THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 44, Issue of November 1, pp. 41701–41705, 2002
Printed in U.S.A.

Induction of Nod2 in Myelomonocytic and Intestinal Epithelial Cells via Nuclear Factor-κB Activation*

Received for publication, June 28, 2002
Published, JBC Papers in Press, August 22, 2002, DOI 10.1074/jbc.M206473200

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Nod2, a member of the Apaf1/Nod protein family, confers responsiveness to bacterial products and activates NF-κB, a transcription factor that plays a central role in innate immunity. Recently, genetic variation in Nod2 has been associated with susceptibility to Crohn's disease. Here, we report that expression of Nod2 is induced upon differentiation of $CD34^+$ hematopoietic progenitor cells into granulocyte or monocyte/macrophages. In peripheral blood cells, the highest levels of Nod2 were observed in $CD14^+$ (monocytes), $CD15^+$ (granulocytes), and $CD40^+$/$CD86^+$ (dendritic cells) cell populations. Notably, stimulation of myeloblastic and epithelial cells with bacterial lipopolysaccharide or TNFα resulted in up-regulation of Nod2. A search for consensus sites within the Nod2 promoter revealed a NF-κB binding element that was required for transcriptional activity in response to TNFα. Moreover, ectopic expression of p65 induced transactivation, whereas that of dominant-negative IκBα blocked the transcriptional activity of the Nod2 promoter. Upon stimulation with TNFα or lipopolysaccharide, both p50 and p65 subunits of NF-κB were bound to the Nod2 promoter. Thus, Nod2 expression is enhanced by proinflammatory cytokines and bacterial components via NF-κB, a mechanism that may contribute to the amplification of the innate immune response and susceptibility to inflammatory disease.

Innate immunity recognizes invading microbes and triggers a host defense response aimed to the clearance of the invading pathogens. Studies of the host defense system in *Drosophila* revealed that Toll receptors play an important role in combating the invasion of pathogens (1). At least ten homologues of *Drosophila* Toll-like receptors (TLRs)[1] have been identified in mammals and shown to participate in the recognition of microbial components and activation of innate immunity, which leads to the development of antigen-specific immune responses (2, 3). Each membrane-associated TLR recognizes specific patterns of microbial components (*i.e.* TLR2 is responsible for the recognition of certain lipoproteins, whereas TLR4 recognizes lipopolysaccharides) (4, 5). TLRs are composed of a cytoplasmic Toll/interleukin-1 receptor domain and extracellular leucine-rich repeats (LRRs) (6). Nods are members of another family of proteins that have been implicated in the intracellular recognition of pathogen components (7). Nod1 and Nod2, the first members of the family to be identified, are composed of an N-terminal caspase recruitment domain, a centrally located nucleotide binding oligomerization domain, and C-terminal LRRs (7). Nod proteins have been shown to recognize bacterial components including bacterial lipopolysaccharides (LPS) and/or peptidoglycan through their LRRs, and this interaction leads to the activation of NF-κB, a transcription factor that plays a central role in innate immunity (7, 8). Studies using genetically modified cells have revealed that Nod1 and Nod2 activate NF-κB by means of the serine/threonine kinase RICK/Rip2 (9). Recently, a frameshift mutation and two nucleotide polymorphisms in the coding region of Nod2 have been associated with susceptibility to Crohn's disease, a chronic inflammatory disorder of the intestinal tract (10–12). The frameshift mutation results in a truncated Nod2 that is deficient in inducing LPS-mediated NF-κB activation (10). In addition, missense mutations in the region encoding the nucleotide binding oligomerization domain of Nod2 have been associated with susceptibility to Blau syndrome, another granulomatous inflammatory disorder (13).

Previous studies showed that Nod1 is broadly expressed in tissues (14), while the expression of Nod2 seems to be more restricted to monocytes (15). Here we report that Nod2 is expressed in monocytes, granulocytes, and dendritic cells and to a lesser extent in T lymphocytes. Significantly, Nod2 levels are up-regulated in myelomonocytic and epithelial cells upon stimulation with TNFα or LPS. Furthermore, we demonstrate that induction of Nod2 by TNFα or LPS is mediated transcriptionally through NF-κB. We suggest that transcriptional regulation of Nod2 by bacterial components and proinflammatory cytokines may play a role in innate immune responses and contribute to susceptibility to inflammatory disease.

