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SARS-CoV-2 Protein Nsp9 Is Involved in Viral Evasion through Interactions with Innate Immune Pathways

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Cite This: *ACS Omega* 2024, 9, 26428−26438

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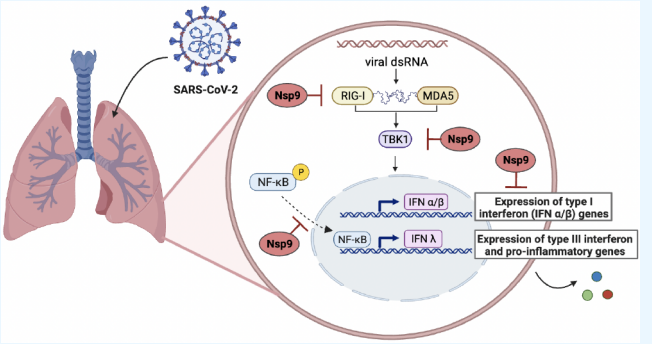
ABSTRACT: The suppression of the host's innate antiviral immune response by SARS-CoV-2, a contributing factor to the severity of disease, has been considerably studied in recent years. Many of these studies have focused on the actions of the structural proteins of the virus because of their accessibility to host immunological components. However, less is known about SARS-CoV-2 nonstructural and accessory proteins in relation to viral evasion. Herein, we study SARS-CoV-2 nonstructural proteins Orf3a, Orf6, and Nsp9 in a mimicked virus-infected state using poly(I:C), a synthetic analog of viral dsRNA, that elicits the antiviral immune response. Through genome-wide expression profiling, we determined that Orf3a, Orf6, and Nsp9 all modulate the host antiviral signaling transcriptome to varying extents, uniquely suppressing aspects of innate immune signaling. Our data suggest that SARS-CoV-2 Nsp9 hinders viral detection through suppression of RIG-I expression and antagonizes the interferon antiviral cascade by downregulating NF-kB and TBK1. Our data point to unique molecular mechanisms through which the different SARS-CoV-2 proteins suppress immune signaling and promote viral evasion. Nsp9 in particular acts on major elements of the host antiviral pathways to impair the antiviral immune response.

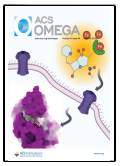


INTRODUCTION

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has proven to be a notable threat to global health, with more than 6.9 million deaths since its emergence in late 2019.[1] To devise therapeutic strategies to counteract SARS-CoV-2 infection and the associated COVID-19 pathology, it is essential to understand how this virus hijacks its host during infection. The SARS-CoV-2 genome is approximately 30 Kb in size with 14 open reading frames (ORFs) encoding 29 viral proteins (Figure 1).[2,3] While structural and nonstructural proteins (Nsps) are typically conserved among coronaviruses, the accessory proteins (Orfs) show more variability in their sequences and associated functions.[4−8] Genetic variability in SARS-CoV-2 proteins may be linked with the severity of COVID-19.[9] Many SARS-CoV-2 nonstructural and accessory proteins have been understudied and may be overlooked as potential druggable targets.[10,11]

In this study, we sought to gain a better understanding of the roles of SARS-CoV-2 protein Nsp9 as well as Orf6 and Orf3a in viral evasion by studying their interaction with antiviral innate immune pathways. The SARS-CoV-2 accessory protein Orf3a shares 73% protein sequence homology with SARS-CoV Orf3a and has a molecular weight of 31 kDa.[12] It is a functional homotetramer residing on the plasma membrane and endomembranes, including endosomes, lysosomes, golgi, and endoplasmic reticulum.[13] Previous studies have implicated Orf3a in the suppression of STAT1 phosphorylation and suppressing interferon (IFN)-alpha induction.[14,15] The SARS-CoV-2 accessory protein Orf6 shares 70% protein sequence homology with that of SARS-CoV Orf6. It is also thought to form dimers and tetramers that reside in the membranes of the endoplasmic reticulum, autophagosome, and lysosomal membranes.[16] SARS-CoV-2 Orf6 has been reported to interact with nuclear pore complex components Nup98 and RAE1, thus hindering STAT1 nuclear transport.[17−19] SARS-CoV-2 Orf6 has also been shown to inhibit nuclear translocation of IRF3 via its interaction with KPNA2 and suggested that this was likely responsible for downstream inhibition of IFN-beta.[14] The SARS-CoV-2 nonstructural protein Nsp9 shares 97% protein sequence homology with SARS-CoV Nsp9 and is a functional homodimer residing on the endoplasmic reticulum.[20] In addition to its essential participation in viral replication as a component of viral polymerase complex,[21] SARS-CoV-2 Nsp9 has been reported to limit the assembly of nucleoporin 62 (NUP62), resulting in impaired nuclear

Received: March 18, 2024
Revised: April 13, 2024
Accepted: April 23, 2024
Published: June 7, 2024



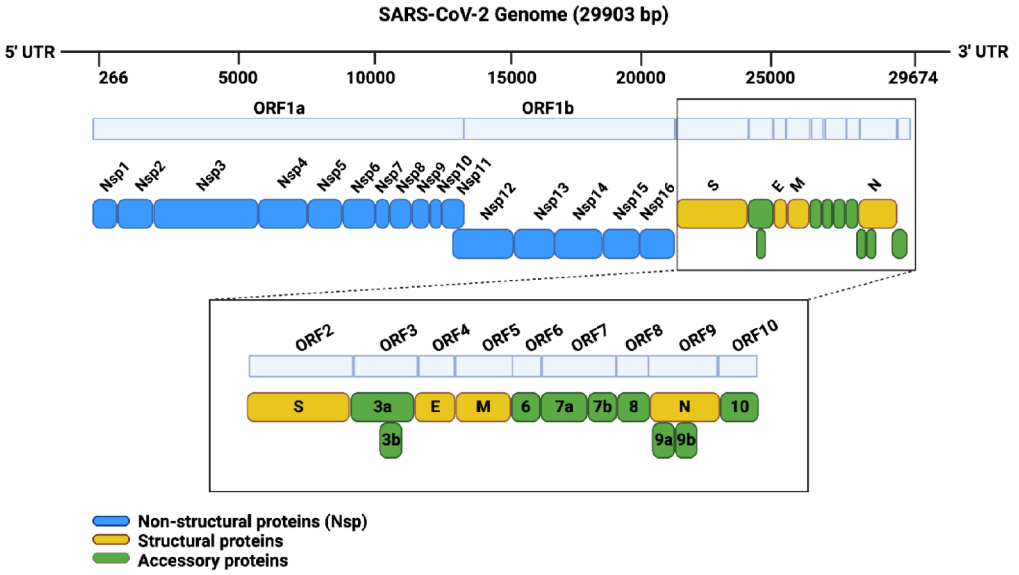


Figure 1. SARS-CoV-2 viral genomic architecture. Two large overlapping ORFs in the 5′ proximal two-third of the genome, ORF1a and ORF1b, encode 16 nonstructural proteins (Nsp1−16). Four additional ORFs of the 3′-terminal region encode four structural proteins [spike (S), envelope (E), nucleocapsid (N), and membrane (M)], which assemble to form the virion.[3] The remaining ORFs encode 9 accessory proteins (ORF3a, 3b, 6, 7a, 7b, 8, 9a, 9b, and 10). Adapted from Kim, D.; Lee, J.Y.; Yang, J.S.; Kim, J.W.; Kim, V.N.; Chang, H. The Architecture of the SARS-CoV-2 Transcriptome. *Cell* 2020, 181, 914−921, doi:10.1016/J.CELL.2020.04.011. Copyright Elsvier 2020

