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The Nuclear IκB Protein IκBNS Selectively Inhibits Lipopolysaccharide-Induced IL-6 Production in Macrophages of the Colonic Lamina Propria[1]

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Macrophages play an important role in the pathogenesis of chronic colitis. However, it remains unknown how macrophages residing in the colonic lamina propria are regulated. We characterized colonic lamina proprial CD11b-positive cells (CLPMϕ). CLPMϕ of wild-type mice, but not IL-10-deficient mice, displayed hyporesponsiveness to TLR stimulation in terms of cytokine production and costimulatory molecule expression. We compared CLPMϕ gene expression profiles of wild-type mice with IL-10-deficient mice, and identified genes that are selectively expressed in wild-type CLPMϕ. These genes included nuclear IκB proteins such as Bcl-3 and IκBNS. Because Bcl-3 has been shown to specifically inhibit LPS-induced TNF-α production, we analyzed the role of IκBNS in macrophages. Lentiviral introduction of IκBNS resulted in impaired LPS-induced IL-6 production, but not TNF-α production in the murine macrophage cell line RAW264.7. IκBNS expression led to constitutive and intense DNA binding of NF-κB p50/p50 homodimers. IκBNS was recruited to the IL-6 promoter, but not to the TNF-α promoter, together with p50. Furthermore, small interference RNA-mediated reduction in IκBNS expression in RAW264.7 cells resulted in increased LPS-induced production of IL-6, but not TNF-α. Thus, IκBNS selectively suppresses LPS-induced IL-6 production in macrophages. This study established that nuclear IκB proteins differentially regulate LPS-induced inflammatory cytokine production in macrophages. *The Journal of Immunology,* **2005, 174: 3650–3657.**

Inflammatory bowel diseases (IBD)[3] including Crohn's disease and ulcerative colitis are chronic immune-mediated disorders for which pathogenesis and etiology are poorly understood (1). A number of animal models of mucosal inflammation have been developed to analyze the pathogenesis of IBD (2, 3). In the course of analyzing these models, many types of cells including T cells, B cells, and epithelial cells have been shown to contribute to the pathogenesis of colitis. Among these cell populations, T cells have been shown to possess effector and regulatory functions in the development of chronic colitis. Both $CD4^+$ Th1 and Th2 cells are critically involved in mucosal immunity as effector cells, and disrupting the balance of Th1/Th2 polarization leads to the development of chronic mucosal inflammation (4). Crohn's disease is considered to be a Th1-dependent inflammatory disease, whereas ulcerative colitis is a Th2-dependent disease (3). In addition, regulatory T cells, such as $CD25^+CD4^+$ T cells, TGF-β-producing Th3 cells, and IL-10-producing type 1 regulatory T cells, all have regulatory functions in prevention of chronic mucosal inflammation and even in amelioration of established colitis (5, 6). B cells also have regulatory functions in mucosal inflammation observed in TCR-α-deficient mice (7). Thus, important roles of adaptive immunity comprising T cells and B cells in mucosal inflammation are well characterized.

However, recent accumulating evidence demonstrates that innate immunity plays crucial roles in controlling Ag-specific adaptive immunity (8–11). Accordingly, the involvement of innate immunity in the development of chronic colitis has been proposed. Abnormal activation of innate immune cells has been shown to initiate the development of chronic colitis in mice lacking Stat3 specifically in macrophages and neutrophils (12). The phenotype observed in the Stat3 mutant mice was very reminiscent of that observed in IL-10-deficient mice, indicating that major target cells of IL-10 in suppressing chronic mucosal inflammation are cells of macrophage lineage (12–14). The mechanisms by which abnormal activity of innate immune cells leads to the development of chronic colitis were further analyzed. In the absence of Stat3, innate immune cells showed increased levels of inflammatory cytokine production through TLR, which are essential for the recognition of microbial components in innate immune cells. Among these cytokines, IL-12p40 is responsible for the exaggerated Th1 cell development and thereby induces Th1-dependent chronic colitis (15). Thus, critical involvement of TLR-dependent activation of innate immunity has clearly been shown in triggering chronic mucosal inflammation. In addition to TLR-mediated activation of innate immunity, NOD2, which is responsible for TLR-independent recognition of microbial components, has been implicated in the pathogenesis of Crohn's disease in human (16–18). Thus, molecules critically involved in innate immune responses, such as TLRs

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[3] Abbreviations used in this paper: IBD, inflammatory bowel disease; CLPMϕ, colonic lamina proprial CD11b-positive cell; ChIP, chromatin immunoprecipitation; siRNA, small interference RNA.

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and NOD2, are associated with the pathogenesis of colitis. However, it remains unclear how activities of these innate immune cell populations are regulated in the intestinal mucosa.

In this study, we isolated CD11b-positive cells from the colonic lamina propria (CLPMϕ), and analyzed their responsiveness to TLR ligands. CLPMϕ of wild-type mice, but not IL-10-deficient mice, showed hyporesponsiveness to TLR ligands. Therefore, we compared CLPMϕ gene expression profiles in wild-type mice with IL-10-deficient mice, which led to identification of genes that are specifically expressed in CLPMϕ of wild-type mice, but not in CLPMϕ of IL-10-deficient mice or wild-type peritoneal macrophages. We further analyzed whether these gene products are capable of inhibiting TLR-mediated responses in macrophages, and found that a member of the IκB family of proteins, IκBNS, inhibits LPS-induced IL-6 production in macrophages.

Materials and Methods

Reagents and cell culture

LPS from *Escherichia coli* (O55:B5) was purchased from Sigma-Aldrich. The mouse macrophage cell line (RAW264.7) and human embryonic kidney 293T cells were cultured in DMEM supplemented with 10% FBS, 100 μg/ml streptomycin, and 10 U/ml penicillin G. Mouse peritoneal macrophages were collected by peritoneal lavage with HBSS at 3 days after i.p. injection of 2 ml of 4% sterile thioglycolate into 8- to 12-wk-old mice. Peritoneal macrophages were cultured in RPMI 1640 medium with 10% FBS, 100 μg/ml streptomycin, and 10 U/ml penicillin G.

Mice

C57BL/6 mice were purchased from the Central Laboratory of Experimental Animals (Tokyo, Japan). IL-10-deficient mice were purchased from The Jackson Laboratory. All experiments using these mice were approved by and performed according to the guidelines of the animal ethics committee of Kyushu University and Osaka University.