EXPERIMENTAL PROCEDURES

Cell Lines—Human leukemia cell lines HL-60, U937, THP-1, K562, and Jurkat and breast cancer cell lines MCF-7 and MDA-MB231 were maintained in RPMI 1640 medium (Seromed Biochrom KG, Berlin, Germany) supplemented with 10% fetal calf serum (FCS) (Flow Laboratories, Irvine, CA). HEK293T cells were grown in Iscove's modified Dulbecco's medium (Invitrogen) supplemented with 10% FCS. Leukemic Mo7e cells were cultured in Iscove's modified Dulbecco's medium-

* This work was supported by Comision Interministerial de Ciencia y Tecnologia Grant SAF-99/0139, by a grant from "Fundacion Marcelino Botin" (proyecto Terapia Genica) (to J. L. F.-L.), and by Grants DK-61707 (to G. N) and GM-60421 (to N. I.) from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Supported by funds from Tokushima University, Japan and the Crohn's and Colitis Foundation of America Fellowship.

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[1] The abbreviations used are: TLR, toll-like receptor; LLR, leucine-rich repeat; NF, nuclear factor; FCS, fetal calf serum; LPS, lipopolysaccharide; IL, interleukin; TNF, tumor necrosis factor; RT, reverse transcriptase; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; G-CSF, granulocyte-colony stimulating factor; M-CSF, monocyte/macrophage-colony stimulating factor; IAP, inhibitor of apoptosis.

10% FCS with 5 ng/ml of recombinant human IL-3 (Immunex, Seattle, WA). Breast cancer MDA-MB435 and SUM159 cell lines were cultured as described elsewhere (16, 17). Normal colon FHC cells were grown in DMEM/HAM'S F-12 with 10% FCS, 10 ng/ml cholera toxin, 5 μg/ml transferrin, 5 μg/ml insulin, and 100 ng/ml hydrocortisone (Sigma).

Primary Cells—Peripheral blood progenitors were obtained from normal donors undergoing mobilization for allogeneic progenitor cell transplantation. All donors signed informed consent according to Guidelines from the Committee for the Protection of Human Subjects at the University of Navarra. $CD34^+$ cells were selected from the peripheral blood mononuclear cell population and induced to undergo granulocyte or monocyte/macrophage differentiation as previously described (18). At the indicated time points, cells were collected for mRNA expression and flow cytometric analysis. Dendritic cells were generated from peripheral blood monocytes and analyzed by flow cytometry as described elsewhere (19).

Gene Reporter Assays—Genomic PCR fragments of 3 kb and 527 bp from the promoter region of Nod2 (Nod2pt), starting 121 bases upstream from the initiation codon, were cloned into *Xho*I and *Hin*dIII sites of the pGL2-basic luciferase reporter vector (Promega). pEF1-BOS-β-gal and pcDNA3-FLAG-DC-CIITA were described previously (15, 20). pYGFP-p65 was a gift of Johannes Schmid (University of Vienna). Expression plasmids to produce NF-AT4, NF-IL3, NF-IL6, cAMP-response element-binding protein, and Elk in pCMV-SPORT6 were obtained from Research Genetics. pcDNA3-p53 and pCMV-c-Myc were a gift of Michael Clarke (University of Michigan). pcDNA3-FLAG-IRF7 and GATA-1 were obtained from David Levy (New York University) and Vishva Dixit (Genentech), respectively. HEK293T cells were cotransfected with 1 μg of pGL2-Nod2pt (527-bp promoter fragment) and 50 ng of pEF1-BOS-β-gal in triplicate by using FuGENE 6 reagent (Roche Molecular Biochemicals) or with 166 ng of expression plasmid of each transcription factor indicated in Fig. 5*C*, 100 ng of pGL2-Nod2 (3-kb promoter fragment) and 73 ng of pEF1BOS-β-gal as previously described (15). When indicated, cells were cotransfected with pGL2-Nod2pt and 1 μg of a vector containing a mutated form of IκBα that inhibits activation of NF-κB (Clontech, Palo Alto, CA). 24 h posttransfection, cells were incubated with 1 μg/ml TNFα (Sigma) for 6 h, and then cell extracts were prepared and analyzed for the relative luciferase activity by a reporter gene assay system (Applied Biosystems, Foster City, CA). Results were normalized for transfection efficiency with values obtained with pEF1BOS-β-gal. Site-directed mutagenesis of the pGL2-Nod2pt vector, containing the 527-bp fragment of the Nod2 promoter, was carried out by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) with the following primers: 5′-CCTTTGTGAATTTCCCTT3-′ and 5′-AAGGGAAATTCA-CAAAGG-3′. The Nod2pt DNA insert was sequenced to verify the mutation.

