

Up-regulation of IL-6 and TNF- α induced by SARS-coronavirus spike protein in murine macrophages via NF- κ B pathway

Wei Wang, Linbai Ye*, Li Ye, Baozong Li, Bo Gao, Yingchun Zeng, Lingbao Kong, Xiaonan Fang, Hong Zheng, Zhenghui Wu, Yinglong She

State Key Laboratory of Virology, College of Life Science, Wuhan University, Wuhan, Hubei 430072, China

Received 3 November 2006; received in revised form 6 February 2007; accepted 8 February 2007

Available online 25 May 2007

Abstract

The clinical picture of severe acute respiratory syndrome (SARS) is characterized by an over-exuberant immune response with lung lymphomononuclear cells infiltration and proliferation that may account for tissue damage more than the direct effect of viral replication. To understand how cells response in the early stage of virus–host cell interaction, in this study, a purified recombinant S protein was studied for stimulating murine macrophages (RAW264.7) to produce proinflammatory cytokines (IL-6 and TNF- α) and chemokine IL-8. We found that direct induction of IL-6 and TNF- α release in the supernatant in a dose-, time-dependent manner and highly spike protein-specific, but no induction of IL-8 was detected. Further experiments showed that IL-6 and TNF- α production were dependent on NF- κ B, which was activated through I- κ B α degradation. These results suggest that SARS-CoV spike protein may play an important role in the pathogenesis of SARS, especially in inflammation and high fever.

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Keywords: SARS-coronavirus; Spike protein; IL-6; TNF- α ; NF- κ B; Macrophages

1. Introduction

Severe acute respiratory syndrome (SARS) caused by a novel human coronavirus (CoV), designated SARS-CoV, is a highly contagious respiratory disease with the lungs as a major target (Peiris et al., 2003). A histology of lung necropsy from SARS patients showed that abundant foamy macrophages and multinucleated syncytial cells were demonstrated (Lee et al., 2003; Nicholls et al., 2003). Thus, cytokines storm has been proposed to be involved in the rapid course of adult respiratory distress syndrome (ARDS) in SARS patients. The likelihood of SARS being an immune-mediated disease is supported by the observation of a transient lymphopenia, especially in CD4⁺ and CD8⁺ T cells early in the SARS episode, and elevated serum levels of inflammatory cytokines in SARS patients (Li et al., 2003b; Wong et al., 2004a; Xie et al., 2003).

SARS-CoV consists of a nucleocapsid (N) core and three membrane proteins of spike (S), membrane (M), and envelope

(E). S protein of SARS-CoV binds to the host receptor, which was identified as angiotensin-converting enzyme 2 (ACE2) (Li et al., 2003a) and mediates membrane fusion. The protein can also induce neutralizing antibodies against SARS-CoV (Chen et al., 2005; He et al., 2004).

NF- κ B is a family of Rel domain-containing proteins present in the cytoplasm of all cells, which includes Rel A, Rel B, c-Rel, p105/p50 and p100/p52. Under resting conditions, NF- κ B consists of a heterotrimer of p50, p65, and I- κ B in the cytoplasm, after activation by a large number of inducers, the I- κ B proteins become phosphorylated, ubiquitinated and, subsequently, degraded by the proteasome. The degradation of I- κ B allows NF- κ B proteins to translocate to the nucleus and bind their cognate DNA binding sites to regulate the transcription of a large number of genes, including antimicrobial peptides, cytokines, chemokines, stress-response proteins and anti-apoptotic proteins. I- κ B degradation is triggered by activation of a complex of multiple kinases that is composed of IKK α and IKK β and a structural regulatory subunit, IKK γ or NEMO. Phosphorylation of IKK α and IKK β is activated by their upstream kinases including NF- κ B-inducing kinase (Ghosh et al., 1998; Karin and Ben-Neriah, 2000; Li and Verma, 2002; Verma et al., 1995). NIK

* Corresponding author. Tel.: +86 27 6875 2372; fax: +86 27 6876 4763.
E-mail addresses: linbaiye@whu.edu.cn, linbaiye@hotmail.com (L. Ye).

as a potential activator of the I- κ B α -kinase complex appeared to have been identified an important element of the NF- κ B activation pathway (Smith et al., 2001).

Interleukin-6 (IL-6) plays a central role in both innate and acquired immune response. IL-6 is the predominate inducer of the acute-phase response, an innate immune mechanism which is triggered by infection and inflammation. IL-6 also plays multiple roles during subsequent development of acquired immunity against incoming pathogens, stimulation of antibody production by B cells, regulation of macrophage (M ϕ) and dendritic cell (DC) differentiation, and response of regulatory T cells to microbial infection. Up-regulation of IL-6 was induced by infection of many viruses, including MHV, HRV, HCMV (Banerjee et al., 2002; Gealy et al., 2005; Griego et al., 2000). Tumor necrosis factor alpha (TNF- α) has multiple biologic effects including the enhanced release of other proinflammatory/chemotactic mediators, up-regulation of adhesion molecules, enhanced migration of eosinophils and neutrophils. TNF- α was detected in increased amounts in sera of patients with SARS (Zhang et al., 2004). These data implicate TNF- α is a potent inflammatory cytokine in the pathogenesis of SARS.

Macrophages are central immunocytes in innate immunity and produce non-specific inflammatory mediators. Many viruses and their membrane proteins can activate macrophages to produce cytokines. Varicella-zoster virus (VZV) induced IL-6 and IL-8 in human monocytes (Wang et al., 2005). Hepatitis B virus Capsid strongly induced TNF- α , IL-6 and IL-12p40 in THP-1 macrophages (Cooper et al., 2005). SARS-CoV failed to productively infect human macrophages, but the interaction between SARS-CoV and human macrophage triggered the production of cytokine (IL-6, IL-12) efficiently (Tseng et al., 2005). And the proinflammatory cytokine IL-6 and the chemokine IL-8 are strongly up-regulated in SARS patients (Hsueh et al., 2004). Recombinant baculovirus displaying SARS-CoV spike protein induced IL-8 release (Chang et al., 2004). Then, we had the hypothesis that the spike protein plays an important role in the production of proinflammatory cytokine at the stage of virus-host cell interaction. In the present study, we examined the ability of S protein of SARS to modulate the proinflammatory cytokines (IL-6, TNF- α) and chemokine (IL-8) production. The murine macrophages cell line (RAW264.7) was incubated with a purified recombinant S protein, then the levels of IL-6, TNF- α and IL-8 in the supernatant were measured by ELISA. Our results showed that S protein induced massive release of IL-6 and TNF- α in an S protein-specific, as well as time- and dose-dependent manner. However, there was no induction of IL-8 was detected. Furthermore, we found that S protein activated NF- κ B signaling pathway via I- κ B α degradation, which was essential for up-regulation of IL-6 and TNF- α .

