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# Japanese Encephalitis Virus infection induces inflammation of swine testis through RIG-I—NF-κB signaling pathway

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ABSTRACT

Japanese Encephalitis Virus (JEV) is an important zoonotic flavivirus transmitted by mosquitos. JEV infection in sows primarily manifests as a reproductive disease such as abortion and transient infertility while in infected boars, it can cause orchitis. Previous studies mainly focused on the pathogenesis of human encephalitis caused by JEV infection, while few concentrations have been made to unveil the potential mechanism of reproductive dysfunction in JEV-infected pigs. In this study, histopathological analysis and immunohistochemistry staining was performed on testis of JEV-infected boars, indicating that JEV could infect testicular cells and cause inflammatory changes in testis. In vitro assays reveal that primary swine testicular cells and swine testis (ST) cells are highly permissive to JEV and significant inflammatory response was shown during JEV infection. Mechanically, we found that JEV infection increases the expression of retinoic acid-inducible gene I (RIG-I) and activates transcription factor NF-κB. Production of pro-inflammatory cytokines was greatly reduced in JEV infected testicular cells after knockout of RIG-I or treatment with the NF-κB specific inhibitor. In addition, activation of NF-κB was also significantly suppressed upon RIG-I knockout. Taken together, our results reveal that JEV could infect boar testicles, and RIG-I—NF-κB signaling pathway is involved in JEV-induced inflammation in swine testicular cells.

## 1. Introduction

Japanese encephalitis virus (JEV), a mosquito-borne flavivirus, was first isolated from a human in Japan in 1935 (Solomon et al., 2000), and in last few years several cases were reported in South and Southeast Asia (Unni et al., 2011). It causes severe central nervous system diseases in humans, which led to 10,000–15,000 deaths annually (Ghosh and Basu, 2009). JEV is mainly transmitted by *Culex*, domestic pigs and water birds. Humans are the dead-end hosts (Burke et al., 1985), because JEV does not spread from person to person through mosquitoes (Vaughn and Hoke, 1992).

Pigs play an important role in JEV transmission, since they live close to human and could carry viremia after infection, which is one of the major cause for JEV transmission to human (Tiroumourougane et al., 2002). JEV spreads among adult pigs and primarily infects reproductive tract leading to abortions in sows and orchitis in boars (Ogasa et al., 1977; Straw et al., 1999). This could threaten the agricultural economy in terms of food security issues (Mansfield et al., 2017).

Inflammation is a key mediator of host response against microbial pathogens (Shrivastava et al., 2016). Pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) or RIG-like receptors (RLRs) sense pathogen-associated molecular patterns (PAMPs), and then induce the production of inflammatory mediators (Poeck and Ruland, 2012). Although a controlled inflammatory response is beneficial, a robust inflammatory response will be harmful. JEV infection can produce IP-10 which contributes to break blood-brain barrier (Wang et al., 2018). JEV infection on glial cells causes releasing of various pro-inflammatory mediators, such as IL-6, TNF-a, and CCL2, by modulation of the ERK/p38MAPK/JNK and AP-1/NF-κB signaling pathways via recognition by TLR3 and RIG-I (Jiang et al., 2014; Ye et al., 2016). These

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**Table 1**
Primer pairs used for plasmid construction, quantitative real-time RT-PCR analyses.

| Primer name | Sequence (5′ → 3′) |
|---|---|
| β-actin-F | CTTCCTGGGCATGGAGTCC |
| β-actin-R | GGCGCGATGATCTTGATCTTC |
| TNF-a-F | GCCCAAGGACTCAGATCATC |
| TNF-a-R | GGCATTGGCATACCCACTCT |
| IL-6-F | CTGCTTCTGGTGATGGCTACTG |
| IL-6-R | GGCATCACCTTTGGCATCTT |
| IL-8-F | AGTTTTCCTGCTTTCTGCAGCT |
| IL-8-R | TGGCATCGAAGTTCTGCACT |
| RANTES-F | ACACCCTGCTGTTTTTCCTACCT |
| RANTES-R | AGACGACTGCTGCCATGGA |
| IL-1β-F | ACAAAAGCCCGTCTTCCTG |
| IL-1β-R | ATGTGGACCTCTGGGTATGG |

Notes: F for forward primer, R for reverse primer.

pro-inflammatory cytokines further recruit leukocytes, which aggravate inflammation and result in irreparable brain damage.

Currently, numerous studies have been done on revealing the pathogenesis of human encephalitis caused by JEV infection. However, the potential mechanisms of JEV caused reproductive dysfunction in pigs remain unclear. In this study, infectivity of JEV on swine testicular cells and the mechanism of JEV-caused inflammation in boar testis were investigated. Our data suggests that JEV can productively infect swine testicular cells and induce inflammation through RIG-I—NF-κB signaling pathway.

## 2. Materials and methods

### 2.1. Tissues, cells and viruses

All pig experimental procedures were approved by the Institutional Animal Care and Use Committee of Huazhong Agricultural University. 6 healthy Large White pigs at the age of 4 months were obtained from the farm in Wuhan, China. 3 pigs were injected with $10^8$ PFU of JEV P3 strain diluted with DMEM. The other 3 pigs in control group were injected with DMEM. Testes were collected and subjected to HE and IHC assays at 7days post infection. The primary testicular cells were isolated from testes of healthy pigs. Testicular parenchyma was cut into small pieces and digested by trypsin (2.5 g/L) at 37 degrees for 20 min. After centrifugation following blowing and filtering, the primary cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (NATOCOR), 100U/ml streptomycin, and 100 U/ml penicillin at 37 °C in a 5% CO2 atmosphere. Swine testis (ST) cells were maintained in Dulbecco modified Eagle medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (NATOCOR), 100 μg/ml streptomycin, and 100 U/ml penicillin at 37 °C in a 5% CO2 atmosphere. JEV P3 strain (GenBank accession no. U47032.1) was passaged in BHK-21 cells, and the titer of the virus was determined by plaque assay.

### 2.2. Virus infection

Primary swine testicular cells or ST cells were plated in 12-well plates. After washing with DMEM, cells were infected with JEV P3 strain for 1.5 h (h). The mock-infected cells were prepared using the same procedures with no virus.