Isolation of CLPMϕ

CLPMϕ was isolated using a protocol modified from an EDTA perfusion method (19). Mice were anesthetized and their peritoneal and pleural cavities were opened for systemic perfusion from left ventricle with 15 ml of HBSS containing 20 mM EDTA. Following perfusion, colons were removed, cut into pieces of 2~3 cm in length, resuspended in HBSS, and shaken with a microbead beater (Biospec Products) at 5000 rpm for 50 s to remove epithelial cells. The colon pieces were then washed with RPMI 1640, mechanically minced and resuspended in RPMI 1640 supplemented with 10% FBS, 2 mg/ml collagenase type II (Invitrogen Life Technologies), 1 mg/ml dispase (Invitrogen Life Technologies), 15 mg/ml DNase (Boehringer), 100 μg/ml streptomycin, and 10 U/ml penicillin G for 30 min at 37°C in a shaking incubator. After filtration of digested tissue with 40-μm nylon mesh, isolated cells were washed with PBS and CD11b-positive cells were purified using MACS selection system using CD11b MicroBeads (Miltenyi Biotec) following manufacturer's instructions.

Measurement of inflammatory cytokines

The cells (5×10^4) were cultured in 96-well plates with 10 or 100 ng/ml LPS for 24 h. The concentrations of TNF-α, IL-6, and IL-12p40 in the culture supernatants were determined by ELISA according to the manufacturer's instructions (Genzyme Techne).

Flow cytometry

Single cell suspension of colonic lamina propria was stained with PE-conjugated anti-CD11b Ab (BD Pharmingen) and biotin-conjugated anti-TLR4/MD-2 Ab (eBioscience), followed by FITC-conjugated streptavidin. Stained cells were analyzed on a FACSCalibur (BD Biosciences).

DNA microarray

Total RNA from wild-type or IL-10-deficient lamina proprial CD11b-positive cells was extracted with an RNeasy kit (Qiagen), followed by mRNA purification with an Oligotex mRNA kit (Amersham Pharmacia Biotech). Double-stranded cDNA was synthesized from 1 μg of mRNA with the SuperScript Choice System (Invitrogen Life Technologies) primed with T7-oligo(dT) 24 primer. These cDNA were used to prepare biotin-labeled cRNA by an in vitro transcription reaction using T7 RNA polymerase in the presence of biotinylated ribonucleotides, according to the manufacturer's protocol (Enzo Diagnostics). The cRNA products were purified using an RNeasy kit (Qiagen), fragmented, and hybridized to Affymetrix Murine Genome U74Av2, Bv2 and Cv2 microarray chips, according to the manufacturer's protocol (Affymetrix). The hybridized chips were stained, washed, and scanned with a GeneArray scanner (Affymetrix).

RT-PCR

Total RNA (1 μg) was primed with random hexamers, followed by reverse transcription with Superscript II (Invitrogen Life Technologies). PCR analysis was performed using recombinant TaqDNA polymerase (Takara Shuzo). Conditions for the reactions were 30 s of denaturation step at 94°C, 30 s of annealing step at 60°C, and 1 min of elongation step at 72°C for 25–30 cycles. Specific primers used were: IκBNS, sense 5′-GCTGTATC CTGAGCCTTCCCTGTC-3′ and antisense 5′-GCTCAGCAGGTCTTC CACAATCAG-3′; IκBζ, sense 5′-GCTCAACCTGGCTTACTTCTAC GG-3′ and antisense 5′-CGGAAGCCTTCTGCTTGTTGCTTC-3′; Bcl-3, sense 5′-GATGCCCATTTACTCTACCCCGAC-3′ and antisense 5′-GC CGGACCATGTCTGGTAATGTGG-3′; CD163, sense 5′-CTTCTTGGAG GTGCTGGATCTCCTG-3′ and antisense 5′-GCTCCCTCTAAGCAAAT CACACCG-3′; macrophage scavenger receptor 2, sense 5′GGTGCTGG AAACAGCTCTTGGAC-3′ and antisense 5′-GCTCAGCAGGTCTTCCA CAATCAG-3′; β-actin, sense 5′-CTATGTGGGTGACGAGGCCCAGAG-3′ and antisense 5′-GGGTACATGGTGGTACCACCAGAC-3′.

Real-time PCR

RAW264.7 cells and murine peritoneal macrophages were treated with 10 ng/ml IL-10 (Genzyme) for 1, 2, 4, or 6 h. Total RNA was isolated with TRIzol (Invitrogen Life Technologies) and treated with DNaseI (Promega). Reverse transcription was performed using MMLV Reverse Transcriptase (Promega) and oligo(dT) primers (Promega). Finally these solutions were directly used as templates for PCR. Quantitative real-time PCR was performed on an ABI 7000 sequence detection system (Applied Biosystems) using TaqMan universal PCR Master Mix (Applied Biosystems), as previously described (20). TaqMan probes mix for IκBNS was purchased from Applied Biosystems. All data were normalized to EF1-α expression in the same cDNA set.

Lentiviral introduction of IκBNS into macrophages

The lentiviral vector, CSII-EF-MCS-IRES-hrGFP (cPPT-containing SIN vector plasmid with multiple cloning sites for cDNA insertion followed by the IRES-GFP sequence under the control of the EF-1α promoter), was used to generate CSII-EF-IκBNS. Woodchuck hepatitis virus posttranslational regulatory element was ligated at the 3′ end of GFP. The lentiviral vectors were cotransfected into 293T cells with pMDLg/pRRE (packaging plasmid), pRSV-Rev (Rev expression plasmid), and pMD.G (VSV-G expression plasmid). Infectious lentiviruses in the culture supernatants were harvested at 48 h after transfection. RAW cells (5×10^5) were cultured with the lentiviruses for 24 h, and then the culture medium was replaced. After 48 h, the cells expressing human recombinant GFP were sorted by FACSVantage SE (BD Biosciences).

Northern blot analysis

The cells were stimulated with 100 ng/ml LPS. Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies), electrophoresed, transferred to a nylon membrane, and hybridized with cDNA probe.

Western blot analysis

Cells (2×10^6) were lysed with lysis buffer containing with 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, and Complete Mini (Roche). The lysates were separated on SDS-PAGE and transferred to polyvinylidene fluoride membrane. The membranes were incubated with anti-Flag M2 Ab (Sigma-Aldrich), anti-IκBα Ab, anti-ERK Ab, anti-p38 Ab (Santa Cruz Biotechnology), anti-phospho-p38 Ab, and anti-phospho-ERK Ab (Cell Signaling Technology). Bound Abs were detected with an ECL system (PerkinElmer).