Reverse Transcriptase (RT)-PCR Analysis—Total RNA was prepared using TRIZOL reagent (Invitrogen). To assess mRNA expression, a semiquantitative RT-PCR method was used as previously described (21). The generated cDNA was amplified by using primers for human Nod2 (5′-AGCCATTGTCAGGAGGCTC-3′ in exon 2, and 5′-CGTCTC-TGCTCCATCATAGG-3′ in exon 4), IL-1β (5′-AAACAGATGAAGTGC-TCCTTCCAGG-3′ and 5′-TGGAGAACACCACTTGTTGCTCCA-3′), c-IAP1 (5′-TGAGCATGCAGACACATGC-3′ and 5′-TGACGGATGAAC-TCCTGTCC-3′), c-IAP2 (5′-CAGAATTGGCAAGAGCTGG-3′ and 5′-C-ACTTGCAAGCTGCTCAGG-3′), TRAIL (5′-TGATCTTCACAGTGCTC-CTGC-3′ and 5′-TGTTGCTTCTTCCTCTGGTCC-3′), and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (21). After 20 (GAPDH), 25 (IL-1β), 28 (c-IAP1, c-IAP2, TRAIL), or 30 (Nod2) amplification cycles, the expected PCR products were size fractionated onto a 2% agarose gel and stained with ethidium bromide. The Nod2 fragment was sequenced to verify the authenticity of the PCR product. Quantitative real-time PCR was performed in a 7000 Sequence Detection System (Applied-Biosystems). The ratio of the abundance of Nod2 transcripts to that of GAPDH transcripts was calculated as 2^n, where n is the C_T (threshold cycle) value of GAPDH minus the C_T value of Nod2, and normalized by the value of the sample with the lowest expression level of Nod2. Specificity of the desired PCR products was determined by melting curve analysis.

Electrophoretic Mobility Shift Assay—HL-60 cells were cultured for 1 h with 10 ng/ml TNFα or 1 μg/ml of LPS from *Salmonella typhimurium* (Sigma) in the presence or absence of 1 μM Bay11–7082 (Calbiochem, La Jolla, CA), an irreversible inhibitor of NF-κB activation (22). Then cells were lysed, and nuclear fractions were resuspended in 20 mM HEPES, pH 7.9, 420 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 20% glycerol. Nuclear extracts (5 μg of total protein) were incubated with a ^{32}P-labeled double-stranded DNA probe from the promoter region of the Nod2 gene (5′-CCTTTGGGAATTTCCCTT-3′). Samples were run on a 5% non-denaturing polyacrylamide gel, in 200 mM Tris borate,

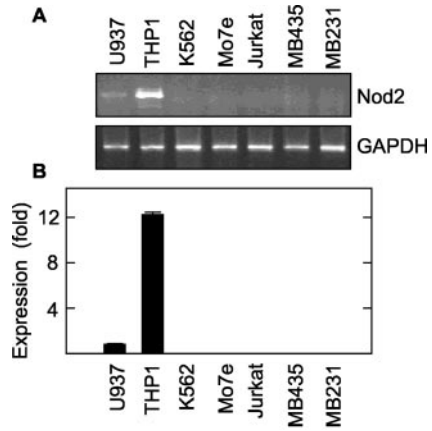

FIG. 1. **Analysis of Nod2 mRNA in hematopoietic cell lines.** Total RNA from myelomonocytic (U937, THP-1, K562), megakaryoblastic (Mo7e), and lymphoblastic (Jurkat) cell lines was obtained and analyzed for Nod2 mRNA levels by semi-quantitative RT-PCR (*A*) and real-time PCR (*B*). Breast cancer epithelial cells (MB435, MB231) were also analyzed as controls. GAPDH mRNA was used as an amplification control. Histograms represent the means ± S.D. of triplicate analyses.

