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Supplementary Information for

Myeloid cells activate iNKT cells to produce IL-4 in the thymic medulla

Haiguang Wang, Elise R. Breed, You Jeong Lee, Lily J. Qian, Stephen C. Jameson, Kristin A. Hogquist*

Kristin A. Hogquist
Email: hogqu001@umn.edu

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Supplementary Material and Methods

Mice

B6 (C57BL/6NCr) mice were purchased from the National Cancer Institute. BALB/cBYJ, CD45.1$^+$/CD45.1$^+$ BALB/cBYJ (CByJ.SJL(B6)-Ptprca/J), Cd1d$^{-/-}$ BALB/c (C.129S2-Cd1tm1Gru/J), *Cd1d*$^{fl/fl}$ B6 (C57BL/6-Cd1d1tm1.1Aben/J), FoxN1Cre B6 (B6(Cg)-Foxn1tm3(cre)Nrm/J), MB1Cre B6 (B6.C(Cg)-Cd79atm1(cre)Reth/EhobJ), CD11cCre B6 (B6.Cg-Tg(Itgax-cre)1-1Reiz/J), *Zbtb46*Cre B6 (B6.Cg-Zbtb46tm3.1(cre)Mnz/J), BDCA2-DTR B6 (C57BL/6-Tg(CLEC4C-HBEGF)956Cln/J) mice were obtained from the Jackson Laboratory. KN2 BALB/cBYJ, *Nur77*^{GFP} BALB/c and KN2 B6 mice have been previously described (1). LysMCre$^+$ *Csf1r*$^{LsL-DTR}$ B6 mice were kindly provided by Dr. Marc Jenkins at University of Minnesota. CD45.1$^+$ CD45.2$^+$ KN2 BALB/cBYJ, *Nur77*^{GFP} KN2 BALB/c, FoxN1Cre$^+$ *Cd1d*$^{fl/fl}$ KN2 F1, MB1Cre$^+$ *Cd1d*$^{fl/fl}$ KN2 F1, CD11cCre$^+$ *Cd1d*$^{fl/fl}$ KN2 F1, *Zbtb46*Cre$^+$ *Cd1d*$^{fl/fl}$ KN2 F1, BDCA2-DTR$^+$ KN2 F1, LysMCre$^+$ *Csfr1*$^{LsL-DTR}$ KN2 F1, LysMCre$^+$ *Csfr1*$^{LsL-DTR}$ F1 mice as well as littermate control mice for each strain were generated through crossbreeding at University of Minnesota. *Cd1d*$^{fl/fl}$ KN2 BALB/c mice were generated through using *Cd1d*$^{fl/fl}$ B6 mice backcrossed to KN2 BALB/c mice for at least 10 generations at University of Minnesota.

Diphtheria toxin treatment

BDCA2-DTR F1 and littermate control mice were injected intraperitoneally (i.p.) with diphtheria toxin (DT) every other day for 9 days (5 injections in total, 500ng of DT in 100μL PBS for the 1st injection on 1st day, 100ng of DT in 100μL PBS for all subsequent injections). LysMCre$^+$ *Csfr1*$^{LsL-DTR}$ F1 and littermate control mice were injected i.p. with diphtherial toxin (DT) every other day for 9 days (5 injections in total, 100ng of DT in 100μL PBS for the all 5 injections). Mice were used the day after the last injections.

Intra-thymic transfer

iNKT enriched thymocytes were prepared through depletion of CD8$^+$ and CD24$^+$ cells (via immunomagnetic selection [Miltenyi]) in thymocytes from CD45.1$^+$ CD45.2$^+$ KN2 BALB/cBYJ mice. Intra-thymic transfers were performed on congenic host mice through ultrasound imaging guidance. Ultrasound imaging guided intra-thymic transfer is described in detail previously (2).

Bone marrow chimera

Bone marrow cells were directly flushed out from the femurs and tibias of donor mice (BALB/c Wt or BALB/c *Cd1d*$^{-/-}$) and depleted of T cells via immunomagnetic depletion (Miltenyi) of CD8$^+$ and CD3$^+$ cells. Recipient mice (BALB/c Wt or BALB/c *Cd1d*$^{-/-}$) received a total lethal dose of 600 rads. Irradiations were done in 2 doses (300 rads each) separated by a period of 3-4 hours. 2 hours after the second irradiation, recipient mouse received at least 4×10^6 of T-cell-depleted bone marrow donor cells through intravenous injection via tail vein. Bone marrow chimeras were used at 8 weeks after introduction of bone marrow donor cells.

Enrichment of iNKT cells

Thymocytes were prepared in single cell suspension and incubated with PE-CD1d-PBS57 tetramer at 4℃ for 30 min followed by immunomagnetic enrichment using anti-PE microbeads per manufacturer's instructions (Miltenyi).

Preparation of lymphocytes and flow cytometric analysis

Thymocytes were prepared in single cell suspension through mashing thymi and filtering through 70 μM cell strainers (FALCON), or digestion with collagenase D (1 mg/mL) at 37℃ for 30 min before mashing and filtration through 70 μM cell strainers. The thymic epithelial cells were prepared as previously described (3). Cells were incubated with Fc block (Tonbo) and mouse serum (Jackson ImmunoResearch) for 15 min at 4℃ before staining with antibodies to surface markers and viability dye (Tonbo) for 45 min at 4℃. For intracellular staining of transcription factors, cells were fixed and permeabilized with a Foxp3 staining buffer kit (eBioscience) after staining with antibodies to surface markers, then were incubated with antibodies to transcription

factors in permeabilization buffer for 45 min at 4℃. Biotinylated PBS57 loaded or unloaded CD1d monomers were from the tetramer core of the US National Institutes of Health and tetramerized with streptavidin-PE at the ratio of 4:1. All antibodies were purchased from eBioscience, BD Biosciences or BioLegend, unless otherwise indicated. See SI Appendix, Suppl. Table 2 for a list of antibodies and other reagents used in this study. Samples were acquired on a BD LSR Fortessa or BD Fortessa X-20 and data were analyzed with FlowJo 10 (Treestar). Samples were SSC-A/FSC-A gated to exclude debris, SSC-A/SSC-W gated to select single cells, then gated to exclude viability dye^{+} dead cells.

