

Calcineurin subunit B upregulates β -interferon production by phosphorylation of interferon regulatory factor 3 via Toll-like receptor 4

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Toll-like receptors (TLRs) function as pattern-reorganization receptors in mammals and play an essential role in innate immunity. After recognizing certain ligands, TLRs activate a cascade of signal pathways to establish a guard environment. For the first time, we report that *in vitro* treatment with recombinant calcineurin subunit B (CNB) upregulated several TLR-related genes. Calcineurin subunit B interacted with the ectodomain of TLR4 *in vitro*. On further investigation, phosphorylation of interferon regulatory factor 3 and degradation of $\text{I}\kappa\text{B-}\alpha$ were observed by CNB stimulation. In addition to pro-inflammatory cytokines, transcription and production of β -interferon were also enhanced after CNB stimulation. Thus, CNB could be further explored as a cancer and virus immunotherapy drug. (Cancer Sci 2012; 103: 515–521)

Innate immunity provides immediate and efficient defense against microbial infection. Toll-like receptors play an important role in innate immunity. They detect microbes, viruses, and endogenous ligands to mediate the induction of genes encoding inflammatory cytokines, chemokines, interferons, and interferon-related molecules.⁽¹⁾ Toll-like receptor 4 is the only member of the TLR family that functions through both the MyD88 and TRIF signal pathways. After receiving certain kinds of stimulation, TLR4 promotes IRF3 and NF- κB translocation to the nucleus to activate IFN- β and pro-inflammatory cytokines transcription. Long-term IFN- β therapy decreases the recurrence rate of hepatocellular carcinoma after curative therapy.⁽²⁾ Interferons do not possess the ability to eliminate viruses or tumors; instead, they activate a cascade of signaling pathways to inhibit virus replication or tumor proliferation.⁽³⁾

Calcineurin is regulated by Ca^{2+} , which in turn dephosphorylates and induces the nuclear localization of cytoplasmic components of the nuclear factor of activated T cells transcription complexes.⁽⁴⁾ Subunit A of calcineurin is a catalytic subunit, whereas CNB is a regulatory subunit that regulates CN's activity by binding to the CNB binding helix.⁽⁵⁾ Subunit B of calcineurin is highly hydrophobic and conserved. CNB^{-/-} mice showed a higher risk for squamous cell carcinoma.⁽⁶⁾ The CNB potentiates the activation of procaspase-3 by accelerating its proteolytic maturation.⁽⁷⁾ In plants, CNB-like proteins have been associated with many fundamental processes, such as salt tolerance or K^+ uptake.^(8,9) These results reveal that CNB might possess some biological functions independent of CNA. To further test the function of CNB alone, CNB has been successfully characterized and purified in our laboratory.⁽¹⁰⁾ Suppression of certain types of carcinoma cells was observed by i.p. injection of CNB into mice pre-inoculated with carcinoma.⁽¹¹⁾ Moreover, CNB activates human PBMC-derived and mice marrow-derived DCs to secrete pro-inflammatory cytokines,⁽¹²⁾ but the underlying mechanisms have not been well described.

Here, for the first time, we report that CNB interacted with TLR4 ectodomain *in vitro*, which in turn led to phosphorylation of IRF3 and degradation of $\text{I}\kappa\text{B-}\alpha$. Secretion of IFN- β and pro-inflammatory cytokines were induced after CNB stimulation. This provides a molecular mechanism for CNB's immune booster and anticancer mechanism.

Materials and Methods

Mice and cell line. Male BALB/c mice, 5–6 weeks old, were purchased from Vital River Laboratory Animal Technology (Beijing, China) and kept under specific pathogen-free and humane conditions. All experiments involving the use of mice were carried out in accordance with protocols approved by the Animal Care and Use Committee of Beijing Normal University (Beijing, China). Raw264.7 cells were cultured in DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) FBS (Invitrogen, Carlsbad, CA, USA) in a humidified atmosphere containing 5% CO_2 at 37°C. U937 cells were cultured in 1640 (Gibco) supplemented with 10% FBS (Invitrogen) in a humidified atmosphere containing 5% CO_2 at 37°C. Sf9 insect cells were maintained in TNM-FH insect medium (Sigma, St. Louis, MO, USA) with 10% (v/v) FBS in a humidified atmosphere at 27°C.

Recombinant CNB and reagents. Construction of vectors, expression, and purification of CNB were accomplished in our previous work.⁽¹⁰⁾ Endotoxin contamination was <8 U/mg as determined by the Limulus amoebocyte lysate assay (Beijing Institute for Drug Control, Beijing, China). Lipopolysaccharide derived from *Escherichia coli* 0111:B4 was obtained from Sigma. Antibodies against TLR4, β -actin, TRAF3, IRF3, and CNB were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against p-IRF3 and $\text{I}\kappa\text{B-}\alpha$ were bought from Cell Signaling Technology (Beverly, MA, USA). Antibodies against HA and Myc tag were obtained from Genetex (Irvine, CA, USA). Horseradish peroxidase-coupled secondary antibodies were from Golden Bridge (Beijing, China). The ELISA kit for mouse IFN- β was from PBL Biomedical Laboratories (Piscataway, NJ, USA). FuGENE HD transfection reagent was purchased from Roche (Mannheim, Germany). The pGL3-Enhancer and pGL3-IFN β -reporter plasmids were gifts from Xuetao Cao (Second Military Medical University, Shanghai, China),⁽¹³⁾ the pGL6-6 \times kB plasmid was from Beyotime (Jiangsu, China), and the pRL-TK-Renilla-luciferase plasmid was a gift from Rui Huang (Beijing Normal University). The dual reporter assay kit was purchased from Promega (Madison, WI, USA). XFI-Myc-IRF3 and pcDNA3-HA-TRAF3 were generous gifts from Rik Derynck (University of Massachusetts, Worcester, MA, USA)⁽¹⁴⁾ and Micheal Karin (University of California San Diego, La Jolla, CA, USA),⁽¹⁾ respectively.