translocation of p65, a subunit of the transcription factor NF-kB.[22] Collectively, these prior discoveries indicate that each of the three proteins may play a role in suppressing innate immune signaling. Here, we sought to study Orf3a, Orf6, and Nsp9 individually in the context of immune signaling specifically by using polyinosinic:polycytidylic acid (poly(I:C)) as the immune stimulant. There have been numerous studies investigating the roles of Orf3a and Orf6 following SARS-CoV-2 infection, with findings suggestive to their participation in viral evasion.[7,13,15−19] We sought to investigate if the SARS-CoV-2 protein, Nsp9, acted in a manner similar to that of Orf3a and Orf6 to modulate the host antiviral response. A greater understanding of SARS-CoV-2 viral evasion through interactions with the host innate pathway is necessary and may unveil potential targets for therapeutic development.[23]

RESULTS

SARS-CoV-2 Orf3a, Orf6, and Nsp9 Modulate the Host Innate Signaling Transcriptome. Consistent with most viral infections, the host innate immune response initiates the expression and production of type I interferons (IFN-alpha and IFN-beta) and type III interferons following SARS-CoV-2 infection.[24−26] This interferon response induces multiple intracellular signaling pathways in order to restrict viral replication, aid in the development of subsequent adaptive immunity, and facilitate viral clearance.[23−28] In this study, we use poly(I:C), a synthetic analog of dsRNA which is a common replication intermediate for all RNA viruses, that elicits an immune response via binding endosomal membrane-associated toll-like receptor 3 (TLR3) or cytosolic receptors melanoma differentiation−association protein 5 (MDA5) and retinoic acid-inducible gene 1 (RIG-I).[29−32] This ultimately induces IRF3, IRF7, and NF-kB-mediated signaling, thus upregulating the downstream expression of type I and III IFNs and pro-inflammatory genes. The subsequent IFN response phase activates the JAK-STAT pathway, a signaling cascade resulting in the expression of interferon-stimulated genes (ISGs) essential for the transduction of the antiviral response.[23−28] This also results in the expression of interleukins and chemokines, which are important markers of immune activation. In the face of these host antiviral defense mechanisms, coronaviruses have developed distinctive evasion tactics, including disruptions of viral sensing, interference with IFN production and signaling, and modulation of ISG expression.[26,32,33] The association between disease severity in COVID-19 patients, characterized by heightened cytokine production and inflammation, is thought to be linked to a compromised or delayed IFN response during the early phase of SARS-CoV-2 infection.[33−37] Through poly(I:C) treatments, we can study the role of Orf3a, Orf6, and Nsp9 individually in a mimicked virus-infected state while avoiding combinatory viral protein effects and isolate independent mechanisms of viral evasion through individual protein expression.

We sought to characterize the roles of SARS-CoV-2 Nsp9 and compare the effects to viral proteins Orf3a and Orf6, specifically in regulating innate immune signaling pathways at the cellular transcriptome level in a mimicked viral-infected state. We performed gene expression profiling, well established for profiling innate immune signaling, for A549 cells overexpressing each viral protein that are also immuno-stimulated with poly(I:C) to mimic viral infection without the complications of other structural protein immune evasion mechanisms. Bioinformatics analysis via ToppGene Suite[38] was performed to identify pathways regulated by these viral accessory proteins. Gene ontology enrichment analysis of genes differentially regulated by more than 1.5 fold in expression, relative to the control pTwist vector, was performed. These data revealed that biological processes related to type I IFN induction were regulated by SARS-CoV-2 Orf3a, Orf6, and Nsp9 (Tables S1−S3). This allowed the identification of differentially regulated genes, grouped into five categories: IFN-stimulated genes (ISGs), JAK-STAT regulatory genes, IFNs and interleukins (IL), type-I IFN pathway genes, and chemokines (Figure 2).

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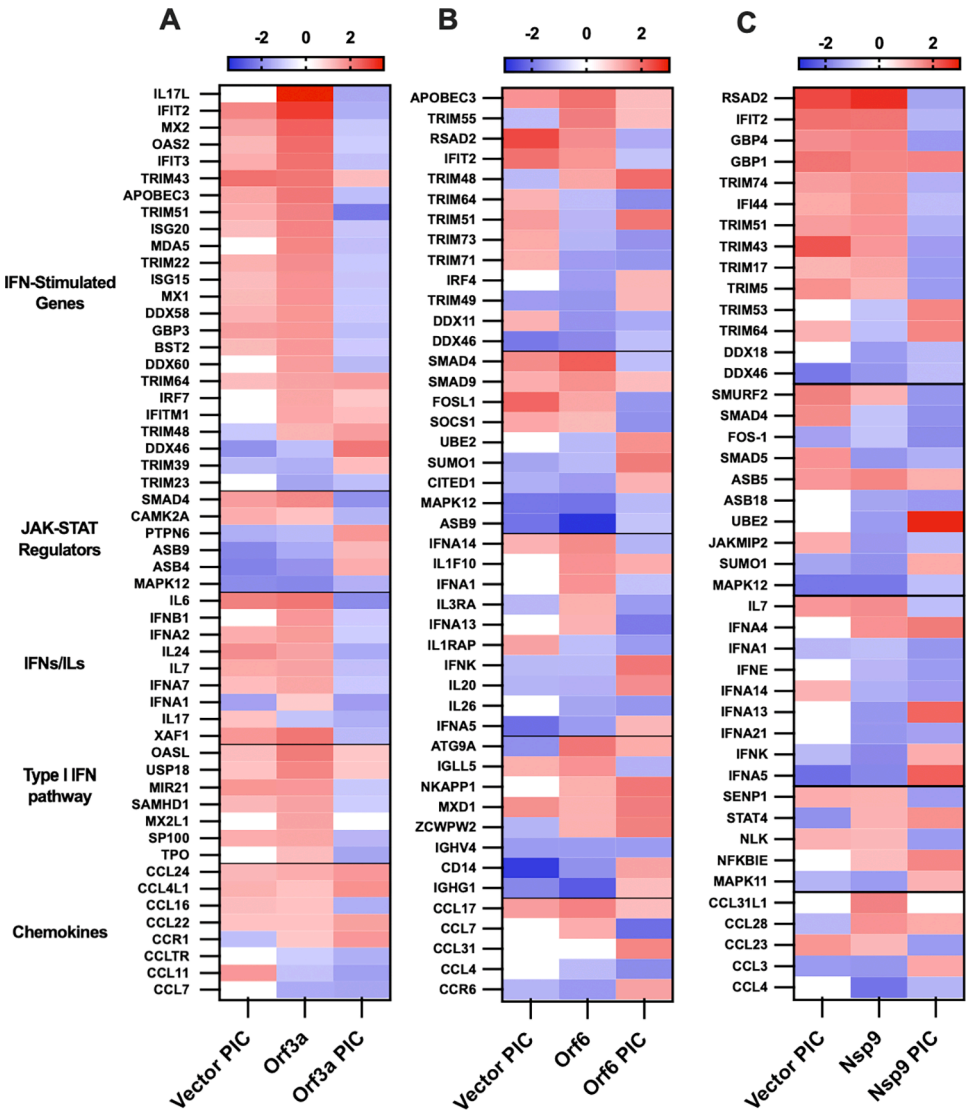

