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ORIGINAL ARTICLE

THE ROLE OF JNK IN THE PROINFLAMMATORY ACTION OF THE SARS-CoV-2 PROTEIN ORF3a

ULOGA JNK U PROINFLAMATORNOM DEJSTVU SARS-CoV-2 PROTEINA ORF3a

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Abstract

Introduction: Coronavirus disease 2019 (COVID-19) is a contagious respiratory infection caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). A significant contributor to the pathogenesis of COVID-19 is the SARS-CoV-2 regulatory protein open reading frame 3a (ORF3a), which drives an excessive inflammatory response associated with adverse clinical outcomes and mortality. Notably, c-Jun N-terminal kinase (JNK), a part of the mitogen-activated protein kinase (MAPK) family, is essential in orchestrating the cellular innate and adaptive immune response initiated by diverse viral infections.
Aim: This study aims to elucidate the involvement of JNK in the proinflammatory response induced by SARS-CoV-2 ORF3a in the H460 non-small cell lung cancer cell line, specifically focusing on the expression of proinflammatory cytokines IL-1β and IL-8.
Material and methods: The H460 cell line was transfected with a DNA plasmid encoding the SARS-CoV-2 ORF3a. Pharmacological inhibition of JNK in H460 cells was achieved using JNK inhibitor SP600215. The expression of p-JNK and JNK was confirmed by immunoblotting, while RT-qPCR was used to quantify IL-1β and IL-8 mRNA levels. Student's t-test was used for statistical analysis.
Results: H460 cells expressing SARS-CoV-2 ORF3a displayed a significant increase in JNK phosphorylation and IL-1β and IL-8 mRNA levels, as verified by immunoblot analysis and RT-qPCR respectively ($p < 0.05$). Subsequently, JNK inhibition in SARS-CoV-2 ORF3a -expressing cells resulted in a significant reduction of IL-1β and IL-8 mRNA expression ($p < 0.05$), thus confirming its involvement in the proinflammatory response elicited by SARS-CoV-2 ORF3a.
Conclusion: The results of the study demonstrated that JNK plays a crucial role in the expression of proinflammatory cytokines IL-1β and IL-8 triggered by SARS-CoV-2 ORF3a. Therefore, targeted JNK inhibition holds promise for mitigating SARS-CoV-2 ORF3a-driven inflammation and providing innovative treatment alternatives for severe COVID-19 cases.

Keywords:
COVID-19,
SARS-CoV-2,
proinflammatory cytokines,
ORF3a, JNK

The authors declare no conflicts of interest.

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Sažetak

Uvod: Koronavirusna bolest 2019 (engl. *Coronavirus disease 2019* - COVID-19) je respiratorno oboljenje izazvano SARS-CoV-2 virusom (engl. *Severe acute respiratory syndrome coronavirus 2* - SARS-CoV-2), koje je odnelo milione života za vreme pandemije 2020. godine i nastavlja da predstavlja značajan izazov za globalno zdravlje ljudi. Protein ORF3a (engl. *Open Reading Frame 3a* - ORF3a) SARS-CoV-2 identifikovan je kao jedan od glavnih pokretača prekomernog inflamatornog odgovora povezanog sa težom kliničkom slikom i smrtnim ishodom usled COVID-19. c-Jun N-terminalna kinaza (engl. *c-Jun N-terminal kinase* - JNK), član familije mitogenom aktiviranih protein kinaza (engl. *Mitogen-activated protein kinases* - MAPK) igra ključnu ulogu u citokinskoj ekspresiji i ćelijskom urođenom i stečenom inflamatornom odgovoru izazvanom različitim virusnim infekcijama.

Cilj: Cilj ovog istraživanja je bio da se ispita uloga JNK u ekspresiji gena za proinflamatorne citokine interleukin 1β (IL-1β) i interleukin 8 (IL-8) na humanoj ćelijskoj liniji nesitnoćelijskog karcinoma pluća H460 koja eksprimira SARS-CoV-2 ORF3a.

Materijal i metode: Ćelijska linija H460 transfektovana je DNK plazmidom koji kodira SARS-CoV-2 ORF3a. Aktivnost JNK u H460 ćelijama farmakološki je inhibirana tretmanom SP600215. Nivo proteina JNK određen je imunoblot metodom, a ekspresija proinflamatornih citokina IL-1β i IL-8 analizirana je na nivou iRNK primenom metode reakcije lančanog umnožavanja sa detekcijom proizvoda u realnom vremenu (RT-qPCR). Statistička analiza je urađena Studentovim t-testom za nezavisne uzorke.

Rezultati: U H460 ćelijama transfektovanim plazmidom koji kodira SARS-CoV-2 ORF3a je utvrđen značajan porast nivoa fosforilacije JNK primenom imunoblot metode ($p < 0,05$). Analiza RT-qPCR je pokazala povećane nivoe ekspresije iRNK za proinflamatorne citokine IL-1β i IL-8 u transfektovanim ćelijama ($p < 0,05$). Naknadna inhibicija JNK je značajno smanjila ekspresiju iRNK za navedene citokine ($p < 0,05$), potvrđujući ulogu JNK u proinflamatornom dejstvu SARS-CoV-2 ORF3a.

