

Supplementary Information

Specialized transendothelial dendritic cells mediate thymic T cell selection against blood-borne macromolecules

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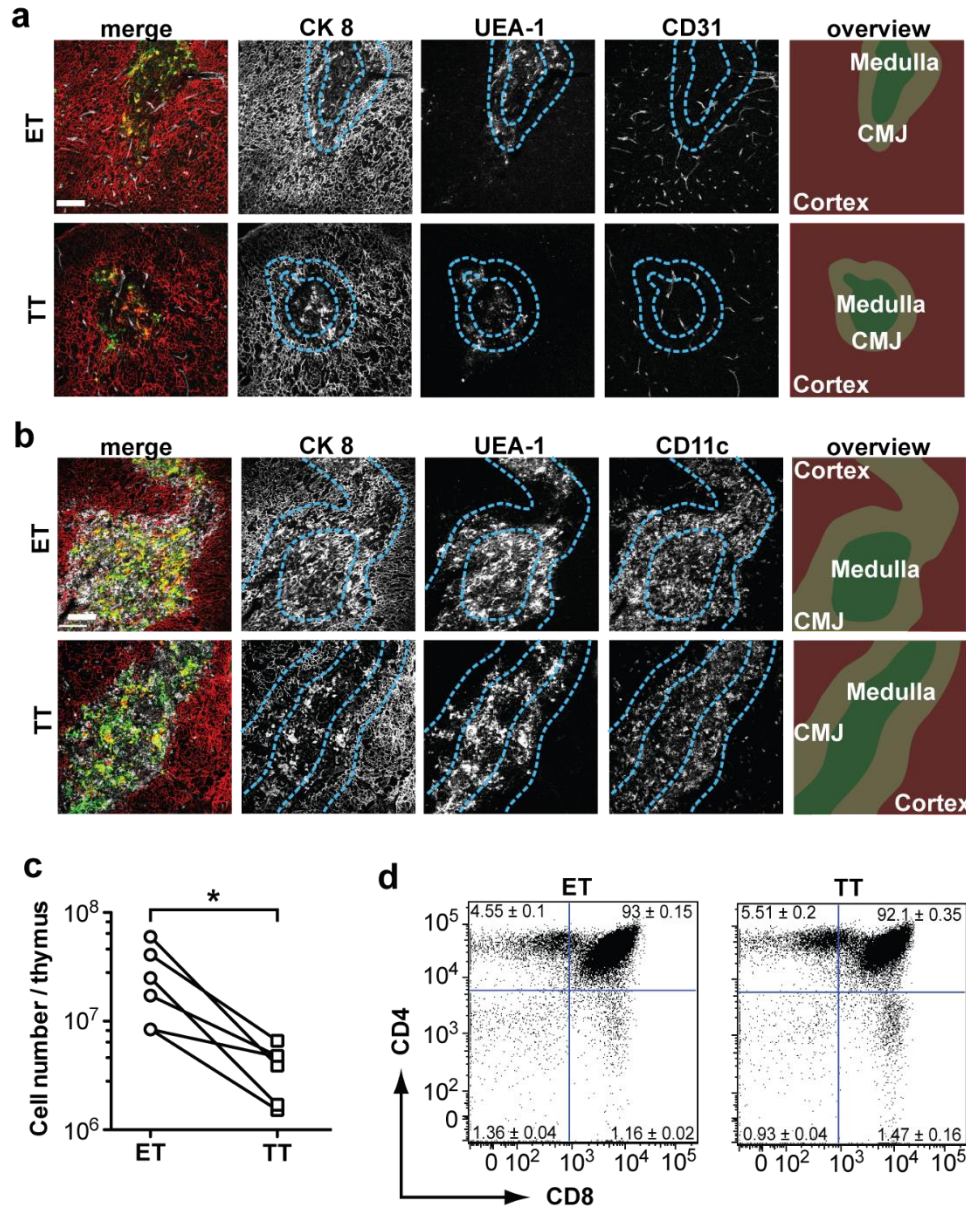
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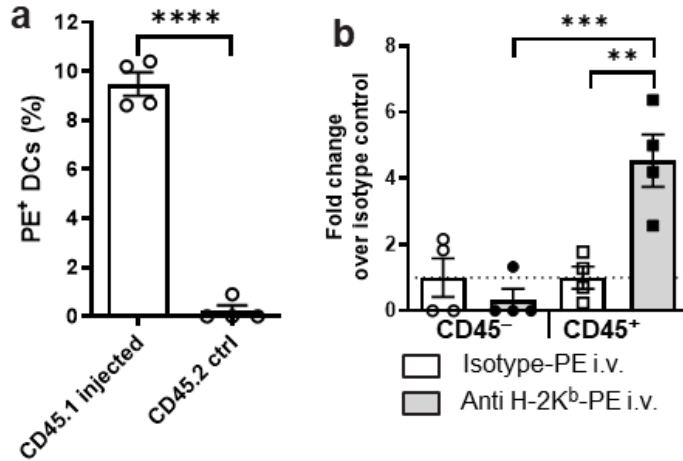
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Supplementary Fig. 1 Comparison of endogenous and transplanted thymi.

