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ORIGINAL RESEARCH

N4BP3 Activates TLR4-NF-κB Pathway in Inflammatory Bowel Disease by Promoting K48-Linked IκBα Ubiquitination

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Purpose: N4BP3 is a ubiquitination-related gene that plays a pivotal role in neurology and neoplasia. Studies have demonstrated its essential function in axonal and dendritic branching, promoting hepatocellular carcinoma and breast cancer. Our previous research reveals that N4BP3 enhances inflammatory responses by modulating the NOD2 signaling pathway. It is crucial to investigate whether N4BP3 regulates inflammatory bowel disease (IBD) through the TLR4 signaling pathway and to elucidate the underlying mechanisms.

Methods: Lipopolysaccharides (LPS) were used to activate the TLR4 pathway in THP-1/Caco-2 cells. THP-1/Caco-2 cells were transfected with either N4BP3 overexpression or knockdown plasmids, generating N4BP3-overexpressing or N4BP3-deficient cell lines. For in vivo studies, colitis was induced in mice using dextran sodium sulfate (DSS). Additionally, negative control and N4BP3-knockdown C57BL/6 mouse models were established via intraperitoneal injection of control or N4BP3-targeting adeno-associated virus (AAV).

Results: LPS stimulation significantly upregulated N4BP3 expression in THP-1/Caco-2 cells compared to sterile water treatment ($P < 0.05$). In N4BP3-overexpressing cells, LPS induction led to significantly higher expression of TNF-α, IL-1β, IL-6, and IL-8 mRNA, as well as phospho-NF-κB p65 protein, compared to wild-type THP-1/Caco-2 cells ($P < 0.05$). Conversely, these inflammatory markers were markedly downregulated in N4BP3-knockdown THP-1 cells following LPS stimulation ($P < 0.05$). In DSS-induced colitis models, N4BP3-knockdown mice showed decreased phospho-NF-κB p65 but increased IκBα protein expression in colonic tissues compared to DSS-treated control mice ($P < 0.05$). Furthermore, we observed interaction between N4BP3 and IκBα, with N4BP3-overexpressing THP-1 cells demonstrating significantly elevated K48-linked ubiquitination levels versus controls.

Conclusion: LPS upregulates N4BP3 expression, which subsequently enhances K48-linked ubiquitination of IκBα, leading to NF-κB pathway activation, and exacerbating IBD progression. These findings suggest N4BP3 as a potential therapeutic target for developing novel IBD treatments.

Keywords: N4BP3, ubiquitination, TLR4, NF-κB, IκBα, inflammatory bowel disease

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Introduction

Inflammatory bowel disease (IBD) is a slow-acting nonspecific intestinal inflammatory disease with a growing prevalence.[1] Although the pathogenesis of IBD has been extensively studied, uncertainties remain to be clarified. Earlier research has shown that IBD is the result of a combination of factors, including susceptibility genes, the immune system, and host microorganisms,[2–5] with intestinal immune disorders playing an important role.[6]

Activation of Toll-like receptors (TLRs) has been demonstrated to promote the intestinal immune imbalance and the progress of IBD.[7] Structurally, TLRs are type I transmembrane glycoproteins characterized by leucine-rich repeat (LRR) domains that recognize both microbe-associated molecular patterns (MAMPs) and damage-associated molecular patterns (DAMPs).[8] In addition, TLRs also contain a structural domain similar to the interleukin I receptor family (TIR), which has been recognized to initiate downstream signaling pathways.[8] Among various TLR subtypes, TLR1-10 is universally expressed in mammals. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are expressed in cell membranes, while TLR3, TLR7, TLR8, and TLR9 are expressed in organelle membranes.[9] Of particular relevance, TLR4 is predominantly expressed on the plasma membrane of monocytes, macrophages, and dendritic cells.[10] It recognizes molecular lipopolysaccharides (LPS) from the bacterial cell wall, then initiates MyD88-dependent intracellular signaling,[11,12] and further activates the signaling molecules IRAK4 and IRAK1, TRAF6, TAK1 and TAB2/3, NEMO, and IKKα/β. Eventually, TLR4 can induce IκBα degradation and release NF-κB into the nucleus (ie, the NF-κB pathway) or activate the MAPK pathway, which stimulates cytokine release and promotes inflammation.[13]

The NF-κB signaling pathway is an important cellular signaling pathway that plays a key role in a variety of physiological and pathological processes in cells. This pathway consists of two distinct branches: the canonical (classical) and non-canonical (alternative) pathways. IκBα is a protein kinase that binds to p50/p65 heterodimers and p65 homodimers in the cytoplasm.[14] IκBα can be degraded by ubiquitinating enzymes, which can lead to the p65 proteins entering into the nucleus, initiating the synthesis of proinflammatory cytokines, and activating the NF-κB signaling pathway.[15] The activation of NF-κB signaling pathway drives the inflammatory pathologies of IBD.[16]

The ubiquitination ligase NEDD4 binding protein 3 (N4BP3) has been identified as an important gene affecting neural development in earlier studies.[17,18] Recently, it has also been associated with multiple tumor diseases, including cervical cancer,[19] hepatocellular carcinoma,[20,21] tongue cancer,[22] breast cancer,[23] hypoxic diseases,[24] and antiviral immunity.[25] In breast cancer, N4BP3 mediates the ubiquitinated degradation of E-calmodulin,[23] and in antiviral immunity, it was demonstrated to interact with and promote the ubiquitination of MAVS.[25]

Ubiquitination is a post-translational modification of proteins.[26] This enzymatic process forms a heteropeptide bond between the lysine residue of a substrate protein and the carboxyl group of glycine on a ubiquitin protein through a series of processes.[27] Thus, ubiquitination can regulate the degradation, transport, and signaling of substrate proteins.[28] Usually, K27-linked ubiquitination is involved in DNA damage responses; K29-linked ubiquitination is an inhibitor of Wnt signaling; K33-linked ubiquitination is associated with protein transport; K48-linked ubiquitination and K11-linked ubiquitination promote the degradation of proteasomes; K63-linked ubiquitination and M1-linked ubiquitination play an important role in the activation of the NF-κB signaling pathway.[26] In addition, ubiquitination modifications can modulate TLR4 receptor-mediated signaling.[29] For example, the ubiquitin ligase TRAF6 can lead to polyubiquitination of its own lysine 63 linkage, thereby recruiting TAK and TAB proteins.[30] Pellino can bind with IRAK1 and cause it to undergo lysine 63-linked polyubiquitination.[31]

Our previous study revealed that N4BP3 activates the NOD2-MAPK/NF-κB pathway in IBD by promoting polyubiquitination of the lysine 63 linkage of the RIPK2 protein.[32] In the study, we tested the expression of N4BP3 in colon tissues of IBD patients and control people. The result showed that N4BP3 expression was significantly higher in the colon of IBD patients than in controls. We constructed N4BP3-overexpressed THP-1 cells and induced them with muramyl dipeptide (MDP). The results showed that MDP-induced TNFα, IL-8 mRNA and phospho (P)-P65, P-ERK, P-JNK, and P-P38 proteins were significantly elevated in THP-1 cells after overexpressing N4BP3. We constructed N4BP3 knockdown C57BL/6 mice and fed them with dextran sodium sulfate (DSS). The results showed that DSS-induced colitis was significantly reduced in mice after knocking down N4BP3. We demonstrated that N4BP3 interacts with RIPK2 and promotes its K63-linked ubiquitination with immunoprecipitation experiments.[32] In this paper, we aimed to further investigate the relationship between N4BP3 and TLR4 in IBD.

Materials and Methods

Cell Culture

The THP-1 cell line was purchased from Procell Life Science & Technology Co., Ltd. and cultured in the RPMI 1640 medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S). The Caco2 cell line was purchased from Suzhou Haixing Biosciences Co., Ltd. and cultured in the DMEM medium containing 10% FBS and 1% P/S. All cells were cultured in an incubator at 5% CO_2, 95% O_2 and 37°C.

