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Research Article

RelA/p65-mediated Innate Immune Response Affecting NDV Replication in CEF

Zhao-xiong WANG [1] Min-hua SUN [2] Yin-feng KANG [2] Peng XIE [2] Tao REN [2]

[1] College of Animal Science, Yangtze University, 88 Jing Mi Road, Jingzhou, Hubei, P. R. CHINA
[2] Key Laboratory of Animal Diseases Control and Prevention of the Ministry of Agriculture; College of Veterinary Medicine, South China Agricultural University, 483 Wu Shan Road, Tian He District, Guangzhou 510642, P. R. CHINA

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Abstract

Newcastle disease virus (NDV) is a non-segmented and single-stranded negative-sense RNA (-)ssRNA virus. Previous studies indicate that NDV elicit hosts to induce strongly innate immune responses. The virus replication can cause hosts to express more type I interferon (IFN) via nuclear kappa B (NF-kB) pathways and further activate IFN-stimulated genes (ISGs) to retard virus growth and induce cells for developing specific protection. This study investigated the role of RelA/p65 in innate immune responses against NDV replication by using the virus infection chicken embryo fibroblast cells (CEF). The result showed that the mRNA and protein levels of RelA/p65 increased markedly in CEF infected with strains of NDV which differed in their virulence, and RelA/p65 translocated from the cytoplasm to the nucleus. Further research indicated that specific siRNA could inhibit expression of RelA/p65, accordingly, mRNA levels of IFN-α, IFN-β and STAT1 decreased significantly and the replication kinetics of 2 NDVs were enhanced after inhibition of RelA/p65. In conclusion, CEF can synthesize more type I IFN via RelA/p65 pathways after NDV infection, which retards NDV replication and spreads in the early phase of viral infection.

Keywords: *NDV, RelA/p65, Interferon, Virus replication, CEF*

CEF'teki NDV Replikasyonunu Etkileyen RelA/p65-güdümlü Doğal İmmun Tepki

Özet

Newcastle hastalığı virüsü (NDV) tek-parçalı ve tek-sarmallı negatif-yönlü RNA (-) SsRNA virüsüdür. Önceki çalışmalar, NDV'nin konakçıların güçlü doğal bağışıklık tepkilerini indüklediğini göstermektedir. Virüs replikasyonu nükleer kappa B (NF-kB) yolakları üzerinden daha fazla tip I (IFN) interferon ekspresyonuna, ve ayrıca IFN-uyarımlı genlerin (ISGs) aktive ederek daha fazla virüs büyümesini geciktirmek ve spesifik korumanın geliştirilmesi için hücrelerin uyarılmasına neden olabilir. Bu çalışmada, tavuk embriyo fibroblast hücreleri (CEF) virüs enfeksiyonu kullanılarak NDV replikasyonuna karşı doğal bağışıklık yanıtlarında RelA/p65'in rolü araştırıldı. Sonuç, RelA/p65'in mRNA ve protein düzeylerinin virülansları farklı NDV suşlarıyla enfekte CEF'de belirgin oranda arttığını ve RelA/p65'in sitoplazmadan çekirdeğe transloke olduğunu gösterdi. Ayrıca, araştırma spesifik siRNA'nın RelA/p65 ekspresyonunu inhibe edebildiğini, buna uygun olarak, IFN-α, IFN-β ve STAT1 mRNA düzeylerinin önemli ölçüde azaldığını ve 2 NDV'nin replikasyon kinetiğinin RelA/p65 inhibisyonu sonrası geliştirildiğini gösterdi. Sonuç olarak; CEF, NDV enfeksiyonu sonrasında RelA/p65 yolakları aracılığıyla daha fazla IFN türü sentezleyebilir ki, bu NDV replikasyonunu geciktirir ve viral enfeksiyonun erken döneminde yayılma gösterir.

Anahtar sözcükler: *NDV, RelA/p65, İnterferon, Virüs replikasyonu, CEF*

INTRODUCTION

Newcastle disease (ND) is recognized as a significant poultry disease; it is caused by Newcastle disease virus (NDV), a member of the Paramyxoviridae family [1]. NDV is a non-segmented and single-stranded negative-sense RNA virus. NDV isolates are usually classified into three groups on the basis of their virulence: non-virulent (lentogenic), mildly virulent (mesogenic) and highly virulent (velogenic). The NDV genome is approximately 15200 nucleotides long, and it composed of six units that encode their corresponding structural proteins: nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), and a

İletişim (Correspondence)
+86 20 85283054; Fax: +86 20 5280242
rentao6868@126.com

RNA-dependent RNA polymerase (L) [1,2]. RNA editing of the P protein produces additional non-structural proteins V and W [3]. NDV V protein can block various components of interferon (IFN) pathways to help the virus replication and spread in host [3-6].

The host's innate immune response to virus infection is an immediate reaction designed to retard virus growth and help the host develop specific protection [7-9]. One key protein that regulates the response is the nuclear transcription factor, NF-kB, which is held quiescent in the cytoplasm when incomplex with IkBa [10,11]. Once in response to virus via transmembrane toll-like receptors (TLRs) or the cytoplasmic sensors (e.g., RIG-I, MDA5, PKR and NOD proteins), IkBa is phosphorylated by IKK and subject to ubiquitin-dependent proteasomal degradation, allowing NF-kB to translocate to the nucleus from the cytoplasm and activate the transcription of a cascade of proinflammatory cytokines and chemokines to induce inflammatory and antiviral responses [12-15]. Of particular interest is RelA/p65, a subunit of the heterodimeric NF-kB protein, which had gained attention for its diverse roles. It plays a central role in initiating the host's antiviral responses by inducing IFNs and ISGs [16,17].