Zaključak: Rezultati ove studije su pokazali da JNK igra značajnu ulogu u ekspresiji proinflamatornih citokina IL-1β i IL-8, indukovanih SARS-CoV-2 proteinom ORF3a. Selektivnom inhibicijom JNK bi se moglo smanjiti potencijalno štetno dejstvo prekomerne inflamacije izazvane proinflamatornim citokinima IL-1β i IL-8 kod teških slučajeva COVID-19.

Ključne reči:
COVID-19,
SARS-CoV-2,
proinflamatorni citokini,
ORF3a, JNK

Introduction

Coronavirus Disease 2019 (COVID-19) is a viral infection caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), which typically manifests as an asymptomatic or mild respiratory illness with nonspecific symptoms (1). In critical instances, the disease can progress to pneumonia and acute respiratory distress syndrome (ARDS), a major contributor to mortality in COVID-19 patients. ARDS is primarily induced by a systemic increase in the production of proinflammatory cytokines, a phenomenon commonly known as a cytokine storm (2,3). Cytokines, which are membrane-bound or secreted glycoproteins, normally regulate numerous biological processes, including inflammation and immune responses during viral infections. Inflammatory-promoting cytokines, such as IL-1β, IL-8 (CXCL8), TNF-α, and IL-6, function as endogenous pyrogens and are essential for adequate activation, differentiation, and migration of immune cells to the infection site. However, during a cytokine storm, the excessive and dysregulated production of these proinflammatory mediators can lead to a disproportionate inflammation that subsequently becomes detrimental to the body, causing tissue damage, and, in some cases, shock and death (3-5).

Recently, the SARS-CoV-2 protein Open Reading Frame 3a (ORF3a) has been established as one of the crucial drivers of the severe inflammatory response observed in COVID-19 (6-8). It is one of the viral regulatory non-structural proteins unique to SARS-CoV and SARS-CoV-2. Although SARS-CoV and SARS-CoV-2 ORF3a share 73% nucleotide sequence homology, SARS-CoV-2 ORF3a has a unique 3D structure that determines its multifunctionality. It is a transmembrane protein known to form homodimers or tetramers, which are localized in the plasma membrane and other cellular compartments, such as the endoplasmic reticulum, the Golgi apparatus, endosomes, and lysosomes. One ORF3a monomer consists of 275 amino acids, with an estimated molecular mass of 31 kDa, and features 3 transmembrane domains (TM1 - TM3) (9). These transmembrane regions are important for their ability to form ion channels, which contribute to cellular dysfunction by altering the ionic balance within host cells. This ion channel activity has been implicated in modulating cellular processes such as apoptosis, autophagy, and the release of viral particles, all of which are critical to the viral replication cycle (7,10-14). Through its interaction with host cell machinery, ORF3a can modulate the host's inflammatory response. Recent studies have highlighted the critical role of ORF3a

in immune response dysregulation during COVID-19, emphasizing its ability to trigger a cytokine storm by activating key inflammatory pathways, including NF-κB and inflammasome signaling. The activation of NF-κB induces the transcription of proinflammatory cytokines such as IL-1β, IL-6, and TNF-α (7,15). Simultaneously, ORF3a activates inflammasomes, which are protein complexes responsible for the cleavage and activation of proinflammatory cytokines, particularly IL-1β and IL-18 (7). This cascade leads to the excessive release of these cytokines, driving a hyperinflammatory response that intensifies inflammation in the lungs and other organs, contributing to the development of ARDS and multiorgan failure in COVID-19 patients (6,7).

Another major cellular inflammatory pathway that contributes to the antiviral immune response during infection is the c-Jun N-terminal kinase (JNK) pathway, also referred to as the stress-activated protein kinase (SAPK) (16). It is a member of the serine/threonine kinase family, specifically the mitogen-activated protein kinases (MAPK). It was initially recognized as the kinase that catalyzes the phosphorylation of the c-Jun protein, which is a part of the activator protein-1 (AP-1) transcriptional complex (17). Since its discovery, it has been recognized as a crucial regulator of a wide range of cellular functions and defense mechanisms, which include cell growth, differentiation, DNA repair, and apoptosis. These processes suggest that JNK plays a significant role in the regulation of various biological and infectious diseases (18,19). There exist 3 isoforms of JNK in mammals — JNK1, JNK2, and JNK3, all encoded by distinct genes. Each gene undergoes alternative splicing, producing a minimum of 10 isoforms with molecular masses ranging from 46 to 55 kDa. The expression of JNK1 and JNK2 is ubiquitous, while JNK3 is primarily expressed in neurons, testes, and the heart (20). Similar to other MAPK family members, the activation of JNK goes through a cascade of phosphorylation events mediated by upstream kinases: MAPK kinases (MAPKK) and MAPK kinase kinases (MAPKKK), respectively. These upstream kinases, in turn, are activated by small GTPases of the Rho family, which respond to diverse stress stimuli. These stimuli include various endogenous and exogenous stressors, such as proinflammatory cytokines, growth factors, environmental factors like UV light, and pathogens, including viruses (21-24). Upon activation, JNK phosphorylates different substrates at serine and threonine residues, thereby modulating cellular transcriptional programs. Targeted solely by JNK, the phosphorylation of serine residues 63 and 73 on the N-terminal domain of c-Jun is necessary for AP-1-mediated transcription. Given the major influence of AP-1 on the expression of cytokines and proinflammatory genes, JNK/c-Jun/AP-1 pathway activation likely represents the central mechanism in the production of numerous proinflammatory mediators in the body during viral infections (25,26).