Receptor Activator

LPS (TLR4 activator) was purchased from MedChemExpress (MCE) corporation (Shanghai, China). Pam3csk4 (TLR1/2 activator) was purchased from MedChemExpress (MCE) corporation (Shanghai, China).

Plasmid Transfection

1) Place an appropriate number of cells in 12-well cell culture plates and wait until the cell confluence reaches 70% to be used for transfection. 2) Prepare the transfection mixture (per well): add 1.5 μL of Lipo3000 (Thermo Scientific, USA) into 25 μL of MEM medium; add 500 ng of DNA plasmid (Public Protein/Plasmid Library, China) and 1 μL of P3000 (Thermo Scientific, USA) into 25 μL of MEM medium; mix the solutions well and rest for 13 minutes. 3) Add the transfection mixture prepared into cell culture plates and perform transfection for 24–72 hours (h).

Both N4BP3 overexpression and knockdown plasmids were purchased from the Public Protein/Plasmid Library. N4BP3 knockdown plasmid sequence: sh1: CGCTTTGACAAGTGCCGCATT; sh2: GCAACGGGAAAGGCTTCCTAT; sh3: GCCAGAAGACAGCAGAGATTA; NC: GTTCTCCGAACGTGTCACGTT. The efficiency of N4BP3 overexpression or knockdown in cells was verified by detecting N4BP3 mRNA or protein expression.

Mouse Model

C57BL/6 mice were randomly allocated into four experimental groups: 1) Blank control group: received sterile drinking water ad libitum; 2) DSS control group: drank 5% DSS solution for 6 days; 3) N4BP3 knockdown group: injected intraperitoneally with N4BP3-targeting adeno-associated virus (AAV-shN4BP3) followed by 5% DSS treatment for 6 days; 4) Negative control group: injected intraperitoneally with negative control AAV (AAV-scramble) followed by 5% DSS treatment for 6 days. All mice were maintained on standard rodent chow throughout the experiment. Three weeks post-viral injection, N4BP3 knockdown efficiency was confirmed by immunohistochemical analysis of colonic tissue sections.

Real-Time Fluorescence Quantitative PCR

1) Collect the cells and mix with 1 mL of Trizol thoroughly. 2) Add 200 μL of trichloromethane into the cells and shake vigorously, then perform centrifugation. 3) Collect 500 μL of supernatant and mix it with 500 μL of isopropanol gently, then perform centrifugation. 3) Discard the supernatant, add 500 μL of anhydrous ethanol, and perform centrifugation. 4) Discard the supernatant, add 10–20 μL of sterile and enzyme-free water. 5) Reversely transcribe the RNA to cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, K1622, USA). 6) Add 5 μL of cDNA, 10 μL of qMIX (Roche, 06924204001, USA), 2 μL of primer, and 3 μL of ultrapure water to eight consecutive tubes, mix well, and place the tubes in a fluorescence quantitative PCR instrument for detection. 7) Analyze the data for Cq values.

Primer sequences (5′→3′): GAPDH Forward primer: AATCCCATCACCATCTTCCA; GAPDH Reverse primer: CCTGCTTCACCACCTTCTTG. N4BP3 Forward primer: AACGAGCCTGCCGACTATG; N4BP3 Reverse primer: ACTTTGCATGGATAGGAAGCC. TNFα Forward primer: AAGCCTGTAGCCCATGTTGT; TNFα Reverse primer: AGTCGGTCACCCTTCTCCA. IL-1β Forward primer: GACGGACCCCAAAAGATGAA; IL-1β Reverse primer: CAGCCACGAGGCTTTTTGTT. IL-8 Forward primer: TTGGCAGCCTTCCTGATTT; IL-8 Reverse primer: TCAAAAACTTCTCCACAACCC.

Immunoprecipitation and Immunoblotting

Immunoprecipitation: 1) Collect cells, add lysate and cocktail to enable full lysis, and perform centrifugation. 2) Take 500 μL of supernatant and mix thoroughly with antibody, then suspend at 4°C for 4–6 h. 3) Add 500 μL of suspension into magnetic beads and suspend for another 4–6 h to obtain the antigen-antibody-magnetic bead mixture. 4) Add an appropriate amount of loading buffer and heat in a thermostat at 100 °C for 5 min. 5) After cooling, collect the loading buffer for immunoblotting.

Immunoblotting/Western blot: 1) Assemble the electrophoresis apparatus properly, connect the power supply after sample loading, and set the program as 160mv, 45 minutes. 2) Place the gel and PVDF membrane after electrophoresis in the transfer apparatus, connect the power supply, and set the program as 200mA, 58 minutes. 3) Wash the PVDF membrane with PBST for 10 minutes and then immerse the PVDF membrane in the protein-free rapid closure solution for 15 min on a shaker. 4) Wash the PVDF membrane with PBST for another 10 minutes and then immerse the PVDF membrane in the primary antibody at 4 °C for 10 h. 5) Wash the PVDF membrane with PBST three times for 10 minutes each, and then immerse the PVDF membrane in the secondary antibody for 50 minutes on a shaker. 6) Wash the PVDF membrane with PBST three times for 10 minutes each. 7) Add an appropriate amount of developing solution dropwise to the PVDF membrane, and develop and fix with a chemiluminescence instrument. 8) Continue with detection of other antibodies after the antibody has been eluted with the antibody stripping solution.

Antibodies and dilutions: GAPDH (Abcam, ab9484, 1:5000), N4BP3 (Proteintech, 16733-1-AP, 1:600), IκBα (Abways, CY5026, 1:1000), P-P65 (CST, 3033, 1:1000), p-P38 (CST, 4511, 1:1000), P-ERK (CST, 4370, 1:2000), P-JNK (CST, 4668, 1:1000), TLR4 (Bioswamp, PAB47910, 1:600), MyD88 (Abways, CY5681, 1:1500), TRAF6 (Bioswamp, RMAB50064, 1:600), IRAK1 (Abways, CY6917, 1:600), IRAK4 (Abways, CY6916, 1:600), TAK1 (Abways, CY7057, 1:1000), NEMO (Abways, CY6843, 1:1000), TNFα (Abcam, ab307164, 1:1000), IL-1β (Abcam, ab283818, 1:1000).

Quantitative analysis of Western blot bands using ImageJ. 1) Launch ImageJ and open the target image file. 2) Convert image to 8-bit format (Image → Type → 8-bit). 3) Perform background subtraction: navigate to "Process" → "subtract background", select "Light background" option. 4) Configure measurement parameters: Select "Analyze" → Set "Measurements", Check: "Area, Mean gray value, Integrated density". 5) Set scale unit to pixels (Analyze → Set Scale → Unit: pixels). 6) Invert image (Edit → Invert). 7) Band quantification: use the rectangular box selection tool to select each band of the immunoblot; Click "Analyze" → "Measure", then integrated density (IntDen) values can be obtained. Protein expression data were normalized by dividing the target protein's IntDen value by that of the loading control.

Immunohistochemical Staining

1) Fix the paraffin sections of embedded colon on slides and heat in a 55 °C oven for 30 minutes. 2) Immerse the sections in xylene for 10 minutes, and then sequentially in anhydrous ethanol, 95% ethanol, 85% ethanol, and distilled water for 5 minutes each. 3) Further immerse the sections in 3% H_2O_2 for 10 minutes. 4) Immerse the sections in the antigen repair solution and heat in a microwave oven. 5) After drying, draw a circle around the outer edge of the tissue, add a drop of sealing solution, and rest for 30 minutes. 6) Discard the sealing solution, add a drop of anti-IκBα (Abways, CY5026, 1:3200) or P-P65 (Epizyme, R013789, 1:3200), and rest at 4°C overnight. 7) Immerse the sections in PBST for 5 minutes, then add the secondary antibody dropwise, and rest at 37 °C for 30 minutes. 8) Immerse the sections in PBST for 5 minutes, and after air-drying, add the DAB color development solution dropwise for 5–15 seconds, followed by double-distilled water. 9) Rinse the sections with water, add a few drops of hematoxylin, rest for 1 minute, and then rinse with water. 10) Immerse the sections sequentially in 85% ethanol, 95% ethanol, and anhydrous ethanol for 4 minutes each and in xylene for 2 minutes. 11) Add a few drops of neutral gum, cover the coverslips, and air-dry the sections for subsequent observation and analysis.

