LETTERS

Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology

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The key role of interleukin (IL)-23 in the pathogenesis of autoimmune and chronic inflammatory disorders is supported by the identification of IL-23 receptor (IL-23R) susceptibility alleles associated with inflammatory bowel disease, psoriasis and ankylosing spondylitis. IL-23-driven inflammation has primarily been linked to the actions of T-helper type 17 (T_H17) cells[1]. Somewhat overlooked, IL-23 also has inflammatory effects on innate immune cells[2] and can drive T-cell-independent colitis. However, the downstream cellular and molecular pathways involved in this innate intestinal inflammatory response are poorly characterized. Here we show that bacteria-driven innate colitis is associated with an increased production of IL-17 and interferon-γ in the colon. Stimulation of colonic leukocytes with IL-23 induced the production of IL-17 and interferon-γ exclusively by innate lymphoid cells expressing Thy1, stem cell antigen 1 (SCA-1), retinoic-acid-related orphan receptor (ROR)-γt and IL-23R, and these cells markedly accumulated in the inflamed colon. IL-23-responsive innate intestinal cells are also a feature of T-cell-dependent models of colitis. The transcription factor ROR-γt, which controls IL-23R expression, has a functional role, because $Rag^{-/-}Rorc^{-/-}$ mice failed to develop innate colitis. Last, depletion of $Thy1^+$ innate lymphoid cells completely abrogated acute and chronic innate colitis. These results identify a previously unrecognized IL-23-responsive innate lymphoid population that mediates intestinal immune pathology and may therefore represent a target in inflammatory bowel disease.

T_H17 cells produce a variety of inflammatory cytokines, including IL-17, IL-17F, IL-22, IL-6 and tumour necrosis factor (TNF)-α, and are implicated in both host defence against extracellular pathogens and the pathogenesis of several inflammatory disorders[1]. T_H17 cells have recently been shown to have flexibility of function, and acquisition of interferon (IFN)-γ production has been linked to their pathogenicity *in vivo*[3,4]. Transforming growth factor-β and IL-6 or IL-21 drive T_H17 cell differentiation[5], and this process is orchestrated by the transcription factor ROR-γt (ref. 6). ROR-γt promotes IL-23R expression, permitting IL-23 to control the expansion and maintenance of T_H17 cells[7]. ROR-γt is also expressed by innate lymphoid cells such as intestinal cells that express natural killer (NK) markers, fetal LTi (lymphoid tissue inducer) cells and adult LTi-like cells[8]. Various innate tissue leukocytes, such as $CD11c^+$ myeloid cells, LTi-like cells and mucosal $NKp46^+$ cells, have been shown to produce IL-22 and/or IL-17 on stimulation with IL-23, but the contribution of these tissue-resident innate immune cells to pathology in the intestine is not known[2,9–11].

We have shown that innate immune colitis in $Rag^{-/-}$ mice after infection with *Helicobacter hepaticus* is IL-23 dependent[12]. To identify the cellular and molecular pathways involved, we first analysed the expression of inflammatory cytokines in this model. Consistent with selective upregulation of IL-23 in the intestine[12] was our observation

