NFIL3 deficient mice develop microbiota dependent, IL-12/23 driven spontaneous colitis

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Abstract

NFIL3 (nuclear factor, IL-3 regulated) is a transcription factor that regulates multiple immunologic functions. In myeloid cells, NFIL3 is IL-10 inducible, and has a key role as a repressor of IL-12p40 transcription. *NFIL3* is a susceptibility gene for the human inflammatory bowel diseases. Here we describe spontaneous colitis in *Nfil3*$^{-/-}$ mice. Mice lacking both *Nfil3* and *Il10* (NIDKO) had severe early-onset colitis, suggesting NFIL3 and IL-10 independently regulate mucosal homeostasis. Lymphocytes were necessary for colitis, as *Nfil3/Rag1* double knockout (NRDKO) mice were protected from disease. However, NRDKO mice adoptively transferred with wild type CD4$^+$ T cells developed severe colitis compared to *Rag1*$^{-/-}$ recipients, suggesting that colitis was linked to defects in innate immune cells. Colitis was abrogated in *Nfil3/Il12b* double-deficient mice, identifying *Il12b* dysregulation as a central pathogenic event. Finally, germ-free *Nfil3*$^{-/-}$ mice do not have colonic inflammation. Thus, NFIL3 is a microbiota-dependent, IL-10-independent regulator of mucosal homeostasis via IL-12p40.

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Keywords

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INTRODUCTION

Genetic variants that confer susceptibility to the human inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC) highlight the importance of innate immune interactions with the enteric microbiota in controlling inflammation (1). A consequence of innate immune activation in IBD is the over-production of, and maladaptive responses to, pro-inflammatory cytokines (2). In this context of human IBD and mouse models of IBD, IL-12 and IL-23 have been shown in multiple contexts to have a central pathogenic role (3–7). IL-12 and IL-23 are heterodimeric cytokines composed of p40-p35 and p40-p19 subunits, respectively. Macrophages and dendritic cells activated by microbes and microbial products are the main cell types that produce IL-12 and IL-23. While IL-12 is decisive in regulating lineage commitment to Th1 development, IL-23 is essential for the maturation and stability of Th17 cells (8, 9). Several genes in the IL-12 and IL-23 expression and signaling pathways, notably *IL23R*, *JAK2*, *STAT3*, and *IL12B* are associated with CD and UC susceptibility (10, 11).

A key step in the normal homeostatic regulation of IL-12 and IL-23 from activated innate cells is inhibition by IL-10 derived from autocrine-paracrine signaling from activated myeloid cells, or from lymphocytes, especially regulatory T cells (12, 13). IL-12 and IL-23 are not the only IL-10 targets; in fact many pro-inflammatory genes implicated in IBD, including *TNF* and *IL1B*, are regulated by IL-10 by a variety of mechanisms (14, 15). However, in the context of intestinal mucosal homeostasis, the IL-10-mediated inhibitory pathway that tonically regulates excessive IL-12 and IL-23 is a central pathway. Defects in the production of or the responsiveness to IL-10 exert profound homeostatic effects in the mucosal immune system (12, 16, 17). Polymorphisms in *IL10* confer susceptibility to UC while complete loss-of-function mutations in the IL-10 receptors cause a rare but severe form of very early onset of IBD (18, 19). Importantly, ablation of IL-12 and IL-23 significantly ameliorates IBD in the IL-10-deficient ($Il10^{-/-}$) mouse model, confirming the essential role of the IL-10 inhibitory pathway that controls IL-12 and IL-23 (20–22).

The basic leucine zipper transcription factor NFIL3 (Nuclear factor, IL-3 regulated, also known as E4BP4) has recently been implicated as a regulator of multiple aspects of immune function. NFIL3 is essential for the development of all natural-killer (NK) cells (23, 24) and CD8α dendritic cells (DCs) (25), cytokine production by Th2 cells (26, 27), and IgE class-switching in B cells (28). IL-10 was reported to regulate NFIL3 mRNA expression over a decade ago (29). Following from that observation, we recently reported that NFIL3 is an essential transcriptional repressor of IL-12p40 in murine macrophages (30, 31). NFIL3 controls *Il12b* transcription by binding to an enhancer located ~10 kb upstream of the canonical *Il12b* promoter (31). An NFIL3 binding element has also been characterized in the proximal *Il12b* promoter region (30). Myeloid cells deficient in NFIL3 overproduce IL-12p40, however, they remain responsive to IL-10-mediated inhibition, arguing that NFIL3 is a necessary component of the IL-10 pathway but alone is not sufficient to block IL-12p40 production (30, 31). We also found that NFIL3 participates in host enteric microbial interactions *in vivo* and the enteric microbiota and IL-10 are co-factors for NFIL3 expression in the intestine. We found that colonic CD11b$^+$ LPMC isolated from $Nfil3^{-/-}$ mice, like bone marrow-derived myeloid cells, made higher amounts of *Il12b* mRNA than WT CD11b$^+$ colonic macrophages. In human IBD, intestinal CD14$^+$ macrophages are major

producers of pro-inflammatory cytokines including IL-12 family members (5). We found that NFIL3 expression was lower in CD14^{+} LPMC isolated from patients with CD and UC compared with non-inflammatory controls undergoing surgical resection (30). Our previous studies also concluded that intestinal NFIL3 expression inversely correlates with higher amounts IL-12p40 in mice and humans (30). Importantly, recent meta-analysis of CD and UC genome-wide association scans identified *NFIL3* as a susceptibility gene for human inflammatory bowel disease (IBD) (32).

Collectively, these studies raised the possibility that NFIL3 would be involved in normal homeostatic regulation of the intestine. Here, we characterize a severe form of spontaneous colitis in *Nfil3*$^{-/-}$ mice that correlated with increased IL-12p40 in serum and colonic tissue. Colitis was dependent on *Il12b*-driven innate and adaptive immune interactions. These results implicate NFIL3 as a central determinant of mucosal immune homeostasis and coupled with the recent human genetic association, an important pathway to elucidate the pathogenesis of IBD.

MATERIALS AND METHODS

Mice

Nfil3$^{-/-}$ mice were generated by gene-targeting strategies as reported (28) and bred with C57BL/6 mice for at least 10 generations. Germ free (GF) *Nfil3*$^{-/-}$ mice were Caesarian derived as previously described (33) and were maintained according to standard techniques in the University of North Carolina National Gnotobiotic Resource Center. WT, *Il10*$^{-/-}$, and *Rag1*$^{-/-}$ mice were obtained from Jackson Laboratory (Bar Harbor, ME). *Il10*$^{-/-}$ and *Rag1*$^{-/-}$ mice were crossed with *Nfil3*$^{-/-}$ mice to generate mice deficient in both genes (*Nfil3*$^{-/-}$*Il10*$^{-/-}$, NIDKO or *Nfil3*$^{-/-}$*Rag1*$^{-/-}$, NRDKO). All experimental mice were genotyped by polymerase chain reaction (PCR) screening before tissue collection, and age-matched littermates were used as controls. Mice were evaluated for colonic inflammation at the age of 12–16 weeks. Another strain of NFIL3-deficient mice (24) was maintained at St. Jude Children's Research Hospital. All animal experiments were in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of North Carolina School of Medicine, the University of Iowa Carver College of Medicine and St. Jude Children's Research Hospital.

Reagents

LPS was purchased from InvivoGen (San Diego, CA). M-CSF was obtained from Peprotech Inc (Rocky Hill, NJ).

Cell isolation

BMDMs were cultured as described (34). Mouse colonic lamina propria mononuclear cells (LPMCs) were isolated by an enzymatic method and separated into CD11b^{+} cells using MACS anti-CD11b microbeads (Miltenyi Biotec, Auburn, CA) (12).

Quantitative RT-PCR

Quantitative real-time RT-PCR was performed as described (35). Primer sequences are available upon request.