Data Analysis

All experiments in this paper were repeated at least three times. The statistical differences between each two groups were compared using a *t*-test, while the statistical differences between more than two groups were compared using one-way ANOVA. $P < 0.05$ indicated statistical significance. The results were plotted using GraphPad Prism 8.0.1.

Results

N4BP3 Expression Is Upregulated After Activation of TLR4 by LPS

To investigate LPS-induced N4BP3 expression, THP-1 cells were treated with 1 μg/mL LPS for different times (0, 1, 3, 6, 12, and 24 h). Quantitative real-time PCR (qPCR) analysis revealed significant upregulation of N4BP3 mRNA levels at 1, 3, and 6 h post-induction compared to untreated controls (0 h) ($P < 0.05$), with peak expression observed at 3 h (Figure 1A).

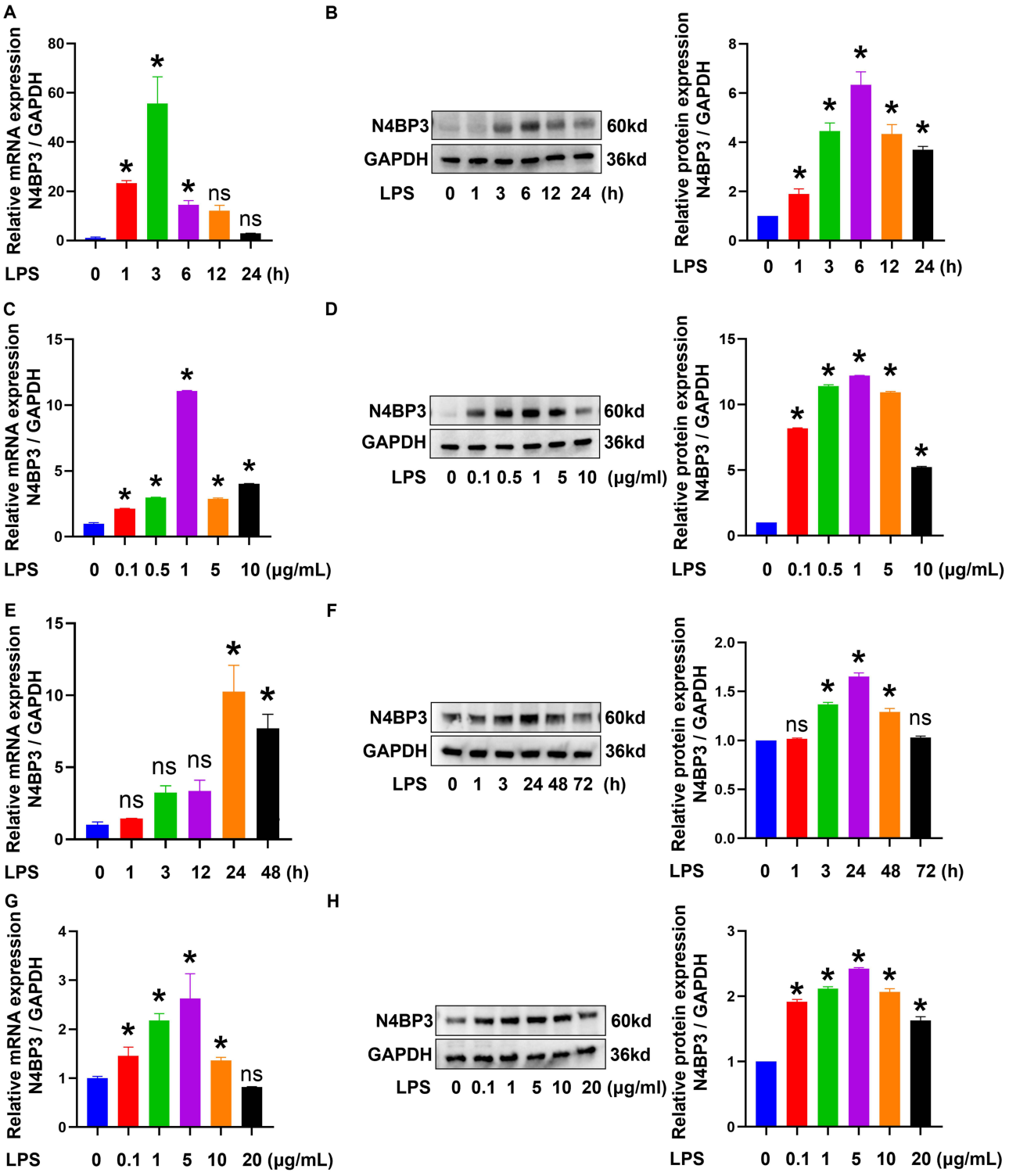

Figure 1 Continued.

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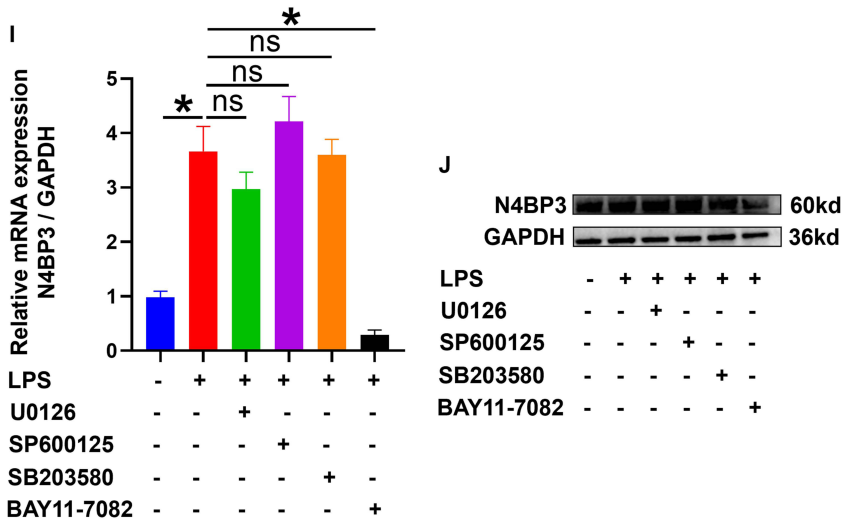

Figure 1 Changes in N4BP3 expression after inducing THP-1/Caco2 cells with LPS. (**A** and **B**) Changes in the expression of N4BP3 mRNA (**A**) and protein (**B**) after inducing THP-1 cells with LPS (1 μg/mL) for different times. (**C** and **D**) Changes in the expression of N4BP3 mRNA (**C**) and protein (**D**) after inducing THP-1 cells with different concentrations of LPS for 3 h. (**E** and **F**) Changes in the expression of N4BP3 mRNA (**E**) and protein (**F**) after inducing Caco2 cells with LPS (10 μg/mL) for different times. (**G** and **H**) Changes in the expression of N4BP3 mRNA (**G**) and protein (**H**) after inducing Caco2 cells with different concentrations of LPS for 24 h. Statistical analysis using paired *t*-test. *P < 0.05 represents a significant difference between the group below the asterisk (*) indicator and the control group (the first group). Indicator "ns" representing no significant difference between the group below the indicator "ns" and the control group (the first group). The error bar represents the mean ± SD. (**I** and **J**) Changes in the expression of N4BP3 mRNA (**I**) and protein (**J**) after inducing THP-1 cells with U0126 (ERK1/2 inhibitor), SP600125 (JNK inhibitor), SB203580 (P38 inhibitor), and BAY11-7082 (NF-κB inhibitor), respectively. Statistical analysis using One-way ANOVA. * P < 0.05, representing a significant difference between the groups at either end of the horizontal line. Indicator "ns" representing no significant difference between the groups at either end of the horizontal line. The error bar represents the mean ± SD.

