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Peer Review File

SARS-CoV-2 infection of human pluripotent stem cell-derived vascular cells reveals smooth muscle cells as key mediators of vascular pathology during infection

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The purpose of this study was to determine the tropism of SARS-CoV-2 with regards to endothelial and perivascular cells using differentiated, hSPC-derived endothelial cells, pericytes, and SMCs. The authors demonstrated that endothelial cells are not productively infected with SARS-CoV-2, whereas both pericytes and SMCs were productively infected with SARS-CoV-2. The study further utilises RNA sequencing to compare transcriptomic changes between endothelial cells and SMCs during SARS-CoV-2 infection. Significant changes in gene expression were observed in SMCs, compared to endothelial cells. In addition, changes in gene expression were observed when endothelial cells were exposed to infected SMC media, compared to mock-infected controls. This suggests that endothelial cells are affected by factors secreted by adjacent SMCs infected with SARS-CoV-2, which may explain why endothelial cells are not productively infected, but switch to an active, inflammatory phenotype during COVID-19.

While the majority of the experiments within the manuscript are technically sound, some of the conclusions are not adequately supported by the results. There is no attempt to rescue any of the effects on endothelial cells by inhibiting the pathways that are identified. Furthermore, the finding that endothelial cells respond to adjacent cell infection is not novel and the manuscript lacks sufficient knowledge gain.

Specific issues
In the introduction, the authors state that SARS-CoV-2 infection been detected in vascular smooth muscle cells and pericytes. The authors do not mention that infection of adjacent epithelial cells in the lower respiratory tract, as well as recruited macrophages and monocytes could be large drivers of the SARS-CoV-2-induced endothelial dysfunction. By not mentioning this large body of work, the authors suggest that endothelial dysfunction during SARS-CoV-2 is only triggered by infection of vascular SMCs, which remains unclear.
Instead of performing a triple culture experiment with ECs, SMCs and pericytes, the authors culture all cell types separately and assess the effect of virus. It would be much more informative to utilise a triple culture system to truly model the autocrine and paracrine signals from these cell types. To what degree do all these cell types express ACE2? Why is CD31 expression induced in pericytes? In Fig. 1C, plaque assay data shows that SARS-CoV-2 preferentially infects perivascular cells rather than ECs. How might the virus disseminate from the respiratory tract to the vasculature?
Why did the authors choose to include pericytes, and state that SARS-CoV-2 preferentially infects them compared to endothelial cells, when from figure 2 onwards they only compare endothelial cells and SMCs?
In Fig. 2C, the authors state that changes in gene expression in endothelial cells live vs. heat-inactivated SARS-CoV-2 were negligible. However, Fig. 2A and C shows that EDIL3 is significantly downregulated in ECs exposed to live virus. What role EDIL3 could be playing in endothelial cells during SARS-CoV-2 infection?
What are the effects of culturing endothelial cells with SMC media alone (ie that has not been pre-conditioned)? As these cells are cultured in highly specialised media, it is possible that components within this media may lead to changes in endothelial cells.

Fig. 3 demonstrates that SMCs exposed to both live and heat inactivated (HI) SARS-CoV-2 resulted in upregulation of interferon alpha and interferon gamma innate immune response pathways. Since the authors have only examined cell responses at 48 hrs post-infection, do the authors propose that the SMCs are mounting a protective anti-viral response or proinflammatory response? It would be useful to assess whether these were upregulated at different timepoints earlier than 48 hrs, and perhaps at 72 hrs.

In Fig. 4, the authors aimed to investigate whether paracrine signalling from nearby-infected SMCs would impact endothelial cells. To achieve this, media from infected SMCs 48 hrs post-infection was added to endothelial cells before harvesting 48 hrs later. The experimental design is not robust enough to mimic paracrine signalling between endothelial cells and SMCs and should be rephrased as "the effect of SMC-secreted factors on endothelial cells".

In Fig. 5E, the authors utilise to brain endothelial cells. The introduction of SARS-CoV-2-induced brain complications such as intracerebral haemorrhage, which are relatively rare, seems outside the theme of the rest of the manuscript. These experiments only utilise a TEER reading and lack any mechanistic insight as to how altered TEER values may occur. While ZO-1 staining is shown in Supp Fig 6A, the magnification is not sufficient to draw conclusions and no quantification is performed. It is unclear why the authors did not investigate the effect of SMC-released factors on the permeability of hPSC-derived endothelial cells.

Fig. 6A shows representative imaging of tissue factor (TF) in infected SMCs. However, the authors did not include a control such as mock-infected SMCs, so it is unclear whether the TF staining is a result of infection, or unspecific staining.

In Suppl. Fig. 7B, the authors determined that treating SMCs with DMS reduced virus production. However, this was only determined using plaque assay and testing cell viability. The authors did not investigate whether treatment with DMS has any downstream effects on SMCs eg. expression of tissue factor, PAI-1, type I IFN, inflammatory signalling, cell death. Furthermore, does DMS have any off-target effects on endothelial cells? This leaves several questions about the effect of DMS as a potential therapeutic strategy.

Minor comments

In the methods, the manuscript does not describe how cells were infected eg. what MOI or PFU. It is only stated how the virus was propagated. The dose of virus must be listed and should also be represented on graphs where PFU is quantitated (eg Fig 1C).

In Fig. 1B, labelling should be made clearer to show which figures/graphs are representing hPSC-derived endothelial cells, pericytes or SMCs.

In Fig. 5A-B, the text size of genes in the plots should be increased.

In Fig. 5C-E, the authors should state whether this data came from 3 independent experiments, as there are 3 data points in Fig. 5C-D, but no data points in Fig. 5E

Reviewer #2 (Remarks to the Author):

Reviewers' comments to: NCOMMS-23-36245:

SARS-CoV-2 infection of human pluripotent stem cell-derived vascular cells reveals smooth muscle cells as key mediators of vascular pathology during infection, by Richards, Khalil, Mooney, Jaenisch et al.

Summary:
The authors present data investigating the role of vascular cell types (endothelial cells, pericytes and smooth muscle cells) derived from human pluripotent stem cells, in SARS-CoV-2 infection and related vascular pathologies, such as blood coagulation/thrombosis. The authors postulate that vascular smooth muscle cells represent a possible site of SARS-CoV-2 infection and propagation. As a result, paracrine signaling from infected vascular smooth muscle cells triggers the pathological and pro-coagulatory processes in endothelial cells. The authors apply an in vitro model of stem cell-derived vascular cells and virus infection, using mainly bulk RNA-seq transcriptome analysis as read out, complemented with immunostaining and protein ELISA assays.

The author's research is timely, since the specific mechanism of SARS-CoV-2 infection remain incompletely understood and controversial. Therefore, the data presented in the manuscript would be a valuable contribution to the scientific community. However, there are several conceptual and technical shortcomings that need to be addressed by the authors prior to a possible consideration for publication. In particular, the manuscript lacks specificity for the host/infected cell type and virus, as well as detail in the data analysis. The conclusions made by the authors about the distinct role of vascular smooth muscle cells in the propagation of SARS-CoV-2 infection and related pathology requires additional investigations, the use of appropriate controls, and mechanistic insights as detailed below.