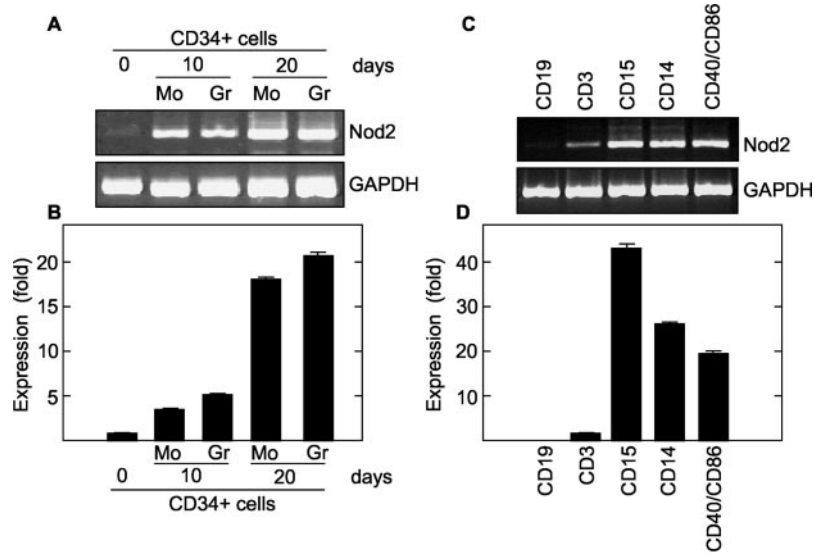

FIG. 2. **Analysis of Nod2 mRNA in primary hematopoietic cells.** Total RNA was purified from $CD34^+$ cells cultured in the presence of M-CSF or G-CSF (*A*, *B*), and immune-purified peripheral blood cell populations (*C*, *D*), and subjected to semiquantitative RT-PCR (*A*, *C*) or real-time PCR (*C*, *D*) analysis. GAPDH mRNA was used as an amplification control. Histograms represent the means ± S.D. of triplicate analyses.

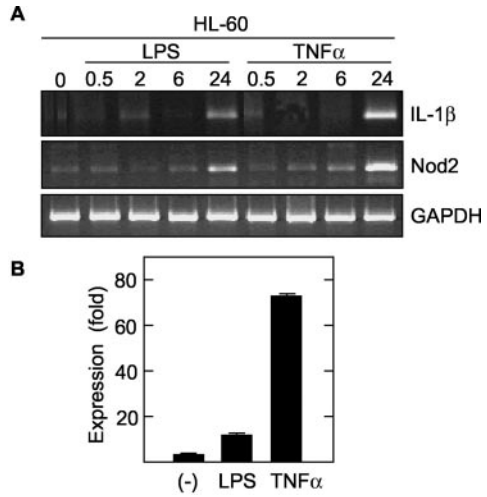

FIG. 3. **Induction of Nod2 mRNA in HL-60 cells treated with TNFα and LPS.** *A*, myeloblastic HL-60 cells were cultured with LPS or TNFα for the indicated time intervals, and then total RNA was extracted and analyzed for the expression of Nod2 and IL-1β by semiquantitative RT-PCR. *B*, real-time PCR analysis of HL-60 cells treated for 24 h with LPS and TNFα. GAPDH mRNA was used as an amplification control. Histograms represent the means ± S.D. of triplicate analyses.

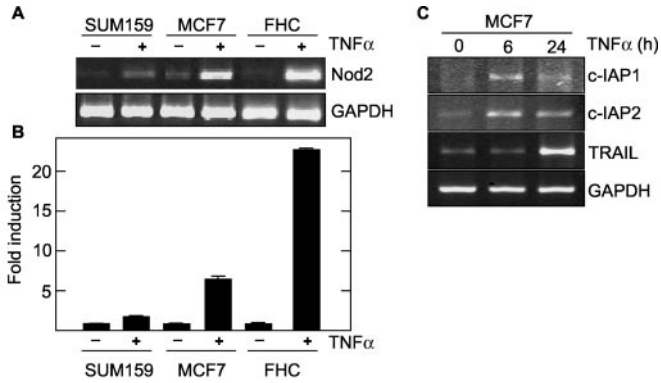

FIG. 4. **Induction of Nod2 mRNA in epithelial cells treated with TNFα.** Epithelial cells derived from breast cancer (SUM159, MCF-7) and normal colon (FHC) were cultured with TNFα for 24 h, and then total RNA was extracted and analyzed for the expression of Nod2 by semiquantitative RT-PCR (*A*) and real-time PCR (*B*). *C*, the levels of c-IAP1, c-IAP2, and TRAIL were analyzed in MCF-7 cells following treatment with TNFα for the indicated time intervals. GAPDH mRNA was used as an amplification control. Histograms represent the means ± S.D. of triplicate analyses.

