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대식세포에서 NF-κB 경로를 통한 IL-6 생산에서의 β-catenin 의 역할

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ABSTRACT

Introduction: β-catenin is known to be involved in various signaling pathways. Recently, the interaction between β-catenin and NF-κB pathway, one of the main pathways for proinflammatory cytokine production in inflammation, has been reported. In this study, I aimed to look at the role of beta-catenin in NF-κB pathway and how that ultimately affects the regulation of IL-6 cytokine. To examine this, a myeloid-specific knockout of *Ctnnb1* (encoding β-catenin), *Ctnnb1*$^{\Delta myeloid}$ mouse strain was used and the level of IL-6 secreted by LPS-stimulated bone marrow-derived macrophages and peritoneal macrophages was examined.

Methods: The *Ctnnb1*$^{\Delta myeloid}$ mice were obtained from breeding a beta-catenin floxed mouse strain *Ctnnb1*$^{fl/fl}$ with LysMcre transgenic mice, LysM^{cre}. The *Ctnnb1*$^{\Delta myeloid}$ mice were confirmed by genotyping and the composition of various immune cells in this strain was screened and compared with that of the wild-type (WT) mouse strain. *Ctnnb1* KO bone marrow-derived macrophages (BMDMs) and peritoneal macrophages (PMs) were isolated to measure the level of IL-6 secreted by these cells *in vitro* after LPS stimulation. The sera IL-6 level in *Ctnnb1*$^{\Delta myeloid}$ and WT mice after intraperitoneal injection of LPS was also measured. Then, the activity of the transcription factor, NF-κB p65 and its related

upstream protein, IκBα, which is responsible for the translocation of p65 were examined in the *Ctnnb1* KO peritoneal macrophages by immunoblot analysis.

Results: The *Ctnnb1*$^{\Delta myeloid}$ mice did not significantly affect the development of the myeloid and lymphoid cells in the lymphoid organs as the percentage and the absolute number of these immune cells between WT and *Ctnnb1*$^{\Delta myeloid}$ mice were similar. The β-catenin also did not affect the morphology and differentiation of BMDMs. However, the function of BMDMs and PMs were different in the *Ctnnb1*$^{\Delta myeloid}$ mice, as the level of IL-6 secreted by *Ctnnb1* KO macrophages was higher than that of WT macrophages after LPS stimulation. This was also true in the sera level of IL-6 as the *Ctnnb1*$^{\Delta myeloid}$ mice showed higher peripheral level of IL-6 at 5 hr post-intraperitoneal injection of LPS. The level of p65 activity and the degradation of IκBα was also higher in the *Ctnnb1* KO PM than the WT PM, suggesting that the β-catenin does contribute to the NF-κB pathway which leads to increased level of IκBα degradation and p65 activity, and hence the level of IL-6 in macrophages.

Conclusion: This study suggests the possible role of β-catenin in NF-κB pathway for IL-6 production in macrophages during inflammation. In macrophages without β-catenin, the activity of p65 was higher than the

normal macrophages and the level of IL-6 was prolonged from certain time point after LPS stimulation, suggesting the role of β-catenin in NF-κB pathway for prolonged IL-6 production during inflammation. The results demonstrate a potential therapeutic target for controlling IL-6 production in chronic IL-6-mediated inflammatory diseases.

Keywords: Beta-catenin, NF-κB pathway, Interleukin-6 (IL-6), Cytokine, Macrophage

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CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

BM	BONE MARROW
BMDM	BONE MARROW-DERIVED MACROPHAGE
CBA	CYTOMETRIC BEAD ARRAY
CD	CLUSTER OF DESIGNATION
DMEM	DULBECCO'S MODIFIED EAGLE'S MEDIUM
FACS	FLUORESCENCE ACTIVATED CELL SORTING
FITC	FLUORESCENCE ISOTHIOCYANATE
IκB	INHIBITOR KAPPA B
IL	INTERLUEKIN
KO	KNOCKOUT
LPS	LIPOPOLYSACCHARIDE
M-CSF	MACROPHAGE-COLONY STIMULATING FACTOR
NF-κB	NUCLEAR FACTOR KAPPA B
PAMP	PATHOGEN ASSOCIATED MOLECULAR PATTERN
PBS	PHOSPHATE BUFFERED SALINE
PE	PHYCOERYTHRIN
PERCP	PERIDININ-CHLOROPHY II PROTEIN COMPLEX
PM	PERITONEAL MACROPHAGE
TG	THIOGLYCOLLATE
TLR	TOLL LIKE RECEPTOR
TNF	TUMOR NECROSIS FACTOR
WT	WILD TYPE

INTRODUCTION

Macrophages have been known as one of the most important players in immune surveillance. They are the most powerful antigen presenting cells that capture antigens and present them to lymphoid cells to elicit subsequent immune responses. Because of their specialty in detecting and killing bacteria and other harmful organisms, macrophages play the most important role in innate immune system, the first line of defense against infections. Macrophages recognize pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors. PAMPs are microbial molecules that are shared by different types of microbes. These include nucleic acids only present in microbes, proteins that are unique to microbes, or lipids and carbohydrates that are only synthesized by microbes. The most well-known substance as one of the PAMPs is lipopolysaccharide (LPS) from gram-negative bacteria. Macrophages capture and engulf LPS via Toll-like receptor 4 (TLR-4), one of the pattern recognition receptors present on the surface of the macrophage (1). Once these microbial substances are bound and recognized by TLR4, TLR dimerization occurs to initiate a subsequent signaling cascade to activate Nuclear Factor kappa B (NF-κB) transcription factor. This ultimately leads to production of pro-inflammatory cytokines, such as

IL-6, TNF-α and IL-1β, which elicit acute inflammatory responses and activate other immune cells to stimulate adaptive immunity.

As a mediator of acute inflammation, interleukin 6 (IL-6) is one of the major pro-inflammatory cytokines of the innate immune system produced by macrophages. IL-6 affects nearly all components of our immune system. Upon stimulation of TLRs, macrophages secrete IL-6 on local endothelial cells and smooth-muscle cells nearby to make them release chemokines, leading to the recruitment of more immune cells around the site of inflammation and hence the resolution of inflammation (2). IL-6 stimulates hepatocytes for synthesis and secretion of acute-phase proteins, such as C-reactive protein, often regarded as a diagnostic marker of inflammation and crucial in the defense mechanism against bacterial pathogens (3, 4). IL-6 secreted by macrophages is also critical in activating the adaptive immunity once the innate immune response has been triggered. Secretion of IL-6 elicits IL-17-producing effector T helper cell (Th17) differentiation with TGF-β and also stimulates B cells to produce IgG antibodies (Abs) (5).

As IL-6 secreted by macrophages is crucial in stimulating our immune system, numerous attempts have been made to target IL-6 for the treatment of inflammatory diseases. Until now, IL-6 receptor neutralizing antibody has been extensively studied for the inhibition of IL-6 signaling in rheumatoid arthritis (6). However, not only the

inhibition of IL-6-induced signaling pathways is important, but also the hampering of signaling pathways that initially induce expression of IL-6 cytokine comes critical in mitigating the IL-6-associated inflammatory responses. For these reasons, there needs be more studies on identifying the molecules that are involved in IL-6 production. These molecules can potentially be targeted as a treatment for inflammatory diseases.

The major signaling pathway of IL-6 production upon stimulation of TLR with PAMPs in macrophages is the NF-κB pathway. NF-κB is the master transcription factor of inflammation that is eventually activated to stimulate the expression of genes encoding many of the proteins involved in inflammatory responses including production of IL-6 cytokine. NF-κB also functions as a master regulator of cell differentiation, proliferation and survival (7, 8). NF-κB signaling pathway can be separated into canonical and non-canonical pathway. Various signals including PAMPs activate the canonical NF-κB signaling (7, 9). Upon stimulation with LPS, the membrane-bound receptor, TLR4 is activated, subsequently leading to the activation of inhibitor of kappa B (IκB) kinase (IKK) complex in the cytoplasm of macrophages. This IKK complex phosphorylates IκBα, which is directly bound to NF- κB RelA (p65). The phosphorylation of IκBα results in ubiquitination and proteasomal degradation of itself, releasing the RelA protein which forms a dimer with p50 or p52 and translocates into the nucleus for

regulation of its target genes. The phosphorylation of IκBα is necessary for the activation of RelA (p65) and its translocation to the nucleus in NF-κB signaling pathway (1, 7, 8). Although the NF-κB signaling pathway is one of the most extensively studied signaling pathways, there still remains a range of other unknown pathways and molecules that interact with this TLR-initiated pathway in macrophages.

β-catenin is a multifunctional protein involved in cell-to-cell adhesion by interacting with E-cadherin (10-12). It also regulates gene transcription associated with cell proliferation, cell survival and differentiation via Wnt/β-catenin pathway (10, 11). As a central mediator of Wnt/β-catenin pathway, β-catenin in the cytoplasm is constitutively made and targeted for degradation by a destruction complex consisting glycogen synthase kinase 3 (GSK-3). With extracellular stimuli such as Wnt proteins through engagement of the co-receptors frizzled (FZD) and low-density lipoprotein receptor-related protein (LRP), the destruction complex is inactivated and β-catenin accumulates in the cytoplasm. This leads to the translocation of β-catenin into the nucleus to associate with T cell factor/lymphoid enhancer factor (TCF/LEF) transcription factor to regulate gene transcription.

