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NF-κB p65 and TCF-4 interactions are associated with LPS-stimulated IL-6 secretion of macrophages

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ABSTRACT

Proinflammatory cytokine plays a central role in host defense and acute inflammatory responses. Both positive and negative correlations of NF-κB and Wnt/β-catenin pathways have been reported depending on cell types in response to inflammatory stimuli for IL-6 cytokine production. Macrophages are vital to the regulation of immune responses and the development of inflammation, but the crosstalk between two pathways has not been elucidated so far in macrophages. We observed a positive cross-regulation between the NF-κB and Wnt/β-catenin pathways for IL-6 production in human macrophages. To verify the functional validity of this interaction, LY294002 or PNU74654, representative blockers of each pathway, were treated. IL-6 secretion was reduced to the basal level by both inhibitor treatments, even when stimulated by LPS. We also found that NF-κB p65 migrated to the nucleus and interacted with the transcription factor TCF-4 in macrophages upon LPS stimulation.

1. Introduction

The innate immune system, which recognizes and responds to pathogens, is primarily composed of macrophages, dendritic cells, natural killer cells, T cells, and B lymphocytes [1,2]. Macrophages are vital to the regulation of immune responses and the development of inflammation. When macrophages are exposed to inflammatory stimuli, they secrete cytokines [3,4]. IL-6 is a keystone cytokine that plays a central role in host defense for controlling local or systemic acute inflammatory responses [5,6]. IL-6 stimulates the inflammatory and autoimmune processes in many diseases, such as diabetes, systemic lupus erythematosus, and rheumatoid arthritis [7–9].

The NF-κB transcription factor plays a key role in regulating the immune response to infection in mammalian cells [10,11]. It is found in an inactive state in the cytoplasm, bound to its inhibitor IκB (Inhibitor of κB). Stimulation with inducers, such as LPS, activates the IκB kinase (IKK) complex that phosphorylates IκB-α, triggering its degradation by the proteasome and allowing free NF-κB to translocate to the nucleus [12–14]. Glycogen synthase kinase-3 beta (GSK-3β) has been shown to regulate TLRs/NF-κB activation for inflammatory responses [15]. As β-catenin is a major substrate of GSK-3β, it might serve as a mediator for cross regulation between these two pathways. In support of this hypothesis, physical interactions of Wnt/β-catenin and NF-κB pathway components have been previously observed [16].

Regarding the cross-regulation of the NF-κB and Wnt/β-catenin pathways, a number of articles suggest both positive and negative correlations [16–20]. In LPS-treated BEAS-2B human bronchial epithelial cells, the siRNA knockdown of β-catenin attenuated not only the Wnt/β-catenin pathway, but also the NF-κB pathway [20]. In other reports, negative cross-regulation of these two signaling pathways has been observed [16,19]. Multiple components are believed to be involved in the pathway interaction. Inflammation-associated carcinogenesis has exhibited a complex role of β-catenin, NF-κB p65, and inducible nitric oxide synthase (iNOS) signaling [21]. Also, regulation of NF-κB p65 activity directly correlates with the β-catenin/TCF pathway in human adipose tissue-derived stem cells and human bone marrow-derived mesenchymal stem cells [22]. However, the crosstalk between NF-κB and Wnt/β-catenin pathways in macrophages, the key player in the regulation of inflammatory immune responses, has not been elucidated so far.

In this study, we sought to determine how NF-κB and Wnt/β-catenin pathways interact in an innate immunity environment using an LPS-stimulated human macrophage cell model.

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2. Materials and methods

2.1. Cell lines and differentiation

Human THP-1 monocyte-like cell lines and TERT-immortalized human embryonic microglial HME3 (CRL-3304) cell lines were purchased from ATCC (Manassas, VA, USA) [23]. All cells were grown in RPMI 1640 medium with 10 % FBS (Corning, Manassas, VA, USA) and 1 % penicillin-streptomycin. For differentiation into macrophages, THP-1 cells were cultured in a medium containing 12.5 ng/mL phorbol-12-myristate 13-acetate (PMA) in 1.75×10^6 cells per 100 mm dish for 72 h in 5 % CO_2 at 37 °C. After fresh media replacement, the cells were further cultured for 48 h until a complete macrophage appearance was observed. Lipopolysaccharide (LPS, 0.125 μg/mL) was inoculated to induce the in vitro inflammatory response (Fig. S1) [9,26], and LY294002 or PNU74654 was inoculated to suppress the inflammatory response [25]. Signal transduction inhibitors were added 30 min before LPS treatment, and neither stimulant nor inhibitor treatment was included in the control group. Unless otherwise stated, reagents, chemicals, and supplies were purchased from Thermo-Fisher Scientific (Waltham, MA, USA).

2.2. Immunocytochemical staining

For immunocytochemical staining, THP-1 macrophages were seeded at 2×10^4 cells/well in a 6-well plate containing a 22 × 22 mm coverslip and were incubated at 37 °C and 5 % CO_2 for 24 h [30]. To observe the IL-6 cytokines present in the cytoplasm, cells were stimulated with LPS and treated with a protein transport inhibitor cocktail (eBioscience, San Diego, CA, USA). The LPS-induced cells were fixed with PBS solution containing 3.7 % paraformaldehyde for 3 min at room temperature, permeabilized with 0.02 % Triton X-100 for 1 min on ice, and blocked with 2 % bovine serum albumin (BSA) overnight at 4 °C. Next, the samples were incubated with primary anti-IL-6 (Clone D3K2N) rabbit mAb (vol/vol 1:200, Cell Signaling Technology, Danvers, MA, USA) for 2 h at room temperature (RT). Subsequently, the cells were incubated in Alexa Fluor® 488 Phalloidin for 30 min, and the nuclei were stained with 4′,6′-diamidine-2′-phenylindole dihydrochloride (DAPI, 1 μg/ml, Roche Applied Science, Mannheim, Germany) for 5 min at RT. After washing several times with PBS, the slides were mounted in the mounting medium. The cells were imaged using a confocal laser scanning microscope (LSM 510 META, Carl Zeiss, Zena, Germany) [30].