Figure 2. SARS-CoV-2 Orf3a, Orf6, and Nsp9 regulate the innate antiviral response. A) A549 cells were transfected with pTwist control vector or Orf3a (A), Orf6 (B), or Nsp9 (C) plasmids independently and immuno-stimulated with PIC (500 ng/mL) after 24 h or left untreated. The heatmap shows genes differentially expressed by more than 1.5 fold (up or down) under each treatment condition, relative to the untreated pTwist control transfected A549 cells.

Overexpression of SARS-CoV-2 Orf3a with and without poly(I:C) treatment led to differential expression of several important ISGs and genes essential to the innate antiviral response (Figure 2A). IL17L and IFIT2 are strongly upregulated in the presence of Orf3a. SARS-CoV-2 infection induces a plethora of chemokines that mediates a strong inflammatory response driven by IL-17 signaling.[39] IFIT2 expression has been shown to induce pro-inflammatory cytokine response both in vitro and in vivo and is highly expressed in patients with severe COVID-19.[40] Our transcriptome analysis suggests that Orf3a contributes to this pro-inflammatory cytokine response through upregulation of IFIT2 and IL-17.

Orf3a in combination with poly(I:C) treatment downregulated the expression of many ISGs, IFNs, and positive regulatory JAK-STAT pathway genes (SMAD4, CAMK2A). During viral infection, the activity of SMAD-containing transcriptional complexes, often containing STATs, can be finely tuned to enhance infectivity and spread.[41,42] Orf3a may be modulating SMAD-STAT transcriptional activity through downregulation of the SMAD4 expression. CAMK2A is a calcium/calmodulin-dependent protein kinase, which in response to IFN-gamma stimulation, catalyzes phosphorylation of STAT1, stimulating the JAK-STAT signaling pathway.[43] Orf3a may be dysregulating JAK-STAT pathway activity through hindering the phosphorylation of STAT1 via CAMK2a.

Orf3a in combination with poly(I:C) treatment upregulated the expression of PTPN6, ASB9, and ASB4 (Figure 2A). PTPN6 (SHP1) is a tyrosine phosphatase that negatively controls the activity of JAK-STAT signaling through dephosphorylation of STAT proteins.[44] Orf3a in the presence of poly(I:C) upregulated PTPN6, possibly suggesting increased dephosphorylation of STAT proteins and negative regulation of downstream STAT signaling. ASB9 and ASB4 are members of the ankyrin repeat and SOCS box-containing (ASB) protein family, in which complex with the cullin and ring box proteins forms E3 ubiquitin ligase complexes.[45] Many viruses hijack members of the cullin-ring E3 ligase family to

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ACS Omega 2024, 9, 26428−26438

avoid degradation by the ubiquitin proteosome system.[45] Our data suggest that Orf3a may be involved in this pathway.

Overexpression of SARS-CoV-2 Orf6 differentially modulated the expression of key ISGs, JAK-STAT pathway regulatory genes, as well as multiple IFNs, chemokines, and type I interferon pathway genes (Figure 2B). Orf6 overexpression in the presence of poly(I:C) downregulated JAK-STAT pathway genes (SMAD4, FOSL1, and SOCS1).[41,42,46,47] Similar to Orf3a, Orf6 may be modulating SMAD-STAT transcriptional activity through the downregulation of SMAD4 expression. FOS-like antigen 1 (FOSL1) protein is a subunit of the transcriptional complex AP-1 and is a downstream target of STAT3.[46] Downregulation of FOSL1 by Orf6 in the presence of poly(I:C) may point at a hindrance in the STAT signaling cascade. Suppressor of the cytokine signaling-1 (SOCS1) protein directly interacts with JAKs to inhibit their tyrosine kinase activities and suppress immune cytokine action.[47,48] A downregulation of SOCS1 by Orf6 in the presence of poly(I:C) suggests possible dismantlement of cytokine regulation, perhaps contributing to the "cytokine storm" characteristic of COVID-19 severity.

Orf6 overexpression in the presence of poly(I:C) upregulated UBE2, SUMO1, and ASB9 (Figure 2B). Ubiquitin-conjugating enzyme E2s (UBE2) have been characterized to negatively modulate the type I IFN pathway and promote RNA virus infection.[49] Our data suggest that Orf6, in the presence of poly(I:C), may act in this manner through increased expression of UBE2. Small ubiquitin-like modifier 1 (SUMO1) protein conjugation to a substrate, known as SUMOylation, greatly impacts host innate immunity through viral manipulation of host machinery and promotes viral replication and pathogenesis.[50] Our data suggest that Orf6 may be involved in this pathway via increased expression of SUMO-1. Similar to Orf3a, Orf6 may be modulating cullin-ring E3 ligase degradation pathways via increased expression of ASB9 with the presence of poly(I:C). These data show that Orf6 affects the relevant pathways similarly to that of Orf3a.

