Supplemental Information for:

Peripheral lymphoid volume expansion and maintenance are controlled by gut microbiota via RALDH$^+$ dendritic cells

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Supplemental Experimental Procedures

Adipose tissue isolation and implanting

Visceral epididymal adipose tissues were surgically removed from 8 week-old SPF C57BL/6 mice and implanted subcutaneously near iLN of 5 week-old GF C57BL/6 mice through standard sterile surgical operation performed in GF isolators. Anesthesia was induced by injection of a mixed zoletil (tiletamine + zolazepam) and xylazine at 50 and 20 mg per kg of body weight, respectively, prior to the surgery.

FITC-dextran gavage

2 week-old SPF C57BL/6 mice were fasted for 4 hrs, orally gavaged with FITC-dextran 2000KD (Sigma) at 0.3 mg per gram of body weight. 6 hrs later, iLN and mLN were collected; collagenase digested to single cells, and stained for CD103 and CD11b before flow cytometry analysis.

Intracellular cytokine stain

SPF C57BL/6 CD4$^+$ T cells were stimulated with plate-bound anti-CD3e (1 μg/ml) + anti-CD28 (0.5ug/ml) antibodies for 3 hrs, followed by monensin + Brefeldin A (eBioscience) for additional 9 hrs. These activated CD4$^+$ T cell were then stained for surface antigen for 30 min in flow cytometry buffer at 4°C. After washing, 1 ml of Permeabilization working solution (eBioscience) was added and the suspension was incubated at 4°C for 30 min. The cells were then pelleted in 100 μl of Permeabilization working solution, and anti IFN-γ and IL-4 antibodies were added for 30 min for cytokine detection by flow cytometry.

Confocal microscopy

Briefly, LN were fixed in 4% PFA overnight at 4ºC, washed in PBS, cryo protected in 20% sucrose in PBS at 4ºC overnight, embedded in OCT (Sakura) and cryo sectioned into 16 μm thick

sections. The sections were blocked for 1 hr (10% FBS in PBS) at room temperature and incubated with primary antibodies (1:50-100) overnight at 4ºC. After washing, secondary antibodies were added for 1 hr at room temperature. Primary antibodies used were: murine PNAd (MECA-79), CCL21 (polyclonal goat IgG), CD3 (17A2), and B220 (RA3-6B2). Secondary antibodies were conjugated with AlexaFluor 488, 555, or 647 (1:200-500). Images were collected using a Zeiss 710 META confocal microscope. All raw images were processed with Image J and HEV lumen areas were calculated by the ruler transformed pixels. Adobe Illustrator was used for figure panel arrangement and labeling.

Multiple cytokine detection

Isolated RALDH$^+$ and RALDH$^-$ cells were cultured *in vitro*, supernatants were collected after 24 hrs. Cytokines were measured with a Luminex 100 platform (Luminex) and analyzed with BioManager software (Bio-Rad). The following multiplexing kits were purchased from Millipore: Mouse Cytokine and Chemokine Panel I (MPXMCYTO-70K) containing Eotaxin, G-CSF, GM-CSF, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, IFN-γ, IP-10, KC, LIF, LIX, CCL2, M-CSF, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNF-α and VEGF. Kits were run according to the manufacturer's instructions.

Real time PCR

RALDH$^+$ and RALDH$^-$ cells were flow cytometry sorted from mLN and pLN of 2 week-old SPF mice. 2 x 10^5 cells were used to extract RNA using an E.Z.N.ATM MicroElute Total kit (Omega bio-tek) according to the manufacturer's protocols, and cDNA was synthesized using a PrimeScript cDNA synthesis kit (Takara). Primers for RALDH2 and GAPDH were purchased from OriGene. Real-time PCR was performed with SYBR Green Master Mix (Takara) on a CFX96 realtime PCR detection system (Bio-Rad). The comparative CT method (Delta CT) was used to assess relative changes in mRNA abundence between samples. Expression of transcripts was normalized to GAPDH. Relative expressionof RALDH2 transcript in one sample of RALDH$^-$ cells (lowest) was set at 1, which served as the base for other relative expressions.

Co-culture and gut homing assay

Briefly, flow cytometry-isolated iLN CD11c$^+$RALDH$^-$, iLN CD103$^+$CD11b$^+$RALDH$^+$ (neo-mi DCs), mLN RALDH$^+$CD11b$^+$CD103$^+$ cells from 2 week-old SPF C57BL/6 were loaded with OVA (200 μg/ml) for 4 hrs, then mixed with CD4$^+$OT-II splenocytes (DC/T ratio 1:4) isolated with EasySep mouse CD4$^+$ T cell enrichment kit (StemCell), and co-cultured for 6 days. Expression of CCR9 and α4β7 was analyzed by flow cytometry on cells in the CD4$^+$ gate. 2 x 10^6 activated OT-II splenocytes were labeled with CFSE and mixed with the same number of purified naive SPF C57BL/6 CD4$^+$ T cells labeled with DDAO and injected i.v. into 6 week-old SPF C57BL/6 mice. After 18 hrs, recipient mouse LN and LP were harvested to measure CFSE/DDAO ratios by flow cytometry. The data is expressed as the Homing Index (HI), as previously described (Hall et al., 2011).