2.3. Enzyme-linked immunosorbent assay (ELISA)

Human inflammatory cytokines IL-6, TNF-α, and IL-1β were quantified in the THP-1 macrophage supernatants using ELISA according to BioLegend's MAX™ kit protocol (BioLegend, San Diego, CA, USA). Briefly, 100 μl of capture antibodies were added to the wells of a 96-well plate and were incubated overnight (16–18 h) at 4 °C. The plates were washed three times with 200 μl PBS with 0.05 % Tween-20 (PBST), and 200 μL 1X Assay Diluent A per well was added to block non-specific binding. In addition, 100 μL/well of the standard or sample was added to the appropriate wells, and 100 μL of the diluted detection antibody solution was added to each well and incubated for 1 h at RT. After four washes, 100 μl/well of substrate solution was added, and the plates were incubated for 30 min at RT in the dark. The reaction was terminated by the addition of 100 μl of stop solution. The optical density at 450 nm was measured using an ELISA Plate Reader within 15 min. The standard curve was created with recombinant human IL-6 at different ranges of concentrations [9].

2.4. RT-PCR

For RT-PCR, THP-1 macrophages were cultured at 1.75×10^6 cell numbers per 100 mm cell culture dishes at 37 °C and 5 % CO2. Then, it was treated with LPS at 0, 10 min, 1 h, 6 h, and 36 h. Then, the cells were recovered, and total RNA was isolated and quantified. The amount of total RNA was about 450ng/ul ~550ng/ul, and the purity ratio was about 1.7 or more (data not shown). Of these, 5 μg each time point was used and converted into 20 μl of cDNA. In the PCR reaction, 4 μl of each cDNA was used as a template to obtain a final reaction product of 50 μl.

The mRNA expression levels of IL-6, TNF-α, and IL-1β in THP-1 macrophages were examined by RT-PCR. Total RNA was prepared using the Trizol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The first-strand cDNA was synthesized using a synthesis kit (Takara Bio, Tokyo, Japan). The primer set sequences for PCR of IL-6, TNF-α, IL-1β, and GAPDH (glyceraldehydes-3-phosphate dehydrogenase) were recorded in Table 1. The reaction mixture was denatured at 98 °C for 30 s and then cycled 35 times at 98 °C/10 s, 58 °C/30 s, and 72 °C/30 s, followed by a final 10 min extension at 72 °C. These products were separated by electrophoresis on a 1 % agarose gel and visualized by ethidium bromide staining in ultraviolet-induced fluorescence [30].

2.5. Western blotting

Cells were lysed with RIPA Lysis Buffer containing protease and phosphatase inhibitors (Sigma-Aldrich, Saint Louis, MO, USA). Subcellular fractionation was performed using NE-PERTM Nuclear and Cytoplasmic Extraction Reagents (Thermos Fisher Scientific, Waltham, MA, USA) as described previously [30]. The protein concentration was quantified by the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, USA). The lysates were separated by SDS/PAGE in a 10 % acrylamide gel and transferred onto a nitrocellulose membrane for immunoblotting. The membrane was blocked with 3 % bovine serum albumin (BSA) and incubated for 12 h at 4 °C with primary antibodies (all from Cell Signaling Technology), including those for Phospho-Akt (Ser473 #9271), Phospho-IκB (Ser32/36 #5210), IκB (#4812), Phospho–NF–κB p65 (Ser536 #3033), NF-κB p65 (#4764), β-Catenin (#9562), TCF-4 (#2569), α-Tubulin (#2144) and fibrillarin (Abcam, Cambridge, UK). The membrane was washed three times with PBST and incubated for 30 min with the appropriate horseradish peroxidase-conjugated secondary antibodies at RT. Finally, vigorous washing was performed with the same washing solution (PBST). After treatment with enhanced chemiluminescence substrate (PerkinElmer, Waltham, MA, USA), the protein bands were sensitized and visualized on X-ray films (Agfa, Mortsel, Belgium) [9,30]. Band intensities were measured using ImageJ [31].

2.6. Immunoprecipitation (IP)

IP was performed with 2.0 μg of antibodies against β-catenin or TCF-4 (GFP as a negative control) in 0.8 mg of whole protein lysates (LPS treatment for 6 h at 0.125 μg/ml). Lysates were first pre-cleared with a nonspecific IgG antibody for 2 h at 4 °C. The pre-cleared lysates were then incubated with antibodies either to β-catenin or TCF-4 overnight at 4 °C and then incubated for 2 h at 4 °C with protein G agarose. The samples were washed three times with PBST and then subjected to immunoblotting as indicated. Cell lysates were separated by SDS/PAGE

Table 1
Sequences of the primers.

Name	Sequence
h IL6 F	5′-ATCTCAGCCCTGAGAAAGGA-3′
h IL6 R	5′-GAGATGAGTTGTCATGTCCT-3′
h TNF α F	5′-AGGCAGTCAGATCATCTTCT-3′
h TNF α R	5′-AGATAGATGGGCTCATACCA-3′
h IL1β F	5′-ATGGCAGAAGTACCTGAGCT-3′
h IL1β R	5′-GAGGTGGAGAGCTTTCAGTT-3′
h GAPDH F	5′-AGCCGCATCTTCTTTTGCGTC-3′
h GAPDH R	5′-TCATATTTGGCAGGTTTTTCT-3′

h, human; F, forward; R, reverse.

in a 10 % acrylamide gel and transferred onto a nitrocellulose membrane for immunoblotting. Antibodies to β-catenin, NF-κBp65, TCF-4, or IκB were used. Performance of IP and loading control was confirmed with precipitating antibody heavy chains [19].

2.7. Statistical analysis

Values in each graph are expressed as the mean ± standard deviation. The data were analyzed using Student's t-test and two-way analysis of variance (ANOVA), followed by Bonferroni's post hoc test. The statistical analysis was performed using the GraphPad Prism software (version 9.0; GraphPad, Inc., La Jolla, CA, USA). The statistical significance was defined as $p < 0.05$.

