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Activation of CpG-ODN-Induced TLR9 Signaling Inhibited by Interleukin-37 in U937 Human Macrophages

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Purpose: Interleukin-37 (IL-37) is an anti-inflammatory cytokine that inhibits a broad spectrum of inflammatory responses in various human cells, including neutrophils, macrophages, and endothelial cells. The aim of this study was to identify the role of IL-37 in toll-like receptor 9 (TLR9) signaling in human macrophages.
Materials and Methods: Human macrophage U937 cells treated with CpG-oligonucleotides (CpG-ODN), recombinant IL-37, or dexamethasone were used in an in vitro study. IL-37 small interfering RNA (siRNA) and TLR9 siRNA were used to silence endogenous IL-37 and TLR9, respectively. Expression levels of phosphorylated nuclear factor-κB (NF-κB), IκBα, IL-37, IL-1β, tumor necrosis factor-α (TNF-α), and IL-6 protein were assessed by real-time quantitative polymerase chain reaction and Western blotting. CpG-ODN-mediated IL-37 expression stimulated by dexamethasone was detected using immunofluorescent analysis.
Results: U937 cells treated with CpG-ODN induced activation of the NF-κB pathway and increased the expression of the pro-inflammatory cytokines IL-1β, TNF-α, and IL-6, but reduced that of IL-37. Recombinant IL-37 attenuated phosphorylation of NF-κB and IκBα and the expression of IL-1β, TNF-α, and IL-6 stimulated by CpG-ODN. Human macrophages transfected with IL-37 siRNA augmented the expression of IL-1β, TNF-α, and IL-6 mRNA and protein in cells treated with CpG-ODN. Dexamethasone markedly inhibited expression of pro-inflammatory cytokines in U937 cells, whereas IL-37 expression was increased with the addition of dexamethasone. Inflammatory responses elicited by CpG-ODN were dependent on an MyD88-TRAF6 pathway. IL-37 inhibited CpG-ODN-induced ubiquitination of TRAF6 in U937 macrophages.
Conclusion: IL-37 inhibits CpG-ODN-mediated inflammatory responses through regulation of a TRAF6- NF-κB pathway in human macrophages.

Key Words: IL-37, TLR9, CpG-ODN, NF-κB, cytokine

INTRODUCTION

Interleukin-37 (IL-37) is a member of the IL-1 gene family and acts as an anti-inflammatory cytokine that broadly inhibits innate immunity. It is considered a unique cytokine, different from other IL-1 gene family cytokines.[1-3] IL-37 is expressed at low concentration in diverse human hematopoietic cells or tissues and is upregulated by inflammatory stimuli, including tumor necrosis factor-α (TNF-α), IL-1β, interferon-γ, and tumor growth factor-β1 (TGF-β1), and toll-like receptor (TLR) agonists, such as lipopolysaccharide (LPS), Pam_3CSK_4, and CpG-oligonucleotides (CpG-ODN).[4,5] IL-37 is associated with the patho-

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genesis of several chronic inflammatory or autoimmune diseases and plays a crucial role in the regulation of inflammation in animal models.[5]

TLR9 is an intracellular receptor for CpG motifs and triggers the induction of inflammatory cytokines responsible for innate immunity.[6,7] The binding of TLR9 and CpG DNA through a TIR domain-containing adaptor, MyD88, is involved in activation of transcription factor nuclear factor-κB (NF-κB), similar to other TLR2- and TLR4-mediated signaling pathways, and induces several inflammatory cytokines, such as TNF-α and IL-12.[8] TLR9 is implicated in diverse autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus (SLE), inflammatory myositis, and systemic sclerosis.[9]

The complex relationship between TLR9 and IL-37 is not clearly understood. While IL-37 is thought to be involved in the pathogeneses of various human diseases, there is some debate on the differential role of IL-37 in autoimmunity and inflammation.[5] Considering IL-37 expression through TLR-9-mediated signaling, Nold, et al.[4] demonstrated that treatment of peripheral blood mononuclear cells (PBMCs) with dinucleotide CpG-ODN (1000 ng/mL) increased the production of IL-37 protein. Whether IL-37 is responsible for regulating the TLR9-induced inflammatory response, however, remains unclear. The aim of this study was to clarify the role of IL-37 in inflammatory responses via regulation of TLR9-mediated signaling in human macrophages.

MATERIALS AND METHODS

Cell culture

Human U937 cells (KCLB, Seoul, Korea) were grown in RPMI-1640 (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; GenDepot, Barker, TX, USA) and 1% penicillin-streptomycin (Sigma-Aldrich, Saint Louis, MO, USA) in a 5% CO_2 humidified atmosphere at 37°C. To differentiate U937 monocytes into a macrophage-like phenotype, the cells were treated with 100 nM of phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) for 24 h.

After exposure to PMA, the medium was changed, and cells were treated with the indicated concentrations of CpG oligodeoxynucleotide (0, 1, 3, or 5 μM). After 24 h, the cells were collected for subsequent experiments. CpG oligodeoxynucleotide (CpG-ODN 2006) was purchased from InvivoGen (San Diego, CA, USA) and dissolved in endotoxin-free water.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Cells were pretreated for 24 h with dexamethasone (1 μg/mL), followed by the addition of CpG-ODN (5 μM). Dexamethasone was dissolved and diluted in 0.1% dimethyl sulfoxide (DMSO). The cells were collected, and total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Then, cDNA was synthesized from 1 μg of RNA using a ReverTra Ace-α-kit (Toyobo, Osaka, Japan) according to the manufacturer's protocols. Quantitative real-time PCR was performed in a MiniOpticon™ real-time PCR system (Bio-Rad, Hercules, CA, USA) with SYBR Green PCR Master Mix (Toyobo) according to the manufacturers' instructions.