Therefore, the objective of this study was to investigate the role of JNK in the expression of proinflammatory cytokines IL-1β and IL-8 stimulated by the SARS-CoV-2 ORF3a protein. Given that the lung epithelium is the primary site of SARS-CoV-2 infection, the non-small cell lung cancer H460 cell line was employed as the model system for this research.

Material and Methods

H460 Cell Culture and Treatments

The human non-small cell lung carcinoma cell line (H460) was acquired from the American Type Culture Collection. Cells were cultured in Petri dishes with diameter of 10 cm (Sarstedt, Nimbrecht, Germany) at 37°C with 5% CO2, using RPMI 1640 medium (Roswell Park Memorial Institute, Sigma Chemicals Co, USA) with 10mM HEPES buffer, supplemented with 2mM L-glutamine, 10% fetal bovine serum, 1mM sodium pyruvate, and 1% antibiotic/antimycotic mix (Capricorn Scientific GmbH, Ebsdorf, Germany). In all experiments, 2×10^6 cells were cultured in a single well of 6-well plates (Thermo Fisher Scientific, Waltham, MA, USA) in 2 ml of complete RPMI 1640 medium and 5 h post-transfection, cells were treated for 24 h with 20 μM of the JNK inhibitor, SP600125 (SP) (Sigma-Aldrich, St. Louis, MO, USA).

Expression of SARS-CoV-2 ORF3a in H460 Cells

The expression of SARS-CoV-2 ORF3a was achieved through the transfection of H460 cells with a plasmid encoding ORF3a (#152159; Addgene, Cambridge, MA, USA). The control plasmid (TWIST) was generated according to a previously described protocol (27). The control plasmid and the plasmid encoding ORF3a protein (2 μg plasmid in 100μL transfection buffer) were introduced into H460 cells by electroporation, using the SF Cell Line 4D-Nucleofector X Kit L and program EH-100 (Lonza, Basel, Switzerland), following the instructions of the manufacturer. Post-transfection, 2×10^6 cells were seeded in a single well of 6-well plates (Thermo Fisher Scientific, Waltham, MA, USA) in 2 ml of complete RPMI 1640 medium and incubated for 24 h. The protein expression of ORF3a in H460 cells was confirmed by immunoblotting.

Immunoblotting

Immunoblotting was performed 24 hours after treatment to analyze protein expression. Cells were lysed in radio-immunoprecipitation assay buffer (R027, Sigma Aldrich, St. Louis, MO, USA), supplemented with protease/phosphatase inhibitors (all obtained from Merck, Darmstadt, Germany). Cell lysates were cooled on ice for 30 minutes before being centrifuged at 14,000 g for 15 minutes at 4°C and the supernatants were collected. Protein concentration was measured using the BCA kit (#C2284-25ML, #B9643-1L, Sigma Aldrich, St. Louis, MO, USA). Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The primary antibodies used comprised rabbit antibodies against phospho-JNK (#4668), JNK (#9252), ORF3a (#34340), and actin (#4967) (Cell Signaling Technology, Cambridge, MA, USA). The secondary

antibody used was anti-rabbit IgG conjugated with horseradish peroxidase (11-035-144; Jackson ImmunoResearch, West Grove, PA, USA). Chemiluminescence was generated using a peroxidase substrate (GE Healthcare, Chicago, IL, USA) and detected with a ChemiDoc system (Bio-Rad, Hercules, CA, USA). The intensity of chemiluminescence was proportional to the amount of protein attached to the nitrocellulose membrane. Densitometric analysis of the protein bands was performed using ImageLab software (Bio-Rad, Hercules, CA, USA). Phosphorylation levels of JNK were presented relative to the total amount of JNK and actin proteins. Signal intensity in ORF3a-transfected cells was presented relative to the signal intensity in cells transfected with the control TWIST plasmid.

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

RT-qPCR was used to measure gene expression for proinflammatory cytokines IL-1β and IL-8, 24 h after treatment. Reagent TRIZol was used for RNA extraction, while reverse transcription was achieved using reverse transcriptase and hexamer primers (all from Thermo Fisher Scientific, Waltham, MA, USA), according to instructions of the manufacturer. The RT-qPCR was carried out on a Mastercycler Realplex2 system (Eppendorf, Hamburg, Germany), using 96-well plates sealed with adhesive film, TaqMan probes/primers and TaqMan PCR Master Mix (all from Thermo Fisher Scientific, Waltham, MA, USA). Both TaqMan probes for human interleukins IL-1β (Hs01555410_m1) and IL-8 (Hs00174103_m1) were used, as well as probes for reference genes hypoxanthine-guanine phosphoribosyltransferase 1 (HPRT1; Hs02800695_m1), TATA-binding protein (TBP; Hs00427620_m1), and 18S ribosomal RNA (RN18S; Hs03928985_g1). The RT-qPCR program was set to 95°C for 1 minute of initial denaturation, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. To calculate ΔCt, the cycle threshold (Ct) values of the interleukin target genes were normalized by subtracting the geometric mean of the Ct values of the reference genes RN18S, TBP, and HPRT1. Relative gene expression was determined using the formula 2-ΔCt. Results were presented as relative to the values in control cells, which were arbitrarily set to 1.

