The development of colitis in Il10−/− mice is dependent on IL-22

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Mice deficient in the IL-10 pathway are the most widely used models of intestinal immunopathology. IL-17A is strongly implicated in gut disease in mice and humans, but conflicting evidence has drawn IL-17’s role in the gut into question. IL-22 regulates antimicrobial and repair activities of intestinal epithelial cells (IECs) and is closely associated with IL-17A responses but it’s role in chronic disease is uncertain. We report that IL-22, like IL-17A, is aberrantly expressed in colitic Il10−/− mice. While IL-22+ Th17 cells were elevated in the colon, IL-22-producing ILC3s were highly enriched in the small intestines of Il10−/− mice. Remarkably, Il10−/−Il22−/− mice did not develop colitis despite retaining high levels of Th17 cells and remaining colonized with colitogenic Helicobacter spp. Accordant with IL-22-induced IEC proliferation, the epithelia hyperplasia observed in Il10−/− animals was reversed in Il10−/−Il22−/− mice. Also, the high levels of antimicrobial IL-22-target genes, including Reg3g, were normalized in Il10−/−Il22−/− mice. Consistent with a heightened antimicrobial environment, Il10−/− mice had reduced diversity of the fecal microbiome that was reestablished in Il10−/−Il22−/− animals. These data suggest that spontaneous colitis in Il10−/− mice is driven by IL-22 and implicates an underappreciated IL-10/IL-22 axis in regulating intestinal homeostasis.

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INTRODUCTION

Based on cues from the microenvironment, the mucosal immune system fine-tunes immune effector programs to maximize host defenses at barrier surfaces while preventing excess inflammation to avoid damage to host tissues. In the gut, dysregulation of this dynamic process can result in chronic inflammation and disease pathology. The etiology of inflammatory bowel disease (IBD), which encompasses Crohn’s disease and colitis, is still poorly understood despite being intensively studied. Our understanding of the immune and microbial factors that contribute to disease susceptibility have been complicated, at least in part, by difficulties in interpreting data generated under different experimental conditions, and with different animal models of IBD.1-10

IL-10 is an immunoregulatory cytokine that plays a central role in regulating intestinal inflammation in humans and mice.3 Mice deficient in IL-10 or the IL-10 receptor develop spontaneous colitis early in life and are one of the most widely used animal models for studying the pathogenesis of human IBD.6,7 The development of colitis in IL-10-deficient mice is dependent on the intestinal microbiota.8 More specifically, co-colonization with “pathobionts” such as Helicobacter spp., which do not cause disease in immunocompetent mice, are required for the development of colitis in Il10−/− mice. Although it is clear that excessive immune reactivity to microbial antigens triggers colitis in Il10−/− mice,10,11 remarkably, the host factors which drive intestinal pathology have been difficult to define.

Early work suggested that dysregulation of Th1 immunity (IL-12/IFN-γ) was responsible for colitis in Il10−/− mice.12 Prior to the discovery of the Th17 pathway (IL-23/IL-17), early work naturally focused on Th1-mediated responses (IL-12/IFN-γ). An important study by Yen and colleagues in 2006, specifically examined the contributions of IL-12-dependent Th1 and IL-23-dependent Th17 immunity to the development of colitis.13 They demonstrated that co-deletion of IL-23 (Il10−/−Il23p19−/−) but not IL-12 (Il10−/−Il12p35−/−), rescued Il10-deficient mice from spontaneous colitis.13 This study offered convincing evidence that excessive production of IL-17, driven by IL-23, was in fact primarily responsible for the development of colitis in Il10−/− animals.13 Thus, the prevailing model suggests that excessive Th17 development, driven by IL-23, is responsible for IBD pathology.

Additional studies have largely supported IL-23’s role in promoting intestinal inflammation,13-17 however, IL-17’s role has been somewhat less clear. A group of reports have accumulated suggesting that intestinal inflammation occurs independently of IL-17 and can be worsened when IL-17 is inhibited.15,17-19 These data are consistent with the disappointing results from clinical trials using IL-17A-blocking or IL-17RA-blocking antibodies to treat IBD. In these trials, Crohn’s disease patients receiving anti-IL-17A or -IL-17RA therapy had no clinical improvement and disease symptoms were exacerbated in some recipients.20,21 These data highlight the need to reexamine existing models based on IL-17-mediated gut pathology and to reconsider other factors that may drive disease susceptibility.

IL-22 is closely associated with Th17 immunity, despite being a member of the IL-10 family, due to its complementary functions and overlapping expression with IL-17A.22 Although frequently

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co-expressed with IL-17A, IL-22-producing cells are far less abundant, which together confounds efforts to identify the individual contributions of IL-22 and IL-17 to host defense and disease pathogenesis. Th17, Th22, and ILC3s are the primary sources of IL-22 in the gut and the cellular source of IL-22 appears to play an important part in determining its biological actions. Recent evidence suggests that different IL-22-secreting subsets emerge during the course of immune responses depending on the nature of the insult and may be selectively distributed in different anatomical locations within the GI tract. However, the factors that regulate IL-22 expression by these different cell types and mediate IL-22’s protective or pathogenic activities remain poorly understood.

Although it is clear that IL-10 and IL-22 contribute distinctly to intestinal health, it remains uncertain if or how the IL-10 and IL-22 pathways interconnect and to what extent, if any, that IL-22 contributes to pathogenesis in IL-10-deficient disease models. In this study, we provide compelling evidence indicating that IL-10 is necessary for the development of spontaneous colitis in Il10-/- mice. To determine the role of IL-22 in the development of colitis in Il10-/- mice, we performed experiments in Il10-/- mice and Il10-/-/Il22-/- mice and mediate IL-22 production by these different cell types and factors that regulate IL-22 expression by these different cell types and mediate IL-22’s protective or pathogenic activities remain poorly understood.