Past research showed that NDV not only could induce IFNα and IFNβ mRNA in macrophages, IFNγ mRNA in peripheral blood mononuclear cells *in vitro* [18-20], but also IFNα and IFNγ mRNA in splenocytes and peripheral blood *in vivo* [21]. Yet this existing knowledge of NDV does not provide a full understanding role of RelA/p65 in the IFN pathway, and whether the pathway affects NDV replication. Here, using an in vitro model of NDV infection in CEF, we examined the global expression of RelA/p65, IFN-α/β and STAT-1, analysed the sub-cellular localisation of RelA/p65 protein in NDV-infected CEF, and determined NDV replication kinetics after inhibition of RelA/p65. The experimental results indicated that two strains of NDV, which differed in their virulence could upregulate the RelA/p65-mediated pathway, increased the expression of IFN-α and IFN-β, and limited the NDVs replication.

MATERIAL and METHODS

Cell Culture and Viruses

CEF and Vero cells were cultured under an atmosphere of 5% CO_2 at 37°C in D-Minimum Essential Medium (D-MEM, GIBCO®) supplemented with 10% fetal bovine serum (FBS) (PAA Laboratories, Ontario, Canada). The velogenic NDV strain GM (of VII genotype) was stored in our laboratory and the lentogenic NDV strain LaSota was a kind gift from the National Reference Laboratory for Newcastle Disease. The GenBank accession numbers of the GM and LaSota strains are DQ486859 and AF077761, respectively. The virus titers were determined on CEF by standard endpoint dilution and expressed as 50% tissue culture infection doses ($TCID_{50}$) per ml. UV-inactivation of viruses were performed by using a UV Stratalinker 2400 (Stratagene, USA) to prevent the expression of NDV hemagglutinin-neuraminadase (HN) viral gene, and evaluated by immuno-fluorescence analysis.

Real-time Quantitative Polymerase Chain Reaction (qPCR)

CEF were infected with NDV strain LaSota or GM at a multiplicity of infection (MOI) of 0.1 $TCID_{50}$/cell when they were grown to 70-80% confluence in 12-well plates. The cells were harvested at 3, 6, 12 and 24 h post infection (h p.i). Total RNA was extracted by using RNeasy® Mini kit (Promega, U.S.) and was used as a template for synthesizing cDNA strands. QPCR was prepared by using the following primers: 5'-AAGCCGACAAAACCACCC-3' (IFN-α-sense), 5'-CAGGAACCAGGCACGAGC-3' (IFN-α-anti-sense), 5'-CCTCAACCAGATC -CAGCATT-3'(IFN-β-sense) and 5'-GGATGAGGCTGTGAGAGGAG-3'(IFN-β-antisense), 5'-TG AACGGATCCGCACCAATA-3' (RelA/p65-sense), 5'-CGTTCAC CCACACCTGGAAG -3 '(RelA/p65-antisense), 5'-GAACGGCTA CATTAGGACTG-3' (STAT1-sense), 5'-GATCCGAGA-TACCT CATCAAAC-3' (STAT1-antisense), 5'-CCTCTCTGGCAAAGTCC AAG -3' (GAPDH-sense), 5'-CATCTGCCCATTTGATGTTG -3' (GAPDH-antisense).

Real time PCR (SYBR green) was performed by the ABI 7500 real-time PCR detection system (Roche Applied Bio Systems, Germany). The Tm and Cp values were calculated using the analysis software provided by Roche Applied Science. The experimental data on the mRNA levels of RelA/p65, IFN-α, IFN-β and STAT1 genes were normalized against internal controls, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) gene. Fold changes in the expression of each gene were compared with expression level in non-infected CEF, and analyzed by a comparative threshold cycle (Ct) method using the formula $2^{-(\Delta\Delta Ct)}$.

Preparation of Cell Extract and Western Blot Analysis

Cells were washed three times with phosphate buffered saline (PBS), and then were lysed by a RIPA buffer containing protease inhibitor PMSF. Lysates were incubated on ice for 10 min and then spun at 12.000xg for 10 min; clarified supernatants were stored at -80°C. The supernatants were diluted 1:1 in a 2×Tris-glycine SDS sample buffer (Invitrogen, U.S.). Proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 10% Tris-glycine gels and then transferred onto nitrocellulose (NC) membranes. The membranes were blocked with 5% skimmed milk and then incubated with anti-RelA p65 antibody which could detect RelA/p65 for chicken (Abcam, U.K.). Following primary incubation, a western blot analysis was performed with a FITC-conjugated goat anti-rabbit antibody (Millipore, Billerica, U.S.) according to the manufacturer's protocol. Protein bands were imaged and quantified with the

Odyssey infrared imaging system (Li-Cor, Odyssey). The GAPDH expression levels, measured in parallel, served as a control.