Colonic tissue explant cultures and histology

Colonic tissue explant cultures were performed as previously described (35). Supernatants were collected after 24 hours for cytokine ELISA. Cytokine values were normalized to dry weight of the colonic tissue explants (pg/ml/100 mg). Slides were prepared for H&E

staining and histological analysis was performed by a pathologist blinded to the study groups (LBB) using criteria reported previously (36).

Flow cytometry

Flow cytometric analysis was performed as previously described (12). Anti-CD3, anti-CD4, anti-NKp46, anti-retinoid-acid receptor-related orphan receptor gamma t (RORχt), anti-forkhead box P3 (Foxp3), anti-IL-17A, and anti-IFN-γ antibodies were purchased from eBioscience (San Diego, California, USA). For intracellular staining, Cytofix/Cytoperm Kit (BD PharMingen) was used according to the manufacturer's instructions. For cytokine staining, LPMCs were stimulated with phorbol myristate acetate (100 ng/ml) and ionomycin (1 µg/ml) (Sigma) in the presence of monensin (Golgistop; BD PharMingen) for 4 hours. Results were analyzed by Beckman-Coulter (Dako) CyAn ADP flow cytometer and Summit (version 4.3) software.

Immunohistochemistry

Mouse colonic tissues were harvested, swiss-rolled, and fixed with 4% paraformaldehyde (Sigma, St. Louis, MO) overnight. Fixed tissues were then embedded in OCT compound (Sakura, Tokyo, Japan) and kept at −80°C. Frozen tissue sections (7 µm) were first blocked for 30 min at room temperature with Blocking Reagent (PerkinElmer, Waltham, MA) and then incubated overnight at 4°C with primary antibodies as described below. Following overnight incubation, sections were washed and then incubated with HRP-conjugated secondary antibody for 1h at room temperature. After washing, sections were treated with tyramide-fluorescein (PerkinElmer), followed by counterstaining with DAPI (Sigma). All stained sections were analyzed by confocal microscope using either the 10× or the 20× objective (TCS SP2, Leica, Wetzlar, Germany). Images were processed with Adobe Photoshop software. The following antibodies were used: anti-CD11c and anti-F4/80 from BD Bioscience (Franklin Lakes, NJ), anti-CD3, anti-NKp46, and anti-B220 from eBioscience (San Diego, CA), HRP-conjugated donkey anti-rat IgG, and HRP-conjugated donkey anti-Armenian hamster IgG (H+L) from Jackson Immunoresearch (West Grove, PA).

ELISA

IL-12p40 concentration was determined by sandwich ELISA (BD Biosciences, San Jose, CA). IL-22 ELISA kit was obtained from eBioscience (San Diego, CA). IL-17A, IL-10, IFN-γ, and TNF-α were measured using the MILLIPLEX MAP Cytokine Kit (Millipore, Billerica, MA).

Induction of colitis by adoptive transfer of T cells

Splenocytes from WT mice were enriched for CD4^{+} cells by positive selection on MACS columns with anti-CD4 microbeads (Miltenyi Biotec). $Rag1^{-/-}$ or $Nfil3^{-/-}Rag1^{-/-}$ (NRDKO) mice were i.p. injected with 1×10^6 CD4^{+} cells. Recipients and control mice were observed for clinical signs of colitis and body weight changes. Clinical score was recorded four weeks post-transfer as previously described (37) (wasting: 0, no wasting; 1, 0.1–10% loss of initial total body weight; 2, >10% loss of initial total body weight; diarrhea: 0, none; 1, soft stool; 2, watery and/or bloody; hunching/bristled fur/skin lesions: 0, unchanged; 1, any positive sign; rectum prolapse: 0, absent; 1, present).

Germ free to conventionalized transition of mice

8–10-week-old GF WT and $Nfil3^{-/-}$ mice were transitioned to conventionalized housing. To re-colonize the GF mice with conventionalized microbiota, each cage was given bedding

from the same donor conventionalized WT cage. Mice were sacrificed after 2 weeks and monitored for colitis as described above.

Statistical analysis

Statistical significance for data subsets was assessed by the two-tailed Student's t test unless specifically described. A p<0.05 was considered significant.

RESULTS

Nfil3$^{-/-}$ mice develop spontaneous Th1/Th17-mediated chronic colitis

On necropsy, colons from 12-week-old *Nfil3*$^{-/-}$ mice were thickened and foreshortened with lack of formed fecal pellets. Colon length was significantly shorter in *Nfil3*$^{-/-}$ compared to WT mice (Fig. 1A). Approximately 20% of *Nfil3*$^{-/-}$ mice developed rectal prolapse by 20 weeks and 50% by 36 weeks of age (Fig. 1B).

Quantitative assessment of colonic histology revealed markedly increased frequency and severity of inflammation in *Nfil3*$^{-/-}$ mice compared to WT mice (Fig. 1C). As in other models of spontaneous colitis, histological severity was variable and penetrance of disease was incomplete (Fig. 1H Compared to colons from WT mice (Fig. 1D), significant elongation of colonic crypts was observed colons from *Nfil3*$^{-/-}$ mice (Fig. 1E). Inflammation was characterized by multifocal aggregates of inflammatory cells that were confined to the mucosa as well as extending transmurally to the submucosa (Fig. 1F). We also observed areas of ulceration (Fig. 1G, arrow). Inflammation in the lamina propria was occasionally organized into *de novo* lymphoid follicles, and multinucleated giant cells were detected (Fig. 1H, arrow). Quantitative assessment of colonic histological severity (n = 25, 12–16 week old *Nfil3*$^{-/-}$ mice) was significantly higher compared to age-matched WT mice (n = 7), but the penetrance of colitis was incomplete (Fig. 1C).

Colonic tissue explants from *Nfil3*$^{-/-}$ mice secreted significantly elevated amounts of pro-inflammatory cytokines IL-12p40, TNF-α, IFNγ and IL-17A compared to WT colonic explants (Fig. 2A). Quantitative real-time RT-PCR from colonic tissue also demonstrated increased mRNA expression for these cytokines (Fig. 2B). Serum IL-12p40, TNF-α, IFNγ and IL-17A were also increased in *Nfil3*$^{-/-}$ mice (Fig. 2C). Increased colonic and serum IL-10 was also detected, suggesting that the loss NFIL3-dependent pathways cannot be compensated by increased IL-10 expression (Fig. 2A, B, C). Moreover, serum IL-12p40 positively correlated with colonic IL-12p40 expression (Fig. 2D) and histologic severity of inflammation (Fig. 2E, F). Consequently, serum IL-12 p40 is a biomarker for histological severity of colitis in this model.

Colonic macrophages are the main source of inflammatory cytokines in *Nfil3*$^{-/-}$ mice

By immunohistochemical analyses, CD3$^+$, B220$^+$. and F4/80$^+$ cells were increased in inflamed colonic lamina propria of *Nfil3*$^{-/-}$ mice compared to WT mice, with the most striking increase in F4/80$^+$ cells (Fig. 3A). There was no significant increase in CD11c$^+$ dendritic cell (DC) populations. B220$^+$ plasma cells were localized inside and outside of secondary lymphoid follicles in *Nfil3*$^{-/-}$ mice in contrast to WT colons where the vast majority of B220$^+$ cells localized within lymphoid structures. CD11b$^+$ lamina propria mononuclear cells (LPMC) are comprised of colonic macrophages (F4/80$^+$ CD11c$^-$ or CD11c^{low}) and DCs (CD11c$^+$ F4/80$^-$) (38). We therefore isolated and analyzed colonic CD11b$^+$ LPMCs from colons of *Nfil3*$^{-/-}$ as a representative colonic macrophage population. *Nfil3*$^{-/-}$ CD11b$^+$ colonic LPMCs expressed higher *Il12b*, *Il23a*, *Tnf*, and *Il10* mRNA compared to WT CD11b$^+$ colonic LPMCs (Fig. 3B). Furthermore, these cytokine mRNAs

were predominantly expressed in CD11b$^+$ LPMCs, whereas expression in the CD11b$^-$ population was limited.