Immunoprecipitation

The cell lysates were precleared with protein G-Sepharose beads (Amersham Pharmacia Biotech) and then incubated with protein G-Sepharose beads together with anti-Flag M2 Ab, anti-p50 Ab, and anti-p65 Ab (Santa Cruz Biotechnology). Immunoprecipitates were separated on SDS-PAGE, transferred to polyvinylidene fluoride membrane, and incubated anti-Flag M2 Ab, anti-p50 Ab, or anti-p65 Ab. Bound Abs were visualized with an ECL system (PerkinElmer).

EMSA

The cells were stimulated with 100 ng/ml LPS for 30 or 60 min. Then, nuclear proteins were extracted and incubated with an end-labeled, double-stranded oligonucleotide containing a NF-κB binding site on the IL-6 promoter in 25 μl of binding buffer (10 mM HEPES-KOH, (pH 7.8), 50 mM KCl, 1 mM EDTA, (pH 8.0), 5 mM $MgCl_2$, and 10% glycerol) for 20 min at room temperature and loaded onto a native 5% polyacrylamide gel. The DNA-protein complexes were visualized by autoradiography. The specificities of the shifted bands were determined by adding Abs specific for p65 and p50 (Santa Cruz Biotechnology).

Luciferase assay

RAW264.7 cells (1×10^5) were transiently transfected with a total 0.5 μg of expression vector, and 100 ng of IL-6 promoter-luciferase construct (21) or TNF-α promoter-luciferase construct (22) using a Superfect transfection reagent (Qiagen). After 24 h, cells were treated with or without 10 ng/ml LPS for 6 h. The luciferase activity was measured using the dual-luciferase reporter assay system (Promega). The Renilla-luciferase reporter gene (20 ng) was used as an internal control.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed essentially with a described protocol (Upstate Biotechnology). In brief, RAW cells were stimulated with 100 ng/ml LPS for 1 or 2 h, and then fixed with formaldehyde for 10 min. The cells were lysed, sheared by sonication, and incubated overnight with specific Ab followed by incubation with protein A-agarose saturated with salmon sperm DNA (Upstate Biotechnology). Precipitated DNA was analyzed by quantitative PCR (35 cycles) using primers 5′-ACTAGCCAGGAGGGAGAACAGAAACTC-3′ and 5′-CA CAAGCAGGAATGAGAAGAGGCTGAG-3′ for the TNF-α promoter and 5′-TAGCAGCAGGTCCAACTGTGCTATCTG-3′ and 5′-AAGC CTCCGACTTGTGAAGTGGTATAG-3′ for the IL-6 promoter.

In another experiment, peritoneal macrophages from wild-type mice and Stat3 mutant mice were pretreated with 10 ng/ml IL-10 for 18 h, then stimulated with 100 ng/ml LPS for 1 h, and used for ChIP assay.

RNA interference

RAW cells (4×10^6) were transfected with 500 pmol of dsRNA using Nucleofector (Amaxa). The target small interference RNA (siRNA), 5′-GUGCAGAUGUUACUGCAAAA-3′, was designed and produced by Dharmacon. The control siRNA was purchased from Dharmacon (catalog no. D-001206-08-05).

Results

Characterization of CD11b-positive cells in the colonic lamina propria

To analyze the function of colonic macrophages, we first isolated CLPMϕ according to procedures described in *Materials and Methods*. Flow cytometric analysis showed that 30–40% of CLPMϕ also expressed CD11c, indicating the presence of both macrophage-lineage cells and dendritic cell-lineage cells in the population. Using highly purified (over 97% purity) CLPMϕ, we analyzed their response to TLR ligands such as LPS and CpG DNA. We first stimulated CD11b-positive cells from the spleen and colonic lamina propria with LPS or CpG DNA, and analyzed for inflammatory cytokine production (Fig. 1*A*). CD11b-positive cells from the spleen produced significant amounts of TNF-α, IL-6, IL-12p40, and IL-10 in response to LPS or CpG DNA. However, TLR ligand-induced increase in production of TNF-α, IL-6, and IL-12p40 was not observed in CLPMϕ, although IL-6 was produced in the absence of stimulation. In addition, IL-10 production was constitutively observed in CLPMϕ. We next analyzed TLR ligand-induced augmentation of surface molecules such as CD40, CD80, CD86, and MHC class II (Fig. 1*B*). These surface molecules were not up-regulated in response to LPS or CpG DNA in CLPMϕ.