Western blot analysis demonstrated a similar temporal pattern for N4BP3 protein expression, showing significant increases at all time points (1–24 h) ($P < 0.05$), with maximal protein levels achieved at 6 h (Figure 1B).

For dose-response experiments, THP-1 cells were exposed to increasing LPS concentrations (0, 0.1, 0.5, 1, 5, and 10 μg/mL) for 3 h. qPCR analysis indicated dose-dependent N4BP3 mRNA induction, with significant upregulation observed at all tested concentrations (0.1–10 μg/mL) compared to untreated controls ($P < 0.05$). The maximal transcriptional response occurred at 1 μg/mL LPS (Figure 1C). Correspondingly, Western blot analysis revealed parallel dose-dependent increases in N4BP3 protein levels, with peak expression similarly observed at 1 μg/mL LPS (Figure 1D).

Subsequently, Caco-2 cells were treated with 5 μg/mL LPS for different times (0, 1, 3, 12, 24, 48, and 72 h). qPCR analysis revealed significant upregulation of N4BP3 mRNA levels at 24 and 48 h post-induction compared to untreated controls ($P < 0.05$), with peak expression observed at 24 h (Figure 1E). Consistent with transcriptional changes, Western blot analysis demonstrated significant increases in N4BP3 protein levels at 3, 24, and 48 h ($P < 0.05$), with maximal protein accumulation also occurring at 24 h (Figure 1F).

For dose-response assessment, Caco-2 cells were exposed to graded LPS concentrations (0, 0.1, 1, 5, 10, and 20 μg/mL) for 24 h. qPCR results showed dose-dependent N4BP3 mRNA induction, with significant upregulation at 0.1–10 μg/mL LPS ($P < 0.05$) and maximal response at 5 μg/mL (Figure 1G). Protein analysis confirmed this pattern, showing significant N4BP3 elevation across all tested concentrations (0.1–20 μg/mL) ($P < 0.05$), with peak expression again observed at 5 μg/mL LPS (Figure 1H).

THP-1 cells were pretreated with specific kinase inhibitors targeting ERK1/2 (U0126, 10 μM), JNK (SP600125, 20 μM), p38 (SB203580, 10 μM), or NF-κB (BAY11-7082, 5 μM) for 1 h prior to LPS stimulation (1 μg/mL, 1 h). qPCR and Western blot analyses revealed that BAY11-7082 pretreatment significantly attenuated LPS-induced N4BP3 expression at both mRNA (Figure 1I) and protein levels (Figure 1J) ($P < 0.05$). In contrast, inhibition of ERK1/2, JNK, or p38 signaling pathways showed no significant effect on LPS-mediated N4BP3 upregulation ($P > 0.05$).

In our previous experiments, we collected colon tissues from Crohn's disease (CD) patients and normal colon tissues (colon tissues from colon cancer patients that were 15 cm from the tumor margins), then detected the expression of

N4BP3 protein with immunohistochemical staining. The results showed that the expression of N4BP3 in the colonic tissues of the CD patients was significantly higher than that of controls.[32]

Overexpression of N4BP3 Promotes the Release of LPS-Induced Cytokines

To investigate the role of N4BP3 in TLR4 pathway activation, we established N4BP3-overexpressing cell lines by transfecting THP-1 and Caco-2 cells with N4BP3 expression plasmids. Following LPS stimulation (1 μg/mL, 4 h), qPCR analysis revealed significantly elevated expression of proinflammatory cytokines in N4BP3-overexpressing cells compared to vector controls ($P < 0.05$).

In THP-1 cells, N4BP3 overexpression markedly enhanced LPS-induced mRNA expression of TNF-α, IL-6, and IL-8 (Figure 2A–D). Similarly, in Caco-2 cells, N4BP3 overexpression significantly potentiated LPS-mediated upregulation of IL-1β, IL-6, and IL-8 mRNA levels (Figure 2E–H). These findings demonstrate that N4BP3 amplifies TLR4-dependent inflammatory responses in both THP-1 and Caco-2 cell lines by promoting cytokine production.

Knockdown of N4BP3 Inhibits the Release of LPS-Induced Cytokines

To further clarify whether and how N4BP3 mediates the pro-inflammatory effects in the TLR4 pathway, we constructed N4BP3-knockdown THP-1 cells by transfecting the cells with knockdown plasmids. Then, quantitative PCR and Western blot were performed to detect the changes in the expression of cytokine mRNAs and proteins after LPS induction. The results showed that among the three types of plasmids investigated, knocking down plasmid 1 (sh1) exhibited the best knockdown efficiency of N4BP3 in THP-1 cells (Figure 3A). Compared to untreated THP-1 cells, the expression of LPS-induced cytokines TNFα, IL-6, and IL-8 mRNAs was significantly downregulated ($P < 0.05$) in N4BP3-knockdown THP-1 cells (Figure 3B–E). In consistency, the expression of TNFα and IL-1β proteins was also significantly downregulated ($P < 0.05$) (Figure 3F). The results further indicate that N4BP3 can mediate the TLR4 pathway to promote inflammation.

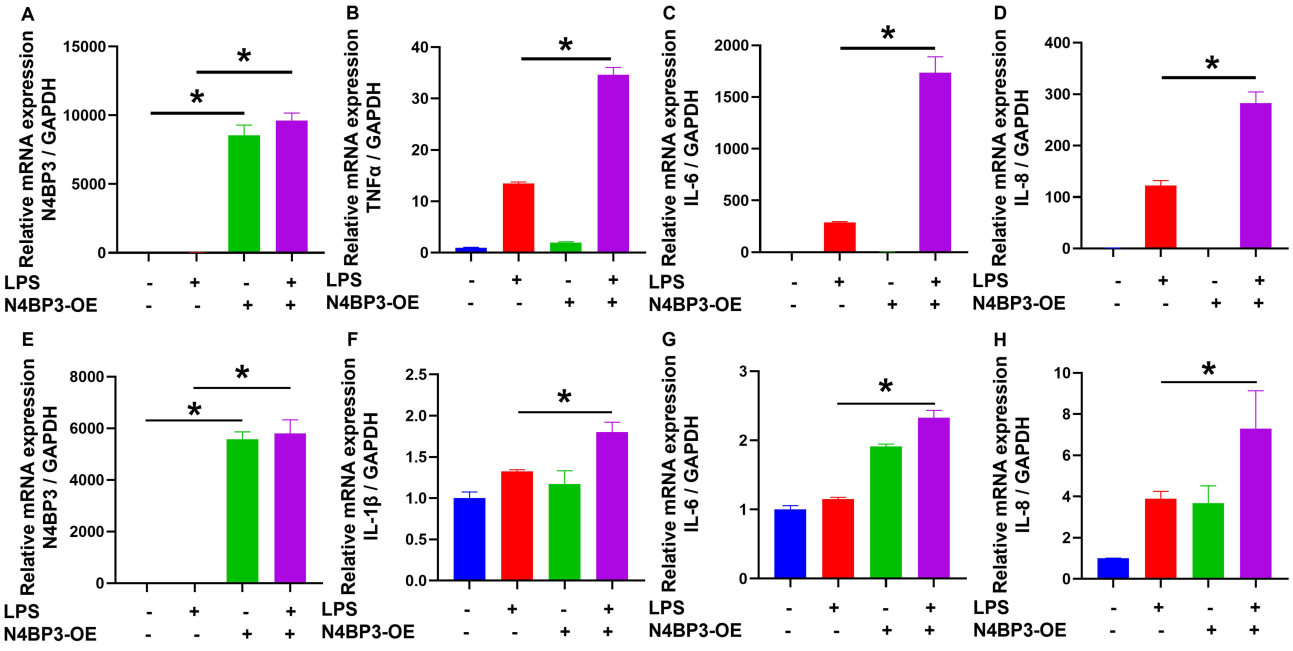

Figure 2 Changes in LPS-induced mRNA expression of cytokines after overexpressing N4BP3 in THP-1/Caco2 cells. (**A**–**D**) Changes in LPS-induced mRNA expression of TNFα (**B**), IL-6 (**C**), and IL-8 (**D**) after overexpressing N4BP3 (**A**) in THP-1 cells. (**E**–**H**) Changes in LPS-induced mRNA expression of IL-1β (**F**), IL-6 (**G**), and IL-8 (**H**) after overexpressing N4BP3 (**E**) in Caco2 cells. Statistical analysis using One-way ANOVA. * $P < 0.05$, representing a significant difference between the data. The error bar represents the mean ± SD.

