Supporting Information

Reardon et al. 10.1073/pnas.1221655110

SI Materials and Methods

Mice. C57BL/6, choline acetyltransferase (ChAT)-GFP, *MyD88*$^{-/-}$, *Rag1*$^{-/-}$, and mice expressing Cre recombinase under the control of the endogenous ChAT promoter (ChAT-Cre), Rosa26LoxP-STOP-LoxP tdTomato (Tomato^{LSL}), CD4 dominant negative TGF-β1 receptor (dnTGFβRII) and mice that underwent a subdiaphragmatic vagotomy (or sham) were purchased from Jackson Laboratories. Mice (6-wk-old) were treated with the synthetic sphingosine 1 phosphate analog FTY720 by oral gavage (1 mg·kg·d). All procedures were approved by the University Health Network Animal Care Committee.

Chemicals. ACh, norepinephrine (NE), and physostigmine bromide were from Sigma–Aldrich, and PHA543613 and cholecystokinins (CCKs) were purchased from Tocris Bioscience. LPS was from Sigma–Aldrich, and all other TLR ligands were purchased from Invivogen.

Cells. The mouse endothelial cell line Bend.3 was purchased from the American Type Culture Collection and seeded at 5×10^4 cells per well (96-well plate).

Microbiota Depletion. Depletion of the intestinal microbiota was accomplished by providing antibiotics [1 g/L ampicillin, 1 g/L neomycin sulfate, and 500 mg/L vancomycin (all from Sigma–Aldrich); 1 g/L metronidazole (US Biologicals)] ad libitum in the drinking water for 4 wk (from weaning to 7 wk of age). Depletion was confirmed by plating of homogenized weighed fecal pellets on 5% sheep's blood agar (37 °C, 24 h).

Sterile Endotoxemia. Mice were bled (50 μL) from the tail vein ("0" time point) and injected with 0.2 mg/kg (i.p.) *Escherichia coli*-derived LPS (Sigma–Aldrich), with blood taken every hour until they were killed at 3 h postinjection. Serum was separated using serum separator collection tubes (BD Biosciences) and stored at −80 °C until use. Peritoneal cavities were washed for 3 h with ice-cold PBS, and cells were then counted and used for flow cytometric staining.

Flow Cytometry and FACS. Cells isolated from the mesenteric lymph node, spleen, intraepithelial lymphocytes, liver, bone marrow, or peritoneum were Fc-blocked and stained with anti-CD4, CD8α, CD8β, CD11b, CD21, CD23, IgM, IgD, CD43, GR1, NK1.1, TCR-αβ, and TCR-γδ. FACS was performed on cells prepared by negative selection, leaving untouched B or T cells. Briefly, cells were stained with anti-CD4 peridinin chlorophyll protein (PerCP), anti-B220 PerCP, or anti-CD23 Allophycocyanin (APC), and sorted on an ARIAIIu (BD Biosciences). With the exception of CD11b (Biolegend), all antibodies were from BD Biosciences.

Intracellular staining for Foxp3 and Helios was performed on FACS-sorted CD4$^+$GFP$^+$ and CD4$^+$GFP$^-$ cells. Isolated cells were reblocked with Fc block and stained using the Foxp3 intracellular staining kit according to the manufacturer's instructions (ebioscience).

IL-10 Secretion Assay. Secretion of IL-10 was assessed using a kit, according to the manufacturer's recommendations (Miltenyi Biotec). Briefly, isolated splenocytes or peritoneal cells were washed, stimulated with phorbol myristate acetate (PMA) (50 ng/mL) and ionomycin (500 ng/mL) for 5 h, and then incubated with capture and detection antibody.

