Virus Research 356 (2025) 199577

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

JNK kinase promotes inflammatory responses by inducing the expression of the inflammatory amplifier TREM1 during influenza a virus infection

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ARTICLE INFO

Keywords:
Influenza A virus
JNK kinase
TREM1
Inflammatory amplifier
Inflammatory responses

ABSTRACT

Since the twentieth century, four influenza pandemics caused by IAV have killed millions of people worldwide. IAV infection could induce acute lung injury mediated by cytokine storms, which is an essential cause of death in critically ill patients. Consequently, it is crucial to explore the regulators and regulatory mechanisms of cytokine storms, which may provide potential drug targets and expand our understanding of acute lung injury. Previous studies have shown that JNK kinase is essential in promoting inflammatory responses during viral infections. In this study, we demonstrated that JNK kinase could regulate the IAV-induced cytokine storms by affecting the expression of pro-inflammatory and anti-inflammatory factors. Further studies revealed that inhibition of JNK kinase activity significantly downregulated the expression of the inflammatory amplifier TREM1. Besides, TREM1 knockdown could significantly inhibit the expression of pro-inflammatory factors. Furthermore, SP600125 is a specific inhibitor of JNK kinase. The results show that TREM1 overexpression reversed the effect of SP600125 treatment on the expression of pro-inflammatory factors. Together, we found that JNK kinase could activate the inflammatory amplifier TREM1 to promote inflammatory responses during influenza A virus infection. These findings may provide some inspiration for subsequent researchers to explore the regulatory mechanisms of cytokine storms induced by emerging viral infections.

1. Introduction

Influenza virus is a well-known common respiratory pathogen. It has the capacity to trigger seasonal epidemics and severe sporadic global pandemics, which leads to human fatalities and substantial economic losses. Influenza viruses can be classified into four types, namely A, B, C, and D. All four influenza pandemics were caused by IAV, which could induce acute lung injury (ALI) mediated by cytokine storms (Tisoncik et al., 2012; Gu et al., 2019; Wei et al., 2022; Chen et al., 2024). ALI, mediated by cytokine storms, is an essential cause of death in critically ill patients. In addition, it is characterized by excessive inflammatory responses and complex regulatory mechanisms. Therefore, there remains an urgent and long-term need to study the host immune responses during IAV infection, especially the excessive inflammatory responses and their regulatory mechanisms.

Inflammation is a double-edged sword, and excessive immune responses may cause immune damage to the body. The mechanism of cytokine storms, also called inflammatory factor storms, is the excessive activation of immune cells and the massive release of intracellular pro-inflammatory and anti-inflammatory factors, including interleukins, chemokines, TNF-α, complement proteins, etc., which carry out a suicidal attack on the source of the infection and the infected cells, resulting in damage to own tissues and cells. In conclusion, cytokine storms are mainly regulated by pro-inflammatory and anti-inflammatory factors. On the one hand, pro-inflammatory factors are represented by IL-6, IL-1β, and IL-8. In addition to these, they also include CCL3, CCL4, CCL20, CXCL2, CXCL3, IL-23, IL-32, and IL-36γ (Qudus et al., 2023; Coperchini et al., 2021; Gao et al., 2021; Ye et al., 2020; Wang et al., 2015; Grebe et al., 2018). Among them, IL-6 is considered an essential indicator of cytokine storms. On the other hand, anti-inflammatory factors include IL-13 and IL-22 (Li et al., 2019; Ivanov et al., 2013). Notably, infections with SARS-CoV-2, SARS-CoV, MERS-CoV, and EBOV have also been reported to induce cytokine storms and even death (Gao et al., 2021; Sun et al., 2020; Wang et al., 2022). Consequently, it is crucial to explore the regulators and regulatory mechanisms of cytokine storms, which may provide potential drug targets. Based on these studies, anti-inflammatory therapy that blocks the progression of cytokine storms to reduce systemic inflammation could be considered an ideal

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https://doi.org/10.1016/j.virusres.2025.199577
Received 20 December 2024; Received in revised form 13 April 2025; Accepted 17 April 2025
Available online 17 April 2025

therapeutic strategy.

It is well known that kinases, as intracellular hub molecules, play an essential role in regulating life processes. For example, RIPK1 kinase inhibitors have been found to be effective in controlling the cytokine storms induced by SARS-CoV-2 infection (Xu et al., 2021). JNK kinase has been shown to regulate the replication of multiple viruses in host cells. In addition, it is also involved in regulating inflammatory responses induced by viral infections (Chen et al., 2021; Li et al., 2022; Sun et al., 2020; Huang et al., 2011). Consequently, JNK kinase inhibitors have attracted considerable interest because of their substantial therapeutic potential (Manning and Davis, 2003). Notably, JNK kinase exerts its biological functions mainly by regulating the expression of downstream genes (Zeke et al., 2016). Interestingly, it was found that JNK kinase could also exert a regulatory role by interacting with BMPR2 (a receptor) (Zeke et al., 2016; Podkowa et al., 2010). This study expands our understanding of the regulatory role of JNK kinase.

TREM1 is a receptor expressed on myeloid cells, and it amplifies immune responses by enhancing the production of pro-inflammatory cytokines such as IL-6 and IL-1β, thereby intensifying the immune reaction (Zhang et al., 2022; Patel et al., 2020). This amplifying effect is crucial in the fight against infection, but in some cases, excessive activation of TREM1 can lead to pathological inflammation. It was regarded as an amplifier of the immune responses, which powerfully promotes the activation of leukocytes in response to microbial products. (Klesney-Tait et al., 2006). Studies have shown that TREM1 could trigger phagocytes to secrete pro-inflammatory factors, amplifying inflammation caused by bacteria and fungi (Colonna, 2003). In addition, it plays an essential role in virus-host interactions (Roe et al., 2014). It is reported that HIV and HCV could amplify inflammatory responses by upregulating TREM1 expression in Kupffer and myeloid cells (Hyun et al., 2019). Besides, reduced morbidity was observed in IAV-infected TREM1 knockout mice (Weber et al., 2014). More importantly, it also serves as a potential biomarker of SARS-CoV-2 infection (de Sa Resende et al., 2021).

In this study, we found that JNK kinase could promote inflammatory responses by inducing the expression of the inflammatory amplifier TREM1 during influenza A virus infection. This study further enriches our understanding of potential hubs in the IAV-host interaction network. It may provide some inspiration for subsequent researchers to explore the regulatory mechanisms of cytokine storms induced by emerging viral infections.

