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# NFIL3 Is a Regulator of IL-12 p40 in Macrophages and Mucosal Immunity

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## Abstract

Regulation of innate inflammatory responses against the enteric microbiota is essential for the maintenance of intestinal homeostasis. Key participants in innate defenses are macrophages. In these studies, the basic leucine zipper protein, NFIL3, is identified as a regulatory transcription factor in macrophages, controlling IL-12 p40 production induced by bacterial products and the enteric microbiota. Exposure to commensal bacteria and bacterial products induced NFIL3 in cultured macrophages and in vivo. The *Il12b* promoter has a putative DNA-binding element for NFIL3. Basal and LPS-activated NFIL3 binding to this site was confirmed by chromatin immunoprecipitation. LPS-induced *Il12b* promoter activity was inhibited by NFIL3 expression and augmented by NFIL3-short hairpin RNA in an *Il12b*-bacterial artificial chromosome-GFP reporter macrophage line. *Il12b* inhibition by NFIL3 does not require IL-10 expression, but a C-terminal minimal repression domain is necessary. Furthermore, colonic $CD11b^+$ lamina propria mononuclear cells from $Nfil3^{-/-}$ mice spontaneously expressed *Il12b* mRNA. Importantly, lower expression of *NFIL3* was observed in $CD14^+$ lamina propria mononuclear cells from Crohn's disease and ulcerative colitis patients compared with control subjects. Likewise, no induction of *Nfil3* was observed in colons of colitis-prone $Il10^{-/-}$ mice transitioned from germ-free to a conventional microbiota. In conclusion, these experiments characterize NFIL3 as an *Il12b* transcriptional inhibitor. Interactions of macrophages with the enteric microbiota induce NFIL3 to



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limit their inflammatory capacity. Furthermore, altered intestinal NFIL3 expression may have implications for the pathogenesis of experimental and human inflammatory bowel diseases.

The gastrointestinal tract represents a complex interface between the enteric microbiota and immune cell populations. A multitude of microorganisms reside in the intestinal lumen separated from the body's largest reservoir of macrophages by a single epithelial cell layer. Macrophages typically produce inflammatory cytokines in response to microbial Ags through pathogen recognition receptors such as TLRs. However, to prevent excessive inflammatory responses to commensal microbes, intestinal macrophages have acquired a unique phenotype, and unlike their systemic counterparts, they do not produce inflammatory cytokines in response to enteric bacteria (1). Consequently, loss of macrophage tolerance to the enteric microbiota is an important and proximal event in the pathogenesis of the inflammatory bowel diseases (IBDs) (2).

IL-12 family members play a central role in experimental models of intestinal inflammation (3, 4) and human IBD (5, 6). IL-12 and IL-23 are heterodimeric cytokines composed of a common p40 subunit in addition to a p35 and p19 subunit, respectively (7). The common subunit IL-12 p40 (*Il12b*) is expressed in macrophages and is highly induced by microbial stimuli such as LPS (8). Therefore, factors that regulate *Il12b* expression in macrophages are likely to be central determinants of intestinal homeostasis and inflammation.

NFIL3 (also called as E4BP4), a mammalian basic leucine zipper transcription factor, was first identified through its ability to bind and repress viral promoter sequences (9). NFIL3 is constitutively expressed in a variety of tissues, including spleen, prostate, testis, ovary, heart, brain, lung, liver, skeletal muscle, and intestine (9). Recently, NFIL3-deficient ($Nfil3^{-/-}$) mice have been independently developed by three groups. Two groups reported defects in NK cell development, with absence of NK cells in $Nfil3^{-/-}$ mice (10, 11). Kashiwada et al. (12) then described that NFIL3 was essential for IgE class switching in B cells. Although these initial reports did not note phenotypic abnormalities in macrophages, they by no means exclude important functional defects. Indeed, NFIL3 was previously identified as an LPS-inducible transcription factor in murine bone marrow-derived macrophages (BMDMs), although functional correlates were not described (13).

In this article, we identify NFIL3 as an inhibitor of *Il12b* transcription in macrophages. Our results suggest that NFIL3 is a physiologic regulator of IL-12/-23 during host-enteric commensal interactions and contributes to intestinal homeostasis.

## Materials and Methods

### Mice

Wild-type (WT) and $Myd88^{-/-}$ mice (C57BL/6 background) in specific pathogen-free (SPF) conditions were used for BMDM derivation. $Nfil3^{-/-}$ mice were generated by gene-targeting strategies, as reported previously (12), and bred with C57BL/6 mice for at least 10 generations. WT and $Il10^{-/-}$ mice (129/SvEv background) were used for all germ-free (GF) transition experiments. GF mice were maintained in the Gnotobiotic Core at the University of North Carolina. Mice housed in GF conditions were transitioned to an SPF environment at 6 wk of age, as previously described (14). 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 and the University of Iowa Carver College of Medicine.

### Reagents

LPS from *Salmonella enteritidis* (Sigma, St. Louis, MO) was repurified by modified phenol extraction as previously described (15). M-CSF was obtained from PeproTech (Rocky Hill, NJ). Blocking rat IgG2a anti–IL-10 mAb and an isotype control were purchased from eBioscience (San Diego, CA).

### Il12b-GFP bacterial artificial chromosome transformed macrophages

Transgenic mice were generated containing a 200-kb *Il12b* bacterial artificial chromosome (BAC) transgene (generated by S.T.S.). Before micro-injection, BAC recombineering in *Escherichia coli* introduced a GFP cDNA into the *Il12b* transcription unit. Next, a transformed macrophage line from the *Il12b*-GFP BAC transgenic mice was created by infection of BMDMs with the murine oncogenic J2 retrovirus (16). Clonal lines were screened by flow cytometry to identify lines that support efficient induction of GFP fluorescence in response to LPS stimulation.

