Supplementary Materials

1. Supplementary Figures

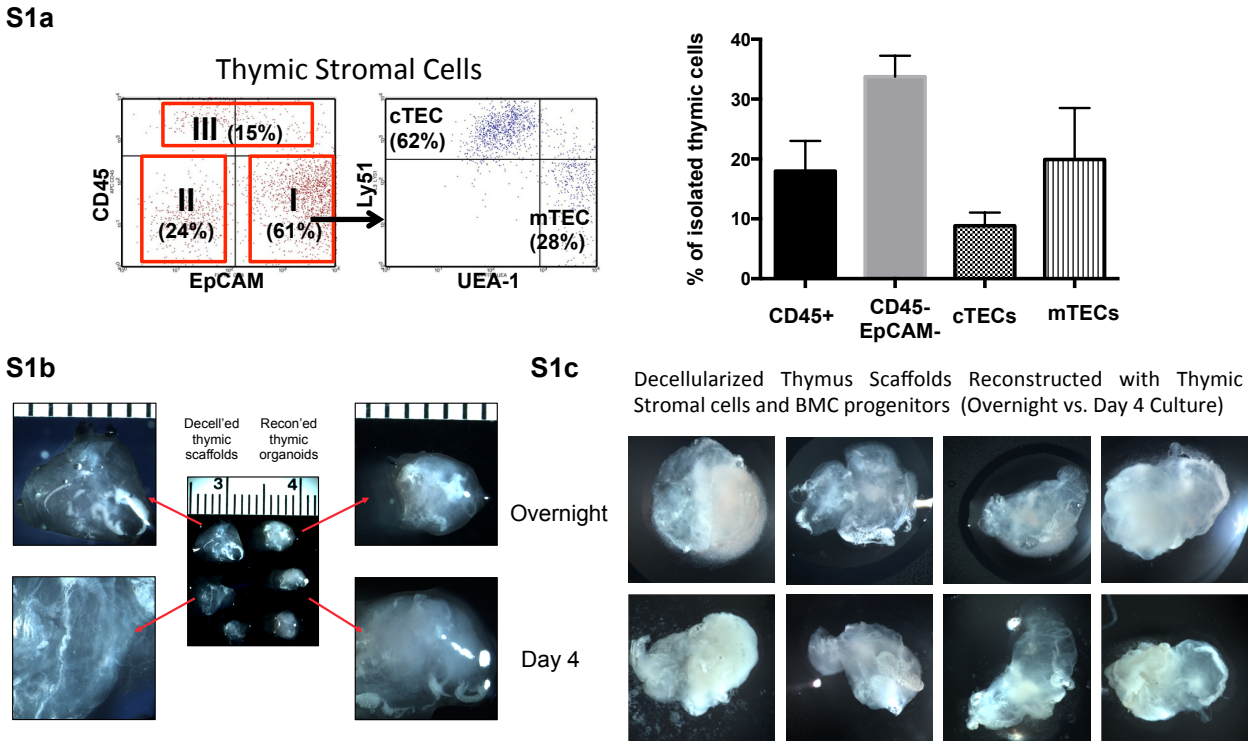


Figure S1. Reconstruction of thymus organoids with decellularized 3-D thymus scaffolds. **S1a.** Thymic stromal cells (TSCs) were enriched from dissociated thymic cells with MACS technology (Miltenyi Biotec), using magnetic bead-conjugated anti-CD45 antibodies to negatively select $CD45^-$ cells. Panel on the left shows a representative flow cytometric (FCM) profile of the MACS isolated cells. The number inside the marked region represents the percentage of cells in the gated population in a typical isolation. I, $CD45^-Epcam^+$ thymic epithelial cells (TECs); II, $CD45^-Epcam^-$ stromal cells including the thymic fibroblasts and endothelial cells; III, residual CD45+ thymocytes. Panel in the middle is a representative FCM profile of Epcam+ TECs of 10-14 day old pups. Bar graph on the right shows the representative compositions of TSCs used for thymus reconstruction (n=6). The predominant population of the CD45+ cells is CD4+CD8+ DP thymoctyes. These cells were co-isolated with the thymic nurse cells of the cTEC subsets, which engulf 7-50 developing DP thymocytes. Data are presented as mean ± SEM. **S1b.** Photoimages of reconstructed thymus organoids. Decellularized thymic scaffolds are shown on the left; scaffolds injected with TSCs in conjunction with BM progenitors are shown on the right. In the middle panel, the length between the two major markers on the ruler that are marked 3 and 4 is 1 centimeter. **S1c.** Photoimages of reconstructed thymus organoids cultured *in vitro* for 24 hours (top panels) and 4-days (bottom panels).