3. Results

3.1. Establishment of an in vitro macrophage model with LPS stimulation

Human monocyte THP-1 cells grow in suspension culture and are originally isolated from an acute monocytic leukemia patient (Fig. 1A) [23]. Treatment with phorbol 12-myristate 13-acetate (PMA) for several days causes the cells to differentiate into a macrophage-like phenotype [24]. In concordance with previous reports, the five-day stimulation resulted in elongated spindle-shaped cells firmly adhered to the substratum, characteristic of macrophages (Fig. 1B). For inflammatory stimulation, fully differentiated macrophage-like THP-1 cells were treated with lipopolysaccharides (LPS). Immunofluorescence staining confirmed the intracellular expression of inflammatory cytokine IL-6 when stimulated by LPS (Fig. 1C). The level of IL-6 in the control THP-1 macrophage culture supernatant was less than 0.16 ng/ml, or 160.0 pg/ml, when measured by ELISA. After LPS stimulation, however, IL-6 level increased rapidly at 6 h and reached 0.9 ng/ml at 36 h, approximately 5.5 times higher than the basal level (Fig. 1D). On the other hand, when THP-1 macrophages were stimulated with EGF, a well-known cell growth stimulant, no significant increase in IL-6 secretion was observed (Fig. 1E).

3.2. Expression patterns of proinflammatory cytokines

RT-PCR was performed to examine the mRNA expression patterns of major proinflammatory factors IL-1β, IL-6, and TNF-α in LPS-stimulated THP-1 macrophages. Peak IL-6 mRNA expression was observed at 6 h. The housekeeping gene was glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Fig. 2A). The amount of mRNA expression was quantified by the intensity fold of the agarose gel (A) as a bar graph (Fig. 2B). When confirmed by ELISA, the secretion pattern of each of the three inflammatory factors increased similarly to the gene expression pattern and lasted up to 36 h (Fig. 2C–E). Compared to IL-6, the level of TNF-α or IL-1β was relatively low, suggesting that IL-6 could be a major cytokine affected by LPS stimulation in the THP-1 macrophage model.

3.3. Positive correlation of NF-κB and Wnt/β-catenin pathways in LPS-stimulated macrophages

LPS stimulation led to activation of the NF-κB pathway in LPS-stimulated THP-1 macrophages, initiated as early as 10 min post-stimulation. Phospho-AKT, which operates upstream of the NF-κB signaling pathway, appeared 10 min after LPS stimulation and gradually decreased until 36 h. Phosphorylated IκB was observed at 10 min but with a significantly decreased IκB protein level. Simultaneously, phosphorylated NF-κB p65 expression level increased markedly and lasted for 1 h (Fig. 3A). The amount of protein was quantified by the intensity fold of the blot (a) as a bar graph (Fig. 3B). In our experimental model, the NF-κB pathway was positively correlated with the Wnt/β-catenin

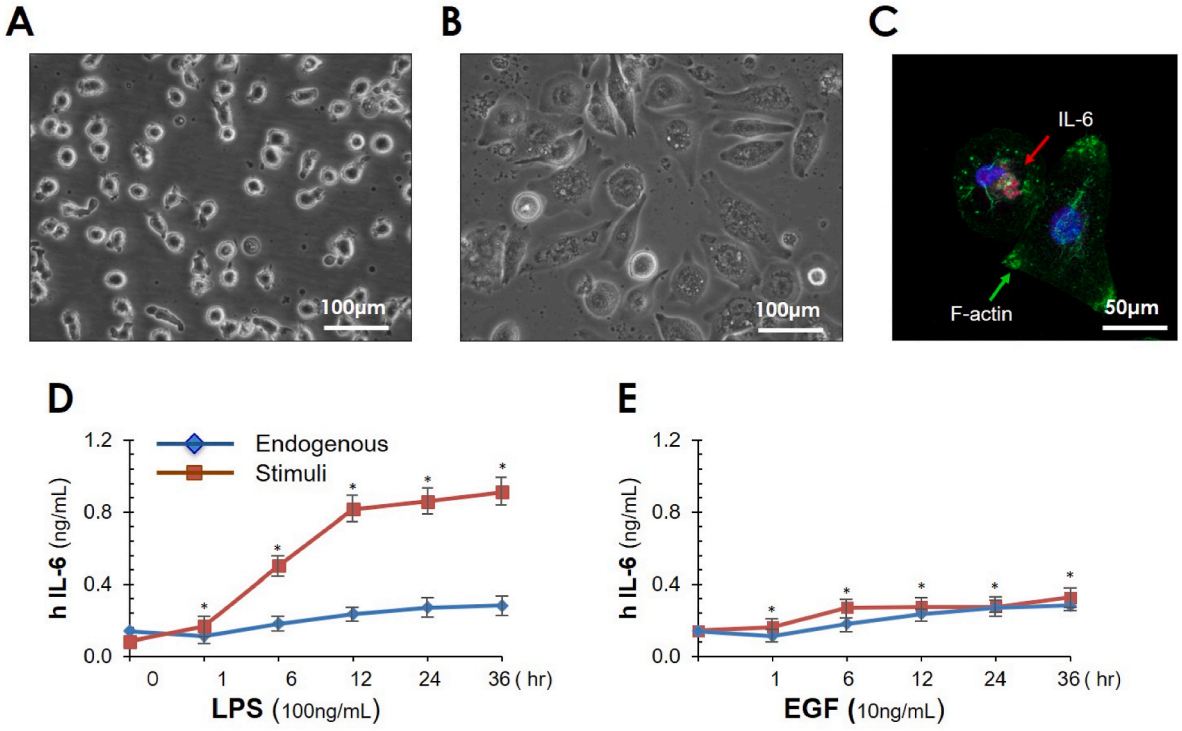

Fig. 1. Establishment of macrophages using human monocytic cell lines and confirmation of IL-6 cytokine secretion by established macrophages by ELISA. (A) THP-1 monocyte cell shape before PMA inoculation. (B) Differentiated into macrophages after five days of PMA inoculation. (C) IL-6 cytokine (red) and cell skeletal structure (green) observed in the cytoplasms by the administration of protein transport inhibitors. (D–E) The amount of IL-6 cytokine secreted from macrophages over time for each stimulating factor by ELISA. LPS stimulated at a concentration of 100 ng/mL. EGF stimulated at a concentration of 10 ng/mL. Statistical significance of differences between groups was assessed using Student's t-tests (*$p \leq 0.05$). PMA: Phorbol-12-myristate-13-acetate, LPS: lipopolysaccharides, EGF: epidermal growth factor. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