### Cell isolation

BMDMs were cultured as described previously (17). Mouse colonic lamina propria mononuclear cells (LPMCs) were isolated by an enzymatic method and separated into $CD11b^+$ cells using anti-CD11b microbeads (Miltenyi Biotec, Auburn, CA) (1). Human $CD14^+$ LPMCs were isolated from surgically resected colons or ilea from patients with IBD (Crohn's disease, $n = 8$; ulcerative colitis, $n = 8$) or control patients ($n = 8$) as described previously (5). Diagnoses of IBD were based on the clinical, radiographic, endoscopic, and histological findings according to established criteria. The degree of inflammation was histologically moderate to severe in all Crohn's disease and ulcerative colitis patients. Control colonic resections were obtained from unaffected areas from patients with colon cancer. Experiments were approved by the Institutional Review Board of Keio University School of Medicine and Yokohama Municipal Hospital, Japan. Written informed consent was obtained from all patients.

### Quantitative RT-PCR

Quantitative real-time RT-PCR was performed as described previously (18). To evaluate the expression of NFIL3-regulated genes, we used an RT2 Profiler PCR array kit (PAMM-073E; SA Biosciences), which profiles inflammatory response cytokines and chemokines. *NFIL3* expression in human $CD14^+$ cells was determined using TaqMan FAM-labeled probe for *NFIL3* obtained from Assays on Demand (Applied Biosystems).

### Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) was performed with ChIP-IT Express kit (Active Motif, Carlsbad, CA) (19). Sample DNAs were also analyzed on 2% agarose gels. Ab for NFIL3 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Primers for NFIL3 binding to the *Il12b* promoter were: forward 5′-TTGCCGCCTCTATTCACCTTAG-3′, reverse 5′-GGAAATGACTTCGGGGTGTG-3′.

### Lentivirus-mediated gene transduction

Lentiviral transduction was optimized for the FuGENE Transfection Reagent (Roche, Indianapolis, IN) in HEK293T cells. Virus was collected at 40 and 60 h after transduction and concentrated with PEG-it virus precipitation solution (System Biosciences, Mountain View, CA). Two hundred micro-liters virus was added to $6 \times 10^5$ BMDMs from WT or $Nfil3^{-/-}$ mice in 12-well plates. Transduced cells were selected by puromycin, and transduction efficiency was confirmed by Western blot, RT-PCR, or both.

### ELISA

IL-12 p40 and IL-10 concentrations were determined by sandwich ELISA (BD Biosciences, San Jose, CA).

### Western immunoblots

Western blot analyses were performed on whole-cell extracts as described previously (17). Anti-NFIL3 Ab was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

### Statistical analysis

Statistical significance for data subsets was assessed by the two-tailed Student *t* test unless specifically described.

## Results

### NFIL3 is induced in macrophages through a MyD88-dependent pathway

Basal expression of *Nfil3* mRNA and protein is detected in murine BMDMs (Fig. 1*A*, 1*B*). Consistent with a prior study (13), LPS induced *Nfil3* mRNA (Fig. 1*A*) and protein (Fig. 1*B*) expression in WT BMDMs. LPS and CpG DNA induced *Nfil3* expression through an MyD88-dependent pathway as induction was abrogated in *Myd88*$^{-/-}$ BMDMs (Fig. 1*A*, Supplemental Fig. 1*A*). *Il10*$^{-/-}$ mice develop spontaneous chronic enterocolitis dependent on the presence of the enteric microbiota (20). Interestingly, as reported previously (13), although basal *Nfil3* expression is detected, *Nfil3* is not induced in LPS-activated *Il10*$^{-/-}$ BMDMs (Fig. 1*C*). This finding suggested that IL-10 is a cofactor for *Nfil3* induction by LPS. Indeed, LPS-induced *Nfil3* expression is abrogated in WT BMDMs in the presence of anti–IL-10 Abs (Fig. 1*D*). Moreover, these results indicate that NFIL3 may play a role in the maintenance of homeostasis to the enteric microbiota. To substantiate this hypothesis, we characterized colonic *Nfil3* expression before and after transition of WT mice from a GF environment to an SPF microbiota. Colonic *Nfil3* expression was significantly induced after colonization, suggesting that NFIL3 participates in physiological host–enteric microbial interactions in vivo (Fig. 1*E*).

### Nfil3 is a regulator of Il12b expression

To determine NFIL3 function in macrophages, we compared gene expression profiles in LPS-activated *Nfil3*$^{-/-}$ and WT BMDMs by PCR array. Among 84 genes interrogated, *Il12b* demonstrated the largest fold increase in *Nfil3*$^{-/-}$ BMDMs compared with WT BMDMs (Fig. 2*A*). Increased *Il12b* expression was validated by gene-specific real-time RT-PCR (Fig. 2*B*, *left*). Moreover, *Nfil3*$^{-/-}$ BMDMs demonstrated increased IL-12/23 p40 protein secretion compared with WT BMDMs (Fig. 2*B*, *right*). Expression of the IL-12 family members *Il12a* and *Il23a* was similar in LPS-activated *Nfil3*$^{-/-}$ and WT BMDMs (Fig. 2*A*, Supplemental Fig. 1*B*). However, consistent with increased *Il12b* expression, increased IL-12 p70 and IL-23 protein secretion is also observed in *Nfil3*$^{-/-}$ BMDMs (Supplemental Fig. 1*B*). There were no significant differences in cell surface phenotypic or activation marker expression on *Nfil3*$^{-/-}$ BMDMs compared with WT BMDMs (Supplemental Fig. 1*C*).