Fetal thymi were transplanted under kidney capsules of recipient mice and endogenous thymi (ET) and transplanted thymi (TT) were analyzed 4-9 weeks later. (a and b) Cryosections of ETs and TTs were stained with anti-cytokeratin 8 mAb (CK8, red, a and b), the lectin UEA-1 that specifically binds to mTECs (green, a and b) and mAbs against CD31 (white, a) or CD11c (white, b). Cortex, medulla and cortex-medulla-junction (CMJ) were identified according to the cytokeratin 8 and UEA-1 staining (Overview, a and b). These images were used to assess the density of CD11c⁺ and CD31⁺ area in the cortex, CMJ and medulla (Fig. 1a and d). n = 5 experiments for each staining, shown are representative confocal micrographs. (c) Absolute cell numbers of the endogenous and transplanted thymi 4 weeks after thymus transplantation. Each pair of symbols represents an individual animal. n = 6 (two-tailed, paired Student's t-test, *: P = 0.0374). (d) Representative FACS plots show the CD4-CD8 profile of thymocytes from ETs and TTs 4 weeks after transplantation.



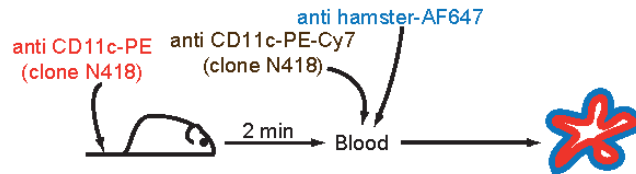
Supplementary Fig. 2 Validation of the in vivo PE⁺ labeling.

(a) CD45.1 mice were injected with anti-CD11c-PE and euthanized 2 minutes later while CD45.2 mice were left untreated. Single cell suspensions of the thymi of two CD45 congenic mice each were prepared in the same dish and PE⁺ DCs among CD45.1⁺ and CD45.2⁺ cells were analyzed by flow cytometry. (b) C57Bl/6 mice were injected i.v. with anti-H-2K^b-PE or isotype-PE and sacrificed 2 minutes later. CD45⁺ and CD45⁻ cells were evaluated for acquisition of the PE label. Data is expressed as fold change of PE⁺ cells over isotype-PE control. Data are representative of 2 (a) and 3 (b) independent experiments with 5 mice per group. P values were calculated using unpaired, two-tailed Students t-test (a) and one-way ANOVA (b). *****P* < 0.0001, ****P* = 0.007, ***P* = 0.003, n.s. not significant; mean ± s.e.m.

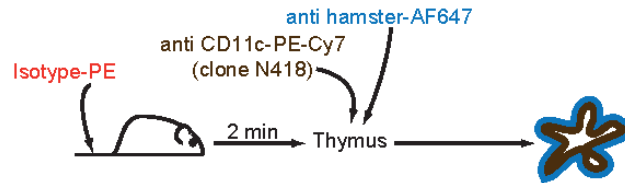
$$\text{in vivo labeling index} = \frac{\text{FI anti CD11c-PE}}{\text{FI anti hamster-AF647}}$$

$$\text{ex vivo labeling index} = \frac{\text{FI anti CD11c-PE-Cy7}}{\text{FI anti hamster-AF647}}$$