Immunofluorescence Staining

CEF were infected by NDV (LaSota or GM) with MOI of 0.1 $TCID_{50}$/cell or were treated by NDV (LaSota or GM)-UV with same titer for 24 h. The cells were fixed with 4% paraformaldehyde for 15 min and incubated with anti-RelA/p65 antibody (Abcam, U.K.) in PBS containing 0.1% BSA at 4°C overnight. Following incubation with FITC-conjugated goat anti-rabbit antibody (Millipore, Billerica, U.S.) for 1 h, the cells were stained with the nuclei, 4′, 6-diamidino-2-phenylindole (DAPI; Sigma) for 10 min. After the cells were washed three times, they were photographed with a laser copolymerization microscope (Olympus, Japan).

RelA/p65 siRNA Treatment

CEF were seeded to 30% confluence into 12-well dishes the day before transfection with small interfering RNA (siRNA). The spectific siRNA (GenePharma, China) for chicken RelA/p65 (the sense strand is 5′- CGUGCACAGU UUCCAGAAUTT-3′ and the antisense strand is 5′-AUUCUGG AAACUGUGCACGTT-3′) and non-targeting siRNA (the sense strand is 5′- UUCUCCGAACGUGUCACGUTT-3′ and the antisense strand is 5′-ACGUGACACGUUCGG AGAATT-3′) was transfected into cells at a concentration of 40 pM using the Lipofectamine RNAi Max reagent (Invitrogen, U.S.), Knockdown efficiency was monitored using qPCR analysis for RelA/p65. The mRNA levels of IFN-α, IFN-β and STAT1 were also measured at 24 h following RelA/p65-siRNA transfection into CEF. Fold changes in the expression of each gene were compared with expression level in normal cells.

NDV Growth Kinetics

RelA/p65-siRNA (40 pM) was transfected into CEF when the cells were grown to 70-80% confluence in 6-well plates. Twenty-four hours later, the cells were infected with NDV strain LaSota with MOI of 1 $TCID_{50}$/cell and GM with MOI of 0.1 $TCID_{50}$/cell, respectively. One h after virus adsorption, the cells were incubated in virus-production medium (basal D-MEM with 2 percent FBS). Supernatants were harvested every 12 h and virus titers were determined by $TCID_{50}$ on Vero cells. Briefly, Vero cells were seeded in 96-well plates at a density of 10^4 cell per well. The following day, Vero cells were infected with 100 uL virus samples of serial tenfold dilutions. One hour after virus adsorption, the cells were incubated in D-MEM with 2% FBS for 7 days, and then analysed for cytopathic effect to determine $TCID_{50}$, as described previously [22].

Statistical Analysis

The statistical significance of experiment data was determined by Student's *t*-test.

RESULTS

NDV up-regulate mRNA Levels of IFN-α, IFN-β, STAT1 and RelA/p65

To decipher how NDV enhanced the transcriptional activity of IFN-α, IFN-β and STAT1, we first examined whether NDV altered their mRNA levels along with that of RelA/p65 in NDV-infected CEF. Our results indicate that two strains of NDV could differentially modulate mRNA levels of the four molecules which are central to the innate antiviral response. As shown in *Fig. 1*, after 3 h of NDV LaSota infection CEF, the mRNA levels of IFN-α, IFN-β, STAT1 and RelA/p65 were strongly increased by 92, 8, 19 and 23 fold, respectively, in comparison with normal control cells. As time went by, the mRNA levels of the four molecules followed an obvious downward trend. The highly virulent strain GM also induced CEF up-regulated mRNA levels of the four molecules compared to the control group, after 3 h of GM infection CEF, the mRNA levels of IFN-α, IFN-β, STAT1 and RelA/p65 were increased by 2.8, 1.4, 1.5 and 12 fold, respectively, in comparison with normal control cells. Compared to strain LaSota, the strain GM induced a lower mRNA level of the four molecules in infected CEF. Taken together, we know that two strains of NDV, which differ in their virulence, upregulated mRNA levels of IFN-α, IFN-β, STAT1 and RelA/p65 in CEF, but there were obvious differences between the two NDVs induction mRNA levels of the four molecules.

NDV up-regulate Protein Level of RelA/p65

Western blot analysis was carried out to demonstrate the protein level of RelA/p65 in NDV-infected CEF. The results showed that NDV could induce CEF to synthesize more RelA/p65 protein. We also found differences in the relative amount of RelA/p65 protein induced by two stains of NDV. In NDV strain LaSota infection CEF, the RelA/p65 protein was increased markedly at 3 h p.i., and the amount reached to peak at 12 h p.i. Compared to the control group, the RelA/p65 protein was expressed at a higher level, which was stably maintained after NDV GM infection *(Fig. 2)*.

NDV Promote RelA/p65 Localization in the Nucleus

It has been reported that RelA/p65 is held quiescent in the cytoplasm in cells. In this study, CEF were infected with the NDV of lentogenic strain LaSota or the velogenic strain GM to reveal the subcellular distribution of RelA/p65 (green) and the nuclei (blue). RelA/p65 showed a predominantly nuclear localization pattern in CEF (*Fig. 3,* up two panels). However, in the mock-infected CEF cells, RelA/p65 was localized predominantly in the cytoplasm (*Fig. 3,* middle panel). In order to further gain insight into the translocation mechanism of RelA/p65, the laser confocal analysis was conducted after CEF cells were treated by UV-inactivated NDV strain GM or LaSota for 24 h and the