PCR was carried out for 40 cycles with denaturation at 94°C for 30 s, annealing from 58°C to 64°C for 1 min, and final extension at 72°C for 30 s using an MJ Mini™ thermal cycler. Relative gene expression analyses were conducted after normalizing to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primer sequences were as follows: IL-1β: 5'-AAA CAG ATG AAG TGC TCC TTC CAG G-3', reverse 5'-TGG AGA ACA CCA CTT GTT GCT CCA-3'; TNF-α: forward 5'-CAA AGT AGA CCT GCC CAG AC-3', reverse 5'-GAC CTC TCT CTA ATC AGC CC-3'; IL-6: forward 5'-ATG AAC TCC TTC TCC ACA AGC GC, reverse 5'-GAA GAG CCC TCA GGC TGG ACT G-3'; IL-37: forward 5'-AGT GCT TAG AAG ACC CGG -3', reverse 5'-AGA GTC CAG GAC CAG TAC TTT GTG A-3'; and GAPDH: forward 5'-GAT GGG- ATT TCC ATT GAT GAC A-3', reverse 5'-CCA CCC ATG GCA AAT TCC-3'.

Small interfering RNA (siRNA) transfection

Cells were seeded in 24-well plates at 2×10^4 cells/well and incubated to 70% confluence. Cells were then transfected with siRNA (human IL-37, human TLR9, or human MyD88) or scrambled siRNA (Invitrogen) using lipofectamine RNAi MAX transfection reagent (Invitrogen) according to the manufacturer's instructions.

Briefly, each siRNA (100 nM IL-37, TLR9, or MyD88 siRNA or 50 nM scrambled siRNA) was diluted in Opti-MEM medium (Gibco Laboratories), and lipofectamine was added. The transfection mixtures were incubated for 10 min at room temperature and added to each well. After 72 h, transfection efficiency was confirmed by qRT-PCR and Western blot analysis. The 72-hour efficiency of 100 nM siRNA for TLR9 or MyD88 performed in this study was found to be inhibit TLR9 expression by about 60%–65% (Supplementary Fig. 1, only online).

Western blot analysis

Cells were pretreated with different doses of recombinant IL-37 (1, 10, or 100 ng/mL) protein and stimulated with CpG-ODN (5 μM). They then were lysed in 50 μL of RIPA buffer containing a protease inhibitor cocktail (Thermo Scientific, Rockford, IL, USA) for 10 min, and the insoluble fraction pellets were removed. The protein lysates were heated at 95°C for 5 min after being mixed with Laemmli protein sample buffer (Bio-Rad, Hercules, CA, USA). The samples were applied on an SDS PAGE gel for electrophoresis at a constant voltage of 50 V for 30 min and then transferred to nitrocellulose membranes (Bio-Rad).

After blocking in TBS buffer with 1% BSA, the membranes were incubated with appropriate primary antibodies at 4°C overnight. The membranes were then washed three times with TBS

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buffer containing Tween-20 (TBST) and incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000 dilution) for 1 h at room temperature. Protein bands were detected with a ChemiDoc XRS imaging system and quantified using Quantity One 1-D analysis software (Bio-Rad).

Immunoprecipitations

Cells were pretreated with 100 ng/mL of recombinant IL-37 protein, with or without CpG-ODN (5 μM). To examine protein interactions, cells were lysed in NP-40 buffer [0.5 mM Tris, pH-7.4, 5M NaCl, 0.2% Nonidet P40, protease inhibitor cocktail 1 tablet (Roche, Penzberg, Germany)], containing a protease inhibitor cocktail)], and centrifuged at 13000 rpm for 10 min at 4°C.

The cell lysates were precleaned with Protein A/G PLUS agarose beads, and immunoprecipitation was performed using anti-TRAF6 and anti-Ub antibodies (Santa Cruz, Dallas, TX, USA) for 24 h at 4°C with gentle rocking. The beads were washed five times with NP-40 buffer and then were resuspended in sample buffer containing 5% β-Mercaptoethanol before being subjected to Western blotting.

Immunofluorescent staining

Cells (5×10^3) were seeded into chamber culture slides (BD Falcon, Franklin Lakes, NJ, USA) and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. After being washed with PBS, the fixed cells were incubated with primary antibodies overnight at 4°C. The cells were then washed three times with cold PBS and cultured with secondary antibody conjugated with goat anti-rabbit IgG-FITC (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 1 h.

Nuclear staining was performed with 4',6-diamidino-2-phenylindole (DAPI; DAKO, Glostrup, Denmark) for 10 min at room temperature in the dark. The cells were mounted on microscopic slides using EverBrite™ Hardset mounting medium (Biotium, Hayward, CA, USA). Immunolabeling was observed under confocal microscopy (Nikon, Tokyo, Japan).

Statistical analysis

Data are presented as means±standard errors of the mean. Nonparametric Mann-Whitney U test was used to assess statistical differences between two groups. A p value less than 0.05 was considered statistically significant. Statistical analysis was performed using GraphPad Prism Version 5.04 software (GraphPad Software, San Diego, CA, USA).

RESULTS

Binding of CpG-ODN to TLR9 induces differential expression of inflammatory cytokines through the NF-κB pathway

Binding of CpG-ODN to TLR9 induced activation of the NF-κB-mediated inflammatory signaling pathway, showing upregulation of phosphorylated NF-κB and IκBα in human macrophage U937 cells (Fig. 1A). This enhanced the expression of pro-inflammatory cytokines IL-1β, TNF-α, and IL-6 in a dose-dependent manner, whereas IL-37 protein expression was markedly inhibited in U937 cells treated with CpG-ODN at dosages of 0, 1, 3, and 5 μM for 24 h. Human macrophages treated with CpG at dosages with 0, 1, 3, and 5 μM for 24 h exhibited markedly induced the expression of TLR9, IL-1β, TNF-α, and IL-6 mRNA, compared to non-treated cells (Fig. 1B). In contrast, IL-37 mRNA expression stimulated with CpG-ODN was decreased in a dose-dependent manner.

