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Depletion of Neural Crest–Derived Cells Leads to Plasma Noradrenaline Decrease and Alters T Cell Development

Naoki Tsunokuma,^{*,1} Doris Narki Tetteh,^{*,1} Kana Isono,^{*} Mari Kuniishi-Hikosaka,^{*,†} Motokazu Tsuneto,^{*,‡} Kenichiro Ishii,[§] and Hidetoshi Yamazaki^{*}

The differentiation of neural crest (NC) cells into various cell lineages contributes to the formation of many organs, including the thymus. In this study, we explored the role of NC cells in thymic T cell development. In double-transgenic mice expressing NC-specific Cre and the Cre-driven diphtheria toxin receptor, plasma noradrenaline and adrenaline levels were significantly reduced, as were thymic T cell progenitors, when NC-derived cells were ablated with short-term administration of diphtheria toxin. Additionally, yellow fluorescent protein⁺ NC-derived mesenchymal cells, perivascular cells, and tyrosine hydroxylase⁺ sympathetic nerves in the thymus significantly decreased. Furthermore, i.p. administration of 6-hydroxydopamine, a known neurotoxin for noradrenergic neurons, resulted in a significant decrease in thymic tyrosine hydroxylase⁺ nerves, a phenotype similar to that of depleted NC-derived cells, whereas administration of a noradrenaline precursor for ablating NC-derived cells or sympathetic nerves rarely rescued this phenotype. To clarify the role of NC-derived cells in the adult thymus, we transplanted thymus into the renal capsules of wild-type mice and observed abnormal T cell development in lethally irradiated thymus with ablation of NC-derived cells or sympathetic nerves, suggesting that NC-derived cells inside and outside of the thymus contribute to T cell development. In particular, the ablation of NC-derived mesenchymal cells in the thymus decreases the number of thymocytes and T cell progenitors. Overall, ablation of NC-derived cells, including sympathetic nerves, in the thymus leads to abnormal T cell development in part by lowering plasma noradrenalin levels. This study reveals that NC-derived cells including mesenchymal cells and sympathetic nerves within thymus regulate T cell development. *The Journal of Immunology*, 2023, 211: 1494–1505.

Neural crest (NC) cells emerge from the neural tube ridge during an early embryonic stage, migrate to the periphery, and contribute to the formation of many organs, including the adrenal gland, thymus, craniofacial region, and heart (1–4). These cells can also differentiate into several cell lineages, including sympathetic and enteric neurons, as well as adrenal medullary, mesenchymal, and perivascular cells (1, 5, 6). During the differentiation of NC cells, *Wnt1* and *P0* are expressed in early migratory NC cells; hence, *P0-Cre*, *Wnt1-Cre*, and *Rosa^{YFP/YFP}* (YFP, yellow fluorescent protein) mice are commonly used to trace NC-derived cells (7–12).

The thymus comprises thymocytes and stromal cells, including epithelial and mesenchymal cells (13, 14). NC cells contribute to the formation of thymic mesenchymal and perivascular cells and sympathetic nerves. Immature T cells differentiate into mature T cells in the thymus through instructive signals from the thymic epithelia and mesenchyme (15). Notably, mesenchymal cells, including NC-derived mesenchymal cells, can participate in embryonic thymic and T cell development (1, 16–21), and surgical deletion of a part of the neural fold that induces NC cells results in immunodeficiency due to hypothalamus or athymus (i.e., DiGeorge syndrome) (22). Although NC-derived cells regulate B cell development in the adult

bone marrow (BM) via various mechanisms, including the expression of hematopoietic factors and contribution to sympathetic innervation (23), their role in T cell development in the adult thymus is not well known.

The adrenal medulla synthesizes catecholamines that include dopamine, noradrenaline, and adrenaline (24). Mouse sympathetic neuronal function was assessed using plasma catecholamine measurements (25–27), and dopamine β -hydroxylase loss was found to be associated with neurologic and immunological disorders and was reversed by administering *L-threo*-3,4-dihydroxyphenylserine (L-DOPS), which converts noradrenaline into adrenaline by *L*-aromatic amino acid decarboxylase (27–30). The adrenal gland releases both adrenaline and noradrenaline into circulation; sympathetic neurons' synaptic vesicles also release noradrenaline into the synaptic cleft (24, 31), where it regulates blood vessel contraction (32). Notably, disrupting sympathetic neurons via 6-hydroxydopamine (6-OHDA) administration induces T cell apoptosis (33), but the effect of 6-OHDA on T cell development remains unknown. While sympathetic neurons regulate the migration of hematopoietic cells between the BM and peripheral blood (34), they release noradrenaline, which acts on adrenaline receptor β (ADRB β)–expressing stromal cells in the BM (34–38) that contribute to the migration of hematopoietic stem

^{*}Department of Stem Cell and Developmental Biology, Mie University Graduate School of Medicine, Tsu, Japan; [†]Laboratory of Molecular Cell Biology, Graduate School of Medicine and Pharmacological Science, University of Toyama, Toyama, Japan; [‡]Division of Regenerative Medicine and Therapeutics, Department of Genetic Medicine and Regenerative Therapeutics, Tottori University, Yonago, Japan; and [§]Department of Nursing, Nagoya University of Arts and Sciences, Nagoya, Japan

¹These authors contributed equally to this work.

ORCIDs: 0000-0003-1043-864X (D.N.T.); 0000-0001-5533-4460 (M.K.-H.); 0000-0001-9006-9148 (K.I.).

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Address correspondence and reprint requests to Dr. Hidetoshi Yamazaki, Department of Stem Cell and Developmental Biology, Mie University Graduate School of Medicine, Tsu, Mie 514-8507, Japan. E-mail address: yamazaki@med.mie-u.ac.jp

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Abbreviations used in this article: ADR β , adrenergic receptor β ; BM, bone marrow; DN, double-negative; DP, double-positive; DT, diphtheria toxin; DTR, DT receptor; ETP, early T cell progenitor; L-DOPS, *L-threo*-3,4-dihydroxyphenylserine; Lin[−], lineage-negative; NC, neural crest; 6-OHDA, 6-hydroxydopamine; PI, propidium iodide; α -SMA, α -smooth muscle actin; TEC, thymic epithelial cell; TH, tyrosine hydroxylase; YFP, yellow fluorescent protein.

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cells by controlling Cxcl12 expression. In addition, reduced noradrenaline affects B cell development (23), but its effect on T cell development remains unclear.

Noradrenergic nerves innervate numerous lymphoid tissues, including BM and thymus (39–41). In the thymus, noradrenergic nerves innervate the corticomedullary junction and connect thymic epithelial cells (TECs) (31, 42, 43). Although NC-derived perivascular cells have been shown to promote the egress of mature thymocytes at the thymic corticomedullary junction (2, 38, 44), whether noradrenergic nerves directly regulate perivascular cells remains unclear.

Considering the contribution of NC-derived cells to the formation of the thymus and adrenal gland, as well as the innervation of thymic noradrenergic nerves, we hypothesized that the depletion of NC-derived cells has an effect on noradrenergic nerves, plasma noradrenaline concentrations, and T cell development. We used the well-established mouse model expressing NC-specific Cre (*Wnt1-Cre*) and Cre-driven diphtheria toxin (DT) receptor (DTR) with YFP reporter to deplete NC-derived cells and 6-OHDA that specifically depletes noradrenergic neurons. As a result, ablation of intrathymic NC-derived cells or noradrenergic nerves induced a significant decrease in thymic T cell progenitors (12, 23, 28, 41, 44, 45).

Materials and Methods

Mice

Wnt1-Cre mice and C57BL/6-*Gt(Rosa)26Sor^{tm1(EYFP)Cos/J}* (*Rosa^{YFP/YFP}*) mice were provided by Drs. H. Sucov (Southern California University) and H. Enomoto (RIKEN, Kobe, Japan), respectively. C57BL/6-*Gt(Rosa)26Sor^{tm1(HBEGF)Awai/J}* (*Rosa^{ΔDTR/DTR}*) and C57BL/6J mice were obtained from The Jackson Laboratory and CLEA Japan, respectively. *Wnt1-Cre* mice were crossed with *Rosa^{YFP/YFP}* and *Rosa^{ΔDTR/DTR}* mice. All mice were kept at the Institute of Laboratory Animals, Mie University; all experimental procedures were approved by the Institutional Animal Care and Use Committee of Mie University (approval no. 24-48) and were performed according to the Mie University guidelines for laboratory animals.

Adrenalectomy

Bilateral adrenal glands were surgically removed from anesthetized 8- to 10-wk-old C57BL/6 mice. Sham surgery was performed as a control using mice of the same age. Three days after surgery, mice were sacrificed and their thymi were isolated and analyzed by flow cytometry (46).

Administration of DT, 6-OHDA, and L-DOPS

In this study, 50 μg/kg DT (Sigma-Aldrich) was i.p. injected into 8- to 10-wk-old *Wnt1-Cre/+;Rosa^{YFP/YFP}*, *Wnt1-Cre/+;Rosa^{YFP/DTR}*, and *Rosa^{YFP/DTR}* mice every 24 h for 3 d (23, 47). In addition, 250 and 120 mg/kg 6-OHDA (Sigma-Aldrich) with 0.01% ascorbic acid (Sigma-Aldrich) were i.p. injected into 8-wk-old C57BL/6 mice and *Wnt1-Cre/+;Rosa^{YFP/YFP}* mice at days 0 and 2, respectively (23). DT-treated mice received i.p. administration of 50, 100, 200, and 1000 mg/kg L-DOPS (Tokyo Chemical Industry) 3–6 h after DT injections (23). 6-OHDA-treated mice received i.p. administration of 50 mg/kg L-DOPS (Tokyo Chemical Industry). PBS, dH₂O, and 0.01% amino acid/dH₂O were injected as vehicles for DT, L-DOPS, and 6-OHDA, respectively. Each mouse was sacrificed 18 h after the final injection, and thymocytes were isolated for analysis by flow cytometry.