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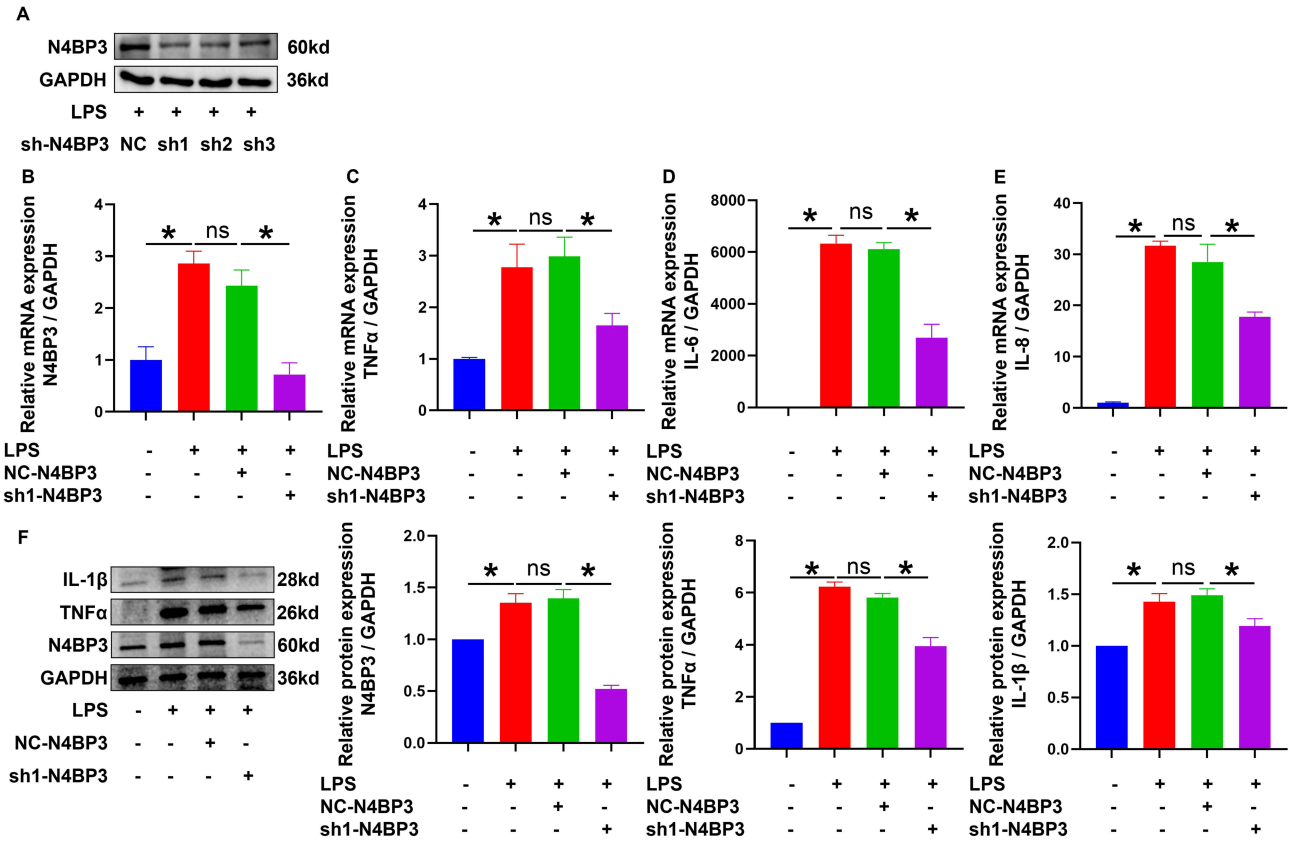

Figure 3 Changes in LPS-induced expression of cytokines after knocking down N4BP3 in THP-1 cells. (**A**) Changes in N4BP3 protein expression after transfection of plasmids containing different knockdown N4BP3 sequences into THP-1 cells. (**B**–**E**) Changes in LPS-induced mRNA expression of TNFα (**C**), IL-6 (**D**), and IL-8 (**E**) after knocking down N4BP3 (**B**) in THP-1 cells. (**F**) Changes in LPS-induced protein expression of TNFα and IL-1β after knocking down N4BP3 in THP-1 cells. Statistical analysis using One-way ANOVA. * $P < 0.05$, representing a significant difference between the groups at either end of the horizontal line. Indicator "ns" representing no significant difference between the groups at either end of the horizontal line. The error bar represents the mean ± SD.

Overexpression of N4BP3 Promotes LPS-Induced Activation of the NF-κB Pathway

While activation of TLR4 was revealed to ultimately promote the production and release of cytokines via the MAPK-ERK/JNK/P38 pathways or the NF-κB pathway, we aimed to further investigate the more specific TLR4-related pathways in which N4BP3 plays a pro-inflammatory role. For this purpose, we constructed N4BP3-overexpressed THP-1 cells and induced the cells with LPS. Then, Western blot was performed to detect the protein expression of signaling molecules in the TLR4 pathway. The results showed that the expression of LPS-induced P-NF-κB p65 protein was significantly upregulated in N4BP3-overexpressed THP-1 cells compared to untreated THP-1 cells ($P < 0.05$) (Figure 4), but there were no significant changes in the expression of p-ERK1/2, p-JNK, and p-P38 ($P > 0.05$) (Figure 4). This suggests that N4BP3 can promote cytokine release via TLR4/NF-κB signaling.

Consistent with previous reports, administration of 3–5% DSS in drinking water induced colitis in C57BL/6 mice.[33] Building on our prior findings that N4BP3 knockdown attenuates DSS-induced colitis,[32] we further investigated this effect through a controlled experimental design. Mice were divided into four groups: (1) blank control group (normal drinking water), (2) DSS group (5% DSS solution), (3) negative control group (AAV-control + 5% DSS), and (4) N4BP3 knockdown group (AAV-shN4BP3 + 5% DSS).

Comparative analysis revealed no significant differences between the DSS group and negative control group in terms of colonic N4BP3 expression levels, disease activity index (DAI), histopathological scores, or proinflammatory cytokine (TNF-α and IL-1β) levels ($P > 0.05$). In contrast, mice with N4BP3 knockdown exhibited significantly reduced N4BP3 expression, attenuated disease severity, improved histopathological scores, and decreased TNF-α and IL-1β levels compared to negative controls ($P < 0.05$). These results confirm that N4BP3 knockdown specifically mitigates DSS-induced colitis progression.

