

• BASIC RESEARCH •

# Inhibition of PMA-induced endothelial cell activation and adhesion by over-expression of domain negative I $\kappa$ B $\alpha$ protein

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## Abstract

**AIM:** NF- $\kappa$ B, regulate the expression of cytokine-inducible genes involving immune and inflammatory responses, will be potential therapy approach for allograft from rejection. In this study, we use pCMV-I $\kappa$ B $\alpha$ M vector to inhibit NF- $\kappa$ B activation and investigate the effect of pCMV-I $\kappa$ B $\alpha$ M in inhibition of T cells adhesion to endothelial cells.

**METHODS:** The NF- $\kappa$ B activity was detected with pNF- $\kappa$ B reporter gene and electrophoretic mobility shift assay. Expression of cell surface molecules was detected by RT-PCR and flow cytometer. The cell-cell adhesion assay was performed to determine the effect of pCMV-I $\kappa$ B $\alpha$ M in inhibition of T cells adhesion to endothelial cells.

**RESULTS:** We could find that NF- $\kappa$ B activity is inhibited by over-expression of non-degraded I $\kappa$ B $\alpha$  protein. Expression of adhesion molecules like ICAM-1, VCAM-1, and P-selectin as well as cell-cell adhesion were inhibited significantly by transfection of the pCMV-I $\kappa$ B $\alpha$ M vector.

**CONCLUSION:** Our results indicate that the pCMV-I $\kappa$ B $\alpha$ M, which inhibit the activity of NF- $\kappa$ B through over-expression of non-degraded I $\kappa$ B $\alpha$  protein, can be used for gene therapy in diseases involving NF- $\kappa$ B activation abnormally like organ transplantation via inhibiting cell adhesion.

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**Key words:** Cytokine-inducible genes; Endothelial cells

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## INTRODUCTION

NF- $\kappa$ B, a DNA binding protein complex, is usually present in the cytosol as an inactive complex. I $\kappa$ B $\alpha$ , an associated protein, renders this complex inactive by shielding the nuclear localization signal (NLS). Upon I $\kappa$ B phosphorylation and its subsequent degradation, the NF- $\kappa$ B subunit P65 will translocate to the nucleus, where it binds to specific DNA sequences in the promoter region of several cytokine-inducible genes and up-regulates their transcription<sup>[1,2]</sup>. Extensive studies have showed that inhibition of NF- $\kappa$ B will be potential therapy approach for allograft from rejection<sup>[3-5]</sup>. The potent immunosuppressive agents FK506 and cyclosporin A (CsA) are reported to switch off gene transcription by inhibiting a key signaling phosphatase, calcinurin, which is involved in the activation of NF- $\kappa$ B<sup>[6,7]</sup>. Glucocorticoid, another major immunosuppressive agent, is also believed to work partly via inhibition of NF- $\kappa$ B activation<sup>[8]</sup>. The detail mechanism about inhibition of NF- $\kappa$ B protecting the graft is not clear till now.

Previous studies have shown that typical procedure of graft rejection is included in three steps: adhesion of alloantigen-activated leukocytes to the vascular endothelial cells, infiltration of alloantigen-activated leukocytes into the graft, activated leukocytes express immune factors which mediate graft tissue destruction<sup>[9]</sup>. The endothelial cell, as the first barrier faced the alloantigen, is thought to be the most important procedure during allograft rejection<sup>[10-13]</sup>. We suppose that NF- $\kappa$ B can be an important factor, which participate in the above three steps especially in the adhesion of leukocytes to the vascular endothelial cells.

In the present study, we focus on the transcriptional factor NF- $\kappa$ B in endothelial cells activation and adhesion to T cells. We use the vector encoding domain negative protein mutated at ser-32 and ser-36 I $\kappa$ B $\alpha$  to inhibit activation of NF- $\kappa$ B.

