

SUPPLEMENTARY INFORMATION

Online-only Methods**Mice**

I29SvEvRag2^{-/-} mice and wild-type C57BL/6, control C57BL/6 *Rag1^{-/-}* and IL-17 deficient mice on a C57BL/6 *Rag1^{-/-}* background (IL-17^{-/-} *Rag^{-/-}* and *Rorc^{-/-} Rag^{-/-}*) were bred and maintained under specific pathogen-free conditions in accredited animal facilities at the University of Oxford. Experiments were conducted in accordance with the UK Scientific Procedures Act of 1986. *Rorc*-deficient mice were kept in specific pathogen-free (SPF) conditions at the animal facility of the Skirball Institute. All animal experiments were performed in accordance with approved protocols for the NYU Institutional Animal Care and Usage Committee.

Quantitation of *H. hepaticus* using real-time PCR

DNA was purified from cecal contents taken from *H. hepaticus*-infected mice using the DNA Stool kit (QIAGEN). *H. hepaticus* DNA was determined using a Q-PCR method based on the *cdtB* gene and performed with a Chromo4 detection system, as previously described¹⁵.

Cell preparation, isolation and FACS sorting

Bone marrow derived dendritic cells (DCs) have been generated as previously described²⁸. Colonic lamina propria (cLP) cells were purified as described in Method Summary. Cells were stained with a combination of α -CD45.2, α -CD11b, α -Ly6C/G

(GR1), α -B220, α -Thy1.2 and α -Ly6A/E antibodies (all from BD Pharmingen) and sorted on a Moflo sorter (DakoCytomation).

Induction of colitis

H. hepaticus NCI-Frederick isolate 1A (strain 51449; American Type Culture Collection) was grown on blood agar plates containing trimethoprim, vancomycin, and polymyxin B (all obtained from Oxoid) under microaerophilic conditions as previously described¹⁵. For *H. hepaticus* infections, bacterial viability was confirmed using fluorescent microscopy with a bacterial live/dead kit (BacLight; Invitrogen), and *129SvEvRag2^{-/-}* mice were infected by oral gavage three times on alternate days with $\sim 5 \times 10^7$ – 2×10^8 CFU *H. hepaticus*. Mice were sacrificed 8 weeks after the first *H. hepaticus* inoculation. Colitis was induced in C57BL/6 mice by infecting with *H. hepaticus* as described above and i.p. injection of 1mg 1B1.2 (α -IL10R) mAb on days 0, 7, 14, and 21 after *H. hepaticus* infection¹⁶. 1week after the last mAb treatment, mice were sacrificed.

To induce acute innate colitis, C57BL/6 *Rag^{-/-}* were administered 100 to 200 μ g of α -CD40 IgG2a mAbs (FGK45) via i.p. injection¹⁷. Mice were sacrificed 7 days post α -CD40 mAb treatment and weight loss was monitored throughout the course of the experiment.

Cytokine detection and intracellular staining

For *in vitro* experiments, (1×10^5 – 3×10^5) spleen or cLP cells we cultured overnight in 100 μ l complete RPMI with or without recombinant IL-12 and IL-23 at a concentration of

10ng/ml. IFN- γ , IL-17, IL-22, TNF- α , MCP-1 and IL-6 levels in culture supernatants were measured using Cytokine Bead Assay (Bender MedSystems) and cytokine concentration was normalized to cell number. For intracellular staining, Brefeldin A (10 μ g/ml, eBiosciences) was added for the last 4 hours of the overnight cultures described above and surface and cytoplasmic stainings were performed as described previously²⁹. Labeled cells were analyzed on a Cyan Flow Cytometer™ (DakoCytomation) using FlowJo™ software (Tree star).

***In vivo* antibody treatment**

To block specific cytokine activity *in vivo*, *H.hepaticus* infected 129SvEvRag2^{-/-} mice received 0.375 mg IL-17 blocking mAb (UCB Celltech), 1 mg of IFN- γ blocking mAb (clone AN18) or 0.375 mg isotype control mAb starting the day of the first inoculation with of *H.hepaticus*. Blocking antibodies were injected i.p. twice per week for the duration of the experiment. To deplete Thy1⁺ cells in the α -CD40 acute colitis model, C57BL6 Rag1^{-/-} mice were administered 1mg of rat α -Thy1 depleting mAb (YTS 154.7.7.10) or the rat isotype control (YKIX 337.217.1) at day -3, 0 and 3. For the depletion of Thy1⁺ cells in the chronic innate model of colitis induced by *H. hepaticus*, 1 mg of α -Thy1 depleting mAb or isotype control was injected i.p once a week starting the day of the first *H.hepaticus* infection.

Quantification of mRNA levels by Real-time PCR

RNA was purified from frozen tissue samples or sorted cells using RNAeasy kits (QIAGEN). Homogenization was performed using a MP Biomedicals Homogenizer.