2 mM EDTA. Gels were dried and visualized by autoradiography. Supershifts were performed using rabbit polyclonal antibodies specific for p50 and p65 NF-κB family members (Santa Cruz Biotechnology, Santa Cruz, CA).

RESULTS

Nod2 Is Expressed in Mature Myelomonocytic and Dendritic Cells—Initial studies showed that Nod2 was expressed primarily in monocytes (15). Consistent with this finding we found that monoblastic U937 cells and the more mature monocyte-like cell line THP-1 expressed Nod2 as assessed by semiquantitative RT-PCR analysis (Fig. 1*A*). The levels of Nod2 mRNA in THP-1 were about 12-fold higher than in U937 cells as determined by real-time PCR (Fig. 1*B*). In contrast, undifferentiated myeloblastic K562 cells, megakaryoblastic Mo7e cells, lymphoblastic Jurkat cells, and two breast cancer cell lines (MB435, MB231) were all negative (Fig. 1, *A* and *B*). To translate these findings to a more physiologically relevant model, we purified $CD34^+$ progenitor cells from peripheral blood, and the selected population was cultured with either G-CSF or M-CSF

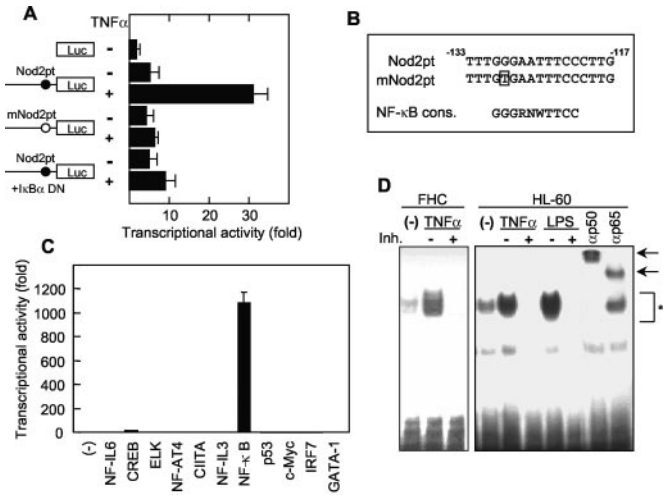

FIG. 5. **Transcriptional activation of the Nod2 promoter.** *A*, HEK293T cells were transfected with a 527-fragment of the Nod2 promoter (Nod2pt) either alone or with a mutated form of IκBα (*IκBα DN*), and a mutant Nod2 promoter (*mNod2pt*) (mutation shown in *panel B*), in the presence of a β-galactosidase reporter vector (pEF1BOS-β-gal). Cells were induced with TNFα for 6 h or left untreated. *C*, HEK293T cells were co-transfected with the indicated transcription factors, a 3-kb fragment of the Nod2 promoter, and pEF1BOS-β-gal. 24 h posttransfection, Nod2 promoter-dependent transcription was determined. Units of luciferase activity were normalized based on values of pEF1BOS-β-gal activity to control for transfection efficiency. Data are presented as the mean of triplicate cultures ± S.D. *D*, FHC and HL-60 cells were stimulated with TNFα or LPS for 60 min in the presence (+) or in the absence (−) of Bay11–7082, an inhibitor of NFκB activation. Electrophoretic mobility shift assay was performed using a radiolabeled probe from the Nod2 promoter. Nuclear extracts from stimulated cells were preincubated with antibodies specific for p50 and p65.