## MATERIALS AND METHODS

### Materials

ECV304, an endothelial cell line, established from the vein of normal human umbilical cord, were obtained from Cell Bank of Chinese Academy of Science. Human Jurkat cell line was obtained from ATCC (American Type Cell Culture, USA) and cultured for adhesion assay. The following

materials were used in this study: pCMV-I $\kappa$ B $\alpha$ , pCMV-I $\kappa$ B $\alpha$ M, pNF- $\kappa$ B Luc reporter vectors were obtained from Clontech (BD Biosciences, NJ, USA). Luciferase assay system, Gel shift assay system (Promega, WI, USA); MuLV reverse transcriptase, TRIzol, RPMI-1640 medium (Gibco, CA, USA); Geneticin (G418), FuGENE 6 transfection reagent, poly (dI-dC), Protease inhibitors cocktail (Roche, Monheim, Germany); Protein assay kit (Bio-Rad, CA, USA). Phorbol myristate acetate (PMA) and all other reagents were purchased from Sigma (Sigma Chemical, USA).

### Cell culture and transfection for ECV

All of the cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin. Cells were maintained in a standard culture incubator with humidified air containing 50 mL/L CO<sub>2</sub> at 37 °C.

We performed transfection according to the instruction manual of FuGENE 6 Transfection Reagent with modifications. Briefly, we seeded  $1 \times 10^5$  cells in 1 mL RPMI 1640 containing 10% FBS medium in 24-well plate and incubated overnight until the ECV cells were 60% confluent. As for stable transfection, added 100  $\mu$ L serum-free medium (SFM) containing 3  $\mu$ L FuGENE 6 and 1  $\mu$ g pCMV-I $\kappa$ B $\alpha$  or pCMV-I $\kappa$ B $\alpha$ M. The plate was incubated for 24 h for expression of reporter gene. Then we added 600 ng/mL G418 in every well and incubated for 7 d. After 7 d incubation, the concentration of G418 was decreased to 300 ng/mL and incubated for more than 3 wk. The over-expression of I $\kappa$ B $\alpha$  protein cells was selected using Western blotting. The cells stably transfected with pCMV-I $\kappa$ B $\alpha$  and pCMV-I $\kappa$ B $\alpha$ M were referenced to ECVWT and ECVMT, separately.

As for transient transfection, 100  $\mu$ L SFM containing 3  $\mu$ L FuGENE 6 and 1  $\mu$ g pNF- $\kappa$ B Luc was added and cells were incubated for 24 h. After treated with PMA for 12 h, the cells were lysed and luciferase activity was measured using luciferase assay system.

### Nuclear protein extraction and electrophoretic mobility shift assay

Nuclear extracts were prepared as previously described<sup>[14]</sup>. Cytoplasmic buffer contained 10 mmol/L HEPES (pH 7.9), 0.1 mmol/L EDTA, 2 mmol/L MgCl<sub>2</sub>, 10 mmol/L KCl, 0.2% NP-40 and protease inhibitors cocktail solution was prepared to the cell pellet and incubated in ice for 10 min before centrifugation at 13 000 *g* for 1 min. The pelleted nuclei were resuspended in nuclear buffer contained 20 mmol/L HEPES pH 7.9, 1.5 mmol/L MgCl<sub>2</sub>, 0.2 mmol/L EDTA, 150 mmol/L NaCl and protease inhibitors. The resuspended nuclei were incubated for 30 min on ice with vortexing interruptedly and centrifuged for 20 min at 13 000 *g*.

The concentrations of samples were measured using protein assay kit with BSA protein standard solution as reference. Samples were stored at -70 °C until use.