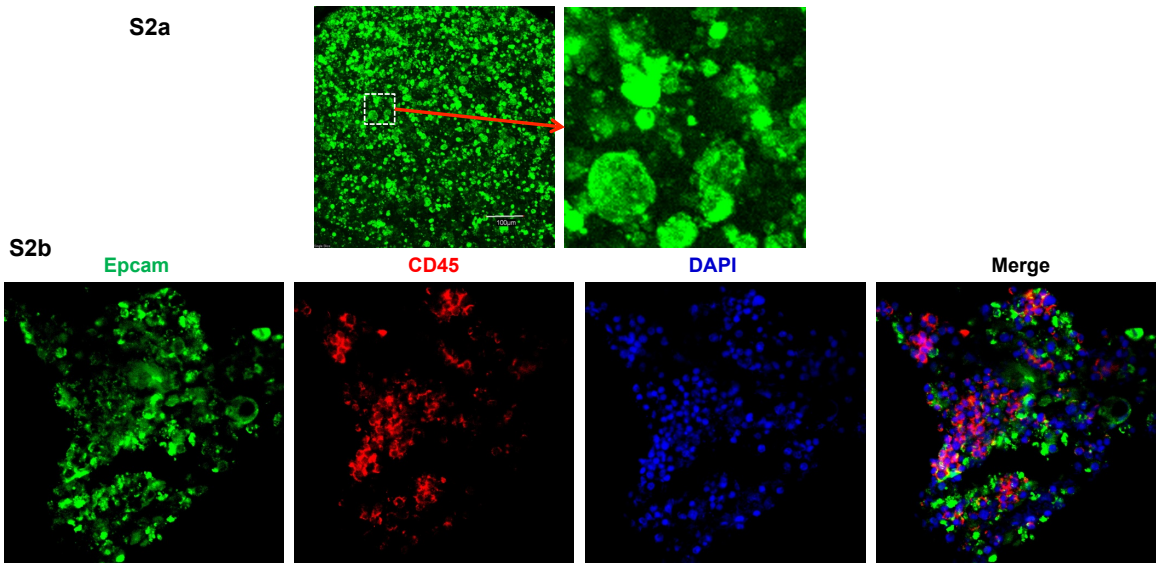


Figure S2. Fluorescence microscopy images of reconstructed thymus organoids. S2a. Fluorescent microscopic images of reconstructed thymus organoids cultured in vitro for 21 days. TSCs were pre-labelled with carboxyfluorescein succinimidyl ester (CFSE). A large number of TSCs were able to retain their CFSE-label, suggesting the integrity of their plasma membrane. *Right panel*, higher magnified image of the dotted areas in the *left panel*. **S2b.** Representative immunohistochemical images of cryosections of reconstructed thymus organoids cultured *in vitro* for 7 days. TECs and bone marrow-derived cells were stained with anti-Epcam (green) and anti-CD45 (red) antibodies, respectively.