2. Materials and methods

2.1. Cell culture

RAW264.7 cells were obtained from China Center for Type Culture Collection (CCTCC) and cultured in RPMI 1640 (Gibco/BRL) supplemented with 10% fetal bovine serum

(FBS; Hyclone), and antibiotics (100 μ g/ml streptomycin and 100 U/ml penicillin) at 37 °C in 5% CO₂.

2.2. Antibodies and plasmid

Anti-S Abs (IMG-542) was from IMGENEX Corporation. The antibody was developed by immunizing rabbits with synthetic peptides corresponding to amino acids 288–303 of the SARS Spike glycoprotein.

Expression vector for dominant-negative NIK (pcDNA3.1(+)-dn-NIK), which has a kinase-dead mutation of K429A/K430A, was kindly provided by Prof. Brian M.J. Foxwell and Alison Davis (Kennedy Institute of Rheumatology Division, Imperial College School of Medicine, London).

2.3. Expression and purification of recombinant spike protein

The spike gene encoding from 262nd to 606th amino acid of SARS-CoV S protein was cloned into pET-his expression vector containing T7 promoter and six histidine fusion tag. After transformation of *E. coli* BL21 (DE3) with the constructed vector, the S proteins were produced by 1 mM IPTG. Cells were resuspended in a buffer containing 50 mM Tris-Cl and 500 mM NaCl (pH 8.0), disrupted by ultrasonic and centrifuged at 12,000 $\times g$ for 15 min. The pellet was dissolved with 8 M urea and the six-histidine-tagged protein was purified by Ni-NTA affinity chromatography. After dialysis against deionized water removing the urea, the purified S protein was performed.

Some soluble S protein was treated by Detoxi-Gel™ Endotoxin Removing Gel (Pierce) to remove the endotoxin according to the manufacturer's instruction.

2.4. Measurement of IL-6, TNF- α and IL-8 in the supernatant of cultured cells

RAW264.7 cells were seeded into 6-well plates at a density of 5 $\times 10^5$ cells/well, and untreated or incubated for indicated times (0, 3, 6, 9, 12, 24 h) or at indicated concentrations (0, 1, 5, 10, 20 μ g/ml) with purified S protein. IL-6, TNF- α and IL-8 in the culture supernatant of RAW264.7 cells were measured by ELISA kits (Bender MedSystems) according to the manufacturer's instructions.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

RAW264.7 cells were incubated with the purified S protein at different concentrations (0, 1, 5, 10, 20 μ g/ml) for 12 h, then total RNA were extracted and treated with DNase I. For reverse transcription (RT), 1 μ g of RNA was incubated with 200 U of Superscript II reverse transcriptase (Invitrogen) and 100 ng random hexanucleotides in 20 μ l of 1 \times RT buffer (Invitrogen) supplied with 1 mM each of the four dextynucleotide triphosphates, 20 U of RNasin, and 10 mM dithiothreitol. The resulting cDNA were amplified by 35 cycles of PCR, with each cycle consisting of 30 s at 94 °C, 30 s at 52 °C, and 30 s at 72 °C. The prime

sequences were as follows: for IL-6, forward (5'-GTT GCC TTC TTG GGA CTG ATG-3') and reverse (5'-CAT ACA ATC AGA ATT GCC ATT GC-3'); for actin, forward (5'-ACA ACG GCT CCG GCA TGT GCA A-3') and reverse (5'-CCA TGT CGT CCC AGT TGG TGA C-3'). The RT-PCR products were analyzed by electrophoresis through 2% agarose gels containing ethidium bromide.

2.6. Transfection and NF- κ B reporter assay

RAW264.7 cells in 96-well plates were cotransfected with NF- κ B firefly luciferase reporter plasmid (0.1 μ g/well) and control Renilla luciferase plasmid (5 ng/well) using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Twenty-four hours after transfection, the cells were stimulated with the purified S protein or untreated for 12 h. Cells were subsequently harvested, lysed and luciferase activity were measured using the dual luciferase reporter assay kit (Promega).

2.7. Western blotting analysis of I- κ B α

Cell lysates were separated by 12% denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using standard techniques and electroblotted onto polyvinylidene difluoride membranes. The membranes were blocked in Tris-buffered saline (10 mM Tris-HCl [pH 8.0], 150 mM NaCl) containing 5% (w/v) dried milk for 2 h at room temperature and then probed with the first antibodies, rabbit anti-I- κ B α polyclonal antibodies (sc-371; Santa Cruz Biotechnology, Santa Cruz, USA) or anti-actin antibody (sc-1616; Santa Cruz Biotechnology) for 2 h at room temperature. After extensive washing, secondary antibodies conjugated with horseradish peroxidase (HRP) were applied onto the blots for at least 1 h at room temperature. The blots were washed four times with TBS containing 0.1% Tween-20. Reagents (Pierce) for enhanced chemiluminescence were applied to the blots and the light signals were detected by X-ray film.

3. Results

3.1. SARS-CoV Spike protein was expressed and purified

The recombinant S protein of SARS-CoV was expressed in *E. coli* strain BL21(DE3) and purified by Ni-NTA affinity column chromatography. We found that expression of S protein was at very high level after 5 h induction with IPTG, and the protein formed inclusion bodies. Our data showed that the purified S protein (38 kDa) was clearly observed in the SDS-PAGE electrophoresis analysis (Fig. 1).