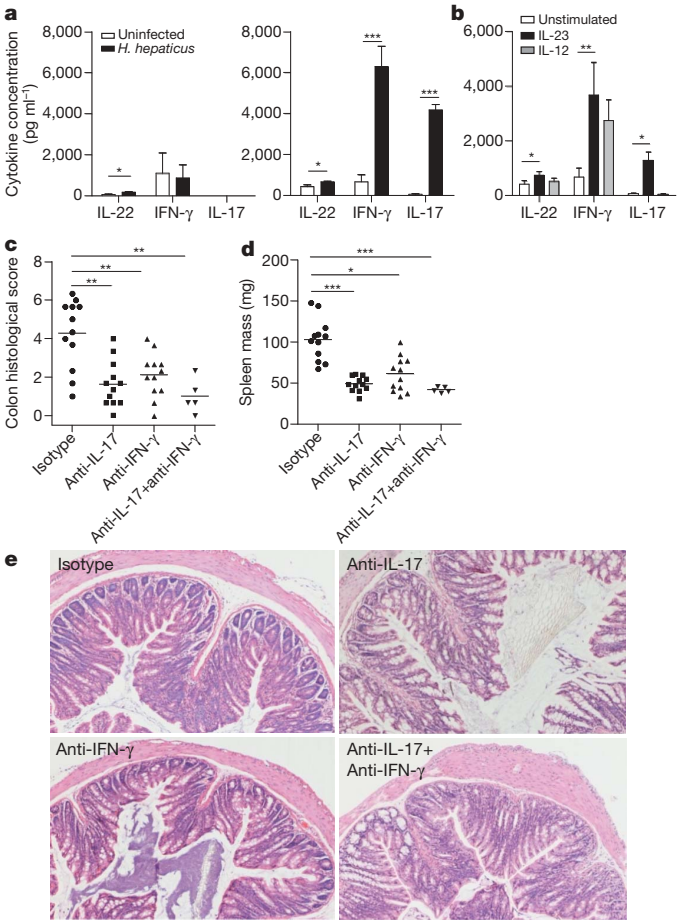

Figure 1 | IL-23-induced IL-17 and IFN-γ are required for *H. hepaticus*-mediated innate colitis. **a**, Cytokine secretion after overnight culture of splenocytes (left) or cLP cells (right) from control or *H. hepaticus*-infected $129SvEvRag^{-/-}$ mice ($n = 6$ per group). **b**, Cytokine secretion by cLP cells from control $129SvEvRag^{-/-}$ mice after overnight culture with IL-12 or IL-23 ($n = 6$). Data are shown as means and s.e.m. **c**–**e**, Colitis scores (**c**), splenomegaly (**d**) and representative colon photomicrographs (magnification × 50) (**e**) from *H. hepaticus*-infected $129SvEvRag^{-/-}$ mice treated with blocking anti-IL-17 and/or anti-IFN-γ or isotype control mAbs. Data represent two pooled experiments ($n = 5$–12 per group). Asterisk, $P < 0.05$; two asterisks, $P < 0.01$; three asterisks, $P < 0.001$.

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of significant increases in the expression of T_H17 and T_H1 signature cytokines, including IL-17, IL-22 and IFN-γ, by colonic lamina propria cells (cLP) from *H. hepaticus*-infected $Rag^{-/-}$ mice but not from spleen cells (Fig. 1a). IL-23-mediated pathology was not associated with an increase in IL-6 (Supplementary Fig. 1). To determine whether IL-23 acts directly on innate cells to induce T_H1 and T_H17 cytokines, cLP cells were isolated from healthy colons of $Rag^{-/-}$ mice and stimulated with IL-12 or IL-23. Addition of IL-23 induced the secretion of IL-17, IL-22 and IFN-γ (Fig. 1b), whereas IL-12 induced IFN-γ only. To determine whether IL-17 and IFN-γ have a functional role in innate colitis, *H. hepaticus*-infected $Rag^{-/-}$ mice were treated with neutralizing anti-IL-17 or anti-IFN-γ monoclonal antibodies (mAbs). Blockade of either IL-17 or IFN-γ was sufficient to decrease colitis significantly (Fig. 1c, e) without affecting colonization with *H. hepaticus* (Supplementary Fig. 2). Similarly, systemic immune activation, assessed by splenomegaly, was also abrogated by IL-17 or IFN-γ blockade (Fig. 1d). Taken together, these results indicate that *H. hepaticus*-induced IL-23 regulates the innate expression of effector cytokines such as IL-17 and IFN-γ that have functional roles in the intestinal innate inflammatory response.

