Stromal Cell Networks Regulate Thymocyte Migration and Dendritic Cell Behavior in the Thymus

Stephanie L. Sanos, Jonathan Nowak, Mathieu Fallet and Marc Bajenoff

*J Immunol* published online 28 January 2011
http://www.jimmunol.org/content/early/2011/01/28/jimmunol.1003563

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2011/01/28/jimmunol.1003563.3.DC1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Stromal Cell Networks Regulate Thymocyte Migration and Dendritic Cell Behavior in the Thymus

Stephanie L. Sanos, Jonathan Nowak, Mathieu Fallet, and Marc Bajenoff

After entry into thymus, T cell progenitors migrate in the cortex and the medulla while completing their education. Recent reports have documented the dynamic and tortuous behavior of thymocytes. However, other than chemokines and/or segregated thymic substrates, the factors contributing to the dynamic patterns of thymocyte movement are poorly characterized. By combining confocal and dynamic two-photon microscopy, we demonstrate that thymocytes continuously migrate on thymic stromal cell networks. In addition to constituting “roads” for thymocytes, we observed that these networks also provide a scaffold on which dendritic cells attach themselves. These results highlight the central role of stromal microanatomy in orchestrating the multiple cellular interactions necessary for T cell migration/development within the thymus. The Journal of Immunology, 2011, 186: 000–000.

The thymus supports the sequential steps leading to T cell maturation and selection (1). Lymphoid progenitors enter the thymus at the corticomedullary junction, where they migrate outward toward the cortex as CD4+CD8− double-negative cells. In the cortex, double-negative cells further mature into CD4+CD8+ double-positive (DP) cells upon rearrangement of their TCR β- and α-chains. Upon completion of the positive selection process, DP cells become either CD4+CD8− or CD4−CD8+ single-positive (SP) cells and migrate to the medulla, where negative selection occurs. At the end of this selection process, mature T cells are exported to the periphery. A key question regarding thymocyte behavior within the thymus is how thymocyte migration is controlled within this densely packed environment.

Thymi have a highly organized architecture composed of distinct cellular compartments and structures, at the heart of which lies a nonhematopoietic cell backbone (2). Among the cells that are critical for generating this backbone are various thymic stromal cells (TSCs), including cortical thymic epithelial cells (cTEC) and medullary epithelial cells (mTEC) as well as nonepithelial stromal cells (1). It is well established that during T cell development, an efficient cross-talk between thymocytes and TSCs is mandatory for their successful maturation (3). In the cortex, DP thymocytes crawl through the cortical environment, seeking to encounter self-MHC–peptide complexes expressed by various TSCs, including cTECs (4). In the medulla, mTECs express the transcription factor autoimmune regulator protein, which regulates the expression of peripheral tissue Ags, a critical step in the establishment of central tolerance as autoimmune regulator–deficient mice present a defined profile of autoimmune diseases (5).

To complete thymocyte education, TSCs also provide chemical cues to them. As an example, SP thymocytes relocate from the cortex to the medulla following TCR-mediated positive selection. This migration is controlled by the induction of CCR7 on newly selected SP thymocytes that become sensitive to its ligands, CCL21 and CCL19, expressed by medullary radioresistant TSCs (6–9).

To date, dynamic imaging studies on intact lymph nodes (LNs) and sliced spleens have documented the crucial role of stromal cells in physically supporting T and B cell migration (10, 11). Studies carried out on sliced thymi have characterized the convoluted thymocyte behavior at steady state and upon negative selection in both medulla and cortex (4, 12, 13). However, whereas it is established that cortical and medullary stromal networks influence thymocyte migration (12), it is still unknown whether they only provide soluble cues directing thymocyte migration, or if they form physical substratums on which thymocytes migrate during their journey in the thymus. Similarly, thymic dendritic cells (DCs) have been reported to be immotile while probing their environment, but to date, it is still unknown whether their behavior is regulated by TSCs (13).

In this study, using whole thymi explants and two-photon (2P) microscopy, we examine the possibility that the extensive networks of TSCs within the cortex and the medulla play central roles in supporting thymocyte migration and regulating DC behavior within the thymus.