We assessed gene expression of inflammatory cytokines in U937 cells transfected with TLR9 siRNA under stimulation with CpG-ODN. Human macrophages transfected with TLR9 siRNA showed much greater induction of IL-37 mRNA expression than non-transfected cells (Fig. 1C). In contrast, IL-1β, TNF-α, and IL-6 mRNA expression by CpG-ODN was inhibited in cells transfected with TLR9 siRNA, compared to non-transfected cells. Consistently, U937 cells transfected with TLR9 siRNA showed suppressed production of IL-1β, TNF-α, and IL-6 protein, compared to non-transfected cells (Fig. 1D).

Recombinant IL-37 inhibits pro-inflammatory cytokines in U937 cells treated with CpG-ODN

The upregulated expression of p-NF-κB and p-IκBα proteins by CpG-ODN was markedly inhibited in a dose-dependent manner by the addition of recombinant IL-37 (Fig. 2A). In addition, IL-37 attenuated protein production of the pro-inflammatory cytokines IL-1β, TNF-α, and IL-6 in human macrophages treated with CpG-ODN. Consistently, recombinant IL-37 potently suppressed mRNA expression of IL-1β, TNF-α, and IL-6, compared to U937 cells treated with only CpG-ODN (Fig. 2B).

Inhibition of IL-37 enhances pro-inflammatory cytokines through NF-κB pathway activation

Silencing endogenous IL-37 using siRNA enhanced production of p-NF-κB and p-IκBα proteins in U937 cells stimulated with CpG-ODN, compared to cells treated with only 5 μM CpG-ODN for 24 h (Fig. 3A). IL-37 siRNA-transfected cells stimulated with CpG showed significant increases in the expression of IL-1β, TNF-α, and IL-6 proteins, compared to non-transfected cells treated with only CpG-ODN. Consistently, U937 cells transfected with IL-37 siRNA showed increased IL-1β, TNF-α, and IL-6 mRNA expression, compared to non-transfected cells treated with only CpG-ODN (Fig. 3B).

IL-37 expression enhanced by dexamethasone blocks the expression of CpG-ODN-induced pro-inflammatory cytokines

The mRNA expression levels of the pro-inflammatory cytokines IL-1β, TNF-α, and IL-6 induced by CpG-ODN were significantly inhibited by dexamethasone (1 μg/mL), compared to those in cells stimulated with only CpG-ODN (Fig. 4A). In contrast to

the responses of the pro-inflammatory cytokines, dexamethasone markedly increased IL-37 mRNA expression, compared to that in U937 cells stimulated with only CpG-ODN. Consistently, the addition of dexamethasone to U937 cells treated with CpG-ODN suppressed expression of IL-1β, TNF-α, and IL-6 proteins and induced IL-37 protein (Fig. 4B). Immunofluorescent analysis showed that the number of IL-37-positive cells in U937 cells stimulated with both dexamethasone and CpG-ODN was significantly higher than that in cells treated only with CpG-ODN (Fig. 4C).

