

IL-23R⁺ innate lymphoid cells induce colitis via interleukin-22-dependent mechanism

A Eken¹, AK Singh², PM Treuting³ and M Oukka^{1,2}

Polymorphisms of interleukin (IL)-23R and signaling components are associated with several autoimmune diseases, including inflammatory bowel diseases (IBD). Similar to T helper type 17 (Th17) lineage, type 3 innate lymphoid cells (ILCs) express retinoic acid–related orphan receptor γ t (Ror γ t) and IL-23R and hence, produce Th17-type cytokines. Recent reports implicated type 3 ILCs in IBD; however, how IL-23R signaling in these cells contributes to pathogenesis is unknown. IL-22, produced in copious amounts by type 3 ILCs, was reported to have both beneficial and pathogenic effects in adaptive, yet only a protective role in innate colitis models. Herein, by employing chronic CD45RB^{high} CD4⁺ T-cell transfer and anti-CD40 antibody-induced acute innate colitis models in Rag1^{-/-} mice, we demonstrated opposite roles for IL-23R in colitogenesis: in the former a protective, and in the latter a pathogenic role. Furthermore, we show that IL-23R signaling promotes innate colitis via IL-22 as neutralization of IL-22 protected mice from colitis and adding back of IL-22 to IL-23R-deficient animals restored the disease. Collectively, our results reveal that similar to its controversial role during chronic or adaptive colitis, IL-22 may also have opposite roles in innate colitis pathogenesis in a context and insult-dependent manner.

INTRODUCTION

Interleukin 23 (IL-23) is a heterodimeric cytokine composed of a specific p19 and a common p40 subunit shared by IL-12.¹ IL-23 is mainly expressed by macrophages and dendritic cells and signals through a heterodimeric receptor comprised of a specific subunit, IL-23R, and the shared IL12R β 1 subunit.² IL-23R signaling involves Jak2/Tyk2 and results in phosphorylation of Stat3 as well as Stat1, 4, and 5.² Expression of IL-23R is regulated by transcription factor retinoic acid–related orphan receptor γ t (Ror γ t) and IL-23R is produced by various adaptive and innate immune cells, including Th17, $\gamma\delta$ T cells, natural killer (NK) T cells, dendritic cells, macrophages, and innate lymphoid cells (ILCs).^{3,4} IL-23 can drive differentiation of Th17 cells from naive CD4⁺ T cells independently of transforming growth factor- β ⁵ and is important for maintenance and expansion of Th17 cells.⁴ IL-23R signaling results in secretion of Th17 signature cytokines IL-17A and F, and IL-22, Th17, $\gamma\delta$ T cells, and Ror γ t⁺ ILC. Th17 cells and their effector cytokines have been implicated in various autoimmune diseases⁶ in humans and extensively studied; however, the role of Ror γ t⁺ IL-23-responsive ILCs in autoimmunity remains elusive.

Genome-wide association studies identified polymorphisms of IL-23R as well as its various signaling components such as JAK2 and STAT3 as susceptibility or resistance factors for inflammatory bowel diseases (IBD).^{7–10} Further studies with both chronic and acute mouse models of IBD revealed a primary role for IL-23/IL-23R signaling and downstream effector cytokines in disease pathogenesis.^{11,12} In this regard, IL-23 cytokine subunits were investigated in detail. IL-23p19 or p40, but not IL-12-specific IL12p35, were shown to be essential for chronic colitis development in IL-10^{-/-} spontaneous colitis models,¹³ CD45RB^{high} CD4⁺ T-cell transfer models,^{13,14} and *Helicobacter hepaticus*–driven colitis.^{14,15} Likewise, IL-23p19 is required for pathogenesis in anti-CD40-induced acute innate colitis model.¹⁶ The role of IL-23 receptor has thus far been tested only in chronic CD45RB^{high} CD4⁺ T-cell transfer¹⁷ and acute dextran sodium sulfate (DSS)-induced colitis¹⁸ models. The former showed that IL-23R expression by CD4⁺ T cells is required for colitis development; DSS model, however, revealed an opposite role for IL-23R signaling in pathogenesis between Rag2-deficient and -sufficient animals.¹⁸ Although T-cell- and B-cell-bearing IL-23R^{-/-} mice were

¹Department of Immunology, University of Washington, Seattle, Washington, USA. ²Seattle Children's Research Institute, Center for Immunity and Immunotherapies, Seattle, Washington, USA and ³Department of Comparative Medicine School of Medicine, University of Washington, Seattle, Washington, USA. Correspondence: M Oukka (moukka@u.washington.edu)

Received 12 October 2012; accepted 22 April 2013; published online 29 May 2013. doi:10.1038/mi.2013.33

protected from the disease, IL-23R^{-/-}Rag2^{-/-} mice had exacerbated DSS-induced colitis, challenging the dominant pathogenic view of IL-23/IL-23R signaling in human IBD. The complexity regarding the role of effector cytokines produced by IL-23R signaling during IBD is also manifold.¹⁹ In one study, IL17A^{-/-} and IL-17R^{-/-} mice were reported to develop less severe DSS²⁰ and trinitrobenzene sulfonate-induced²¹ colitis, respectively. Another DSS study defined IL-17F as pathogenic and IL-17A as protective.²² Neutral, pathogenic,²³ and protective²⁴ roles have been reported for IL-17A in CD45RB^{high} CD4⁺ T-cell-transfer models. Lastly, IL-22, a member of the IL-10 family Th17 cytokine that acts on epithelial cells,²⁵ was shown to confer protection from DSS- and CD45RB^{high} naive CD4⁺ T cell-driven colitis,²⁶ whereas a recent study²⁷ demonstrated a pathogenic role for IL-22 in CD45RB^{low} memory CD4⁺ T-cell-induced chronic colitis.

Adult Rorγt⁺ ILCs, which have recently been named as type 3 ILCs, constitute a sizeable fraction of the intestinal lymphoid population in mice and share similar phenotypic and transcriptional profile with fetal lymphoid tissue inducer (LTi) cells.^{28,29} The resemblance of these LTi-like (or adult LTi) cells to Th17 lineage and their ability to produce IL-17A, IL-17F, and IL-22 sparked investigations as to their role in protective immunity and during disease pathogenesis.³⁰ Indeed, recent studies in mice revealed that Rorγt⁺ ILCs are not only required for protection from *Citrobacter rodentium* infection³¹ but are also instrumental in colitis mediated by innate cells.^{32,33} Buonocore *et al.*³² showed that colonic Thy1⁺Rorγt⁺ ILCs were increased and produced large quantities of IL-17A and IFN-γ after *Hepaticus* infection in Rag^{-/-} mice and are required for colitogenesis. Moreover, that same group showed that anti-CD40-induced innate colitis was also Rorγt and IFN-γ dependent, whereas a subsequent report introduced the notion of IFN-γ secreting NKp46⁺Rorγt⁻ ex-LTi cells and their involvement in colitogenesis.³³ Accumulating evidence suggests that type 3 ILCs may be involved in human IBD.³⁴ Thus, further study is required to better understand the role of these cells and their effector cytokines in autoimmune settings.

In this report, by utilizing CD45RB^{high} CD4⁺ T-cell-induced chronic and anti-CD40-induced acute innate colitis models in IL-23R^{GFP} Rag1^{-/-} knock-in mice, we explored the colitogenic role of IL-23R signaling exclusively in innate cells. We discovered that the absence of IL-23R in this compartment protects mice from innate cell-driven colitis, whereas it exacerbates T-cell-driven colitis. Lastly, we provide evidence, for the first time, that IL-22 produced by type 3 ILCs can promote colitis development in an innate context.

RESULTS

IL-23R deficiency exacerbates T-cell-driven colitis but protects from innate cell-induced colitis

Although the absence of IL-23R signaling in T cells in transferring colitis to Rag^{-/-} mice has been studied,¹⁷ how a defect of IL-23R in the non-T-cell compartment affects colitis development by adaptive immune cells has not been addressed. To study this, we transferred CD45RB^{high}NK1.1⁻

Foxp3⁻ CD4⁺ T cells into IL-23R^{-/-} Rag^{-/-} double knockout or Rag^{-/-} mice, which lack T and B cells. As previously^{26,35} described, recipient mice developed wasting disease starting week 3, post-T-cell transfer. Moreover, IL-23R^{-/-} Rag^{-/-} mice had significantly less weight loss by the end of week 5, compared with IL-23R-sufficient animals (**Figure 1a**). In contrast to the pathogenic role of IL-23R in systemic weight loss, IL-23R^{-/-} Rag^{-/-} mice developed exacerbated colitis. Thus, IL-23R was protective, as indicated by reduced weight loss, histopathology, and colitis scores (**Figure 1b,c**). T-cell-driven colitis histology was characterized by moderate-to-severe infiltration of mononuclear cells (macrophages with lesser lymphocytes; colitis score = 13) with cryptitis and abscessation, loss of goblet cells, and minimal-to-moderate epithelial proliferation with a prominent and edematous submucosa (**Figure 1c**). IL-23R^{-/-} Rag^{-/-} and Rag^{-/-} mice showed comparable levels of IL-17A, IFN-γ, and diminished IL-22 secretion in IL-23R^{-/-} Rag^{-/-} colon and ileum (**Supplementary Figure S1a** online). These results suggest that exacerbated colitis may be due to loss of IL-22 production in the absence of IL-23R. Indeed, a report by Zenewicz *et al.*²⁶ showed that IL-22 has a protective role in CD45RB^{high} CD4⁺ T-cell-transfer models.