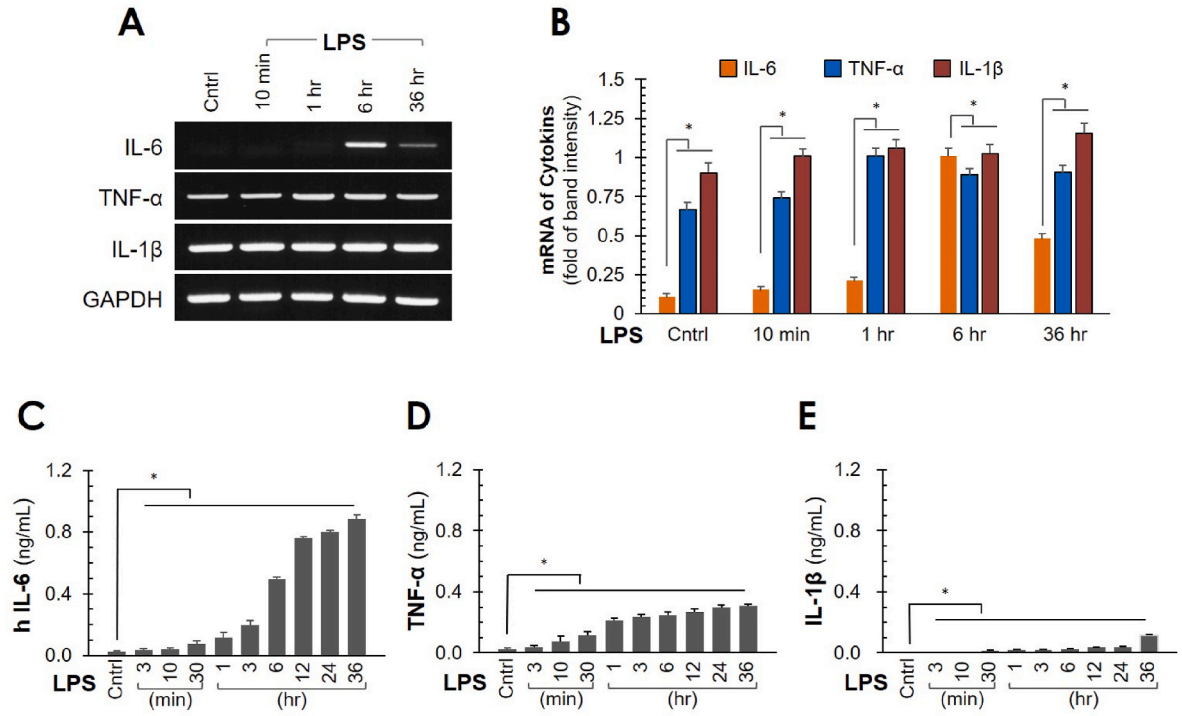

Fig. 2. Identification of gene expression patterns and protein secretion patterns of several pro-inflammatory cytokines by inflammation induction from established THP-1 macrophages. (A) Time-dependent gene expression patterns of proinflammatory cytokines by RT-PCR, and (B) the amount of mRNA expression was quantified by the intensity fold of the agarose gel as a bar graph. (C-E) Time-dependent secretion patterns of proinflammatory cytokines by ELISA. Data are expressed as the mean ± standard deviation of at least three independent experiments. Statistical significance of differences between groups was assessed using Student's t-tests (*$p \leq 0.05$).

pathway. Upon LPS stimulation, protein levels of both β-catenin and TCF-4 were increased at 10 min, peaked at 6 h, and decreased until 36 h, indicating activation of the Wnt/β-catenin pathway (Fig. 3A and B).

LY294002 was originally developed as a PI3K pathway inhibitor but is also known to inhibit the NF-κB pathway. In a previous report using THP-1 cells, LY294002 specifically decreased the NF-κB expression level in the nucleus, resulting in inhibition of the NF-κB pathway [25]. In concordance with previous findings, LY294002 reduced LPS-mediated IL-6 secretion to the basal level. Additionally, the Wnt/β-catenin pathway inhibitor PNU74654, which blocks the binding of β-catenin and TCF-4, also decreased IL-6 secretion, exhibiting a positive contribution of the Wnt/β-catenin pathway to LPS-mediated NF-κB activation (Fig. 3C). Positive cross-regulation of the two pathways may be specific to IL-6 secretion in our macrophage model, as expression of TNF-α, another proinflammatory cytokine, was not affected by LY294002 or PNU74654 (Fig. 3D).

For molecular level confirmation of NF-κB and Wnt/β-catenin pathway crosstalk, NF-κB p65, β-catenin, and TCF-4 were analyzed by immunoblotting. Both pathway inhibitors reduced the expression levels of NF-κB p65 and TCF-4. β-catenin expression was diminished only by PNU74654 but not by LY294002 (Fig. 3E). The amount of protein was quantified by the intensity fold of the blot (E) as a bar graph (Fig. 3F). A positive correlation of the NF-κB and Wnt/β-catenin pathways was also confirmed in a human microglial cell line HMC3. Blockage of IL-6 secretion but not TNF-a secretion was observed by treating LPS-stimulated HMC3 cells with LY294002 or PNU74654, indicating the involvement of both signaling pathways in LPS-driven IL-6 expression (Fig. 3G and H).