RNA purity and quantification was determined using a Nanodrop spectrophotometer (Nanodrop Technologies). cDNA synthesis was performed using a reverse transcriptase kit (Superscript III) with Oligo dT (both obtained from Invitrogen). Q-PCR reactions were performed using the following primers, together with FAM/TAMRA- or VIC/TAMRA-labeled probes: HPRT primers, 5'-GACCGGTCCCGTCATGC-3' and 5'-TCATAACCTGGTTCATCATCGC-3', and probe, 5'-ACCCGCAGTCCCAGCGTCGTC-3'; IL-23p19 primers, 5'-AGCGGGACATATGAATCTACTAAGAGA-3' and 5'-GTCCTAGTAGGGAGGTGTGAAGTTG-3', and probe, 5'-CCAGTTCTGCTTGCAAAGGATCCGC-3'; IL-12p40 primers, 5'-GACCATCACTGTCAAAGAGTTTCTAGAT-3' and 5'-AGGAAAGTCTTGTTTTTGAATTTTTTAA-3', and probe, 5'-CCACTCACATCTGCTGCTCCACAAGAAG-3'; IL-17A primers, 5'-GCTCCAGAAGGCCCTCAG-3' and 5'-CTTTCCCTCCGCATTGACA-3', and probe, 5'-ACCTCAACCGTTCCACGTCACCTG-3'; *Rorc* primers, 5'-CCGCTGAGAGGGCTTCAC-3' and 5'-TGCAGGAGTAGGCCACATTACA-3', and probe, 5'-AAGGGCTTCTTCCGCCGAGCCAGCAG-3'; IL-23R primers, 5'-ATGGTCTTGGGTACAGTATCG-3' and 5'-CCATCTGGATGATATAGTGATA-3' and probe, 5'-CGTCCATCATTTCCAGGGTG-3'; *Tbx21* and *AHR* gene expression were assessed using an Applied Biosystems TaqMan® Gene Expression Assay. Qiagen SYBR Green-based gene Expression Assay was used for detection of the following genes: *LT α* , 5'-GTACCCAACAAGGTGAGCAGC-3' and 5'-CCAGGACAGCCCATCCACT-3'; *LT β* , 5'-ACGCTTCTTCTTGGCTCGC-3' and 5'-

ACCTCATAGGCGCTTGGATG-3'; TRANCE, 5'-CGCTTCCCGATGTTTCATG-3'
and 5'-GGTTAACCAAGATGGCTTCTATTACC-3'; CXCR5, 5'-
CCTCGACTGTAGAGCAGAAGTTACTG-3' and 5'-
ATATGGATGACCTGTACAAGGAACTG-3'. Qiagen QuantiTect pre-designed primers
were used for detection of HPRT gene expression.

Assessment of intestinal inflammation

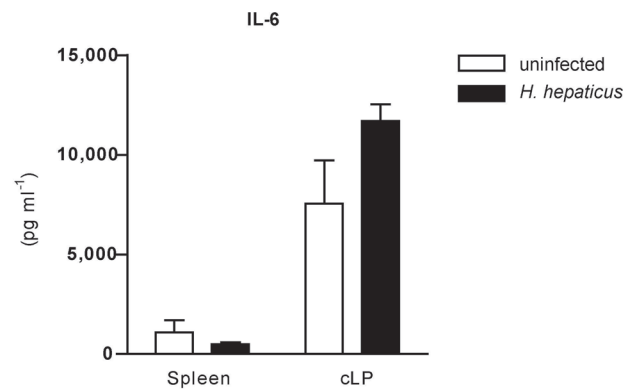
Mice were sacrificed when symptoms of clinical disease (weight loss or diarrhoea) became apparent in control groups, usually 6–8 wk after initiation of experiments for the *H.hepaticus* chronic innate colitis and 1 week for the α -CD40 driven acute colitis. Samples of the proximal colon (α -CD40 colitis) or the cecum, and proximal, mid-, and distal colon (*H.hepaticus* thyphlocolitis) were taken and immediately fixed in buffered 10% formalin (3.6% w/v formaldehyde). 4–5 μ m paraffin-embedded sections were stained with H&E, and inflammation was assessed as previously described³⁰. Each sample was graded semiquantitatively from 0 to 4, and typical features of each grade are as follows: 0 = normal; 1 = mild epithelial hyperplasia; 2 = pronounced hyperplasia with substantial leukocytic infiltrates; 3 = severe hyperplasia and infiltration with marked decrease in goblet cells; and 4 = severe hyperplasia, severe transmural inflammation, ulceration, crypt abscesses, and severe depletion of goblet cells. Cecae and colons were assessed separately, and three separate colon sections from each sample were examined. Scores for each criterion were added to give an overall inflammation score for each sample of 0–12. The total colonic score was calculated as the average of the individual scores from the sections of proximal colon, mid-colon, and distal colon for the *H*

hepaticus model. In the graphs shown, each point corresponds to an individual mouse. Micrographs show sections of mid-colon and ceacum for the *H.hepaticus* model and proximal-colon for the α -CD40 model.