To identify IL-23-responsive innate immune cells present in the inflamed intestine, we used a cell sorting approach. Using leukocyte lineage (Lin) markers CD11b, GR1 and B220, we found that cytokine-expressing cells were $CD45^+Lin^-$ and distinct from common innate cell populations (Supplementary Fig. 3). To identify these cells, we performed intracellular cytokine staining in combination with cell-surface marker expression on IL-23-stimulated cLP cells from colitic mice. We found that IL-23 not only enhanced the frequency of IL-17^+IFN-γ^- cells but also increased the frequency of IL-17^+IFN-γ^+ cLP cells, whereas the frequency of IL-17^-IFN-γ^+ cells did not increase (Fig. 2a). Analysis of surface markers showed that the vast majority of the cLP IL-17-secreting cells expressed high levels of Thy1 (Fig. 2b). Lin^-Thy1^+ cells in $Rag^{-/-}$ mice include a population of cells required for the organogenesis of secondary lymphoid organs (SLOs), termed LTi/LTi-like cells[13]. Similarly to classical LTi-like cells, IL-17-expressing cells were found to be IL-$7R^+CD44^+NKp46^-CCR6^+CD25^+$ROR-$\gamma^+$ (Fig. 2c). They also expressed LTi-related genes such as those encoding lymphotoxin (LT)-α and LT-β, TNF-related activation-induced cytokine (TRANCE) and CXCR5, recently found to be important for the recruitment of LTi-like cells during inflammation (Supplementary Fig. 4) (refs 13, 14). However, IL-17-expressing innate lymphoid cells were also phenotypically distinct from LTi-like cells: they were $CD4^-$c-Kit^- and also expressed SCA-1 (Fig. 2c, d), suggesting heterogeneity amongst $Thy1^+$ innate lymphoid cells in the intestine.

To characterize $Thy1^{high}SCA\text{-}1^+$ innate lymphoid cells further, this population was sorted from the inflamed colon of *H. hepaticus*-infected $Rag^{-/-}$ mice. Even in the absence of cytokine stimulation, the $Thy1^{high}SCA\text{-}1^+$ population produced IL-17, IL-22 and IFN-γ *ex vivo*, in contrast to most cLP cells (Fig. 2d). In addition, cytokine secretion was enhanced on stimulation with IL-23 (Fig. 2d). Consistent with their production of T_H17 signature cytokines and IFN-γ in response to stimulation with IL-23 was our observation that $Thy1^{high}SCA\text{-}1^+$ cells expressed higher levels of mRNA encoding IL-23R, ROR-γt and Tbx21 but not of mRNA encoding aryl hydrocarbon receptor (AHR) (Fig. 2e).

$Thy1^{high}SCA\text{-}1^+$ innate lymphoid cells were present at low frequency in the $Rag^{-/-}$ colon but increased significantly during intestinal inflammation (Fig. 3a). Because there is about a tenfold increase in leukocytes in inflamed colons compared with controls[15], this represents a roughly 100-fold increase in the total number of $Thy1^{high}SCA\text{-}1^+$ cells. There was also a marked increase in the proportion of $Thy1^{high}SCA\text{-}1^+$ cells that produced IL-17 *de novo* from *H. hepaticus*-infected mice and after IL-23 stimulation, suggesting not only accumulation but also activation of this population in the inflamed intestine (Fig. 3b). The presence of $Thy1^{high}SCA\text{-}1^+$ innate lymphoid cells was not restricted to the colon: these cells were also observed in the small intestine (Supplementary Fig. 5a). Furthermore, they were present in other tissues in which *H. hepaticus* has been shown to mediate immune