3.4. NF-κB p65 and TCF-4 form a complex in the nucleus upon LPS stimulation

For an inflammatory response to the stimuli, NF-κB translocation to the nucleus is an important feature of canonical NF-κB pathway activation. In line with this, LPS-stimulated cells respond by secreting proinflammatory cytokines, requiring NF-κB nuclear translocation [26]. In our macrophage cell line model, increased expression levels of β-catenin and TCF-4 proteins were observed in response to LPS stimulation. Based on this finding, we sought to see if any interactions with the NF-κB complex occurs with β-catenin or TCF-4 proteins upon LPS stimulation. Since NF-κB nuclear translocation is a leading event of NF-κB pathway activation, we performed an immunoprecipitation assay in each subcellular fraction. Interestingly, NF-κB p65 and TCF-4 binding was observed in the nuclear fraction only when LPS stimulation was present. Additionally, the LPS-independent cytosolic NF-κB p65/β-catenin complex was observed. The LPS-independent β-catenin/TCF-4 nuclear complex was also found, indicating canonical Wnt/β-catenin pathway activation (Fig. 4A and B).

4. Discussion

Crosstalk between NF-κB and Wnt/β-catenin pathways in inflammation has been suggested previously by various groups [17,18]. However, both positive and negative correlations have been observed depending on the cell or tissue types or inflammation status [27]. In our in vitro model, a positive correlation between the two pathways was observed. Both NF-κB blockers and Wnt/β-catenin blockers attenuated LPS-induced IL-6 production, indicating positive cross-regulation between the two pathways in our cell model. PNU74654, the Wnt/β-catenin pathway inhibitor used in this study, acts as a blocker by disrupting β-catenin/TCF-4 binding downstream of the pathway.

In this study, the relationship between proinflammatory cytokines (IL-1β, IL-6, and TNF-α) and NF-κB-WNT/β-Catenin signaling pathway was well known in Figs. 2 and 3. Fig. 2 showed a significant increase in the same time as the IL-6 cytokine secretion of macrophages caused by LPS stimuli was more than two IL-1β and TNF-α. However, if the