Lastly, Nsp9 overexpression with and without poly(I:C) treatment induced differential expression of multiple ISGs, JAK-STAT regulatory genes, IL7, IFNA4, as well as essential chemokines (Figure 2C). Nsp9 overexpression, in combination with poly(I:C) treatment, downregulated the expression of JAK-STAT pathway genes (SMAD4, FOSL1)[41,42,46] and upregulated the expression of UBE2[49] and SUMO1[50] (Figure 2C). SARS-CoV-2 Nsp9 may be modulating pathways associated with these protein coding genes in the same manner as that of Orf3a and Orf6, as described previously. Collectively, genome-wide expression profiling suggests that in a mimicked viral-infected state, SARS-CoV-2 Orf3a, Orf6, and Nsp9 modulate the cellular transcriptome to favor the inhibition of the JAK-STAT pathway, thus potentially preventing the downstream expression of ISGs necessary for sequential induction of the antiviral innate immune response.

SARS-CoV-2 Orf3a, Orf6, and Nsp9 Antagonize the Host Innate Antiviral Response. Next, we sought to compare gene expression profiles of A549 cells overexpressing SARS-CoV-2 Orf3a, Orf6, and Nsp9 viral proteins and subsequently immuno-stimulated with poly(I:C) to identify common innate antiviral-related genes being regulated in a mimicked virus-infected state. Overexpression of Orf3a, Orf6, and Nsp9 elicited a broad signature of cytokines and ISGs attributed to type I and III interferon responses (Figure 3A–D).

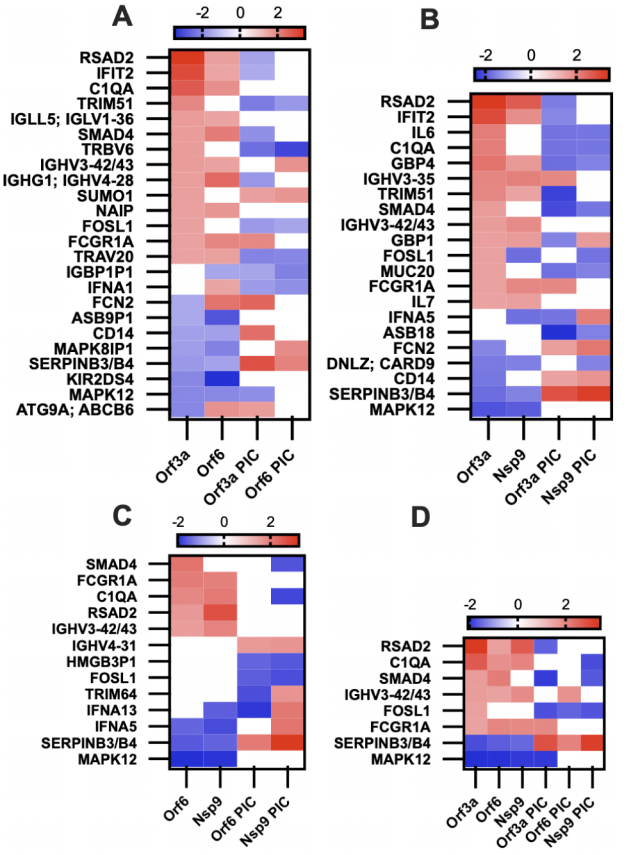

Figure 3. SARS-CoV-2 Orf3a, Orf6, and Nsp9 regulate similar innate antiviral-related genes. A) Heatmap shows common genes differentially expressed by more than 1.5 fold (up or down) by Orf3a or Orf6 (A), Orf3a or Nsp9 (B), or Orf6 or Nsp9 (C) or Orf3a, Orf6 or Nsp9 (D) under each treatment condition relative to the pTwist vector control-transfected A549 cells.

Overexpression of both Orf3a and Orf6 regulate common gene characteristics of interferon stimulation (IFIT2),[51] immunoglobulin regulation (IGLL5, IGHV3, IGHG1, and IGBP1), and SMAD4, a proviral host factor (Figure 3A).[41,52] Following poly(I:C) treatment, both Orf3a and Orf6 downregulate the expression of IFNA1, a type I interferon essential for the innate antiviral response (Figure 3A).[53] Overexpression of Orf3a and Nsp9 showed similarities in regulating the expression of cytokines (IL6, IL7) (Figure 3B), whereas Orf6 and Nsp9 showed similarities in regulating the expression of type I interferon genes (IFNA13, IFNA5)[53] (Figure 3C). Following overexpression of Orf3a, Orf6, and Nsp9, a short overlapping list of innate antiviral-related genes appeared (Figure 3D).

All three viral proteins upregulated the expression of the ISG RSAD2, responsible for encoding an antiviral interferon-inducible protein (Figure 3D).[54] Following the overexpression of Orf3a, Orf6, and Nsp9, all viral proteins downregulated the expression of SERPINB3/4, responsible for encoding a serine protease inhibitor known to enhance the host immune cell function, as well as MAPK12, which encodes a kinase involved in cascades evoked by pro-inflammatory cytokines (Figure 3D).[55,56] Following poly(I:C) treatment, all three proteins downregulated the expression of FOSL1, suggesting hindrance in the STAT signaling cascade as described earlier (Figure 3D).[57] Together, genome-wide expression profiling suggests

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that SARS-CoV-2 Orf3a, Orf6, and Nsp9 modulate the cellular transcriptome in a manner favorable to viral evasion through antagonizing the host innate antiviral response, albeit with some differences, as is expected.

Nsp9 Antagonizes IFN Induction by Targeting NF-kB and TBK1. Previous studies on SARS-CoV-2 accessory proteins have reported several roles for Orf6 and Orf3a in antagonizing IFN viral detection.[14,15] In the literature, Orf6 has been suggested to inhibit STAT1 nuclear translocation through interaction with nuclear pore complex components Nup98 and RAE1, ultimately antagonizing IFN signaling.[16] Orf3a suppresses STAT1 nuclear translocation and ISRE promoter activity during IFN-I induction.[14,15] We sought to unveil whether Nsp9 interfered with IFN viral detection by similar or different mechanisms, using Orf3a and Orf6 accessory proteins as positive controls. Individual viral proteins were expressed with a C-terminal FLAG tag to facilitate the detection of their expression (Figure 4A). We overexpressed

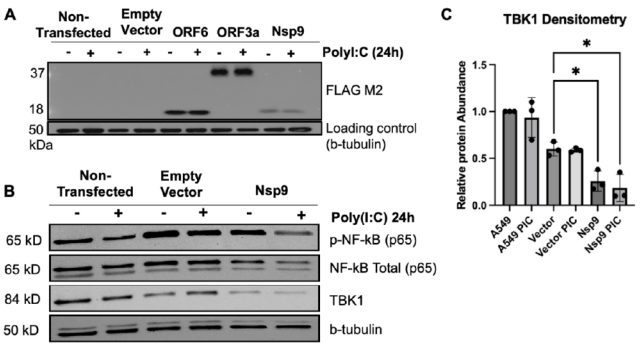

Figure 4. SARS-CoV-2 Nsp9 Western blot analysis. A) SARS-CoV-2 Orf6, Orf3a, and Nsp9 expression. A549 cells were reverse transfected with 2 μg Orf6, Orf3a, or Nsp9 expression vectors and then lysed for protein detection by Western blotting. B) Western blotting of p-NF-kB, NF-kB, and TBK1 protein expression in 24 h poly I:C-treated A549 cells transfected with Nsp9 or empty vector control plasmids. B-tubulin is used as a control for protein expression. C) Densitometry analysis showing relative TBK1 abundance in 24 h poly I:C (PIC)-treated A549 cells transfected with Nsp9 or empty vector control plasmids compared to nontransfected and nontreated A549 cells (n = 3). Error bars represent standard error of the mean, * = $p < 0.05$.