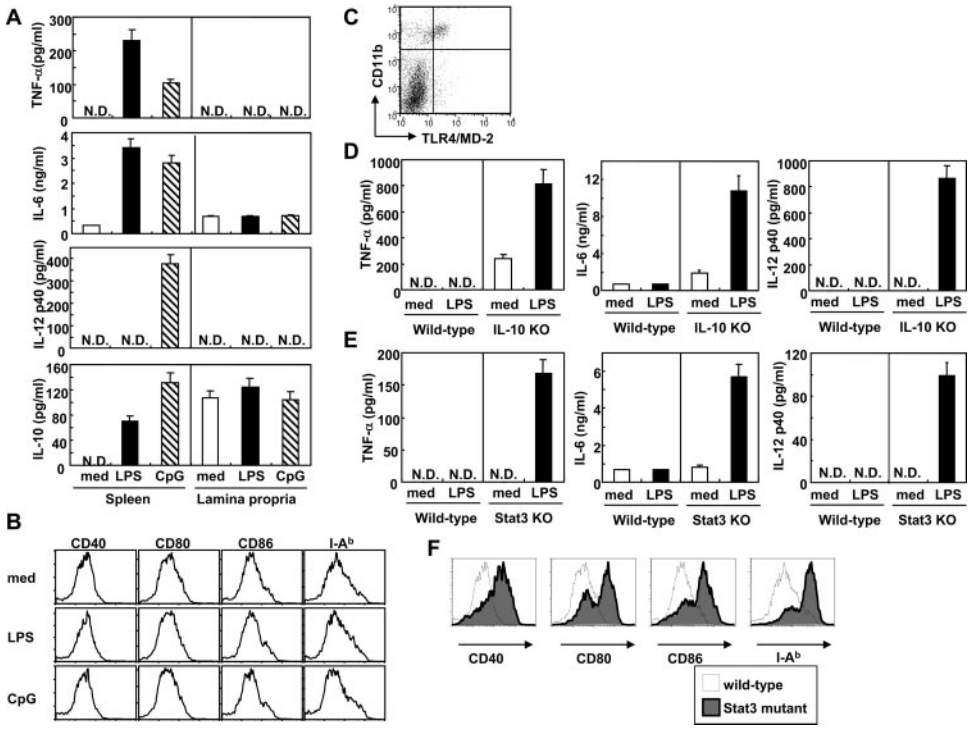

FIGURE 1. Characterization of CD11b-positive cells in the colonic lamina propria (CLPMϕ). *A*, CD11b-positive cells were isolated from the spleen and the colonic lamina propria, then stimulated with 100 ng/ml LPS or 10 nM CpG DNA for 24 h. Concentrations of TNF-α, IL-6, IL-12p40, and IL-10 in the culture supernatants were measured by ELISA. N.D., Not detected. *B*, The cells were also analyzed for surface expression of CD40, CD80, CD86, and MHC class II by flow cytometry. *C*, Colonic lamina proprial cells were stained with anti-CD11b and anti-TLR4/MD-2 Abs. *D*, CLPMϕ were isolated from IL-10-deficient mice, in which chronic colitis was already developed, and were analyzed for LPS-induced production of TNF-α, IL-6, and IL-12p40 by ELISA. *E*, CLPMϕ were isolated from 4- to 5-wk-old Stat3 mutant mice, in which chronic colitis was not developed yet, and analyzed for LPS-induced production of TNF-α, IL-6, and IL-12p40 by ELISA. *F*, CLPMϕ isolated from 4- to 5-wk-old wild-type and Stat3 mutant mice were analyzed for surface expression of CD40, CD80, CD86, and MHC class II by flow cytometry.

Thus, CLPMϕ were refractory to TLR ligands in terms of inflammatory cytokine production and costimulatory molecule expression. Surface expression of TLR4-MD-2 complex on CLPMϕ was observed (Fig. 1*C*). Therefore, the hyporesponsiveness to TLR ligands was not due to the lack of TLR4 expression in CLPMϕ (Fig. 1*C*).

We next isolated CLPMϕ from IL-10-deficient mice, in which chronic colitis has already developed, and analyzed for inflammatory cytokine production in response to TLR ligands (Fig. 1*D*). Although CLPMϕ from wild-type mice did not show LPS-induced production of TNF-α and IL-6, CLPMϕ from IL-10-deficient mice produced small amounts of TNF-α and IL-6 even when cultured with media alone, and the production was robustly enhanced in response to LPS. CLPMϕ from mice lacking Stat3 in macrophage (Stat3 mutant mice) also showed increased TNF-α and IL-6 production in response to LPS (data not shown). Even in CLPMϕ from young (4- to 5-wk-old) IL-10-deficient or Stat3 mutant mice, which have not developed colitis yet, LPS stimulation resulted in increased production of TNF-α and IL-6, indicating that enhanced production of inflammatory cytokines was not due to environmental effects, but intrinsic to CLPMϕ themselves (Fig. 1*E*). Surface expression of CD40, CD80, CD86, and MHC class II was upregulated in CLPMϕ from young Stat3 mutant mice (Fig. 1*F*). Thus, CLPMϕ from IL-10-deficient or Stat3 mutant mice showed enhanced inflammatory response even before colitis was developed. These findings suggest that under normal conditions, CLPMϕ become tolerant to TLR ligand stimulation, and failure to establish tolerance correlates with the development of chronic colitis.

Identification of genes that are specifically expressed in CLPMϕ

In the next set of experiments, we tried to reveal the mechanisms for differential responses to TLR ligands seen in CLPMϕ of wild-type and IL-10-deficient mice. DNA microarray analysis using mRNA purified from CLPMϕ of wild-type mice and IL-10-deficient mice led to identification of several genes that are selectively expressed in wild-type CLPMϕ, but not in IL-10-deficient CLPMϕ (data not shown). These genes include IκBNS, Bcl-3, macrophage scavenger receptor 2, and CD163. RT-PCR analysis confirmed that these genes were expressed in wild-type CLPMϕ, but not in IL-10-deficient CLPMϕ or wild-type peritoneal CD11b-positive cells (Fig. 2*A*). CD163 is a member of the scavenger receptor cysteine-rich superfamily, and was shown to be an IL-10-inducible gene in monocytes/macrophages (23–25). Bcl-3 has been shown to be induced by IL-10 in macrophages and is responsible for suppression of LPS-induced TNF-α production (22). In addition to Bcl-3, IκBNS was selectively expressed in wild-type CLPMϕ. Like Bcl-3, IκBNS is a member of the nucleus-localized IκB family proteins bearing ankyrin-repeats (26). We analyzed whether IκBNS expression is induced by IL-10 in the RAW macrophage cell line and peritoneal macrophages. Real-time RT-PCR analysis showed that IκBNS mRNA was induced within 1 h of IL-10 treatment in both RAW cells and peritoneal macrophages, indicating that like Bcl-3, IκBNS is an IL-10-inducible gene in these cells (Fig. 2, *B* and *C*). Because Bcl-3 was shown to inhibit LPS-induced TNF-α production and IκBNS is structurally related to Bcl-3, we decided to analyze the role of IκBNS in macrophages.

IκBNS inhibits IL-6 production in macrophages

To analyze the role of IκBNS in macrophages, we introduced IκBNS together with GFP into RAW264.7 cells using a lentiviral vector system (22, 27). A lentiviral vector containing GFP alone was used as control in all experiments. RAW264.7 cells were infected with lentivirus, and after 2 days of culture, GFP-positive cells were isolated by FACS sorting. Following stimulation with LPS, the production of TNF-α and IL-6 in the culture supernatants was analyzed (Fig. 3*A*). RAW cells expressing GFP alone secreted significant amounts of IL-6 in response to LPS. However, LPS-induced secretion of IL-6 was severely reduced in cells expressing IκBNS/GFP. Similar amounts of TNF-α were produced by RAW cells expressing GFP alone and IκBNS/GFP in response to LPS. We also analyzed the LPS-induced mRNA expression of IL-6, TNF-α, and IL-1β (Fig. 3*B*). Introduction of IκBNS/GFP resulted in severely impaired LPS-induced IL-6 mRNA expression. However, even in cells expressing IκBNS/GFP, LPS-induced mRNA expression of TNF-α and IL-1β was not impaired. Thus, lentiviral expression of IκBNS in macrophages resulted in specific inhibition of LPS-induced IL-6 production.