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BaculoGold Linearized virus genome DNA was purchased from BD Pharmingen (Franklin Lakes, NJ, USA). pAcGP67a-hTLR4-ectodomain (amino acids 27–631) plasmid was a kind gift from J.O. Lee (KAIST, Daejeon, Korea).⁽¹⁵⁾ The plasmid was modified by standard molecular methods to add 6× His tag at the C-terminus of hTLR4, and was named pAcGP67a-His-hTLR4-ectodomain.

Enzyme-linked immunosorbent assay. The ELISA for mouse IFN- β was carried out according to the manufacturer's instructions. Mice were i.p. injected with 20 mg/kg CNB or 0.5 mg/kg LPS (body weight) dissolved in PBS, or PBS alone (control group) every day for 1 week, and blood was collected from the cut tail on the day 7. Data were based on serum samples from the cut tail blood. Raw264.7 cells were seeded in 96-well plates at 1×10^5 cells per well. After culture for 24 h, cells were incubated with 1 μ M or 3 μ M TAK-242 (Invitrogen) for 4 h, subsequently stimulated with 8 μ M CNB for 12 h. The concentrations of IFN- β in culture medium were determined with mouse IFN- β ELISA kits.

GeneChip assay. The method for GeneChip assay has been reported previously.⁽¹⁶⁾ In brief, total RNA of U937 cells treated or untreated with 1 μ M CNB for 3 h was prepared by Trizol (Invitrogen, Gaithersburg, MD, USA) according to the recommended protocol. The NucleoSpin RNA clean-up kit (Macherey-Nagel, Düren, Germany) was used to further purify the total RNA. The quality of RNA was confirmed by use of a formaldehyde agarose gel and the concentration of RNA was determined by reading the absorbance at 260/280 nm. The procedure for labeling, hybridization, and washing was carried out as previously described.⁽¹⁶⁾ After drying, the slides were scanned for the fluorescence intensity of both the Cy5 and Cy3 Fluors using LuxScan 10KA confocal laser scanner and analyzed by LuxScan 3.0 (CapitalBio Company, Beijing, China). The biological interpretation of the gene clusters was further completed by KEGG pathways annotation.

Assay of luciferase reporter gene. For the IFN- β reporter assay, Raw264.7 cells were cotransfected with a mixture of 100 ng IFN- β luciferase reporter plasmid, 10 ng pRL-TK-Renilla-luciferase plasmid, and various amounts of IRF3 or TRAF3 construct. For the κ B reporter assay, Raw264.7 cells were cotransfected with a mixture of 100 ng κ B luciferase reporter plasmid and 10 ng pRL-TK-Renilla-luciferase plasmid. Either CNB or LPS (1 μ g/mL) was added to the medium 6 h post transfection. The luciferase activity in the cells was measured with a Dual-Luciferase Reporter Assay system according to the manufacturer's instructions (Promega) 24 h post transfection. Empty vector was added to each sample to obtain a final concentration of 0.02 μ g/ μ L. Data were normalized for transfection efficiency by division of firefly luciferase activities with that of Renilla luciferase.

Quantitative PCR and immunoblot analysis. Raw264.7 cells were stimulated with LPS (1 μ g/mL) or CNB. Total RNA was extracted with an RNA extraction kit according to the manufacturer's instructions (Biotek, Beijing, China). The quality of RNA was confirmed by use of a formaldehyde agarose gel and the concentration of RNA was determined by reading the absorbance at OD 260/280 nm. The 7500 Realtime PCR cycler (ABI, Foster City, CA, USA), RevertAid First Strand synthesis kit (Fermentas, Vilnius, Lithuania) and SYBR Green Mix (Toyobo, Osaka, Japan) were used for quantitative PCR analysis. Data were normalized to GAPDH expression in each sample. The primers used for IFN- β , RANTES, TAK-1, TBK-1, and GAPDH have been described.^(17–19)

His-tag pull-down assay and IRF3 dimerization assay. In brief, 2 μ g pAcGP67a-His-hTLR4-ectodomain (amino acids 27–631) was cotransfected with 0.5 μ g BD BaculoGold Linearized Baculovirus DNA into Sf9 cells using FuGENE HD transfection reagent (Roche). Cotransfection efficiencies were assayed by

end-point dilution assay according to the manufacturer's instructions. Virus titer higher than 2×10^8 was used for further protein production. Cells were pelleted by centrifugation. Supernatant (20 mL) was incubated with 40 μ L Ni Sepharose High Performance resin (GE Healthcare, San Diego, CA, USA) at 4°C overnight in a sterile tube. The resin was then centrifuged at 2000g and washed five times in ice-cold buffer A (20 mM Tris, 20 mM imidazole, 120 mM NaCl, and protease inhibitor cocktail [pH 7.4]). The resin was then boiled for 5 min for detection of hTLR4. The infected supernatant was incubated with CNB for 12 h in buffer A, then incubated with Ni resin for 2 h, and washed five times with buffer A. The resin was boiled for 5 min in 2× SDS loading buffer for SDS gel separation and blotting analysis. The IRF3 dimerization assay has been described previously.⁽²⁰⁾ In brief, 5×10^6 Raw264.7 cells were stimulated with 4 μ M CNB or 1 μ g/mL LPS for 2 h or 1 h, respectively. The cells were lysed in Western and IP Buffer (Beyotime), and the lysates were electrophoresed on 7% polyacrylamide native gels containing 1% deoxycholate. Both IRF3 monomer and dimer were detected by Western blotting assay.