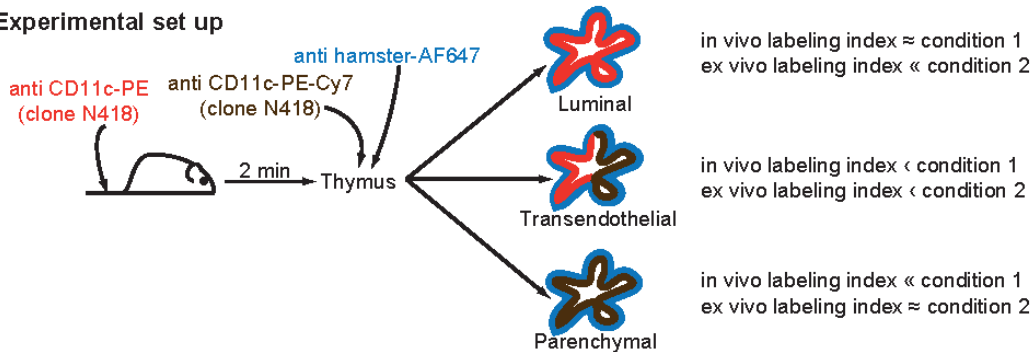
Condition 1: Luminal localization



Condition 2: Parenchymal localization

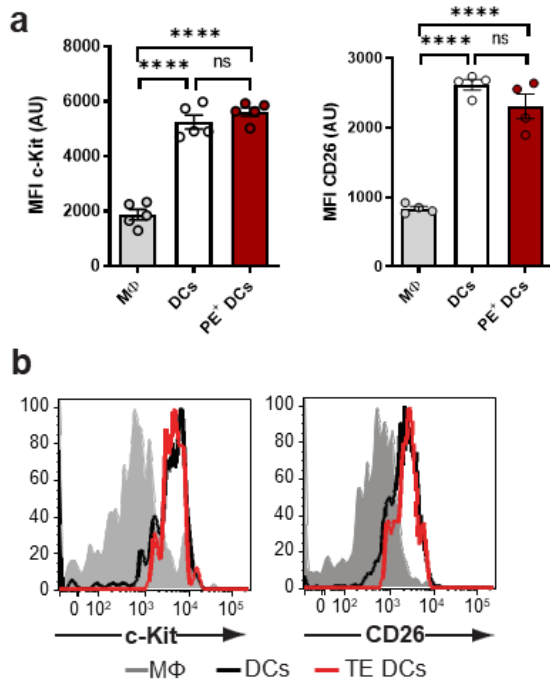


Experimental set up



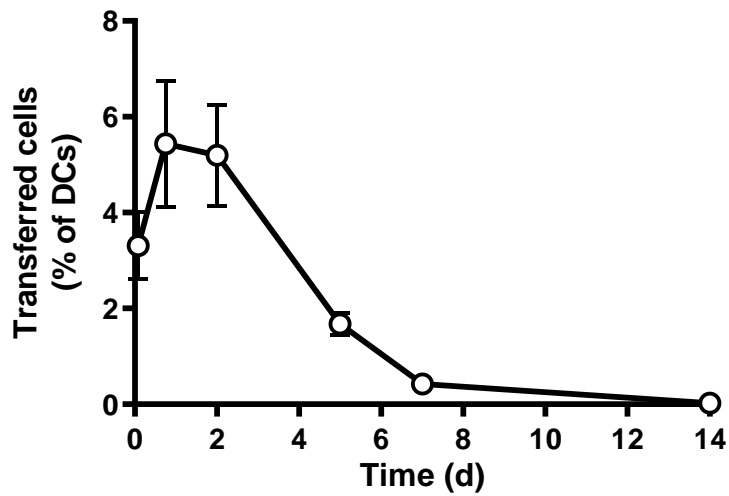
Supplementary Fig. 3 Schematic of competitive CD11c staining to identify cell localization.

Staining indexes were determined by applying a competitive staining protocol whereby mice were injected with anti-CD11c-PE (clone N428) IV, sacrificed 2 minutes later and single cell suspensions were stained ex vivo with anti-CD11c-PE-Cy7 (clone N418) and anti-hamster-AF647. In vivo and ex vivo staining indexes were calculated by dividing each cell's fluorescence intensity (FI) for the respective in vivo (PE) or ex vivo stain (PE-Cy7) by the FI of anti-hamster-AF647 to normalize for differences in the overall expression of CD11c. Lu-DCs are identified PE⁺ PE-Cy7⁻, Par-DCs are PE⁻ PE-Cy7⁺ and TE-DCs have an intermediate phenotype. Blood borne DCs can be used to identify Lu-DCs (Condition 1) and isotype-PE injected thymic DCs can act as a surrogate for Par DCs (Condition 2). With this gating strategy one can then identify Lu-DCs, Par-DCs and TE-DCs (Experimental setup).