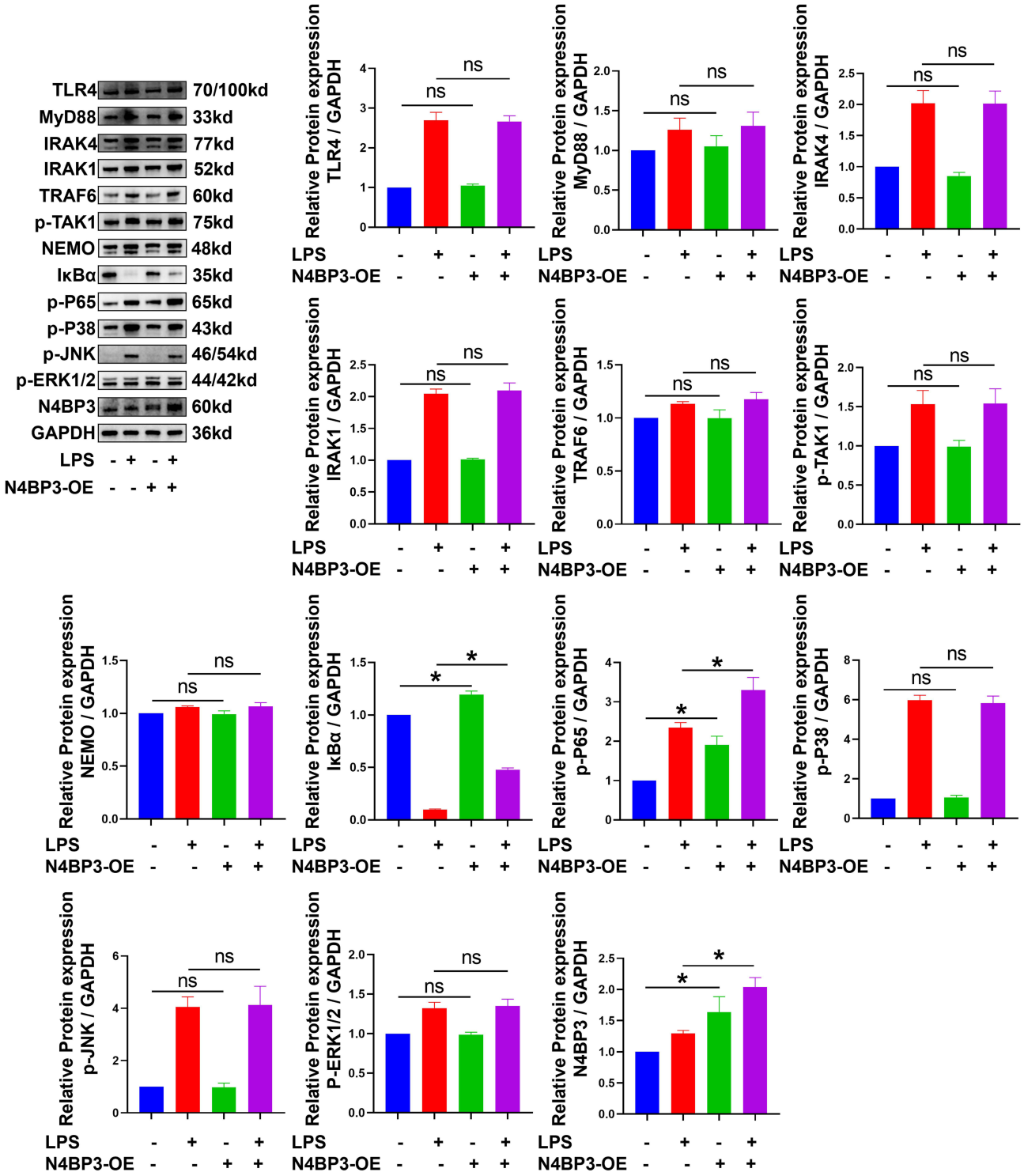

Figure 4 Changes in LPS-induced protein expression of signaling molecules after overexpressing N4BP3 in THP-1 cells. Changes in LPS-induced protein expression of TLR4, MyD88, IRAK1, IRAK4, TRAF6, p-TAK1, NEMO, IκBα, p-P65, p-ERK, p-JNK, and p-P38 after overexpressing N4BP3 in THP-1 cells. Statistical analysis using One-way ANOVA. *P < 0.05, representing a significant difference between the groups at either end of the horizontal line. Indicator "ns" representing no significant difference between the groups at either end of the horizontal line. The error bar represents the mean ± SD.

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The colon tissues of N4BP3 knockdown group mice and negative control group mice were embedded in paraffin. We detected the expression of p-P65 protein and IκBα protein with immunohistochemical staining. The results showed that the expression of IκBα protein was significantly upregulated ($P < 0.05$) (Figure 5A–C) while the expression of p-P65 protein was significantly downregulated ($P < 0.05$) (Figure 5D–F) in the colonic tissues of N4BP3 knockdown mice compared to the negative control group. This suggests that N4BP3 can activate NF-κB signaling in IBD.

