

Supplementary Information for

**IL-23 Activates Innate Lymphoid Cells to Promote
Neonatal Intestinal Pathology**

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Supplementary Procedure

Supplementary Figure S1

Supplementary Figure S2

Supplementary Figure S3

Supplementary Figure S4

Supplementary Figure S5

Supplementary Figure S6

Supplementary Figure S7

Supplementary Figure S8

Supplementary Figure S9

Supplementary Figure S10

Supplementary Figure S11

Supplementary Figure S12

Supplementary Figure S13

Supplementary Procedure

Antibodies. For flow cytometry, the following fluorochrome-conjugated anti-mouse antibodies were used: Thy-1.2 (53-2.1), Sca-1 (D7), CD45 (30-F11), MHC Class II (M5/114.15.2), CD11b (M1/70), CD11c (N418), CD3e (145-2C11), CD45R (B220) (RA3-6B2), CD127 (A7R34), CD4 (GK1.5), Ki-67 (SolA15), ROR γ t (AFKJS-9), CD152 (CTLA-4)(UC10-4B9), CD335 (NKp46) (29A1.4), CD44 (IM7), CD25 (PC61.5), CD117 (2B8), IL-22 (1H8PWSR), IL-17A (eBio17B7), IFN- γ (XMG1.2), GM-CSF (MP1-22E9), CD282 (TLR2) (6C2) were from eBioscience; lineage cocktail (CD3, B220, CD11b, Gr-1, Ter119 $^{-}$) (145-2C11, RA3-6B2, M1/70, RB6-8C5, TER-119), Gr-1(RB6-8C5) were from BD Biosciences; CCR6 (FAB590P) was from R&D Systems; CD218a (IL-18R α) (BG/IL18RA) and CD195(CCR5) (HM-CCR5) were from Biolegend. Streptavidin PE-Cyanine7 was from eBioscience. For immunofluorescence staining, purified anti-mouse IL-22 rabbit polyclonal antibody and anti-mouse S100A9 (2B10) were from Abcam; anti-mouse ICAM-1 (3E2) was from BD Biosciences; anti-mouse Thy-1 (G7) was from eBioscience. Alexa Fluor 488-conjugated Donkey antibody to rabbit IgG (A21206), Alexa Fluor 488-conjugated Donkey antibody to goat IgG (A11058), Alexa Fluor 488-conjugated goat antibody to rat IgG (A11006), and Alexa Fluor 594 Streptavidin conjugates (S-32356) were from Invitrogen. Alexa Fluor 594-conjugated goat antibody to Armenian hamster IgG (127-175-160) was from Jackson.

Antibiotic treatment. For eradication of intestinal bacterial flora in newborn V23 mice, the parental mice (V19 and V40 mice) were treated with a 'cocktail' of

antibiotics containing ampicillin 1g/L, metronidazole 1g/L, neomycin 1g/L, and vancomycin 0.5g/L (Sigma Aldrich) in the drinking water starting after birth and continuing until give birth to V23 mice. Antibiotic treatment was renewed every week. Newborn V23 mice were analyzed at the age of P4.

Flow cytometry and sorting. The small intestine, large intestine, and mesenteric lymph nodes of embryonic and P0 mice were micro-dissected using a stereo microscope and further digested with 2 mg/ml collagenase D (Roche) Cell suspension was passed through a 70- μ m cell strainer and mononuclear cells were isolated. All cells were first pre-incubated with anti-mouse CD16/CD32 for blockade of Fc γ receptors, then were washed and incubated for 40 min with the appropriate monoclonal antibody conjugates in a total volume of 200 μ l PBS containing 2 mM EDTA and 2% (vol/vol) bovine serum. Propidium iodide (Sigma) or DAPI (Invitrogen) was used to distinguish live cells from dead cells during cell analysis and sorting. Stained cells were analyzed on a FACS Canto or LSR II machine using Diva program (BD Bioscience) or purified with a MoFlo Astrios cell sorter (DakoCytomation). Cells were > 98% pure after sorting. Data were analyzed with FlowJo software (TreeStar).

Cell cultures. All cultures were performed in DMEM (Gibco) supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μ g/ml streptomycin, at 37°C and in 5% CO₂. Mouse lymphocytes isolated from the tissues/sorted cells (1×10^6 / 1×10^4 ml) were cultured in 48-well/ 96-well plates in DMEM medium. Cells were

analyzed after stimulated with recombinant mouse IL-23 (R&D Systems) at 10ng/ml or vehicles as control for 72 hr.

Cell stimulation and intracellular staining. Intracellular cytokine expression was measured after cells were stimulated for 6 hr with phorbol 12-myristate 13-acetate (PMA) (50 ng/ml), and ionomycin (1 µg/ml, both from Sigma-Aldrich) in the presence of monensin (2µM) (eBioscience) for the final 4 hr at 37°C culture. For detection of IL-22, IL-17, GM-CSF, IFN-γ and CTLA4, cells were fixed and permeabilized according to manufacturer's protocol (eBioscience). For detection of intracellular RORγt, a Foxp3 Staining Buffer Set (eBioscience) was used for fixation and permeabilization of the cells.

Enzyme-linked immunosorbent assay. IL-23 was quantified in intestinal homogenates from V23 and WT mice by enzyme-linked immunosorbent assay (ELISA) according to standard manufacturer's recommendations (Biolegend). IL-22 was quantified in supernatants of sorted cells (1×10^4 cells) that were cultured for 72 hr in the presence or absence of IL-23 (20ng/ml, R&D Systems) by ELISA according to standard manufacturer's recommendations (eBioscience).