To exclude possible indirect effects of genetic deletion in *Nfil3*$^{-/-}$ BMDMs, *Nfil3* gene expression was silenced by lentivirus-mediated short hairpin RNA (shRNA) transduction of WT BMDMs (Supplemental Fig. 2*A*). LPS-activated, *Nfil3*-silenced WT BMDMs demonstrated significantly higher *Il12b* expression compared with control scrambled-shRNA–expressing BMDMs (Fig. 2*C*).

To begin to understand how NFIL3 inhibits *Il12b*, we overexpressed NFIL3 in WT BMDMs. NFIL3-expressing, lentivirus-transduced WT BMDMs demonstrated significantly less LPS-induced *Il12b* (Fig. 2*D*, *left graph*; Supplemental Fig. 2*B*) mRNA and IL-12 p40 protein (Fig. 2*D*, *right graph*) compared with control GFP lentivirus-transduced BMDMs. Two NFIL3 proteins with mutations in a minimal repression domain (9, 21) were constructed by substituting Lys-330 and Lys-332 to Glu (mutated NFIL3) and deleting the C-terminal domain from 296 and 462 (truncated NFIL3), respectively (Supplemental Fig. 2*C*). Expression levels of WT and mutant constructs after lentivirus transduction in BMDMs were similar (Supplemental Fig. 2*D*). Both NFIL3 mutant proteins did not fully suppress LPS-activated *Il12b* expression compared with intact NFIL3 in WT (Fig. 2*E*), suggesting that the inhibitory effect of NFIL3 on *Il12b* is dependent on the minimal repression domain, previously shown to be essential for NFIL3 interactions with the TATA-binding protein Dr1 (21). Moreover, NFIL3 expressed in *Nfil3*$^{-/-}$ BMDMs demonstrated inhibition of LPS-activated *Il12b* expression in a concentration-dependent manner, whereas expression of mutant proteins (mutated NFIL3 and truncated NFIL3) did not affect LPS-induced *Il12b* expression (Fig. 2*F*).

## NFIL3 inhibition of Il12b is independent of IL-10

Whether inhibition of *Il12b* by NFIL3 is mediated through IL-10 was next examined. IL-10 production was similar between WT and *Nfil3*$^{-/-}$ BMDMs, suggesting that increased IL-12/23 p40 was not due to impaired IL-10 expression (Fig. 2*A*, 3*A*). To confirm this finding, NFIL3 was overexpressed in *Il10*$^{-/-}$ BMDMs. Similar to experiments in WT BMDMs (Fig. 2*D*), NFIL3 also inhibited LPS-activated *Il12b* mRNA (Fig. 3*B*, *left graph*) and IL-12 p40 protein (Fig. 3*B*, *right graph*) in *Il10*$^{-/-}$ BMDMs. Likewise, the minimal repression domain was also required for the inhibition of *Il12b* by NFIL3 (Fig. 3*C*). Basal NFIL3 expression is detected in *Il10*$^{-/-}$ BMDMs (Fig. 1*C*). Consequently, knockdown of NFIL3 by shRNA resulted in increased LPS-induced *Il12b* in the absence of IL-10 (Fig. 3*D*). Overall, these results suggest that the inhibitory effect of NFIL3 on *Il12b* does not require IL-10.

## NFIL3 interacts with the Il12b promoter and inhibits promoter activity

Using a bioinformatics approach (http://www.cbrc.jp/research/db/TFSEARCH.html), we identified a putative consensus NFIL3 binding site in the *Il12b* promoter from −269 to −279 upstream of the transcription start site. Furthermore, this binding sequence resides in an evolutionary conserved region through multiple species including human and mouse (Supplemental Fig. 3*A*; http://ecrbrowser.dcode.org), suggesting that it is functionally important. ChIP experiments from WT BMDMs revealed basal occupancy of NFIL3 on this binding element (Fig. 4*A*). NFIL3 occupancy to this promoter region is enhanced in LPS-activated BMDMs by semiquantitative agarose gel electrophoresis with polyclonal goat IgG as a negative control (Fig. 4*A*). Moreover, as expected, no NFIL3 binding to this region is detected in ChIP experiments from *Nfil3*$^{-/-}$ BMDMs (Fig. 4*B*). To demonstrate a direct effect of NFIL3 on *Il12b* promoter activity, we used a transformed macrophage line derived from an *Il12b* BAC transgenic mouse where a GFP reporter gene was introduced into the *Il12b* transcription unit. Lentiviral overexpression of NFIL3 in LPS-activated *Il12b*-GFP macrophages resulted in diminished numbers of GFP-expressing cells (Fig. 4*C*, Supplemental Fig. 3*B*). Expression of mutant NFIL3 proteins (mutated NFIL3 and truncated NFIL3) did not alter the number of LPS-activated GFP$^{+}$ cells (Fig. 4*C*). Moreover, in LPS-activated, *Nfil3* shRNA-transduced *Il12b*-GFP macrophages, increased numbers of GFP$^{+}$ cells were observed compared with scrambled-shRNA–transduced cells (Fig. 4*D*, Supplemental Fig. 3*C*).