Localization of IFNγ and IL-17A expression in CD3$^+$CD4$^+$ colonic lamina propria T cells was examined by intracellular cytokine staining in WT and *Nfil3*$^{-/-}$ mice. Both IFN-γ- and IL-17A-producing CD4$^+$ T cells are significantly increased in *Nfil3*$^{-/-}$ mice (Fig. 3C, D). The percentage of Foxp3$^+$CD4$^+$ regulatory T cells did not differ between WT and *Nfil3*$^{-/-}$ colonic LPMCs (Fig. 3E).

Nfil3$^{-/-}$ mice have decreased numbers of colonic NK cells and lymphoid tissue inducer cells (LTi)

Nfil3$^{-/-}$ mice lack peripheral NK1.1$^+$ NK cells (23, 24). However it is unclear whether these mice also lack intestinal NK cells, which express NKp46 (39). The lamina propria CD3$^-$NK1.1$^+$ NK cell population was significantly decreased, while CD3$^+$NK1.1$^+$ NK T cells were similar in number in *Nfil3*$^{-/-}$ compared to WT mice (Fig. 4A). The transcription factor retinoid-acid receptor-related orphan receptor gamma t (RORγt) is implicated in NK marker-expressing mucosal lymphoid cell development and function (39, 40). Therefore, RORγt expression was assessed in the colonic NKp46$^+$ cell population. Both RORγt$^+$ and RORγt$^-$ CD3$^-$NKp46$^+$ colonic lamina propria ILC populations were significantly decreased in number in *Nfil3*$^{-/-}$ compared to WT mice (Fig. 4B). Lymphoid tissue inducer (LTi) cells, defined as CD3$^-$ RORγt$^+$, are required for the development of secondary lymphoid tissue in the gut (41). In contrast to CD3$^+$RORγt$^+$ cells that include IL-17A-producing T cells, the CD3$^-$ RORγt$^+$ LTi population was significantly decreased in *Nfil3*$^{-/-}$ compared to WT mice. Both CD4$^+$ and CD4$^-$ populations were decreased among CD3$^-$NKp46$^-$RORγt$^+$ LTi cells in *Nfil3*$^{-/-}$ colons, even though de novo colonic mucosal lymphoid structures are present in *Nfil3*$^{-/-}$ mice (Fig. 1H). RORγt$^+$ CD3$^-$NKp46$^+$ mucosal lymphoid cells are a major source of the homeostatic cytokine IL-22 (NK22 cells) (39, 40). Therefore, expression and production of colonic IL-22 was examined in *Nfil3*$^{-/-}$ mice. By contrast to other pro- and anti-inflammatory (IL-10) cytokines (Fig. 2A, B), IL-22 production in colonic explant culture and *Il22* mRNA expression were not increased in *Nfil3*$^{-/-}$ colonic tissue (Fig. 4C).

IL-10 and NFIL3 independently but cooperatively regulate IL-12p40 in BMDMs and *in vivo*

We have previously shown that IL-10 is required for LPS-induced NFIL3 expression in BMDMs and that NFIL3 partly mediates IL-10 inhibition of *Il12b* (30, 31). Moreover, *Il10*$^{-/-}$ mice demonstrated impaired induction of *Nfil3* in the colon by the microbiota (30). However, inhibition of *Il12b* by NFIL3 does not require IL-10, since overexpression of NFIL3 in *Il10*$^{-/-}$ BMDMs effectively inhibited IL-12p40 (30). Therefore, whether IL-10 and NFIL3 independently regulate IL-12p40 in cells and *in vivo* was next addressed. The inhibitory effect of IL-10 on IL-12p40 in WT and *Nfil3*$^{-/-}$ BMDMs was compared. Recombinant IL-10 inhibited LPS-induced IL-12p40 to a similar extent in WT and *Nfil3*$^{-/-}$ BMDMs (Fig. 5A). We next generated *Nfil3* $\times$ *Il10* double knockout (NIDKO) mice, and hypothesized that colitis would be similar in severity in NIDKO mice compared to single knockouts if NFIL3 and IL-10 functions are completely dependent on each other. IL-12p40 production from activated macrophages was dramatically increased in NIDKO BMDMs compared to either single knockout cells (Fig. 5B). However, there was no difference in IL-10 mediated IL-12p40 inhibition in *Il10*$^{-/-}$ and NIDKO BMDMs (Fig. 5C), suggesting that inhibition of IL-12p40 by IL-10 does not require NFIL3. These data were ascertained independently with BMDMs from two different strains of NIDKO and single knockout mice derived at UNC and St. Jude Children's Research Hospital.

Importantly, NIDKO mice had severe colitis with 100% penetrance (Fig. 5D) and higher colonic IL-12p40 expression (Fig. 5E) at 5 weeks of age (when these mice were euthanized

due to severity of illness) compared to single knockout mice at 12–16 weeks of age. All double knockout mice developed severe diarrhea and 50% (3/6) developed rectal prolapse by 5 weeks of age. By comparison, 7 and 12 percent of $Il10^{-/-}$ (n=28, data not shown) and $Nfil3^{-/-}$ (n=41, see Fig. 1B), respectively, developed rectal prolapse in mice at 16 weeks of age. Colon length was shorter in NIDKO mice compared to $Nfil3^{-/-}$ and $Il10^{-/-}$ mice (Fig. 5F). Interestingly, histological colonic inflammation was more severe in $Nfil3^{-/-}$ mice than $Il10^{-/-}$ mice on a C57BL/6 background (Fig. 5D). A separate colony of NIDKO mice generated at St. Jude Children's Research Hospital confirmed these findings; even though the St. Jude $Nfil3^{-/-}$ mice do not get colitis, combined loss of IL-10 and NFIL3 caused a dramatic acceleration in colitis. These findings demonstrate that NFIL3 and IL-10 coordinately but independently regulate mucosal homeostasis.

$Nfil3^{+/+}$ T cells are sufficient to induce colitis in $Nfil3$/$Rag1$ double knockout mice

In an adoptive transfer model of colitis in $Rag1^{-/-}$ recipients, Motomura *et al.*, demonstrated that naive $Nfil3^{-/-}$ CD4$^+$T cells were potent in colitis induction potential compared to WT CD4$^+$ T cells (27), mediated by defective IL-10 production from $Nfil3^{-/-}$ T cells. However, since IL-10 production was increased in $Nfil3^{-/-}$ colons (Fig. 2A, B, C), other mechanisms underlie colitis development in the $Nfil3^{-/-}$ background. Therefore, $Nfil3^{-/-}$ mice were crossed to $Rag1^{-/-}$ mice (NRDKO) to study whether lymphocytes are necessary for the colitis development in $Nfil3^{-/-}$ mice. NRDKO mice had no clinical signs of colitis (data not shown). Colonic histological scores (Fig. 6A) and colonic IL-12p40 (Fig. 6B) were comparable to WT and $Rag^{-/-}$ mice, showing that lymphocytes are necessary for colitis development.

We next asked whether innate-T cell interactions are necessary for the development of colitis in $Nfil3^{-/-}$ mice. WT ($Nfil3^{+/+}$) unfractionated CD4$^+$ T cells were adoptively transferred into $Rag1^{-/-}$ or NRDKO mice. Unfractionated CD4$^+$ cells did not cause severe pathology when transferred to $Rag1$ single knockout recipients (Fig. 6C–G), as previously described (42). However, NRDKO recipients develop severe colitis clinically (Fig. 6C, D, E) and histologically (Fig. 6F, G), correlating with increased intestinal IL-12p40 (Fig. 6H) and IFN-γ (Fig. 6I). These results suggest that T cells are necessary for the development of colitis in $Nfil3^{-/-}$ mice; however the lack of NFIL3 in accessory cell populations is essential for intestinal inflammation. Furthermore, transferred Tregs in the unfractionated CD4$^+$ population cannot rescue disease when NFIL3 is absent in non-lymphocyte cells.