Immunofluorescence

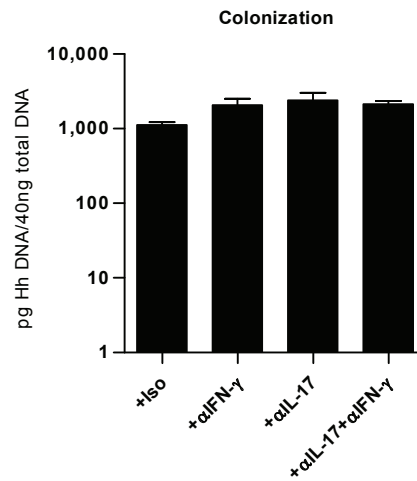
Cryosections were fixed in 4% paraformaldehyde. Endogenous peroxidase was inhibited using 3% H₂O₂ as well as 2% sodium azide. After blocking with 10% normal goat serum, sections were incubated with Armenian hamster anti-CD3 (clone 145-2C11, eBioscience) followed by goat anti-Armenian hamster-POD (Jackson ImmunoResearch Laboratories) and tyramide signal amplification (PerkinElmer). After this staining, peroxidase was inhibited as described above. Thy1.2 positive cells were stained using FITC labelled Thy1.2 (clone 53-2.1, eBioscience) followed by peroxidase labelled mouse anti-FITC IgG (Jackson ImmunoResearch Laboratories). Alternatively, anti-Thy1.2 staining (clone 53-2.1, eBioscience) was performed followed by incubation with peroxidase labelled donkey anti-rat (Jackson ImmunoResearch Laboratories, multiple species absorbed). Tyramide signal amplification (PerkinElmer) has been performed. Cell nuclei have been stained using DAPI.

28. Lutz, M.B. *et al.* An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J Immunol Methods* **223**, 77-92 (1999).
29. Openshaw, P. *et al.* Heterogeneity of intracellular cytokine synthesis at the single-cell level in polarized T helper 1 and T helper 2 populations. *J Exp Med* **182**, 1357-1367 (1995).
30. Izcue, A. *et al.* Interleukin-23 restrains regulatory T cell activity to drive T cell-dependent colitis. *Immunity* **28**, 559-570 (2008).



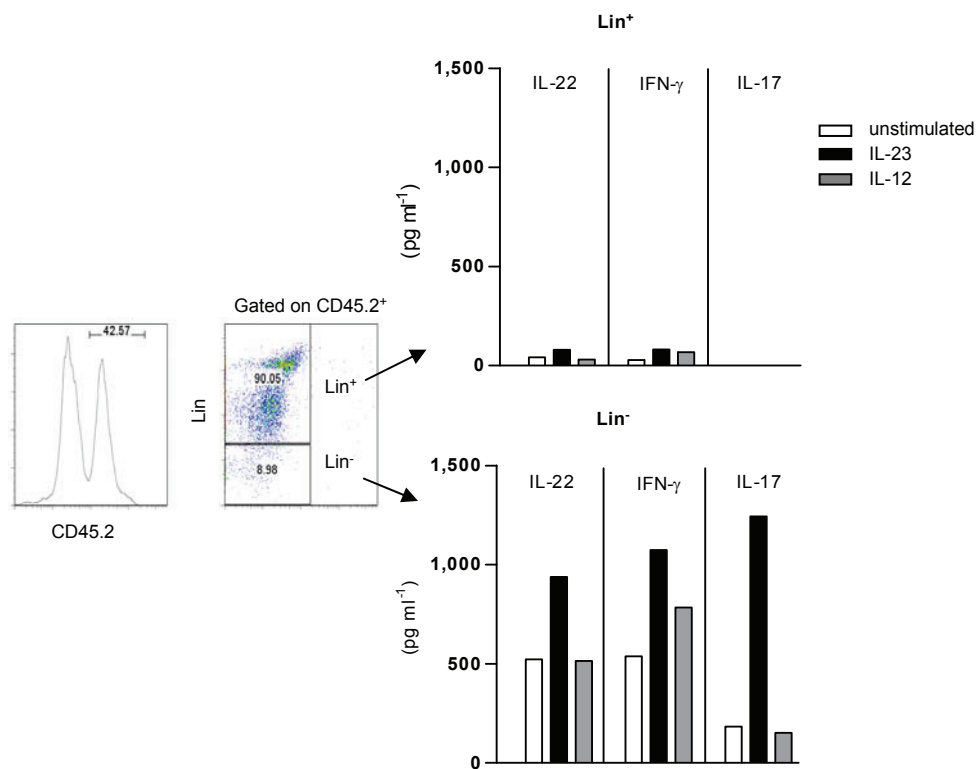
Supplementary figure 1: No induction of IL-6 following *H. hepaticus* infection.

IL-6 secretion following overnight culture of splenocytes or cLP cells from control or *H. hepaticus*-infected 129SvEvRag^{-/-} mice. Data represents mean \pm s.e.m. (n=6).



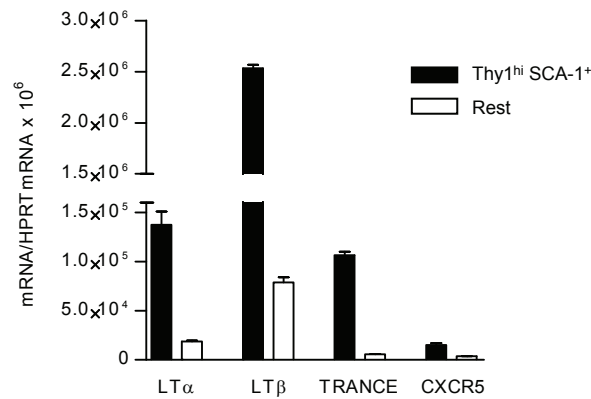
Supplementary figure 2: IL-17 and IFN- γ blockade does not affect *H.hepaticus* colonization.