### Induction of NFIL3 by commensal bacteria is impaired in murine and human IBD, which correlates with increased Il12b expression

To study NFIL3 regulation of *Il12b* in mucosal innate immunity, we isolated colonic $CD11b^+$ LPMCs from WT and $Nfil3^{-/-}$ mice, and examined for *Il12b* expression. $CD11b^+$ LPMCs from $Nfil3^{-/-}$ mice show higher spontaneous *Il12b* expression than $CD11b^+$ LPMCs from WT mice (Fig. 5*A*). *Il12b* was predominantly expressed in $CD11b^+$ LPMCs in WT and $Nfil3^{-/-}$ mice, with very low expression in the $CD11b^-$ LPMC population. Next, colitis-prone $Il10^{-/-}$ mice were transitioned from GF to SPF microbiota, and *Nfil3* expression was examined in the colon before and 3 d after transition. Unlike WT mice where colonic *Nfil3* expression is significantly induced (Fig. 1*E*), in $Il10^{-/-}$ mice, colonic *Nfil3* is not induced after transition to SPF microbiota (Fig. 5*B*).

In humans with IBD, intestinal $CD14^+$ macrophages are important producers of proinflammatory cytokines including IL-12 family members (5, 22). *NFIL3* expression was significantly lower in $CD14^+$ LPMCs isolated from patients with Crohn's disease and ulcerative colitis compared with noninflammatory control subjects (Fig. 5*C*). It has previously been demonstrated in the same samples that IL-12 p40 production is increased in $CD14^+$ LPMCs from IBD patients; thus, NFIL3 expression inversely correlates with greater levels of IL-12 p40 (5, 6).

## Discussion

Macrophages contribute to the functional integrity of the intestinal mucosa in health and disease through monitoring of luminal contents, prominently the enteric microbiota. Innate responses are rapid and directed toward conserved structures on infectious agents recognized by germline-encoded pattern recognition receptors such as TLRs and Nod-like receptors (2). During physiologic conditions, the default innate immune response to the microbiota is induction of anti-inflammatory pathways to maintain homeostasis. Defects in this innate immune "tolerance" to the enteric microbiota are central events in the development of IBD. However, molecular events mediating physiologic innate homeostasis and chronic intestinal inflammation are incompletely understood. In this study, NFIL3 is identified as a regulatory transcription factor in macrophages, controlling macrophage IL-12 p40 production on interaction with the enteric microbiota. Exposure to commensal bacteria and bacterial products induced NFIL3 in cultured macrophages and in vivo. Furthermore, colonic $CD11b^+$ LPMCs from $Nfil3^{-/-}$ mice spontaneously expressed *Il12b* mRNA. This implicates an important role of microbiota-induced NFIL3 in the maintenance of intestinal homeostasis through regulation of IL-12 p40.

$Il10^{-/-}$ mice experience development of spontaneous colitis characterized by overexpression of *Il12b* in the colon (3). Interestingly, unlike WT mice, $Il10^{-/-}$ mice transitioned from GF to SPF microbiota demonstrated attenuated induction of colonic *Nfil3*. We also show that IL-10 is a cofactor for induction of *Nfil3* by bacterial products in BMDMs. Consequently, the absence of IL-10 in vivo leads to loss of homeostasis and the development of spontaneous colitis through multiple mechanisms, one of which may be attenuation of *Nfil3* induction. In fact, lower expression of *NFIL3* was also observed in $CD14^+$ LPMCs from Crohn's disease and ulcerative colitis patients. This is consistent with the recent reports indicating the importance of not directly IL-10, but IL-10–related functional pathways in both ulcerative colitis and Crohn's disease, through genetic studies (23, 24). Altered functional IL-10 pathway may be crucial for the lower expression of NFIL3 in the intestinal macrophages, resulting in higher IL-12 p40, which is essential for enhanced Th1/Th17 response in patients with IBD. Therefore, how NFIL3 is regulated in macrophages will provide insight into its function and may guide new therapeutic strategies of IBD. Our findings indicate that IL-10 is a cofactor for induction of NFIL3 expression in BMDMs and

in vivo. However, the inhibitory function of NFIL3 on *Il12b* is independent of IL-10. IL-10 is a well-characterized and biologically significant inhibitor of IL-12 p40 (25). Therefore, the identification of NFIL3 as an inhibitor of LPS-induced *Il12b* in macrophages, independent of the functional effects of IL-10, opens new avenues to understand the regulation of this central inflammatory gene. NFIL3 induction by microbial stimuli such as LPS may limit the extent and duration of *Il12b* expression during physiologic innate immune responses.

Nonetheless, precise molecular mechanisms of NFIL3-mediated *Il12b* inhibition remain to be elucidated. As it interacts with *Il12b* promoter, NFIL3 may recruit a corepressor. One candidate is the TATA-binding protein Dr1. We show that the inhibitory effect of NFIL3 on *Il12b* is dependent on amino acids 330 and 332, essential for interactions with Dr1 (21). Moreover, interactions between NFIL3 and other transcription factors may ultimately determine the complexities of *Il12b* regulation. Many reported proteins recognize similar DNA sequences, such as CREB, ATF3, and HLF (9). Indeed, ATF3 previously has been demonstrated to be a regulator of *Il12b* transcription (26). Based on the described transcriptional regulatory functions of NFIL3, we speculate that NFIL3 regulates *Il12b* through chromatin modifications. This hypothesis will need to be tested in future studies through detailed mapping and mutagenesis of NFIL3 sites within the endogenous *Il12b* locus, because of limitations of transient transfection of minimal promoter constructs to address these questions.