Cell isolation and flow cytometry

LN single cell suspension was achieved by frosted cover slide grinding. PP were excised from small intestine, digested in 5% FBS RPMI 1640 with 0.1% collagenase I /0.05% dispase (Sigma) for 1 hr at 37ºC. The remaining tissue was incubated with 1 mM DTT, 5mM EDTA, 5% FBS PBS for additional 30 min at 37ºC before intraepithelial lymphocyte removal. Residual intestinal tissue was digested for LP cell isolation. RALDH activity in individual cells was measured using an Aldefluor kit according to the manufacturer's protocol. 7.5 μM of DEAB was added in different tubes at 37ºC for 15 min as Aldefluor fluorescent baseline control. For conventional flow cytometry analysis, primary antibodies at 1 μg/ml were used to stained target cells at 4ºC for 30 min followed by fluorophore-conjugated secondary antibodies and washed three times with FASC buffer (2% FBS + PBS). Flow cytometry was performed on Accuri C6 (BD Biosciences) and analyzed using FlowJo software (Tree Star). Percentage values were charted with Graphpad Prism.

Adoptive cell transfer

Pooled-single cell suspension of iLN and aLN were either first purified for marker-positive populations as described or directly labeled with 5 μM CFSE for 15 minutes at 37°C. After washing twice with PBS containing 5% FBS, cells at the indicated number per mouse were injected into the tail vein of recipients. Lymphocyte transfer to GF mice was conducted in GF isolator (over the fixed gloves). After the injection suspensions were prepared, they were passed into the isolator through germicidal dip tank filled with 3% Peracetic acid. After tail vein injection, the mice were maintained in GF isolator until analysis. The GF status was confirmed at the time of sacrifice by enlarged cecum, microbial culture and 16s rDNA PCR amplification of feces (not shown). BMDCs were produced from C57BL/6 bone marrow in the presence of 5 ng/ml of GMC-CSF and IL-4 and harvested after a 5 day culture. The cells were stained by Aldefluor and the negative population was sorted for the assay.

Cell sorting

For isolation of pLN (neo-mi DCs) and mLN $CD103^+CD11b^+RALDH^+$ cells and their $RALAH^-$ control, 2 week-old C57BL/6 iLN, aLN and mLN were digested in 5% FBS RPMI 1640 with 0.1% collagenase I /0.05% dispase for 1 hr at 37ºC. After washing twice with 5% FBS RPMI 1640, single cells were labeled with Aldefluor fluorescent substrate for 15 minutes at 37°C in the provided assay buffer. Aldefluor-labeled cells were incubated with PE or APC-conjugated anti-mouse CD103, CD11b or CD11c antibodies at 4ºC to prevent the reduction of Aldefluor fluorescence. Labeled lymphocytes were sorted by Influx cell sorter (BD) at 4ºC. Occasionally, mLN or LP from 6 week-old C57BL/6 mice were used for isolation of $RALDH^+CD11b^+CD103^+$ cell, as indicated. In all experiment, a separate cell suspension was treated with DEAB and used as Aldefluor fluorescent baseline control.

β-galactosidase activity

Isolated RALDH reporter and control CD1 LN were digested into single cells, incubated with DDAOG (10 μM) in Hanks' buffer for 2 hrs at 37°C. The cells were washed three times with Hanks' buffer before flow cytometry analysis. Upon excitation, DDAO generates a far-red-shifted fluorescent signal that can be analyzed by Flow cytometry.

cDNA library preparation and Illumina sequencing

Total tissue RNA from pLN of 5 week-old GF or SPF C57BL/6 was extracted using the TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. Poly $(A)^+$ RNA was purified from 5 μg of pooled total RNA using oligo(dT) magnetic beads, sheared into short fragments, and primed for cDNA library synthesis using the TruSeq RNA sample preparation kit per Illumina protocol. After quantitation using a TBS-380 mini-fluorometer (PicoGreen), the samples were clustered (TruSeq paired-end cluster kit, v3-cBot-HS; Illumina) and sequenced on the HiSeq2000 platform (100 bp, TruSeq SBS kit v3-HS 200 cycles; Illumina).

Annotations for the entire data set were obtained with Tophat, and followed by statistical analysis with Cufflink (Cuffdiff module). FKPM values with X^2 less than 0.05 were extracted and logarithmized by 2. The heat maps were generated by HemI (Heatmap Illustrator) software.

DEAB treatment and anti-MAdCAM-1 antibody injection

2 week-old SPF C57BL/6 mice were injected i.p. with DEAB (100 μg/ per gram of weight, dissolved in 50 ul DMSO) 3 to 6 times (3 times/week) and the control groups were injected with DMSO alone. For MAdCAM-1 blocking, anti-MAdCAM-1antibody or rat IgG was injected i.p. into neonatal mice (40 μg/mouse) for four times every other day starting at 6 days of age.