Recently, studies exploring the significance of β-catenin in inflammation is increasing. It was shown by Suryawanshi A, *et al.,* that β-catenin knockout in dendritic cells enhanced the disease progression

of EAE by increasing the level of IL-17 and the percentage of effector T cells (13). Moreover, evidences that β-catenin in the cytoplasm may interact with other signaling pathways or molecules are emerging. Current findings suggest that β-catenin can modulate the immune response by interacting with NF-κB signaling pathway. Bin *et al.,* suggests that the reduction of NF-κB signaling was achieved by CBP-mediated Wnt-3A and IL-1β stimulation in chondrocytes (14).

However, this negative regulation of NF-κB signaling by β-catenin can be controversial since positive regulation of NF-κB signaling by β-catenin has also been reported. Studies reveal a negative regulation of β-catenin on NF-κB activity and expression of the target gene including IL-6 in liver, breast and colon cancer cells (15, 16), but such interaction was not seen in head and neck cancer cells (17), implying that the role of β-catenin may vary depending on the cell type. Also, the ligand of a target gene that is activated downstream of the Wnt/β-catenin pathway increased NF-κB signaling in colorectal cancer cells, suggesting an indirect interaction between β-catenin and NF-κB (18). Based on these evidences, the involvement of β-catenin in the regulation of NF-κB signaling has raised the possibility that this protein may play an important role in modulating inflammatory responses in a heavily context-dependent manner.

Since NF-κB-mediated immune responses can either be positively or negatively regulated by β-catenin depending on the cell type, the role of β-catenin in IL-6 production via NF-κB pathway in a various cellular context is still an intriguing question to dwell upon. Also, unlike TNF-α and IL-1β, which are part of the primary response genes that are rapidly induced upon innate stimulus without any additional de novo protein synthesis, IL-6 gene is a secondary response gene that requires additional involvement of proteins for its transcriptional activation (33). This suggests a possibility that additional protein, like β-catenin may interact with proteins in NF-κB pathway for IL-6 production specifically. It is also needless to say that more extensive studies on how β-catenin regulates IL-6 production in different contexts are required for better understanding of this molecular interaction and its physiological significance.

The purpose of this study is to discover the role of β- catenin in IL-6 production via NF-κB pathway in activated macrophages stimulated by LPS. To specifically see the effect of β-catenin on the regulation of NF-κB and its target gene in macrophages, a myeloid specific knockout of β-catenin, *Ctnnb1*$^{\Delta myeloid}$ (cKO) mice were used. Two different types of macrophages, bone marrow-derived macrophages and peritoneal macrophages were isolated from each group to measure the different levels of IL-6 secreted by these macrophages to LPS stimuli.

Then the protein levels of molecules that are responsible for IL-6 production, the transcription factor NF-κB p65 and its associated upstream molecule, IκBα were further examined in these β-catenin conditional KO macrophages. The results demonstrated a prolonged secretion of IL-6 by macrophages in the absence of β-catenin, as the secretion of IL-6 from 3 hrs until 12 hrs post LPS stimulation was higher in the *Ctnnb1* KO macrophages than in the WT macrophages. This increment was also observed in protein levels of NF-κB RelA (p65) and its upstream molecule, IκBα in the cell lysates, implying that the prolonged secretion of IL-6 cytokine in the absence of β-catenin is due to the higher activity of NF-κB and the level of IκBα phosphorylation than that of WT. This led to the next question that β-catenin may physically interact with one of the molecules in the upstream of NF-κB signaling to regulate the phosphorylation of IκBα and ultimately control the level of IL-6 in activated macrophages. This study has illustrated the importance of β-catenin in controlling the level of IL-6 in macrophages to LPS by negatively regulating the activity of NF-κB and the phosphorylation of IκBα.

MATERIALS AND METHODS

Animals and models

Animals were bred in a pathogen-free animal facility at Seoul National University. 10-12-week-old female C57BL/6 (B6) mice were used in all experiments according to procedures approved by the Seoul National University Institutional Animal Care and Use Committee (IACUC no. SNU-180226-6 and SNU-190617-7)

The myeloid specific β-catenin conditional knockout mice, *Ctnnb1*$^{\Delta myeloid}$ (cKO) were obtained by breeding *Ctnnb1* floxed mice, *Ctnnb1*$^{fl/fl}$ with LysMcre transgenic mice, LysM^{cre} with B6 background.

Ctnnb1$^{fl/fl}$ mice were provided by Professor Jin Woo Kim (Korea Advanced Institute of Science and Technology).

LysM^{cre} mice were provided by Professor Suk-JO Kang (Korea Advanced Institute of Science and Technology).

Genotyping

Mouse genomic DNA was obtained from tail biopsies following 40minute digestion at 85℃ in Tail Lysis buffer (Fiat International Ltd., Seoul, Korea), followed by heat inactivation. PCR was performed with these samples using the primer pairs provided by the Jackson Laboratory

to distinguish between the wild type and the myeloid specific β-catenin knockout mice (Primer pair 1 and 2). Primer pair 1 was used to detect wild-type allele (221 bp) or mutant allele (324 bp). PCR conditions were as follows: 35 cycles at 94℃ for 30 s, 60℃ for 50 sec, 72℃ for 1 min.

Primer pair 1:

5’ TACACTATTGAATCACAGGGACTT 3’

3’ CACCATGTCCTCTGTCTATTC 5’

Primer pair 2:

5’ AAGGTAGAGTGATGAAAGTTGTT 3’

3’ CACCATGTCCTCTGTCTATTC 5’

Flow Cytometric analysis of myeloid and lymphoid cells

Three 10-12-week-old female wild type (WT) and *Ctnnb1*$^{\Delta myeloid}$ mice were sacrificed with CO2. The abdomen was cut opened and the axillary, brachial and inguinal lymph nodes were isolated from the mice. After removing the lymph nodes, the spleen was also isolated. The isolated lymph nodes and spleen were homogenized with frosted glass and strained with 70 μm cell strainer in sterile PBS. After centrifugation at 500 $\times$ *g* for 10 min, the splenocytes were treated with Red Blood Cell Lysing Buffer (Sigma-Aldrich, St. Louis, MO) for 5 min and then washed with sterile PBS. After cell counting, approximately 1 $\times$ 10^5

cells/mL of lymph nodes and spleen were stained with both types of Ab cocktails in FACS buffer for 40 min at 4℃ in the dark. To avoid non-specific binding, the cells were previously incubated with 2.4G2 for 10 min at 4℃. Then, the samples were washed and resuspended with FACS buffer (0.5% BSA, 0.1% sodium azide in 1× PBS) for flow cytometric analysis (BD Canto II).

Peripheral blood was harvested from retro-orbital plexus of the wild type and the knockout mice. Then the blood was treated with Red Blood Cell Lysis Buffer (Sigma) for 5 min at room temperature. The blood was incubated with 2.4G2 (anti-mouse Fc receptor Ab) at 4℃ for 10 min and washed with FACS buffer to avoid non-specific binding of the Ab. Then the sample was treated with both types of Ab cocktails in FACS buffer for 40 min at 4℃. After washing twice with FACS buffer, the blood sample was analyzed by flow cytometry (BD Canto II).

Ab cocktails for myeloid cell analysis include anti-CD11b (FITC), anti-Ly6G (PE), anti-Ly6C (PerCp-Cy5.5), anti-F4/80 (PE-Cy7), and anti-CD45 (APC-Cy7) Abs. All antibodies were purchased from Biolegend (San Diego, CA).

Ab cocktails for lymphoid cell analysis include anti-CD4 (PE-Cy7), anti-CD8 (APC-Cy7), anti-CD19 (PerCP-Cy5.5) and anti-CD49b (FITC) Abs. All antibodies were purchased from Biolegend.

Bone Marrow-Derived Macrophage Differentiation

BMDCs were isolated from the tibias and femurs of the 10-week-old wild type B6 mice and myeloid specific β-catenin knockout mice. The femurs and tibias were separated from the mice. All the muscles and fats were removed with the epiphysis intact. The ends of the femurs and tibias were trimmed and the bone marrow cells were flushed out with ice cold PBS using a 24G needle. All cells were collected and passed through a 70 μm cell strainer to make a single cell suspension. BMDCs were washed once by PBS and then counted with a hemocytometer and resuspended with DMEM complete media at 1×10^6 cells/mL. Bone marrow progenitor cells were seeded onto a 10 cm culture dish at 1×10^6 cells/mL density and cultured with DMEM complete media (DMEM, Gibco Laboratories, Waltham, MA) supplemented with 10% FBS (FBS, Gibco Laboratories), L-glutamine, 2-ME, HEPES, penicillin/streptomycin, 10 ng/ml M-CSF (Cat#416-ML, R&D systems, Minneapolis, MN) at 37℃ in 5% CO_2 for seven days. On Day3, fresh 5 ml complete media was added. On day 7, adherent cells were harvested by trypsin (Gibco Laboratories) and cell scraping. Then the cells were

centrifuged and seeded onto a new 48 well-plate for 1 day, followed by LPS stimulation for cytokine analysis.

Thioglycolate-elicited Peritoneal Macrophage Isolation

Thioglycolate-elicted peritoneal macrophages were isolated from wild type and conditional knockout mice 4 days after peritoneal injection of 3% thioglycolate broth media. On day 4, mice were sacrificed with CO_2 without cervical dislocation to avoid internal bleeding. The skin of the abdomen was removed to expose the peritoneal wall. 10 ml of ice cold PBS using a 10 ml syringe with 24G needle was injected carefully into the peritoneal cavity. After massaging the abdomen for 30 sec, the peritoneal fluid was recovered as much as possible using a 10 ml syringe with 20G needle. The recovered fluid was put through a 70 μm cell strainer for a single suspension and then centrifuged 5 min at 300 $\times g$ at 4℃. The cells were resuspended in 10 ml DMEM complete media and seeded onto a 10 cm culture dish for overnight culture, followed by LPS stimulation for cytokine and protein analysis.