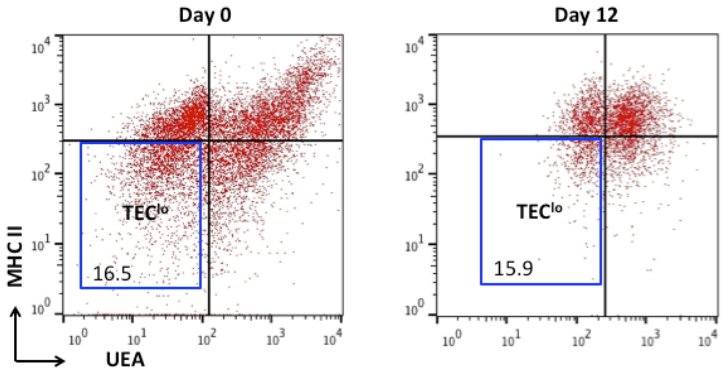


Figure S3. Frequencies of TEPCs in reconstructed thymus organoids cultured *in vitro*. Reconstructed thymus organoids were cultured in RPMI-10, supplemented with human keratinocyte growth supplement (HKGS, with 0.2% v/v bovine pituitary extract, 1 µg/mL recombinant human insulin-like growth factor-I, 0.18 µg/mL hydrocortisone, 5 µg/mL bovine transferrin, and 0.2 ng/mL human epidermal growth factor, Life Technologies, Grand island, New York, USA) for 12 days. To determine the frequencies of TEPC-containing TEC^{lo} population, thymus organoids were digested with Dispase I (1U/mL, Roche Life Science, Indianapolis, Indiana, USA) for three times (5 min each) at 37^{o}C, followed by Liberase TM digestion (6 min, once). Cells from three thymus organoids were pooled and stained with anti-CD45, anti-EpCAM, anti-MHC II antibodies and UEA1-FITC. Panels show the representative flow cytometric (FCM) profiles of the $CD45^{-}EpCAM^{+}$ TEC population at Day 0 (*left*) and Day 12 (*right*). Similar percentages of the TEPC-containing TEC^{lo} populations (numbers inside the boxes with blue lines) were found. The *in vitro* culture experiment was repeated once, with similar result.

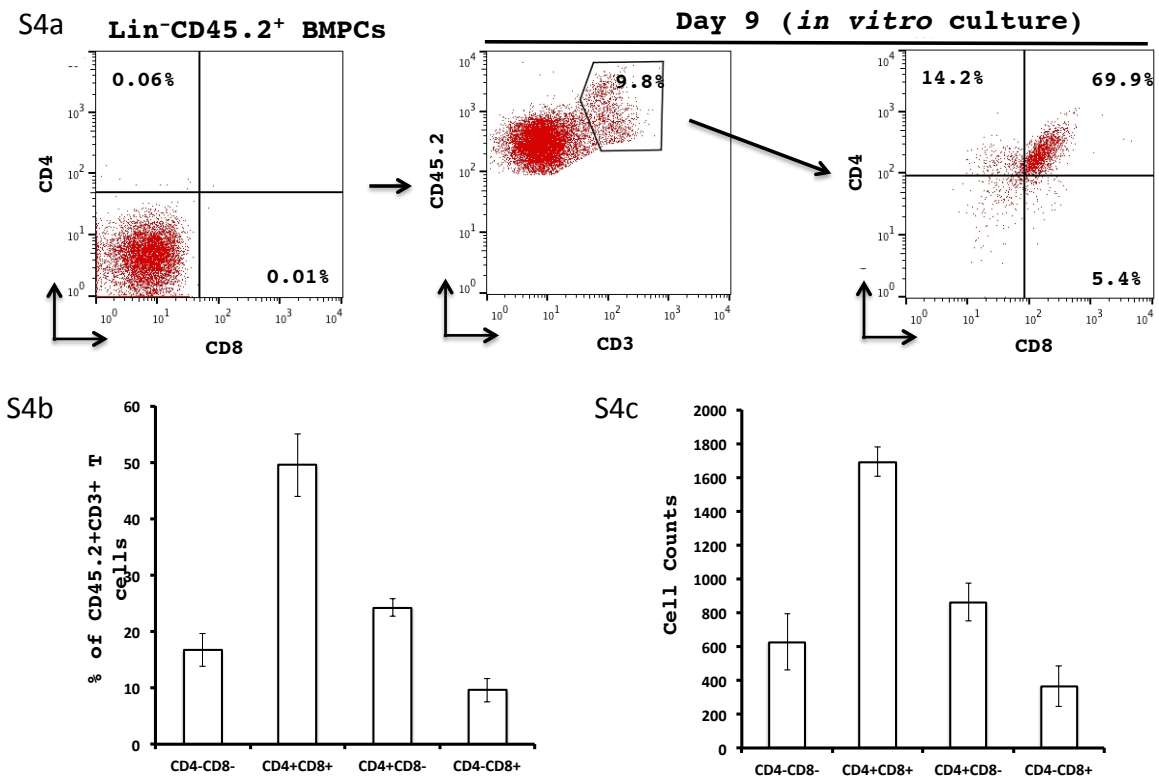


Figure S4. Reconstructed thymus organoids support T-cell differentiation *in vitro*. Cell mixtures including B6 Lin$^-$ BM progenitor cells (BMPCs) and B6.CD45.1 TSCs at 1:1 ratio were injected into the decellularized thymus scaffolds. The reconstruct the thymus organoids (n=5) were cultured in vitro for 9 days in the presence of 2ng/ml IL-7 and the differentiation of T-cell lineage was analyzed with FCM. **S4a.** Representative FCM dot blots show isolated BMPCs (*Left panel*), CD3+CD45.2+ T-cells after 9-days of culture (boxed population in the *Middle panel*), and SP and DP T-cells (*Right panel*). **S4b.** The composition of CD4-CD8- DN cells, CD4+CD8+ DP cells and CD4+CD8-/CD4-CD8+ SP cells in the developing T-cell (CD3+CD45.2+) population in a reconstructed thymus organoid (n=5). **S4c.** The absolute numbers of DN, DP and SP cells in one thymus organoid (n=5). Data were presented as Mean±SEM. The experiment was repeated once.