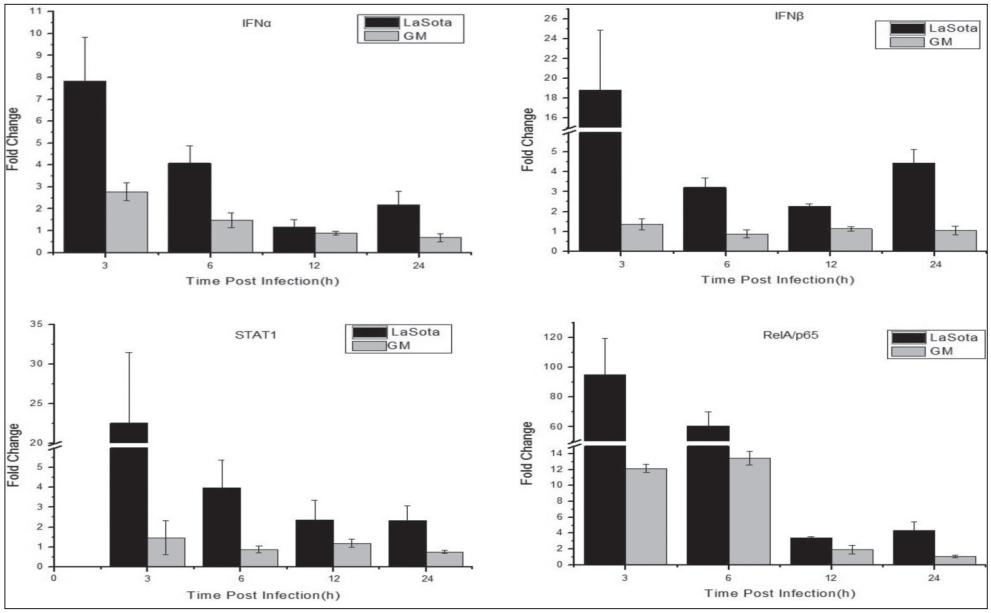


Fig 1. Two stains of NDV alter mRNA levels of IFN-α, IFN-β, STAT1 and RelA/p65 in CEF. After CEF were infected with NDV LaSota or GM at a MOI of 0.1 $TCID_{50}$/cell, the cells were harvested at 3, 6, 12 and 24 h p.i. Total RNA was extracted and analyzed by qPCR on mRNA levels of IFN-α, IFN-β, STAT1 and RelA/p65. The data were normalized by the internal control, the GAPDH gene, and the fold-change was relative to uninfected cells

Şekil 1. İki NDV suşu CEF'teki IFN-α, IFN-β, STAT1 düzeylerini ve RelA/p65'i değiştirir. CEF'nin 0.1 MOI $TCID_{50}$/hücre dozda NDV LaSota veya GM ile enfekte edilmesinin ardından, hücreler enjeksiyon sonrası (p.i) 3, 6, 12 ve 24. saatte toplandı. Total RNA mRNA üzerindeki IFN-α, IFN-β, STAT1ve RelA/p65 düzeyleri qPCR ile eksrakte edildi ve analiz edildi. Data iç kontrol ile normalize edildi, GADPH geni ve kat-değişimi non-enfekte hücrelere yakın idi

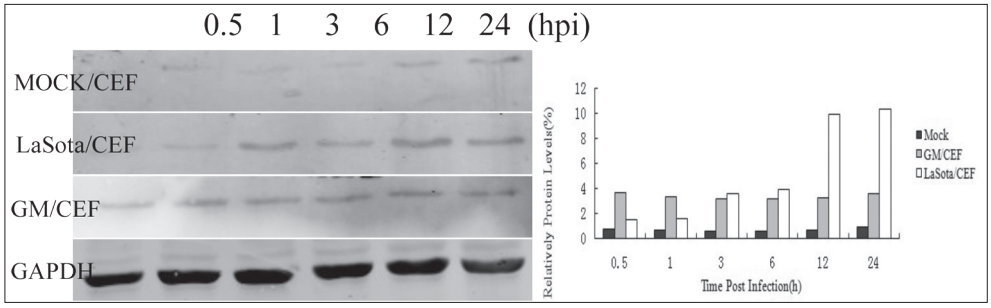


Fig 2. NDV alters the protein expression levels of RelA/p65. CEF were infected with the indicated strains of NDV LaSota or GM (MOI of 0.1 $TCID_{50}$/cell). Total cellular proteins were harvested at 0.5, 1, 3, 6, 12 and 24 h p.i and separated by SDS-PAGE. A western blotting analysis of proteins resolved by SDS-PAGE was performed with antibodies of RelA/p65 and GAPDH protein. Proteins were quantified with the Odyssey infrared imaging system. RelA/p65 protein band intensities were normalized to GAPDH

Şekil 2. NDV RelA/p65 protein ekspresyon düzeylerini değiştirir. CEF, belirtilen NDV LaSota veya GM (0,1 $TCID_{50}$/hücre MOI) suşları ile enfekte edildi. Toplam hücresel proteinler enjeksiyon sonrası 0.5, 1, 3, 6, 12 ve 24. saatte toplandı ve SDS-PAGE ile ayrıldı. SDS-PAGE ile yeniden çözülmüş proteinlerin RelA/p65 ve GAPDH protein antikorları ile Western blot analizi gerçekleştirildi. Proteinler Odyssey kızılötesi görüntüleme sistemi ile ölçüldü. RelA/p65 protein bandı yoğunlukları GAPDH için normalize edildi

results showed that RelA/p65 located predominantly in the cytoplasm (*Fig. 3*, lower two panels).

