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Original Research

Vaccinia virus Tiantan strain blocks host antiviral innate immunity and programmed cell death by disrupting gene expression

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ABSTRACT

The vaccinia virus Tiantan (VTT) is widely utilized as a smallpox vaccine in China and holds significant importance in the prevention of diseases stemming from poxvirus infections. Nevertheless, few studies have investigated the influence of VTT infection on host gene expression. In this study, we constructed time series transcriptomic profiles of HeLa cells infected with both VTT and western reserve (WR) strains. We observed similar patterns of viral gene expression, while the expression levels of host genes varied between the two strains. There was an immediate and significant repression of host gene expression, particularly in genes associated with oxidative phosphorylation. Conversely, genes involved in nerve growth factor (NGF)-stimulated transcription were significantly activated. The upregulation of genes linked to the ribonucleic acid (RNA)-induced silencing complex (RISC) suggested a potential role for posttranscriptional regulation in the interaction between the vaccinia virus and the host. In the later stages of infection, pathways such as extracellular matrix organization, neutrophil degranulation, complement and interferon responses, translation, and programmed cell death are largely inhibited. A significant number of host genes exhibit correlations with changes in the expression levels of viral genes. The host genes that are negatively correlated with viral genes are mainly enriched in pathways associated with translation and the response to viral infection. This study significantly contributes to advancing our understanding of the dynamics between the vaccinia virus and the host, improving the application of VTTs and facilitating the development of effective vaccines against diseases such as smallpox and monkeypox.

1. Introduction

Poxviruses, such as smallpox, monkeypox, cowpox, and vaccinia viruses, constitute a family of double-stranded deoxyribonucleic acid (DNA) viruses that can infect humans [1]. The smallpox virus is one of the deadliest infectious diseases in human history. Recently, the monkeypox virus has spread out of Africa and is widely prevalent globally [2–4]; thus, the development of vaccines and drugs is crucial for epidemic prevention and control. As vaccinia virus (VACV) has low pathogenicity, it is widely used for vaccination to prevent smallpox infection. The VACV genome is composed of approximately 200,000 base pairs and contains approximately 200 genes encoding a variety of functional proteins [5]. These viral proteins play important roles during infection, participating in biological processes such as virus genome replication, protein synthesis, and interference with host immune responses [6]. By studying the interaction mechanism of VACV, a key pathogen for the prevention and control of diseases caused by poxvirus infection, with respect to the host, we can better understand the infection, replication, and pathogenesis of poxvirus. These findings are highly important for the prevention and control of diseases caused by infection with smallpox, monkeypox, and related poxviruses.

After poxvirus infects host cells, a replication factory is built, and viral genes start transcription and translation in a specific order [7,8]. Early viral proteins are responsible for inhibiting host antiviral pathways and replicating the viral genome, whereas middle and late

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HIGHLIGHTS

Scientific question

To our best knowledge, few systematic studies have reported the changes in host gene expression after vaccinia virus Tiantan (VTT) infection. This study aimed to reveal the dynamic process of reshaping the expression network of the host's innate immune system at various stages of infection through time-series transcriptome analysis.

Evidence before this study

Studies on the host-virus interaction mechanism of vaccinia virus (VACV) based on the western reserve (WR) have revealed that the VACV suppresses the expression of host genes related to innate immunity. Infection of the replication-deficient modified vaccinia virus Ankara (MVA) strain can induce the type I interferon (IFN-I) pathway.

New findings

VTT and WR have similar gene expression patterns, widely suppressing host gene expression and regulating the expression of different host genes. VTT disrupts the host immune response to facilitate replication, underscoring the critical virus-host interactions in poxvirus infections.

Significance of the study

The outcomes of this study are significant for advancing our understanding of the interaction between the VTT and the host, enhancing the utilization of the VTT strain as novel viral vector or effective vaccine targeting smallpox and monkeypox.

proteins participate in viral particle assembly and envelope formation [9]. In addition, poxvirus infection affects numerous signal transduction pathways in host cells. Virus-encoded proteins can block host immune responses [10,11], interfere with apoptotic signals [12], and manipulate host protein synthesis [13,14]. Poxvirus infection causes many changes in host gene expression. Recent transcriptomic analysis revealed significant changes in the expression of thousands of genes in infected cells [15–17], leading to the global shutoff of host genes and the suppression of the expression of some genes related to cell growth and metabolism [16,17].

Moreover, viral infections can induce the activation of host immune responses, leading to notable upregulation of the expression of host immune-related genes, such as interferons and inflammation-related genes [18–20]. Signaling pathways associated with viral infections have been identified as crucial for regulating gene expression. VACV infection can trigger innate immune signaling pathways, such as the cGAS-STING and NF-κB pathways, leading to immune modulation and inflammatory responses. Recent single-cell transcriptome sequencing studies revealed distinct patterns of gene expression regulation in the innate immune pathway between infected and bystander (i.e., uninfected) cells [18,19]. Furthermore, integrated analysis of the transcriptome and proteome indicated that VACV modulates the expression of host proteins through the regulation of translation and degradation processes [21,22]. This finding suggests that the mechanism underlying VACV gene expression and its interaction with the host may be more intricate than previously known.

Compared with those of other strains, the genes of VACV exhibit rapid evolutionary changes [6], with the vaccinia virus Tiantan (VTT) strain showing variations in gene content due to gains and losses (unpublished data). In this study, we utilized a HeLa cell infection model and transcriptome analysis to investigate the gene expression patterns of host and viral genes at early, middle, and late infection stages. Both the VTT and western reserve (WR) strains presented similar expression profiles, primarily suppressing host transcription but eliciting distinct host responses. Notably, antiviral pathways are suppressed as a consequence of viral proteins compromising the host immune response. Multiple host genes associated with vital biological processes were downregulated, and their expression levels were correlated with those of viral genes. This study integrated publicly available data from WR infection proteomics to conduct a preliminary in silico screening of host factors that may affect VTT replication. These findings also indicate a potential role for ribonucleic acid (RNA) interference in the interaction between viruses and host cells.

2. Materials and methods

2.1. Viruses and cells

VTT, the smallpox virus vaccine strain in China, which was originally obtained from the Chinese Food and Drug Inspection Institute (formerly the Chinese Biological Products Inspection Institute), was transferred as a gift to the Virus Disease Prevention and Control Institute of the Chinese Center for Disease Control and Prevention (formerly known as the Institute of Virology under the Chinese Academy of Preventive Medicine) for scientific investigation and was assigned the code 7601. The representative monoclonal virus strain was isolated and named Vaccinia Tiantan TK^+ (VTT-TK^+). The WR strain was a gift from Bernard Moss's laboratory. The conditions for the cultivation of HeLa cells were as follows: cell culture medium was prepared by adding 10 % fetal bovine serum (FBS), 1 % non-essential amino acids (NEAA), and 1 % PBS to dulbecco's modified eagle medium (DMEM), and the cells were incubated in a 37 °C incubator with 5 % CO_2.

2.2. Viral infection

The HeLa cells were revived and utilized from the fourth generation. Upon reaching 80 % confluence, the cells were infected with VTT or WR at a multiplicity of infection (MOI) of 5. The infected cells were then incubated in a 37 °C incubator with 5 % CO_2 for 1 h. The medium was discarded, and the cells were washed twice with PBS to eliminate dead cells. 15 mL of maintenance solution (consisting of DMEM + 2% FBS) was added, marking this time point as 0 h. At various time intervals, the cell–virus mixtures were collected and subjected to high-speed centrifugation at 4 °C and 1,000 × g for 5 min. The supernatant was completely removed, and 1 mL of precooled TRIzol was added to the cell pellet for cell lysis. The lysed cells were then transferred to a 1.5 mL centrifuge tube, rapidly frozen in liquid nitrogen, and stored at −80 °C.

