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Supporting Online Material for

Acetylcholine-Synthesizing T Cells Relay Neural Signals in a Vagus Nerve Circuit

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SUPPORTING ONLINE MATERIAL

MATERIALS AND METHODS

Mice

Animals were housed at 25°C on a 12-hour light/dark cycle, and acclimatized for at least one week before conducting experiments. Water and regular rodent chow were available *ad libitum.* Male BALB/c and BALB/c nude (nu/nu) mice 8 to 12 weeks-old were obtained from Taconic. ChAT(BAC)-eGFP mice (B6.Cg-Tg(RP23-268L19-EGFP)2Mik/J) were obtained from The Jackson Laboratory, and bred on premises. Cells pooled from ChAT(BAC)-eGFP male and female mice were used for functional and phenotypic characterization. All experiments were performed under protocols approved by the Institutional Animal Care and Use Committee of the Feinstein Institute for Medical Research, North Shore-LIJ Health System.

Immunofluorescence

Spleens were fixed in 4% paraformaldehyde and immersed in 30% sucrose overnight at 4°C. Tissue was embedded in O.C.T. compound (Tissue-Tech) and 10 μm-thick slices were obtained. Sections were mounted on Superfrost microscope slides (Fisherbrand). Heat-induced antigen retrieval was performed using the citrate buffer method (10mM citric acid, 0.05% Tween 20, pH 6.0). Slides were incubated overnight at 4°C with primary antibodies diluted in staining solution (PBS, 0.1% Triton-X 100, and 1% BSA). Antibodies used were: rabbit anti-CD3 (Abcam), rabbit anti-tyrosine hydroxylase (Millipore), and rabbit anti-synaptophysin (Abcam). eGFP was detected with a biotin-conjugated goat anti-GFP antibody (Abcam). In some sections, GFP fluorescence was directly visualized in non-fixed tissue. Slides were washed, and incubated at room temperature for 1 hour in PBS containing the following secondary reagents: DyLight488-conjugated donkey anti-rabbit (Jackson ImmunoResearch), Cy3-conjugated donkey anti-rabbit (Jackson ImmunoReserach) or Cy3-streptavidin (Amersham). Slides were washed and mounted (Vectashield), and visualized using a Zeiss Axiovert 20 inverted fluorescence microscope. Pictures were taken and analyzed using the Axio Vision V5 software.

Flow cytometry and cell sorting

Total spleen cell suspensions were obtained from ChAT(BAC)-eGFP mice. An enriched T cell suspension (purity >90%) was obtained by negative selection using magnetic columns (Pan T cell kit, Miltenyi). In another set of experiments, cell suspensions were obtained from inguinal node and Peyer's patch. Cells were then stained with combinations of the following antibodies: CD3-eFluor450 (eBioscience), CD4-Pacific Orange (Invitrogen), CD8-PerCP (Invitrogen), CD25-Pacific Blue (eBioscience), CD44-PE (eBioscience), and CD62L PE-Cy7 (eBioscience). Cells were acquired with a LSRII flow cytometer (Becton Dickinson). Data was analyzed using FlowJo (Tree Star). For cell sorting experiments, an enriched $CD4^+$ T cell suspension was obtained by negative selection ($CD4^+$ T cell isolation kit II, Miltenyi) of spleen cells harvested

from ChAT(BAC)-eGFP mice. The enriched $CD4^+$ cell suspension was then stained with anti-CD62L PE-Cy7 (eBioscience) and anti-CD19 APC (BD Biosciences) antibodies. After gating out $CD19^+$ and $CD62L^{high}$ cells, a ChAT-$eGFP^-$ and a ChAT-$eGFP^+$ fraction were collected using a FACSAria cell sorter (Becton Dickinson). The resulting fractions were $CD4^+CD44^{high}CD62L^{low}$ChAT-$eGFP^-$ and $CD4^+CD44^{high}CD62L^{low}$ChAT-$eGFP^+$.