Inhibition of RelA/p65 down-regulates IFN-α, IFN-β and STAT1

To confirm the role of RelA/p65 in the NF-kB signal, we inhibited RelA/p65 in CEF by specific siRNA. The specific siRNA treatment resulted in significant decline of RelA/p65 by 78 percent at the mRNA level in CEF compared to normal cells. In order to examine whether RelA/p65 regulates the NF-kB signal, mRNA levels of IFN-α, IFN-β and STAT1 were also detected. Real time PCR showed that inhibition of RelA/p65 also led to the down-regulation of the mRNA level of IFN-α, IFN-β and STAT1 by 38, 55 and 61 percent *(Fig. 4)*. Compared to normal cells, the fold change of mRNA levels of RelA/p65, IFN-α, IFN-β and STAT1 were not apparent after treatment cells with non-targeting siRNA of RelA/p65.

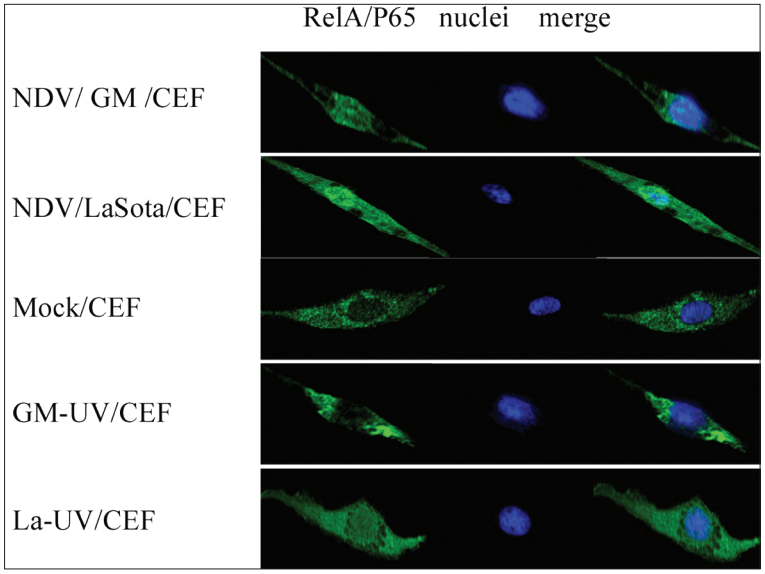
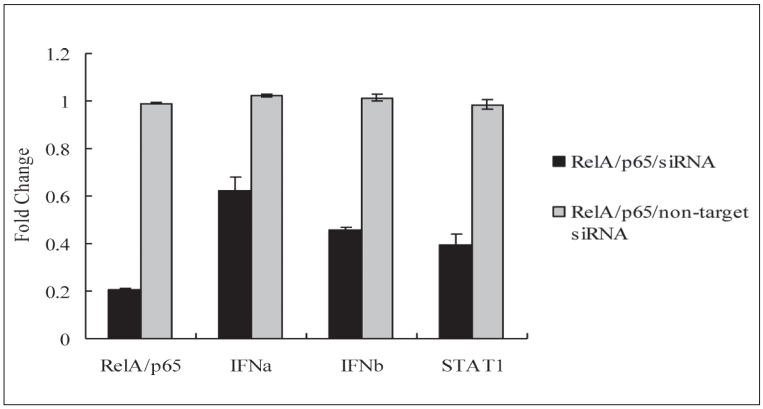


Fig 3. Subcellular localization of RelA/p65 protein in CEF. CEF were infected by NDV strain LaSota or GM with MOI of 0.1 $TCID_{50}$/cell, or were treated with UV-inactivated NDV (NDV-UV) strain LaSota or GM for 24 h. After cells were fixed with 4% paraformaldehyde for 15 min, they were incubated with rabbit RelA/p65 antibody at 4°C overnight and then stained with FITC-conjugated goat anti-rabbit antibody. To stain the nuclei, DAPI was added to CEF at room temperature for 10 min. The cells were observed under a laser copolymerization microscope

Şekil 3. CEF'de RelA/p65 proteininin hücre-içi lokalizasyonu. CEF ya 0.1 TCID50/hücre MOI doz NDV LaSota veya GM suşu ile enfekte edildi, ya da 24 saat süreyle UV ile etkisizleştirilmiş NDV (NDV-UV) LaSota veya GM suşu ile muamele edildi. Hücreler 15 dak. boyunca %4 paraformaldehit ile fikse edildikten sonra, gece boyunca tavşan RelA/p65 antikoru ile 4°C'de inkübe edildikten sonra FITC-konjügeli keçi anti-tavşan antikoru ile boyandı. Çekirdekleri boyamak amacıyla CEF'e 10 dak. için oda sıcaklığında DAPI ilave edildi. Hücreler bir lazer kopolimerizasyon mikroskobu altında gözlendi

Fig 4. Inhibition of RelA/p65 down-regulates the mRNA level of IFN-α, IFN-β, and STAT1. When CEF were grown to 70-80% confluence in 12-well plates, the cells were treated with 40 pM oligonucleotide of RelA/p65 siRNA or control siRNA for 24 h. Then the cells wereæ collected to extract total RNA, the mRNA levels of RelA/p65, IFN-α, IFN-β and STAT1 were determined by qPCR, and the fold-change was relative to normal CEF

