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Intranuclear interactomic inhibition of NF-κB suppresses LPS-induced severe sepsis

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ABSTRACT

Suppression of nuclear factor-κB (NF-κB) activation, which is best known as a major regulator of innate and adaptive immune responses, is a potent strategy for the treatment of endotoxic sepsis. To inhibit NF-κB functions, we designed the intra-nuclear transducible form of transcription modulation domain (TMD) of RelA (p65), called nt-p65-TMD, which can be delivered effectively into the nucleus without influencing the cell viability, and work as interactomic inhibitors via disruption of the endogenous p65-mediated transcription complex. nt-p65-TMD effectively inhibited the secretion of pro-inflammatory cytokines, including TNF-α, IL-1β, or IL-6 from BV2 microglia cells stimulated by lipopolysaccharide (LPS). nt-p65-TMD did not inhibit tyrosine phosphorylation of signaling mediators such as ZAP-70, p38, JNK, or ERK involved in T cell activation, but was capable of suppressing the transcriptional activity of NF-κB without the functional effect on that of NFAT upon T-cell receptor (TCR) stimulation. The transduced nt-p65-TMD in T cell did not affect the expression of CD69, however significantly inhibited the secretion of T cell-specific cytokines such as IL-2, IFN-γ, IL-4, IL-17A, or IL-10. Systemic administration of nt-p65-TMD showed a significant therapeutic effect on LPS-induced sepsis model by inhibiting pro-inflammatory cytokines secretion. Therefore, nt-p65-TMD can be a novel therapeutics for the treatment of various inflammatory diseases, including sepsis, where a transcription factor has a key role in pathogenesis, and further allows us to discover new functions of p65 under normal physiological condition without genetic alteration.

1. Introduction

Sepsis is one of the most serious and elusive clinical syndrome caused by severe systemic inflammatory response to infection [1]. The transcriptional regulatory factor NF-κB plays critical roles in modulating the expression of various immunoregulatory mediators involved in the development of sepsis-induced multiple organ failure [2]. Signaling pathways initiated by engagement of Toll-like receptors (TLRs) or cytokine receptors, including those for tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 lead to nuclear translocation of NF-κB and enhanced transcription of genes responsible for the expression of the inflammatory mediators associated with the pathogenesis of sepsis [3,4]. Thus, the development of new therapeutics specifically targeting NF-κB is essential to ameliorate the high mortality of severe sepsis.

Five mammalian members of the NF-κB family have been identified: RelA (p65), RelB, c-Rel, NF-κB1 (p50 and its precursor p105), and NF-κB2 (p52 and its precursor p100) [5,6]. The different members of the NF-κB family have the N-terminal Rel-homology domain (RHD), which is responsible for DNA-binding, homo- or hetero-dimerization, and interaction with inhibitory IκB. The unique pairing among NF-κB family members exerts different

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transcriptional abilities: p50-p65 heterodimers can strongly promote expression of NF-κB-dependent genes whereas p50-p50 homodimers have the suppressive activities on gene activation [7,8]. For this reason, suppression of LPS-induced NF-κB activation, particularly p65, is likely to be most effective in improving the symptoms of sepsis.

In this study, we demonstrated that the nt-p65-TMD is delivered into the nucleus effectively *in vitro* and *in vivo* without any cellular cytotoxicity, and directly targets the endogenous p65 in interactomic inhibitory manner. The transduced nt-p65-TMD specifically not only suppresses transcriptional activity of NF-κB and IL-2, but also inhibits the secretion of the cytokines associated with T cell activation such as IL-2, IFN-γ, IL-4, IL-17A, and IL-10 via inhibition of p65-mediated gene expression. However, the signaling events upstream of NF-κB activation such as induction of tyrosine phosphorylation of various signal mediators and CD69 expression, and NFAT-mediated transcription were not influenced by nt-p65-TMD. We revealed the *in vivo* therapeutic effect of nt-p65-TMD in LPS-induced sepsis model. Based on our results, nt-p65-TMD might be a novel protein drug candidate for the treatment of various inflammatory diseases.