LPS Stimulation

In vitro

After overnight seeding, 1×10^6 cells/mL macrophage cells were stimulated with LPS 100 ng/mL for 1 hr, 3 hr, 5 hr, 8 hr, 12 hr and 24 hr.

For cytokine analysis, the supernatant was collected for CBA analysis whereas cell lysates were collected for western blot analysis.

In vivo

10-12-week-old female WT and *Ctnnb1*$^{\Delta myeloid}$ mice were intraperitoneally injected with LPS (0.125 μg/g).

Flow cytometric analysis

Supernatant from macrophage culture after LPS stimulation was collected at different time points (0 hr, 1 hr, 3 hr, 5 hr, 8 hr, 12 hr, 48 hr). IL-6 was detected by multiplexed bead-based immunoassays (CBA kits- BD Biosciences, Franklin Lakes, NJ).

Peripheral blood was harvested from retro-orbital plexus of the WT and the *Ctnnb1*$^{\Delta myeloid}$ mice after LPS stimulation at different time points. IL-6 was detected by multiplexed bead-based immunoassays (CBA kits- BD Biosciences).

In general, 1×10^5 cells/mL bone marrow derived macrophages and peritoneal macrophages were harvested for confirmation of surface marker protein, CD45+ and F4/80+. To prevent non-specific binding, the sample was incubated with 2.4G2 (anti-mouse Fc receptor Ab) at room temperature for 10 min and washed with FACS buffer. Cells were then

stained with anti-CD45 and anti-F4/80 Abs, purchased from BioLegend, for 40 minutes at 4℃ in dark. After incubation, cells were washed twice with FACS buffer and resuspended for flow cytometric analysis (BD Canto II).

For intracellular staining, peritoneal macrophages were harvested and stained with extracellular surface markers, CD45+ and F4/80+. Then the cells were fixed and permeabilized using the ebioscience kit according to the manufacturer's protocol. After fixation and permeabilization, cells were stained with anti-IL6 Ab at room temperature for 30 min. Cells were washed twice with 1X Perm buffer (eBioscience, San Diego, CA) and resuspended with FACS buffer for flow cytometric analysis (BD Canto II). For isotype staining, anti- mouse IgG Ab was used.

Immunoblotting

In general, 5×10^6 peritoneal macrophage cells were lysed in lysis buffer (1× RIPA, 1× Phosphatase inhibitor, 1× Protease inhibitor in ddH20) at certain time points after LPS stimulation. After adding the lysis buffer directly to the cells in the 10 cm petri dish and incubating it on ice for 5 min, the adherent cells were scraped using a plastic cell scraper. The lysate was transferred to a 1.5 mL microtube and centrifuged for 15 min at 14,000 $\times g$ at 4℃. Supernatant was collected followed by protein quantification for western blot analysis.

With the cell lysate samples, the cell protein content was measured using the bicinchoninic acid (BCA) assay (Pierce, Waltham, MA), according to the manufacturer's protocol.

To assess the protein levels of phosphorylated NF-κB p65 and IκBα proteins by Western blotting, the cell lysate samples were diluted in 4× Laemmle sample buffer (277.8mM Tris-HCl, pH6.8, 44.4% (v/v) glycerol, 4.4% LDS, 0,02% bromophenol blue, and 10% 2-mercaptoethanol; Bio-Rad, Hercules, CA) and boiled for 5 min. Approximately 15 μg of protein samples were separated by 10% SDS PAGE and transferred to PVDF membranes (Bio-Rad). The membrane was blocked with 5% BSA in PBST at room temperature for 2 hr. Then the membrane was incubated with anti-phosphorylated NF-κB p65 (1:1000 in 5% BSA; Cell Signaling Technologies, Beverly, MA), anti-IκBα (1:1000 in 5% BSA; Cell Signaling Technologies) and anti-β-actin (1:1000 in 5% BSA; Thermofisher, Waltham, MA) overnight at 4℃. After primary Ab incubation, the membrane was washed with PBST (0.1% Tween 20) for 30 min and incubated with anti-rabbit IgG Ab conjugated with HRP (1:2000 in PBST; Bioss Antibodies Inc, Woburn, MA) for 40 min at room temperature. The membrane was thoroughly washed for 30 min with PBST and enhanced chemiluminescent HRP substrate was added to the blot for HRP signal detection by Amersham Imager 600 (GE

healthcare, Chicago, Illinois). The intensity of the band was measured by Image J and the relative band density was calculated by normalization to β-actin, the house-keeping protein.

Statistical analysis

All statistical analysis were conducted using GraphPad prism 6 (GraphPad Software, La Jolla, CA). All the data were assessed using the unpaired two-tailed student's *t* test. Values were expressed as mean ± S.E.M. (standard error of the mean). The *p*-values were shown as: *level of significance, $p<0.05$; **level of significance, $p<0.01$

RESULTS

1. Confirmation of myeloid specific β-catenin conditional knockout mice, *Ctnnb1*$^{\Delta myeloid}$ (cKO) and its characterization of the immune cell composition

To find out the effect of β-catenin specifically in macrophages, I first looked for an appropriate mouse strain. To obtain *Ctnnb1*$^{\Delta myeloid}$ mice (cKO), a β-catenin floxed mouse with a LysMcre transgenic mouse were crossbred. The genotype of these *Ctnnb1*$^{\Delta myeloid}$ mice was confirmed by PCR analysis (Figure 1A). By obtaining gDNA from tail lysis, one 300 bp band for β-catenin floxed gene and a 700 bp band for LysMcre transgene were confirmed from the *Ctnnb1*$^{\Delta myeloid}$ mice. A 200 bp band for the wild type β-catenin gene and a 350 bp band for the wild type Lysozyme gene were also confirmed from the WT mice. The gDNA from peritoneal macrophage, elicited by thioglycolate solution was obtained and both types of primer pairs were used for gene confirmation (Figure 1B). By using the primer pair 1, a single band at 500 bp in *Ctnnb1*$^{\Delta myeloid}$ mice and at 3 kb in WT mice was confirmed. From the primer pair 2, a band at 300 bp in *Ctnnb1*$^{\Delta myeloid}$ mice and at 220 bp in WT mice was confirmed. By obtaining a myeloid specific β-catenin conditional knockout mice from mating *Ctnnb1*$^{fl/fl}$ mice and LysM^{cre} transgenic mice, an appropriate mouse strain for this study was confirmed and used.

Next, I wanted to see whether the absence of β-catenin in macrophages has altered the overall composition of immune cells in the lymphoid organs of WT and *Ctnnb1*$^{\Delta myeloid}$ mice. So, I analyzed the percentage and absolute cell number of the myeloid and lymphoid cells in total leukocytes from the blood, spleen, and lymph nodes of the *Ctnnb1*$^{\Delta myeloid}$ mice and compared with those of WT mice (Figure 2A-I). There were no significant differences observed in the compositions of myeloid immune cells between blood, spleen and lymph nodes of *Ctnnb1*$^{\Delta myeloid}$ and WT mice (Figure 2G-I). The percentage and absolute cell count of the lymphoid cells such as CD4+, CD8+ T cells and CD19+ B cells were either lower or higher respectively in the blood, spleen and lymph nodes of the *Ctnnb1*$^{\Delta myeloid}$ mice than those of WT. Hence, the β-catenin deficiency did not affect the development of myeloid cells.

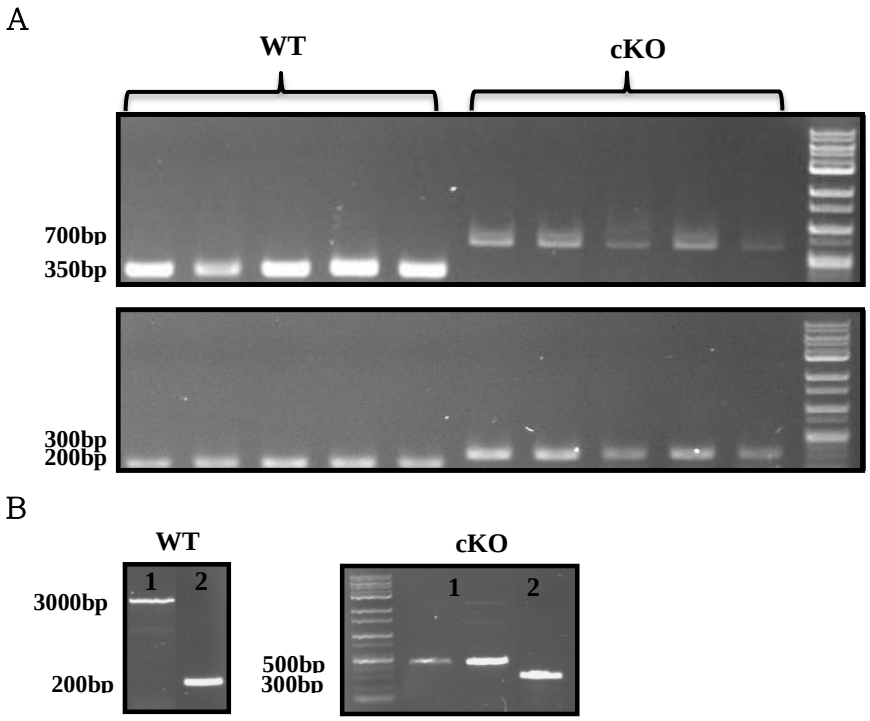
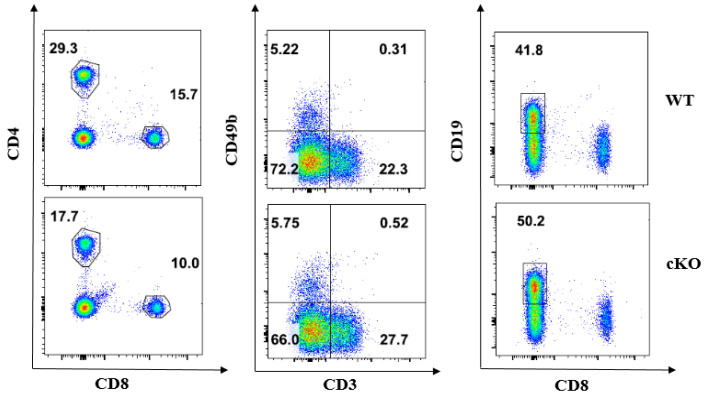