Spleen lymphocyte and T cell culture for acetylcholine determination

Total spleen cell suspensions were obtained from BALB/c mice. Cells were incubated for 2 hours in complete RPMI medium at 37°C. Non-adherent cells were collected and washed once in PBS, and incubated in 96-well plates in PBS for 15 min at 37°C in the presence or absence of norepinephrine (Sigma-Aldrich). In another set of experiments, $CD4^+CD44^{high}CD62L^{low}$ChAT-$eGFP^-$ and $CD4^+CD44^{high}CD62L^{low}$ChAT-$eGFP^+$ were incubated in PBS for 15 min at 37°C. Cells were spun down and supernatants were kept frozen at -80°C. Acetylcholine was determined by mass spectrometry.

T cell stimulation and quantification of cytokine production

Spleen $CD4^+CD44^{high}CD62L^{low}$ChAT-$eGFP^-$ or $CD4^+CD44^{high}CD62L^{low}$ChAT-$eGFP^+$ cells obtained by cell sorting were incubated in complete RPMI medium in 96-well plates in the presence or absence of plate-bound anti-CD3 antibody (10 μg/mL) (clone 145-2C11, BD Biosciences) for 48 hours. ChAT-eGFP expression was then determined by flow cytometry. Cytokine levels were determined by cytometric bead array immunoassay using the mouse Th1/Th2/Th17 cytokine kit (BD Biosciences).

Adrenergic receptor mRNA quantification

Total RNA was extracted from spleen $CD4^+$ChAT-$eGFP^+$ cells (obtained by cell sorting, see above) and mouse cardiomyocytes (used as positive control for adrenergic receptors β_1, β_2, and β_3) using the RNeasy Mini Kit (Qiagen), including on-column DNase treatment (Qiagen), and reverse transcribed to cDNA using iScript cDNA synthesis kit (Biorad). Semi-quantitative measurements of mRNA levels were obtained using the reaction mix Light Cycler 480 Probes Master (Roche) in the LightCycler 480 System (Roche) using the following primers (Invitrogen) and probes (Roche Universal Probe Library): *Adrb1*, left 5′-cctagagggcaaaccttgtg-3′, right 5′-tgcacagagtgaggtagaggac-3′, probe #18; *Adrb2*, left 5′-cgagctgagtgtgcaggac-3′, right 5′-gactcctggaagcttcattca-3′, probe #76; *Adrb3*, left 5′-cagccagccctgttgaag-3′, right 5′-ccttcatagccatcaaacctg-3′, probe #13; *Actb*, left 5’-cgtgaaaagatgacccagatca-3’, right 5’-tggtacgaccagaggcatacag-3’, probe 5’-tcaacaccccagccatgtacgtagcc-3’. Values were calculated from a standard curve and β-actin was used as reference gene.

Adoptive transfer

$CD4^+CD44^{high}CD62L^{low}$ChAT-eGFP$^-$ and $CD4^+CD44^{high}CD62L^{low}$ChAT-eGFP$^+$ cells obtained by cell sorting (see above) were transferred to BALB/c nude mice by i.p. injection (1.5×10^5 cells/200 μL of sterile PBS). Five days after transfer of cells mice were subjected to vagus nerve stimulation and endotoxemia. In a separate experiment, $CD4^+CD44^{high}CD62L^{low}$ChAT-eGFP$^-$ and $CD4^+CD44^{high}CD62L^{low}$ChAT-eGFP$^+$ cells were labeled with CellTrace Violet (Invitrogen). Briefly, cells (10^6 cells/mL) were incubated in PBS containing CellTrace Violet (100nM) for 30 min at 37°C. Cells were then resuspended in complete medium and incubated for 30 min at 37°C. Cells were washed and transferred to nude mice (2×10^6/50 μL of sterile PBS). Total spleen cells were analyzed by flow cytometry for eGFP and CellTrace Violet fluorescence five days later. In another set of experiments, an enriched suspension of $CD4^+$ T cells (purity >90%) was obtained from mouse spleens by negative selection using magnetic columns ($CD4^+$ T cell Isolation kit II, Miltenyi). A pool of siRNAs directed against ChAT (s63922, s63923, s63924, Ambion) or scrambled siRNA (4390843, Ambion) at 300 nM was electroporated into the cells using the Amaxa Mouse T cell Nucleofection kit (Lonza) and kept in culture. One day later, cells were transferred to BALB/c nude mice (2.5×10^5 cells/200 μL of sterile PBS). Three days later, mice were subjected to vagus nerve stimulation and endotoxemia. ChAT expression levels were investigated at day 4 by protein immunoblot of transfected cells kept in culture.