To study how IL-23R deficiency in the non-T-cell compartment affects colitogenesis induced by innate cells, we employed a colitis model that has been recently described by Uhlig *et al.*¹⁶ in which colitis is triggered by infusing mice on Rag-deficient background with an agonistic anti-CD40-specific antibody. Anti-CD40 infusion of Rag^{-/-} mice was reported to lead to acute IL-23-dependent colitis and IL-23-independent wasting disease.¹⁶ In this particular model, inflammation of the gastrointestinal tract occurs through activation of dendritic cells and subsequent production of IL-23 and other pro-inflammatory cytokines. Therefore, we assessed the susceptibility of IL-23R^{-/-} Rag^{-/-} and Rag^{-/-} mice to CD40-induced colitis by infusing a single dose of anti-CD40-specific antibody. As in the first model, IL-23R^{-/-} Rag^{-/-} mice were protected from wasting disease (**Figure 1d**). However, unlike the T-cell-transfer model, IL-23R^{-/-} Rag^{-/-} mice developed milder colitis compared with Rag^{-/-} mice (**Figure 1e,f**). The colitis in Rag^{-/-} + anti-CD40 was histologically characterized by extensive areas of increased lamina propria mononuclear and lesser polymorphonuclear inflammatory cells (colitis score = 12) with occasional areas of marked mixed inflammation in the attached mesentery, whereas in the IL-23^{-/-}Rag^{-/-} colons, the inflammation was milder and more segmental with regions of relatively spared mucosa adjacent to colitis regions (**Figure 1f**). Recently, Buonocore *et al.*³² reported that Rag^{-/-}Rorγt^{-/-} mice lacking type 3 ILCs were protected from anti-CD40-induced colitis and have identified a subset of ILCs that are Thy1.2⁺Sca1⁺Rorγt⁺ to be critical for colitis pathogenesis. However, the Thy1⁺Sca1⁺ population is heterogeneous and is composed of various subsets of cells that may have different functions (**Supplementary Figure S2b** online). More recently, by using elegant cell-transfer experiments, Vonarbourg *et al.*³³ have shown that among

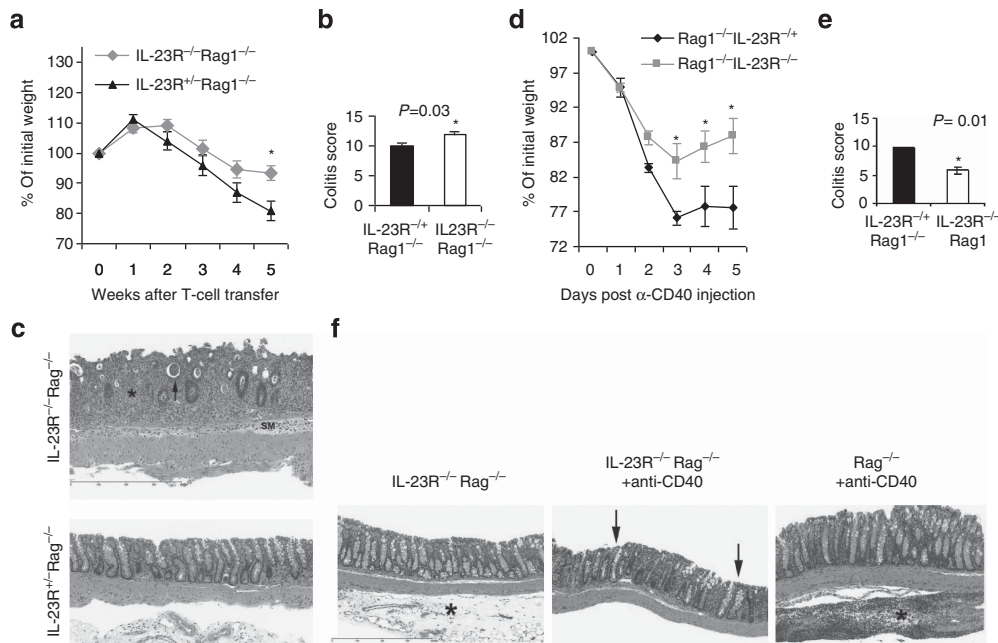


Figure 1 Interleukin (IL)-23R deficiency exacerbates T-cell driven but protects from innate cell-induced colitis. **(a)** IL-23R^{-/-} Rag1^{-/-} and Rag1^{-/-} mice were intraperitoneally injected with 5×10^5 CD45RB^{high} CD4⁺ NK1.1⁻ Foxp3YFP⁻ T cells and weight loss was measured relative to initial weight. **(b)** At the end of week 5, colons were harvested, stained with hematoxylin and eosin (H&E) and scored for colitis. **(c)** Colitic changes included loss of crypts and goblet cells, crypt abscesses (arrow), and increased lamina propria inflammatory mononuclear cells (*) with a prominent and edematous submucosa (SM). All panels, original magnification $\times 20$; bar in upper panel is 400 μ m. **(d)** IL-23R^{-/-} Rag1^{-/-} and Rag1^{-/-} mice were intraperitoneally injected anti-CD40 mAb for acute colitis, weight loss was measured relative to initial weight. **(e)** At the end of day 7, colons were harvested, stained with H&E, and scored for colitis. **(f)** Representative histology where (*) indicates attached mesentery (left and right panel; not present in the center panel). Arrows indicate segmental areas of normal mucosa. All panels, original magnification $\times 20$; bar in left panel is 400 μ m. *P* value < 0.05 are shown with "*" in **a**, **b**, **d**, and **e**. The experiments were performed with 4–5 animals in each group and repeated 2–3 times.

all type 3 ILCs, only the ones that express NKp46 and NK1.1 could induce colitis when transferred into IL-2R γ c^{-/-} Rag1^{-/-} mice, which have very few type 3 ILCs and are resistant to CD40-induced colitis. They showed that this NKp46⁺ subset arose from an NKp46⁻ Ror γ t⁺ precursors that are different from conventional NK cells and express high levels of IL-23R. Intriguingly, they also found that mice depleted of NK1.1-expressing cells were resistant to the development of CD40-induced colitis. They argued that this NKp46⁺ subset promotes colitis only once it loses ROR γ t expression and switches from IL-22 production to IFN- γ production via IL-23-dependent mechanisms.³³

First, we attempted to confirm the published data by using the same experimental approach as the Diefenbach's group: to study the development of colitis in mice that were depleted of NK1.1-expressing cells.³³ In our experiments using animals housed in our facility, mice that were depleted of NK1.1-expressing cells developed colitis with the same severity as control mice (unpublished data). Therefore, the identity of the subset of cells that express IL-23R and trigger colitis has yet to be identified.

IL-23R deficiency does not impact intestinal IL-23R⁺ ILC expansion but reduces IFN- γ , IL-22, IL-17A, and IL-17F production in the colon during colitis

The IL-23R^{-/-} mouse has IRES-GFP (internal ribosomal entry site-green fluorescent protein) knocked in to IL-23R locus

after exon 8 and can be used as a reporter of IL-23R⁺ innate cells in the Rag-deficient background.³ IL-23RGFP⁺ ILCs were detectable in various organs, including colon, small intestine (SI), spleen, mesenteric, and peripheral lymph nodes in the steady state (**Supplementary Figure S2a** online). We characterized IL-23R⁺ ILCs by staining for various markers described in the literature (**Supplementary Figure S2b,c** online). In the colon, 37–49% of IL-23R⁺ ILCs expressed CD4, 23–33% expressed NK cell marker NKp46, 36–47% expressed NK1.1, 52–76% expressed CCR6, 86–100% expressed Sca-1, and lastly, both colon and SI IL-23R⁺ ILCs cells expressed inducible T-cell costimulator and CD25 (**Supplementary Figure S2c** online). Thy1.2⁺ IL-23R⁺ ILCs were negative for CD11c and CD11b markers indicating a non-DC non-macrophage population (unpublished data). We did not observe a drastic difference between IL-23R-sufficient and -deficient Rag1^{-/-} mice with respect to representation of these colonic IL-23R⁺ ILC subsets after several gut isolations (**Supplementary Figure S2a–c** online). This data suggest that IL-23R is dispensable for the development of these ILC subsets and may have an important role in their effector function during colitis. We also took advantage of this reporter mice and probed the dynamics of IL-23R⁺ ILCs before and after anti-CD40 injection (**Figure 2a**). Interestingly, 2 days after anti-CD40 infusion, we observed a dramatic increase in the percentage of GFP⁺ cells early on during colitis in both IL-23R^{-/-} Rag1^{-/-} and Rag1^{-/-} mice (**Figure 2a** and **Supplementary Figure S3** online). However, in