2.3. Nucleic acid extraction and library construction

The collected cell sediment was removed, defrosted on ice, and shaken on a vortex. When the liquid in the tube appeared clear, 250 μL of chloroform was added, the mixture was shaken well, and the mixture was incubated on ice for 5 min. The mixture was then transferred to a precooled centrifuge and centrifuged at 12,000 rpm at 4 °C for 20 min. 350 μL of clear liquid from the top layer was carefully added to a new 1.5 mL centrifuge tube, an equal volume of isopropanol was added, the mixture was mixed well, and the mixture was allowed to stand on ice for 10 min. The mixture was then centrifuged again at 12,000 rpm at 4 °C for 15 min. The RNA precipitate was removed from the centrifuge tube, and 1 mL of precooled 75 % ethanol was discarded to wash the precipitate. The mixture was centrifuged again at 12,000 rpm at 4 °C for 10 min. All the liquid was discarded, and the precipitate was dried

with the cap open in a laminar flow hood, after which 20 μL of nuclease-free water was added to dissolve the RNA precipitate. Total RNA from samples at different time points was sent to Novogene Beijing for transcriptome library construction via the Illumina NovaSeq 6000 sequencing platform, and the sequencing strategy was based on PE150. The transcriptomic dataset has been uploaded to the Genome Sequence Archive (GSA) database (CRA015707).

2.4. Transcriptome data analysis

The raw sequencing reads were quality controlled by FastQC, and the low-quality fragments and adapters were removed via fastp v0.23.2 [23]; STAR v2.7.9a was used to align the reads to the hg38 and VTT reference genomes (GenBank: AF095689.1) [24]; the transcripts were quantified with featureCounts and Kallisto v0.50.1 [25,26]; differential expression analysis was conducted with DESeq2 [27]; and pathway and gene ontology (GO) enrichment analysis was performed with Metascape [28]. The read counts and viral messenger RNA (mRNA) proportions are provided in Supplementary Table 1**.** The number of viral differentially expressed genes (vDEGs) between the two strains was determined based on a log10 TPM (transcripts per million) difference greater than one. Host differentially expressed genes (hDEGs) were defined as those with a $|\log2\ FC\ (fold\ change)| > 1$ and a false-discovery rate (FDR) < 0.05. The analysis of the correlation between host and virus gene expression was conducted via the Pearson correlation test, with *P* values adjusted for multiple hypothesis testing. The adjusted values for significantly correlated virus-host gene pairs should be less than 0.05.

2.5. Mapping hDEGs to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways

The related pathways were selected from the enrichment analysis results of the differentially expressed host genes and processed via the ggkegg v1.0.2 package [29]. All pathway annotation information used was downloaded directly from the following website: https://www.kegg.jp.

2.6. Statistical analysis

All the statistical analyses were completed in R v4.1.1. The differences in the copy numbers of the VTT genome were examined using the Student's *t*-test. $P < 0.05$ were considered to indicate significant differences. Plotting was performed with the package ggplot2.

3. Results

3.1. VTT and WR share similar expression patterns and induce host transcription shutoff

To construct a dynamic profile of host and viral gene expression after VTT and WR infection, we conducted transcriptomic analysis at multiple stages postinfection (Fig. 1A). The reverse transcription-quantitative polymerase chain reaction (RT–qPCR) and tissue culture infectious dose 50% ($TCID_{50}$) results revealed that VTTs completed one replication cycle within 12 h (Fig. 1B). Hence, we designated 0–2 hours postinfection (hpi), 4–6 hpi, and 8–12 hpi as the early, middle, and late stages of VTT infection, respectively. We also determined the expression profiles of HeLa cells infected with the WR strain at 0 hpi, 4 hpi, and 8 hpi. All replicates of the experimental groups were well clustered, indicating the high reliability of the sequencing data (Fig. 1C). The viral gene expression level largely increased after infection (Fig. 1D-E), whereas the expression of host genes largely decreased (Fig. 1F). VTT and WR viral gene expression exhibited significant temporal specificity (Fig. S1A-B). We identified the viral genes with the highest expression levels during each infection period (Fig. 1E). During the initial stage of infection, *B3* and *E3*, which inhibit the signaling of the host's innate immune system, are expressed immediately (Fig. 1E and S1A-B). Moreover, *I3* and *H5*, which are vital for VACV DNA replication and the regulation of late gene expression, presented high levels of transcription (Fig. 1E and S1A-B). The upregulation of VP11 (*F17*) and virion structure-related genes originating from the largest genomic fragment, designated with the prefix letter A, is notably rapid during the late stages of replication in both VTT and WR. (Fig. 1E). Notably, *A31* and *A46* play important roles in immune evasion by inhibiting dsRNA binding and NF-κB signaling at the early stage.

The expression levels of viral genes were highly similar in the early and late stages, with few vDEGs between the VTTs and WRs (Fig. S2A). In the early stages, the *A11-A19* cluster tended to exhibit greater expression in the WR strain than in the VTT strain. Conversely, the genes *L1*, *ankyrin-like* (newly identified in VTT), *C12*, and *B-ORF-C12* (the paralogous gene of *C12*) presented elevated expression levels in VTT (Fig. S2A). In the WR-infected samples, the *C10* gene presented almost no expression, whereas its paralog gene, *B-ORF-C10*, presented a similar expression level in both strains. Five vDEGs (*A8*, *A11*, *E1*, *G2*, and *D9*) were identified in the late stage (Fig. S2A). The expression profiles of the viral and host genes are provided in Supplementary Table 2.

3.2. Vaccinia virus infection causes dysregulation of host gene expression

Upon infecting the host, VACV replicates its genome and produces mature virus particles via host transcription and translation mechanisms. We identified hDEGs between infected and uninfected samples. The results revealed a significant increase in the number of hDEGs with prolonged infection duration (Fig. 2A). By comparing the vDEGs and hDEGs between groups, we observed a noteworthy cumulative impact of differentially expressed genes (DEGs) associated with viral replication. Specifically, many hDEGs in the ongoing infection phase remained differentially expressed in the subsequent infection phases (Fig. 2B-C). Notably, the number of hDEGs sharply increased in the middle and late stages of infection, suggesting a rapid onset of disruption in transcription regulation in both the VTT and WR infection groups. Unlike the viral genes, hDEGs were largely different between WRs and VTTs, especially in the late infection stage. The correlation of host gene expression levels was weaker in the late stage ($\rho = 0.79$; $P<0.001$) than in the early stage ($\rho = 0.85$; $P<0.001$; Fig. S2B).

The hDEGs were split into eight clusters, named C1–8, according to the fold changes (Fig. 2D). The genes from C5 and C8 presented the most similar expression change patterns in samples infected with VTT and those infected with WR. Genes from C5 (n = 516) were downregulated synchronously, exhibiting enrichment in pathways such as focal adhesion, oxidative phosphorylation, interferon response, and lysosome. C8 (n = 623) included genes upregulated after WR and VTT infection and enriched in RNA splicing, nerve growth factor (NGF)-stimulated transcription, viral infection, DNA repair, and RNA binding pathways. C6 (n = 2,009) and C7 (n = 1,619) were mostly upregulated in WR- and VTT-infected cells, respectively. C3 (n = 1,434) and C4 (n = 1,230) were strongly downregulated during VTT infection, whereas C1 (n = 699) and C2 (n = 987) were strongly downregulated during WR infection. C3, C4, and C5 were enriched in the oxidative phosphorylation pathway (Fig. 2D), implying that VTT and WR repress the pathway through different factors (Fig. S3). The activity of antiviral-related pathways (C2) was largely reduced, whereas RNA-processing pathways were activated during infection (C7 and C8).