IκBNS associates with NF-κB p50 and enhances its DNA-binding activity

Because Bcl-3 has a regulatory function on NF-κB activity, we next examined LPS-induced NF-κB activation in cells expressing IκBNS/GFP. We first analyzed LPS-induced degradation of IκBα by Western blot analysis (Fig. 4*A*). LPS stimulation induced degradation of IκBα in cells expressing IκBNS/GFP as well as cells expressing GFP alone. LPS-induced phosphorylation of ERK1/2 and p38 was not impaired in cells expressing IκBNS/GFP, indicating that expression of IκBNS did not affect LPS signaling pathway in the cytoplasmic compartment (Fig. 4*B*). We next analyzed whether the DNA-binding activity of NF-κB was altered in cells expressing IκBNS (Fig. 4*C*). LPS stimulation predominantly enhanced DNA-binding activity of p50/p65 heterodimers in cells transduced with GFP alone. In cells expressing IκBNS/GFP, the DNA-binding activity of p50/p50 homodimers became evident even before LPS stimulation, and the DNA-binding activity of

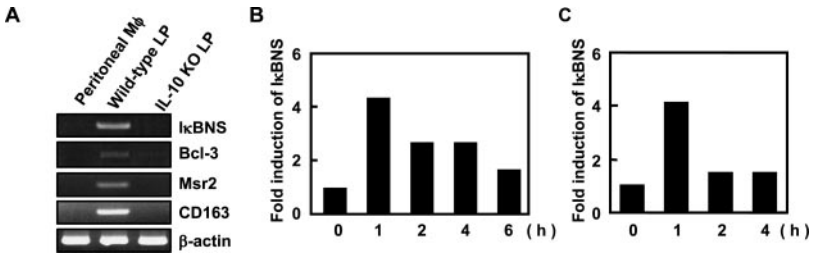

FIGURE 2. Identification of genes that are selectively expressed in CLPMϕ. *A*, Total RNA was purified from CLPMϕ of wild-type or IL-10-deficient mice, and peritoneal CD11b-positive cells (Mϕ), and then analyzed for expression of IκBNS, IκBζ, Bcl-3, macrophage scavenger receptor 2 (Msr2), and CD163 by RT-PCR. *B* and *C*, RAW264.7 cells (*B*) and peritoneal macrophages (*C*) were treated with 10 ng/ml IL-10 for the indicated periods. IκBNS and EF1-α mRNA were measured by quantitative real-time PCR. Expression of IκBNS was normalized to housekeeping gene EF1-α. Data were expressed as relative fold induction of IκBNS compared with nontreated condition.

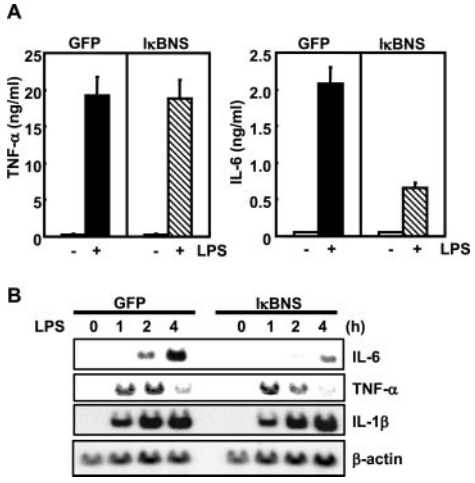

FIGURE 3. Lentiviral introduction of IκBNS results in impaired LPS-induced IL-6 production in macrophages. *A*, RAW cells were infected with lentivirus expressing IκBNS/GFP or GFP alone. After infection for 24 h, RAW cells were washed and additionally incubated for 2 days. Then, GFP-positive cells were purified by FACS sorting, and stimulated with 100 ng/ml LPS for 24 h. Concentrations of TNF-α and IL-6 in the culture supernatants were determined by ELISA. *B*, GFP-positive cells (GFP alone and IκBNS/GFP) were purified, stimulated with LPS (100 ng/ml) for the indicated period, and then total RNA extracts were analyzed for the mRNA expression of IL-6, TNF-α, and IL-1β. Hybridization with the β-actin probe confirmed even loading of RNA in each lane.

p50/p50 homodimers remained dominant even after LPS stimulation. The specificity of the bands was confirmed by supershifts using anti-p50 and anti-p65 Abs. Thus, cells expressing IκBNS showed altered DNA-binding activity of NF-κB. Previous studies showed that Bcl-3 preferentially interacted with p50 subunit of NF-κB (28). Therefore, we next analyzed whether IκBNS associates with NF-κB. RAW cells were lentivirally introduced with Flag-tagged IκBNS and subjected to coimmunoprecipitation analysis using Abs that detect endogenous p50 or p65 subunit (Fig. 4*D*). Flag-tagged IκBNS that coimmunoprecipitated with p50, but not p65, was detected by anti-Flag Ab (Fig. 4*D*, *left*). Conversely, p50, but not p65, coimmunoprecipitated with IκBNS (Fig. 4*D*, *right*). Thus, these findings indicate that like Bcl-3, IκBNS specifically associates with p50 subunit of NF-κB in macrophages.

IκBNS inhibits LPS-induced activation of the IL-6 promoter

We next analyzed the mechanism by which IκBNS specifically inhibits IL-6 production in macrophages. We first examined the effect of transient overexpression of IκBNS on LPS-induced activation of the IL-6 and TNF-α promoters using a reporter gene assay. Ectopic expression of IκBNS suppressed LPS-induced transcriptional activity of the IL-6 promoter in RAW cells in a dose-dependent manner (Fig. 5*A*). In contrast, IκBNS expression had no effect on LPS-induced transactivation of the TNF-α promoter (Fig. 5*B*). Thus, IκBNS has an inhibitory effect on the LPS-induced activation of the IL-6 promoter, but not the TNF-α promoter.