Figure 1. Confirmation and characterization of myeloid specific conditional β-catenin knockout mice, ***Ctnnb1*$^{\Delta myeloid}$ (cKO)** A. PCR analysis of genomic DNA by tail lysis from *Ctnnb1*$^{\Delta myeloid}$ and WT mice. Genomic DNA was amplified with PCR and analyzed in a 1% agarose gel. DNA ladder 1 kb. The PCR amplifies a 700 bp for LysM^{cre} and 350 bp for LysM^{wt} (wild type) as shown in the upper gel image. The PCR amplifies a 300 bp for homozygote β-catenin floxed allele (*Ctnnb1*$^{fl/fl}$) and a 200 bp for wild type (*Ctnnb1*$^{wt/wt}$). For *Ctnnb1*$^{\Delta myeloid}$ mice, 700 bp for LysMcre gene and 300 bp for β-catenin floxed gene were confirmed. For WT mice, bands at 350 bp and 200 bp were confirmed. (*n*=5) B. PCR analysis of genomic DNA obtained from peritoneal macrophage cell lysates from *Ctnnb1*$^{\Delta myeloid}$ and WT mice; *lane*1 of *Ctnnb1*$^{\Delta myeloid}$ and WT shows gDNA amplified with primer pair 1; *lane*2 shows gDNA amplified with primer pair 2. The primer pair 1 amplifies 500 bp in *Ctnnb1*$^{\Delta myeloid}$ mice and 3 kb in WT mice. The primer pair 2 amplifies 300 bp in *Ctnnb1*$^{\Delta myeloid}$ mice and 200 bp in WT mice. 1 kb DNA ladder was used.