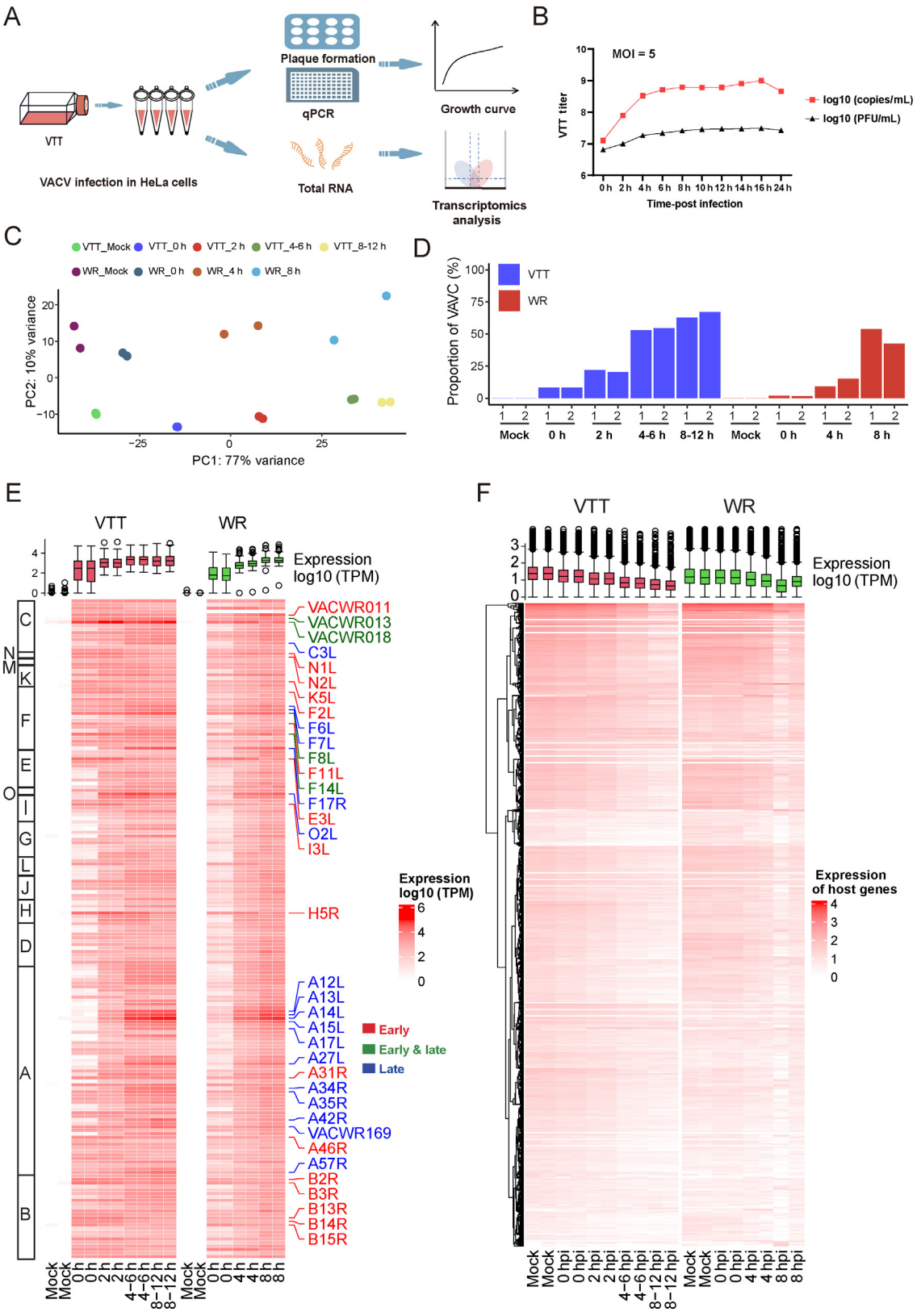

Fig. 1. Dynamics of VTT and WR infection growth and gene expression changes in HeLa cells. A) Experimental process. B) Growth kinetics of VTT in HeLa cells. C) Principal component analysis of gene expression levels in the infected and uninfected groups. D) Proportions of VTT RNA in different infection groups. Changes in the expression levels of viral (E) and host (F) genes. Abbreviations: VTT, vaccinia virus Tiantan; WR, western reserve; TPM, transcripts per million; VACA, vaccinia virus; q-PCR, quantitative polymerase chain reaction; RNA, ribonucleic acid; MOI, multiplicity of infection.