Flow cytometric analysis and cell sorting

Thymocytes were isolated from the thymi. For stromal cells, thymi were minced and incubated with 0.1 mg/ml collagenase D (Roche), 2.4 U/ml Dispase (Life Technologies), and 0.1 mg/ml DNase I (Wako) in 10% FBS (Life Technologies)/HBSS (Nissui) for 2 h at 37°C. Single-cell suspensions were blocked using rat IgG, and cells were stained with the following Abs. Allophycocyanin-Cy7-conjugated rat anti-mouse Epcam1 (G8.8; BioLegend); allophycocyanin-conjugated CD140a (APA5; BioLegend), CD140b (APB5; BioLegend), CD117 (c-Kit, 2B8; BioLegend), and CD31 (390; BioLegend); PE-Cy7-conjugated CD31 (390; BioLegend), CD45 (30-F11; BioLegend), and Ter119 (BioLegend); and Pacific Blue-conjugated I-A/I-E (M5/114.15.2; BioLegend). PE-conjugated rat anti-mouse CD4 (GK1.5; BioLegend), CD8a (53-6.7; BioLegend), B220 (RA3-6B2; BioLegend), CD11b (Mac-1; M1/70; BioLegend), Ly-6G/C (Gr-1, RB6-8C5; BioLegend), and Ter119 (BioLegend) Abs were used as lineage markers, and allophycocyanin-Cy7-conjugated

mouse anti-mouse CD45.2 (104; BioLegend) or FITC-conjugated mouse CD45.1 (A20; BioLegend) was used to detect host- and donor-derived hematopoietic cells. Rat IgG (Sigma-Aldrich) was used for blocking nonspecific Ab binding, and propidium iodide (PI; Dojindo) was used for the exclusion of PI⁺ dead cells. Pacific Blue-conjugated annexin V (BioLegend) was used to detect early apoptotic cells. FACSCanto II and FACSARIA (BD Biosciences) were used as the cell analyzer and cell sorter; all data were analyzed with FlowJo software (Tree Star).

Histochemistry and immunohistochemistry

The thymus was fixed in 4% paraformaldehyde and embedded in paraffin (Wako), and sections were stained with H&E (Leica). For immunohistochemistry, fixed samples were embedded in super cryoembedding medium (Section-Lab), and frozen sections were prepared with a cryostat (CM3050S; Leica) using Kawamoto's film methods (48). Sections were blocked using 1% BSA (Sigma-Aldrich) and stained with the following Abs. Purified rat anti-mouse pan-endothelial cell Ag (MECA32; BioLegend), DyLight 649-conjugated donkey anti-rat IgG (Invitrogen), Cy3-conjugated mouse monoclonal anti-α-smooth muscle actin (α-SMA, 1A4; Sigma-Aldrich), rabbit anti-mouse purified anti-tyrosine hydroxylase (TH) (AB152; EMD Millipore), and Alexa Fluor 647-donkey anti-rabbit IgG (Life Technologies) were used. Rabbit IgG (Invitrogen) and rat IgG (BioLegend) were used as isotype controls. DAPI (Sigma-Aldrich) was used to detect nuclear. Images were captured using microscopy (BX-X700; Keyence). For estimation and counting, the number of images on each section was analyzed using ImageJ.

Measurement of catecholamines

Whole blood samples were drawn from mice, and plasma was collected to measure plasma adrenaline, noradrenaline, and dopamine using HPLC (SRL 23).

Adult thymic organ culture with DT

Thymi from lethally irradiated 8- to 10-wk-old *Wnt1-Cre/+;Rosa^{YFP/YFP}*, *Wnt1-Cre/+;Rosa^{YFP/DTR}*, and *Rosa^{YFP/DTR}* mice were incubated in the presence of 100 ng/ml DT (Sigma-Aldrich) with RPMI 1640 medium containing 10% FCS, 1× nonessential amino acid (Life Technologies), 1 mM pyruvic acid (Roche), 2 mM L-glutamic acid (Life Technologies), and 10 mM HEPES (pH 8.0), 10⁻⁷ M 2-ME (Wako) at 37°C for 4 d (49). Before culture and 4 d after culture, YFP⁺ cell reduction was checked under a fluorescence microscope. Flow cytometry was used to analyze YFP⁺ cell reduction 4 d after culture.

Thymus transplantation under renal capsule

Adult thymi isolated from lethally irradiated (4.75 Gy, twice; interval 3 h) 8- to 10-wk-old *Wnt1-Cre/Rosa^{YFP/DTR}*, *Rosa^{YFP/YFP}*, and C57BL/6 mice (CD45.2) were transplanted under renal capsules in the anesthetized C57BL/6 (CD45.1) mice (50). Before transplantation, irradiated thymi were cultured, as described above. Thymi were isolated from renal capsules 12 or 21 d after transplantation and were analyzed. To investigate the role of sympathetic nerves outside the thymus, 6-OHDA was administered to C57BL/6 mice (CD45.2) prior to thymus transplantation, and 3 d later, normal thymi from C57BL/6 mice (CD45.1) were transplanted. Thymi were then isolated and analyzed 5 d after transplantation.

Genotyping

Genomic DNA samples were prepared from tail tips, and PCR was performed with rTaq polymerase (Toyobo). Primers used for PCR were as follows: Cre, forward, 5'-GGACATGTTTCAGGGATCGCCAGGCG-3', reverse, 5'-GCAT AACCAGTGAAACAGCATTGCTG-3'; Rosa, common forward, 5'-AAAG TCGCTCTGAGTTGTTAT-3', wild-type reverse, 5'-GGAGCGGGAGAAA TGGATATG-3', mutant reverse, 5'-AAGACCGCGAAGAGTTTGTGTC-3'; Dtr, forward, 5'-TAGGAGAGGTGGTTATGATGTGGAA-3', reverse, 5'-GGAG CCGGAATGGATATG-3'.

RT-PCR

Total RNA was prepared with TRIzol reagent (Invitrogen), and cDNA synthesis was performed with reverse transcriptase (ReverTraAce; Toyobo) using oligo(dT) primers (Toyobo). RT-PCR was performed with gene-specific primers. The primers used for RT-PCR are listed in Supplemental Fig. 1.

Statistical analysis

Data are shown as means ± SD. The statistical significance of the differences between two groups was assessed using a two-tailed Student *t* test (**p* < 0.05 and ***p* < 0.01), and differences between three groups were assessed using a Tukey test (**p* < 0.05 and ***p* < 0.01). Bar graphs were generated

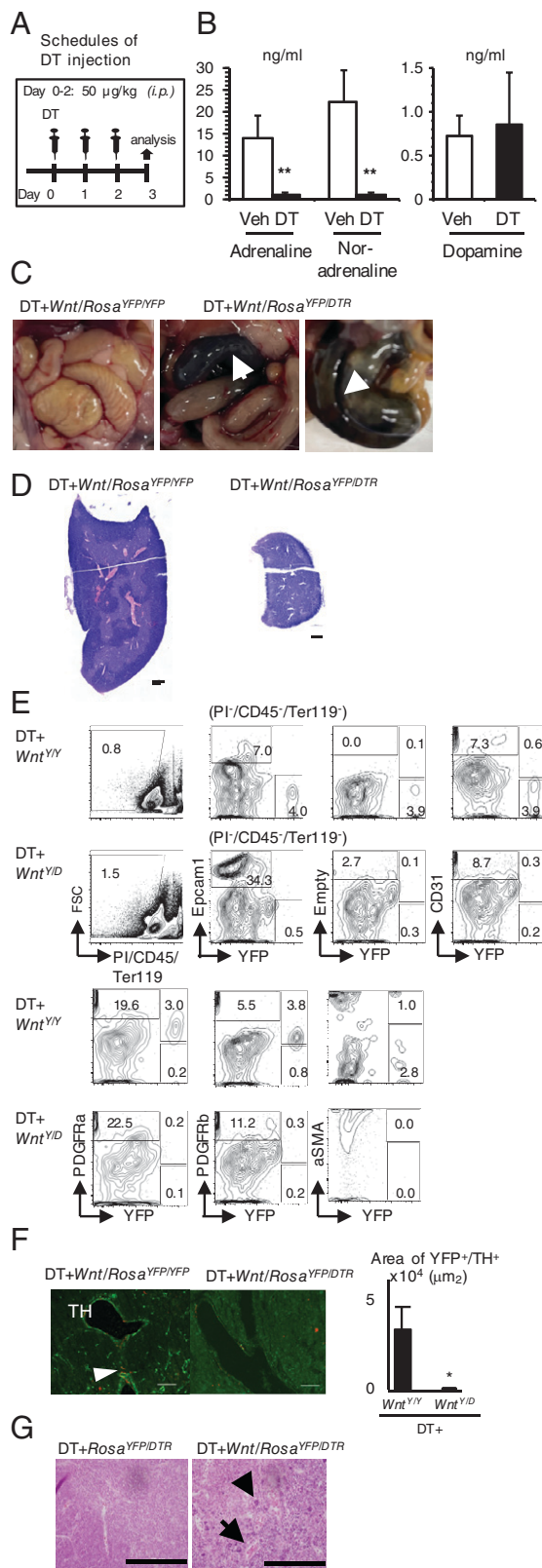


FIGURE 1. Significant reduction in plasma noradrenaline and adrenaline and proportion of NC-derived mesenchymal cells and the area of YFP⁺/TH⁺ cells from *Wnt1-Cre/+;Rosa^{YFP/DTR}* mice administered DT. **(A)** Schedule of DT injections. **(B)** Plasma adrenaline, noradrenaline, and dopamine concentrations in PBS (vehicle [Veh]) or DT-treated *Wnt/Rosa^{YFP/DTR}* mice by HPLC. **(C)** Photograph of the colon of DT-administered *Wnt/Rosa^{YFP/YFP}* and *Wnt/Rosa^{YFP/DTR}* (DT+*Wnt/Rosa^{YFP/DTR}*) mice. White arrow and arrowhead point to colon disease. Scale bar, 300 µm. **(D)** H&E-stained sections of thymus from DT+*Wnt/Rosa^{YFP/YFP}* mice or DT+*Wnt/Rosa^{YFP/DTR}* mice.

with DeltaGraph (Poladigital), and dot plots were generated with GraphPad Prism 6 (MDF).

Results

Reduced plasma noradrenaline and adrenaline levels in DT-administered *Wnt1-Cre/Rosa^{YFP/DTR}* mice

Owing to NC cell contribution to the formation of the adrenal medulla, which synthesizes noradrenaline and adrenaline, we hypothesized that NC cell depletion would affect the adrenal medulla and reduce plasma noradrenaline concentration, probably resulting in alterations in thymic T cell development. Interestingly, the ablation of NC-derived cells in *Wnt1-Cre/Rosa^{YFP/DTR}* mice via DT injection (hereafter abbreviated DT+*Wnt/Rosa^{YFP/DTR}*) (Fig. 1A) markedly reduced the plasma concentrations of noradrenaline and adrenaline (Fig. 1B). NC-derived cell depletion also induced a severe colon-like disease (Fig. 1C) comparable to Hirschsprung disease, corroborating previous studies that observed a similar phenotype following NC-derived cell depletion (51).