Protein immunoblot

Protein was obtained using the protein extraction reagent (T-PER, ThermoScientific) containing protease inhibitors (MiniPREP, Roche). SDS protein electrophoresis and immunoblot were performed using a standard protocol. Antibodies used were rabbit anti-choline acetyltransferase (Pierce) and mouse anti-β-actin (Novus Biologicals). Secondary antibodies were detected using goat anti-rabbit IRDye 680 (Licor), and goat anti-mouse IRDye800 (Licor). Blotting was visualized using the Odyssey infrared imaging system (Licor).

Vagus nerve stimulation

Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), i.p. A midline cervical incision was made and the left cervical vagus nerve was isolated and placed on a bipolar platinum electrode (Plastics One). Electrical stimulation was delivered at 1 V, 2 ms, 5 Hz for 2.5 min by a stimulation module (STM100A) controlled by the AcqKnowledge software (Biopac Systems). In sham-operated animals only a cervical incision was made. Two hours after vagus nerve stimulation, endotoxin (LPS from *Escherichia coli*, 0111:B4; Sigma) was injected (10 mg/kg, i.p.). Mice were euthanized 90 min later and serum was obtained for determination of TNF, IL-6 and IL-10 concentration by ELISA (R&D Systems) or by cytometric bead array immunoassay using the mouse Th1/Th2/Th17 cytokine kit (BD Biosciences). Mean absolute serum TNF values in pg/mL were: BALB/c sham: 1083; BALB/c VNS: 637; nude sham: 256; nude VNS: 341; ChAT-eGFP$^-$ sham: 885; ChAT-eGFP$^-$ VNS: 1113; ChAT-eGFP$^+$ sham: 721; ChAT-eGFP$^+$ VNS: 497; scrambled siRNA sham: 679; scrambled siRNA VNS: 352; ChAT siRNA sham: 664; ChAT siRNA VNS: 562.

Microdialysis

BALB/c mice were anesthesized with urethane (2 g/kg) and xylazine (8 mg/kg), i.p., and placed on a heating pad. A 1 cm-long incision was made in the left flank to expose the spleen. A linear probe (BASi) containing a dialysis membrane (diameter 360 μm, 5 mm-long) was inserted parallel to the longitudinal axis of the spleen through a 25 gauge needle. After removing the needle, the spleen was returned to the abdominal cavity and the skin was sutured. Ringer's solution (Baxter) without acetylcholinesterase inhibitors was perfused with a syringe pump (Harvard Aparatus) at a constant flow rate of 1 μL/min. Sample collection started 30 min after probe insertion, allowing for dialysis membrane activation. Samples were collected every 10 min until the end of the experiment. Baseline acetylcholine release was determined in 3 samples collected prior to any surgical manipulation of the vagus nerve. The vagus nerve was stimulated for 5 min at 5 V, 2 ms, and 5 Hz.

