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14th September 2026

Dear Editor and Reviewers,

We sincerely thank you and the reviewers for the careful evaluation of our manuscript entitled "**SARS-CoV-2 ORF8 exploits host miRNA networks to rewire post-transcriptional regulation**". We greatly appreciate the constructive comments and suggestions, which have helped us substantially improve the quality, clarity, and mechanistic interpretation of the study.

In response to the reviewers' comments, we extensively revised the manuscript. Below, we provide a detailed point-by-point response to all reviewer comments in blue font. All changes in the revised manuscript have been highlighted in yellow.

REVIEWER 2

Comment 1: *Lines 52--53. Please describe the mechanism by which ORF8 interferes with MHC-I presentation and the interferon type I signalling pathway. This statement is currently declarative. Adding 1-2 sentences describing the mechanism would significantly enhance the information contained in the introduction.*

Response 1: We agree that integrating these mechanistic details strengthens the Introduction. We have revised the text to outline the established roles of ORF8 in MHC-I downregulation and type I IFN pathway inhibition. We have added the following text to the Introduction: "*Multiple studies have demonstrated that ORF8 interferes with major histocompatibility complex class I (MHC-I) presentation by directly binding surface MHC-I molecules and targeting them for lysosomal and autophagic degradation [5]. Furthermore, ORF8 impairs type I interferon (IFN) antiviral responses through a dual mechanism: it directly antagonizes host innate sensing factors to prevent the nuclear translocation and phosphorylation of IRF3, while also dampening the downstream STAT1/STAT2 pathway to suppress interferon-stimulated gene (ISG) transcription [6–8], thereby reducing cytotoxic T cell recognition and promoting immune evasion.*" (Lines 52-59).

Comment 2: *Lines 66-67. This paragraph discusses the roles of miRNAs in immune regulation... It is also appropriate to mention here the results of a recent in silico study that conducted a systems analysis of microRNA regulatory networks in viral infections and identified microRNAs regulating individual stages of the immune response 10.3390/ijms262010100.*

Response 2: We thank the reviewer for highlighting this recent study. We have incorporated this reference into the text to better support our rationale for analysing microRNA networks. We have added the following sentence and citation: "*Recent in silico systems analyses have demonstrated the power of studying miRNA regulatory networks to understand the complex transcriptional and post-transcriptional control of the host immune response during viral infections, further underscoring the importance of investigating how viral proteins can manipulate these networks [ref: 10.3390/ijms262010100].*" (Lines 70-74).

Comment 3: *Line 97. Please justify the choice of the A549 cell line. Why was this particular line chosen and not, for example, primary respiratory epithelial cultures or the Calu-3 line, which is more physiologically relevant for SARS-CoV-2? Furthermore, was it directly proven that the cells actually express ORF8 at the protein level (Western blot or immunofluorescence)?*

Response 3: We agree that clarifying our model choice and confirming protein expression are critical points (also raised by Reviewer 1).

Regarding protein expression, we have verified ORF8 expression by Western blot in the transduced cells compared to controls (as shown in Author Response Figure 1 in our response to Reviewer 1, Major Point 2). Additionally, successful expression in this exact A549 lentiviral system were previously validated by confocal immunofluorescence in our earlier published study (López-Ayllón et al., Front. Immunol. 2023 [20]).

Furthermore, we have expanded Section 4.1 to justify the use of A549 cells based on their stability and suitability for high-throughput multi-omics analyses. "*A549 cells were selected as a well-established and highly tractable model for studying lung epithelial cell biology and host-pathogen interactions. Their high transduction efficiency and robust growth properties make them ideal for generating stable, homogeneous cell populations for controlled mechanistic and multi-omics studies.*" (Lines 532-535).

