Biochemical and Biophysical Research Communications 438 (2013) 133–139

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

miR-122 targets NOD2 to decrease intestinal epithelial cell injury in Crohn's disease

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ARTICLE INFO

Article history:
Received 2 July 2013
Available online 19 July 2013

Keywords:
IBD
Crohn's disease (CD)
miR-122
NOD2

ABSTRACT

Crohn's disease (CD) is one of the two major types of inflammatory bowel disease (IBD) thought to be caused by genetic and environmental factors. Recently, miR-122 was found to be deregulated in association with CD progression. However, the underlying molecular mechanisms remain unclear. In the present study, the gene nucleotide-binding oligomerization domain 2 (NOD2/CARD15), which is strongly associated with susceptibility to CD, was identified as a functional target of miR-122. MiR-122 inhibited LPS-induced apoptosis by suppressing NOD2 in HT-29 cells. NOD2 interaction with LPS initiates signal transduction mechanisms resulting in the activation of nuclear factor κB (NF-κB) and the stimulation of downstream pro-inflammatory events. The activation of NF-κB was inhibited in LPS-stimulated HT-29 cells pretreated with miR-122 precursor or NOD2 shRNA. The expression of the pro-inflammatory cytokines TNF-α and IFN-γ was significantly decreased, whereas therelease of the anti-inflammatory cytokines IL-4 and IL-10 was increased in LPS-stimulated HT-29 cells pretreated with miR-122 precursor, NOD2 shRNA or the NF-κB inhibitor QNZ. Taken together, these results indicate that miR-122 and its target gene NOD2 may play an important role in the injury of intestinal epithelial cells induced by LPS.

1. Introduction

Crohn's disease (CD), one of the 2 major forms of inflammatory bowel disease (IBD), is a chronic inflammatory disorder of unknown etiology that primarily affects the gastrointestinal tract. Genetic factors play an important role in determining IBD susceptibility [1–3]. Alterations in susceptibility genes associated with IBD, such as NOD2 [4–6], ATG16L1 [7] and IRGM [8], are closely related to the development of CD.

Pattern recognition receptors such as Toll-like receptors (TLR) and nucleotide-binding oligomerization domain (NOD) proteins play important roles in mediating the innate host response to infection. Intracellular NOD2 (CARD15), which contains two N-terminal caspase activating and recruiting domains (CARD), functions as a sensor that recognizes bacterial factors such as lipopolysaccharide (LPS) and triggers a signaling cascade that results in the activation of the nuclear factor-κB (NF-κB) pathway [9–11]. NOD2 signaling activates the IκB kinase (IKK) complex through receptor-interacting protein-2 (RIP-2). The IKK enzyme complex phosphorylates the NF-κB inhibitor IκB, resulting in its ubiquitination and degradation and the release of NF-κB. Free NF-κB translocates to the nucleus and binds to promoter elements to stimulate the transcription of a large number of target genes, including those involved in immune and inflammatory responses and apoptosis [12]. The *CARD15* gene is the first CD susceptibility gene identified, and its overexpression in the inflamed colon of CD patients indicates its contribution to the pathogenesis of this disease [13]. NOD2 polymorphisms have been associated with CD, and intestinal macrophages isolated from CD patients harboring disease-associated alleles of NOD2 produce elevated levels of NF-κB targets including the pro-inflammatory cytokine tumor necrosis factor α (TNF-α) [14,15].

MicroRNAs (miRNAs) are evolutionary highly conserved small (18–25) non-coding RNAs that play a role in the regulation of most cell processes, and have recently emerged as biomarkers for specific diseases [16,17]. Deregulation of miRNAs is associated with the pathogenesis of several diseases including cancer, and tumor-associated miRNAs can function as tumor suppressors or oncogenes depending on whether they target oncogenes or tumor suppressor genes [18,19]. Aberrant expression of miRNAs has been implicated in the pathogenesis of IBD, and their potential as diagnostic and therapeutic tools for IBD is being increasingly demonstrated [20]. Several upregulated and downregulated miRNAs have been detected in patients with ulcerative colitis and CD [21]. MicroRNA-122 (miR-122), which is mainly found in the liver,

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http://dx.doi.org/10.1016/j.bbrc.2013.07.040

accounts for approximately 70% of all liver miRNAs and is known to regulate the expression of genes that control cell cycle, differentiation, proliferation and apoptosis [22–24]. A recent study identified miR-122 as one of six miRNAs differentially expressed in samples from CD patients, where it was downregulated in association with CD progression from dysplasia to cancer [25]. However, the precise molecular roles of miR-122 in CD remain largely unknown.