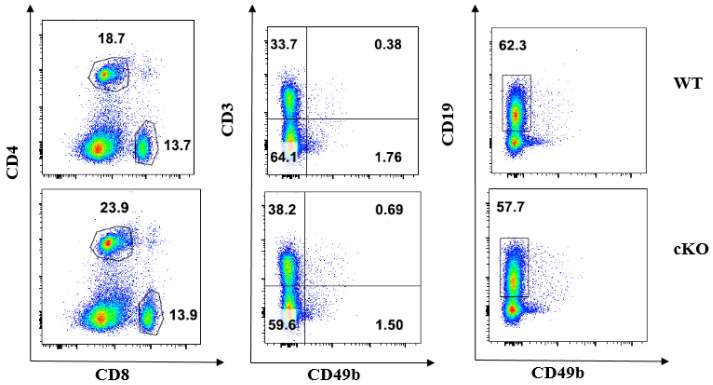
A

Blood



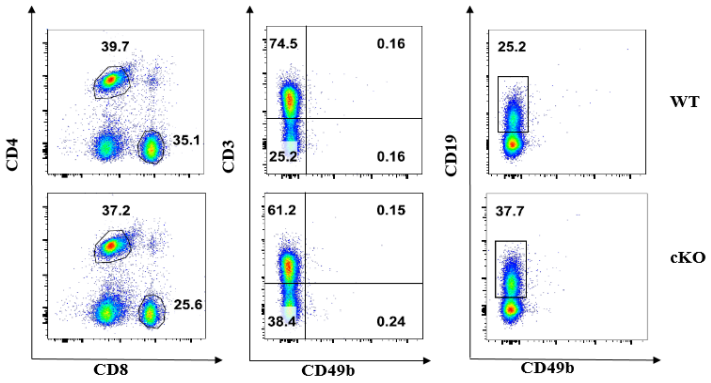
B

Spleen



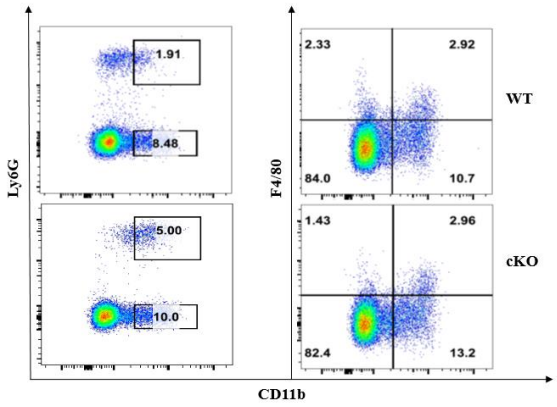
C

Lymph Node



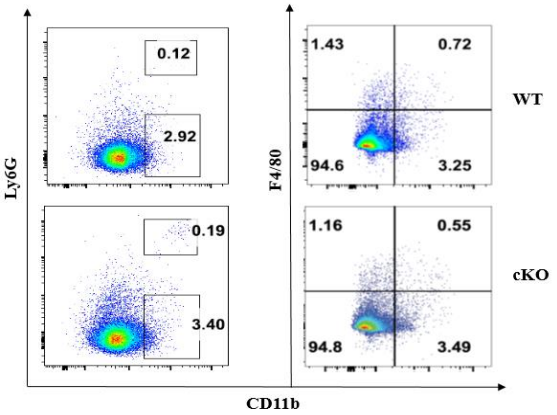
D

Blood



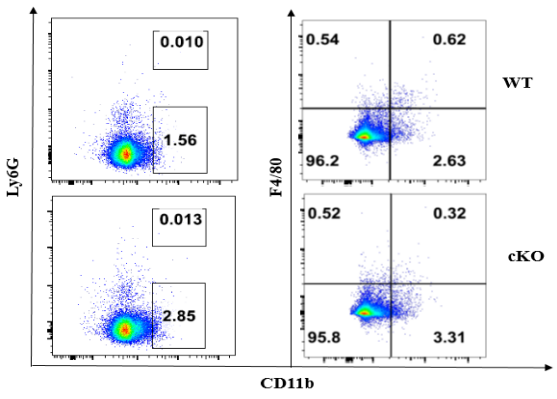
E

Spleen



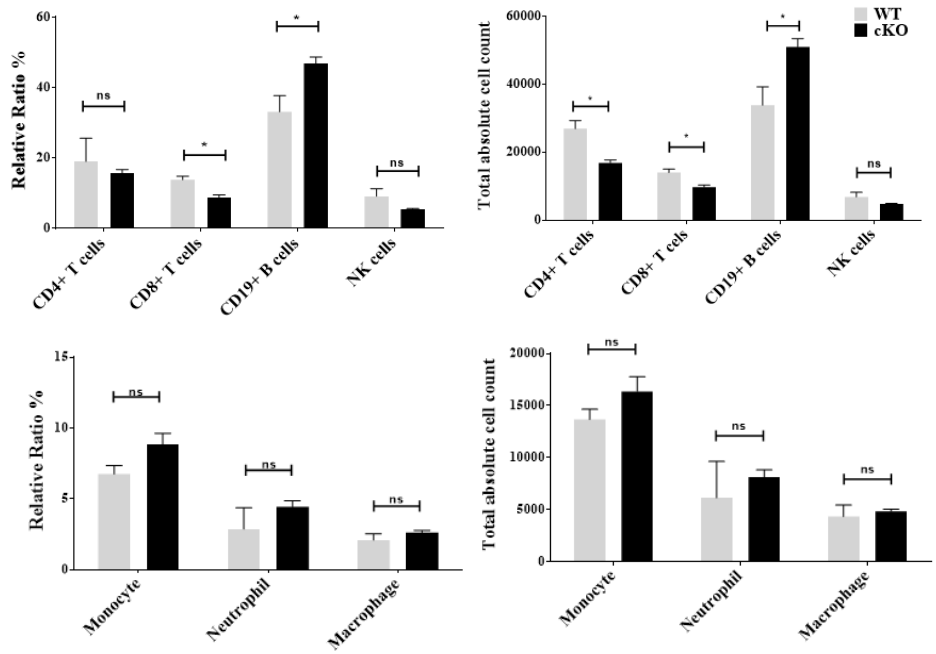
F

Lymph node



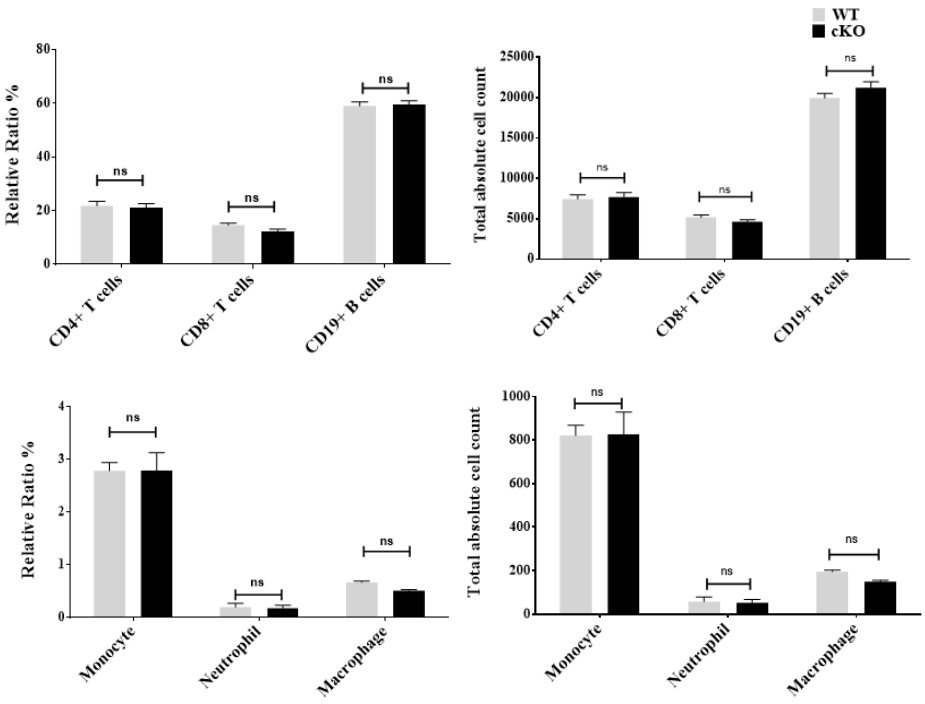
G

Blood



H

Spleen



I

Lymph node

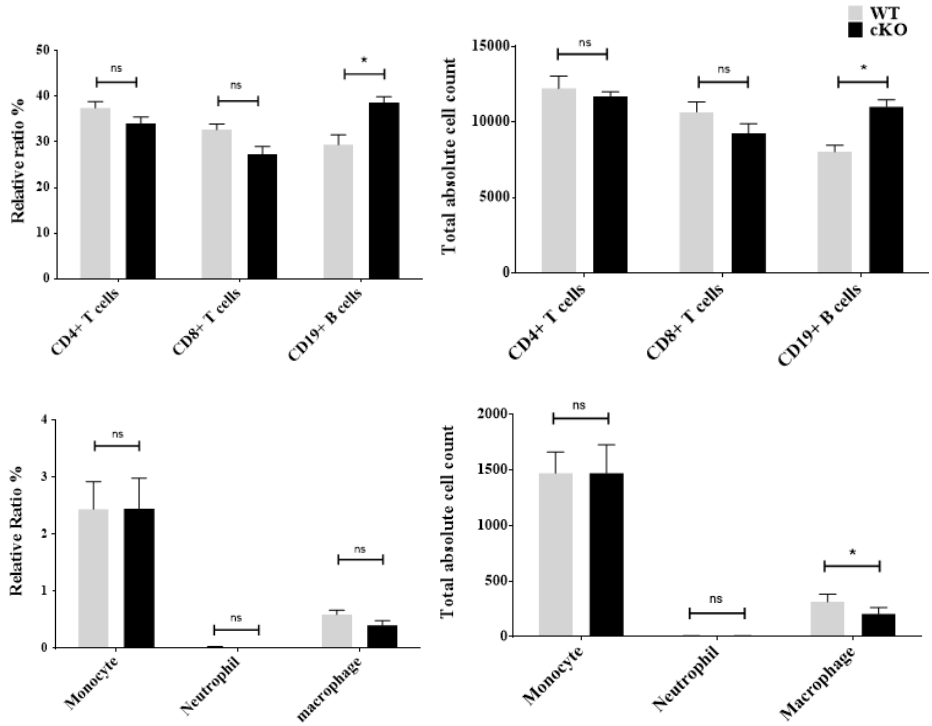


Figure 2. Composition of lymphoid and myeloid immune cells of *Ctnnb1*$^{\Delta myeloid}$ (cKO) and WT mice (A-C). Representative flow cytometric dot plots showing the percentage of lymphoid cells (CD49b+NK cells, CD4+ T cells, CD8+ T cells and CD19+ B cells) in blood, spleen and lymph nodes of WT and cKO mice. (D-F). Representative flow cytometric dot plots showing the percentage of myeloid cells (monocytes, neutrophils and macrophages) in blood, spleen and lymph nodes of WT and cKO mice. (G-I). Bar graphs showing the percentage and absolute cell number of myeloid cells and lymphoid cells in total leukocytes in peripheral blood, spleen and lymph nodes of WT and cKO mice. The data are from three independent experiments (n = 9). (G-I mean±s.e.m.). $^{*}p < 0.05$; ns: not significant.

2. The effect of β-catenin deficiency in IL-6 secretion of LPS-stimulated bone marrow derived macrophages (BMDMs).

To further examine the effect of β-catenin on the function of macrophages, bone marrow progenitor cells from WT and *Ctnnb1*$^{\Delta myeloid}$ mice were isolated and cultured with macrophage-colony stimulating factor (M-CSF) to differentiate them into macrophages on Day 7 (Figure 3A). The bone marrow-derived macrophages (BMDMs) from WT and *Ctnnb1*$^{\Delta myeloid}$ mice on Day 7 showed similar morphology, implying that β-catenin deficiency did not affect the morphology of BMDMs. Also, the surface markers of these BMDMs were checked by flow cytometry, and more than 95% of the cells expressed both CD11b and F4/80, confirming that the absence of β-catenin did not affect the differentiation of BMDMs (Figure 3B).

Then, the secretion of IL-6 from WT and *Ctnnb1*$^{\Delta myeloid}$ macrophages was examined. Because IL-6 is one of the most abundantly produced pro-inflammatory cytokines from activated macrophages, BMDMs were stimulated with lipopolysaccharide and the level of IL-6 secretion in the cell supernatant at various time points was measured. Notably, the IL-6 level from the *Ctnnb1* KO BMDMs was higher than that of the WT BMDMs from 8 hrs until 48 hrs after LPS stimulation (Figure 3C). Between 0 hrs and 5 hrs post LPS stimulation, the IL-6 level

of *Ctnnb1* KO BMDMs did not significantly differ from the IL-6 level of the WT BMDMs. Also, there was no difference in the proliferation of these two groups as the cell number of WT and *Ctnnb1* KO macrophages were similar during the 48 hrs of LPS stimulation (Figure 3D). This was an interesting finding that the absence of β-catenin did not change the morphology, the differentiation and the proliferation of BMDMs, but did affect the function of the macrophage, the production of IL-6 cytokine when stimulated by LPS.

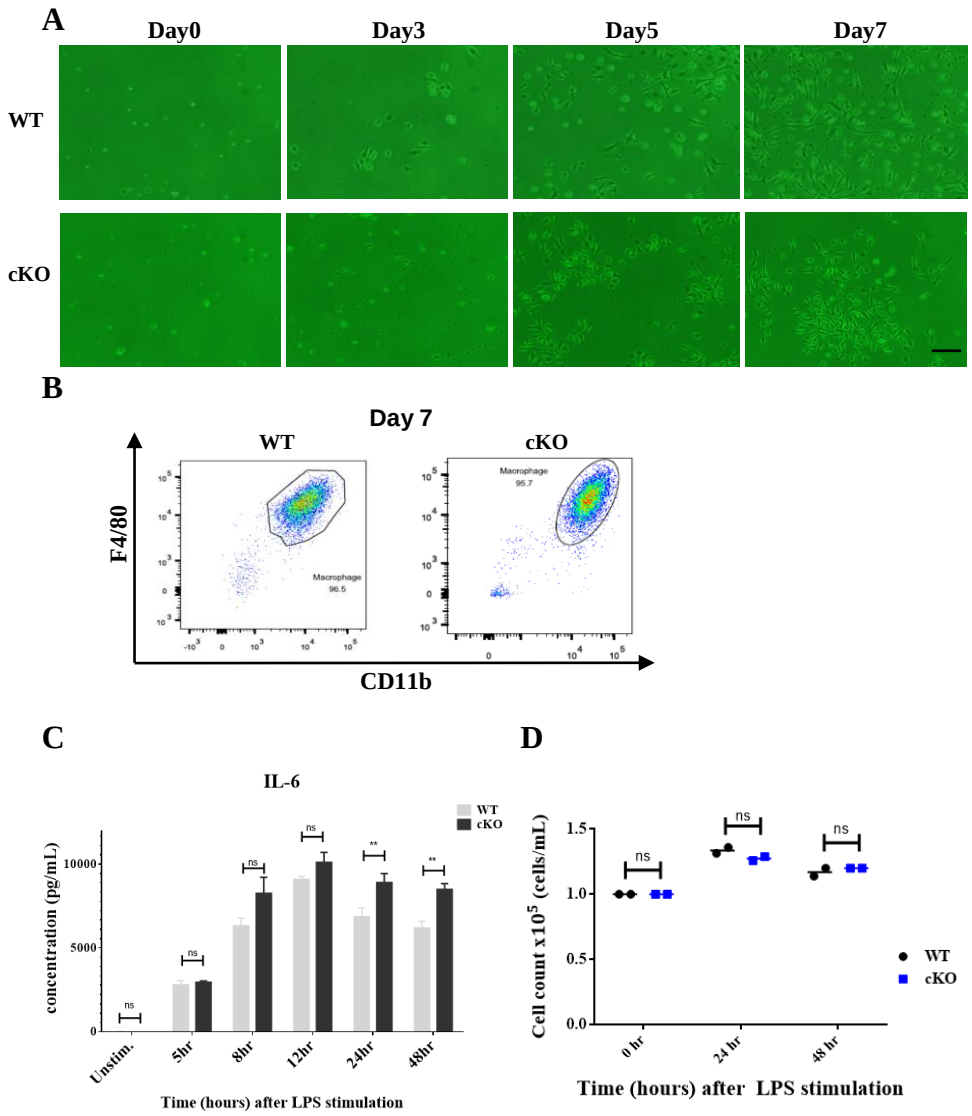


Figure 3. The level of IL-6 secreted by bone marrow-derived macrophages (BMDM) of WT and *Ctnnb1*$^{\Delta myeloid}$ (cKO) mice stimulated by LPS. A. Microscopic image of BMDM (100×) of WT and cKO mice during differentiation. Scale bar, 1 μm. B. Representative FACS dot plots of CD11b+ and F4/80+ BMDM of WT and cKO mice at day 7. C. Level of IL-6 (pg/mL) from BMDM culture supernatant after 0 hr, 5 hr, 8 hr, 12 hr, 24 hr, 48 hr post LPS stimulation (100 ng/mL). The data are from three independent experiments (n = 6). D. Cell number of WT and cKO BMDM during LPS stimulation. (c, mean ± s.e.m.). $^{**}p$ < 0.01; ns: not significant.