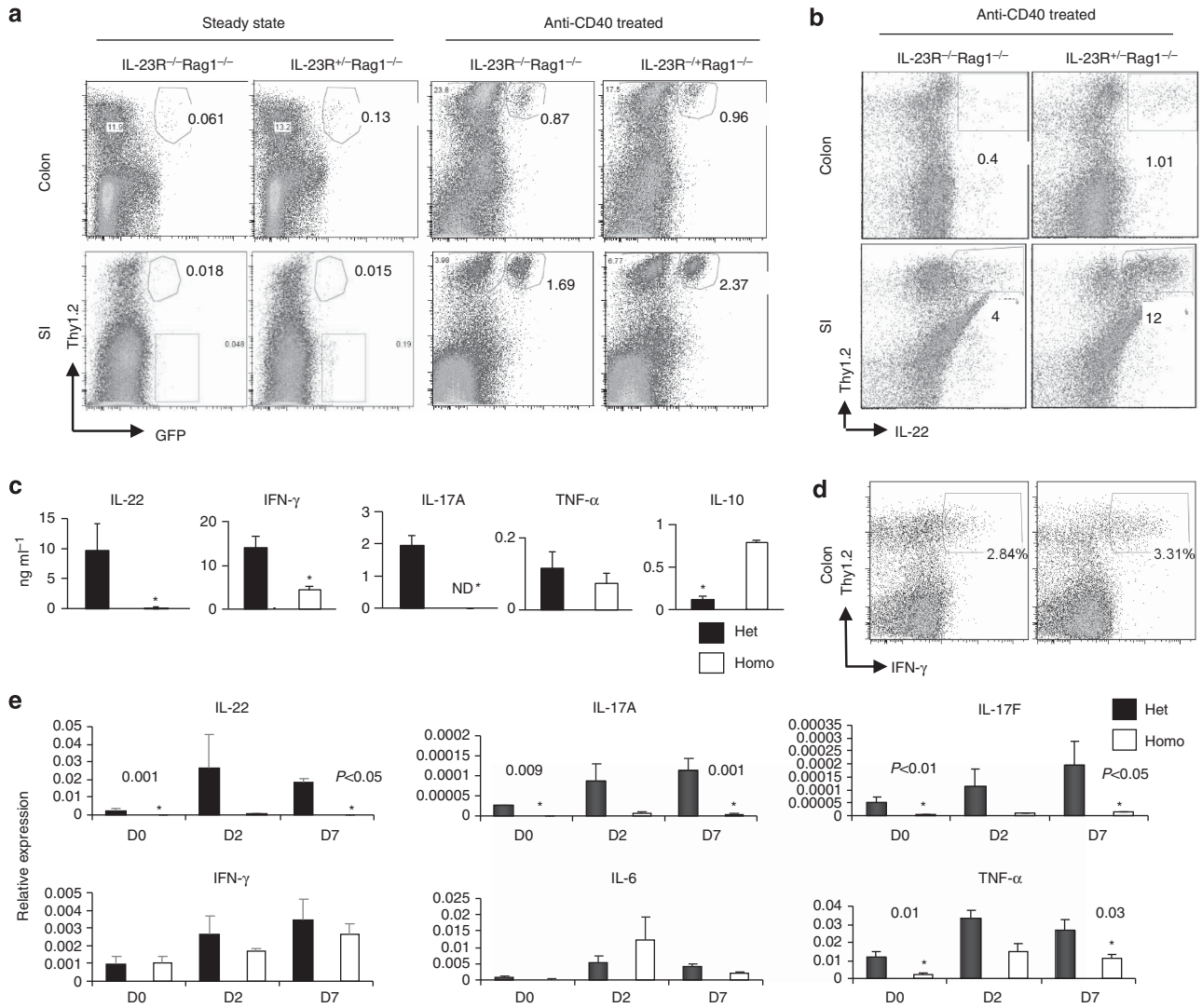


Figure 2 Interleukin (IL)-23R deficiency does not impact intestinal IL-23R⁺ innate lymphoid cell expansion but reduces interferon (IFN)- γ , IL-22, IL-17A, and IL-17F production in the colon during colitis. **(a)** Colon or small intestine lamina propria (cLP or SI LP) cells were purified from untreated mice or mice treated with anti-CD40 antibody (at day 2), then stained for indicated cell surface markers. **(b)** cLP or SI LP cells were stimulated with PMA-ionomycin for 4 h and intracellular cytokine staining was performed for IL-22 and **(d)** IFN- γ . **(c)** Proximal colon (1 cm) from anti-CD40-injected IL-23R^{-/-} Rag1^{-/-} and control Rag1^{-/-} mice were harvested at day 7 and cultured for 48 h and indicated cytokines were measured from supernatants via enzyme-linked immunosorbent assay. Het: IL-23R^{+/-} Rag1^{-/-}, Homo: IL-23R^{-/-} Rag1^{-/-}. **(e)** mRNA expression of indicated genes in the colons were measured via quantitative real-time PCR before (0) or 2 and 7 days after anti-CD40 injection. Het: IL-23R^{+/-} Rag1^{-/-}, Homo: IL-23R^{-/-} Rag1^{-/-}. *P value < 0.05. The experiments were performed with 3–5 animals in each group and repeated 2–3 times. GFP, green fluorescent protein; ND, not detected; TNF, tumor necrosis factor.

the steady state, and 2 days after anti-CD40 infusion, colonic lamina propria (cLP) lymphocytes of Rag^{-/-} and IL-23R^{-/-}Rag^{-/-} mice contained comparable absolute numbers of IL-23R⁺ ILCs that were also Thy1.2⁺ (**Supplementary Figure S2d** online), despite the fact that the absolute number of colon LP lymphocytes was reduced 2 days after colitis induction. Therefore, increased representation of IL-23R-expressing cells during colitis was not due to the expansion or recruitment of IL-23R⁺ ILCs in the colon but was rather due to increased cell death during the development of colitis. We also observed that SI lamina propria contained fewer IL-23R⁺ ILCs in the steady state compared with cLP (**Figure 2a**). However, the absolute number of IL-23R⁺

ILCs in the SI was greatly enhanced during colitis due to proliferation or recruitment (**Figure 2a**). The expansion of IL-23R⁺ ILCs in the SI was not dependent on IL-23 as the percentage and the absolute number of IL-23R⁺ ILCs was comparable in IL-23R^{-/-}Rag^{-/-} and Rag^{-/-} mice. Therefore, we concluded that IL-23R is dispensable for enrichment of type3 ILCs in the SI (**Figure 2a**).

Next, we compared the effector cytokines produced by *ex vivo* cultures of the colons and SI of IL-23R^{-/-}Rag^{-/-} and Rag^{-/-} mice a week after colitis induction. IL-22, IFN- γ , and IL-17A cytokines were significantly downregulated and IL-10 was upregulated in IL-23R^{-/-}Rag^{-/-} mice, consistent with reduced severity of colitis compared with Rag^{-/-} mice

(Figure 2c). Real-time quantitative PCR data obtained from colons on days 0, 2, and 7 of colitis supported the IL-22 and IL-17A enzyme-linked immunosorbent assay (ELISA) results and revealed a reduction in IFN- γ , although not at a significant level (Figure 2e). In addition, we observed a significant reduction in IL-17F and tumor necrosis factor (TNF)- α mRNA levels in IL-23R^{-/-} Rag^{-/-} colons. We further confirmed the reduction of IL-22 and IFN- γ production by Thy1.2⁺ cells isolated from IL-23R^{-/-} Rag^{-/-} mice cLP via intracellular cytokine staining (Figure 2b,d).

Lastly, we sorted Thy1.2⁺IL-23RGFP⁺, Thy1.2⁺IL-23RGFP⁻, Thy1.2⁻IL-23RGFP⁻, and Thy1.2⁻IL-23RGFP^{low} lymphocytes from cLP on day 5 after induction of colitis. We found that Thy1.2⁺IL-23RGFP⁺ lymphocyte population was the main source of IL-23R, Ror γ t, and IL-22 and that IL-22 was diminished in this subset of cells isolated from IL-23R^{-/-} Rag^{-/-} mice colons (Supplementary Figure S4a,b online). Using intracellular cytokine staining, we found that 70% of IL-22⁺ Thy1.2⁺ ILCs were also Ror γ t⁺ (Supplementary Figure S4c online). Interestingly, IFN- γ mRNA levels were comparable between IL-23R^{-/-} and wild-type (WT) Thy1.2⁺ lymphocyte subsets despite the intracellular cytokine data showing a reduction in IFN- γ protein, which suggests a translational regulation of IFN- γ by Thy1.2⁺ ILCs rather than a transcriptional one. Collectively, these findings reveal that although the abundance of IL23R⁺ ILCs are unaltered in the absence of IL-23R during innate colitis, there is a pro-inflammatory environment in the colons of IL-23R-sufficient mice to which these cells may contribute via IL-22, IL-17A, IL-17F, and IFN- γ .

IL-22 is pathogenic during CD40-induced colitis

IFN- γ has been reported to have a role in colitis pathogenesis induced by anti-CD40.^{32,33} Moreover, IL-17A-deficient Rag^{-/-} mice developed colitis to the same degree as Rag^{-/-} mice.³² Thus, we directed our focus on less explored IL-22 and IL-17RA, the receptor subunit of IL-17A, IL-17C, IL-25, and IL-17F. Blocking the IL-17R pathway via antibody injection did not change the severity of anti-CD40-induced colitis, consistent with the reports on Rag^{-/-}IL-17A^{-/-} knockout mice (Figure 3a,b). However, IL-17RA has also been reported to pair with IL-17RB or IL-17RE, which makes up the receptor complex for IL-25 (IL-17E)³⁶ or IL-17C,³⁷ respectively. Additionally, injection of IL-17A and IL-17F neutralizing antibodies had no impact on the severity of anti-CD40-induced colitis as shown by pathology scores (Figure 3b lower panel). Neutralization of IL-22 in IL-23R^{-/-} Rag^{-/-} mice via daily injections, however, significantly reduced the weight loss, colon pathology, and colitis scores caused by anti-CD40 injection (Figure 3c-e and Supplementary Figure S5 online). Histologically, in the anti-IgG-treated colon, the inflammation was severe (colitis score = 12) with loss of crypts and goblet cells, cryptitis, and crypt abscesses and increased lamina propria inflammatory mononuclear cells. Milder changes were noted in the anti-IL-22-treated colon. To ensure that the neutralization was specific and the effect was

caused exclusively by IL-22 neutralization, we cloned IL-22 cDNA onto pBSHCRHP-A plasmid and injected via hydrodynamic delivery into IL-23R^{-/-} Rag^{-/-} mice, which have no detectable IL-22. One dose of injection into IL-23R^{-/-} Rag^{-/-} mice restored expression of IL-22 in blood and colon lasting as long as 10 days (Figure 4c, lower panel IL-22 and unpublished data). IL-23R^{-/-} Rag^{-/-} mice that received the IL-22 plasmid developed severe colitis after CD40 injection, compared with empty plasmid recipients (Figure 3f-h). In the IL-22 plasmid-treated colon, the inflammation was severe, with dense infiltration of the lamina propria with mononuclear cells and loss of crypts and goblet cells, cryptitis, and crypt abscesses. By contrast, the empty vector-treated colon had minimal-to-mild lamina propria inflammation (Figure 3g). Of note, IL-22 plasmid-recipient IL-23R^{-/-} Rag^{-/-} mice developed colitis only if they were treated with anti-CD40 (Supplementary Figure S6b online), suggesting that IL-22 alone is insufficient for the pathogenesis and requires possibly the cytokine burst provided by CD40 ligation of antigen-presenting cells. This set of experiments collectively showed that in this particular model of innate colitis IL-22, but not IL-17, has a pathogenic role.