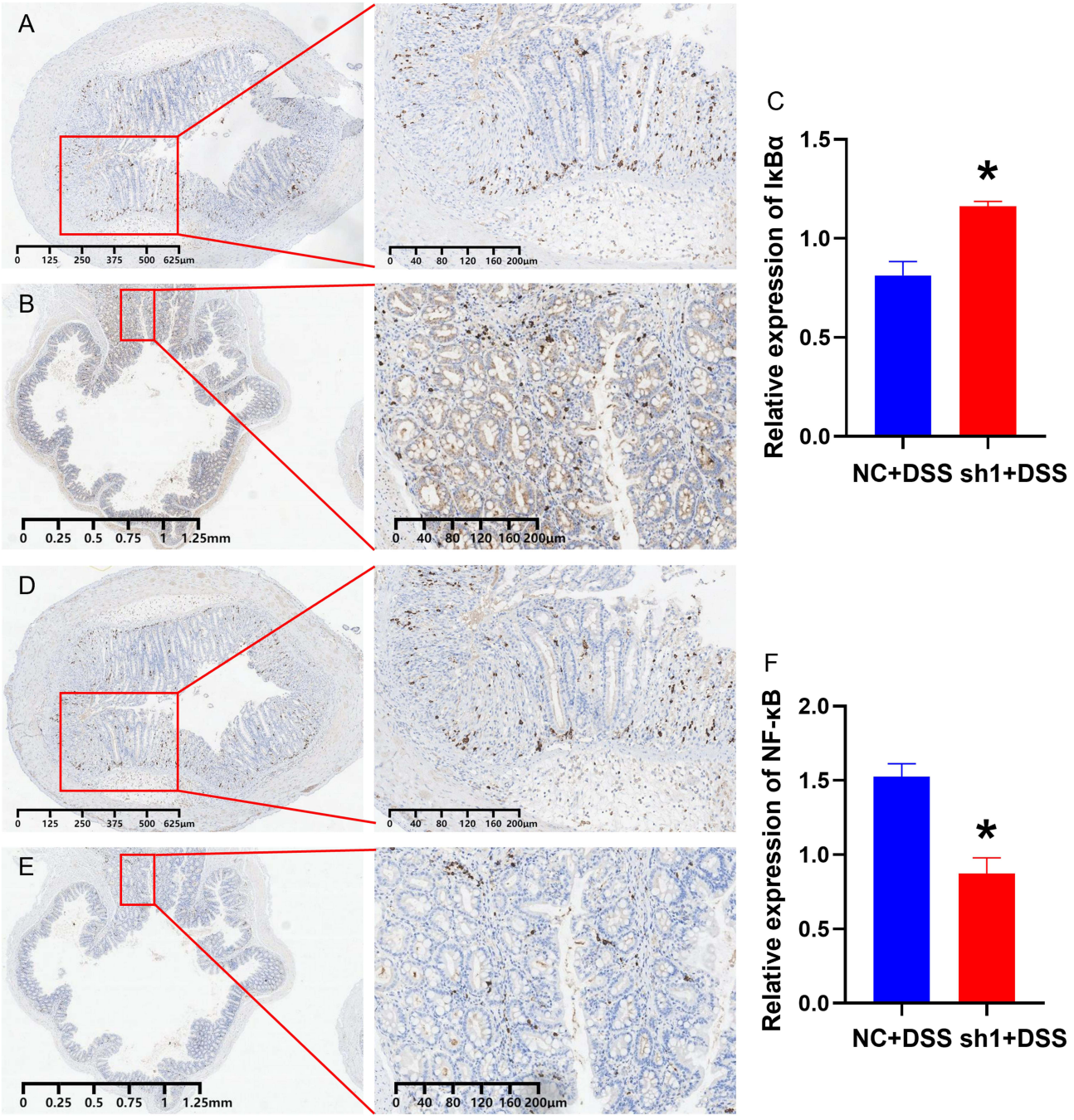

Figure 5 Expression of IκBα protein and p-P65 protein in colonic tissues of mice. (**A**) Expression of IκBα protein in colon tissues of negative control mice. (**B**) Expression of IκBα protein in colon tissues of N4BP3 knockdown mice. (**C**) Relative expression of IκBα protein in colon tissues of negative control mice and N4BP3 knockdown mice. (**D**) Expression of p-P65 protein in colon tissues of negative control mice. (**E**) Expression of p-P65 protein in colon tissues of N4BP3 knockdown mice. (**F**) Relative expression of p-P65 protein in colon tissues of negative control mice and N4BP3 knockdown mice. Statistical analysis using paired *t*-test. *$P < 0.05$, representing a significant difference between the data. The error bar represents the mean ± SD.

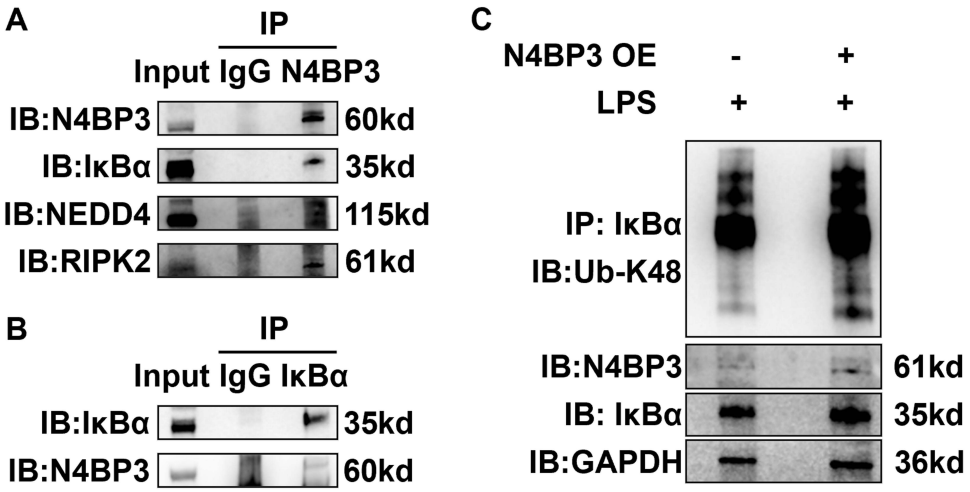

Figure 6 Target protein and ubiquitination modification assays for N4BP3 interactions. (**A** and **B**) Expression of different proteins detected by immunoblotting after immunoprecipitating LPS-induced THP-1 cells overexpressing N4BP3 with the N4BP3 antibody (**A**) and the IκBα antibody (**B**). (**C**) Expression of different proteins detected by immunoblotting after immunoprecipitating LPS-induced control THP-1 cells and THP-1 cells overexpressing N4BP3 with the IκBα antibody.

N4BP3 Interacts with IκBα and Promotes Its Ubiquitination

To explore the specific pathways by which N4BP3 promotes inflammation, we examined the expression of key signaling molecules in the TLR4 pathway. After overexpressing N4BP3 in THP-1 cells, it was found that both the expression of LPS-induced p-P65 and its upstream IκBα protein were significantly upregulated ($P < 0.05$) (Figure 4), whereas all other upstream molecules were not significantly affected ($P > 0.05$) (Figure 4). Therefore, we hypothesized that N4BP3 can interact with IκBα.

Further, we constructed N4BP3-overexpressed THP-1 cells and induced the cells with LPS. The proteins were then extracted and added with the IgG antibody, N4BP3 antibody, and IκBα antibody, respectively, for immunoprecipitation and immunoblotting in order to detect the protein expression of N4BP3 antibody, and NEDD4. The results showed that the IκBα protein could be pulled down after immunoprecipitation of the N4BP3 antibody compared to precipitation of the IgG antibody (Figure 6A), while the N4BP3 protein could be pulled down after immunoprecipitation of the IκBα antibody (Figure 6B). This indicated that there are interactions between N4BP3 and IκBα.

Subsequently, we induced both untreated THP-1 cells and N4BP3-overexpressed THP-1 cells with LPS. Then, the proteins were extracted and immunoprecipitated with the IκBα antibody, and immunoblotting was performed to detect the expression of ubiquitin-K48, N4BP3, and IκBα. The results showed that the K48 ubiquitination level of IκBα was significantly upregulated in LPS-induced THP-1 cells overexpressing N4BP3 compared to untreated THP-1 cells (Figure 6C), suggesting that N4BP3 can promote the K48-linked ubiquitination of IκBα.

Discussion

Our previous study has revealed that LPS-induced NF-κB signaling was significantly upregulated after overexpressing N4BP3, which is believed to be associated with RIPK2 activation. In this paper, we induced THP-1 cells with LPS and immunoprecipitated the N4BP3 antibody. The result confirmed that N4BP3 could indeed interact with RIPK2. Besides, it was also found that the N4BP3 expression was significantly upregulated after TLR4 activation. Both N4BP3-overexpressed THP-1 cells and Caco2 cells could significantly upregulate LPS-induced cytokines, suggesting that N4BP3 is able to promote LPS-induced inflammatory responses. However, the upregulated fold of IL-1β, IL-6, and IL-8 in Caco2 cells was not as much as in THP-1 cells, which may be due to the lower expression of the TLR4 receptor in Caco2 cells. Further, we detected the expression of key signaling molecules in the TLR4 signaling pathway by Western blot in LPS-induced THP-1 cells overexpressing N4BP3, and the results demonstrated that N4BP3 enhanced the NF-κB pathway but had no significant effect on the MAPK pathway. Moreover, after inducing N4BP3-overexpressed THP-1 cells with LPS, only the expression of IκBα was significantly altered among the upstream signaling molecules.