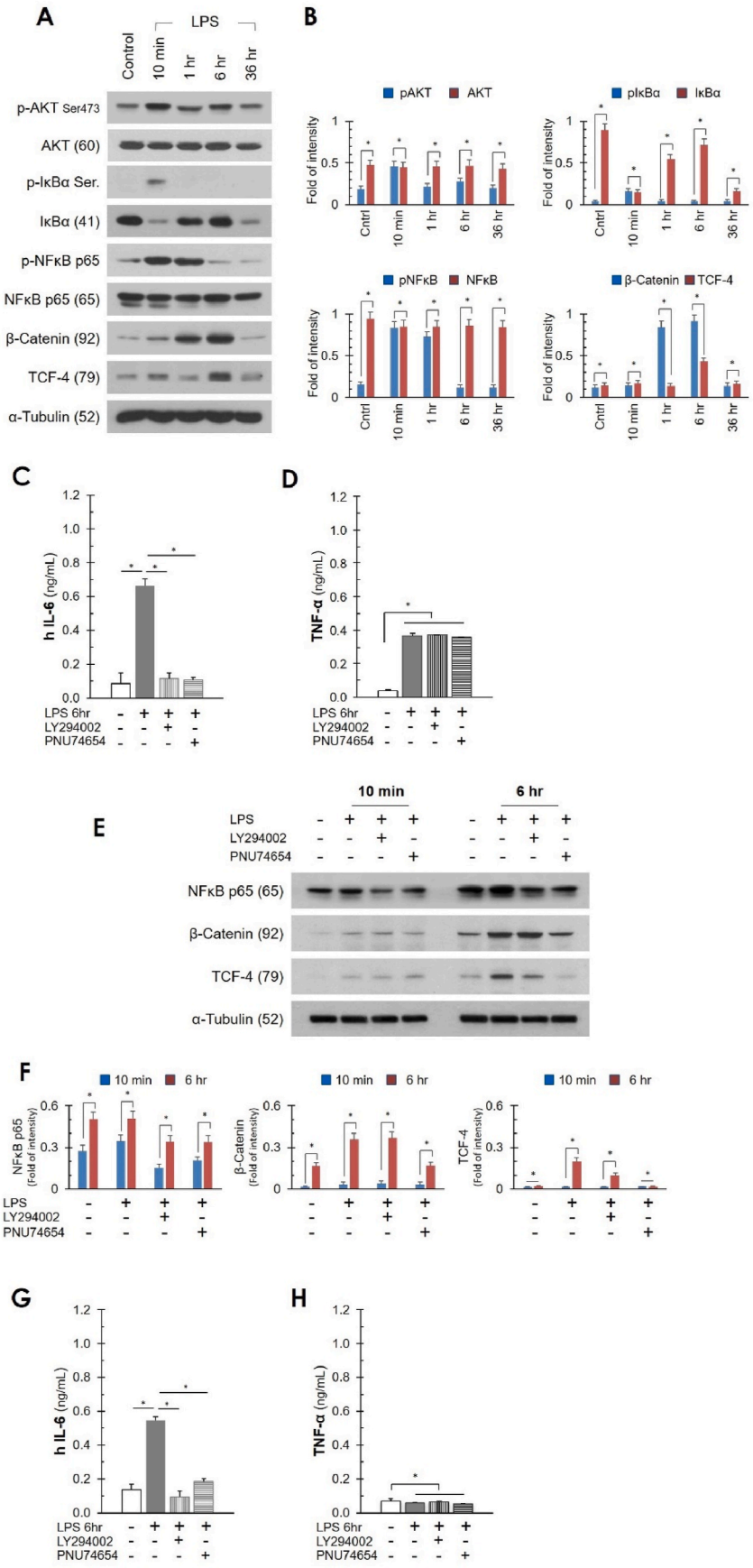

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Fig. 3. Cell signaling transduction assay of THP-1 macrophages and microglial cell line. (A) Western blot assay of time-dependent expression patterns of major signaling molecules in the NF-kB signaling pathway and the Wnt/β-catenin pathway onto THP-1. (B) The amount of protein was quantified by the intensity fold of the blot as a bar graph. (C–D) Patterns of IL-6 and TNF-α secretion using phosphatidylinositol 3 (PI3) kinase inhibitors (LY294002) and Wnt/β-Catenin pathway inhibitors (PNU74654) by ELISA onto THP-1. (E) Western blot assay of the expression patterns of signaling transduction molecules over time using cell signaling pathway inhibitors onto THP-1. (F) The amount of protein was quantified by the intensity fold of the blot as a bar graph. (G–H) Effects of the above cell signaling inhibitors on the regulation of IL-6 and TNF-a secretion onto microglial cell lines. The molecular weight (kDa) is indicated in parentheses on the left of each blot. Data are expressed as the mean ± standard deviation of at least three independent experiments. Statistical significance of differences between groups was assessed using Student's t-tests (*p ≤ 0.05).

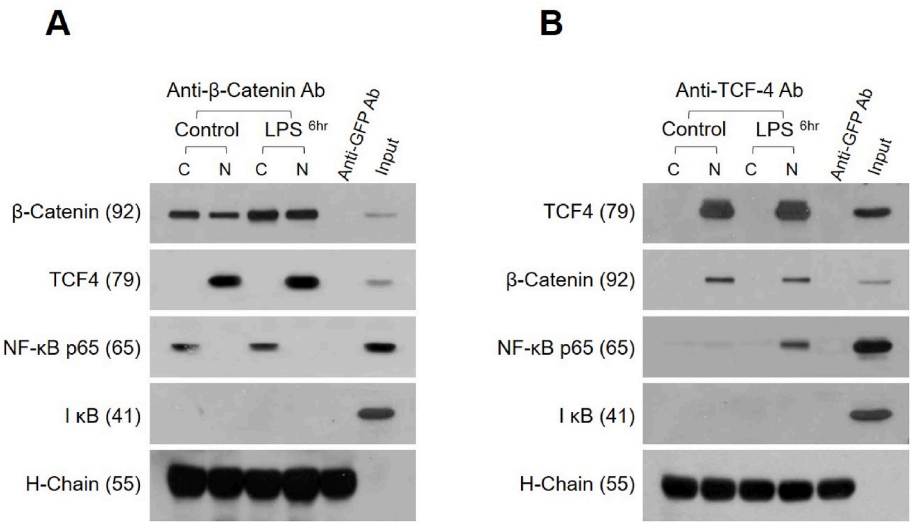

Fig. 4. Immunoprecipitation analysis (IP) using subcellular fractions to confirm cross-talk between NF-κB and WNT/β-Catenin signaling pathway. After 6 hours of stimulation with LPS, THP-1 macrophages were divided into nuclear and cytoplasmic proteins, and IP was performed for each component protein. (A) IP was performed with an anti-β-Catenin antibody, and (B) IP was performed with an anti-TCF-4 antibody. The molecular weight (kDa) is indicated in parentheses on the left of each blot. The protein fractions used for IP are shown in Fig. S2 supplementary data. Ab: antibody, C: cytosolic fraction, N: nuclear fraction.

inhibitory factor of the two signaling systems, LY294002 and PNU74654, is present, the amount of IL-6 cytokine secretion is significantly reduced, while the amount of secretion of TNF-α is not affect. This suggests that the signaling pathway of NF-κB-WNT/β-catenin is specifically involved in the IL-6 cytokine secretion control.

A previous in vivo study using LGK294, an upstream Wnt/β-Catenin pathway inhibitor LGK294, reduced the NF-κB target gene expression and rescued mice from LPS-associated death. This finding also supports the positive correlation of NF-κB and Wnt/β-catenin pathways and the full involvement of the Wnt/β-catenin pathway from upstream to downstream [28]. These knockdown/inhibition studies, including ours, collectively support the functional role of the Wnt/β-catenin pathway in the cross-activation of the NF-κB pathway. Additionally, a higher β-catenin expression level upon LPS stimulation was observed in our cell model; in concordance, liver tissue β-catenin expression was increased in the above-mentioned LPS-injected mice [28].

Of the protein binding complexes screened by immunoprecipitation, NF-κB p65/TCF-4 was the only protein complex that appeared upon LPS stimulation. As expected, the binding was in the nucleus, where activated NF-κB p65 initiates its target gene expression in the canonical NF-κB pathway. Of our IP-tested protein binding targets, the NF-κB p65/β-catenin complex was the only one found in the cytosol. As this interaction was independent of LPS stimulation and was not observed in the nuclear fraction, therefore, the event seems irrelevant to the cellular inflammatory response. There is an existing report on the NF-κB p65/β-catenin complex, and the interaction is believed to inhibit the NF-κB pathway [19]. Briefly, in HEK 293 human embryonic kidney cells, the NF-κB p65/β-catenin complex has been observed but was present only when whole cell lysate was provided, implying indirect binding involving other proteins.

The NF-κB p65/TCF-4 complex, however, behaved differently compared to the NF-κB p65/β-catenin complex. The binding was only present when stimulated by LPS, indicating a potential role of the complex in the NF-κB pathway. It is important that the complex was observed only in the nucleus, where activated NF-κB p65 and p50 translocation occurs. In support of this, the NF-κB p65/TCF-4 complex has been recently observed in human lung carcinoma cells, contributing to more efficient binding to the NF-κB binding sites of the DNA and increasing MMP-15 expression following NF-κB activation. Furthermore, in this human lung carcinoma model, TCF-4 knockdown prevented the translocation of NF-κB p65, suggesting the possible involvement of TCF-4 in cellular trafficking [29]. During the inflammatory response of macrophages, a similar NF-κB p65/TCF-4 interaction-driven mechanism may play a positive role in connecting the NF-κB and Wnt/β-catenin pathways. We plan to conduct more detailed experiments to discover how this nuclear complex contributes functionally to the positive crosstalk of the NF-κB and Wnt/β-catenin pathways.