Statistical analyses. Data are expressed as the means \pm SE of three independent experiments. Statistical significance was determined with the two-tailed Student's *t*-test, with a P-value of less than 0.05 considered statistically significant.

Results

Subunit B of calcineurin upregulates several TLRs and apoptosis-related genes. To address the possible pathways stimulated by CNB, we generated two RNA samples from U937 cells treated or untreated with CNB, and analyzed the data by gene chip (Fig. 1A,B). Among the 897 assayed genes, 30 genes were upregulated and two genes were downregulated. Analysis using the KEGG database showed that 43% of the upregulated genes were TLR pathway-related genes and 34% were apoptosis-related genes. Genes with a twofold change or higher are listed in Table 1. These results accorded with our previous findings that DCs stimulated with CNB enhanced the secretion of cytokines tumor necrosis factor- α , IL-12p70, IL-1 β , and IL-6 and chemokines IL-8, IP-10, MIP-1 α , and MIP-1 β by ELISA or qPCR.⁽¹²⁾

Activation of IRF3 by CNB stimulation in Raw264.7 and U937 cells. IRF3 is an essential transcription factor downstream of

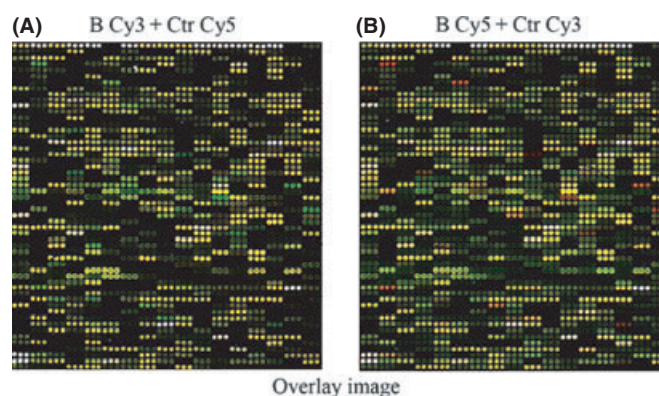


Fig. 1. Expression of various Toll-like receptor-related genes was upregulated by subunit B of calcineurin (CNB) stimulation in U937 cells. (A) Microarray-based expression profiling experiments using a two-color design: total RNA extracted from CNB-induced U937 cells labeled with Cy3 (green); and that of medium control with Cy5 (red). Green dot, gene is upregulated; red dot, gene is downregulated; yellow, gene is not sensitive to CNB stimulation. B, CNB-treated group; Ctr, control group. (B) Fluorophore exchange in microarray analysis. Green, control group treated with Cy3; red, CNB-treated group labeled with Cy5.

Table 1. Genes upregulated by *in vitro* treatment with recombinant calcineurin subunit B

Gene symbol	GenBank accession no.	Ratio	Signaling pathway in KEGG
<i>NF-κB</i>	NM_003998	3.500	hsa04010, hsa04210, hsa04620, hsa04660
<i>MIP-1α</i>	NM_002983	2.228	hsa04060, hsa04620
<i>MIP-1β</i>	NM_002984	2.769	hsa04060, hsa04620
<i>IκB-α</i>	NM_020529	3.200	hsa04210, hsa04620, hsa04660, hsa04662, hsa04920, hsa05120, hsa05215, hsa05220, hsa05225
<i>TNF-α</i>	NM_000594	4.108	hsa04010, hsa04060, hsa04210, hsa04350, hsa04620, hsa04640, hsa04650, hsa04660, hsa04664, hsa04920, hsa04930, hsa04940, hsa05010, hsa05060
<i>TRAF3</i>	AF110908	2.700	hsa04620, hsa05222
<i>IKK-ε</i>	NM_014112	2.200	hsa04620
<i>IL-8</i>	NM_000584	11.68	hsa04060, hsa04620, hsa05120, hsa05219
<i>NF-κB-2</i>	S76638	3.767	hsa04662, hsa04920, hsa05120, hsa05212, hsa05215, hsa05220, hsa05221, hsa05222
<i>TRAF1</i>	NM_005658	2.996	hsa05222
<i>ADRB2</i>	M15169	3.398	hsa04020, hsa04080
<i>GADD45</i>	L24498	2.199	hsa04010, hsa04110, hsa04115
<i>NFκBIE</i>	NM_004556	2.711	hsa04660, hsa04662, hsa04920
<i>Collagenase-1</i>	NM_002421	2.714	hsa03320, hsa05219
<i>Fas/Apo-1</i>	NM_000043	2.133	hsa04010, hsa04060, hsa04115, hsa04210, hsa04650, hsa04940
<i>ICAM1</i>	NM_000201	4.035	hsa04514, hsa04650, hsa04670
<i>IFNGR-2</i>	NM_005534	2.801	hsa04060, hsa04630, hsa04650
<i>MKP-3</i>	NM_001946	2.098	hsa04010
<i>IAP-1</i>	AF070674	2.058	hsa04210, hsa04510, hsa05222
<i>IRS2</i>	AF073310	2.155	hsa04910, hsa04920, hsa04930
<i>IRAK2</i>	NM_001570	2.315	hsa04210
<i>Mcp-1</i>	NM_002982	5.991	hsa04060