Statistical Analysis

Student's t-test was applied for statistical analysis on an extremely small number of samples ($n \leq 5$), with a p-value < 0.05 considered statistically significant (28).

Results

SARS-CoV-2 ORF3a Activates JNK In H460 Cells

To investigate whether ORF3a activates JNK, phosphorylated JNK (pJNK) and total JNK (JNK) protein levels were measured in H460 cells expressing SARS-CoV-2 ORF3a using the immunoblot method. Immunoblot analysis demonstrated a significant increase in pJNK levels in H460 cells expressing SARS-CoV-2 ORF3a compared to control cells transfected with the TWIST plasmid ($p < 0.05$) (**Figure 1**).

Inhibition Of JNK Reduces The IL-1β And IL-8 Proinflammatory Cytokine Gene Expression In H460 Cells Transfected With SARS-CoV-2 ORF3a

To better understand the functional impacts of JNK activation by ORF3a, a specific JNK inhibitor SP600215 was employed. As expected, immunoblot analysis confirmed that treatment with SP600215 resulted in a significant decline in pJNK levels in SARS-CoV-2 ORF3a expressing cells compared to untreated cells ($p < 0.05$) (**Figure 2**).

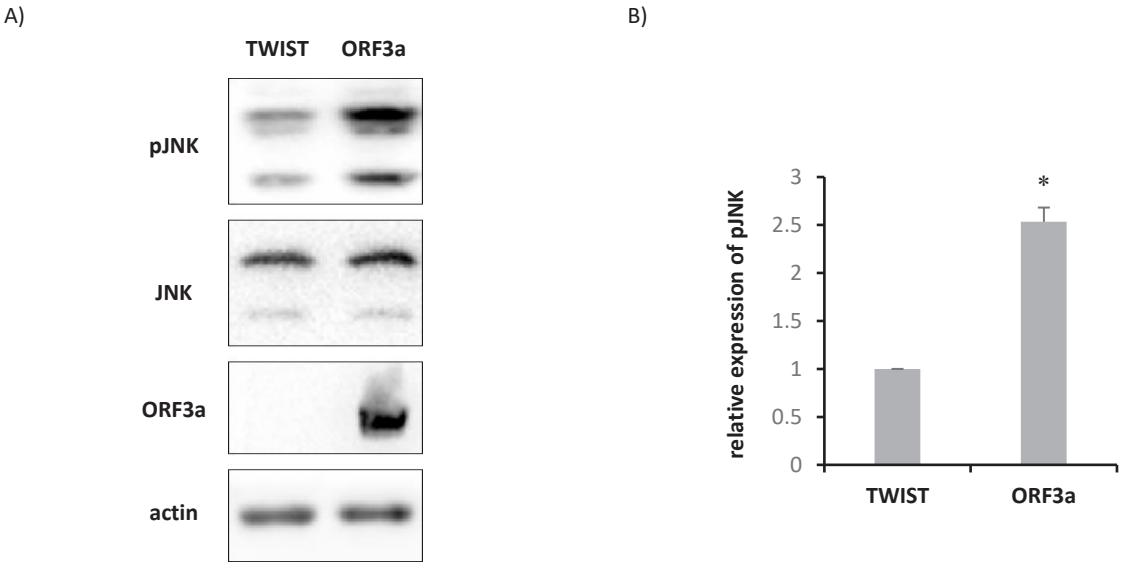

Figure 1. SARS-CoV-2 ORF3a induces JNK activation in H460 cells

Phosphorylated JNK (pJNK), total JNK (JNK), ORF3a and actin were detected in H460 cells transfected with the control TWIST plasmid or the plasmid encoding SARS-CoV-2 ORF3a. A) Representative immunoblots from one of 3 different experiments are shown. B) Densitometric analysis results of the blots are displayed as the mean ± standard deviation of 3 different experiments. *$p < 0.05$ specifies a statistically significant difference compared to cells transfected with the TWIST control plasmid