Materials and Methods

Mice

All mice were housed and bred in pathogen-free conditions at the animal facility of the Centre d’Immunologie de Marseille Luminy (Marseille, France). C57BL/6 and C57BL/6 Rag2−/− mice were purchased from Charles River Laboratories France (L’Arbresle, France). C57BL/6 ubiquitin–GFP mice [strain 4353 (14)] and C57BL/6 actin–cyan fluorescent protein (CFP) mice [strain 4218 (15)] were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in the Centre d’Immunologie de Marseille Luminy animal facilities. C57BL/6 CD11c–yellow fluorescent protein (YFP) mice were a gift from M. Nussenzweig (The Rockefeller University, New York, NY) (16). For the generation of chimeras, C57BL/6 ubiquitin–GFP Rag2−/− mice were γ-irradiated twice with 500 rad (4-h interval) from a cesium...
source and were reconstituted with $2 \times 10^6$ bone marrow cells isolated from various animal strains, as described in this study.

**Abs**

RA3-6B2 Ab specific for B220, 17A2 specific for the CD3 complex, RM4-5 specific for CD4, and 53-6-7 specific for CD8 were purchased from BD Pharmingen (San Diego, CA). CDR-1 specific for cortical stromal cells is a gift from B. Kyewski (German Cancer Research Center, Heidelberg, Germany). The Abs specific for collagen IV, K5, and K8 were purchased from Abcam (Paris, France). CCL21/6Ckine affinity-purified polyclonal Ab was purchased from R&D Systems Europe (Lille, France). Isotype control Abs were purchased from BD Biosciences Pharmingen and R&D Systems Europe. Primary Abs were visualized by direct coupling to Pacific blue, allophycocyanin, Alexa fluor-488, -568, -647, or through the use of Alexa fluor-488, -568, -647, or biotin-coupled secondary Abs. No signals were obtained using isotype control stainings.

**Immunostaining**

Organs were fixed by periodate-lysine-parafomaldehyde solution (0.05 M phosphate buffer containing 0.1 M l-lysine [pH 7.4], 2 mg/ml NaIO$_4$, and 10 mg/ml parafomaldehyde) for 12 h, washed in phosphate buffer, and dehydrated in 30% sucrose in phosphate buffer. In some cases, animals were anesthetized with xylazine/ketamine and given an intracardiac injection of 15 ml periodate-lysine-parafomaldehyde. After excision from perfused animals, organs were further treated, as mentioned above. Tissues were snap frozen in Tissue-Tek (Sakura Finetek). Frozen sections (30 μm) were cut and then stained with the indicated Abs, as previously described (17). Immunofluorescence confocal microscopy was performed using a Leica SP5 confocal microscope. Separate images were collected for each fluorochrome and overlaid to obtain a multicolor image. Final image processing was performed using Imaris software (Bitplane) and Adobe Photoshop.

**Quantification of cell densities in the medullary and cortical areas**

Immunofluorescence images were segmented into medullary and cortical areas according to CDR-1 and K5 stainings or GFP+ TSC network patterns. The numbers of pixels of each area as well as the numbers of GFP+ TSC bodies and CD11c–YFP+ cells were measured using ImageJ software (National Institutes of Health). Finally, densities of cells/mm$^2$ cortex and medulla were calculated. For each condition, a minimum of six different fields acquired in five different animals was counted, representing an area of interest ≥6 mm$^2$.

**2P microscopy**

Freshly isolated thymi from chimeric mice were mounted on a custom-made piece of metal and perfused with serum-free cell tissue culture media heated...
at 37°C and bubbled with 5% CO₂/95% O₂. Imaging was performed using a Zeiss LSM-7 MP system fitted with a ×20 water immersion lens (NA = 1.0, Plan-Apochromat; Zeiss). The 2P laser was a Spectra-Physics Mai-Tai Deepsee tuned to 860 nm. Images in movies were collected with typical voxel size = 0.43 × 0.43 × 2–4 μm and a volume dimension = 220 × 220 × 12 μm, unless indicated otherwise. Images were typically collected between 50 and 160 μm below the capsule. This volume collection was repeated every 12–20 s to create four-dimensional data sets that were then processed with Imaris software (Bitplane) and Adobe AfterEffect (Adobe). Supplemental videos created from these image stacks are either single slices or maximum intensity projections and play at ×120 real time. Immobile cells were defined as round stationary cells moving with a speed <2 μm/min⁻¹.