In order to gain insight into how IL-22 drives pathogenesis, we compared the cytokine profiles of intestines harvested on day 7 after induction of colitis in mice that received the neutralizing anti-IL-22 antibody and mice that received isotype control antibody. ELISA results obtained from *ex-vivo* colon cultures revealed a significant reduction in IFN- γ and a significant increase in IL-10 after IL-22 neutralization (Figure 4a). Although levels were not significantly measurable, we observed a decrease in IL-6 levels. Similarly, ileum cultures also produced significantly less IFN- γ and IL-6. Interestingly, we found significantly higher TNF- α and slightly elevated IL-17A after IL-22 neutralization, suggesting that these cytokines are not likely candidates for IL-22-driven colitis pathogenesis. IL-22 message levels were also unaffected by neutralization. However, various IL-22-driven genes, notably antimicrobial peptides RegIII γ (Figure 4c), RegIII β , and S100A8 (unpublished data), were downregulated, confirming that neutralizing antibody effectively blocked IL-22-mediated signaling. Restoration of IL-22 in IL-23R^{-/-} Rag^{-/-} mice via hydrodynamic delivery of IL-22-expressing plasmid corroborated our results with neutralization experiments. RegIII γ is an antimicrobial peptide, which exclusively kills Gram-positive bacteria.³⁸ Selective disruption of microbial community is speculated to result in colon pathology.²⁷ IL-22 alone or in combination with IL-17A and IL-17F enhances its expression.³⁹ Because RegIII γ was the most drastically altered transcript in IL-23R^{-/-} Rag^{-/-} mice and was restored upon IL-22 plasmid injections, to rule out a possible direct or indirect role for RegIII γ in mediating IL-22-driven colitis, we cloned RegIII γ onto pBSHCRHP-A plasmid and delivered it into IL-23R^{-/-} Rag^{-/-} mice, which were subsequently injected with anti-CD40. Both RegIII γ plasmid- and mock plasmid-recipient

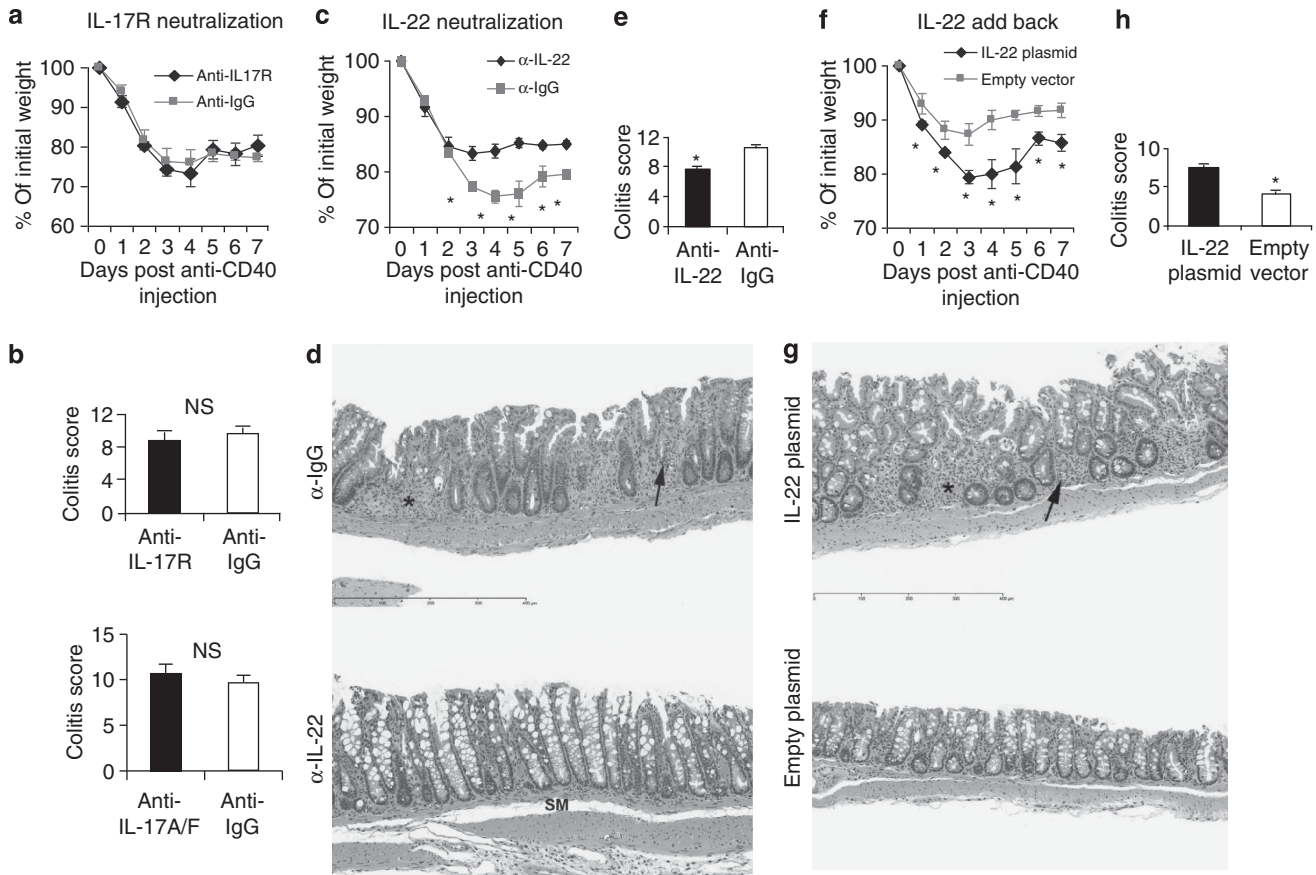


Figure 3 Interleukin (IL)-22 is pathogenic during α -CD40-induced colitis. **(a)** Anti-CD40-injected $Rag^{-/-}$ mice were injected with either IL-17RA blocking or isotype immunoglobulin G (IgG) daily until day 5 of colitis. Weight loss was measured relative to initial weight; **(b)** colons were harvested at day 7 of colitis and were scored after hematoxylin and eosin (H&E) staining. **(c)** Anti-CD40-injected $Rag^{-/-}$ mice were injected with either IL-22 neutralizing or isotype IgG antibodies daily until day 5 of colitis. Weight loss was measured relative to initial weight; **(d)** colons were harvested at day 7 of colitis and stained with H&E. Histology included loss of crypts and goblet cells, cryptitis (arrow), and increased lamina propria inflammatory mononuclear cells (*). Lower left panel: the submucosa (SM) widened due to fixation and not due to edema was shown. All panels, original magnification $\times 20$; bar in upper panel is 400 μ m. **(e)** Colitis scores of **d**. **(f)** IL-23R $^{-/-}$ $Rag^{-/-}$ mice were hydrodynamically injected with either IL-22 or mock-expressing plasmid 2 days before anti-CD40 injection. Weight loss post-anti-CD40 treatment was measured relative to initial weight; **(g)** colons were harvested at day 7 of colitis and stained with H&E. Labels and magnifications as in **g**. **(h)** Colitis score of **g**. * P value < 0.05 in **c**, **e**, **f**, and **h**. The experiments were performed with 4–5 animals in each group and repeated 2–3 times.

mice developed colitis to the same degree and had similar wasting disease. Unlike IL-22 plasmid injection, RegIII γ plasmid delivery did not restore colitis in IL-23R $^{-/-}$ $Rag^{-/-}$ mice, suggesting that IL-22 drives colitis through means other than RegIII γ and downstream molecules (**Figure 4d,e**). RegIII γ plasmid used for these experiments was indeed functional as IL-23R $^{-/-}$ $Rag^{-/-}$ mice that received RegIII γ plasmid was resistant to *Citrobacter rodentium* infection compared with empty vector recipients marked by reduced weight loss and bacterial translocation/burden (**Supplementary Figure S6a** online and unpublished data). Altogether, these data reveal that IL-23R-dependent colitis is, at least in part, mediated by IL-22, likely through its direct or indirect actions on IFN- γ and IL-10 but not through induction of RegIII γ expression.

IL-22 requires innate cells to drive pathogenesis but not p40

The only source of IL-22 in the colon and SI of $Rag^{-/-}$ mice is IL23R $^{+}$ Ror γ t $^{+}$ type3 ILCs. IL-22 appeared to be necessary for

full phenotype of anti-CD40-induced colitis from data above. To test whether IL-22 is sufficient to promote anti-CD40-induced colitis in the absence of these cells, we utilized IL-2R γ c $^{-/-}$ $Rag^{-/-}$ mice, which lack colonic IL-23R $^{+}$ Ror γ t $^{+}$ cells and NK cells in addition to T and B cells due to lack of common γ c. IL-2R γ c $^{-/-}$ $Rag^{-/-}$ mice have functional macrophages and dendritic cells, although a reduction in absolute number and subsets of DCs have been reported⁴⁰ in the absence of IL-2R γ c $^{-/-}$. Therefore, these cells can be activated by anti-CD40 injection. However, IL-2R γ c $^{-/-}$ $Rag^{-/-}$ mice do not develop colitis after anti-CD40 injection, suggesting that other innate cells are required for colitogenesis in addition to antigen-presenting cells. Provision of IL-2R γ c $^{-/-}$ $Rag^{-/-}$ mice with IL-22-expressing plasmid also did not promote colitis or wasting disease, indicating that IL-22 alone is not sufficient for pathogenesis (**Figure 5a,b**). Therefore, we concluded that the presence of either IL-23R $^{+}$ Ror γ t $^{+}$ ILCs or NK cells are required for IL-22-mediated colitis.