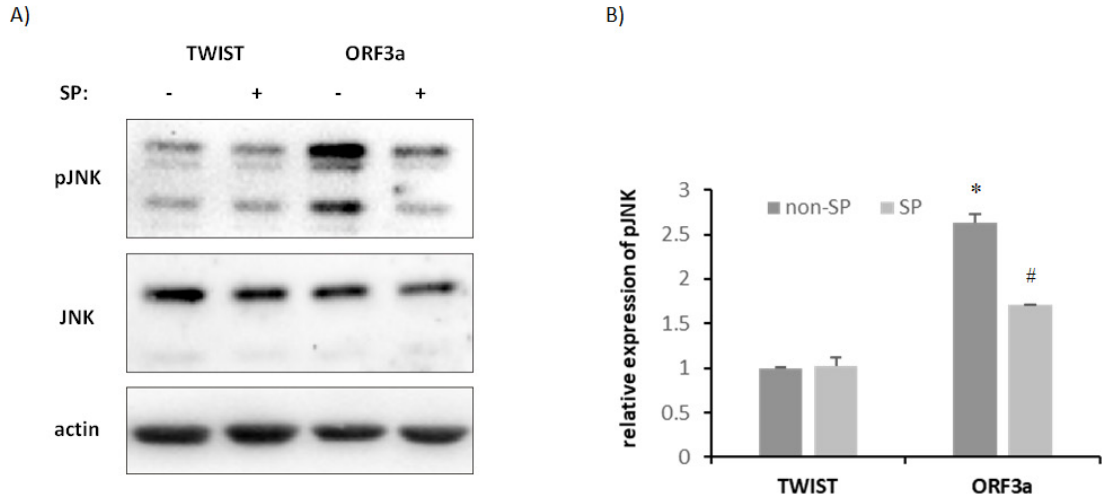

Figure 2. Pharmacological inhibition of JNK in H460 cells transfected with SARS-CoV-2 ORF3a reduces JNK phosphorylation

All pJNK, JNK, and actin were detected in H460 cells transfected with the control TWIST plasmid or the plasmid encoding SARS-CoV-2 ORF3a in the absence or presence of SP600125 (SP) using the immunoblot method. A) Representative immunoblots from one of 3 different experiments are shown and B) Densitometric analysis results of the blots are displayed as the mean ± standard deviation of 3 different experiments. *p < 0.05 specifies a statistically significant difference compared to cells transfected with the TWIST control plasmid, while #p < 0.05 specifies a statistically significant difference compared to cells transfected with the plasmid encoding SARS-CoV-2 ORF3a

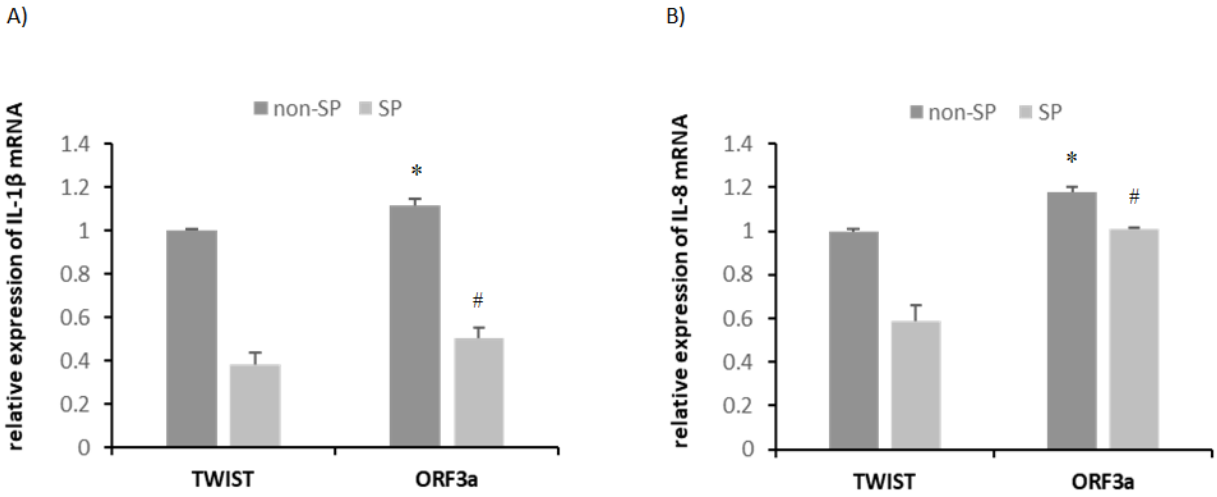

Figure 3. Inhibition of JNK reduces IL-1β and IL-8 expression in H460 cells transfected with SARS-CoV-2 ORF3a

Quantitative representation of mRNA expression for the proinflammatory cytokine A) IL-1β and B) IL-8 in H460 cells transfected with the control TWIST plasmid or the plasmid encoding SARS-CoV-2 ORF3a in the absence or presence of SP. Results are displayed as the mean ± standard deviation of triplicates from one experiment. *p < 0.05 specifies a statistically significant difference compared to cells transfected with the TWIST control plasmid, while #p < 0.05 specifies a statistically significant difference compared to cells transfected with the plasmid encoding SARS-CoV-2 ORF3a

Concurrently, the expression of IL-1β and IL-8 cytokines was measured using RT-qPCR. The analysis demonstrated a substantial rise in mRNA levels of IL-1β and IL-8 in H460 cells transfected with ORF3a compared to cells transfected with the TWIST plasmid ($p < 0.05$) (**Figure 3**). Importantly, mRNA expression of these cytokines was significantly reduced in SARS-CoV-2 ORF3a-transfected cells treated with SP600215 compared to untreated controls ($p < 0.05$) (**Figure 3**).

