sphere provides the smallest possible area \((14.7 \pm 4.2 \text{ mm}^2)\) (Fig. 4F). The random model, being also composed of spheres, leads to a comparable value. However, both areas are 3- to 5-fold lower than the experimentally measured fractal CMJ area. Hence, a fractal medulla ensures a substantial CMJ area, together with short distances from any cortical location, thus favoring efficient thymocyte migration. Immunodeficient conditions such as Rag2\(^{-/-}\) and irradiated thymi present defects in these two parameters, defining a new signature of their deficiency.
FIGURE 3. FOR3D of WT thymic lobes after TBI and following BMT. (A) Thymocytes isolated from untreated mice (WT), irradiated mice 3 d after TBI and mice reconstituted 1, 2, or 3 wk after WT BMT, were analyzed for CD4 and CD8 expression by flow cytometry. (B) Histograms show the numbers of DN, DP, CD4+, and CD8+ thymocytes for the indicated conditions. TBI denotes 3 d after TBI; w1, w2, and w3 denote 1, 2, and 3 wk after WT BMT, respectively. (C) Representative images of thymic sections costained for K14 (red) and K8 (green) and counterstained with DAPI (blue), with same scale for each condition. Scale bar, 1 mm. (D) FOR3D of thymic lobes depicting the subcapsular region (blue), the cortical compartment (green), and the medullary compartment (red) for each condition at the same scale. All axes are graduated in millimeters. (E) Histograms show quantification of cortical and medullary volumes as well as CMJ area for the indicated conditions. (F) FOR3D of thymic lobes for each condition, with medullae color coded according to their volume, from red (smallest) to yellow (largest). Scale bar, 1 mm. (G) Medullary volumes were measured by FOR3D. Horizontal lines represent the geometric mean and SD, derived from three thymic lobes for each condition (see Supplemental Fig. 2), as indicated by different colors. Dashed circles denote the largest medulla of each lobe. In (B), (E), and (G), means and standard deviations are derived from three mice per condition. Statistical significance, from one-way ANOVA with post hoc Bonferroni test: *p < 0.05, **p < 0.01, ***p < 0.001.
The CMJ is enriched in Aire\(^+\) mTECs, particularly in neonates

The thymic medulla is specialized for the deletion of autoreactive SP thymocytes. The mTECs play a pivotal role in this process by expressing peripheral self-antigens regulated in part by the transcription factor Aire. It has been suggested that Aire\(^+\) mTECs are located at the CMJ, in addition to the medulla (30, 31). A preferential localization at the CMJ was notably reported by in situ hybridization (32), but this specific pattern has not yet been quantified. Furthermore, a recent study indicated that Aire is particularly important during the perinatal period for preventing the development of multiorgan autoimmunity (33). Because we observed that the medulla exhibits a complex topology leading to a large CMJ area, we quantified the density of Aire\(^+\) mTECs located at the CMJ and in the medulla in neonates and adult thymic sections by costaining for K14 and Aire (Fig. 5A). The CMJ region was defined as an external fringe 80 \(\mu m\) in width surrounding the medulla. The density of Aire\(^+\) mTECs is higher at the CMJ than in the medulla at both stages of development (Fig. 5B). Moreover, both regions exhibit a higher density at the neonate stage than in the adult. Enrichment in Aire\(^+\) cells at the CMJ was observed for a majority (~60\%) of medullae (Supplemental Fig. 3A). This enrichment was further analyzed by computing the percentage of Aire staining relative to the distance from the CMJ external border. The gradual enrichment in Aire\(^+\) cells follows a sharp gradient, culminating in a 2-fold increase at the CMJ in neonates and to a lower extent in adults (Fig. 5C). This gradient is firmly established within a few micrometers from the CMJ border. The increased density in Aire\(^+\) mTECs in neonates compared with adults mice suggests that the frequency of these cells decreases in adults. To confirm, we quantified the frequency of Aire\(^+\) mTECs within the total mTEC population in neonates and adults by flow cytometry (Fig. 5D, 5E). The frequency of Aire\(^+\) mTECs was 2× higher in neonates than in adults, in accordance with the quantification of Aire\(^+\) cell density observed by immunohistology (Fig. 5B, 5C). Preferential localization of Aire\(^+\) cells at the CMJ was further confirmed by FOR3D of thymi costained for K14 and Aire (Fig. 5E; Supplemental Videos 11, 12). Computing in 3D the distance of Aire\(^+\) cells from the CMJ led to a distribution biased toward lower values, denoting that Aire\(^+\) cells are preferentially located at the border rather than in the inner regions of the thymic cortex.