In the present study, we identified and characterized NOD2 as a functional downstream target of miR-122 and examined the roles of miR-122 and NOD2 in the injury of intestinal epithelial cells in CD.

2. Materials and methods

2.1. Cell culture

The HT-29 human intestinal epithelial cell line (American Type Culture Collection, ATCC) was cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 1 mg/mL streptomycin at 37 °C and 5% CO_2.

2.2. Dual luciferase reporter gene assay

The wild-type and a mutant 3′UTR of NOD2 were cloned into the pGL3-control vector (Promega, Shanghai, China) between the KpnI and XhoI enzyme sites to generate PGL3-NOD2 3′UTR-wild and PGL3-NOD2 3′UTR-mut. The pRL-CMV vector (Promega) was used as an internal control. Cells were seeded in 24-well plates and cultured for 24 h before being transfected with the miR-122 precursor and control precursor molecules (Ambion). After 24 h, cells were co-transfected with PGL3-NOD2 3′UTR-wild and PGL3-NOD2 3′UTR-mut using Lipofectamine (Invitrogen, Carlsbad, CA). Cells were lysed and the relative luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega).

Primer sequences were as follows:

NOD2-3′UTR wild-F,
5′-GGGTACCCGGAGGATGTTCGTCTCAGTTTG-3′;
NOD2-3′UTR wild-R,
5′-CCTCGAGGAAGTTCACGGCCATGTTGTC-3′;
NOD2-3′-UTR mut-F,
5′-GGGTACCCTATGACCTGTGACCAGCTGGGATC-3′;
NOD2-3′-UTR mut-R,
5′-GGGTACCCGGAGGATGTTCGTCTCAGTTTG-3′.

2.3. Cell transfection and RNA interference

HT-29 cells were pretreated with the control miR precursor, miR-122 precursor (Ambion) or NOD2 shRNA for 2 h using Lipofectamine 2000 (Invitrogen, CA, USA) following the instructions of the manufacturer or as indicated followed by stimulation with LPS (500 ng/mL; *Eschericia coli*, Sigma) for 16 h or left untreated. NOD2 small hairpin RNAs (shRNA) were purchased from Sigma (St. Louis, MO).

2.4. mRNA isolation and quantitative real-time RT-PCR

Total RNA was isolated using the TRIzol® reagent (Invitrogen). Complementary DNA (cDNA) was synthesized using the M-MLV RT enzyme kit (Invitrogen), oligo dT primers (Sigma) and RNase inhibitor (Applied Biosystems, Foster City, CA). RT-PCR was performed using the ABI 7900 HT system (Applied Biosystems). For PCR, 2 μL cDNA, 150 nM of forward and reverse primers and 6 μL SYBR Premix Ex Taq II (TaKaRa, Dalian, China) were mixed in a total volume of 12 μL. The PCR reaction was as follows: 15 min at 95 °C (initial denaturation); 20° temperature transition rate upto 95 °C for 30 s, 45 s at 56 °C, 45 s at 72 °C, repeated 40 times (amplification). PCR was evaluated by melting curve analysis following the manufacturer's instructions.

RT-PCR primer sequences were as follows:

NOD2 forward 5′-CAGCCTCCGCAAGCACTTCCACT-3′ and reverse 5′-CTCCACGCCAATGTCACCCACAG-3′;
TNF-α (F) 5′-AGGGCTCCAGGCGGTGCTTGTT-3′ and (R) 5′-AGACGGCGATGCGGCTGATGGT-3′;
IFN-γ (F) 5′-TTCAGATGTAGCGGATAATGG-3′ and (R) 5′-ATGTATTGCTTTGCGTTGGAC-3′;
IL-4 (F) 5′-TGACCGTAACAGACATCTTTGC-3′ and (R) 5′-CTCTGGTTGGCTTCCTTCACA-3′;
IL-10 (F) 5′-CAACCTGCCTAACATGCTTCG-3′ and (R) 5′-GGCATTCTTCACCTGCTCCAC-3′;
GAPDH (F) 5′-GAAGGTGAAGGTCGGAGTC-3′ and (R) 5′-GAAGATGGTGATGGGATTTC-3′.