Ratio, describes fold change. hsa04620, Toll-like receptor-related signaling pathway in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

TLR. To test whether CNB promotes activation of IRF3, we added purified CNB to the medium of U937 or Raw264.7 cells. IRF3 phosphorylation was observed in both cell lines (Fig. 2A,B). The time to reach the peak for IRF3 phosphorylation differed between Raw264.7 cells (approximately 30 min) and U937 cells (maintained 1–2 h). We detected the constitutive formation of the IRF3 dimer and its further augmentation by CNB stimulation in Raw264.7 cells. Consistent with IRF3 phosphorylation blotting, the IRF3 dimer was detected 1 h post CNB stimulation (LPS was used as a positive control)⁽²¹⁾ (Fig. 2C). The protein level of TRAF3 was detected by blotting. It was previously shown that CNB cannot promote TRAF3 degradation, which is different from LPS (Fig. 2D).⁽¹⁾ These data indicated that CNB activated a signaling pathway leading to activation of IRF3.

Degradation of IκB-α by CNB stimulation. Our previous research also implied that CNB undertook the NF-κB pathway.⁽¹²⁾ Therefore, we examined IκB-α expression levels after CNB stimulation. The degradation of IκB-α was observed in a time-dependent manner (Fig. 3A). To confirm this result, we transfected κB reporter plasmid into Raw264.7 cells. The CNB enhanced expression of the κB reporter gene in a dose-dependent and time-dependent manner in Raw264.7 cells (Fig. 3B,C).

Effects of CNB on IFN-β transcription. Given the results that CNB promoted both phosphorylation of IRF3 and degradation of IκB-α, we tested whether CNB enhanced IFN-β transcription. The indicated amounts of recombinant CNB were added to the medium of Raw264.7 cells, and the mRNA level for IFN-β was detected using qPCR. The IFN-β mRNA level was upregulated by CNB stimulation in a time-dependent way (Fig. 4A). The time point to reach the peak was approximately 1 h post CNB stimulation, which was consistent with previous result (30 min to reach the peak for p-IRF3) (Fig. 2B). As expected, the mRNA level of another IRF3-related gene, *RANTES*, was also upregulated by CNB stimulation (Fig. 4B). The IFN-αn mRNA (IFN-αn was designed to detect the mRNAs for IFN-α1, α2, α7, α11,

and α12 subtypes) was not detected as stimulated with either LPS or CNB (data not shown) by qPCR, consistent with a previous report.⁽²²⁾

Expression of the IFNβ reporter gene was enhanced by CNB in a dose-dependent way in Raw264.7 cells (Fig. 4C). To further test the function of IRF3 and TRAF3 in CNB stimulated pathways, we transfected Raw264.7 cells with different amounts of IRF3 or TRAF3 constructs. Cells were stimulated with indicated amounts of CNB 6 h post transfection. Activation of the IFNβ reporter gene was also further upregulated by IRF3 or TRAF3 (Fig. 4D,E). Protein expression of IRF3 (lower) or TRAF3 (upper) was detected in whole cell lysates by Western blotting (Fig. 4F). These results indicated that CNB increased IRF3/TRAF3-mediated transcription of genes encoding IFN-β.

Involvement of TAK-1 and TBK-1 in CNB signal pathway. Nuclear translocation of IRF3 and degradation of IκB-α both require signal-dependent phosphorylation. Previous reports indicated that TBK-1 and TAK-1 were essential for IRF3 phosphorylation and NF-κB activation, respectively.^(23,24) To seek kinases involved in the CNB signal pathway, TAK-1 and TBK-1 mRNA were analyzed after CNB stimulation. After CNB stimulation, TAK-1 and TBK-1 were upregulated 1.7-fold and 2.2-fold, respectively (Fig. 5A,B). Thus, TBK-1 and TAK-1 might be responsible for IRF3 phosphorylation and NF-κB activation in the CNB stimulated pathway.

Production of IFN-β upregulated by CNB. To further investigate the function of CNB *in vivo*, we i.p. injected mice with CNB, LPS, or PBS for a period of 7 days. The level of IFN-β in serum was measured by ELISA (Fig. 6A). Subunit B of calcineurin showed a mild degree of IFN-β induction (35.75 ± 6.78 pg/mL) compared to LPS treatment (215.37 ± 20.76 pg/mL). A TLR4-specific inhibitor, TAK-242, was used to test whether CNB activates IFN-β expression through TLR4.⁽²⁵⁾ As expected, inhibition of TLR4 by TAK-242 attenuated the production of IFN-β *in vitro* (Fig. 6B). This result indicated that CNB probably undertook the TLR4 signal pathway.