Quantitative RT-PCR and Quantitative RT-PCRarray. Sorted cells were centrifuged, and pellets were resuspended in TRIZOL (Invitrogen). cDNA was prepared from isolated RNA using an iScript kit (Bio-Rad Laboratories) for quantitative RT-PCR (qRT-PCR) with the following primers from the PrimerBank (1) (5′-3′): *ChAT*: forward (F)-GGCCATTGTGAAGCGGTTTG and reverse (R)-GCCAGGCGGTTGTTTAGATACA, and *gapdh*: F-AGGTCGGTGTGAACGGATTTG and R-TGTAGACCATGTAGTTGAGGTCA. Data analysis was conducted using the $-2\Delta\Delta$CT method normalizing to *gapdh*. cDNA was also used in the mouse neurotransmitter qRT-PCR array (SABiosciences) and was performed and analyzed according to the manufacturer's instructions.

Immunosuppression Assays. Colitis was induced by the transfer of FACS-purified CD4$^+$CD45RB^{hi} effector T cells (4×10^5 cells per mouse). The capacity of immunoregulatory CD4$^+$CD45RB^{low} or CD4$^+$ ChAT-GFP$^+$ or B220$^+$ ChAT-GFP$^+$ (2×10^5 cells per mouse) cells to protect against the development of colitis was assessed following cotransfer of cells into recipient C57BL/6 *Rag1*$^{-/-}$ mice. Colitis was assessed by macroscopic and histological scores published previously (2).

In Vitro Differentiation. Naive CD4$^+$CD62L$^+$ T cells were isolated from spleens and lymph nodes and enriched according to the manufacturer's instructions (Miltenyi Biotec). T cells were primed for 72 h with anti-CD3 + anti-CD28 (BD Biosciences) and differentiated as follows: T helper (Th) 1 cells: IL-12 (4 ng/mL) and recombinant human (rh) IL-2 (50 units; both from Peprotech), and Th2 cells: IL-4 (10 ng/mL; Peprotech), anti–IFN-γ (5 μg/mL; BD Biosciences), and rhIL-2 (50 units). For Th17 cell differentiation, primed Th cells were cultured in anti–IFN-γ (5 μg/mL), recombinant mouse (rm) IL-6 (30 ng/mL; Peprotech), rhTGF-β1 (1 ng/mL; R&D Systems), rmIL-23 (10 ng/mL; Peprotech), and 50 units of rhIL-2 (BD Biosciences).

MS. Cell pellets from FACS-sorted samples were lysed in ice-cold methanol (MeOH). For ACh released by stimulated cells, cells were allowed to recover in vitamin-supplemented (containing choline; Invitrogen) R10 media for 30 min, washed twice in PBS (with $Ca^{2+}$and Mg^{2+}), resuspended, and added to wells containing physostigmine bromide (10 μM) ± NE and CCKs for 15 min. Following centrifugation, supernatants were mixed with ice-cold MeOH and centrifuged at high speed (14,000 × *g*) to clarify. Supernatants were stored at −80 °C until analysis. Samples were spiked with deuterated-ACh internal standard (Sigma–Aldrich) and analyzed by LC-tandem MS on an AB Sciex API 4000 equipped with an Agilent 1100 HPLC instrument.

1. Wang X, Spandidos A, Wang H, Seed B (2012) PrimerBank: a PCR primer database for quantitative gene expression analysis. *Nucleic Acids Res* 40(D1): D1144–D1149.

2. Reardon C, Sanchez A, Hogaboam CM, McKay DM (2001) Tapeworm infection reduces epithelial ion transport abnormalities in murine dextran sulfate sodium-induced colitis. *Infect Immun* 69(7):4417–4423.