$Nfil3$/$Il12b$ double knockout mice do not develop colitis

Genetic deletion of $Il12b$ rescues several forms of intestinal inflammatory disease because IL-12 and IL-23 production is ablated (20, 43). Therefore, a key test of the relationship between NFIL3 and IL-12p40 is to determine if loss of $Il12b$ rescues colitis in $Nfil3^{-/-}$ mice. We therefore generated $Nfil3 \times Il12b$ double knockout (DKO) mice and observed that these mice did not develop colitis up to 4 months of age (Fig. 7A, B), confirming that increased IL-12p40 production in $Nfil3^{-/-}$ mice is responsible for the development of colitis and of the inflammatory T cell phenotype.

Colitis development in $Nfil3^{-/-}$ mice is dependent on the enteric microbiota

$Nfil3^{-/-}$ mice in our colony at the University of North Carolina develop significant spontaneous colitis, but mice from the founder colony at the University of Iowa (28) and a colony at St. Jude Children's Research Hospital (31) do not develop colitis. Indeed, in a histologic analysis of 14 $Nfil3^{-/-}$ mice 6–8 months in age from the University of Iowa and St. Jude Children's Research Hospital, no colitis was detected (data not shown), whereas 10 age-matched mice from the UNC colony manifest histologic colitis (data not shown). We assayed for *Helicobacter* spp. in the feces of Iowa and UNC mice. Non-*H. hepaticus* (*H.*

hepaticus is routinely screened by both facilities) *Helicobacter* spp. were present in both colonies (data not shown), leading to the conclusion that other components of the intestinal microbiota are necessary for colitis development (although interactions of other enteric microbes with *Helicobacter* spp. are possible).

To definitively confirm the microbiota dependence for colitis development, *Nfil3*$^{-/-}$ mice were derived germ free (GF). Importantly, GF *Nfil3*$^{-/-}$ mice did not manifest colitis at 6 months of age (Fig. 7C, left) and colonic explant cultures produce low levels of IL-12p40 (Fig. 7D, left). However, upon transition to conventional housing (GF-CNV), *Nfil3*$^{-/-}$ mice demonstrated histologic colitis (Fig. 7C, right), and colonic explant cultures produced increased IL-12p40 (Fig. 7D, right) at 14 days, compared to similarly transitioned GF-CNV WT mice.

DISCUSSION

Here we report that NFIL3 deficiency, akin to IL-10 deficiency (17), results in the development of spontaneous colitis in mice. Immunohistochemistry showed that F4/80$^+$ macrophages are predominant amongst the infiltrating inflammatory cells in the colons of inflamed *Nfil3*$^{-/-}$ mice. Colonic inflammation in *Nfil3*$^{-/-}$ mice correlated with increased colonic expression of innate derived cytokines TNF and IL-12p40, both central to human IBD pathogenesis. Moreover, colonic CD11b$^+$ cells, comprising mainly macrophages, are the major source of increased IL-12p40 in *Nfil3*$^{-/-}$ mice. Increased expression of IFN-γ and IL-17 in *Nfil3*$^{-/-}$ mice is consistent with the hypothesis that NFIL3 deficiency leads to a pathogenic inflammatory Th1 and Th17 immune response. This inflammatory T cell phenotype is driven by innate interactions with the enteric microbiota, as *Nfil3*$^{-/-}$ mice crossed with *Il12b*$^{-/-}$ mice do not develop colitis. Two recent papers reported that NFIL3 regulates Th2 cytokine production in T cells but does not affect Th1 and Th17 cytokines (26, 27), providing further evidence that the lack of NFIL3 in T cells does not directly explain the increased Th1/Th17 response in *Nfil3*$^{-/-}$ mice.

The enteric microbiota and IL-10 are co-factors for NFIL3 expression in the intestine (29, 30). Indeed, we found colonic CD11b$^+$ LPMC isolated from *Nfil3*$^{-/-}$ mice produced higher amounts of *Il12b* mRNA than WT CD11b$^+$ colonic macrophages. In human IBD, intestinal CD14$^+$ macrophages are the main producers of pro-inflammatory cytokines including IL-12 family members. We previously demonstrated that *NFIL3* expression was lower in CD14$^+$ LPMC isolated from patients with CD and UC compared with non-inflammatory controls undergoing surgical resection (30), and intestinal *NFIL3* expression inversely correlated with higher amounts IL-12p40 in mice and humans (30). Increased colonic expression of IL-10 was detected in *Nfil3*$^{-/-}$ mice, suggesting that even in the presence of abundant IL-10, the loss of the downstream NFIL3 pathway cannot be overcome by increased local amounts of IL-10. This notion is consistent with our data showing that IL-10 and NFIL3 mediate cooperative but independent functions in macrophages and *in vivo*; IL-10 potently inhibited *Il12b* to the same extent in WT, *Nfil3*$^{-/-}$, and *Nfil3*$^{-/-}$ × *Il10*$^{-/-}$ macrophages arguing that IL-10-dependent, NFIL3-independent pathways can regulate IL-12p40 production. Furthermore, *Nfil3*$^{-/-}$ × *Il10*$^{-/-}$ mice developed severe colitis at an early age, demonstrating non-overlapping homeostatic functions of NFIL3 and IL-10. However, the NFIL3-independent pathways are not sufficient to prevent colitis in mice able to express IL-10.

Although T cells were necessary for colitis development, the critical role of innate immune contributions in directing the inflammatory T cell response was conclusively demonstrated through several lines of experimental evidence. First, *Nfil3*$^{-/-}$ × *Il12b*$^{-/-}$ mice were protected from colitis. Moreover, *Nfil3*$^{-/-}$ × *Rag1*$^{-/-}$ mice developed worse colitis than *Rag1*$^{-/-}$ mice when adoptively transferred with unfractionated WT CD4$^+$ T cells. NFIL3

was shown to be essential for NK cell development; therefore, $Nfil3^{-/-}$ mice lack peripheral NK1.1$^+$ NK cells (23, 24). Recently, Lang *et al*., demonstrated that the presence of NK cells limited T cell expansion *in vivo*, and $Nfil3^{-/-}$ mice failed to regulate the expansion of IFNγ-producing CD8$^+$ cells (44). Thus it is possible that systemic NK cell deficiency leads to an insufficient regulation of T cell responses in $Nfil3^{-/-}$ mice. We demonstrated that colonic lamina propria from $Nfil3^{-/-}$ mice show a severe paucity of both RORγt$^+$ and RORγt$^-$ NKp46$^+$ mucosal NK cells, and it was recently demonstrated that $Nfil3^{-/-}$ mice lack intraepithelial NKp46$^+$ ILC1 in the small intestine (45). Therefore, it is also possible that a local mucosal NK cell deficiency plays a role in the pathogenesis of $Nfil3^{-/-}$ colitis that requires further analysis. Interestingly, amongst multiple colonic cytokines we examined in this study, IL-22 was the only cytokine that was not increased. IL-22 is largely produced by RORγt$^+$NKp46$^+$ cells in the gut (NK22 cells) and is implicated in intestinal immune homeostasis (39, 46). Therefore, lack of IL-22 induction may reflect the inability of $Nfil3^{-/-}$ mice to properly respond to and resolve inflammation through IL-22. Further study is needed to unravel the role of mucosal NK cells and IL-22 in the development of spontaneous colitis in $Nfil3^{-/-}$ mice. Taken together, our study suggests that lack of NFIL3 in non-lymphocyte populations is essential for the development of colitis in $Nfil3^{-/-}$ mice through subsequent Th1/Th17-biased acquired immune responses. Macrophages and mucosal NK cells may play key roles in this process, and an imbalance in IL-12p40 and IL-22 may be factors bridging innate immune dysfunction and T cell polarization described in this study.