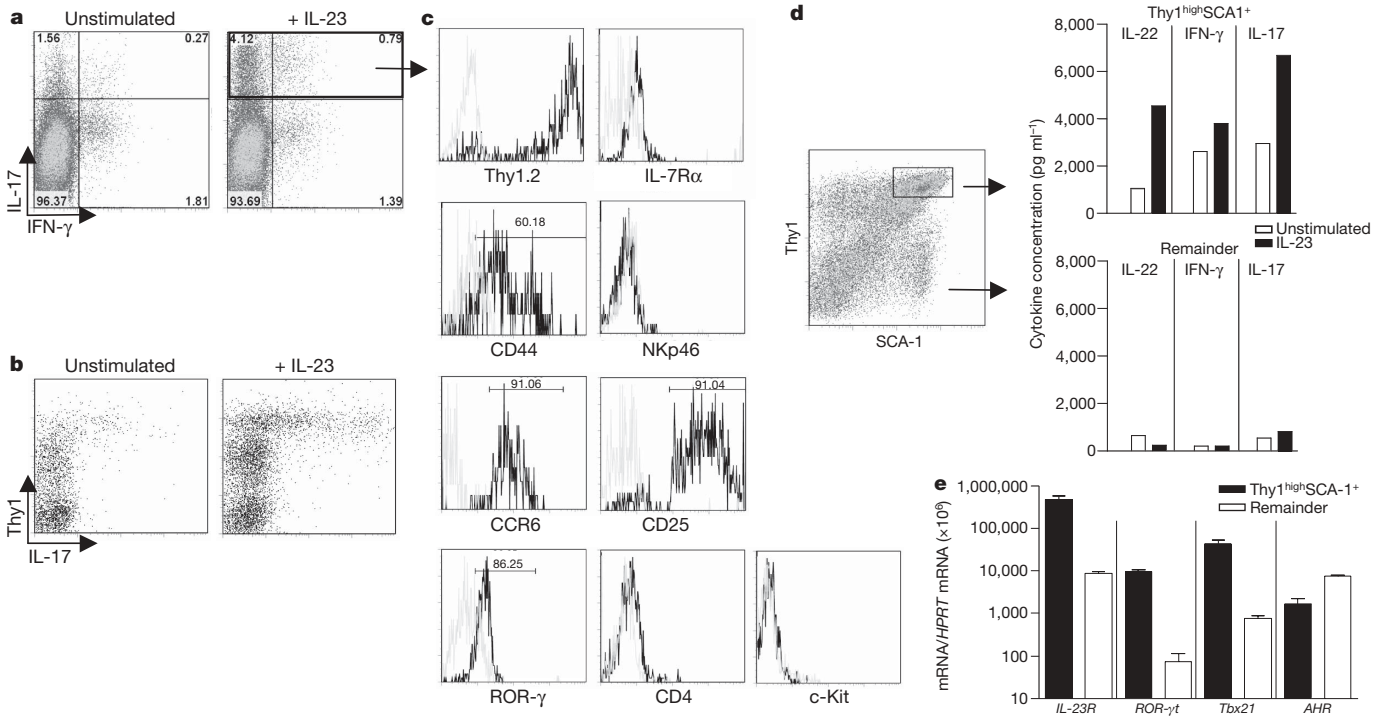

Figure 2 | IL-23-responsive innate lymphoid cells in inflamed colon are $Thy1^{high}SCA\text{-}1^+ROR\text{-}\gamma t^+$. **a**, **b**, IL-17 and IFN-γ (**a**) and Thy1 and IL-17 (**b**) expression in Lin^- cLP cells from *H. hepaticus*-infected 129SvEv$Rag^{-/-}$ mice after overnight culture with or without IL-23. **c**, Phenotypic analysis of Lin^-IL-17^+ cLP cells from *H. hepaticus*-infected 129SvEv$Rag^{-/-}$ mice, using specific antibodies (black line) and isotype controls (grey line). **d**, Cytokine secretion by sorted $Thy1^{high}SCA\text{-}1^+$ or the remaining cLP cells (remainder) isolated from *H. hepaticus*-infected 129SvEv$Rag^{-/-}$ mice after overnight culture with or without IL-23. **e**, *IL-23R*, *ROR-γt*, *AHR* and *Tbx21* mRNA expression by unstimulated cells isolated as in **d**. Data are shown as means and s.e.m. Results are representative of at least two independent experiments.