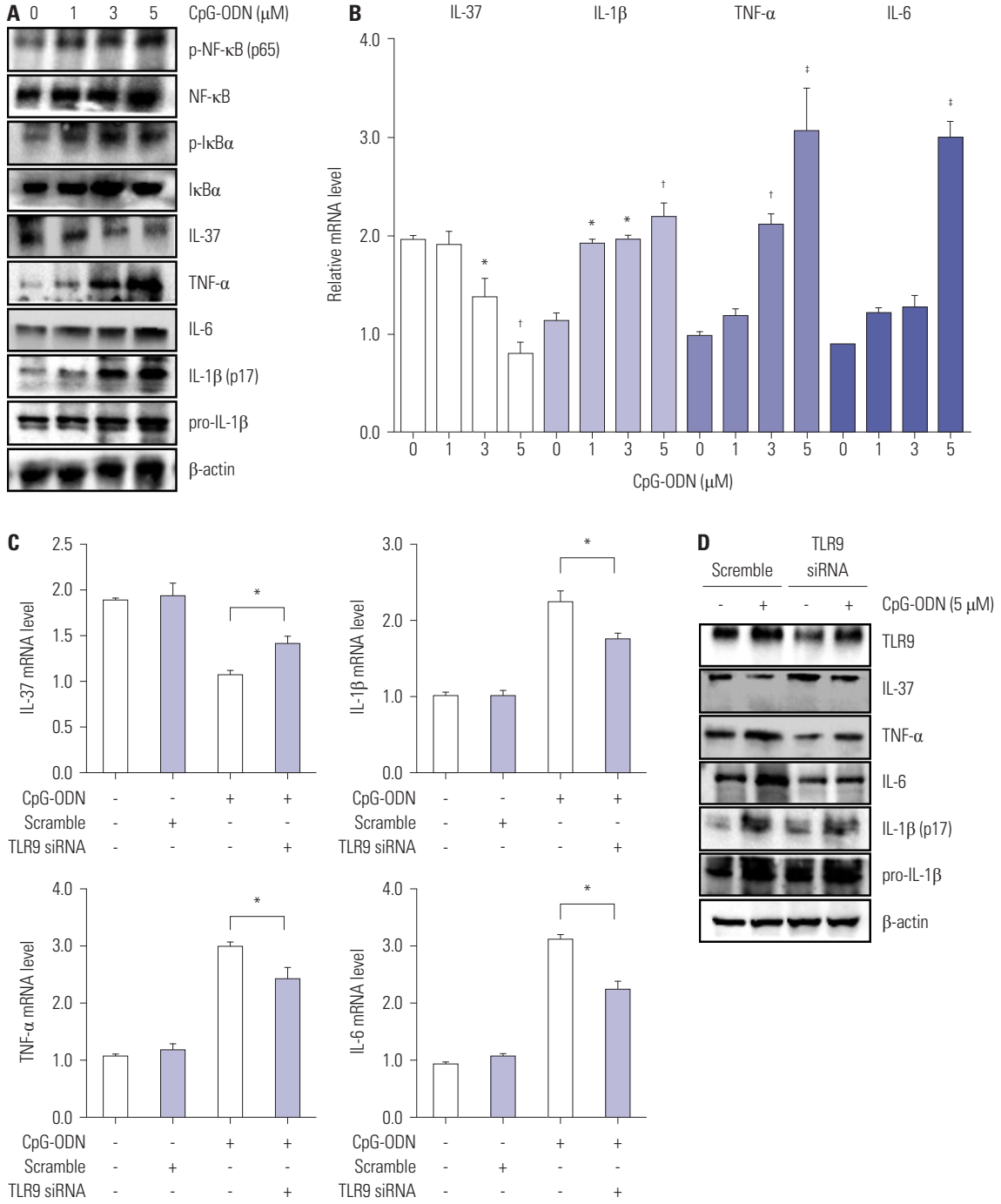

Fig. 1. CpG-ODN binding to TLR9 induced inflammatory response through the NF-κB pathway. (A) CpG-ODN (0 to 5 μM) activates NF-κB components and IL-1β, TNF-α, and IL-6, and attenuated IL-37 proteins in U937 cells. (B) Human macrophages treated with CpG-ODN (0 to 5 μM) increased mRNA expression of IL-1β, TNF-α, and IL-6, but suppressed IL-37 mRNA expression. *p<0.05, †p<0.01, and ‡p<0.001 versus cells treated without CpG-ODN. (C) IL-37 mRNA expression in U937 cells transfected with TLR9 siRNA was increased following 5 μM CpG-ODN stimulation, while IL-1β, TNF-α, and IL-6 mRNA expression was attenuated. *p<0.05 versus cells transfected without TLR9 siRNA. (D) Knockdown of TLR9 increased IL-37 protein production, whereas the production of IL-1β, TNF-α, and IL-6 proteins was reduced under stimulation of 5 μM CpG-ODN. CpG-ODN, CpG-oligonucleotides; TLR9, toll-like receptor 9; NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor-α; IL, interleukin.

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IL-37 blocks ubiquitination of TRAF-6 stimulated by CpG-ODN in U937 cells

Enhanced expression of the pro-inflammatory cytokines IL-1β, TNF-α, and IL-6 in U937 cells stimulated by CpG-ODN was dependent on MyD88 (Fig. 5A). Cells transfected with MyD88 siRNA showed suppressed production of these pro-inflammatory cytokines. CpG-ODN significantly induced ubiquitination of TRAF6 in a dose-dependent manner (Fig. 5B). Furthermore, IL-37 (100 ng/mL) markedly blocked CpG-ODN-induced TRAF6 ubiquitination.

DISCUSSION

IL-37 is thought to play a broad role in suppressing innate inflammation, activating the anti-inflammatory pathway, and in-

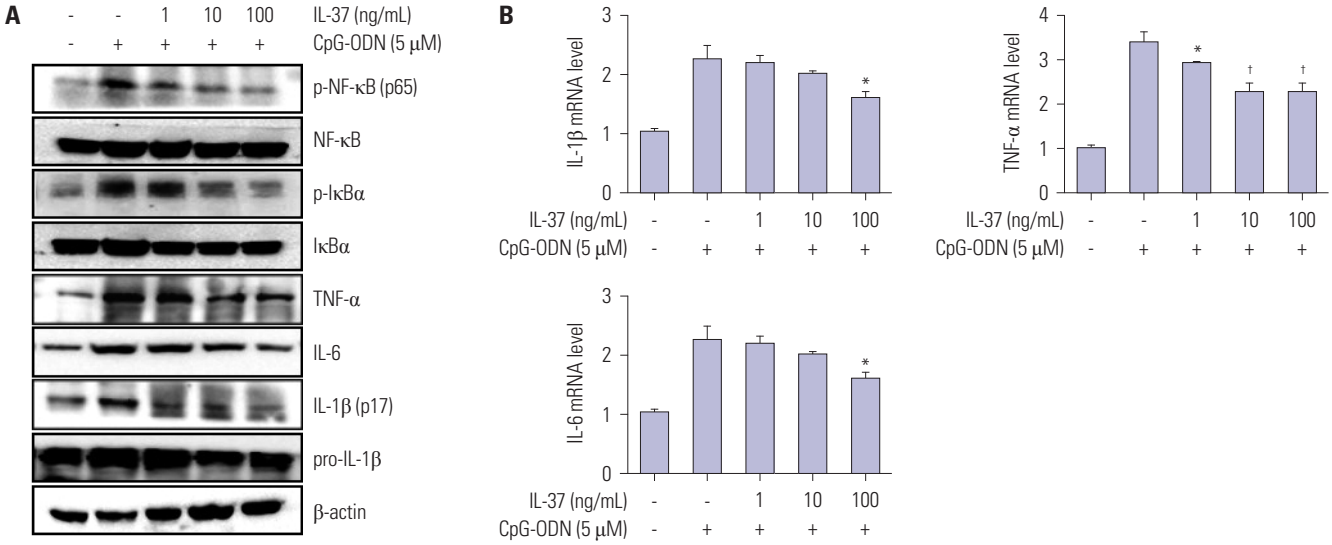

Fig. 2. IL-37 inhibits activation of the NF-κB pathway and pro-inflammatory cytokines induced by CpG-ODN. (A) Immunoblot assay shows that recombinant IL-37 dose-dependently reduces phosphorylation of NF-κB components and attenuates the production of IL-1β, TNF-α, and IL-6 proteins induced by CpG-ODN (5 μM). (B) In real-time PCR analysis, IL-1β, TNF-α, and IL-6 mRNA following 5 μM CpG-ODN were inhibited in U937 cells treated with IL-37. *p<0.05 and †p<0.01 versus cells treated with only CpG-ODN. CpG-ODN, CpG-oligonucleotides; NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor-α; IL, interleukin.