Comments:
1. The authors differentiate human stem cells into endothelial cells, pericytes and smooth muscle cells. The delineation of endothelial cells from pericytes and smooth muscle cells is convincing, however, the demarcation of pericytes from smooth muscle cells remains confusing. For example, the expression of PDGFRA is normally attributed to fibroblasts and not pericytes (doi:10.1038/s41586-021-03549-5), NG2/CSPG4 as well as PDGFRB should be expressed by pericytes and smooth muscle cells. The data presented for validation of cell type differentiation in Figure 1B does show minimal comparison between the three differentiation protocols, but mainly the positivity of the selected cell type. Some discrepancy between mRNA-expression (middle panels) with staining (right panel) data are present e.g., pericytes have aSMA (ACTA2) and PECAM1 mRNA signal, whereas staining appears low (Fig 1 and S1). In the mRNA-expression panels for smooth muscle cells and pericytes, the authors show data for "SM22A" and "TAGLN", which are two synonyms for the same gene: TAGLN. The data looks nearly identical. Thus, one should be removed.

2. After the initial test for susceptibility of SARS-CoV-2 infection the authors continue to analyze smooth muscle cells, however the rationale behind this decision is not clear, since also pericytes can be infected (Fig 1C). According to the literature, in vivo pericytes are suggested to be a major site for SARS-CoV-2 infection in the vasculature e.g., doi:10.1093/cvr/cvaa078, doi: 10.3390/ijms222111622. Considering this background, a comparison of how pericytes and smooth muscle cells react to infection by SARS-CoV-2 and if there would be differences in the paracrine signaling to endothelial cells could improve the impact of the manuscript and provide specificity for certain host cell types. Further, the authors do not provide any mechanistic details to the observed tropism for smooth muscle cells and pericytes. The suggested host cell surface receptor for the SARS-CoV-2 spike protein is ACE2. How is the expression of ACE2 in the different stem cell-derived cell types which the authors investigate? What is the expression of related

molecules, such as TMPRSS2 or NRP1 that are suggested to participate in the cell-entry of SARS-CoV-2?

3. The authors postulate that infection of smooth muscle cells may be one of the initiating factors for the development of vascular pathologies, however, the authors do not provide any comparative data. Only the effect of conditioned medium from smooth muscle cell medium after infection was tested, but not compared to conditioned medium from any other cell type, for example pericytes (as already mentioned above). Neither the specificity of the reaction of smooth muscle cells to SARS-CoV-2 infection was tested. Further, the authors do not address the question how the response of smooth muscle cells to infection with SARS-CoV-2 differs from the response to infection by other viruses (for example other human corona viruses, or viruses that do not lead to similar vascular pathologies/coagulopathy) and if there would be a difference in the paracrine activation of endothelial cells? Is the observed reaction of smooth muscle cells a common response to (any) virus infection? Such comparative analysis and deciphering of the mechanistic details that are specific for SARS-CoV-2 infection could increase the impact of the manuscript.

4. The main analysis method for the response of cells to SARS-CoV-2 infection is bulk RNA-seq. The authors mainly rely on pathway analysis, which is an appropriate analysis to get an overview and condensed view of such complex datasets. Nevertheless, the data holds the potential to also investigate at a more detailed level, such as single genes or groups of genes. The authors investigate tissue factor (gene: F3) expression and activity, as well as PAI-1 (gene: SERPINE1) in more detail, but the link to their own transcriptome analysis becomes not clear. Further, both molecules are well known players in coagulation and inflammation, hence novelty remains limited. SERPINE1 appears to be upregulated by conditioned medium from both SARS-CoV-2 infected smooth muscle cells and smooth muscle cells exposed to heat inactivated SARS-CoV-2 (Fig 5A,B), which would suggest that SERPINE1 expression is a general response and likely not SARS-CoV-2 infection-specific. The authors' approach has the potential to shed light to the complex mechanisms of SARS-CoV-2 related pathologies and to reveal new targets for potential therapeutic interventions, however comparative studies and more detailed analysis are warranted to improve the impact of the manuscript.

5. Overall, the manuscript text and especially the figure captions lack detail. For example, the authors should give the specific PDGF ligand(s) used, -A, -B, -C, or -D, as well as which PDGF-receptor was analyzed (for example for the IF staining in Fig 1). The figure captions do not contain sufficient information to understand the figures and what data is presented. The understanding of the figures would highly benefit from more detailed explanations within the figure-panels and the related caption.

Reviewer #3 (Remarks to the Author):

In this research, Richards et al utilized hPSC-derived smooth muscle cells, endothelial cells, and pericytes to model the vascular complications that arise due to SARS-CoV-2 infection. The team reported that smooth muscle cells (SMCs) are notably susceptible to SARS-CoV-2 infection. Using RNA-seq, they highlighted the prominent molecular changes occurring within these infected cells, encompassing an

amplified inflammatory response and the heightened expression of critical players in the coagulation cascade. Additionally, the researchers demonstrated that human endothelial cells, when exposed to the secretome of infected SMCs, generate hemostatic factors that potentially contribute to vascular dysfunction, indicating a mechanism of vascular damage that operates independently of direct infection by SARS-CoV-2. Overall, the manuscript is well-organized. Below are some issues that need to be addressed.

Major concerns:
1. To reinforce the validation of cell identities, incorporating additional markers would be beneficial. For instance, is there an upregulation of ETV2 in the endothelial cells produced using the current protocol? Including primary human cells as controls in the qRT-PCR analysis presented in Figure 1B would aid in a more comprehensive interpretation of the data.
2. For Figures 4B and 4C, a comparison of gene expression between ECs exposed to live SARS-CoV-2 SMC CM and those exposed to HI SARS-CoV-2 SMC CM would be insightful.

Minor issues:
1. It is essential to include at least three biological replicates for the experiments depicted in Figures 1C, S1A, and S1B.
2. The scale bar appears to be missing in Fig. 1B.
3. The gene names in the volcano plots featured in Figures 2A, 2B, 2C, 2D, 4B, 4C, S2, S4, and S5 are currently too small to read clearly, adjustment for better visibility is recommended.

Point-by-point reviewer response

Reviewer #1 (Remarks to the Author):

Specific issues:

In the introduction, the authors state that SARS-CoV-2 infection been detected in vascular smooth muscle cells and pericytes. The authors do not mention that infection of adjacent epithelial cells in the lower respiratory tract, as well as recruited macrophages and monocytes could be large drivers of the SARS-CoV-2-induced endothelial dysfunction. By not mentioning this large body of work, the authors suggest that endothelial dysfunction during SARS-CoV-2 is only triggered by infection of vascular SMCs, which remains unclear.

We acknowledge that our initial manuscript did not adequately discuss the role of adjacent epithelial cells, recruited macrophages, and monocytes in SARS-CoV-2-induced endothelial dysfunction. In response, we have revised the introduction to include this broader context and cite relevant studies (Varga et al., 2020; Ackermann et al., 2020; Blanco-Melo et al., 2020; Merad and Martin, 2020). This will provide a more comprehensive overview of the mechanisms involved in endothelial dysfunction during SARS-CoV-2 infection.