2.5. Total protein extracts

Cells were treated as indicated and stimulated or not with LPS (0.5 μg/mL). Cells were scraped into PBS, 10 mM NaF, and 1 mM Na_3VO_4 and centrifuged at 2000 rpm. The pellets were homogenized in lysis buffer (50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% NP-40) containing protease and phosphatase inhibitors (1 mM PMSF, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin A, 10 mM NaF, 1 mM Na_3VO_4). Extracts were sonicated, centrifuged at 12,000 rpm for 30 min and the supernatant was used for immunoblotting. Protein concentration was determined with the Bradford method (BioRad Laboratories, CA, USA) using bovine serum albumin as a standard.

2.6. Nuclear and cytosolic extracts

Cells were treated as indicated, scraped into PBS–PMSF, centrifuged at 2500 rpm and resuspended in 150 μL lysis buffer (10 mM Hepes, 10 mM KCl, 2 mM $MgCl_2$, 500 μM DTT, 1 mM PMSF, 5 μg/mL aprotinin, 5 μg/mL leupeptin, 5 μg/mL pepstatin A, 500 μM Na_3VO_4, 1 mM NaF) followed by incubation with 1% NP-40. Lysates were centrifuged at 14,000 rpm for 15 min and supernatants were collected as the cytosolic fraction. Pellets were resuspended in buffer B (20 mM Hepes, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 200 μM EDTA, 500 μM DTT, plus protease and phosphatase inhibitors and shaken on ice for 30 min. Lysates were centrifuged at 14,000 rpm for 15 min and the supernatants (nuclear extracts) were diluted in buffer C (20 mM Hepes, 20% glycerol, 50 mM KCl, 200 μM EDTA, 500 μM DTT, 1 mM PMSF). Protein concentration was determined using the Bradford method. Cytosolic and nuclear extracts were confirmed by immunoblotting against an anti-histone antibody as a marker of the nuclear fraction.

2.7. Western blots

Aliquots containing 20 μg of protein lysate were subjected to 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). Membranes were blocked in a solution containing 5% milk in Tris-buffered saline Tween-20 (TBS-T) and incubated in primary antibodies against NOD2 (Abcam), cleaved and total caspase-3 (Cell Signaling Technology), cleaved and total caspase-9 (Cell Signaling Technology), phosphoserine IκBα (S32, Cell Signaling, Beverly, MA), IκB (C-21, Santa Cruz Biotechnology, Santa Cruz, CA), β-actin (Abcam), NF-κB (p65, Cell Signaling Technology) and anti-histone deacetylase (HDAC, Cell Signaling

Technology) overnight at 4 °C. All antibodies were used at a 1:1000 dilution in TBS-T. After incubation in the corresponding secondary antibodies for 1 h at room temperature, immunoreactive proteins were detected using the enhanced chemiluminescence light detecting kit (Amersham Biosciences).

2.8. Analysis of apoptosis by Annexin V-FITC and PI

The Annexin V-FITC Apoptosis Detection kit (Calbiochem) and PI (Sigma) were used to assess the antiapoptotic effect of miR-122. HT-29 cells (1×10^6 cells/mL) were treated as indicated, washed twice with PBS, suspended in Annexin-V binding buffer and incubated with Annexin-V-FITC/PI in the dark for 10 min. Cells were analyzed by flow cytometry using FloMax software and the fraction of cells in different quadrants was quantified using quadrant statistics.

2.9. Cytokine ELISAs

HT-29 cells were pretreated with the control miR precursor, miR-122 precursor, NOD2 shRNA or the NF-κB inhibitor 6-amino-4-(4-phenoxyphenylethylamino)quinazoline (QNZ, 10 mM, Enzo Life Sciences International Inc) for 2 h as indicated followed by stimulation with LPS (500 ng/mL) for 16 h or left untreated. The cytokines TNF-α, IFN-γ, IL-4 and IL-10 were measured in culture supernatants using enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems.