In humans, intestinal macrophages express low levels of cell surface innate immune receptors such as TLR4 and CD14, which contributes to their anti-inflammatory functional phenotype. However, in IBD patients, CD14-expressing macrophages are increased in number, and these cells produce proinflammatory cytokines including IL-12/23, in response to enteric bacteria (5). $CD14^+$ LPMCs have been demonstrated to directly activate the Th1/Th17 immune response in Crohn's disease (27). Our results in human intestinal $CD14^+$ LPMCs suggest that NFIL3 may be important for intestinal macrophages to acquire a hyporesponsive phenotype, and impaired NFIL3 expression may shift tolerogenic functions to proinflammatory.

Through a gene expression array, NFIL3 was identified as a fairly specific regulator of *Il12b*. However, *Il6* was another proinflammatory cytokine gene upregulated in LPS-activated $Nfil3^{-/-}$ BMDMs (Fig. 2*A*). The *Il6* promoter also contains putative binding sites for NFIL3. IL-6 is essential for the development of Th17 cells (28, 29). Therefore, NFIL3 may serve as a molecular link between innate and adaptive immunity, shaping Th1 and Th17 responses in vivo.

NFIL3 joins a growing list of factors important for the maintenance of intestinal homeostasis. It is plausible that NFIL3 has a much broader role in anti-inflammatory innate immune responses than described in this study, perhaps through gene regulation in concert with other proteins that share the same binding sequences. In conclusion, these experiments implicate NFIL3 as a microbiota-induced *Il12b* transcriptional inhibitor, involved in the maintenance of intestinal homeostasis.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations used in this article

| | |
|---|---|
| **BAC** | bacterial artificial chromosome |
| **BMDM** | bone marrow-derived macrophage |
| **ChIP** | chromatin immunoprecipitation |
| **GF** | germ-free |
| **IBD** | inflammatory bowel disease |
| **LPMC** | lamina propria mononuclear cell |
| **shRNA** | short hairpin RNA |
| **SPF** | specific pathogen-free |
| **WT** | wild-type |

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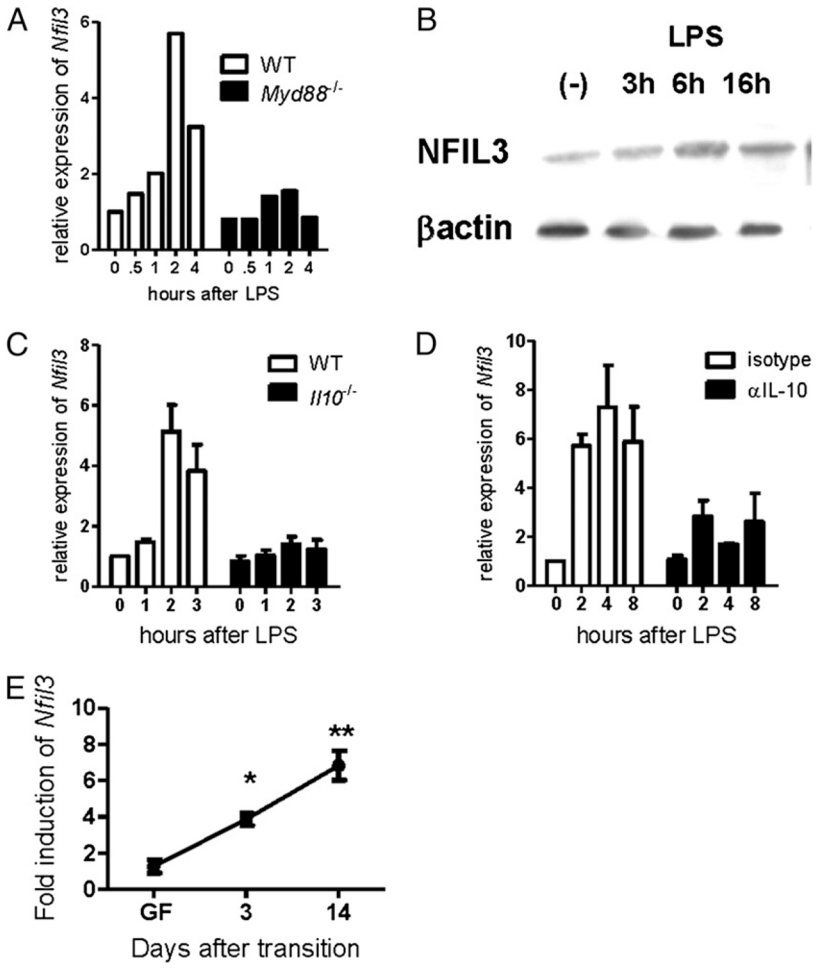


**FIGURE 1.**

*Nfil3* is induced by bacterial products in cultured BMDMs and the enteric microbiota in vivo. *A*, WT and *Myd88*$^{-/-}$ BMDMs were stimulated with LPS (10 ng/ml) for 0.5, 1, 2, and 3 h, and *Nfil3* expression was analyzed by real-time RT-PCR. Results are expressed as fold induction versus unstimulated WT BMDMs normalized to β-actin (representative from three independent experiments). *B*, WT BMDMs were analyzed for NFIL3 protein by Western immunoblot. WT BMDMs were stimulated with LPS (100 ng/ml) for 3, 6, and 16 h. Results are representative from four independent experiments. *C*, WT and *Il10*$^{-/-}$ BMDMs were stimulated with LPS for 1, 2, and 3 h, and kinetics of *Nfil3* expression was analyzed by real-time RT-PCR. Results are expressed as fold induction versus unstimulated WT BMDMs normalized to β-actin (mean ± SEM from four independent experiments). *D*, WT BMDMs were stimulated with LPS in the presence of anti–IL-10 Ab (10 μg/ml) or isotype control Ab (10 μg/ml) for 2, 4, and 8 h, and kinetics of *Nfil3* expression was analyzed by real-time RT-PCR. Results are expressed as fold induction versus unstimulated WT BMDMs normalized to β-actin (mean ± SEM from four independent experiments). *E*, GF WT mice were transitioned to an SPF enteric microbiota and colonic *Nfil3* expression was analyzed before and 3 and 14 d postcolonization. Results are expressed as fold induction versus GF colons normalized to β-actin (mean ± SEM from three independent experiments). *$p < 0.05$, **$p < 0.01$ versus GF.

