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


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 II12b 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 II12b promoter activity was inhibited by NFIL3 expression and augmented by NFIL3-short hairpin RNA in an II12b-bacterial artificial chromosome-GFP reporter macrophage line. II12b 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 II12b 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 II10-/-/- mice transitioned from germ-free to a conventional microbiota. In conclusion, these experiments characterize NFIL3 as an II12b transcriptional inhibitor. Interactions of macrophages with the enteric microbiota induce NFIL3 to limit their inflammatory capacity. Furthermore, altered intestinal NFIL3 expression may have implications for the pathogenesis of experimental and human inflammatory bowel diseases. The Journal of Immunology, 2011, 186: 4649–4655.

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 (II12b) is expressed in macrophages and is highly induced by microbial stimuli such as LPS (8). Therefore, factors that regulate II12b 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, 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). Kashvada 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 macro-
phages, 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 \( \text{II} 12b \) transcription in macrophages. Our results suggest that NFIL3 is a physiological regulator of IL-12/IL-23 during host-enteric commensal interactions and contributes to intestinal homeostasis.

Materials and Methods

*Mice*

Wild-type (WT) and \( \text{Myd88}^{-/-} \) mice (C57BL/6 background) in specific pathogen-free (SPF) conditions were used for BMDM derivation. \( \text{Nfil3}^{-/-} \) mice were generated by gene-targeting strategies, as reported previously (12), and bred with C57BL/6 mice for at least 10 generations. WT and \( \text{Il} 10^{-/-} \) 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).

\( \text{II} 12b \)–GFP bacterial artificial chromosome transformed macrophages

Transgenic mice were generated containing a 200-kb \( \text{II} 12b \) bacterial artificial chromosome (BAC) transgene (generated by S.T.S.). Before microinjection, BAC recombineering in *Escherichia coli* introduced a GFP cDNA into the \( \text{II} 12b \) transcription unit. Next, a transformed macrophage line from the \( \text{II} 12b \)-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, \( n = 8 \)) or control patients (\( n = 8 \)) as described previously (17). Anti-NFIL3 Ab was obtained from Santa Cruz Biotechnology (Santa Cruz, 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 \( \text{Nfil3} \) mRNA and protein is detected in murine BMDMs (Fig. 1A, 1B). Consistent with a prior study (13), LPS induced \( \text{Nfil3} \) mRNA (Fig. 1A) and protein (Fig. 1B) expression in WT BMDMs. LPS and CpG DNA induced \( \text{Nfil3} \) expression through a MyD88-dependent pathway as induction was abrogated in \( \text{Myd88}^{-/-} \) BMDMs (Fig. 1A, Supplemental Fig. 1A). \( \text{Il} 10^{-/-} \) mice develop spontaneous chronic enterocolitis dependent on the presence of the enteric microbiota (20). Interestingly, as reported previously (13), although basal \( \text{Nfil3} \) expression is detected, \( \text{Nfil3} \) is not induced in LPS-activated \( \text{Il} 10^{-/-} \) BMDMs (Fig. 1C). This finding suggested that IL-10 is a cofactor for \( \text{Nfil3} \) induction by LPS. Indeed, LPS-induced \( \text{Nfil3} \) expression is abrogated in WT BMDMs in the presence of anti–IL-10 Abs (Fig. 1D).

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 \( \text{Nfil3} \) expression before and after transition of WT mice from a GF environment to an SPF microbiota. Colonic \( \text{Nfil3} \) expression was significantly induced after colonization, suggesting that NFIL3 participates in physiological host–enteric microbial interactions in vivo (Fig. 1E).

**NFIL3 is a regulator of \( \text{II} 12b \) expression**

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

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. 2D, left graph; Supplemental Fig. 2B) mRNA and IL-12 p40 protein (Fig. 2D, 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 to 462 (truncated NFIL3), respectively (Supplemental Fig. 2C). Expression levels of WT and mutant constructs after lentivirus transduction in BMDMs were similar (Supplemental Fig. 2A).

Both NFIL3 mutant proteins did not fully suppress LPS-activated Il12b expression compared with intact NFIL3 in WT (Fig. 2E), 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. 2F).

**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. 2A, 3A). To confirm this finding, NFIL3 was overexpressed in Il10−/− BMDMs. Similar to experiments in WT BMDMs (Fig. 2D), NFIL3 also inhibited LPS-activated Il12b mRNA (Fig. 3B, left graph) and IL-12 p40 protein (Fig. 3B, right graph) in Il10−/− BMDMs. Likewise, the minimal repression domain was also required for the inhibition of Il12b by NFIL3 (Fig. 3C). Basal NFIL3 expression is detected in Il10−/− BMDMs (Fig. 1C). Consequently, knockdown of NFIL3 by shRNA resulted in increased LPS-induced Il12b in the absence of IL-10 (Fig. 3D). 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. 3A; http://ecbrowser.dcode.org), suggesting that it is functionally important. ChIP experiments from WT BMDMs revealed basal occupancy of NFIL3 on this binding element (Fig. 4A). 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. 4A). Moreover, as expected, no NFIL3 binding to this region is detected in ChIP experiments from Nfil3<sup>−/−</sup>BMDMs (Fig. 4B). 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. 4C, Supplemental Fig. 3B). Expression of mutant NFIL3 proteins (mutated NFIL3 and truncated NFIL3) did not alter the number of LPS-activated GFP<sup>+</sup> cells (Fig. 4C).

Moreover, in LPS-activated, Nfil3 shRNA-transduced Il12b-GFP macrophages, results are expressed as fold induction versus unstimulated WT BMDMs normalized to β-actin (mean ± SEM from three independent experiments). *p < 0.05 versus LPS-stimulated GFP-expressing BMDMs were calculated. F, WT and Nfil3<sup>−/−</sup>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.
macrophages, increased numbers of GFP+ cells were observed compared with scrambled-shRNA–transduced cells (Fig. 4D, Supplemental Fig. 3C).

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. 5A). 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. 1E), in Il10−/− mice, colonic Nfil3 is not induced after transition to SPF microbiota (Fig. 5B).

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. 5C). It has previously been demonstrated in the same sample 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. Inmate 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 in 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.

**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).
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 BMDMs (Fig. 2A). The Il6 promoter also contains putative binding sites for NFIL3. Il6 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.

Disclosures

The authors have no financial conflicts of interest.

References