2. Materials and methods

2.1. Generation and purification of nucleus-transducible form of nt-p65-TMD

The FLAG-tagged p65-DBD (p65-TMD) that encode amino acids (1–187) conjugated with Hph-1-PTD were amplified by PCR from the full-length mouse p65 (1–551) and (kindly provided by B. Park, Yonsei University, Seoul, Republic of Korea), and inserted into the pET-28a(+) vector (Novagen). A p65 mutant was generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) in which two residues essential for base-specific contacts, Tyr 36 and Glu 39, were mutated to Ala and Asp, respectively. The cloned DNA was transformed into *Escherichia coli* BL21 CodonPlus (DE3)-RIPL strain (Invitogen) for proteins expression. The proteins expression was induced for 5 h at 37 °C with 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG; Duchefa). After harvesting, cells were resuspended and sonicated in lysis buffer (10 mM imidazole, 50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0). Soluble fraction of lysates was obtained by centrifugation (13,000 rpm for 10 min at 4 °C), and then mixed with Ni-NTA resin (Qiagen) for 1 h at 4 °C. The proteins were loaded to HisTrap chromatography columns (Bio-Rad). After non-specifically bound proteins were washed with wash buffer (30 mM imidazole, 50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0), and the target proteins were eluted with elution buffer (250 mM imidazole, 50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0). The eluted proteins were desalted by PD-10 Sephadex G-25 (GE Healthcare) supplemented with 10% glycerol PBS. To eliminate the endotoxin such as LPS, the desalted proteins were once again mixed with SP SepharoseTM Fast Flow (GE Healthcare) in ion-exchange chromatography binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 6.0) for 1 h at 4 °C. Bound proteins were completely washed with SP wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 6.0), and the target proteins were eluted by SP elution buffer (50 mM NaH_2PO_4, 2 M NaCl, pH 6.0). The eluted proteins were desalted by PD-10 Sephadex G-25 supplemented with 10% glycerol PBS and the aliquots were to be stored at −80 °C.

2.2. Cell cultures

Jurkat T cells and mouse splenocytes were cultured in RPMI 1640 medium (Lonza) supplemented with 10% FBS (Lonza), 2 mM L-glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin. BV2 microglia, HeLa, and HEK293T cells were cultured in DMEM medium (Lonza) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin, 1 mM sodium pyruvate and non-essential amino acids (NEAA; Invitrogen) and incubated at 37 °C in a 5% CO_2 incubator.

2.3. In vitro intra-nuclear transduction kinetics of nt-p65-TMD

BV2 and Jurkat T cells (2×10^6) were treated with proteins in concentration- (1 h) and time-dependent manner, washed with PBS, and lysed with RIPA buffer (Sigma–Aldrich). Protein extracts were separated by SDS-PAGE, and then the separated proteins were transferred to poly vinylidine difloride (PVDF; Millipore) transfer membrane. The membrane was blocked in 5% Bovine Serum Albumine (BSA; Amresco) in Tris-buffered saline with tween-20 (TTBS) for 1 h at room temperature and then incubated with anti-FLAG (1:3000 dilution; Sigma–Aldrich) and anti-β-actin (1:5000 dilution; Sigma–Aldrich) in 5% BSA for 2 h at room temperature. After probing with anti-mouse IgG (1:10,000 dilution; Sigma–Aldrich) and anti-rabbit IgG (1:10,000 dilution; Sigma–Aldrich) antibodies in TTBS for 1 h at room temperature, the target protein in the membrane was detected by chemiluminescence using WESTZOL plus (iNtRON Biotechnology).

2.4. Immunocytochemistry

BV2 and HeLa cells (5×10^4) seeded on Lab-Tek II chamber slides (Nunc) were treated with 5 μM of nt-p65-TMD for 1 h. The cells were washed twice with PBS, then fixed with 4% formaldehyde, permeabilized with 0.2% Triton-X-100 (Sigma–Aldrich) and blocked with blocking solution (1% BSA in PBS base). The transduced nt-p65-TMD was stained with FITC-conjugated anti-FLAG (1:200 dilution; Sigma–Aldrich) antibody, and the nucleus was stained with 4′-6-Diamidino-2-phenylindole (1:10,000 dilution; DAPI; Invitrogen) and analyzed by confocal microscopy (Carl Zeiss LSM510).

2.5. Cytotoxicity assay

BV2 cells (1×10^4) and splenocytes (1×10^5) in 96-well plate were treated with various concentration (0.01–5 μM) of nt-p65-TMD for 24 h. Then, CCK-8 solution (Dojindo Molecular Technologies) was added to each well of plate, and then the samples were incubated for 4 h. The cell viability was determined by measuring the absorbance at 450 nm using a microplate reader (Bio-rad). The absorbance measured in this assay correlates with the number of living cells.

2.6. Luciferase assay

Both 1 μg of full-length Rela cloned into pEGFP-N1 plasmid and 1 μg of *NFKB1*- or *Il2*-luciferase (kindly provided by E.-S. Hwang, Ewha Womans University, Seoul, Republic of Korea) reporter genes were co-transfected into HEK293T cells (4×10^5/well). After 3 h incubation, the cells were treated with nt-p65-TMD and incubated 24 h. Jurkat T cells (5×10^6) were transiently transfected with 20 μg of either *NFKB1*- or *NFATC1*-luciferase (kindly provided by K.-C. Chung, Yonsei University, Seoul, Republic of Korea) reporter genes by electroporation (260 V, 975 μF). Transfected cells were cultured for 20 h and were treated with nt-p65-TMD for 1 h, and then stimulated with anti-CD3 (1 μg/ml; BD Pharmingen) and anti-CD28 (1 μg/ml; BD Pharmingen) for 6 h. The luciferase activity was performed according to the manufacturer's instructions and the value was normalized by Renilla activity measured with a luminometer

(Promega), and the relative measurement was calculated, compared to the absorbance of the highest value.