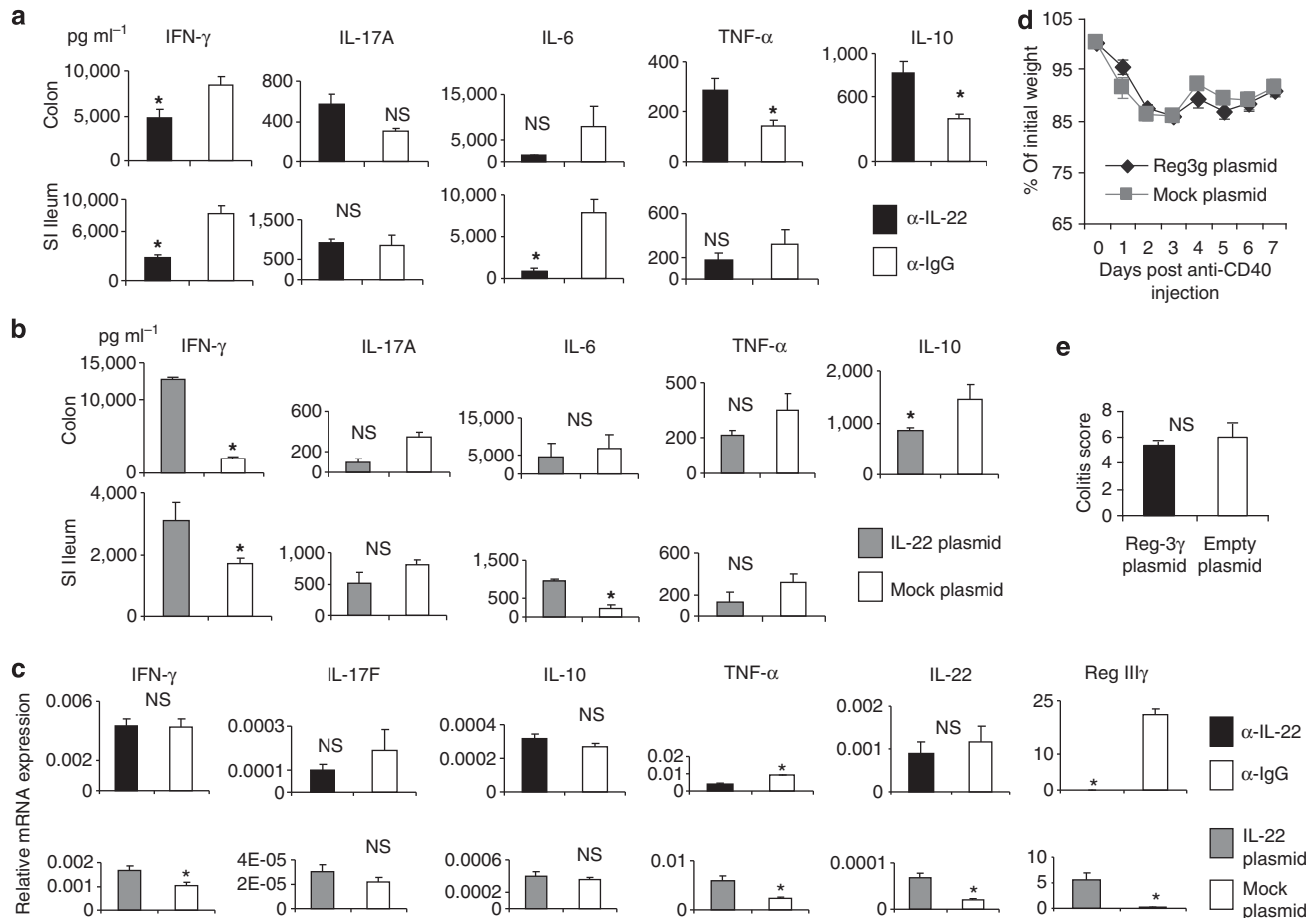


Figure 4 Modulation of interleukin (IL)-22 alters expression of pro-inflammatory cytokines. (a) Proximal colons and ileum were harvested at day 7 of colitis from Rag^{-/-} mice that received neutralizing IL-22 or isotype immunoglobulin G antibodies and 1 cm piece was cultured for 48 h *ex vivo*; the rest of the colon is used for quantitative PCR (qPCR). Indicated cytokines were measured from supernatants via enzyme-linked immunosorbent assay (ELISA). (b) Proximal colon and ileum of colitic mice that received either IL-22 expressing or mock plasmid 2 days before CD40 injection were harvested at day 7 and cultured as in a, leftover colon was used for qPCR. Cytokine levels were measured via ELISA. (c) Proximal colons saved from a and b were used to detect expression of indicated mRNA levels. (d) IL-23R^{-/-}Rag^{-/-} mice were hydrodynamically injected with either RegIII γ or mock-expressing plasmid 2 days before anti-CD40 injection. Weight loss post-anti-CD40 treatment was measured relative to initial weight; colons were harvested at day 7 of colitis after hematoxylin and eosin staining (e) colitis was scored. **P* value < 0.05. The experiments were performed with 4–5 animals in each group and repeated 2–3 times. IFN, interferon; NS, not significant; SI, small intestine; TNF, tumor necrosis factor.

Although IL-22 expression is induced as a result of IL-23/IL-23R signaling, IFN- γ could be driven by both IL-12/IL-12R and IL-23/IL-23R axes. Furthermore, IFN- γ neutralization was reported to alleviate anti-CD40-induced colitis.¹⁶ Therefore, we neutralized both IL-12 and IL-23 signaling by infusing mice with common anti-p40 subunit-specific antibody and assessed whether restoration of IL-22 expression via hydrodynamic plasmid delivery would be sufficient to promote colitis in the absence of IL-23 and IL-12 signaling. Indeed, Rag^{-/-} mice that received IL-22 plasmid developed significantly more colitis and wasting disease after CD40 injection compared with the mock plasmid recipients even in mice in which p40 had been neutralized (Figure 5c,d). Colitis in the IL-22 plasmid-treated mice was characterized by inflammatory cell infiltration and mucosal hyperplasia, similar to the pathology above. Interestingly, mice that received IL-22 plasmid and anti-p40 had significantly less IL-10 and increased IFN- γ in their colons compared with mice that received mock plasmid and anti-p40

neutralizing antibody. Collectively, these data clearly show that IL-22 can drive colitis in the absence of IL-12- and IL-23-mediated signaling.

IL-22 promotes neutrophil recruitment and downregulates matrix metalloproteinase (MMP)-2 and MMP-9

IL-22 was previously shown to orchestrate the recruitment of neutrophils and macrophages in asthma⁴¹ and DSS-induced colitis models.¹⁸ Therefore we examined the Gr1⁺ CD11b⁺ neutrophil counts in colon LP after colitis induction via anti-CD40 antibody infusion. As expected, IL-23R^{-/+} Rag^{-/-} mice colon LP had significantly more Gr1⁺ CD11b⁺ cells compared with that of IL-23R^{-/-} Rag^{-/-} after colitis induction (Figure 6a). Moreover, IL-22 plasmid-recipient IL-23R^{-/-} Rag^{-/-} mice also had significantly more neutrophil recruitment to the colon compared with empty vector-recipient mice (Figure 6b). In accord with neutrophil recruitment data, IL-23R^{-/-} Rag^{-/-} mice that received

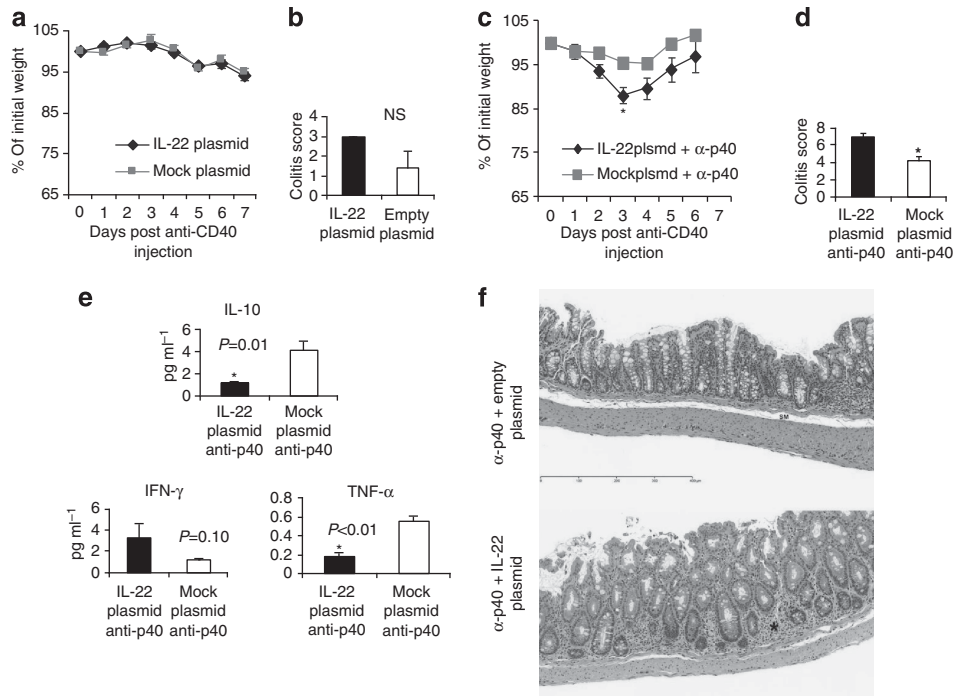


Figure 5 Interleukin (IL)-22 requires innate cells to drive pathogenesis but not p40. (a) $Rag^{-/-}$ IL2R γ $C^{-/-}$ mice were hydrodynamically injected with either IL-22 or mock-expressing plasmid 2 days before anti-CD40 injection. Weight loss post-anti-CD40 treatment was measured relative to initial weight; colons were harvested at day 7 of colitis after hematoxylin and eosin staining (b) colitis was scored. (c) $Rag^{-/-}$ mice were hydrodynamically injected with either IL-22 or mock-expressing plasmid 2 days before anti-CD40 injection. In addition, both groups received anti-p40 neutralizing antibody at days 0, 2, 4, and 6. Weight loss post-anti-CD40 treatment was measured relative to initial weight; colons were harvested at day 7 of colitis. (d) Colitis was scored after (f) representative histology. Note that the widened submucosa (SM) is artifactual due to fixation; increased lamina propria inflammatory mononuclear cells (*) as indicated. All panels, original magnification $\times 20$; bar in upper panel is 400 μ m. (e) One centimeter from proximal colon of each animal was cultured 48 h and indicated cytokines were measured via enzyme-linked immunosorbent assay. * P value < 0.05 in c–e. The experiments were performed with 4–6 animals in each group and repeated 2–3 times. NS, not significant.