Results
Visualization of TSCs using bone marrow chimera
To investigate the dynamics of thymocyte interactions with TSCs, we first needed to generate mice possessing fluorescent TSCs. To this aim, we generated chimeric mice expressing fluorescent TSCs by using wild-type (Wt) bone marrow cells to reconstitute irradiated Rag2⁻/⁻ ubiquitin–GFP mice in which the fluorescent protein is expressed by all cells (14). Animals were allowed to reconstitute for a minimum of 6 wk. To confirm that GFP-expressing cells within the cortex and the medulla of chimeric animals represented radioresistant TSCs, thymic sections from such animals were stained for Keratin 5 (K5, medulla) and CDR-1 expression, indicating that this network was indeed formed by medullary TSCs (Fig. 1A, Supplemental Fig. 1A). Surrounding these medullary islands, we identified the cortical TSCs as being easily identified in chimeric thymi solely based on their locations, arrangements, and morphologies.

Dynamic interactions of thymocytes with TSCs
As a first approach to determine the possible influence of TSCs on thymocyte migratory behavior, chimeric animals were anesthetized and perfused with a fixative, a protocol that conserves the characteristic elongated shape of motile leukocytes in fixed tissues (10). Such fixed thymi were sectioned; stained with Sytox-Red (a nucleic acid stain), anti-CD8, and anti-CD4 Abs; and analyzed using confocal microscopy. In the chimeric thymi, two regions of interest were easily identified. Deep in the tissue (typically >200 μm under the capsule), GFP⁺ cells created a three-dimensional network positive for K5 and CCL-21 expression, indicating that this network was indeed formed by medullary TSCs (Fig. 1A, Supplemental Fig. 1B, 1C). Interestingly, GFP⁺ syncitiums were frequently observed in medullary regions (Fig. 1, Supplemental Fig. 1) (18). Surrounding these medullary islands, we identified the cortical TSCs as being a second network of GFP⁺ cells recognized by anti-K8 (Supplemental Fig. 1B) and CDR-1 Abs (Fig. 1A). As expected, CD4 and CD8 staining revealed that DP-expressing cells were confined to the cortex, whereas SP CD4⁺ or CD8⁺ cells resided in the medulla (Fig. 1B, Supplemental Fig. 1A). In addition to expressing various markers and being segregated in the thymus, cortical and medullary TSC networks appeared structurally different when observed via the GFP channel. In the cortex, TSC bodies were sparse (350 cells/mm², n = 1084) and formed dense and winding networks of thin and extended processes reminiscent of the follicular DCs (Fig. 1B, Supplemental Fig. 2). In contrast, medullary TSC bodies were more numerous (1400 cells/mm², n = 2426), bigger, and formed sheetlike structures interconnected via large and short processes reminiscent of the fibroblastic reticular cells (FRCs) observed in secondary lymphoid organs (10) (Fig. 1B, Supplemental Fig. 2). Altogether, these observations indicate that cortical and medullary TSCs can easily be identified in chimeric thymi solely based on their locations, arrangements, and morphologies.