### 2.3. RNA extraction and quantitative real-time RT-PCR

Total RNA of cells was extracted individually by Trizol reagent (Invitrogen), and 1 μg of RNA was used to synthesize cDNA using a first-strand cDNA synthesis kit (Toyobo). Quantitative real-time RT-PCR was performed using a 7500 real-time PCR system (Applied Biosystems) and SYBR green PCR master mix (Toyobo). Data were normalized to the level of β-actin expression in each sample. Primers used are listed in Table 1.

### 2.4. Plaque assay

ST cells were infected with JEV at 0.01 MOI. At 12, 24, 36, 48, 60 and 72 h post-infection, cell suspensions were harvested, serially diluted with DMEM, and then used to inoculate monolayers of BHK cells. After 1.5 h of absorption, the cells were washed with DMEM and cultured for 5 days in DMEM containing 3% fetal bovine serum and 1.5% sodium carboxymethyl cellulose (Sigma). The cells were then stained with crystal violet for 2 h, followed by fixation with 10% formaldehyde overnight. Visible plaques were counted, and the viral titers were calculated. All data are expressed as means of triplicate samples.

### 2.5. Antibodies

Mouse monoclonal antibodies against JEV E and NS5 proteins were generated in our laboratory (Chen et al., 2012). The purified E or NS5 protein was injected into the mouse, and the spleen cells were fused with the murine myeloma cells. The positive hybridoma cells were selected for intraperitoneal injection into the mouse and ascites was collected. The IgG was purified by commercial kit. Commercially obtained antibodies used were: rabbit polyclonal anti-DDX58 (ABclonal Technology), rabbit polyclonal anti-phospho-jun (ABclonal Technology), rabbit monoclonal anti-phospho-NF-κB p65 (Cell Signaling Technology), mouse monoclonal anti-GAPDH (ABclonal Technology).

### 2.6. H&E staining and immunohistochemistry

Swine testes samples were fixed in buffered formalin and processed for routine H&E. For immunohistochemistry staining, sections were incubated overnight at 4 °C with primary antibodies against JEV E, followed by application of the polymer for 30 min. The slides were counterstained with hematoxylin.

### 2.7. Western blotting

Total cellular lysates were prepared using radioimmunoprecipitation assay buffer (Sigma) containing protease inhibitors (Roche) and phosphatase (Roche). Protein concentrations were determined using a BCA protein assay kit (Thermo Scientific). Equal protein quantities were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Millipore) using a Mini Trans-Blot Cell (Bio-Rad). Blots were probed with the relevant antibodies, and proteins were detected using enhanced chemiluminescence reagent (Thermo Scientific)

### 2.8. Construction of RIG-I knockout cell line using CRISPR/Cas9 technology

The specific sgRNA which targeting pig RIG-I gene using sgRNAcas9 software (www.biootools.com). Two oligos for the sgRNA of RIG-I 5′ -CACCGAAACAACAAGGGCCCGACAG-3′ and 5′ -AAACCTGTCGGGCCC TTGTTGTTTC-3′, were cloned into the lentiCRlSPRv2 plasmid (Addgene, #52961). The virus was packaged in HEK 293 T cells. In 6-well plate, 140 μl opti-MEM I (invitrogen), 800 ng lentiCRlSPRv2 (Contains sgRNA), 800 ng pSPAX2 (Addgene #12260) and 400 ng pMD2.G (Addene #122591) mix well, and then add 8 u L Fugene HD transfection reagent (150 μ1 total). 48 h later, spun at 3000 rpm for 15 min to harvest the supernatant. Put ST cells in 24-well plate, 24 h later, add lentivirus with 8 ug/mL polybrene spun at 1000 g for 30 min at 32 ℃. At 24 h post-infection, puromycin (2.5 μg/ml) was added for selection. Cell clones with RIG-I knock out were confirmed by western blotting.