The consensus oligonucleotide of NF- $\kappa$ B was obtained commercially from Santa Cruz and the sequence was shown as the following: NF- $\kappa$ B (5'-AGT TGA GGG GAC TTT CCC AGG C-3'). EMSA were performed according to Holmes's protocol<sup>[15]</sup>. Briefly, NF- $\kappa$ B consensus oligonucleotide was radiolabeled with [ $\gamma$ -<sup>32</sup>P]ATP in the presence of T4

polynucleotide kinase. Five micrograms nuclear extract was incubated with labeled probe in 1  $\mu$ g of poly(dI-dC) for 30 min. Complexes were separated on a 5% polyacrylamide gel in 0.5 $\times$ TBE and dried using dryer (Bio-Rad, USA) and autoradiographed at -80 °C.

### Reverse transcription polymerase chain reaction

To study cytokine gene expression patterns, we used reverse transcription-polymerase chain reaction (RT-PCR), as previously described. Total RNA was extracted from ECV cells using TRIzol reagent according to the manufacturer's recommendations. For cDNA synthesis, 4  $\mu$ g total RNA was reverse transcribed with MuLV reverse transcriptase. Primer sequences and reaction conditions: Sequences of the primers used for RT-PCR analysis are described in Table 1. Amplifications were performed under the following conditions: 95 °C for 2 min, 94 °C for 45 s, 56 °C for 45 s, 72 °C for 45 s, totally 32 cycles. The final extension step was performed by one cycle at 72 °C for 10 min. Twenty-five microliters of reaction system was used including: 2  $\mu$ L cDNA template, 1  $\mu$ L sense primer, 1  $\mu$ L anti-sense primer, 2  $\mu$ L 25 mmol/L MgCl<sub>2</sub>, 1  $\mu$ L dNTP and 1.5 u Taq DNA polymerase. Reaction products were run by electrophoresis on a 1.5% agarose gel for 30-40 min at 100 V in 0.5 $\times$ TBE buffer, and visualized with ethidium bromide staining under UV light. Relative expression level of ICAM-1 and VCAM-1 were defined as optical density ratio (Target gene/GAPDH) analyzed by Kodak digital science scanning system.

**Table 1** Sequences of primers for amplified cDNA of the ICAM-1, VCAM-1, and GAPDH

Genes	Primers	Sequences	Amplifiers
ICAM-1	Sense	5'-CAGTGACCATCTACAGCTTCCGG-3'	555 bp
	Anti-sense	5'-GCTGCTACCACAGTGATGATGACAA-3'	
VCAM-1	Sense	5'-ACCCTCCCAAGGCACACAG-3'	533 bp
	Anti-sense	5'-GTAAGTCTATCTCCAGCCTGTC-3'	
GAPDH	Sense	5'-ATGGCACCGTCAAGGCTGAG-3'	225 bp
	Anti-sense	5'-GCAGTGATGGCATGGACTGT-3'	

### Analysis of the expression of cell adhesion molecules using flow cytometer

ECVMT and ECVWT cells were treated with PMA (50 nmol/L) for 12 h. Then cells were harvested and washed thrice using cold PBS. Then the cells were incubated with FITC-labeled CD54(ICAM-1), FITC-labeled CD62 (VCAM-1) or PE-labeled CD106(P-selectin) antibodies. The fluorescence densities were determined using flow cytometer (Coulter, USA) and the experiment was repeated thrice.

### Adhesion of Jurkat cells to ECV304

We used Jurkat cells for lymphocyte-endothelial cell adhesion assay. The adhesion procedure was performed according to Roy<sup>[16]</sup>. Briefly, monolayers of cells were seeded at a density of  $10^4$  cells/well in 96-well plate (Becton Dickinson, NJ, USA). After 24 h of seeding, the cells were treated with PMA (50 nmol/L) for 12 h. Before cell-cell adhesion assay, the ECV monolayers were washed thrice

with PBS. Jurkat T-cells ( $2 \times 10^5$  cells/well) were co-cultured with ECV monolayer for 2 h in a culture incubator. After the co-culture period, the non-adherent Jurkat T-cells were removed by washing each well four times with PBS carefully. Jurkat cell adhesion was determined by visual counting under a phase-contrast microscope. Both Jurkat cells and ECV were counted in five fields of each well at  $100\times$  magnification by two individuals, and the average values were taken. The adhesion rate is expressed as the number of attached Jurkat cells per 100 ECV cells.