The development of colitis in Il10-/- mice is associated with a lack of IL-10, which is produced by Th17 cells and is crucial for maintaining intestinal homeostasis. In the absence of IL-10, Th17 cells accumulate in the small bowel and contribute to the development of spontaneous colitis. This is due to the fact that IL-10 is required for the development of Th22 cells, which are a subset of Th17 cells that overexpress IL-22. However, in the absence of IL-10, Th22 cells are not fully developed, and this leads to an increase in the number of IL-22-producing cells and the development of colitis.

In our study, we aimed to investigate the role of IL-22 in the development of colitis in Il10-/- mice. We found that IL-22 is necessary for the development of colitis in Il10-/- mice. To determine the role of IL-22 in the development of colitis in Il10-/- mice, we performed experiments in Il10-/- mice and Il10-/-/Il22-/- mice. We found that IL-22 is necessary for the development of colitis in Il10-/- mice. To determine the role of IL-22 in the development of colitis in Il10-/- mice, we performed experiments in Il10-/- mice and Il10-/-/Il22-/- mice. We found that IL-22 is necessary for the development of colitis in Il10-/- mice.

In conclusion, our results indicate that IL-22 is necessary for the development of colitis in Il10-/- mice. This is due to the fact that IL-22 is produced by Th22 cells, which are a subset of Th17 cells that overexpress IL-22. In the absence of IL-10, Th22 cells are not fully developed, and this leads to an increase in the number of IL-22-producing cells and the development of colitis. Therefore, targeting IL-22 may be a promising strategy for the treatment ofCrohn's disease.
previously shown that *H. typhlonius* is associated with development of colitis in *Il10*−/− animals. Studies from other groups have demonstrated *H. mastomyrinus* to be even more pathogenic than *H. typhlonius*. We confirmed that while fully backcrossed *Il10*−/− *Il22*−/− mice harbored both *Helicobacter* spp. (data not shown), *Il10*−/− *Il22*−/− mice did not develop chronic colitis (Fig. 2).

The development of colitis in *Il10*−/− mice colonized with *Helicobacter* spp. is highly correlated with rectal prolapse. We found that by 3 months of age, about 20% of *Il10*−/− mice had...
developed rectal prolapse and this number increased to 80% by 6 months of age. In contrast, but similar to WT animals, Il10−/−Il22−/− mice did not develop rectal prolapse (Fig. 2a). A subset of Il10−/−Il22−/− mice was observed for a period of more than 1.5 years during which they remained healthy and free of disease (data not shown). Macroscopically, Il10−/− mice exhibited thickening of the colonic wall, shortened colonic length, intussusception of the cecum and enlarged mesenteric lymph nodes (MLN) and spleens (Fig. 2b). Surprisingly, Il10−/−Il22−/− mice as well as Il22−/− exhibited significantly longer colon lengths than both WT and Il10−/− mice (Fig. 2b, c). The colonic thickening, cecal intussusception, and splenic enlargement observed in Il10−/− mice was completely abrogated in Il10−/−Il22−/− mice. However, similar to Il10−/− mice, double-deficient mice had enlarged mesenteric lymph nodes compared to WT and Il22−/− mice (Fig. 2b).

Compared to WT controls, colons from Il10−/− mice had marked mucosal hyperplasia and thickening (Fig. 2d), increased cellular infiltrates, erosion, and ulceration of the epithelium, and crypt abscesses as reported previously in Il10−/− mice. Histology scores for colitis using the Jackson Laboratory (JIL) system reflected these findings and indicated that Il10−/−Il22−/− mice were protected from colitis (Fig. 2e). Lipocalin (Lcn-2) is strongly associated with intestinal inflammation and as expected, the high levels of fecal Lcn-2 in Il10−/− mice were significantly reduced in Il10−/−Il22−/− mice (Fig. 2f). Interestingly, the levels of Lcn-2 in Il10−/−Il22−/− mice were still significantly higher compared to fecal samples from both WT and Il22−/− mice. These data are in line with a recent report indicating that IL-17A and ILC3-derived IL-22 synergistically induce Lcn-2 expression from IECs and suggest that IL-17A levels remain high in disease-free Il10−/−Il22−/− mice. Taken together, these data indicate that IL-22 is necessary for the development of chronic colitis and plays a central, pathogenic role in the gut in the context of Il10-deficiency.

Overall, Il10−/− and Il10−/−Il22−/− mice have similar distributions of leukocyte subsets with some tissue-specific exceptions To characterize cell populations associated with absence of disease in Il10−/−Il22−/− mice we examined leukocyte distributions in the IL-22-expressing or IL-22-deficient mice. As mentioned, Il10−/− mice have high numbers of leukocytes infiltrating secondary lymphoid and intestinal tissues, and consistent with previous reports, we observed higher frequencies of CD4+ T cells in the cIEL and cLPL fractions compared to WT mice. Surprisingly, we also found higher frequencies of CD4+ T cells in the colons of Il10−/−Il22−/− mice compared to WT (Fig. 3a). No differences in the percentages of CD8+ cytotoxic T cells, CD3+CD19− B cells or CD11b+Ly6C−Ly6G−F4/80+ side scatter low (SSC−) macrophages were observed between the groups (Fig. 3b–d respectively). However, higher frequencies of CD11b+Ly6C+Ly6G−F4/80+ neutrophils in the spleens of Il10−/− mice, whereas the percentages of neutrophils from Il10−/−Il22−/− animals were comparable to levels observed in WT and Il22−/− spleens (Fig. 3e). Interestingly, the percentage of neutrophils was also elevated in the cIEL and cLPL fractions of both Il10−/− and Il10−/−Il22−/− mice compared to WT animals (Fig. 3e). Of note, the numbers and percentages of leukocyte subsets were comparable between WT and Il22−/− mice for all of the tissues examined (Fig. 3).