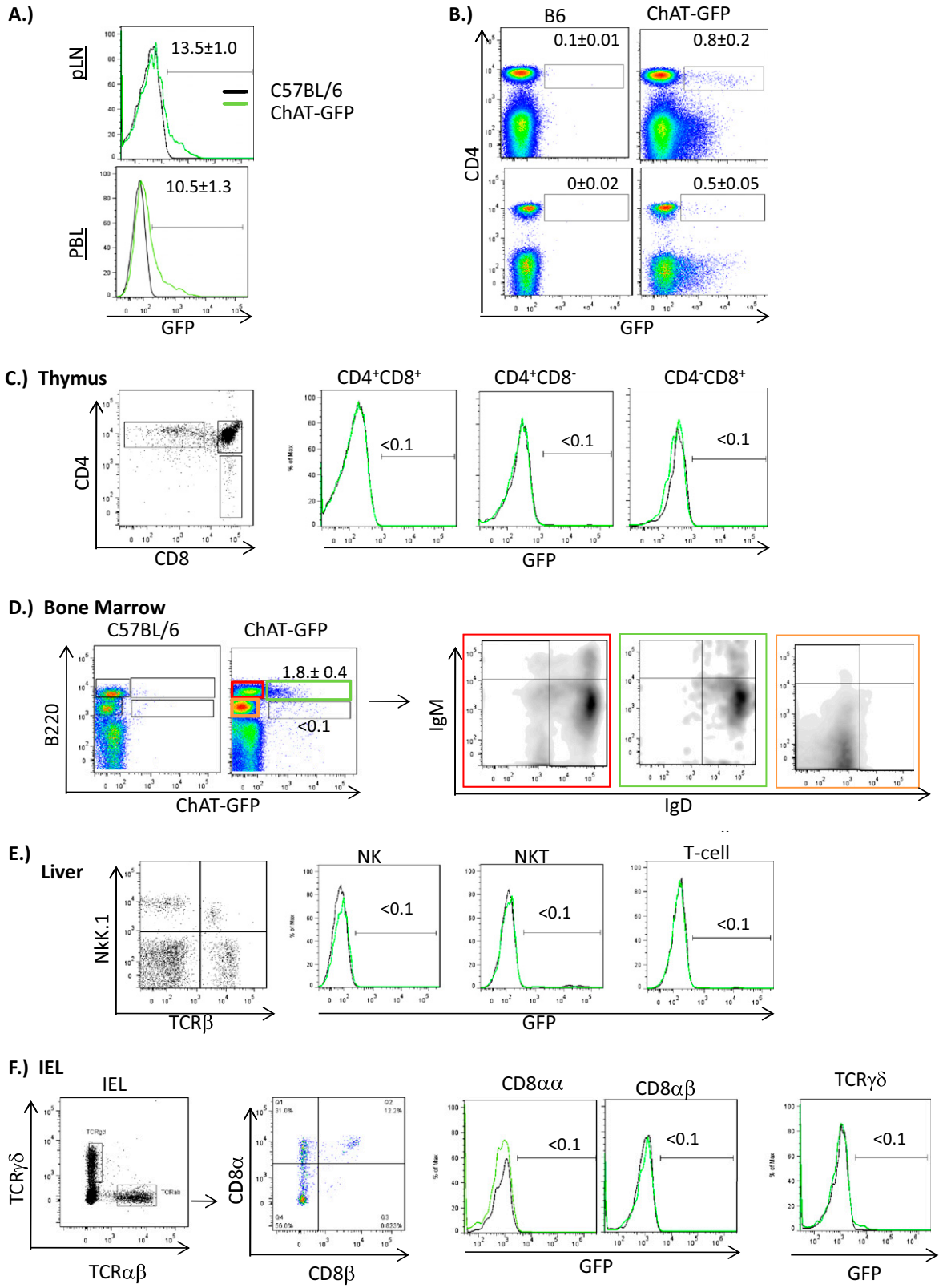

Fig. S1. ChAT expression in peripheral lymphoid tissues. Flow cytometry revealed ChAT-GFP^{+} B220^{+}CD23^{+} B cells (*A*) and CD4^{+} T cells (*B*) in the peripheral lymph node (pLN) and blood (PBL). (*C*) No appreciable ChAT-GFP^{+} cells were detected in the CD4^{+}CD8^{-}, CD4^{-}CD8^{-}, or CD4^{+}CD8^{+} thymocytes. (*D*) In bone marrow, ChAT-GFP expression was restricted to recirculating (B220^{hi}IgD^{+}) cells. (*E*) Populations of natural killer (NK), NK T cells (NKT), or T cells in the liver also indicated no ChAT-GFP^{+} cells. (*F*) Staining of isolated intraepithelial lymphocytes (IEL) revealed no TCR-αβ CD8-αα or TCR-αβ CD8-αβ, or TCR-γδ GFP^{+} cells. Representative results from three to six mice in at least three independent experiments are shown.