Colitis in $Nfil3^{-/-}$ mice is dependent on the presence and composition of the enteric microbiota. $Nfil3^{-/-}$ mice demonstrated incomplete penetrance of colitis in the UNC facility, and there was a marked difference in penetrance and severity of disease between institutions maintaining the same strain of $Nfil3^{-/-}$ mice (University of Iowa) (28) or at St. Jude Children's Research Hospital where a different $Nfil3^{-/-}$ mouse is maintained (24, 31). Importantly, the four described NFIL3-deficient mice (23, 24, 27, 28) have the same phenotypes, arguing that the microbiota at a given location is a key non-genetic factor in determining whether colitis develops. Many of the currently available murine models of IBDs also exhibit incomplete penetrance and variable severity of disease (47). Hence, compositional differences between enteric microbiota are the most plausible environmental determinants for the development of colitis in different models at different sites. Importantly, our GF $Nfil3^{-/-}$ mice do not manifest colitis at 6 months of age, and colonic explant cultures produce low levels of IL-12p40, conclusively demonstrating a role for the microbiota in colitis pathogenesis in this model. Non-*H. hepaticus* (*H. hepaticus* is routinely screened by all facilities) *Helicobacter* spp. were ubiquitously present in the UNC and Iowa colonies, leading to the conclusion that other components of the intestinal microbiota are necessary for colitis development, although interactions of distinct enteric microbes with *Helicobacter* spp. leading to colitis are possible. Moreover, components of the enteric microbiota that colonize $Nfil3^{-/-}$ (and by inference WT) mice at the UNC facility not present in the Iowa facility are colitogenic in $Nfil3^{-/-}$ mice. Further studies to characterize the enteric microbial communities in WT and in $Nfil3^{-/-}$ mice at different facilities with different colitis penetrance are likely to provide important insights into gene-environment relationships relevant to human IBD pathogenesis.

Absence of colitis development in other institutions also raised a critical question for the essential role for *Nfil3* in the development of colitis. Therefore, we also generated *Nfil3/Il10* DKO in St. Jude Children's Research Hospital. The DKO demonstrate more severe and more consistent colitis than the $Il10^{-/-}$ counterparts as we saw at UNC, despite the absence of clinically apparent colitis in *Nfil3* singles (data not shown). The DKOs also needed to be euthanized at an early age due to severity of disease with very high prevalence of rectal prolapse. This clear contrast of penetrance between *Il10/Nfil3* DKO (complete penetrance)

and singles (incomplete) across the different institutions elegantly shows the compensating/ interacting roles of IL-10 and NFIL3, and provides a model for elucidating multi-hit mechanisms by multiple IBD susceptibility genes.

Several genes in pathways regulating the expression and/or function of the cytokines IL-10, IL-12 and IL-23 contribute to IBD susceptibility (*IL10, IL23R, IL12B, IL12RB1, STAT3, JAK2, TYK2, HCK*) (10, 18, 32). Work from our group and others have shown that other IBD susceptibility genes are functionally embedded in these pathways (*IFNG, IRF1, REL, CEBPB*) (32, 48–51). Moreover, the *NFIL3* risk polymorphism is relatively common, present in 70% of IBD cases; thus, approximately 50% of cases are homozygous for the risk allele and 10% homozygous for the protective allele. Hence, we speculate that NFIL3 may be one gene of many that affects *IL12B* expression in human IBD, with polymorphisms leading to an incremental increase in IBD susceptibility. Moreover, there may be rare genetic variants of *NFIL3*, akin to recently described IL-10 and IL-10 receptor polymorphisms, which underlie a severe clinical phenotype such as very early onset IBD (19).

Collectively, our results show that excessive IL-12p40 production in *Nfil3*$^{-/-}$ colonic macrophages in response to the enteric microbiota is a key trigger that leads to an exaggerated intestinal Th1/Th17 response, resulting in colitis development. As *NFIL3* is a human IBD susceptibility gene, the *Nfil3*$^{-/-}$ mouse is a new model to understand how a genetic defect in a transcriptional repressor in innate immune cells leads to dysregulated T cell inflammatory responses to the enteric microbiota. Furthermore, NFIL3 may be a useful therapeutic target for human IBD given its central homeostatic functions in the intestines.

Acknowledgments

ABBREVIATIONS

BMDMs	bone marrow derived macrophages
DC	dendritic cells
DKO	double knockout
Foxp3	forkhead box P3
IBD	inflammatory bowel diseases
LPMCs	lamina propria mononuclear cells
LTi	lymphoid tissue inducer
NFIL3	nuclear factor, IL-3 regulated
RORγt	retinoid-acid receptor-related orphan receptor gamma t
WT	wild-type