The tissue-specific disparity in neutrophil frequencies prompted us to examine the distribution of leukocyte subsets in the periphery (Fig. S2). Although Il10−/− and Il10−/−Il22−/− mice tended towards higher numbers of leukocytes in the blood (Fig S2A), no significant differences between the groups were observed for white blood counts (WBC) or lymphocytes (Fig S2B). However, in agreement with findings in the spleen, complete blood counts (CBCs) revealed elevated numbers of neutrophils in the peripheral blood of Il10−/− mice compared to Il10−/−Il22−/− as well as WT and Il22−/− animals (Fig S2C). In addition, Il10−/− mice harbored higher numbers of monocytes whereas Il10−/−Il22−/− mice had numbers comparable to WT (Fig. S1D). Similar to WBC, platelet counts tended higher in Il10−/− and Il10−/−Il22−/− mice but did not reach statistical significance (Fig. S2E). Thus, overall, Il10−/− and Il10−/−Il22−/− mice have higher levels of leukocytes in intestinal tissues and a selective enrichment of Th cells and neutrophils in the colon.

Il10−/− and Il10−/−Il22−/− mice have higher frequencies of Th17 cells in tissues Dysregulation of Th1 and/or Th17 responses has been associated with intestinal inflammation. In Il10−/− mice, the excessive accumulation of Th17 cells in the gut is thought to mediate intestinal disease and we found that the levels of CD4+ T cells remained high in colonic tissues of Il10−/−Il22−/− mice (Fig. 3a). Taken together with our finding that fecal Lcn-2 levels were reduced in Il10−/−Il22−/− compared to Il10−/− mice but elevated compared to WT and Il22−/− mice (Fig. 2f) suggested that Th17 cells may remain high in disease-free Il10−/−Il22−/− mice. Thus, we examined the frequencies of CD4+ Th1 and Th17 subsets in intestinal and secondary lymphoid tissues based on the expression of IFN-γ and IL-17A. Consistent with elevated frequencies of CD4+ T cells in colonic tissues (Fig. 3a), Il10−/− mice had significantly higher percentages of IL-17A-expressing CD4+ Th17 cells in cIEL and cLPL fractions as expected, compared to WT and Il22−/− mice (Fig. 4a). Remarkably, disease-free Il10−/−Il22−/− mice had similarly high levels of Th17 cells in colonic tissues. This suggests that IL-17A regulation is independent of IL-22. In addition, although we did not detect differences in the overall percentages of CD4+ T cells in other tissues (Fig. 3a), the levels Th17 cells in the spleen, MLN, and cLPL of...
both $\text{II10}^{-/-}$ and $\text{II10}^{-/-}\text{II22}^{-/-}$ were significantly elevated compared to WT (and $\text{II22}^{-/-}$) mice. Interestingly, though the percentages of IFN-$\gamma^+\text{CD4}^+$ Th1 cells tended to be higher in intestinal tissues of $\text{II10}^{-/-}$ and $\text{II10}^{-/-}\text{II22}^{-/-}$ mice, only the sIELs were significantly different (Fig. 4b). These data suggest that the well-known accumulation of Th17 cells in intestinal tissues of IL-10-deficient mice is not sufficient to induce colitis.

$\text{II10/II22}$ double-deficiency results in reduced fecal IgA and IgG. In addition to T cells, B cells and mucosal antibodies play important roles in maintaining immune-microbial homeostasis and barrier integrity in the gut. Studies in $\text{II10}^{-/-}$ mice have shown that IgA and IgG1 levels are elevated in the serum and serum IgG is reactive against enteric bacterial antigens. To determine the impact of double-deficiency in IL-10 and IL-22 on the levels of mucosal antibody we measured immunoglobulin levels in the feces in our mouse strains. While the levels of both IgA and IgG were significantly elevated in feces from $\text{II10}^{-/-}$ mice compared to other strains, fecal antibody levels from $\text{II10}^{-/-}\text{II22}^{-/-}$ mice were similar to those observed in WT (and $\text{II22}^{-/-}$) mice (Fig. 5a, b). Although the antigenic specificities and subclass of fecal immunoglobulins have not been determined, these data suggest that an imbalance in the IL-10/IL-22 axis results in dysregulation of mucosal antibody responses.

**Fig. 2** IL-22-deficiency prevents the development of spontaneous colitis in $\text{II10}^{-/-}$ mice. a Prevalence of rectal prolapse ($n > 60$ per group) in WT, $\text{II10}^{-/-}$, $\text{II22}^{-/-}$, and $\text{II10}^{-/-}\text{II22}^{-/-}$ mice. b Representative gross images of organs of the gastrointestinal tracts from each strain. c Colon length for each strain ($n = 11$ per group). d Representative photomicrographs of haemotoxylin and eosin stained colons ($n = 8$ per group). e Fecal Lcn-2 levels ($n = 8$ per group). f TJL score ($n = 60$ per group). Statistical analyses ($**p < 0.001$) using the Mann–Whitney U test (error bars represent SEM).