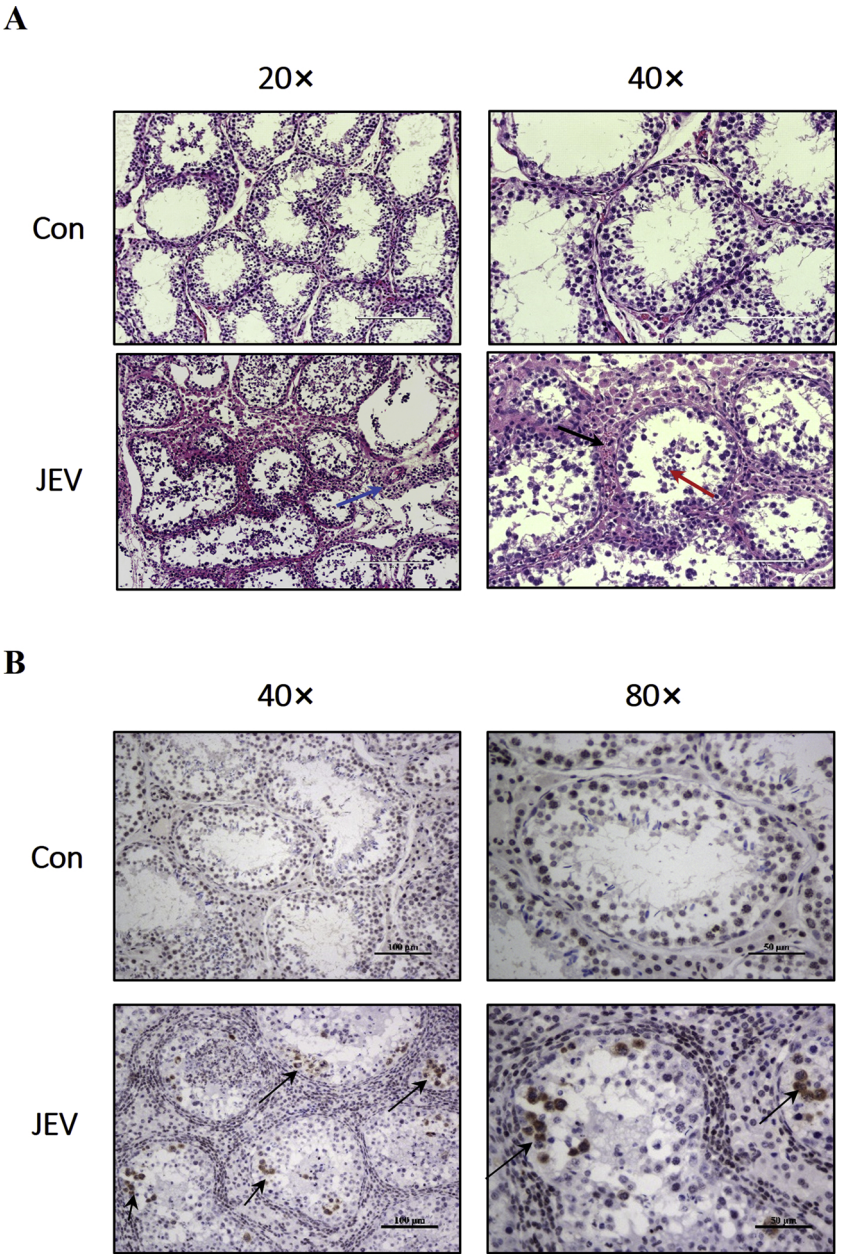


**Fig. 1.** Histopathological changes of the testes in boars infected with JEV. (A) Swine testes samples were fixed in buffered formalin and processed for routine H&E. Photomicrographs of H&E stained swine testes from JEV infected and uninfected control. Inflammatory cell infiltration and hemorrhages are indicated by arrows. (B) Immunohistochemistry staining was performed by using mAb against JEV E. JEV E positive expressed cells are indicated by arrows. Con is for Control group, JEV is for Japanese encephalitis virus infected group. 20×, 40×, 80× for magnification.

## 3. Results

### 3.1. JEV infects testis and leads to orchitis in swine

To characterize JEV infection, testes from JEV infected or uninfected swine were isolated. Histopathological analysis showed that cells in the seminiferous tubules are disorderly arranged, and a large number of spermatogonium are shed in the center of the seminiferous tubules (red arrow). There are a lot of red blood cells in the testicular stroma (black arrow) and inflammatory cells infiltration around the blood vessels (blue arrow). (Fig. 1A). These indicate severe damage of swine testes caused by JEV infection. IHC analysis was subsequently performed to detect JEV antigen in swine testes. The tissue sections were incubated with monoclonal antibody against JEV E and positive signals were mainly detected in spermatogenic cells and sertoli cells of JEV-infected swine (Fig. 1B), indicating that these cells may be the major target cells of JEV infection in swine testis.

### 3.2. Primary swine testicular cells and ST cells are permissive to JEV

To determine the infectivity of JEV to testicular cells, primary swine testicular cells and ST cells were infected with 5 MOI JEV. Viral protein was detected clearly in both JEV-infected primary swine testicular cells and ST cells at 12 h, 24 h and 36 h post-infection (Fig. 2A). Furthermore, western blotting was performed with cells infected with 1, 2, 5 or 10 MOI of JEV. Increasing amount of NS5 protein was detected with increasing MOI in both primary swine testicular cells and ST cells (Fig. 2B). Primary swine testicular cells and ST cells were then exposed to JEV at a MOI of 0.01, and viral replication was quantified by plaque assay. The kinetic of JEV growth showed that virus titer significantly