2. Materials and methods

2.1. Cells and viruses

Human lung adenocarcinoma epithelial cells (A549) were maintained in Dulbecco's Modified Eagle's medium (DMEM; Gibco) containing 100 IU/mL penicillin (Gibco), 100 μg/mL streptomycin, 0.2 % $NaHCO_3$, and 10 % fetal bovine serum (FBS; Gibco) at 37 °C with 5 % CO2. Influenza A virus A/WSN/1933 (H1N1) was inoculated into 10-day-old specific-pathogen-free (SPF) chicken embryos for virus propagation in our laboratory.

2.2. siRNA-mediated silencing

A549 cells were transfected with 100 nM of target genes siRNA or negative control (NC.si) using Lipofectamine 2000 (Invitrogen, USA) in 6-well plates and 24-well plates, according to the manufacturer's instructions. Negative control (NC.si) and siRNAs were acquired from Genepharma (Shanghai, China). The siRNAs designed for target genes were presented as follows: si-TREM1: 5′-GGUCAUUUGUACCCUAGGCTT-3′, si-JNK: 5′-GGAGGAGAGAACCAAGAAUTT-3′, and si-NC: 5′-UUCUCCGAACGUGUCACGUTT-3′.

2.3. Plasmid construction

Full-length DNA fragments encoding TREM1 were amplified by PCR and the PCR products were inserted into the vector pcDNA 3.1. All plasmid constructs were verified by Sanger sequencing. All primers and sequences used in this article were available from the authors upon request.

2.4. RT-PCR and qRT-PCR

Total RNA was extracted from A549 cells using TRIzol reagent (catalog number R0016; Beyotime). For the detection of mRNA, vRNA, and cRNA, oligo(dT)20, vRNA primers, and cRNA primers were used to generate cDNAs with 2 μg of total RNA per sample according to the HiScript II 1st-strand cDNA synthesis kit (Vazyme, China). RT-PCR and qRT-PCR were conducted using 2 × Taq Plus Master Mix (Vazyme, China) and the AceQ qPCR SYBR Green Master Mix (Vazyme, China). A template cDNA of 1 μL was used in the RT-PCR reaction system, and the reaction procedure was as follows: 95 °C for 5 min; 95 °C for 15 s, 56–58 °C for 15 s, 72 °C for 30 s, 30–35 cycles; 72 °C for 10 min; and storage at 4 °C. Besides, qRT-PCR was conducted using the cDNAs and gene-specific primers with the AceQ qPCR SYBR Green Master Mix on a Roche LightCycler 96, according to the manufacturer's instructions using the following cycling program: 95 °C for 5 mins, 40 cycles at 95 °C for 10 s, and 60 °C for 30 s. The primers are presented in Table S1. The $2-\Delta\Delta Ct$ method was used to determine the relative levels of candidate genes.

2.5. Drug treatment

JNK inhibitor SP600125 (#8177) was purchased from Cell Signaling Technology. For inhibition of protein activity, cells were treated with the SP600125 or dimethyl sulfoxide (DMSO) as a vehicle control for 2 h, and then the cells were infected with IAV for 24 and 48 h in the presence of inhibitors.

2.6. Western blotting

Cells were washed twice with ice-cold PBS and lysed in cold lysis buffer (1 % Triton X-100, 1 mM phenylmethylsulfonyl difluoride [PMSF] in PBS) for 30 min. Cell lysates were separated by SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane (Amersham, 10,600,001). The membranes were then blocked with 5 % nonfat milk for 2 h. Next, the membranes were incubated with primary antibodies and horseradish peroxidase-conjugated secondary antibodies. Finally, the membranes were incubated with enhanced chemiluminescence (ECL) reagent (Vazyme, China), and the protein signals were analyzed using an Amersham Imager 600 (GE Healthcare). Proteins were detected with the following antibodies: anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (10,494–1-AP; Proteintech), anti-p-JNK antibody (sc-6254; Santa), anti-JNK antibody (sc-137,019; Santa), and anti-TREM1 antibody (11,791–1-AP; Proteintech).

2.7. ELISA assay

The concentration of IL-6 in cell culture supernatant was measured using the ELISA kit according to the manufacturer's instructions. The ELISA kit for IL-6 was purchased from BYabscience.

2.8. RNA-seq analysis

A549 cells cultured in 6-well plates were pretreated with 20 μM SP600125 or DMSO as a vehicle control for 2 h and subsequently infected with WSN (MOI = 1). Infected and uninfected cells were collected at 24hpi. Total RNA was extracted for RNA-seq analysis. The

concentration and integrity of the extracted RNA were evaluated by Agilent 2200 Bioanalyzer (Agilent Technologies, USA). RNA samples with RNA Integrity Number ≥ 7 were utilized for library construction. The libraries were generated utilizing the TruSeq RNA Sample Preparation Kit (Illumina, USA) according to the manufacturer's instructions. Thereafter, the samples were subjected to sequencing on the Illumina HiSeq XTen (Illumina, USA). The construction and sequencing of libraries were conducted by Shanghai OE Biotech Co. (Shanghai, China). The analysis of differentially expressed genes (DEGs) was conducted utilizing the DEGSeq algorithm, and DEGs with a p-value ⟨ 0.05, an FDR < 0.05, and a fold change ⟩ 2 were selected for GO and KEGG pathway enrichment analyses, respectively.

2.9. Plaque assays

The plaque assays were employed to assess the viral titer. Briefly, viral supernatants were serially 10-fold diluted using DMEM and inoculated onto an MDCK cell monolayer. After incubation at 37 °C for 1 h, the cells were overlaid with 2x DMEM containing 2 % SeaPlaque agarose (Lonza) and 2 μg/mL TPCK-trypsin and then placed upside down at 37 °C for 48 h, followed by the detection of the quantity of plaques.

2.10. Statistical analysis

Data are presented as mean ± standard deviation (SD) obtained from at least three independent experiments unless otherwise indicated. Statistical analysis was performed using GraphPad Prism software version 7.0 (GraphPad Software, Inc.). Student's t-test was used to analyze the data. Significant differences are indicated by asterisks as follows: *, P < 0.05; **, P < 0.01; ***, P < 0.001.