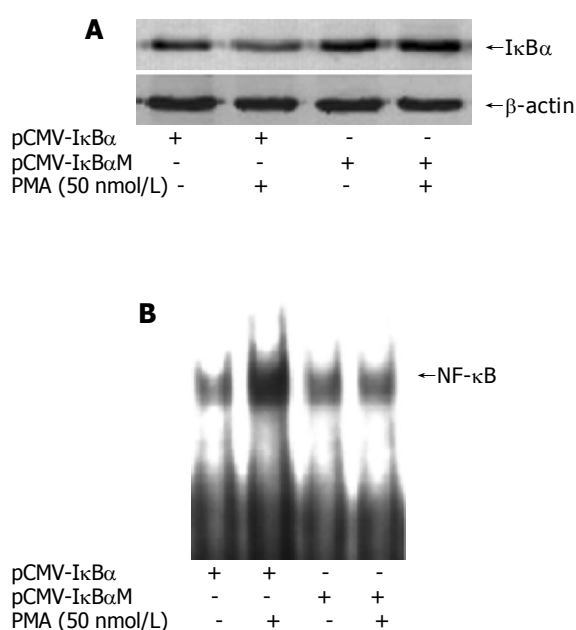
### Statistical analyses

Results are presented as mean  $\pm$  SD of at least three separate experiments. Differences between means of groups were determined by Student's *t*-test. The level of significance was set at  $P < 0.05$  or  $P < 0.01$ .

## RESULTS

### Over-expression of domain negative I $\kappa$ B $\alpha$ inhibit NF- $\kappa$ B activation

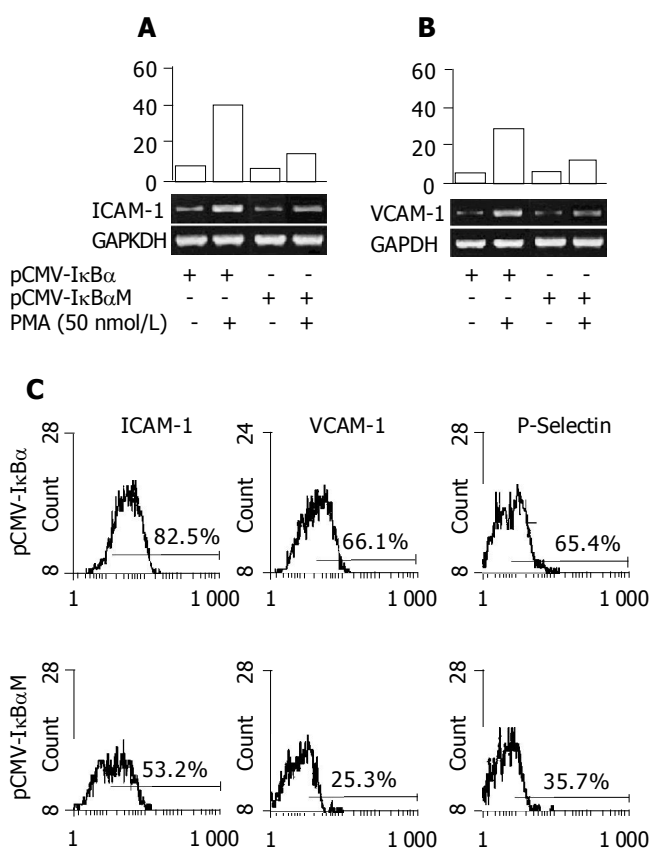
We stably transfected the ECV304 cells with the pCMV-I $\kappa$ B $\alpha$ M vector, which encoded domain negative I $\kappa$ B $\alpha$  protein mutated at ser-32 and ser-36 and also with the pCMV-I $\kappa$ B $\alpha$  as control. Firstly, we extracted the total protein after being treated with PMA (50 nmol/L) for 45 min. The expression level of I $\kappa$ B $\alpha$  was determined with specific antibody. As shown in Figure 1A, I $\kappa$ B $\alpha$  level was higher in ECVMT cells after treated with PMA compared with ECVWT cells. To determine the NF- $\kappa$ B activity further, the nuclear proteins were extracted after treated with PMA for 45 min. These proteins were applied to EMSA. Figure 1B showed us that pCMV-I $\kappa$ B $\alpha$ M could inhibit the activity of NF- $\kappa$ B significantly.