2.7. Measurement of pro-inflammatory cytokines secretion

BV2 cells were pre-treated with nt-p65-TMD for 1 h prior to stimulation with LPS (1 μg/ml; *E. coli* serotype O55:B5; Sigma–Aldrich) for 24 h. To measure the secretion of LPS-induced cytokines in the serum, mice were intra-peritoneally injected with nt-p65-TMD two times at 2 h and 14 h after LPS (20 mg/kg) administration, and then serum was collected at 24 h after LPS. The culture supernatants and serum were analyzed for secreted TNF-α, IL-1β, or IL-6 by ELISA kit (all purchased from eBioscience) according to the manufacturer's instructions.

2.8. Phosphorylation of intracellular signaling proteins

Jurkat T cells (5×10^5) were treated with 2 μM of nt-p65-TMD for 1 h, and then the cells were stimulated with anti-CD3 (2.5 μg/ml) and anti-CD28 (2.5 μg/ml) antibodies for 5 min at 37 °C, the cell lysates were analyzed by immunoblotting with anti-phospho-ZAP-70 (Tyr319) (1:1000 dilution), -phospho-p38 MAPK (Thr180/Tyr182) (1:1000 dilution), -phospho-SAPK/JNK (Thr183/Tyr185) (1:1000 dilution), and -phospho-p44/42 (ERK1/2) (1:2000 dilution) (all purchased from Cell Signaling Technology) antibodies, and β-actin (1:5000 dilution) was used as lysate control.

2.9. Analysis of T cell activation

C57BL/6 mouse splenocytes (2×10^6) were treated with nt-p65-TMD for 1 h, and then stimulated with plate-bound anti-CD3 (1 μg/ml) and soluble anti-CD28 (1 μg/ml) antibodies for 72 h. The supernatants were collected after the indicated periods of cell culture and were analyzed for secreted IL-2, IFN-γ, IL-4, IL-17A, or IL-10 by ELISA kit (all purchased from eBioscience) according to the manufacturer's instructions. The cells were stained with FITC-conjugated anti-CD69 (BD Pharmingen) antibody, and then were analyzed by FACScaliber (BD Biosciences).

2.10. Animals

All animals were treated in accordance with the Guidelines and Regulations for the Use and Care of Laboratory Animals of Yonsei University (Seoul, Republic of Korea). Female C57BL/6 or male BALB/c mice (Orient Bio, Republic of Korea) was purchased and housed individually in a specific pathogen-free barrier facility under standard sterile conditions.

2.11. LPS-induced sepsis model

For LPS-induced sepsis model, male BALB/c mice (6 to 8-week-old) were intra-peritoneally injected with LPS (20 mg/kg). The nt-p65-TMD (1 mg/kg or 5 mg/kg) or mnt-p65-TMD (5 mg/kg) was intra-peritoneally injected two times at 2 h and 14 h after LPS administration. The survival rate was monitored for 6 days.

2.12. Statistical analysis

All values and error bars were calculated using triplicate, and represent the mean ± s.e.m. of three independent experiments. Statistical analysis of group differences was determined using an unpaired two-tailed Student's *t*-test, with the exception of the survival data. The Kaplan–Meier plot for survival rate was analyzed using the log rank test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ were considered statistically significant.

3. Results

3.1. Generation of nucleus-transducible forms of p65-TMD

The N-terminus of p65 has the TMD comprising DNA-binding amino acid residues and isotype-specific sequences that may play key roles in the functional specificity of p65 [9]. To modulate p65-mediated NF-κB functions, we designed a novel therapeutic strategy to deliver p65-TMD efficiently into the nucleus of the cells *in vitro* and *in vivo*, and thereby, the delivered nt-p65-TMD competitively interferes with the transcriptional activity of endogenous p65 at the promoter of p65-target genes. The nt-p65-TMD was generated by the fusion of p65-TMD with Hph-1-PTD (Protein Transduction Domain) [10,11] (Fig. 1A and B). To examine the intracellular transduction efficiency of nt-p65-TMD into cells, we treated BV2 or Jurkat T cells with nt-p65-TMD. nt-p65-TMD was effectively transduced into the cells in a concentration-dependent manner while p65-TMD without PTD was not transduced (Fig. 1C). The delivered nt-p65-TMD remained inside the cells up to 48 h after transduction (Fig. 1D), and the majority of nt-p65-TMD was detected in the nucleus as early as 1 h after transduction, which was analyzed by confocal microscopy (Fig. 1E). Importantly, neither nt-p65-TMD nor the control proteins resulted in cytotoxicity in BV2 cells or mouse splenocytes (Fig. 1F). These results suggest that nt-p65-TMD is efficiently transduced into the nucleus of the cells without any adverse effects.