**FIGURE 4.** Distance from cortex to medulla in experimental and modelized thymi. (A) The FOR3D results obtained on a thymic lobe (see Fig. 1) allow delimitation of the cortex (white) and medulla (black), illustrated by the representative binary image (top panel) corresponding to the middle of the thymus. The Euclidean distance from every cortical location to the nearest medullary position was computed and represented as a distance map (pseudocolors, middle panel) and histogram (bottom panel), with the average value \(d\) indicated by the red dashed line. For comparison, the same computation was performed for two immunodeficient conditions as well as for two mathematical models: Rag2\(^{-/-}\) thymi (B), thymi from TBI mice (C), theoretical spherical thymi exhibiting a single and concentric medulla (D), and theoretical spherical thymi with small medullae randomly located in 3D (E). Sizes of the modeled medullae in (D) and (E) were set to the mean total volume experimentally determined in WT conditions (5.5 mm\(^3\)). Scale bar, 1 mm. (F) The histograms depict the mean distance from cortical localization to the CMJ (left) and the CMJ area (right), as measured by FOR3D for the indicated experimental conditions or mathematical models. Means and standard deviations are derived from three measures. Statistical significance, from one-way ANOVA with post hoc Bonferroni test for comparison with WT: **\(p < 0.01\), ***\(p < 0.001\).
medulla. Importantly, the CMJ constitutes the first region that SP thymocytes encounter when migrating to the medulla and is where autoreactive thymocytes are deleted. The substantial CMJ area, because of the complex medullary topology, together with the local enrichment in Aire+ cells, enhances the crucial importance of this privileged interface for the induction of T cell tolerance.

The emergence of the central medulla is promoted by CD4+ thymocytes via CD80/86 and LTα signaling

It is well described that the formation of the thymic medulla is controlled by the development of SP thymocytes (24, 34–36). We investigated the subset of SP thymocytes responsible for this complex 3D medullary organization. Thymic lobes from WT, β2m−/− (lacking CD8+ thymocytes), and H2-Aa−/− (lacking CD4+ thymocytes) mice were sectioned, costained for K8 and K14, and reconstructed by FOR3D (Fig. 6A–C). Thymic lobe volumes were comparable for all conditions (Fig. 6F). In β2m−/− and WT thymi, medullary volumes and fractal dimensions (Fig. 6A, 6B, 6F; Supplemental Fig. 4; Supplemental Video 13) were also similar. Notably, a central compartment accounted for the majority of the medullary volume (asterisk in Fig. 6A, 6B). In contrast, H2-Aa−/− thymi lacked any large medullary compartment, leading to a noticeably reduced volume (Fig. 6C, 6F; Supplemental Fig. 4; Supplemental Video 14). Isolated islets displayed various sizes, with the largest one accounting for only 0.08 ± 0.07 mm³ (20.2 ± 1.4%, n = 3) of the total volume, compared with 2.0 ± 1.5 mm³ (73.6 ± 33.1%, n = 3) for WT mice. We further investigated the molecular mediators responsible for this complex architecture. SP thymocytes interact with mTEC via several signaling axes of the TNF family, such as CD40, receptor activator of NF-κB (RANK), and LTβ receptor (LTβR) (25, 37, 38). Previous studies suggested that only the LTα1β2–LTβR axis was correlated with a defect in medullary architecture (23, 39). We analyzed the 3D architecture in LTα−/− thymi and found a topology similar to that in H2-Aa−/−...
thymi, characterized by isolated islets without any large central compartment (Fig. 6D, Supplemental Video 15). Islet and total medullary volumes displayed values intermediate between those of H2-Aa<sup>2</sup>/<sup>2</sup> and WT thymi (Fig. 6F). In H2-Aa<sup>2</sup>/<sup>2</sup>, the fractal dimension was increased to a value closer to 3, denoting a topology of reduced complexity (Supplemental Fig. 4C). We previously reported that Ag-specific interactions between CD4<sup>+</sup> thymocytes and mTECs determine the cellularity of mature Aire<sup>+</sup> mTECs (31). In this context of cellular interactions, we therefore hypothesized a possible involvement of the CD28–CD80/86 co-stimulatory pathway in controlling the 3D medullary organization. FOR3D indeed revealed that CD80/86<sup>2</sup>/<sup>2</sup> thymi also presented a medulla mostly devoid of any major compartment, as for H2-Aa<sup>2</sup>/<sup>2</sup> and LTα<sup>2</sup>/<sup>2</sup> mice (Fig. 6E, 6F; Supplemental Video 16). Because LTα<sup>2</sup>/<sup>2</sup> and CD80/86<sup>2</sup>/<sup>2</sup> thymi exhibit similar phenotypes, we hypothesized that CD80/86 could regulate LTα expression. We thus compared LTα expression in WT and CD80/86<sup>2</sup>/<sup>2</sup> medullae by immunofluorescence. We observed a significant decrease of LTα protein expression in CD4<sup>+</sup> SP thymocytes from CD80/86<sup>2</sup>/<sup>2</sup> medullae (Fig. 6G), showing that LTα is upregulated upon CD28–CD80/86 engagement. Altogether, these results indicate that CD4<sup>+</sup> thymocytes are required for the establishment of a proper medullary topology with a major compartment through the CD80/86 and LTα signaling pathways.