Reference

Hall, J.A., Cannons, J.L., Grainger, J.R., Dos Santos, L.M., Hand, T.W., Naik, S., Wohlfert, E.A., Chou, D.B., Oldenhove, G., Robinson, M.*, et al.* (2011). Essential role for retinoic acid in the promotion of CD4(+) T cell effector responses via retinoic acid receptor alpha. Immunity *34*, 435-447.

Figure S1: related to Figure 1

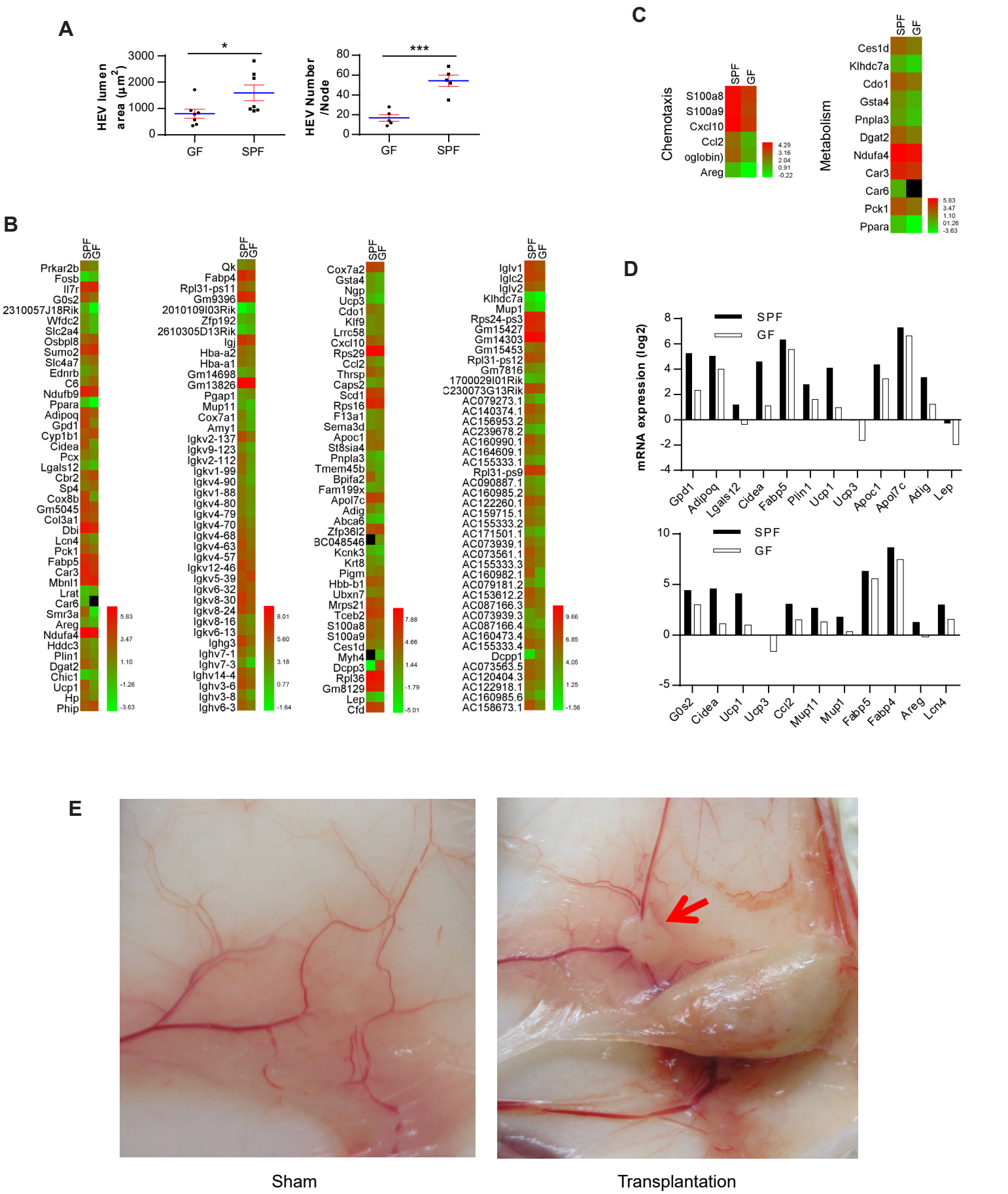

Figure S1 (related to Figure 1). Adipose tissue regulation and RA signaling are involved in pLN volume and cellularity development

(A) Related to Figure 1B, the HEV^{+} area size per lumen and HEV number per node are shown. **(B)** The overall heatmap showing the comparison between 5 week-old C57BL/6 GF and SPF mice. mRNA from pLN (pooled) was extracted and subjected to RNAseq mRNA trasncriptome analysis as indicated in the methods. **(C)** Additional functional groups that were different in GF and SPF mice: chemotaxis and metabolism. **(D)** The same data used in Figure 1C are displayed in bar graph. **(E)** The visceral fat tissues were harvested under sterile conditions and transplanted to inguinal area of GF mice under GF confinement. Photos were taken 2 weeks after the operation and the sham-operated site is shown as the control. The arrow indicates the expanded LN. All operated GF mice were determined to remain GF by enlarged colon, feces culture and lack of 16S RNA by PCR at the time of photographing.