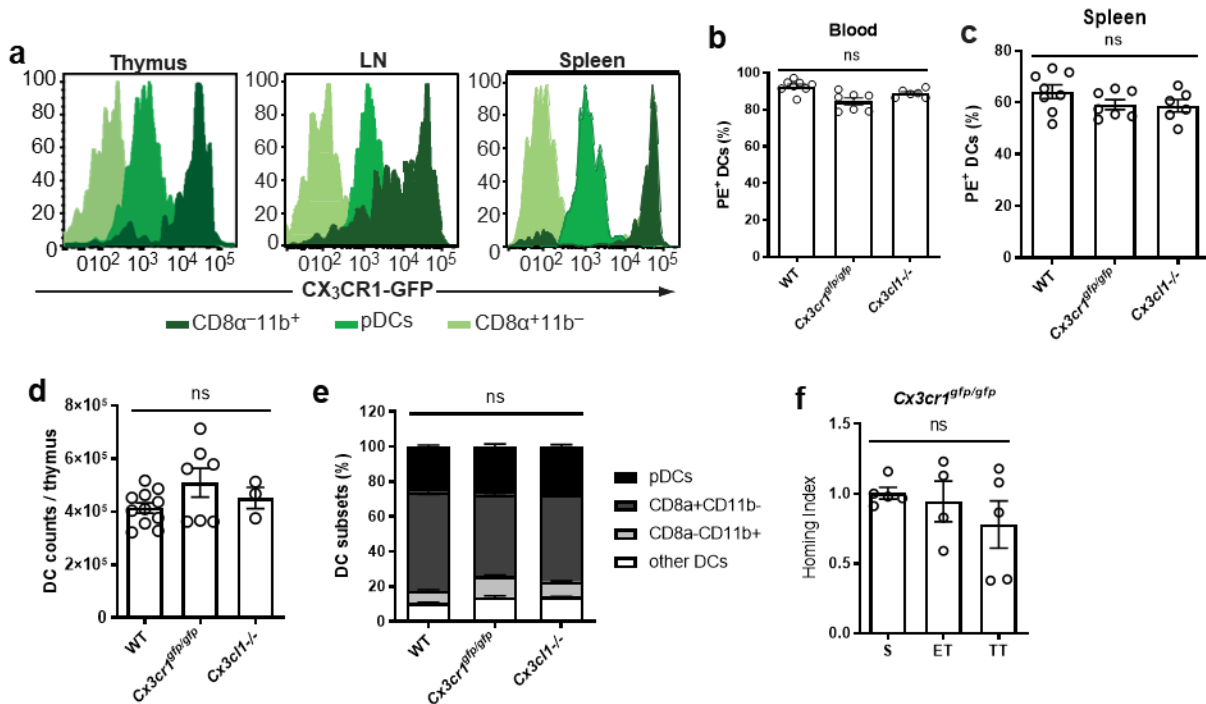
Supplementary Fig. 4 Analysis of thymic macrophages and DCs

(a and b) Thymic macrophages (Mφ), parenchymal denritic cells (Par-DCs) and PE⁺ DCs were evaluated for c-Kit (left panel) and CD26 (right panel) expression. (a) mean fluorescence intensities (MFI) of each cell population, (b) Representative FACS histogram; TE DCs: transendothelial dendritic cells, n=3 independent experiments with 5 mice/each for c-Kit and 4 mice/each for CD26 (one-way ANOVA). Bars and error bars represent mean ± SEM. ****: $P < 0.0001$, n.s.: not significant