FIGURE 2. Thymocytes closely interact with TSC networks. Ubiquitin–GFP Rag2⁻/⁻ mice were irradiated and reconstituted with Wt bone marrow cells. Six to 8 wk later, chimera were perfused with a fixative solution. The 20-μm–thick thymus sections were examined using confocal microscopy after staining with sytox red (nuclei), anti-CD4 (white), and anti-CD8 (blue) Abs to differentiate SP and DP thymocytes in the cortex (A) and the medulla (B). Attention was focused on thymocytes harboring an elongated shape typical of motile cells (*). Each panel represents a single slice of a whole z stack. C, Histograms indicate the percentage of elongated medullary SP (CD4 or CD8) and cortical DP thymocytes adjacent to or stretched along GFP⁺ TSCs. Data are representative of three different experiments (two mice per experiment).
The cortical and medullary TSC networks are quite dense. Therefore, the observed association between thymocytes and TSCs might not reflect specific interactions, but merely a close proximity of the two, based on a random distribution of thymocytes packed within the TSC networks. To investigate the dynamics of thymocyte interactions with TSC networks, we reconstituted irradiated Rag2⁻/⁻ ubiquitin–GFP⁺ mice with a mixture of Wt (97%) and CFP-expressing (3%) bone marrow cells. In these chimeric thymi, a small population of CFP⁺ thymocytes developed among the vast majority of unlabeled thymocytes and radioresistant GFP⁺ TSCs. Thymi were harvested 6 wk after reconstitution, quickly mounted on a heated stage, perfused with 5% CO₂/95% O₂, and imaged by 2P microscopy. Under these conditions, recorded thymocyte velocities in the cortex (11.4 ± 3.1 μm/min, n = 801 tracks) and in the medulla (12 ± 1.5μm/min⁻¹, n = 268 tracks) were in agreement with measurements obtained from sliced thymi experiments (12, 13). As cortical TSCs can be found just beneath the capsule and possess unique shapes, they were easily identified and imaged. In contrast, medullary regions were usually too deep to be imaged by 2P microscopy (>200 μm), explaining the thymic slice models developed by other groups to visualize these areas. However, we observed that this rule was not mandatory, as few medullary areas were present within <100 μm below the capsule of Wt and chimeric thymi, a sufficiently small distance to image them by 2P microscopy, provided one can identify them (Supplemental Fig. 3). In our setup, we identified medullary regions based on several features, as described earlier (Fig. 1, Supplemental Fig. 2). Analysis of 4D (x, y, z, and time) data sets suggested that migrating CFP⁺ thymocytes actively crawled on GFP⁺ cortical and medullary TSCs, following and morphologically adapting to the paths established by the cell bodies and extended processes of these nonhematopoietic cells (Fig. 3A, 3B, Supplemental Videos 1–4). These data therefore suggest that thymocytes actively follow the TSCs, rather than finding pathways around potential obstructions.

To assess quantitatively whether thymocytes actively followed the paths laid out by the TSC networks, we assumed that if TSCs provided guidance for cell movement, then any directional turns made by a thymocyte should always be associated with a corresponding turn or branch of a supporting TSC. Conversely, a lack of correspondence between thymocyte directionality and TSC pathways would indicate that spontaneous turns or physical impediments posed by the many other cells packed in the thymus environment accounted for thymocyte directional changes. Analysis of thymocyte turns in the cortex and the medulla revealed a 91.5% correlation (343 of 375 cells, cortex) and 98.8% correlation (160 of 162 cells, medulla) between changes in thymocyte direction and the presence of thymocyte-associated GFP⁺ TSCs running at the corresponding angle in thymus (Fig. 3C). Occasionally, some thymocytes in the processed image stacks seemed

![FIGURE 3. Thymocytes crawl on TSCs. Ubiquitin–GFP Rag2⁻/⁻ mice were irradiated and reconstituted with a mixture of Wt (~97%) and CFP⁺ (~3%) bone marrow cells. Six to 8 wk later, intact thymic lobes were imaged using 2P microscopy. Data show intravital snapshots of thymocytes (red) moving over time either on cortical (A) or medullary (B) green fibers in a 12-μm–thick volume. The last panel of each snapshot series represents the superposition of all thymocyte positions over time. See also Supplemental Videos 1–4. Histograms (C) indicate the numbers of motile thymocytes showing turns in dynamic four-dimensional data sets with respect to their location on or off GFP-marked stromal fibers in chimeric thymi. Data are representative of at least five different experiments (three mice per experiment).](http://www.jimmunol.org/DownloadedFrom)
to be moving in an empty space in which no TSC processes were visible. When such behavior was observed in the intermediate z planes in the imaged volume, detailed analysis revealed that the thymocytes were still in contact with faint GFP+ extensions of TSCs extending under or above the field of view (data not shown). Finally, we observed that some thymocytes paused their migration for few minutes during the imaging sessions. During this stationary phase, 74 (of 77 in the cortex) and 38 (of 39 in the medulla) were associated with GFP+ TSCs.