DT administration reduced the proportion of NC-derived YFP⁺ cells, PDGFRα⁺, PDGFRβ⁺, α-SMA⁺ perivascular cells, and the area of YFP⁺/TH⁺ sympathetic nerves in the thymus

Notably, the thymus size was strikingly reduced following DT administration (Fig. 1D). To ascertain that NC cells contribute to the formation of mesenchymal cells, perivascular cells, and sympathetic nerves in the thymus, we performed flow cytometry analysis and found that NC-derived YFP⁺ cells did not express Epcam1 (epithelial marker) (Fig. 1E), nor did they express CD45, CD31, or I-A/I-E (MHC class II); 4–8% of CD45⁺/Ter119⁺ cells were found to be NC-derived YFP⁺ cells, with most of these cells expressing PDGFRα and PDGFRβ (Fig. 1E, Supplemental Fig. 1A). Interestingly, these NC-derived YFP⁺ cells expressed hematopoietic factors *Cxcl12*, *Scf*, and *Dll1* more robustly than CD45⁺/Ter119⁺/CD31⁺ cells or CD45⁺/Ter119⁺/CD31⁺/MHC and Ii⁺ cells (TECs) (Supplemental Fig. 1B). Thus, YFP⁺ NC-derived thymic mesenchymal cells expressed hematopoietic factors similar to those of the thymic epithelium, suggesting their possible roles in T cell development.

Next, we investigated the effect of DT injection on these cells and found that the proportion of NC-derived YFP⁺ cells, YFP⁺/PDGFRα⁺ or PDGFRβ⁺ mesenchymal cells, and YFP⁺/α-SMA⁺ perivascular cells decreased significantly after DT administration by flow cytometry (Fig. 1E). In addition, we measured the areas of the YFP⁺/TH⁺ cells in DT+*Wnt/Rosa^{YFP/DTR}* and DT+*Wnt/Rosa^{YFP/YFP}* thymi. The areas of YFP⁺/TH⁺ cells in the DT+*Wnt/Rosa^{YFP/DTR}* thymi were reduced significantly (Fig. 1F).

(E) Flow cytometric analysis of the proportion of YFP⁺ cells, YFP⁺/Epcam1⁺ cells, YFP⁺/CD31⁺ cells, YFP⁺/PDGFRα⁺ cells, YFP⁺/PDGFRβ⁺ cells, and YFP⁺/α-SMA⁺ cells in the CD45⁺/Ter119⁺/PI⁺ fraction of thymi from DT-treated *Wnt/Rosa^{YFP/YFP}* (DT+*Wnt/Rosa^{YFP/Y}*) mice or DT-treated *Wnt/Rosa^{YFP/DTR}* (DT+*Wnt/Rosa^{YFP/D}*) mice. **(F)** Immunohistochemistry analysis of stained sections of thymus from DT+*Wnt/Rosa^{YFP/YFP}* mice or DT+*Wnt/Rosa^{YFP/DTR}* mice with Ab against TH thymi. The positive cell areas of YFP⁺/TH⁺ cells of thymus from each group are shown. Scale bar, 200 µm. White arrowhead points to positive cells stained with Ab (red) and YFP (green). All error bars represent the SD of the means. **p* < 0.05, ***p* < 0.01.

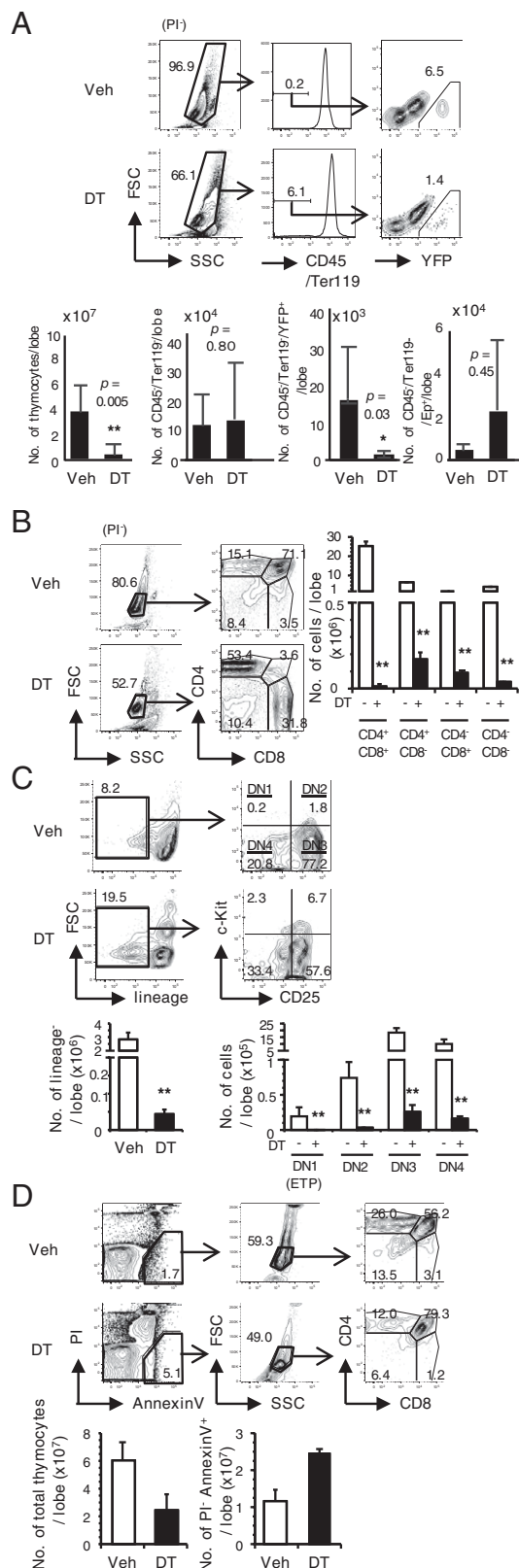


FIGURE 2. T cells and T cell progenitors were significantly reduced in the thymi of DT-administered *Wnt1-Cre/+; Rosa^{YFP/DTR}* mice. **(A)** Flow cytometric analysis of the percentages and numbers of thymocytes and CD45⁺/Ter119⁺, CD45⁺/Ter119⁺/YFP⁺, or CD45⁺/Ter119⁺/Epcam1⁺ cells in the thymi from *Wnt/Rosa^{YFP/DTR}* mice treated with either vehicle (Veh) or DT. **(B)** Flow cytometric analysis of the percentages and numbers of CD4⁺/CD8⁺ (DP) T cells, CD4⁺/CD8⁺ or CD4⁺/CD8⁺ (single-positive [SP]) T cells, and CD4⁺/CD8⁺ (DN) T cells in the thymus from each group. **(C)** Flow cytometric analysis of the percentages and numbers of lineage-negative

Considering the aforementioned findings, DT treatment successfully eliminated NC-derived YFP⁺ thymic cells. In addition, blood vessel dilatation was found at the corticomedullary junction in DT-treated mice (Fig. 1G) due to the absence of NC-derived cells around the perivascular cells, resulting in blood vessel dilation and probably abnormal blood vessel functioning.

NC-derived cell depletion decreased the number of thymic T cell progenitors

Informed by the results above, we examined the effect of eliminating NC-derived cells on T cell development. Despite the significantly reduced number of thymocytes, NC-derived cell depletion had no effect on the number of CD45⁺/Ter119⁺ or CD45⁺/Ter119⁺/Epcam1⁺ cells in the thymus (Fig. 2A), but it reduced the number of CD4⁺/CD8⁺ T, CD4⁺/CD8⁺ or CD4⁺/CD8⁺ T, and CD4⁺/CD8⁺ (double-negative [DN]) T cells in DT+*Wnt/Rosa^{YFP/DTR}* thymi (Fig. 2B). To study the effect of eliminating NC-derived cells on early T cell progenitors (ETPs), we fractionated CD4⁺/CD8⁺ (DN) T cells using CD25 and c-Kit Abs. Because the focus of this study was on ETPs in the DN1 fraction, we used c-Kit and CD25 instead of CD44 and CD25 (52–56). The numbers of T cell progenitors in DN1-like ETPs and DN2–4 fractions and lineage-negative (Lin⁺) cells were significantly reduced in DT-*Wnt/Rosa^{YFP/DTR}* thymi (Fig. 2C). These results indicate that NC-derived cell ablation reduces the number of thymic NC-derived cells while altering thymic T cell development.

To investigate whether DT treatment directly induced thymic apoptosis, the proportion of PI/annexin⁺ cells representing early apoptosis was measured in the thymi of DT+*Wnt/Rosa^{YFP/DTR}* mice. The number of thymocytes decreased 36 h after DT administration, but the proportion and numbers of PI/annexin⁺ cells in the thymi of DT+*Wnt/Rosa^{YFP/DTR}* mice increased (Fig. 2D). Consequently, 80% of the PI/annexin⁺ cells were possibly the CD4⁺/CD8⁺ T cell fraction (Fig. 2D). These findings suggest that an increase in early apoptosis induces significant thymocyte loss.

Adrenalectomized mice did not show abnormalities in T cell development

As demonstrated in Figs. 1 and 2, NC-derived cell depletion affected thymic T cell development. However, whether these T cell development alterations were due to the depletion of NC-derived cells inside (intrathymic) or outside (extrathymic) the thymus, as well as whether the observed decrease in adrenaline and noradrenaline (Fig. 1B) following DT administration affected the number of thymic T cells, was unclear. To better understand the effect of noradrenaline and adrenaline decrease on T cell development, we performed an adrenalectomy and assessed its effect on thymocytes. A decrease in plasma adrenaline alone occurred 3 d after adrenalectomy, as expected (Fig. 3A). However, the numbers of thymocytes, CD4⁺/CD8⁺ T cells, and CD4⁺/CD8⁺ T cells between adrenalectomized and sham-operated mice did not differ significantly (46) (Fig. 3B–D). These findings indicate that reduced plasma adrenaline did not induce the T cell development alterations observed in Fig. 1B.