3. The effect of β-catenin knockout in IL-6 secretion in LPS-activated peritoneal macrophages.

To examine if the absence of β-catenin in peritoneal macrophages is also critical in its IL-6 secretion, thioglycolate-elicited peritoneal macrophages from the peritoneal cavity of *Ctnnb1*$^{\Delta myeloid}$ and WT mice were isolated. The surface markers of these isolated macrophages were also confirmed, and more than 70% of the cell population was identified to be CD45+ and F4/80+ peritoneal macrophages (Figure 4A). To increase the purity of peritoneal macrophages, peritoneal macrophages were cultured overnight on 10 cm culture dish to only obtain adherent cells.

To further investigate the effect of β-catenin knockout on the IL-6 production in a time-dependent manner, the level of IL-6 in the cell culture supernatant at various time points after LPS stimulation was measured. Remarkably, the level of IL-6 from *Ctnnb1* KO peritoneal macrophages was higher than that of the WT peritoneal macrophages from 8 hrs to 12 hrs after LPS stimulation (Figure 4B). This result was also similar to the result obtained from BMDMs in that the *Ctnnb1* KO macrophages showed prolonged higher level of IL-6 to LPS stimulation. However, the increase in IL-6 level was not due to the increased cell proliferation of *Ctnnb1* KO macrophages during the 12 hrs of incubation,

because the cell number of WT and *Ctnnb1* KO macrophages were similar throughout the time point (Figure 4C). The intracellular level of IL-6 in the peritoneal macrophages by FACS after 12 hrs post LPS stimulation was also checked. Even though the data was not statistically significant, the result demonstrated that the *Ctnnb1* KO peritoneal macrophages showed overall higher mean fluorescence intensity of IL-6 intracellularly than that of WT (Figure 4D).

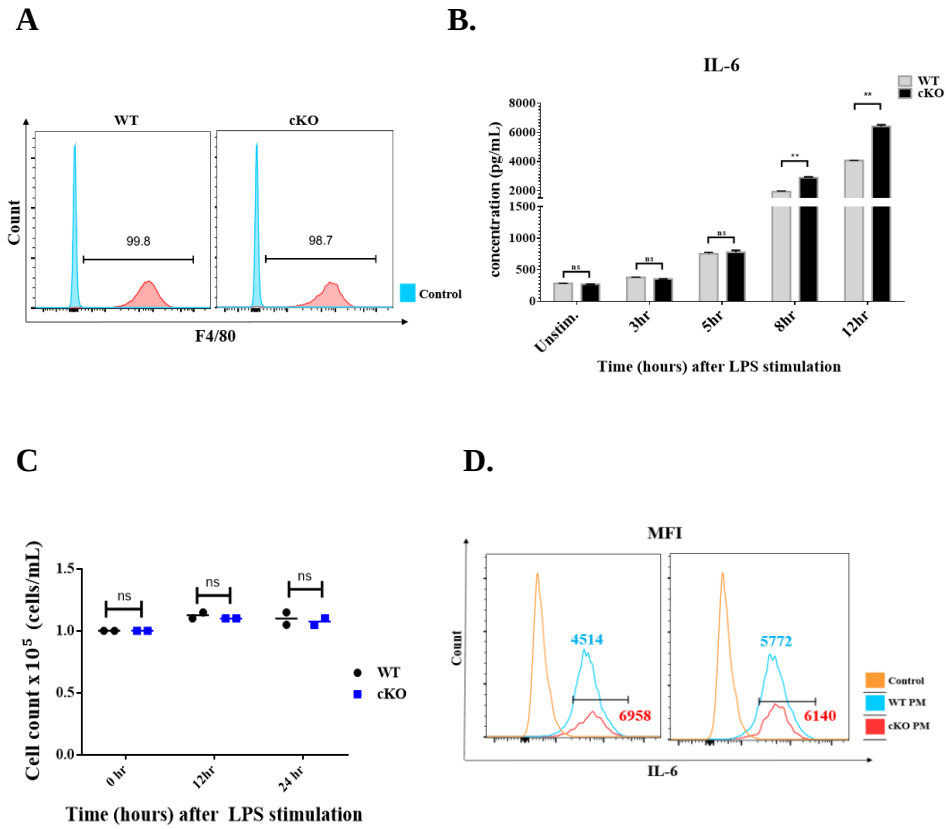


Figure 4. The level of IL-6 secreted by thioglycolate (TG)-elicited peritoneal macrophage (PM) of WT and *Ctnnb1*$^{\Delta myeloid}$ (cKO) mice stimulated by LPS. A. Representative FACS histogram of (CD45+ gated) F4/80+ PM of WT and cKO mice. B. Level of IL-6 (pg/mL) from PM culture supernatant after 0 hr, 5 hr, 8 hr, 12 hr, 24 hr, 48 hr post LPS stimulation (100 ng/mL). The data are from three independent experiments ($n = 6$). C. Cell number of WT and cKO BMDM during LPS stimulation, D. Representative FACS histogram showing intracellular staining of IL-6 in PM of WT and cKO mice after 12 hr post LPS stimulation. The numbers show the mean fluorescence intensity of each group. (n=2) (B, mean ± s.e.m.). $^{**}p < 0.01$; ns: not significant.

4. The effect of myeloid-specific β-catenin knockout on the level of sera IL-6.

Until now, I have explored the effect of β-catenin on IL-6 production specifically in macrophages. However, it is also important to consider the comprehensive role of β-catenin in macrophages and what that brings to the overall system of an organism during inflammation. I wanted to see whether the absence of β-catenin in macrophages is critical in regulating the overall level of IL-6 during inflammation. So, the sera level of IL-6 in *Ctnnb1*$^{\Delta myeloid}$ and WT mice at various time points after intraperitoneal injection of LPS *in vivo* was measured. The *Ctnnb1*$^{\Delta myeloid}$ mice showed higher IL-6 level at 5 hrs than the WT mice after LPS stimulation (Figure 5A). Because this is the peripheral level of IL-6, and IL-6 can be secreted from cells other than macrophages such as epithelial cells and neutrophils (5), I cannot simply say that the change in level of sera IL-6 is directly due to the β-catenin knockout in macrophages. After 24 hrs post injection, the level of serum IL-6 in both *Ctnnb1*$^{\Delta myeloid}$ and WT mice returned to the basal level.

A

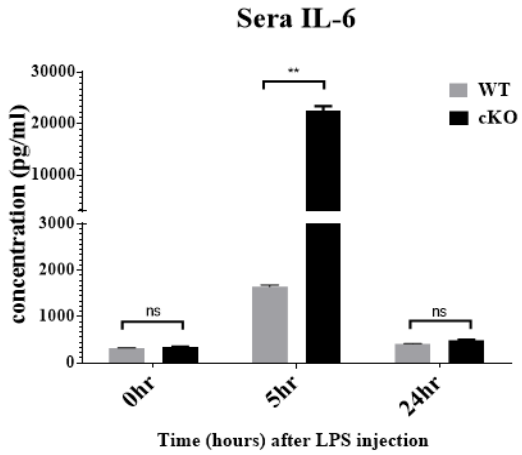


Figure 5. Level of sera IL-6 from *Ctnnb1*$^{\Delta myeloid}$ (cKO) and WT mice after LPS stimulation (*i.p.*) A. The level of sera IL-6 (pg/mL) in cKO and WT mice at 0 hr, 5 hr and 24 hr post LPS intraperitoneal injection (0.125 μg/g). The data are from two independent experiments ($n = 4$). (A, mean ± s.e.m.). $^{**}p < 0.01$; ns: not significant.

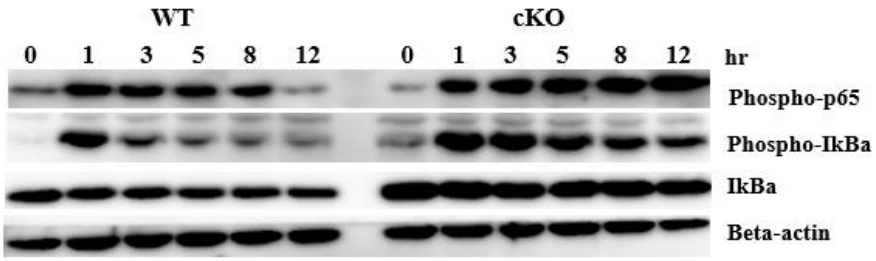
5. The prolonged level of IL-6 is due to the increased activity of NF-κB p65 and phosphorylation of IκBα

One can simply think that the increased level of IL-6 in BMDMs, peritoneal macrophages and peripheral blood is related to the activity of the transcription factor NF-κB, since it is the main transcription factor responsible for production of pro-inflammatory cytokines such as IL-6 in macrophages during inflammation (7, 8). Hence, I next studied the level of p65 activity and its related upstream proteins such as IκBα in peritoneal macrophages. I isolated and cultured peritoneal macrophages from the peritoneum of WT and *Ctnnb1*$^{\Delta myeloid}$ mice to obtain cell lysates for western blot analysis. The representative western blot shows that the protein level of phosphorylated p65 in *Ctnnb1* KO peritoneal macrophage was generally higher than the level of p65 in WT macrophages (Figure 6A).