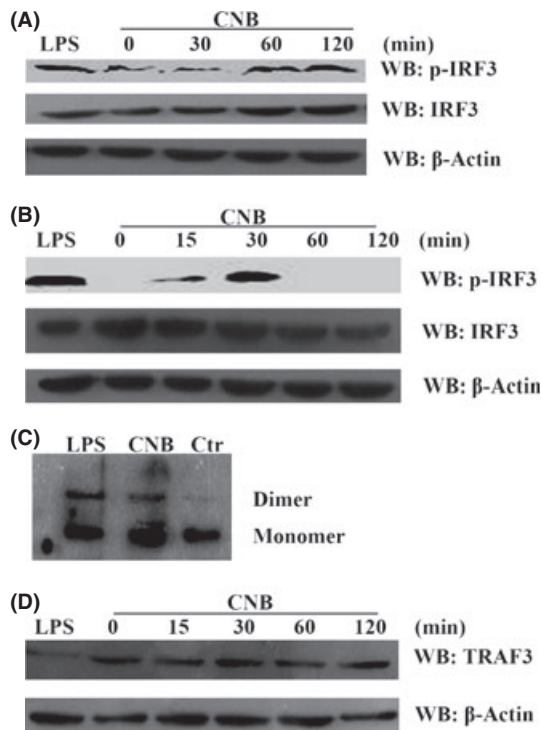


Fig. 2. Phosphorylation and dimerization of interferon regulatory factor 3 (IRF3) were induced by subunit B of calcineurin (CNB) stimulation. (A) Detection of p-IRF3 in U937 cells. Cells were treated with either 1 $\mu\text{g}/\text{mL}$ lipopolysaccharide (LPS) for 30 min as a positive control or 1 μM CNB for 0 min, 30 min, 1 h, or 2 h. WB, Western blotting. (B) Detection of p-IRF3 in Raw264.7 cells. Cells were treated with either 1 $\mu\text{g}/\text{mL}$ LPS for 30 min as a positive control or 2 μM CNB for 0 min, 15 min, 30 min, 1 h, or 2 h. IRF3 and β -actin in the whole cell lysates were used as endogenous controls. (C) Dimerization of IRF3 in Raw264.7 cells. Cells were treated with 1 $\mu\text{g}/\text{mL}$ LPS for 1 h, 2 μM CNB for 2 h, or left untreated (Ctrl, control). Cell lysates were separated by native PAGE and detected by WB. (D) Detection of tumor necrosis factor receptor-associated factor 3 (TRAF3) degradation. The samples were the same as in (B).

Interaction between CNB with TLR4 *in vitro*. Activation of IRF3 is found to confer a TLR3/TLR4 specificity, which mediates a specific gene program for innate immunity response.⁽²⁶⁾ Moreover, from previous results (Fig. 6B), we speculated that CNB might interact with TLR4. To further address this hypothesis, we expressed the His-tagged ectodomain of TLR4 (amino acids 27–631) in Sf9 insect cells. The TLR4 ectodomain expression was monitored by SDS-PAGE (Fig. 7A) or Western blotting using anti-TLR4 or anti-His antibody (Fig. 7B). As expected, CNB interacted with the TLR4 ectodomain, as shown by the His-tag pull-down assay *in vitro* (Fig. 7C).

Discussion

Our previous results have shown that the phagocytic activities of peritoneal macrophages and the natural killer activities of murine spleen lymphocytes increased after CNB injection.⁽²⁷⁾ Subunit B of calcineurin also inhibits the growth of H22 liver ascites cancer cells and S180 solid cancer cells *in vivo*.⁽¹¹⁾ Moreover, CNB activates human PBMC-derived and mice marrow-derived DCs to secrete pro-inflammatory cytokines.⁽¹²⁾ To elucidate the molecular mechanism, we designed a gene chip assay. From the assay, we found that genes for IKK ϵ , TRAF3, and NF- κ B were upregulated by CNB stimulation in U937 cells (Fig. 1). The TRAF3–TBK1–IKK ϵ –IRF3 complex is of great importance to both TLR-dependent and TLR-independent production of type I IFN production.^(28,29) IKK ϵ can form a heterodimer with TBK1, and may act as a kinase to phosphorylate IRF3.^(23,28) Both TRAF3 and IRF3 are uniquely required for the TRIF-dependent interferon response.⁽²⁹⁾ The regulatory elements for IFN- β included κ B sites, IRES sites, and AP-1 sites, which bind NF- κ B, IRFs, or c-Jun/ATF2 heterodimer, respectively.⁽³⁰⁾ Previous reports have also implied that CNB undertakes NF- κ B signal pathways to activate DCs to produce several pro-inflammatory cytokines.⁽¹²⁾ To further investigate the immune booster mechanism, we carried out experiments to detect NF- κ B and IRF3 activation. As expected, CNB promoted degradation of I κ B- α in both Raw264.7 cells (Fig. 3) and U937 cells (Wu Wu, Beijing Normal University, Beijing, China,