Immunofluorescence and histocytometric analysis

For CD1d tetramer based immunofluorescence, the fresh thymic lobe was incubated with PE-CD1d-PBS57 tetramer (50 µL of 1×PBS containing 2% FBS + 2 µL of CD1d tetramer in 96 well plate) at 4℃ overnight, then washed with PBS for 3 times, then fixed in 4% paraformaldehyde (PFA) at 4℃ for 1 hour followed by incubation in 30% (w/v) sucrose for overnight. Tissues were further snap frozen in Optimal Cutting Temperature Compound (Tissue-Tek) and stored at -80℃ before sectioning (5-7 µm) on a Cryostat (Leica). The immunofluorescence was performed in CAS block solution (Invitrogen) as follows: 1 hour blocking in CAS block solution at room temperature (RT), 2 hours incubation at RT with primary antibodies mixture including anti-PE antibody (Goat polyclonal to R Phycoerythrin, Abcam; or rabbit whole antisera, Novus Biologicals) and other antibodies, wash 3 times, 1 hour incubation at RT with secondary antibodies mixture (Donkey anti Goat AF555, Invitrogen; or Goat-anti-Rabbit-AF555, Invitrogen), wash 3 times, mount overnight in mounting media containing DAPI (ProLong® Diamond Antifade Mountant with DAPI). For human CD2 (huCD2) immunofluorescence, the tissue was stained with anti-huCD2 (RPA2) antibody followed by tyramide-based amplification per manufacturer's instructions (PerkinElmer). The images were collected on a Leica DM6000B Epi-Fluorescent microscope. The histocytometric analysis to quantify localization was performed in ImageJ, Flowjo and Prism as previously described (4, 5).

Author contributions

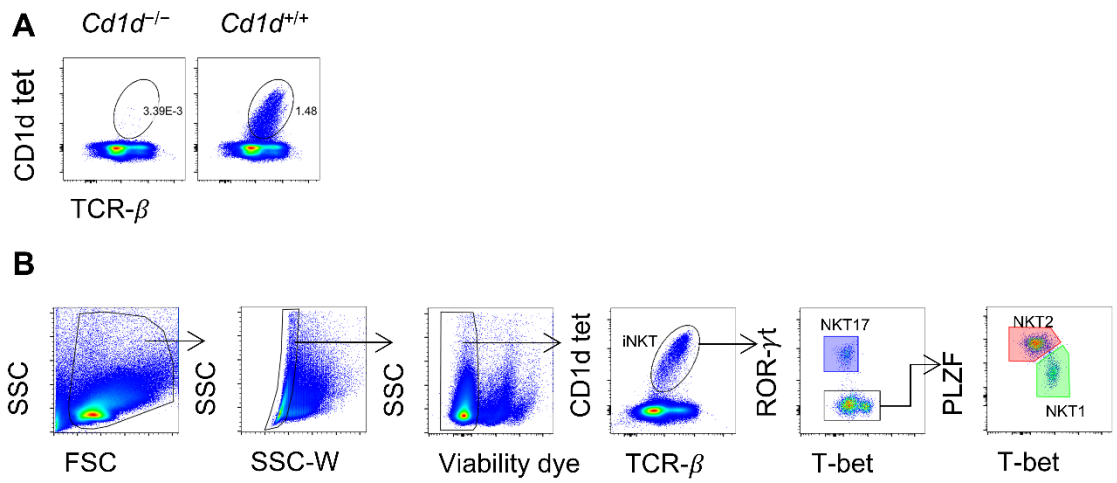
H.W. designed and performed the research, analyzed the data, wrote and edited the paper. Y.J.L and L.J.Q performed the research. E.R.B contributed a new analytic tool and analyzed the data. S.C.J provided insights to the research. K.A.H designed and supervised the research, edited the paper.

Conflict of interest

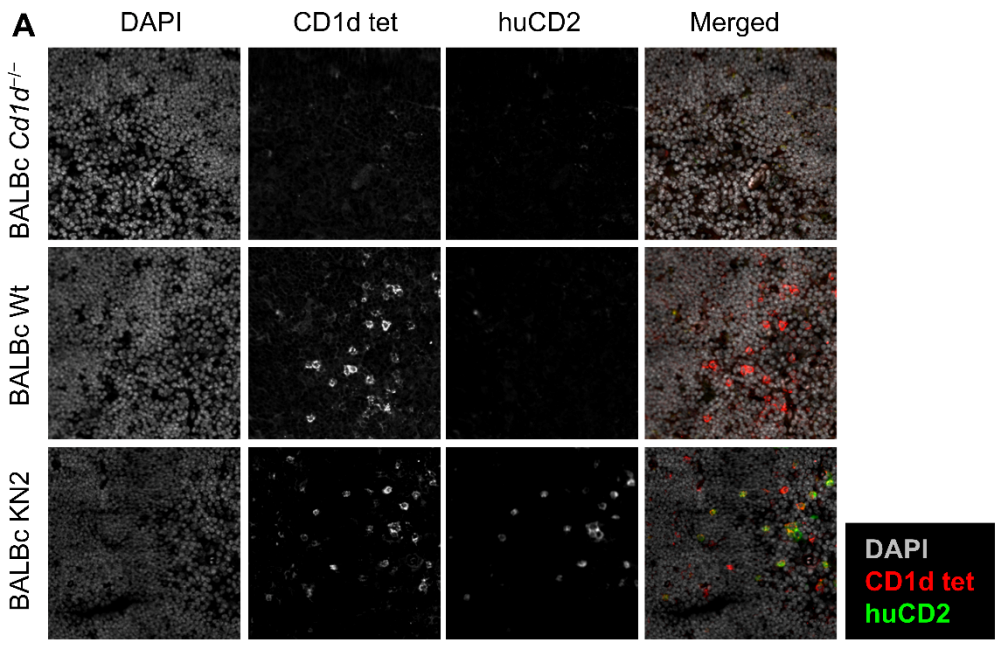
The authors declare no conflict of interest.

Acknowledgements

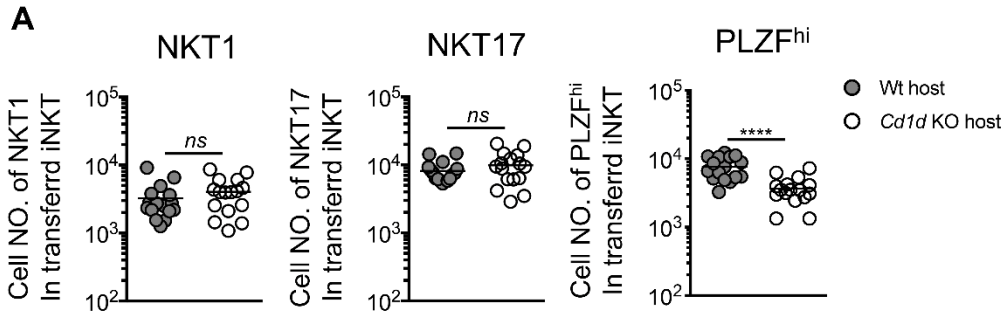
We thank Ms. Jane Ding for expert technical assistance, Dr. Hristo Georgiev for critical reading of the manuscript as well as all present and past members in Hogquist & Jameson lab for insightful discussions. This work was supported by AI39560 (K.A.H.), F30 AI131483, T32 AI007313, and T32 GM008244 (E.R.B) and UMN dissertation fellowship (H.W.).