**FIGURE 6.** CD4<sup>+</sup> thymocytes are required for 3D medullary organization through CD80/86 and LTα signals. (A–E) Representative images of thymic sections, costained for K14 (red) and K8 (green) and counterstained with DAPI (blue) (upper panel). FOR3D rendering using Matlab (middle panel) and Imaris, with medullae color coded according to their volume, from red (smallest) to yellow (largest) (lower panel) of a WT, β<sub>2m</sub><sup>−/−</sup>, H2-Aa<sup>−/−</sup>, LTα<sup>−/−</sup>, or CD80/86<sup>−/−</sup> thymus. Same scale used for each condition. Scale bar, 1 mm. All axes are graduated in millimeters. The asterisk denotes the largest medulla for WT and β<sub>2m</sub><sup>−/−</sup> mice. (F) Histograms show quantification of volumes for thymic lobe, total medulla, mean medullary islet, and largest medulla for the indicated conditions. Mean and SD are derived from three thymic lobes (see Supplemental Fig. 4). Statistical significance, from one-way ANOVA with post hoc Bonferroni test: *p < 0.05. (G) Thymic sections from WT or CD80/86<sup>−/−</sup> mice were costained for K14 (blue), CD4 (green), and LTα (red) and imaged by confocal microscopy with identical settings for each condition. LTα staining was evaluated by computing the mean value of the fluorescence intensity (n = 3 medullae for WT mice and n = 6 medullae for CD80/86<sup>−/−</sup> mice). Scale bar, 40 μm.
Discussion
Reconstructing the 3D architecture of lymphoid organs provides a better understanding of the topology of their specific compartments. It also allows quantification of defined structures in terms of number and volume, such as B cell follicles in LNs or medullary islets in the thymus, as shown in this article. Furthermore, by allowing 3D rotation, this approach demonstrated that certain compartments are connected through highly curved structures, whereas others are disconnected. This geometric distribution is difficult to assess using two-dimensional sections, particularly for thymic medullary islets, which exhibit a highly complex topology. Diagrams depicting the medullary geometry of the thymus in textbooks or academic websites oversimplify its organization, underestimating its complexity by depicting either a central compartment or a collection of small medullae. Our results firmly establish the existence of an intricate central compartment that is either subtly connected or not connected to a hundred smaller peripheral islets, with a broad distribution of volumes. The coexistence of individual medullae in agreement with the observation that mTEC islets develop from single precursors (40).

Thymic function can be affected by numerous conditions, such as SCIDs or therapeutic treatments such as chemotherapy and TBI. We investigated the 3D corticomedullary organization in Rag2−/− immunodeficient mice and during thymic recovery after TBI and BMT. Rag2−/− mice exhibit an early defect in T cell development and thus lack adequate mTEC development (26). We found that the thymus of these animals is devoid of the large central medulla observed in healthy animals and exhibits only 10 small, unconnected islets with a noncomplex topology. These observations indicate that the intricate 3D organization of the medulla is tightly controlled by the development of SP thymocytes. We further applied FOR3D to decipher to which extent the corticomedullary organization is affected upon TBI and subsequent BMT. A severe thymic atrophy was observed after lethal TBI, which was characterized by a substantial decrease in cortical and, to a lesser extent, medullary volumes. Despite these massive effects, the fundamental corticomedullary organization characterized by a large central medulla and surrounding islets was surprisingly conserved. This finding could be explained by the fact that before TBI, mTECs have been already educated through interactions with SP thymocytes, allowing preservation of their overall organization, which was not the case in Rag2−/− mice. However, the number of islets and medullary complexity were reduced after TBI. Upon BMT, the cortical volume started to increase already after 1 wk, correlating with the reappearance of DP thymocytes, whereas the medullary volume increased after 2 wk, correlating with the development of SP thymocytes. These rearrangements also correlate with a recovery in topological complexity, as assessed by fractal dimensions. Overall, a major difference between the two immunodeficient conditions studied in this article is the quasi-absence of medulla in Rag2−/− thymi, whereas TBI essentially affects the cortex. Nevertheless, in both cases, the exquisite intricacy between cortex and medulla is affected, which can be directly related to defects in thymocyte development and migration.