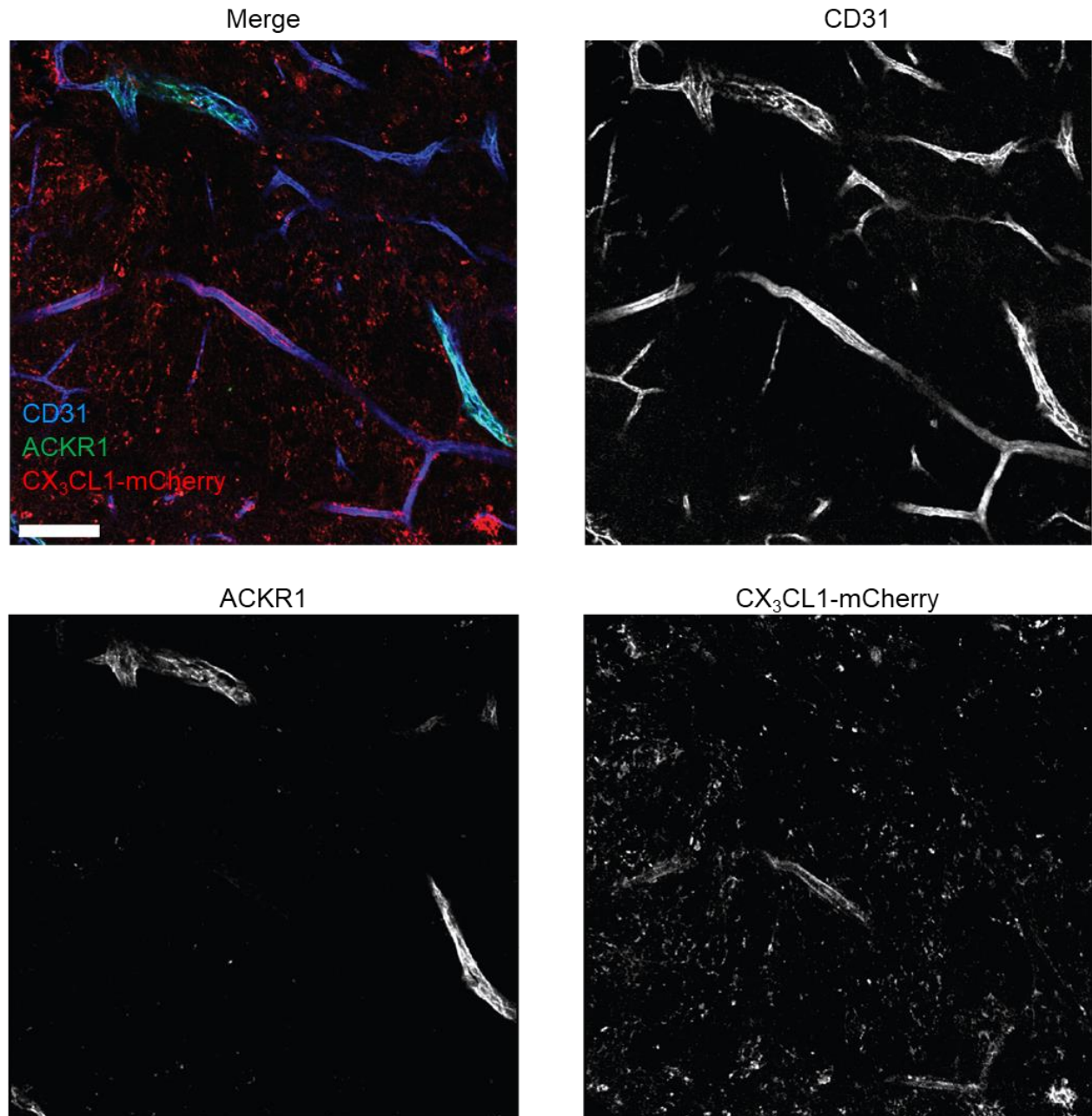
Supplementary Fig. 5 Timeline of the percentage of adoptively transferred DCs in the thymus.

Homing of 1×10^7 adoptively transferred, splenic, in vivo Flt3L expanded DCs from WT mice into CD45 congenic recipient mice was assessed by flow cytometry. The percentage of homed DCs among all DCs in the thymus was evaluated at the indicated time points post transfer. Bars and error bars represent mean \pm SEM. n = 5 animals (5 days and 14 days) and n = 10 animals (2hrs, 18 hrs, 2 days and 7 days)