2.10. Statistical analysis

Data were expressed as the mean ± SE of at least three independent experiments. Differences were analyzed using two-sided Student's *t*-tests, and statistical significance was set at $P < 0.05$.

3. Results

3.1. miR-122 directly regulates NOD2 expression by targeting the 3′UTR of NOD2

A single binding site for miR-122 in the 3′UTR of NOD2 was identified by bioinformatics analysis, which suggested that NOD2 is a target gene for miR-122 (Fig. 1A). To confirm NOD2 as a target of miR-122, HT-29 cells were co-transfected with a miR-122 precursor or control precursor and the luciferase reporter constructs pGL3-NOD2 3′UTR-wild and pGL3-NOD 3′UTR-mut and luciferase activity was determined. The results showed that overexpression of miR-122 significantly decreased the luciferase activity of the wild-type NOD2 3′UTR by 47.6% relative to control precursor-transfected cells, whereas it had no effect on the mutant construct. No significant differences in luciferase activity were detected between cells transfected with miR-122 precursor and control precursor (Fig. 1B). These findings confirmed that NOD2 is a target gene of miR-122.

To examine the effect of miR-122 on the LPS-induced expression of NOD2, the mRNA and protein levels of NOD2 were measured in HT-29 cells (Fig. 2A and B). NOD2 expression was significantly induced by LPS at the mRNA and protein levels, and this effect was significantly inhibited by overexpression of the miR-122 precursor or transfection with NOD2 shRNA, indicating that miR-122 downregulates LPS-induced NOD2 expression in HT-29 cells.

3.2. miR-122 inhibits LPS-induced apoptosis by suppressing NOD2 in HT-29 cells

HT-29 cells pretreated with the control miR precursor, miR-122 precursor or NOD2 shRNA were exposed to LPS (500 ng/mL) and assessed for apoptosis. LPS significantly increased HT-29 cell apoptosis by approximately 27% compared to untreated controls, and

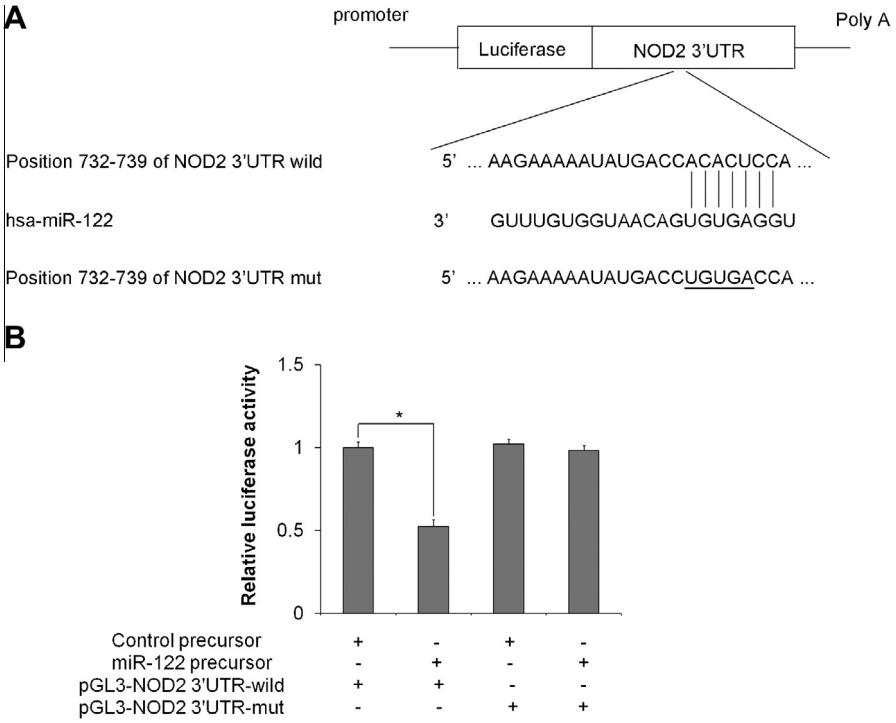

Fig. 1. NOD2 is a target gene of miR-122. (A) Schematic diagram of the plasmids pGL3-NOD2 3′UTR-wild and pGL3-NOD2 3′UTR-mut. The wild-type and mutated sequences of the target sites of miR-122 on the 3′UTR of NOD2 are shown. (B) Analysis of the relative luciferase activity in HT-29 cells cotransfected with pGL3-NOD2 3′UTR-wild (or pGL3-NOD2 3′UTR-mut) and miR-122 precursor (or control precursor). *$P < 0.05$.