3.2. nt-p65-TMD specifically inhibits p65-mediated transcription

To examine whether nt-p65-TMD can inhibit the functions of NF-κB and its downstream transcriptional activity, HEK293T cells were cotransfected with the plasmids expressing wild-type p65 and NF-κB- or IL-2-promoter luciferase reporter gene. The transfected cells were then treated with nt-p65-TMD, and the luciferase activity was measured. The nt-p65-TMD significantly inhibited the p65-mediated both NF-κB- and IL-2-promoter luciferase activities in a dose-dependent manner, whereas the non-transducible p65-TMD could not affect these activities. Also, we generated mutant form of nt-p65-TMD in which two amino acid residues essential for DNA binding, Tyr 36 and Glu 39, were mutated to Ala and Asp, respectively. Interestingly, the nt-p65-TMD mutant could not bind to the NF-κB- and IL-2-promoter, and failed to attenuate the luciferase activity (Fig. 1G and H). Thus, nt-p65-TMD can specifically inhibit the transcriptional activity of endogenous p65 on its target genes.

3.3. nt-p65-TMD inhibits LPS-induced pro-inflammatory cytokines secretion from BV2 microglia cells

To investigate the anti-inflammatory effect of nt-p65-TMD, we determined the pro-inflammatory cytokines secretion, including TNF-α, IL-1β, or IL-6 by stimulating BV2 cells with LPS in the presence of nt-p65-TMD. The production of TNF-α, IL-1β, or IL-6 by LPS was increased over basal levels after stimulation with LPS for 24 h, whereas nt-p65-TMD significantly inhibited their production in BV2 cells (Fig. 2A–C). These results indicated that the nt-p65-TMD may act as a modulator of pro-inflammatory cytokines secretion from BV2 cells stimulated by LPS.

3.4. nt-p65-TMD specifically inhibits NF-κB transcriptional activity, but neither TCR proximal signaling events nor NFAT transcriptional activity by T cell activation

To determine whether the transduced nt-p65-TMD can influence the induction of tyrosine phosphorylation of various