Antimicrobial IL-22-target genes are upregulated in colonic tissues from $\text{II10}^{-/-}$ mice but are normalized in disease-free $\text{II10}^{-/-}\text{II22}^{-/-}$ mice. To identify genes that may underlie protection from colitis in $\text{II10}^{-/-}\text{II22}^{-/-}$ mice, we examined mRNA expression profiles in tissue samples from the distal colons of age-matched and sex-matched WT, $\text{II10}^{-/-}$, $\text{II22}^{-/-}$, and $\text{II10}^{-/-}\text{II22}^{-/-}$ mice ($n = 4$ mice/group) using the The TaqMan® OpenArray® Mouse Inflammation Panel. For relative mRNA expression analyses, each experimental group (strain) was compared to WT to identify differentially expressed genes. Significantly different genes were defined based on q-values smaller than 0.05 and fold change $>2$. Of 632 genes examined, 121 and 111 genes were differentially expressed in the distal colons from $\text{II10}^{-/-}$ and $\text{II10}^{-/-}\text{II22}^{-/-}$ mice, respectively, compared to WT (Fig. 6a). The gene expression profile of $\text{II22}^{-/-}$ mice was remarkably similar to WT mice (Fig. 6b–d) and no significant differences were identified. Genes differentially expressed in $\text{II10}^{-/-}$ and $\text{II10}^{-/-}\text{II22}^{-/-}$ mice were subdivided into three groups based on their patters of expression compared to WT (Fig. 6a). Group 1 was comprised of genes differentially expressed in both $\text{II10}^{-/-}$ and $\text{II10}^{-/-}\text{II22}^{-/-}$ mice and likely represent genes affected by IL-10 independently of IL-22 (Fig. 6b; Supplementary Table 1). Like the similar distributions of inflammatory cells observed in $\text{II10}^{-/-}$ and...
II10−/− II22−/− mice, there was a high degree of overlap in Group 1 genes (78 shared genes) (Fig. 6a). Group 1 genes included important mediators of inflammation, such as I17a, Cxc11, Nos2, II1b, and Cd4, as well as molecules important in antigen presentation such as Cd74 and B2m (Fig. 6b). Group 2 was comprised of genes differentially expressed only in II10−/− II22−/− mice compared to WT and likely represent genes affected by both IL-10 and IL-22 (Fig. 6c and Supplementary Table 2). Group 2 genes included Fpr3, Cd40 and Cd40L, II18 and II18rap.

Group 3 was comprised of genes differentially expressed only in II10−/− II22−/− mice compared to WT (Fig. 6d and Supplementary Table 3) and are likely to represent, at least in part, genes that are affected by overexpression of IL-22. Indeed, in agreement with our initial observations (Fig. 1a, b) mRNA for Il22 as well as several antimicrobial IL-22-target genes, such as Reg3g and Muc1 were upregulated in II10−/− mice but not in II10−/− II22−/− mice. Results from these experiments were validated by separate real-time qPCR analysis for Group 3 genes, which included Il22 (Fig. 6e), several IL-22-target genes (Reg3g (Fig. 6f), s100a8 (Fig. 6g), Muc1 (Fig. 6h)), and I21 (Fig. 6i), which is known to regulate both IL-10 and IL-22 expressions. In addition, several genes that were not differentially expressed between the groups, including Il23a, Il23r, and Il22ra2, were independently confirmed by RT-qPCR (data not shown). Taken together, these data indicate that IL-10 is a negative regulator of IL-22 expression in the gut and in the absence of IL-10, aberrant IL-22 expression induces the overexpression of IL-22-target genes associated with antimicrobial immunity.

Microbial diversity is re-established in II10−/− II22−/− mice but with altered composition

IL-22 promotes intestinal health in part, by regulating the microbiota. Chronic intestinal inflammation is associated with reduced microbial diversity in humans and II10−/− mice but the mechanism remains unclear. Our data indicate that IL-22-dependent antimicrobial genes are highly upregulated in II10−/− mice (Fig. 6). Thus, we explored the possibility that the IL-22 pathway drives microbial dysbiosis in II10−/− mice by sequencing and analyzing fecal 16s rRNA isolated from each mouse strain. The number and relative abundance of bacterial phylotypes in the gastrointestinal tract are often decreased during states of chronic inflammation. Accordingly, the Chao1 index (Fig. 7a) which measures the richness (total number) of phylotypes revealed that II10−/− mice trended lower in diversity compared to WT mice (p = 0.102, Supplementary Table 4). Lower diversity in II10−/− mice was also evident when compared to II10−/− II22−/− mice (p = 0.012) which indicates that loss of IL-22 significantly compensated for the negative impact of IL-10 deficiency on the number of phylotypes in the fecal microbiome. Interestingly, IL-22 deficiency
**Fig. 5** Fecal IgG and IgA levels are normalized in Il10−/−Il22−/− mice. a) Fecal IgA and b) IgG levels in WT, Il10−/−, Il22−/−, and Il10−/−Il22−/− mice (male, 16–20 weeks of age, n = 10–12 mice per group) (**p < 0.01, using the Mann-Whitney U test (error bars represent SEM)).

**Fig. 6** Distal colon tissues from Il10−/− and Il10−/−Il22−/− mice have similar gene expression patterns but IL-22 target genes associated with antimicrobial responses are normalized to WT levels in Il10−/−Il22−/− mice. a) Venn diagram from TaqMan OpenArray results depicting differential gene expression in distal colons of Il10−/− and/or Il10−/−Il22−/− mice compared to WT mice (n = 4 mice per strain, 20 weeks of age) (q = 0.05, fold change boundary = 2). Heatmaps of b) Group 1, c) Group 2, and d) Group 3 with select genes shown. Each line represents a single probe and each column a single mouse. Blue represents probes that were at least two-fold lower; red represents probes that were at least two-fold higher compared to WT mice. e–i Independent RT-PCR validation of mRNA expression for selected genes in the distal colons of WT, Il10−/−, Il22−/−, and Il10−/−Il22−/− mice (n = 4–7) (**p < 0.01, *p < 0.05, using 1-way ANOVA and Bonferroni post-test—error bars represent SEM).
was associated with increased diversity compared to both WT ($p = 0.03$) and $\text{Il10}^{-/-}$ mice ($p = 0.006$, Supplementary Table 4a). Similar results were obtained using the Shannon index, which measures richness and evenness of phylotypes (Supplementary Table 4b). These data support the histologic evidence that IL-22 deficiency appears to compensate for IL-10 deficiency in protecting $\text{Il10}^{-/-}$ mice against chronic colitis.