Dynamic interactions of DCs with TSCs

It is well established that both thymocyte/TSC and thymocyte/DC interactions in the medulla are important to ensure an efficient negative selection of potentially autoreactive thymocytes (13). Dynamic studies have shown that medullary thymic DCs are nonmotile (19) and exhibit stable bodies while extending their dendrites (13). However, to date, information on thymic DC localization regarding TSCs as well as on DC/TSC dynamic interactions is lacking. Therefore, we set out to investigate the behavior of DCs relative to TSCs. To this aim, we reconstituted irradiated Rag2\(^{-/-}\) ubiquitin–GFP mice with a mixture of CD11c–YFP (97%) and CFP (3%) bone marrow cells to generate a mouse in which DCs, TSCs, and thymocytes would express different colors. First, thymic sections from chimera were observed by confocal microscopy to analyze the location of thymic DCs regarding TSCs (Fig. 4A). As previously observed (13), the density of DCs was higher in the medulla (1002 cells/mm\(^2\), \(n = 1004\)) than in the cortex (431 cells/mm\(^2\), \(n = 1110\)). A closer examination revealed that thymic DCs laid on the TSC networks in both the cortex and the medulla, extending their dendrites along TSCs processes, a result reminiscent of their LN counterparts attached to the FRC network (10). We next investigated the behavior of thymic DCs in chimeric animals by 2P microscopy. Analysis of four-dimensional data sets revealed that whereas medullary and cortical YFP+ DCs remained immotile, they slowly probed their environment while maintaining their cell bodies attached to the GFP+ TSCs (Fig. 4B, Supplemental Videos 5 and 6).

FIGURE 4. Thymic DCs lay on TSC networks. Ubiquitin–GFP Rag2\(^{-/-}\) mice were irradiated and reconstituted with a mixture of CD11c–YFP (97%) and CFP (3%) bone marrow cells. Six to 8 wk later, thymi were either sectioned and observed by confocal microscopy (A) or further processed for 2P imaging (B). Data show confocal and dynamic 2P pictures of immobile CD11c–YFP cells (red) laying on GFP+ TSCs (green or yellow) while probing their immediate environment with their dendrites (arrowheads). See also Supplemental Movies 5 and 6. Data are representative of three different experiments (three mice per experiment).

FIGURE 5. Summary. Cortical and medullary stromal cells form dense three-dimensional meshworks structurally different, but functionally akin. In addition to constituting an anchoring scaffold to DCs, TSC networks form substrata on which thymocytes migrate. As a consequence, thymocytes constantly crawl on TSCs and frequently encounter the numerous DCs laying on their paths, a behavior that likely fosters the crucial interactions required to complete their education.
Discussion
Several reports have documented thymocyte and DC dynamic behavior in the thymus at steady state, but also during positive and negative selection. In these reports, thymocytes were reported to be highly motile, moving randomly and tortuously in their respective areas among immobile DCs (13). However, to date, the nature of the cellular mechanisms regulating this active migration remains unknown.

In 2002, reaggregate thymic organ culture (RTOC) systems have been used to study the dynamics of thymocyte and stromal cell interactions. In this setup in which the only cells present in the RTOC were stromal cells and thymocytes, the authors observed that motile thymocytes were in contact with stromal cells. Although this observation indicated that thymocytes possess the ability to migrate along TSCs, it did not prove that such mechanism regulates the motility of thymocytes in real thymi, composed of many more numerous, various cell subsets and extracellular matrix molecules than the ones present in RTOC systems. As a comparison, lymphocytes robustly migrate in three-dimensional collagen matrices, whereas these molecules remain inaccessible to them in secondary lymphoid organs, preventing a generalization of this phenomenon to the complex environments of lymphoid organs (20).

More recently, Ehrlich et al. (12) analyzed the behavior/ segregation of DP and SP thymocytes deposited on vibratome-sliced thymic sections. Although these authors demonstrated that medullary and cortical stromal cells govern thymocyte localization/ migration, they did not evaluate whether TSCs only provide soluble cues directing thymocyte migration or if they form physical substrata on which thymocytes migrate during their journey in the thymus.