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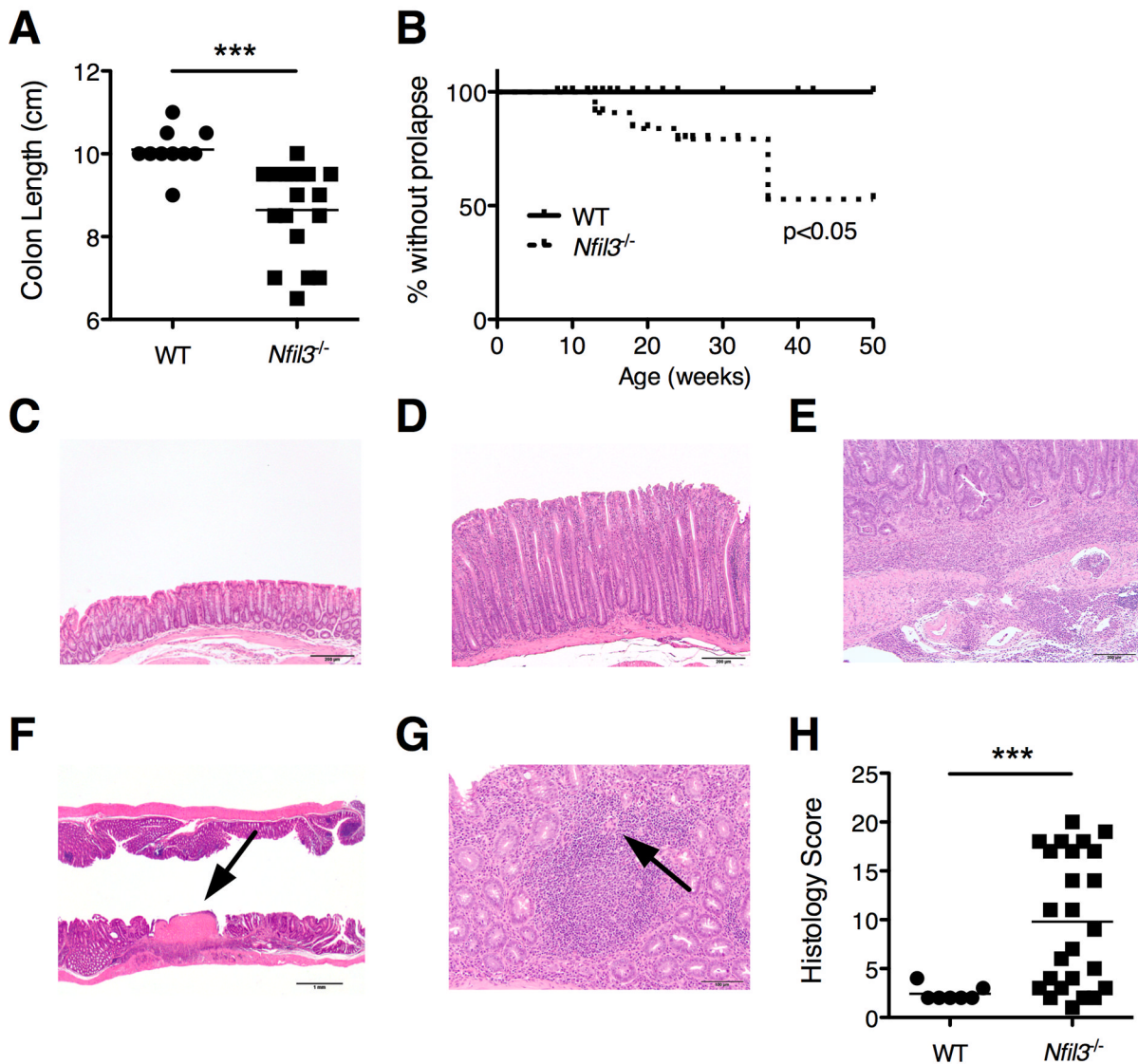
Figure 1. *Nfil3*−/− mice develop spontaneous colitis
(A) Colon length of 12–18 week-old *Nfil3*−/− (*n* = 14) and age-matched WT (*n* = 8) mice was measured. *, p<0.05 versus WT. **(B)** Prevalence of rectal prolapse was plotted (WT, *n* = 25; *Nfil3*−/−, *n* = 41). p<0.05 versus WT. **(C)** Histological severity (WT, *n* = 4; *Nfil3*−/−, *n* = 14) was scored based on Berg's score. **, p<0.005 versus WT. **(D, E)** Colonic epithelium, H&E (20× magnification). Photomicrographs of colonic mucosa taken at the same magnification compared to WT mouse colons **(D)** demonstrate significant mucosal hypertrophy with crypt elongation and decreased goblet cells in the *Nfil3*−/− **(E)** mouse colons. **(F)** Colon, H&E (40× magnification). Inflammation in *Nfil3*−/− colons was characterized by multifocal aggregates of inflammatory cells that extended transmurally to the submucosa **(G)** Colon, H&E (4× magnification). Areas of ulceration **(arrow)** were also occasionally observed. **(H)** Colon, H&E (40× magnification). In *Nfil3*−/− the inflammation in the lamina propria was occasionally organized into *de novo* lymphoid follicles and occasional multinucleated giant cells **(arrow)** were observed.
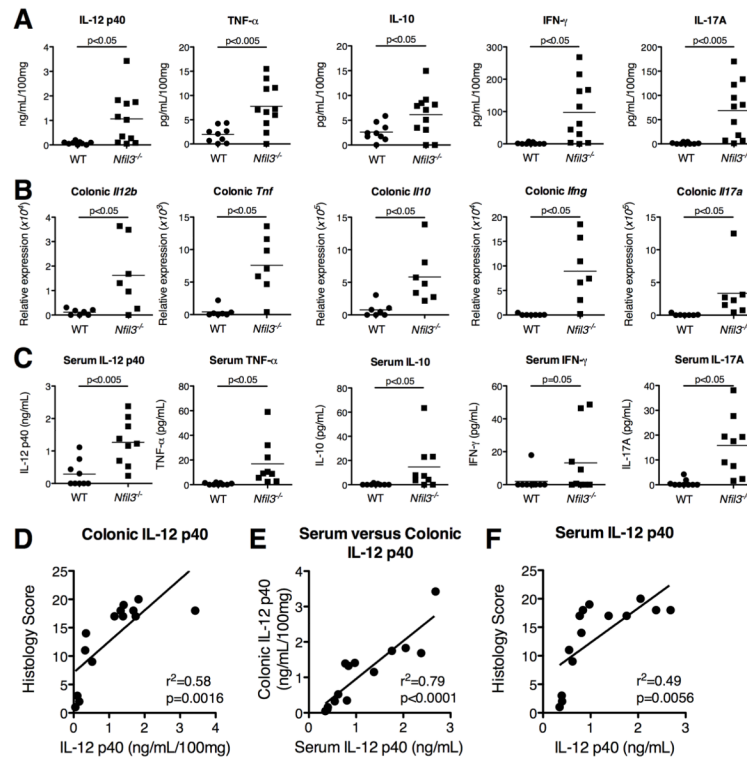
Figure 2. Characterization of cytokine profile in the colon and serum in *Nfil3*⁻/⁻ mice
(**A**) Colonic explants (WT, *n* = 9; *Nfil3*⁻/⁻, *n* = 11) were assayed for spontaneous secretion of cytokines. Colons were isolated from WT and *Nfil3*⁻/⁻ mice, cleaned, and processed for intestinal tissue explant cultures as described in Experimental Procedures. Tissue fragments (approximately 0.05 g dry weight) were incubated in 1 ml of RPMI 1640 supplemented with 1% antibiotics and 10% FBS. Supernatants were collected after 24 h and spontaneous secretion of cytokines was measured by Multiplex. Values were normalized to dry weight of the colonic explants (pg/ml/100 mg). (**B**) Colonic mRNA from WT (*n* = 7) and *Nfil3*⁻/⁻ (*n* = 7) mice was extracted and expression of *Il12b*, *Tnf*, *Ifng*, *Il17* and *Il10* mRNA was assessed by real-time RT-PCR. Results are expressed as relative expression normalized to β-actin. (**C**) Serum cytokine concentration in WT (*n* = 9) and *Nfil3*⁻/⁻ (*n* = 9) mice was measured by Multiplex. (**D**) Correlation of serum and colonic tissue explant IL-12p40 in 12–18 week old *Nfil3*⁻/⁻ mice (*n* = 11). R^2=0.8883, p<0.0001. (**E**) Correlation of histological score and colonic tissue explant IL-12p40 in 12–18 week old *Nfil3*⁻/⁻ mice (*n* = 11). R^2=0.625, p<0.005. (**F**) Correlation of serum IL-12p40 and histological score in 12–18 week old *Nfil3*⁻/⁻ mice (*n* = 11). R^2=0.7914, p<0.0005.
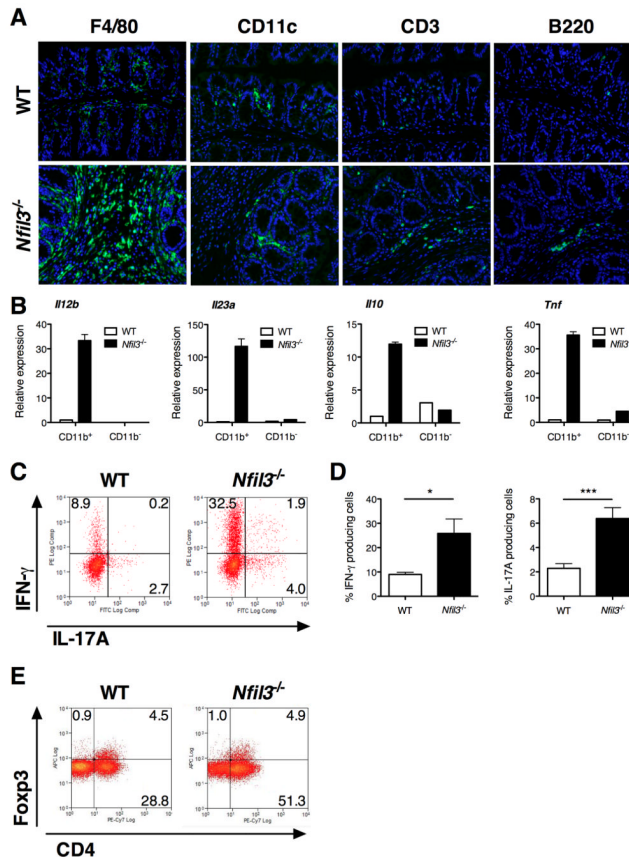
Figure 3. Characterization of increased inflammatory cell infiltration in the colon of *Nfil3*−/− mice