Effect of 6-OHDA administration on T cell development

Informed by the preceding data, we examined the effects of noradrenergic neuron ablation on T cell development via 6-OHDA injection, a

(Lin⁺) cell and T cell progenitors from each group, DN1-like ETPs (Lin⁺/c-Kit⁺/CD25⁺), DN2 (Lin⁺/c-Kit⁺/CD25⁺), DN3 (Lin⁺/c-Kit⁺/CD25⁺), and DN4 (Lin⁺/c-Kit⁺/CD25⁺). **(D)** Flow cytometric analysis of the percentages and numbers of thymocytes and PI/annexin V⁺ cells per lobe from the thymus of each group. All error bars represent the SD of the means. The experiments in (A)–(C) (*n* = 3 per group) were repeated three times, and one representative experiment is presented. The experiment in (D) was repeated twice, and one of the experiments is presented (Veh, *n* = 2; DT, *n* = 2). **p* < 0.05, ***p* < 0.01.

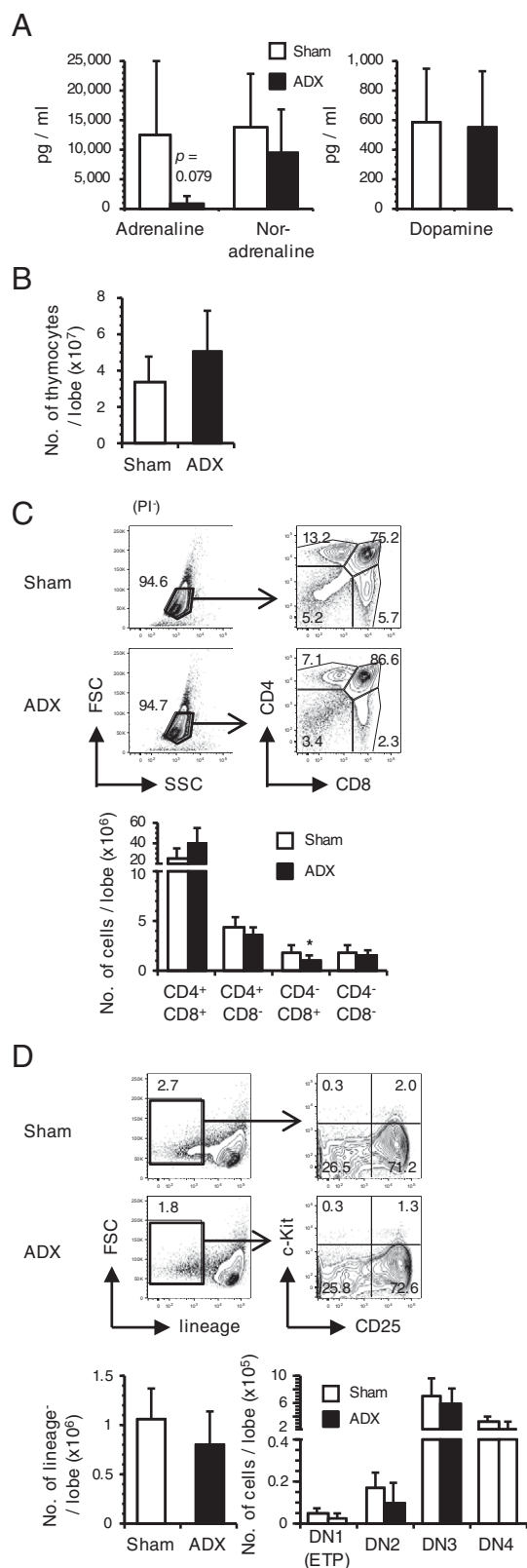


FIGURE 3. Adrenalectomized mice showed no abnormalities in T cell development. **(A)** Plasma concentrations of adrenaline, noradrenaline, or dopamine were measured in each group 3 d after surgery ($n = 6$ per group). **(B)** Number of thymocytes from C57BL/6 mice that underwent sham surgery (Sham) or adrenalectomy (ADX) 3 d after surgery ($n = 3$ per group). **(C)** Flow cytometric analysis of the percentages and numbers of CD4⁺/CD8⁺ (DP) T cells, CD4⁺ or CD8⁺ (single-positive [SP]) T cells, and CD4⁻/CD8⁻ (DN) T cells in the thymus from each group ($n = 3$ per group). **(D)** Flow cytometric analysis of the percentages and numbers of lineage-negative (Lin⁻)

neurotoxin for noradrenergic neuron depletion (Fig. 4A). Similar to results obtained on NC-derived cell depletion, short-term i.p. injection of 6-OHDA affected the thymus (Fig. 4B). Furthermore, immunohistochemical data demonstrated that the areas of the thymus and TH⁺ cells, but not α -SMA⁺, MECA32⁺, and Epcam1⁺ cells, in the 6-OHDA-treated thymi were significantly reduced (Fig. 4C, 4D). Moreover, the plasma noradrenaline level, but not adrenaline or dopamine, was reduced in the 6-OHDA-treated mice (Fig. 4E). Flow cytometric analysis showed that the numbers of thymocytes, CD4⁺/CD8⁺ T cells, CD4⁺/CD8⁻, or CD4⁻/CD8⁺ T cells, and CD4⁻/CD8⁻ T cells were also significantly reduced by 6-OHDA treatment (Fig. 4F). In addition, whereas the numbers of DN1-like ETPs and DN2–4 and Lin⁻ fractions between 6-OHDA- and PBS-treated mice differed significantly (Fig. 4G), the numbers of CD45⁻/Ter119⁻ cells and CD45⁻/Ter119⁻/YFP⁺ cells but not CD45⁻/Ter119⁻/Epcam1⁺ cells between 6-OHDA- and PBS-treated *Wnt/Rosa^{YFP/DTR}* mice did not differ (Fig. 4H). These results indicate that depletion of noradrenergic nerves or noradrenaline probably induced the marked decrease in T cell progenitors observed in the DT+ *Wnt/Rosa^{YFP/DTR}* thymi (Fig. 2B, 2C).

L-DOPS administration barely rescued the phenotype of DT-treated Wnt-1 Cre/Rosa^{YFP/DTR} or 6-OHDA-treated mice

L-DOPS is a known noradrenaline precursor that is converted into noradrenaline following administration to mice. To fully understand the effect of noradrenaline on T cell development, L-DOPS was i.p. administered into DT-treated mice and its effect on T cell development was evaluated. Increasing the concentration of L-DOPS administration gradually improves plasma noradrenalin but has no effect on T cell development (Supplemental Fig. 2). The number and ratio of thymocytes, CD4⁺/CD8⁺, CD4⁺/CD8⁻, CD4⁻/CD8⁺, and CD4⁻/CD8⁻ T cells and Lin⁻ cells, and DN3–4 cells did not differ significantly in the DT+/- and DT/L-DOPS+/- treated *Wnt/Rosa^{YFP/DTR}* mice (Supplemental Fig. 2). Next, we examined the effect of L-DOPS on 6-OHDA-treated mice. Unfortunately, the numbers of thymocytes and CD4⁺/CD8⁺, CD4⁺/CD8⁻, CD4⁻/CD8⁺ T, and CD4⁻/CD8⁻ T cells did not differ significantly between L-DOPS/6-OHDA and 6-OHDA mice (Supplemental Fig. 3B, 3C), nor did the numbers of Lin⁻ cells and DN1-like ETPs and DN2–4 (Supplemental Fig. 3D). L-DOPS injection partially restored plasma noradrenaline levels but had little effect on T cell abnormalities. These results indicate that noradrenaline secreted from noradrenergic nerves may not directly affect thymic T cell development.

Role of noradrenaline via adrenaline receptors in T cell development

Noradrenergic nerves innervate the thymus (39–41), and sympathetic nerves regulate the migration of hematopoietic cells between the BM and peripheral blood. In addition, these sympathetic nerves could release noradrenaline, which acts on adrenaline receptors (ADRB¹, 2, and 3) expressed on stromal cells (34, 37, 57, 58). To further assess the effect of noradrenaline and ADRB¹ antagonists on T cell development, we administered propranolol (ADRB¹ and ADRB² antagonists) and CYP (ADRB³ antagonist) to C57BL/6 mice continuously for 14 d and examined the effect on T cell development (Fig. 5A). Except for the number of CD4⁺/CD8⁻ T cells, no difference was observed in the numbers of thymocytes and T cell progenitors between mice treated with antagonist and vehicle (Fig. 5B). Considering these findings, the decrease in T cell progenitors observed

cells and T cell progenitors (DN1-like ETPs and DN2–4) from each group 3 d after surgery ($n = 3$ per group). Experiments were performed twice, and the sum of the data is represented. All error bars represent the SD of the means. * $p < 0.05$.