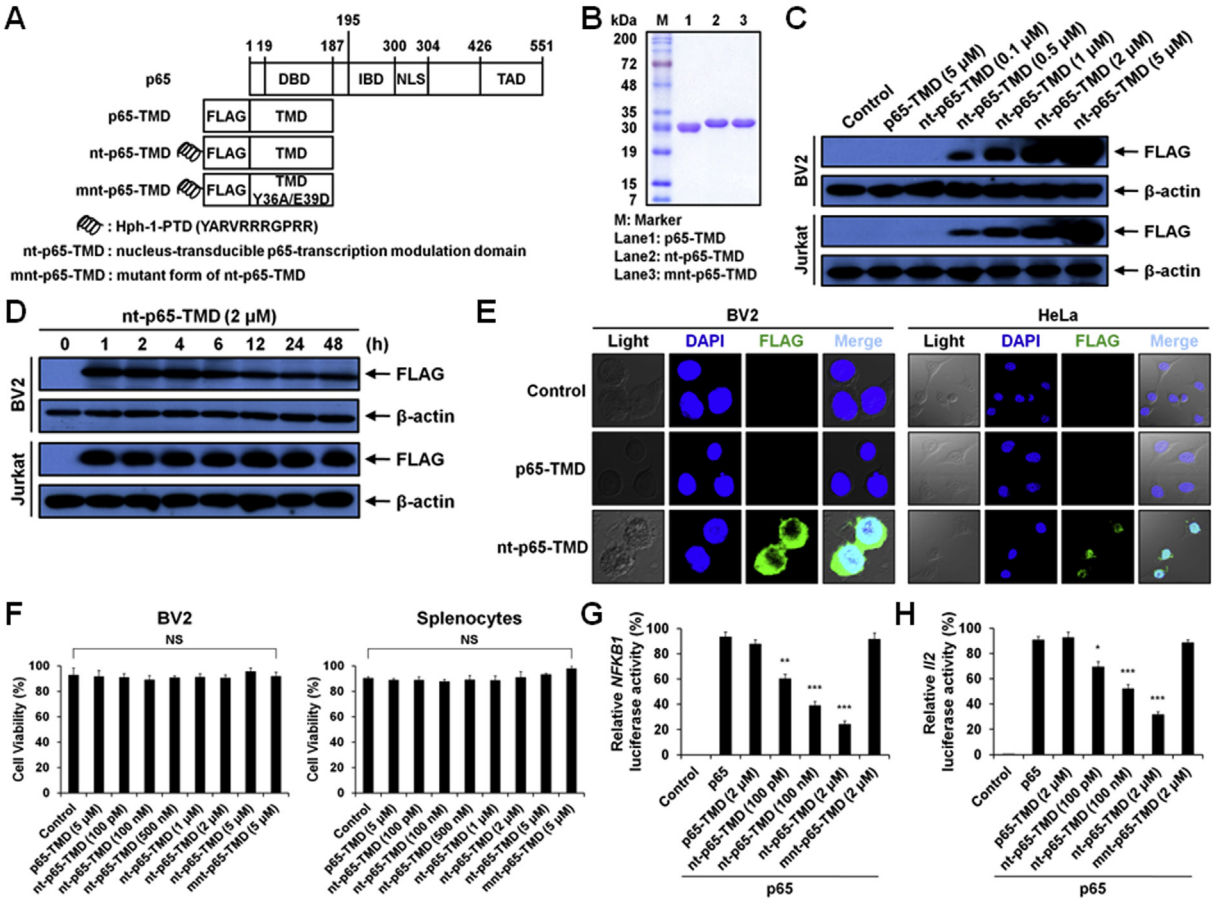

Fig. 1. Generation of nt-p65-TMD, a nucleus-transducible form of interactomic inhibitor of p65. (A) Construct of nt-p65-TMD and its derivatives: non-transducible p65-TMD (p65-TMD), nucleus-transducible p65-TMD (nt-p65-TMD), and a mutant form of nt-p65-TMD (mnt-p65-TMD) without DNA-binding capacity. (B) The recombinant proteins were purified under native condition, and its identity was confirmed by SDS-PAGE. (C, D) Concentration- and time-dependent delivery of nt-p65-TMD into the BV2 and Jurkat T cells. nt-p65-TMD was detected by immunoblot analysis with anti-FLAG antibody. β-actin was used as lysate control. (E) Intranuclear localization of nt-p65-TMD was analyzed by intracellular staining with FITC-conjugated anti-FLAG antibody at 1 h of post-transduction followed by analysis using confocal microscopy. (F) BV2 cells and mouse splenocytes were treated with various concentrations of nt-p65-TMD for 24, and cell viability was measured by CCK-8 assay. (G, H) Functional specificity of nt-p65-TMD was examined in HEK293T cells co-transfected with the vectors expressing wild-type p65 and luciferase driven by *NFKB1*- or *Il2*-promoter. After 24 h, luciferase activity was measured by luminometer. Error bars represent mean ± s.e.m. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ were considered statistically significant. NS, not significant.

intracellular signaling proteins proximal to TCR complex, Jurkat T cells were treated with nt-p65-TMD for 1 h. Then, the cells were stimulated with anti-CD3 and anti-CD28, and the tyrosine phosphorylation of ZAP-70, p38, JNK, or ERK was analyzed by phosphotyrosine immunoblot. nt-p65-TMD did not affect the tyrosine phosphorylation of ZAP-70, p38, JNK, or ERK (Fig. 2D). To demonstrate the functional specificity of nt-p65-TMD, we transiently transfected Jurkat T cells with NF-κB- or NFAT-promoter luciferase reporter gene in which the expression of luciferase gene is induced by T cell activation. nt-p65-TMD was transduced into the transfected Jurkat T cells, followed by the activation with anti-CD3 and anti-CD28, and then the luciferase activity was measured. The nt-p65-TMD significantly inhibited the NF-κB transcriptional activity in a dose-dependent manner, but did not affect the NFAT transcriptional activity (Fig. 2E and F). These results suggest that nt-p65-TMD can specifically inhibit the NF-κB transcriptional activity in T cells.

3.5. nt-p65-TMD inhibits the secretion of cytokines upon T cell activation

To investigate whether nt-p65-TMD suppresses the secretion of cytokines associated with T cell activation, the splenocytes isolated from C57BL/6 mice were treated with nt-p65-TMD for 1 h. The cells were stimulated with anti-CD3 and anti-CD28, and then the level of CD69 induction on the surface and the secretion of T cells proliferation cytokine (IL-2) or inflammatory cytokines such as IFN-γ, IL-4, IL-17A, or IL-10 were analyzed. nt-p65-TMD did not affect the induction of CD69, which is known to be an NF-κB-independent marker of T cell activation [12] (Fig. 3A), but significantly inhibited the secretion of IL-2, IFN-γ, IL-4, IL-17, or IL-10 (Fig. 3B–F). Thus, nt-p65-TMD effectively modulates the expression of activation-induced cytokines in T cells by suppressing of TCR-induced NF-κB activation, but neither the activation events upstream of NF-κB activation nor NF-κB-independent ones.

3.6. nt-p65-TMD protects mice from LPS-induced sepsis

To assess the therapeutic effect of nt-p65-TMD on NF-κB-mediated sepsis model we intra-peritoneally injected the mice with nt-p65-TMD (1 mg/kg or 5 mg/kg) or mutant form of nt-p65-TMD (5 mg/kg) at 2 h and 14 h after LPS (20 mg/kg) administration, and their survival rate was monitored up to 6 days. Treatment with nt-p65-TMD significantly increased the survival rate of these mice in a dose-dependent manner compared with that of untreated mice (Fig 4A). To investigate the effect of nt-p65-TMD on LPS-induced