Figure S2: related to Figure 2

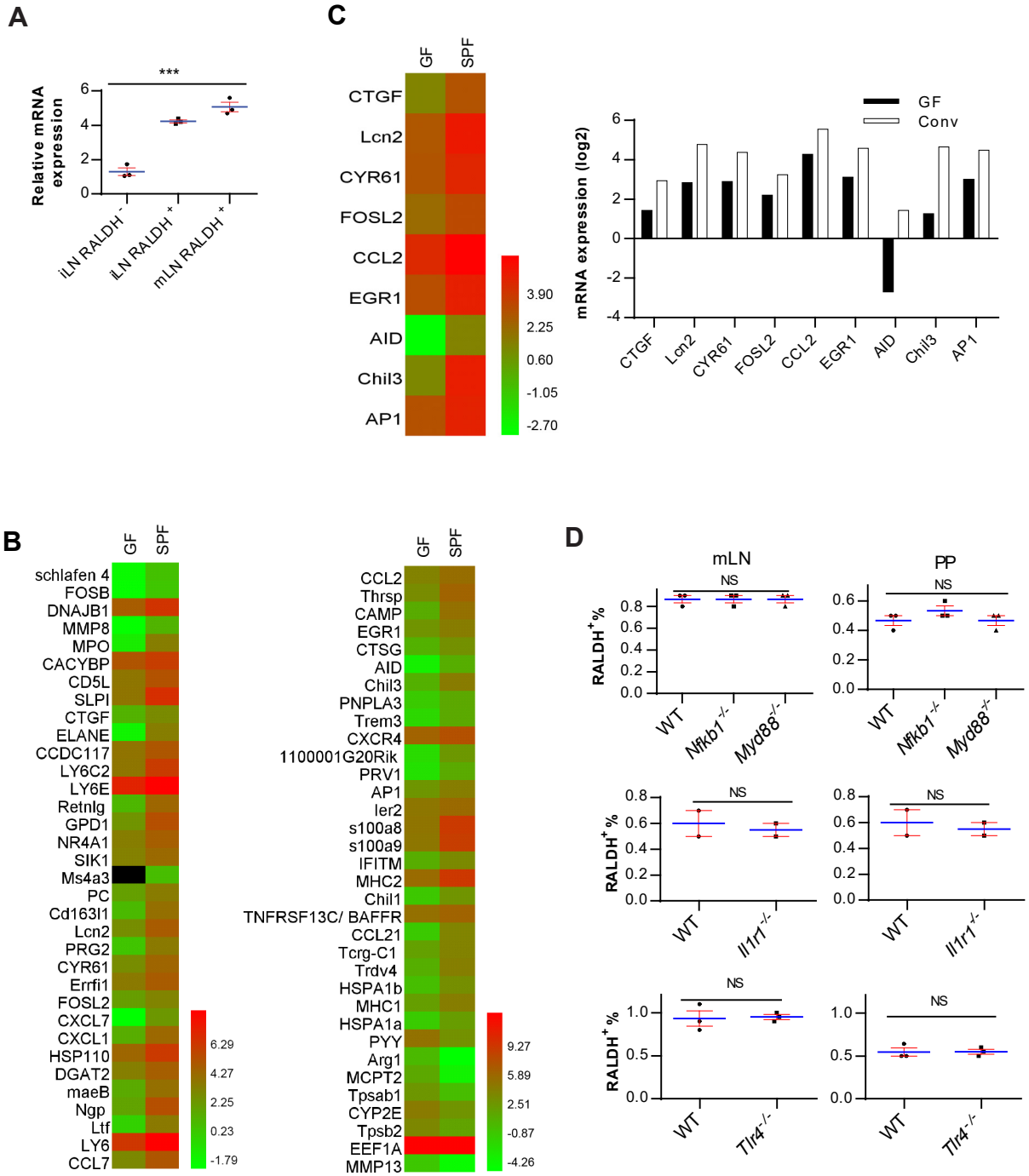

Figure S2 (related to Figure 2). RALDH activities are robust in neonatal iLN and mLN

(A) Quantitative PCR for RALDH2 in mRNA isolated from $RALDH^+$ cells in iLN and mLN of 2 week-old SPF mice. **(B)** The overall heatmap showing the comparisons of mRNA expression levels in pooled pLN from 5 week-old ICR GF and identical GF mice 1 week after cohousing with SPF mice. **(C)** Left panel: the heatmap for genes associated with RA signaling, derived from B. Right panel: the same data used in the left panel are displayed in bar graph. **(D)** The presence of $CD45^+RALDH^+$ cells in the mLN and PP was not affected by the absence of NFkb (classical), Myd88, TLR4 and IL-1 receptor. The percentage of these cells in 2 week-old *Nfkb1*$^{-/-}$, *Myd88*$^{-/-}$, *Tlr4*$^{-/-}$ and *Il1r1*$^{-/-}$ SPF mice were compared with WT mice.