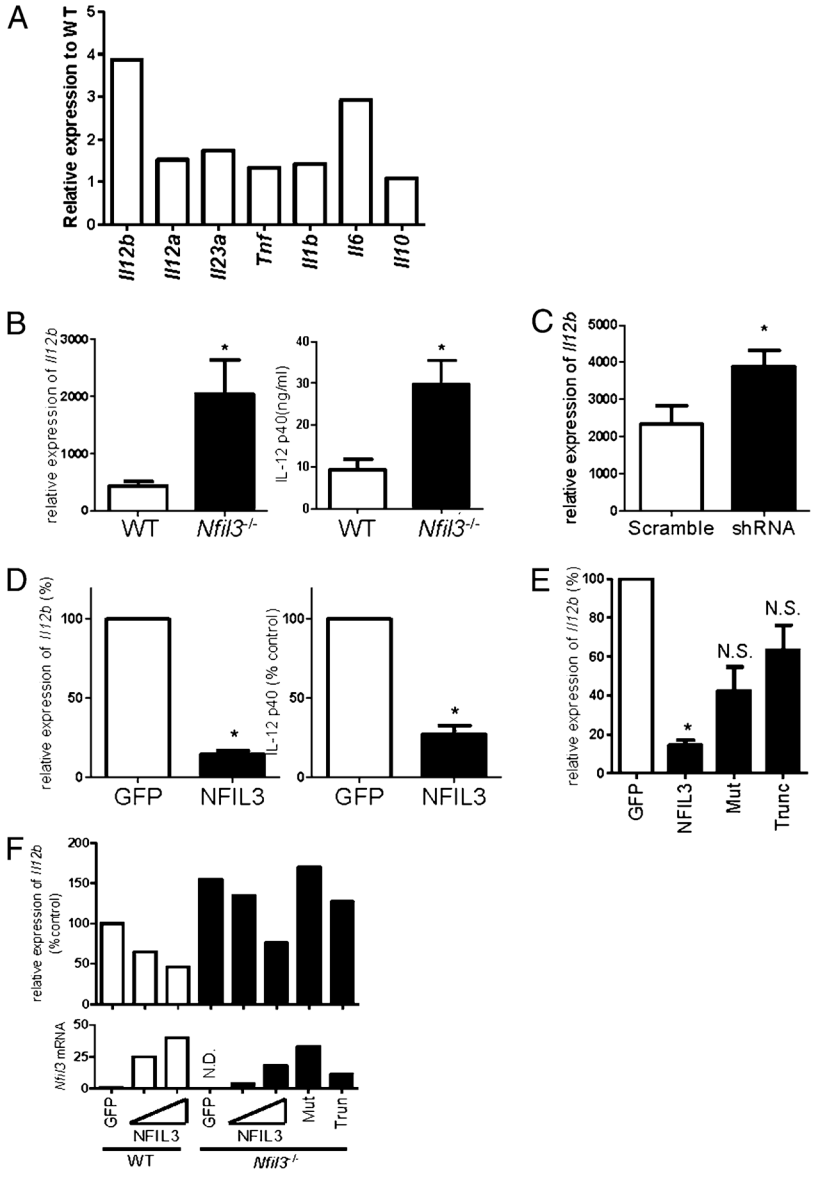


**FIGURE 2.**

*Nfil3*$^{-/-}$ macrophages demonstrate increased *Il12b* expression. *A*, WT and *Nfil3*$^{-/-}$ BMDMs were stimulated with LPS (10 ng/ml) for 4 h, and mRNA expression was quantified by PCR array (SA Biosciences). Relative mRNA expression for *Il12a*, *Il12b*, *Il23a*, *Tnf*, *Il6*, and *Il10* in *Nfil3*$^{-/-}$ BMDMs compared with WT BMDMs are shown. Results are representative from two independent experiments. *B*, WT and *Nfil3*$^{-/-}$ BMDMs were stimulated with LPS (10 ng/ml), and *Il12b* mRNA (4 h post-LPS) and IL-12 p40 protein (24 h post-LPS) were analyzed by real-time RT-PCR and ELISA. *Il12b* results are expressed as fold induction versus unstimulated WT BMDMs. All results represent mean ± SEM of four independent experiments. *$p < 0.05$ versus LPS-stimulated WT BMDMs. *C*, WT BMDMs transduced with lentiviral expressed *Nfil3* shRNA and control scrambled shRNA were stimulated with LPS for 3 h for *Il12b* expression analysis. Results are expressed as fold induction versus unstimulated BMDMs transduced with scrambled shRNA (mean ± SEM from four independent experiments). *$p < 0.05$ versus scrambled shRNA transduced BMDMs stimulated with LPS. *D*, WT BMDMs transduced with lentivirus to express NFIL3 or GFP