(**A**) Fluorescence immunohistochemistry in colonic tissues from WT and *Nfil3*−/− mice was performed for F4/80, CD11c, CD3, and B220 (Green). Specimens were counterstained with DAPI (Blue). (**B**) Expression relative to β-actin of *Il12b*, *Il23a*, *Tnf* and *Il10* transcripts in CD11b+ and CD11b− LPMC from WT and *Nfil3*−/− mice. Results represent 3 independent experiments. (**C**) LPMCs were isolated from WT and *Nfil3*−/− colons and stimulated with phorbol myristate acetate (100 ng/ml) and ionomycin (1 mg/ml) in the presence of monensin for 4 hours. Representative patterns for intracellular IFN-g and IL-17 in gated CD3+CD4+ lymphocytes from 3 independent experiments are shown. (**D**) Quantification of IFN-γ- and IL-17A-positive cells in CD3+CD4+ lymphocytes from WT and *Nfil3*−/− LPMCs. Data are presented as percentage of IFN-γ- or IL-17A-positive cells in gated CD3+CD4+ lymphocytes (mean ± SEM from 3 independent experiments). *, p<0.05; ***, p<0.0005 versus WT. (**E**) Representative patterns for intracellular FoxP3 staining in gated CD3+CD4+ lymphocytes from 3 independent experiments are shown.
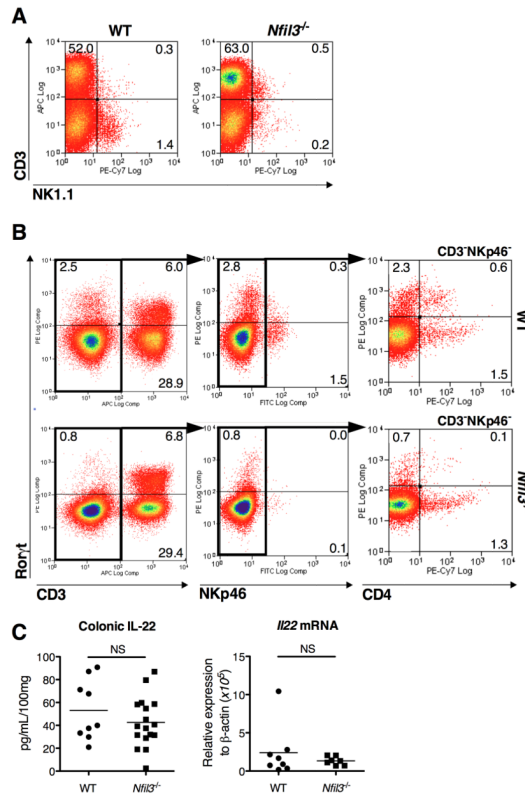
Figure 4. *Nfil3*$^{-/-}$ mice show decreased mucosal NK cells and LTi subpopulation
(**A**) CD4$^+$ Colonic LPMCs were isolated and analyzed for CD3 and NK1.1. Lymphocytes were gated by forward and side scatter and CD45 expression. (**B**) Colonic LPMCs were isolated and analyzed for CD3, NKp46 and CD4 in combination with intracellular RORγt by flow cytometry. Lymphocytes were gated by forward- and side-scatter and further gated in CD3-negative cells (middle), CD3-negative NKp46-negative cells (right). (**C**) Colonic explant cultures were analyzed for IL-22 expression by ELISA (WT, *n* = 6; *Nfil3*$^{-/-}$, *n* = 7) and colonic tissue was analyzed for *Il22* expression by RT-PCR (WT, *n* = 6; *Nfil3*$^{-/-}$, *n* = 7). NS, not significant versus WT.
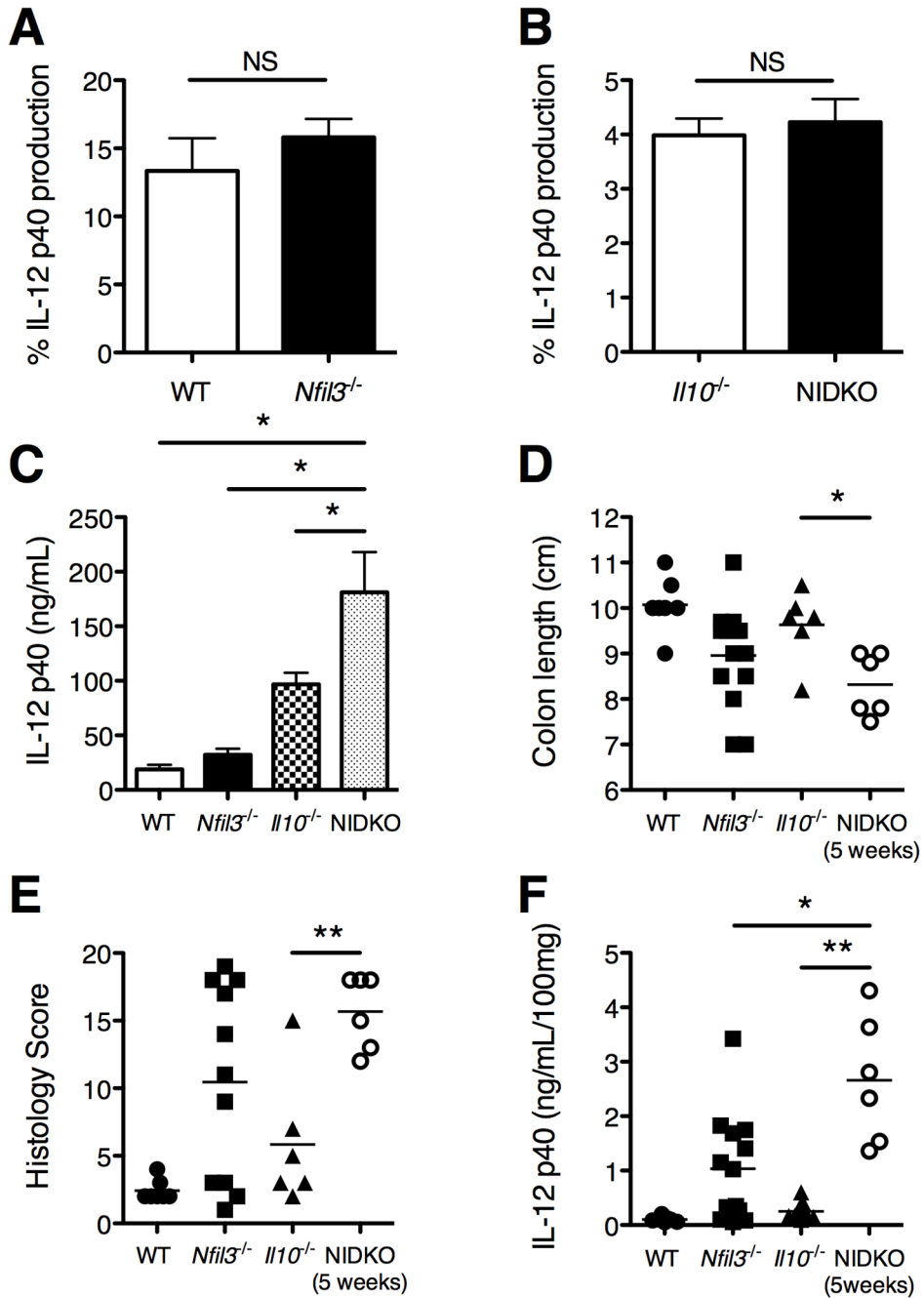
Figure 5. IL-10 and NFIL3 independently regulate IL-12p40 in BMDMs and *in vivo*

(**A**) WT and *Nfil3⁻/⁻* BMDMs were stimulated with LPS (10 ng/ml) in the presence or absence of IL-10 (1 ng/ml). Supernatants were harvested 24 hours post-LPS stimulation and analyzed for IL-12p40 by ELISA. Results are presented as percent IL-12p40 by LPS in the presence of IL-10 relative to LPS alone (mean ± SEM from 4 independent experiments). NS, not significant versus WT. (**B**) WT, *Nfil3⁻/⁻*, *Il10⁻/⁻*, and *Nfil3/Il10⁻/⁻* double knockout (NIDKO) BMDMs were stimulated with LPS (10 ng/ml) for 24 hours and IL-12p40 in the supernatants were analyzed by ELISA. Results are presented as mean ± SEM from at least 4 independent experiments. *, p<0.05 versus NIDKO. (**C**) *Il10⁻/⁻* and NIDKO BMDMs were