Orf3a, Orf6, and Nsp9 in A549 cells and subsequently immuno-stimulated with poly(I:C) to mimic viral infection. Via Western blotting, we observed no change in p-STAT1 (Y701), p-STAT1 (S727), and total STAT1 protein abundance in the poly(I:C)-treated Orf6 sample compared to the poly(I:C)-treated vector control sample (Figure S1A). Via Western blotting, we observed no change in p-STAT1 (Y701), p-STAT1 (S727), and total STAT1 protein abundance in the poly(I:C)-treated Orf3a sample compared to the poly(I:C)-treated vector control sample (Figure S1B).

The transcription factor NF-kB regulates multiple aspects of innate and adaptive immune functions and serves as a pivotal mediator of the host's innate immune response. The activation of NF-kB is known to balance antiviral activity, as well as differentially regulate the expression of type I IFNs, cytokines, and chemokines, essential for antiviral activity.[58] One group found that viral protein 4b of MERS-CoV hindered the expression of pro-inflammatory genes via blocking NF-kB transport, which implicates the antiviral role of this transcription factor during viral infection.[58] We found that the overexpression of Orf3a, in combination with poly(I:C) treatment, led to a lower protein abundance of both p-NF-kB and NF-kB, compared to the poly(I:C)-treated vector control sample (Figure S1B). Through Western blot analysis, we also show that the overexpression of Nsp9 in combination with poly(I:C) treatment results in a lower abundance of both p-NF-kB and NF-kB, compared to the poly(I:C)-treated vector control sample (Figure 4B). A decrease in p-NF-kB and NF-kB abundance suggests that type I IFN expression and production will be downregulated. We then probed for TBK1, a kinase of the RIG-I-like receptor pathway,[59] and observed a slight decrease in protein abundance in the poly(I:C)-treated Nsp9 sample compared to the poly(I:C)-treated vector control sample (Figure 4B). We performed densitometry on replicates of the Nsp9 Western blots probing for TBK1, a kinase responsible for the phosphorylation of IRF3 and the downstream induction of type I IFN expression (Figure 4B). TBK1 protein abundance was normalized to nontransfected and nontreated A549 cells. We found that there to be a significant decrease in relative TBK1 abundance in the Nsp9-transfected cells compared with the control vector-transfected cells (Figure 4C). Likewise, there is a significant decrease in relative TBK1 abundance in poly(I:C)-treated Nsp9-transfected cells compared to that of the control vector-transfected cells (Figure 4C). Together, our data suggest that SARS-CoV-2 Nsp9 may prevent the induction of type I IFN expression by targeting NF-kB and TBK1. Collectively, our Western blot analysis supports the notion that these accessory proteins employ viral evasion strategies by suppressing the activity or function of transcription factors essential for the expression of antiviral-related genes.

Nsp9 Hinders Viral Detection through RIG-I and Antagonizes the IFN Antiviral Cascade. Given our previous finding that SARS-CoV-2 Nsp9 targets NF-kB and TBK1, we sought to investigate whether Nsp9 targeted other IFN viral detection pathway components. We overexpressed Nsp9 and empty vector control plasmids in A549 cells and subsequently immuno-stimulated with poly(I:C) to mimic viral infection. Expression levels of various ISGs in pTwist control and Nsp9 samples were characterized via quantitative RT-qPCR (Figure 5). Nsp9 overexpression, in combination with poly(I:C) treatment, lead to a significant downregulation in expression of RIG-I, responsible for the viral RNA pattern-recognition receptor RIG-I (Figure 5A).[60] This suggests that in a mimicked viral-infected state, Nsp9 hinders viral detection via RIG-I as well as subsequent RIG-I-mediated signaling. Nsp9 overexpression, in combination with poly(I:C) treatment, led to a significant upregulation of TRIM27 (Figure 5B), a multifunctional E3 ligase known to interact with TBK1[61] and IFN-B.[18] Knowing that TRIM27 degrades TBK1,[61] and given the increase in TRIM27 expression in Nsp9 poly(I:C)-treated samples (Figure 5B), it is expected that we see a lower protein abundance of TBK1 (Figure 4C). As TBK1 is responsible for the phosphorylation of IRF3 and induction of type I-IFN expression, it is justifiable that we observed a decreased expression of IFN-B via qPCR analysis (Figure 5C). Nsp9 overexpression, in combination with poly(I:C) treatment, also resulted in a significant upregulation of PTPN11, a negative regulator of the JAK-STAT pathway (Figure 5D).[62] This implies that Nsp9 also hinders the JAK-STAT pathway activity. These findings suggest that SARS-CoV-2 Nsp9 plays a role in the regulation of the viral sensing pathways (RIG-I/TRIM27/TBK1; NF-kB) as well as the antiviral IFN response (Jak/

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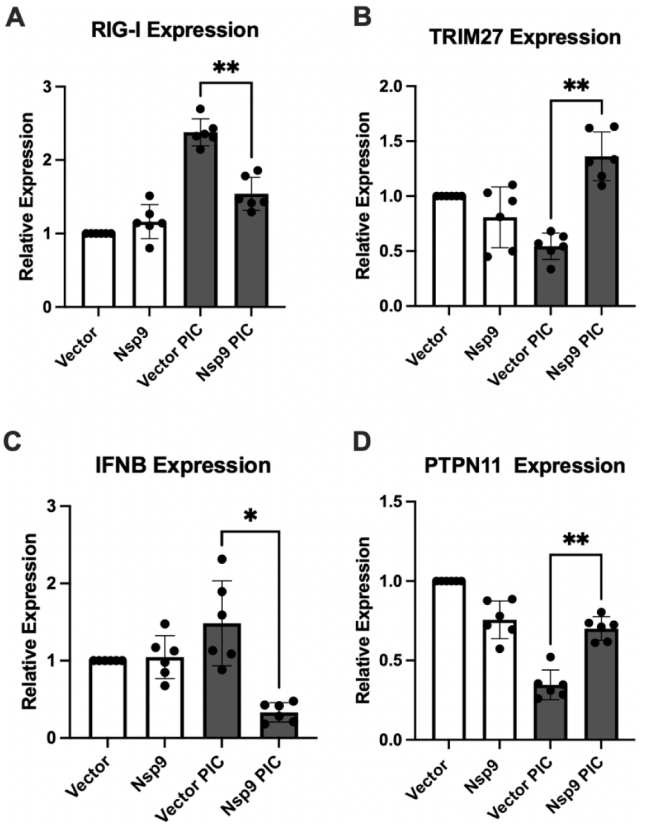