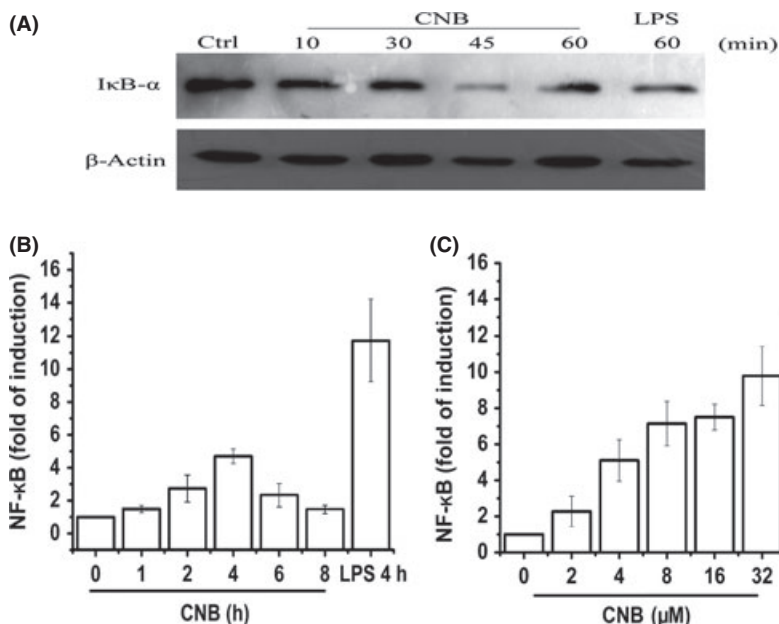


Fig. 3. Nuclear factor- κ B (NF- κ B) activated by subunit B of calcineurin (CNB) stimulation. (A) Detection of I κ B- α degradation. RAW264.7 cells were treated with 1 $\mu\text{g}/\text{mL}$ lipopolysaccharide (LPS) for 60 min as a positive control (Ctrl) or 4 μM CNB for 0 min, 10 min, 30 min, 45 min, or 1 h. (B) Luciferase assay of the induction of NF- κ B-related genes in lysates of Raw264.7 cells transfected with 100 ng pGL6-6 \times κ B reporter plasmid and 10 ng pTK-Renilla-luciferase. Six hours post transfection, 4 μM CNB was added to the medium. Activity of κ B was detected at different time points. Empty control vector was added to each sample to ensure transfection of the same amount of DNA in each. Luciferase activity was normalized to Renilla luciferase activity. (C) Transfection procedure was the same as (B). Different amounts of CNB (0, 2, 4, 8, 16, or 32 μM) were added after transfection. Activity of κ B was detected at 4 h post stimulation. LPS was used as a positive control.

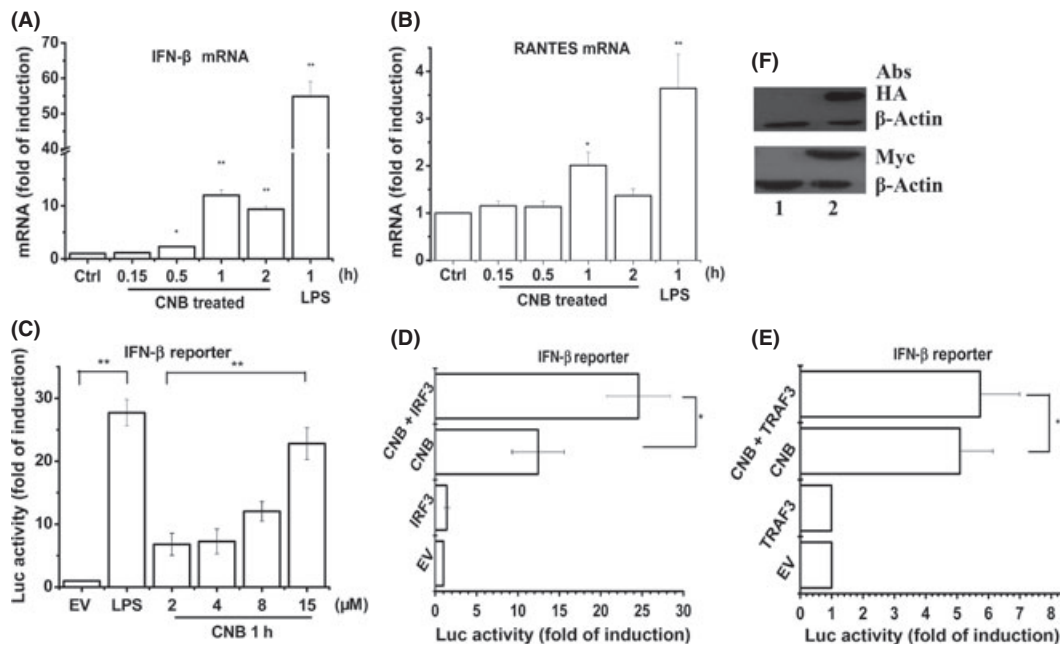


Fig. 4. Subunit B of calcineurin (CNB) enhanced transcription of genes encoding β -interferon (IFN- β) in Raw264.7 cells in a tumor necrosis factor receptor-associated factor 3/interferon regulatory factor 3 (TRAF3/IRF3)-dependent and dose-dependent way. (A,B) Real-time PCR of IFN- β and RANTES mRNA in RAW264.7 cells stimulated with CNB or lipopolysaccharide (LPS; 1 μ g/mL) for the indicated time (horizontal axis), presented as "fold of induction" relative to GAPDH. (C,D,E) Luciferase assay of the induction of genes encoding IFN- β in lysates of Raw264.7 cells transfected with 100 ng pGL3-IFN β reporter plasmid and 10 ng pTK-Renilla-luciferase, and 100 ng IRF3 expression plasmid (D), or 100 ng TRAF3 expression plasmid (E). Empty control vector (EV) was added to each sample to ensure transfection of the same amount of DNA in each. Luciferase activity was normalized to Renilla luciferase activity. (F) Detection of IRF3 (lower, lane 2) or TRAF3 (upper, lane 2) expression in the lysates of (D) or (E). Lane 1, EV control. * $P < 0.05$; ** $P < 0.01$.