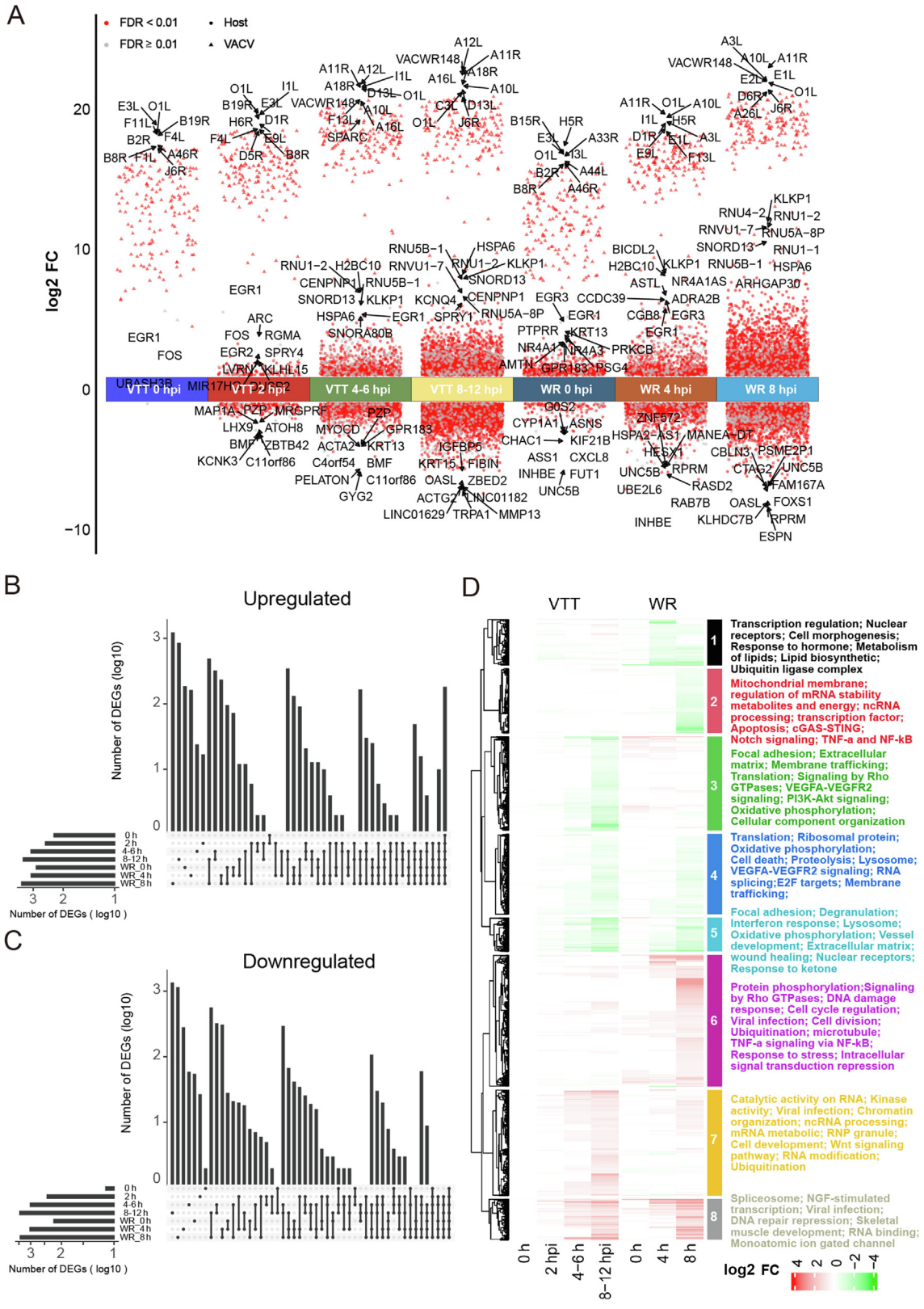

Fig. 2. Dynamic differences in host and viral gene expression profiles after VACV infection. A) Alterations in the expression levels of differentially expressed genes at all stages within each infection group. The intersection of upregulated (B) and downregulated (C) viral and host genes among the infection groups. D) Clustering of host genes on the basis of expression changes and enrichment of biological categories for each cluster. Abbreviations: VTT, vaccinia virus Tiantan; WR, western reserve; VACA, vaccinia virus; DEGs, differentially expressed genes; FC, fold change; FDR, false-discovery rate; hpi, hour postinfection.

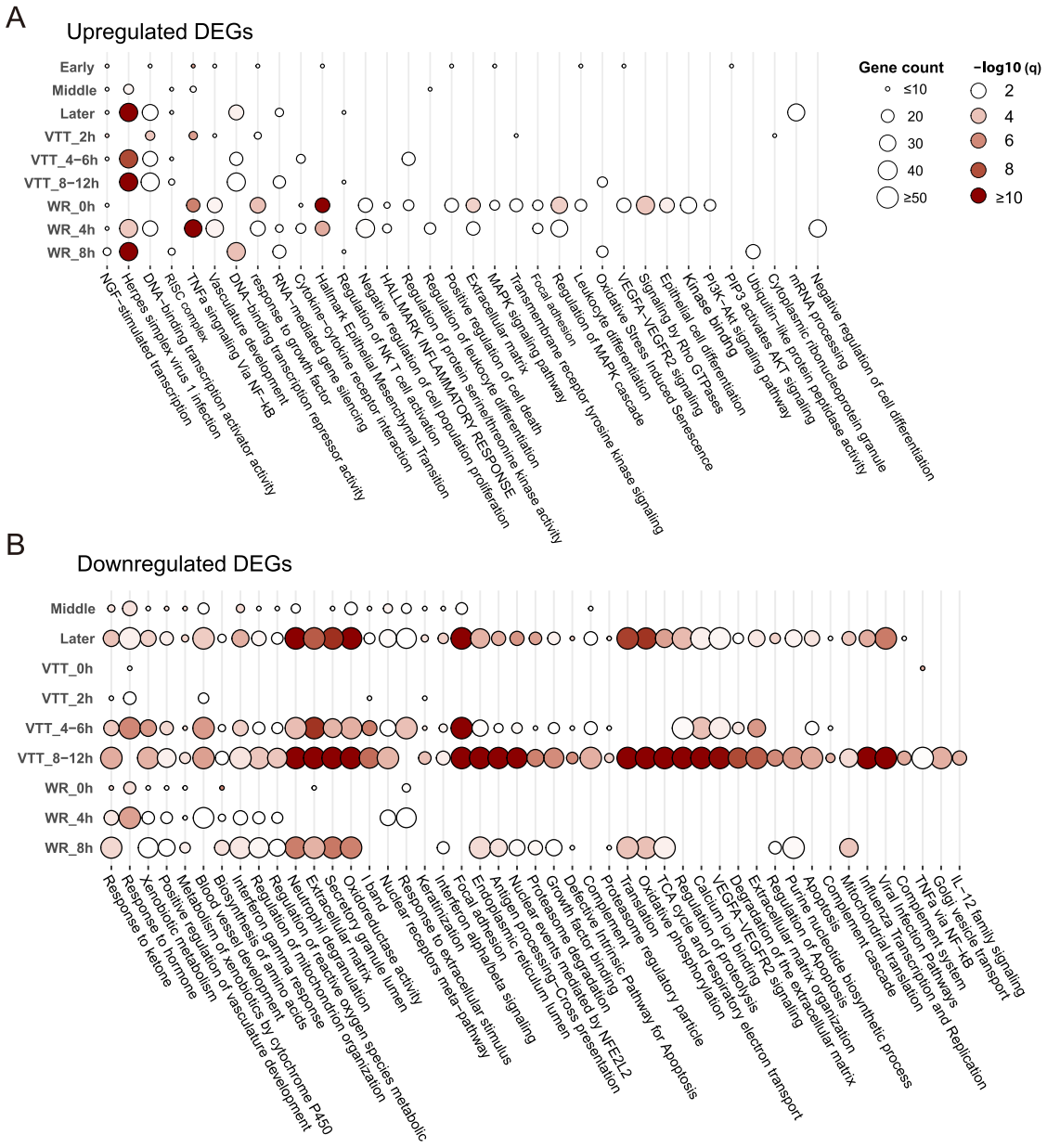

Fig. 3. Functional category enrichment of the differentially expressed genes. Enriched categories of upregulated (A) and downregulated (B) genes in VTT infection. The size and color of the circle correspond to the number of genes enriched in the functional pathway and the significance values after multiple testing corrections, respectively. Early, middle, and late refer to the union of DEGs at 2 hpi, 4 hpi, and 8 hpi, respectively, with VTT and WR. The VTT-infected groups at 0 hpi and 2 hpi were combined into the early-stage infection group. Abbreviations: hpi, hour post infection; VTT, vaccinia virus Tiantan; WR, western reserved; DEGs, differentially expressed genes; TCA, tricarboxylic acid; TNF, tumour necrosis factor; NGF, nerve growth factor.

3.3. Dynamic changes in the biological pathways influenced by VACV infection

To elucidate the influence of VTT and WR infection on host biological pathways, we conducted an integrated analysis of the pathways enriched with hDEGs. The genes enriched in pathways related to NGF-stimulated transcription, tumor necrosis factor (TNF)-α signaling via NF-κB, cell growth, differentiation, and kinase signaling exhibited marked transcriptional activation during the initial stages of infection (Fig. 3A). Antivirus and cytokine interactions and DNA binding transcription pathways are activated in the middle and late stages of infection. In addition, genes related to the RNA-mediated gene silencing complex were induced at the late infection phase. At 0 hpi, WRs induced the upregulation of more host genes (n = 521) compared to VTTs (n = 3). Consequently, the hDEGs from the WR 0 hpi group were enriched in a greater number of categories, particularly pathways related to the response to growth factors, epithelial-mesenchymal transition, regulation of the MAPK cascade, and signaling by Rho GTPases.