Suppl. Figure 1. Detection and gating strategy of iNKT subsets.
(**A**) Representative flowcytometry plots of PBS57-CD1d tetramer staining in thymocytes from BALB/c *Cd1d*$^{-/-}$ and *Cd1d*$^{+/+}$ mouse. (**B**) Flowcytometric gating strategy to identify thymic iNKT cells and iNKT subsets (NKT1, green; NKT2, red; NKT17, blue).

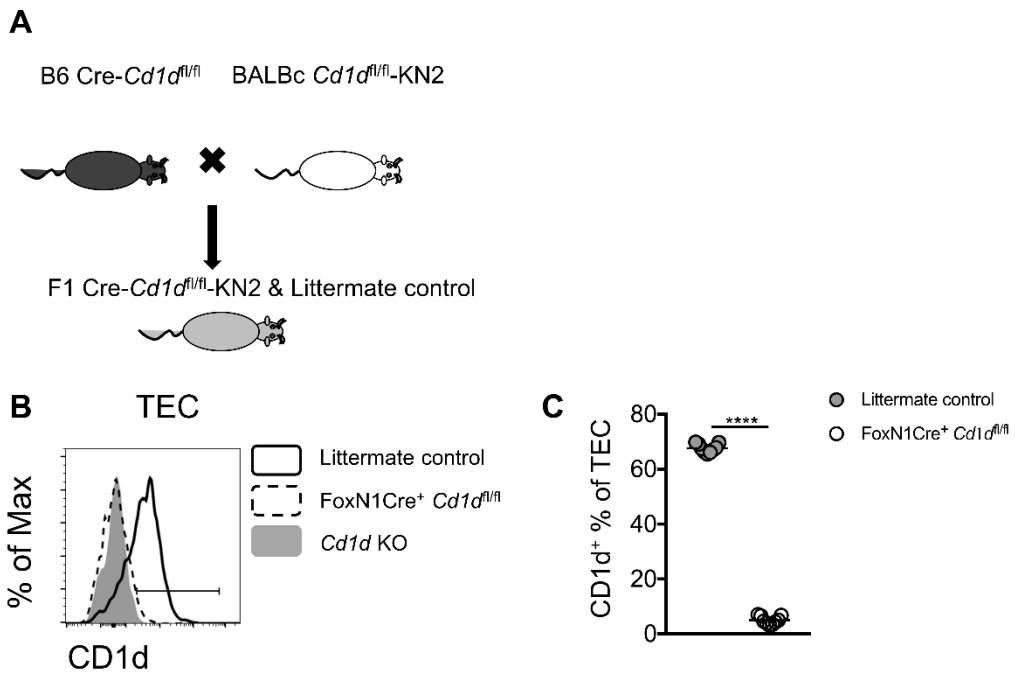


Suppl. Figure 2. Specific detection of IL-4 producing NKT2 cells *in situ*.
(**A**) Immunofluorescent microscopy of thymic sections of BALB/c *Cd1d*$^{-/-}$, Wt or KN2 mice, stained with DAPI, PBS57-CD1d tetramer and huCD2.



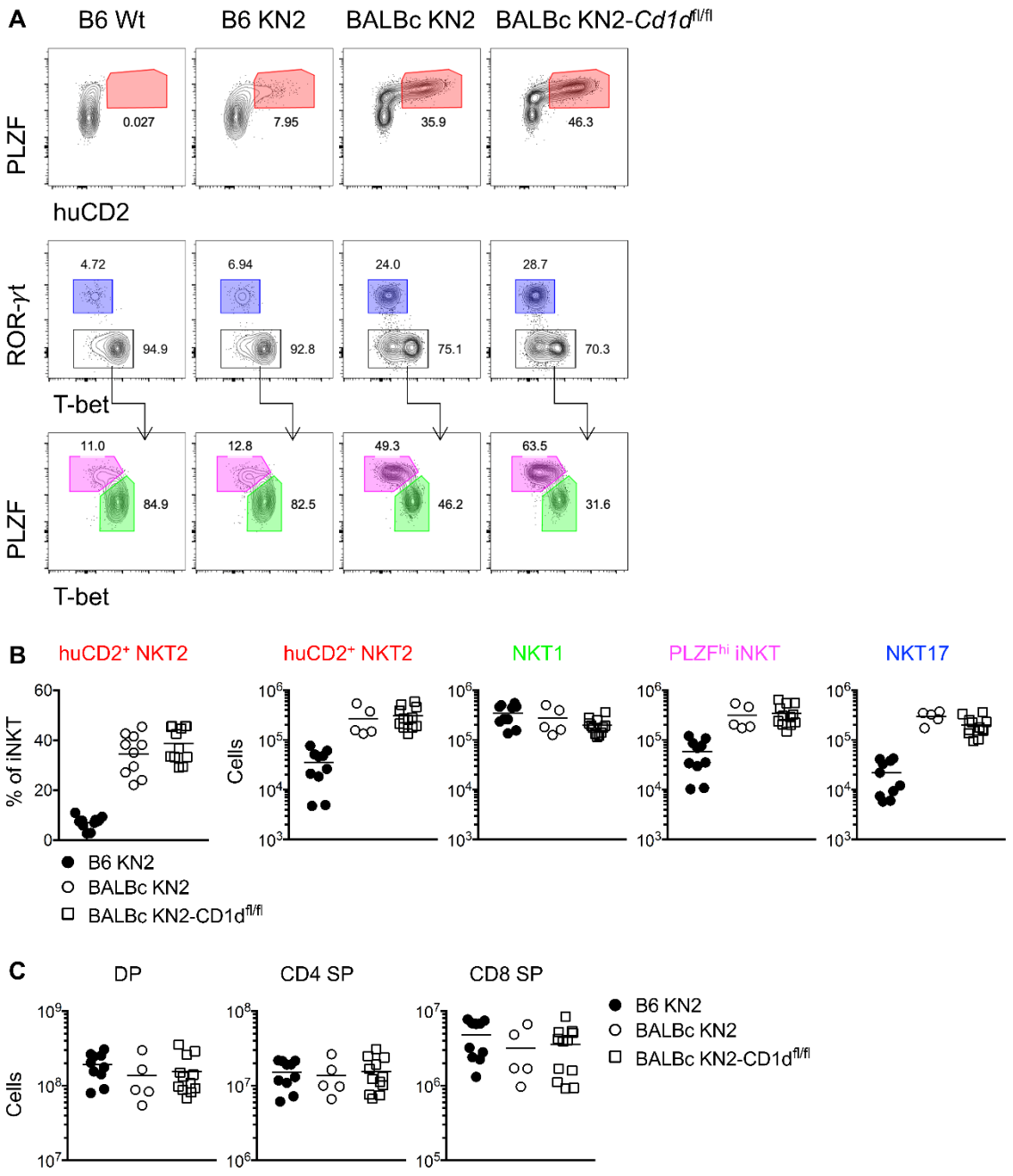
Suppl. Figure 3. Intra-thymic transfer into *Cd1d*$^{-/-}$ host does NOT affect donor NKT1 & NKT17 in short term