3.2. Spike protein stimulated IL-6 and TNF- α release from RAW264.7 cells

To investigate whether S protein can induce cytokine expression in macrophages, we first measured the level of IL-6, TNF- α and IL-8 in the supernatant of RAW264.7 treated with S protein. After exposing RAW264.7 cells to the purified S protein

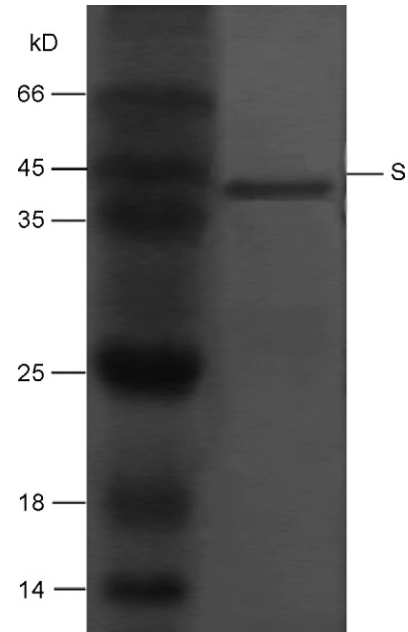


Fig. 1. Expression and purification of recombinant S protein of SARS-CoV. Recombinant S protein was expressed in *E. coli* after 5 h induction with IPTG, purified by Ni-NTA affinity column, separated by 12% SDS-PAGE and visualized by Coomassie Brilliant Blue staining.

at a concentration ranging from 0 to 20 μ g/ml and at a time ranging from 0 to 24 h, the levels of IL-6, TNF- α and IL-8 in the culture supernatant of RAW264.7 cells were measured using enzyme-linked immunosorbent assay kits. Recombinant S protein induced the production of IL-6 and TNF- α in the culture supernatant of RAW264.7 cells in a dose- and time-dependent manner (Fig. 2A–D). Increasing concentrations of S protein resulted in release of increasing amounts of IL-6 and TNF- α into the supernatant. Treatment of RAW264.7 cells with about 5 μ g/ml S protein was sufficient for full induction of TNF- α , and even 1 μ g/ml S protein significantly induced TNF- α production in RAW264.7 cells. IL-6 release was detectable at 6 h and sustained throughout the experiment (up to 24 h), and induction of TNF- α was observed as early as 3 h and increased further at 9 h to remain stable for up to 24 h of treatment. There was no up-regulation of IL-8 in RAW264.7 incubated with S protein, and it was not due to the problem of the IL-8 ELISA kit because of the positive control of IL-8 standard substance (data not shown). To further determine whether IL-6 induction occurred at the transcriptional level, IL-6 mRNA levels were evaluated by semiquantitative RT-PCR. S protein strongly induced the transcription level of IL-6 in a dose-dependence manner, which was consistent with the release of IL-6 in the supernatants (Fig. 2E). These results demonstrate that the recombinant SARS-CoV S protein is a highly potent inducer of IL-6 and TNF- α in RAW264.7 macrophages.

We found that the IL-6 and TNF- α production were not affected in RAW264.7 cells stimulated with S protein pretreated by Detoxi-Gel™ Endotoxin Removing Gel (Pierce). And RAW264.7 cells were treated with pre-boiled S protein, subsequent IL-6 and TNF- α release decreased by 85–90% and 65–80%, respectively. These results ruled out the possibility of