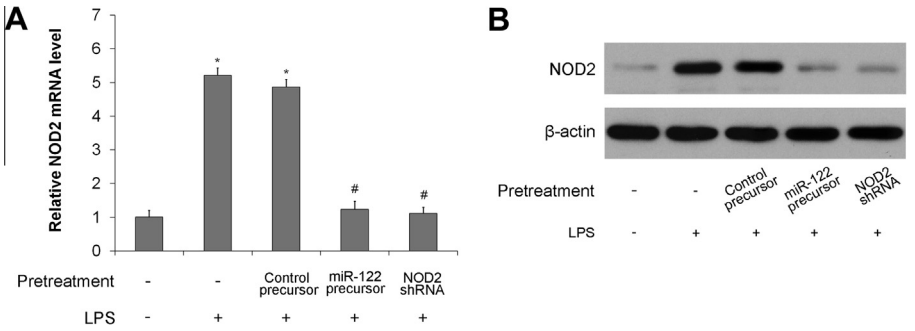

Fig. 2. miR-122 inhibits LPS-induced NOD2 expression in HT-29 cells. HT-29 cells were pretreated with the control miR precursor, miR-122 precursor or NOD2 shRNA for 2 h as indicated followed by stimulation with LPS (500 ng/mL) for 16 h or left untreated. The effect of miR-122 on mRNA (A) and protein (B) levels of NOD2 was evaluated in HT-29 cells. $^{*}P < 0.05$ compared with the untreated cells; $^{\#}P < 0.05$ compared with the cells stimulated by only LPS.