Instead of performing a triple culture experiment with ECs, SMCs and pericytes, the authors culture all cell types separately and assess the effect of virus. It would be much more informative to utilise a triple culture system to truly model the autocrine and paracrine signals from these cell types.

We agree that a triple culture system would be informative and appreciate the reviewer's insights. However, due to the challenges of working in a BSL3 facility and the complexity of triple co-cultures, we focused on separate cultures of ECs, SMCs, and pericytes. This allowed us to isolate and analyze the specific effects of SARS-CoV-2 on each cell type. While a triple culture could offer further insights into the complex physiology of vascular complications in COVID-19, our approach has the advantage of identifying the individual contributions of distinct populations of mural cells to vascular pathology induced by SARS-CoV-2. We have added the reviewer's comments and suggestions to the discussion as future investigation warranted by our results.

To what degree do all these cell types express ACE2?

We have now added in the expression levels of ACE2 in **Figure 2C** from RNA sequencing of the starting cell populations. The data indicate the highest expression in SMCs, followed by PCs, and then ECs, which reflects our infectivity data. Additionally, the serine protease TMPRSS2, which is responsible for cleavage of the SARS-CoV-2 spike protein to facilitate entry following receptor binding, was expressed in both mural cell populations. Further, we now show NRP1 expression is highest in ECs and lower in PCs. This result was perhaps not surprising given the known role of NRP1 in endothelial angiogenesis[1]. NRP1 can also bind to the SARS-CoV-2 Spike protein. Critically, previously studies have shown that expression of NRP1 alone is not sufficient to make cells susceptible to SARS-CoV-2 infection[2].

Why is CD31 expression induced in pericytes?

CD31 is crucial for cell adhesion and transmigration of vascular cells and previous studies have shown that mural cells, including pericytes, express CD31 during vascular development as well as under pathological conditions[3, 4],likely as part of their essential role in vascular stability and remodeling particularly in inflammation and vascular pathology. Furthermore, embryonic stem cell-derived pericytes express CD31, indicating it as a feature of

pericyte differentiation and activation[5]. Notably, the levels of CD31 expression in our stem cell derived pericytes were significantly lower than in our stem cell derived ECs **(Figure 1B).**

In Fig. 1C, plaque assay data shows that SARS-CoV-2 preferentially infects perivascular cells rather than ECs. How might the virus disseminate from the respiratory tract to the vasculature?

We appreciate the reviewer's response and agree that this is a highly relevant point regarding understanding the role mural cell tropism contributes to COVID19 vascular complications and was not included in the initial manuscript. The dissemination of SARS-CoV-2 from the respiratory tract to the vasculature likely involves several mechanisms. SARS-CoV-2 is known to cause a systemic viral infection, with the virus detected in the bloodstream and in multiple tissues beyond the respiratory tract[6, 7]. One of the hallmarks of COVID-19 vasculopathy is the disruption of endothelial integrity[8]. Our data shows that exposure of ECs to SARS-CoV-2 activates reactive oxygen signaling in the absence of productive EC infection. Excessive ROS signaling has been shown to contribute to a reduction in EC barrier integrity[9]. This initial reduction in barrier function may provide an access point for SARS-CoV-2 to infect the perivascular cells surrounding ECs. The ability of the virus to reach perivascular cells in vivo is supported by data showing pericyte infection[8, 10]. We have added text to the discussion to reflect the existing data to suggest our *in vitro* mechanistic findings are plausibly supported by *in vivo* observations

Why did the authors choose to include pericytes, and state that SARS-CoV-2 preferentially infects them compared to endothelial cells, when from figure 2 onwards they only compare endothelial cells and SMCs?

The reviewer raises a good point that characterizing both mural cell populations would add to the knowledge of vascular complications during SARS-CoV-2 infection. While our primary focus was on endothelial cells and smooth muscle cells, we acknowledge the importance of pericytes in this context. We have now included corresponding data regarding the infection of pericytes throughout the manuscript to provide a more comprehensive analysis of the vascular pathology induced by SARS-CoV-2 (Figure 3C, Figure S10, Figure S12). This addition aims to enhance the overall understanding of the roles of different mural cells in the infection process.

In Fig. 2C, the authors state that changes in gene expression in endothelial cells live vs. heat-inactivated SARS-CoV-2 were negligible. However, Fig. 2A and C shows that EDIL3 is significantly downregulated in ECs exposed to live virus. What role EDIL3 could be playing in endothelial cells during SARS-CoV-2 infection?

We acknowledge this oversight regarding EDIL3 was absent from our original analysis. This is particularly relevant as EDIL3, or EGF-like repeats and discoidin I-like domains 3, plays a significant role in endothelial cell adhesion and angiogenesis and its downregulation in endothelial cells exposed to live SARS-CoV-2 might be expected to impair these functions via weakened cell-cell adhesion, exacerbating the vascular complications associated with COVID-19 (Wautier & Wautier, 2013). These interpretations are importantly supported by our new data showing increased FITC-dextran permeability in ECs exposed to live SARS-CoV-2 **(Figure 4C)**. We have revised our discussion in the manuscript to include discussion of these data with respect to the observed downregulation of EDIL3 below to highlight the gene's role in endothelial cells and its potential impact during SARS-CoV-2 infection

What are the effects of culturing endothelial cells with SMC media alone (ie that has not been pre-

conditioned)? As these cells are cultured in highly specialised media, it is possible that components within this media may lead to changes in endothelial cells.

We thank the reviewer for this comment. For clarification the media that was conditioned by infected SMCs is "infection media" which is EC media lacking VEGF. We have added this clarification to the manuscript. In addition, we have added additional data and analysis to characterize gene expression changes in ECs exposed to infection media compared to control ECs in their standard maintenance media. While we observed about 150 genes with significant changes in expression, we do not observe changes in the inflammatory or coagulation pathways that we observe upregulated with infection media conditioned by infected SMCs (Figure S8).

Fig. 3 demonstrates that SMCs exposed to both live and heat inactivated (HI) SARS-CoV-2 resulted in upregulation of interferon alpha and interferon gamma innate immune response pathways. Since the authors have only examined cell responses at 48 hrs post-infection, do the authors propose that the SMCs are mounting a protective anti-viral response or proinflammatory response? It would be useful to assess whether these were upregulated at different timepoints earlier than 48 hrs, and perhaps at 72 hrs.

We thank the reviewer for their comment. We have included additional data on the transcriptional response of SMCs at 72 hours post infection **(Figure S4C)**. Our results show that SARS-CoV-2 infection results in a sustained activation of inflammatory signaling pathways with robust activation of IFNα and IFNγ signaling at 72 hours post-infection.

In Fig. 4, the authors aimed to investigate whether paracrine signalling from nearby-infected SMCs would impact endothelial cells. To achieve this, media from infected SMCs 48 hrs post-infection was added to endothelial cells before harvesting 48 hrs later. The experimental design is not robust enough to mimic paracrine signalling between endothelial cells and SMCs and should be rephrased as "the effect of SMC-secreted factors on endothelial cells".

