Lung Epithelial Cell Transcriptional Regulation as a Factor in COVID-19 Associated Coagulopathies

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Some of the results of these studies have been previously reported in the form of a preprint (bioRxiv, [07 July 2020] https://doi.org/10.1101/2020.07.06.182972).

This article has an online data supplement, which is accessible from this issue's table of content online at <u>www.atsjournals.org</u>.

ABSTRACT

SARS-CoV-2 has rapidly become a global pandemic. In addition to the acute pulmonary symptoms of COVID-19 (the disease associated with SARS-CoV-2 infection), pulmonary and distal coagulopathies have caused morbidity and mortality in many patients. Currently, the molecular pathogenesis underlying COVID-19 associated coagulopathies are unknown. Identifying the molecular basis of how SARS-CoV-2 drives coagulation is essential to mitigating short and long term thrombotic risks of sick and recovered COVID-19 patients. We aimed to perform coagulation focused transcriptome analysis of *in vitro* infected primary respiratory epithelial cells, patient derived bronchial alveolar lavage (BALF) cells, and circulating immune cells during SARS-CoV-2 infection. Our objective was to identify transcription mediated signaling networks driving coagulopathies associated with COVID-19. We analyzed recently published experimentally and clinically derived bulk or single cell RNA sequencing datasets of SARS-CoV-2 infection to identify changes in transcriptional regulation of blood coagulation. We also confirmed that the transcriptional expression of a key coagulation regulator was recapitulated at the protein level. We specifically focused our analysis on lung tissue expressed genes regulating the extrinsic coagulation cascade and the plasminogen activation system. Analyzing transcriptomic data of *in vitro* infected normal human bronchial epithelial (NHBE) cells and patient derived BALF samples revealed that SARS-CoV-2 infection induces the extrinsic blood coagulation cascade and suppresses the plasminogen activation system. We also performed in vitro SARS-CoV-2 infection experiments on primary human lung epithelial cells to confirm that transcriptional upregulation of Tissue Factor, the extrinsic coagulation cascade master regulator, manifested at the protein level. Further, infection of NHBEs with influenza A virus (IAV) did not drive key regulators of blood coagulation in a similar manner as SARS-CoV- 2. Additionally, peripheral blood mononuclear cells (PBMCs) did not differentially express genes regulating the extrinsic coagulation cascade or plasminogen activation system during SARS-CoV-2 infection, suggesting that they are not directly inducing coagulopathy through these pathways. The hyper-activation of the extrinsic blood coagulation cascade and the suppression of the plasminogen activation system in SARS-CoV-2 infected epithelial cells may drive diverse coagulopathies in the lung and distal organ systems. Understanding how hosts drive such transcriptional changes with SARS-CoV-2 infection may enable the design of host-directed therapeutic strategies to treat COVID-19 and other coronaviruses inducing hyper-coagulation.



INTRODUCTION:

In December 2019, the novel SARS-CoV-2 coronavirus emerged in Wuhan China.¹ It has since spread globally causing societal shutdowns with nearly 110 million global cases and 2.5 million deaths.^{2,3} Severe cases are often complicated by acute respiratory distress syndrome (ARDS) and hyper-inflammation, with many patients requiring mechanical ventilation and ICU admission due to hypoxia and pneumonia.^{4,5} However, the pathology of COVID-19 also impacts organ systems and tissues beyond the lung, including the kidneys, gut, liver, and brain,^{6–9} and many of the most concerning distal pathologies have been associated with increased blood coagulation and clotting.^{8,10–14}

Previous work has shown that increases in pro-coagulant biomarkers are associated with greater mortality rates for patients suffering acute lung injury (ALI).^{15–17} Additionally, modulation of blood coagulation and fibrinolysis have previously been proposed to treat ALI.¹⁸ Coagulopathies concomitant to ALI and ARDS have been hypothesized to emerge due to interactions of inflammation and the extrinsic coagulation cascade, much of which is through interaction with the vascular endothelium.¹⁹ While some valid clinical reports have reported rare instances of coagulopathy complications occurring with influenza infection or influenza like illness, case controlled studies have not identified a significant association with these infections and pulmonary embolisms or deep vein thromboses.^{20,21} Even among a study focusing only on the pulmonary pathology of lethal 2009 pandemic swine flu patients in Brazil, 6 out of 21 patients had microthrombi and only 4 had pulmonary embolism.²² Coagulopathies have been observed in coronavirus infection at far greater rates compared to other viral infections driving pulmonary inflammation. For instance, a study of pulmonary pathology of 20 lethal SARS-CoV patients in Toronto observed fibrin thrombi in 17 out of 20 patients. 12 out of 20 of these emboli resulted in