3. Results

3.1. mRNA expression profiles and biological function of JNK kinase during IAV infection analyzed by functional enrichment

Previous studies have shown that JNK kinase could regulate the replication of multiple viruses in cells. Furthermore, it is also involved in regulating inflammatory responses induced by viral infections (Chen et al., 2021; Li et al., 2022; Sun et al., 2020; Huang et al., 2011). SP600125 is a specific inhibitor of JNK kinase. To further explore the biological function of JNK kinase during IAV infection, RNA-seq analysis was performed to compare the differentially expressed genes in the mock-treated and two types of IAV-infected cells. Data analysis revealed that 236 genes were upregulated, and 150 genes were downregulated (fold change > 2, p < 0.05) in IAV-infected cells treated with SP600125 compared with the controls (Fig. 1A–C). Regulating the expression of so many genes suggests that JNK kinase possesses the potential to regulate many biological functions. A Venn diagram showed the intersection of differentially expressed genes among the three groups (Fig. 1D). KEGG pathway enrichment analysis demonstrated that the TNF signaling pathway, IL-17 signaling pathway, Toll-like receptor signaling pathway, and NF-kappa B signaling pathway were significantly enriched (Fig. 1E). Interestingly, several metabolic pathways were significantly enriched, highlighting the potential of metabolic regulation, which deserves further study in the future (Fig. 1E and F).

3.2. JNK kinase regulates the IAV-induced cytokine storms by affecting the expression of pro-inflammatory and anti-inflammatory factors

The corresponding KEGG enrichment map (WSN_SP600125-vs-WSN) illustrated that SP600125 treatment significantly reduced the expression of pro-inflammatory factors IL-6, IL-1β, IL-1α, and IL-8 at 24hpi (Fig. S1). Next, RT-PCR and qRT-PCR were performed to validate the KEGG pathway analysis results (Fig. 2A–E). The qPCR data showed that the mRNA levels of IL-6, IL-1β, IL-1α, and IL-8 were decreased to 47.84 %, 41.44 %, 48.42 %, and 48.55 %, respectively (Fig. 2B–E). These results suggest that JNK kinase induces the expression of pro-inflammatory factors such as IL-6 during IAV infection.

To further explore the biological function of JNK kinase during IAV infection, GO enrichment analysis was also performed in addition to KEGG pathway analysis. Interestingly, the GO enrichment analysis revealed that inflammatory signaling pathways were highly enriched (Fig. 3A). Given that many pro-inflammatory and anti-inflammatory factors are enriched, we speculate that JNK kinase mainly regulates inflammatory responses during IAV infection. Next, these enriched pro-inflammatory and anti-inflammatory factors were clustered and shown in a heat map (WSN_SP600125-vs-WSN) (Fig. 3B). Notably, pro-inflammatory factors were predominant (over 85 %). They include IL-6, IL-1β, IL-1α, IL-8, IL-23, IL-32, IL-36γ, CCL3, CCL4, CCL20, CXCL2, and CXCL3.

Next, RT-PCR and qRT-PCR were performed to validate the differential expression of these pro-inflammatory factors (Fig. 3C–K). Interestingly, we found that these enriched factors included the anti-inflammatory factors IL-13 and IL-22. The results showed that SP600125 treatment significantly increased the expression of IL-13 and IL-22 (Fig. 3C, 3L, and 3M). IL-22 has been reported to reduce lung inflammation during IAV infection (Ivanov et al., 2013). Besides, IL-13 is an anti-inflammatory factor during IAV infection (Li et al., 2019). Therefore, we suggested that JNK kinase could amplify the inflammatory responses by inhibiting the expression of anti-inflammatory factors. Some studies have demonstrated that kinases are essential in regulating the cytokine storms induced by viral infections. RIPK1 kinase inhibitors could effectively control the cytokine storms induced by SARS-CoV-2 infection (Xu et al., 2021). Furthermore, JAK inhibitors are a potential therapeutic strategy for cytokine storms (Spinelli et al., 2020). In conclusion, these results suggest that JNK kinase regulates the IAV-induced cytokine storms by affecting the expression of pro-inflammatory and anti-inflammatory factors.

3.3. JNK kinase induces the expression of the inflammatory amplifier TREM1 during IAV infection

Studies have shown that JNK kinase exerts its biological functions mainly by regulating the expression of downstream genes (Statello et al., 2021; Liu et al., 2019; Gil and Ulitsky, 2020). Given this, we present the top 30 differentially expressed genes between two groups (WSN_SP600125-vs-WSN) as a heat map (Fig. 4A and S2A). Interestingly, we found that the most significantly upregulated by JNK kinase was TREM1, an inflammatory amplifier. The results of RT-PCR, western blotting, and qRT-PCR showed that SP600125 treatment significantly decreased JNK phosphorylation and the expression of TREM1, suggesting that JNK kinase induces the expression of the inflammatory amplifier TREM1 during IAV infection (Fig. 4B–D). Besides, the inhibitory effect of SP600125 on TREM1 expression increased gradually with the increase of SP600125 dose (Fig. 4E). In eukaryotic cells, pre-mRNA is transcribed from a gene's DNA template. Pre-mRNA splicing removes the introns and ligates the exons to form a mature mRNA (Ouyang et al., 2014; Liu et al., 2019). We conjectured that JNK kinase may regulate TREM1 pre-mRNA levels. Next, we designed specific primers to examine the pre-mRNA levels of TREM1. We found that SP600125 treatment significantly reduced the preTREM1 levels at 24hpi (Fig. S2B). Furthermore, JNK knockdown drastically inhibited the expression of IL-6, IL-1β, and TREM1 (Fig. S2C-G). Therefore, we speculate that the role of JNK kinase in regulating inflammatory responses may be related to TREM1.

For the purpose of further investigating TREM1 expression during IAV infection, qRT-PCR was performed to measure the TREM1 mRNA levels at the specific time points following infection. The results showed that TREM1 was induced by IAV infection in a time-dependent fashion (Fig. 4F). Moreover, A549 cells were infected with IAV at different MOIs. The TREM1 mRNA levels were determined by qRT-PCR. The data