We appreciate the distinction the reviewer raises and have corrected references to our model mimicking paracrine signaling between ECs and SMCs and replaced with the suggested wording.

In Fig. 5E, the authors utilise to brain endothelial cells. The introduction of SARS-CoV-2-induced brain complications such as intracerebral haemorrhage, which are relatively rare, seems outside the theme of the rest of the manuscript. These experiments only utilise a TEER reading and lack any mechanistic insight as to how altered TEER values may occur. While ZO-1 staining is shown in Supp Fig 6A, the magnification is not sufficient to draw conclusions and no quantification is performed. It is unclear why the authors did not investigate the effect of SMC-released factors on the permeability of hPSC-derived endothelial cells.

We thank the reviewer for these comments. We have added additional data investigating the impact of SMC secreted factors on the permeability of ECs **(Figure 6E)**. While we agree that intracranial hemorrhage is a rare complication of COVID-19, recent data has demonstrated that breakdown of the BBB may contribute to the neurological complications associated with long COVID[11]. Our data suggests that factors released from infected mural cells could contribute to this breakdown. We have edited our manuscript to include a discussion of this recent manuscript. For clarification, our goal with showing ZO-1 staining was not to propose that changes in ZO-1 were responsible for loss in barrier function, but rather to show a known marker of BMECs, we have added this clarification to the text. While investigation of the molecular mechanism behind the reduction of barrier

function is outside the scope of this manuscript, we have added a discussion of the possible link to the reduction in EDIL expression.

Fig. 6A shows representative imaging of tissue factor (TF) in infected SMCs. However, the authors did not include a control such as mock-infected SMCs, so it is unclear whether the TF staining is a result of infection, or unspecific staining.

We thank the reviewer for their comment. In the updated manuscript we show TF staining in mock infected cells in **Figure 7B**.

In Suppl. Fig. 7B, the authors determined that treating SMCs with DMS reduced virus production. However, this was only determined using plaque assay and testing cell viability. The authors did not investigate whether treatment with DMS has any downstream effects on SMCs eg. expression of tissue factor, PAI-1, type I IFN, inflammatory signalling, cell death. Furthermore, does DMS have any off-target effects on endothelial cells? This leaves several questions about the effect of DMS as a potential therapeutic strategy.

We observed the treatment with DMS increases the activation of innate immune signaling in SMCs infected with SARS-CoV-2, which may explain the observe reduced viral replication in DMS treated cells **(Figure S16B**). However, as our new data show activation of inflammatory signaling in SMCs may directly contribute to their effect on EC coagulation cascades (**Figure S13A)** it is unclear if DMS treatment during SARS-CoV-2 infection would reduce the severity of the vascular complications**.** Our goal with including the DMS data was to show the potential of our hPSC-derived vascular cells to be used as a platform for testing potential antiviral drugs and not necessarily as a specific treatment for the vascular complications associated with infection. A full investigation of all potential off target effects of DMS on vascular cells will be critical for potential future studies on this drug as a potential anti-viral treatment. As preliminary characterization we have included data on the viability of ECs treated with doses of DMS that we observed reduced viral infection of SMCs while not impacting SMC viability **(Figure S16C).**

Minor comments:
In the methods, the manuscript does not describe how cells were infected eg. what MOI or PFU. It is only stated how the virus was propagated. The dose of virus must be listed and should also be represented on graphs where PFU is quantitated (eg Fig 1C).

We thank the reviewer for their comment. We have added the infectious dose used to all figure legends as well as to the methods section.

In Fig. 1B, labelling should be made clearer to show which figures/graphs are representing hPSC-derived endothelial cells, pericytes or SMCs.

We appreciate the reviewer's suggestion; we have added additional labeling to **Figure 1C-E**.

In Fig. 5A-B, the text size of genes in the plots should be increased.

We appreciate the reviewer's comment. Unfortunately, by increasing the text size in these volcano plots the gene names overlap and become difficult to read. We have increased the text size on all gene set specific volcano plots. We can reduce the number of gene labeled if the reviewer feels that will improve the clarity of the figure.

In Fig. 5C-E, the authors should state whether this data came from 3 independent experiments, as there are 3 data points in Fig. 5C-D, but no data points in Fig. 5E.

We thank the reviewer for the comment. We have added this clarification to the text.

Reviewer #2 (Remarks to the Author):

Comments:

1. The authors differentiate human stem cells into endothelial cells, pericytes and smooth muscle cells. The delineation of endothelial cells from pericytes and smooth muscle cells is convincing, however, the demarcation of pericytes from smooth muscle cells remains confusing. For example, the expression of PDGFRA is normally attributed to fibroblasts and not pericytes (doi:10.1038/s41586-021-03549-5), NG2/CSPG4 as well as PDGFRB should be expressed by pericytes and smooth muscle cells. The data presented for validation of cell type differentiation in Figure 1B does show minimal comparison between the three differentiation protocols, but mainly the positivity of the selected cell type. Some discrepancy between mRNA-expression (middle panels) with staining (right panel) data are present e.g., pericytes have aSMA (ACTA2) and PECAM1 mRNA signal, whereas staining appears low (Fig 1 and S1). In the mRNA-expression panels for smooth muscle cells and pericytes, the authors show data for "SM22A" and "TAGLN", which are two synonyms for the same gene: TAGLN. The data looks nearly identical. Thus, one should be removed.

We agree with the reviewer that the initial characterization of the hPSC-derived endothelial and mural cell populations was insufficient. As such, we have added substantial additional characterization and comparison of these cells to each other, hPSC-ECs and hPSCs, as well as primary vascular cells using bulk RNA sequencing **(Fig. 1B-C)**. These data showing gene expression of canonical genes from the original qPCR data with duplicate labels had been remade with the table of reads in **Figures 1B and S1J** and now show each canonical gene expression value in the context of all populations for comparison to each other in addition to the starting hPSCs **(Fig. 1B)** and to primary cells **(Supp Fig. S1J)** with duplicate genes removed**.** We have also further strengthened the hPSC-derived vascular cell characterizations using this sequencing data with PCA comparisons of these cell populations to each other and to primary counterparts **(Fig. 1C)**. These results show that the stem cell-derived ECs group with HUVECs, while the stem cell-derived SMCs and PCs cluster with bronchial SMCs and primary PCs. We then further resolved the mural cell populations in separate PCA analyses without hPSCs and ECs and Euclidean distance comparisons and showed that hPSC-SMCs are closer to BSMCs and hPSC-PCs to primary PCs **(Figs. 1C and S1G-H)**.