Acetylcholine quantification

Acetylcholine concentration was determined by chromatography followed by mass spectrometry. Chromatographic separation was achieved using an Aquity BEH C18 column (2.1x50 mm, 1.7 μm; Waters Corporation) which was maintained at oven temperature of 25ºC. Pumping was performed by using the UPLC system (Waters) at a flow rate of 0.5 mL/min. Samples were loaded in the Waters Aquity UPLC autosampler. Temperature of the autosampler was set at 10ºC. 5 μL of sample extract was injected and the eluent was monitored by tandem mass spectrometry with an electrospray ionization (ESI) interface of Vantage Triple Quadrupole mass spectrometer (Thermo). The chromatographic data was acquired and processed using the Xcalibur software Version 3.07. The positive ions were monitored in the multiple reaction monitoring (MRM) mode. The following ion transitions (m/z) were monitored: 146.0 (parent) and 87.0 (product) for acetylcholine and 150.0 (parent) and 91.0 (product) for 1,1,2,2-acetylcholine-D4 that was used as the internal standard.

Statistical analysis

Data are expressed as mean ± SEM. Differences between means were determined using two-tailed *t* test or ANOVA with repeated measures using the Dunnett post hoc test. *P* values < 0.05 were considered significant.

Fig. S1

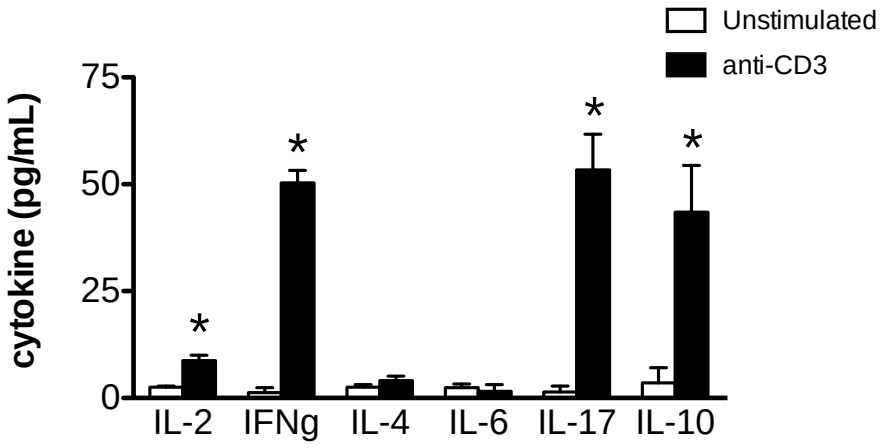


Fig. S1. Cytokine secretion profile of spleen CD4$^+$ ChAT-eGFP$^+$ T cells. Spleen CD4$^+$ CD44^{high} CD62L^{low} ChAT-eGFP$^+$ T cells were obtained by cell sorting and were incubated in the presence or absence of plate-bound anti-CD3. Cytokine levels were determined in supernatants 48 hours later. Data was obtained from pooled cells stimulated in duplicate. Results are expressed as mean ± SEM. * $P < 0.05$ compared to unstimulated cells (two-tailed *t* test).