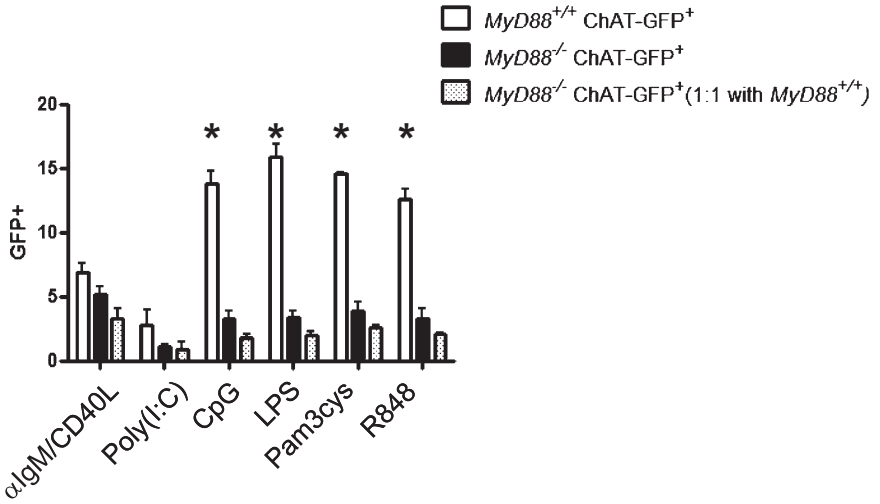

Fig. S2. B cell-intrinsic MyD88 signaling is required for ChAT-GFP induction. To determine if the requirement for MyD88-dependent signaling in ChAT-GFP expression was cell-intrinsic, B220^{+}CD23^{+} cells were FACS-sorted from *MyD88*$^{+/+}$ ChAT-GFP^{+} and *MyD88*$^{-/-}$ ChAT-GFP^{+} mice. *MyD88*$^{-/-}$ cells were labeled with e670 proliferation dye, and *MyD88*$^{+/+}$ cells were labeled with violet proliferation dye. Cells were cultured either alone or mixed 1:1 in media containing anti-IgM/CD40 (10 μg/mL and 5 μg/mL, respectively), polyinosinic:polycytidylic acid [poly(I:C); 100 μg/mL], CpG (10 μM), LPS (10 μg/mL), Pam3cys (1 μM), or R848 (1 μg/mL), and they were assessed for GFP after 16 h (n = 3 mice, two independent experiments). *P < 0.05 by ANOVA.