We also acknowledge the reviewer's point regarding the canonical expression of PDGFRA in fibroblasts, and specifically myofibroblasts; however, we argue the role of PDGFRA in pericytes vs. fibroblasts (myo) is less clear for this setting given the established varied roles of PDGFRA in hPSC and embryonic/developmental cells[12, 13], artifactual in vitro expression, and its expression in both specialized vascular niches[13, 14]and disease states[15-17]. More specifically, PDGFRA in developmental and embryonic cells is considered a pan-mesenchymal marker[12] as well as a marker of partial fibroblast-like transitions in *in vitro* expanded primary mural cells[18] due to possible artifacts like high mechanical stiffness[19] or aberrations in TGFb1 signaling[20] that can be associated with *in vitro* cultures[21, 22], for which we observe potential evidence of here in our sequencing data from expanded primary-derived brain vascular PCs and bronchial SMCs **(Supp Fig. S1J)**. Additionally, there still exists debate about what definitive mural cell markers are, particularly in the case of hPSC-derived or early/non-contractile pericyte contexts of what is perivascular mesenchyme vs. a "pericyte." Notably, perivascular cells have shown expression of PDGFRA in subpopulations in adipose tissue[14], diverging developmental perivascular cells that contribute to embryonic hematopoiesis[13], and in dysregulated PCs in disease states[17].

Nevertheless, we acknowledge the importance of better characterizing the phenotypes of our mural cell populations and have added substantial new genetic characterization and comparison to primary mural cells using bulk RNA sequencing **(Figs. 1B-C and S1J).** These new data again show PDGFRB and CSPG4

expression in both smooth muscle cells (SMCs) and pericytes (PCs), and higher levels of PDGFRA expression in PCs. These results align with our original PCR results and previous findings, highlighting PDGFRB and CSPG4 as markers for both cell types with higher gene expression levels for ACTA2, CNN1, and MYH11 in SMCs, well-established markers for smooth muscle cells, reflecting their contractile function and suggestive of phenotypic characteristics in larger vessels[23]. While the presence of high PDGFRB with CSPG4 with low ACTA2 and CNN1, represent a canonical definition of PCs[24] we now also show ANGPT1 expression in PCs, a perivascular- specific mural marker involved in maintaining vascular stability via interaction with TIE2 receptors on ECs[25, 26], that more strongly distinguishes these cells from PDGFRα-expressing fibroblasts[25, 26].

Lastly, to further demonstrate the mural potential of these two cell populations, we have provided new microfluidic functional data showing that both cell populations can support robust microvascular network (MVN) formation with hPSC-derived ECs and that PCs and SMCs closely associate with the networks **(Fig. S1I)**. We have added all these new data and a discussion of their results to the manuscript, as well as specific comments regarding limitations of precisely identifying what in vivo counterpart of perivascular cells we can claim these cells to be. We appreciate the reviewer's critique and the opportunity to strengthen our characterization data of the vascular cells derived from our defined protocol.

2. After the initial test for susceptibility of SARS-CoV-2 infection the authors continue to analyze smooth muscle cells, however the rationale behind this decision is not clear, since also pericytes can be infected (Fig 1C). According to the literature, in vivo pericytes are suggested to be a major site for SARS-CoV-2 infection in the vasculature e.g., doi:10.1093/cvr/cvaa078, doi: 10.3390/ijms222111622. Considering this background, a comparison of how pericytes and smooth muscle cells react to infection by SARS-CoV-2 and if there would be differences in the paracrine signaling to endothelial cells could improve the impact of the manuscript and provide specificity for certain host cell types. Further, the authors do not provide any mechanistic details to the observed tropism for smooth muscle cells and pericytes. The suggested host cell surface receptor for the SARS-CoV-2 spike protein is ACE2. How is the expression of ACE2 in the different stem cell-derived cell types which the authors investigate? What is the expression of related molecules, such as TMPRSS2 or NRP1 that are suggested to participate in the cell-entry of SARS-CoV-2?

We thank the reviewer for these comments and agree that it was an oversight to not include additional follow up on the response of pericytes to SARS-CoV-2 infection. We have added in additional data showing the transcriptional response of pericytes to SARS-CoV-2 infection **(Fig.3C)**. Our results show that although pericytes are susceptible to SARS-CoV-2 infection, the response to infection differs from what we observed in SMCs following infection. Notably, we do not observe induction of the inflammatory pathways that were induced in infected SMCs. We also have now included data on the relative expression levels of ACE2, TMPRSS2 and NRP1 in SMCs, PCs, and ECs (**Fig. 2C).** Our results show that SMCs express the highest levels of ACE2 and TMPRSS2. PCs express variable levels of ACE2 and intermediate levels of TMPRSS2, where as ECs had very low levels of both ACE2 and TMPRSS2, which is consistent with data on primary EC ACE2 expression[27]. The data parallels what we observed with our infectivity studies (Fig 2A). Further, we now show NRP1 expression is highest in ECs and lower in PCs. This result was perhaps not surprising given the known role of NRP1 in endothelial angiogenesis[1]. NRP1 can also bind to the SARS-CoV-2 Spike protein. Notably, previously studies have shown that expression of NRP1 alone is not sufficient to make cells susceptible to SARS-CoV-2 infection[2]

3. The authors postulate that infection of smooth muscle cells may be one of the initiating factors for the development of vascular pathologies, however, the authors do not provide any comparative data. Only the effect of conditioned medium from smooth muscle cell medium after infection was tested, but

not compared to conditioned medium from any other cell type, for example pericytes (as already mentioned above). Neither the specificity of the reaction of smooth muscle cells to SARS-CoV-2 infection was tested. Further, the authors do not address the question how the response of smooth muscle cells to infection with SARS-CoV-2 differs from the response to infection by other viruses (for example other human corona viruses, or viruses that do not lead to similar vascular pathologies/coagulopathy) and if there would be a difference in the paracrine activation of endothelial cells? Is the observed reaction of smooth muscle cells a common response to (any) virus infection? Such comparative analysis and deciphering of the mechanistic details that are specific for SARS-CoV-2 infection could increase the impact of the manuscript.

We agree with the reviewer's comment and acknowledge that the novelty of our finding with respect to the impact of SMC conditioned media on ECs is strengthened by comparing the effect to ECs treated with media conditioned by another cell type. In the revised manuscript we now include data on the impact of media conditioned by SARS-CoV-2 infected pericytes **(Fig. S10 and Fig. S12)**. Treatment of ECs with infected pericyte conditioned media resulted in induction of inflammatory signaling, however, there was little overlap in the specific genes that were strongly induced **(Table 1).** The pericytes exhibit similar but more muted effects than SMCs, which corresponds with the additional analysis of ACE2 expression shown in response to comment 2 from this reviewer. Notably, pericytes show lower levels of SERPINE1 (log2FC=3.53 vs. 0.37 in SMCs vs. PCs) In addition, treatment of ECs with media conditioned by infected pericytes did not result in increased release of pro-clotting factors vWF and SERPINE1.

In response to the reviewer's request, we also conducted a comparative bulk RNA sequencing study with another human coronavirus in SMCs **(Fig S9)**. We selected SARS-CoV-2 variant BA5.1 (Omicron), a strain with enhanced virulence in humans but a marked reduction or absence of vasculopathy observed in human patients. We corroborated the new RNA sequencing analysis with repeat functional analysis of SERPINE1 and VWF production in response to BA5.1 in SMCs (absent of significant differences) **(Fig S11)** Our findings show no upregulation of the coagulation cascade or SERPINE1, suggesting the specificity of the original strain of SARS-CoV-2 in inducing these effects in SMCs.