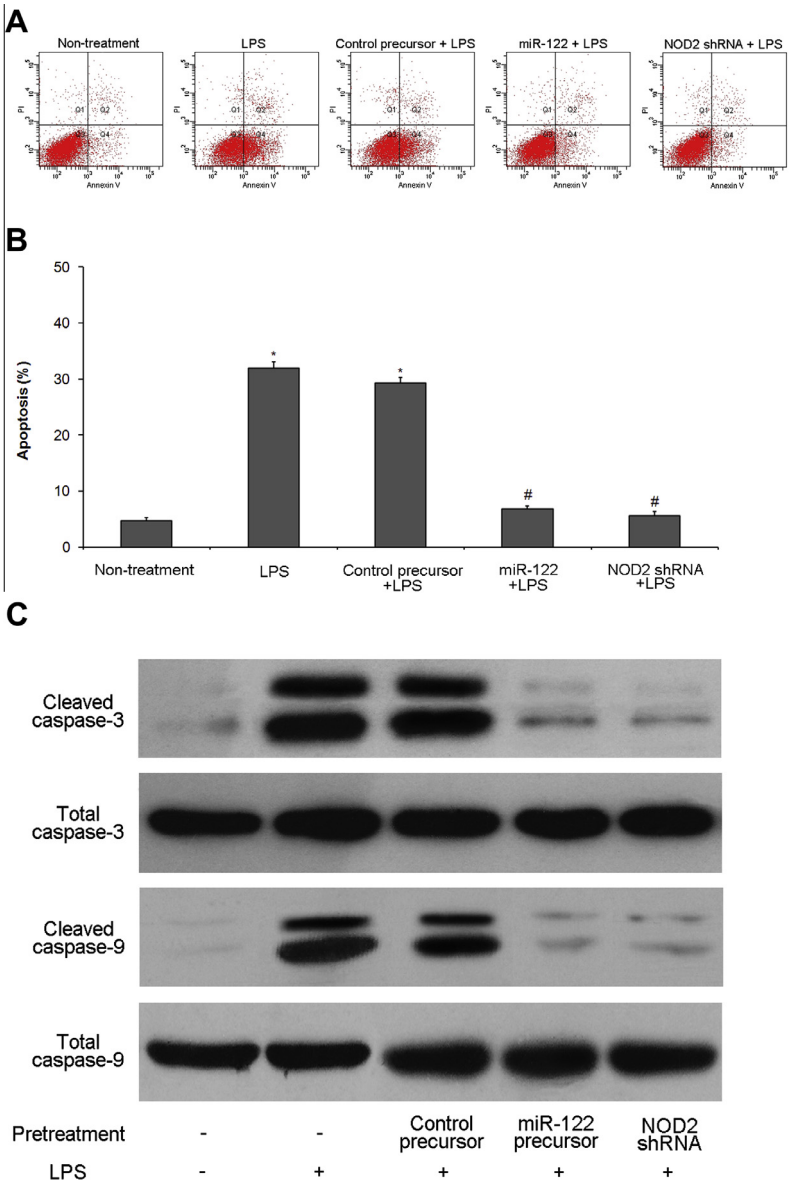

Fig. 3. Antiapoptotic effects of miR-122 in LPS-stimulated HT-29 cells. HT-29 cells were pretreated with the control miR precursor, miR-122 precursor or NOD2 shRNA for 2 h as indicated followed by stimulation with LPS (500 ng/mL) for 16 h or left untreated. (A) LPS-induced apoptosis of HT-29 cells was determined by Annexin V/propidium iodide (PI) staining. (B) The fraction of apoptotic cells was calculated and plotted. $^{*}P < 0.05$ compared with the untreated cells; $^{\#}P < 0.05$ compared with the cells stimulated by only LPS. (C) Western blot analysis of the LPS-induced cleavage of caspase-3 and -9 and its inhibition in HT-29 cells pretreated with miR-122 precursor or NOD2 shRNA.

LPS induced apoptosis was inhibited by overexpression of the miR-122 precursor or NOD2 shRNA (Fig. 3A and B). To identify downstream effectors in the apoptotic signaling pathway, caspase-3/9 cleavage was examined by Western blotting. LPS treatment induced cleavage of caspase-3 and caspase-9, and this effect was inhibited by transfection with the miR-122 precursor or NOD2

shRNA (Fig. 3C). These results indicated that overexpression of miR-122 blocked LPS-induced caspase-3 and -9 dependent apoptosis by down-regulating NOD2 in intestinal epithelial cells.

3.3. miR-122 regulates the levels of key inflammatory cytokines by targeting the NOD2-induced NF-κB signaling pathway in LPS-stimulated HT-29 cells

The effect of miR-122 overexpression on NOD2-induced NF-κB activation was examined in HT-29 cells. As seen in Fig. 4A, overexpression of miR-122 or NOD2 silencing significantly reduced LPS-induced IκB-phosphorylation and IκBα-degradation and prevented the nuclear translocation of NF-κB in HT-29 cells, suggesting that intestinal epithelial cells respond to LPS stimulation through NOD2 induction of NF-κB signaling activity.

We next examined the effect of miR-122 on the expression of the pro-inflammatory cytokines TNF-α and IFN-γ and the anti-inflammatory cytokines IL-4 and IL-10. As shown in Fig. 4B and C, LPS treatment significantly increased the mRNA and protein levels of TNF-α, IFN-γ, and the anti-inflammatory cytokine IL-10, but only slightly increased the level of IL-4 in HT-29 cells. Pretreatment with the miR-122 precursor, NOD2 shRNA or the NF-κB inhibitor 6-amino-4-quinazoline (QNZ) significantly decreased the expression of the pro-inflammatory cytokines TNF-α and IFN-γ and significantly increased the anti-inflammatory cytokines IL-4 and IL-10 at the mRNA and protein levels in LPS-stimulated HT-29 cells.

