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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 corticomedullary 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 signals. This comprehensive three-dimensional view 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.
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 implicating 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 costimulation. These findings are confirmed by background subtraction.

Materials and Methods

Mice

C57BL/6 mice (Charles River) were maintained in a conventional animal facility. Rag2−/− (16), β2m−/− (17), H2-Aa−/− (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 105 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 anti-EpCAM (Miltenyi Biotec) by AutoMACS. CD45−/− thymocytes were isolated and further stained with anti-Aire (clone 5H12; eBioscience) and anti-Ly51 (clone 6C3; Pharmingen), and 10% DAPI and Alexa Fluor 488 (ex. BP470/40–em. BP515/80). A picture of each section was saved with a pixel size of 1.3 μm.

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 AF64; Covance Research) and either biotin-conjugated anti-keratin 8 (clone C51; ProGene) or Alexa Fluor 488–conjugated anti-Aire (clone 5H12; eBioscience) or hamster anti-LTα (clone AEF3; Pharmingen). Thymic slices were then incubated for 10 min in blocking buffer (5% 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 Axiscan MRm camera, and shift-free filter-sets: DAPI [excitation (ex.) BP255/50–emission (em.) BP255/50], 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 Pannoramic 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 \( I_{\text{mean}} \) with a second-order equation (Supplemental Fig. 1B). Frame intensities were then corrected by normalizing according to the fit results \( I_0 \):

\[
I_0(x, y, z) = I(x, y, z) \left( \frac{I_{\text{fit}}(Z)}{I_{\text{mean}}(Z)} \right)^n.
\]

The exponent \( n \) allows tuning this normalization. Because organs are not totally spherical, \( n \) 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 macrocellular 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. II, 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 thresholded 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 interface 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-zone 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 volumes and numbers. The percentages of volume occupied by the B- and T-zones in the LNs were 18 ± 6% and 71 ± 25%, respectively (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. Collectively, 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 usefulness for reconstructing lymphoid organs in 3D.

FIGURE 1. Overview of the FOR3D process. This function block diagram depicts the operations iteratively applied to images acquired by fluorescence microscopy of immunolabeled thymic sections, costained with Abs against the mTEC-specific marker K14 (red) and the cTEC-specific marker K8 (green) and counterstained for nuclei (DAPI, blue). Each block corresponds to a main task (A–I), with software used and eventual subfunction indicated below (left column). Note that equalize_stack (C), filter_stack (D, E), and rendering_3D (F–H) are homemade functions, whereas medfilt2, smooth3, graythresh, isosurface, and view are original Matlab functions. Each step is illustrated by representative results: collection of all images (middle column; scale bar, 10 mm) and a representative one (right column; scale bar, 1 mm). The last steps (G–I) combine all images within a single 3D thymic lobe (axes graduated in millimeters). Dashed boxes (B, C) denote sections typically corrected by the intensity equalization step. The thresholded image (F) was color coded with partial transparency, in agreement with the isosurface (G) and 3D (H). In the last 3D view, obtained by Imaris (I), individual medullae are color coded according to their volume, from red (small medullae) to yellow (large central medulla), as indicated by the color bar.
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−/− thymic section, stained as described for WT thymi (D). Scale bar, 1 mm. (H) FOR3D of a Rag2−/− 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 (C), (F), and (I), horizontal lines represent the geometric mean and SD, derived from 4 LNs and 3 WT or 3 Rag2−/− thymic lobes, respectively, as indicated by different colors. Statistical significance, from unpaired Student t test p value between WT and Rag2−/− 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 \((D = 3\), 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: \(25.9 \pm 8.8\ mm^3, n = 3\)). Furthermore, as performed for B-zones in LNs, this procedure permitted the quantification of individual medullary islets (Fig. 2F). We observed, on average, \(171.7 \pm 32.5\ mm^3\) medullary islets per thymic lobe. Individual medullary volumes were distributed logarithmically over several orders of magnitude, with a geometric mean volume of \(1.6 \times 10^{-4}\ mm^3\) (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 costained 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 \(20.7 \pm 9.3\ mm^3\), 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, with a geometric mean volume of \(6.5 \times 10^{-4}\ mm^3\) (geometric SD 3.8, Fig. 2I). Collectively, these results demonstrate the complexity and highly structured 3D organization of the medulla in healthy conditions, which is largely lost in Rag2:\(^{-/-}\) immunodeficient mice, directly correlating the complex medullary organization with the development of SP thymocytes.