To our knowledge, our results constitute the first report of dynamic interaction between thymocytes and thymic DCs regarding the endogenous natural TSC networks. As summarized in our model (Fig. 5), our results demonstrate that cortical and medullary thymocytes are continuously crawling on medullary and cortical TSCs, and that such interactions are maintained even when thymocytes pause their migration for few minutes. These data provide both a cellular and structural explanation for previous 2P reports, suggesting that thymocyte motility is random and that the crawling thymocytes frequently make sudden turns in the thymus. The TSC networks, by virtue of their dimensions and the presence of frequent crossing points, support this apparent random migration. Importantly, our results imply that thymocytes are indeed crawling on the very cells they have to scan during their thymic journey to complete their education/maturition (i.e., cTECs and mTECs). In the medulla, the frequency of mTECs that express a given tissue-restricted Ag is estimated to be in the range of 1–5%, which raises questions about how and how such a restricted expression pattern allows complete tolerization to the complex environments of lymphoid organs (20).

These data provide both a cellular and structural explanation for previous 2P reports, suggesting that thymocyte motility is random and that the crawling thymocytes frequently make sudden turns in the thymus. The TSC networks, by virtue of their dimensions and the presence of frequent crossing points, support this apparent random migration. Importantly, our results imply that thymocytes are indeed crawling on the very cells they have to scan during their thymic journey to complete their education/maturition (i.e., cTECs and mTECs). In the medulla, the frequency of mTECs that express a given tissue-restricted Ag is estimated to be in the range of 1–5%, which raises questions about how and how such a restricted expression pattern allows complete tolerization to the complex environments of lymphoid organs (20).

Our results demonstrate that cortical and medullary thymocytes are continuously crawling on medullary and cortical TSCs, and that such interactions are maintained even when thymocytes pause their migration for few minutes. These data provide both a cellular and structural explanation for previous 2P reports, suggesting that thymocyte motility is random and that the crawling thymocytes frequently make sudden turns in the thymus. The TSC networks, by virtue of their dimensions and the presence of frequent crossing points, support this apparent random migration. Importantly, our results imply that thymocytes are indeed crawling on the very cells they have to scan during their thymic journey to complete their education/maturition (i.e., cTECs and mTECs). In the medulla, the frequency of mTECs that express a given tissue-restricted Ag is estimated to be in the range of 1–5%, which raises questions about how and how such a restricted expression pattern allows complete tolerization to the complex environments of lymphoid organs (20).

Our results demonstrate that cortical and medullary thymocytes are continuously crawling on medullary and cortical TSCs, and that such interactions are maintained even when thymocytes pause their migration for few minutes. These data provide both a cellular and structural explanation for previous 2P reports, suggesting that thymocyte motility is random and that the crawling thymocytes frequently make sudden turns in the thymus. The TSC networks, by virtue of their dimensions and the presence of frequent crossing points, support this apparent random migration. Importantly, our results imply that thymocytes are indeed crawling on the very cells they have to scan during their thymic journey to complete their education/maturition (i.e., cTECs and mTECs). In the medulla, the frequency of mTECs that express a given tissue-restricted Ag is estimated to be in the range of 1–5%, which raises questions about how and how such a restricted expression pattern allows complete tolerization to the complex environments of lymphoid organs (20).

Acknowledgments
We thank P. Pierre for active involvement in the acquisition of a 2P microscope at the Centre d’Immunologie de Marseille Luminy and G. Lauvau and E. Narni-Mancinelli for suggestions on the manuscript.

Disclosures
The authors have no financial conflicts of interest.

References


Supplementary Figures Legends:

Figure S.1: Visualization of thymic stromal cells using ubiquitin-GFP chimeras.
Ubiquitin-GFP Rag2<sup>−/−</sup> mice were irradiated and reconstituted with Wt bone marrow cells. Six to eight weeks later, 20 µm thick thymus sections from chimeric mice were examined using confocal microscopy after staining with anti-CD4 (red) and anti-CD8 (green) Abs (A) or anti-K8 (red, cortex) and anti-K5 (blue, medulla) Abs (B) or anti-CCL21 (C). Medulla (M); Cortex (C). Pictures are representative of 2 different experiments (2 mice per experiment).