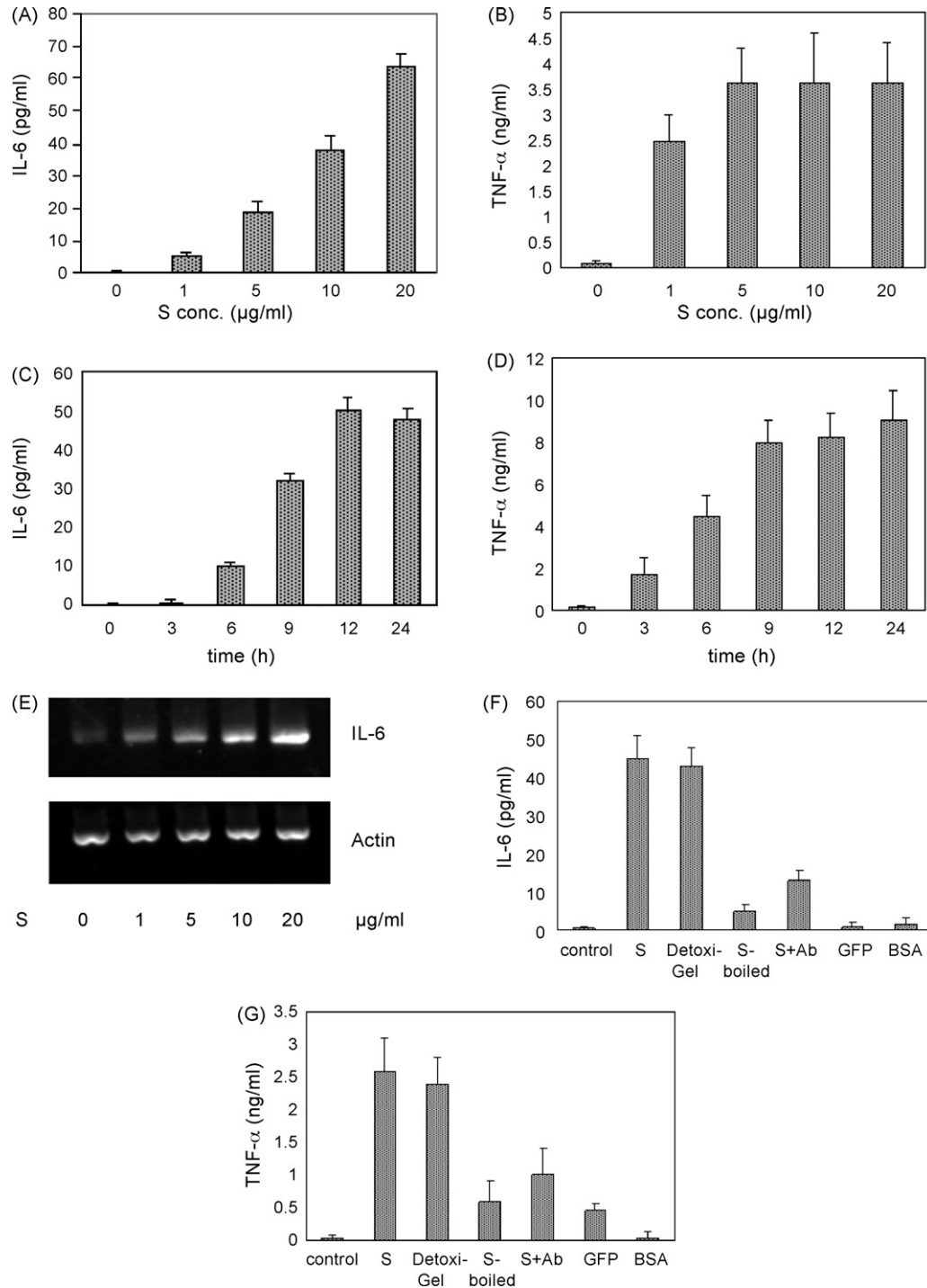


Fig. 2. Purified S protein of SARS-CoV promotes IL-6 and TNF- α induction in murine macrophages. IL-6 and TNF- α were induced by S protein in a dose-dependent manner. RAW264.7 cells were incubated with the indicated concentrations of S protein for 12 h, IL-6 and TNF- α (A and B) production were determined by ELISA. IL-6 and TNF- α were induced by S protein in a time-dependent manner. RAW264.7 cells were incubated with 20 μ g/ml S protein for the indicated times, IL-6 and TNF- α (C and D) production were determined by ELISA. (E) Transcriptional induction of IL-6 by S protein. RAW264.7 cells were incubated with the indicated concentrations of S protein for 12 h, and the mRNA levels of IL-6 were tested by RT-PCR. Data shown are representative of three independent experiments. IL-6 and TNF- α were induced in an S protein-specific manner. RAW264.7 cells were untreated, incubated with 20 μ g/ml S protein, 20 μ g/ml S protein pre-treated by Detoxi-Gel™ Endotoxin Removing Gel, 20 μ g/ml S protein pre-boiled for 1 h or pre-incubated with anti-S antibody for 2 h at 37 °C, 20 μ g/ml GFP and 20 μ g/ml BSA. IL-6 and TNF- α (F and G) production were determined by ELISA. All bars represent the means \pm S.E. of three independent experiments.

the induction of cytokines being due to endotoxin contamination instead of S protein. And, pre-treated S protein with anti-S protein antibody neutralized subsequent S protein-induced IL-6 and TNF- α release in RAW264.7 cells by 60–70% and 50–65%,

respectively. The RAW264.7 cells treated with GFP and BSA did not induce IL-6 and TNF- α (Fig. 2F and G). These data showed that the activation of macrophages appeared to be the S protein-specific.

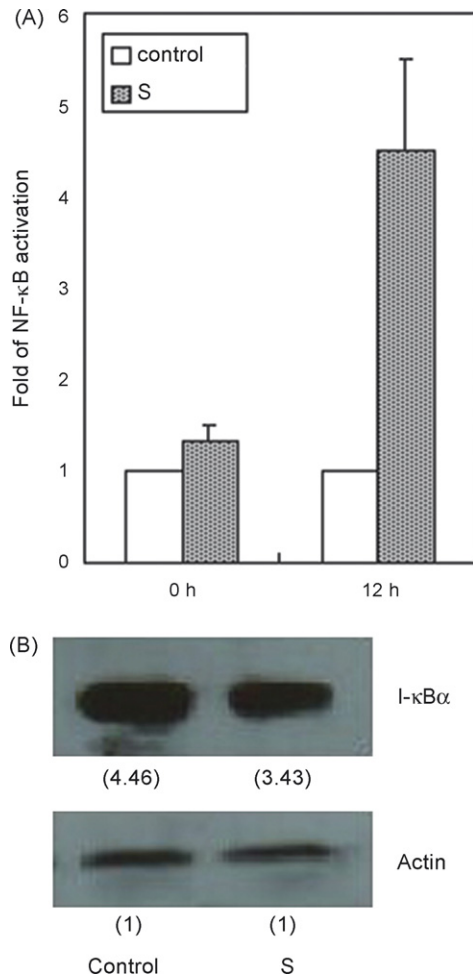


Fig. 3. NF- κ B activation by purified S protein of SARS-CoV. (A) RAW264.7 cells in a 96-well plate were transfected with NF- κ B-luciferase reporter plasmid (0.1 μ g/well). A Renilla reporter plasmid (5 ng/well) was cotransfected for normalization. Twenty-four hour after transfection, the cells were either treated with S protein (10 μ g/ml) or left untreated. After 12 h, the cells were lysed, and luciferase activity was quantified. The results were expressed as the mean \pm S.E. of three independent experiments. (B) RAW264.7 cells in a 6-well plate were treated with S protein (10 μ g/ml) or left untreated for 1.5 h. Total cell lysates were analyzed by Western blotting using anti-I- κ B α polyclonal antibodies (upper panel) and anti-actin polyclonal antibodies (lower panel). The density of the protein band was determined by using Bio-Rad Quantity One imaging software. The values in parentheses are density values of I- κ B α relative to actin. Results of one of three independent experiments are shown.

3.3. Spike protein induced NF- κ B activation

To investigate the role of NF- κ B in mediating S protein-induced cytokines expression, we next analyzed the ability of purified S protein to activate the NF- κ B. RAW264.7 cells were transfected with an NF- κ B-luciferase reporter plasmid. At 24 h posttransfection, cells were stimulated with purified S protein (10 μ g/ml) or control medium for 12 h before a luciferase assay was performed. As shown in Fig. 3A, the purified S protein significantly increased NF- κ B activity to approximately 5-fold compared with the control.