Histology. Tissue was dissected, fixed in 10% phosphate-buffered formalin and then processed for paraffin sections. Five-micrometer sections were stained with hematoxylin and eosin.

Transmission electron microscopy. Representative small intestinal tissue specimens from mice were removed from the animal and minced with a double edged razor blade in a drop of glutaraldehyde into 1 - 2 mm³ cubes and then placed directly into a solution of 3% glutaraldehyde buffered with sodium cacodylate at pH 7.4. After 3 hr the tissue was placed in buffer and then subjected to routine embedding protocol consisting of graded steps of ethanol through propylene oxide and into Embed 812. One-micrometer plastic sections were cut, stained with methylene blue and azure II, and observed by light microscopy. Representative areas were identified; blocks trimmed and ultra thin sections were cut, stained with uranyl acetate and lead citrate and observed with H7650 TEM with digital imaging.

Immunofluorescence staining. Tissue samples were embedded in OCT buffer (Sakura) and snap frozen in 2-methylbutane (Merck) chilled in dry ice. Alternatively, tissues were fixed in 1.6% paraformaldehyde (Electron Microscopy Science) containing 20% sucrose for 20 h at 4°C. Cryostat sections (8 µm) were fixed in acetone, blocked, and incubated with primary Abs in a humidified atmosphere for 1 h at room temperature. After washing, conjugated secondary Abs were added and then incubated for 35 min. Cell nuclei have been stained using DAPI. The slides were next washed and mounted with Fluoromount-G (Southern Biotech). Images were captured using a Nikon fluorescence microscope.

***In vivo* antibody treatment.** To deplete Thy1⁺ cells in newborn V23 mice, pregnant mothers were administered intravenously at embryonic day E15.5, E16.5, E17.5 and E18.5 with 1 mg rat anti-Thy1 mAb or 1 mg isotype control mAb (both from BioXcell). After pups born, we continued to administrate 100 µg anti-Thy1 mAb or 100 µg isotype control per mouse i.p. everyday as shown in the figure.

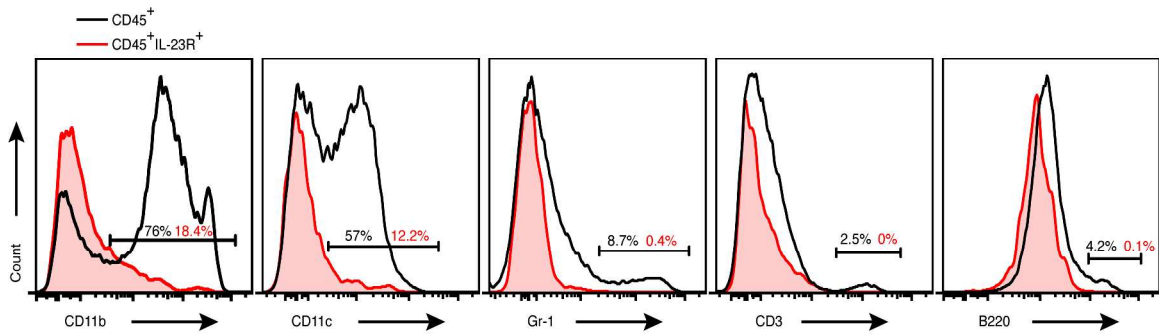
Laser-capture microdissection. Frozen tissue sections (10 µm in thickness) were cut under RNase-free conditions. On the day of microdissection, sections were stained with a HistoGene Frozen Section Staining Kit according to the manufacturer's protocol (Applied Biosystems). For dehydration, slides were incubated for 30s consecutively in 75%, 95% and 100% (vol/vol) ethanol and then for 5 min in xylene. After the dehydration procedure, sections were air-dried. Samples of intestine tissue were captured from the stained slides on Arcturus CapSure Macro LCM caps by using an ArcturusXT Microdissection microscope (Applied Biosystems).

Quantitative Real-time PCR. Total RNA was extracted from cells on CapSure Macro LCM caps (Applied Biosystems) with a PicoPure RNA isolation kit according to the manufacturer's protocol (Arcturus; Applied Biosystems). Total RNA from tissues/sorted cells was extracted using the RNeasy mini/micro Kit (Qiagen) according to the manufacturer's instructions. Complementary DNA (cDNA) was generated with Superscript III (Invitrogen). Quantitative PCR was performed using SYBR Green Dye (Roche) on the 7500 Real Time System

(Applied Biosystems) machine. Results were normalized to the housekeeping gene Ubiquitin. Relative expression levels were calculated as $2^{-(Ct(\text{Ubiquitin})-Ct(\text{gene}))}$. Primers were designed using primer express 2.0 software (System Applied Biosystems).

Barrier function assay. In brief, E18.5 embryos were dissected from the uterus, after which the abdominal cavity was opened. A 30-gauge needle was inserted into the upper portion of the duodenum near the junction with the stomach, and 0.2 ml of sulfo-NHS-biotin (1 mg/ml; Sigma) was injected into the lumen of the intestine. The embryos were incubated in a humidified chamber for 30 min at room temperature. The tissue was washed in PBS for 5 min, fixed overnight at 4°C with 4% paraformaldehyde and 20% sucrose in PBS, embedded in OCT compound, and sectioned. The sections were incubated for 1 h at room temperature with a 1:400 dilution of Alexa Fluor 594- conjugated streptavidin (Invitrogen) and then processed for fluorescence microscopy.