to induce granulocyte or monocyte/macrophage maturation, as previously described (18). The granulocytic cell population ($CD34^-$ $CD15^+$) increased to more than 85% after 20 days of culture, and showed morphologic features of mature granulocytes.[2] When the cells were cultured in the presence of M-CSF, a clear pattern of monocyte/macrophage maturation was observed, and by day 20 the majority of cells were mature monocytes/macrophages as determined by morphology and immunophenotype (83% of the cells were $CD34^-$ $CD14^+$). We then analyzed the levels of Nod2 mRNA in these cell populations and found that $CD34^+$ progenitors expressed low levels of Nod2, which increased 3- to 5-fold by day 10 and 17- to 20-fold by day 20 of culture in both monocytic and granulocytic lineages (Fig. 2, *A* and *B*). Thus, Nod2 is up-regulated during myelomonocytic differentiation. Then we analyzed the expression of Nod2 in peripheral blood populations of B cells (91.4% $CD19^+$ cells), T cells (99.0% $CD3^+$ cells), granulocytes (84.0% $CD15^+$ cells), monocytes (99% $CD14^+$ cells), and monocyte-derived dendritic cells (81.1% $CD40^+$/$CD86^+$ cells). The mRNA analysis showed that B lymphocytes expressed undetectable and T cells low levels of Nod2, whereas granulocytes, monocytes, and dendritic cells expressed about 42-, 26-, and 19-fold more Nod2 mRNA than T-cells, respectively, as determined by semiquantitative RT-PCR and real-time PCR analyses (Fig. 2, *C* and *D*). Thus, within the hematopoietic system, myelomonocytic and dendritic cells express the highest levels of Nod2.

Nod2 mRNA Is Induced in Hematopoietic and Epithelial Cell Lines by Lypopolysacharide and Tumor Necrosis Factor—It has been described that Nod2 activates NF-κB (15), a transcriptional factor involved in the induction of inflammatory re-

[2] O. Gutierrez, F. Prosper, and J. L. Fernandez-Luna, unpublished results.

sponses. Interestingly, some of the target genes of NF-κB (IL-1β, TNFα), in turn activate NF-κB (23). Based on these observations, we first analyzed the expression of Nod2 mRNA in myeloblastic HL-60 cells after treatment with two NF-κB activators, LPS and TNFα. Nod2 was weakly expressed in unstimulated HL-60 cells, but after treatment for 24 h with either LPS or TNFα, the mRNA levels of Nod2 increased about 8- and 70-fold, respectively (Fig. 3, *A* and *B*). This expression pattern was similar to that of the IL-1β gene (Fig. 3*A*), a known target of NF-κB. We then analyzed whether Nod2 could also be induced in non-hematopoietic cells. As shown in Fig. 4*A*, the expression levels of Nod2 mRNA were very low in two breast cancer cell lines, SUM159 and MCF-7, and in FHC (an epithelial cell line derived from normal colon). By 24 h of stimulation with TNFα, the levels of Nod2 mRNA in SUM159 and MCF-7 were increased about 1.5- and 6-fold respectively (Fig. 4, *A* and *B*). Up-regulation of Nod2 mRNA levels was more prominent (23-fold) in FHC colon cells (Fig. 4*B*). In control experiments, the levels of NF-κB-regulated genes such as TRAIL and members of the inhibitor of apoptosis family (c-IAP1, and c-IAP2) were increased after treatment with TNFα (Fig. 4*C*).

The Promoter Region of Nod2 Contains a NF-κB-Consensus Sequence That Is Responsive to TNFα and LPS—Based on the up-regulation of Nod2 in response to NF-κB activators, we searched for consensus sites within the Nod2 promoter region and found a putative NF-κB recognition sequence 121 bases upstream from the initiation codon (Fig. 5*B*). To assess the transcriptional activity of the Nod2 promoter, a 527-bp fragment containing the NF-κB consensus site of the Nod2 promoter (Nod2pt) was cloned into a promoterless luciferase vector (Nod2pt-luciferase), and this construct was transiently transfected into HEK293T cells. Stimulation of the cells with TNFα induced the transcriptional activity about 5-fold when compared with unstimulated cells (Fig. 5*A*). To assess the relevance of the putative NF-κB site, we mutated an essential base within this sequence motif (Fig. 5*B*) in the Nod2 promoter fragment. In contrast to the wild-type promoter, there were no significant differences between TNFα-stimulated and unstimulated cells when the mutant Nod2-luciferase construct was transfected into HEK293T cells (Fig. 5*A*). Moreover, activation of the Nod2 promoter was dependent on endogenous NF-κB, as demonstrated by the ability of an IκBα dominant negative mutant to block transactivation of the Nod2 promoter induced by TNFα (Fig. 5*A*). We then tested whether other transcriptional factors could transactivate the Nod2 promoter using a 3-kb fragment of the promoter in a luciferase reporter construct. Overexpression of the p65 subunit of NF-κB induced the activity of the Nod2 promoter about 1000-fold, whereas expression of NF-IL6, cAMP-response element-binding protein, Elk, NF-AT4, CIITA, NF-IL3, p53, c-Myc, IRF-7, or GATA-1 induced very little or no transactivation (Fig. 5*C*). Based on these data, we tested if NF-κB binds to the Nod2 promoter in response to TNFα and LPS by using an electrophoretic mobility shift assay. Treatment of colon FHC cells with TNFα or myeloid HL-60 cells with either TNFα or LPS resulted in significant increase of the NF-κB-DNA complex (Fig. 5*D*), which was inhibited by treatment with Bay11–7082, an inhibitor of NF-κB activation. Furthermore, the induced DNA binding complex was shifted by incubation of the nuclear extracts with anti-p50 or -p65 antibody, indicating that the protein-DNA complex contained NF-κB (Fig. 5*D*). In addition, we tested the NF-κB binding ability of the same DNA probe carrying a mutated Nod2 promoter sequence and found no electrophoretic shift of the radiolabeled probe.[2]