To further confirm that S protein can activate NF- κ B, we analyzed I- κ B α by Western blotting. I- κ B α was found to be decreased by 30% after incubation with purified S protein for

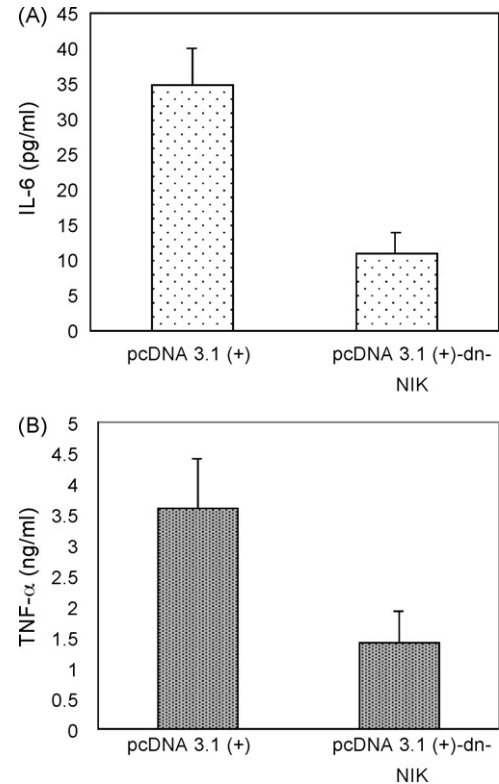


Fig. 4. IL-6 and TNF- α induction by purified S protein of SARS-CoV requires NF- κ B. RAW264.7 cells in a 24-well plate were transfected with either pcDNA3.1(+) (empty vector) or pcDNA3.1(+)-dn-NIK (expression plasmid for dominant negative mutant NIK). At 24 h posttransfection, the cells were treated with S protein (10 μ g/ml) for 12 h, IL-6 and TNF- α (A and B) production in the supernatant were determined by ELISA. The means and standard error of the means are shown from three independent experiments.

1.5 h, compared with the control situation (Fig. 3B, top). Equal loading of protein samples was shown by reprobing the blot with an anti-actin antibody (Fig. 3B, bottom). Together with the luciferase assay, our results clearly demonstrated that S protein induced NF- κ B activation and I- κ B α degradation.

3.4. NF- κ B was essential for up-regulation of IL-6 and TNF- α in purified spike protein-stimulated RAW264.7 cells

To determine whether S protein-induced IL-6 and TNF- α production is dependent on NF- κ B, the effect of inhibition of NF- κ B activation on IL-6 and TNF- α release in RAW264.7 incubated with S protein was investigated. Transfection with dominant-negative NIK (pcDNA3.1(+)-dn-NIK), which inhibit NF- κ B activation, led to a strong inhibition of purified S protein-induced release of IL-6 and TNF- α in RAW264.7 cells (Fig. 4), indicating that NF- κ B is required for effective induction of IL-6 and TNF- α by S protein.

4. Discussion

Patients with severe acute respiratory syndrome (SARS) were reported to have elevated levels of cytokines in blood and cytokine gene expression in peripheral blood mononuclear cells (PBMC) (Sheng et al., 2005; Yu et al., 2005; Zhang et

al., 2004). Previous reports have shown that live SARS-CoV induced a production of IL-6 and TNF- α in human M ϕ and DC, importantly, the ability of SARS-CoV modulating M ϕ and DC functions was independent of viral replication (Law et al., 2005; Tseng et al., 2005). The mechanism of SARS-CoV infection inducing the release of cytokines remains obscure. Since the S protein can bind to the receptor, we sought to investigate whether the S protein can interact with macrophages then up-regulate the release of cytokines/chemokines. In the current study, we found that incubation of RAW264.7 with truncated SARS-CoV S protein led to production of IL-6 and TNF- α which were depended on NF- κ B activation. We expressed the truncated S protein incorporating amino acids 262–606 instead of the full-length spike protein in our study. As we all known, the full-length spike protein consists of S1 domain and S2 domain, and S1 domain is responsible for the attachment to the target cells (Li et al., 2005; Wong et al., 2004b). So we tried to express the full-length S1 protein at the beginning, but it could not be expressed in BL21(DE3). The probably reason is that there is a large hydrophobic region in N-terminal peptidase domain, which is not necessary for the binding with receptor. Then we tried to express the truncated S1 protein reserving the receptor-binding domain (RBD) and lacking the hydrophobic region in BL21(DE3). Fortunately, the truncated protein was expressed at a very high level as inclusion bodies and it was sufficient for our study.

In our study, we found massive release of IL-6 and TNF- α , but no induction of IL-8 in RAW264.7 stimulated with S protein, while the over-expression of IL-8 is one of the major phenomena in SARS patients and human macrophages. First, our study just focused on the response of macrophages to S protein of SARS-CoV in vitro, while over-expression of IL-8 in patients was one of quite complicated responses of the whole human's immune system to the virus. We cannot explain all the symptoms in the patients by our relative simple study. Second, the RAW264.7 is a murine macrophage cell line. It may be different in S protein inducing cytokines in murine macrophages from that in human macrophages.

It has been demonstrated that viral infection triggered the NF- κ B pathway, resulting in transcription activation of a large number of proinflammatory genes, including cytokine and chemokines (Zhu et al., 1996). To delineate the mechanisms of S protein inducing cytokine production, we first measured NF- κ B activation by luciferase assay in S protein-induced RAW264.7 increased 5-fold compare to the control. Activation of NF- κ B depends on degradation of I- κ B, which normally sequesters NF- κ B in the cytoplasm, inhibiting its function (Ghosh et al., 1998; Li and Verma, 2002; Verma et al., 1995). To further investigate the involvement of the NF- κ B signaling pathways in S protein-induced cytokine regulation, we examined I- κ B α degradation in S protein-incubated cells by western blotting analysis. Consistent with our luciferase results, the level of I- κ B α in macrophages incubated with S protein was reduced at 1.5 h after incubation. We also found S protein induced IL-6 and TNF- α production through an NF- κ B-dependent mechanism in RAW264.7, because inhibition of NF- κ B activity caused inhibition of IL-6 and TNF- α production. Mizutani et al. (2004) found

that p38 MAPK was activated in SARS-CoV-infected cells (Vero E6), and SARS-CoV infection induced apoptotic cell death. Lee et al. (2004) showed that SARS patients had a higher intracellular phosphor-p38 level in total leukocytes than normal controls. Some studies have demonstrated p38 MAPK activation resulted in accumulation of IL-6 and TNF- α mRNAs and an increase in production of IL-6 and TNF- α (Banerjee et al., 2002; Eliopoulos et al., 1999; He et al., 2004; Lee et al., 2005; Wang et al., 2005). In our study, we did not examine the effects of S protein on activation of the p38 MAPK, but according to the above studies, it is possible that incubation RAW264.7 with S protein can also activate p38 MAPK pathway leading to the production of IL-6 and TNF- α , future studies will address this issue.