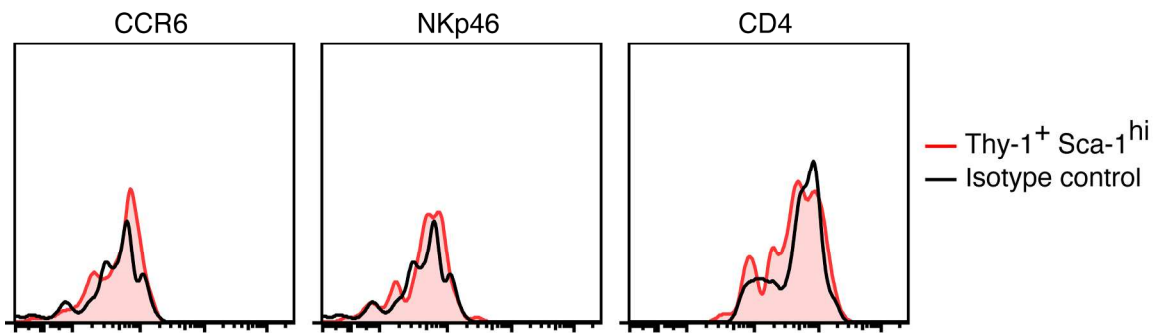
Supplementary Figures



Supplementary Figure S1:

IL-23R⁺ GFP expression in the gut of embryonic *Il-23r^{+/GFP}* mice.

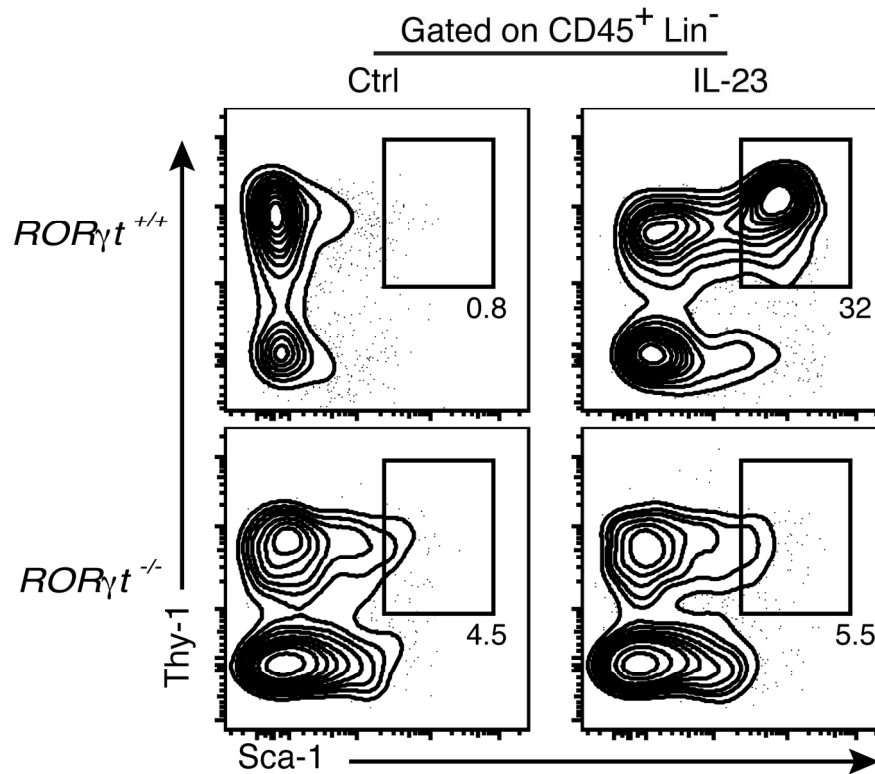
Flow cytometric analysis of IL-23R⁺ GFP expression in the leukocytes from the intestine of *Il-23r^{+/GFP}* mice at embryonic day E18.5.



Supplementary Figure S2:

Characterization of CD45⁺Lin⁻Thy1⁺Sca-1^{hi} population induced by IL-23 stimulation *in vitro*.

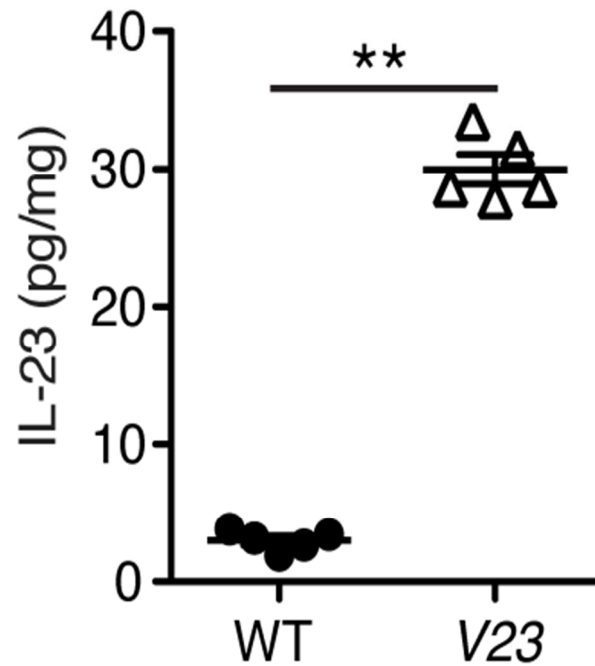
In vitro stimulated CD45⁺Lin⁻ leukocyte subpopulations from the intestine of *Il-23r^{+/+}* mice at embryonic day E18.5 with IL-23 (10ng/ml) for 72 hr. Cells were stained with indicated markers or isotype control. Histograms are electronically gated on CD45⁺Lin⁻Thy1⁺Sca-1^{hi} population.