(**A**) Cell number of transferred donors NKT1 (left), NKT17 (middle) and PLZF^{hi} iNKT (right) cells 9 days after intra-thymic transfer into BALB/c Wt or BALB/c *Cd1d* KO host mice. Data are pooled from 5 experiments. n=16 (BALB/c Wt), 16 (BALB/c *Cd1d* KO). Each dot represents an individual mouse and horizontal bars indicate mean values. Unpaired t test, ^{ns}p=0.3013 (NKT1) or 0.2534 (NKT17); ****p<0.0001 (PLZF^{hi} iNKT).

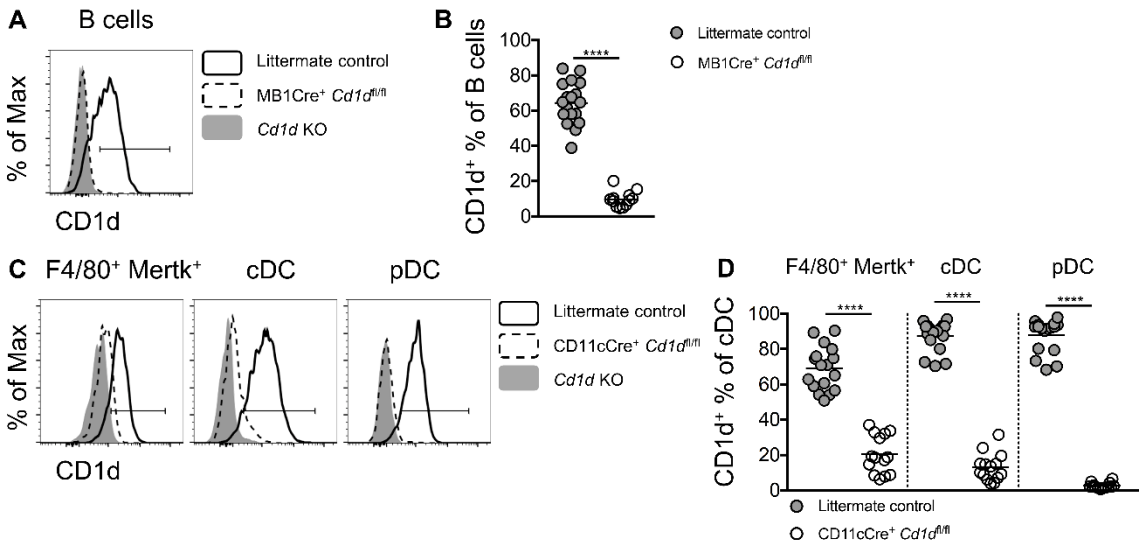


Suppl. Figure 4. Experimental strategy of generating F1 Cre^{+}-*Cd1d*$^{fl/fl}$-KN2 mouse & targeting of Cd1d with FoxN1Cre

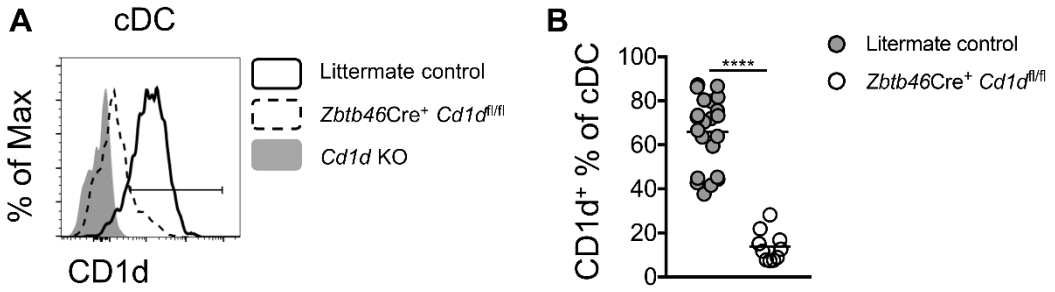
(**A**) Experimental scheme showing generation of F1 Cre^{+}-*Cd1d*$^{fl/fl}$-KN2 or Littermate control mice through cross breeding of B6 Cre^{+}-*Cd1d*$^{fl/fl}$ with BALB/c *Cd1d*$^{fl/fl}$-KN2 mice. (**B**) Representative flowcytometry histogram showing expression of CD1d in thymic epithelial cells (TECs, EpCAM^{+} CD45^{-}) from F1 littermate control, F1 FoxN1Cre *Cd1d*$^{fl/fl}$ and BALB/c *Cd1d* KO mice. (**C**) Frequency of CD1d^{+} cells of TECs from F1 littermate control or F1 FoxN1Cre^{+} *Cd1d*$^{fl/fl}$ mice. Each dot represents an individual mouse and horizontal bars indicate mean values. Data are pooled from 3 experiments. n=9 (Littermate control), 9 (FoxN1Cre^{+} *Cd1d*$^{fl/fl}$). Unpaired t test, ****p<0.0001.