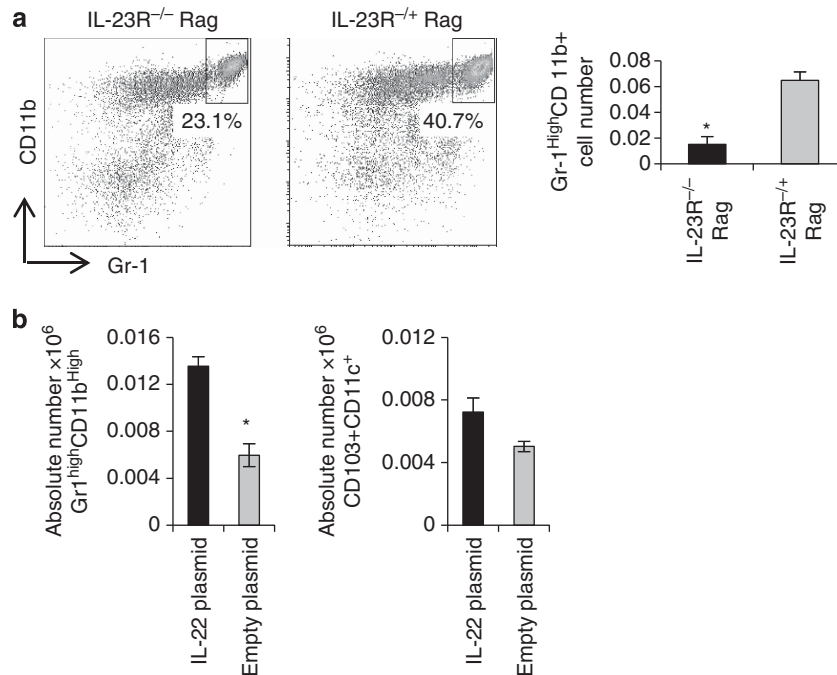


Figure 6 Neutrophil recruitment is interleukin (IL)-22 dependent. (a) IL-23R $^{-/-}$ Rag $^{-/-}$ and IL-23R $^{-/-}$ Rag $^{-/-}$ mice colon lamina propria (LP) lymphocytes were stained with Gr-1 and CD11b antibodies following the day of anti-CD40 antibody injection and the absolute number of infiltrating neutrophils quantified. (b) Absolute number of infiltrating neutrophils (Gr-1 high CD11b high) or CD103 $^{+}$ CD11c $^{+}$ dendritic cells were quantified from colon LP of IL-22 plasmid or empty plasmid–recipient IL-23R $^{-/-}$ Rag $^{-/-}$ mice following the day of anti-CD40 antibody injection. * P < 0.05 .

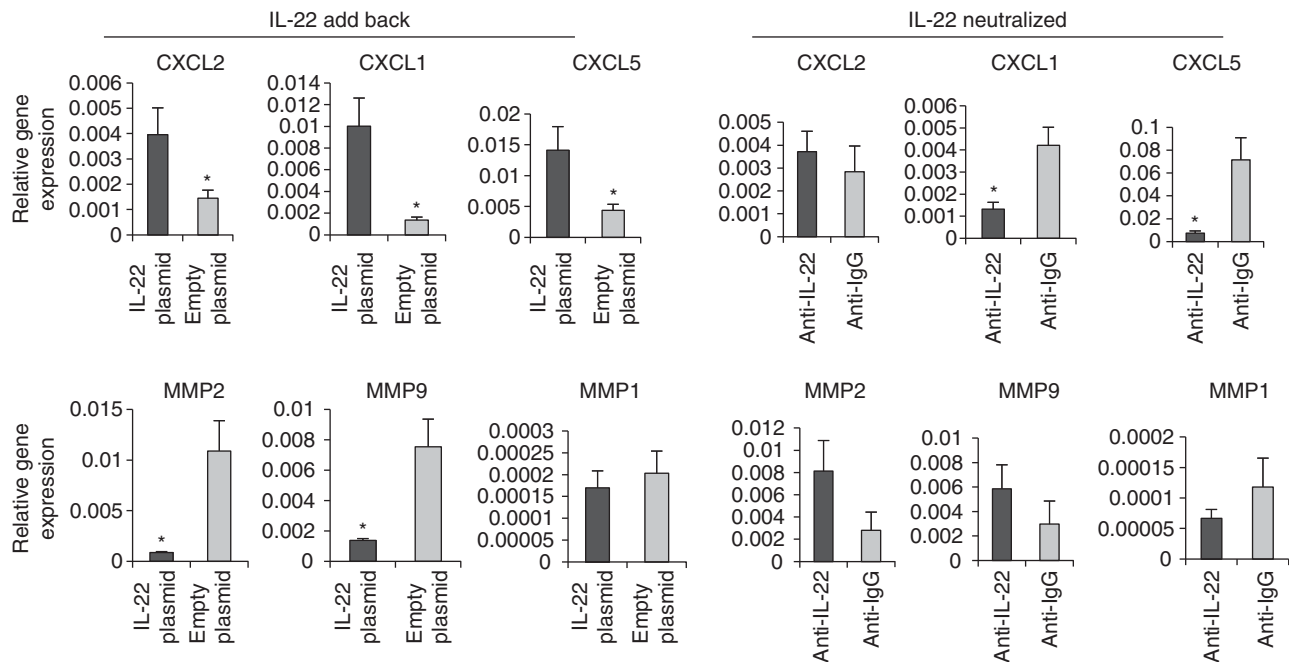


Figure 7 Interleukin (IL)-22 upregulates neutrophil-recruiting chemokines, downregulates matrix metalloproteinase (MMP)-2 and MMP-9. Relative mRNA expression of indicated genes in the colon lamina propria at the end of day 7 of anti-CD40-induced colitis. CXCL, C-X-C motif chemokine ligand 1; IgG, immunoglobulin G. * $P < 0.05$.

IL-22 plasmid expressed higher levels of neutrophil attractant chemokines C-X-C motif chemokine ligand 1 (CXCL1), CXCL2, and CXCL5 compared with mock plasmid-recipient (Figure 7). Similarly, when IL-22 was neutralized in IL-23R^{-/+} Rag^{-/-} mice, expression levels of CXCL2 and CXCL5 were decreased (Figure 7). Additionally, IL-23R^{-/-} Rag^{-/-} mice colons also expressed significantly decreased CXCL5 and reduced CXCL2 and CXCL1 mRNA (did not reach the significance). Altogether these results suggest that IL-22 may contribute to colon pathology in anti-CD40 setting, in part, through mobilization of neutrophils.

To further gain insight into the mechanisms of IL-22-dependent pathology, we also investigated MMP-2 and -9 levels. MMPs have an important role in tissue remodeling⁴² and are implicated in various pathological conditions, such as arthritis and IBD.⁴³ In fact, MMP levels were reported to be upregulated in human IBD.^{43,44} Moreover, studies with MMP-2-deficient mice revealed that MMP-2 has a protective role during DSS-induced and *Salmonella enterica* subsp. serovar *Typhimurium*-driven enteritis as those mice developed more severe mucosal injury.⁴⁵ Comparison of colon LP of IL-22 plasmid-recipient IL-23R^{-/-} Rag^{-/-} mice to empty vector recipients after colitis induction revealed a drastic reduction in MMP-2 and MMP-9 levels (Figure 7). Although we observed a similar trend (decrease in MMP-2 and MMP-9) in the IL-22-neutralized IL-23R^{-/+} Rag^{-/-} group compared with IgG recipients, this was not statistically significant. We then compared MMP-2 and -9 levels in colitic IL-23R^{-/-} Rag^{-/-} and IL-23R^{-/+} Rag^{-/-} animals and did not detect a difference. This suggests that expression of MMP-2

and -9 could be induced independently of IL-23R, and large quantities of IL-22 may negatively regulate their expression.

DISCUSSION

IBD is a complex disease involving both genetic and dietary components. Inflammation in the bowels is a result of collaborative work between innate and adaptive immune cells. IL-23/IL-23R signaling axis and mutations in this pathway emerged as a major genetic contributor to IBD pathogenesis based on data provided by genome-wide association and animal model studies. Because IL-23R is expressed by Th17 cells, and is important for their maintenance as well as differentiation from CD4⁺ naive T cells, most studies pertaining to IL-23/IL-23R axis-IBD relation focused on the adaptive arm of immunity, exclusively Th17 cells. Although IL-23R is expressed by Rorγt⁺ ILCs in both mice and humans, their contribution to IBD has only recently been questioned. Herein, by utilizing IL-23R^{-/-} Rag^{-/-} mice, which harbor IL-23R^{-/-} type 3 ILCs, we report opposite effects of IL-23R deficiency in the innate compartment on colitis development in two distinct models of IBD. IL-23R deficiency in Rag^{-/-} mice exacerbated CD45RB^{high} Foxp3⁻ T-cell-transfer-induced chronic colitis, whereas it conferred a protection from anti-CD40 antibody-induced acute colitis. We further showed that IL-23-dependent colitis pathogenesis is mediated, at least in part, by IL-22 in anti-CD40 antibody-induced acute colitis.

We propose that exacerbated colitis in IL-23R^{-/-} Rag^{-/-} mice that received CD45RB^{high} Foxp3⁻ T cells compared with IL-23R-sufficient counterparts is most likely due to reduced IL-22 levels in the small and large intestines of these mice and that our data corroborate the results obtained by a previous

report performed using IL-22^{-/-}Rag^{-/-} mice. Five weeks following the transfer of CD45RB^{high} Foxp3⁻ T cells, IFN- γ and IL-17A cytokine levels were comparable. However, IL-22 protein was higher in Rag^{-/-} mice compared with IL-23R^{-/-}Rag^{-/-} animals, although engrafted T cells were IL-23R-sufficient and a source of IL-22. By transferring IL-22^{-/-}CD45RB^{high} Foxp3⁻ T cells into IL-22^{-/-}Rag^{-/-} mice, Zenewicz *et al.*²⁶ previously showed that when IL-22 is defective in both recipient Rag^{-/-} mice and engrafted cells, colitis worsened. Although their result indicated that the deficiency of IL-22 in the recipient mice alone could be compensated by transferred WT T cells, our data with IL-23R^{-/-}Rag^{-/-} mice showed exacerbated colitis even if the engrafted cells were IL-23R-sufficient. A possible explanation may be that the difference in pathology may become more discernible at later time points following T-cell transfer. Supporting this view, we observed that the weight loss difference between IL-23R^{-/-}Rag^{-/-} and Rag^{-/-} mice only became significant at week 5, although weight loss inversely correlates with the severity of the colonic pathology.

Type 3 ILCs have been characterized in most studies based on their expression of transcription factor Ror γ t. IL-23R, turned on by Ror γ t, may also be used to track these cells *in vivo*, yet has never been comprehensively utilized. Our findings with IL-23R^{GFP} reporter mice corroborate the previous studies⁴⁶ in a number of ways, as described in the Results section: colonic IL-23R⁺ ILCs were all Thy1.2⁺, Sca-1⁺ and 30–50% of these cells were CD4⁺. Also, 30% of these cells express NKp46 or NK1.1 NK cell markers. Thy1.2⁺IL-23R⁺ ILCs were the only IL-22 source and the only major Ror γ t⁺ cells.

Anti-CD40-induced colitis was described as a means of inducing colitis via IL-23 produced by dendritic cells.¹⁶ Both p40 and p19 subunits of IL-23, but not IL-12p35 were shown to be required for the development of colitis.¹⁶ In a subsequent report, Ror γ t^{-/-}Rag^{-/-} mice were shown to be resistant to colitis mediated by anti-CD40 infusion, implicating Ror γ t⁺ ILCs as inducers of innate cell-driven colitis.³² However, the mechanism by which IL-23R induced colitis remained unknown. Our results have identified the cytokine IL-22 as one of the targets of IL-23R-mediated signaling involved in the pathology of colitis.