DISCUSSION

In the present study, we have focused on the expression and transcriptional regulation of Nod2 in different cell populations. Our results show that under unstimulated conditions Nod2 is primarily expressed in myelomonocytic and dendritic cells, an expression pattern that is similar to that described for TLR4 and TLR2 (24, 25). Surprisingly, stimulation of myelomonocytic cells with LPS or TNFα and intestinal epithelial cells with TNFα up-regulated or induced Nod2 gene expression. Furthermore, the mechanism of Nod2 regulation involves transcriptional activation of the Nod2 promoter through NF-κB. Because Nod2 activates NF-κB and this response is likely to mediate the induction of cytokines including TNFα, up-regulation of Nod2 may be part of a positive regulatory loop induced through inflammatory cytokines or bacterial components. A similar regulatory mechanism has been recently described for TLR2 (26). Although TLR2 is expressed at very low levels in unstimulated human epithelial cells, its expression is enhanced by bacterial pathogens through a NF-κB-dependent pathway (26). There is mounting evidence that NF-κB signaling in response to pathogens mediates protection of the host against invading microbes. For example, mice deficient in RICK (a factor required for Nod2 signaling) are more susceptible to *Listeria monocytogenes* (27). It is interesting to note that the expression of RICK, a kinase that is required for Nod2 signaling, is also induced by LPS stimulation presumably via NF-κB (9). Thus, enhanced expression of Nod2 and its binding partner RICK may facilitate the response of the host to pathogens. Intestinal epithelial cells have evolved mechanisms to prevent inappropriate activation of inflammatory responses in the microbe-rich environment of the gut (28). Consistent with this, we found that unstimulated epithelial cells from normal colon express low levels of Nod2. However, expression of Nod2 was induced following treatment with TNFα. It will be important, therefore, to assess the expression of Nod2 in epithelial tissues characterized by increased levels of proinflammatory cytokines (*i.e.* Crohn's disease). Down-regulation of Nod2 in epithelial cells may contribute to the maintenance of a state of hyporesponsiveness toward comensal microflora, which would be beneficial to the host. However, proinflammatory stimuli derived from enteropathogens or other pathogenic bacteria could activate NF-κB through several mechanisms including TLR or TNFα receptor signaling. Under these conditions, activation of NF-κB could induce the expression of Nod2, which in turn activates NF-κB establishing a positive feedback loop that may contribute to the secretion of proinflammatory cytokines and chemokines at epithelial sites.

Current therapy for Crohn's disease includes blockage of TNF signaling (29). This therapy is effective in reducing active disease in patients with Crohn's disease but no permanent cure has been achieved. Anti-TNF therapy could inhibit the mechanism of Nod2 up-regulation in monocyte/dendritic cells and epithelial cells. This may be particularly significant in patients in which Nod2 is functional and could contribute to the increased incidence of opportunistic infections associated with anti-TNF therapy (30). The finding that Nod2 gene can be up-regulated by activation of NF-κB in both immune-competent and epithelial cells is consistent with an emerging concept in which a microbial-epithelial-immune circuit is important for intestinal epithelium homeostasis (31) and mucosal defense.

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