Fig. S2

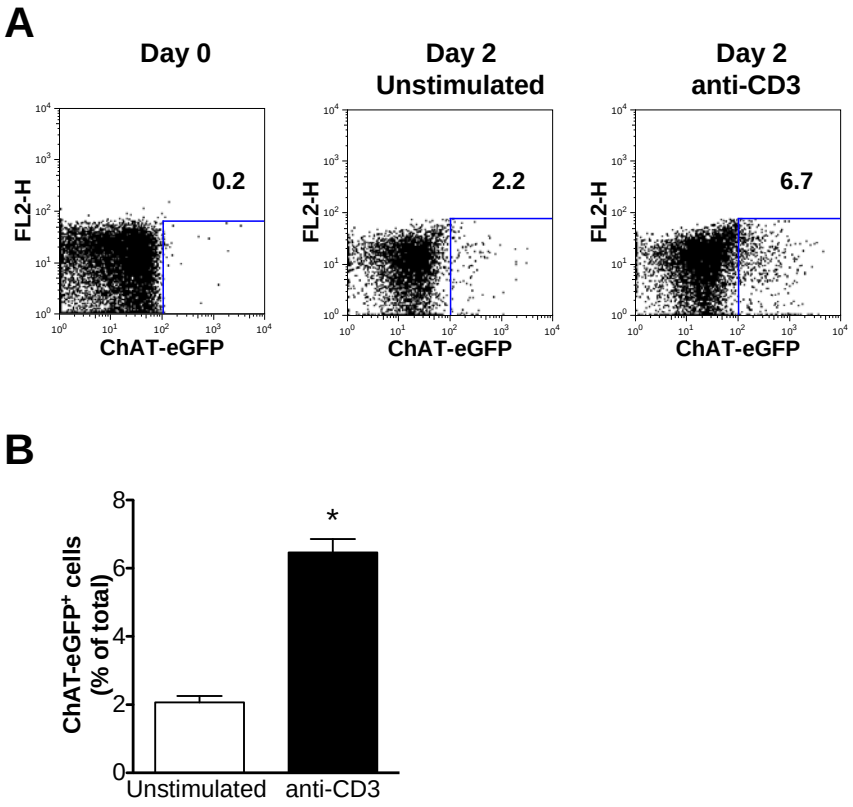


Fig. S2. Stimulation with anti-CD3 antibody induces ChAT-eGFP expression in spleen T cells. Spleen $CD4^{+}$ $CD44^{high}$ $CD62L^{low}$ ChAT-eGFP^{-} cells obtained by cell sorting were stimulated with plate-bound anti-CD3 and ChAT-eGFP expression was determined 48 hours later. *(A)* Representative dot plots showing expression of ChAT-eGFP immediately after cell sorting (day 0), and after 48 hours in unstimulated and stimulated cells. *(B)* Quantification of ChAT-eGFP expression. Plots are representative of data obtained from pooled cells stimulated in triplicate. Results are expressed as mean ± SEM. * $P < 0.05$ compared to unstimulated cells (two-tailed *t* test).

Fig. S3

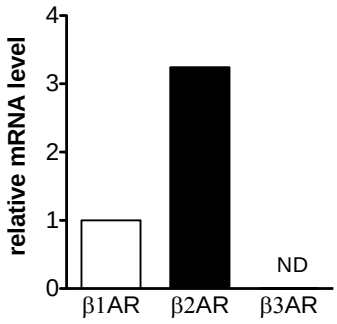
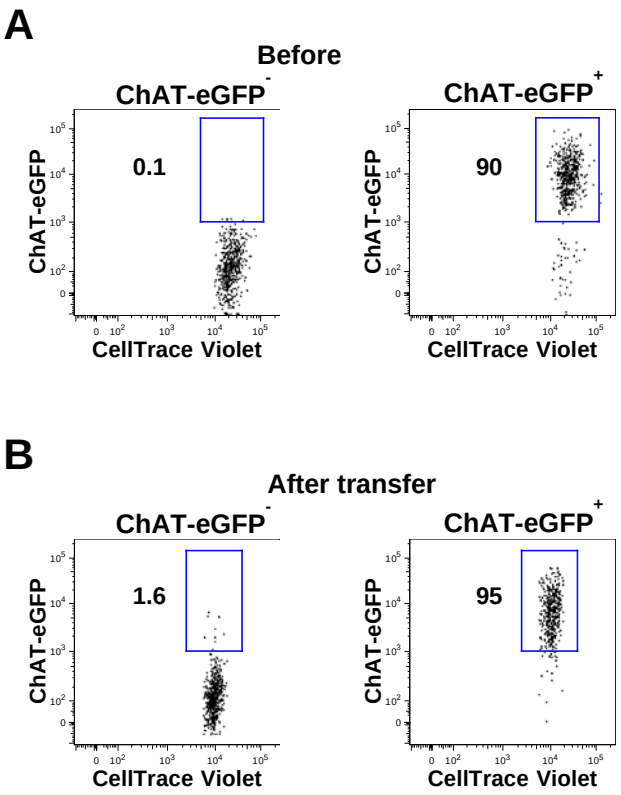


Fig. S3. Spleen CD4$^+$ ChAT-eGFP$^+$ T cells express adrenergic receptors β1 and β2. Spleen CD4$^+$ CD44^{high} CD62L^{low} ChAT-eGFP$^+$ cells were obtained by cell sorting and mRNA expression of adrenergic receptors (AR) β1, β2, and β3 was determined by qPCR. *ND*, not detected.