### 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 4-fold, compared with those in WT thymi, with a geometric mean volume of \(8.8 \times 10^{-4}\ mm^3\) (geometric SD 3.7%, Fig. 3A). 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 distance 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.

BMT from WT donors to irradiated WT mice allowed gradual recovery of thymopoiesis and thymic organization over time. This recovery was assessed by flow cytometry, showing the sequential reappearance of DP thymocytes by 1 wk after BMT, followed by the development of CD4\(^{+}\) and CD8\(^{+}\) SP thymocytes after 2 and 3 wk (Fig. 3A, 3B). FOR3D revealed that the increase in thymic size after 1 wk was 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 \((D_f \approx 2.2,\) 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 \(114 \pm 42\ mm\) \((n = 3,\) 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 \((0.42 \pm 0.23\ mm^2)\), compared with the WT CMJ area \((40.5 \pm 10.6\ mm^2)\), whereas thymi after TBI display an intermediate area \((22.3 \pm 8.8\ mm^2)\). Compared with the WT medullary volume, a sphere provides the smallest possible area \((14.7 \pm 4.2\ mm^2)\) (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⁺ cells are 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 μm 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 (Fig. 5F; Supplemental Fig. 3B, 3C) led to a distribution biased toward lower values, denoting that Aire⁺ cells are preferentially located at the border rather than in the inner region.

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**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 modelized medullae in (D) and (E) were set to the mean total volume experimentally determined in WT conditions (5.5 mm³). 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.

FIGURE 5. Aire+ mTECs are enriched at the CMJ in the thymus. (A) Thymic sections from neonate or adult WT mice were costained for K14 (red) and Aire (green) and imaged by confocal microscopy. M denotes the medulla, and CMJ denotes the corticomedullary junction, which was defined by an 80-μm-thick zone surrounding the medullae. Scale bar, 100 μm. (B) The density of Aire+ cells in the center of medullae and at the CMJ was measured using Metamorph. Each symbol represents an individual confocal image. (C) The percentage of area exhibiting Aire fluorescence intensity above threshold was averaged over all pixels, in either the medulla (M) or the cortex (C), being at the same distance from the CMJ outer border, which was defined as the limit of K14 staining (vertical dashed line at 0). Curves represent the mean and SD (plain and dashed lines, respectively), derived from 12 confocal images for neonates and adults. (D) Representative flow cytometry profiles for the expression of EpCAM and Aire by CD45−EpCAM−Ly51−mTECs from neonate and adult WT mice. Numbers represent the percentages of cells within the indicated gates. (E) The histogram summarizes quantifications of the percentages of Aire+ mTECs observed in total mTECs. (F) FOR3D of neonate (top) and adult (bottom) WT thymic lobes costained for K14 (red) and Aire (green) and counterstained with DAPI (blue) depicts Aire+ cell 3D localization within and at the border of the medulla. (G) The distribution of distances from Aire+ cells within the medulla to the CMJ was computed in 3D for neonate and adult thymi and compared with a spherical medulla modelized with homogenously distributed cells. Mean values ± are indicated by dashed lines. In (B) and (E), means and standard deviations are derived from three mice per condition. Statistical significance, from unpaired Student t test p value: *p < 0.05, **p < 0.01, ***p < 0.001.
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-Aα<sup>−/−</sup> and WT thymi (Fig. 6F). In H2-Aα<sup>−/−</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>−/−</sup> thymi also presented a medulla mostly devoid of any major compartment, as for H2-Aα<sup>−/−</sup> and LTα<sup>−/−</sup> mice (Fig. 6E, 6F; Supplemental Video 16). Because LTα<sup>−/−</sup> and CD80/86<sup>−/−</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>−/−</sup> medullae by immunofluorescence. We observed a significant decrease of LTα protein expression in CD4<sup>+</sup> SP thymocytes from CD80/86<sup>−/−</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, β2m<sup>−/−</sup>, H2-Aα<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 β2m<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 Web sites 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 is 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 ∼20 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 LTα−/− mouse model, because studies on two-dimensional sections suggested that this cytokine could influence medullary architecture (23, 39). Of interest, the absence of LTα led to an intermediate phenotype between that of WT mice and that of mice lacking CD4+ thymocytes. LTα−/− 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 LTα 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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