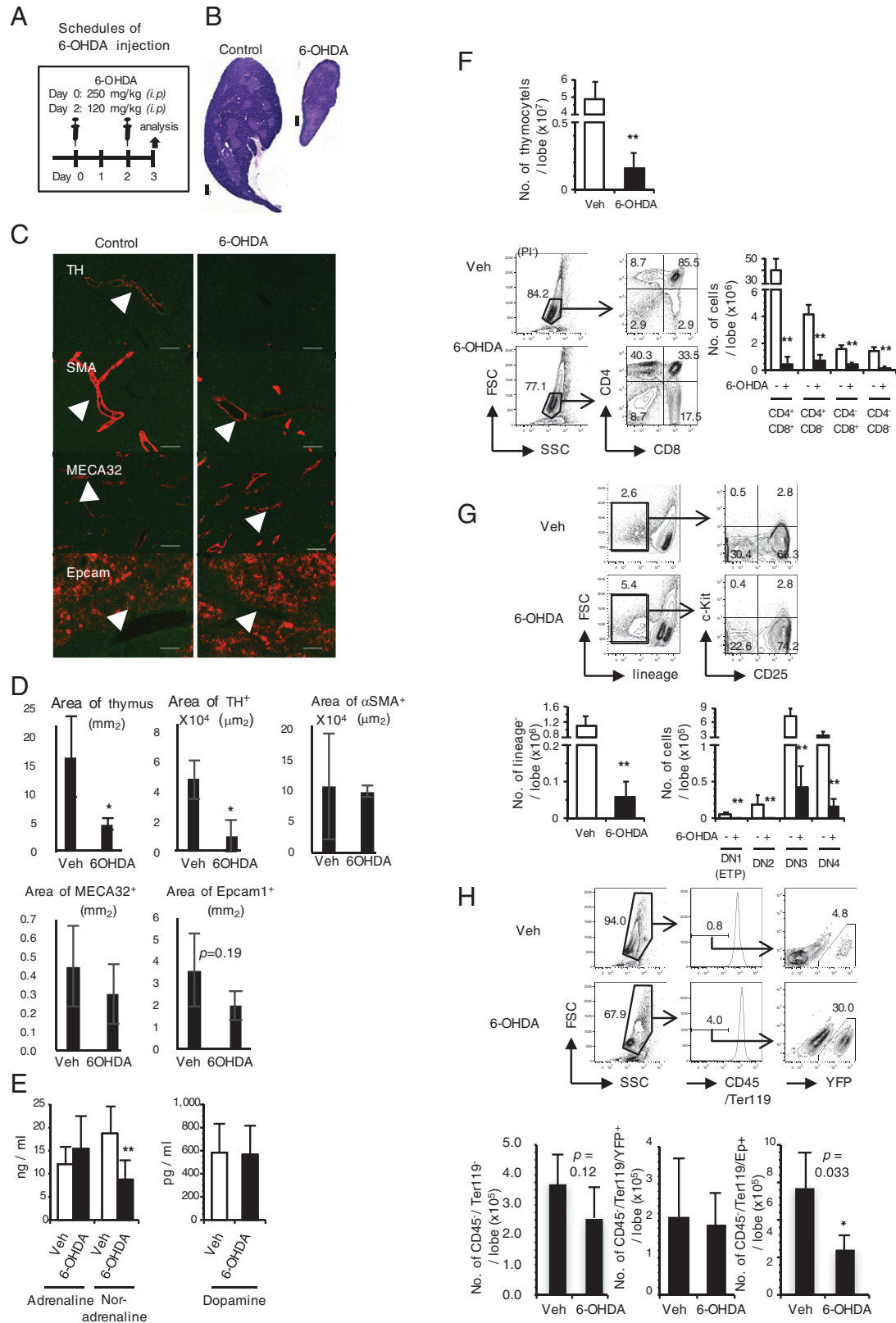


FIGURE 4. 6-OHDA administration depleted TH⁺ and markedly reduced T cell progenitor cells in the thymus. **(A)** Schedule of 6-OHDA injections. **(B and C)** Effect of 6-OHDA administration on thymus. **(B)** H&E-stained sections of thymus from each group. Scale bar, 300 μm. **(C)** Immunohistochemical analysis of stained sections with Ab against TH, α-SMA, MECA32, and EpCAM1 of thymus from each group. Scale bars, 200 μm. White arrowheads point to positive stains with each Ab. **(D)** Positive cell areas of TH, α-SMA, MECA32, and EpCAM1 of the thymus from each group. All error bars represent the SD of the means. **(E)** Plasma concentrations of adrenaline, noradrenaline, and dopamine by HPLC from each group. **(F)** Number of thymocytes per lobe from *Wnt1-Cre/+; Rosa^{YFP/DTR}* mice treated with either vehicle (Veh) or 6-OHDA. Flow cytometric analysis of the percentages and numbers of CD4⁺/CD8⁺ (DP) T cells, CD4⁺/CD8⁻ or CD4⁻/CD8⁺ (single-positive [SP]) T cells, and CD4⁻/CD8⁻ (DN) T cells in the thymocytes of each group. **(G)** Flow cytometric analysis of the (Figure legend continues)

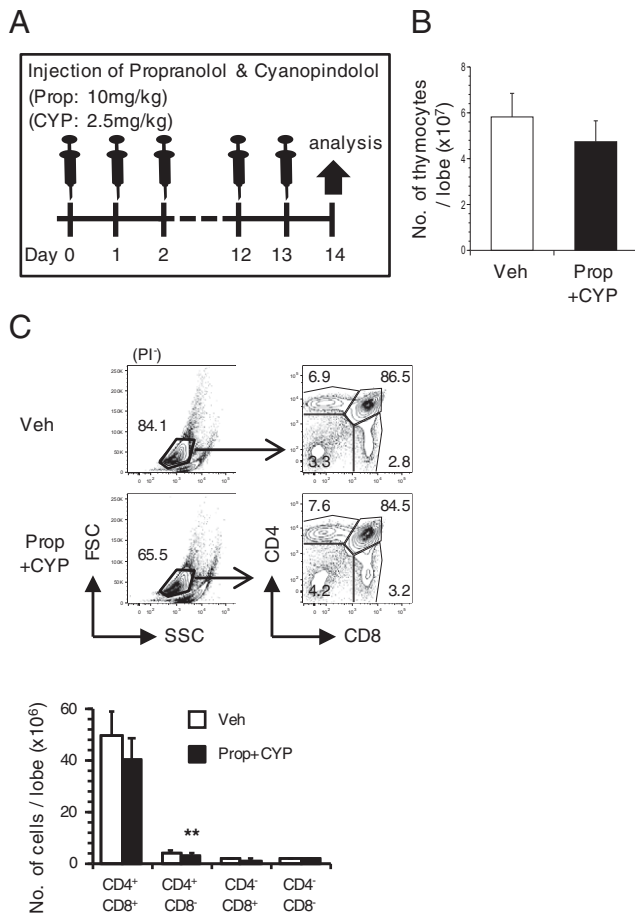


FIGURE 5. Administration of ADRB antagonists, propranolol, and cyanopindolol had no noticeable impact on T cell development. **(A)** Schedule of propranolol (Prop) and cyanopindolol (CYP) injections. **(B)** Number of thymocytes per lobe from 6- to 8-wk-old C57BL/6 mice treated with vehicle (Veh) or Prop + CYP ($n = 3$ per group). **(C)** Flow cytometric analysis of the percentages and numbers of CD4⁺/CD8⁺ (DP) T cells, CD4⁺/CD8⁻ or CD4⁻/CD8⁺ (single-positive [SP]) T cells, and CD4⁻/CD8⁻ (DN) T cells in the thymus from each group ($n = 3$ per group). The experiments were repeated twice, and one representative experiment is presented. All error bars represent the SD of the means. $**p < 0.01$.

in DT+*Wnt/Rosa*^{YFP/DTR} mice cannot be solely attributed to the reduction or inhibition of noradrenaline. Because sympathetic nerves release multiple molecules, noradrenaline may not be the only molecule contributing to thymic T cell development.

Depletion of NC-derived cells within the thymus (intrathymic) before transplantation markedly affected T cell development

To examine whether intrathymic depletion of NC-derived cells was the direct cause of the observed alterations in CD4⁺/CD8⁺ T cells, we isolated the thymus from *Wnt/Rosa*^{YFP/DTR} (CD45.2) donor mice and transplanted it under the renal capsules of C57BL/6 (CD45.1) host mice (Fig. 6A). Nine days after transplantation, DT was injected into the host mice to deplete NC-derived cells. Twelve days following DT administration, NC-derived YFP⁺ cells were significantly reduced in the donor *Wnt/Rosa*^{YFP/DTR} (CD45.2) thymus; however, the numbers of thymocytes, T cells, and nonhematopoietic

cells were unaltered (Fig. 6A). These results suggest that ablation of NC-derived cells within or inside the thymus (intrathymic) after transplantation had a negligible effect on T cell development. This study employed thymic transplantation techniques to investigate thymic function, despite that these methods are generally employed for the embryonic thymus (59–61). The transplantation of normal adult thymi was successful, as demonstrated in Fig. 6a, but it was challenging to determine the role of host-derived hematopoietic cells due to the large number of graft-derived hematopoietic cells in the untreated adult thymus. The presence of a large population of T cell progenitors in the donor thymus, which inhibited the invasion or infiltration of host-derived T cell progenitors into the transplanted thymus, may account for the results observed in Fig. 6A.

To better understand the potential mechanism underlying intrathymic depletion of NC-derived cells on T cell development, we irradiated donor (*Wnt/Rosa*^{YFP/DTR} or *Wnt/Rosa*^{YFP/YFP}) mice to reduce their T cell progenitors before transplantation. The postirradiated adult thymus reduced in size as irradiation caused a considerable loss of many hematopoietic cells (Supplemental Fig. 4). After 3 wk of transplantation, the postirradiated thymus was ~10-fold larger and effectively supported T cell development. That is, host-derived hematopoietic cells migrated into the transplanted thymus and induced ~93% of CD4⁺/CD8⁺ T cells (Supplemental Fig. 4). Subsequently, DT was injected to deplete NC-derived cells 8 d after transplantation, and 3 d later, that is 11 d following the transplantation, the donor (*Wnt/Rosa*^{YFP/DTR}) thymi had a significantly lower number of NC-derived YFP⁺ cells than the control (*Wnt/Rosa*^{YFP/YFP}) thymi (Fig. 6B), but the numbers of thymocytes and CD4⁺/CD8⁺ T cells did not differ significantly. Despite the decrease in T cell progenitors in the donor mice caused by irradiation, these results were comparable to those obtained in Fig. 6A. This shows that depletion of NC-derived cells inside the thymus (intrathymic) after transplantation has no significant effect on T cell development.

To further elucidate the effect of intrathymic depletion of NC-derived cells on T cell development, we isolated the thymi from lethally irradiated DT-treated donor (*Wnt/Rosa*^{YFP/DTR} or *Wnt/Rosa*^{YFP/YFP}) mice and transplanted them under the renal capsules of host (C57BL/6) mice. NC-derived cells were depleted via DT administration before transplantation. Eleven days following transplantation, in addition to the decrease in YFP⁺ cells in the donor (*Wnt/Rosa*^{YFP/DTR}) thymi, the numbers of thymocytes and CD4⁺/CD8⁺ T cells were significantly reduced (Fig. 6C). These results reveal that depletion of NC-derived cells within or inside the thymus (intrathymic) before transplantation has a significant effect on T cell development.