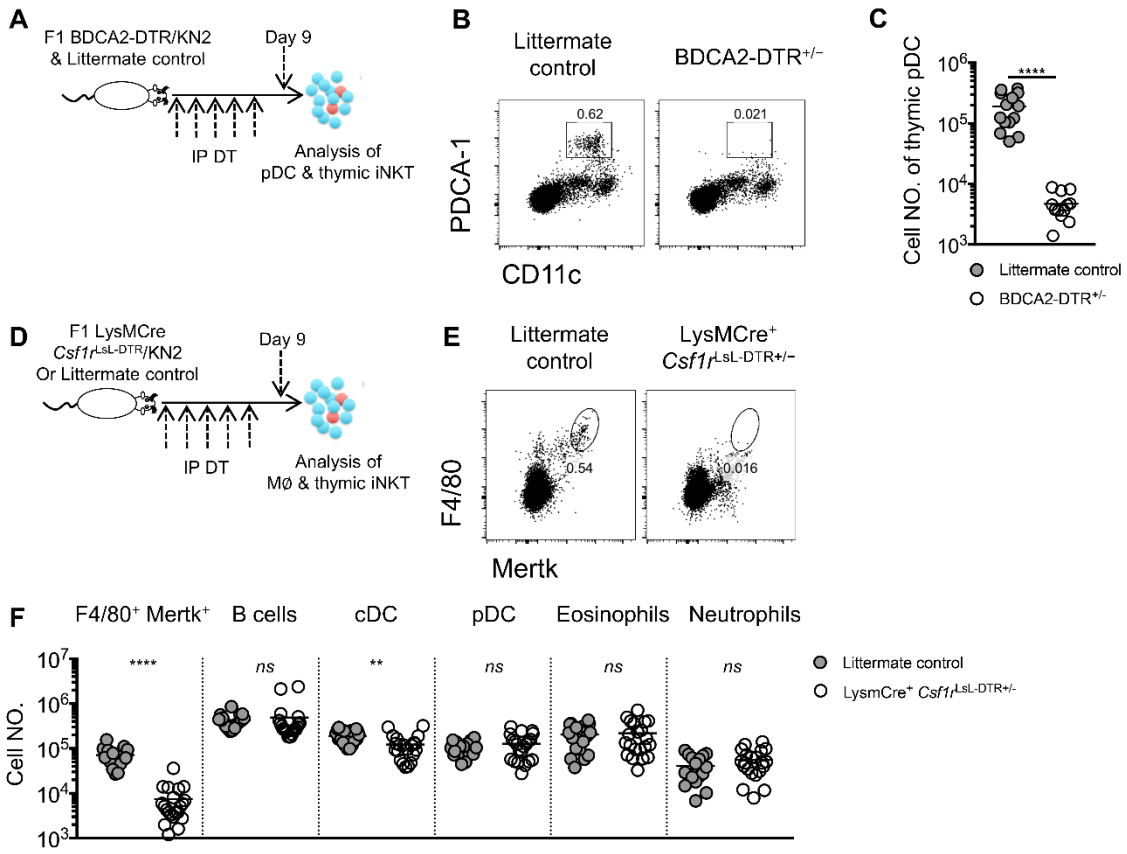
Suppl. Figure 5. B6 *Cd1d*$^{fl/fl}$ mouse backcrossed to BALB/c KN2 mouse.
(**A**) Expression of huCD2 and PLZF (upper row), T-bet and ROR-γt (middle row) in thymic iNKT cells, and expression of T-bet and PLZF in ROR-γt^{-} (lower row) thymic iNKT cells from B6 Wt, B6 KN2, BALB/c KN2 and BALB/c *Cd1d*$^{fl/fl}$-KN2 mice. (**B**) Frequency of huCD2^{+} NKT2 cells (far left panel 1, red) and cell number of huCD2^{+} NKT2 cells (panel 2, red), NKT1 cells (panel 3, green), PLZF^{high} cells (panel 4, pink) and NKT17 cells (panel 5, blue) in thymic iNKT cells from B6 KN2, BALB/c KN2 or BALB/c *Cd1d*$^{fl/fl}$-KN2 mice. Data are pooled from 4 (Frequency of huCD2^{+} NKT2 cells) or 3 (Cell number of huCD2^{+} NKT2, NKT1, PLZF^{high}, NKT17 cells) experiments. n=10 (B6 KN2), 10 and 5 (BALBc KN2), 12 (BALBc KN2-CD1d$^{fl/fl}$). (**C**) Cell number of double positive (DP) (far left), CD4 single positive (SP) (middle) and CD8 single positive (SP) (far right) thymocytes from B6 KN2, BALB/c KN2 or BALB/c *Cd1d*$^{fl/fl}$-KN2 mice. Data are pooled from 3 experiments. n=10 (B6 KN2), 5 (BALBc KN2), 12 (BALBc KN2-CD1d$^{fl/fl}$).



Suppl. Figure 6. Targeting of *Cd1d* with MB1Cre & CD11cCre, respectively.
(**A**) Representative flowcytometry histogram showing expression of CD1d in thymic B cells from F1 littermate control, F1 MB1Cre^{+} *Cd1d*$^{fl/fl}$ and BALB/c *Cd1d* KO mice. (**B**) Frequency of CD1d^{+} cells of thymic B cells from F1 littermate control or F1 MB1Cre^{+} *Cd1d*$^{fl/fl}$ mice. Data are pooled from 5 experiments. n=18 (Littermate control), 12 (MB1Cre^{+} *Cd1d*$^{fl/fl}$). Each dot represents an individual mouse and horizontal bars indicate mean values. Unpaired t test, ****p<0.0001. (**C**) Representative flowcytometry histogram showing expression of CD1d in thymic macrophages (F4/80^{+} Mertk^{+}, left), cDC (middle) and pDC (right) from F1 littermate control, F1 CD11cCre *Cd1d*$^{fl/fl}$ and BALB/c *Cd1d* KO mice. (**D**) Frequency of CD1d^{+} cells of thymic macrophages (F4/80^{+} Mertk^{+}, left), cDC (middle) and pDC (right) from F1 littermate control or F1 CD11cCre^{+} *Cd1d*$^{fl/fl}$ mice. Data are pooled from 7 experiments. n=17 (Littermate control), 14 (CD11cCre^{+} *Cd1d*$^{fl/fl}$). Each dot represents an individual mouse and horizontal bars indicate mean values. Unpaired t test, ****p<0.0001.