While the level of phosphorylated p65 in WT PMs decreased significantly after 3 hrs post LPS stimulation, the level of phosphorylated p65 in *Ctnnb1* KO PMs increased more than that of WT PMs from 3 hrs and continued to maintain its high level until 12 hrs after LPS stimulation (Figure 6A and 6B). Another factor that needs to be noted was the IκBα protein, which is closely related to the translocation of p65 (1, 20). Phosphorylation of IκBα leads to the dissociation of p65 in the cytosol,

and eventually promotes the translocation and activation of p65 as a transcription factor in the nucleus. Hence, the protein level of phosphorylated IκBα was also observed in the WT and *Ctnnb1* KO PMs after LPS stimulation, in parallel with the p65 activity. Surprisingly, the protein level of phosphorylated IκBα in the *Ctnnb1* KO PM was notably higher than that of WT PMs from 1 hr post LPS stimulation (Figure 6A). This suggests that β-catenin somehow affects the phosphorylation of IκBα, eventually leading to increased level of p65 activity and the prolonged secretion of IL-6 in LPS-stimulated macrophages.

A



B

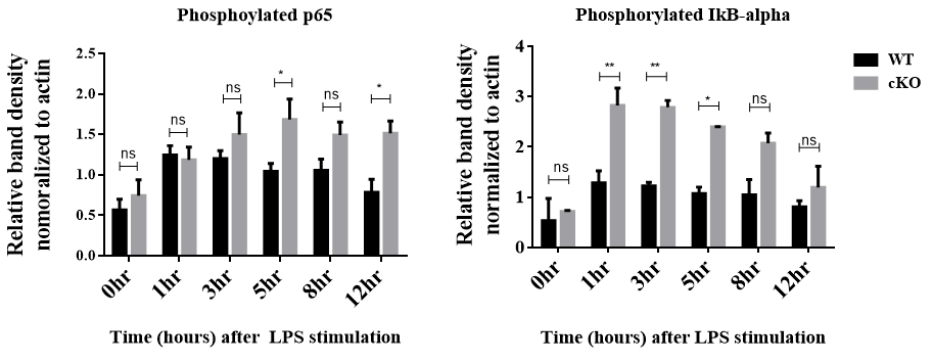


Figure 6. The activity of transcription factor NF-κB and IκBα in LPS-stimulated peritoneal macrophages of *Ctnnb1*$^{\Delta myeloid}$ (cKO) and WT mice A. Representative immunoblot analysis of LPS-stimulated (100 ng/mL) peritoneal macrophages of cKO and WT mice. B. Bar graphs showing relative band density of phosphorylated p65 and IκBα normalized to beta-actin. The data are from five independent experiments or representative of five independent experiments with similar results (n = 5). (B, mean±s.e.m.). $^{*}p< 0.05$; $^{**}p < 0.01$; ns: not significant.

DISCUSSION

This study demonstrated the role of β-catenin as a negative regulator of IL-6 production in activated macrophages by increasing the activity of NF-κB p65, which was due to the increased phosphorylation of IκBα. This finding led to the question that β-catenin itself may indirectly and/or directly interact with one of the components of the NF-κB signaling pathway to modulate the inflammatory response of stimulated macrophages exposed to bacterial stimuli. I first observed an increased secretion of IL-6 from LPS-induced bone marrow-derived macrophages and peritoneal macrophages of the WT and *Ctnnb1*$^{\Delta myeloid}$ mice. There was no significant difference between the levels of IL-6 secreted by *Ctnnb1* KO macrophages and WT macrophages until 3 hrs post LPS stimulation. However, the *Ctnnb1* KO macrophages showed higher level of IL-6 from 5 hrs onwards than the WT macrophages, implying that the absence of β-catenin somehow affected the mechanism underlying IL-6 production after a certain time. To further investigate the mechanism behind this regulation, I examined the protein levels of NF-κB p65 and the phosphorylated form of IκBα in the same context. As expected, I observed a higher increase in the level of phosphorylated p65 in the *Ctnnb1* KO peritoneal macrophages from 3 hrs than the WT macrophages. Since the level of phosphorylated IκBα positively

correlates with the activity of p65 (20), I also examined the level of IκBα and its phosphorylation level. The level of phosphorylated IκBα was significantly and generally higher over time in the *Ctnnb1* KO macrophages than those of WT.

IL-6 is a cytokine that can be secreted by many other cell types, such as endothelial cells, osteoblasts and muscle cells under bacterial infection (5, 6, 21). To see the overall effect of β-catenin on the production of IL-6 by macrophages *in vivo*, it would have been better if sera levels of IL-6 was examined in a macrophage-mediated inflammatory disorder such as sepsis.

It is also notable from the *in vivo* results that this increased and prolonged secretion of sera IL-6 is not due to the increased cell number or relative ratio of the myeloid cells in the lymphoid organs of the *Ctnnb1* $^{\Delta myeloid}$ mice, since the immune profiling data (Figure 2G-I) suggests no significant difference in the cell number nor the relative ratio between WT and *Ctnnb1*$^{\Delta myeloid}$ mice. However, based on the results, there is evidence that the conditional knockout of β-catenin in macrophages may have affected the development of T cells and B cells in indirect ways, changing their percentage and cell number in the lymphoid organs (Figure 2G-I). This was something that could be further studied on.

What was intriguing from these results is that the loss of β-catenin in these activated macrophages did not remarkably increase the

level of IL-6 from the start, but rather prolonged its IL-6 secretion after certain time. This may suggest the importance of β-catenin in the homeostatic control of IL-6 in murine peritoneal macrophages. The homeostatic control of IL-6 is physiologically important since a sustained production of pro-inflammatory cytokines can lead to lethal chronic inflammation (22-24). In a situation where cells are exposed to bacterial stimuli, the immune system works to fight against this infection, by secreting pro-inflammatory cytokines like IL-6. However, the secretion of IL-6 cannot be prolonged eternally as the homoeostatic mechanism works to keep the equilibrium by self-regulation. Hence, it is physiologically natural that the level of IL-6 and the activity of p65 decreases over time after LPS stimulation in macrophages. The absence of β-catenin in peritoneal macrophages and its following outcome imply that the homeostatic control of IL-6 secretion and the activity of p65 were somehow partially dependent on β-catenin interacting with components of NF-κB signaling pathway.

These results can be supported by other studies that also claim the importance of β-catenin as a negative regulator of NF-κB activity. *Bowen et al*., also demonstrated an enhanced activity of p65 in hepatocytes when β-catenin was knocked out after d-galactosamine and LPS stimulation (25). The inhibitory effect of β-catenin on NF-κB activity was also found in cells other than macrophages, including

chondrocytes (14), fibroblasts (26), and osteoblasts (20). From the data, the activity of p65 is regulated by the level of phosphorylated IκBα. Studies also support this result in that LPS-induced IκBα phosphorylation and activation of p65 were both decreased in osteoblast with GSK-3 inhibitor or in hepatocytes with higher β-catenin levels (20, 27). However, they suggest that β-catenin did not directly bind to IκBα in these cells, implying that β-catenin may interact with molecules other than IκBα in the upstream of NF-κB signaling to regulate phosphorylation and degradation of IκBα.

While there are many studies that support the results on the role of β-catenin as a negative regulator of IL-6 production and NF-κB signaling, positive effects of β-catenin on NF-κB activity have also been reported. Intriguingly, in contrast to the study in which deletion of β-catenin in hepatocytes increased RelA protein levels and target gene expression, accumulation of β-catenin in the cytoplasm induced by E-cadherin disassembly and GSK-3β inactivation triggered gene expression of NF-κB-dependent inducible nitric oxide synthase (iNOS) in hepatocytes (28). In human bronchial epithelial cells, depletion of β-catenin by siRNA decreased NF-κB activation and pro-inflammatory cytokine expression including IL-6 to LPS stimulation (29). Based on these evidences, it is highly probable that under certain conditions, opposing effects of β-catenin on NF-κB activity might be observed in

different cell types, even among different stimuli. NF-κB-mediated immune responses can either be positively or negatively regulated by β-catenin depending on the context of genes or cell type.

To further search for the key molecule in the NF-κB pathway that directly interacts with β-catenin, it is necessary to investigate the changing levels of molecules that function upstream of IκBα in the same context. There is possibility that β-catenin may interact with IκB kinase (IKK) complex, which is a molecule in NF-κB pathway that directly phosphorylates IκBα after LPS stimulation (7, 9). A study done by Lamberti *et al*., demonstrates the effect of IKK-α on the β-catenin-dependent transcriptional activity, in which IKK-α and bind and phosphorylate β-catenin via *in vitro* and *in vivo* assays (30). Carayol *et al*., also illustrates the interaction of IKK-α with β-catenin (31). This study shows that IKK-α inhibits β-catenin degradation in the cytoplasm by Siah-1 pathway and also interacts with β-catenin to inhibit ubiquitination of β-catenin. Although these studies reveal the direct interaction between IKK complex and β-catenin in the cytoplasm, the authors of the studies only observed the effect of the interaction on the protein levels and stabilization of β-catenin, and hence the transcriptional activity of the Wnt/β-catenin pathway. The effect of the interaction between IKK complex and β-catenin on NF-κB pathway and the IL-6 production is still an intriguing question to explore in further studies.