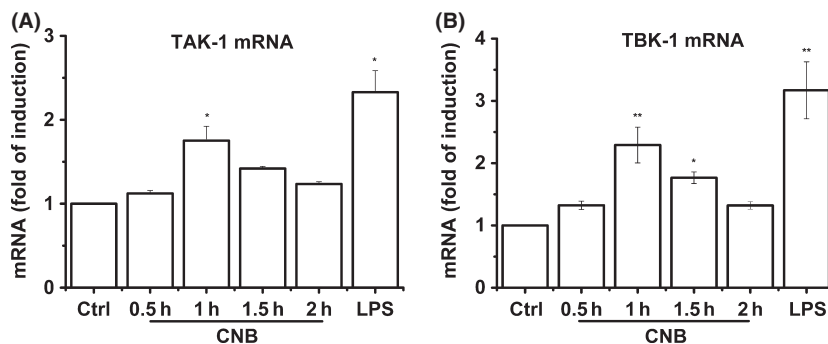


Fig. 5. Analysis of transforming growth factor β -activated kinase 1 (TAK-1) and Tank binding kinase 1 (TBK-1) mRNA level by subunit B of calcineurin (CNB) stimulation. (A,B) Real-time PCR of TAK-1 and TBK-1 mRNA in RAW264.7 cells stimulated with CNB or lipopolysaccharide (LPS; 1 μ g/mL) for the indicated time (horizontal axis), presented as "fold of induction" relative to GAPDH.

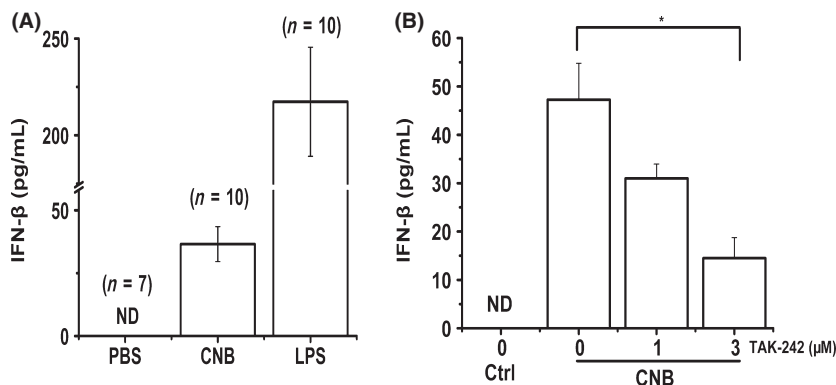


Fig. 6. Enzyme-linked immunosorbent assay of β -interferon (IFN- β). (A) Effect of subunit B of calcineurin (CNB) *in vivo*. Mice were i.p. injected with PBS (control), lipopolysaccharide (LPS), or CNB. β -interferon in serum samples from cut tail blood was measured by ELISA. (B) Effect of transforming growth factor β -activated kinase 242 (TAK-242) on CNB-induced IFN- β production. RAW264.7 cells were incubated with 1 or 3 μ M TAK-242 for 4 h then stimulated with CNB for 12 h. The concentrations of IFN- β in the culture medium were determined by ELISA. Ctrl, control group; ND, not detected. * $P < 0.05$.

unpublished data). Phosphorylation and dimerization of IRF3 was also observed after CNB stimulation (Fig. 2). Transcription of IFN- β in Raw264.7 cells was enhanced in a TRAF3- and

IRF3-dependent way by CNB stimulation (Fig. 4). All of these results indicate that CNB stimulation may lead to IFN- β production as well as pro-inflammatory cytokines.

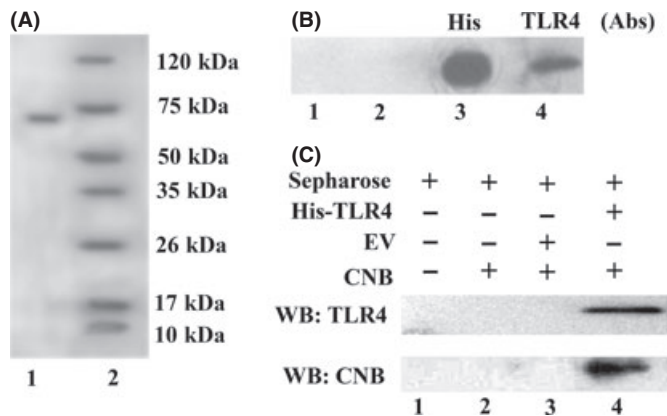


Fig. 7. Subunit B of calcineurin (CNB) interacts with Toll-like receptor 4 (TLR4) *in vitro*. (A) SDS-PAGE detection of TLR4 ectodomain. Lane 1, 20 mL supernatant of hTLR4-infected Sf9 cells was incubated with 40 μ L Ni Sepharose and washed five times with buffer (20 mM Tris, 20 mM imidazole, 120 mM NaCl [pH 7.4]); resin was boiled with 2 \times SDS loading buffer for TLR4 ectodomain detection. Lane 2, protein marker. (B) Western blotting detection of TLR4 ectodomain. Lane 1, protein marker; lane 2, supernatant of Sf9 without infection; lanes 3,4, supernatant of Sf9 infected with His-tag hTLR4 ectodomain baculo-virus expression vectors (BEVs), detection of His-tag hTLR4 ectodomain with either anti-His mAb (lane 3) or TLR4 (H180) pAbs (lane 4). (C) His-tag pull-down assay. Lane 1, mock, resin only. Ten micrograms of CNB was incubated with resin only (lane 2), uninfected Sf9 supernatant (lane 3), or pAcGP67a-hTLR4/BEVs-infected Sf9 supernatant (lane 4) overnight at 4°C. Then Ni resin was added to each sample, incubated for 2 h at 4°C, then washed five times. Resin was boiled for 5 min with 2 \times SDS loading buffer. The interaction was detected by Western blotting. EV, empty control vector.