**Figure 1** Determining NF- $\kappa$ B activity in ECV304WT and ECV304MT cells. **A**: I $\kappa$ B $\alpha$  level were detected after treated with PMA (50 nmol/L) for 12 h; **B**: EMSA was performed to determine the activity of NF- $\kappa$ B after treated with PMA (50 nmol/L) for 12 h. Band of NF- $\kappa$ B was marked.

### Down-regulation adhesion molecules expression under inhibition of NF- $\kappa$ B

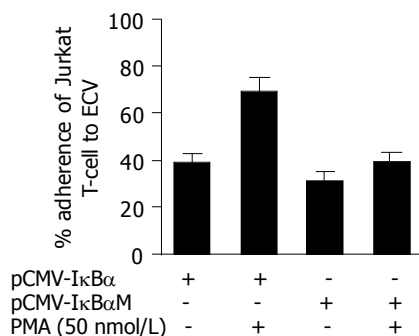
Adhesion molecules expression in endothelial cells is dependent, at least in part, on the activation of NF- $\kappa$ B<sup>[1]</sup>. To determine whether the pCMV-I $\kappa$ B $\alpha$ M can inhibit the expression of adhesion molecules, we performed RT-PCR to determine the mRNA level of adhesion molecules in the total RNA extracted from the cells treated with PMA (50 nmol/L) for 12 h. The results were shown as ratio compared to housekeeping gene GAPDH expression. As shown in Figures 2A and B, we can see that the ICAM-1 and VCAM-1 mRNA levels are inhibited significantly in ECVMT cells compared to ECVWT cells (40.3% *vs* 18.7% for ICAM-1 and 27.3% *vs* 16.7% for VCAM-1). We also compare the protein levels of various adhesion molecules by flow cytometer. The cells were harvested after stimulation with PMA (50 nmol/L) for 12 h. As shown in Figure 2C, the pCMV-I $\kappa$ B $\alpha$ M can inhibit the expression of adhesion molecules including ICAM-1, VCAM-1 and P-selectin. As for ICAM-1, 82.5% cells expressed ICAM-1 in ECVWT cells after being treated with PMA (50 nmol/L) compared 53.2% positive cells in ECVMT cells. Almost the same results were found in VCAM-1 and P-selectin expression between ECVMT cells and ECVWT cells (25.3% *vs* 66.1% for VCAM-1; 65.4% *vs* 35.7% for P-selectin).



**Figure 2** Down-regulation of PMA-induced adhesion molecules expression in ECV cells transfected with pCMV-I $\kappa$ B $\alpha$ M compared with pCMV-I $\kappa$ B $\alpha$ . RT-PCR analysis was performed with primers specific for ICAM-1 (**A**) and VCAM-1 (**B**). **C**: The expression of ICAM-1, VCAM-1 and P-selectin were detected using specific antibodies by flow cytometry. Results are representative of at least three independent experiments.