Comment 4: *Line 182. Two studies were selected for comparison with clinical data [42,43]. First, justify the selection of these two studies—why these two and not other available publications on miRNA profiles in COVID-19? Second, two studies are insufficient for a reliable comparison; consider including additional cohorts. Third, provide additional information on the clinical status of the patients in each study: were they hospitalized? Did they require intensive care? Did they survive the infection? What was the spectrum of disease severity? This information is critical for interpreting signature matches, as miRNA profiles can vary significantly depending on disease severity.*

Response 4: We thank the reviewer for this thorough critique. The studies by de Gonzalo-Calvo et al. and Farr et al. were selected because they are two of the most comprehensive and well-cited studies that performed global miRNA profiling in

hospitalized COVID-19 patients, making them ideal benchmarks for clinical miRNA signatures. We agree that two studies are insufficient for a definitive comparison. Our goal was to make a preliminary, hypothesis generating comparison. We have now rephrased our interpretation to clearly frame this as an exploratory observation. We also state that this comparison is limited by the small number of studies and the heterogeneity in patient cohorts and methodologies.

The comparison section (2.3) has been rephrased to emphasize the exploratory nature of the analysis and we have added the requested details on patient severity for both studies to provide context. The following details have been added: "*The study by de Gonzalo-Calvo et al. analysed hospitalized COVID-19 patients, including those with severe disease requiring mechanical ventilation [44]. The study by Farr et al. profiled a cohort of patients with a spectrum of disease severity, from mild to critical, also requiring hospitalization [45].*" (Lines 198-201).

Comment 5: *Discussion Section. The text does not discuss whether ORF8 influences miRNA biogenesis (Drosha, Dicer, DGCR8, Exportin-5). Given that ORF8 causes large-scale reorganization of the miRNA landscape, a discussion of possible mechanisms at the level of the miRNA biogenesis machinery would strengthen the study. Is this due to a direct interaction of ORF8 with components of microRNA biogenesis, or is the effect mediated by transcriptional changes?*

Response 5: This is an excellent and critical point. Our study identifies the phenotypic outcome (miRNA changes) but does not prove the mechanism by which ORF8 causes these changes. We have added a discussion on this topic.

We have added a new paragraph to the Discussion section: "*An important question raised by these findings is the precise mechanism by which ORF8 drives this widespread miRNA remodelling. This landscape alteration could occur through several non-mutually exclusive pathways. ORF8 may modulate the host transcriptional program, thereby altering the expression of primary miRNA transcripts or their key transcription factors. Alternatively, it could directly interact with or disrupt components of the core miRNA biogenesis machinery, such as the Microprocessor complex (Drosha/DGCR8), the nuclear export factor Exportin-5, or cytoplasmic Dicer. Finally, potential effects on mature miRNA stability cannot be ruled out, and future biochemical studies will be required to distinguish among these possibilities.*" (Lines 413-421)

Comment 6: *Limitations Section. The authors use ORF8 from the Wuhan-Hu-1 variant, but ORF8 is one of the most variable proteins in SARS-CoV-2 (including deletions, such as in the B.1.1.529/Omicron variant, and stop codon mutations). While the authors mention this as a direction for future research, a more detailed discussion of the extent to which the findings of this study apply to currently circulating variants in which ORF8 is functionally altered or missing deserves further consideration.*

Response 6: We thank the reviewer for highlighting the variability of ORF8 across SARS-CoV-2 lineages. We have expanded the limitations paragraph to acknowledge that our study used the Wuhan-Hu-1 ORF8 sequence and that our findings may not be uniformly applicable to variants with altered or absent ORF8.

We have added this limitation to the Discussion and noted that further studies using representative ORF8 variants are needed to determine the conservation of the observed miRNA regulatory effects: *"Finally, the extent to which ORF8 alone accounts for the miRNA changes observed in COVID-19 patients remains unknown, as other viral and host factors likely contribute. In addition, this study used the Wuhan-Hu-1 ORF8 sequence, whereas ORF8 is highly variable among SARS-CoV-2 lineages, including deletions and stop-codon mutations. Therefore, our findings may not be uniformly applicable to variants with altered or absent ORF8, and further studies using representative ORF8 variants will be required to determine the conservation of these miRNA regulatory effects".* (Lines 505-511).