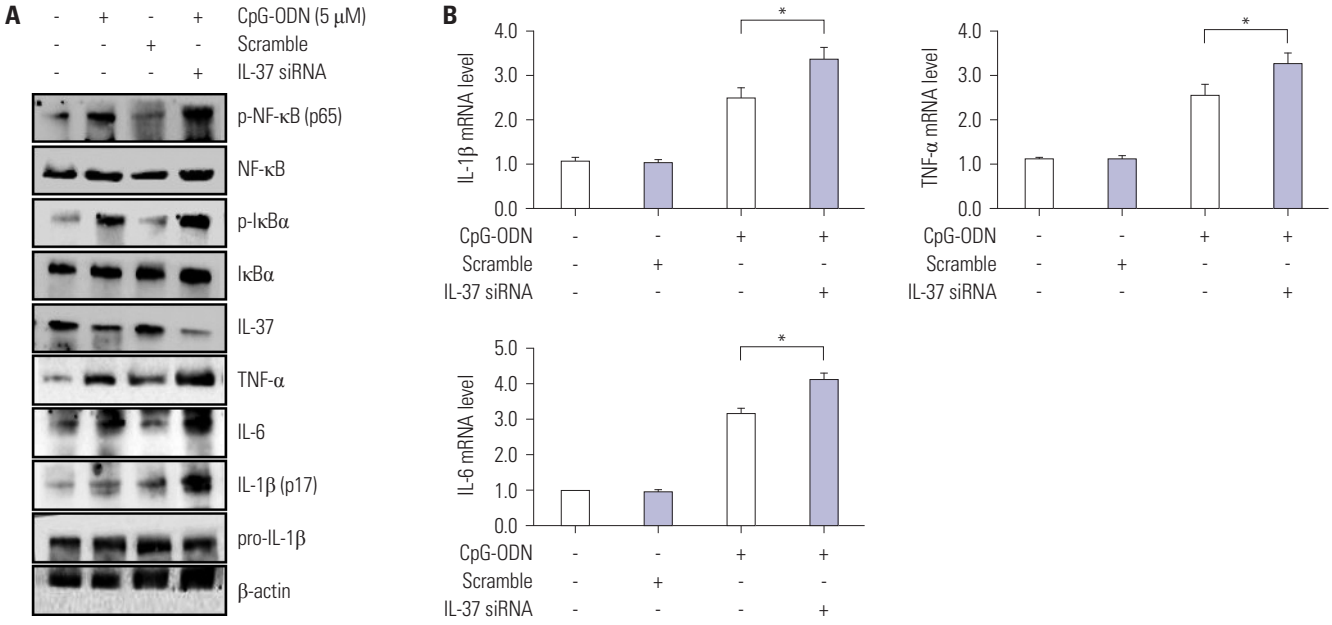

Fig. 3. Knockdown of IL-37 augments activation of NF-κB pathway and pro-inflammatory cytokines. (A) In the immunoblotting assay, human macrophages transfected with IL-37 siRNA after stimulation with CpG-ODN show upregulated NF-κB pathway signaling and pro-inflammatory cytokines, but reduced IL-37 expression. (B) Human macrophages transfected with IL-37 siRNA exhibit increased gene expression of pro-inflammatory cytokines, compared to non-transfected cells, under stimulation of 5 μM CpG-ODN. *p<0.05 versus cells treated transfected without IL-37 siRNA. CpG-ODN, CpG-oligonucleotides; NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor-α; IL, interleukin.