Şekil 4. RelA/p65 inhibisyonu IFN-a, IFN-p ve STAT1 mRNA düzeyini aşağı-yöne doğru çevirir. CEF 12-yuvalı plakalar içinde %70-80 kaynaşmaya kadar büyütüldüğünde, hücreler RelA/p65 siRNA'nın 40 uM oligonükleotidi veya kontrol siRNA ile 24 saat boyunca işlemden geçirildi. Daha sonra hücreler, toplam RNA elde etmek için toplandı, RelA/p65, IFN-a, IFN-p ve STAT1 mRNA düzeyleri qPCR ile belirlendi ve kat-değişimi normal CEF'e yakındı



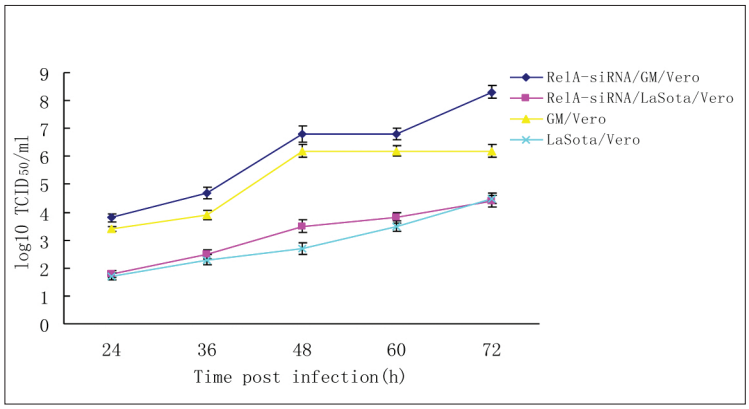


Fig 5. Inhibition of RelA/p65 enhancement of NDV production kinetics. When CEF were grown to 70-80% confluence in 6-well plates, the cells were treated with 40 pM oligonucleotide of RelA/p65 siRNA or control siRNA for 24 h and were infected by NDV strain GM with MOI of 0.1 and LaSota with MOI of 1 $TCID_{50}$/cell, respectively. The cell supernatant was collected at 24, 36, 48, 60 and 72 h p.i., and $TCID_{50}$ was determined on Vero cells

Şekil 5. NDV üretim kinetiğinin RelA/p65 artışını engellemesi. CEF 6-yuvalı plakalar içinde %70-80'lik bir kaynaşmaya kadar büyütüldüğünde, hücreler 40 uM RelA/p65 siRNA oligonükleotidi veya kontrol siRNA ile 24 saat boyunca işlemden geçirildi ve sırasıyla 0.1 MOI NDV LaSota ve 1 $TCID_{50}$/hücre MOI GM suşu ile enfekte edildi. Hücre süpernatantı enjeksiyon sonrası 24, 36, 48, 60 ve 72. saatte toplandı ve Vero hücrelerindeki $TCID_{50}$ belirlendi

Inhibition of RelA/p65 Enhance NDV Replication

To investigate the effect of RelA/p65 signals on virus production, after CEF were treated with RelA/p65 siRNA for 24 h, the cells were infected with NDV strain GM or LaSota. The cell supernatants were collected to examine the virus-production kinetics on Vero cells. In our study, whether the NDV strain velogenic GM or lentogenic LaSota, if RelA/p65 had been inhibited in advance, the virus-production kinetics were higher than that of the control group 36 h p.i *(Fig. 5)*.

DISCUSSION

The early response to a virus causes host activation of transcription factors NF-κB, interferon regulatory factors (IRFs) and activator protein-1 (AP-1) (ATF-2/c-jun) through pattern recognition receptors (PRRs), recognized pathogen-associated molecular patterns (PAMPS), and further results in secretion IFNs [23-25]. Once type I IFN are synthesized, they act in both an autocrine and paracrine manner during the innate immune response to retard virus replication by

activating the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signal transduction cascade, leading to the activation of ISGs to further limit virus replication [7,26,27].

As a subunit of the heterodimeric NF-kB protein, RelA/p65 plays an essential role in immune response. Virus entry in the host can lead to the activation of RelA/p65 and then the activation of IFN-α/β genes via the RelA/p65 signal [28-30]. However, the research of the RelA/p65 signal in animal virus infections has been limited to mammals or their cell, it is rare to study the effect of the signal on viral replication in birds, especially, the action of the RelA/p65 signal on NDV replication in CEF. Based on these considerations, we chose cell CEF as the primary cell to research the change of the RelA/p65 signal after NDV infection, and chose Vero cell which is deficient in interferon production to support efficient production of NDV for studying anti-viral responses.

In vitro or *in vivo* experiments suggest that a pronounced and rapid innate immune response may be induced by NDV. In this study, we measured mRNA levels of IFN-α, IFN-β, STAT1 and RelA/p65 in NDV-infected CEF. Our study showed that the mRNA levels of RelA/p65, IFN-α and IFN-β are increased obviously after lentogenic or velogenic NDV infection CEF, and correspondingly, the mRNA level of STAT1 was also increased. These results are in agreement with previous studies [20,21], but we also found that lentogenic and velogenic NDV have different ability to induce CEF express IFN-α, IFN-β, STAT1 and RelA/p65. An important reason perhaps interrelated to V protein of NDV which functions as an IFN-α/β antagonist by targeting STAT1 for degradation [2,5,6]. The difference of V proteins derived from the velogenic NDV-GM and the lentogenic NDV-LaSota maybe have different ability to target STAT1, and which is worthy of further study. The other reason could be interrelated to apoptosis. NDV is known to cause apoptosis in different cell types including CEF cells, Vero cells and peripheral blood mononuclear cells, NDV-induced apoptosis required virus entry and replication [31-33], different apoptosis rate induced by lentogenic and velogenic NDV maybe leading to differences of the protein production synthesized by CEF.