We next performed ChIP assays to further investigate how IκBNS specifically regulates IL-6 promoter activity. RAW cells constitutively expressing Flag-IκBNS were stimulated with LPS for 1 or 2 h, and ChIP assays were performed using Abs that detect Flag or endogenous p50 and p65 (Fig. 5*C*). In RAW cells expressing IκBNS, both IκBNS and p50 were recruited to the IL-6 promoter before LPS stimulation. The recruitment of IκBNS and p50 to the IL-6 promoter was stably observed even after LPS stimulation. Furthermore, LPS-induced recruitment of p65 to the IL-6 promoter was reduced in RAW cells expressing IκBNS. In contrast, IκBNS was not recruited to the TNF-α promoter, and LPS-induced recruitment of p50 and p65 was normally observed at the TNF-α promoter, indicating that IκBNS expression did not have any effect on the TNF-α promoter. Taken together, these results suggest that IκBNS suppresses the IL-6 promoter activity by selective recruitment to the IL-6 promoter with NF-κB p50.

We addressed whether the altered recruitment of p50 and p65 to the IL-6 promoter was observed in IL-10-pretreated primary macrophages, in which IκBNS expression was induced. Peritoneal macrophages from wild-type mice were pretreated with IL-10 for 18 h, then stimulated with LPS for 1 h, and analyzed by ChIP assay (Fig. 6). In nonpretreated macrophages, recruitment of p50 and p65 to the IL-6 promoter was observed only after LPS stimulation. However, in IL-10 pretreated cells, p50 was recruited to the IL-6 promoter even before LPS stimulation and LPS-induced recruitment of p65 was severely impaired. In addition, IL-10-mediated alteration of NF-κB recruitment to the IL-6 promoter was not observed in Stat3-deficient macrophages, in which IL-10 signaling was abolished. Thus, IκBNS-mediated alteration of NF-κB recruitment to the IL-6 promoter correlates with changes mediated by IL-10 in macrophages, indicating that IκBNS mediates IL-10-induced inhibition of IL-6 production.

Inhibition of IκBNS expression results in increased IL-6 production in macrophages

To further clarify the involvement of IκBNS in suppression of LPS-induced IL-6 production in macrophages, we used siRNA to block expression of IκBNS in RAW cells. RAW cells were transfected with control (nonspecific) siRNA or IκBNS siRNA. After transfection, the cells were stimulated with LPS and analyzed for expression of IκBNS, IL-6, and TNF-α. IκBNS mRNA expression was induced by LPS as well as IL-10 in RAW cells (Fig. 7*A*). Introduction of IκBNS siRNA resulted in reduced IκBNS mRNA expression to ~30% (Fig. 7*A*). In these cells, LPS-induced TNF-α mRNA expression was not significantly altered. However, LPS-induced IL-6 mRNA expression was increased to ~200% of the level found in control cells. LPS-induced production of TNF-α and IL-6 was also analyzed by ELISA (Fig. 7*B*). IκBNS knockdown in RAW cells had no effect on LPS-induced TNF-α production. However, in cells transfected with IκBNS siRNA, LPS-induced IL-6 production was increased by about 2-fold compared with cells transfected with control siRNA. We also analyzed activity of the IL-6 promoter in cells transfected with IκBNS siRNA (Fig. 7*C*). LPS-induced activation of the IL-6 promoter, but not the TNF-α promoter, was increased in cells with reduced IκBNS expression. Thus, siRNA-mediated reduction of IκBNS expression in macrophages enhanced LPS-induced activation of the IL-6 promoter and production of IL-6. Taken together, these data demonstrate that IκBNS negatively regulates LPS-induced IL-6 production in macrophages.

Discussion

In this study, we first characterized CLPMϕ. Several studies in animal models of IBD and human IBD patients indicate that cells of macrophage lineage play an important role in intestinal mucosal immune responses. Aberrant activation of macrophages due to the absence of Stat3 led to the development of chronic colitis (12). Increased CD40L-induced production of IL-12 in mucosal dendritic cells was also demonstrated in mice with colitis (29). In humans, mucosal macrophages from IBD patients showed higher expression of several surface molecules such as CD14, CD16, and HLA-DR (30–32). Furthermore, mucosal macrophages from IBD patients showed enhanced activity, such as the release of oxygen

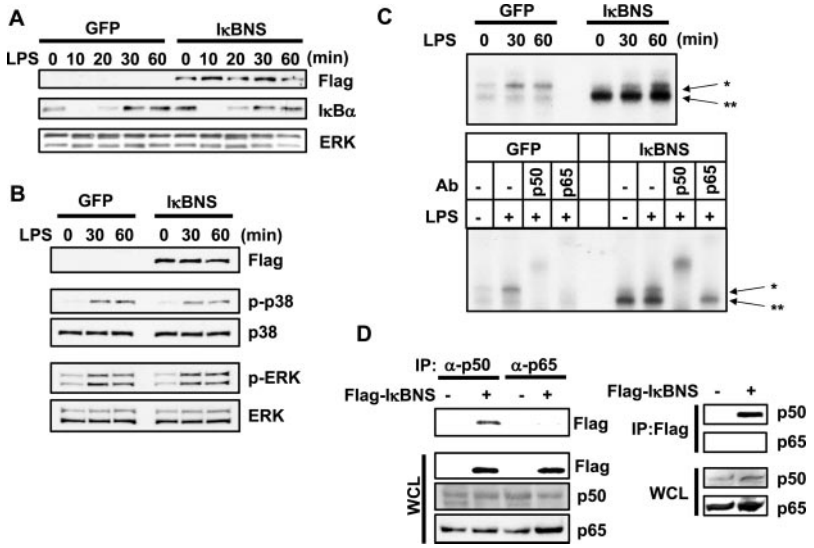

FIGURE 4. Impaired NF-κB activity in IκBNS-introduced macrophages. *A*, GFP-positive cells (GFP alone and IκBNS/GFP) were purified, stimulated with LPS (100 ng/ml) for the indicated period (min), and the total cell lysates were analyzed for expression of Flag-IκBNS, IκBα, and ERK by Western blot analysis. *B*, Western blots showing the extent of phosphorylation of p38 and ERK MAPKs at the indicated time period after LPS stimulation in RAW cells expressing GFP alone or IκBNS/GFP. *C*, RAW cells introduced with GFP alone or IκBNS/GFP were stimulated with 100 ng/ml LPS for the indicated time period. Nuclear extracts were subjected to EMSA using the NF-κB binding site of the IL-6 promoter as a probe. The specificities of the shifted bands were determined by adding specific Abs to p50 and p65. Two types of NF-κB bindings to the probe, p50/p65 heterodimer (*) and p50/p50 homodimer (**), are indicated. *D*, RAW cells introduced with GFP alone or IκBNS/GFP were lysed and immunoprecipitated (IP) with anti-p50 or anti-p65 Abs (*left*). The immunoprecipitated lysates were subsequently immunoblotted with anti-Flag Ab. The same lysates were immunoprecipitated with anti-Flag Ab, and blotted with anti-p50 or anti-p65 Abs (*right*).

radicals and mature IL-1β, and presentation of Ag (33–35). Thus, enhanced activity of mucosal macrophages is associated with the pathogenesis of colitis.