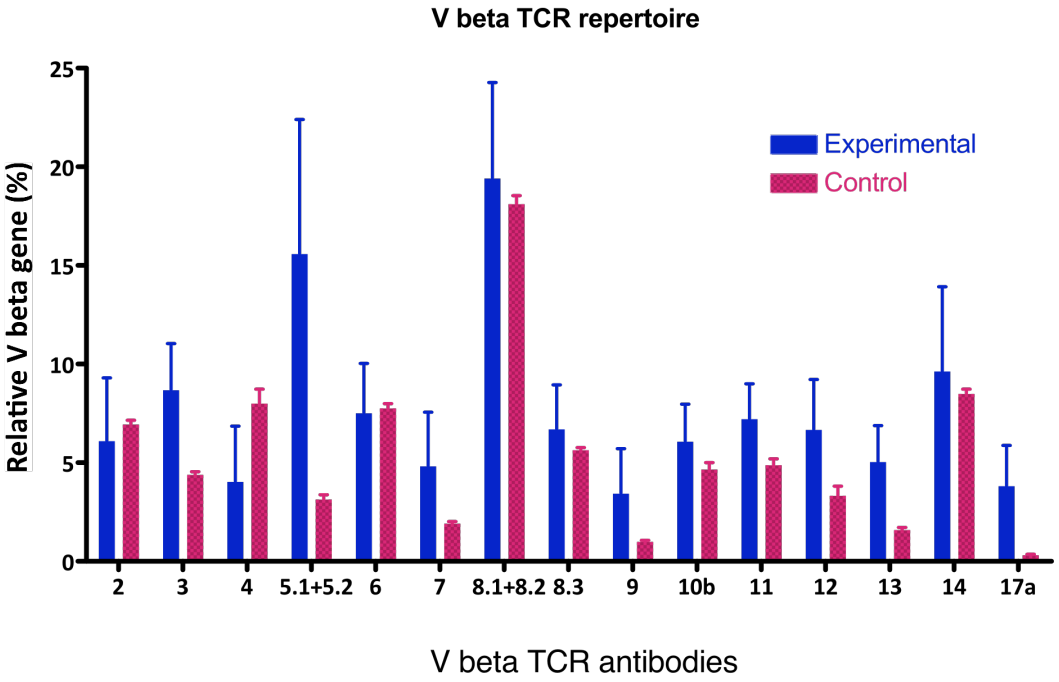
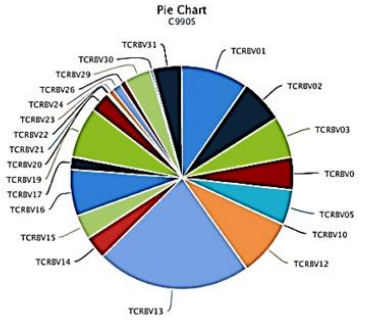
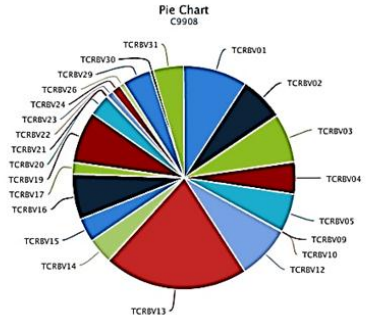


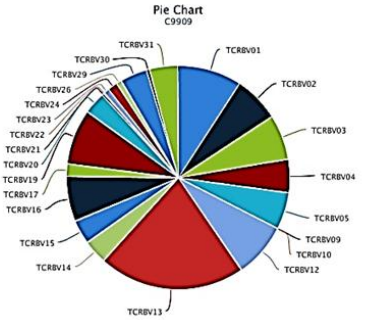
Figure S5. FCM analyses of the diverse distribution of T-cell receptor (TCR) Vβ genes. T-cells harvested from the spleens of thymus organoid transferred Tot.B6.nude mice (Blue bars, n=5) were stained with anti-CD4 antibody and mAbs against various mouse Vβ chains, and analyzed by FCM. The percentage of CD4+ T-cells expressing each specific TCR Vb chain is shown, in comparison to that of immunocompetent wildtype B6 controls (Red bars, n=5).