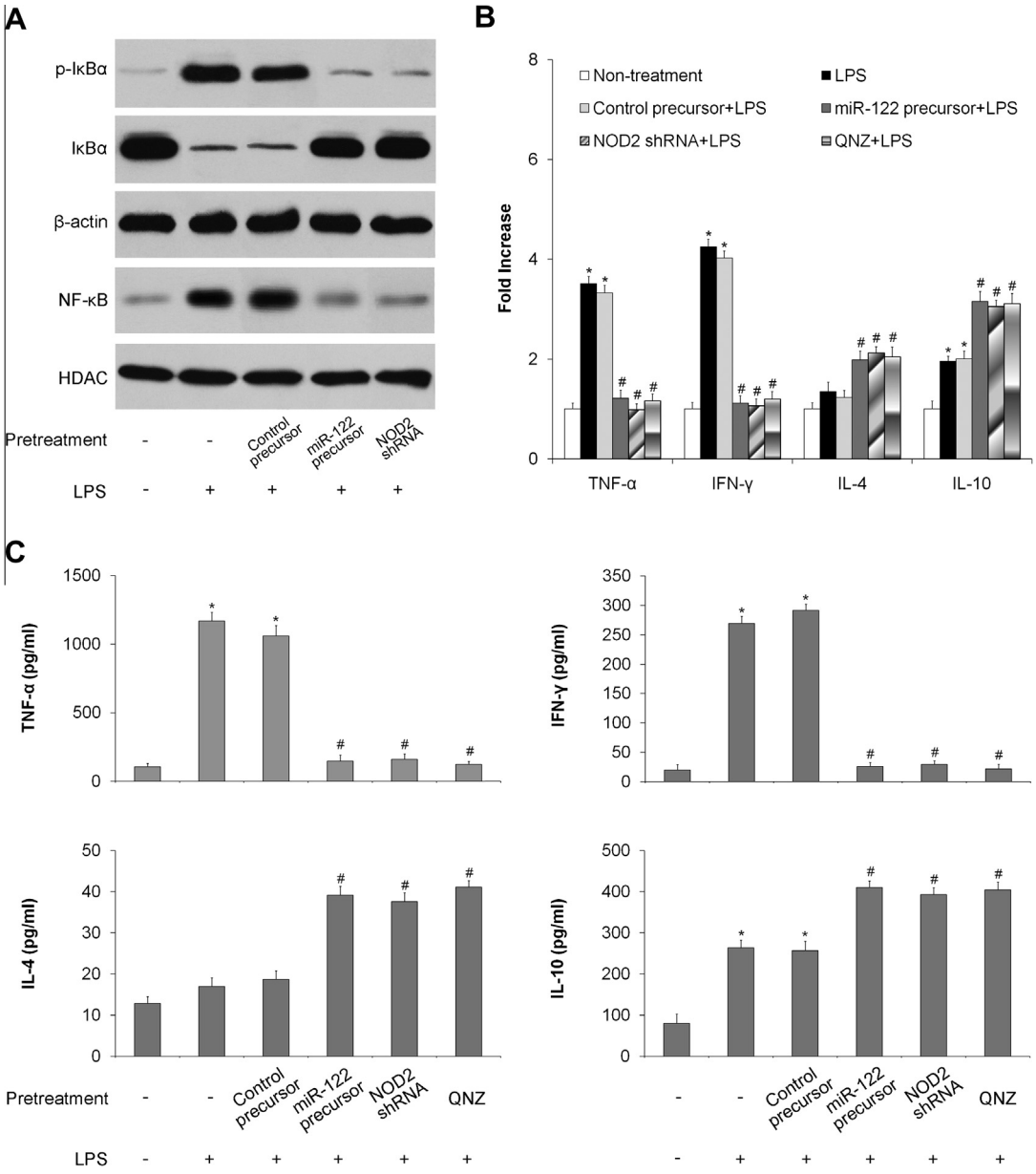

Fig. 4. miR-122 regulates the levels of key inflammatory cytokines through the NOD2-induced NF-κB signallig pathway in LPS-stimulated HT-29 cells. (A) NOD2-induced nuclear factor-kB (NF-κB) activities in LPS-stimulated HT-29 cells. Cells pretreated with control miR precursor, miR-122 precursor or NOD2 shRNA for 2 h were exposed to LPS (500 ng/mL) for 16 h or left untreated. Cytosolic and nuclear extracts were prepared and used to analyze phospho-IκB-α (Ser 32), IκB-α degradation and NF-κB nuclear translocation. β-actin and histone deacetylase (HDAC) were used as cytosolic and nuclear controls, respectively. The mRNA (B) and protein (C) levels of key inflammatory cytokines were analyzed in LPS-stimulated HT-29 cells. Cells were pretreated for 24 h with control miR precursor, miR-122 precursor, NOD2 shRNA or the NF-kB specific inhibitor QNZ (10 μM) and then stimulated with LPS (500 ng/mL) for 16 h. $^{*}P < 0.05$ compared with the untreated cells; $^{\#}P < 0.05$ compared with the cells stimulated by only LPS.