Figure S3: related to Figure 3

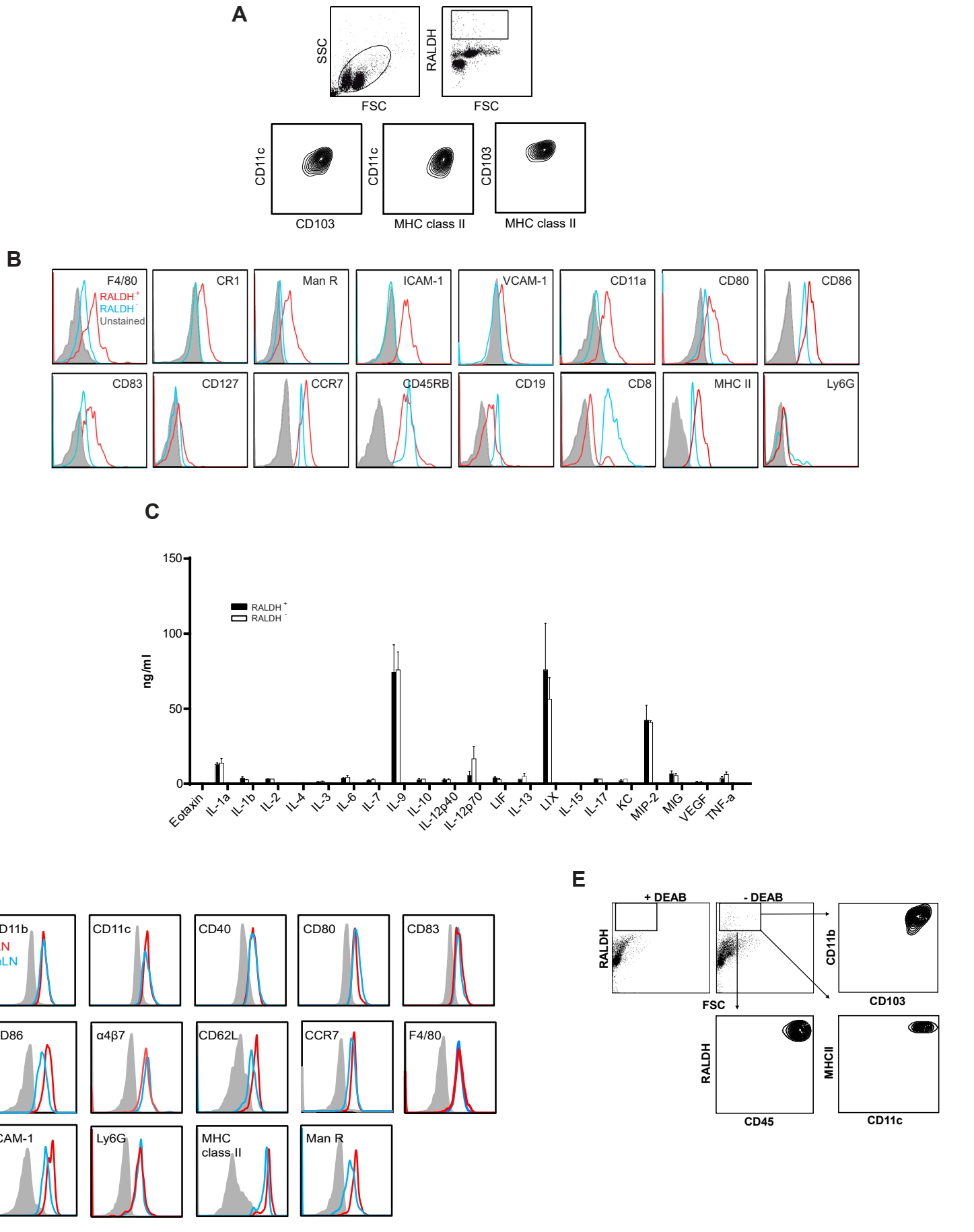

Figure S3 (related to Figure 3). Phenotypical characterization of $CD45^+RALDH^+$ cells

(A) FACS analysis of $CD45^+RALDH^+$ cells. The top panels show the FSC and SSC gating and the population in the oval in the left panel was analyzed in the right panel. The $RALDH^+$ box in the top right was stained by three different pairs of antibodies in the bottom panels. **(B)** Supplemental to Figure 3E, shown are the additional staining for F4/80, complement receptor 1 (CR1), mannose receptor (MR), ICAM-1, VCAM-1, CD11a, CD80, CD86, CD83, CD127, CCR7, CD45RB, CD19, CD8, MHC class II and Ly6G. The lack of heterogeneity in the expression of specific markers in $RALDH^-$ cells was due to the suboptimal gating in our pre-gating to isolate $RALDH^+$ cells for best analysis of respective markers on these cells. **(C)** Supplemental to Figure 3F, shown are the additional cytokine comparisons that did not reach statistical significance. **(D)** $CD45^+RALDH^+$ cells from iLN and mLN of 2 week-old SPF mice were stained for a panel of surface markers to identify any differences in expression. Secondary antibody-only staining of $CD45^+RALDH^+$ cells was used as the background control. **(E)** Similar to A except that only 2 week-old LN were used and CD11b expression was also analyzed.