Real time PCR quantification of *H. hepaticus* DNA in cecal contents of *H. hepaticus* infected 129SvEvRag^{-/-} mice treated with α -IL-17 and/or α -IFN- γ blocking antibodies or isotype control. Bars represent the mean \pm s.e.m. (n=5-6).



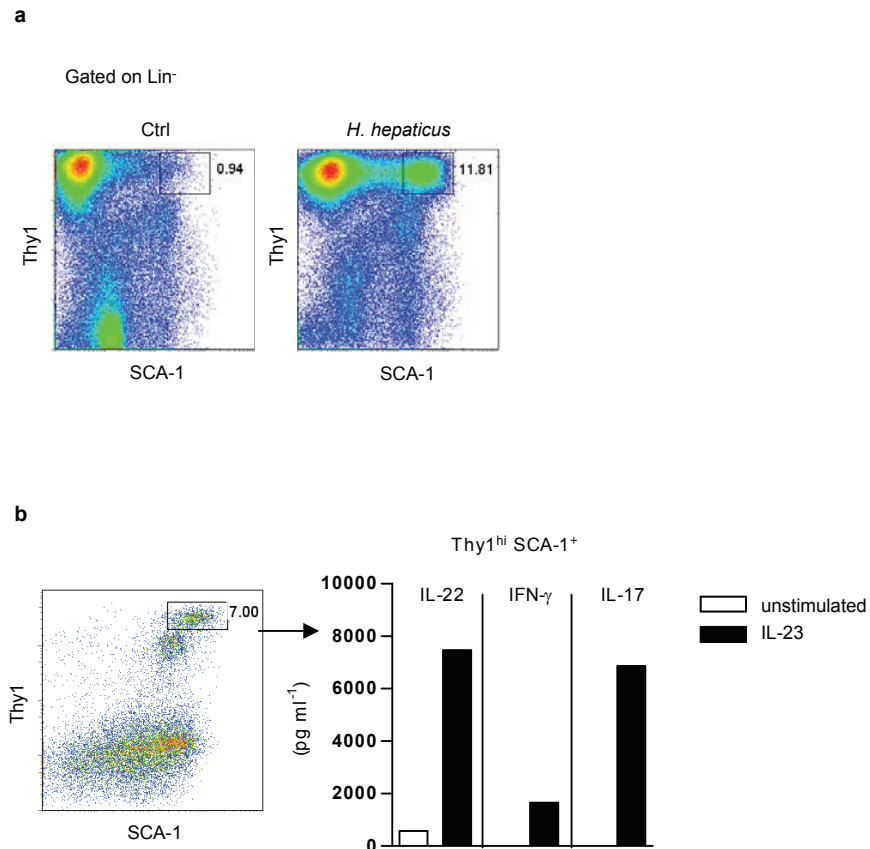
Supplementary figure 3: IL-23 drives cytokine production by CD45.2⁺ Lin⁻ cLP cells.

Cytokine secretion by CD45.2⁺Lin⁺ and CD45.2⁺Lin⁻ cLP cells isolated from *H. hepaticus* infected 129SvEvRag^{-/-} mice following overnight culture in the presence or absence of IL-12 or IL-23. Results represent one of three independent experiments with similar results.