Supplementary Figure S3:

Development of IL-23 responsive Thy1⁺Sca-1^{hi} ILC3s requires ROR γ t.

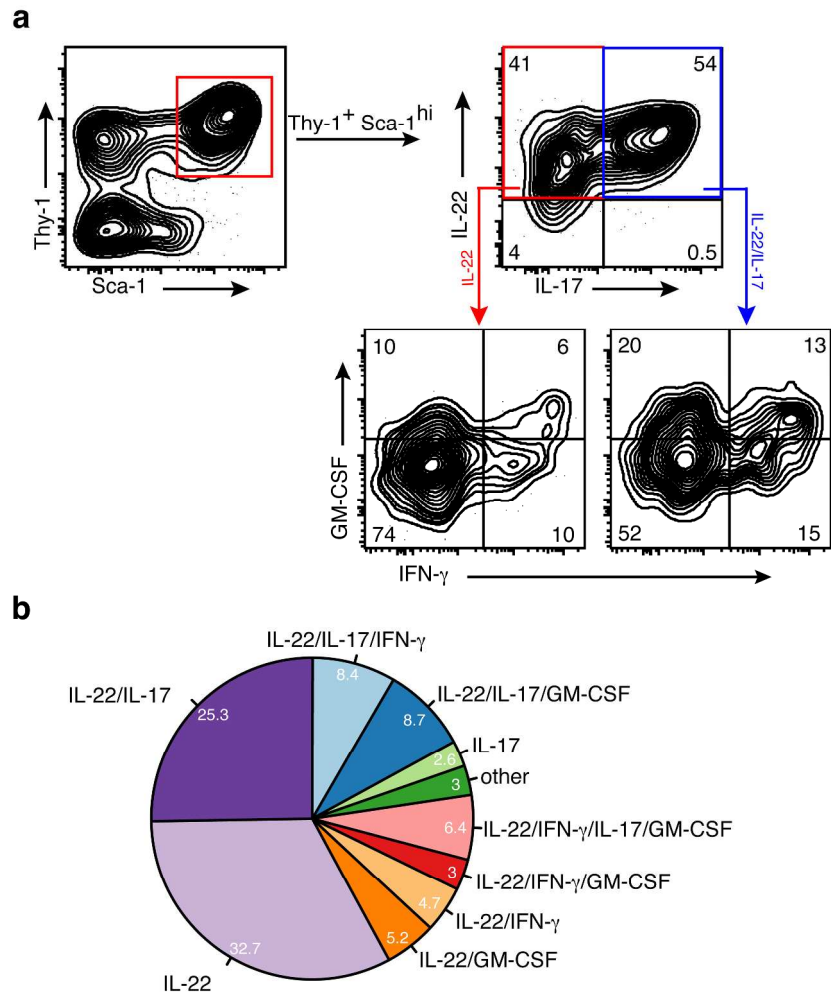
In vitro stimulated CD45⁺Lin⁻ leukocyte subpopulations from the intestine of *Rorc*(γ)^{+/+} and *Rorc*(γ)^{-/-} mice at embryonic day E18.5 with IL-23 (10ng/ml) or vehicle control for 72 hr. Representative flow cytometry plots showing CD45⁺Lin⁻ Thy1⁺Sca-1^{hi} population after culture of three independent experiments.



Supplementary Figure S4:

ELISA analysis of IL-23 expression level in the small intestine.

ELISA assay of IL-23 in the intestinal homogenates from WT and V23 mice at P0. Data are shown as means \pm s.e.m., $n = 5$ per group. ** $P < 0.01$, nonparametric Mann-Whitney test. Data are representative of two independent experiments with similar results.

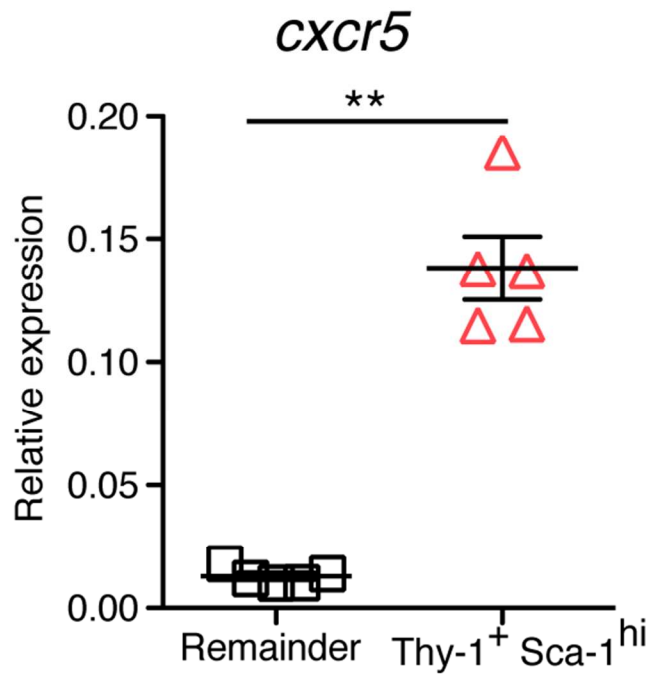


Supplementary Figure S5:

FACS analysis of cytokine expression pattern in Thy1⁺Sca-1^{hi} ILC3s.

(a) Intracellular staining analysis of IL-22, IL-17, IFN- γ and GM-CSF expression in Thy1⁺Sca-1^{hi} ILC3s present in the small intestine of V23 mice at P0.