In healthy conditions, SP thymocytes migrating from the cortex to the medulla encounter the CMJ, which is enriched in Aire+ mTECs. Remarkably, we found that this region is particularly dense in Aire+ mTECs in neonates, compared with adults. This finding is consistent with the fact that Aire was described as important during the perinatal period for preventing the emergence of autoimmune disorders (33). Aire+ mTECs, by expressing peripheral self-antigens, play a critical role in the deletion of T cells bearing self-reactive TCRs (25, 41). Therefore, the CMJ represents not only the privileged site of homing and export of thymocytes from the vasculature (11, 42) but also the interface where SP thymocytes reach the medulla. We found that the medulla exhibits a fractal topology, which is characterized by islet volumes spanning over several orders of magnitude. This feature substantially increases the surface of the CMJ interface by almost 10×. Fractal geometry also ensures that the average distance from any location within the cortex to the nearest medulla remains reasonably low, as experimentally measured for the medullary geometry by FOR3D. For comparison, in a nonfractal model with a single central medulla, this average distance increases substantially. A random medulla model exhibits a more complex geometry and hence also ensures that any location in the cortex remains sufficiently close to the medulla. Considering pseudo-Brownian motion with an average motility of 7 μm²/min, as measured by two-photon microscopy (13, 14), the distance for a thymocyte randomly located in the cortex to reach the CMJ is covered in ~7.8 ± 1 h, instead of 194 ± 12 h for the spherical medulla model. Of note, thymocytes undergo partially directed, instead of strictly Brownian, motion, being guided by stromal network and chemotactic cues such as CCL19 and CCL21 (2, 43). Directed motion, compared with erratic motion, results in a reduced migration time. However, thymocytes also undergo sequestration in specific niches in a chemokine-dependent manner, leading to an overall residency time of a few days (42, 44). Even if linear or random behaviors, together with transient arrests, directly have an impact on motion duration, this will similarly affect cell motion for experimental and theoretical configurations. This effect will hence not qualitatively have a bearing on the conclusion that a complex medulla shape ensures reduced distance and duration for thymocytes to encounter the CMJ. Fractal geometry, together with the enrichment in Aire+ cells at the CMJ, stresses the importance of harboring a substantial CMJ area for favoring fast and efficient interactions between SP thymocytes and mTECs and should thus be crucial for adequately educating thymocytes and avoiding autoimmune disorders.

We investigated the cellular and molecular mediators dictating this intricate 3D medullary topology. Formation and homeostasis of the medulla are known to rely on signaling crosstalk between mTECs and SP thymocytes (24, 25). We show that proper 3D medullary organization is specifically controlled by CD4+ thymocytes, which are necessary and sufficient for inducing the emergence of the major medulla, hence generating a consequent CMJ area. We further deciphered the signals mediating this effect of CD4+ thymocytes on medullary organization. We first used the LTool−/− mouse model, because studies on two-dimensional sections suggested that this cytokine could influence medullary architecture (23, 39). Of interest, the absence of LTo led to an intermediate phenotype between that of WT mice and that of mice lacking CD4+ thymocytes. LTool−/− thymi were also characterized by the lack of a large central medulla, leading to a lower reduction of medullary volume. This finding suggests that additional molecular mediators, which remain to be identified, could be expected to contribute to the effect induced by CD4+ thymocytes. Because SP thymocytes interacting with mTECs control their development, we investigated the implication of costimulatory molecules, expressed by mTECs (27, 31). Previous studies ruled out a direct effect of the CD40L–CD40 axis on the medullary architecture (45). Nevertheless, the role of the CD28–CD80/86 axis had not been investigated so far. In this article, we show that CD80/86 are required for upregulating LTool expression in CD4+ thymocytes, leading to the establishment of a proper 3D medullary organization, notably through the emergence of a large central medulla. Therefore, during thymic development, growing
mTEC islets (40) are expected to join together, leading to the emergence of large structures and ultimately to a major medulla.