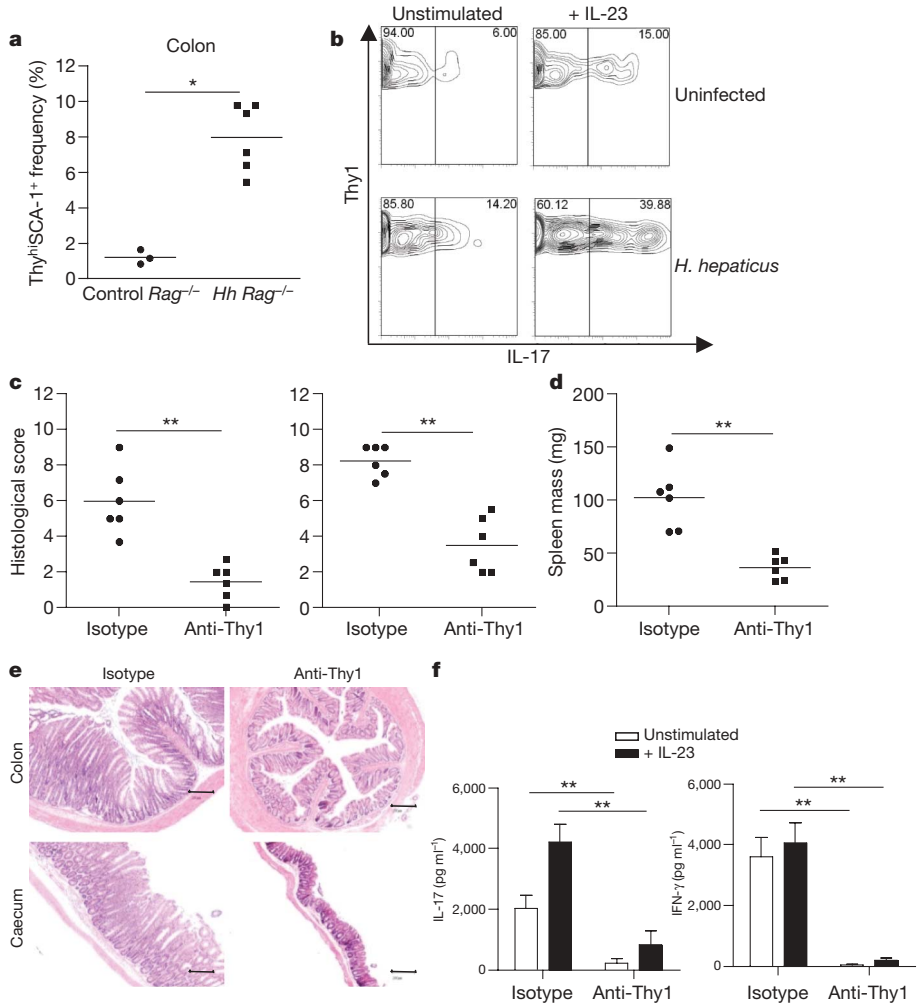

Figure 3 | Thy1^{high} innate lymphoid cells drive *H. hepaticus*-induced innate intestinal inflammation. **a**, **b**, Frequency of Thy1^{high}SCA-1$^+$ cells ($n = 3$–6) (**a**) and IL-17 expression among Thy1^{high} cells (**b**) after culture with or without IL-23 in cLP cells from control or *H. hepaticus*-infected 129SvEv*Rag*$^{-/-}$ (*Hh Rag*$^{-/-}$) mice. Data are representative of two independent experiments. **c**–**e**, Colitis (**c**, left) and typhlitis (**c**, right) scores, splenomegaly (**d**), and representative photomicrographs (magnification ×50; scale bars, 200 μm) (**e**) from *H. hepaticus*-infected mice treated with anti-Thy1 or isotype control mAbs ($n = 6$ per group). **f**, IL-17 and IFN-γ secretion by cLP cells from the mice described above, following stimulation with or without IL-23. Data are shown as means and s.e.m. ($n = 6$). Asterisk, $P < 0.05$; two asterisks, $P < 0.01$.

pathology such as the liver[15], where they also responded to IL-23 by secreting IL-22, IFN-γ and IL-17 (Supplementary Fig. 5b). A similar population of Lin$^-$CD3ε$^-$Thy1^{high} cells was also present in the colon of immunocompetent mice both at steady state and during intestinal inflammation induced by infection with *H. hepaticus* plus concomitant blockade of IL-10R (ref. 16) (Supplementary Fig. 6a). Lin$^-$CD3ε$^-$Thy1$^+$ cells again expressed *IL-23R*, *ROR-γt* and *Tbx21* (Supplementary Fig. 6b) and secreted IL-17, IFN-γ and IL-22 in response to stimulation with IL-23 (Supplementary Fig. 6c). Immunohistological analyses demonstrated that CD3ε$^-$Thy1$^+$ cells were localized mainly within leukocytic clusters and were often present in close association with CD3$^+$ T cell infiltrates (Supplementary Fig. 7). However, some CD3ε$^-$Thy1$^+$ cells were also observed scattered throughout the lamina propria (Supplementary Fig. 7).