These results indicated that overexpression of miR-122 down-regulates the expression of pro-inflammatory cytokines and promotes the release of anti-inflammatory cytokines through NOD2-induced NF-κB signaling to reduce the injury of intestinal epithelial cells induced by LPS.

4. Discussion

In the present study, we showed that miR-122 targets the Crohn's disease susceptibility gene NOD2 in intestinal cells, downregulating the LPS-induced expression of NOD2 at the mRNA and protein levels. The role of NOD proteins as intracellular receptors for bacterial factors, in particular LPS, was proposed based on the presence of domains similar to those of plant R proteins [26]. The specific responsiveness of NOD2 to LPS was demonstrated in the HEK293T cell line, in which LPS simulated NF-κB activation in cells expressing wild-type but not mutant NOD2 [9]. These results supported the presence of a detection system for bacterial factors in the epithelial surface of the gut. Genetic studies further demonstrated a link between NOD2 deficiency and the susceptibility to CD, underscoring the importance of NOD-like receptors in the control of inflammatory responses in the intestine [27].

The intracellular recognition of LPS by NOD2 activates the NF-κB pathway, which induces the expression of proinflammatory genes [11]. In addition, NOD2 belongs to the caspase recruitment domain (CARD) subfamily, which as part of the death domain (DD) superfamily is involved in the assembly of oligomeric signaling complexes that function in apoptosis and inflammation [28]. In the present study, we identified a NOD2 binding domain in miR-122 and hypothesized that miR-122 may act on LPS-induced apoptosis and inflammation in the intestine by down-regulating its target NOD2. We showed that miR-122 inhibited LPS induced NF-κB activation, apoptosis and the expression of pro-inflammatory cytokines, and it promoted the expression of anti-inflammatory cytokines by targeting NOD2. The association of NOD2 with the apoptotic pathway has been demonstrated previously. For example, the cellular inhibitors of apoptosis cIAP1 and cIAP2 function in innate immunity signaling activated by NOD2, modulating NOD-dependent production of pro-inflammatory cytokines [29]. A recent study showed that the pro-apoptotic protein Bid, which binds most Bcl-2 family members, interacts directly with NOD2 [30], although the role of NOD proteins in BID-dependent apoptosis is not clear.

The control of innate immune signaling in the intestine is of critical importance to maintain a balance between the inflammatory response against bacteria and the maintenance of tissue integrity. Furthermore, a detection system for bacterial LPS is important in organs such as the intestine, in which triggering of inflammatory responses through surface receptors such as TLR4 could be detrimental [9]. Therefore, the regulation of the activity of epithelial receptors such as NOD2 could have applications in diseases in which dampening the pro-inflammatory response of the innate immune system may be beneficial [31]. However, a critical issue is the control of NOD2 activity without a detrimental effect on host defenses. The results of the present study indicate that miR-122 may be a potential modulator of the LPS-induced inflammatory response through the regulation of NOD2. Furthermore, miR-122 may have a protective effect against the progression from IBD to colorectal cancer (CRC), which is supported by the identification of several miRNAs dysregulated in IBD and the specific identification of miR-122 as one of several genes downregulated in association with the progression from dysplasia to cancer [13,14].

In conclusion, in the present study, we identified NOD2 as a target of miR-122 and showed that miR-122 downregulates LPS-induced NOD2 expression and inhibits LPS-induced apoptosis, NF-κB activation and the expression of pro-inflammatory cytokines. Our results indicate that miR-122 may act as a modulator of the inflammatory response of intestinal epithelial cells in CD by down-regulating the expression of its target gene NOD2, identifying miR-122 as a potential target for the treatment of IBD.

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