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pulmonary infarction.²³ From the extraordinarily large pool of SARS-CoV-2 clinical data, it is clear that severe COVID-19 pathology is associated with and partially driven by coagulopathies. One study from Tongji Hospital of Huazhong University found disseminated intravascular coagulation occurred in 71.4% of patients, with elevated D-dimer, pro-thrombin time, and fibrinogen degradation products in the blood.²⁴ A clinical study in the Netherlands reported in 31% of COVID-19 ICU patients suffered coagulation complications, 81% of which were pulmonary emboli and 27% of which were deep vein thrombi.²⁵ It has become standard practice for blood thinning treatments to be administered prophylactically to minimize the risk of COVID-19 associated coagulopathies and clinical trials to investigate the efficacy of common anti-coagulants at mitigating COVID-19 are ongoing.²⁶⁻²⁸ (ClinicalTrials.gov Identifiers: NCT04333407 & NCT04365309).

Blood coagulation is primarily regulated by three highly interconnected molecular signaling pathways, platelet activation, the coagulation cascade, and fibrinolysis.^{29–37} The extrinsic blood coagulation pathway is effected through the initial activity of the protein tissue factor, which drives the cascading activation of several zymogen coagulation factors including factor VII, factor V, and factor X. The result of the extrinsic coagulation cascade is conversion of prothrombin into thrombin, which crosslinks fibrin into a mesh essential for clot formation. The activation of this zymogen cascade is similarly balanced by endogenously encoded inhibitors, including tissue factor pathway inhibitor and activated protein C (APC). Plasmin suppresses coagulation via proteolytic degradation of this cross-linked fibrin mesh in blood clots.

Most hypotheses propose that COVID-19 coagulopathies are indirectly induced by acute inflammation and cytokine secretion associated with SARS-CoV-2, but the precise mechanisms underlying coagulopathies remain elusive.^{38,39} Identifying the cellular source of signal

transducers initially driving coagulopathy will be critical in understanding and mitigating SARS-CoV-2-induced coagulopathies. To this end, we performed post-hoc analysis of publicly available transcriptomics datasets of SARS-CoV-2-infected normal human bronchial epithelial cells (NHBEs), COVID-19 patient bronchoalveolar lavage fluid (BALF) and COVID-19 peripheral blood mononuclear cells (PBMCs), with the goal of identifying possible etiologies of SARS-CoV-2 induced coagulopathies.^{40–42} Our study demonstrates that changes to the lung epithelium directly caused by SARS-CoV-2 infection may directly contribute to the induction of coagulopathy seen in COVID-19 patients via modulation of the extrinsic coagulation cascade and plasminogen activation system. The altered transcriptional profile of the lung epithelium and increased production of TF protein as a result of SARS-CoV-2 infection is a likely contributor to COVID-19 associated coagulopathies in the lung and a possible contributor to systemic coagulopathies. Lung epithelial cells, as the primary target of SARS-CoV-2, may play a key role in the initiation of coagulopathies observed during COVID-19. Such changes likely occur in upstream or in concert with SARS-CoV-2 induced changes in lung endothelial cells, platelets, and immune cell driven inflammation thrombosis circuits that also drive coagulopathies. These findings do not rule out coagulation defects driven by immune cells and the vascular endothelium, but suggests the lung epithelium as an additional factor driving COVID-19 patient coagulopathy.