The most important finding of this study is that IL-23R signaling drives colitis and systemic wasting disease, at least in part, via IL-22 in an anti-CD40 setting. We demonstrated this in a number of ways: when IL-22 was neutralized in IL-23R^{-/-}Rag^{-/-} mice, colitis and wasting disease were ameliorated. When IL-22 was injected back to IL-23R^{-/-}Rag^{-/-} mice, colitis and wasting disease was restored, despite the absence of IL-23R. Furthermore, IL-22 was able to induce colitis even when both IL-12R and IL-23R axes were inhibited via p40 neutralization. IL-22 was shown to protect mice from DSS-induced acute innate colitis,²⁶ CD45RB^{high} Foxp3⁻ T-cell-driven chronic colitis,²⁶ an ulcerative colitis model,⁴⁷ as well as ConA-induced hepatitis.⁴⁸ We propose that IL-22 protects against colitis through increased mucus production or increased epithelial cell proliferation/regeneration and repair.

IL-22 was also shown to drive pathogenesis in various models. IL-23-driven acanthosis and dermatitis⁴⁹ was shown to occur via IL-22. Moreover, a recent report that utilized CD45RB^{low} Foxp3⁻ T-cell-transfer-induced chronic colitis showed that IL-22 promotes colitis in this model²⁷ and proposed that IL-22-driven epithelial hyperplasia in the colon may cause pathology as opposed to CD45RB^{high} T-cell-driven colitis where the same mechanism is beneficial. Although hyperplasia may, to some extent, explain IL-22-driven pathology in our innate colitis model, our data indicate that perturbations in IL-10 and IFN- γ levels produced by IL-22-responsive epithelial cells as well as enhanced neutrophil recruitment are operational in determining the pathogenicity of IL-22 in this context. In line with this, we observed higher IFN- γ and less IL-10 in IL-23R^{-/-}Rag^{-/-} mice colitic colons compared with Rag^{-/-} mice. Moreover, neutralization of IL-22 mimicked IL-23R deficiency and adding IL-22 back via plasmid delivery elevated IFN- γ and reduced IL-10 levels. IL-22-dependent changes in IFN- γ levels, though not studied extensively, were reported in CD45RB^{low} T-cell-driven colitis model²⁷ and are parallel to our findings, yet more extensive studies are required for a definitive mechanism. Furthermore, reduced MMP-2 levels in IL-23R^{-/-}Rag^{-/-} mice that received IL-22 plasmid suggest that in this setting lack of MMP-2 may also contribute to pathogenesis possibly through decreased tissue remodeling due to a loss in MMP-2 levels; however, this explanation does not apply to IL-23R^{-/-}Rag^{-/-} vs. IL-23R^{-/-}Rag^{-/+} groups where we saw comparable MMP-2 expression.

In addition to differences in IFN- γ and IL-10, we observed reduced IL-17A cytokine production in the colon of colitic IL-23R^{-/-}Rag^{-/-} mice compared with Rag^{-/-} animals. A previous report demonstrated that IL-17A determines whether IL-22 will be pathogenic or protective in a bleomycin-induced asthma model.⁴¹ However, our data with blockade of IL-17R signaling and IL-17A/F neutralization suggested that neither IL-17A nor IL-17F can have a part in IL-22-driven pathogenesis during anti-CD40-driven colitis, as neither IL-17R blockade nor IL-17A/F neutralization improved colitis. Furthermore, modulation of IL-22 did not impact IL-17A or IL-17F levels, further supporting this point.

Throughout this study, we report opposite roles of IL-23R signaling in two distinct models of IBD. We showed that absence of IL-23R exacerbates CD45RB^{high} Foxp3⁻ T-cell-driven chronic colitis and protects from anti-CD40-induced acute innate colitis. We also provide evidence that IL-22 produced by type3 ILCs is, at least partly, responsible for IL-23R-dependent colitis development in anti-CD40 models. To our knowledge, this is the first demonstration that IL-22 can be pathogenic in an acute/innate colitis mouse model and suggests that similar to its bimodal role during chronic/adaptive colitis, IL-22 may also have opposite roles in innate colitis pathogenesis in a context- and insult-dependent manner. Given that IL-22 expression is elevated during Crohn's disease and ulcerative colitis in humans,⁵⁰ these findings will prove useful when designing therapeutic strategies that target IL-23 and IL-22 pathways for human IBD.

METHODS

Mice. IL23R^{-/-}Rag1^{-/-} mice were generated as previously described.³ IL2Rγc^{-/-}Rag2^{-/-} mice were purchased from Taconic (B10;B6-Rag2^{tm1Fwa} II2rg^{tm1Wjl}), bred, and maintained under specific pathogen-free conditions. All mice were on C57BL/6J background and were tested positive for *H. hepaticus*. All experiments were approved by the Institutional Animal Care and Use Committee of Seattle Children's Research Institute.

Anti-CD40-induced colitis. Seven-to-twelve-week-old sex-matched female and male mice were injected intraperitoneally with 200 μg anti-CD40 (clone FGK4.5, Bioexcell, West Lebanon, NH) on day 0. Mice were killed on day 2 or day 7. Colon and SI were removed, cleaned, and processed as described.³¹ To neutralize IL-22 or IL-17A/F, or block IL-17R, mice were injected intraperitoneally on days 0 (2 h before anti-cd40 injection), 1, 2, 3, 4, and 5 with 200 μg anti-IL-22 or IL-17A and IL-17F (Genentech, South San Francisco, CA) or 250 μg anti-IL-17R (Genentech), as control Rat IgG2a or phosphate-buffered saline. Phosphate-buffered saline and Rat IgG2a gave identical results and were interchangeably used.

T cell-driven colitis. CD45RB^{high} CD4⁺ NK1.1⁻ Foxp3YFP⁻ T cells were sorted from the Foxp3 reporter mice, which expresses yellow fluorescent protein from an IRES in Foxp3⁺ cells by Foxp3 promoter. The cells (5 × 10⁵) were injected intraperitoneally, and mice were monitored and weighed for 5 weeks post injection and killed. Intestines were collected for further experiments.

Flow cytometry. For flow cytometry, colon and SI lamina propria lymphocytes were isolated as described previously³¹ and surface stained with the appropriate antibodies. For intracellular cytokine staining, single-cell suspensions from lamina propria were cultured directly in RPMI containing 10% fetal bovine serum and Golgi plug for 4–5 h or restimulated in RPMI containing 10% fetal bovine serum with 50 ng ml⁻¹ of phorbol 12-myristate 13-acetate and 1 μg ml⁻¹ of ionomycin in the presence of Golgi plug. Cells were then fixed and stained according to instructions by the manufacturer, using an intracellular cytokine staining kit (eBioscience, San Diego, CA).

Following antibodies were used for staining: Thy1.2⁻ APC/PE-Cy7/FITC (allophycocyanin/ phycoerythrin-Cy7/fluorescein isothiocyanate), Thy1.1-PerpCy5.5, NKp46-AlexaFlour647/PE, CCR6-AlexaFlour647, CD127-APC, Sca-1-PerCpCy5.5, IL-22-APC, IL-17A-PE, and IFN-γ-PE Cy7.

Cloning and hydrodynamic gene delivery. IL-22 and RegIIIγ cDNAs were amplified using following primers from total cDNA generated from colonic total RNA reverse transcribed by iScript cDNA synthesis kit (Philadelphia, PA). cDNA were cloned into a TOPO vector validated, amplified, and blunt-end cloned into pBSHCRHP-A backbone.⁵¹ For hydrodynamic injections, 15 μg plasmid was resuspended in 2 ml of saline and intravenously injected in 5–10 s. IL-22 protein and RegIIIγ mRNA production of the constructs were validated from the blood or colons of mice that received the hydrodynamic plasmid injections. Two days post-gene delivery, the expression of genes could be detected; thus we induced colitis on day 2 of plasmid injection.

Quantitative reverse transcription-PCR. Colons were collected in Trizol and homogenized with the Pro200 Homogenizer (Pro Scientific, Oxford, CT) and total RNA was extracted. Taqman one-step RT-PCR (Applied Biosystems, Foster City, CA) or SYBER green q-PCR was performed with a 7500 Real Time PCR System according to the instructions of the manufacturer (Applied Biosystems). Expression of the tested genes were normalized to the housekeeping ribosomal protein L19(rPL19) mRNA. Arbitrary relative expression units were calculated by division of expression of the gene of interest by rP L19 mRNA expression. Primer and probe sequences for each target are available upon request.

ELISA. One centimeter proximal or distal colon and 1 cm of ileum were cultured in RPMI containing 10% fetal bovine serum in 24-cell plates for 24–48 h. Supernatants were taken and used for ELISA. Assay was performed according to the manufacturer's guidelines. IL-22, TNF-α and GM-CSF Ready-Set-Go! ELISA kits were purchased from eBioscience. IL-17A and IFN-γ ELISA Max Set Standard were purchased from Biologend (San Diego, CA).

Histology. Colonic strips were fixed in 10% phosphate-buffered formalin for overnight and transferred in to 70% ethanol and processed routinely with paraffin embedding, 4–5 μm sectioning, and staining with hematoxylin and eosin. Sections were scored by Piper M Treuting masked to treatment groups after Burich *et al.* 2001⁵² with modifications (**Supplementary Table 1** online). Lesions that were individually scored (0–4 increasing severity) included: (1) degree of inflammation, (2) severity of mucosal epithelial proliferation, and (3) the percentage of the section affected in any manner and (4) percentage affected in the most severe manner. The colitis score was derived by summing the individual lesion and the extent scores (max score = 16). Note that mesenteric (mesocolic) inflammation was noted but not scored.

Barrier integrity. Performed as previously described.⁵³ Briefly, overnight fasted mice were gavaged with 200 μl of FITC-dextran beads (75 ml ml⁻¹) and 90 min later blood serum was prepared for fluorimetric detection of FITC translocation to blood.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at <http://www.nature.com/mi>

ACKNOWLEDGEMENTS

We would like to thank Mallory Fry for technical help and mouse genotyping; Brian Johnson from Histology and Imaging Core Facility at UW for his help with histology, and Devin Margolies for assistance with animal husbandry, Amgen and Genentech for providing, respectively, IL-17RA- and IL-22-specific antibodies. This work is supported by The Crohn's and Colitis Foundation of America.