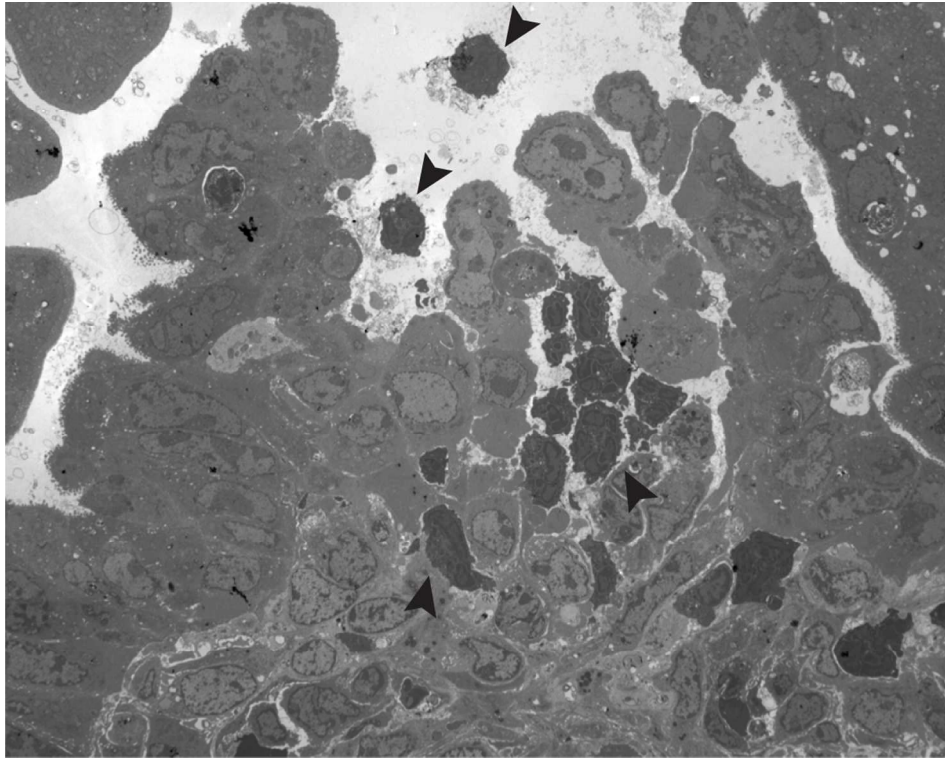
(b) Pie chart summarizing the proportion of cytokine expression pattern (%) in Lin⁻Thy1⁺Sca-1^{hi} ILC3s in the V23 mice. Data are representative of at least three independent experiments.



Supplementary Figure S6:

Quantitative RT-PCR analysis of *Cxcr5* mRNA expression in the Thy1⁺Sca-1^{hi} ILC3s.

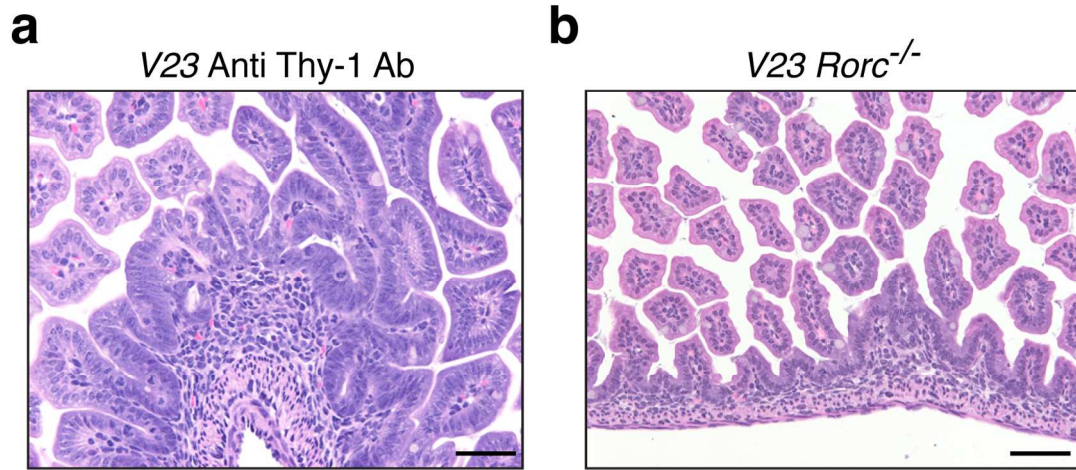
Thy1⁺ Sca-1^{hi} cells and remainder Lin⁻ cells are indicated in Figure 4C. Data are shown as means ± s.e.m., n = 5 per group. ** $P < 0.01$, nonparametric Mann-Whitney test. Data are representative of two independent experiments with similar results.



Supplementary Figure S7:

Transmission electron micrograph of the lesions in the small intestine.