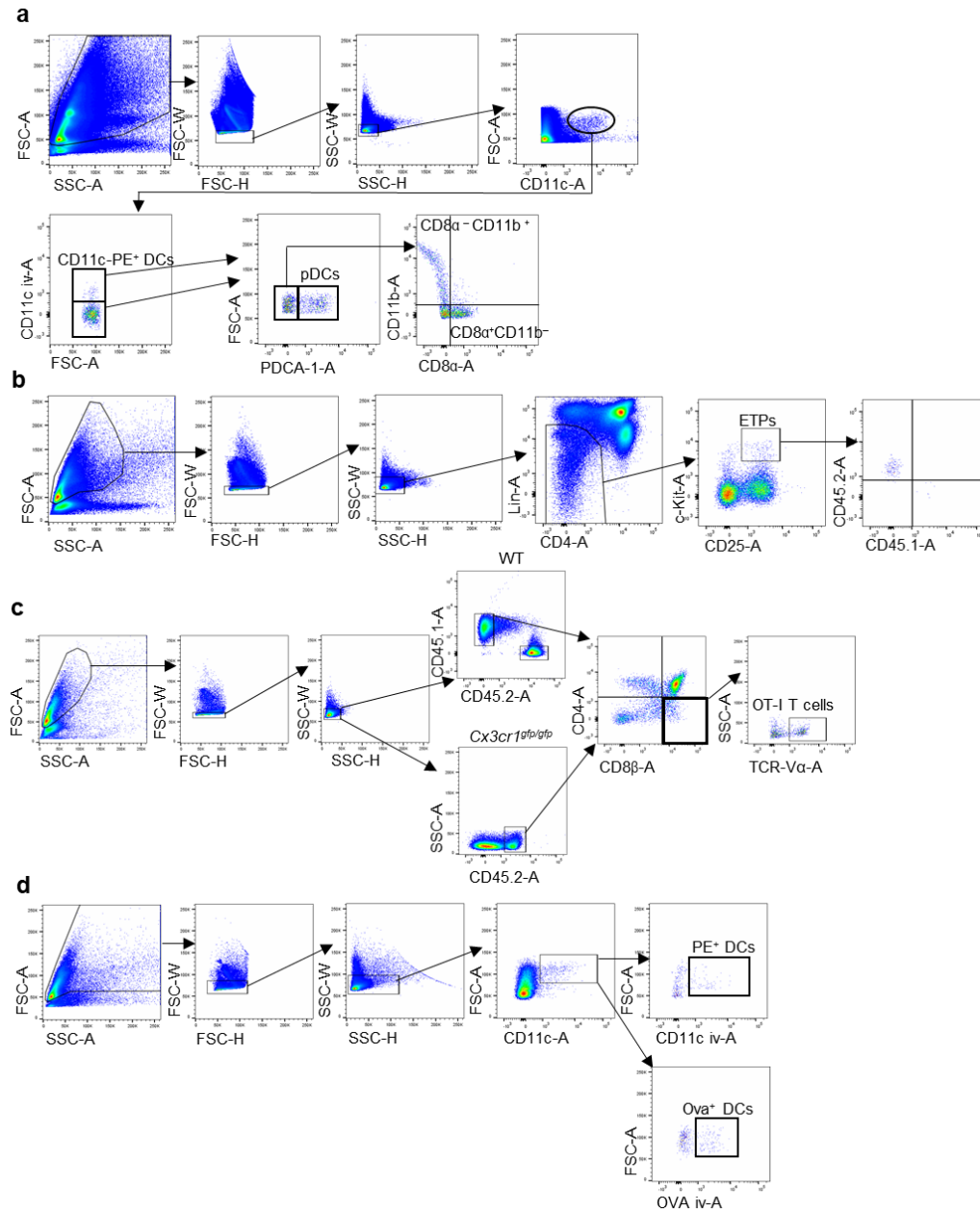
Supplementary Fig. 6 Characterization of *Cx3cr1*^{gfp/+} and *Cx3cl1*^{-/-} mice.

(a) CX₃CR1 expression on DC subsets was evaluated by flow cytometry using *Cx3cr1*^{gfp/+} reporter mice. The representative histograms show the CX₃CR1-GFP expression in CD8α⁻CD11b⁻ (light green), CD8α⁻CD11b⁺ dendritic cells (DCs, dark green) and plasmacytoid dendritic cells (pDCs, mid-green) of *Cx3cr1*^{gfp/+} animals in thymus (left panel), lymph node (LN, middle panel) and spleen (right panel). n = 3 experiment with 5 mice each. (b and c) WT (n=15), *Cx3cr1*^{gfp/gfp} (n=7) and *Cx3cl1*^{-/-} (n=3) were injected IV with anti-CD11c-PE, sacrificed 2 minutes later and PE⁺ DCs in blood (b) and spleen (c) were analyzed by flow cytometry (one-way ANOVA), n.s.: not significant. (d) Absolute cell numbers of whole thymi of sex and age matched WT, *Cx3cr1*^{gfp/gfp} and *Cx3cl1*^{-/-} mice. n = 3 independent experiments with 3 mice (one-way ANOVA), n.s.: not significant. (e) Subset composition of thymic DCs in sex and age matched WT, *Cx3cr1*^{gfp/gfp} and *Cx3cl1*^{-/-} mice. n = 3 independent experiments with 3 mice (two-way ANOVA), n.s.: not significant. (f) Recruitment of circulating WT and *Cx3cr1*^{gfp/gfp} DCs to spleen (S), TT and ET was assessed by competitive homing assays. A 1:1 mixture of differentially labeled splenic, in vivo Flt3L expanded DCs from WT and *Cx3cr1*^{gfp/gfp} donors was injected IV into mice that were previously transplanted with a fetal thymus. The homing index was determined after 18h by FACS analysis of the indicated recipient tissue. Results were pooled from 2 independent experiments, n = 5 mice/group (one-way ANOVA). Bars and error bars represent mean ± SEM. n.s.: not significant.



Supplementary Fig. 7 Thymic expression pattern of CX₃CL1.

Representative confocal micrograph of three ETs from CX₃CL1-mCherry reporter mice shows the expression of CD31 (white), ACKR1 (green) and CX₃CL1 (red). The scale bar indicates 100 μ m.