Figure S4: related to Figure 4

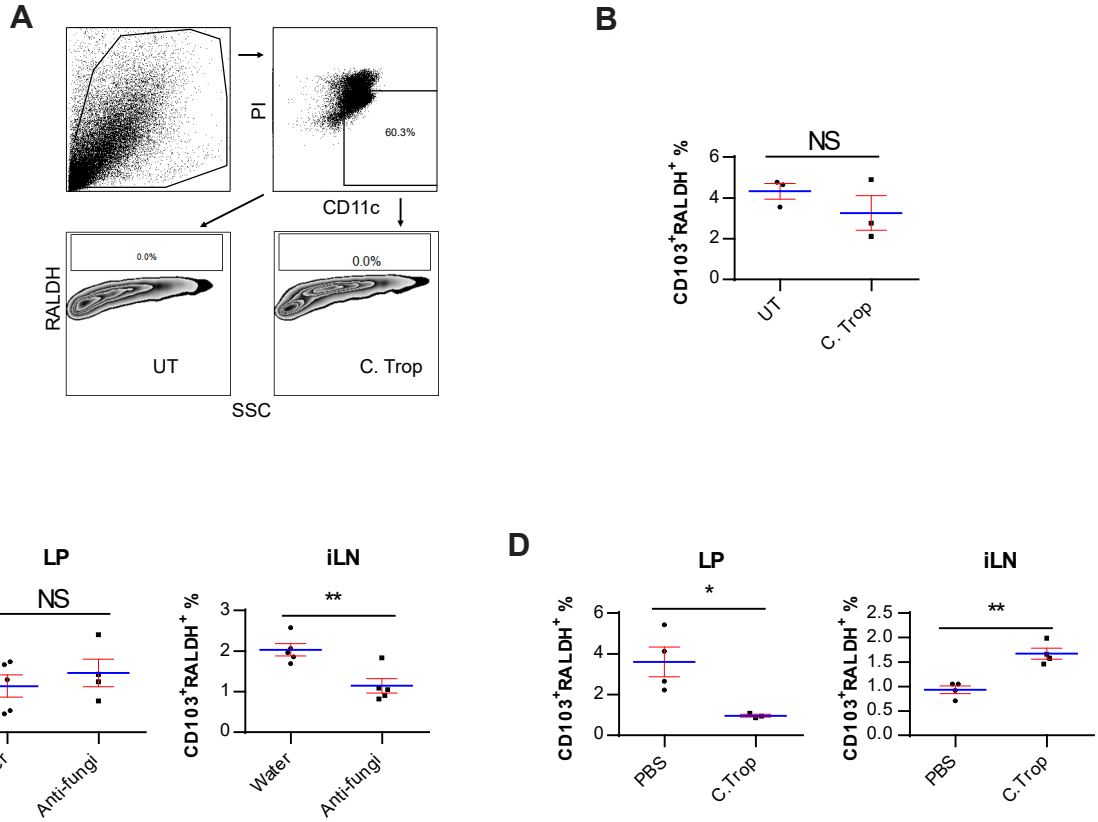

Figure S4 (related to Figure 4). $CD103^+RALDH^+$ DCs from the gut are the likely the driving force of pLN development

(A) 10^5 Purified $CD103^+CD11c^+RALDH^-$ LP cells were either untreated or incubated with 5 x 10^5 boiled *C. tropicalis* for 24 hrs. The resulting $CD11c^+/PI^-$ population was stained for RALDH expression. Triplicates were analyzed and no $RALDH^+$ cell was found. **(B)** Similar to A except 10^6 BMDCs were either untreated or incubated with the same number of boiled *C. tropicalis* for the same duration. Chart graphs to summarize multiple repeats are shown. **(C)** LP and iLN of control or anti-fungi-treated (for 2 weeks) mice were gated on $CD11c^+$ and stained for $CD103^+RALDH^+$ expression. **(D)** 2 week-old SPF mice were force-fed with PBS or 10^8 live *C. tropicalis* in PBS suspension. 48 hrs later, the percentage of $CD103^+RALDH^+$ cells in LP and iLN was determined by FACS.