Discussion

The results of immunoblot analysis demonstrated that ORF3a significantly upregulates JNK phosphorylation, thereby activating the JNK signaling cascade in H460 cells. These findings are consistent with previous studies showing the crucial involvement of JNK in c-Jun/AP-1 inflammatory pathway activation during viral infections (29-31). Previous research has also indicated the function of JNK signaling in modulating the inflammatory response to coronaviruses such as SARS-CoV and MERS-CoV (32). Furthermore, Kanzawa et al. (33) suggested that JNK is involved in signal transduction initiated by the SARS-CoV ORF3a expression in HEK293T cells. However, there has been no prior work on the effect of SARS-CoV-2 ORF3a on the activation of the JNK signaling pathway. Therefore, this is the initial study that confirms SARS-CoV-2 ORF3a-dependent activation of JNK in H460 cells.

The upregulation of IL-1β and IL-8 mRNA levels in H460 cells by ORF3a, as revealed by RT-qPCR analysis,

further underscores the significance of this accessory protein in driving inflammation. Firstly, IL-1β is an important mediator of systemic inflammation and a known contributor to the cytokine storm in critically ill COVID-19 patients (34-36). Similarly, IL-8 is important for neutrophil migration to infection sites, contributing to tissue damage and worsening respiratory complications (37). Prior research has established that SARS-CoV-2 infection leads to increased IL-8 and IL-1β expression in cells of respiratory epithelium (38, 39). The study conducted by Xu et al. (40) showed that ORF3a increases IL-1β expression through the inflammasome in a tumor cell culture of respiratory epithelium (A549). Gowda et al. (41) reported that ORF3a induces IL-8 and IL-1β expression in bronchial epithelial cells (BEAS-2B) and tumor cell lines of respiratory epithelium (A549, H1299). The results of our study align with these findings, corroborating that ORF3a directly influences cytokine dysregulation in COVID-19.

To further investigate the functional impact of JNK activation on cytokine expression induced by ORF3a, SP600215 was employed - a specific JNK inhibitor. As confirmed by immunoblot analysis, SP600215 effectively suppressed JNK activity, and this inhibition was associated with a substantial reduction of IL-1β and IL-8 mRNA levels in ORF3a-transfected H460 cells confirmed by RT-qPCR analysis. Therefore, the results of the study demonstrate that ORF3a robustly activates the JNK signaling pathway in H460 cells, thereby driving the upregulation of cytokines IL-8 and IL-1β. This JNK-mediated expression of cytokines may be attributed to SARS-CoV-2 ORF3a-induced endoplasmic reticulum stress and oxidative stress, known triggers of JNK activation (42-44). Notably, JNK could also promote the activation of other inflammatory pathways induced by ORF3a, such as NLRP3 inflammasomes, which drive IL-8 and IL-1β upregulation (7, 40, 45). Given this intricate network of interactions, further research is necessary to clarify the mechanisms by which JNK and ORF3a interact.

Furthermore, these results suggest that pharmacological inhibition of JNK, using agents such as SP600215, could provide a new potential treatment option to alleviate hyperinflammation in COVID-19. As a chemical compound, SP600215, with a structure of anthra[1,9-cd] pyrazol-6-(2H)-one, was detected through high-throughput screening of Celgene's proprietary chemical library as a selective inhibitor of JNK activity (46). It was originally used as a tool compound to study the JNK pathway. The study conducted by Han et al. (47) on the animal model of arthritis was the first to reveal the potential of SP600125 in disease applications. Despite its usefulness in research, SP600125 itself has not advanced significantly beyond research settings into clinical trials. Concerns about off-target effects, toxicity, or other adverse reactions could be factors in why SP600125 has not proceeded further. Given our results, further investigation into the potential of JNK inhibition for therapeutic purposes is necessary (48).

While the study focused on H460 cells, it would be valuable to expand this research to other cell types relevant to COVID-19 pathology, such as lung epithelial or immune cells, to better understand the broader implications of JNK activation in different tissue environments. Moreover, considering the complex cytokine landscape of COVID-19, investigating the role of JNK in regulating other proinflammatory mediators could offer deeper insights into its broader immunomodulatory function of ORF3a during SARS-CoV-2 infection.

Conclusion

In conclusion, the research provides novel evidence that the SARS-CoV-2 ORF3a protein upregulates the JNK activity in H460 cells, thereby increasing the IL-8 and IL-1β proinflammatory cytokines expression. The pharmacological inhibition of JNK significantly reduces this cytokine expression, highlighting the role of ORF3a in cytokine dysregulation and JNK as a promising therapeutic target for controlling inflammation in COVID-19. Further investigation into more selective JNK inhibitors and expanding research into additional cytokines and cell types will be crucial for advancing therapeutic approaches aimed at mitigating inflammation and enhancing the clinical prognosis of COVID-19 patients.