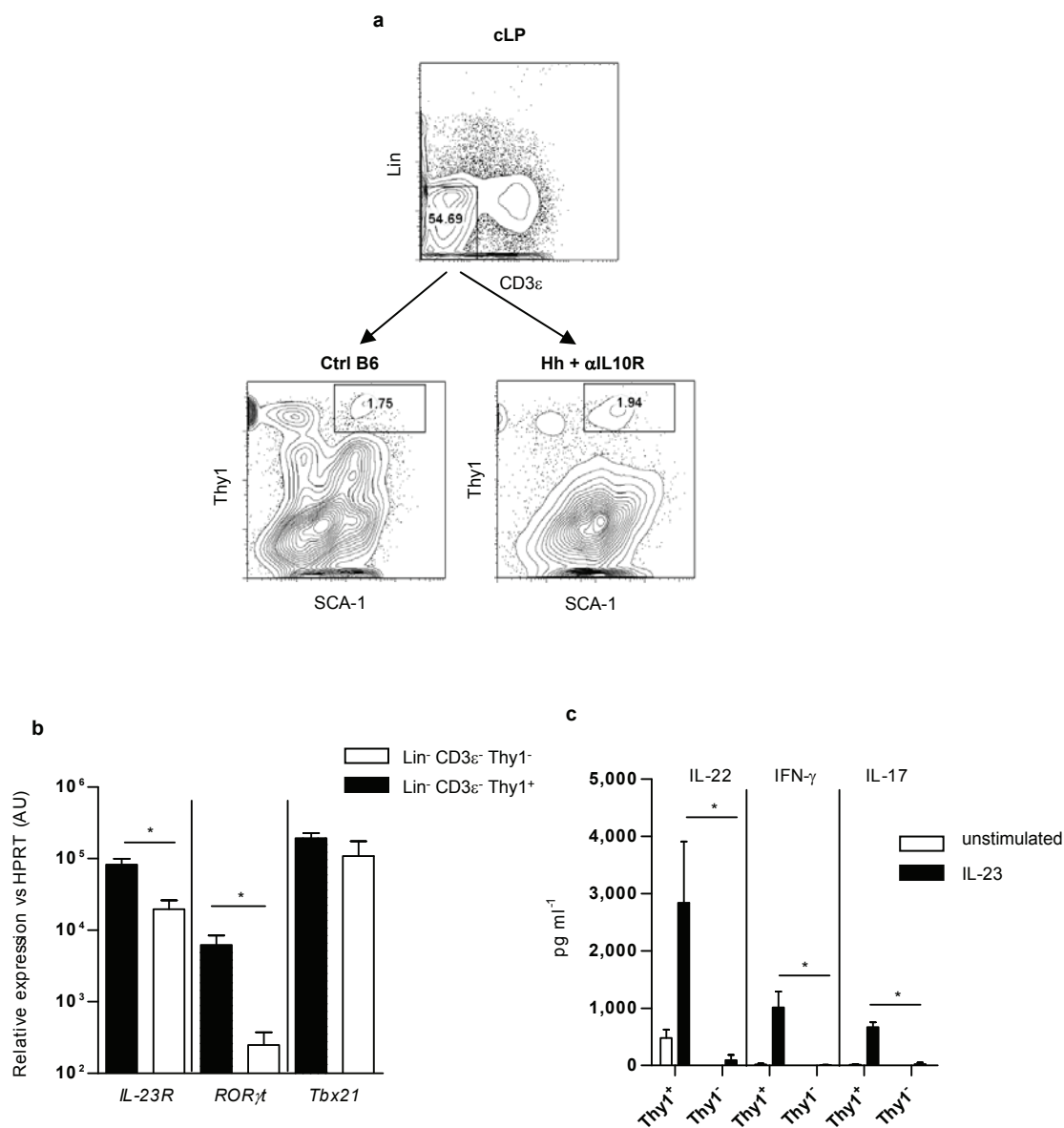
Supplementary figure 4: Thy1^{hi} SCA-1⁺ cLP cells have an LTi-like gene expression profile.

Expression of LTi associated genes among Thy1.2^{hi} SCA-1⁺ innate lymphoid cells or the remaining population of cLP cells (Rest) isolated from *H. hepaticus* infected 129SvEvRag^{-/-} mice. Gene expression was assessed by qRT-PCR and normalized to HPRT using the 2^{- Δ Ct} method. Data represent the mean of experimental triplicates \pm s.e.m. Results represent one of two independent experiments with similar results.



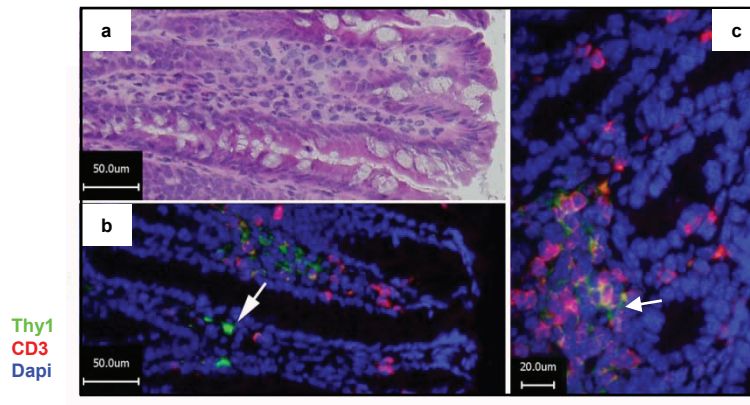
Supplementary Figure 5: Thy1^{hi} SCA-1⁺ innate lymphoid cells are present in the small intestine and inflamed liver.

a, Increased frequency of Thy1^{hi}SCA-1⁺ cells in the small intestine lamina propria of *H. hepaticus* infected mice compared to controls (Ctrl) and **b**, Cytokine secretion by sorted Thy1^{hi}SCA-1⁺ liver cells from *H. hepaticus*-infected 129SvEvRag^{-/-} mice following overnight culture with or without IL-23. Results are representative of 3 independent experiments.



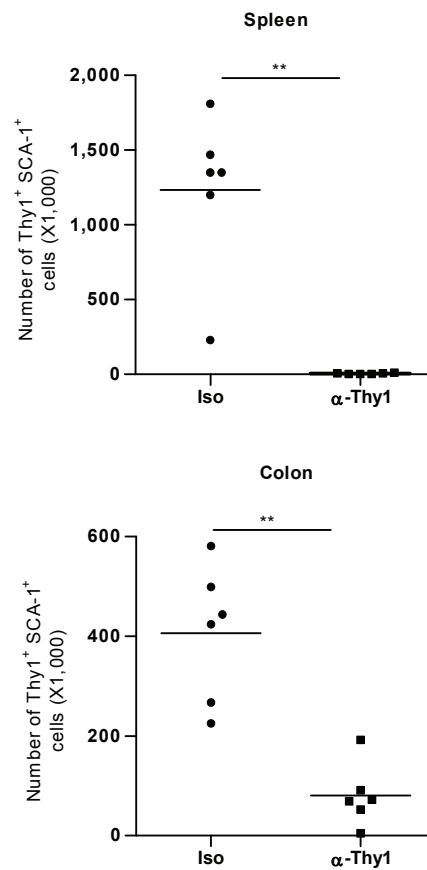
Supplementary figure 6: IL-23 responsive Lin⁻ CD3 ϵ ⁻ Thy1⁺ innate lymphoid cells are present in immune competent mice with intestinal inflammation.