Figure S5: related to Figure 5

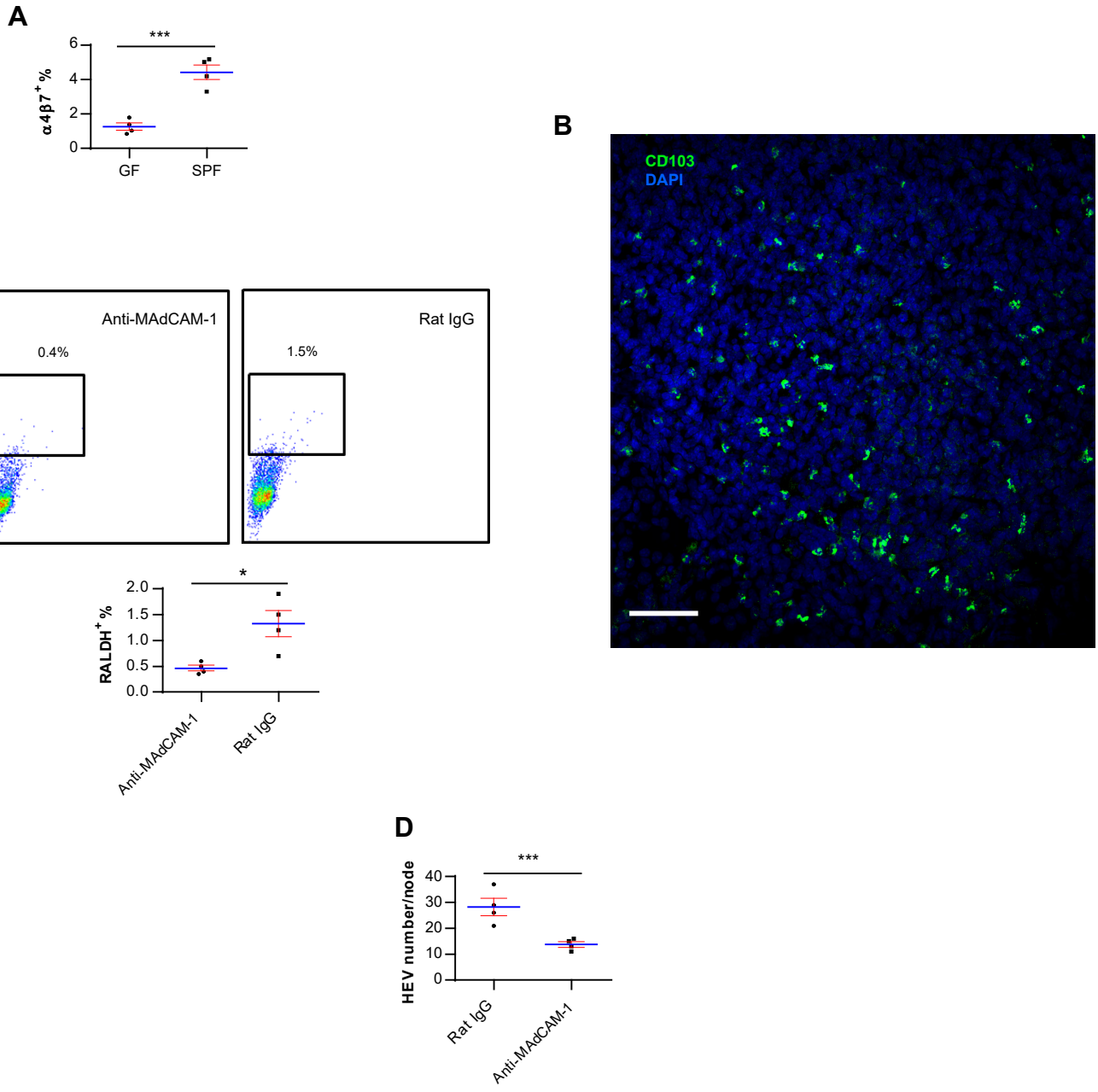

Figure S5 (related to Figure 5). Neo-mi DCs imprint pLN lymphocytes for gut homing

(A) Lymphocytes from iLN of GF and SPF mice were stained for α4β7 expression. The positive percentage values were graphed. **(B)** The detection of $CD103^+$ cells in iLN of 2 week-old *Rag1*$^{-/-}$ mice. **(C)** CFSE-labeled neo-mi DCs were i.v. injected into anti-MAdCAM-1-pretreated or control 2 week-old SPF mice. 24 hrs later, the presence of these cells in iLN was analyzed by FACS. The lower panel shows the result from multiple assays. **(D)** Related to Figure 5C. Operations were identical to Figure 5A with anti-MAdCAM-1 injection starting from the birth. HEV numbers from 2 week-old iLN were counted. Rat Ig was used as control.