To determine whether Thy1^{high}SCA-1$^+$ innate lymphoid cells had a functional role in *H. hepaticus* innate immune-driven typhlocolitis, we depleted these cells by injection of an anti-Thy1 mAb during the course of infection (Supplementary Fig. 8). Efficient depletion of Thy1$^+$ cells led to the abrogation of both colitis and typhlitis (Fig. 3c, e). IL-23-dependent systemic immune activation was also ablated, as shown by the significantly decreased splenomegaly (Fig. 3d). Absence of intestinal inflammation after depletion of Thy1$^+$SCA-1$^+$ correlated with the abrogation of both the spontaneous and IL-23-induced production of IL-17 and IFN-γ by cLP cells (Fig. 3f).

We next assessed the role of Thy1^{high}SCA-1$^+$ cells in another distinct model of IL-23-driven innate intestinal inflammation. Injection of agonistic anti-CD40 mAb into *Rag*$^{-/-}$ mice induces an IL-23-dependent acute innate immune colitis that is accompanied by an IL-23-independent systemic inflammatory response[17]. We found that depletion of Thy1$^+$ cells completely abrogated anti-CD40-induced innate colitis (Fig. 4a, b) but did not affect the IL-23-independent systemic wasting disease (Fig. 4c). In this acute innate colitis model there was no detectable production of IL-17 by Thy1$^+$ cells from the inflamed colon even after stimulation with IL-23 (Supplementary Fig. 9a), and IL-17 was not required for the development of colitis (Supplementary Fig. 9b, c). In contrast, intestinal Thy1^{high}SCA-1$^+$ cells were found to be the major source of IFN-γ during anti-CD40-induced innate colitis, with IL-23 further enhancing their production of IFN-γ (Supplementary Fig. 9d). Furthermore, depletion of Thy1$^+$SCA-1$^+$ cells was associated with a significant decrease in the production of IFN-γ, IL-22 and TNF-α but not that of MCP-1 or IL-6 in the colon (Fig. 4d). In this innate model, activation of Thy1^{high}SCA-1$^+$ by the agonistic anti-CD40 mAb seems to be indirect, because no CD40 expression was observed on these cells (Supplementary Fig. 10).

ROR-γt is required for the development of LTi cells and IL-22-producing LTi-like cells[8,18,19]. Indeed, we found high expression of *ROR-γt* by Thy1^{high}SCA-1$^+$ compared to the remainder of cells isolated

