Online-only Methods

Mice

129SvEvRag2−/− mice and wild-type C57BL/6, control C57BL/6 Rag1−/− and IL-17 deficient mice on a C57BL/6 Rag1−/− background (IL-17−/− Rag2−/− and Rorc−/− Rag2−/−) 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 described.15

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 described\(^{15}\). 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 \(\sim 5 \times 10^7\)–\(2 \times 10^8\) 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_ infection\(^{16}\). 1 week after the last mAb treatment, mice were sacrificed.

To induce acute innate colitis, C57BL/6 _Rag\(^{-/-}\)_ were administered 100 to 200 µg of α-CD40 IgG2a mAbs (FGK45) via i.p. injection\(^{17}\). 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, (1x10^5-3x10^5) spleen or cLP cells we cultured overnight in 100µl 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 (10µg/ml, eBiosciences) was added for the last 4 hours of the overnight cultures described above and surface and cytoplasmic stainings were performed as described previously. 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-Thyl 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-Thyl 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'-TCATAACCTGGTTTCATCATCGC-3', and probe, 5'-ACCCGCAGTCCAGCGTGCAG-3'; IL-23p19 primers, 5'-AGCGGGACATATGAATCTACTAAGGAGA-3' and 5'-GTCCTAGTAGGAGGTGAAATGGT-3', and probe, 5'-CCAGTTCCTGCTTGCAGCTCCAG-3'; IL-12p40 primers, 5'-GACCATCACTGTCAAAGAGTTTCTAGAT-3' and 5'-AGGAAAGTCTTTTGGAAATTTTTGA-3', and probe, 5'-CCACTCACATCTGCTCCACAAGAGA-3'; IL-17A primers, 5'-GCTCCAGAAGGCCCTCAG-3' and 5'-CTTTCCCTTCCGCGGATGC-3', and probe, 5'-ACCTAACCGTTCCACGTCACCCT-3'; \textit{Rorc} primers, 5'-CCGCTGAGAGGGCTTCAC-3' and 5'-TGCAGGAGTAGGCCACATTACA-3', and probe, 5'-AAGGGGCTTCTTGTCCCGCAGCCAGC-3'; \textit{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: LTα, 5'-GTACCCAAACAGGTGACGACG-3' and 5'-CCAGGACAGCCCATCCACT-3'; LTβ, 5'-ACGCTTTCTTTGCTCGG-3' and 5'-
ACCTCATTAGGCGCTTGGATG-3’; TRANCE, 5’-CGCTTCCCGATGTTTCATG-3’
and 5’-GGTTAACAAGATGCTTCTATTACC-3’; CXCR5, 5’-
CCTCGACTGTAGAGCAGAAAGTTACTG-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 caecum, 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 µm 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 caecum 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.


**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<sup>+</sup> mice. Data represents mean ± 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 ± 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<sup>hi</sup> SCA-1<sup>+</sup> cLP cells have an LTi-like gene expression profile.

Expression of LTi associated genes among Thy1.2<sup>hi</sup> SCA-1<sup>+</sup> innate lymphoid cells or the remaining population of cLP cells (Rest) isolated from *H. hepaticus* infected 129SvEvRag<sup>−/−</sup> mice. Gene expression was assessed by qRT-PCR and normalized to HPRT using the 2<sup>−ΔΔCT</sup> method. Data represent the mean of experimental triplicates ± s.e.m. Results represent one of two independent experiments with similar results.
**Supplementary Figure 5:** Thy1\textsuperscript{hi} SCA-1\textsuperscript{+} innate lymphoid cells are present in the small intestine and inflamed liver.

**a,** Increased frequency of Thy1\textsuperscript{hi}SCA-1\textsuperscript{+} cells in the small intestine lamina propria of *H. hepaticus* infected mice compared to controls (Ctrl) and **b,** Cytokine secretion by sorted Thy1\textsuperscript{hi}SCA-1\textsuperscript{+} liver cells from *H. hepaticus*–infected 129SvEvRag\textsuperscript{-/-} mice following overnight culture with or without IL-23. Results are representative of 3 independent experiments.
Supplementary figure 6: IL-23 responsive Lin⁻CD3ε⁻ 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), CD3ε, Thy1 and SCA-1 and analyzed for the presence of Lin⁻CD3ε⁻ Thy1⁻ SCA-1⁺ cells by FACS (a) or sorted based on Thy1 expression and analyzed for *IL-23R, RORγt* 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 ± s.e.m. (n=3 to 4). *P<0.05; unpaired t test (b,c).
Supplementary figure 7: Localization of Thy1⁺CD3ε⁻ innate lymphoid cells in immune competent mice with intestinal inflammation.

Representative photomicrographs showing the presence of CD3ε⁻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 CD3ε costaining (b,c). Arrows indicate representative CD3ε⁻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 Thy1hiSCA-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 RORγ 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<sup>−/−</sup> or Rag<sup>−/−</sup>Rorc<sup>−/−</sup> 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 ± s.e.m. (n=11-13). *P<0.05; ***P<0.001.
Supplementary Figure 12. A conserved IL-23/RORγt inflammatory axis in innate and adaptive lymphoid cells in the intestine.

The IL-23/RORγt 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 RORγt 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/RORγt 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.