These data, combined with the original observations of HI particles eliciting a strong inflammatory response in the absence of infection in SMCs, a lack of coagulation and SERPINE1 upregulation in BA5.1 infection in SMCs, and the muted transcriptional responses and non-observed functional responses in PCs, all suggest a unique specificity of the early 2020 strain of SARS-CoV-2 to impact SMCs. Our hypothesis is that this interaction generates an inflammatory response in exposed ECs that triggers hallmark pathways known to influence the vasculopathies observed in early SARS-CoV-2 human patients[28, 29].

4. The main analysis method for the response of cells to SARS-CoV-2 infection is bulk RNA-seq. The authors mainly rely on pathway analysis, which is an appropriate analysis to get an overview and condensed view of such complex datasets. Nevertheless, the data holds the potential to also investigate at a more detailed level, such as single genes or groups of genes. The authors investigate tissue factor (gene: F3) expression and activity, as well as PAI-1 (gene: SERPINE1) in more detail, but the link to their own transcriptome analysis becomes not clear. Further, both molecules are well known players in coagulation and inflammation, hence novelty remains limited. SERPINE1 appears to be upregulated by conditioned medium from both SARS-CoV-2 infected smooth muscle cells and smooth muscle cells exposed to heat inactivated SARS-CoV-2 (Fig 5A,B), which would suggest that SERPINE1 expression is a general response and likely not SARS-CoV-2 infection-specific. The authors' approach has the potential to shed light to the complex mechanisms of SARS-CoV-2 related pathologies and to

reveal new targets for potential therapeutic interventions, however comparative studies and more detailed analysis are warranted to improve the impact of the manuscript.

We acknowledge the reviewer's critique regarding the need for comparative data and specificity of the smooth muscle cells' (SMCs) response to SARS-CoV-2 infection. To address these concerns, we have expanded our analyses to include comparative data from infection control media, other coronaviruses, and pericytes, as well as additional functional data with cytokine stimulation.

In response to the reviewer's comment, we investigated if direct activation with the inflammatory cytokines IFN-α and IFN-γ could result in the release of factors that promote coagulation signaling in ECs. We preformed these studies in both SMCs and PCs as our data showed that specifically infection of SMCs caused upregulation in release of vWF and SERPINE1 from ECs. We observed slight upregulation of vWF and SERPINE1 in ECs exposed to media from SMCs treated with IFN-α but not IFN-γ, and no response in ECs exposed to media from inflammatory cytokine-treated PCs. Notably, while these results point to inflammatory signaling in SMCs being a factor in the activation of coagulation cascades in nearby ECs, the effects overall were minor compared to the original SMC infection exposure experiment suggesting that infection may either produce a stronger or more prolonged activation of inflammatory signaling or there are additional factors activated outside of IFN-α or IFN-γ gamma signaling which contribute to induction of coagulation signaling in nearby ECs.

We thank the reviewer for their critique and suggestions, in which we agree that the now more comprehensive and comparative analyses strengthen the findings in our manuscript.

5. Overall, the manuscript text and especially the figure captions lack detail. For example, the authors should give the specific PDGF ligand(s) used, -A, -B, -C, or -D, as well as which PDGF-receptor was analyzed (for example for the IF staining in Fig 1). The figure captions do not contain sufficient information to understand the figures and what data is presented. The understanding of the figures would highly benefit from more detailed explanations within the figure-panels and the related caption.

We thank the reviewer for their comment. We have added clarification of the specific PDGF ligand used for the differentiations (PDGFbb). In addition, we have added clarification of the receptor that was detected in IF staining and flow cytometry in **Figure 1**.

Reviewer #3 (Remarks to the Author):

In this research, Richards et al utilized hPSC-derived smooth muscle cells, endothelial cells, and pericytes to model the vascular complications that arise due to SARS-CoV-2 infection. The team reported that smooth muscle cells (SMCs) are notably susceptible to SARS-CoV-2 infection. Using RNA-seq, they highlighted the prominent molecular changes occurring within these infected cells, encompassing an amplified inflammatory response and the heightened expression of critical players in the coagulation cascade. Additionally, the researchers demonstrated that human endothelial cells, when exposed to the secretome of infected SMCs, generate hemostatic factors that potentially contribute to vascular dysfunction, indicating a mechanism of vascular damage that operates independently of direct infection by SARS-CoV-2. Overall, the manuscript is well-organized. Below are some issues that need to be addressed.

Major concerns:

1. To reinforce the validation of cell identities, incorporating additional markers would be beneficial. For instance, is there an upregulation of ETV2 in the endothelial cells produced using the current protocol? Including primary human cells as controls in the qRT-PCR analysis presented in Figure 1B would aid in a more comprehensive interpretation of the data.

We agree with the reviewer's comment and have added in additional bulk RNA sequencing analysis directly comparing our hPSC-derived ECs, SMCs, and PCs to primary ECs (HUVECs), primary PCs, and primary bronchial smooth muscle cells (BSMCs) **(Figs. 1C and S1G-J)**. We also, per the reviewer's request show ETV2 expression in our hPSC-derived ECs **(Fig. S1D)**, which indicate higher levels of expression relative to HUVECs and hPSCs, indicating upregulation as suspected

2. For Figures 4B and 4C, a comparison of gene expression between ECs exposed to live SARS-CoV-2 SMC CM and those exposed to HI SARS-CoV-2 SMC CM would be insightful.

We thank the reviewer for their comment. A comparison of gene expression changes between ECs exposed to live SARS-CoV-2 SMC CM and those exposed to HI SARS-CoV-2 SMC CM is included in **Figure S7**. Specific changes in genes belonging to the IFN-α and IFN-γ response genes sets are shown in **Figure S7B**.

Minor issues:

1. It is essential to include at least three biological replicates for the experiments depicted in Figures 1C, S1A, and S1B.

In combination with addressing several comments from reviewer 2 regarding better characterization and identification of our hPSC-derived vascular populations we have replaced the original qPCR of hallmark genes in 1C with triplicate replicates of bulk RNA seq analysis and analyses in the new **Figures. 1B-C and S1J**. As additionally requested, we have added replicate differentiation flow cytometry data to the **Figures S1A and S1B** showing the effects of the different inhibitors and coating substrates on EC differentiation efficiency.

2. The scale bar appears to be missing in Fig. 1B.

We thank the reviewer for this comment. We have added additional scale bars to the figure which is now **Figure 1C-E**.

3. The gene names in the volcano plots featured in Figures 2A, 2B, 2C, 2D, 4B, 4C, S2, S4, and S5 are currently too small to read clearly, adjustment for better visibility is recommended.

We appreciate the reviewer's comment. Unfortunately, by increasing the text size in these volcano plots the gene names overlap and become difficult to read. We have increased the text size on all gene set specific volcano plots. We can reduce the number of gene labeled if the reviewer feels that will improve the clarity of the figure.

1. Fantin, A. *et al.* NRP1 acts cell autonomously in endothelium to promote tip cell function during sprouting angiogenesis. *Blood* **121**, 2352-2362 (2013).
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29. Xu, S.W., Ilyas, I. & Weng, J.P. Endothelial dysfunction in COVID-19: an overview of evidence, biomarkers, mechanisms and potential therapies. *Acta Pharmacol Sin* **44**, 695-709 (2023).