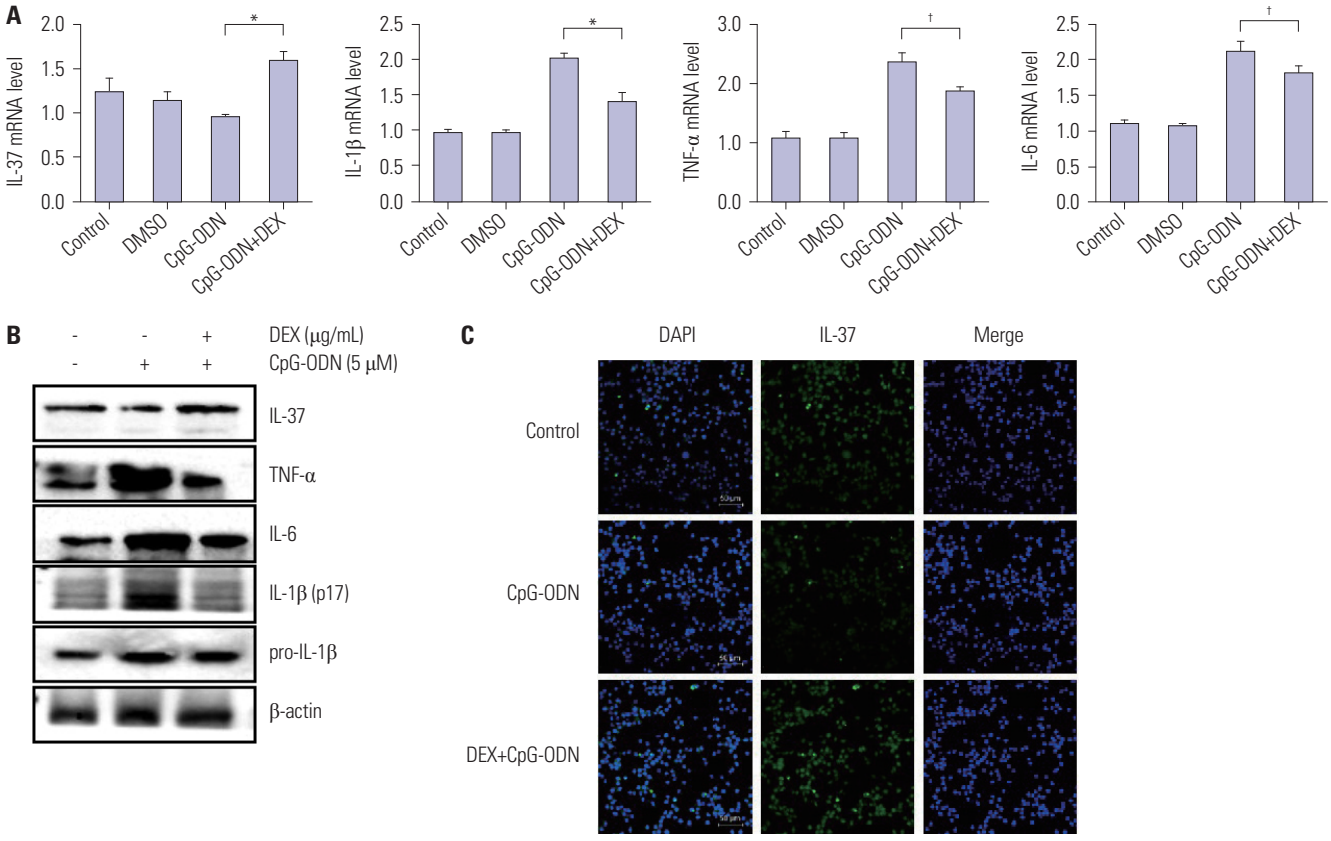

Fig. 4. Dexamethasone differentially regulates inflammatory cytokines following CpG-ODN stimulation. (A) Human macrophages treated with DEX (1 μg/mL) and CpG-ODN (5 μM) show attenuated gene expression of pro-inflammatory cytokine, but increased IL-37 gene expression, compared to only CpG-ODN stimulation. *p<0.01 and †p<0.05 versus cells treated with only CpG-ODN. (B) Immunoblots show that DEX in U937 cells treated with CpG-ODN reduces pro-inflammatory cytokines, but augments IL-37 protein production. (C) Immunofluorescent staining shows that IL-37 staining cells were increased in stimulation with DEX (1 μg/mL) and CpG-ODN (5 μM), rather than cells treated with only CpG-ODN. DMSO, dimethyl sulfoxide; DEX, dexamethasone; CpG-ODN, CpG-oligonucleotides; TNF-α, tumor necrosis factor-α; IL, interleukin.

hibiting adaptive immunity,[1,2] although mechanisms for the action and function of endogenous IL-37 have not been elucidated. Research has established that IL-37 binds to a chain of the IL-18 receptor (IL-18Rα) and recruits the orphan decoy IL-1R8, but not the IL-18Rβ chain, which leads to decreased expression of its adaptor proteins, MyD88, IRAK4, and TRAF6. This inhibits activation of inflammatory signal pathways, including the NF-κB, ERK1/2, JNK, p38MAPK, and TAK1 pathways.[5,10,11] In reflection of the anti-inflammatory effects of IL-37, Nold, et al.[4] demonstrated that LPS-induced endotoxemia is attenuated significantly in transgenic mice using FLAG-in IL-37b (IL-37 tg), compared to negative wild-type mice. In addition, IL-37 tg mice showed markedly inhibited generation of LPS-induced pro-inflammatory cytokines IL-6, IL-1β, IL-17, and IFN-γ compared to negative wild-type mice.[4] In the present study, we confirmed that activation of MyD88-TRAF6-NK-κB pathways and production of pro-inflammatory cytokines IL-1β, TNF-α, and IL-6 induced by CpG-mediated TLR9 activation is tightly regulated in human macrophages. In addition, we discovered that dexamethasone suppresses CpG-mediated pro-inflammatory cytokines and restores IL-37 expression. Altogether, our results indicated that the anti-inflammatory effect of IL-37 is related with inhibition of CpG-mediated ubiquitination of TRAF6.

IL-37 can be induced or upregulated by several TLR ligands, including LPS for TLR4 and Pam3CSK4 for TLR2, through diverse signal transduction pathways.[4] Considering interactions between IL-37 and TLR ligands, human PBMCs treated with LPS (10 and 1000 ng/mL) and Pam_3CSK_4 (10 ng/mL) for 20 h exhibited increased synthesis of IL-37.[4] PBMCs transfected with IL-37 siRNA after incubation with LPS or Pam_3CSK_4 consistently induced greater IL-1β and IL-6 secretion than those with scrambled siRNA.[4] In contrast, another TLR ligand, flagellin for TLR5, did not increase expression of the IL-37b isoform protein in a mouse macrophage RAW cell line.[4] Considering the relationship between TLR9 and IL-37, CpG for the TLR9 ligand increased the synthesis of IL-37 protein in PBMCs and RAW cells.[4] There are insufficient data about the role of IL-37 in regulation of the CpG-TLR9 signal pathway. We investigated whether IL-37 has an inhibitory effect on the CpG-TLR inflammatory signal pathway and its related production of pro-inflammatory cytokines in U937 cells stimulated by CpG. In doing so, we observed that U937 cells treated with CpG (5.0 μM) for 24 h demonstrated decreased IL-37 mRNA and protein in a dose-dependent manner as analyzed by RT-PCR and immunoblotting assay. Research has