In vitro depletion of NC-derived cells (intrathymic) before transplantation strikingly affected T cell development

To eliminate any other potential systemic influence of DT administration on the depletion of NC-derived cells in the thymus, thymus-specific NC-derived cell depletion was performed. To deplete NC-derived cells, the thymi of lethally irradiated donor (*Wnt/Rosa*^{YFP/DTR}) mice (CD45.2) were cultured in the presence of DT (Fig. 6D). Following 4 d of successful depletion of NC-derived cells (Fig. 6E), the thymi from the donor (*Wnt/Rosa*^{YFP/DTR}) mice (CD45.2) were transplanted under the renal capsules of C57BL/6 host mice (CD45.1). Twenty days after transplantation, the numbers of thymocytes, host-derived CD4⁺/CD8⁺ T cells, host-derived Lin⁻ cells, and DN1-like ETPs and DN2–4 cells were significantly reduced

percentages and number of lineage-negative (Lin⁻) cells and T cell progenitors (DN1-like ETPs and DN2–4) from each group. **(H)** Flow cytometry analysis of the percentages and number of CD45⁻/Ter119⁻ cells and CD45⁻/Ter119⁻/YFP⁺ cells and CD45⁻/Ter119⁻/Epcam1⁺ cells per thymic lobe from each group. The experiments were repeated three times in (D) and (F)–(H) ($n = 2$ –4 per group; Veh, $n = 8$; DT, $n = 10$) and four times in (E) ($n = 2$ –4 per group; Veh, $n = 11$; 6-OHDA, $n = 14$), and the sum of all experiments is presented. All error bars represent the SD of the means. $*p < 0.05$, $**p < 0.01$.

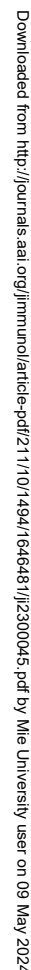


FIGURE 6. In vivo and in vitro depletion of NC-derived cells within the thymus (intrathymic) before transplantation had a significant effect on T cell development. **(A)** Transplantation of thymus from *Wnt1-Cre/+;Rosa^{YFP/DTR}* mice (CD45.2) under renal capsules of C57BL/6 (CD45.1) mice. DT or vehicle (Veh; PBS) was i.p. administered to mice three times 9 d after transplantation, and these mice were analyzed 3 d after the injection. The numbers of thymocytes, CD45.1⁺/CD45.2⁺ cells, and CD45.1⁺/CD45.2⁺/YFP⁺ cells and CD4⁺/CD8⁺ (DP), CD4⁺ or CD8⁺ (single-positive [SP]), and CD4⁺/CD8⁺ (DN) T cells in donor (graft)-derived CD45.2⁺ fractions per lobe and of each group ($n = 4$ per group) are shown. **(B)** Transplantation of thymus from lethally irradiated *Wnt/Rosa^{YFP/DTR}* mice (CD45.2) under the renal capsules of C57BL/6 (CD45.1) mice. Eight days after transplantation, DT was i.p. administered to mice three times, and these mice were analyzed 3 d after injection. Flow cytometric analyses of the percentages and numbers of thymocytes, host-derived CD45.1⁺ CD4⁺/CD8⁺ (DP) T cells, and CD45⁺/Ter119⁺/YFP⁺ cells per lobe in each group ($n = 5$ per group) are shown. **(C)** Transplantation of thymus from lethally irradiated DT-treated *Wnt/Rosa^{YFP/DTR}* mice (CD45.2) under the renal capsules of C57BL/6 (CD45.1) mice. Three days before transplantation, (Figure legend continues)

in DT-*Wnt/Rosa^{YFP/DTR}* thymi but not the ratio of double-positive (DP) T cells (Fig. 6F, 6G, Supplemental Fig. 4). The number of thymocytes in the irradiated thymus without culture and in the irradiated thymus 4 d after culture was about the same, revealing that the culture had no apparent effect on the cell viability (Supplemental Fig. 4). These results indicate that the removal of NC-derived cells from the thymus decreases thymocyte number and T progenitor cell engraftment but does not significantly affect T cell development with respect to the ratio of the T cell population.

Depletion of intrathymic or extrathymic noradrenergic nerves affected T cell development

To better comprehend the effect of extrathymic or intrathymic depletion of the noradrenergic nerve on T cell development, we transplanted thymi from C57BL/6 donor mice under the renal capsules of 6-OHDA-treated C57BL/6 host mice. Five days after transplantation, the numbers of thymocytes and CD4⁺/CD8⁺ T cells in the host (6-OHDA-treated) thymi were reduced significantly, but only moderate changes were observed in the donor (C57BL/6) thymi (Fig. 7A). Although the effect of the absence of neural connections in the host (6-OHDA-treated) thymus on T cell development in the donor (C57BL/6) thymus was unclear, our findings indicated that depletion of the noradrenergic nerve outside the thymus (extrathymic) via 6-OHDA injection before transplantation affected thymic T cell development.

Moreover, to further investigate the effect of intrathymic depletion of noradrenergic nerves on T cell development, we isolated the thymi from lethally irradiated 6-OHDA-treated donor (C57BL/6) mice (CD45.2) and transplanted them under the renal capsules of host (C57BL/6) mice (CD45.1). The numbers of thymocytes and CD4⁺/CD8⁺, CD4⁺/CD8⁻, and CD4⁻/CD8⁻ T cells and the ratio of CD4⁺/CD8⁺ T cells were significantly reduced in 6-OHDA-treated mice 11 d after transplantation (Fig. 7B). These results suggest that sympathetic nerves in the thymus (intrathymic) are important for T cell development despite the lack of a neural connection.

Discussion

In this study, the ablation of NC-derived cells by short-term DT administration decreased YFP⁺/PDGFRα⁺ and PDGFRβ⁺ and α-SMA⁺ mesenchymal cells and plasma noradrenaline and adrenaline levels, thymic TH⁺ sympathetic nerves, and the number of thymocytes in DT-treated *Wnt/Rosa^{YFP/DTR}* mice. To examine the effects of adrenaline on thymocytes, adrenalectomized mice were derived (46). These mice showed only reduced plasma adrenaline and no T cell abnormalities; 6-OHDA-treated mice showed decreased plasma noradrenaline alone and a thymic phenotype comparable to that of DT-treated *Wnt/Rosa^{YFP/DTR}* mice.

To clarify whether reducing plasma noradrenaline directly caused T cell impairment, we used short-term L-DOPS (noradrenaline precursor) treatment to rescue T cell abnormalities (28); however, after 3 d of treatment, gradually increasing the concentration of L-DOPS

elevated plasma noradrenaline levels accordingly but barely improved T cell development in mice administered DT or 6-OHDA. It has been reported that L-DOPS administration improved neurologic abnormalities and tissue noradrenaline levels in dopamine β-hydroxylase-deficient mice (24, 29, 30). However, no report is available on the improvement of immunological abnormalities by L-DOPS. The relatively short treatment time may not have been sufficient to reverse the phenotype. Alternatively, YFP⁺/TH⁺ sympathetic nerves in the thymi of *Wnt/Rosa^{YFP/DTR}* mice were abrogated by DT; the preservation of thymic-resident sympathetic nerves that release noradrenaline in synaptic vesicles may be more essential than the restoration of plasma noradrenaline levels for T cell improvement. In addition, because the continuous administration of ADRB antagonists did not affect the number of thymocytes, a factor distinct from noradrenaline released by sympathetic neurons may be essential for T cell development.

Given NC cell involvement in the development of several organs other than the thymus, determining whether the phenotypes observed following NC depletion were global or intrathymic was difficult. Notably, noradrenergic nerves innervate numerous lymphoid tissues, including the thymus, and sympathetic nerves are located in the thymic corticomedullary junction (31, 39, 40, 42). Consistent with a previous report, TH⁺/YFP⁺ cells were innervated in the thymic corticomedullary junction, and depletion of NC-derived cells in the DT-*Wnt/Rosa^{YFP/DTR}* mice showed vasodilation in the thymus (42). These results suggest that NC-derived cells and sympathetic nerves are involved in the regulation of thymic vascular function.

In this study, we observed that thymic organ culture and thymic transplantation methods were feasible approaches to examining the thymic function of the adult thymus (59–61). The number of thymocytes in the postirradiated thymus with or without culture was about the same, and both transplanted thymi developed with an almost 10-fold number of thymocytes after 3 wk of transplantation and supported T cell development with the induction of 93% of CD4⁺/CD8⁺ cells. This suggests that postirradiated adult thymus transplantation is suitable for studying the role of NC-derived cells or TH⁺ sympathetic nerves on T cell development.

Depletion of NC-derived cells including PDGFRα⁺, PDGFRβ⁺, and α-SMA⁺ mesenchymal cells by DT administration and T cells in the thymus by irradiation before transplantation was observed to affect host-derived T cell development 11 d after transplantation. However, no effect was observed in the irradiated thymic depletion of NC-derived cells by DT on day 9 after transplantation. Furthermore, the numbers of thymocytes, host-derived T cells, and T cell progenitors were significantly reduced 20 d after transplantation of the irradiated thymi that had been cultured in the presence of DT to remove NC-derived cells inside the thymus. This suggests that depletion of NC-derived cells inside (intrathymic) the thymus before transplantation induces T cell abnormalities and that NC-derived cells are involved in the early stage of thymus development. These results are also consistent with the previous study by Bockman and Kirby (4), which highlighted the importance of thymic NC-derived

DT was i.p. administered to mice three times, and 11 d after transplantation these mice were analyzed. Flow cytometric analyses of the percentages and numbers of thymocytes, CD45⁻/Ter119⁻/YFP⁺ cells and host-derived CD45.1⁺ CD4⁺/CD8⁺ (DP) T cells, CD4⁺/CD8⁻ or CD4⁻/CD8⁺ (SP) T cells, and CD4⁻/CD8⁻ (DN) T cells per lobe in each group ($n = 5$ per group) are shown. (D) Adult thymic organ culture in the presence of DT. Three days after lethal irradiation, the thymus was cultured on filters in the presence of DT. Four days after the culture, the thymus was analyzed by flow cytometry. (E) Proportions of CD45⁻/Ter119⁻/YFP⁺ cells in the thymus of DT-treated *Wnt/Rosa^{YFP/YFP}* (DT+ *Wnt^{Y/Y}*) mice or *Wnt/Rosa^{YFP/DTR}* (DT+ *Wnt^{Y/D}*) mice. (F) Thymi (CD45.2) cultured for 4 d were transplanted under the renal capsules of C57BL/6 (CD45.1) mice. Twenty days after transplantation, the transplanted thymi were isolated and analyzed. Flow cytometric analyses of the percentages and numbers of thymocytes, host-derived CD45.1⁺ cells, and CD45.1⁺ CD4⁺/CD8⁺ (DP) T cells, CD4⁺ or CD8⁺ (SP) T cells, and CD4⁻/CD8⁻ (DN) T cells per lobe of each group ($n = 4$ per group) are shown. (G) Flow cytometric analysis of the percentages and numbers of lineage-negative (Lin⁻) cells and T cell progenitors (DN1-like ETPs and DN2–4) from each group. The experiment was repeated twice independently, and one representative experiment is presented. All error bars represent the SD of the means. * $p < 0.05$, ** $p < 0.01$.