During the initial stages of infection, the genes whose expression was suppressed were enriched in response to hormones and ketones, in response to I bands, and the development of blood vessels (Fig. 3B). Notably, these pathways continued to be enriched in the middle and late stages of infection. During the middle and late stages of infection, the downregulated genes were enriched in various pathways, including the extracellular matrix, neutrophil degradation, secretion granule lumen, amino acid biosynthesis, interferon response, translation, oxidative phosphorylation, and apoptosis pathways. Interestingly, while the late infection stage presented a similar number of

downregulated genes between WRs and VTTs, hDEGs in VTTs were enriched in additional functional categories. Specifically, pathways related to viral infection, proteolysis regulation, and the complement response were also suppressed in the VTT infection groups. In addition, there is substantial inhibition of pathways associated with type I interferon (IFN-I), leukocyte migration, translation, oxidative phosphorylation, and programmed cell death. These results imply that VTT could increase viral replication by influencing antiviral innate immune pathways.

3.4. Changes in antiviral innate immune pathways during VTT infection

To assess the activation status of the host's key antiviral pathways in response to viral infection, we determined the changes in the expression of crucial genes involved in cytoplasmic double-stranded DNA recognition and the host's antiviral response. The expression levels of cyclic guanosinc monophosphate (GMP)-adenosine monophosphate (AMP) synthase (*cGAS*) increased by 3.68-fold and 4.69-fold at 4 hpi and 8 hpi, respectively, in the VTT-infected groups (Fig. S4A). Similarly, in the WR infection groups, *cGAS* expression levels were upregulated at 4 hpi (2.27-fold) and 8 hpi (3.58-fold) (Fig. S4B). Conversely, the expression of genes involved in degrading cytoplasmic DNA, such as *SAMHD1* and *DNASE2*, was suppressed in both the VTT (Fig. S4C) and WR infection groups (Fig. S4D), potentially impacting the cGAS-STING axis. Moreover, the expression of genes in the *DDX41*-, *IFI16*-, and *MAVS*-related modules, which are pivotal factors of the STING pathway, decreased in the later stages of infection, indicating potential transcriptional inhibition of the cGAS–STING axis (Fig. S4C–D).

In response to the viral invasion, the host NF-κB pathway becomes activated, leading to the upregulation of IFN-I and cytokines. This activation triggers a cascade of inflammatory responses aimed at hindering viral replication, ultimately disrupting viral maturation and dissemination. While the expression level of *TBK1* remained unchanged, the expression levels of the *IKK* family genes (*CHUK*, *IKBKB*, and *IKBKG*) exhibited varying alterations. Specifically, *CHUK* (*IKKA*) expression continued to increase following VTT and WR infection (Fig. S4A-B), whereas the expression of *IKBKB* and *IKBKG* decreased during the middle and late stages of infection (Fig. S4C-D). The transcription factors IRF3 and IRF7 undergo phosphorylation, leading to the activation of downstream interferon-related gene transcription. Interestingly, the transcription levels of these two genes remained unaltered following VTT infection and were downregulated at the late phase of WR infection. While TBK1 exhibited no significant changes, the expression levels of its binding factor TBKBP1 were markedly upregulated during VTT infection and downregulated during WR infection.

The JAK-STAT axis serves as a crucial pathway in response to interferon signals, with its core genes exhibiting contrasting trends in expression following VTT and WR infection. For example, during the early stages of infection, the expression of *Jak2* and *Tyk2* significantly increased (Fig. S4A-B), whereas the expression of suppressor of cytokine signaling 3 (*SOCS3*) decreased (Fig. S4C-D), consequently enhancing the activation of this pathway. However, the expression of *Jak1* significantly decreased. VACV infection might decrease the expression of *Jak1*, impacting the formation of the kinase heterodimer and inhibiting the JAK-STAT pathway. Moreover, the expression of *STAT1/3* and *OAS1* decreased (Fig. S4C-D), whereas the expression of *STAT2/5B/6*, members of the same STAT family, significantly increased. Notably, the expression of *NXF1* (nuclear RNA export factor 1) and *SRSF* (serine and arginine-rich splicing factor) family genes significantly changed, which might affect host mRNA splicing and nuclear export processes. The presence of both upregulated and downregulated genes in antiviral innate immunity pathways signifies a complex situation in which the host's innate immunity is triggered concurrently with the virus's interference with the host's antiviral defenses during infection.

3.5. VTT infection suppresses host cell programmed death

Previous studies have shown that after VACV infection of the host, the necrosis and apoptosis pathways are predominantly triggered [18]. VACV encodes proteins that inhibit cell death [12]. We found that in the later stages of infection, HeLa cells exhibited clear indications of cell death. To elucidate the regulatory impact of VTT on cell death, we analyzed alterations in the expression levels of pivotal factors involved in the TNF-α and apoptosis pathways. The results revealed the upregulation of MAPK8/FOS family genes at all time points after VTT and WR infection (Fig. 4A and S5A-B), whereas a substantial decrease in the expression of numerous pivotal apoptosis genes was detected during the late stage. Although the transcription of TNF did not change significantly, its key downstream effector, TNF receptor superfamily member 1A (*TNFRSF1A*), was significantly downregulated in the middle and late phases of infection (Fig. S5C-D), which is consistent with previous proteomic datasets [9]. In addition, the expression of the TNF pathway inhibitor BCL2-associated athanogene 4 (*BAG4*) increased, which may further inhibit TNF-α-mediated cell death (Fig. S5A-B).

The PI3K–Akt axis serves as a downstream component of TNFR2 signaling, phosphorylating IKKs and subsequently phosphorylating IκBα. This process ultimately inhibits the NF-κB pathway, allowing host cells to evade cell death. The impact of the VTT infection-induced reduction in PIK3C-related gene expression on the apoptosis of HeLa cells requires further investigation. Caspase family proteins play a central role in programmed cell death, and the expression levels of *Casp4* and *Casp9* are downregulated in the middle and late stages of VTT infection (Fig. 4B and S5C-D), hindering the formation of the apoptosome [30]. The expression levels of several proinflammatory genes, including *Jun* and *Tp53,* also decreased (Fig. 4B). The Fas-associated death domain (FADD) acts as an upstream regulator by binding to Fas and other molecules. A decrease in FADD expression during the middle stage of infection may significantly affect the suppression of apoptosis. In addition, many host genes related to the upstream region of the programmed cell death pathway were also dysregulated (Fig. 4C). Therefore, infection with VTT can disrupt the expression of crucial genes involved in the classical programmed cell death pathway, leading to the suppression of host cell apoptosis. Discrepancies are observed in the gene expression patterns linked to host-programmed cell death triggered by the WR and VTT strains, warranting additional research to elucidate the mechanisms driving this variation. Infection with VACV has the potential to interfere with the expression of essential genes associated with the classical programmed cell death pathway, resulting in the inhibition of host cell apoptosis. This effect is thought to stem from the extensive influence of viral protein expression on the host cellular machinery.