DISCLOSURE

The authors declared no conflict of interest.

© 2014 Society for Mucosal Immunology

REFERENCES

- Oppmann, B. *et al.* Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* **13**, 715–725 (2000).
- Parham, C. *et al.* A receptor for the heterodimeric cytokine IL-23 is composed of IL-12Rβ1 and a novel cytokine receptor subunit, IL-23R. *J. Immunol.* **168**, 5699–5708 (2002).
- Awasthi, A. *et al.* Cutting edge: IL-23 receptor gfp reporter mice reveal distinct populations of IL-17-producing cells. *J. Immunol.* **182**, 5904–5908 (2009).
- Zhou, L. *et al.* IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat. Immunol.* **8**, 967–974 (2007).
- Ghoreschi, K. *et al.* Generation of pathogenic T(H)17 cells in the absence of TGF-β signalling. *Nature* **467**, 967–971 (2010).
- Tesmer, L.A., Lundy, S.K., Sarkar, S. & Fox, D.A. Th17 cells in human disease. *Immunol. Rev.* **223**, 87–113 (2008).
- Wellcome Trust Case Control Consortium. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature* **447**, 661–678 (2007).
- Barrett, J.C. *et al.* Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. *Nat. Genet.* **40**, 955–962 (2008).
- Duerr, R.H. *et al.* A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. *Science* **314**, 1461–1463 (2006).

10. Eleftherohorinou, H. *et al.* Pathway analysis of GWAS provides new insights into genetic susceptibility to 3 inflammatory diseases. *PLoS One* **4**, e8068 (2009).
11. Morrison, P.J., Ballantyne, S.J. & Kullberg, M.C. Interleukin-23 and T helper 17-type responses in intestinal inflammation: from cytokines to T-cell plasticity. *Immunology* **133**, 397–408 (2011).
12. Duvallet, E., Semerano, L., Assier, E., Falgarone, G. & Boissier, M.C. Interleukin-23: a key cytokine in inflammatory diseases. *Ann. Med.* **43**, 503–511 (2011).
13. Yen, D. *et al.* IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. *J. Clin. Invest.* **116**, 1310–1316 (2006).
14. Hue, S. *et al.* Interleukin-23 drives innate and T cell-mediated intestinal inflammation. *J. Exp. Med.* **203**, 2473–2483 (2006).
15. Kullberg, M.C. *et al.* IL-23 plays a key role in Helicobacter hepaticus-induced T cell-dependent colitis. *J. Exp. Med.* **203**, 2485–2494 (2006).
16. Uhlig, H.H. *et al.* Differential activity of IL-12 and IL-23 in mucosal and systemic innate immune pathology. *Immunity* **25**, 309–318 (2006).
17. Ahern, P.P. *et al.* Interleukin-23 drives intestinal inflammation through direct activity on T cells. *Immunity* **33**, 279–288 (2010).
18. Cox, J.H. *et al.* Opposing consequences of IL-23 signaling mediated by innate and adaptive cells in chemically induced colitis in mice. *Mucosal Immunol.* **5**, 99–109 (2012).
19. Kanai, T., Mikami, Y., Sujino, T., Hisamatsu, T. & Hibi, T. RORgammat-dependent IL-17A-producing cells in the pathogenesis of intestinal inflammation. *Mucosal Immunol.* **5**, 240–247 (2012).
20. Ito, R. *et al.* Involvement of IL-17A in the pathogenesis of DSS-induced colitis in mice. *Biochem. Biophys. Res. Commun.* **377**, 12–16 (2008).
21. Zhang, Z., Zheng, M., Bindas, J., Schwarzenberger, P. & Kolls, J.K. Critical role of IL-17 receptor signaling in acute TNBS-induced colitis. *Inflamm. Bowel Dis.* **12**, 382–388 (2006).
22. Yang, X.O. *et al.* Regulation of inflammatory responses by IL-17F. *J. Exp. Med.* **205**, 1063–1075 (2008).
23. Leppkes, M. *et al.* RORgamma-expressing Th17 cells induce murine chronic intestinal inflammation via redundant effects of IL-17A and IL-17F. *Gastroenterology* **136**, 257–267 (2009).
24. O'Connor, W. Jr. *et al.* A protective function for interleukin 17A in T cell-mediated intestinal inflammation. *Nat. Immunol.* **10**, 603–609 (2009).
25. Sonnenberg, G.F., Fouser, L.A. & Artis, D. Border patrol: regulation of immunity, inflammation and tissue homeostasis at barrier surfaces by IL-22. *Nat. Immunol.* **12**, 383–390 (2011).
26. Zenewicz, L.A. *et al.* Innate and adaptive interleukin-22 protects mice from inflammatory bowel disease. *Immunity* **29**, 947–957 (2008).
27. Kamanaka, M. *et al.* Memory/effector (CD45RB(lo)) CD4 T cells are controlled directly by IL-10 and cause IL-22-dependent intestinal pathology. *J. Exp. Med.* **208**, 1027–1040 (2011).
28. Schmutz, S. *et al.* Cutting edge: IL-7 regulates the peripheral pool of adult ROR gamma + lymphoid tissue inducer cells. *J. Immunol.* **183**, 2217–2221 (2009).
29. Kim, M.Y. *et al.* Neonatal and adult CD4 + CD3- cells share similar gene expression profile, and neonatal cells up-regulate OX40 ligand in response to TL1A (TNFSF15). *J. Immunol.* **177**, 3074–3081 (2006).
30. Takatori, H. *et al.* Lymphoid tissue inducer-like cells are an innate source of IL-17 and IL-22. *J. Exp. Med.* **206**, 35–41 (2009).
31. Sonnenberg, G.F., Monticelli, L.A., Elloso, M.M., Fouser, L.A. & Artis, D. CD4(+) lymphoid tissue-inducer cells promote innate immunity in the gut. *Immunity* **34**, 122–134 (2011).
32. Buonocore, S. *et al.* Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology. *Nature* **464**, 1371–1375 (2010).
33. Vonarbourg, C. *et al.* Regulated expression of nuclear receptor ROR-gammat confers distinct functional fates to NK cell receptor-expressing RORgammat(+) innate lymphocytes. *Immunity* **33**, 736–751 (2010).
34. Geremia, A. *et al.* IL-23-responsive innate lymphoid cells are increased in inflammatory bowel disease. *J. Exp. Med.* **208**, 1127–1133 (2011).
35. Powrie, F., Leach, M.W., Mauze, S., Caddle, L.B. & Coffman, R.L. Phenotypically distinct subsets of CD4+ T cells induce or protect from chronic intestinal inflammation in C. B-17 scid mice. *Int. Immunol.* **5**, 1461–1471 (1993).
36. Rickel, E.A. *et al.* Identification of functional roles for both IL-17RB and IL-17RA in mediating IL-25-induced activities. *J. Immunol.* **181**, 4299–4310 (2008).
37. Song, X. *et al.* IL-17RE is the functional receptor for IL-17C and mediates mucosal immunity to infection with intestinal pathogens. *Nat. Immunol.* **12**, 1151–1158 (2011).
38. Cash, H.L., Whitham, C.V., Behrendt, C.L. & Hooper, L.V. Symbiotic bacteria direct expression of an intestinal bactericidal lectin. *Science* **313**, 1126–1130 (2006).
39. Liang, S.C. *et al.* Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J. Exp. Med.* **203**, 2271–2279 (2006).
40. Yamaoka, K., Min, B., Zhou, Y.J., Paul, W.E. & O'Shea, J.J. Jak3 negatively regulates dendritic-cell cytokine production and survival. *Blood* **106**, 3227–3233 (2005).
41. Sonnenberg, G.F. *et al.* Pathological versus protective functions of IL-22 in airway inflammation are regulated by IL-17A. *J. Exp. Med.* **207**, 1293–1305 (2010).
42. Vu, T.H. & Werb, Z. Matrix metalloproteinases: effectors of development and normal physiology. *Genes Dev.* **14**, 2123–2133 (2000).
43. Baugh, M.D. *et al.* Matrix metalloproteinase levels are elevated in inflammatory bowel disease. *Gastroenterology* **117**, 814–822 (1999).
44. Kirkegaard, T., Hansen, A., Bruun, E. & Brynskov, J. Expression and localisation of matrix metalloproteinases and their natural inhibitors in fistulae of patients with Crohn's disease. *Gut* **53**, 701–709 (2004).
45. Garg, P. *et al.* Selective ablation of matrix metalloproteinase-2 exacerbates experimental colitis: contrasting role of gelatinases in the pathogenesis of colitis. *J. Immunol.* **177**, 4103–4112 (2006).
46. Satoh-Takayama, N. *et al.* Microbial flora drives interleukin 22 production in intestinal NKp46+ cells that provide innate mucosal immune defense. *Immunity* **29**, 958–970 (2008).
47. Sugimoto, K. *et al.* IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis. *J. Clin. Invest.* **118**, 534–544 (2008).
48. Zenewicz, L.A. *et al.* Interleukin-22 but not interleukin-17 provides protection to hepatocytes during acute liver inflammation. *Immunity* **27**, 647–659 (2007).
49. Zheng, Y. *et al.* Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. *Nature* **445**, 648–651 (2007).
50. Andoh, A. *et al.* Interleukin-22, a member of the IL-10 subfamily, induces inflammatory responses in colonic subepithelial myofibroblasts. *Gastroenterology* **129**, 969–984 (2005).
51. Miao, C.H., Ye, X. & Thompson, A.R. High-level factor VIII gene expression in vivo achieved by nonviral liver-specific gene therapy vectors. *Hum. Gene Ther.* **14**, 1297–1305 (2003).
52. Burich, A. *et al.* Helicobacter-induced inflammatory bowel disease in IL-10- and T-cell-deficient mice. *Am. J. Physiol. Gastrointest. Liver Physiol.* **281**, G764–G778 (2001).
53. Lin, J.E. *et al.* GUCY2C opposes systemic genotoxic tumorigenesis by regulating AKT-dependent intestinal barrier integrity. *PLoS One* **7**, e31686 (2012).