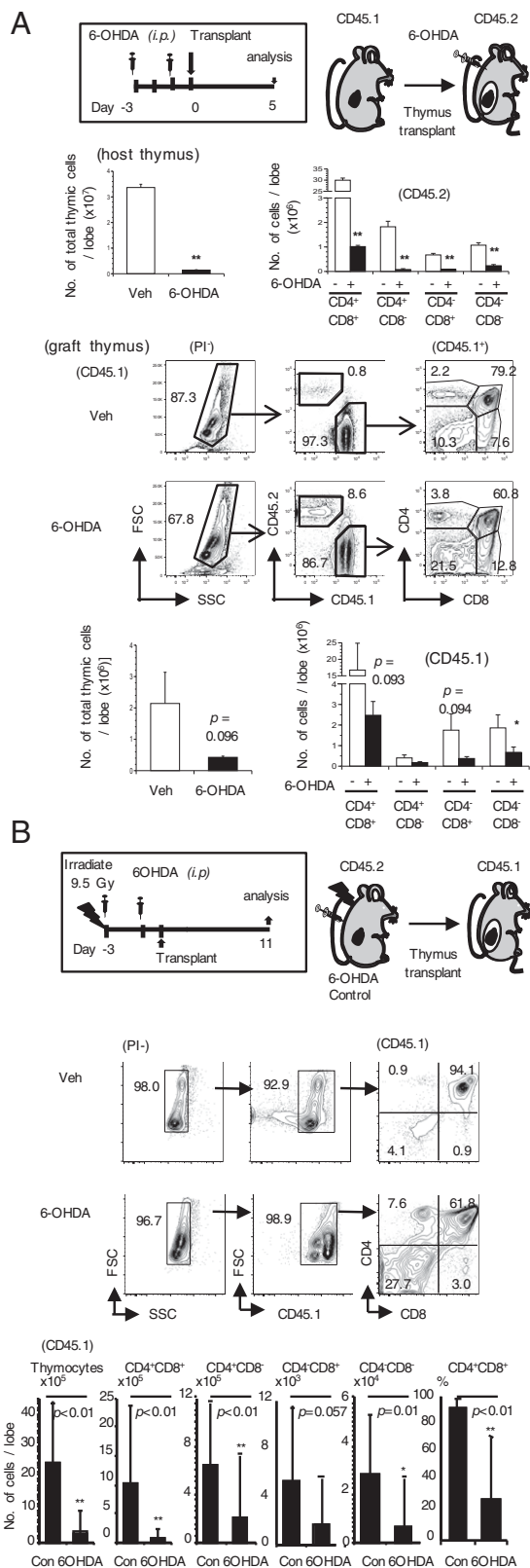


FIGURE 7. Depletion of intrathymic or extrathymic noradrenergic nerves affected T cell development. **(A)** Schedule of 6-OHDA injections. Thymus from C57BL/6 (CD45.1) mice was transplanted under renal capsules of 6-OHDA-treated C57BL/6 (CD45.2) mice. Numbers of thymocytes and host-derived CD45.2⁺ CD4⁺/CD8⁺ (DP) T cells, CD4⁺ or CD8⁺ (single-positive [SP]) T cells, and CD4⁺/CD8⁺ (DN) T cells per lobe in the host thymus from mice (CD45.2) administered vehicle or 6-OHDA are shown. Flow cytometric analysis is shown of the percentages and numbers of thymocytes and donor-derived CD45.1⁺ (graft) CD4⁺/CD8⁺ (DP) T cells, CD4⁺ or CD8⁺ (SP)

mesenchyme for thymic development and function. Notably, the removal of NC-derived cells from the thymus decreases the thymocyte number and T cell progenitor cell engraftment but does not significantly affect T cell development concerning the proportion of the T cell population. This is in line with the finding that in partial DiGeorge syndrome, owing to abnormal NC cells, the thymus becomes abnormally small, but there is no significant difference in T cell development with respect to the ratio of T cell populations (62). The decrease in the thymocyte number is presumably because T cell progenitor cells always migrate to the thymus at a steady state and their migration is impaired by removal of NC-derived cells rather than by an abnormality in T cell development caused by removal of NC-derived cells.

Notably, irradiation affects the number of thymocytes and thymic epithelium but has little effect on the thymic mesenchyme (63, 64). Similar to TECs, NC-derived cells strongly express *Il7*, *Cxcl12*, and *Scf*, which are important for T cell development (61, 65–68), and irradiation may affect these factors produced by epithelial cells. These results suggest that NC-derived thymic mesenchymal cells compensate for the reduction of these factors produced by irradiated epithelial cells (62). Furthermore, it was reported that thymus development depends on direct interaction of NC-derived mesenchyme with thymic epithelia, and that thymic mesenchyme is required for the proliferation and maturation of thymic epithelia (4, 20). Therefore, both irradiation and ablation of NC-derived mesenchymal cells may have a significant effect on the production of these factors, and TECs and may explain abnormal T cell development.

In the thymus, an increase in T cell apoptosis was observed after DT administration. 6-OHDA was shown to induce T cell apoptosis (33). This result corroborates previous findings that inhibition of TH⁺ sympathetic nerves induced thymocyte apoptosis (69, 70). In the DT model, T cells do not express DTR, and NC-derived cells expressing DTR are lost upon DT treatment; nevertheless, the number of thymic T cells was markedly reduced. Although decreased noradrenalin may induce T cell apoptosis, as YFP⁺ NC-derived mesenchymal cells in *Wnt/Rosa^{YFP/YFP}* thymi strongly express stem cell factor, a known survival factor for hematopoietic cells, stem cell factor reduction due to NC-derived cell loss may partly contribute to thymic T cell survival (71).

DT administration led to a reduction in the numbers of YFP⁺/PDGFR α ⁺ or YFP⁺/PDGFR β ⁺ mesenchymal cells, YFP⁺/ α -SMA⁺ perivascular cells, and YFP⁺/TH⁺ sympathetic nerves, whereas 6-OHDA treatment only led to a reduction in thymic TH⁺ sympathetic nerves. Nevertheless, 6-OHDA treatment resulted in a more severe abnormality of T cell development than did DT administration in the thymus. The observed phenomenon may potentially be attributed to the elimination of both YFP⁺ NC-derived and YFP⁺ sympathetic nerves by 6-OHDA administration. It is also possible that 6-OHDA administration affected the number of TECs producing these hematopoietic factors, as we show in Fig. 4H.

T cells, and CD4⁺/CD8⁺ (DN) T cells per lobe of a graft thymus in each group (lower) ($n = 3$ per group). **(B)** Transplantation of lethally irradiated thymus from C57BL/6 (CD45.2) mice under the renal capsules of C57BL/6 (CD45.1) mice. Three days before transplantation, 6-OHDA was administered twice i.p. into C57BL/6 (CD45.2) mice, and 11 d after transplantation, these thymi were isolated and analyzed. Flow cytometric analysis is shown of the percentages and numbers of thymocytes and host-derived CD45.1⁺ CD4⁺/CD8⁺ (DP) T cells, CD4⁺ or CD8⁺ (SP) T cells, and CD4⁺/CD8⁺ (DN) T cells per lobe in the transplanted thymi of each group. The experiments were repeated twice, and the sum of the experiments is presented. All error bars represent the SD of the means. * $p < 0.05$, ** $p < 0.01$.

The findings that the depletion of NC-derived cells including sympathetic nerves may contribute to abnormal phenotypes in T cell development are (to our knowledge) novel and suggest that NC-derived cells regulate T cell development. The data from this study and our previous report (23) suggest that NC-derived cells including sympathetic nerves play an important role in BM and thymus function and that sympathetic nerve abnormalities may also have a significant effect on T and B cell development. Although the relationship between autonomic neuronal dysfunction and immune dysfunction (72) remains unclear, future studies should explore the relationship between autonomic neuronal failure including the reduction of sympathetic nerves and abnormalities of B and T cell development, as well as factors other than noradrenaline secreted by the sympathetic nerves that are involved in T cell development.

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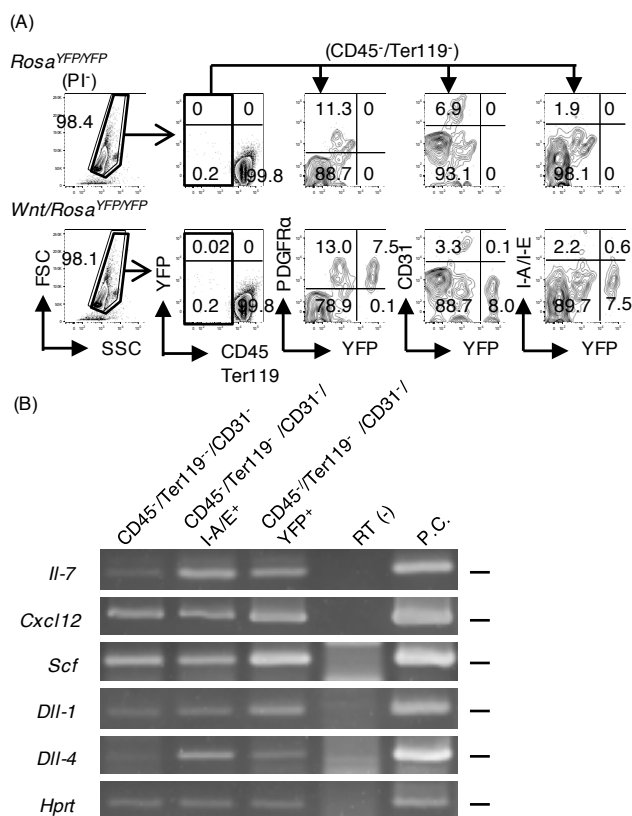
Disclosures

The authors have no financial conflicts of interest.