Supplementary Figure 8. Gating strategies used for phenotypic analysis

(a) pDCs were identified as single cells that express CD11c and PDCA-1. CD8α⁻CD11b⁺ and CD8α⁺CD11b⁻ DCs were gated from the PDCA-1 negative population. (b) ETPs in parabiotic mice were gated as single cells that are Lin⁻ (Gr-1, CD11b, B220, CD3, NK1.1, Terr119) CD4⁻ and express c-Kit and CD25. The percent of endogenous vs partner derived ETPs was calculated based on the CD45.1 and CD45.2 expression. (c) OT-I thymocytes in in vivo deletion experiments were detected by gating on single cells that are CD45.1⁺CD45.2⁻ that express TCR Vα for CX₃CR1 sufficient animals or CD45.2⁺ that express TCR Vα for *Cx3cr1^{gfp/gfp}* animals. (d) BLT mice were either injected with CD11c-PE, isotype-PE or Ova-AF488, respectively. Human dendritic cells were analyzed by gating on CD11c⁺ single cells and then evaluation of positivity of foe PE or AF488, respectively.

| Antibody | Clone | Vendor | Catalogue number | dilution factor | Format / conjugate |
|--------------------|--------------|----------------|-------------------------|------------------------|---------------------------|
| Anti- human CD11c | 3.9 | Biolegend | 301622 | 100 | Alexa Fluor® 647 |
| Anti- human CD11c | Bu15 | Biolegend | 337218 | 100 | APC/Cyanine7 |
| Anti- human CD11c | 3.9 | Biolegend | 301606 | 200 | PE |
| Anti- human CD31 | WM59 | Biolegend | 303110 | 100 | Alexa Fluor® 488 |
| Anti- human CD31 | WM59 | Biolegend | 303112 | 100 | Alexa Fluor® 647 |
| Anti- mouse B220 | RA3-6B2 | BioLegend | 103226 | 400 | Alexa Fluor® 647 |
| Anti- mouse B220 | RA3-6B2 | BioLegend | 103224 | 400 | APC/Cyanine7 |
| Anti- mouse B220 | RA3-6B2 | BioLegend | 103206 | 400 | FITC |
| Anti- mouse B220 | RA3-6B2 | BioLegend | 103208 | 400 | PE |
| Anti- mouse B220 | RA3-6B2 | BioLegend | 103222 | 400 | PE/Cyanine7 |
| Anti- mouse B220 | RA3-6B2 | BioLegend | 103236 | 400 | PerCP/Cyanine5.5 |
| Anti- mouse CD11c | N418 | BioLegend | 117313 | 100 | Alexa Fluor® 488 |
| Anti- mouse CD11c | N418 | BioLegend | 117312 | 400 | Alexa Fluor® 647 |
| Anti- mouse CD11c | HL3 | BD Biosciences | 553801 | 400 | FITC |
| Anti- mouse CD11c | HL3 | BD Biosciences | 557401 | 400 | PE |
| Anti- mouse CD11c | N418 | BioLegend | 117308 | 400 | PE |
| Anti- mouse CD11c | N418 | BioLegend | 117318 | 400 | PE/Cyanine7 |
| Anti- mouse CD11c | HL3 | BD Biosciences | 561022 | 400 | PE/Cyanine7 |
| Anti- mouse CD24 | M1/69 | BD Biosciences | 553260 | 200 | Biotin |
| Anti- mouse CD24 | 30-F1 | BioLegend | 138504 | 400 | PE |
| Anti- mouse CD25 | PC61 | BioLegend | 102008 | 400 | PE |
| Anti- mouse CD26 | H194-112 | BioLegend | 137806 | 200 | FITC |
| Anti- mouse CD3 | 17A2 | BioLegend | 100206 | 400 | PE |
| Anti- mouse CD31 | 390 | BioLegend | 102414 | 100 | Alexa Fluor® 488 |
| Anti- mouse CD31 | 390 | BioLegend | 102416 | 100 | Alexa Fluor® 647 |
| Anti- mouse CD4 | GK1.5 | BioLegend | 100412 | 400 | APC |
| Anti- mouse CD4 | GK1.5 | BioLegend | 100422 | 400 | PE/Cyanine7 |
| Anti- mouse CD4 | RM4-5 | BioLegend | 100540 | 400 | PerCP/Cyanine5.5 |
| Anti- mouse CD45 | 30-F11 | BioLegend | 103106 | 400 | PE |
| Anti- mouse CD45 | 30-F11 | BioLegend | 103114 | 400 | PE/Cyanine7 |
| Anti- mouse CD45.1 | A20 | BioLegend | 110708 | 400 | PE |
| Anti- mouse CD45.1 | A20 | BioLegend | 110730 | 400 | PE/Cyanine7 |
| Anti- mouse CD45.2 | 104 | BioLegend | 109824 | 400 | APC/Cyanine7 |
| Anti- mouse CD45.2 | 104 | eBioscience | 47-0454 | 400 | APC-eFluor 780 |
| Anti- mouse CD45.2 | 104 | BioLegend | 109828 | 400 | PerCP/Cyanine5.5 |
| Anti- mouse CD62L | MEL-14 | BioLegend | 104421 | 400 | Alexa Fluor® 647 |
| Anti- mouse CD69 | MEL-14 | BioLegend | 104412 | 400 | APC |
| Anti- mouse CD8α | 53-6.7 | BioLegend | 100714 | 400 | APC/Cyanine7 |
| Anti- mouse CD8α | 53-6.7 | BioLegend | 100706 | 400 | FITC |
| Anti- mouse CD8α | 53-6.7 | BioLegend | 100734 | 400 | PerCP/Cyanine5.5 |