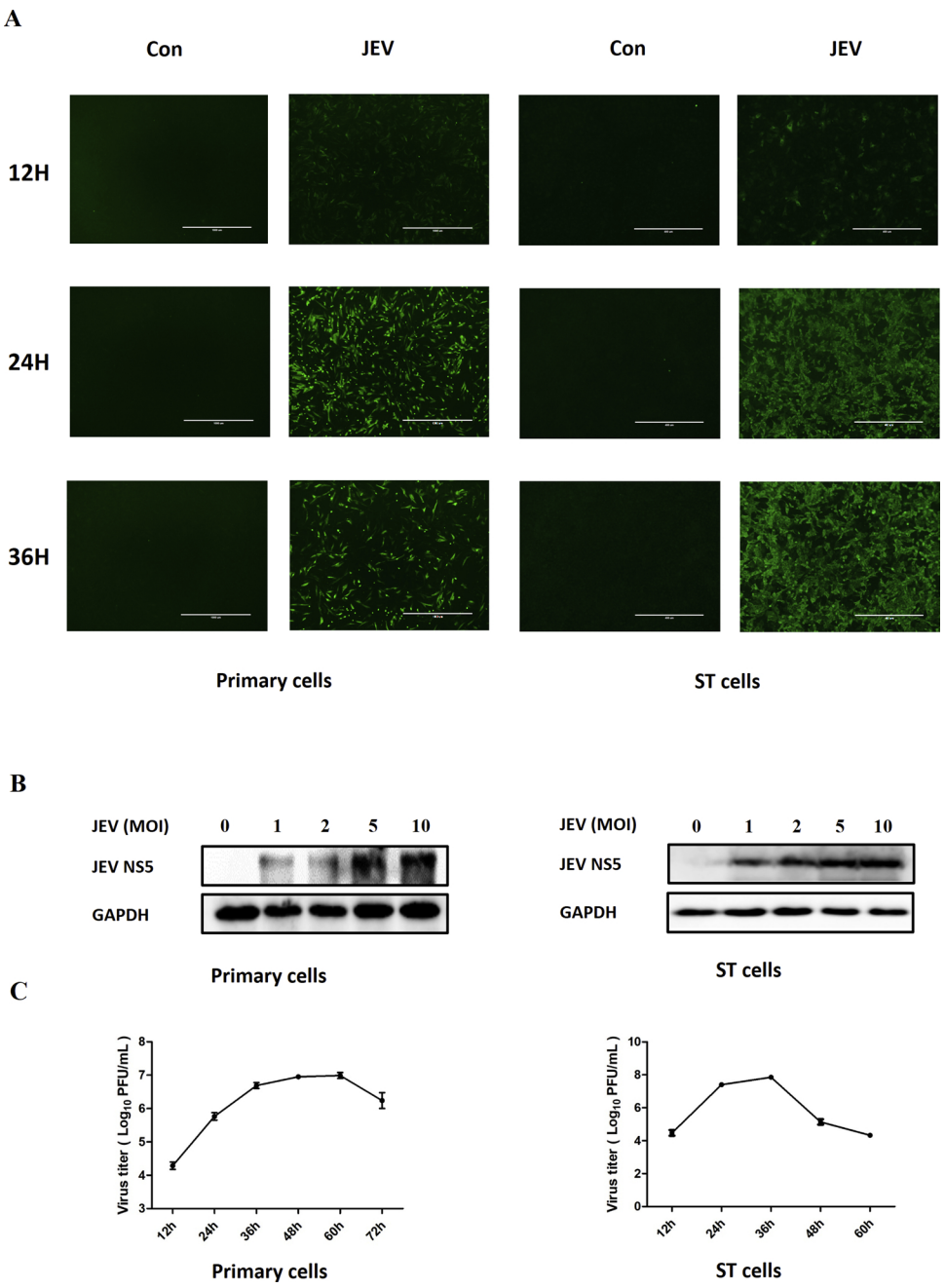


**Fig. 2.** Primary swine testicular cells and ST cells were susceptible to JEV. (A) Primary swine testicular cells and ST cells were infected with JEV at a MOI of 5, IFA was performed by using anti-NS5 antibody at 12 h, 24 h, and 36 h post infection. (B) Primary swine testicular cells and ST cells were infected with JEV at indicated MOI. Cell lysates were collected at 24 hpi and western blotting was performed to detect the expression of JEV NS5. (C) Primary swine testicular cells and ST cells were infected with JEV at a MOI of 0.01. The supernatant of cells were collected at indicated time points to determine virus titer by plaque assay. Con is for Control group, JEV is for Japanese encephalitis virus infected group.

increased upto 36 h post-infection in ST cells, while it reached a plateau at 60 h post-infection in primary cells (Fig. 2C). Taken together, these results suggest that JEV could productively infect both primary swine testicular cells and ST cells.

### 3.3. JEV induces differential productions of inflammatory cytokines in swine testicular cells

To examine JEV-induced inflammation in swine testes, quantitative real-time RT-PCR was performed to detect expression of pro-inflammatory cytokines IL-1β, IL-6, IL-8, RANTES, and TNF-α in primary swine testicular cells and ST cells. As shown in (Fig. 3A), mRNA levels of pro-inflammatory cytokines were increased significantly in primary swine testicular cells and ST cells upon JEV infection. The expression kinetics of these cytokines were further characterized in ST cells by harvesting samples at different time points of post-infection. Increasing amount of JEV NS5 protein was detected according the time course (Fig. 3B). The highest mRNA levels of TNF-α and IL-6 were found at 24 h post-infection, while the highest mRNA levels of IL-8 and RANTES were found at 36 h post-infection (Fig. 3C). These data suggest that JEV infection induces the production of inflammatory cytokines in swine testicular cells.

### 3.4. RIG-I contributes to JEV-mediated inflammation responses in swine testicular cells

Activation of various pattern recognition receptors (PRRs) represents critical effectors of the antiviral immune response by inducing the release of cytokines and chemokines. Previous studies reported that RIG-I, TLR3, TLR4, and TLR7 are mainly involved in immune response or in pathogenesis of flaviviruses. To investigate which PRRs play the most important role to recognize viral components and induce antiviral response during JEV infection in swine testicular cells, the expression of PPRs i.e., RIG-I, TLR3, TLR4, and TLR7 were analyzed with quantitative real-time RT-PCR.