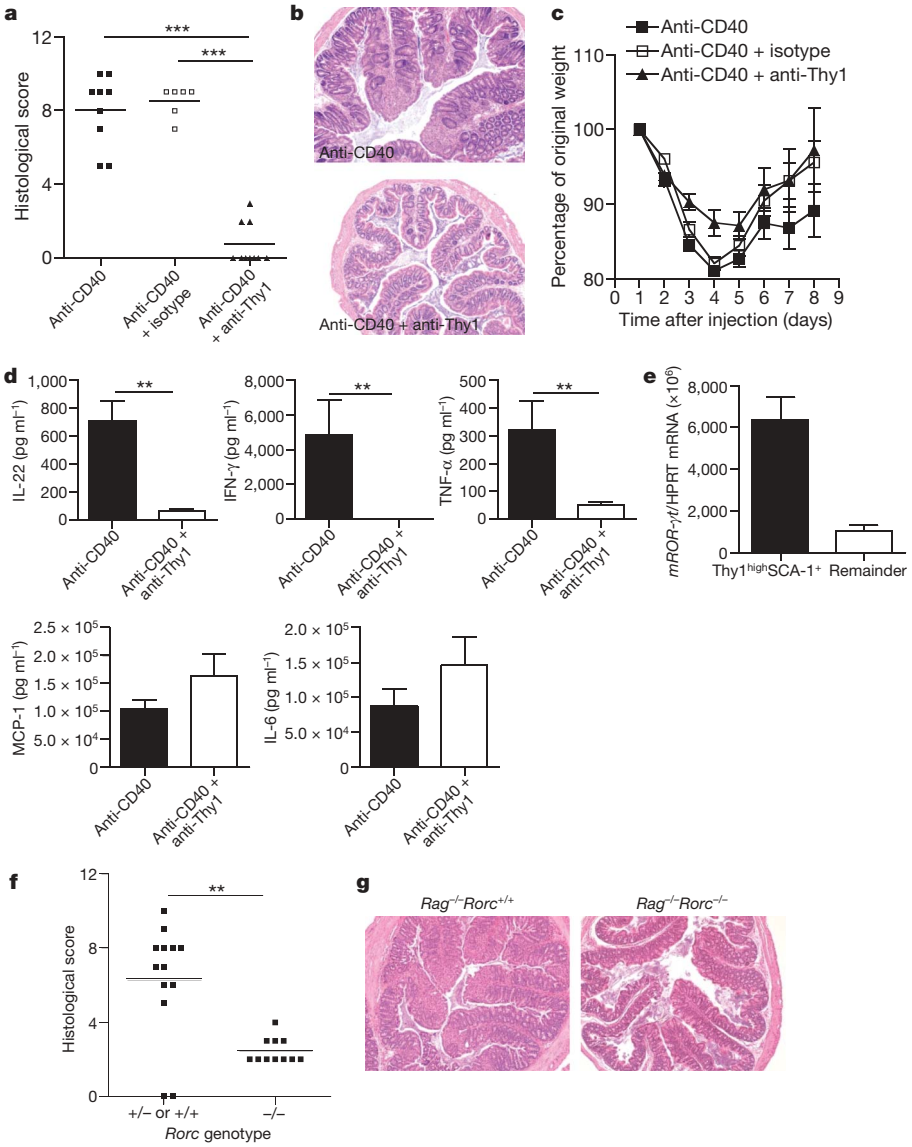

Figure 4 | ROR-γt-expressing Thy1^{high} innate lymphoid cells are required for anti-CD40-induced innate intestinal inflammation. **a**–**d**, Proximal colitis scores (**a**), colon photomicrographs (magnification ×50) (**b**), weight loss (**c**) and cLP cytokine secretion after overnight culture (**d**) in anti-CD40-treated C57BL/6 *Rag*$^{-/-}$ mice injected with or without anti-Thy1 or isotype control mAb. **e**, *ROR*-γt mRNA expression by sorted Thy1^{high}SCA-1^{+} cells or the remaining cLP cells from anti-CD40-treated mice. Results are representative of two independent experiments. **f**, **g**, Proximal colitis scores (**f**) and photomicrographs (magnification ×50) (**g**) from anti-CD40-treated C57BL/6 *Rag*$^{-/-}$ or C57BL/6 *Rag*$^{-/-}$*Rorc*$^{-/-}$ mice. Data represent pooled results from two experiments (**a**–**c**, $n = 6$–10; **f**, **g**, $n = 11$–13). In **c**–**f**, data are shown as means and s.e.m. ($n = 5$). Two asterisks, $P < 0.01$; three asterisks, $P < 0.001$.

from the colons of mice treated with anti-CD40 (Fig. 4e). Because ROR-γt is important for IL-23R expression[7] we next assessed whether it was required for the development of anti-CD40-induced innate colitis. Although treatment of *Rag*$^{-/-}$*Rorc*$^{-/-}$ mice with anti-CD40 mAb elicited potent systemic inflammation and wasting disease comparable to that observed in *Rag*$^{-/-}$ mice (data not shown), *Rag*$^{-/-}$*Rorc*$^{-/-}$ mice developed only mild intestinal inflammation (Fig. 4f, g). Attenuated colitis was associated with decreased production of IL-22, IFN-γ and TNF-α by cLP cells in *Rag*$^{-/-}$*Rorc*$^{-/-}$ mice (Supplementary Fig. 11a). Although we found no differences between *Rag*$^{-/-}$ and *Rag*$^{-/-}$*Rorc*$^{-/-}$ mice in the intestinal expression of the IL-23p19 subunit after anti-CD40 treatment, there was a significantly decreased colonic expression of IL-23R in *Rag*$^{-/-}$*Rorc*$^{-/-}$ mice (Supplementary Fig. 11b). Thus, ROR-γt may be important in the control of IL-23R and inflammatory cytokine expression in innate lymphoid cells.