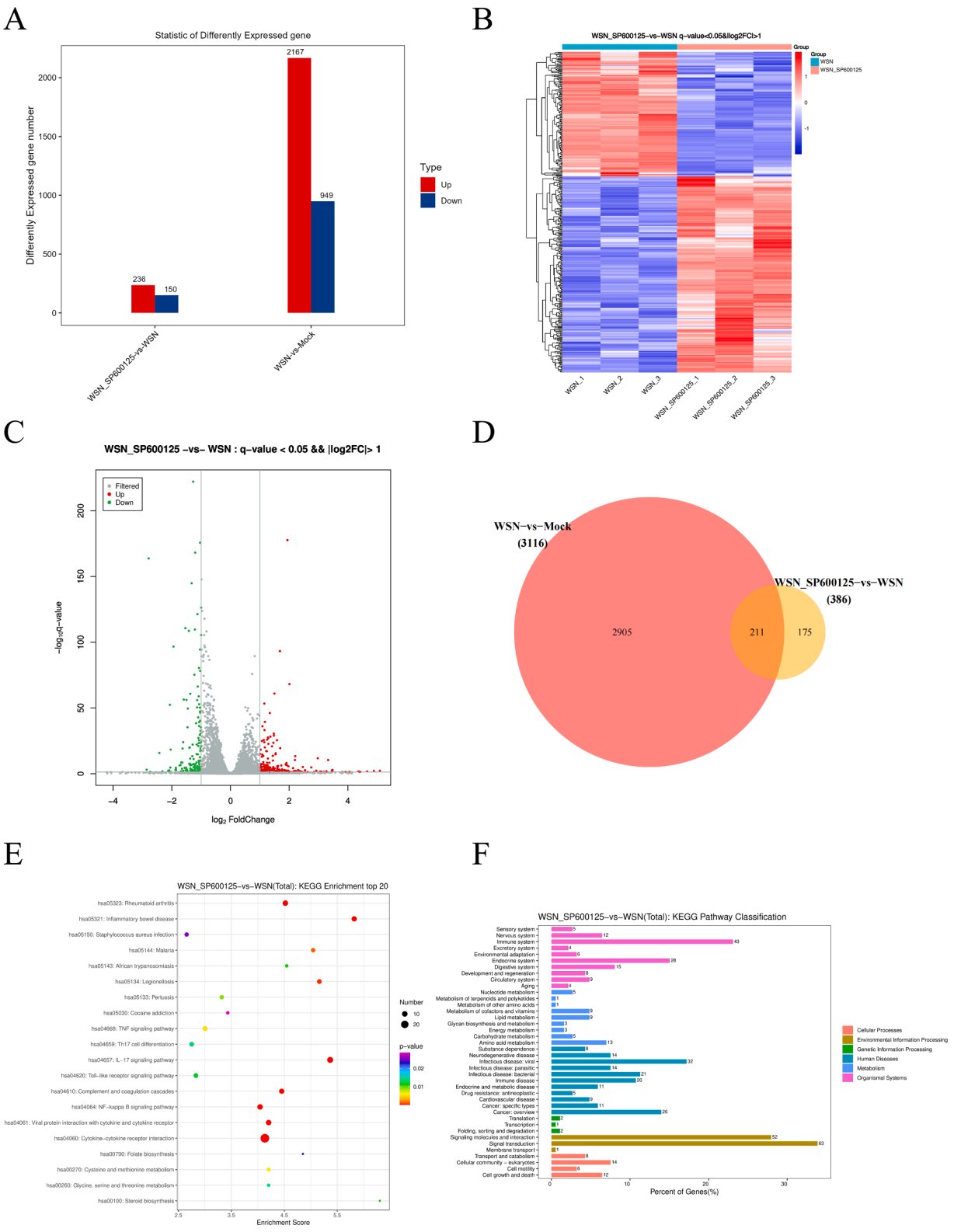

Fig. 1. mRNA expression profiles and biological function of JNK kinase during IAV infection analyzed by functional enrichment.
(A) A549 cells were pretreated with 20 μM SP600125 or DMSO as a vehicle control for 2 h and subsequently infected with WSN (MOI = 1). Infected and uninfected cells were collected at 24hpi. Total RNA was extracted for RNA-seq analysis. A bar graph showing the number of differentially expressed mRNAs between two groups. (B) A cluster heat map showing the mRNAs with significantly different expressions between two groups (WSN_SP600125-vs-WSN). (C) A volcano map showing the mRNAs with significantly different expressions between two groups (WSN_SP600125-vs-WSN). (D) A Venn diagram showing the number of differentially expressed mRNAs between two groups and the intersection. (E) A bubble plot showing the top20 enriched KEGG pathways (WSN_SP600125-vs-WSN). (F) KEGG classification diagram (WSN_SP600125-vs-WSN).