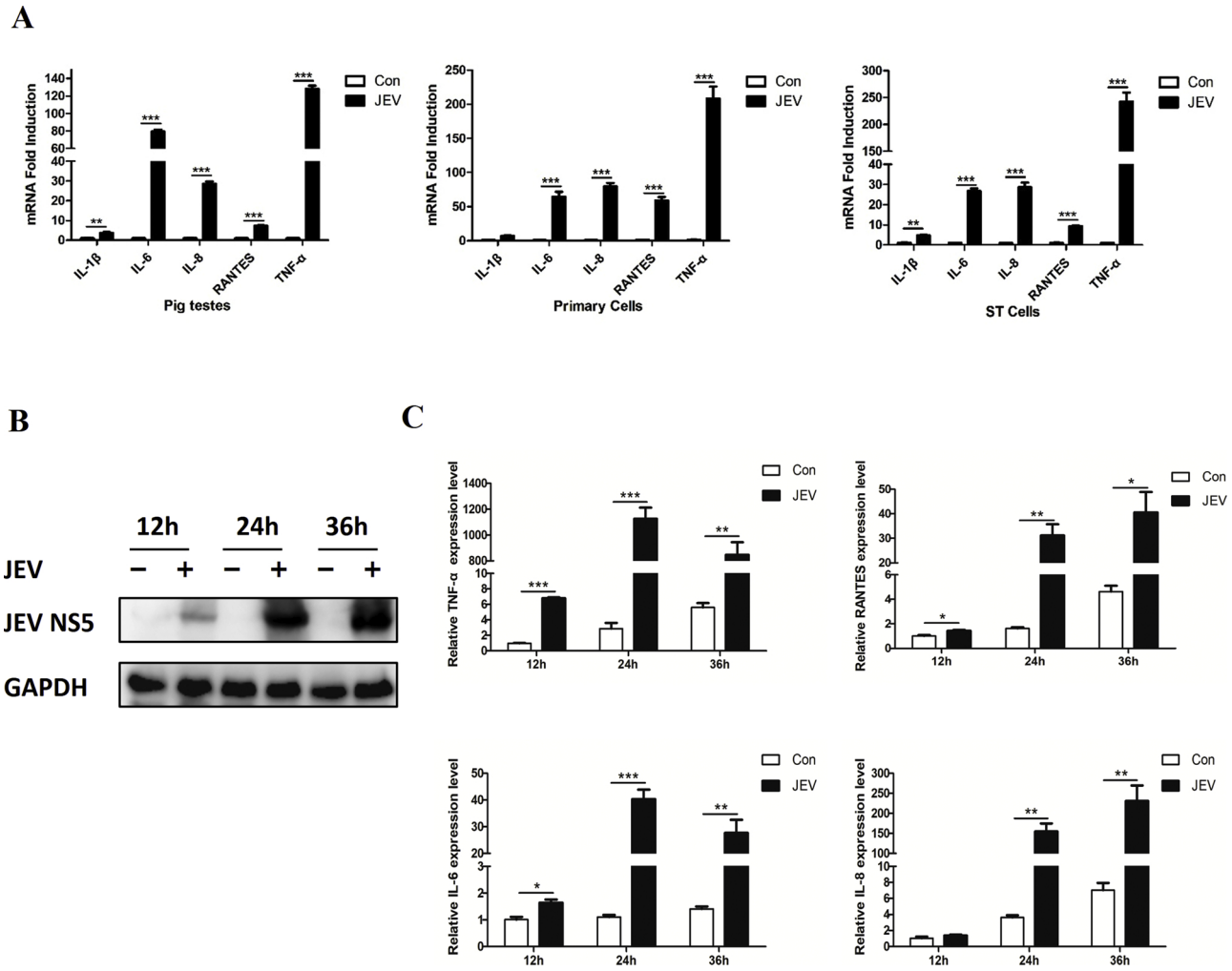


**Fig. 3.** JEV infection induces the production of inflammatory cytokines in primary swine testicular cells, and ST cells. (A) Primary swine testicular cells and ST cells were infected with JEV at a MOI of 5. Samples were harvested at 24 h after infection. Total RNA were extracted from pig testes and cell samples, including JEV infected and control groups. Quantitative real-time RT-PCR was performed to determine mRNA levels of inflammatory factors in pig testes, primary swine testicular cells, and ST cells. (B and C) ST cells were infected with JEV at a MOI of 5, and cell samples were harvested at different time points. The expression of JEV NS5 protein were detected by Western blotting (B) and mRNA levels of inflammatory factors were measured by quantitative real-time RT-PCR (C). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

In JEV- or mock-infected primary swine testicular cells and ST cells, as shown in (Fig. 4A), the level of RIG-I mRNA was most significantly up-regulated in both primary swine testicular cells and ST cells, although TLR3 level was also increased in primary cells. In contrast, no obvious change in the expression of other PPRs was observed in both cells. To further confirm the result, protein level of RIG-I in ST cells was examined by Western blotting, the result shown that up-regulation of RIG-I expression in ST cells after infection with JEV (Fig. 4B).

To elucidate the function of RIG-I in the JEV-induced inflammation in swine testes, RIG-I knockout (RIG-I KO) ST cell line was generated using CRISPR/Cas9 technology (Fig. 4C). The RIG-I knockout ST cells and negative control cells (NC) were infected with JEV and the mRNA expression of inflammatory factors were detected by quantitative real-time RT-PCR. The levels of inflammatory cytokines induced by JEV infection was markedly reduced in RIG-I knockout ST cells compared with those in wild-type control cells (Fig. 4D).

### 3.5. JEV infection activates NF-κB signaling promoting inflammation in swine testicular cells

To investigate the role of key transcriptional factors of inflammatory cytokines during JEV infection in swine testicular cells, the activation of NF-κB and AP-1 was evaluated by western blotting to detetct phosphorylation of p65 and c-Jun after JEV infection (Fig. 5A). The result showed that level of phosphorylated p65 was significantly up-regulated, whereas the phosphorylation of AP-1 was almost unchanged after JEV infection, suggesting that JEV infection could activate NF-κB but not AP-1 in swine testicular cells. This result was further confirmed by immunofluorescence assay which showed that JEV promotes the nuclear localization of p65 (Fig. 5C).

To verify the effect of NF-κB activation on JEV-induced inflammation, QNZ, a specific inhibitor of NF-κB was added into mock or JEV-infected ST cells (Fig. 5B). As expected, phosphorylation and nuclear translocation of p65 was obviously suppressed by QNZ treatment (Fig. 5B and C), and levels of inflammatory cytokines was also reduced in QNZ-treated cells during JEV infection (Fig. 5D), indicating that NF-κB signaling plays a critical role on JEV-induced inflammatory response in swine testicular cells.

### 3.6. RIG-I regulates NF-κB activation during JEV infection

To validate the role of RIG-I on NF-κB activation, phosphorylation and nuclear translocation of NF-κB subunit p65 were detected in RIG-I knockout ST cells. RIG-I knockout ST cells and wide-type control cells