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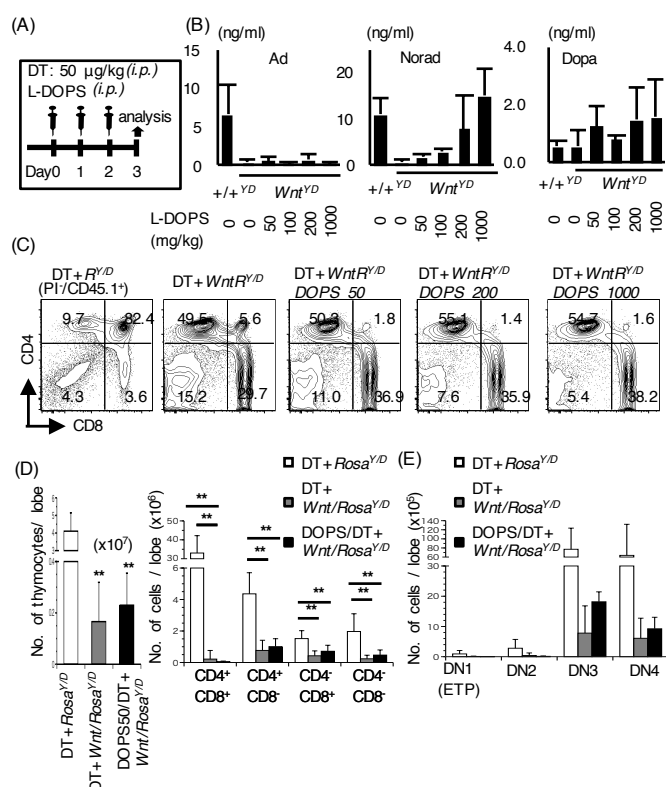
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Supplemental Figure 1. Characterization of YFP⁺ cells in the thymus of *Wnt1-Cre*⁺; *Rosa*^{YFP/YFP} mice

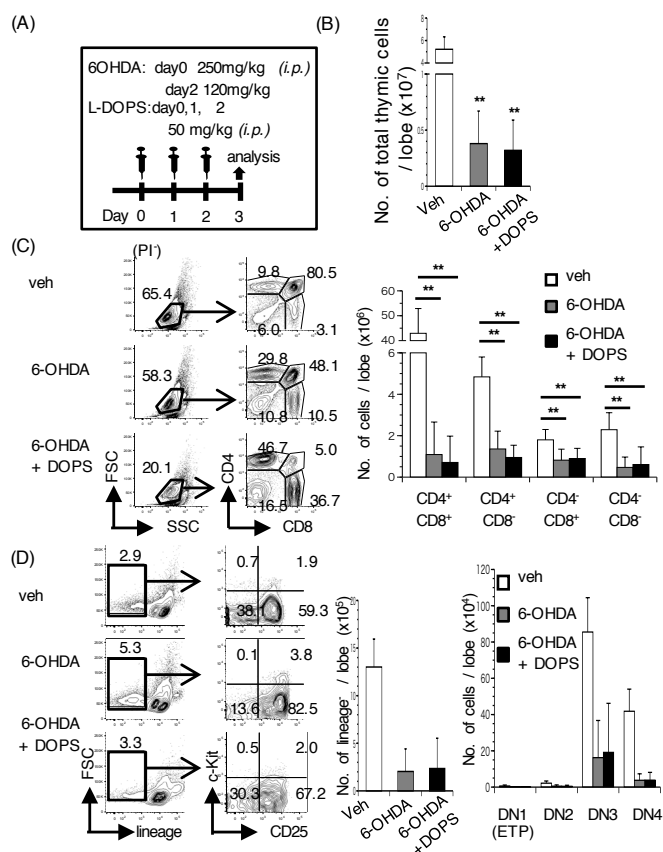
(A) Flow cytometric analysis of the thymus from *Rosa*^{YFP/YFP} mice and *Wnt1-Cre*⁺; *Rosa*^{YFP/YFP} (*Wnt/Rosa*^{YFP/YFP}) mice. Expression of YFP, CD45, Ter119, PDGFR α , CD31, and I-A/I-E on cells from the thymus. (B) Expression of *Il-7*, *Cxcl12*, *Scf*, *Dll-1*, and *Dll-4* in each fraction from *Wnt1-Cre*⁺; *Rosa*^{YFP/YFP} mice by RT-PCR. *Hprt* was used as an internal control. RT- means RNA without treatment with reverse transcriptase. ST2 stromal cells (for *Il7*, *Cxcl12*, and *Scf*), OP9-DLL1 (for *Dll-1*), and whole thymus (*Dll-4*) were used as positive controls, respectively. The primers (5'-3') used for RT-PCR were as follows:

Il7: Fwd, TTGGAATTCCTCCACTGATCCT
Rev: TCGGGCAATTACTATCAGTTCC;
Cxcl12: Fwd:
GCTCTGCATCAGTGACGGTAAAC, Rev:
GCAATATCGTACCATATGCTATGGC; *Scf*:
Fwd: GGAAAATAGTGGATGACCTCGTGT,
Rev: GTCAGATGCCACCATAAAGTCC; *Dll-1* Fwd: TTGGGCTTCTCTGGCTTCA, Rev:
AGGAGGCACAGTCATCCACA; *Dll-4* Fwd:
AGCTGGAAGTGGACTGTGGT, Rev:
TAGAGTCCCTGGGAGAGCAA; *Hprt*: Fwd
GTAATGATCAGTCAACGGGGGAC, Rev:
CCAGCAAGCTTGCAACCTTAACCAT.

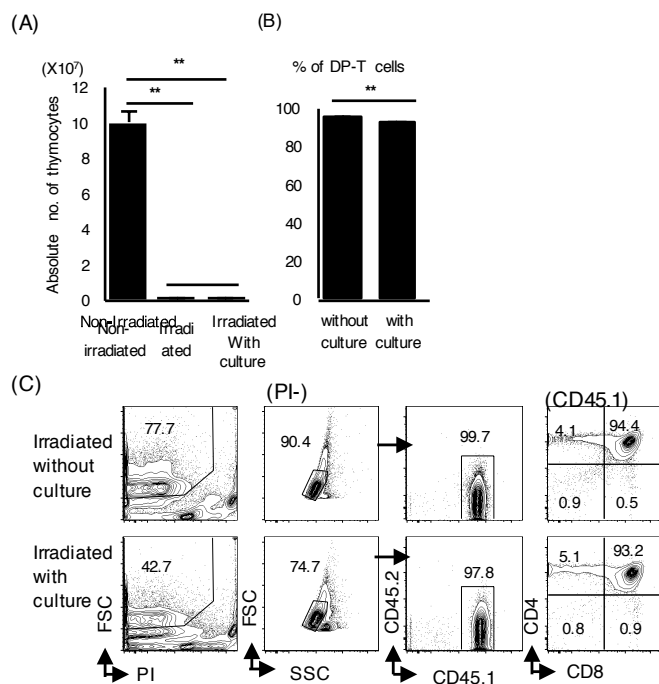


Supplemental Figure 2. L-DOPS administration barely rescued the phenotype of DT-treated *Wnt/Rosa*^{YFP/DTR} mice

(A) Schedule of L-DOPS and DT injections. (B) Plasma concentrations of adrenaline, noradrenaline, and dopamine by HPLC from each group. The experiments were repeated, and the sum of the experiments was presented. (DT-*Rosa*^{YFP/DTR}, $n = 15$ ($n = 1-3$ /group, 9 times), DT-*Wnt/Rosa*^{YFP/DTR}, $n = 15$ ($n = 1,3,4$ /group, 8 times) and DOPS/DT-*Wnt/Rosa*^{YFP/DTR}, $n = 5$ ($n = 2-3$ /group, twice). (C) Flow cytometric analysis of the proportion of CD4⁺/8⁺ (DP) T cells, CD4⁺/8⁻ or CD4⁻/8⁺ (SP) T cells, and CD4⁻/8⁻ (DN) T cells in the thymocytes of each group. ($n = 3$ /group). (D) The numbers of thymocytes and CD4⁺/8⁺ (DP) T cells, CD4⁺/8⁻ or CD4⁻/8⁺ (SP) T cells, and CD4⁻/8⁻ (DN) T cells from DT-*Rosa*^{YFP/DTR} mice, DT-*Wnt/Rosa*^{YFP/DTR} mice and L-DOPS(50mg/kg)/DT-*Wnt/Rosa*^{YFP/DTR} mice ($n = 3$ /group). (E) T-cell progenitors (DN 1-4) from DT-*Rosa*^{YFP/DTR}, DT-*Wnt/Rosa*^{YFP/DTR} and L-DOPS (50mg/kg)/DT-*Wnt/Rosa*^{YFP/DTR} mice ($n = 3$ /group). The experiments were repeated 12 times (A, B, C; $n = 1-3$ /group, DT-*Rosa*^{YFP/DTR}; $n = 18$, DT-*Wnt/Rosa*^{YFP/DTR}; $n = 21$, L-DOPS/DT-*Wnt/Rosa*^{YFP/DTR}; $n = 7$), and the sum of all experiments was presented. All error bars represent the SD of the means; * $p < 0.05$, ** $p < 0.01$.



Supplemental Figure 3. L-DOPS administration barely rescued the phenotype of DT-treated 6-OHDA-treated mice (A) Schedule of 6-OHDA and L-DOPS injections. (B) Number of thymocytes per lobe from Veh (PBS), 6-OHDA and L-DOPS/6OHDA-treated mice. (C) Flow cytometric analysis of the numbers of CD4⁺/8⁺ (DP) T cells, CD4⁺/8⁻ or CD4⁻/8⁺ (SP) T cells, and CD4⁻/8⁻ (DN) T cells in the thymocytes of each group. (n = 3/group). (D) Flow cytometric analysis of the number of lineage-negative cells (Lin⁻) and T-cell progenitors (DN 1–4) from each group.



Supplemental Figure 4. The number of thymocytes from irradiated thymus with or without culture and transplanted thymus 21 days after transplantation under renal capsules.

(A) Absolute number of thymocytes per lobes from non-irradiated or post-irradiated thymus with or without culture for 4 days. (n = 6/group). (B-C) The proportion of CD4⁺/8⁺ (DP) T cells from post-irradiated thymus 21 days after transplantation under renal capsules. (C) Flow cytometric analysis of the proportion of CD4⁺/8⁺ (DP) T cells, CD4⁺/8⁻ or CD4⁻/8⁺ (SP) T cells, and CD4⁻/8⁻ (DN) T cells in the thymocytes of post-irradiated thymus 21 days after transplantation (n = 4/group). All error bars represent the SD of the means; ***p* < 0.01.