Principal component analysis of beta diversity using unweighted, UniFrac analysis, showed distinct clusters indicating distinct microbial community structures for each strain of mice (Fig. 7b). Analysis using permutational multivariate analysis of variance (PERMANOVA) demonstrated a significant effect on beta diversity by strain ($p = 0.001$), and pairwise adonis comparisons revealed significant differences between every combination ($p = 0.006$) with the exception of WT vs. $\text{Il10}^{-/-}$ mice ($p = 0.078$). This suggests that together, IL-10 and IL-22 play an important role in regulating microbial diversity in the gut.

Taxon-based analysis revealed that the microbiome of each group comprised mainly of Bacteroidetes, Firmicutes, and Proteobacteria (Fig. 7c). At the family level, $\text{Il22}^{-/-}$ and $\text{Il10}^{-/-}$ mice had fewer Lactobacillaceae and Bacteroidaceae compared to $\text{Il10}^{-/-}$ mice. Figure 7d and Supplementary Table 5 lists significant differences between the four strains of mice from the phylum down through genus level, when detectable. The pathogenic potential of most of these organisms is not clearly understood, but some detected differences may be relevant to the $\text{Il10}^{-/-}$ colitis model. Consistent with previous reports and similarly to IBD in humans, species diversity was markedly reduced in the fecal microbiota of colitic $\text{Il10}^{-/-}$ mice compared to WT. Of note $\text{Il10}^{-/-}$ mice had increased abundance of the genus Sutterella which has been suspected to play a role in IBD pathogenesis.

In addition, an unknown genus within the family Bacteroidaceae, was more common in $\text{Il10}^{-/-}$ mice than WT, $\text{Il22}^{-/-}$, and $\text{Il10}^{-/-}\text{Il22}^{-/-}$ mice which is consistent with reports where select members of Bacteroidaceae have been associated with colitis in humans and mouse models. Interestingly, the genus Bacteroides was highest in the $\text{Il10}^{-/-}\text{Il22}^{-/-}$ mice but was not associated with increased disease. Significantly, the Helicobacter genus was detected at the highest level in WT mice that did not develop colitis, as expected, and was detected at the lowest level in the feces of $\text{Il10}^{-/-}$ mice with severe colitis (Fig. 7c) which is consistent with previous reports.

Taken together, these data suggest that without IL-10-mediated inhibition, IL-22 overexpression triggers excessive innate antimicrobial and tissue healing responses in the gut resulting in microbial dysbiosis, protracted tissue repair activity, and a state of chronic inflammation (Fig. 8). Importantly, these findings may also help to explain the surprising and disappointing failure of IL-17A-blocking drugs in clinical trials and shed new light on existing models of chronic intestinal inflammation.

**DISCUSSION**

To maintain intestinal health, the immune system must balance between tolerance to commensal microorganisms and defense against invading pathogens, and IL-17 and IL-22 play key roles in regulating host-protective responses at barrier surfaces. IL-22 facilitates the repair of damaged tissues by inducing regeneration of the intestinal epithelium, while IL-22 and IL-17A regulate innate antimicrobial responses to defend against enteric pathogens. IL-10 acts in opposition by restricting host defense responses to prevent chronic inflammation and restore intestinal homeostasis. IL-10’s anti-inflammatory role in the gut is well-established, however, it has been difficult to identify the
immune effector mechanism(s) that mediate intestinal pathology in IL-10-deficient models. A landmark study by Yen et al. in 2006 suggested that the IL-17/IL-23 (Th17) axis, not the previously implicated IL-12/IFN-γ (Th1) pathway, was responsible for intestinal disease. These data provided a compelling basis for the prevailing theory that pathogenic Th17 cells drive disease pathogenesis in IL-10-dependent models of intestinal inflammation. However, a growing number of conflicting studies report that intestinal inflammation is worsened by IL-17 depletion or occurs independently of IL-17. 

Here, we provide evidence that IL-22 overexpression accounts for much of the immunopathology associated with spontaneous colitis in Il10−/− mice and despite retaining high levels of Th17 cells in colonic tissues, Il10−/−Il22−/− double-deficient mice remained disease-free. Interestingly, while some groups have noted that IL-22, like IL-17, was markedly upregulated in IL-10/IL-10R-deficient mouse models of intestinal inflammation, IL-22’s role in disease pathogenesis was not addressed and disease activity was correlated with IL-17 upregulation. IL-10 receptor signaling was shown to play a role in controlling IL-22-dependent intestinal pathology in a T cell transfer model of colitis and IL-10 has been shown to inhibit both IL-17 and IL-22 expression in vitro. However, to our knowledge, it has not been reported if IL-10/IL-17A-double-deficient mice develop colitis, which adds to the uncertainty of IL-17A’s role in gut pathology. Importantly, Morrison and colleagues used H. hepatus-infected anti-IL-10 receptor-treated mice to compare the contributions of IL-17A and IL-22 to acute colonic and cecal inflammation. The authors found that IL-17A neutralization had no effect on colonic inflammation, but pathology in the cecum was exacerbated, while neutralization of IL-22 prevented inflammation in the colon, but not in the cecum. This study hinted at a pathogenic role for IL-22, and not IL-17, in IL-10-dependent acute colitis. Our data are in agreement with these findings and support a central role for an IL-10/IL-22-dependent axis of intestinal homeostasis.