stimulated with LPS (10 ng/ml) in the presence or absence of IL-10 (1 ng/ml) for 24 hours and analyzed for IL-12p40 by ELISA. Results are presented as percent IL-12p40 by LPS in the presence of IL-10 relative to LPS alone (mean ± SEM from 4 independent experiments). NS, not significant versus $Il10^{-/-}$. **(D)** Quantitative histological scores from WT ($n = 4$), $Nfil3^{-/-}$ ($n = 14$), $Il10^{-/-}$ ($n = 6$) and NIDKO ($n = 6$) mice were given. **, p<0.005 versus NIDKO. **(E)** Comparison of spontaneous secretion of IL-12p40 by colonic explants from 12–18 week old WT ($n = 7$), $Nfil3^{-/-}$ ($n = 13$), Il10$^{-/-}$ ($n = 6$) and 5-week-old NIDKO ($n = 6$) mice. Values were normalized to dry weight of the colonic explants (ng/ml/100 mg). *, p<0.05; **, p<0.005 versus NIDKO. **(F)** Colon lengths from WT ($n = 8$), $Nfil3^{-/-}$ ($n = 14$), $Il10^{-/-}$ ($n = 6$) and NIDKO ($n = 6$) mice were measured. *, p<0.05 versus NIDKO.
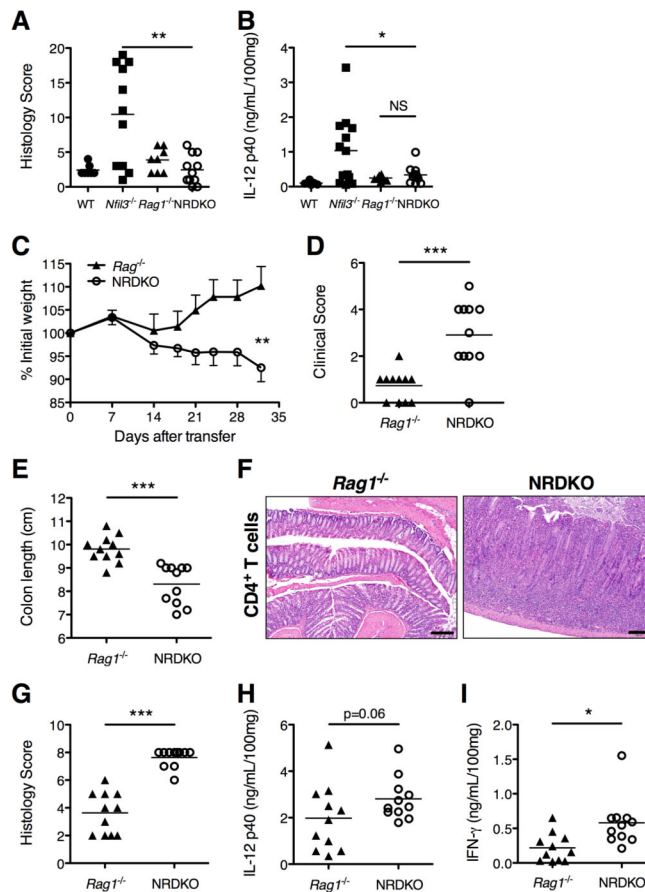
Figure 6. Lymphocytes are necessary for the development of colitis in *Nfil3*$^{-/-}$ mice
(A) *Nfil3/Rag1* double knockout (NRDKO) mice were generated and histological scores were assigned at 16 weeks of age (WT, *n* = 4; *Nfil3*$^{-/-}$, *n* = 14; *Rag1*$^{-/-}$, *n* = 8; NRDKO, *n* = 11). **, p<0.005 versus NRDKO. **(B)** Comparison of spontaneous secretion of IL-12p40 by colonic explants from WT (*n* = 7), *Nfil3*$^{-/-}$ (*n* = 13), *Rag1*$^{-/-}$ (*n* = 8) and NRDKO (*n* = 11) mice. *, p<0.05; NS, not significant versus NRDKO. **(C–I)** *Rag1*$^{-/-}$ (*n* = 7) and NRDKO (*n* = 6) mice were given 1×10^6 unfractionated CD4$^+$ T cells by i.p. injection and development of colitis was monitored over 4 weeks. **(C)** Body weight expressed as a percentage of the total body weight on Day 0 of the T cell transfer was measured. ***, p<0.0005 versus *Rag1*$^{-/-}$. **(D)** Clinical scores were measured at time of sacrifice as described in Experimental Procedures. ***, p<0.0005 versus *Rag1*$^{-/-}$. **(E)** Colon lengths were measured. ***, p<0.0005 versus *Rag1*$^{-/-}$. **(F)** Colon, H&E (10× magnification). Representative photomicrographs from *Rag1*$^{-/-}$ and NRDKO mice given unfractionated CD4$^+$ T cells. **(G)** Quantitative histological scores were given. ***, p<0.0005 versus *Rag1*$^{-/-}$. **(H)** Comparison of spontaneous secretion of IL-12p40 by colonic explants from *Rag1*$^{-/-}$ (*n* = 7) and NRDKO (*n* = 6) mice given unfractionated CD4$^+$ T cells. *, p<0.05 versus *Rag1*$^{-/-}$. **(I)** Comparison of spontaneous secretion of IFN-γ by colonic explants. ***, p<0.0005 versus *Rag1*$^{-/-}$.
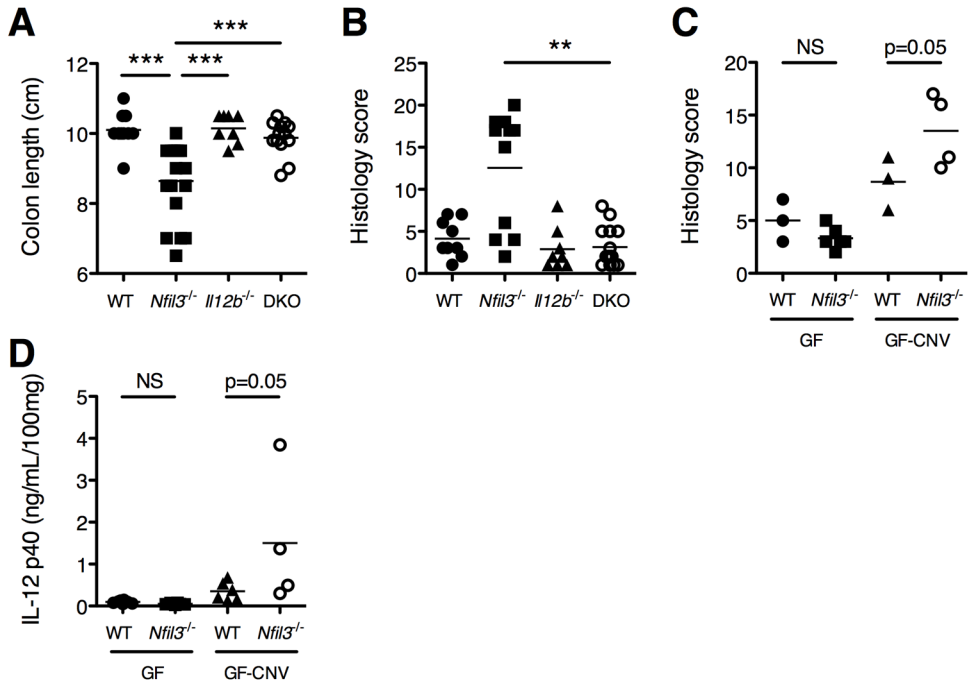
Figure 7. Development of colitis in *Nfil3*−/− mice requires the microbiota and is driven by IL-12p40

(A,B) *Nfil3/Il12b* double knockout (DKO) mice were generated and monitored for colitis development. **(A)** Colon lengths were measured at 16 weeks of age (WT, *n* = 8; *Nfil3*−/−, *n* = 14; *Il12b*−/−, *n* = 8; DKO, *n* = 13). ***, p<0.0005 versus *Nfil3*−/−. **(B)** Histological scores were assigned at 16 weeks of age (WT, *n* = 4; *Nfil3*−/−, *n* = 14; *Il12b*−/−, *n* = 8; DKO, *n* = 13). **, p<0.005 versus DKO. **(C,D)** Germ free (GF) *Nfil3*−/− were generated and monitored for colitis (WT, *n* = 12; *Nfil3*−/−, *n* = 6). Additionally, GF WT (*n* = 6) and *Nfil3*−/− (*n* = 4) were transitioned to conventional housing (GF to CNV) and monitored for colitis development for 14 days. **(C)** Histological scores were assigned. **, p<0.005 versus GF-CNV WT. **(D)** Comparison of spontaneous secretion of IL-12p40 by colonic explants. *, p<0.05 versus GF-CNV WT.