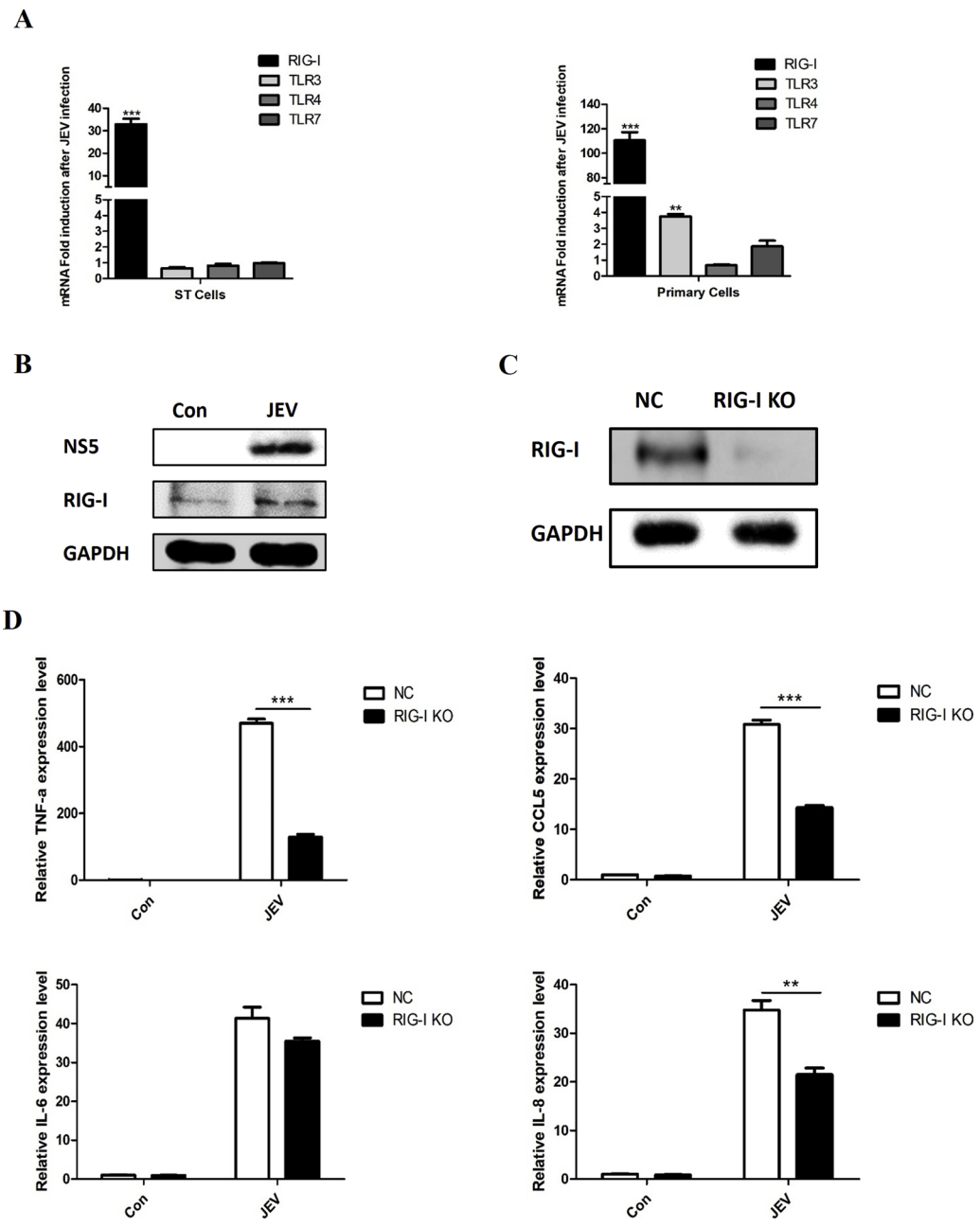


**Fig. 4.** RIG-I contributes to JEV-mediated inflammation responses in swine testicular cells. (A) Primary swine testicular cells and ST cells were infected with JEV at 5 MOI, and samples were harvested at 24 h post infection, quantitative real-time RT-PCR was performed to detect mRNA levels of pattern recognition receptors (PRRs) in primary swine testicular cells and ST cells. (B) ST cells were infected with JEV at 5 MOI. Western blotting was performed to detect RIG-I at 24 h post JEV infection. (C) RIG-I knockout (RIG-I KO) ST cell line was generated using CRISPR/Cas9 technology as described. RIG-I protein in NC and RIG-I knockout cells were detected by western blotting. (D) NC and RIG-I knockout ST cell line were infected with JEV at 5 MOI and cell samples were harvested at 24 h after infection. mRNA levels of inflammatory factors were measured by quantitative real-time RT-PCR. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

were infected with JEV at 5 MOI and samples were harvested at 24 h after infection. As compared to negative control, the phosphorylation level of p65 was significantly reduced in RIG-I knockout cells (Fig. 6A). Consistently, the nuclear translocation of p65 induced by JEV infection was apparently suppressed in RIG-I deficient cells (Fig. 6B). These results revealed that JEV could activate NF-κB signaling through recognition of RIG-I.

## 4. Discussion

Pigs are considered to be JEV amplifying hosts and make the virus available to mosquitoes of the genus *Culex* (Ravanini et al., 2012). Swine testes which are major target for JEV can undergo inflammation, enlargement and sperm necrosis after infection (Zhang et al., 2015). However, the molecular mechanism of JEV induces inflammatory response in swine testicular cells has been rarely reported. In our study, we demonstrated for the first time that swine testicular cells are susceptible to JEV and RIG-I—NF-κB pathway plays a critical role in JEV-induced inflammatory response in swine testicular cells.

To explore the mechanism of inflammatory response, primary swine testicular cells and ST cells, a sertoli cell line, were used for JEV infection and the expression of inflammatory cytokines was analyzed. Our results suggest that JEV could infect ST cells and induce strong inflammatory response, which reveals that the sertoli cell may be at least one type of cells that cause inflammation in testis during JEV infection. This finding is supported by previous reports which demonstrated that

sertoli cells participate in the inflammatory response to invading pathogens, such as ZIKA virus and Mumps virus (Sheng et al., 2017; Wu et al., 2016).

The blood-brain barrier (BBB) is constituted by specialized vascular endothelium which interacts with astrocytes, neurons and pericytes. It protects the brain from the environmental influences and harmful molecules of the systemic circulation (Tajes et al., 2014). However, inflammatory chemokines and cytokines during JEV infection could damage the integrity of the tight junctions between microvascular endothelial cells and trigger BBB disruption in the CNS (Li et al., 2015). The blood-testis barrier (BTB) formed by coexisting tight junction, basal ectoplasmic specializations, desmosomes, and gap junctions between Sertoli cells. BTB is similar to blood-brain barrier, which creates an unique microenvironment for the completion of meiosis and it is one of the tightest tissue barriers in the mammalian body (Mruk and Cheng, 2015). Our IHC results reveal that JEV can cross the blood-testis barrier and infect testicular cells. However, it is needed to be explored whether JEV triggers the disruption of blood-testis barrier or not.

JEV can cause neuroinflammation, including immune cell infiltration and neuronal degeneration, which has been known as a key factor of JEV pathogenesis in human (Misra and Kalita, 2010). Our previous studies demonstrated that RIG-I and TLR3 participate in JEV-caused