Colitis was induced in C57BL/6 mice after infection with *H. hepaticus* and treatment with α -IL10R mAbs (**a-c**). Pooled cLP cells isolated from healthy C57BL/6 and/or colitic mice were stained for Lin (CD11b, GR1, B220), CD3 ϵ , Thy1 and SCA-1 and analyzed for the presence of Lin⁻CD3 ϵ ⁻ Thy1^{hi} SCA-1⁺ cells by FACS (**a**) or sorted based on Thy1 expression and analyzed for *IL-23R*, *ROR γ t* and *Tbx21* mRNA (**b**) or stimulated with or without IL-23 for cytokine detection (**c**). Data showed in **a** are representative of 2 to 4 individual mice. (**b,c**) Data represents mean \pm s.e.m. (n=3 to 4). *P<0.05; unpaired t test (**b,c**).



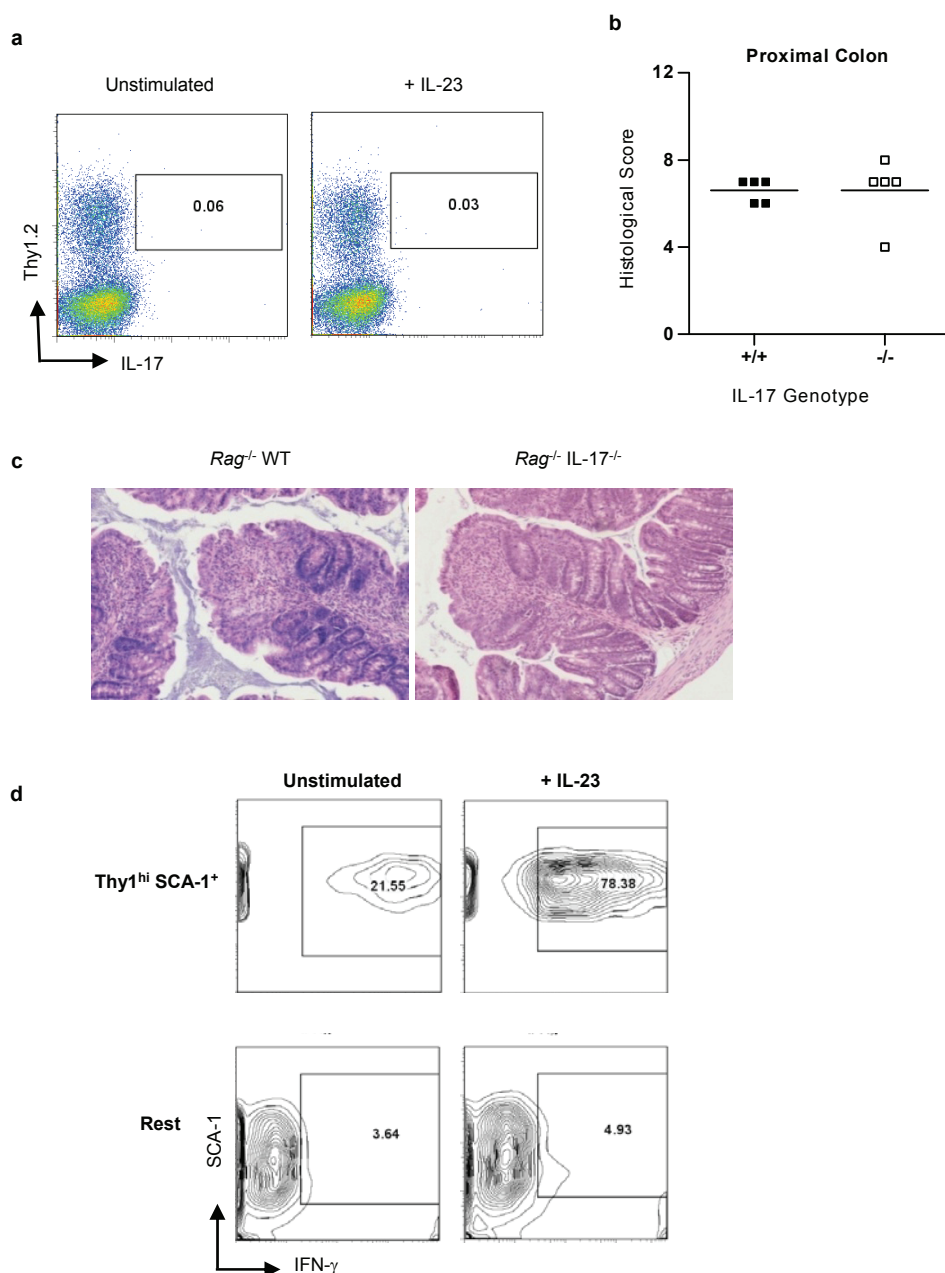
Supplementary figure 7 : Localization of Thy1⁺CD3 ϵ ⁻ innate lymphoid cells in immune competent mice with intestinal inflammation.