The β-catenin/TCF-4 interaction observed in our model may not be related to NF-κB pathway crosstalk but may represent the activation of a canonical Wnt/β-catenin pathway. In our experimental model, the small molecule PNU74654 successfully inhibited the LPS-induced secretion of IL-6. Interestingly, we determined that PNU74654 disruption also led to decreased protein levels of β-catenin and TCF-4 in the cell. There is a previous report of xenopus egg extracts that β-catenin and TCF-3 bound to each other, and disruption of the complex led to the degradation of β-catenin [30], suggesting mutual stabilization of β-catenin and TCF-4 proteins in the binding form in our cell model.

However, the use of NF-κB pathway blocker LY294002 did not affect LPS-induced β-catenin expression but diminished TCF-4 expression in our immunoblotting experiments. To our knowledge, there is no report of reduced TCF-4 expression triggered by NF-κB pathway inhibition. We

suspect that this phenomenon further supports our hypothesis that LPS independent NF-κB p65/β-Catenin binding is irrelevant to the NF-κB pathway, whereas LPS-dependent NF-κB p65/TCF-4 binding plays an important role in NF-κB pathway activation. In other words, NF-κB pathway blockage may lead to reduced translocation of NF-κB p65 into the nucleus and less interaction between NF-κB p65 and TCF-4. Mutual stabilization of the two proteins by binding may be a mechanism to keep the TCF-4 level constant in the nucleus, as with β-catenin and TCF-3. Even a scenario where TCF-4 is involved in activated NF-κB p65 nuclear translocation is possible, as suggested previously in lung carcinoma cells [29].

In conclusion, a human macrophage model was developed using a PMA-induced THP-1 cell line, and this cell model secreted IL-6 upon LPS stimulation. A positive correlation of NF-κB and β-catenin pathways to include IL-6 expression was suggested. LPS-stimulated NF-κB p65/TCF-4 binding was also observed in the nucleus, suggesting that this protein complex may play a pivotal role in linking NF-κB and β-catenin pathways in macrophage responses to inflammatory stimuli.

CRediT authorship contribution statement

Ji-Youn Kim: Writing – original draft, Investigation. **Hyeon-Gun Jee:** Investigation, Conceptualization. **Ju Yeong Kim:** Methodology, Formal analysis. **Tai-Soon Yong:** Validation, Supervision. **Soung-Hoo Jeon:** Writing – review & editing, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2024.101659.