### Inhibit the adhesion of T cells to ECV cells

We then evaluated the effect of pCMV-I $\kappa$ B $\alpha$ M on the adhesion of human T lymphocyte to ECV304 cells as mentioned in Methods section. After the ECV cells were treated with PMA (50 nmol/L) for 24 h, the Jurkat T cells were added and co-incubated for further 2 h. Then we calculated the adherent cells using phase-contrast microscope after non-adherent cells were washed away using cold PBS (Figure 3). We found that the pCMV-I $\kappa$ B $\alpha$ M could inhibit the adhesion of Jurkat T cells to ECV cells from  $71.4 \pm 5.2\%$  to  $42.2 \pm 3.7\%$  after being treated with PMA for 12 h ( $P < 0.05$ ).



**Figure 3** Adhesion of human Jurkat T-cells to PMA-activated endothelial (ECV) cells is inhibited by transfection with pCMV-I $\kappa$ B $\alpha$ M compared with pCMV-I $\kappa$ B $\alpha$ . ECV cells were activated with PMA (50 nmol/L) for 12 h. Cells were washed thrice with PBS and then co-cultured with Jurkat T-cells for 2 h. Then the cells were washed with PBS thrice. Jurkat cell adhesion was determined by visual counting under a phase-contrast microscope.  $P < 0.05$  when compared ECVMT with ECVWT.

## DISCUSSION

The adhesion of leukocyte to the vascular endothelial cells is a critical step in the immunological response and involves recruitment and infiltration of leukocytes to the site of tissue injury or allograft. The endothelial cell, as the first barrier faced the alloantigen, will be activated upon stimulation like cytokines (e.g., PMA, IL-1, TNF- $\alpha$ ) *in vitro* as well as *in vivo* at sites of allograft. Activated endothelial cells express adhesion molecules to assist adhesion between activated T cells and endothelial cells<sup>[18,19]</sup>. Among these molecules are P- and E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1), on the endothelial cells, and their respective counter receptors, P-selectin glycoprotein ligand-1 (PSGL-1), leukocyte function-associated antigen-1 (LFA-1) and very late antigen-4 (VLA-4), on the leukocytes<sup>[17]</sup>. Activated endothelial cells also can secrete chemokines such as IL-6, IL-8 and MCP-1<sup>[20,21]</sup>, which can recruit leukocyte. ICAM-1, which has been studied extensively, binds to its ligand LFA-1 on lymphocytes and promotes lymphocytes binding to the endothelium and facilitates the lymphocytes to invade the graft<sup>[22-24]</sup>. Blockade of ICAM-1, either with monoclonal antibodies or with antisense oligodeoxynucleotides, has been shown to decrease reperfusion injury and prolong the survival of allograft<sup>[25-27]</sup>.

Activation of endothelial cells requires multiple

transcriptional factors. Nuclear factor  $\kappa$ B, one of such transcription factors, is held in the cytoplasm by inhibitory I $\kappa$ B proteins (I $\kappa$ Bs) and regulates many genes involving immune and inflammatory pathways such as various proinflammatory cytokines, adhesion molecules and apoptosis-associated factors<sup>[8,28]</sup>. Accumulating evidence clearly demonstrates that blocking the activity of NF- $\kappa$ B might arrest the progression of acute rejection by interrupting the activation of genes of major inflammatory cytokines and adhesion molecules<sup>[29]</sup>. We use the pCMV-I $\kappa$ B $\alpha$ M vector, which encodes domain negative nondegraded I $\kappa$ B $\alpha$  protein mutated at ser-32 and ser-36 to inhibit the activation of NF- $\kappa$ B. We found that the adhesion molecules including ICAM-1, VACM-1 and P-selectin were down-regulated under inhibition of NF- $\kappa$ B. We also mimic the procedure of cell-cell adhesion *in vitro* between endothelial cells and T cells. The results told us that inhibition of NF- $\kappa$ B could inhibit endothelial cell activation and decrease cell adhesion.

In conclusion, we propose that pCMV-I $\kappa$ B $\alpha$ M vector can be used as novel immunological strategy. Further explorations were needed to verify the effect of pCMV-I $\kappa$ B $\alpha$ M to protect the allograft from rejection *in vivo*.

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