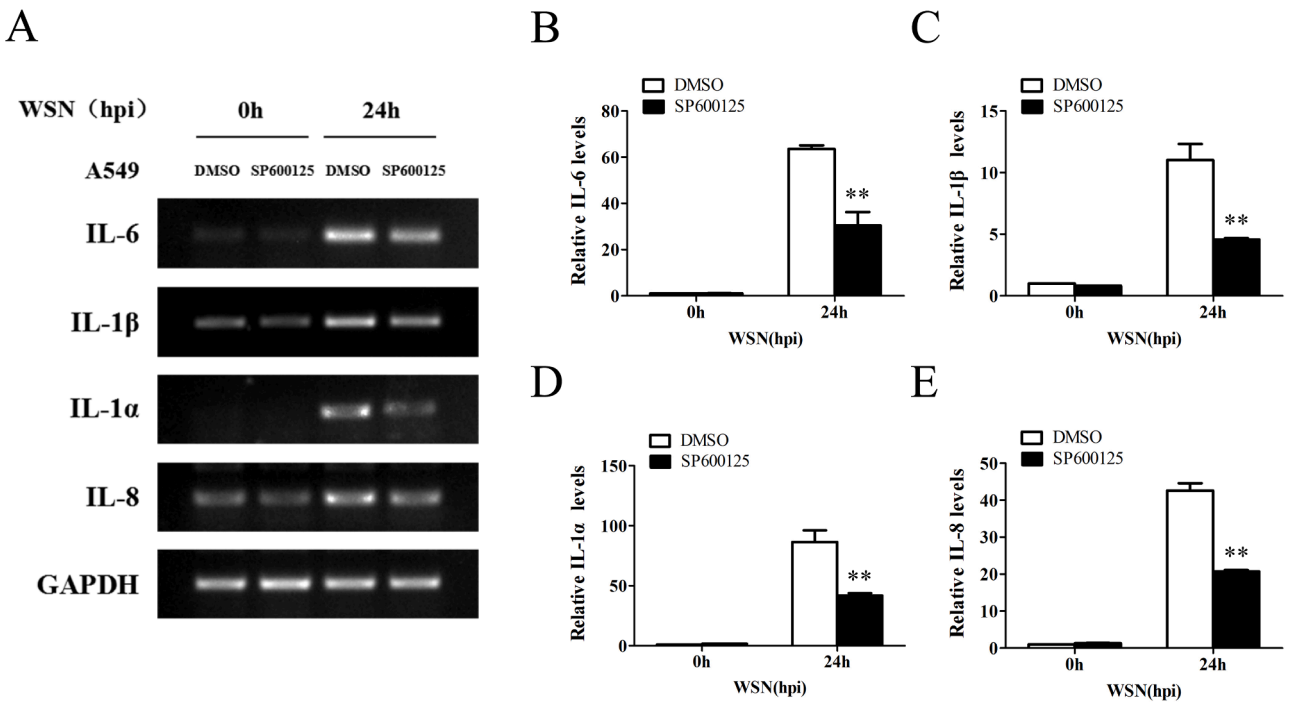

Fig. 2. JNK kinase induces the expression of pro-inflammatory factors IL-6, IL-1β, IL-1α, and IL-8 during IAV infection.
(A) A549 cells were pretreated with 20 μM SP600125 or DMSO as a vehicle control for 2 h and subsequently infected with WSN. Total RNA was extracted at 24hpi. Real-time polymerase chain reaction (RT-PCR) was performed to detect the expression of IL-6, IL-1β, IL-1α, and IL-8. (B-E) Quantitative real-time polymerase chain reaction (qRT-PCR) was conducted to validate the expression of IL-6 (B), IL-1β (C), IL-1α (D), and IL-8 (E). Data represent means ± standard deviations (n = 3; **, $P < 0.01$).

demonstrated that TREM1 was upregulated in an IAV dose-dependent manner, with a 300-fold increase at the highest infectious dose (Fig. 4G). Together, these results imply that TREM1 expression is induced in A549 cells after IAV infection.

3.4. TREM1 amplifies the inflammatory responses during IAV infection

It has been suggested that TREM1 plays an essential role in amplifying the inflammatory responses induced by microbial infections (Colonna, 2003; Roe et al., 2014; Hyun et al., 2019). Therefore, we speculate that TREM1 could also amplify the inflammatory responses during IAV infection. To examine this hypothesis, A549 cells plated in 24-well plates underwent transfection with si-NC and si-TREM1 and were then subjected to infection with IAV. The results of western blotting and qRT-PCR showed that TREM1 was knocked down by siRNA in A549 cells (Fig. 5A, S2H, S2I). To gain a more comprehensive understanding of the functional role of TREM1 during IAV infection, we analyzed the interference efficiency of si-TREM1 in A549 cells seeded in 6-well plates. The interference efficiency of si-TREM1 is about 60 % (Fig. 5B). Consistent with the conjecture, we found that the mRNA level of pro-inflammatory factors was significantly reduced in the TREM1 knockdown cells compared to that in the control cells. These pro-inflammatory factors include IL-6, IL-1β, IL-1α, IL-8, CCL3, CCL4, CCL20, CXCL2, CXCL3, IL-23, IL-32, and IL-36γ (Fig. 5C–N). These data indicate that TREM1 could also amplify the inflammatory responses during IAV infection. Besides, the results indicated that TREM1 overexpression increased IL-6 expression and reversed the effect of SP600125 treatment on the expression of IL-6 and IL-1β (Fig. 5O-Q and S2J-K). Based on these results, we suggest that the inflammatory amplifier TREM1 could be induced by JNK kinase to amplify the inflammatory responses induced by IAV infection.

4. Discussion

Previous studies have shown that JNK kinase regulates inflammatory responses during viral infections (Li et al., 2022; Sun et al., 2020; Huang et al., 2011). In this study, we demonstrated that JNK kinase could regulate IAV-induced cytokine storms by affecting the expression of pro-inflammatory and anti-inflammatory factors. Further studies revealed that inhibition of JNK kinase activity significantly downregulated the expression of the inflammatory amplifier TREM1. Besides, TREM1 knockdown could significantly inhibit the expression of pro-inflammatory factors such as IL-6, IL-1β, IL-1α, and IL-8. Additionally, the results indicated that TREM1 overexpression reversed the effect of SP600125 treatment on the expression of pro-inflammatory factors. Therefore, we hypothesize that JNK kinase could amplify the inflammatory responses by positively regulating the expression of the inflammatory amplifier TREM1 during IAV infection. These results expand our understanding of JNK kinase amplifying the inflammatory responses induced by viral infection. Notably, experimental results indicate that SP600125 treatment inhibited WSN replication at 24hpi to a certain extent (Fig. S3A). TREM1 knockdown or TREM1 overexpression did not regulate WSN replication (Fig. S3B and S3C). Besides, SP600125 treatment still regulated the expression of IL-6, IL-1β, and TREM1 at 48hpi but might not regulate WSN replication (Fig. S3D-S3G). TREM1 overexpression reversed the effect of SP600125 treatment on IL-6 expression (Fig. S3H). The above results indicate that the regulatory effect of JNK kinase on the expression of IL-6, IL-1β, and TREM1 during WSN infection is independent of viral replication.

The treatment experience of COVID-19 patients may provide meaningful guidance for rational clinical medication of JNK kinase and TREM1, especially the delivery time of anti-inflammatory drugs. Inflammation is a double-edged sword during SARS-CoV-2 infection. In the early stages of infection, inflammation is a beneficial host response and may contribute to viral clearance. Therefore, early drug administration may impede the immune system from clearing the virus and