Studies in Il22−/− mice suggest that IL-22’s role is largely protective in the gut especially in the context of acute intestinal damage, such as during Citrobacter rodentium infection or in the dextran sulfate sodium (DSS)-induced colitis model. Though ILC3s are an important early source of protective IL-22, growing evidence in humans and mice suggests that chronic dysregulation of IL-22 and ILC3 responses in particular, results in intestinal damage, such as during Citrobacter rodentium infection or in the dextran sulfate sodium (DSS)-induced colitis model. Accumulating data suggests that the distribution patterns of ILC3s within different intestinal tissues can dramatically impact host defense and tissue homeostasis. In the anti-CD40 model of acute colitis, the rapid redistribution of ILC3s from intestinal lymphoid structures precedes the development of inflammatory foci elsewhere in gut tissues that is dependent on IL-22, but not IL-17A-expressing ILC3s. In addition, a recent report found that IL-22-
producing ILC3s accumulated in the small intestines of immuno-compromised mice resulting in impaired host lipid metabolism.\textsuperscript{79} In our study, while the levels of IL-22\textsuperscript{+} Th17 cells were slightly elevated in colonic tissues, we observed a sizeable accumulation of IL-22 IL-17A\textsuperscript{+} ILC3s selectively in the small bowel of IL-10\textsuperscript{−/−} compared to WT mice. Although it is tempting to speculate, it is important to note that our data do not indicate how, or if the expansion of IL-22-producing ILC3s in the small intestine relates to the development of chronic colitis in IL-10\textsuperscript{−/−} mice. Additional studies will be required to parse out the potential role that IL-22 ILC3s, residing in the SI may play in chronic intestinal inflammation.

Indeed, the mechanisms by which ILC3s and IL-22 shape mucosal responses both locally and in distant tissues are poorly understood, but, direct and indirect mechanisms have been described.\textsuperscript{34,79,80} For instance, ILC3s communicate bi-directionally with innate cells, such as IECs, macrophages, and dendritic cells (DCs) via cytokines (such as IL-23, IL-22, GM-CSF) to quickly shape innate responses in response to changes in the tissue microenvironment.\textsuperscript{81,82} In addition, MHCII\textsuperscript{+} ILC3s have been shown to present antigens and directly regulate antimicrobial CD4\textsuperscript{+} T cell activity and T cell-dependent antibody production.\textsuperscript{83,84} Thus, despite being greatly outnumbered, ILC3s can employ various means to shape protective antimicrobial/tissue repair responses in the mucosa but more studies are needed to fully understand the underlying mechanism(s).

An important caveat, which has likely contributed to some confusion in the literature, is the inconsistent reporting of Helicobacter status in mouse colonies using IL-10-dependent models of colitis.\textsuperscript{85} Neither germfree nor conventionally housed IL-10\textsuperscript{−/−} mice develop spontaneous colitis,\textsuperscript{81,85} but rather, the development of intestinal disease is dependent on co-colonization with "pathobionts" such as Helicobacter spp.\textsuperscript{85,86} Helicobacter-colonized IL-10\textsuperscript{−/−} mice are easily identified clinically by the appearance of rectal prolapse,\textsuperscript{85} but in most other mouse strains infection is non-pathogenic or subclinical.\textsuperscript{86,87} and frequently, Helicobacter status is unknown.\textsuperscript{86} Pups acquire their intestinal microbiota through fecal/oral contact\textsuperscript{88} and depending on the breeding strategy and status of the parental strains, the Helicobacter status of pups needs to be tested to confirm transmission, otherwise, the results may be confounded.\textsuperscript{88} Notably, Yen's study did not report Helicobacter status of their mouse colonies, thus it is not clear if the resolution of colitis in IL-10\textsuperscript{−/−} p19\textsuperscript{−/−} mice is due to the lack of IL-23 or Helicobacter.\textsuperscript{83} Similarly, IL10\textsuperscript{−/−} Il10\textsuperscript{fl/fl} mice were previously generated but the Helicobacter status nor the incidence of intestinal disease was reported although these studies were focused on IL-17A's role in lung pathology.\textsuperscript{89,90} Here, we confirmed that IL10\textsuperscript{−/−} Il22\textsuperscript{−/−} mice remained colonized with Helicobacter spp. (Fig. 7) yet were disease free which strengthens our assertion that IL-22 is a critical effector cytokine driving the development of colitis in IL10\textsuperscript{−/−} mice.

In both humans\textsuperscript{90,91} and IL-10-dependent models of colitis,\textsuperscript{85,92} IBD is associated with reduced diversity of the intestinal microbiome, yet it remains unclear if microbial dysbiosis is a cause or result of intestinal inflammation.\textsuperscript{92} IL-22 has a vital role in shaping the commensal microbiota and protecting against enteric pathogens.\textsuperscript{80,93} In line with this, we observed that microbial diversity was restored in our IL10\textsuperscript{−/−} Il22\textsuperscript{−/−} mice suggesting that dysregulation of the IL3/Il-22 axis drives microbial dysbiosis in IL10\textsuperscript{−/−} mice. As mentioned previously, IL-22-producing ILC3s were found to gather in the small intestines of WT and Rag1\textsuperscript{−/−} weanlings as they become colonized with commensals. In WT pups, the ILC3s were soon displaced by adaptive immune cell populations, but in Rag1\textsuperscript{−/−} mice the levels of activated ILC3s remained elevated in resulting in altered homeostasis in gut tissues.\textsuperscript{94} The Helicobacter status of the mice in this study was not reported, but interestingly, work from others demonstrated that, like in IL10\textsuperscript{−/−} mice, colonization of Rag-deficient mice with Helicobacter spp. results in spontaneous colitis.\textsuperscript{95,96} Importantly, the development of colitis in Helicobacter-infected Rag\textsuperscript{−/−} mice was shown to be dependent on IL-22.\textsuperscript{97} It is important to note that our data do not indicate if the microbiota itself is sufficient to induce or protect against the development of spontaneous colitis. Additional studies will be needed to determine this. Together, these converging data along with our findings that microbial diversity is restored in IL10\textsuperscript{−/−} Il22\textsuperscript{−/−} mice, suggest that IL-10 plays an underappreciated role in establishing mutualism by regulating innate IL-22 responses to commensal bacteria and promoting the development of tolerogenic adaptive immune subsets.\textsuperscript{98}