S6a

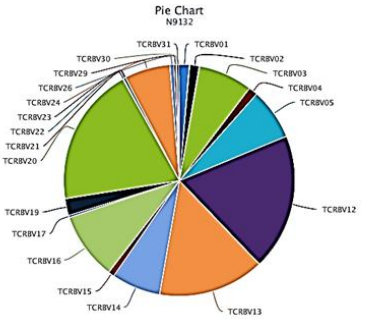
B6 TCRβ gene V family

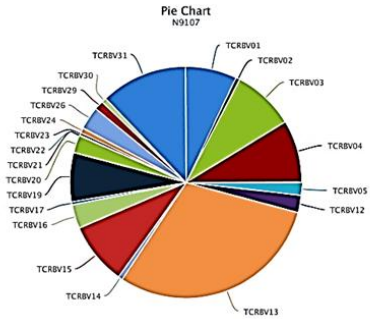


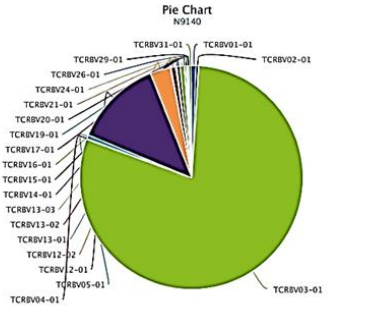




Tot.B6.nude TCRβ gene V family

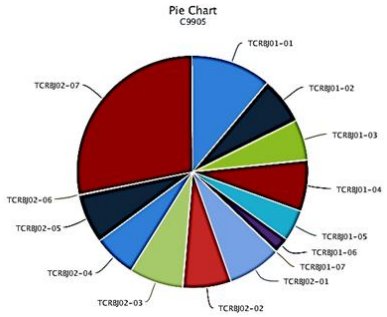


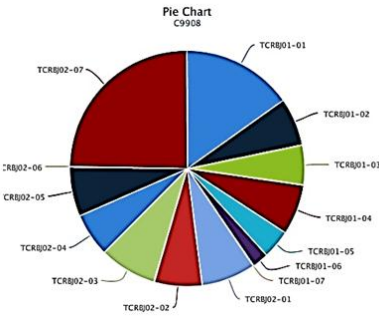


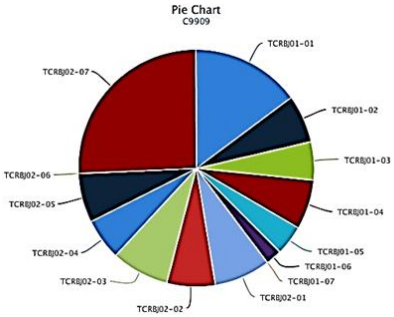


S6b

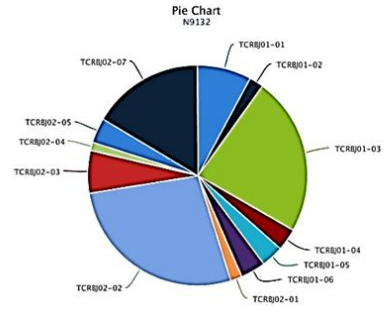
B6 TCRβ gene J region

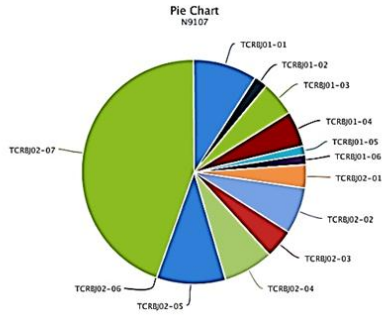


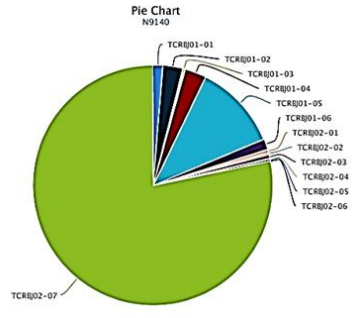




Tot.B6.nude TCRβ gene J region







S6c

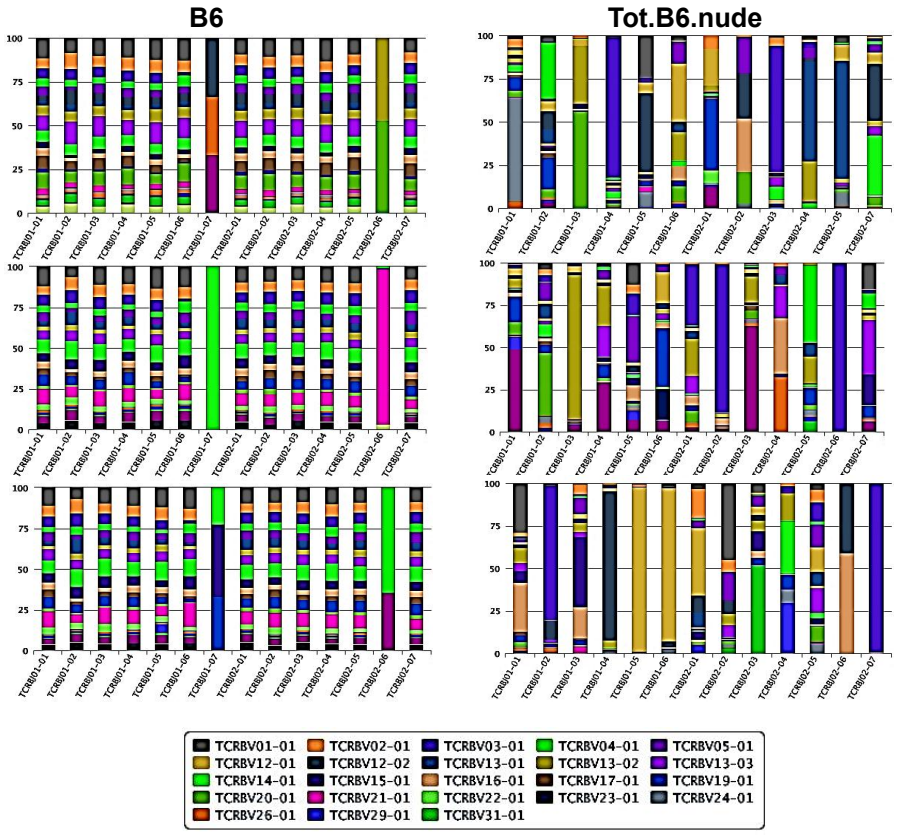


Figure S6. Diversity of TCR Vβ genes in Tot.B6.nude mice determined by next-generation-sequencing-spectratyping (NGS-S). T-cells were harvested from the spleens of Tot.B6.nude mice (n=3) with Pan T-cell isolation kit (Miltenyi Biotec), and were used to extract genomic DNA (DNeasy blood and tissue kit, Qiagen) for NGS-S (Adaptive Biotechnologies, Seattle, WA). T-cells isolated from wildtype B6 mice (n=3) were used as controls. The data were analyzed with the immunoSEQ Analyzer software (www.immunoseq.com, Adaptive Biotechnologies). Pie charts show the distributions of T-cells expressing TCR β genes of various V (**S6a**) and J (**S6b**) families. Each color represents one specific V or J family, and each sector corresponds to the percentage of T-cells expressing TCR β genes that belong to the