Figure 5. SARS-CoV-2 Nsp9 qPCR analysis. A) qPCR analysis showing regulation of RIG-I (A), TRIM27 (B), IFN-B (C), or PTPN11 (D) expression in Nsp9 nontreated and poly I:C-treated samples compared to empty vector control (n = 6). Error bars represent standard error of the mean, * = $p < 0.05$.

STAT). Collectively, these findings suggest that Nsp9 may contribute to viral evasion through modulating the expression of components essential to viral detection and the success of the antiviral IFN response.

DISCUSSION

It is widely known that viruses suppress the host immune response in order to establish infection in the host cells. Viruses perform (i) production of IFN-binding proteins, (ii) degradation of JAK-STAT components, (iii) suppression of activation (phosphorylation) of pathway components, (iv) inhibition of nuclear translocation of activated transcription factor, and (v) induction of host JAK-STAT negative regulators.[25] The specifics of innate immune evasion strategies of SARS-CoV-2, including the individual roles of accessory and nonstructural proteins, have been under investigation in recent years.[32] Despite these efforts, it remained unclear how Nsp9 may contribute to host immune evasion during the SARS-CoV-2 infection. In this study, we used poly(I:C) to mimic viral infection and investigate how Nsp9, as well as Orf3a and Orf6, modulates the antiviral signaling transcriptome. Our genome-wide expression profiling data suggest that these viral proteins modulate the cellular transcriptome to favor the inhibition of the JAK-STAT pathway and hindrance of the host antiviral IFN response. The inhibitory effect of Orf6 on innate immune signaling regulation has been broadly attributed in the literature to its ability to block nucleocytoplasmic transport and therefore affect general expression levels of a broad class of antiviral genes, subsequently impacting their ability to function in signaling pathways. While it is possible that Orf6 has more direct effects on the genes mentioned in our transcriptomics analysis, the function of Orf6 is also essential to consider. In regard to our transcriptome analysis, it is important to consider that the regulation of some genes may be beneficial to the virus in early infection prior to dsRNA accumulation but detrimental once the antiviral response is triggered or vice versa.

Our Western blot analysis revealed that Nsp9 targets transcription factor NF-kB, a crucial regulator of type I IFN expression and downstream antiviral activity. It is important to consider the implications of one study that suggests that activation of NF-kB is essential for SARS-CoV-2 replication.[69] While it is true that several SARS-CoV-2 proteins enhance the NF-kB signaling cascade, it is also has been shown in the literature that several SARS-CoV-2 proteins (Nsp5 and Orf9ab) act in the opposite manner to inhibit the NF-kB signaling cascade.[70,71]

Through quantitative RT-qPCR, we demonstrated that RIG-I transcription is downregulated in the presence of Nsp9, implying compromised viral detection and downstream RIG-I mediated signaling. Some studies have pointed to the important and potentially independent role of MDA5 in the antiviral innate immune response to SARS-CoV-2 in lung epithelial cells;[63,64] however, RIG-I is also involved in viral dsRNA detection and both receptors play crucial roles in type I IFN expression following SARS-CoV-2 infection.[65] Our data also revealed that Nsp9 upregulated TRIM27 and PTPN11 expression, lowered TBK1 abundance, and downregulated the expression of IFN-B. We speculate that Nsp9 targets TBK1 indirectly through regulation of PTPN11,[62] which inhibits TBK1 phosphatase activity and TRIM27,[61] an E3 ligase that degrades TBK1 via K48-linked ubiquitination. Interestingly, one group found that Nsp9 targets TBK1 but comes to the opposite conclusion in which Nsp9 induces, rather than inhibits, IFN signaling in HEK293T and L292 cells.[68] As SARS-CoV-2 primarily infects human lung tissue, we believe the use of A549 cells in our study to be a more representative model of Nsp9's role during SARS-CoV-2 infection. Nsp9 has been found to interact with RNA-dependent RNA polymerase (RdRp/Nsp12) to contribute to the SARS-CoV-2 replication and transcription complex, an essential component for viral replication.[4] Given its ability to bind RNA, Nsp9's indirect interactions may be through binding of host noncoding RNAs such as microRNAs or siRNAs. There is little shown in the literature regarding direct Nsp9-host protein interactions, and this topic warrants further investigation.[72]

Together, our findings point to a novel mechanism by which Nsp9 antagonizes IFN signaling through targeting multiple components of the innate antiviral host response (Figure 6A). Pieced together with the known interactions of Orf3a and Orf6 with STAT1, Figure 6 illustrates how these viral proteins target the host's antiviral response to undergo viral evasion. Through this study, in combination with work by other groups,[13,15−18] it is evident that Nsp9, Orf3a, and Orf6 play important roles in SARS-CoV-2 viral evasion. Notably, Nsp9, Orf3a, and Orf6 do not act in isolation in the context of viral infection. While our data suggest that these proteins play a role in viral evasion, there are other factors at play, including other viral binding partners. Nsp9 has been shown to directly interact with the host RNA-binding protein SND1, which remodels Nsp9 occupancy and alters the covalent linkage of Nsp9 to viral RNA.[72] Interaction with viral or host binding partners may