(control) were stimulated with LPS (10 ng/ml). *Il12b* mRNA (3 h post-LPS) and IL-12 p40 protein (24 h post-LPS) were determined. Results for mRNA are expressed as fold induction versus unstimulated GFP transduced BMDMs normalized to β-actin (mean ± SEM from three independent experiments). *$p < 0.05$ versus LPS-stimulated GFP-transduced BMDMs. *E*, WT BMDMs transduced with lentivirus that expressed WT, mutated, and truncated NFIL3 were stimulated with LPS for 4 h. See text for description of protein constructs. Results are expressed as percent induction versus LPS-stimulated GFP-transduced BMDMs normalized to β-actin (mean ± SEM from three independent experiments). *$p < 0.01$ versus LPS-stimulated GFP-expressing BMDMs were calculated. *F*, WT and *Nfil3*$^{-/-}$ BMDMs transduced with different titers of lentivirus-expressed NFIL3 constructs were stimulated with LPS for 4 h and analyzed for *Il12b* and *Nfil3* expression. *Il12b* expressions are presented as percent induction versus LPS-stimulated GFP-transduced WT BMDMs normalized to β-actin. *Nfil3* expression is presented as relative induction versus unstimulated GFP-transduced WT BMDMs normalized to β-actin.

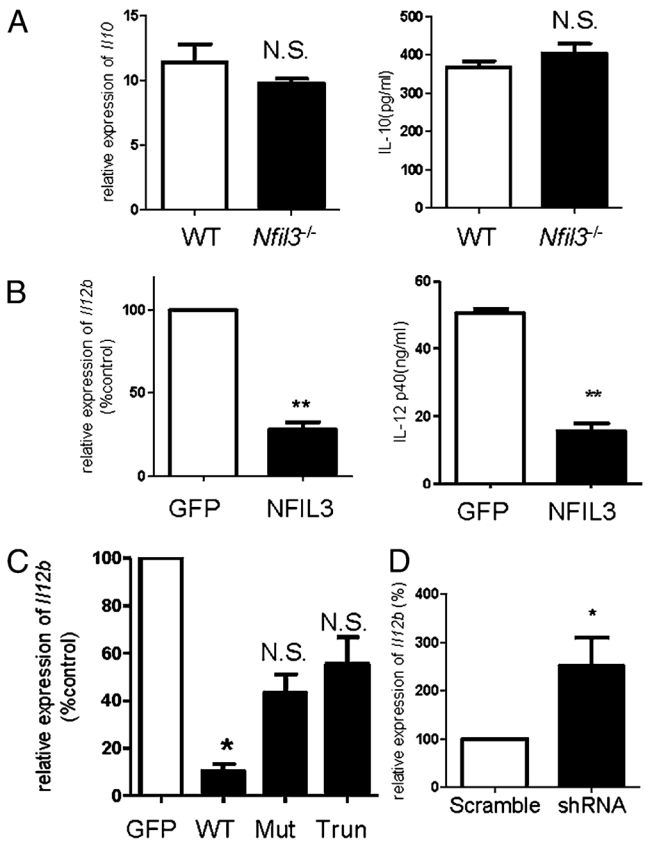


**FIGURE 3.**

NFIL3 inhibition of *Il12b* is independent of IL-10. *A*, WT and *Nfil3*$^{-/-}$ BMDMs were stimulated with LPS (10 ng/ml), and *Il10* mRNA (4 h post-LPS) and IL-10 protein (24 h post-LPS) were analyzed by real-time RT-PCR and ELISA. *Il10* results are expressed as fold induction versus unstimulated WT BMDMs. Results represent mean ± SEM of four independent experiments. *B*, *Il10*$^{-/-}$ BMDMs transduced with lentivirus to express NFIL3 or GFP were stimulated with LPS (10 ng/ml). *Il12b* mRNA (3 h post-LPS, *left panel*) and IL-12 p40 protein (24 h post-LPS, *right panel*) were determined. Results for mRNA are expressed as fold induction versus unstimulated GFP-transduced BMDMs normalized to β-actin (mean ± SEM from three independent experiments). $^{**}p < 0.01$ versus LPS-stimulated GFP-transduced BMDMs. *C*, *Il10*$^{-/-}$ BMDMs transduced with lentivirus that expressed WT, mutated (Mut), and truncated (Trunc) NFIL3 were stimulated with LPS for 4 h. Results are expressed as percent induction versus LPS-stimulated GFP-transduced BMDMs normalized to β-actin (mean ± SEM from three independent experiments). $^{*}p < 0.01$ versus LPS-stimulated GFP-expressing BMDMs. *D*, *Il10*$^{-/-}$ BMDMs transduced with lentiviral that expressed *Nfil3* shRNA and control scrambled shRNA were stimulated with LPS for 3 h for *Il12b* expression analysis. Results are expressed as fold induction versus unstimulated BMDMs transduced with scrambled shRNA (mean ± SEM from four independent experiments). $^{*}p < 0.05$ versus scrambled shRNA-transduced BMDMs stimulated with LPS by paired *t* test.