On the basis of the above research, Western blot analysis was carried out to demonstrate the protein level of RelA/p65 in NDV-infected CEF. The results showed that NDV could induce CEF to synthesize more RelA/p65 protein, but different virulent NDVs have different capabilities to activating RelA/p65-mediated signaling pathways. After completing above study, we performed immunofluorescence staining to confirm the subcellular localization of RelA/p65. Immunofluorescence staining demonstrated that RelA/p65 protein had a predominantly nuclear localization pattern in NDV-infected cells. While for the mock-infection CEF and NDV-UV treatment group, the RelA/p65 protein was mainly distributed to the cytoplasm. The study showed that NDV could activate the RelA/p65 signal and induce the RelA/p65 protein shift from the cytoplasm to the nucleus while inactivated NDV did not exert this function in CEF, which suggesting that the nuclear translocation of RelA/p65 is probably related to virus replication.

In order to further determine the role of the RelA/p65 signal to different NDV replications, we knocked down RelA/p65 with a specific siRNA in CEF. Real time PCR showed that the inhibition of RelA/p65 also led to down-regulating the mRNA level of IFN-α, IFN-β and STAT1. The results further indicated that RelA/p65 played an important role in the process of anti-viral actions. Subsequent studies revealed that the NDV production kinetics were enhanced after the inhibition of RelA/p65 in CEF. The results indicated that a RelA/p65-mediated signaling pathway could limit NDV replication and that RelA/p65 played an important role in the process of anti-viral actions.

This study showed that a RelA/p65-mediated signaling pathway could be activated after CEF were infected by NDV, and this change in the signal could limit the replication of NDV. Therefore, this finding will provide a theoretical foundation of the pathogenic mechanism in RelA/p65-mediated hosts' innate immune responses caused by NDV.

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REFERENCES

1. Alexander DJ: Newcastle disease and other avian paramyxoviruses. *Rev Sci Tech,* 192, 443-462, 2000.

2. Huang Z, Panda A, Elankumaran S, Govindarajan D, Rockemann DD, Samal SK: The hemagglutinin-neuraminidase protein of Newcastle disease virus determines tropism and virulence. *J Virol,* 78, 4176-4184, 2004. DOI: 10.1128/JVI.78.8.4176-4184.2004

3. Steward M,Vipond IB, Millar NS, Emmerson PT: RNA editing in Newcastle disease virus. *J Gen Virol,* 74, 2539-2547, 1993. DOI: 10.1099/0022-1317-74-12-2539

4. Huang Z, Krishnamurthy S, Panda A, Samal SK: Newcastle disease virus V protein is associated with viral pathogenesis and functions as an alpha interferon antagonist. *J Virol,* 77, 8676-8685, 2003. DOI: 10.1128/JVI.77.16.8676-8685.2003

5. Nishio M, Tsurudome M, Ito M, Garcin D, Kolakofsky D, Ito Y: Identification of paramyxovirus V protein residues essential for STAT protein degradation and promotion of virus replication. *J Virol,* 79, 8591-8601, 2005. DOI: 10.1128/JVI.79.13.8591-8601.2005

6. Park MS, Garcia-Sastre A, Cros JF, Basler CF, Palese P: Newcastle disease virus V protein is a determinant of host range restriction. *J Virol,* 77, 9522-9532, 2003. DOI: 10.1128/JVI.77.17.9522-9532.2003

7. Honda K, Takaoka A,Taniguchi T: Type I interferon gene induction by the interferon regulatory factor family of transcription factors. *Immunity,* 25, 349-360, 2006. DOI: 10.1016/j.immuni.2006.08.009

8. Sharma S, tenOever BR, Grandvaux N, Zhou GP, Lin R, Hiscott J: Triggering the interferon antiviral response through an IKK-related pathway. *Science,* 300, 1148-1151, 2003. DOI: 10.1126/science.1081315

9. Yoneyama M, Suhara W, Fukuhara Y, Fukuda M, Nishida E, Fujita

T: Direct triggering of the type I interferon system by virus infection: Activation of a transcription factor complex containing IRF-3 and CBP/p300. *EMBO J,* 7, 1087-1095, 1998. DOI: 10.1093/emboj/17.4.1087

10. Chakraborty JB, Mann DA: NF-kappaB signaling: embracing complexity to achieve translation. *J Hepatol,* 52, 285-291, 2010. DOI: 10.1016/j.jhep.2009.10.030

11. Chen LF, Greene WC: Shaping the nuclear action of NF-kappaB. *Nat Rev Mol Cell Biol,* 5, 392-401, 2004. DOI: 10.1038/nrm1368

12. Kato H, Takeuchi O, Sato S: Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature,* 441, 101-105, 2006. DOI: 10.1038/nature04734

13. Liu P, Jamaluddin M, Li K, Garofalo RP, Casola A, Brasier AR: Retinoic acid-inducible gene I mediates early antiviral response and Toll-like receptor 3 expression in respiratory syncytial virus-infected airway epithelial cells. *J Virol,* 81, 1401-1411, 2007. DOI: 10.1128/JVI.01740-06