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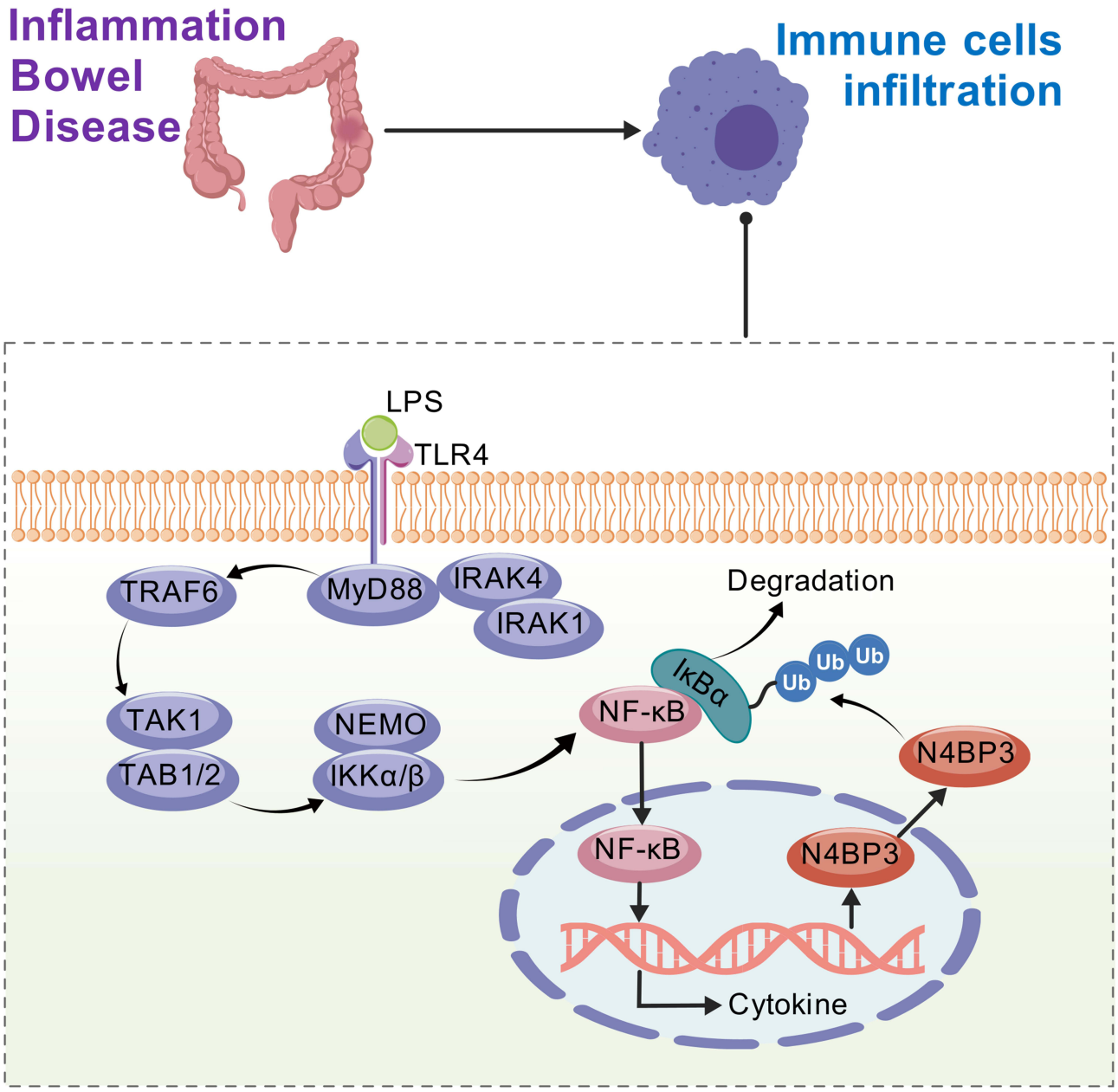

Figure 7 Schematic diagram of the mechanism by which N4BP3 activates TLR4-NF-κB signaling. In inflammatory bowel disease, the activation of TLR4 in macrophages promotes the expression of N4BP3 via the NF-κB pathway. N4BP3 can further promote the ubiquitination of IκBα K48 linkages, facilitate the entry of NF-κB into the nucleus, and promote the production and release of cytokine. This figure was created with BioGDP.com.

Upon activation of the TLR4 pathway, IκBα is phosphorylated on Ser32 or Ser36, and then undergoes Lys48-linked ubiquitination of Lys21 or Lys22; this process will release NF-κB into the nucleus to stimulate cytokine synthesis and release.[34,35] The Siah E3 ubiquitin protein ligase 1 (SIAH1) has been shown to promote the ubiquitination of IκBα, activate NF-κB signaling, and promote cardiomyocyte pyroptosis, therefore exacerbating the progression of inflammatory cardiomyopathy.[36] The ubiquitinating enzyme β-TrCP can bind to IκBα and promote its ubiquitinated degradation,[37,38] while another ubiquitinating enzyme, NEDD4L, can induce the ubiquitination of IκBα in an IKK-2-dependent manner, which promotes NF-κB signaling and exacerbates diabetic retinopathy.[39] In addition, the binding of the deubiquitinating enzyme USP14 to IκBα was reported to reduce the K48 ubiquitination of IκBα and inhibit TNFα-induced cell death in the head and neck squamous cell carcinoma (HNSCC).[40] The deubiquitinating enzyme USP39 can reduce K48-linked ubiquitination of IκBα and inhibit inflammatory responses.[41] In line with the existing studies, our

results indicate that N4BP3 can bind to IκBα and promote K48-linked ubiquitination of IκBα, thereby facilitating NF-κB signaling and exacerbating LPS-induced inflammatory responses and DSS-induced colitis.

Interestingly, we found that N4BP3 also promotes the NF-κB signaling pathway and promotes pro-inflammatory cytokine release through other TLRs. We transfected THP-1 cells with the N4BP3 overexpression plasmid and induced TLR1/2 receptors with Pam3csk4. The results showed that THP-1 cells overexpressing N4BP3 significantly up-regulated Pam3csk4-induced TNFα, IL-8 mRNA, and P-P65 protein expression ($P < 0.05$) (Supplementary Figure 1).

Hexu Han et al found that N4BP3 increased transducer and activator of transcription 3 (STAT3) and the activity of the STAT3 signaling pathway by interacting with lysine acetyltransferase 2B (KAT2B) and regulating the distribution of acetyl histone H3 (Lys27) (H3K27ac) in its promoter region, which promotes microvascular proliferation and accelerates malignant progression of tumors in hepatocellular carcinoma.[21] A genetic predisposition to overactivation of STAT3 in monocytes and epithelial compartments compromises innate defenses, allowing low-level bacterial infections to fester and eventually cause disease, which includes IBD.[42] This suggests that n4bp3 may promote IBD by mediating STAT3 or other signaling pathways. The NF-κB signaling pathway also plays an important role in diseases such as tumors, which suggests that N4BP3 may regulate tumors such as HCC through the NF-κB signaling pathway.

Common biomarkers of IBD include C-reactive protein, erythrocyte sedimentation rate, leucine-rich α2 glycoprotein, fecal calprotectin, the fecal immunochemical test and antineutrophil cytoplasmic antibodies, anti-Saccharomyces cerevisiae antibodies. These biomarkers play an important role in diagnosing IBD and predicting treatment outcomes.[43] Detecting the expression of N4BP3 in the blood from IBD patients and normal populations, analyzing the correlation between the expression of N4BP3 in the blood and IBD, which may provide a new method for the diagnosis of IBD.

Traditional treatments for IBD include aminosalicylates, corticosteroids, immunosuppressants, and cyclosporine.[44] Despite improvements, it still fails to cure many IBD patients. While targeted therapies offer more possibilities for the treatment of patients with IBD. Currently, targeted therapies are used on a mass of IBD patients, which include anti-tumor necrosis factor antibodies, anti-IL-12 and anti-IL-23 antibodies, anti-α4β7 integrin antibodies, and sphingosine-1-phosphate receptor agonists.[45] Anti-N4BP3 antibodies may provide a new targeted therapy, and we expect that an inhibitor of N4BP3 can be developed in the near future, as it currently does not exist.

Conclusion

In conclusion, our study revealed an important role of the ubiquitination-associated protein N4BP3 in IBD through cellular and mouse experiments. Mechanistically, N4BP3 promotes both the NOD2-MAPK/NF-κB signaling pathway through ubiquitination of RIPK2 and the TLR4-NF-κB signaling pathway through ubiquitination of IκBα, which promote the release of pro-inflammatory cytokines and inflammation of the colon (Figure 7). Our findings are expected to enrich the current pathogenic network of IBD, provide future research direction-developing N4BP3 inhibitors and a new therapeutic implication-targeting N4BP3 in IBD.

Data Sharing Statement

All data and material are included in this manuscript.

Ethics Approval

The paraffin blocks embedded with mouse colon tissue used in this study were preserved from our previous study which was performed in line with the principles of the Declaration of Helsinki and granted by the Ethics Committee of University of South China (NO. 2023094). This study was proved by the Laboratory Animal Ethics Committee of University of South China (NO. 2024605).

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors have no relevant financial or non-financial interests to disclose.

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