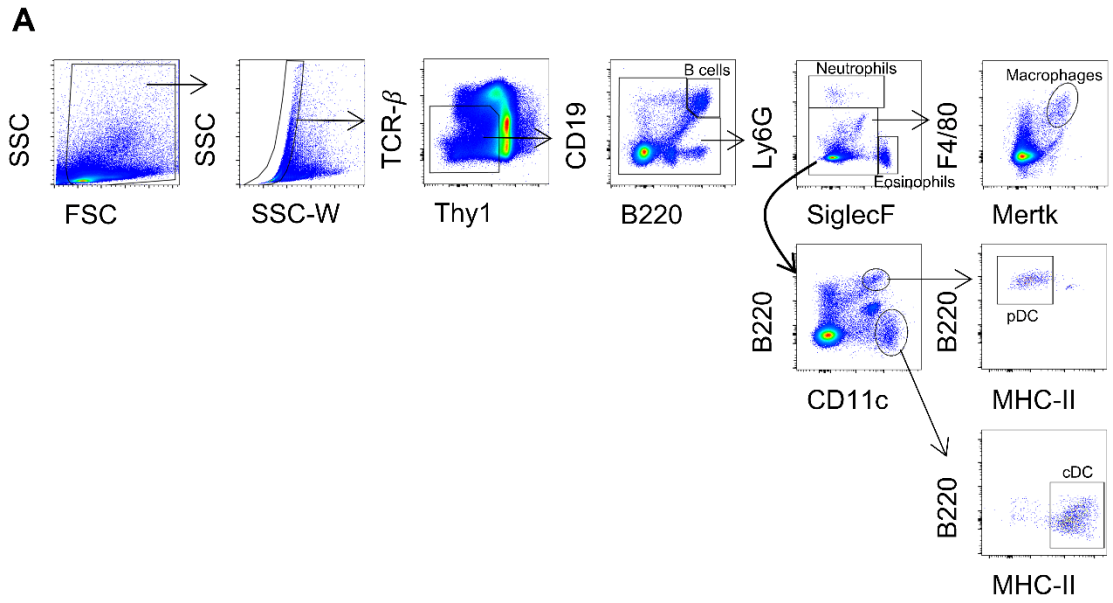


Suppl. Figure 7. Targeting of *Cd1d* with *Zbtb46*Cre.
(**A**) Representative flowcytometry histogram showing expression of CD1d in thymic cDC from F1 littermate control, F1 *Zbtb46*Cre^{+} *Cd1d*$^{fl/fl}$ and BALB/c *Cd1d* KO mice. (**B**) Frequency of CD1d^{+} cells of thymic cDC from F1 littermate control or F1 *Zbtb46*Cre^{+} *Cd1d*$^{fl/fl}$ mice. Data are pooled from 7 experiments. n=22 (Littermate control), 10 (*Zbtb46*Cre^{+} *Cd1d*$^{fl/fl}$). Each dot represents an individual mouse and horizontal bars indicate mean values. Unpaired t test, ****p<0.0001.

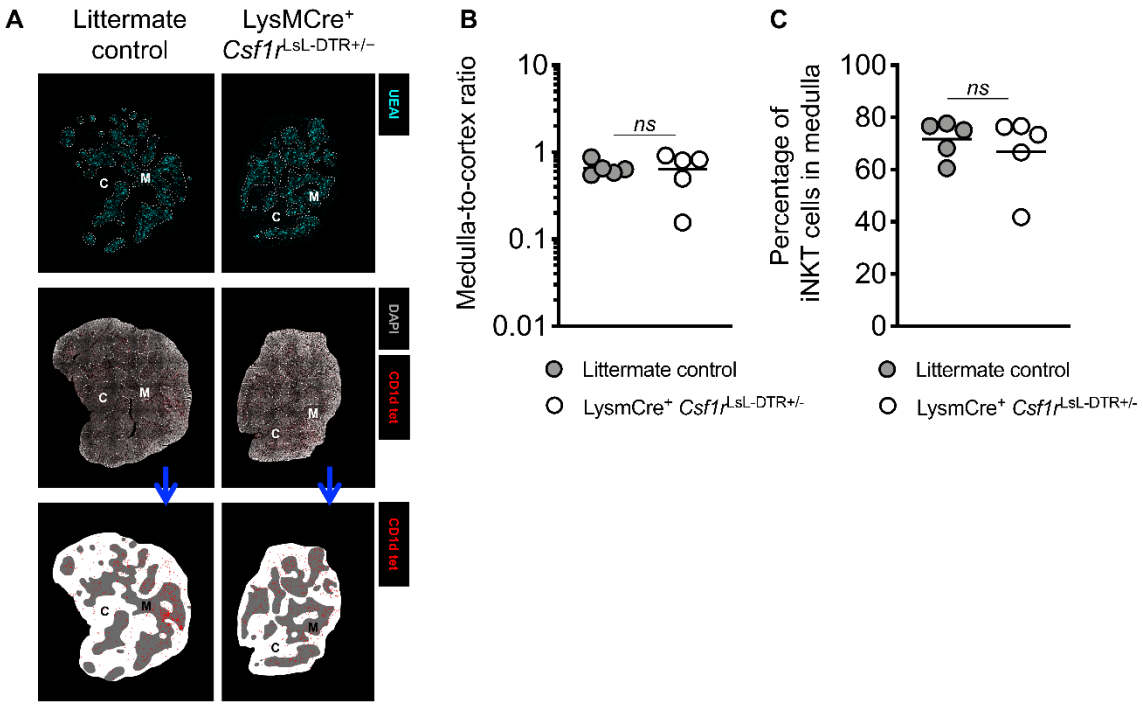


Suppl. Figure 8. Depletion of pDC and macrophages/phagocytes in respective transgenic mouse models.