Figure S.2: Structural comparison of thymic and lymph node stromal cells networks.
Ubiquitin-GFP Rag2<sup>−/−</sup> mice were irradiated and reconstituted with Wt bone marrow cells. Six to eight weeks later, 20 µm thick thymus and lymph node sections from chimeric mice were examined using confocal microscopy after staining with anti-CDR1 (blue, cortex) and anti-Keratin-5 (red, medulla) Abs (thymus) or anti-CD3 (blue, T cell zone) and anti-B220 (red, B cell zone) Abs (lymph node). Inserts represent magnified views of either the cortico-medullary junction (thymus) where cortical (C) and medullary regions (M) are visible or T/B interface (lymph node) where FRCs and FDCs networks are present. Pictures are representative of 3 different experiments (2 mice per experiment).

Figure S.3: Superficial medullary regions.
Ubiquitin-GFP Rag2<sup>−/−</sup> mice were irradiated and reconstituted with Wt bone marrow cells. Six to eight weeks later, 20 µm thick thymus from chimeric mice were examined using confocal microscopy. Data show that medullary areas (M) surrounded by cortical regions (C) can be present <100µM under the capsule and therefore accessible to 2P imaging. (B) Partial reconstruction of a Wt thymus stained for K5 (green) and sytox red (red- nuclei) showing that the
presence of superficial medullary regions is not restricted to chimeric thymi. Pictures are representative of 2 experiments (2 mice per experiment).

**Video 1: Thymocytes crawling along cortical thymic stromal cell networks: example 1.**
Ubiquitin-GFP Rag2°/° mice were irradiated and reconstituted with a mixture of Wt (~97%) and CFP⁺ (~3%) bone marrow cells. Six to eight weeks later, intact thymic lobes were imaged using 2-P microscopy. Data show dynamic images of thymocytes (red) migration along the cortical stromal cell networks (green). Videos parameters: z stack 6 µm; interval 12s.. Video is representative of at least 5 experiments (3 mice per group).

**Video 2: Thymocytes crawling along cortical thymic stromal cell networks: example 2.**
This movie shows another example of thymocytes behavior (red) within the cortical stroma network (green) of Ubiquitin-GFP Rag2°/° chimeras mentionned in video 1. Acquisition parameters were the same that the ones indicated in Video 1. Video is representative of at least 5 experiments (3 mice per group).

**Video 3: Thymocytes crawling along medullary thymic stromal cell networks: example 1.**
Ubiquitin-GFP Rag2°/° mice were irradiated and reconstituted with a mixture of Wt (~97%) and CFP⁺ (~3%) bone marrow cells. Six to eight weeks later, intact thymic lobes were imaged using 2-P microscopy. Data show dynamic images of thymocytes (red) migration along the medullary stromal cell networks (green). Videos parameters: z stack 12µm; interval 20s. Video is representative of at least 5 experiments (3 mice per group).
Video 4: Thymocytes crawling along medullary thymic stromal cell networks: example 2.

This movie shows another example of thymocytes behavior (red) within the medullary stroma network (green) of Ubiquitin-GFP Rag2\(^{+/0}\) chimeras mentionned in video 1. Acquisition parameters were the same that the ones indicated in Video 3. Video is representative of at least 5 experiments (3 mice per group).

Videos 5: Behavior of thymic DCs.

Ubiquitin-GFP Rag2\(^{+/0}\) mice were irradiated and reconstituted with CD11c-YFP\(^{+}\) (~97%) and CFP\(^{+}\) (~3%) bone marrow cells. Six to eight weeks later, 20 µm thick thymus sections from chimeric mice were examined using 2-P microscopy. Data show dynamic images of thymocytes (blue) and DCs (red) behaviour regarding thymic stromal cell networks (yellow). Arrowheads indicate non motile DCs actively extending their dendrites. Videos parameters: z stack 6 µm; interval 20s. Video is representative of at least 3 experiments (3 mice per group).

Video 6: Behavior of thymic DCs: a closer look.

These videos were performed according to the legend describing video 5. Data show high magnification views of immotile DCs attached to cortical and medullary TSC networks while probing away their immediate vicinity with their dendrites (arrowheads). Videos parameters: single slice; interval 20s. Video is representative of at least 3 experiments (3 mice per group).