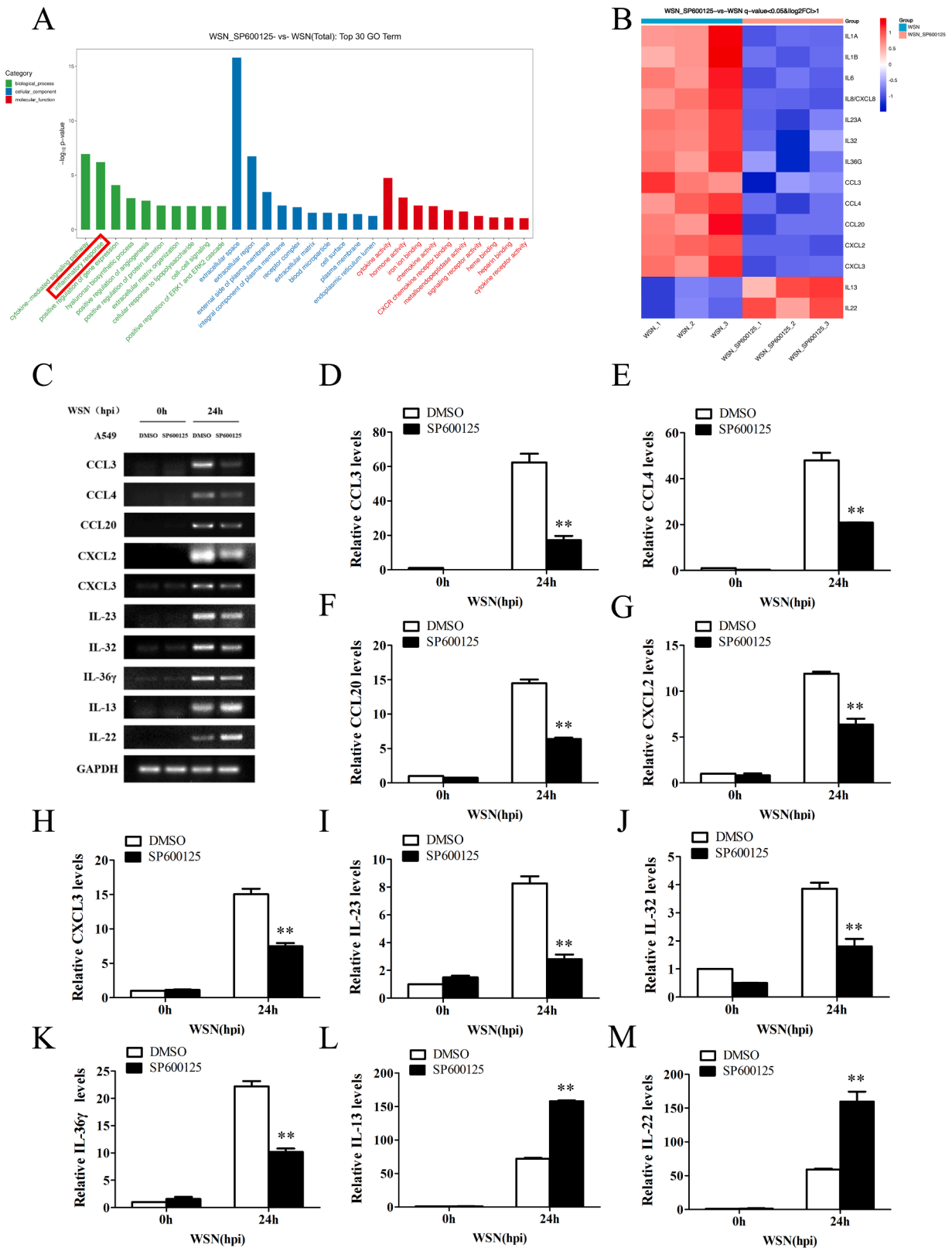

Fig. 3. JNK kinase regulates the IAV-induced cytokine storms by affecting the expression of pro-inflammatory and anti-inflammatory factors.
(A) Top30 GO enrichment terms (WSN_SP600125-vs-WSN). (B) A heat map showing these pro-inflammatory and anti-inflammatory factors with significantly different expressions between two groups. (C) RT-PCR was performed to validate the expression of CCL3, CCL4, CCL20, CXCL2, CXCL3, IL-23, IL-32, IL-36γ, IL-13, and IL-22. (D-M) qRT-PCR was conducted to validate the expression of CCL3 (D), CCL4 (E), CCL20 (F), CXCL2 (G), CXCL3 (H), IL-23 (I), IL-32 (J), IL-36γ (K), IL-13 (L), and IL-22 (M). Data represent means ± standard deviations (n = 3; **, $P < 0.01$).