METHODS:

Xiong et al. – Bulk RNA-seq analysis of BALF and PBMCs from SARS-CoV-2 infected patients

Bulk BALF and PBMC transcriptomics data were generated through RNA-sequencing of purified cells from SARS-CoV-2 infected patients as described in Xiong *et al.*⁴⁰ Raw sequencing

data were accessed via the Chinese Academy of Science's Genome Sequence Archive (GSA) (COVID+ BALF - GSA Accession CRP001417 ; PBMCs – GSA Accession CRA002390). Three BALF control samples isolated from healthy volunteers were downloaded from a publicly available NCBI dataset at sample accession SRR10571724, SRR10571730, and SRR10571732.⁴³

Blanco-Melo et al. – Bulk RNA-seq analysis of SARS-CoV-2 infected NHBEs cultured in-vitro

Normal human bronchial epithelial (NHBE) cells were cultured under non-differentiating conditions in bronchial epithelial growth media. SARS-CoV-2 isolate USA-WA1/2020 (NR-52281) was used to infect NHBE ($5x10^5$) cells at a multiplicity of infection (MOI) of 2 for 24 hours and or mock infected in their culture media prior to RNA purification, library preparation, and sequencing as described in as described in Blanco-melo *et al.*⁴¹ Raw sequencing data were accessed via the National Center for Biotechnology Information's Gene Expression Omnibus (GEO Accession - GSE63473).

Blanco-Melo et al. –Bulk RNA-seq analysis of H1N1 infected NHBEs cultured in vitro

Normal human bronchial epithelial (NHBE) cells were infected with A/Puerto Rico/8/1934 (PR8) influenza A virus at a MOI of 3 for 12 hours prior to RNA purification, library preparation, and sequencing as described in Blanco-Melo *et al.*⁴¹ Raw sequencing data were accessed via the National Center for Biotechnology Information's Gene Expression Omnibus (GEO Accession - GSE63473).

Bulk RNA-seq analysis pipeline

The analysis pipeline described below was used to analyze NHBE, PBMC, and BALF bulk RNA-seq data sets. Sequencing reads were downloaded in their raw fastq format. Read adapter and quality trimming was performed using Trim Galore! and sequencing quality was confirmed using the FASTQC and MULTIQC.^{44,45} Sequence alignment to the GRCh38 reference transcriptome was performed using Salmon and differential gene expression analysis was performed using DESeq2.^{46,47} DESeq2 adjusted p-values are produced by the Wald test and corrected for multiple hypothesis testing using the Benjamini-Hochberg method. See supplemental data for full differential expression results of each bulk RNA-seq data set. (Supplemental Data Files 1-4) Gene plots and heat maps were generated in R using the pheatmap or ggplot2 R packages.^{48,49}

SARS-CoV-2 infection of NHBEs cultured in vitro for quantification of Tissue Factor protein

NHBEs were cultured in Pneumacult-Ex media (STEMCELL - #05008) using the standard formulation recommended by the manufacturer. NHBEs (8.5x10⁴) per well were plated onto tissue culture treated 24 well plates and grown to 85% confluence before infection. NHBEs were then infected with SARS-CoV-2 (USA-WA1/2020) at a MOI of 2 for 24 hours before supernatant and cell extract samples were collected for quantification of tissue factor by enzyme linked immunosorbent assay (ELISA). ELISA based quantification of tissue factor protein levels was performed using a Human Tissue Factor ELISA Kit (Abcam - ab220653) according to manufacturer's instructions.

Liao et al. – Single cell RNA-seq analysis of COVID-19 patient bronchoalveolar lavage fluid

BALF samples were obtained from 13 COVID-19 patients in Shenzhen Third People's Hospital from January to February, 2020. Patient disease severity was stratified as moderate, severe, or critical based on the "Diagnosis and Treatment Protocol of COVID-19 by the National Health Commission of China".⁵⁰ Single cell RNA-seq libraries were generated using Chromium Single Cell V(D)J reagent kits (10x Genomics; PN-1000006, PN-100014, PN-100020, PN_1000005) according to the manufacturer's protocol. Three healthy control BALF samples were also processed, and a fourth additional healthy control sample included from the gene expression omnibus (GSE128033). Refer to Liao *et al.* for further methodological detail. scRNA-seq data were accessed as a fully annotated Seurat R data file (rds format) deposited by the study authors at http://cells.ucsc.edu/covid19-balf/nCoV.rds. QC, visualization, and analysis were performed using Seurat, an R package for processing scRNA-seq data.

RESULTS:



Figure 1: The gene expression profile of differentially expressed genes within the enriched the regulation of blood coagulation GO term for SARS-CoV-2 infected NHBE cells.(A) Heatmap of selected genes in the regulation of blood coagulation GO term. Bolded genes represent differentially expressed genes as calculated by DESeq2 (P.adj < .05). (B) Pathway map of the extrinsic blood coagulation cascade (right) and the plasminogen activation system (left) with overlaid expression values. Blue asterisks indicate upregulation, black asterisks indicate no change, and red asterisks indicate down regulation.