Fig. S4



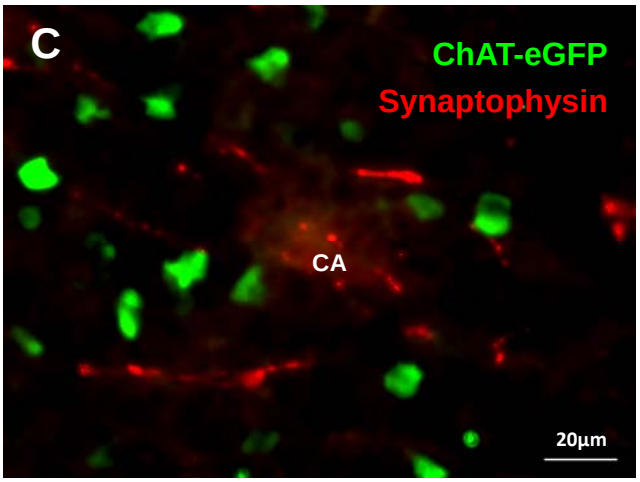


Fig. S4. $CD4^+$ $CD44^{high}$ $CD62L^{low}$ T cells retain ChAT-eGFP expression level and are found in proximity to splenic nerve endings in white pulp after adoptive tranfer. *(A* and *B)* Labeled (CellTrace Violet) spleen $CD4^+$ $CD44^{high}$ $CD62L^{low}$ ChAT-eGFP$^-$ or $CD4^+$ $CD44^{high}$ $CD62L^{low}$ ChAT-eGFP$^+$ cells were adoptively transferred into nude mice, and ChAT-eGFP expression was evaluated in cells harvested from spleen 5 days later. *(C)* ChAT-eGFP$^+$ cells (green) and synaptophysin$^+$ cells (red) in spleen white pulp after transfer of $CD4^+$ $CD44^{high}$ $CD62L^{low}$ ChATeGFP$^+$ cells to BALB/c nude mice, x400 magnification. *CA*, central artery.

Fig. S5

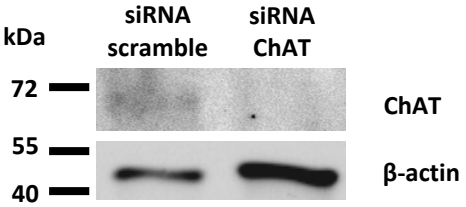


Fig. S5. Choline acetyltransferase (ChAT) knockdown in spleen CD4$^+$ T cells. Spleen CD4$^+$ T cells were transfected with ChAT-specific or scrambled control siRNA. ChAT protein expression (68 kDa band) was verified by protein immunoblot.