Literature

1. Zhou P, Yang XL, Wang XG, Hu B, Zhang L, Zhang W, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. Nature. 2020; 579(7798):270-3.
2. Cron RQ, Goyal G, Chatham WW. Cytokine Storm Syndrome. Annu Rev Med. 2023; 74:321-37.
3. Karki R, Kanneganti TD. Innate immunity, cytokine storm, and inflammatory cell death in COVID-19. J Transl Med. 2022; 20(1):542.
4. Darif D, Hammi I, Kihel A, El Idrissi Saik I, Guessous F, Akarid K. The pro-inflammatory cytokines in COVID-19 pathogenesis: What goes wrong? Microb Pathog. 2021; 153:104799.
5. Mohseni Afshar Z, Barary M, Babazadeh A, Tavakoli Pirzaman A, Hosseinzadeh R, Alijanpour A, et al. The role of cytokines and their antagonists in the treatment of COVID-19 patients. Rev Med Virol. 2023; 33(1):e2372.
6. Zhang J, Hom K, Zhang C, Nasr M, Gerzanich V, Zhang Y, et al. SARS-CoV-2 ORF3a Protein as a Therapeutic Target against COVID-19 and Long-Term Post-Infection Effects. Pathogens. 2024; 13(1):75.
7. Zhang J, Ejikemeuwa A, Gerzanich V, Nasr M, Tang Q, Simard JM, et al. Understanding the Role of SARS-CoV-2 ORF3a in Viral Pathogenesis and COVID-19. Front Microbiol. 2022; 13:854567.
8. Zhang J, Li Q, Cruz Cosme RS, Gerzanich V, Tang Q, Simard JM, et al. Genome-Wide Characterization of SARS-CoV-2 Cytopathogenic Proteins in the Search of Antiviral Targets. mBio. 2021; 13(1):e0016922.
9. Kern DM, Sorum B, Mali SS, Hoel CM, Sridharan S, Remis JP, et al. Cryo-EM structure of SARS-CoV-2 ORF3a in lipid nanodiscs. Nat Struct Mol Biol. 2021; 28(7):573-82.
10. Castaño-Rodriguez C, Honrubia JM, Gutiérrez-Álvarez J, DeDiego ML, Nieto-Torres JL, Jimenez-Guardeño JM, et al. Role of Severe Acute Respiratory Syndrome Coronavirus Viroporins E, 3a, and 8a in Replication and Pathogenesis. mBio. 2018; 9(3): e02325-17.
11. Miller AN, Houlihan PR, Matamala E, Cabezas-Bratesco D, Lee GY, Cristofori-Armstrong B, et al. The SARS-CoV-2 accessory protein Orf3a is not an ion channel, but does interact with trafficking proteins. eLife. 2023; 12:e84477.