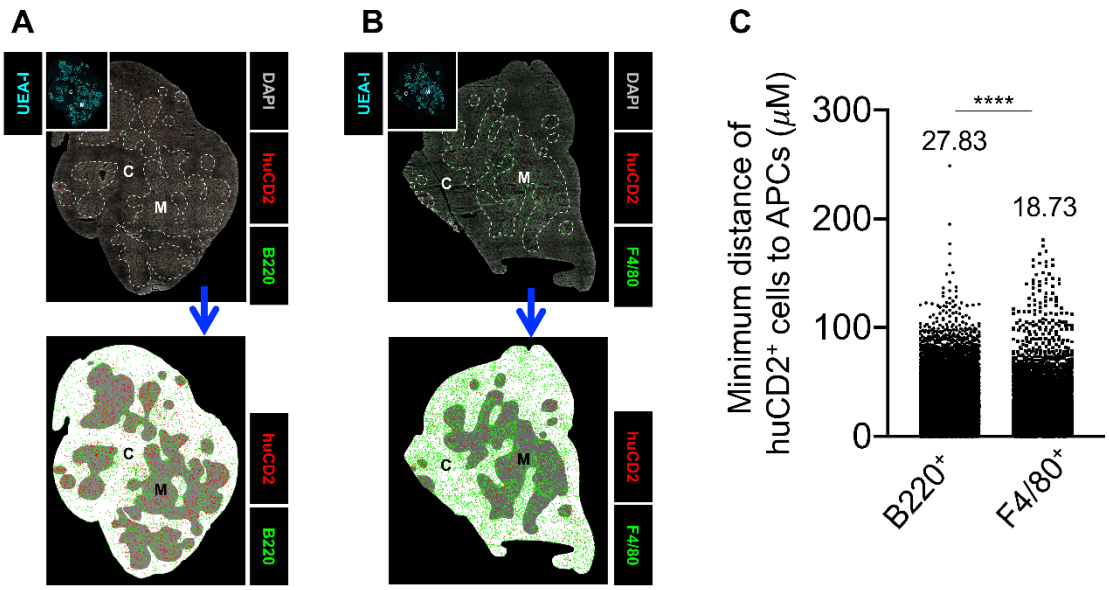
(**A**) Experimental scheme showing the F1 littermate control and F1 BDCA2-DTR KN2 mice were injected intraperitoneally (i.p.) with DT every other day for 5 times in a 9-day course, and the thymic iNKT cells and pDC were analyzed at day 9. (**B**) Expression of CD11c and PDCA-1 in thymic cells and gating of pDC ($CD11c^{intermediate}$ $PDCA\text{-}1^{+}$) from F1 littermate control and F1 $BDCA2\text{-}DTR^{+/-}$ KN2 mice after a 9-day course of DT treatment. (**C**) Cell number of thymic pDC in F1 littermate control and F1 $BDCA2\text{-}DTR^{+/-}$ KN2 mice after a 9-day course of DT treatment. Data are pooled from 3 experiments. n=14 (Littermate control), 12 ($BDCA2\text{-}DTR^{+/-}$). Each dot represents an individual mouse and horizontal bars indicate mean values. Unpaired t test, ****p<0.0001. (**D**) Experimental scheme showing the F1 littermate control and F1 BDCA2-DTR KN2 mice were injected intraperitoneally (i.p.) with DT every other day for 5 times in a 9-day course, and the thymic iNKT cells and myeloid cells were analyzed at day 9. (**E**) Expression of Mertk and F4/80 in thymic cells and gating of macrophages/phagocytes ($F4/80^{+}$ $Mertk^{+}$) from F1 littermate control and F1 $LysMCre^{+}$ $Csf1r^{LsL\text{-}DTR+/-}$ KN2 mice after a 9-day course of DT treatment. (**F**) Cell number of thymic $F4/80^{+}$ $Mertk^{+}$ macrophages/phagocytes, B cells, cDC, pDC, eosinophils and neutrophils in F1 littermate control and F1 $LysMCre^{+}$ $Csf1r^{LsL\text{-}DTR+/-}$ KN2 mice after a 9-day course of DT treatment. Data are pooled from 6 experiments. n=17 (Littermate control), 21 ($LysMCre^{+}$ $Csf1r^{LsL\text{-}DTR+/-}$). Each dot represents an individual mouse and horizontal bars indicate mean values. Unpaired t test, ****p<0.0001, **p=0.0087, ^{ns}p=0.6902 (B cells), 0.2220 (pDC), 0.7613 (Eosinophil), 0.1556 (Neutrophil).



Suppl. Figure 9. Gating strategy of myeloid cell populations in the thymus.
(**A**) Flowcytometric gating strategy to identify thymic B cells (CD19^{+} B220^{+}), neutrophils (Ly6G^{+} SiglecF^{-}), eosinophils (Ly6G^{-}, SiglecF^{+}), cDC (CD11c^{high}, MHC-II^{high}), pDC (CD11c^{intermediate}, B220^{+}, MHC-II^{low}) and macrophages/phagocytes (F4/80^{+} Mertk^{+}).



Suppl. Figure 10. Depletion of macrophages does not disrupt thymic medulla-to-cortex ratio as well as localization of iNKT cells.
(**A**) Immunofluorescent microcopy of thymic section of F1 littermate control and F1 LysMCre^{+} *Csf1r*$^{LsL\text{-}DTR+/-}$ mice after a 9-day course of DT treatment (upper row), stained with DAPI, UEA-I, CD1d tetramer, the white dash lines outline the UEA-I^{+} medullary region. The staining of UEA-I (top row) was used to distinguish cortex (UEA-I^{-}, marked as C) and medulla (UEA-I^{+}, marked as M), outlined by the white dash line. The staining of DAPI and CD1d tetramer was used to identify iNKT cells (middle row). Localization of iNKT cells (DAPI^{+} CD1d tetramer^{+}) analyzed using histocytometric algorithm (bottom row). (**B**) Medulla-to-cortex ratio of the thymus from F1 littermate control and F1 LysMCre^{+} *Csf1r*$^{LsL\text{-}DTR+/-}$ mice after a 9-day course of DT treatment. The medulla-to-cortex ratio was calculated as (UEA-I^{+} medulla area)/(total thymus arear - UEA-I^{+} medulla area). Data are from 1 experiment. n=5 (littermate control), 5 (LysMCre^{+} *Csf1r*$^{LsL\text{-}DTR+/-}$). Each dot represents an individual mouse and horizontal bars indicate mean values. Unpaired t test, ^{ns}p=0.9070. (**C**) Percentage of CD1d tetramer^{+} iNKT cells localized in thymic medulla from F1 littermate control and F1 LysMCre^{+} *Csf1r*$^{LsL\text{-}DTR+/-}$ mice after a 9-day course of DT treatment. Data are from 1 experiment. n=5 (littermate control), 5 (LysMCre^{+} *Csf1r*$^{LsL\text{-}DTR+/-}$). Each dot represents an individual mouse and horizontal bars indicate mean values. Unpaired t test, ^{ns}p=0.5370.



Suppl. Figure 11. Localization of thymic IL-4$^+$ cells together with APCs, B220$^+$ or F4/80$^+$ cells.

(**A**) Immunofluorescent microcopy of thymic section of BALB/c KN2 mice (upper row), stained with DAPI, UEA-I, huCD2 and B220, the white dash lines outline the UEA-I$^+$ medullary region. The staining of UEA-I (upper left) was used to distinguish cortex (UEA-I$^-$, marked as C) and medulla (UEA-I$^+$, marked as M), outlined by the white dash line. Localization of IL-4 producing cells (huCD2$^+$) together with B220$^+$ cells analyzed using histocytometric algorithm (lower row). (**B**) Immunofluorescent microcopy of thymic section of BALB/c KN2 mice (upper row), stained with DAPI, UEA-I, huCD2 and F4/80, the white dash lines outline the UEA-I$^+$ medullary region. The staining of UEA-I (upper left) was used to distinguish cortex (UEA-I$^-$, marked as C) and medulla (UEA-I$^+$, marked as M), outlined by the white dash line. Localization of IL-4 producing cells (huCD2$^+$) together with F4/80$^+$ cells analyzed using histocytometric algorithm (lower row). (**C**) Minimum distance of each huCD2$^+$ cells to the nearest APC, a B220$^+$ or F4/80$^+$ cell. The numbers above columns indicate the average. Each dot represents a huCD2$^+$ cell and horizontal bars indicate mean values. Unpaired t test, ****p<0.0001.