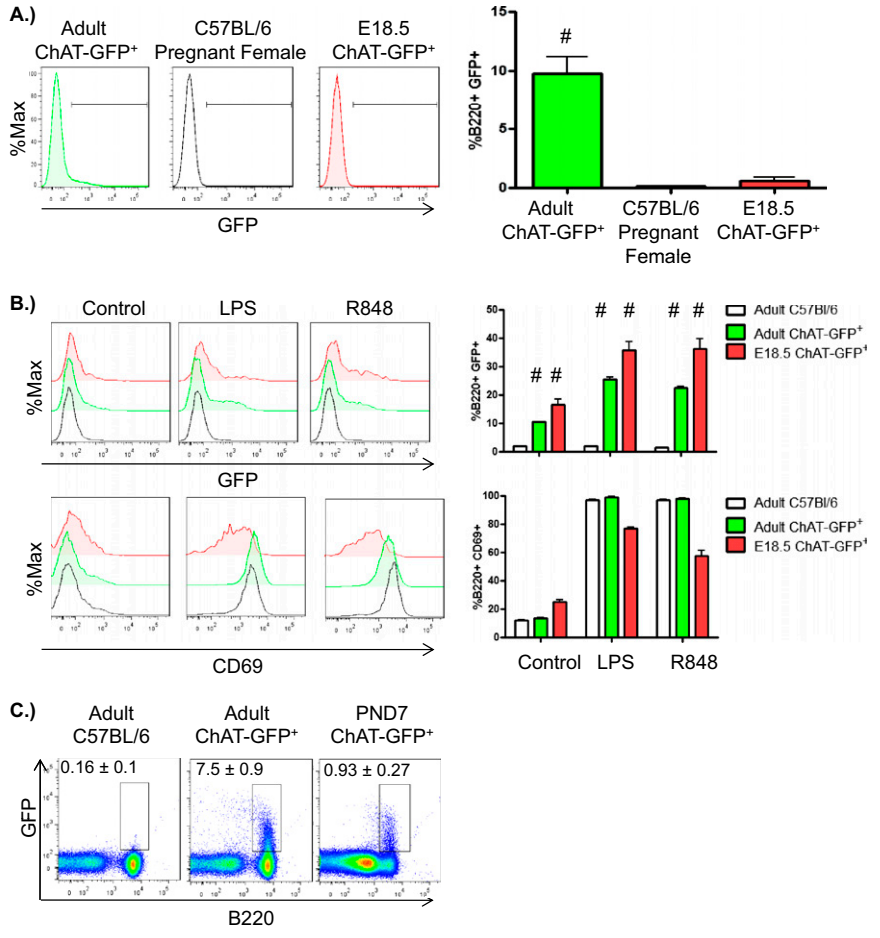

Fig. S3. ChAT expression in lymphocytes occurs after birth. (*A*) Splenocytes were isolated and assessed for GFP expression in B220^{+} B cells from adult (10-wk-old) ChAT-GFP^{+} female or pregnant C57BL/6 mice and ChAT-GFP^{+} fetuses [embryonic day (E) 18.5]. These splenocytes were cultured overnight (16 h) ± LPS (10 μg/mL) or R848 (1 μg/mL). (*B*) ChAT-GFP (*Upper Left* and *Upper Right*) and B-cell activation by CD69 expression were assessed by flow cytometry. %Max, maximum percentage. (*C*) Expression of ChAT after birth was assessed at postnatal day 7 and compared with C57BL/6 or ChAT-GFP^{+} adult mice. All data are representative of three to four adults, 14 fetuses, and eight postnatal day 7 mice in two to three independent experiments. #P < 0.001 by ANOVA.