12. Su J, Shen S, Hu Y, Chen S, Cheng L, Cai Y, et al. SARS-CoV-2 ORF3a inhibits cGAS-STING-mediated autophagy flux and antiviral function. J Med Virol. 2023; 95(1):e28175.
13. Jiao S, Miranda P, Li Y, Maric D, Holmgren M. Some aspects of the life of SARS-CoV-2 ORF3a protein in mammalian cells. Heliyon. 2023; 9(8):e18754.
14. Chen D, Zheng Q, Sun L, Ji M, Li Y, Deng H, et al. ORF3a of SARS-CoV-2 promotes lysosomal exocytosis-mediated viral egress. Dev Cell. 2021; 56(23):3250-63.e5.
15. Neufeldt CJ, Cerikan B, Cortese M, Frankish J, Lee JY, Plociennikowska A, et al. SARS-CoV-2 infection induces a pro-inflammatory cytokine response through cGAS-STING and NF-κB. Commun Biol. 2022; 5(1):45.
16. Rincón M, Davis RJ. Regulation of the immune response by stress-activated protein kinases. Immunol Rev. 2009; 228(1):212-24.
17. Davis RJ. MAPKs: new JNK expands the group. Trends in biochemical sciences. 1994; 19(11):470-3.
18. Ip YT, Davis RJ. Signal transduction by the c-Jun N-terminal kinase (JNK)--from inflammation to development. Curr Opin Cell Biol. 1998; 10(2):205-19.
19. Sabapathy K. Role of the JNK pathway in human diseases. Prog Mol Biol Transl Sci. 2012; 106:145-69.
20. Bode AM, Dong Z. The functional contrariety of JNK. Molecular carcinogenesis. 2007; 46(8):591-8.
21. Davis RJ. Signal transduction by the JNK group of MAP kinases. Cell. 2000; 103(2):239-52.
22. Weston CR, Davis RJ. The JNK signal transduction pathway. Curr Opin Cell Biol. 2007; 19(2):142-9.
23. Cui J, Zhang M, Zhang YQ, Xu ZH. JNK pathway: diseases and therapeutic potential. Acta Pharmacol Sin. 2007; 28(5):601-8.
24. Chen J, Ye C, Wan C, Li G, Peng L, Peng Y, et al. The Roles of c-Jun N-Terminal Kinase (JNK) in Infectious Diseases. Int J Mol Sci. 2021; 22(17):9640.
25. Bennett BL. c-Jun N-terminal kinase-dependent mechanisms in respiratory disease. Eur Respir J. 2006; 28(3):651-61.
26. Zeke A, Misheva M, Reményi A, Bogoyevitch MA. JNK Signaling: Regulation and Functions Based on Complex Protein-Protein Partnerships. Microbiol Mol Biol Rev. 2016; 80(3):793-835.
27. Paunovic V, Vucicevic L, Misirkic Marjanovic M, Perovic V, Ristic B, Bosnjak M, et al. Autophagy Receptor p62 Regulates SARS-CoV-2-Induced Inflammation in COVID-19. Cells. 2023; 12(9):1282.
28. De Winter JC. Using the Student's t-Test with Extremely Small Sample Sizes. Practical assessment, research & evaluation. 2013; 18(10):n10.
29. Chu WM, Ostertag D, Li ZW, Chang L, Chen Y, Hu Y, et al. JNK2 and IKKbeta are required for activating the innate response to viral infection. Immunity. 1999; 11(6):721-31.
30. Li XM, Sun SZ, Wu FL, Shi T, Fan HJ, Li DZ. Study on JNK/AP-1 signaling pathway of airway mucus hypersecretion of severe pneumonia under RSV infection. Eur Rev Med Pharmacol Sci. 2016; 20(5):853-7.
31. Ludwig S, Ehrhardt C, Neumeier ER, Kracht M, Rapp UR, Pleschka S. Influenza virus-induced AP-1-dependent gene expression requires activation of the JNK signaling pathway. J Biol Chem. 2001; 276(14):10990-8.
32. Hemmat N, Asadzadeh Z, Ahangar NK, Alemohammad H, Najafzadeh B, Derakhshani A, et al. The roles of signaling pathways in SARS-CoV-2 infection; lessons learned from SARS-CoV and MERS-CoV. Arch Virol. 2021; 166(3):675-96.
33. Kanzawa N, Nishigaki K, Hayashi T, Ishii Y, Furukawa S, Niiro A, et al. Augmentation of chemokine production by severe acute respiratory syndrome coronavirus 3a/X1 and 7a/X4 proteins through NF-kappaB activation. FEBS letters. 2006; 580(30):6807-12.
34. Fawzy S, Ahmed MM, Alsayed BA, Mir R, Amle D. IL-2 and IL-1β Patient Immune Responses Are Critical Factors in SARS-CoV-2 Infection Outcomes. J Pers Med. 2022; 12(10):1729.
35. Conti P, Caraffa A, Gallenga CE, Ross R, Kritas SK, Frydas I, et al. Coronavirus-19 (SARS-CoV-2) induces acute severe lung inflammation via IL-1 causing cytokine storm in COVID-19: a promising inhibitory strategy. J Biol Regul Homeost Agents. 2020; 34(6):1971-5.
36. Mardi A, Meidaninikjeh S, Nikfarjam S, Majidi Zolbanin N, Jafari R. Interleukin-1 in COVID-19 Infection: Immunopathogenesis and Possible Therapeutic Perspective. Viral Immunol 2021; 34(10):679-88.
37. Hasanvand A. COVID-19 and the role of cytokines in this disease. Inflammopharmacology. 2022; 30(3):789-98.
38. Barilli A, Visigalli R, Ferrari F, Bianchi MG, Dall'Asta V, Rotoli BM. Immune-Mediated Inflammatory Responses of Alveolar Epithelial Cells: Implications for COVID-19 Lung Pathology. Biomedicines. 2022; 10(3):618.
39. Bridges JP, Vladar EK, Huang H, Mason RJ. Respiratory epithelial cell responses to SARS-CoV-2 in COVID-19. Thorax. 2022; 77(2):203-9.
40. Xu H, Akinyemi IA, Chitre SA, Loeb JC, Lednicky JA, McIntosh MT, et al. SARS-CoV-2 viroporin encoded by ORF3a triggers the NLRP3 inflammatory pathway. Virology. 2022; 568:13-22.
41. Gowda P, Patrick S, Joshi SD, Kumawat RK, Sen E. Glycyrrhizin prevents SARS-CoV-2 S1 and Orf3a induced high mobility group box 1 (HMGB1) release and inhibits viral replication. Cytokine. 2021; 142:155496.
42. Zhang X, Yang Z, Pan T, Long X, Sun Q, Wang PH, et al. SARS-CoV-2 ORF3a induces RETREG1/FAM134B-dependent reticulophagy and triggers sequential ER stress and inflammatory responses during SARS-CoV-2 infection. Autophagy. 2022; 18(11):2576-92.
43. Liu L, Du J, Yang S, Zheng B, Shen J, Huang J, et al. SARS-CoV-2 ORF3a sensitizes cells to ferroptosis via Keap1-NRF2 axis. Redox Biol. 2023; 63:102752.
44. Kaneto H, Matsuoka TA, Nakatani Y, Kawamori D, Miyatsuka T, Matsuhisa M, et al. Oxidative stress, ER stress, and the JNK pathway in type 2 diabetes. J Mol Med (Berl). 2005; 83(6):429-39.
45. Wang X, Zhao Y, Wang D, Liu C, Qi Z, Tang H, et al. ALK-JNK signaling promotes NLRP3 inflammasome activation and pyroptosis via NEK7 during Streptococcus pneumoniae infection. Mol Immunol. 2023; 157:78-90.
46. Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, et al. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc Natl Acad Sci USA. 2001; 98(24):13681-6.
47. Han Z, Boyle DL, Chang L, Bennett B, Karin M, Yang L, et al. c-Jun N-terminal kinase is required for metalloproteinase expression and joint destruction in inflammatory arthritis. J Clin Invest. 2001; 108(1):73-81.
48. Han SY. c-Jun N-Terminal Kinase Signaling Inhibitors Under Development. Toxicol Res. 2008; 24(2):93-100.