3.6. Posttranscriptional level regulation associated with VACV infection

The RNA-mediated gene silencing pathway was activated in the upregulated hDEGs during the late phase of infection. Therefore, we investigated the regulatory pathways at the transcriptional level and observed that both VTT and WR infections upregulated genes associated with the RNA-induced silencing complex (Fig. 4D). Specifically, the expression levels of *Dicer1*, *Ago2*, and *Ago3* increased. Moreover, the levels of specific microRNA clusters, such as *MIR17HG*, *MIR155HG*, *MIR23AHG*, and *MIR137HG*, were also increased (Fig. S6-A-D). Interestingly, we noted that VTT and WR infections did not impact the region where microRNA (miRNA) precursors are located (Fig. S6A). Previous research has demonstrated a general decrease in mature microRNA expression following VACV infection [31]. Hence,

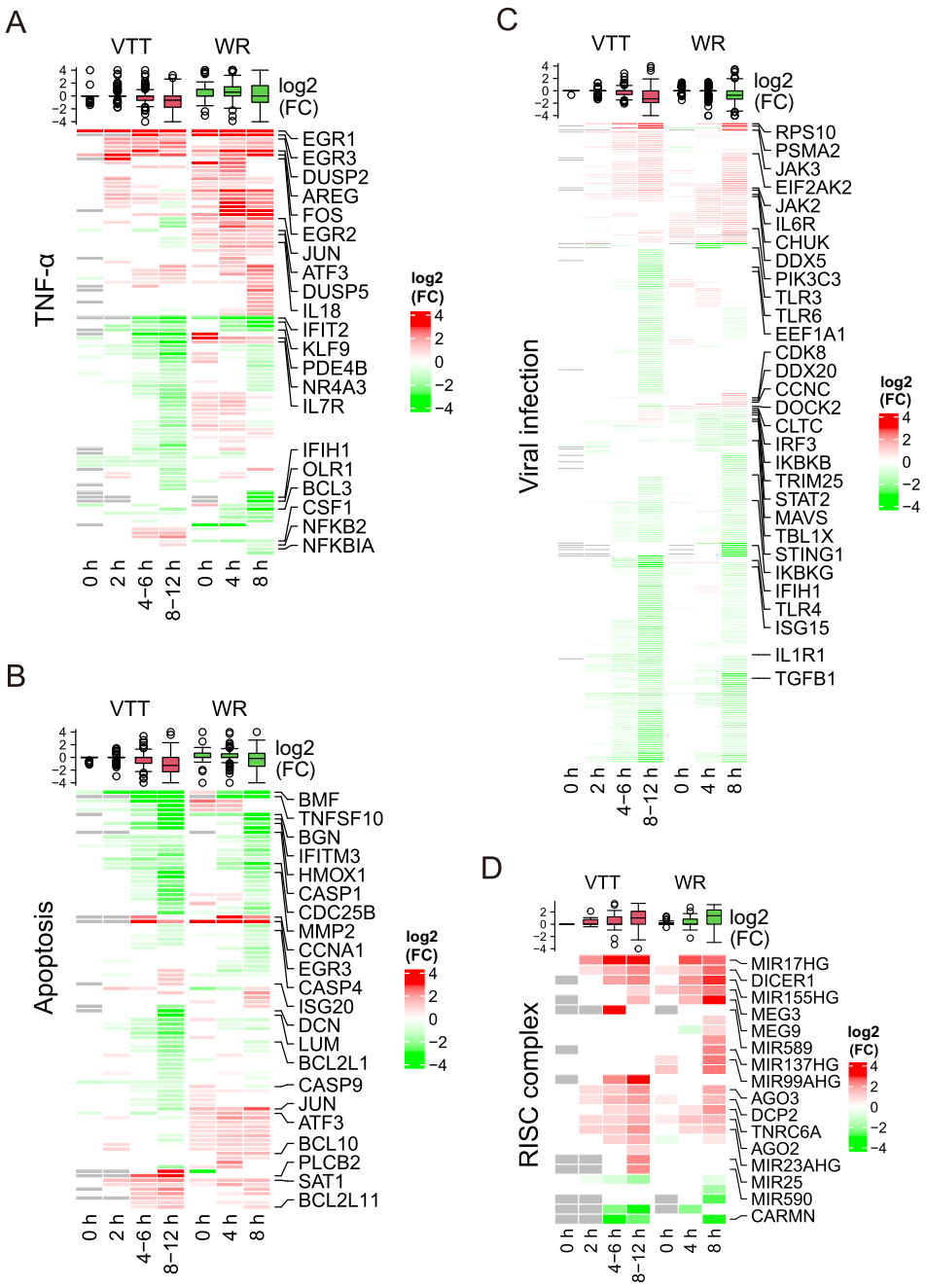

Fig. 4. Inhibition of programmed cell death and activation of RISC-related categories. Changes in the transcription levels of genes related to the TNF-α, apoptosis, antiviral, and RISC pathways. The pathway information for TNF-α (A) and apoptosis (B) was sourced from the hallmark database, with the index numbers M5890 and M5902. The accession number for the RISC functional pathway is GO:0016442; the antiviral pathway is from the Reactome database with accession number R-HSA-9824446. Red, green, white, and gray represent upregulated, downregulated, not changed, and not detected, respectively. Abbreviations: VTT, vaccinia virus Tiantan; WR, western reserve; TNF, tumor necrosis factor; RISC, RNA-induced silencing complex; FC, fold change.

this study tentatively proposes an association between VACV infection and the RNA-induced silencing pathway, although the regulatory effects of VACV infection remain ambiguous.

3.7. Numerous host genes exhibit correlations with variations in the expression levels of viral genes

We performed a correlation analysis to investigate the potential relationship between changes in host and viral gene expression levels across different infection groups. These findings indicated significant correlations in the changes in expression levels among hundreds of host-viral gene pairs. Specifically, the expression trends of the viral genes *A31* and *F11* showed negative and positive correlations with 315 and 99 host gene expression level changes, respectively (Fig. 5A; Supplementary Table 3). For example, *HDAC5* and *RPL35* exhibited significant negative correlations with *A31*, whereas *Eif4a1* and *C5,* and *Cox7c* and *A53* were also negatively correlated (Fig. 5B). Additionally, the expression of *Exo1* and *DEK* was positively correlated with that of *A48* and *F11*, respectively. Functional enrichment analysis of the positively and negatively correlated host genes revealed that genes positively correlated with viral genes are associated primarily with cell division, mRNA processing, the G2M check-