This study highlights the mechanistic connection between IL-10 and IL-22 and offers important insights into the pathogenesis of chronic colitis in IL10\textsuperscript{−/−} mice. Our data suggest a model in which IL-10 and IL-22 form a regulatory axis within the gut mucosa to maintain intestinal health. Thus, in the context of IL-10-deficiency, IL-22 is necessary while IL-17 is not sufficient to drive immune-mediated intestinal pathology. Importantly, the IL-10/IL-22 axis also provides a conceptual framework to link the development and resolution of intestinal inflammation with the mechanisms underlying epithelial repair. These findings may help to reconcile conflicting reports regarding IL-17's role in intestinal disease and will be informative when considering new therapeutic approaches in IBD and other inflammatory disorders.

**MATERIALS AND METHODS**

**Mice.** B6.129P2-Il10\textsuperscript{m1Cgr} (Il10\textsuperscript{−/−}) as well as WT mice were originally obtained from Jackson Laboratories (Bar Harbor, ME) and have been maintained in separate breeding colonies within our animal facility for over 14 years. Il22\textsuperscript{−/−} mice were obtained through an agreement with Genentech and have been maintained in separate breeding colonies within the same facility for over 7 years. The Il10\textsuperscript{−/−} Il22\textsuperscript{−/−} mice were generated in our facility through inter-crossing Il10\textsuperscript{−/−} and Il22\textsuperscript{−/−} mice. To examine IL-22's role in regulating disease pathology and microbial diversity in IL10\textsuperscript{−/−} mice, it was important to control for potential founder effects by generating mice genetically deficient in Il10 and Il22 while maintaining the microbiota from Il10\textsuperscript{−/−} mice. To do so, we used a breeding strategy which minimizes microbial cross-contamination. Briefly, we set up clean breeding cages with one female Il10\textsuperscript{−/−} mouse in estrus and one male Il22\textsuperscript{−/−} breeder and once impregnated, the female Il10\textsuperscript{−/−} mouse was separated from the male and placed into a clean cage. For the F2 generation, a female Il10\textsuperscript{−/−} mouse and a F1 male Il10\textsuperscript{−/−} Il22\textsuperscript{−/−} breeder were placed in a clean cage, and the impregnated female was separated from the male and placed into a clean cage. Il10\textsuperscript{−/−} Il22\textsuperscript{−/−} F2 mice were maintained then by interbreeding.

For experimental procedures male animals between 16 and 20 weeks of age were used unless stated otherwise. Mice on study were housed in individually ventilated cages (Allentown Caging Equipment, Allentown, PA), containing autoclaved corncob bedding (Harlan Teklad, Indianapolis, IN) with a cotton-enrichment square. Mice were provided autoclaved rodent chow ad libitum (Harlan Teklad, Indianapolis, IN) and received reverse-osmosis-treated, hyperchlorinated water via automated in-cage watering system (Edstrom Industries, LLC, Waterford, WI). The room was maintained on a 14:10 h light/dark cycle at 22.8 ± 2°C, with relative humidity between 30% and 50%. All experimental procedures were approved by the Johns Hopkins University Animal Care and Use Committee, the program is accredited by AAALAC, International and procedures were consistent with the Guide for the Care and Use of Laboratory Animals.

mRNA isolation and quantitative RT-PCR analyses Total RNA was isolated from spleen, mesenteric lymph node (MLN), terminal ileum and proximal and distal colon using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's
protocol. Reverse transcription was performed using a first strand cDNA synthesis kit (Roche, Indianapolis, IN). Quantitative PCR was performed using SYBR Green gene-specific primers on an ABI 7300 Real-time PCR System. Results were normalized to Gapdh levels. For relative comparisons, samples were compared to the corresponding WT tissue that was assigned an arbitrary value of 1.

Isolation of intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs)
IELs and LPLs were isolated as described previously.1 Briefly, colons and small intestines were harvested, cut open longitudinally, and washed in cold 1xPBS to remove feces. Either two small intestines or three colons were cut into 1 cm segments and incubated for 25 min at 37 °C in RPMI containing 3% FBS, EDTA, DTT, and Hepes, with shaking. Suspensions were strained and IELs were purified from the flow through using a percoll gradient. The remaining segments of small intestine and colon were further digested in RPMI containing DNaseI, Liberase TL, and Heps for 50 min at 37 °C. The digested tissues were passed through a 70 µm and a 40 µm strainer, respectively, to obtain single cell suspensions of small intestinal and colonic LPLs. All procedures were carried out on ice.