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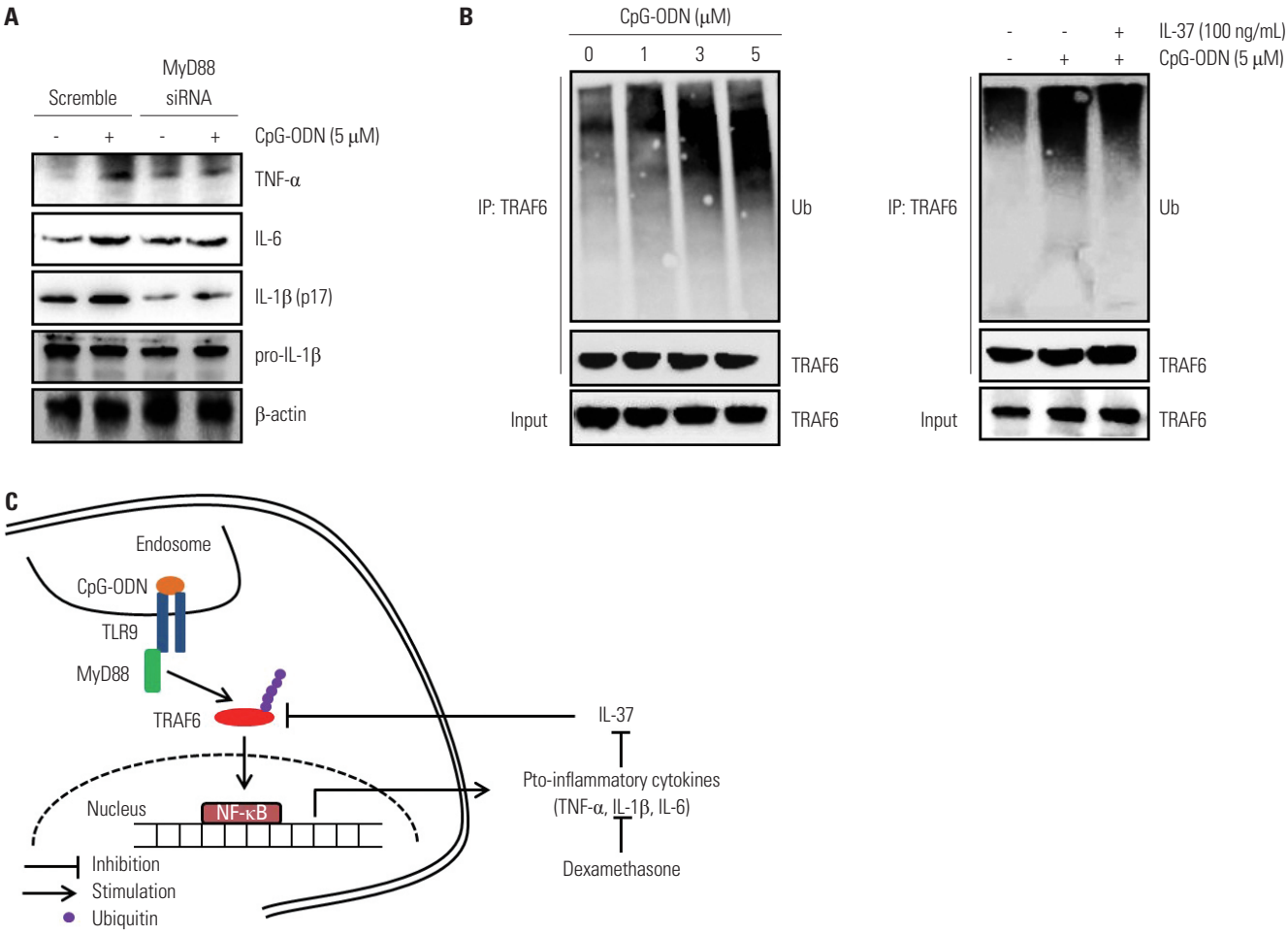

Fig. 5. IL-37 suppresses ubiquitination of TRAF6 induced by CpG-ODN. (A) Under stimulation with CpG-ODN (5 μM), pro-inflammatory cytokines in U937 cells transfected with MyD88 siRNA were suppressed, compared with non-transfected cells. (B) CpG-ODN stimulates ubiquitination of TRAF6 in a dose-dependent manner. Ubiquitination of TRAF6 in U937 cells treated with IL-37 (100 ng/mL) was suppressed. (C) Schematic illustration for the regulatory effect of IL-37 on CpG-ODN-mediated inflammatory responses. Binding to CpG-ODN and TLR9 induces the MyD88-TRAF6-NF-κB pathway to induce inflammatory cytokines in U937 cells. Deletion of pro-inflammatory cytokines by dexamethasone augments IL-37 expression in human macrophages. IL-37 potently blocks TRAF6 ubiquitination. CpG-ODN, CpG-oligonucleotides; TLR9, toll-like receptor 9; NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor-α; IL, interleukin.

shown that mature IL-37 cleaved by caspase-1 binds to Smad3. This complex is translocated to the nucleus to suppress signal transduction proteins, thereby blocking TLR-induced inflammatory responses.[12] Some studies have demonstrated that CpG-ODN inhibits the Smad signal pathway.[13,14] Based on this evidence, it can be proposed that decreases in IL-37 expression by CpG-ODN are involved in the negative feedback of aberrant expression of mature IL-37 by impeding the formation of mature IL-37 and Smad3 complex by CpG-ODN. Conflicting results regarding TLR9 and IL-37-mediated inflammatory responses need to be verified through additional studies in consideration of the experimental cells used in the study and various experimental conditions.