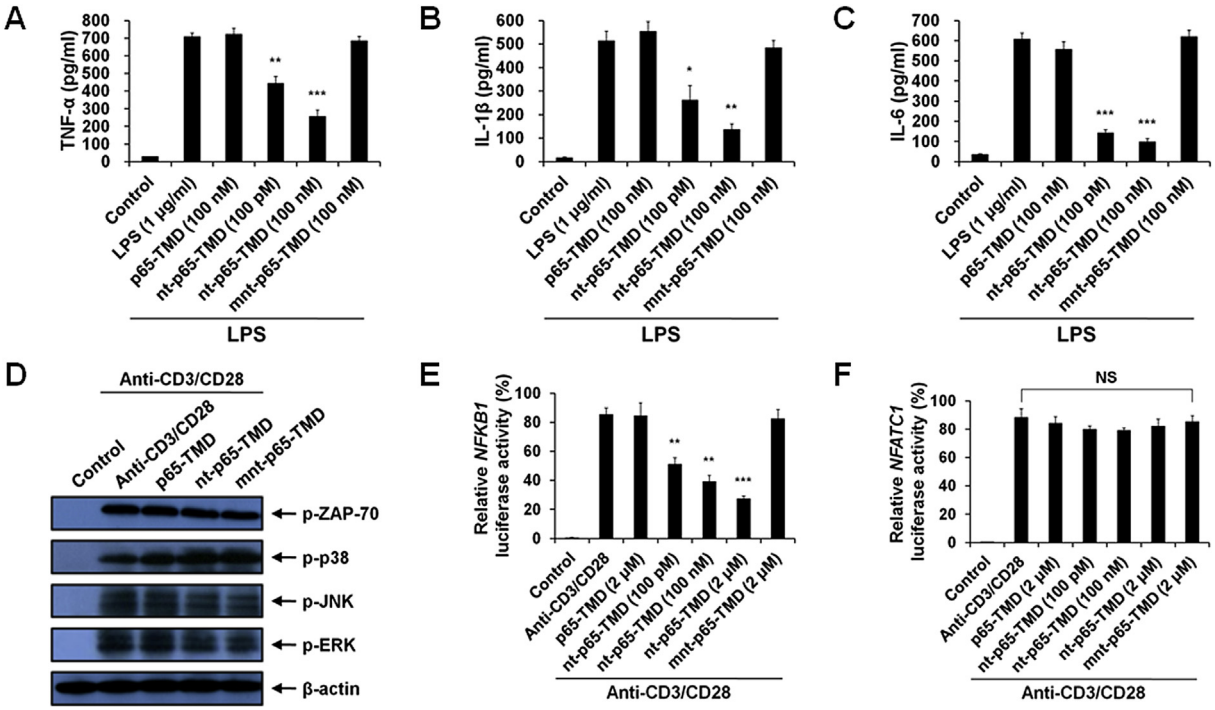

Fig. 2. nt-p65-TMD inhibited both LPS-induced pro-inflammatory cytokines secretion in BV2 cells and NF-κB transcriptional activity in T cells. (A–C) BV2 cells were pre-treated with nt-p65-TMD for 1 h prior to stimulation with LPS (1 μg/ml) for 24 h. The level of TNF-α (A), IL-1β (B), or IL-6 (C) in the culture medium was measured by ELISA. (D) After Jurkat T cells were treated with nt-p65-TMD for 1 h, and then stimulated with anti-CD3 and anti-CD28 for 5 min at 37 °C, the tyrosine phosphorylated form of each protein in the cell lysate was detected by immunoblot analysis with anti-phospho-ZAP-70, -p38 MAPK, -phospho-SAPK/JNK, or -phospho-p44/42 (ERK1/2) antibodies, and β-actin was used as lysate control. (E, F) Jurkat T cells were transfected with *NFKB1*- or *NFATC1*-luciferase reporter genes by electroporation. Transfected cells were treated with nt-p65-TMD for 1 h, and then stimulated with anti-CD3 and anti-CD28 for 6 h. Luciferase activity was measured by luminometer. Error bars represent mean ± s.e.m. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ were considered statistically significant. NS, not significant.