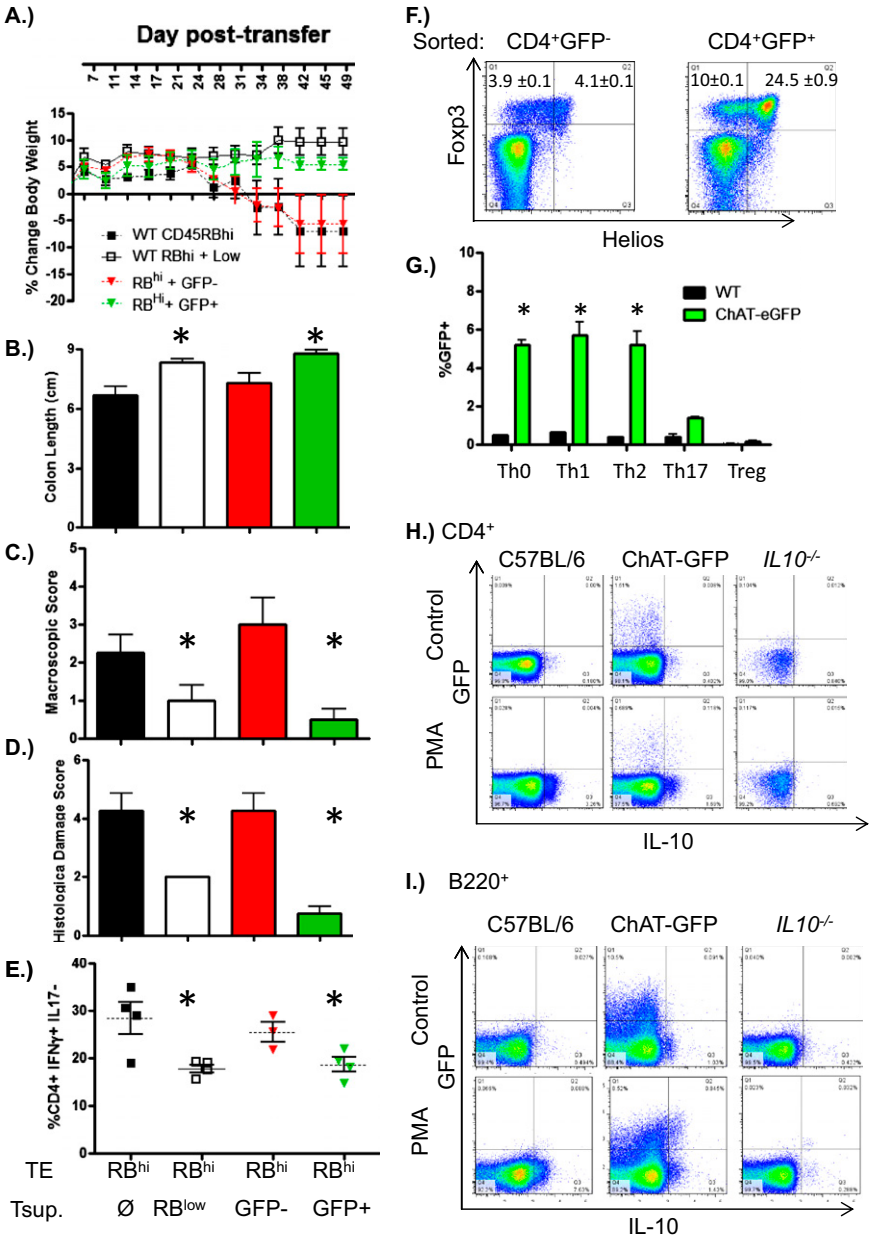

Fig. S4. CD4$^+$ ChAT-GFP$^+$ T-cell population is enriched for natural regulatory T cells (nTregs). The regulatory capacity of CD4$^+$ ChAT-GFP$^+$ T cells was assessed by means of the adoptive transfer model of colitis, using CD4$^+$CD45RB^{hi} T cells transferred into *Rag1*$^{-/-}$ recipients. Suppression was tested by cotransfer of FACS-sorted CD4$^+$ ChAT-GFP$^-$ T cells, ChAT-GFP$^+$ T cells, or CD4$^+$CD45RB^{lo} T cells. Colitis was assessed by weight loss (*A*), at the termination of the experiment by colon length (*B*), by macroscopic score (*C*), by histological damage score (*D*), and by IFN-γ–producing CD4$^+$ T cells in the mesenteric lymph node (MLN) (*E*). TE, Effector T cells; Tsup., suppressor T cells. (*F*) To determine the nature of suppression, intracellular staining for FoxP3 and Helios was performed on FACS-sorted CD4$^+$ ChAT-GFP$^+$ and ChAT-GFP$^-$ T cells. (*G*) In separate experiments, sorted GFP$^-$ cells were subjected to in vitro differentiation under control (anti-CD3, CD28) and T helper (Th)1, Th2, Th17, or Treg polarizing conditions. Secretion of IL-10 was assessed in unstimulated (control, *Upper*) and phorbol myristate acetate (PMA)/ionomycin (50 ng/mL and 500 ng/mL, *Lower*)-stimulated conditions in splenic CD4$^+$ cells (*H*) and B220$^+$CD23$^+$ cells (*I*) from C57BL/6, ChAT-GFP$^+$, and *IL-10*$^{-/-}$ mice. All data are representative of two experiments, with three to four mice per experiment (*A–D*) and four to five mice per experiment (*E–I*). *$P < 0.05$ by ANOVA.