FOR3D should be relevant for deciphering the 3D organization of complex tissues under healthy or pathological conditions. For instance, tumor vascularization, also exhibiting a fractal pattern, is a critical step in cancer evolution, which helps in quantifying diagnosis (46). The precise geometric aspect of vascularization and the impact of antiangiogenesis agents, putatively altering this fractal topology, may be clinically relevant for evaluating potential therapeutic effects. FOR3D is open source software available upon request for academic research and adaptable for innovative tasks. Finally, this procedure could be applied to determining the specific localization of different cell types and evaluating the degree of organization in WT and mutant conditions, hence opening new perspectives for preclinical studies.

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Disclosures

The authors have no financial conflicts of interest.

References


Mean fluorescence intensity

z plane
A. WT  β2m−/−  H2-Aa−/−  LTα−/−  CD80/86−/−

B. WT  β2m−/−  H2-Aa−/−  LTα−/−  CD80/86−/−

C. WT  β2m−/−  H2-Aa−/−  LTα−/−  CD80/86−/−

Fractal dimension

Dj

thymic lobe  medulla

Box size (mm)
**Supplemental Figure 1**

**FOR3D settings**

(A) A graphical user interface allows users to verify and eventually adjust the parameters used for running the `rendering_3D` function in Matlab, which automatically process images to generate the 3D reconstruction. The program only requires five input parameters, which may be adjusted or left to their default values:

- Image name, corresponding to a multi-tiff format. Matlab directory should be set according to data localization.
- Fluorescence thresholds for each channel, either automatically or manually estimated.
- Pixel size, according to microscope settings.
- Slice width, defined by cryosection thickness.
- Equalization factor, from 0 (none) to 1 (full correction).

Pixel size and slice width are used to compute the image sub-sampling factor required to optimize voxel proportions (Supplemental Methods). Note that default values may be easily modified by editing the associated Matlab function `FOR3D_dialog_box`. (B) Equalization of fluorescence intensities over thymic slices. The graph depicts the mean fluorescent values for each z plane and each color channel (K14, red; K8, green and DAPI, blue). The effect of equalization (see Figure 1C) is illustrated before (solid lines, $\alpha$ set at 0), after partial (dashed lines, $\alpha$ set at 0.2 as used by default) and after full (dotted lines, $\alpha$ set at 1) correction.

**Supplemental Figure 2**

**3D rendering and fractal analysis of thymic lobes in WT, after TBI and BMT**

3D rendering was performed by FOR3D using Matlab (A) and Imaris (B) for thymi in WT conditions, after TBI or after subsequent BMT, in triplicate for each condition. Thymic slices were labeled with K14 (red), K8 (green) and DAPI (blue). Axes are graduated in mm and all graphs are displayed at the same scale. In Imaris, individual medullae are color encoded according to their volume, from red (smallest islets) to yellow (major compartment). (C) Fractal dimension was assessed for the outline of the medulla (corresponding to the CMJ) and for the entire lobe, as defined by FOR3D, using the Matlab function `boxcount`. This tool allows evaluation of the number of boxes required to cover the object of interest, as a function of the box size. For a fractal object, the slope of the logarithm of this function is constant. It exhibits at least a plateau over a given range, and this value can be regarded as a definition of
the fractal dimension. In all conditions, the slope of the medulla curve (red) consistently displayed a plateau from ~10 μm to ~1 mm (horizontal dash line), whereas the slope of the thymic lobe curve (green) never displayed any plateau. The fractal dimension \( D_f \) defined by the mean value over the plateau, is hence only given for the medulla, with \( n=3 \) lobes for each condition.

**Supplemental Figure 3**

**Aire\(^+\) mTECs are preferentially located at the CMJ**

(A) Thymic sections from neonate or adult WT mice were co-stained with antibodies against the mTEC marker K14 (red) and Aire (green). Large-scale images were acquired by Mirax at 20x magnification. Aire\(^+\) mTEC enrichment at the CMJ is indicated by arrowheads. M stands for medullary islets. (B) Representative thymic sections, located in the middle of the organ, from neonate (upper left image) or adult (lower left image) thymic lobes depict the regions used for mapping Aire\(^+\) cell localization. The thresholded sections (middle images) show the positions of medulla (K14, red) and Aire\(^+\) cells (green). The maps (right images) encode in pseudo-colors the distances from any location within the medulla to the CMJ. (C) 3D rendering was stretched by 3 fold along the optical z-axis (lower images) to localize Aire\(^+\) cell with better precision.