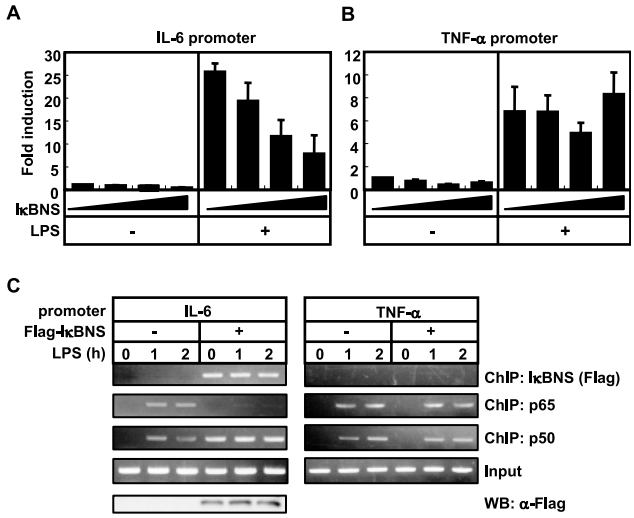

FIGURE 5. IκBNS is specifically recruited to the IL-6 promoter. *A* and *B*, RAW cells were transiently cotransfected with the IL-6 promoter-luciferase or TNF-α promoter-luciferase construct (10 ng), together with an IκBNS expression vector (0, 0.05, 0.25, or 0.5 μg) as indicated. After 24 h of transfection, cells were treated with or without 10 ng/ml LPS for 6 h and then the luciferase activities were measured. The data are representative of three independent experiments yielding similar results. Data are expressed as relative fold activation compared with the nonstimulated (−) set. *C*, RAW cells that constitutively express Flag-IκBNS were stimulated with 100 ng/ml LPS for 1 or 2 h, and ChIP assays were performed with anti-Flag, anti-p50, or anti-p65 Abs. The immunoprecipitated IL-6 promoter (*left*) or TNF-α promoter (*right*) was detected by PCR with promoter-specific primers. Data are representative of three independent experiments. The expression of Flag-IκBNS by Western blot analysis is shown at *bottom*.

Recently, TLRs have been shown to be essential for activation of innate immune cells, such as macrophages and dendritic cells, through the recognition of microbial components (8–11). Involvement of TLR-dependent responses in the pathogenesis of IBD has further been postulated because chronic colitis was greatly reduced in mice lacking Stat3 and TLR4 (15). In the present study, we demonstrated that CLPMϕ in normal mice, unlike CD11b-positive cells from the spleen, are refractory to TLR stimulation in terms of inflammatory cytokine production and costimulatory molecule expression. Furthermore, CLPMϕ from IL-10-deficient or Stat3 mutant mice, even in the absence of colitis, showed significant responses to TLR stimulation, indicating that the responsiveness of CLPMϕ to TLR ligands correlates with the pathogenesis of chronic colitis in these mutant mice.

We further analyzed the molecular basis for the hyporesponsiveness of CLPMϕ by comparing gene expression profiles of CLPMϕ from wild-type mice and IL-10-deficient mice. DNA microarray analysis led to the identification of several genes that are selectively expressed in CLPMϕ of wild-type mice, but not in peritoneal macrophages or CLPMϕ of IL-10-deficient mice. Among these genes, CD163 and Bcl-3 were reported to be induced by IL-10 in macrophages or monocytes (22, 23, 36). In addition, we found that IκBNS is selectively expressed in CLPMϕ of wild-type mice, and further is induced by IL-10 in peritoneal macrophages and RAW cells. IκBNS was originally identified as a gene that is induced upon TCR stimulation and affects NF-κB activity in T cells (26). Although IκBNS was suggested to be involved in negative selection of thymocytes, the role of IκBNS in macrophages remained unclear. In addition, although IκBNS was shown to be localized in the nucleus and preferentially associates with p50 subunit of NF-κB, the mechanism by which IκBNS inhibits NF-κB activity remains unknown. Because IκBNS is structurally related to Bcl-3, we postulated that IκBNS has a regulatory role in macrophages, and analyzed the effect of IκBNS expression in the RAW macrophage cell line. Lentiviral expression and siRNA-mediated knockdown of IκBNS in RAW cells demonstrated

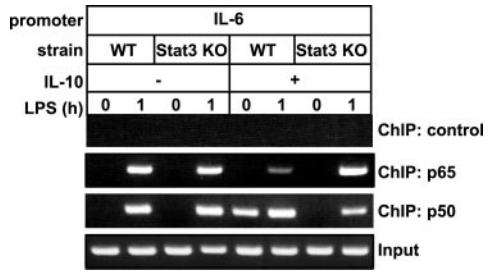

FIGURE 6. IL-10-induced alteration in recruitment of p50 and p65 to the IL-6 promoter. Peritoneal macrophages from wild-type or Stat3 mutant mice were cultured for 18 h in the presence or absence of 10 ng/ml IL-10, then stimulated with 100 ng/ml LPS for 1 h, and ChIP assays were performed with anti-p50, anti-p65 Abs, or control rabbit Ig. The immunoprecipitated IL-6 promoter was detected by PCR. Representative of three independent experiments.

that this molecule suppresses LPS-induced IL-6 production. Similar to the case of Bcl-3, which is specifically recruited to the TNF-α promoter together with the p50 subunit of NF-κB thereby inhibiting LPS-induced TNF-α production (22), IκBNS suppressed LPS-induced activation of the IL-6 promoter through constitutive recruitment to the promoter together with p50. Thus, nuclear IκB proteins Bcl-3 and IκBNS differentially modulate TLR-mediated gene induction in macrophages.