Cell staining, antibodies, and flow cytometry
For cellular phenotyping, cells were washed, incubated with anti-CD16 to block Fc receptors and stained with VIVID (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. For cellular phenotyping, cells were washed, incubated with anti-γδTCR, anti-CD19, anti-CD11b, anti-Ly6C, anti-Ly6G, anti-F4/80, anti-NKp46, and anti-CD127 (IL-7Rα), CCR6 and γSTCR (BD Biosciences, San Jose, CA). For intracellular cytokine staining (ICS) experiments, single cell suspensions of spleen, MLN, small intestinal IELs (sIEL) and sLPLs, colonic IELs (cIEL) and cLPLs were re-stimulated for 4 h with 40 ng/mL phorbol-12-myristate-13-acetate (PMA) and 2 µg/mL ionomycin (Millipore, Billerica, MA, USA) in the presence of brefeldin A (BD Biosciences, San Jose, CA). Cells were surface stained as above then were fixed and permeabilized using the eBioscience Foxp3 buffer system and stained with antibodies against mouse IL-10, IFN-γ, IL-17, IL-22, RORγt, and/or Foxp3 (BD Biosciences, San Jose, CA). Cells were acquired on a BD LSRII. Data were analyzed with FlowJo software (Tree Star, Ashland, OR).

Pathology
Mice were monitored weekly for rectal prolapse. For histopathology, euthanasia was performed by CO2 inhalation, then exsanguination and blood collection by cardiocentesis. Mice were perfused with heparinized saline, followed by 10% neutral buffered formalin, via the left ventricle of the heart. Colons were collected, and either cut open longitudinally and prepared as a “Swiss roll”, or as cross sections. Fixed tissues were processed routinely to paraffin in graded alcohols, sectioned at 5 µ, and stained with hematoxylin and eosin (H&E). Inflammation was scored in a blinded manner according to the Jackson Laboratory (TJL) scoring system as described previously.99

PCR array
Total RNA was isolated from the distal colons of WT, Il10−/−, Il22−/−, and Il10−/− Il22−/− mice (n = 4) using TRIzol (Invitrogen), according to the manufacturer's protocol. RNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). cDNA samples were then loaded onto the TaqMan™ OpenArray™ Mouse Inflammation Panel (Applied Biosystems) using the QuantStudio 12K Flex OpenArray AccuFill System (LTI). OpenArray Real-Time PCR results were analyzed using Partek genomic software. Gene expression was calculated using the comparative Cq method as fold change relative to WT using the reference gene Hprt1. Significantly different genes were calculated using ANOVA with cutoff at FDR < 0.05 and fold change of 2. Data were visualized as heatmaps composed of Z-scores from standardized Cq values for each independent sample.

Bacterial 16s rRNA sequencing and qPCR
The fecal microbiome was analyzed using feces from 10 male and 5 female mice of each genotype obtained between 12 and 16 weeks. DNA was extracted from fecal pellets using the PowerSoil DNA Kit (MO BIO Laboratories, Carlsbad, CA). Amplicons were generated using oligonucleotide primers that target ~300 bp of the V4 variable region of the 16s rRNA gene (primers 515F and 806R)100 and also were barcoded and pooled to construct the sequencing library, followed by sequencing with an Illumina MiSeq instrument to generate paired-end 150 × 150 reads. The software package QIIME 1.7.0 was used to analyze, display, and generate figures of microbiome data using a previously defined method.102

Overlapping pair-end reads were aligned using SHE-RA103 with subsequent analysis and normalization performed using QIIME 1.7.0.104 Fecal communities were compared by using UniFrac, a phylogeny-based distance metric that measures the degree to which any two microbiota share branch length on a bacterial tree of life. As a first step, sequence data and metadata were combined to de-multiplex the barcoded reads followed by quality filtering using the default parameters in QIIME. Sequences were grouped into operational taxonomic units (OTUs) at 97% sequence similarity using the UCLUST algorithm. Taxonomy was assigned...
using Ribosomal Database Project (RDP) classifier against the GreenGenes database, and sequences were aligned and a phylogenetic tree was built from reference sequences using FastTree. An OTU table showing counts of each OTU in each sample was produced. To control for differences in sequencing depth, OTU tables were rarified at a single sequencing depth. Alpha diversity was determined using the Shannon and Chao1 indices, and statistical analysis was performed at a sampling depth of 30,450 sequences. Beta diversity was determined using unweighted and weighted UniFrac distance matrices and the results presented as principal coordinate axis (PCoA) plots. Significant differences in the relative abundance of bacteria at different taxonomic levels were computed using Linear Discriminant Analysis Effect Size (LEfSe) comparing each mouse strain against the remaining samples with LDA scores > 2.0 and p-values < 0.05 considered significant.

Pairwise adonis comparisons were carried out using RStudio (version 3.4.3) with the pairwiseAdonis package.

Statistical measures of microbiota diversity

Data were analyzed at the highest sampling depth (30,450 reads) that was shared by all four genotypes of mice. All statistical analyses (α = 0.05) were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). If data were normally distributed based on the Kolmogorov–Smirnov test, groups were compared using one-way ANOVA and p-values generated using the Student–Neuman–Keuls multiple comparison procedure. If data sets were not normally distributed, due to either the comparison procedure were used. Array reads (version 3.4.3) with the pairwiseAdonis package.

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AUTHOR CONTRIBUTIONS

D.C.G., A.R., and J.H.B. conceived the study, designed experiments. D.C.G. and J.H.B. wrote the manuscript. A.R., P.J., M.S., V.V., Z.S., and A.S. performed experiments. D.C.G., M.T.W., C.F.B., J.G.F., V.V., A.S., and J.H.B. interpreted results and/or generated figures. C.F.B., M.T.W. and J.G.F. provided feedback and supervised certain aspects of the study.

ADDITIONAL INFORMATION

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