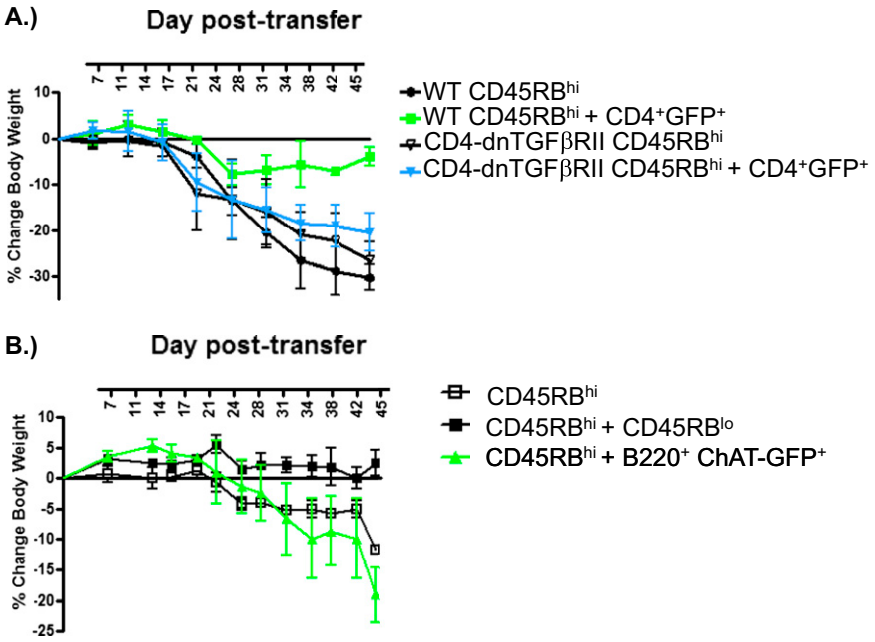

Fig. S5. ChAT$^+$ T cells provide suppression by TGF-β, and ChAT$^+$ B cells fail to prevent colitis. As before, FACS-sorted CD4$^+$CD45RB^{hi} T cells were injected into *RAG1*$^{-/-}$ recipient mice and monitored for disease. (*A*) To test if TGF-β produced by CD4$^+$ ChAT$^+$ prevented colitis, pathogenic effector T cells from WT or mice with disrupted TGF-β signaling were used [CD4 dominant negative TGF-β1 receptor (dnTGFβRII)]. (*B*) Ability of ChAT$^+$ B cells to prevent colitis was assessed using this model of colitis (n = 3 to 4 mice per group).

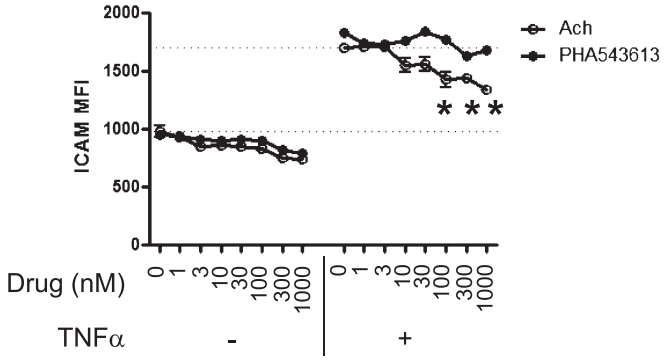

Fig. S6. Endothelial intracellular adhesion molecule (ICAM) expression is not reduced by nicotinic α7-receptors. Bend3 endothelial cells were pretreated (16 h) with physostigmine bromide (10 μM) and ACh (0–1,000 nM) or PHA543613 (0–1,000 nM) before LPS stimulation (1 ng/mL, 4 h). Cells were trypsinized and assessed for surface ICAM-1 by flow cytometry. Data presented as mean ± SD of duplicates from three independent experiments. MFI, mean fluorescence intensity. *$P < 0.05$ (vs. "0 ACh").