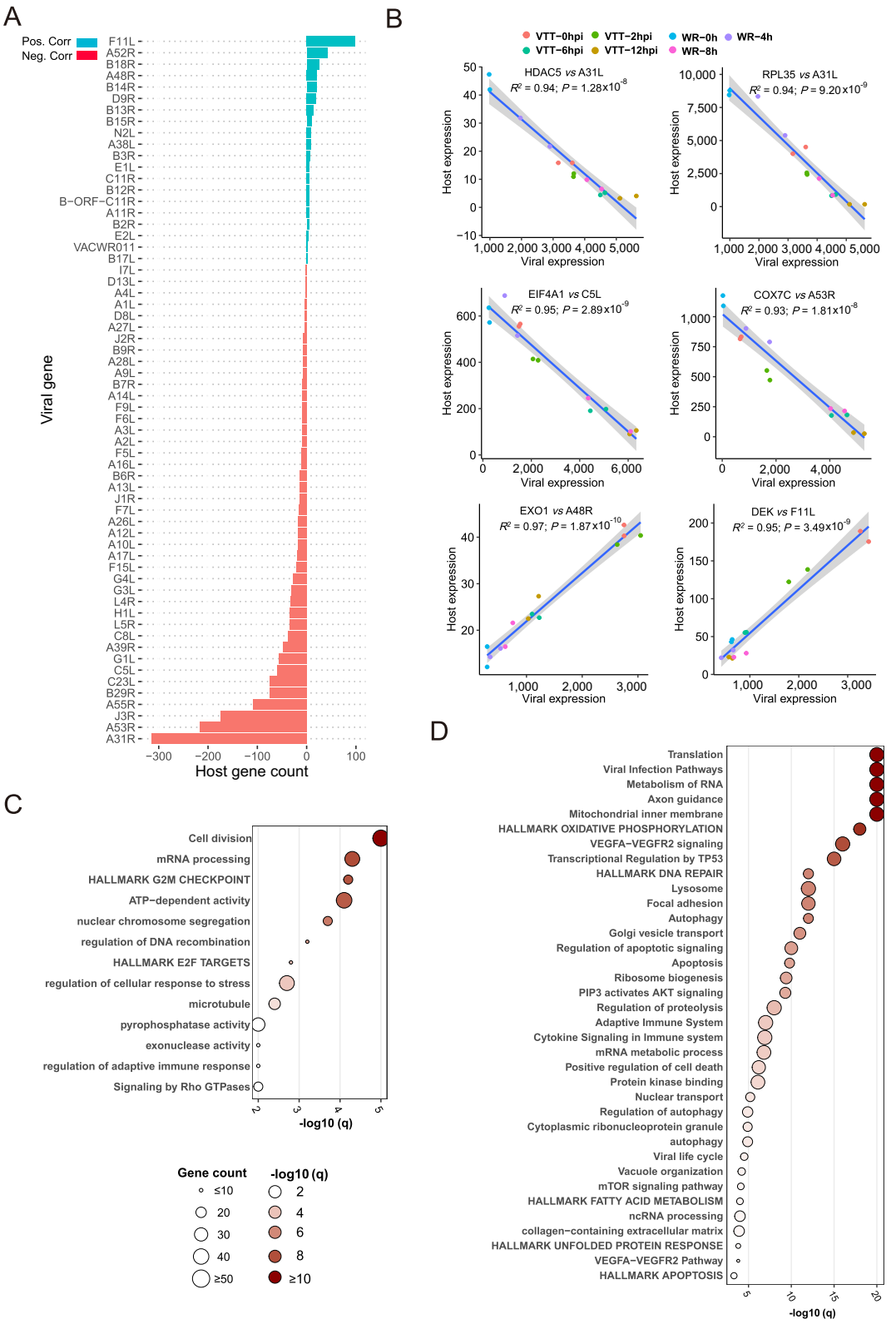

Fig. 5. Host gene expression changes are strongly correlated with virus gene expression. A) Distribution of host gene numbers highly correlated with virus gene expression levels. B) Highly related host-virus genes, for example. The functional pathways enriched by genes positively (C) and negatively (D) correlated with changes in virus gene expression levels. The size and color of the circle correspond to the number of genes enriched in the functional pathway and the significance values after multiple testing corrections, respectively. Abbreviations: VTT, vaccinia virus Tiantan; WR, western reserve.

point, and E2F target genes (Fig. 5C). Conversely, host genes that were negatively correlated with viral genes were significantly enriched in translation, virus infection, RNA metabolism, and oxidative phosphorylation. Furthermore, these inversely correlated genes were also associated with TP53-regulated transcription, lysosomes, autophagy, and apoptosis (Fig. 5D).

4. Discussion

The VTT vaccine is extensively utilized in China for smallpox prevention. Nonetheless, a detailed understanding of its genomic sequence and host interaction mechanisms needs to be improved, which has hindered progress in the prevention and control of smallpox and other orthopoxvirus infections. This study presents a systematic analysis of the temporal changes in the transcriptome and regulation of the host immune system in HeLa cells upon infection with the VTT and WR strains of VACV. After infection, the proportion of viral transcripts rapidly increases. During the early stage of infection, the virus genes involved in evading the host immune system are expressed first, similar to other DNA viruses [32–34]. For example, *E3* is rapidly expressed following infection, leading to a reduction in dsRNA accumulation to prevent eIF2α phosphorylation [35]. The late viral protein VP11 (F17) dysregulates mTOR to block host antiviral responses [36] and block cGAS-dependent interferon-stimulated genes (ISGs) responses [37], thus diminishing the recognition of the host's innate immune system by the extensively replicated viral genome and ultimately inhibiting immune activation.

With the formation of virus replication factories and genome replication, the maturity and release of large amounts of virus particles disrupt essential pathways crucial for host antiviral defense mechanisms and normal physiological functions. N. Yang et al. [10] discovered that the E5 protein from VACV induces the ubiquitination and proteasome-mediated degradation of cGAS through direct interaction. Moreover, the deletion of E5 significantly enhances the production of IFN-I in dendritic cells. Proteomic analysis of WR virus infection revealed that the expression of *cGAS* did not significantly increase following VACV infection; however, a noteworthy increase in its expression was observed upon the addition of MG132 [9]. We analyzed the expression of key factors involved in host antiviral and cell death pathways. These results indicate that the expression of key factors related to the host's recognition of viral infection pathways becomes dysregulated following VTT infection. For example, the expression of *IKK* family proteins, which regulate the NF-κB pathway, showed opposite trends. *Jak1*, a key component of the JAK-STAT pathway, was significantly downregulated, possibly resulting in the inhibition of this pathway. R.P. Sumner et al. [38] reported that A49 effectively prevents the degradation of IκBα, thereby inhibiting the NF-κB pathway. Previous studies have shown that E3 and C6 inhibit the phosphorylation of IRF3 and ORF7 [39,40], thereby reducing the expression of host interferons and downstream genes. Notably, C6 has also been found to act on TBKBP1 and AZI2 to inhibit TBK1-mediated phosphorylation [40]. Recently, M. El-Jesr et al. [41] summarized the activation and antagonism of cytosolic DNA sensing by VACV, contributing additional insight to this research field. Additionally, we found that VTT infection may affect the maturation and nuclear export of host mRNA-related pathways. G. Sivan et al. [42] reported that the nuclear pore complex was necessary for effective VACV replication. However, it remains to be seen whether this process involves mRNA transport, indicating the need for future research.

Interestingly, in the middle and late stages of VTT infection, the expression levels of critical cell death factors involved in extensive cell death were notably decreased, potentially hindering the assembly of apoptotic bodies. These findings suggest that certain viral proteins may inhibit the host cell death pathway at the transcriptional level to ensure efficient replication of VACV. Notably, during VACV infection, the virus typically controls the host translation system for its benefit [43], resulting in potential discrepancies between host gene transcription levels and protein expression levels. Furthermore, VACV is equipped with multiple proteases and can manipulate host protein degradation pathways, effectively antagonizing host restriction factors. Therefore, variations in transcription levels may not directly correlate with changes in protein abundance. After 18 h of infection of fibroblasts with the WR strain, L. Soday et al. [9] reported that the levels of only 265 and 70 host proteins decreased and increased, respectively, by more than twofold. Furthermore, L. Soday et al. [9] compared the transcriptional data of WRs, revealing variations in the patterns of viral gene transcription and protein expression. Future research should prioritize investigating the regulation of translation levels following VACV infection in hosts to reconcile discrepancies between transcription and protein expression. Future studies should delve into the mechanisms of translation regulation, posttranslational modifications, and protein degradation pathways to explore the interactions between the virus and the host.

During the middle and late stages of viral replication, numerous viral proteins involved in assembling mature virus particles or participating in the virus particle maturation process are upregulated. The variation in the expression levels of highly expressed viral genes throughout different infection stages aligns with earlier observations in WR strain infections [7,16]. However, proteomics experiments from different stages of WR infection revealed that proteins of some early genes with high mRNA expression were not highly expressed in early infection samples [21], suggesting that posttranscriptional regulatory mechanisms need further investigation.