Angiotensin-converting enzyme 2 (ACE2) has been identified as a functional receptor for SARS-CoV, and the tissue distribution of ACE2 has been studied extensively (Hamming et al., 2004; To and Lo, 2004). Although immune cells lack ACE2, SARS-CoV can infect human macrophages and dendritic cells (Law et al., 2005; Tseng et al., 2005; Yilla et al., 2005) without producing infectious virus particles, suggesting that SARS-CoV can entry the immune cells via other receptors. Toll-like receptors (TLRs), a major class of molecular pattern recognition receptors, are activated by direct interaction of the extracellular domain of the receptor with a pathogen-associated molecular pattern, resulting in activation of NF- κ B, IRF3, and MAPK signaling pathways. TLR family is responsible for activation of innate immunity in response to various pathogens, including certain viruses (Boehme and Compton, 2004). TLR2 interacts with viruses or their components, including measles virus, hepatitis C virus, and several herpesviruses, including cytomegalovirus and herpes simplex virus (HSV) (Bieback et al., 2002; Compton et al., 2003; Dolganiuc et al., 2004; Kurt-Jones et al., 2004). Activation of TLR2 by these viruses is followed by the production of inflammatory cytokines, including IL-6, IL-8, and TNF- α . In future studies, we will examine whether TLRs participate in the induction of IL-6 and TNF- α by SARS-CoV S protein. When S protein pre-treated with anti-S antibody and then incubated RAW264.7 cells, the cytokine induction reduced strongly. The result showed that anti-S antibody could block the interaction between S protein and the receptor in the immune cells resulting in reduced production of IL-6 and TNF- α . Pre-boiled S protein cannot induce IL-6 and TNF- α efficiently. This result ruled out the possibility that contamination of endotoxin is responsible for cytokine induction. Because heat treatment for 1 h can destroy the functional structure of protein, thereby abolish the protein-induced activation effect on macrophages, while the similar heat treatment do not reduce the LPS-mediated stimulation (Cooper et al., 2005; Lee et al., 2001; Pathak et al., 2006). And S protein-induced IL-6 and TNF- α release were not affected by Detoxi-Gel™ Endotoxin Removing Gel treatment, further conforming the cytokines production were induced by S protein instead of endotoxin contamination.

IL-6, a pleiotropic factor, is a downstream target protein of NF- κ B, on the other hand, IL-6 can also influence NF- κ B activation. Neutrophils were incubated with IL-6 for 30 min, I- κ B degradation was observed (Lindemans et al., 2006). IL-6 up-regulated TLR2 expression in vitro in monocytes (Pons

et al., 2006). We have a hypothesis that SARS-CoV interact with macrophages through the interaction between spike protein and the TLR on the cell surface, leading to activate multiple intracellular pathways involved inflammation, including activation of NF- κ B and production of IL-6 and TNF- α in our study. IL-6 could activate NF- κ B and up-regulate TLR expression via autocrine/paracrine effects, then aggravate the inflammation.

In summary, we firstly demonstrated that the interaction between SARS-CoV S protein and murine macrophages could induce cytokines IL-6 and TNF- α release, while most of our current knowledge of inducing innate immunity and inflammation focused on the whole live virus. S protein triggered the activation of NF- κ B, and then initiated the cytokine release. Our results may partly explain the clinical observation of dramatic cytokines storm and inflammation responses in SARS patients, and could help in the development of novel vaccine immunogens and therapeutics for prevention and treatment of SARS.

Acknowledgement

We thank Prof. Brian M. J. Foxwell and Alison Davis for providing us with plasmid pcDNA3.1(+)-dn-NIK.