| | | | | | |
|--|-------------|----------------|------------|-----|------------------------------|
| Anti- mouse CD8 β | YTS156.7.7 | BioLegend | 126612 | 400 | Alexa Fluor [®] 647 |
| Anti- mouse c-Kit | 2B8 | eBioscience | 47-1171-82 | 200 | APC-eFluor 780 |
| Anti- mouse c-Kit | 2B8 | eBioscience | 12-1171-82 | 200 | FITC |
| Anti- mouse EpCAM | G8.8 | BioLegend | 118212 | 100 | Alexa Fluor [®] 647 |
| Anti- mouse Flt3 | A2F10 | BioLegend | 135308 | 200 | Biotin |
| Anti- mouse Gr-1 | RB6-8C5 | BioLegend | 108407 | 400 | PE |
| Anti- mouse Ly51 | 6C3/BP-1 | BioLegend | 108308 | 200 | PE |
| Anti- mouse MHCII | M5/114.15.2 | BD Biosciences | 107628 | 200 | APC/Cyanine7 |
| Anti- mouse NK1.1 | PK136 | BioLegend | 108708 | 400 | PE |
| Anti- mouse PDCA-1 | 927 | BioLegend | 127014 | 400 | Alexa Fluor [®] 647 |
| Anti- mouse PDCA-1 | 129C1 | BioLegend | 127106 | 400 | Alexa Fluor [®] 647 |
| Anti- mouse PDCA-1 | 927 | BioLegend | 127006 | 400 | Biotin |
| Anti- mouse PDCA-1 | 927 | BioLegend | 127010 | 400 | PE |
| Anti- mouse PDCA-1 | 927 | eBioscience | 46-3172-82 | 400 | PerCP/Cyanine5.5 |
| Anti- mouse TCR V α 2 | B20.1 | BioLegend | 127812 | 400 | Alexa Fluor [®] 647 |
| Anti- mouse TCR V α 2 | B20.1 | BioLegend | 127808 | 400 | PE |
| Anti- mouse TCR V α 2 | B20.1 | BioLegend | 127814 | 400 | PerCP/Cyanine5.5 |
| Anti- mouse Ter119 | TER-119 | BD Biosciences | 553673 | 400 | PE |
| Anti- mouse VCAM-1 | MK2.7 | BioXcell | BE0027 | 100 | |
| Anti- mouse α L integrin | M17/4 | BioXcell | BE0006 | 100 | |
| Anti- mouse/human CD11b | M1/70 | BioLegend | 101217 | 400 | Alexa Fluor [®] 488 |
| Anti- mouse/human CD11b | M1/70 | BioLegend | 101218 | 400 | Alexa Fluor [®] 647 |
| Anti- mouse/human CD11b | M1/70 | BioLegend | 101216 | 400 | PE/Cyanine7 |
| Anti- mouse/human CD11b | M1/70 | BioLegend | 101228 | 400 | PerCP/Cyanine5.5 |
| Anti- mouse/human α 4 integrin | PS/2 | BioXcell | BE0071 | 100 | |
| hamster IgG and purified neutralizing mAbs to mouse P-selectin | RB40.34 | BD Biosciences | 553742 | 100 | |
| InVivoMAb anti-mouse CD16/CD32 | 2.4G2 | BioXcell | BE0307 | 100 | |
| Streptavidin | | BioLegend | 405204 | 400 | PE |
| Streptavidin | | BioLegend | 405214 | 400 | PerCP/Cyanine5.5 |

Supplementary table 1. List of antibodies

Commercially available unconjugated and fluorochrome-conjugated antibodies are listed, providing information on the antibody specificity, clone, vendor, catalogue number, used dilution factor and the format / conjugate for each antibody.