REVIEWERS' COMMENTS

Reviewer #2 (Remarks to the Author):

Reviewer comments to NCOMMS-23-36245A;
SARS-CoV-2 infection of human pluripotent stem cell-derived vascular cells reveals smooth muscle cells as key mediators of vascular pathology during infection,
by Richards A., Khalil A., et al.

In the revised version of their manuscript, the authors have added a substantial body of work to strengthen the data and claims of the manuscript. Thereby, the authors have substantially strengthened the manuscript and have addressed all my comments. Especially, the comparative experiments add valuable information about the specific effect of active SARS-CoV-2 infection of SMC, and the paracrine effect of this infection on endothelial cells. The authors may consider highlighting the results obtained from thecomparison of SMC infected with active SARS-CoV-2 compared to heat-inactivated SARS-CoV-2 (now in Supplemental Figure 4A, B, and Supplemental Figure S7A, B), since these analyses may identify the most relevant gene expression responses following active SARS-CoV-2 infection. Further, there are some minor points to be addressed by the authors.

Minor:
General for many volcano plots: The gene name labels in the volcano plots are often too small to read and overlap. The authors may consider reducing the number of labeled genes together with a larger type-set and instead presenting the list of differentially expressed genes in an accompanying supplementary table.

Figure 1: The authors use different denominations for PDGFRa/b (in the figure panels) or PDGFR-A/-B in the figure legend.

Figure 2: The authors write in the legend 'Titers of EC 0 h.p.i. samples were below the limit of detection…', but in the barplot (A) there are values shown for the 0 h.p.i. time point. Maybe the authors refer to the 48 h.p.i. time point?

Figure 4: The measurement of the CellROX green signal in cell cultures of ECs appears to come from one single experiment (at least no independent repetitions are mentioned) and the measurement of single cells in the culture (five cells from three fields; 15 data points) appear to be the basis for the statistical calculations (A). Instead, the authors should perform statistics on at least three independent experiments, using the mean signal from each experiment calculated as shown now in A.

Figure 5: In the Figure legend the authors write '(C)/(E) … compared to ECs exposed to control EC (Control)'. This is confusing, and not clear from the manuscript text or methods. What does ECs exposed to control EC refer to?

Supplemental Figure 3: From the volcano plot, only few genes appear to be differentially expressed, however the GSEA analysis in A left panel suggests a high number of differentially expressed genes. What

is the input to the GSEA analysis and how does it differ from the data presented in the volcano plot?

Supplemental Figure 11: There is a discrepancy between the data presented in the figure and the figure legend. Four datapoints are shown in the quantification of SERPINE1, whereas the legend says 'Three independent experiments…'
Further, why is the bar for Mock SMC exposed not at 1.0 for the vWF quantification (left panel)?

Supplemental Figure 13: There is a discrepancy between the data presented in the figure and the figure legend. Five datapoints are shown in the quantifications of SERPINE1 (A and B), whereas the legend says 'Three independent experiments…'

Reviewer #3 (Remarks to the Author):

All previous concerns have been addressed.

Reviewer #4 (Remarks to the Author):

In the manuscript, the authors use a stem cell derived model to determine the effects of SARS-CoV-2 on vascular pathology. While the authors have addressed the main concerns outlined by the original review making the reported data robust, there is still concerns on linking the findings to the molecular mechanism between paracrine action of SARS-CoV-2 infected SMC on EC. Below are some points to consider:
• Authors confirm, through plague assay and dsRNA measurements, SARS-CoV-2 infection in SMC and PA but not in EC. However, EC exposed to live virus does show changes to gene expression related to ROS. To be sure there is no replication of virus in EC, protein levels of the virus need to be measured in EC cell lysates (Nucleocapsid protein antibody).
• While the data confirms some of the current literature that SARS-CoV-2 endothelium dysfunction is induced indirectly via SMC, further detail in the discussion on how the upregulation of certain genes reported both in SMC and EC are related in vivo is required.

REVIEWERS' COMMENTS

Reviewer #2 (Remarks to the Author):

Reviewer comments to NCOMMS-23-36245A;
SARS-CoV-2 infection of human pluripotent stem cell-derived vascular cells reveals smooth muscle cells as key mediators of vascular pathology during infection,
by Richards A., Khalil A., et al.

In the revised version of their manuscript, the authors have added a substantial body of work to strengthen the data and claims of the manuscript. Thereby, the authors have substantially strengthened the manuscript and have addressed all my comments. Especially, the comparative experiments add valuable information about the specific effect of active SARS-CoV-2 infection of SMC, and the paracrine effect of this infection on endothelial cells. The authors may consider highlighting the results obtained from the comparison of SMC infected with active SARS-CoV-2 compared to heat-inactivated SARS-CoV-2 (now in Supplemental Figure 4A, B, and Supplemental Figure S7A, B), since these analyses may identify the most relevant gene expression responses following active SARS-CoV-2 infection. Further, there are some minor points to be addressed by the authors.

We thank the reviewer for their comment. We agree that our studies comparing active SARS-CoV-2 infection to exposure to “dead” heat-inactivated virions has the potential to identify the specific pathological consequences that are unique to active infection by SARS-CoV-2. We have added the following text to the discussion to highlight these observations.

“Our comparison of the transcriptional response in smooth muscle cells (SMCs) to live versus heat-inactivated SARS-CoV-2 revealed the specific upregulation of several inflammatory genes, including HELZ2, MX1, and IFI6, during active infection. These genes play key roles in orchestrating immune responses to viral infections and are notably upregulated in patients with severe COVID-19[54-56]*. The selective elevation of these genes following exposure to live virus suggests that active SARS-CoV-2 infection enhances antiviral signaling in SMCs beyond what is triggered by viral proteins alone. HELZ2, MX1, and IFI6 are also induced in endothelial cells (ECs) after exposure to factors secreted from SARS-CoV-2-infected SMCs. Since ECs are not directly infected by SARS-CoV-2, this data implies that inflammatory signals from neighboring SMCs are sufficient to activate expression in ECs. All three genes are interferon-stimulated genes (ISGs), whose expression is induced by interferon (IFN) signaling*[57-59]*, we hypothesize that infected SMCs may secrete IFNs, which then activate these antiviral pathways in nearby ECs*.”

Minor:
General for many volcano plots: The gene name labels in the volcano plots are often too small to read and overlap. The authors may consider reducing the

number of labeled genes together with a larger type-set and instead presenting the list of differentially expressed genes in an accompanying supplementary table.

We thank the reviewer for their comment we have increased the size of the text labeling the genes in all volcano plots.

Figure 1: The authors use different denominations for PDGFRa/b (in the figure panels) or PDGFR-A/-B in the figure legend.

We have corrected the format of the labels in the figure panel to match the figure legend.

Figure 2: The authors write in the legend 'Titers of EC 0 h.p.i. samples were below the limit of detection…', but in the barplot (A) there are values shown for the 0 h.p.i. time point. Maybe the authors refer to the 48 h.p.i. time point?