References

- Banerjee, S., Narayanan, K., Mizutani, T., Makino, S., 2002. Murine coronavirus replication-induced p38 mitogen-activated protein kinase activation promotes interleukin-6 production and virus replication in cultured cells. *J. Virol.* 76 (12), 5937–5948.
- Bieback, K., Lien, E., Klagge, I.M., Avota, E., Schneider-Schaulies, J., Duprex, W.P., Wagner, H., Kirschning, C.J., Ter Meulen, V., Schneider-Schaulies, S., 2002. Hemagglutinin protein of wild-type measles virus activates toll-like receptor 2 signaling. *J. Virol.* 76 (17), 8729–8736.
- Boehme, K.W., Compton, T., 2004. Innate sensing of viruses by toll-like receptors. *J. Virol.* 78 (15), 7867–7873.
- Chang, Y.J., Liu, C.Y., Chiang, B.L., Chao, Y.C., Chen, C.C., 2004. Induction of IL-8 release in lung cells via activator protein-1 by recombinant baculovirus displaying severe acute respiratory syndrome-coronavirus spike proteins: identification of two functional regions. *J. Immunol.* 173 (12), 7602–7614.
- Chen, Z., Zhang, L., Qin, C., Ba, L., Yi, C.E., Zhang, F., Wei, Q., He, T., Yu, W., Yu, J., Gao, H., Tu, X., Gettie, A., Farzan, M., Yuen, K.Y., Ho, D.D., 2005. Recombinant modified vaccinia virus Ankara expressing the spike glycoprotein of severe acute respiratory syndrome coronavirus induces protective neutralizing antibodies primarily targeting the receptor binding region. *J. Virol.* 79 (5), 2678–2688.
- Compton, T., Kurt-Jones, E.A., Boehme, K.W., Belko, J., Latz, E., Golenbock, D.T., Finberg, R.W., 2003. Human cytomegalovirus activates inflammatory cytokine responses via CD14 and Toll-like receptor 2. *J. Virol.* 77 (8), 4588–4596.
- Cooper, A., Tal, G., Lider, O., Shaul, Y., 2005. Cytokine induction by the hepatitis B virus capsid in macrophages is facilitated by membrane heparan sulfate and involves TLR2. *J. Immunol.* 175 (5), 3165–3176.
- Dolganic, A., Oak, S., Kodys, K., Golenbock, D.T., Finberg, R.W., Kurt-Jones, E., Szabo, G., 2004. Hepatitis C core and nonstructural 3 proteins trigger toll-like receptor 2-mediated pathways and inflammatory activation. *Gastroenterology* 127 (5), 1513–1524.
- Eliopoulos, A.G., Gallagher, N.J., Blake, S.M., Dawson, C.W., Young, L.S., 1999. Activation of the p38 mitogen-activated protein kinase pathway by Epstein-Barr virus-encoded latent membrane protein 1 coregulates interleukin-6 and interleukin-8 production. *J. Biol. Chem.* 274 (23), 16085–16096.
- Gealy, C., Denson, M., Humphreys, C., McSharry, B., Wilkinson, G., Caswell, R., 2005. Posttranscriptional suppression of interleukin-6 production by human cytomegalovirus. *J. Virol.* 79 (1), 472–485.
- Ghosh, S., May, M.J., Kopp, E.B., 1998. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu. Rev. Immunol.* 16, 225–260.
- Griego, S.D., Weston, C.B., Adams, J.L., Tal-Singer, R., Dillon, S.B., 2000. Role of p38 mitogen-activated protein kinase in rhinovirus-induced cytokine production by bronchial epithelial cells. *J. Immunol.* 165 (9), 5211–5220.
- Hamming, I., Timens, W., Bulthuis, M.L., Lely, A.T., Navis, G.J., van Goor, H., 2004. Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. *J. Pathol.* 203 (2), 631–637.
- He, Y., Zhou, Y., Liu, S., Kou, Z., Li, W., Farzan, M., Jiang, S., 2004. Receptor-binding domain of SARS-CoV spike protein induces highly potent neutralizing antibodies: implication for developing subunit vaccine. *Biochem. Biophys. Res. Commun.* 324 (2), 773–781.
- Hsueh, P.R., Chen, P.J., Hsiao, C.H., Yeh, S.H., Cheng, W.C., Wang, J.L., Chiang, B.L., Chang, S.C., Chang, F.Y., Wong, W.W., Kao, C.L., Yang, P.C., 2004. Patient data, early SARS epidemic, Taiwan. *Emerg. Infect. Dis.* 10 (3), 489–493.
- Karin, M., Ben-Neriah, Y., 2000. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annu. Rev. Immunol.* 18, 621–663.
- Kurt-Jones, E.A., Chan, M., Zhou, S., Wang, J., Reed, G., Bronson, R., Arnold, M.M., Knipe, D.M., Finberg, R.W., 2004. Herpes simplex virus 1 interaction with Toll-like receptor 2 contributes to lethal encephalitis. *Proc. Natl. Acad. Sci. U.S.A.* 101 (5), 1315–1320.
- Law, H.K., Cheung, C.Y., Ng, H.Y., Sia, S.F., Chan, Y.O., Luk, W., Nicholls, J.M., Peiris, J.S., Lau, Y.L., 2005. Chemokine up-regulation in SARS-coronavirus-infected, monocyte-derived human dendritic cells. *Blood* 106 (7), 2366–2374.
- Lee, C.H., Chen, R.F., Liu, J.W., Yeh, W.T., Chang, J.C., Liu, P.M., Eng, H.L., Lin, M.C., Yang, K.D., 2004. Altered p38 mitogen-activated protein kinase expression in different leukocytes with increment of immunosuppressive mediators in patients with severe acute respiratory syndrome. *J. Immunol.* 172 (12), 7841–7847.
- Lee, D.C., Cheung, C.Y., Law, A.H., Mok, C.K., Peiris, M., Lau, A.S., 2005. p38 mitogen-activated protein kinase-dependent hyperinduction of tumor necrosis factor alpha expression in response to avian influenza virus H5N1. *J. Virol.* 79 (16), 10147–10154.
- Lee, N., Hui, D., Wu, A., Chan, P., Cameron, P., Joynt, G.M., Ahuja, A., Yung, M.Y., Leung, C.B., To, K.F., Lui, S.F., Szeto, C.C., Chung, S., Sung, J.J., 2003. A major outbreak of severe acute respiratory syndrome in Hong Kong. *N. Engl. J. Med.* 348 (20), 1986–1994.
- Lee, S.E., Choi, S.E., Kim, J.H., Kim, K.S., Kang, Y., 2001. Activation of mouse macrophage by soluble endogenous murine leukemia virus (MuLV) envelope protein. *Immunol. Lett.* 79 (3), 221–227.
- Li, F., Li, W., Farzan, M., Harrison, S.C., 2005. Structure of SARS coronavirus spike receptor-binding domain complexed with receptor. *Science* 309 (5742), 1864–1868.
- Li, Q., Verma, I.M., 2002. NF-kappaB regulation in the immune system. *Nat. Rev. Immunol.* 2 (10), 725–734.
- Li, W., Moore, M.J., Vasilieva, N., Sui, J., Wong, S.K., Berne, M.A., Somasundaran, M., Sullivan, J.L., Luzuriaga, K., Greenough, T.C., Choe, H., Farzan, M., 2003a. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature* 426 (6965), 450–454.
- Li, Z., Guo, X., Hao, W., Wu, Y., Ji, Y., Zhao, Y., Liu, F., Xie, X., 2003b. The relationship between serum interleukins and T-lymphocyte subsets in patients with severe acute respiratory syndrome. *Chin. Med. J. (Engl.)* 116 (7), 981–984.
- Lindemans, C.A., Coffey, P.J., Schellens, I.M., de Graaff, P.M., Kimpen, J.L., Koenderman, L., 2006. Respiratory syncytial virus inhibits granulocyte apoptosis through a phosphatidylinositol 3-kinase and NF-kappaB-dependent mechanism. *J. Immunol.* 176 (9), 5529–5537.
- Mizutani, T., Fukushi, S., Saijo, M., Kurane, I., Morikawa, S., 2004. Phosphorylation of p38 MAPK and its downstream targets in SARS coronavirus-infected cells. *Biochem. Biophys. Res. Commun.* 319 (4), 1228–1234.