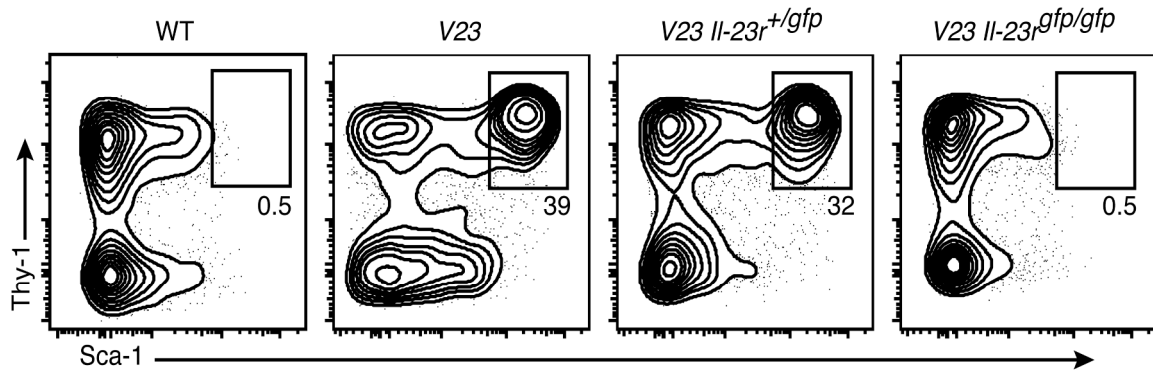
Transmission electron micrograph of the lesions from the small intestine at P0 shows neutrophils disrupting the adjacent epithelium. Representative data from three small intestines with twenty sections for each small intestine from three independent experiments are shown. Arrows indicate neutrophils migrated from the lamina propria to the lumen and disrupted the epithelium.



Supplementary Figure S8:

Representative H&E staining of small intestine from V23 receiving anti-Thy1 antibody and *V23Rorc*^{-/-} mice.

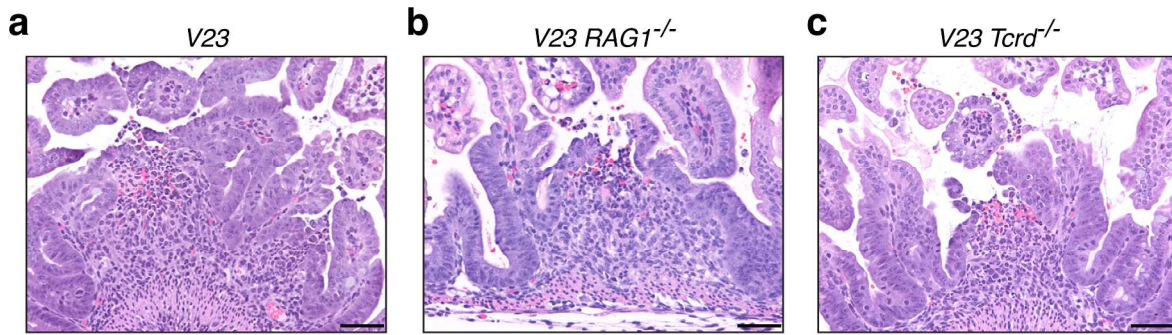
Representative H&E stained section of the small intestine of V23 receiving anti-Thy1 antibody (a) and *V23Rorc*^{-/-} (b) mice at P0. Scale bars, 100 μ m.



Supplementary Figure S9:

IL-23-IL-23R signaling is required for $Thy1^+Sca-1^{hi}$ ILC3s development *in vivo*.

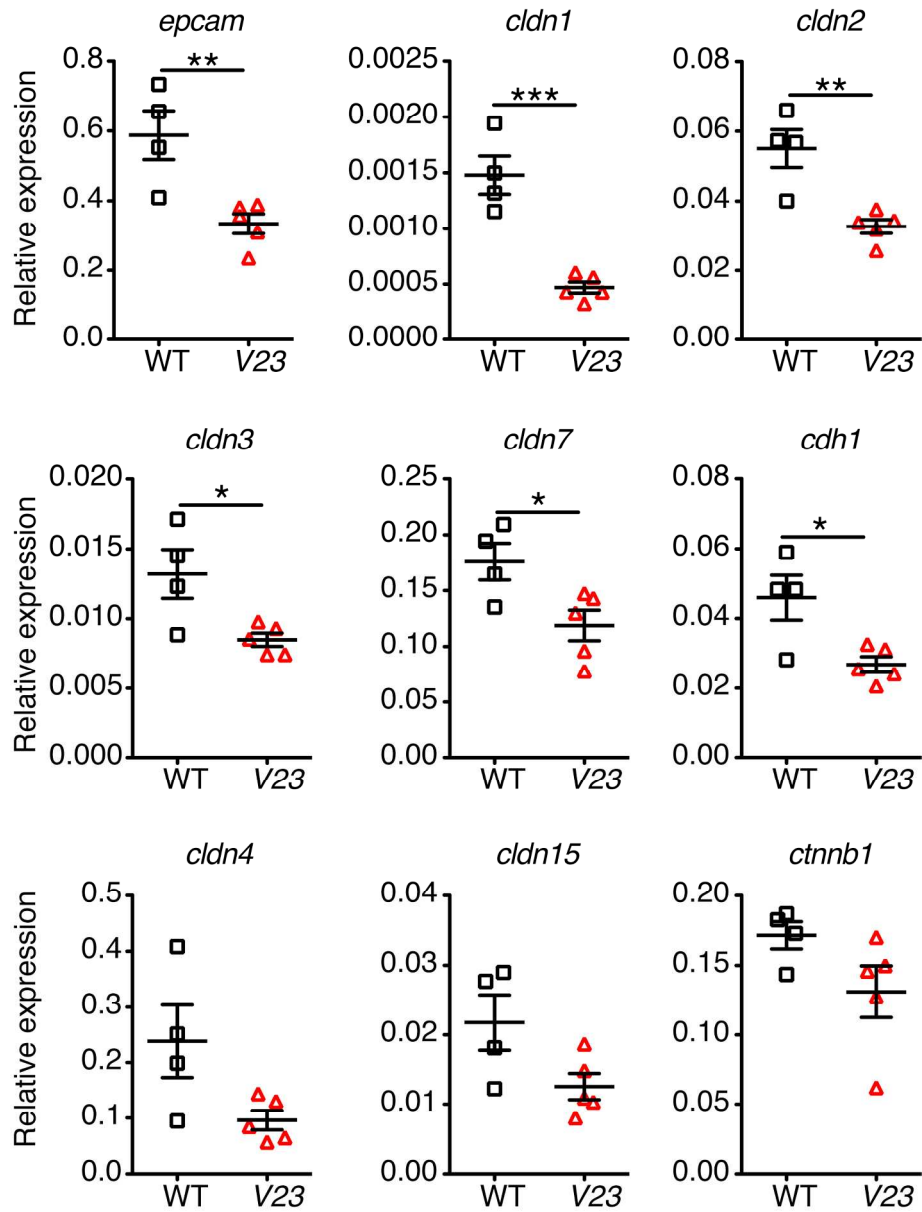
Flow cytometry plot showing the proportion of $Lin^-Thy1^+Sca-1^{hi}$ cells in the small intestine from V23, *Il-23r^{+/gfp}*, and V23 *Il-23r^{gfp/gfp}* mice at P0. Dot plots show cells gated on $CD45^+Lin^-$. Data are representative of three independent experiments.