Suppl. Table 1. Cell type specificities of Cre and DTR transgenic mouse strains used in this study

Cell type specificities of Cre and DTR transgenic mouse strains used in this study		
Mouse strains	**Cell type specificity**	**The Jackson Laboratory Stock NO.**
FoxN1Cre	thymic epithelial cells (TEC) and keratinocytes	018448
MB1Cre	B cells	020505
CD11cCre	classical dendritic cells (cDC), plasmacytoid dendritic cells (pDC), Macrophages	008068
*Zbtb46*Cre	classical dendritic cells (cDC)	028538
BDCA2-DTR	plasmacytoid dendritic cells (pDC)	014176
LysMCre^{+} *Csf1r*$^{LSL\text{-}DTR+/-}$	macrophages, monocytes	LysMCre: 004781 *Csf1r*$^{LSL\text{-}DTR}$: 24046

Suppl. Table 2. Key recourses table

Key Resources Table				
Reagent type (species) or resource	**Designation**	**Source or reference**	**Identifiers**	**Additional information**
antibody	anti-CD4	BD Biosciences	Cat # 563331	(1:400)
antibody	anti-CD8α	BD Biosciences	Cat # 563786	(1:400)
antibody	anti-CD24	BioLegend	Cat # 101824	(1:200)
antibody	anti-CD44	TONBO Biosciences	Cat # 80-0441-U025	(1:200)
antibody	anti-human CD2	BioLegend	Cat # 309218	5μL/test
antibody	anti-TCRβ	BD Biosciences	Cat # 563221	(1:200)
antibody	anti-PD-1	BioLegend	Cat # 135213	(1:100)
antibody	anti-CD45.1	BioLegend	Cat # 110738	(1:200)
antibody	anti-CD45.2	eBioscience	Cat # 11-0454-81	(1:200)
antibody	anti-CD45	eBioscience	Cat # 25-0451-82	(1:200)
antibody	anti-B220	BioLegend	Cat # 103244	(1:200)
antibody	anti-CD11c	eBioscience	Cat # 47-0114-82	(1:200)
antibody	anti-CD11b	eBioscience	Cat # 47-0112-82	(1:200)
antibody	anti-F4/80	eBioscience	Cat # 47-4801-82	(1:200)
antibody	anti-CD122	BD Biosciences	Cat # 562960	(1:100)
antibody	anti-PLZF	BD Biosciences	Cat # 563490	(1:200)
antibody	anti-ROR-γt	BD Biosciences	Cat # 562684	(1:200)
antibody	anti-T-bet	BioLegend	Cat # 644824	(1:200)
antibody	anti-Mertk	BioLegend	Cat # 151504	(1:200)
antibody	anti-CD1d	eBioscience & BD Biosciences	Cat # 12-0011-81 & 553846	(1:200)
antibody	anti-CD19	TONBO Biosciences	Cat # 60-0193-U100	(1:200)
antibody	anti-PDCA-1	BioLegend	Cat # 127106	(1:200)
antibody	anti-Thy1.2	eBiosiences	Cat # 47-0902-82	(1:400)
antibody	anti-SiglecF	BD Biosciences	Cat # 740280	(1:200)
antibody	anti-Ly6G	BioLegend	Cat # 127610	(1:200)
antibody	anti-MHC-II	BioLegend	Cat # 107653	(1:1000)
antibody	anti-EpCAM	BioLegend	Cat # 118214	(1:200)
antibody	Goat-anti-R Phycoerythrin (PE)	Abcam	Cat # ab34721	(1:200)
antibody	whole rabbit sera anti-R Phycoerythin (PE)	Novus Biologicals	Cat # NB120-7011	(1:200)
antibody	Donkey-anti-Rabbit AF555 & Goat-anti-Rabbit AF555	Invitrogen	Cat # A31572 & A21429	(1:400)
antibody	Donkey-anti-Goat AF555	Invitrogen	Cat # A21432	(1:400)
Other	Ulex Europaeus Agglutinin I (UEA I)	VECTOR LOBORATORY	FL-1061	(1:200)
Other	Strepavidin PE	Invitrogen	Cat # S21388	
commercial assay or kit	Viability dye Ghost Dye™ Red 780	TONBO Biosciences	Cat # 13-0865-T100	(1:500)
commercial assay or kit	Magnetic columns	Miltenyi	Cat # 130-042-401	
commercial assay or kit	anti-PE microbeads	Miltenyi	Cat # 130-048-801	
commercial assay or kit	tyramide amplification kit	PerkinElmer	Cat # NEL745001KT	

chemical compound, drug	Collagenase D	Roche	Cat # 11088882001	1mg/mL
chemical compound, drug	Mouse serum	Jackson ImmunoResearch	Cat # 015-000-120	5µL/test
chemical compound, drug	DNase I	Roche	Cat # 10104159001	100 U/ml
chemical compound, drug	Liberase TH	Roche	Cat # 5401127001	0.05% [w/v]
chemical compound, drug	Diptherial toxin	Sigma-Aldrich	Cat # D0564	Diluted in PBS
software, algorithm	Prism 7	GraphPad	https://www.graphpad.com/	
software, algorithm	ImageJ	ImageJ	https://imagej.nih.gov/ij/	
software, algorithm	FlowJo v10	TreeStar - Flowjo	https://www.flowjo.com/solutions/flowjo	

Reference

1. Lee YJ, Holzapfel KL, Zhu J, Jameson SC, & Hogquist KA (2013) Steady-state production of IL-4 modulates immunity in mouse strains and is determined by lineage diversity of iNKT cells. *Nature immunology* 14(11):1146-1154.
2. Wang H*, et al.* (2018) Ultrasound Guided Intra-thymic Injection to Track Recent Thymic Emigrants and Investigate T Cell Development. *Bio-protocol* 8(23):e3107.
3. Xing Y & Hogquist KA (2014) Isolation, identification, and purification of murine thymic epithelial cells. *Journal of visualized experiments : JoVE* (90):e51780.
4. Gerner MY, Kastenmuller W, Ifrim I, Kabat J, & Germain RN (2012) Histo-cytometry: a method for highly multiplex quantitative tissue imaging analysis applied to dendritic cell subset microanatomy in lymph nodes. *Immunity* 37(2):364-376.
5. Lee YJ*, et al.* (2015) Tissue-Specific Distribution of iNKT Cells Impacts Their Cytokine Response. *Immunity* 43(3):566-578.