specific V or J family, respectively. Upper and lower panels show data of T-cells isolated from B6 wildtype mice (n=3) and Tot.B6.nude mice (n=3). **S6c**, histograms show the paired expression of V- and J-family regions in TCR β genes (left panels, B6 mice; right panels, Tot.B6.nude mice). Each bar is a composite of percentages of T-cells expressing each V family region (y-axis) in the subset of T-cells expressing a specific J-family.

2. Captions for Supplementary videos

SV1. 3-D animation of the reconstructed thymus organoid. Thymus organoids reconstructed with decellularized thymus scaffolds supplemented with BMPCs were cultured in vitro for 7-days. Cryosections (10 μm) were stained with antibodies against CD4 or CD8 (red) and Epcam (green), and counterstained with Hoechst 33342 dye (blue).

SV2. Representative 3-D composition of images of cryosections (20 μm) of 7-day thymus organoids (same condition as SV1) stained with antibodies against Epcam (green), and counterstained with Hoechst 33342 dye (blue). Shown is the region of clusters of Epcam+ cells.

SV3. Representative composite images of cryosections (20 μm) of reconstructed thymus organoid (same condition as SV1) stained with antibodies against CD4 or CD8 (red) and Epcam (green), and counterstained with Hoechst 33342 dye (blue). Shown are sequential images of 2 μm depth along the *z*-axis.