Supplementary Figure S10:

Representative H&E staining of small intestine from *V23RAG1^{-/-}* and *V23 Tcrd^{-/-}* mice.

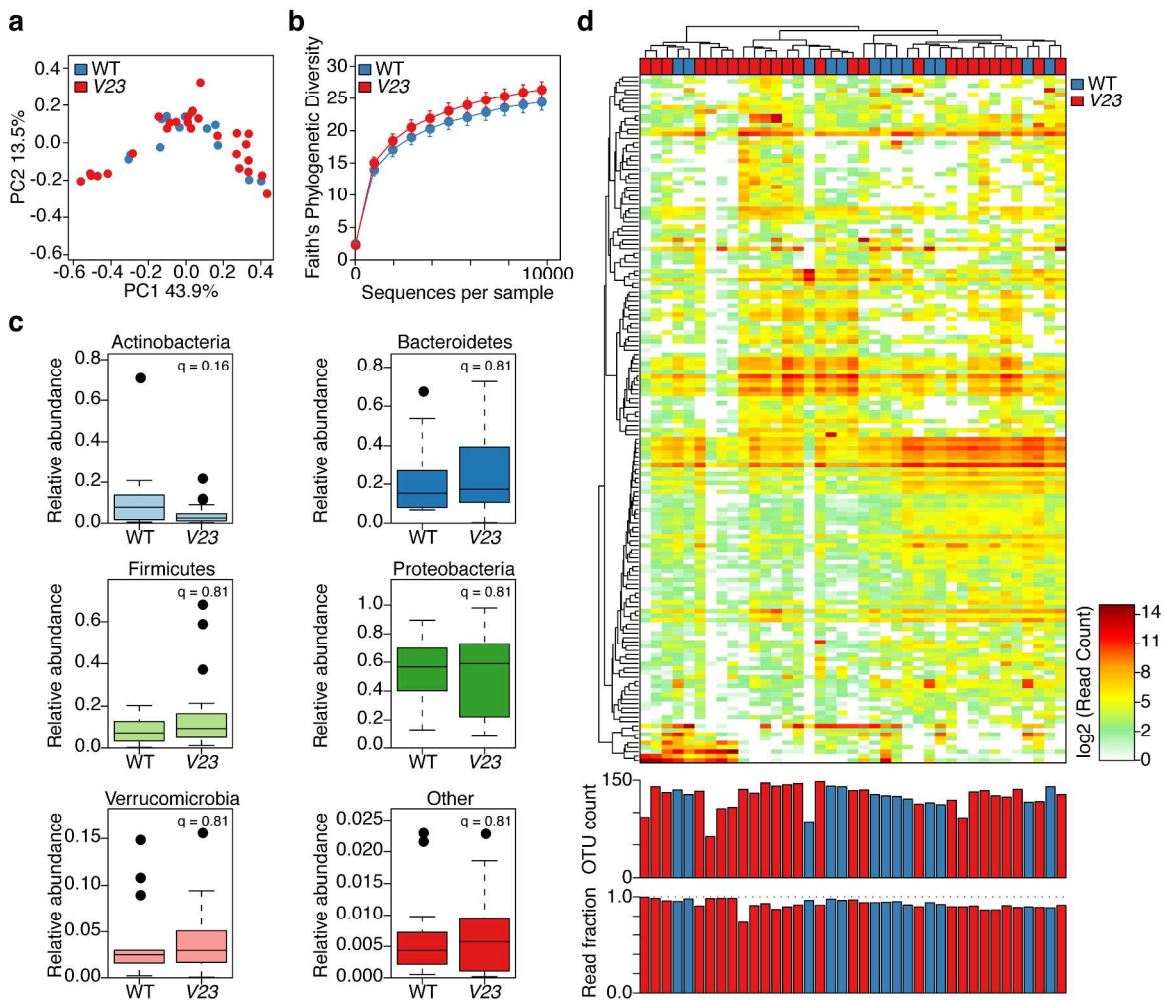
Representative H&E stained section of the small intestine of *V23* (a), *V23RAG1^{-/-}* (b) and *V23 Tcrd^{-/-}* (c) mice at P0. Scale bars, 100 μ m.



Supplementary Figure S11:

Junction associated genes transcription levels in sorted epithelial cells.

Relative expression levels of junction associated genes in sorted CD45⁻ cells from the SI of WT and V23 at E18.5. Data are shown as means \pm s.e.m., $n = 4-5$ per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, nonparametric Mann-Whitney test.