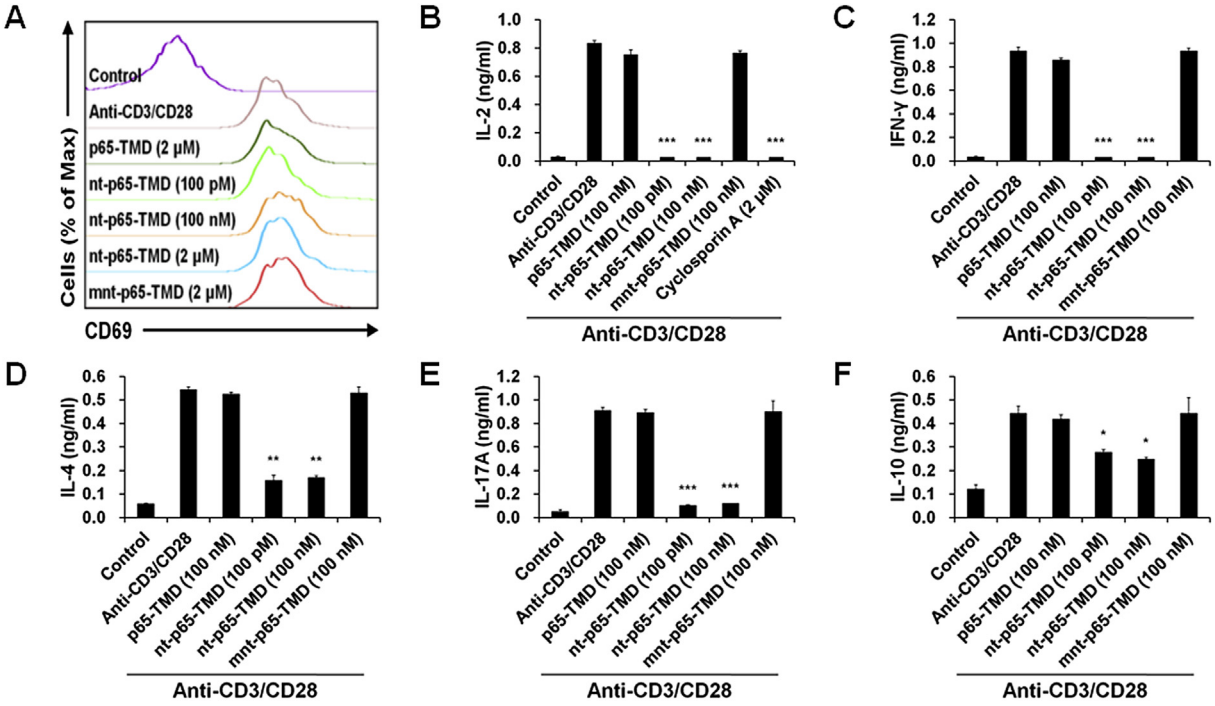

Fig. 3. Inhibition of cytokines secretion from TCR-stimulated splenocytes by nt-p65-TMD. (A–F) Mouse splenocytes were treated with nt-p65-TMD for 1 h followed by TCR stimulation with anti-CD3 and anti-CD28 for 72 h. The cells were stained with anti-CD69 antibody and analyzed by FACS (A), and the level of IL-2 (B), IFN-γ (C), IL-4 (D), IL-17A (E), or IL-10 (F) in the culture medium was analyzed by ELISA. Error bars represent mean ± s.e.m. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ were considered statistically significant.