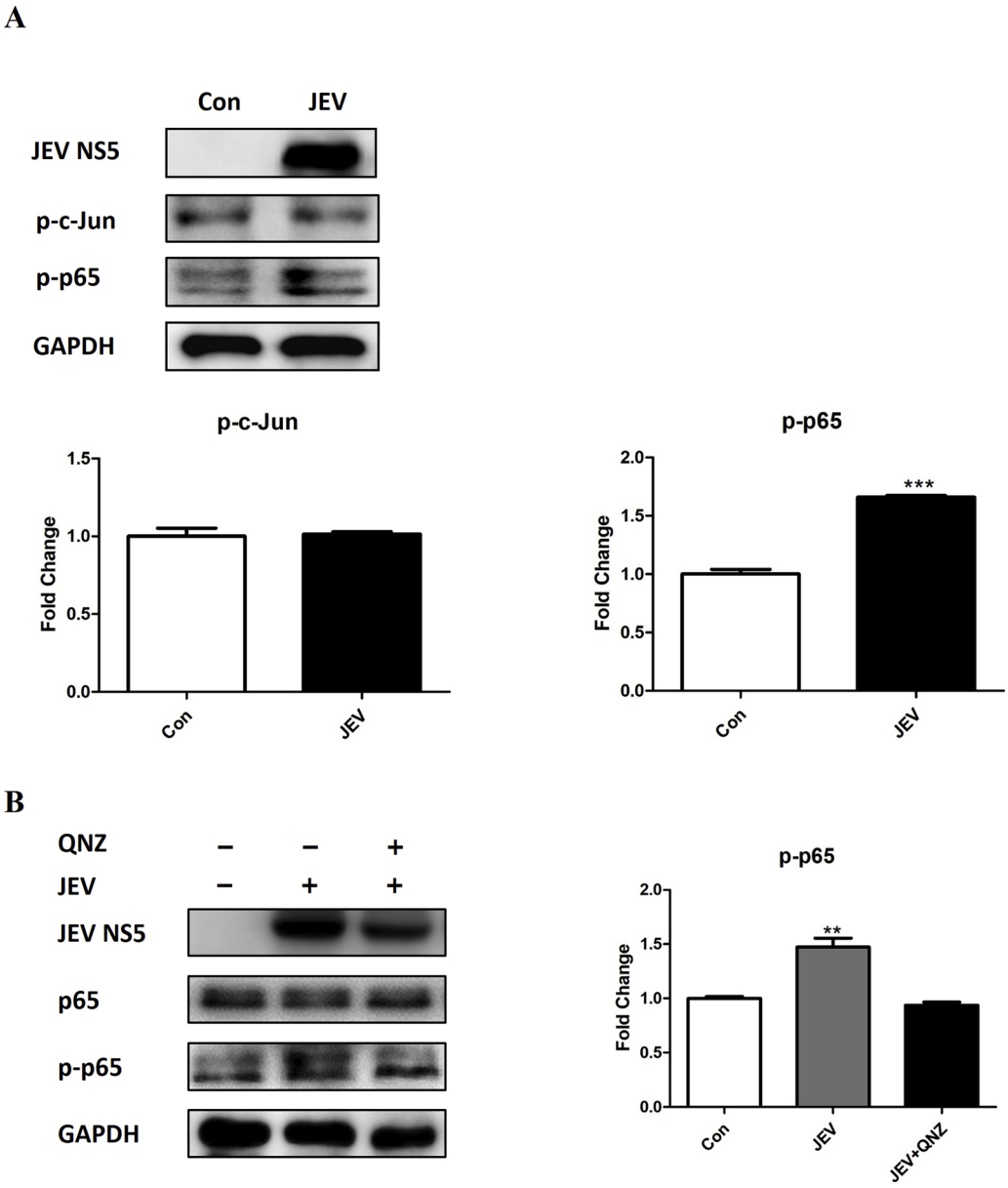


**Fig. 5.** NF-κB activation mediates JEV induces inflammation in ST cells. (A) ST cells were infected with JEV at MOI of 5 and cell samples were harvested at 24 h post-infection. The expression of phosohor-p65 (p-p65) and phosohor-c-Jun (p-c-Jun) were detected by western blotting. (B) ST cells were infected with JEV at 5 MOI followed by treatment of QNZ (1 μM). The expression of p-p65 were detected by western blotting, (C) and the subcellular localization of p65 was determined by IFA and observed by confocal microscope. (D) mRNA levels of inflammatory factors were measured by quantitative real-time RT-PCR. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.,.