14. Loo YM, Fornek J, Crochet N, Bajwa G, Perwitasari O, Martinez-Sobrido L, Akira S, Gill MA, Garcı´a-Sastre: Distinct RIG-I and MDA5 signaling by RNA viruses in innate immunity. *J Virol,* 82, 335-345, 2008. DOI: 10.1128/JVI.01080-07

15. Zhang G, Ghosh S: Toll-like receptor-mediated NF-kappaB activation: Aphylogenetically conserved paradigm in innate immunity. *J Clin Invest,* 107, 13-19, 2001. DOI: 10.1172/JCI11837

16. Kiernan R, Bres V, Ng RW, Coudart MP, Messaoudi S: Post-activation turn-off of NF-kappaB-dependent transcription is regulated by acetylation of p65. *J Biol Chem,* 278, 2758-2766, 2003. DOI: 10.1074/jbc.M209572200

17. Schmitz ML, Baeuerle PA: The p65 subunit is responsible for the strong transcription activating potential of NF-kappaB. *EMBO J,* 10, 3805-3817, 1991.

18. Ahmed KA, Saxena VK, Ara A, Singh KB, Sundaresan NR, Saxena M, Rasool TJ: Immune response to Newcastle disease virus in chicken lines divergently selected for cutaneous hypersensitivity. *Int J Immunogenet,* 34, 445-455, 2007. DOI: 10.1111/j.1744-313X.2007.00722.x

19. Sick C, Schultz U, Mu ¨nster U, Meier J, Kaspers B, Staeheli P: Promoter structures and differential responses to viral and nonviral inducers of chicken type I interferon genes. *J Biol Chem,* 273, 9749-9754, 1998. DOI: 10.1074/jbc.273.16.9749

20. Cary AR, Leonardo S, Ingrid C, Corrie CB, Darrell RK, David LS, Daniel JK, Patti JM, Claudio LA: Virulent Newcastle disease virus elicits a strong innate immune response in chickens. *J Gen Virol,* 92, 931-939, 2011. DOI: 10.1099/vir.0.025486-0

21. Mo CW, Cao YC, Lim BL: The *in vivo* and *in vitro* effects of chicken interferon alpha on infectious bursal disease virus and Newcastle disease virus infection. *Avian Dis,* 45, 389-399, 2001. DOI: 10.2307/1592978

22. Hamilton MA, Russo RC, Thurston RV: Trimmed Spearman-Kärber CPE method for estimating median lethal concentrations in toxicity bioassays. *Environ Sci Technol,* 13, 714-719, 1977. DOI: 10.1021/es60130a004

23. Dey N, Liu T, Garofalo RP, Casola A: TAK1 regulates NF-KappaB and AP-1activation in airway epithelial cells following RSV infection. *Virology,* 418, 93-101, 2011. DOI: 10.1016/j.virol.2011.07.007

24. Hayden MS, Ghosh S: Shared principles in NF-kappaB signaling. *Cell,* 132, 344-362, 2008. DOI: 10.1016/j.cell.2008.01.020

25. Hiscott J: Convergence of the NF-kB and IRF pathways in the regulation of the innate antiviral response. *Cytokine Growth Factor Rev,* 18, 483-490, 2007. DOI: 10.1016/j.cytogfr.2007.06.002

26. Pansky A, Hildebrand P, Fasler-Kan E: Defective Jak-STAT signal transduction pathway in melanoma cells resistant to growth inhibition by interferon-alpha. *Int J Cancer,* 85, 720-725, 2000. DOI: 10.1002/(SICI)1097-0215(20000301)85:5<720::AID-IJC20>3.0.CO;2-O

27. Thoennissen NH, Iwanski GB, Doan NB, Okamoto R, Lin P, Abbassi S, Song JH, Yin D, Toh M, Xie WD, Said JW, Koeffler HP: Cucurbitacin B induces apoptosis by inhibition of the JAK/STAT pathway and potentiates antiproliferative effects of gemcitabine on pancreatic cancer cells. *Cancer Res,* 69, 5876-5884, 2009. DOI: 10.1158/0008-5472.CAN-09-0536

28. Chen LF, Mu Y, Greene WC: Acetylation of RelA at discrete sites regulates distinct nuclear functions of NF-kappaB. *EMBO J,* 21, 6539-6548, 2002. DOI: 10.1093/emboj/cdf660

29. Sethi G, Sung B, Aggarwal BB: Nuclear factor-kappaB activation: From bench to bedside. *Exp Biol Med,* 233, 21-31, 2008. DOI: 10.3181/0707-MR-196

30. Vallabhapurapu S, Karin M: Regulation and function of NF-kappaB transcription factors in the immune system. *Annu Rev Immuno,* I27, 693-733, 2009. DOI: 10.1146/annurev.immunol.021908.132641

31. Geisler F, Algul H, Paxian S, Schmid RM: Genetic inactivation of RelA/p65 sensitizes adult mouse hepatocytes to TNF-induced apoptosis *in vivo* and *in vitro. Gastroenterology,* 132, 2489-2503, 2007. DOI: 10.1053/j.gastro.2007.03.033

32. Lam KM: Apoptosis in chicken embryo fibroblasts caused by Newcastle disease virus. *Vet Microbiol,* 47, 357-363, 1995. DOI: 10.1016/0378-1135(95)00111-5

33. Lam KM: Newcastle disease virus-induced apoptosis in peripheral blood mononuclear cells of chickens. *J Comp Pathol,* 114, 63-71, 1996. DOI: 10.1016/S0021-9975(96)80063-6