Stimulation of LPS caused degradation of TRAF3 in Raw264.7 cells and upregulation of RANTES in A549 cells, which has been reported previously.^(1,31) Subunit B of calcineurin does not promote degradation of TRAF3, which is different from LPS (Fig. 2). TRAF3 is a key regulator for balancing type I interferon and pro-inflammatory cytokines production. K48 ubiquitination of TRAF3 induced by LPS is designed for proteasomal degradation, whereas K63 ubiquitination of TRAF3 is essential for IFN production.⁽¹⁾ TRAF3^{-/-} cells are defective in type I IFN responses activated by several different TLRs.⁽²⁸⁾ TRAF3 associates with the TLR adaptors TRIF and IRAK1, as well as downstream IRF3/7 kinases TBK1 and IKK ϵ , suggesting that TRAF3 serves as a critical link between TLR adaptors and downstream regulatory kinases important for IRF activation. We speculated that cells receiving CNB stimulation might “preferentially” promote K63 ubiquitination of TRAF3 for type I IFN production, which needs further research.

The MKK4/7-JNK or TBK-1/IKK ϵ complex functions as an upstream kinase for IRF3.^(23,32) TAK1 forms a complex with TAB 1, TAB 2, and TAB 3. The activated TAK1 complex phosphorylates I κ B kinases, which activates NF- κ B.⁽³³⁾ Thus, we detected TAK-1 and TBK-1 mRNA levels after CNB stimulation by qPCR (Fig. 5). The mRNA levels for TAK-1 and TBK-1 were both upregulated after addition of CNB. These results implied that the kinases for IRF3 and I κ B- α phosphorylation in our CNB stimulation pathways were TBK-1 and TAK-1, respectively.

β -interferon itself does not kill the tumor or virus. Instead, it initiates a cascade of signal pathways to inhibit virus replication or tumor cell proliferation.⁽³⁾ β -interferon eliminates activated DCs by caspase-3/11 activation, which plays an essential role in

re-establishing homeostasis.⁽³⁴⁾ Antitumor effects mediated by IFN- β have also been reported.^(34–36) β -interferon KO mice showed a higher risk for sustained inflammation, cytokine production, and tissue damage with consequent chronic neurological deficits.⁽³⁷⁾ Injection of CNB upregulated IFN- β production to a mild degree compared to LPS-stimulated mice (Fig. 6). This result explained the efficient antitumor and immune booster effects of CNB.^(11,12)

Activation of IRF3 confers a TLR3/4, rather than TLR2/9, specificity.⁽²⁶⁾ Moreover, from our gene chip assay results, approximately half of the genes changed by twofold or more were TLR-related genes (Fig. 1). To seek the membrane receptor for CNB, we focused mainly on TLRs. An atypical member of the leucine-rich repeats family, TLR4 is composed of N-terminal, central, and C-terminal domains. The primary function of these motifs provides a versatile structural framework for the formation of protein–protein interactions. The leucine-rich repeat domain of TLR4 interacts with a large range of hydrophobic ligands such as HSP70/60, MD-2, fusion protein, envelope protein, and flavolipin.^(38,39) The structural basic of TLR4 also provides a possibility for our highly hydrophobic CNB to bind. We validated our speculation by pull-down assay (Fig. 7). Moreover, CNB binds to endogenous TLR4 by IP experiment in U937 cell lines and TAK-242 (TLR4-specific inhibitor) pre-incubation attenuates CNB stimulated signals (Wu Wu, Beijing Normal University, Beijing, China, unpublished data; Fig. 6B). Indeed, CNB interacted with TLR4 and activated a signal cascade.

In conclusion, CNB upregulates IFN- β and several pro-inflammatory cytokines by activation of the IRF3 and NF- κ B signal pathways through TLR4 signal pathways. Our findings imply that CNB could be useful for enhancing immune response and preventing cancer and virus infections.

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Disclosure Statement

The authors have no conflict of interest.

Abbreviations

CNA	subunit A of calcineurin
CNB	subunit B of calcineurin
DC	dendritic cell
IFN- β	β -interferon
IL	interleukin
IP	immunoprecipitation
IRF3	interferon regulatory factor 3
KEGG	Kyoto Encyclopedia of Genes and Genomes
LPS	lipopolysaccharide
MIP	macrophage inflammatory protein
NF- κ B	nuclear factor κ B
qPCR	quantitative PCR
TAB	TAK1-binding protein
TAK1	transforming growth factor β -activated kinase 1
TBK1	Tank-binding kinase 1
TLR	Toll-like receptor
TRAF3	tumor necrosis factor receptor-associated factor 3
TRIF	TIR-domain-containing adapter-inducing interferon- β

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