Fig. S6

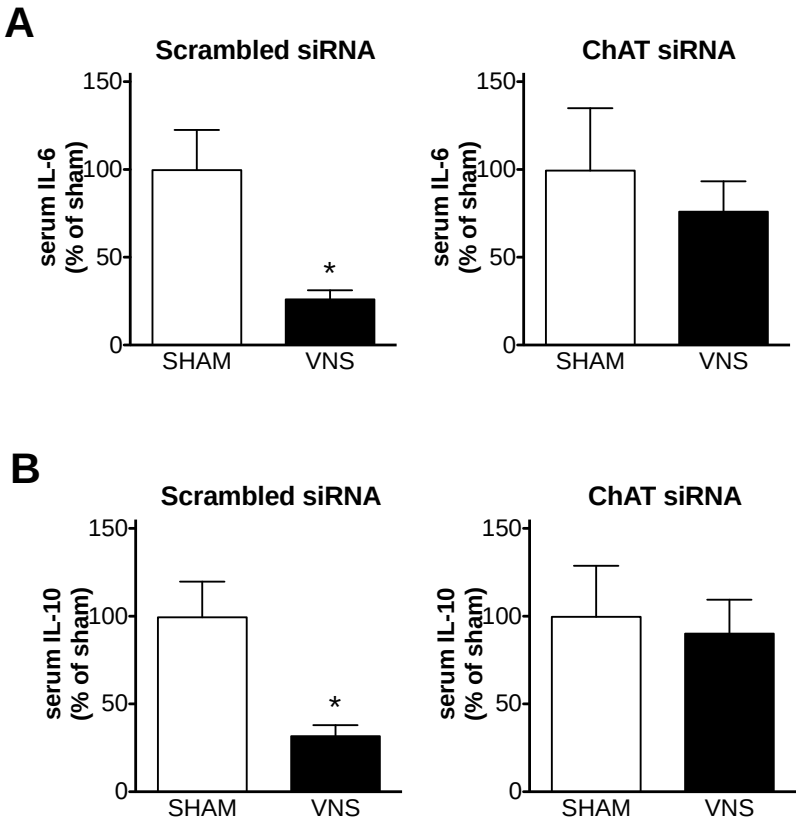


Fig. S6. Attenuation of serum IL-6 and IL-10 levels by vagus nerve stimulation is dependent on ChAT expression in T cells. BALB/c nude mice receiving spleen CD4$^+$ cells transfected with control scramble siRNA or spleen CD4$^+$ cells transfected with ChAT siRNA were subjected to sham surgery or vagus nerve stimulation followed by endotoxin injection. Serum IL-6 *(A)* and IL-10 *(B)* were determined 90 minutes after endotoxin administration. Results are expressed as mean ± SEM. * $P < 0.05$ compared to the respective sham group (two-tailed *t* test).

Fig. S7

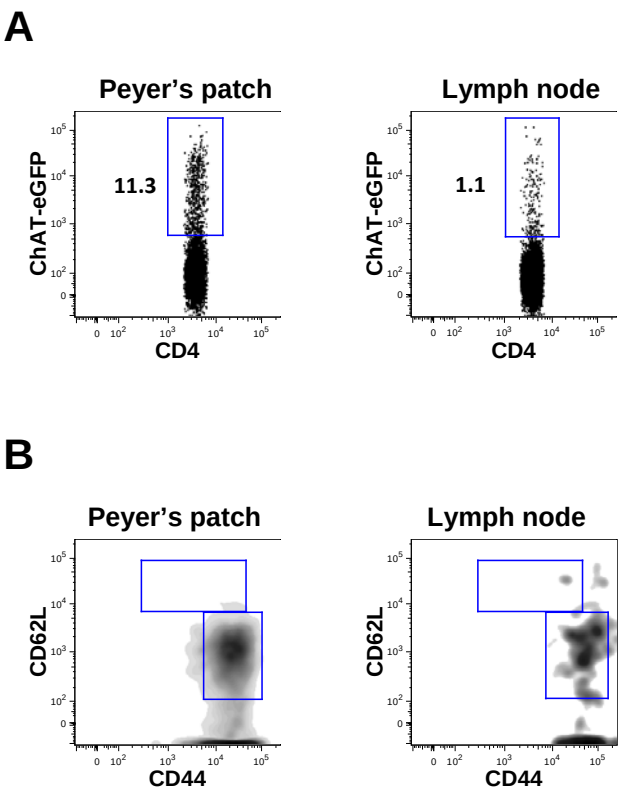


Fig. S7. CD4$^+$ ChAT-eGFP$^+$ T cells are present in lymph nodes and Peyer's patches and express a memory T cell phenotype. *(A)* ChAT-eGFP expression in CD4$^+$ cells obtained from inguinal lymph nodes and Peyer's patches. *(B)* Expression of CD44 and CD62L in CD4$^+$ ChAT-eGFP$^+$ cells from inguinal lymph node and Peyer's patch. Plots are representative of tissue obtained from 2 mice.

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