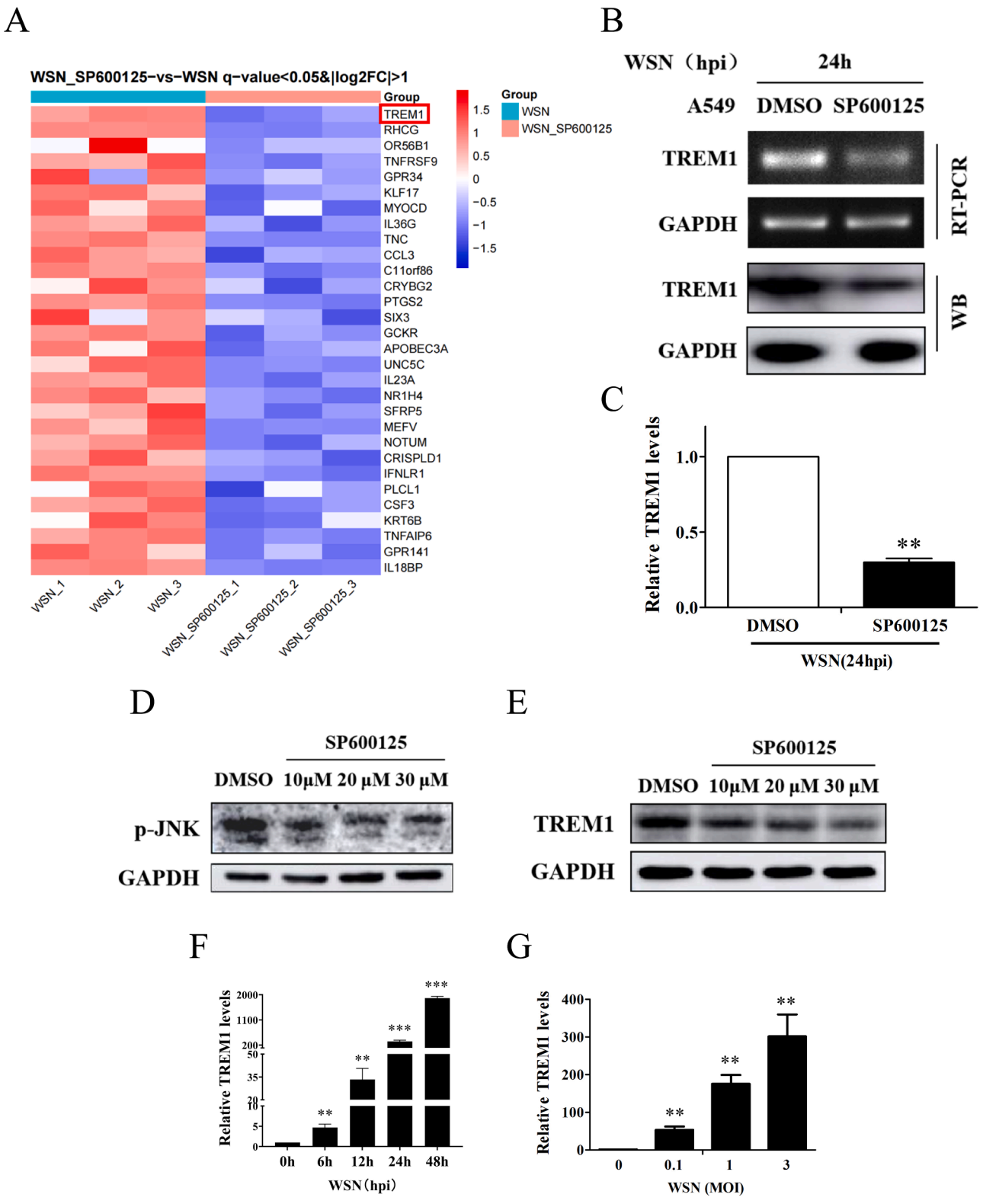

Fig. 4. JNK kinase induces the expression of the inflammatory amplifier TREM1 during IAV infection.
(A) A heat map showing the top 30 downregulated differentially expressed genes between two groups (WSN_SP600125-vs-WSN). (B, C) RT-PCR, western blotting, and qRT-PCR were conducted to validate the expression of TREM1, respectively. (D, E) A549 cells were pretreated with SP600125 (10, 20, or 30 μM) or DMSO as a vehicle control for 2 h and subsequently infected with WSN. Western blotting was performed to detect the phosphorylation level of JNK kinase and the expression of TREM1. (F) qRT-PCR was performed to examine the differential expression of TREM1 in A549 cells infected with or without WSN. (G) A549 cells were infected with various multiplicities of infection (MOI) of WSN for a period of 24 h. The expression level of TREM1 was detected by qRT-PCR. Data represent means ± standard deviations (n = 3; **, $P < 0.01$; ***, $P < 0.001$).

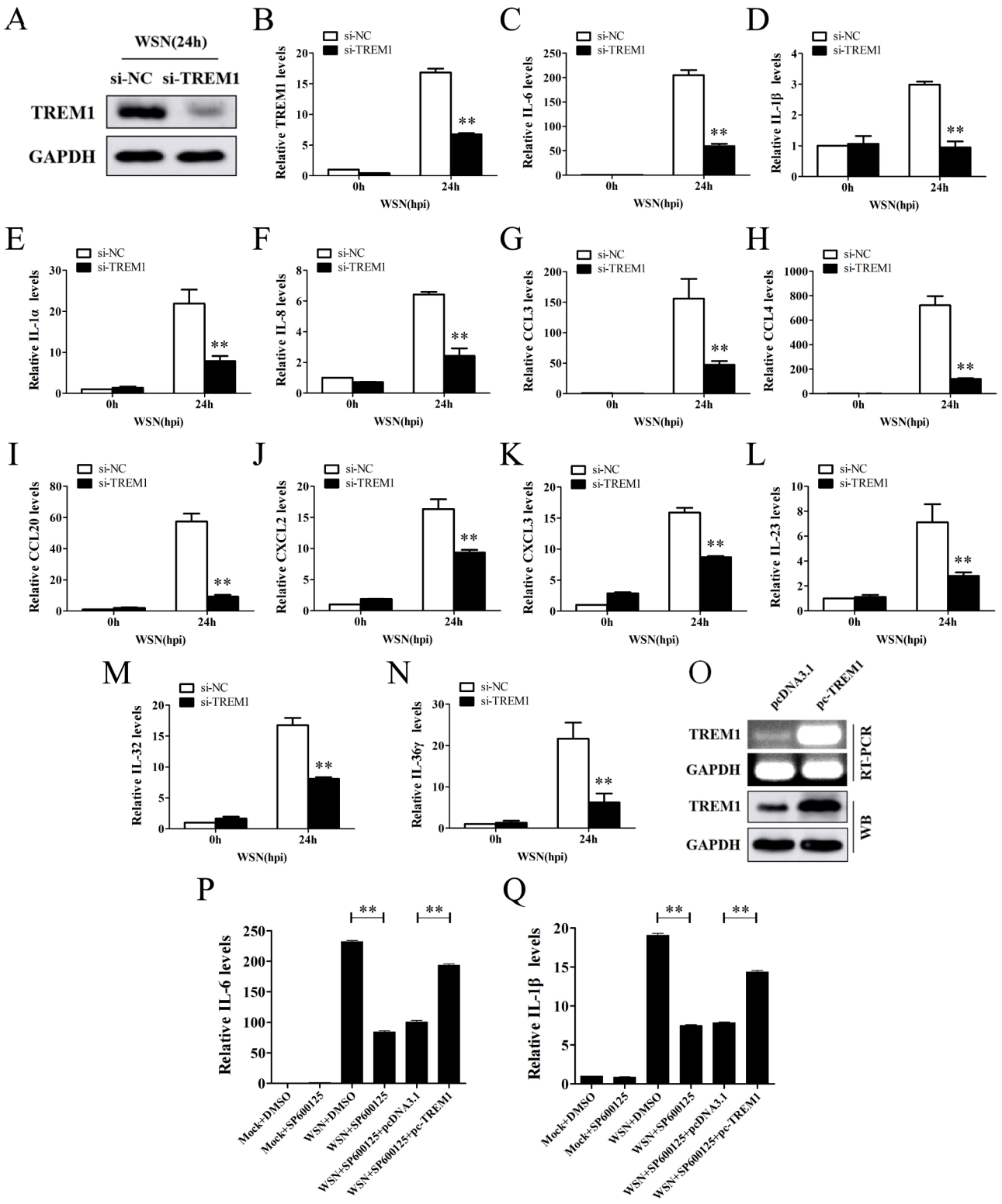

Fig. 5. TREM1 amplifies the inflammatory responses during IAV infection.
(A-N) A549 cells seeded in 24-well plates and 6-well plates were transfected with si-NC/si-TREM1 and then infected with WSN (MOI = 1). Western blotting and qRT-PCR were performed to detect the expression of TREM1 in A549 cells (A, B). qRT-PCR was conducted to detect the expression of IL-6 (C), IL-1β (D), IL-1α (E), IL-8 (F), CCL3 (G), CCL4 (H), CCL20 (I), CXCL2 (J), CXCL3 (K), IL-23 (L), IL-32 (M), and IL-36γ (N). (O) A549 cells underwent transfection with pcDNA3.1/pcDNA3.1-TREM1 for 36 h. The overexpression efficiency of TREM1 in A549 cells was determined by RT-PCR and western blotting. (P, Q) The mRNA expression of pro-inflammatory factors in each group at 24hpi was measured by qRT-PCR. Data represent means ± standard deviations (n = 3; **, $P < 0.01$).

promote its spread. In the advanced stages, the inflammatory responses become detrimental, worsening the disease in 10 to 15 percent of patients. Consequently, the delivery of anti-inflammatory drugs at the start of cytokine storms may effectively prevent the development of ARDS and multiple organ failure (Zoulikha et al., 2022). The treatment experience of COVID-19 patients gives us insight into the importance of the timing of anti-inflammatory drug delivery, which is an essential guide for the delivery of anti-inflammatory drugs targeting JNK kinase or TREM1. Besides, the translational potential of targeting JNK kinase or TREM1 for the treatment of IAV-induced cytokine storms requires further investigation, including the assessment of drug efficacy and safety in preclinical and clinical settings.