VACV infection leads to a decrease in host gene transcription. We analyzed genes related to transcription pathways at different time points and found that VTT and WR infections caused significant dysregulation of host transcription factors, especially zinc finger and ZBTB family proteins (Fig. S7A). For example, *ZBTB6* was upregulated throughout the infection process by both strains, whereas *ZNF750* and *TBX2* were significantly upregulated in the late stages of VACV infection. In contrast, *ZBED* and *NR2F* family genes were significantly downregulated in the late stages of infection. Dysregulation of these transcription factors may contribute to the chaos of the host transcription system. However, the impact of these changes on VACV infection and its upstream regulatory factors remains to be studied. Furthermore, the differential changes in the expression levels of transcription factors between the two strains suggest that they may restructure the host transcription system in different ways.

hDEGs induced by VACV infection were significantly enriched in the MAPK pathway (Fig. S5). As an important signaling pathway through which cells respond to external stimuli, the MAPK cascade plays both promoting and inhibitory roles in viral infection. Previous studies have shown that the MAPK pathway is activated after vaccinia virus infection [44]. To further analyze the transcriptional impact of VACV infection on the factors in this pathway, we studied the changes in the expression levels of key factors. We found that (1) the pathway exhibited significant transcriptional dysregulation characteristics and (2) the overall trend of host gene changes was consistent between the two strains, but VTT and WR induced greater downregulation and upregulation of host genes, respectively, in the late stage of infection (Fig. S7B). The expression of important natural immune signal transduction factors, such as *TLR4*, *Myd88*, and *Traf7*, is inhibited. In addition, the expression of kinases such as *MAPK3*, *MAP4K2*, and *PAK4* is also inhibited. However, the expression of several factors, such as *Jak2* and *HIPK3*, tended to increase. The impact of changes in the expression of these factors on VACV infection requires further in-depth research.

L. Klaas et al. [45] recently reported that the ZBP1-RIPK3 axis mediates programmed cell death in macrophages induced by some modified vaccinia virus Ankara (MVA) infections. However, our study revealed no transcription of *Zbp1* after HeLa cell infection, with significant downregulation of caspase family genes, mainly *Casp1/4/9/10,* suggesting that the cell apoptosis signaling pathway induced by VTT infection in HeLa cells may significantly differ from that induced by infection with other vaccinia virus strains or cell types, possibly because the vaccinia virus encodes many antihost apoptosis-related genes, such as N1 and other BCL2-like proteins, which may redun-

dantly function together to exert inhibitory effects. The specific cell apoptosis response mechanism activated by the host may be influenced by factors such as the timing of viral protein expression and amino acid mutations. Further research is needed to investigate the impact of these factors on the regulation of programmed cell death in host cells during VACV infection.

To analyze the key factors influencing VACV infection, we identified host and viral DEGs with the greatest fold changes across various infection stages (Fig. S8A-B). Early growth response gene 1 (*EGR1*) and *Fos* were significantly upregulated during the immediate early stage (Fig. S8B), which is consistent with proteomic data [9]. The introduction of MG132 (a proteasome inhibitor) increased EGR1 levels, suggesting the potential involvement of the VACV-mediated protein degradation signaling pathway in EGR1 processing (Fig. S8C). Owing to its double-stranded DNA-binding function, EGR1 serves as a transcription factor responsible for regulating cell survival, differentiation, and apoptosis. During viral infection, EGR1 expression can be triggered by type I interferons, leading to the activation of downstream ISGs [46]. In addition, it can inhibit foot-and-mouth disease virus replication by increasing the phosphorylation level of TBK1 [47]. However, a recent study revealed that it can enhance the replication of VACV [48]. FOS abundance also increased after VACV infection [9], and its expression further increased in the presence of proteasome inhibitors (Fig. S8C). The underlying molecular mechanism driving these expression changes and their impact on VACV replication remains unclear. In the middle and late stages of infection, the expression of numerous host genes was upregulated (Fig. S8C). TNF receptor superfamily member 10 d (TNFRSF10D) and CDC-like kinase 1 (CLK1) were significantly upregulated at both the transcriptional and protein levels (Fig. S8C). Remarkably, CLK1 expression notably increased after proteasome pathway inhibition, suggesting the potential regulation of CLK1 protein degradation during VACV infection. However, knocking down *clk1* via RNA interference did not influence VTT replication (Fig. S8D); therefore, the impacts of the upregulation of *CLK1* and other hDEGs remain to be elucidated.

Although this study identified many host genes with significant differences at the transcriptional level, we failed to screen for host factors directly affecting the replication of the VACV. Using RNAi screening, P.M. Beard et al. [49] identified approximately 300 genes that promote or inhibit VACV replication; however, most of these genes did not exhibit significant changes in transcription levels after VACV infection. Recently, A. Matia et al. [50] identified dozens of host factors that alter virus replication, including *B2M*, on the basis of genome-wide CRISPR genetic screens. We also analyzed these screened host factors and found that their transcription levels did not significantly differ throughout the infection cycle. Owing to the complexity of the VACV genes themselves and the regulatory network affecting host gene expression, studies solely at the bulk-transcriptional level may be insufficient to identify genes significantly associated with VACV replication. In particular, the heterogeneity in gene expression between infected cells and bystander cells [18,19], the low correlation between changes in gene expression between mRNAs and proteins [22], and the differences in the migration of host factors between organelles and between organelles and the VACV replication factory [19] may affect the interaction between the VACV and the host. Future studies should combine genome-wide screening, single-cell transcriptomics, translation, proteomics, and other multiomics technologies to screen for key host factors that may regulate VACV-host interactions efficiently. The transcriptional profiles of the two vaccinia virus variants provided in this study under equal conditions will also contribute to this work.

5. Conclusion

In this study, a transcriptome atlas of VTT and WR infection in HeLa cells was constructed. Through analysis of DEGs from the host- and VACV-infected groups, we found that viral gene transcription exhibited a clear temporal pattern. After infection with VTT and WR, similar trends were observed in changes in virus gene expression; however, there were notable differences in host DEGs, suggesting the necessity of investigating the host interaction mechanisms of VTT. Both the WR and VTT resulted in widespread downregulation of host genes, particularly in pathways related to oxidative phosphorylation. Notably, both strains disrupted key factors in the host antiviral innate immune pathways. VTT induces extensive disruptions in more biological pathways, including programmed cell death pathways, in the later stages of infection. Moreover, during the late stage of VACV infection, the activation of gene silencing-related pathways indicates the potential involvement of posttranscriptional regulation in virus-host interactions. The outcomes of this study are significant for advancing our understanding of the interaction between the vaccinia virus and the host, enhancing the utilization of the VTT strain, and for the creation of effective vaccines targeting smallpox and monkeypox.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

Author contributions

Changcheng Wu: Writing – review & editing, Writing – original draft, Visualization, Methodology, Formal analysis, Data curation, Conceptualization. **Zhongxian Zhang:** Visualization, Formal analysis, Data curation. **Zhaoqing Li:** Methodology, Data curation. **Ruorui Li:** Methodology. **Shuting Huo:** Resources, Methodology. **Han Li:** Methodology. **Roujian Lu:** Resources, Funding acquisition. **Houwen Tian:** Resources, Methodology. **Wenling Wang:** Methodology. **Li Zhao:** Resources. **Baoying Huang:** Resources, Funding acquisition. **Yao Deng:** Resources, Methodology. **Wenjie Tan:** Writing – review & editing, Writing – original draft, Project administration, Investigation, Conceptualization.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bsheal.2024.08.001.

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