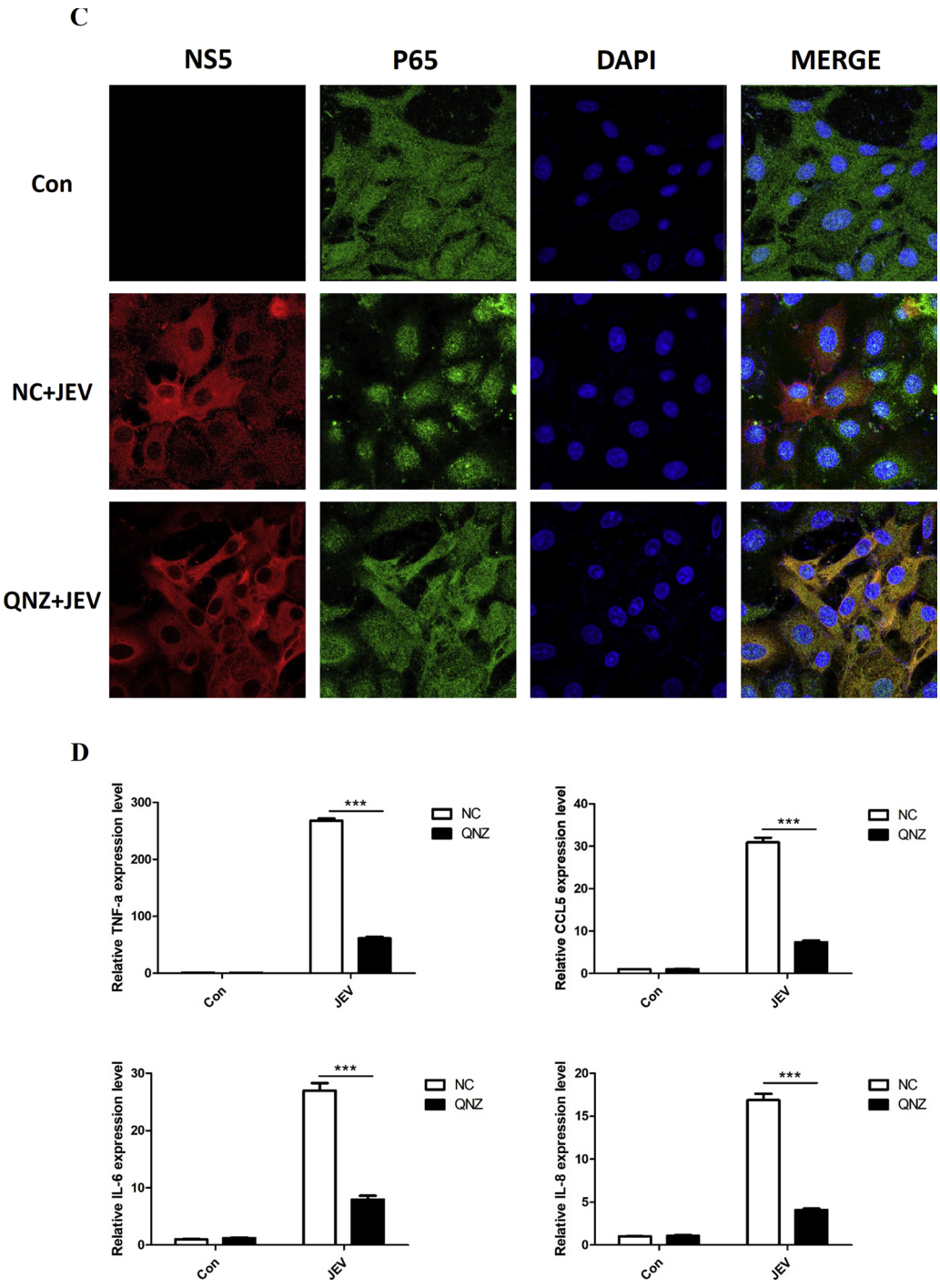


**Fig. 5.** (*continued*)

neuroimflammation and JNK1 and PAK4 signaling play important role in promoting the inflammatory response in JEV-infected glial cells (He et al., 2017; Jiang et al., 2014; Ye et al., 2016). Noncoding RNAs (ncRNAs), including microRNA and long nocoding RNA (LncRNA), have also been found to regulate inflammatory response in CNS during JEV infection (Li et al., 2017). Although neuroinflammation caused by JEV has been studied extensively, but the researches of swine orchitis are at its infancy stages. In this study, we found that JEV infection activates transcription factor NF-κB through recognition of RIG-I in swine testicular cells. This result is partially consistent with our previous study which demonstrated that RIG-I/TLR3-NF-κB/AP-1signaling pathways play critical role in JEV-mediated inflammatory response in glial cells. In this study, the role of TLR3, which also recognizes viral double-strand RNA, in JEV-induced orchitis was not investigated, since the expression of TLR3 is only up-regulated in JEV-infected primary swine testicular cells but not in ST cells. Therefore, we cannot exclude

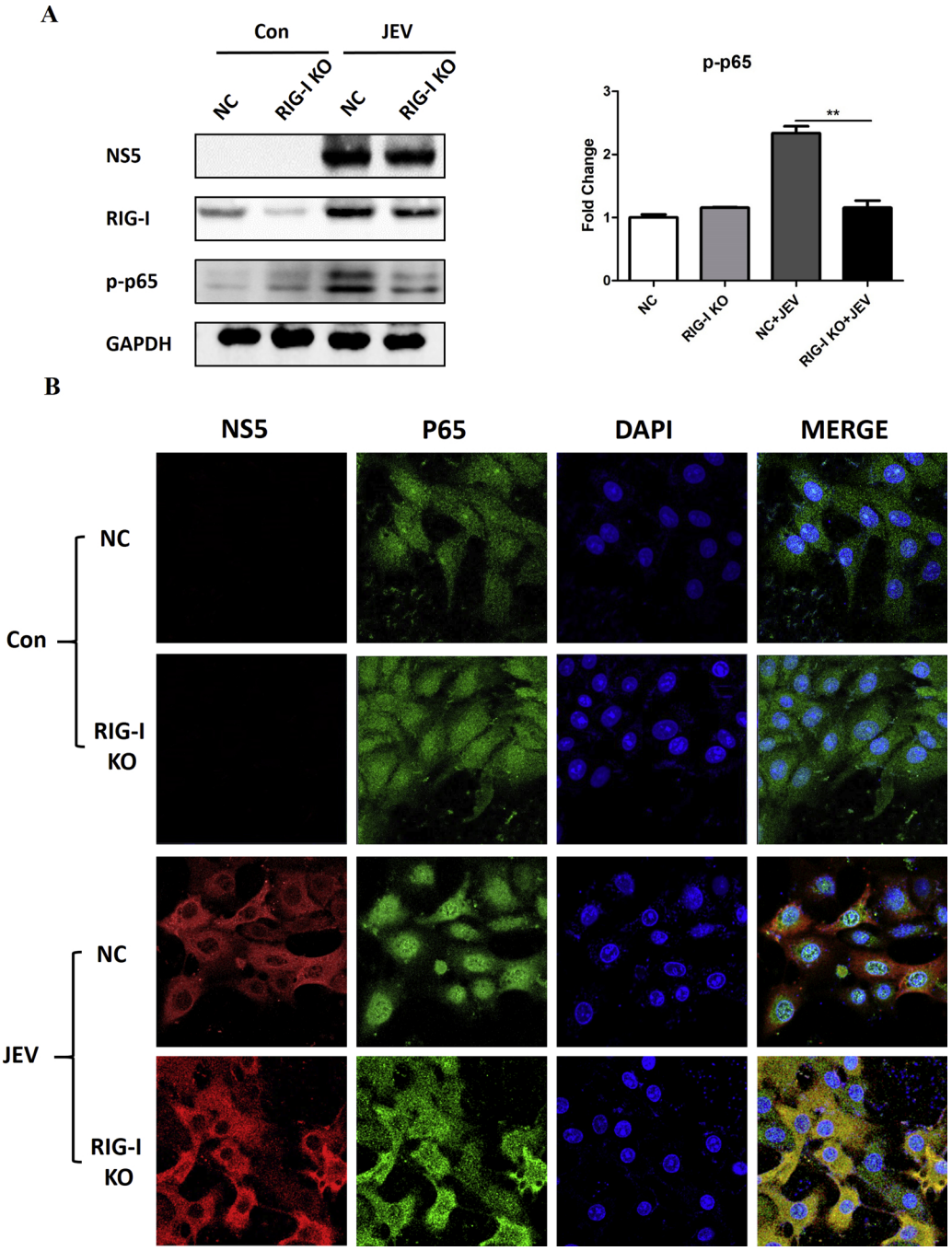


**Fig. 6.** RIG-I knockout attenuates NF-κB activation induced by JEV infection. NC and RIG-I knockout ST cell line were infected with JEV at a MOI of 5, and samples were harvested at 24 h after infection. (A) The expression of p-p65 and RIG-I were detected by western blotting, (B) and the subcellular localization of p65 was determined by IFA and observed by confocal microscope. **$p < 0.01$.

the role of TLR3 in triggering orchitis in JEV-infected pigs. It is also worthwhile to note that AP-1 which was reported as a key transcriptional factor of inflammatory response in JEV-infected glia was not activated in swine testicular cells during JEV infection, revealing distinct signaling pathways involved in JEV-induced inflammation in different cell types.

Taken together, our study demonstrated that JEV can infect swine testis cells and cause inflammatory response both in vivo and in vitro. RIG-I—NF-κB signaling pathway is involved in JEV-induced inflammation in swine testis. This study may enrich our understanding of JEV pathogenesis in pigs and provide potential therapeutic targets for JEV-caused swine orchitis.

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