Exploring TREM1 ligands during IAV infection is of great significance for understanding its inflammatory response mechanism. At present, although its specific ligand has not been determined, multiple potential ligands have been discovered in other disease studies. High mobility group protein B1 (HMGB1) has been mentioned in multiple studies as a damage-associated molecular pattern protein (DAMP) that can directly interact with TREM1, induce inflammatory responses, and play an important role in diseases such as cerebral hemorrhage and liver injury (Tammaro et al., 2017; Gao et al., 2019; Li et al., 2024). It may also have a similar role in the process of IAV infection. Peptidoglycan recognition protein 1 (PGLYRP1) can form a complex with peptidoglycan to activate TREM1 and participate in the inflammatory response caused by bacterial infection (Fan et al., 2024; Li et al., 2025; Zhang et al., 2022; Kouassi et al., 2018). Its role in IAV infection remains to be explored. Extracellular cold inducible RNA binding protein (eCIRP) and extracellular actin have also been confirmed to be ligands of TREM1, playing important roles in inflammatory diseases and sepsis, respectively (Li et al., 2024; Fan et al., 2024; Zhang et al., 2022). These potential ligands discovered in other diseases provide important clues for studying the ligands of TREM1 during IAV infection, and further exploration is needed to investigate their relationship with TREM1 during IAV infection in the future.

While our study demonstrates that SP600125, a specific JNK inhibitor, significantly modulates the inflammatory response at the cellular level, it is essential to acknowledge the limitations inherent to using an in vitro system. Cell culture models, although invaluable for dissecting molecular pathways and understanding specific mechanisms, do not fully replicate the complex interactions present in a living organism. For example, the immune response in vivo involves not only individual cells but also complex tissue environments, systemic factors, and multiple cell types interacting simultaneously. The use of SP600125 in our in vitro experiments allowed us to investigate its effects on inflammation-related pathways. Nevertheless, the findings from these experiments may not fully translate to in vivo conditions, where factors such as drug metabolism, tissue distribution, and multi-organ interactions play critical roles. To address these limitations and strengthen the conclusions drawn from this study, future research will involve conducting in vivo experiments using mouse models. These studies will enable us to validate the regulation of SP600125 on inflammatory response in a more physiologically relevant context and assess the broader implications of inhibition of JNK activity on systemic inflammation.

Besides, although this study has achieved some results, the detailed molecular mechanisms underlying the regulation of TREM1 pre-mRNA levels by JNK kinase remain to be fully elucidated. Investigating the specific transcriptional and post-transcriptional regulators involved could provide a more comprehensive understanding of this regulatory process. Several references have reported that the TREM1 promoter contains multiple potential binding motifs for AP-1 (Hosoda et al., 2015; Hosoda et al., 2011; Peng et al., 2016). Furthermore, JNK kinase participates in regulating AP-1 activity (Chen et al., 2021; Zhou et al., 2015; Sui et al., 2014). Therefore, in the future, it is necessary to further determine whether there is a connection between JNK, AP-1, and TREM1, for example, to determine whether the TREM1 promoter responds to IAV infection and is suppressed by inhibiting JNK. Moreover, exploring the potential crosstalk between JNK kinase and other signaling pathways during IAV infection may uncover additional regulatory mechanisms and potential therapeutic targets. In addition, further clarification of TREM1 ligands is still needed in the future, which will improve the understanding of related mechanisms and enhance the comprehensiveness and reliability of the study. The current study is mainly focused on A549 cells. Nonetheless, it is well known that different cell types may exhibit significant differences in immune responses following IAV infection. Future research could be extended to macrophages, other cell types of the respiratory epithelium such as bronchial epithelial cells, and immune cell subsets like T lymphocytes and NK cells. Investigations into the expression changes and interaction relationships of JNK kinase and TREM1 in these cells are warranted. It is essential to determine whether the regulatory pattern of JNK kinase on TREM1 is consistent across different cell types and whether the amplifying effect of TREM1 on pro-inflammatory factors is cell-specific.

In conclusion, we found that the inflammatory amplifier TREM1 could be induced by JNK kinase to amplify the inflammatory responses during IAV infection. This study further enriches our understanding of potential hubs in the IAV-host interaction network. It may provide some inspiration for subsequent researchers to explore the regulatory mechanisms of cytokine storms induced by emerging viral infections. Besides, animal experiments are needed to further explore the role of JNK kinase in regulating inflammatory response at the animal level in the future.

Glossary

IAV	Influenza A virus
ALI	Acute lung injury
JNK	c-Jun N-terminal kinase
TREM1	Triggering receptor expressed on myeloid cells-1
TNF-α	Tumour necrosis factor-alpha
ILs	Interleukins
CCLs	Cys-Cys motif chemokines
CXCLs	Cys-X-Cys motif chemokines
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SARS-CoV	Severe acute respiratory syndrome coronavirus
MERS-CoV	Middle East respiratory syndrome
EBOV	Ebola virus
RIPK1	Receptor-interacting protein kinase 1
BMPR2	Bone morphogenetic protein receptor 2
HIV	Human immunodeficiency virus
HCV	Hepatitis C virus
JAK	Janus kinase
MOI	Multiplicities of infection
COVID-19	Coronavirus disease 2019
ARDS	Acute respiratory distress syndrome

Funding statement

This work was supported by the National Key Research and Development Programme of China (Grant number: 2021YFD1800205), the National Natural Science Foundation of China (Grant numbers: 31772775, 32272992), the State Key Laboratory of Veterinary Biotechnology (SKLVBF202103), and the Fundamental Research Funds for the Central Universities (YDZX2023005).

CRediT authorship contribution statement

Na Chen: Writing – review & editing, Writing – original draft, Conceptualization. **Jiayu Jin:** Writing – review & editing. **Bingchen Qiao:** Writing – review & editing. **Zihe Gao:** Visualization. **Yusen Tian:** Visualization. **Jihui Ping:** Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2025.199577.

Data availability

Data will be made available on request.

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