- Nicholls, J.M., Poon, L.L., Lee, K.C., Ng, W.F., Lai, S.T., Leung, C.Y., Chu, C.M., Hui, P.K., Mak, K.L., Lim, W., Yan, K.W., Chan, K.H., Tsang, N.C., Guan, Y., Yuen, K.Y., Peiris, J.S., 2003. Lung pathology of fatal severe acute respiratory syndrome. *Lancet* 361 (9371), 1773–1778.
- Pathak, S.K., Basu, S., Bhattacharyya, A., Pathak, S., Banerjee, A., Basu, J., Kundu, M., 2006. TLR4-dependent NF-kappaB activation and mitogen- and stress-activated protein kinase 1-triggered phosphorylation events are central to *Helicobacter pylori* peptidyl prolyl cis-, trans-isomerase (HP0175)-mediated induction of IL-6 release from macrophages. *J. Immunol.* 177 (11), 7950–7958.
- Peiris, J.S., Lai, S.T., Poon, L.L., Guan, Y., Yam, L.Y., Lim, W., Nicholls, J., Yee, W.K., Yan, W.W., Cheung, M.T., Cheng, V.C., Chan, K.H., Tsang, D.N., Yung, R.W., Ng, T.K., Yuen, K.Y., 2003. Coronavirus as a possible cause of severe acute respiratory syndrome. *Lancet* 361 (9366), 1319–1325.
- Pons, J., Saulea, J., Regueiro, V., Santos, C., Lopez, M., Ferrer, J., Agusti, A.G., Bengoechea, J.A., 2006. Expression of Toll-like receptor 2 is up-regulated in monocytes from patients with chronic obstructive pulmonary disease. *Respir. Res.* 7, 64.
- Sheng, W.H., Chiang, B.L., Chang, S.C., Ho, H.N., Wang, J.T., Chen, Y.C., Hsiao, C.H., Hseuh, P.R., Chie, W.C., Yang, P.C., 2005. Clinical manifestations and inflammatory cytokine responses in patients with severe acute respiratory syndrome. *J. Formos. Med. Assoc.* 104 (10), 715–723.
- Smith, C., Andreaskos, E., Crawley, J.B., Brennan, F.M., Feldmann, M., Foxwell, B.M., 2001. NF-kappaB-inducing kinase is dispensable for activation of NF-kappaB in inflammatory settings but essential for lymphotoxin beta receptor activation of NF-kappaB in primary human fibroblasts. *J. Immunol.* 167 (10), 5895–5903.
- To, K.F., Lo, A.W., 2004. Exploring the pathogenesis of severe acute respiratory syndrome (SARS): the tissue distribution of the coronavirus (SARS-CoV) and its putative receptor, angiotensin-converting enzyme 2 (ACE2). *J. Pathol.* 203 (3), 740–743.
- Tseng, C.T., Perrone, L.A., Zhu, H., Makino, S., Peters, C.J., 2005. Severe acute respiratory syndrome and the innate immune responses: modulation of effector cell function without productive infection. *J. Immunol.* 174 (12), 7977–7985.
- Verma, I.M., Stevenson, J.K., Schwarz, E.M., Van Antwerp, D., Miyamoto, S., 1995. Rel/NF-kappa B/I kappa B family: intimate tales of association and dissociation. *Genes Dev.* 9 (22), 2723–2735.
- Wang, J.P., Kurt-Jones, E.A., Shin, O.S., Manchak, M.D., Levin, M.J., Finberg, R.W., 2005. Varicella-zoster virus activates inflammatory cytokines in human monocytes and macrophages via Toll-like receptor 2. *J. Virol.* 79 (20), 12658–12666.
- Wong, C.K., Lam, C.W., Wu, A.K., Ip, W.K., Lee, N.L., Chan, I.H., Lit, L.C., Hui, D.S., Chan, M.H., Chung, S.S., Sung, J.J., 2004a. Plasma inflammatory cytokines and chemokines in severe acute respiratory syndrome. *Clin. Exp. Immunol.* 136 (1), 95–103.
- Wong, S.K., Li, W., Moore, M.J., Choe, H., Farzan, M., 2004b. A 193-amino acid fragment of the SARS coronavirus S protein efficiently binds angiotensin-converting enzyme 2. *J. Biol. Chem.* 279 (5), 3197–3201.
- Xie, J., Han, Y., Li, T.S., Qiu, Z.F., Ma, X.J., Fan, H.W., Lu, W., Liu, Z.Y., Wang, Z., Wang, H.L., Deng, G.H., 2003. Dynamic changes of plasma cytokine levels in patients with severe acute respiratory syndrome. *Zhonghua Nei Ke Za Zhi* 42 (9), 643–645.
- Yilla, M., Harcourt, B.H., Hickman, C.J., McGrew, M., Tamin, A., Goldsmith, C.S., Bellini, W.J., Anderson, L.J., 2005. SARS-coronavirus replication in human peripheral monocytes/macrophages. *Virus Res.* 107 (1), 93–101.
- Yu, S.Y., Hu, Y.W., Liu, X.Y., Xiong, W., Zhou, Z.T., Yuan, Z.H., 2005. Gene expression profiles in peripheral blood mononuclear cells of SARS patients. *World J. Gastroenterol.* 11 (32), 5037–5043.
- Zhang, Y., Li, J., Zhan, Y., Wu, L., Yu, X., Zhang, W., Ye, L., Xu, S., Sun, R., Wang, Y., Lou, J., 2004. Analysis of serum cytokines in patients with severe acute respiratory syndrome. *Infect. Immun.* 72 (8), 4410–4415.
- Zhu, Z., Tang, W., Ray, A., Wu, Y., Einarsson, O., Landry, M.L., Gwaltney Jr., J., Elias, J.A., 1996. Rhinovirus stimulation of interleukin-6 in vivo and in vitro. Evidence for nuclear factor kappa B-dependent transcriptional activation. *J. Clin. Invest.* 97 (2), 421–430.