https://doi.org/10.1021/acsomega.4c02631
ACS Omega 2024, 9, 26428−26438

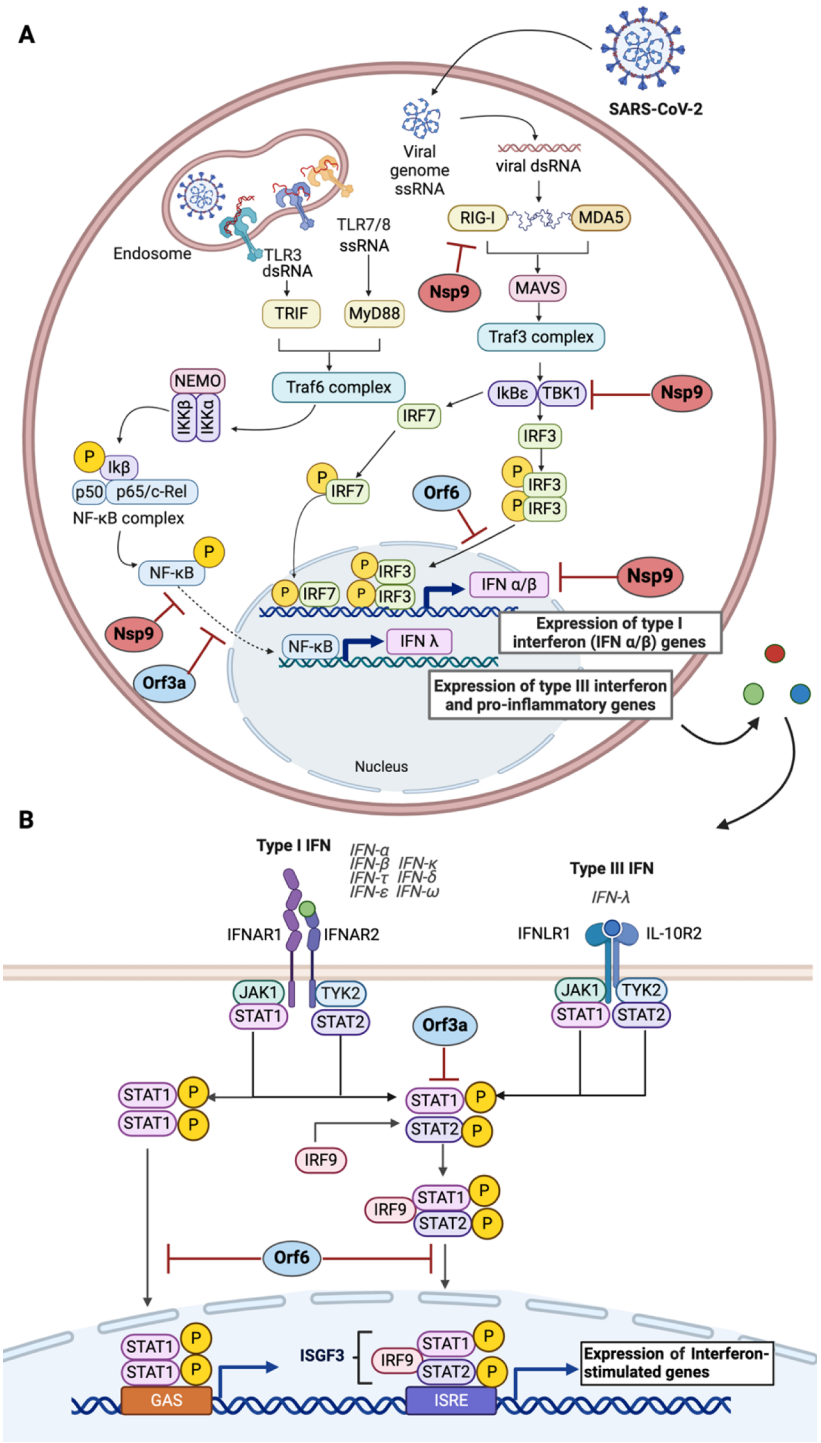

Figure 6. SARS-CoV-2 Orf3a, Orf6, and Nsp9 in the antiviral response. A) Upon cell entry, SARS-CoV-2 releases its genomic ssRNA, allowing for subsequent transcription and replication. TLR3 in endosomes detects SARS-CoV-2 dsRNA, while viral ssRNA is recognized by TLR7. This activates downstream adaptor proteins TRIF and MyD88, respectively. This leads to recruitment of the TRAF6 complex, which activates NEMO, the regulatory subunit of this IKK complex. IKKB is associated with p50 and p65, forming the NF-kB complex. When IKK-beta is phosphorylated, it dissociates from NF-kB, unmasking its nuclear localization signal, allowing it to migrate to the nucleus, pass through nucleoporin62, and function as a transcription factor. Viral dsRNA can also be recognized by cytosolic pattern recognition receptors RIG-I and MDA5, which induces the activation of common adaptor MAVS (mitochondrial antiviral signaling protein) and recruits the TRAF3 complex. This recruits kinases TBK1 and IKK epsilon, which phosphorylate and hence activate transcription factors IRF3 and IRF7. Transcription factors then translocate across the nucleus and induce the expression of type I and III IFNs as well as other cytokines, chemokines, and pro-inflammatory genes. B) In the subsequent IFN action phase, the expressed and secreted IFNs bind to their respective IFN receptors on adjacent cells in an autocrine or paracrine manner. Despite interaction with their unique receptors, type I and III IFNs activate the same JAK-STAT pathway cascade following phosphorylation of the STATs. In combination with IRF9, phosphorylated STAT1 and STAT2 form the trimeric complex ISGF3. This complex is translocates to the nucleus where it binds to IFN-stimulated response elements (ISREs) to promote the transcription of hundreds of ISGs.

https://doi.org/10.1021/acsomega.4c02631
ACS Omega 2024, 9, 26428−26438

alter the function of Nsp9, Orf3a, and Orf6 in viral invasion following SARS-CoV-2 infection. It is important to note that the C-terminus of Orf6 is essential for its interaction with Nup98/Rae1; therefore, it is possible that the C-terminal FLAG tag on our Orf6 variant is affecting the function of Orf6.

It is important to note the relationship between SARS-CoV and SARS-CoV-2 Nsp9 and Orf3a and Orf6 protein sequence homology. While protein homology suggests that functional conservation provides insight into shared biological mechanisms such as viral entry, replication, and immune evasion strategies, differences in protein sequence between SARS-CoV and SARS-CoV-2 can also be informative as they may contribute to changes in these viral properties. SARS-CoV Nsp9 is a single-stranded RNA-binding protein abundant in the replication complex and is thought to stabilize nascent nucleic acid during replication or transcription, thus providing protection from nucleases.[66] SARS-CoV Orf3a is a multifunctional protein with several proposed roles, including viroporin activity, induction of apoptosis, innate immune evasion, and antagonization of the host immune response.[67] SARS-CoV Orf6 is thought to contribute to virulence and inhibits nuclear import of STAT1.[67] Owing to their degree of sequence conservation, the roles of and essentiality of Orf3a, Orf6, and Nsp9 within SARS-CoV-2 are likely to mimic the behavior of homologous proteins within previously studied SARS-CoV.

Given that effective diagnostic and therapeutic strategies against SARS-CoV-2 rely heavily on comprehending the mutual interplay between the virus and its host, these viral proteins should be further investigated as potential therapeutic targets for COVID-19.