Cytokines IL-1β, IL-18, TNF, and TGF-β1 trigger increased expression of IL-37 in human PBMCs, although the exact mechanism thereof has not been confirmed.[4] Nevertheless, clinical evidence has consistently shown that IL-37 levels in PBMCs or in serum in autoimmune and inflammatory diseases, including rheumatoid arthritis, SLE, and ankylosing spondylitis, are significantly higher than those in healthy controls. In addition, IL-37b levels have been shown to be positively correlated with disease activity or inflammatory cytokines, including IL-17A, TNF-α, IL-18, IL-18BP, IFN-γ, and IL-6.[15-17] In ankylosing spondylitis, serum IL-37 levels were found to be associated significantly with pro-inflammatory cytokines, such as IL-6, TNF-α, and IL-17, and with clinical disease activity index.[17] Zhao, et al.[15] demonstrated a parallel decrease in pro-inflammatory cytokines and IL-37 in rheumatoid arthritis upon disease-modifying anti-rheumatic drug treatment. Similarly, glucocorticoid treatment was shown to significantly reduce plasma levels and mRNA expression of IL-37, compared to pretreatment in SLE patients.[16] This suggests that IL-37 levels are tightly regulated by pro-inflammatory cytokines. However, the exact pathogenic mechanism between IL-37 and these pro-inflammatory cyto-

kines awaits discovery.

The main finding of this study is the disequilibrium between some pro-inflammatory cytokines, including IL-1β, TNF-α, and IL-6, and the anti-inflammatory cytokine IL-37 during the CpG-induced inflammatory process. Interestingly, when pro-inflammatory cytokines were markedly increased by stimulation with CpG, IL-37 mRNA and protein levels were simultaneously reduced. Earlier clinical observations have revealed decreased IL-37 mRNA and protein levels in PBMCs, serum, sputum, or skin derived from patients with inflammatory diseases, such as asthma, Behçet's disease, and intervertebral disc degeneration, and the level of IL-37 in active disease activity was much lower than that in healthy controls or patients with inactive disease.[18-20] Glucocorticoid treatment has been found to increase IL-37 mRNA expression in PBMCs in Behçet's disease patients and to further increase IL-37 in a steroid dose-dependent manner.[19] Considering that IL-1β, IL-16, TNF-α and TGF-β1 are significantly increased in degenerative intervertebral disc disease, it can be assumed that decreases in IL-37 mRNA and protein levels in degenerative discs result from an increase in these inflammatory cytokines.[20] It has been suggested that decreased IL-37 levels are closely associated with disease activity and pro-inflammatory cytokines in some inflammatory diseases, contradictory to results in other studies.[15-17] Here, we provide experimental evidence that dexamethasone suppresses CpG-induced increases in the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α in U937 cells while upregulating IL-37 expression. This suggests that pro-inflammatory cytokines act as crucial factors that negatively regulate IL-37 expression.

TLRs play a role in the regulation of innate immunity: sensing endogenous danger signals or microbial pathogens, triggering signal pathways, and initiating the expression of gene products related with innate immunity.[21] Production of many TLR-mediated pro-inflammatory cytokines is dependent on activation of NF-κB and MAPK signaling.[21,22] Among TLRs, TLR9 is responsible for intracellular recognition of CpG motifs.[23] Upon stimulation of CpG, NF-κB pathway activation is needed for induction of TLR9-mediated pro-inflammatory cytokines and chemokines.[24,25] Consistently, our study showed that CpG binding to TLR9 induced the NF-κB signaling pathway and stimulated production and secretion of the pro-inflammatory cytokines IL-1β, TNF-α, and IL-6 in human macrophages. In addition, the reduced expression of these pro-inflammatory cytokines in human macrophages transfected with TLR9 siRNA reflected downregulation of the NF-κB signaling pathway stimulated by CpG.

Various autoimmune diseases are induced by immune responses to self-antigens. Particular autoimmune diseases mediated by TLR9 include SLE and Sjogren's syndrome. Meanwhile, however, there has been a debate about the protective or regulatory effect of TLR9 in the pathogenesis of SLE. Immune complexes that play roles similar to CpG and TLR9 have been found to induce activation of dendritic cells, cause differentiation of T- and B-lymphocytes, and stimulate secretion of pro-inflammatory cytokines to create an inflammatory response in SLE.[26] Others have shown that TLR9 is involved in Epstein-Barr virus infection, triggering the pathological immune response of systemic sclerosis.[27] This is presumably due to activation of NF-κB signaling pathway through TLR9. Additionally, researchers have indicated that the expression of TLR9 in the salivary epithelial gland cells of Sjogren's syndrome patients is higher than that of controls and contributes to increased expression of pro-inflammatory cytokines.[28] Based on these results, we propose that the anti-inflammatory effect of IL-37 could be a potential target for treating various autoimmune diseases involving TLR9.

The use of siRNA to inhibit target molecules can be considered as a limitation in this study. siRNA for TLR9 and MyD8 was used to determine their effect on the CpG-induced NF-κB signaling pathway. The knockdown efficacy of siRNA for TLR9 and MyD8 was about 60%–65%, so there was a limit to completely blocking this pathway. Incomplete blockage of these target molecules can lead to expression of other downstream cytokines, and it is difficult to exclude paracrine effects from cytokines on our results.

In conclusion, CpG-mediated TLR9 activation induced the pro-inflammatory cytokines IL-1β, TNF-α, and IL-6 through the NF-κB signaling pathway in human macrophages, whereas IL-37 expression was inhibited through blocking of TRAF6 ubiquitination (Fig. 5C). In addition, recombinant IL-37 addition or IL-37 enhancement by corticosteroid treatment potently attenuated expression of CpG- and TLR9-mediated pro-inflammatory cytokines. Based on these findings, we suggest that IL-37 inhibits ubiquitination of TRAF6 upon activation of TLR9 and is unlikely to be directly involved in the regulation of NF-κB signaling.

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AUTHOR CONTRIBUTIONS

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