We thank the reviewer for catching this error. We have corrected the figure legend to say 48 h.p.i.

Figure 4: The measurement of the CellROX green signal in cell cultures of ECs appears to come from one single experiment (at least no independent repetitions are mentioned) and the measurement of single cells in the culture (five cells from three fields; 15 data points) appear to be the basis for the statistical calculations (A). Instead, the authors should perform statistics on at least three independent experiments, using the mean signal from each experiment calculated as shown now in A.

We appreciate the reviewer's comment. We have updated Figure 4 to show the mean relative florescence of all conditions (compared to the average mock value) for three independent experiments.

Figure 5: In the Figure legend the authors write '(C)/(E) … compared to ECs exposed to control EC (Control)'. This is confusing, and not clear from the manuscript text or methods. What does ECs exposed to control EC refer to?

We thank the reviewer for catching this error. We have corrected the text and the figure legend. The text now reads, "Volcano plot of differentially expressed genes in ECs treated with media from SMCs exposed to heat-inactivated SARS-CoV-2 *(HI SMC CM)* compared to control ECs *(Control)*."

Supplemental Figure 3: From the volcano plot, only few genes appear to be differentially expressed, however the GSEA analysis in A left panel suggests a high number of differentially expressed genes. What is the input to the GSEA analysis and how does it differ from the data presented in the volcano plot?

The enrichment scores computed by GSEA are not directly related to differentially expressed genes. The determination of whether a gene is differentially expressed depends upon chosen thresholds for fold-change and significance after applying a statistical model to the counts data, but GSEA does not take into account these thresholds. Instead, it computes an enrichment score partly based upon whether genes within a given gene-set exhibit a consistent up- or down-regulation, even if none of the individual genes are significantly differentially expressed. While there can be a clear correspondence between "differentially expressed" genes and gene-set enrichments, this is not always the case when changes in individual gene expression are below the defined thresholds.

Supplemental Figure 11: There is a discrepancy between the data presented in the figure and the figure legend. Four datapoints are shown in the quantification of SERPINE1, whereas the legend says 'Three independent experiments…' Further, why is the bar for Mock SMC exposed not at 1.0 for the vWF quantification (left panel)?

We thank the reviewer for bringing our attention to this mistake. There was an error in the values plotted for the Mock vWF condition. We have corrected the error and the Average Fold Change for Mock SMC exposed ECs is now 1.0. This error does not impact our conclusions from the data. We have also edited the figure legend to reflect the correct number of independent experiments that were analyzed for the SERPINE1 quantitation.

Supplemental Figure 13: There is a discrepancy between the data presented in the figure and the figure legend. Five datapoints are shown in the quantifications of SERPINE1 (A and B), whereas the legend says 'Three independent experiments…'

We thank the reviewer altering us to the error. There was a mistake in the data and two data points were included in the SERPINE1 data that were technical replicates and not biological replicates. We have corrected the figure and rerun the statistical analysis. This error does not impact the conclusions reached in the manuscript.

**Please see additional "Note to Reviewers" at the end of this document*

Reviewer #4 (Remarks to the Author):

In the manuscript, the authors use a stem cell derived model to determine the effects of SARS-CoV-2 on vascular pathology. While the authors have addressed the main concerns outlined by the original review making the reported data robust, there is still concerns on linking the findings to the molecular mechanism between paracrine action of SARS-CoV-2 infected SMC on EC. Below are some points to consider:

• Authors confirm, through plague assay and dsRNA measurements, SARS-CoV-2

infection in SMC and PA but not in EC. However, EC exposed to live virus does show changes to gene expression related to ROS. To be sure there is no replication of virus in EC, protein levels of the virus need to be measured in EC cell lysates (Nucleocapsid protein antibody).

We agree with the reviewer that adding additional conformation that our ECs are not infected by SARS-CoV-2 strengthens our hypothesis that the changes in EC gene expression following SARS-CoV-2 exposure occur in the absence of productive infection. While a western blot for the nucleocapsid protein could provide this additional conformation, the results could be complicated by the fact that the nucleocapsid protein is present in the inoculating virus making it difficult to conclusively rule out a low level of infection. In our revised manuscript we have included additional data examining levels of the positive and negative sense SARS-CoV-2 genome in ECs exposed to live SARS-CoV-2 or heat-inactivated SARS-CoV-2. A negative sense copy of the viral genome is produced only during viral genomic replication and therefore can be used to distinguish actively replicating virus from input virus. Our results show that ECs exposed to live and heat-inactivated SARS-CoV-2 show similarly low levels of positive and negative sense viral genomes (Supplemental Fig. 3). Conversely, SMCs exposed to live SARS-CoV-2 show robust amplification of both the positive and negative sense SARS-CoV-2 genome. Collectively, these data support our hypothesis that no productive infection occurs in ECs.

• While the data confirms some of the current literature that SARS-CoV-2 endothelium dysfunction is induced indirectly via SMC, further detail in the discussion on how the upregulation of certain genes reported both in SMC and EC are related in vivo is required.

We thank the reviewer for this suggestion. To address this comment, we have added the following text to the discussion.

“To assess the in vivo relevance of our model system, we explored several pathways that could underlie the vascular complications seen in COVID-19, such as hypercoagulability and bleeding disorders leading to exsanguination—events our data also detected[8, 35, 37, 60, 61]. Notably, we observed an upregulation of interferon-stimulated genes and coagulation mediators like SERPINE1 (encoding for plasminogen activator inhibitor-1) and vWF in ECs exposed to conditioned media from SARS-CoV-2-infected SMCs. These are both key factors known to mediate thromboembolic events[35] and have been observed at elevated levels in severe COVID-19 patients associated with high thrombotic risk[35, 37] suggesting fidelity of the model findings. In addition to this EC dysfunction driven by paracrine signaling, infected SMCs in our model showed increased Tissue Factor activity, a primary driver of the intrinsic coagulation cascade[43, 45], further aligning with in vivo reports of its upregulation in lung tissue from severe COVID-19 cases[45] and persistent systemic inflammation[44]. Beyond SMC-driven effects, our model indicated that SARS-CoV-2 particles directly impair EC barrier function even

without productive infection, potentially explaining the paradoxical bleeding and exsanguination observed in severe cases[60]. We identified significant disruptions in ROS signaling in ECs following viral exposure, consistent with increased vascular permeability seen in severe COVID-19[57] and supported by elevated NADPH oxidase activity in the microvasculature of patients[62]. Our concurrent observation of EIDL3 downregulation, with its potential role in weakening tight junctions and compromising vascular integrity[53], suggests a novel mechanism by which SARS-CoV-2 may induce EC dysfunction. Given the alignment of our collective findings with established observations in COVID-19 patients, further studies using human vascular tissue are warranted to validate these possible EIDL3-dependent mechanisms."

*Note to reviewers:
While compiling the source data for this submission we noted an error in the data plotted for DMS % inhibition data (Supplemental Fig. 16A). We have corrected this error and put in an updated graph. The changes do not alter the conclusions reached in the manuscript.