IκBNS and Bcl-3 are differentially recruited to specific promoters through association with p50 homodimers. But, it remains unclear how the specific recruitment of these nuclear IκB proteins are induced. Nuclear IκB proteins do not seem to have the ability to directly bind the promoters. Therefore, association with p50 may cause modulation of the DNA binding specificity through unknown mechanisms. Similar specific recruitment was also demonstrated in IκBζ, which is structurally related to IκBNS and Bcl-3 (21, 37–39). In the case of IκBζ, the specific recruitment to the IL-6 promoter led to activation of the promoter (21, 37). Thus, several lines of evidence demonstrate that nuclear IκB proteins specifically regulate expression of NF-κB target genes.

In normal condition, both IκBNS and IκBζ have regulatory roles in the production of IL-6 in macrophages. IκBNS suppresses LPS-induced IL-6 production, whereas IκBζ promotes IL-6 production (21). Thus, although the ankyrin-repeats of both molecules are highly conserved, they have opposite functions. IκBNS consists of 327 amino acids and the ankyrin-repeat covers almost the entire protein. In contrast, IκBζ is a protein with 629 amino acids and possesses an N-terminal region of ~300 amino acids in addition to the C-terminal ankyrin-repeat. The full-length IκBζ has no inhibitory effect on NF-κB activity, but introduction of the C-terminal ankyrin-repeat of IκBζ resulted in impaired NF-κB activation (37). Thus, the ankyrin-repeat of IκBζ has an inhibitory effect on NF-κB activity, but the N-terminal region may activate NF-κB with unknown mechanisms.

IκBNS and Bcl-3 are expressed in CLPMϕ of wild-type mice, but not the CLPMϕ of IL-10-deificient mice. In addition, expression of IκBNS and Bcl-3 is induced by IL-10 treatment in peritoneal macrophages and RAW cells. Therefore, expression of IκBNS and Bcl-3 in CLPMϕ of wild-type mice might be induced by IL-10. Indeed, constitutive production of IL-10 was observed in CLPMϕ of wild-type mice. Additionally, IL-10 might be provided from other types of cells such as IL-10-producing type 1 regulatory T cells or epithelial cells. There might be other explanations of the characteristics of CLPMϕ of wild-type mice. IκBNS and Bcl-3 were also found to be induced by LPS stimulation, but their expression was not observed in CLPMϕ of IL-10-deficient or Stat3 mutant mice. Therefore, CLPMϕ of wild-type mice might be a distinct cell population from macrophages residing in other tissues, and IL-10 might be involved in the development of these unique CLPMϕ or in the recruitment of CLPMϕ to the colonic lamina propria. Constitutive expression of both IκBNS and Bcl-3 in CLPMϕ may suppress exaggerated inflammatory responses in the intestinal mucosal surface. A more precise characterization of

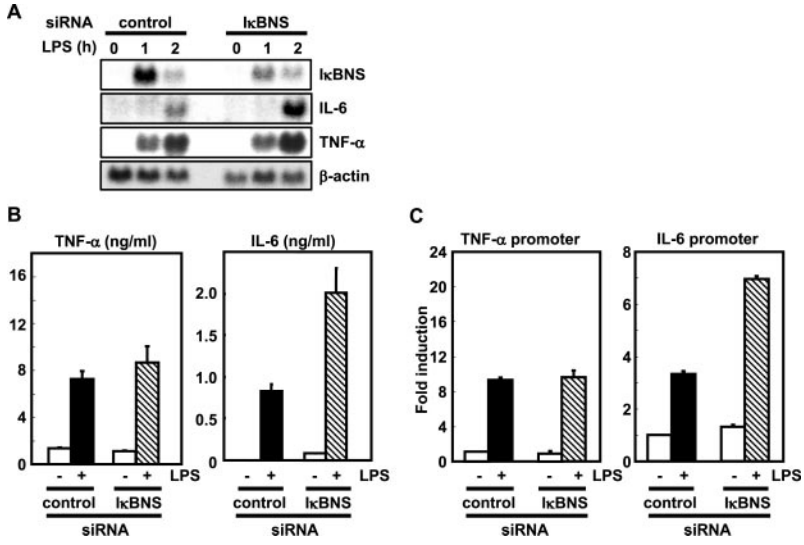

FIGURE 7. IκBNS is required for suppression of LPS-induced IL-6 expression. *A*, RAW cells were transfected with 500 pmol IκBNS siRNA or control siRNA as indicated. Three hours later, the cells were stimulated with 100 ng/ml LPS for 1 or 2 h. Total RNA was prepared and analyzed for expression of IκBNS, IL-6, TNF-α, and β-actin by Northern blotting. Data are representative of three independent experiments. *B*, After transfection of IκBNS siRNA or control siRNA, RAW cells were stimulated with 100 ng/ml LPS for 24 h. Concentrations of TNF-α and IL-6 in the culture supernatants were determined by ELISA. *C*, RAW cells were transfected with 500 pmol IκBNS siRNA or control siRNA together with 100 ng of IL-6 or TNF-α promoter reporter plasmid. Three hours after transfection, the cells were stimulated with 100 ng/ml LPS for 6 h, and the luciferase activities were measured. Representative of three independent experiments yielding similar results. Data are expressed as relative fold activation compared with the nonstimulated (−) set.

CLPMϕ of wild-type mice will be required to answer to this question.

CLPMϕ are tolerant to TLR stimulation and show no inflammatory cytokine production. However, Bcl-3 and IκBNS are likely to be involved in suppression of the specific cytokine such as TNF-α and IL-6, respectively. Therefore, there might be other mechanisms that suppress TLR responses. NOD2, a member of the NOD family of proteins, might be involved in one of such mechanisms. NOD2 is implicated in the negative regulation of TLR2-mediated NF-κB activation (40). Mutations in NOD2 have been shown to be associated with Crohn's diseases (16, 17).

In this study, we showed that nuclear IκB proteins, such as Bcl-3 and IκBNS, differentially modulate LPS-induced inflammatory cytokine production in macrophages. CLPMϕ in wild-type mice are tolerant to TLR stimulation, which may contribute to prevention of chronic intestinal inflammation. Several mechanisms are involved in the hyporesponsiveness of CLPMϕ, and selective inhibition of LPS responses by nuclear IκB proteins may explain a part of these mechanisms. Further studies will be required to understand regulations of CLPMϕ activity, which will definitely be useful for the development of an effective cure for IBD.

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Disclosures

The authors have no financial conflict of interest.

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