Taken together, our results identify a novel innate lymphoid cell population that accumulates in the inflamed colon and directly mediates IL-23-dependent acute and chronic innate immune-mediated colitis through the production of inflammatory cytokines. This innate lymphoid population bears many hallmarks of the colitogenic T-cell response, including the expression of ROR-γt and the production of IL-17 and IFN-γ. IL-17 production by innate lymphoid cells was a feature of chronic bacteria-induced colitis but not acute anti-CD40-induced disease, suggesting that the tissue IL-23-driven innate lymphoid response shows flexibility of function and is shaped by environmental factors. The remarkable conservation in function between IL-23-driven αβ and γδ T-cell responses[20,21] and IL-23-driven innate lymphoid responses indicates that T_H17-associated effector functions can be generated in the complete absence of TCR-mediated signals. Indeed, the production of IL-17 and IFN-γ by innate lymphoid cells may represent a primitive tissue inflammatory response that is activated in response to microbe-induced cytokines such as IL-23 (Supplementary Fig. 12). The tissue innate lymphoid response may extend beyond IL-23-driven responses, because non-B/non-T Lin^{-} and Lin^{-}c-Kit^{+}SCA-1^{+} cells have been shown to be a

source of T_H2-type cytokines in response to IL-25 and IL-33, respectively, in parasitic helminth infection models[22,23]. Intestinal inflammatory innate lymphoid cells share some features of *Rorc*-dependent LTi cells that mediate lymphoid organogenesis in the fetus as well as those of adult LTi-like cells that produce IL-22 or IL-17 in response to IL-23 or zymosan challenge[2]. Whether these represent distinct tissue innate lymphoid populations or different functional states of the same population is not known. It is notable that hyperplastic lymphoid aggregates have been observed in the colon of patients with inflammatory bowel disease[24,25]. Furthermore, we have found a similar IL-23-responsive IL-17-producing innate lymphoid cell (ILC) population in the inflamed intestine of patients with inflammatory bowel disease (A. Geremia, C. Arancibia and F.P., unpublished observations). Intestinal innate lymphoid populations may therefore contribute to chronic intestinal inflammation through effects on organogenesis as well as inflammatory cytokine production. Further characterization of IL-23-responsive ILC in the human intestine is required to establish a role in the pathogenesis of inflammatory bowel disease.

METHODS SUMMARY

Isolation of murine cells. cLP cells were purified as described[26]. In brief, colon tissue was cut into 0.5-cm pieces and incubated in RPMI medium containing 10% heat-inactivated FCS (Gibco-BRL) and 5 mM EDTA to remove epithelial cells. The remaining tissue was further digested with complete RPMI medium containing 15 mM HEPES and 100 U ml^{-1} collagenase VIII (Sigma Chemical Co.). For liver-cell isolation, livers were perfused at a rate of 2–3 ml min^{-1}, first with PBS and then with PBS containing 100 U ml^{-1} of type VIII collagenase. Livers were then cut into small pieces and digested for a further 1 h at 37 °C with complete RPMI medium containing 15 mM HEPES and 100 U ml^{-1} collagenase VIII. Liver cells and LP cells were then layered on a 30%/40%/75% Percoll gradient (Amersham Pharmacia Biotech). After centrifugation at 600*g* for 20 min, cells were recovered at the 40%/75% Percoll interface. Cells were stained with a combination of anti-CD45.2, anti-CD11b, anti-Ly6C/G (GR1), anti-B220, anti-CD90.2 (anti-Thy1.2), anti-Ly6A/E (SCA-1) and anti-CD3ε antibodies (all from BD Pharmingen) and sorted on a Moflo sorter (DakoCytomation).
Reagents. Cytokine detection in culture supernatants was quantified with a Flow Cytomix Cytokine Bead Assay (Bender MedSystems). Recombinant mouse IL-12 was obtained by transfecting COS7 with the cDNA encoding the p35 and p40 as described previously[27]. Recombinant mouse IL-23 was purchased from R&D. Both recombinant cytokines were used at 10 ng ml^{-1} for overnight stimulation of cells. Anti-IFN-γ and anti-IL-17 antibodies for intracellular staining were obtained from BD Pharmingen, and anti-ROR-γ antibody came from eBiosciences. Surface antibodies against CD90.2, CD3ε, CD45.2, CD11b, Ly6C/G, B220, Ly6A/E (SCA-1), CCR6, CD25, CD44, CD117 (c-Kit), CD11c and CD4 were purchased from BD Pharmingen. Antibodies against NKp46 and CD127 (IL-7Rα) were obtained from R&D and eBiosciences, respectively.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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