However, there are several aspects to consider when it comes to studying β-catenin and NF-κB pathway. β-catenin is not only involved in the Wnt/β-catenin pathway, but it also interacts with the intracellular domain of E-cadherin, a cell adhesion molecule that forms adherens junctions between cells (12). From the study done by Solanas *et al.,* the β-catenin and E-cadherin complex in mesenchymal cells interacted with RelA and prevented its nuclear translocation and hence downregulating its activity (12). Therefore, it must be considered that there may be another factor, such as E-cadherin, that is involved in the interaction of β-catenin and NF-κB, and hence the regulation of IL-6 secretion.

Another point to consider is that the protein levels of NF-κB and IκBα should be confirmed in cells other than thioglycolate-elicited peritoneal macrophages since these cells were previously exposed to stimuli other than LPS, the thioglycolate broth. Clearly, these elicited peritoneal macrophages show different phagocytic activity than the resident peritoneal macrophages (32). This may be important especially in studies related to NF-κB pathway because the role of β-catenin in NF-κB pathway can vary depending on different cellular contexts and the activity of RelA is very sensitive to various types of stress induced.

In summary, I have demonstrated the negative role of β-catenin on IL-6 production to LPS by interacting with NF-κB pathway in stimulated macrophages. The secretion of IL-6 in β-catenin KO

macrophages was higher and more prolonged than the WT macrophages from 5 hrs post LPS stimulation. This was due to the increased protein levels of activated p65 and phosphorylated IκBα overall in the *Ctnnb1* KO macrophages. From these results and other supporting studies, I could postulate that β-catenin may directly interact with IKK complex to increase the activity of NF-κB p65 to eventually prolong the secretion of IL-6 in activated macrophages. However, I could also draw a conclusion that this negative regulation of β-catenin on NF-κB pathway is context-dependent. Therefore, I have shown the effect of β-catenin on IL-6 production and the potential interaction with the component of NF-κB pathway, specifically in LPS-induced macrophages. This raises the significance of β-catenin as a potential cellular therapeutic target for IL-6-mediated chronic inflammation or macrophage-mediated inflammatory disorders.

SUMMARY

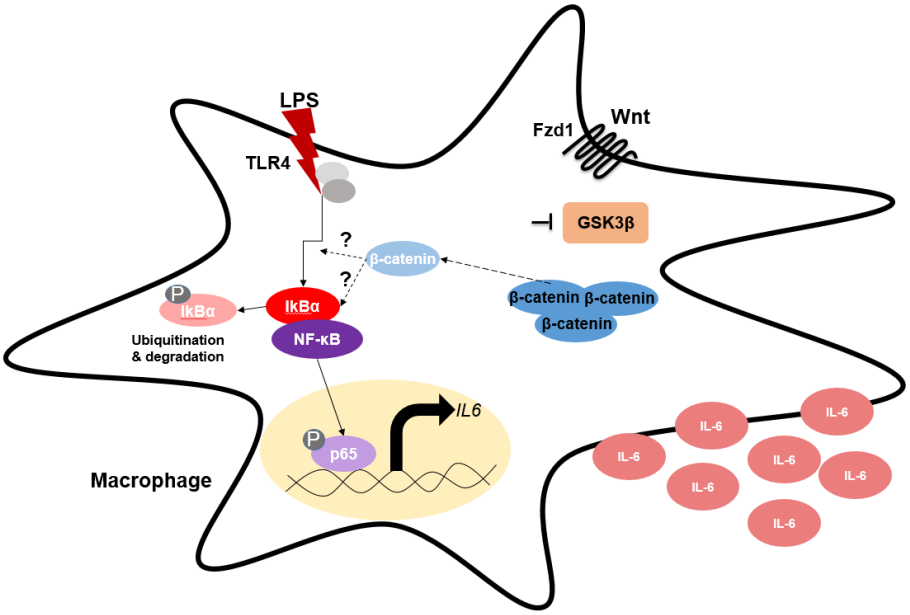


Figure 7. A schematic diagram illustrating the interaction between β-catenin and NF-κB pathway in LPS-stimulated macrophages. Smooth arrows represent experimentally confirmed interaction; dashed arrows represent predicted interaction in this study.

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A

	Cell types	Antibodies/markers
Myeloid cells	Monocytes	CD11b+, CD115+, Ly6C(hi), Ly6G-
	Macrophages	CD11b+,F4/80+, Ly6G+, Ly6C-
	Neutrophils	CD11b+, Ly6G+Ly6C+
Lymphoid cells	B cells	CD45, CD19+,
	T cells	CD45, CD4, CD8

Supplementary Table 1. A. Table showing the surface markers of each immune cell type for flow cytometric analysis.

국문 초록

서론: 베타카테닌은 세포에서 다양한 신호 전달 경로에 기여한다. 최근 연구들은 베타카티닌이 염증상태에서 사이토카인 생산에 가장 중요한 경로 중 하나인 NF-κB 경로와 상호작용한다고 주장한다. 이 연구에서는 NF-κB 경로에서 베타카테닌의 역할을 알아내고, 베타카티닌이 없음으로 인해 궁극적으로 IL-6 생산에 일어나는 변화에 대해서 알아보고자 한다. 이를 알아보기 위해 골수에서만 특이적으로 베타카테닌이 발현되지 않은 conditional knockout 마우스를 얻었고, 염증상태에서 자극된 대식세포에 의해 분비된 중요한 염증성 사이토카인 중 하나인 IL-6의 수치를 골수 유래 대식 세포 및 복강 대식세포에서 조사하였다.

방법: 베타카테닌이 대식세포에서 발현되지 않은 마우스를 얻기 위해, β-catenin floxed 마우스를 LysMcre transgenic 마우스와 교배 시켰다. 베타카티닌이 골수 유래세포에서 발현되지 않은 마우스의 유전자를 확인 및 분석하고, 이 마우스의 다양한 면역 세포의 구성을 일반 마우스와 비교 및 분석했다. 확인된 *Ctnnb1*$^{\Delta myeloid}$ 마우스를 이용하여 골수 유래 대식세포와 복막

대식세포를 쥐에서 분리하여 균의 지질다당류로 자극을 준 후 *Ctnnb1*이 발현되지 않은 대식세포에 의해 분비 된 IL-6의 수치를 측정했다. 또한, 마우스에게 균의 지질다당류를 복막으로 주사 시킨 후 *Ctnnb1*$^{\Delta myeloid}$ 마우스의 혈청 IL-6 수치를 측정했다. 그리고 마지막으로, 마우스의 복막 대식세포에서 IL-6 사이토카인을 만들어내는데 중요한 전사인자 NF-κB p65 및 IκBα의 레벨을 immunoblot으로 분석했다.

결과: 베타카테닌 녹아웃은 골수 및 림프구 세포의 발달 및 성장에 영향을 미치지 않았으며 *Ctnnb1*$^{\Delta myeloid}$ 마우스와 일반마우스 사이의 골수면역 세포의 절대적 숫자와 비율은 비슷했다. 베타카테닌이 결핍된 골수 유래 대식세포는 일반 마우스에서 분리된 골수 유래 대식세포와 비교했을 때 형태, 분화 그리고 증식에 큰 차이는 없었다. 그러나 균의 지질다당류로 자극 후 대식세포에 의해 분비 된 IL-6의 수치는 베타카티닌이 결핍된 대식세포에서 8시간대부터 더 높게 나왔다. 그러므로, 베타카티닌이 결핍된 골수 유래 대식세포와 복강대식세포의 IL-6 생산 능력은 일반 대식세포와는 다르다는 것을 알 수 있었다. 또한, 지질다당류를 복강 내로 주사하였을 때, *Ctnnb1*$^{\Delta myeloid}$ 마우

스의 IL-6의 혈청 수치가 5시간대에서 일반 마우스보다 높게 나온 것을 관찰했다. p65의 활성 수준과 그와 관련된 IκBα 단백질의 인산화 수준도 일반 복강대식세포보다 베타카테닌이 결핍된 복강대식세포에서 일반적으로 더 높고, 지질다당류로 자극 후 시간이 지날수록 더 오래 지속되는 것을 관찰했다. 이러한 결과는 베타카테닌이 IκBα의 인산화 및 p65의 활성 레벨을 간접적으로 억제한다는 것을 암시했다. 따라서 베타카테닌이 NF-κB경로와 상호작용하여 궁극적으로 활성화된 대식세포에서 IL-6의 생산을 억제한다는 단서를 얻을 수 있었다.

결론: 본 연구는, 염증 시 대식세포에서 IL-6 생산을 위한 NF-κB 경로에서의 베타카티닌의 역할을 보여줬다. 베타카티닌이 없는 대식세포에서는 지질다당류로 자극이 되면 p65의 활성이 증가하고, 특정 시점에서 IL-6의 수치가 베타카티닌이 있는 일반 대식세포보다 더 증가했다. 이는 베타카티닌이 대식세포에서 외부자극에 노출되고 특정 시점 이 후에 발생하는 항상성 제어 메커니즘에 의한 IL-6 분비 조절을 간접적으로 억제한다는 것을 보여줬다. 본 연구의 결과는 IL-6 사이토카인이 중점으로 역할 하는 만성 염증 질환이나 질병에서 베타카티닌이

잠재적인 치료 목표물이 될 수 있다는 것을 보여주었다고 생각한다.

주요어: 베타카티닌, NF-κB 경로, 인터루킨-6, 사이토카인, 대식세포

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