References

[1] R. Medzhitov, P. Preston-Hurlburt, C.A. Janeway Jr., A human homologue of the Drosophila Toll protein signals activation of adaptive immunity, Nature 388 (1997) 394–397, https://doi.org/10.1038/41131.
[2] T. van der Poll, C.V. Keogh, X. Guirao, W.A. Buurman, M. Kopf, S.F. Lowry, Interleukin-6 gene-deficient mice show impaired defense against pneumococcal pneumonia, J. Infect. Dis. 176 (1997) 439–444, https://doi.org/10.1086/514062.
[3] J.L. Stow, P.C. Low, C. Offenhäuser, D. Sangermani, Cytokine secretion in macrophages and other cells: pathways and mediators, Immunobiology 214 (2009) 601–612, https://doi.org/10.1016/j.imbio.2008.11.005.
[4] B. Geckin, G. Kilic, P.A. Debisarun, K. Föhse, A. Rodríguez-Luna, P. Fernández-González, A.L. Sánchez, J. Domínguez-Andrés, The fungal-derived compound AM3 modulates pro-inflammatory cytokine production and skews the differentiation of human monocytes, Front. Immunol. 14 (2023) 1165683, https://doi.org/10.3389/fimmu.2023.1165683.
[5] C.A. Hunter, S.A. Jones, IL-6 as a keystone cytokine in health and disease, Nat. Immunol. 16 (2015) 448–457, https://doi.org/10.1038/ni.3153.
[6] N. Toyama, T. Ono, T. Ono, T. Nakashima, The interleukin-6 signal regulates orthodontic tooth movement and pain, Biochem. Biophys. Res. Commun. 684 (2023) 149068, https://doi.org/10.1016/j.bbrc.2023.09.096.
[7] O.P. Kristiansen, T. Mandrup-Poulsen, Interleukin-6 and diabetes: the good, the bad, or the indifferent? Diabetes 54 (Suppl 2) (2005) S114–S124, https://doi.org/10.2337/diabetes.54.suppl_2.s114.
[8] K. Ohl, K. Tenbrock, Inflammatory cytokines in systemic lupus erythematosus, J. Biomed. Biotechnol. 2011 (2011) 432595, https://doi.org/10.1155/2011/432595.
[9] I. Caiello, G. Minnone, D. Holzinger, T. Vogl, G. Prencipe, A. Manzo, F. De Benedetti, R. Strippoli, IL-6 amplifies TLR mediated cytokine and chemokine production: implications for the pathogenesis of rheumatic inflammatory diseases, PLoS One 9 (2014) e107886, https://doi.org/10.1371/journal.pone.0107886.
[10] X. Li, Y. Song, X. Wang, C. Fu, F. Zhao, L. Zou, K. Wu, W. Chen, Z. Li, J. Fan, Y. Li, B. Li, S. Zeng, X. Liu, M. Zhao, L. Yi, J. Chen, S. Fan, The regulation of cell homeostasis and antiviral innate immunity by autophagy during classical swine fever virus infection, Emerg. Microb. Infect. 12 (2023) 2164217, https://doi.org/10.1080/22221751.2022.2164217.
[11] J. Guan, Y. Fan, S. Wang, F. Zhou, Functions of MAP3Ks in antiviral immunity, Immunol. Res. (2023) 1–19, https://doi.org/10.1007/s12026-023-09401-4.
[12] S. Ghosh, M. Karin, Missing pieces in the NF-kappaB puzzle, Cell 109 (Suppl) (2002) S81–S96, https://doi.org/10.1016/s0092-8674(02)00703-1.
[13] N. Silverman, T. Maniatis, NF-kappaB signaling pathways in mammalian and insect innate immunity, Genes Dev. 15 (2001) 2321–2342, https://doi.org/10.1101/gad.909001.
[14] D. Thanos, T. Maniatis, Virus induction of human IFN beta gene expression requires the assembly of an enhanceosome, Cell 83 (1995) 1091–1100, https://doi.org/10.1016/0092-8674(95)90136-1.
[15] M. Martin, K. Rehani, R.S. Jope, S.M. Michalek, Toll-like receptor-mediated cytokine production is differentially regulated by glycogen synthase kinase 3, Nat. Immunol. 6 (2005) 777–784, https://doi.org/10.1038/ni1221.
[16] K. Nejak-Bowen, A. Kikuchi, S.P. Monga, Beta-catenin-NF-κB interactions in murine hepatocytes: a complex to die for, Hepatology 57 (2013) 763–774, https://doi.org/10.1002/hep.26042.
[17] Y. Hu, Z. Wang, C. Fan, P. Gao, W. Wang, Y. Xie, Q. Xu, Human gingival mesenchymal stem cell-derived exosomes cross-regulate the Wnt/β-catenin and NF-κB signalling pathways in the periodontal inflammation microenvironment, J. Clin. Periodontol. 50 (2023) 796–806, https://doi.org/10.1111/jcpe.13798.
[18] X. Zhao, B. Ma, H. Zhu, J. Bai, L. Liu, X. Li, J. Cai, B. Wang, L. Wang, Y. Pang, H. Zhang, Y.I. Yang, N. Li, M. Chen, PI3K/Akt and Wnt/β-catenin signaling cross-regulate NF-κB signaling in TNF-α-induced human Lgr5(+) intestinal stem cells, Anticancer Res. 42 (2022) 3325–3340, https://doi.org/10.21873/anticanres.15820.
[19] J. Deng, S.A. Miller, H.Y. Wang, W. Xia, Y. Wen, B.P. Zhou, Y. Li, S.Y. Lin, M. C. Hung, beta-catenin interacts with and inhibits NF-kappa B in human colon and breast cancer, Cancer Cell 2 (2002) 323–334, https://doi.org/10.1016/s1535-6108(02)00154-x.
[20] J. Jang, J.H. Ha, S.I. Chung, Y. Yoon, B-catenin regulates NF-κB activity and inflammatory cytokine expression in bronchial epithelial cells treated with lipopolysaccharide, Int. J. Mol. Med. 34 (2014) 632–638, https://doi.org/10.3892/ijmm.2014.1807.
[21] Q. Du, X. Zhang, J. Cardinal, Z. Cao, Z. Guo, L. Shao, D.A. Geller, Wnt/beta-catenin signaling regulates cytokine-induced human inducible nitric oxide synthase expression by inhibiting nuclear factor-kappaB activation in cancer cells, Cancer Res. 69 (2009) 3764–3771, https://doi.org/10.1158/0008-5472.Can-09-0014.
[22] C. Hyun Hwa, J. Hye Joon, S. Ji Sun, B. Yong Chan, J. Jin Sup, Crossregulation of beta-catenin/Tcf pathway by NF-kappaB is mediated by lzts2 in human adipose tissue-derived mesenchymal stem cells, Biochim. Biophys. Acta 1783 (2008) 419–428, https://doi.org/10.1016/j.bbamcr.2007.08.005.
[23] S. Tsuchiya, M. Yamabe, Y. Yamaguchi, Y. Kobayashi, T. Konno, K. Tada, Establishment and characterization of a human acute monocytic leukemia cell line (THP-1), Int. J. Cancer 26 (1980) 171–176, https://doi.org/10.1002/ijc.2910260208.
[24] T. Tenno, Regulation of tissue factor expression in myeloid and monocytic leukaemia cells, Acta Univ. Upsal. (2001).
[25] D. Avni, Y. Glucksam, T. Zor, The phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 modulates cytokine expression in macrophages via p50 nuclear factor κB inhibition, in a PI3K-independent mechanism, Biochem. Pharmacol. 83 (2012) 106–114, https://doi.org/10.1016/j.bcp.2011.09.025.
[26] J. Sakai, E. Cammarota, J.A. Wright, P. Cicuta, R.A. Gottschalk, N. Li, I.D.C. Fraser, C.E. Bryant, Lipopolysaccharide-induced NF-κB nuclear translocation is primarily dependent on MyD88, but TNFα expression requires TRIF and MyD88, Sci. Rep. 7 (2017) 1428, https://doi.org/10.1038/s41598-017-01600-y.
[27] B. Ma, M.O. Hottiger, Crosstalk between Wnt/β-catenin and NF-κB signaling pathway during inflammation, Front. Immunol. 7 (2016) 378, https://doi.org/10.3389/fimmu.2016.00378.
[28] J. Jang, J. Song, H. Lee, I. Sim, Y.V. Kwon, E.H. Jho, Y. Yoon, LGK974 suppresses lipopolysaccharide-induced endotoxemia in mice by modulating the crosstalk between the Wnt/β-catenin and NF-κB pathways, Exp. Mol. Med. 53 (2021) 407–421, https://doi.org/10.1038/s12276-021-00577-z.
[29] Y. Liu, Y. Xu, S. Guo, H. Chen, T cell factor-4 functions as a co-activator to promote NF-κB-dependent MMP-15 expression in lung carcinoma cells, Sci. Rep. 6 (2016) 24025, https://doi.org/10.1038/srep24025.
[30] E. Lee, A. Salic, M.W. Kirschner, Physiological regulation of [beta]-catenin stability by Tcf3 and CK1epsilon, J. Cell Biol. 154 (2001) 983–993, https://doi.org/10.1083/jcb.200102074.