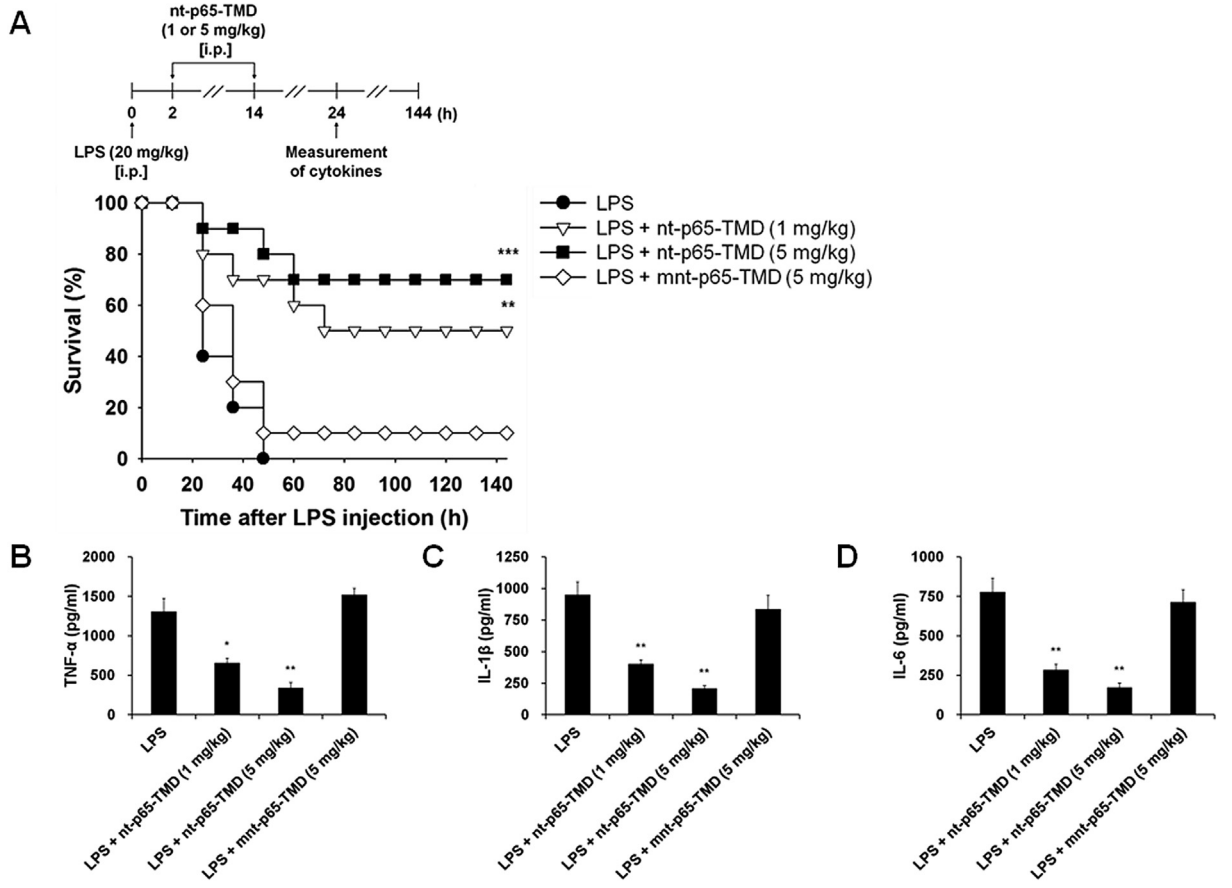

Fig. 4. Therapeutic effects of nt-p65-TMD in LPS-induced sepsis mice. (A–D) Mice were intraperitoneally injected with nt-p65-TMD (1 mg/kg or 5 mg/kg) or mnt-p65-TMD (5 mg/kg) two times at 2 h and 14 h after LPS (20 mg/kg) administration. The survival rate was monitored up to 6 days (A) and the serum was collected at 24 h after LPS and then analyzed for the secreted TNF-α, IL-1β, or IL-6 by ELISA (B–D). All results are mean ± s.e.m. (n = 10 mice per group). The Kaplan–Meier plot for survival rate was analyzed using the log rank test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ were considered statistically significant.

pro-inflammatory cytokines secretion we measured the concentrations of TNF-α, IL-1β, or IL-6 in the serum of LPS-injected mice. Mice treated with nt-p65-TMD had significantly lower level of serum concentration of TNF-α, IL-1β, or IL-6 than those in the untreated mice (Fig 4B). These results suggest that nt-p65-TMD may have therapeutic potential to promote survival rate in sepsis animal model by suppressing pro-inflammatory cytokines secretion.

4. Discussion

Sepsis is regarded as a major clinical problem in modern medical environment with limited treatment options [13]. Despite decades of intense research, severe sepsis continues to be associated with an extremely high mortality rate. Thus, it is essential to identify the new therapeutics for the treatment of sepsis based on its pathogenesis. NF-κB occupies a central role in the onset of sepsis, endotoxemia-induced increases in pro-inflammatory mediators and organ dysfunction. There are multiple cellular activators associated with sepsis, including bacterial products and cytokines which can result in nuclear translocation of NF-κB [7]. NF-κB is essentially involved in the transcriptional regulation of many pro-inflammatory mediators, which play important role in the progress of sepsis. Therefore, modulation of NF-κB is an attractive therapeutic option in the treatment of sepsis. On the basis of these concepts, we hypothesized that the therapeutic administration of nt-p65-TMD can significantly increase survival rate in sepsis mice model.

In this study, we developed a novel therapeutic strategy to suppress the functions of endogenous p65 in interactomic and competitive manner by intranuclear delivery of TMD of p65 *in vitro* and *in vivo*. nt-p65-TMD is the fusion protein between TMD of p65 and a human origin Hph-1-PTD that can be delivered effectively into the nucleus without influencing the cell viability (Fig. 1A–F). nt-p65-TMD, not nt-p65-TMD without DNA-binding capacity (mnt-p65-TMD), significantly inhibited p65-mediated NF-κB or IL-2 gene transcription mainly through the competition with endogenous p65 binding to promoter (Fig. 1G and H).

Microglia cells are the resident macrophage-like population within the central nervous system (CNS), which serve as antigen-presenting cells (APCs) and pro-inflammatory effector following activation [14]. Increased inflammatory cytokines in the CNS have been observed especially after brain damage due to several insults: infection, traumatic brain injury and cerebral ischemia. Here we demonstrated that nt-p65-TMD significantly inhibited production of TNF-α, IL-1β, or IL-6 by LPS in BV2 microglia cells (Fig. 2A–C).

In addition, the NF-κB plays important roles as a major regulator in modulation of T cell development, activation, differentiation, and survival through the transcriptional regulation of cytokine expression [15]. Our results suggest that nt-p65-TMD did not affect phosphorylation of ZAP-70, p38, JNK, or ERK as well as transcriptional activity of NFAT, whereas nt-p65-TMD significantly inhibited the transcriptional activity of NF-κB in a dose-dependent manner in T cells (Fig. 2D–F). We also revealed that nt-p65-TMD did not affect the induction of CD69 on the surface which is known to be NF-κB-

independent, but significantly inhibited secretion of IL-2, IFN-γ, IL-4, IL-17, or IL-10 in T cells by suppressing of TCR-induced NF-κB activation (Fig. 3). Our *in vivo* results demonstrated that control group injected with a lethal dose LPS (20 mg/kg) nearly died within 48 h. The treatment of the sepsis-induced mice with low dose nt-p65-TMD (1 mg/kg) resulted in 50% survival rate, and the sepsis-induced mice treated with high dose nt-p65-TMD (5 mg/kg) showed significantly higher therapeutic effect on the survival improvement of LPS-induced sepsis mice (Fig. 4).

In conclusion, nt-p65-TMD can be regarded as a novel and highly specific therapeutics for the treatment of severe sepsis, and further allows us to discover new function of p65 in other immune cells as well as animals under normal physiological conditions without genetic alteration. This novel strategy can be easily applied in development of novel therapeutics for the treatment of various inflammatory diseases, where a specific transcription factor has a key role in pathogenesis.

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