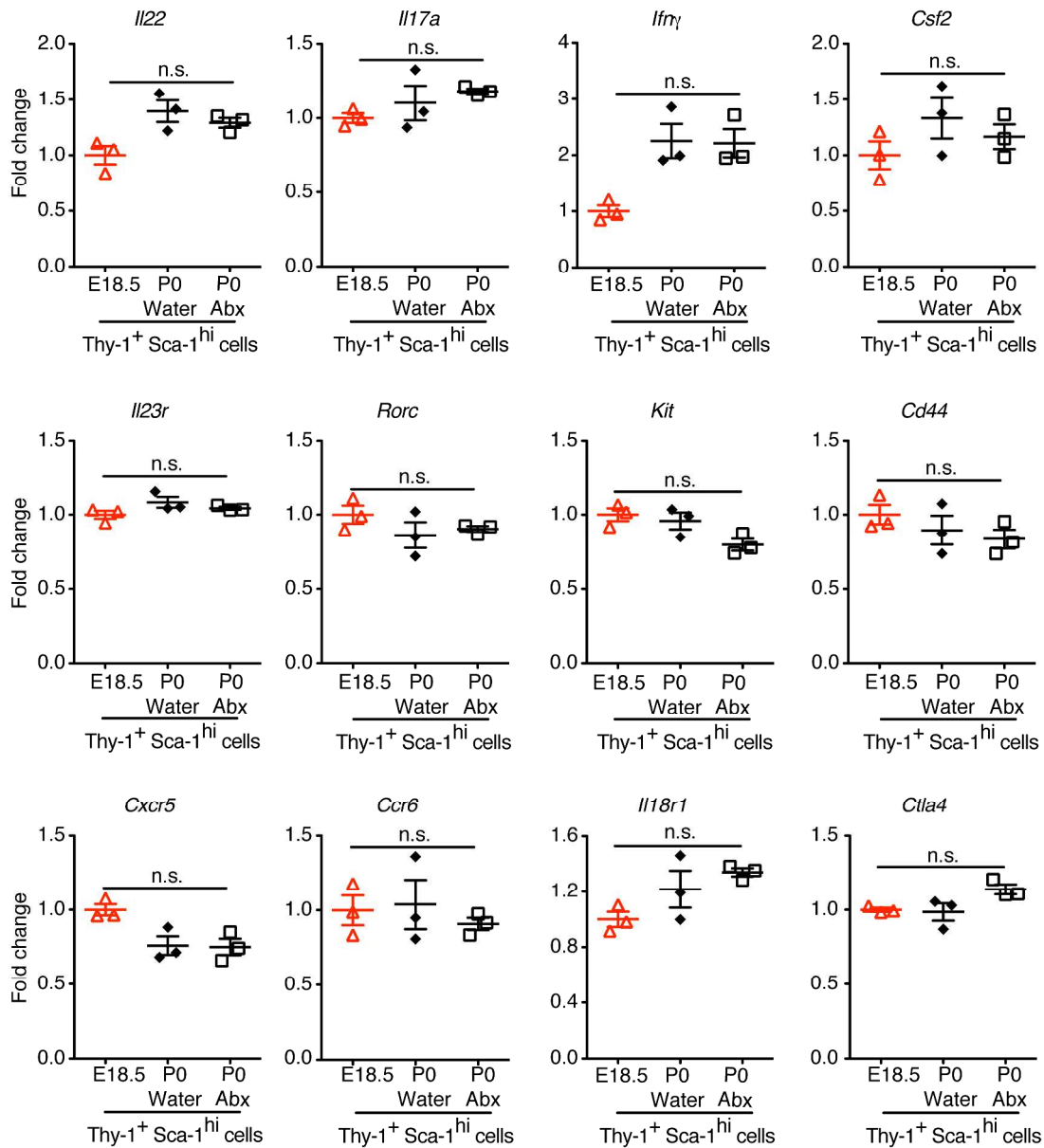


Supplementary Figure S12:

Microbiota of V23 mice are not different from WT mice.

The microbiome of V23 mice ($n = 26$) and WT ($n = 13$) littermate controls at P0 were analyzed by 16S rDNA sequencing. (a) No clustering of V23 and WT littermate controls in Weighted Unifrac analysis. (b) Similar alpha diversity in WT and V23 mice. (c) No significant difference in the relative abundance of the phyla present in V23 mice and WT littermates. The most abundant phyla are shown; low abundant ($< 1\%$) phyla were grouped in 'Other'. (d) Hierarchical clustering of the abundance profiles of all 156 OTUs detected after filtering (top). Rows in the heatmap represent individual OTUs. None of these OTUs showed a significant

change in abundance between WT and V23 mice ($q < 0.05$, fold > 1.5). Bar plots (bottom) represent the number of OTUs above in the individual samples and fraction of total sample reads accounted for by the set of 156 filtered OTUs. Sample groups are colored according to the legend.



Supplementary Figure S13:

Quantitative RT-PCR analysis of ILC3s signature genes expression in the sorted $\text{Thy-1}^+ \text{Sca-1}^{\text{hi}}$ ILC3s.

ILC3s signature gene expression of sorted $\text{Lin}^- \text{Thy-1}^+ \text{Sca-1}^{\text{hi}}$ cells from the small intestine of non-treated (water) and antibiotic treated (Abx) neonatal V23 mice as well as prenatal V23 mice. Data are calculated as fold change over ILC3s from

E18.5 V23 mice. Data are shown as means \pm s.e.m., n = 3 per group. N.s., not significant, nonparametric Mann-Whitney test.