Table S1. Neurotransmitter receptors identified by RT-PCR array: FACS-sorted GFP$^+$ B2 cells

Rank	Gene	Name	GFP$^+$ /GFP$^-$
1	Anxa9	Annexin A9	17.80
2	Ache	Acetylcholinesterase	14.19
3	Cckar	Cholecystokinin A receptor	12.73
4	Cckbr	Cholecystokinin B receptor	12.73
5	Chat	ChAT	12.72
6	Brs3	Bombesin-like receptor 3	12.66
7	Tacr3	Tachykinin receptor 3	11.05
8	Chrna3	Cholinergic receptor, nicotinic, α-polypeptide 3	9.98
9	Pgr15l	G protein-coupled receptor 15-like	8.90
10	Galr3	Galanin receptor 3	6.18
11	Sstr1	Somatostatin receptor 1	6.05
12	Htr3a	5-Hydroxytryptamine (serotonin) receptor 3A	5.88
13	Tacr1	Tachykinin receptor 1	4.86
14	Galr1	Galanin receptor 1	4.47
15	Npy6r	Neuropeptide Y receptor Y6	4.42
16	Chrnd	Cholinergic receptor, nicotinic, δ-polypeptide	4.25
17	Chrng	Cholinergic receptor, nicotinic, γ-polypeptide	4.15
18	Chrm4	Cholinergic receptor, muscarinic 4	3.65
19	Chrm2	Cholinergic receptor, muscarinic 2, cardiac	3.63
20	Gabrd	$GABA_A$ receptor, subunit δ	3.55
21	Gusb	Glucuronidase, β	3.49
22	Chrne	Cholinergic receptor, nicotinic, ε-polypeptide	3.28
23	Gabrr2	$GABA_C$ receptor, subunit ρ2	3.03
49	Chrna7	Cholinergic receptor, nicotinic, α-polypeptide 7	0.72

Table S2. Neurotransmitter receptors identified by RT-PCR array: FACS sorted GFP$^-$ B-cells

Rank	Gene	Name	GFP$^-$/GFP$^+$
1	Gpr83	G protein-coupled receptor 83	28.94611
2	Chrna2	Cholinergic receptor, nicotinic, α-polypeptide 2 (neuronal)	12.62599
3	Gabrq	$GABA_A$ receptor, subunit θ	11.92184
4	Mc2r	Melanocortin 2 receptor	7.641956
5	Gabra6	$GABA_A$ receptor, subunit α6	6.152375
6	Chrna4	Cholinergic receptor, nicotinic, α-polypeptide 4	5.637961
7	Glrb	Glycine receptor, subunit β	4.561553
8	Drd1a	Dopamine receptor D1A	4.360062
9	Gabrb2	$GABA_A$ receptor, subunit β2	4.307477
10	Hprt1	Hypoxanthine guanine phosphoribosyl transferase	4.182413
11	Drd2	Dopamine receptor D2	4.146011
12	Gabra1	$GABA_A$ receptor, subunit α1	3.993553
13	Nmur1	Neuromedin U receptor 1	3.926089
14	Maoa	Monoamine oxidase A	3.724661
15	Nmur2	Neuromedin U receptor 2	3.605688
16	Chrnb4	Cholinergic receptor, nicotinic, β-polypeptide 4	3.536628
17	Gabrg2	$GABA_A$ receptor, subunit γ2	3.527477
18	Drd5	Dopamine receptor D5	3.499417
19	Chrm1	Cholinergic receptor, muscarinic 1, CNS	3.391319
20	Npy5r	Neuropeptide Y receptor Y5	3.330397
21	Chrnb1	Cholinergic receptor, nicotinic, β-polypeptide 1 (muscle)	3.165792
41	Chrna7	Cholinergic receptor, nicotinic, α-polypeptide 7	1.382388