CONCLUSION

Herein, we discover that the SARS-CoV-2 protein Nsp9 has strong effects on IFN viral detection and induction. Our results show that Nsp9 hinders viral detection through the suppression of RIG-I expression. Simultaneously, Nsp9 antagonizes the viral sensing cascade by downregulating NF-kB and TBK1. These results are important in identifying mechanisms that SARS-CoV-2 may evade and host responses that give rise to larger, general tissue damage and fibrosis through failed or inefficient antiviral pathways. It is interesting and relevant that SARS-CoV-2 proteins suppress immune signaling. Nsp9 as a protein involved in viral replication also modulates the activity of major elements of the host antiviral pathways to impair several key components of the cellular antiviral immune response.

MATERIALS AND METHODS

Plasmids and Cell Lines. A549 adenocarcinoma human alveolar basal epithelial cells (CCL-185) were obtained from the ATCC and cultured in Dulbecco's modified Eagle medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; PAA Laboratories). SARS-CoV-2 nonstructural and accessory protein expression vectors were purchased from Addgene (MA, USA): pTwist-CMV-Nsp9−3xFlag (pGBW-m4133209), pTwist-CMV-ORF6−3xFlag (pGBW-m4252831), and pTwist-CMV-ORF3a- 3xFlag (pGBW-m4133902). The pTwist-CMV control vector was made by restriction enzyme digestion of the pTwist-CMV-Nsp2 (pGBW-m4134143) expression vector, excising the inset and relitigating the plasmid DNA. The plasmid was verified by sequencing.

Transfections. A549 cells were seeded in six-well plates (750−000 cells/mL) and were reverse transfected with 2μg of the following plasmid DNA: pTwist-CMV vector, pTwist-Nsp9−3xFlag, pTwist-ORF6−3xFlag, or pTwist-ORF3a-3xFlag. Plasmid DNA was transfected using Lipofectamine 2000 transfection reagent (Invitrogen), according to the manufacturer's protocol, and Opti-MEM media. The cells were grown at 37 °C and 5% CO_2. At 48 h post-transfection, the cells were lysed using RLT Plus lysis buffer (RNeasyPlus kit, Qiagen, Mississauga, ON) for RNA or protein analysis using lysed with 1x sodium dodecyl sulfate (SDS) lysis buffer (50 mM Tris-HCl [pH 6.8], 2% SDS, and 10% glycerol).

Poly I:C Treatment. 24 h post-transfection, the innate immune response was induced by treating the cells with 500 ng/mL of poly(I:C)-LMW LyoVec (InvivoGen), a dsRNA analog. The cells were grown at 37 °C 5% CO_2 for another 24 h.

Microarray Analysis. RNA isolation from transfected and treated cells was performed using the Norgen total RNA isolation kit (Norgen Biotek, Thorold, ON). Samples were prepared as required by the Microarray Analysis and Genomic Expression Facility of The Centre for Applied Genomics in Toronto, Ontario. For microarray gene profiling, Affymetrix Human Gene ST.2.0 arrays were utilized. Affymetrix Expression Console and Transcriptome Analysis COnsole (v3.0) were utilized to analyze and normalize the obtained data. Pathway enrichment and gene ontology analysis were conducted using the ToppGene Suite.

Immunoblotting. Following transfection with plasmid DNA and poly I:C treatment, cells were washed with PBS and lysed with RIPA buffer in addition to Roche Diagnostic protease inhibitor mixture. The protein concentration of each sample was quantified using the Bio-Rad DC Protein Assay according to the manufacturer's protocol. Protein samples were prepared by adding 4X Laemmli Buffer and heated at 95 °C. 30 μg of protein was loaded per well of an SDS- PAGE gel (12% resolving gel, 4% stacking gel). Proteins were then transferred to a PVDF membrane using Bio-Rad Trans-Blot Turbo Transfer system according to the manufacturer's protocol. The resulting PVDF membranes were blocked for 1 h in 5% milk-TBST and incubated for approximately 16−20 h at 4 °C with primary antibodies including mouse anti-FLAG M2 (1:4000, Sigma-Aldrich, F1804). The membranes were then incubated with secondary antibodies, either HRP-conjugated goat antimouse (1:20000) or donkey antirabbit (1:10000) (Jackson ImmunoResearch Laboratories). Clarity Max Western ECL Substrate (Bio-Rad) was used to visualize protein bands according to the manufacturer's protocol.

Quantitative RT-PCR. For the RNA analysis, RNA isolations were performed using an RNA RNeasyPlus kit (Qiagen, Mississauga, ON). For the RT-qPCR analysis, isolated RNA was quantified using a NanoDrop instrument (Thermo Fisher Scientific). Isolated RNA (500 ng) was reverse-transcribed using the iScript reverse transcription kit (Bio-Rad) according to the manufacturer's protocol. Quantitative PCR was then performed in the iCycler (Bio-Rad), using iQ SYBR Green SSO Advanced Supermix (Bio-Rad) according to the manufacturer's protocol. The runs were analyzed by using a CFX Connect Real-Time PCR Detection System (Bio-Read, Hercules, CA). The $2^{-\Delta\Delta Ct}$ method was used for the analysis of the levels of mRNA and to evaluate the relative fold changes in mRNA expression, with 18S rRNA levels being used for normalization. Relative expression was calculated to

https://doi.org/10.1021/acsomega.4c02631
ACS Omega 2024, 9, 26428−26438

evaluate the expression changes between the mock or poly(I:C)-LMW Lyovec treated samples. Unless otherwise stated, data were presented as the fold change relative to the pTwist-CMV expression vector.

Statistical Analysis. In this study, data are presented as the mean of replicates, with error bars representing the standard error of the mean. Statistical analysis and graphing were done using Prism 9 software. All datasets were assessed for normality before analysis. Unless otherwise stated, the statistical analysis was done using a one-way ANOVA, and $p < 0.05$ was considered significant.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c02631.

> Regulation of biological pathways and processes in Nsp9, Orf3a, and Orf6 nontreated and poly(I:C)-treated samples relative to empty vector control (1.5> and ← 1.5-fold change); Western blot showing SARS-CoV-2 Orf6, Orf3a, and Nsp9 expression in A549 cells; Western blotting of p-STAT1 (Y701), p-STAT1 (S727) and STAT1 protein expression in 24 h poly I:C-treated A549 cells transfected with Orf6 and empty vector control plasmids; Western blotting of NF-kB (p65), p-STAT1 (Y701), p-STAT1 (S727), and STAT1 protein expression in 24 h poly I:C-treated A549 cells transfected with Orf3a and empty vector control plasmids (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank the Natural Sciences and Engineering Council of Canada (NSERC) for funding in the form of a discovery grant to JPP. EL acknowledges NSERC CREATE for support through a training grant.

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https://doi.org/10.1021/acsomega.4c02631
ACS Omega 2024, 9, 26428−26438