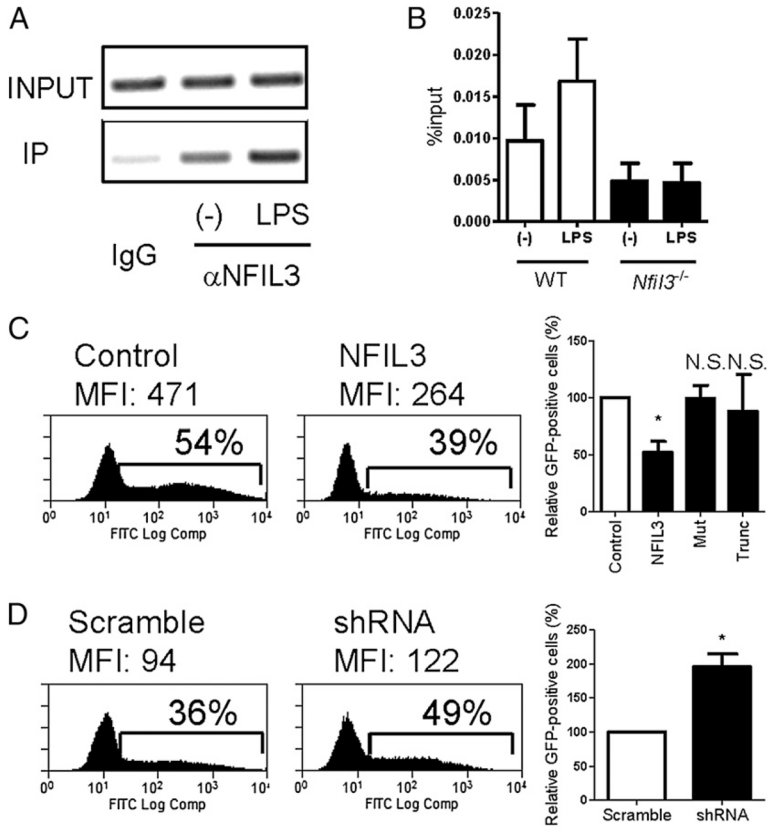


**FIGURE 4.**

NFIL3 inhibits *Il12b* through interactions with the promoter. *A*, Recruitment of NFIL3 on the *Il12b* promoter was analyzed by ChIP. Induction of NFIL3-binding activity to the *Il12b* promoter was confirmed by ChIP with PCR products visualized on a 2% agarose gel. WT BMDMs were stimulated with or without LPS (100 ng/ml) for 3 h. Polyclonal goat IgG (*first lane*) is a negative control. This result is representative of three independent chromatin preparations. *B*, In separate experiments, WT and *Nfil3*$^{-/-}$ BMDMs were stimulated with or without LPS (100 ng/ml) for 3 h. Quantitative results are presented as enrichment (percentage input DNA) of NFIL3 binding to the *Il12b* promoter and are representative of three independent chromatin preparations. *C*, *Il12b*-GFP transformed macrophages transduced with empty (control), WT, 2-aa mutated (Mut) or truncated (Trunc) NFIL3 were stimulated with 100 ng/ml LPS for 24 h and analyzed by FACS. Representative histograms with mean fluorescent intensity are given. Percentage of GFP$^{+}$ cells relative to control is also shown (mean ± SEM from three independent experiments). *$p < 0.05$ versus control. *D*, *Il12b*-GFP macrophages were transduced with scrambled or NFIL3-shRNA. Cells were stimulated with 10 ng/ml LPS for 24 h and analyzed by FACS. Representative histograms with mean fluorescent intensity are given. Data are presented as percentage of GFP$^{+}$ cells relative to scrambled shRNA-treated cells (mean ± SEM from three independent experiments). *$p < 0.05$ versus scrambled shRNA.

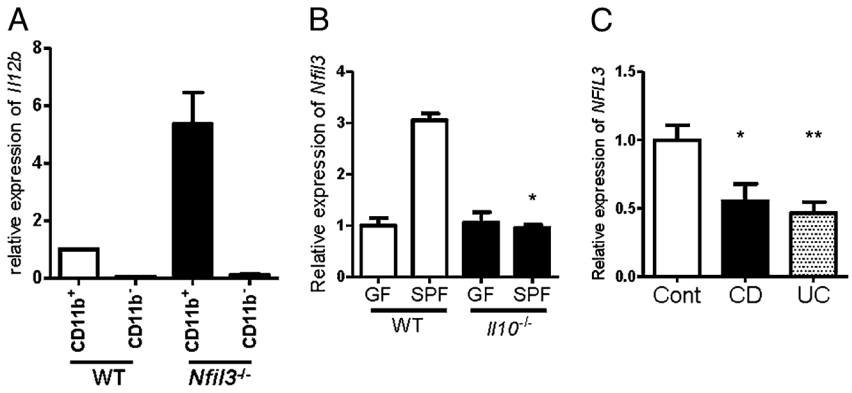


**FIGURE 5.**

Induction of NFIL3 by commensal bacteria is impaired in murine and human IBD. *A*, Colonic CD11b$^+$ and CD11b$^-$ LPMCs from WT and *Nfil3*$^{-/-}$ mice were analyzed for *Il12b* expression. Results are presented as expression relative to WT 11b$^+$ LPMCs normalized to β-actin (mean ± SEM from three independent experiments). *B*, GF WT and colitis-prone *Il10*$^{-/-}$ mice were transitioned to an SPF enteric microbiota, and colonic *Nfil3* expression was analyzed 3 d postcolonization. Results are expressed as fold induction versus WT GF colons normalized to β-actin (mean ± SEM from three independent experiments). *$p < 0.05$ versus GF. *C*, *NFIL3* expression in human CD14$^+$ LPMCs was examined (control [cont], $n = 8$; Crohn's disease [CD], $n = 8$; ulcerative colitis [UC], $n = 8$). Results are presented as relative expression compared with controls normalized to β-actin (mean ± SEM). *$p < 0.05$, **$p < 0.01$ versus control (Dunnett's multiple-comparison test).