**Supplemental Figure 4**

**3D rendering and fractal analysis of thymic lobes in WT, β2m\(^+\), H2-Aa\(^+\), LTα\(^−\) and CD80/86\(^−\) mice**

FOR3D was performed in triplicate for each condition, allowing evaluation of fractal dimensions, as described for Supplemental Figure 2.
Movie legends

Movie 1

FOR3D successive steps
Each step of FOR3D image processing is shown for each slice of the thymic lobe depicted in Figure 1 and Movie 2: (A) raw image, (B) registering, (C) intensity equalization, (D) 2D median filtering, (E) 3D Gaussian filtering and (F) thresholding (see Figure 1A-F). The entire lobe (DAPI, blue), the cortical compartment (K8, green) and the medullary compartment (K14, red) were immunolabeled and acquired by fluorescence microscopy. Scale bar: 1 mm.

Movie 2

3D rotation of a WT thymic lobe
FOR3D of a WT thymic lobe depicting the entire lobe (DAPI, blue), the cortical compartment (K8, green) and the medullary compartment (K14, red) (see Figure 1H). Rotation allows viewing its organization under various angles. All axes are graduated in mm.

Movie 3

3D rotation of a WT thymic lobe emphasizing medullary compartment sizes
FOR3D of a WT thymic lobe (see Movie 2) depicting the entire lobe (DAPI, blue), the cortical (K8, green) and medullary compartments (K14). The medullary compartment was segmented with the threshold set by FOR3D and encoded according to medullary sizes with pseudo-colors (see color-bar) from red (smallest medullae) to yellow (largest medulla).

Movie 4

3D rotation of a WT skin-draining LN
FOR3D of a WT inguinal LN (DAPI, blue) depicting B cell zones (IgD, red) and T cell zone (CD3, green) (see Supplemental Figure 2B). Rotation allows viewing its organization under various angles. All axes are graduated in mm.
Movie 5

3D rotation of a WT thymic lobe
FOR3D of a WT thymic lobe depicting the entire lobe (DAPI, blue), the cortical compartment (K8, green) and the medullary compartment (K14, red) (see Figure 2B). Rotation allows viewing its organization under various angles. All axes are graduated in mm.

Movie 6

3D rotation of a Rag2⁻/⁻ thymic lobe
FOR3D of a Rag2⁻/⁻ thymic lobe depicting the entire lobe (DAPI, blue), the cortical compartment (K8, green) and the medullary compartment (K14, red) (see Figure 2D). Rotation allows viewing its organization under various angles. All axes are graduated in mm.

Movie 7

3D rotation of a WT thymic lobe three days after TBI
FOR3D of a thymic lobe isolated from a sublethally irradiated WT mouse, depicting the entire lobe (DAPI, blue), the cortical compartment (K8, green) and the medullary compartment (K14, red) (see Figure 3E). Rotation allows viewing its organization under various angles. All axes are graduated in mm.

Movie 8

3D rotation of an irradiated WT thymic lobe one week after BMT
Same legend as for the previous movie

Movie 9

3D rotation of an irradiated WT thymic lobe two weeks after BMT
Same legend as for the previous movie

Movie 10

3D rotation of an irradiated WT thymic lobe three weeks after BMT
Same legend as for the previous movie
Movie 11

3D rotation of a neonate thymic lobe stained for K14 and Aire\(^+\) cells
FOR3D of a thymic lobe isolated from a neonate WT mouse, depicting the center of a lobe (DAPI, blue), the medullary compartment (K14, red) and Aire\(^+\) mTEC (green) (see Figure 5F). Rotation allows viewing cell localizations under various angles. All axes are graduated in mm.

Movie 12

3D rotation of an adult thymic lobe stained for K14 and Aire\(^+\) cells
Same legend as for the previous movie

Movie 13

3D rotation of a β2m\(^-\) thymic lobe
FOR3D of a β2m\(^-\) thymic lobe depicting the entire lobe (DAPI, blue), the cortical compartment (K8, green) and the medullary compartment (K14, red) (see Figure 6). Rotation allows viewing its organization under various angles. All axes are graduated in mm.

Movie 14

3D rotation of a H2-Aa\(^+\) thymic lobe
Same legend as for the previous movie

Movie 15

3D rotation of a LTα\(^+\) thymic lobe
Same legend as for the previous movie

Movie 16

3D rotation of a CD80/86\(^+\) thymic lobe
Same legend as for the previous movie