Figure S6: related to Figure 7

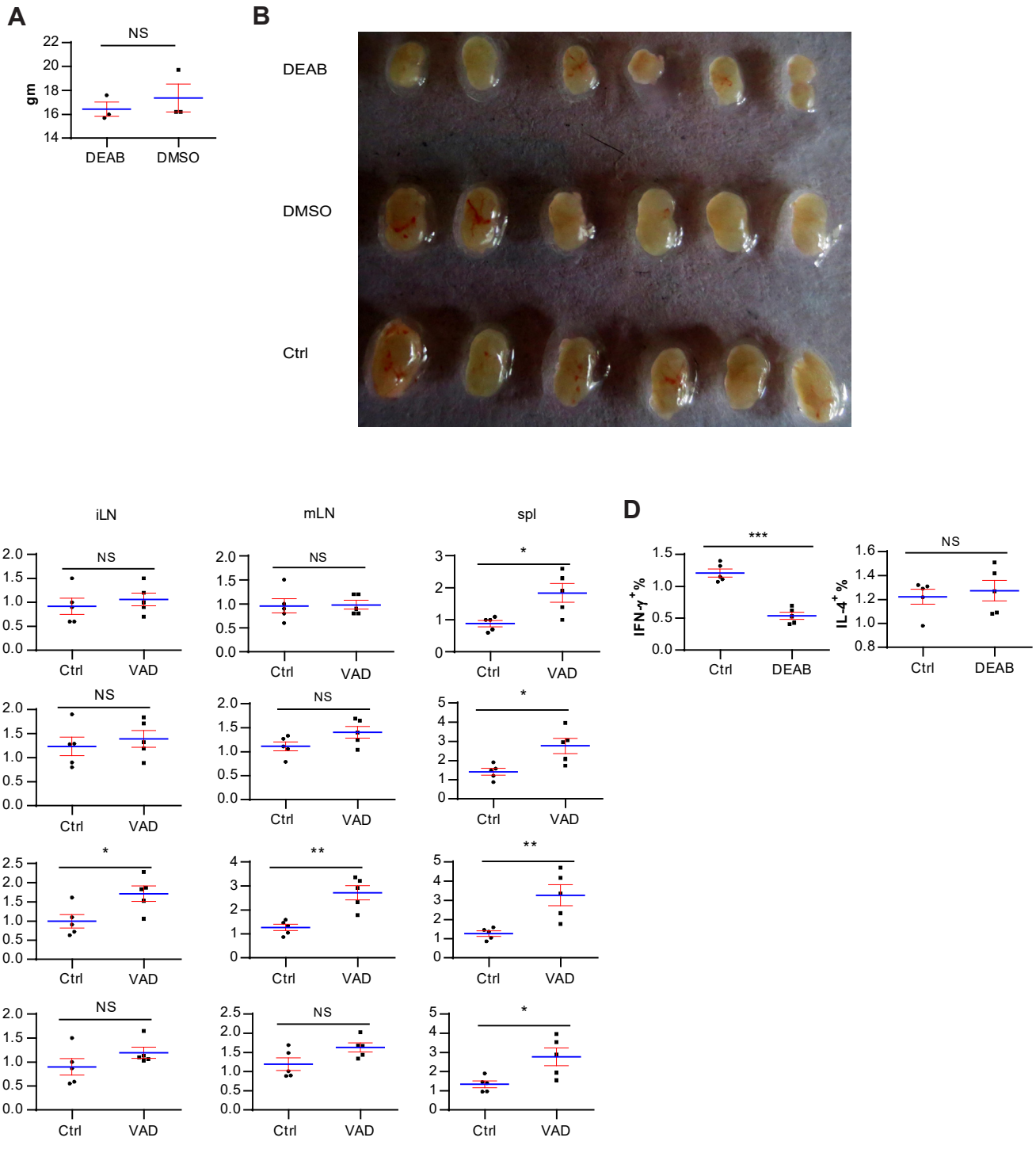

Figure S6 (related to Figure 7). Vitamin A deficiency is associated with the lack of tonal homing of neo-mi DCs to pLN

(A) The body weight of DEAB-treated SPF mice 6 days from the first dose (one dose every other day) in comparison with the untreated mice. **(B)** Sizes of iLN 6 days into DEAB treatment; untreated and DMSO-treated are displayed as controls. **(C)** CFSE-labeled total lymphocytes from the pooled pLN of VAD and control fed mice were i.v. injected into 5 week-old SPF mice; their and the $CD4^+$, $CD8^+$, $CD19^+$ subset percentages in iLN, mLN and the spleen were determined after 24 hrs. **(D)** 5 week-old SPF mice were i.p. injected with DEAB 3 times once every other day, or received one injection and then rested for two day (to induce a LN structural attrition as in the 3 injection group, yet allowing $RALDH^+$ cells to recover). The iLN were isolated and single cell suspension was stimulated with plate-bound anti-CD3. 24 hrs later, intracellular IFN-γ^+ and IL-4^+ $CD4^+$ cells were analyzed by FACS and the percentage values are shown.

Supplemental Data S1. Related to Figure 1. RNAseq results of GF vs SPF C57BL/6 mice.

The total dataset for the RNAseq described in Figure 1, with statistical analyses provided in the table

Supplemental Data S2. Related to Figure 2. RNAseq results of GF vs SPF ICR mice.

Similar to Supplemental Data S1 with data from ICR mice.