Representative photomicrographs showing the presence of CD3 ϵ ⁻Thy1⁺ innate cells in inflamed colon sections from *H. hepaticus* infected C57BL/6 WT treated with α -IL10R (**a-c**). H&E staining (**a**) and immunofluorescence showing Thy1 and CD3 ϵ costaining (**b,c**). Arrows indicate representative CD3 ϵ ⁻ Thy1⁺ innate cells. X200 (**a,b**); X300 (**c**).



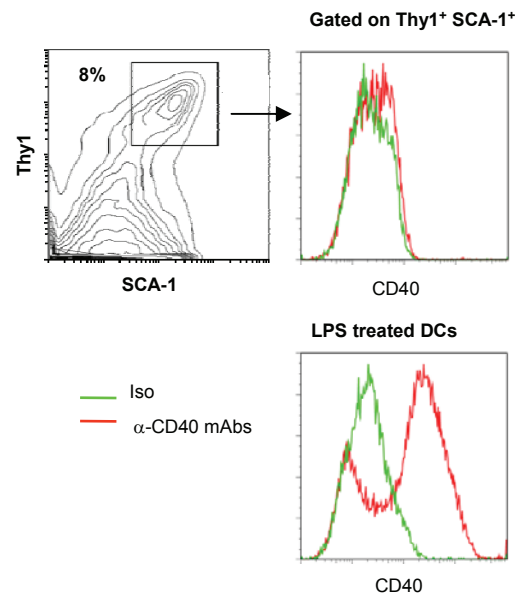
Supplementary figure 8: α -Thy1 antibody depletes systemic and mucosal Thy1⁺SCA-1⁺ ILCs

Thy1⁺SCA-1⁺ cell number in the spleen (top) and colon (bottom) in *H. hepaticus* infected 129SvEvRag^{-/-} mice treated with a depleting α -Thy1 or isotype control mAb. Each symbol represents an individual mouse and bars represent the mean (n=6). **P<0.01.



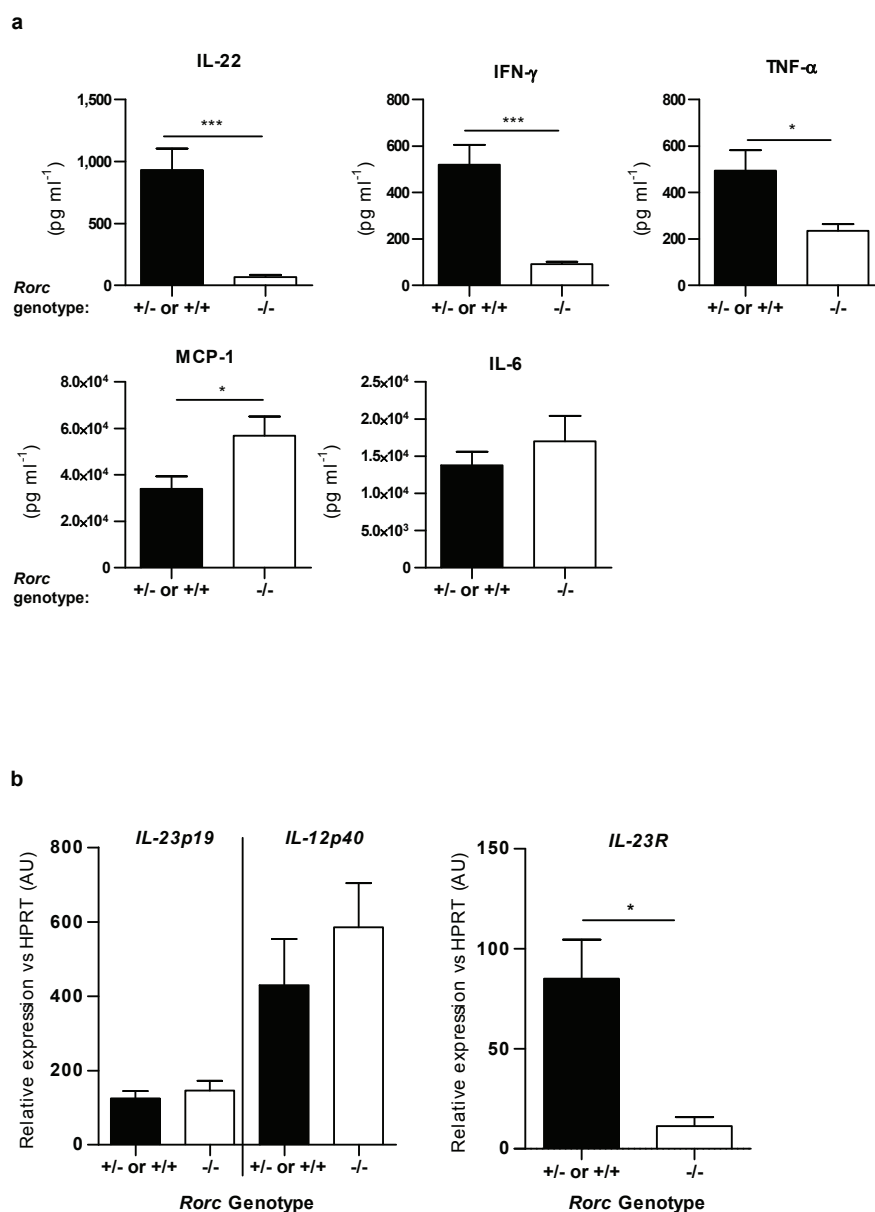
Supplementary figure 9: IL-17 is dispensable for α -CD40 mediated innate intestinal inflammation.

a, IL-17 expression among Thy1⁺ cLP cells from α -CD40 treated C57BL/6 *Rag*^{-/-} mice from overnight *ex vivo* cultures with or without IL-23 stimulation. **b**, Colitis scores and **c**, Representative photomicrographs of proximal colon of α -CD40 treated C57BL/6 IL-17^{-/-} *Rag*^{-/-} and WT *Rag*^{-/-}. **d**, IFN- γ expression analyzed by FACS among Thy1^{hi}SCA-1⁺ and the remaining population of cLP cells (Rest) from overnight cultures with or without IL-23 stimulation. Results represent one of two independent experiments with similar results.



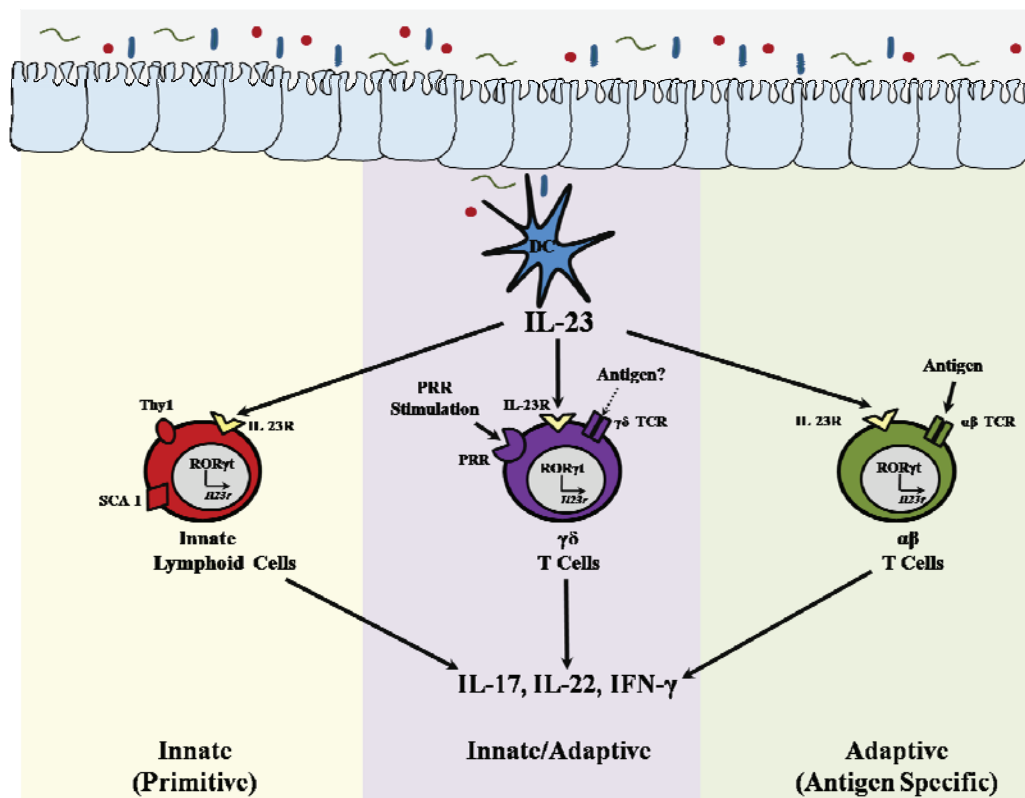
Supplementary figure 10: CD40 expression on colonic Thy1⁺SCA-1⁺ innate lymphoid cells.

cLP cells isolated from *H. hepaticus* infected 129SvEvRag^{-/-} mice were stained for Thy1 and SCA-1 and analyzed for CD40 expression by FACS. LPS treated bone marrow derived DCs were used as positive control for CD40 expression.



Supplementary figure 11: Absence of ROR γ is associated with reduced IL-23-induced cytokine production in the colon.

a, Cytokine production in overnight cultures of cLP cells isolated from α -CD40 treated $Rag^{-/-}$ or $Rag^{-/-}Rorc^{-/-}$ mice. **b**, *IL-23p19*, *IL-12p40* and *IL-23R* gene expression in the colons of mice described in **a**. Data represent pooled results from two independent experiments. Bars represent the mean \pm s.e.m. (n=11-13). *P<0.05; ***P<0.001.



Supplementary Figure 12. A conserved IL-23/ROR γ t inflammatory axis in innate and adaptive lymphoid cells in the intestine.

The IL-23/ROR γ t axis appears to represent a highly conserved tissue inflammatory response that drives IL-17, IL-22 and IFN- γ production by diverse lymphoid cells present in the intestine. Our results describe an ILC population that constitutively express high levels of both *IL-23R* and *ROR γ t* and produce IL-17, IL-22 and IFN- γ in response to IL-23 stimulation, suggesting that this response evolved prior to adaptive immunity. Although this identifies the IL-23/ROR γ t pathway as a conserved immune axis, the requirements for the induction of this response may differ within each of these distinct lymphoid cell compartments and further functional and phenotypic heterogeneity may exist.