Coagulation pathway gene expression in normal human bronchial epithelial cells is impacted by infection with SARS-CoV-2

To determine the impact SARS-CoV-2 infection has on the coagulation cascade we examined gene transcription regulating of hemostasis and thrombosis, including the extrinsic coagulation pathway and the plasminogen activation system. We identified a dozen differentially expressed genes that are part of the Regulation of Blood Coagulation gene ontology (GO) term (GO:0030193) (Figure 1A).

Visualization of gene expression directionality on pathway maps for the extrinsic blood coagulation cascade and plasminogen activation pathway (Figure 1B and Supplemental Table 1), illustrate how infected respiratory epithelial cells may drive coagulopathy in COVID-19. Most notably, the extrinsic coagulation cascade master signal transducer, tissue factor (F3), is significantly transcriptionally upregulated while balancing inhibitory proteins are unmodified or significantly downregulated. Additionally, while plasminogen activating proteins are significantly upregulated, plasminogen activating inhibitors and localizing receptors also increase. Podoplanin and endothelin-1 are also notably upregulated in the context of SARS-CoV-2 infection of NHBEs.



Upregulation of tissue factor in NHBEs infected with SARS-CoV-2

Figure 2: (A) Violin plot depicting raw counts of reads mapping to key regulators of the extrinsic blood coagulation cascade in mock infected and SARS-CoV-2 infected NHBE cells as performed by Blanco-Melo et al. Raw counts were normalized to library size in the DESeq2 software package. Adjusted P values for all differentially expressed genes were also calculated within DESeq2. Images were generated using GGPlot2 in the R studio environment. (B-C) ELISA measurements quantifying tissue factor in lysate and supernatant respectively. Samples were isolated at 24 hours post-infection (MOI 2) in NHBEs. Plotted values are the mean of 2 technical replicates ± standard deviation. P values were determined using and unpaired two-tailed t-test

One epithelial expressed factor that is known to be a major driver of coagulopathy is increased expression of *F3* in SARS-CoV-2 infected NHBEs (Figure 2A). *F3* encodes the Tissue Factor protein (TF), which is the master regulator responsible for the initiation of the extrinsic coagulation cascade. Tissue factor gene expression was found to be significantly upregulated in SARS-CoV-2 infected NHBEs as performed by Blanco-Melo *et al.* To further confirm the upregulation of TF by NHBEs during SARS-CoV-2 infection at the protein level, we replicated the NHBE infection experiments performed by Blanco-Melo as described in the methods section. The average amount of tissue factor associated with the cellular fraction of NHBEs increased by

62.34%. (Figure 2B). Similarly, the average amount of tissue factor that was released from NHBEs into the culture supernatant increased by 64.1% with SARS-CoV-2 infection. (Figure 2C)







Extrinsic coagulation cascade signaling is regulated by the balance of TF with endogenously encoded inhibitors. The first inhibitor in this cascade is *TFPI*, which encodes the Tissue Factor

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Pathway Inhibitor (TFPI) protein. TFPI suppresses coagulation by inhibiting tissue factor's activation of coagulation factor VII, the first zymogen along the cascade. *TFPI* transcription is not significantly different in SARS-CoV-2 infected NHBEs (Figure 3A). TFPI is additionally responsible for inhibiting the activation of coagulation factor V via inhibition of factor X. Significant increases in the amount of factor V has been correlated with greater COVID-19 disease severity and coagulopathy risk.⁵⁰ Maintenance of homeostasis between TF and TFPI is essential to limit clotting. An increase of TF without corollary increases of TFPI could contribute to coagulopathies in COVID-19 patients through unimpeded tissue factor and coagulation factor X signaling.⁵¹

Decreased expression of PROS1 in NHBEs infected with SARS-CoV-2

The *PROS1* gene which encodes Protein S, was also downregulated in SARS-CoV-2 infected NHBEs (Figure 3B). The primary function of Protein S is to antagonize the coagulation cascade by complexing with Protein C. The complex (known as Activated Protein C) inhibits the maturation of pro-coagulation factors Va and VIIIa, suppressing thrombin maturation and activity. The activity of both protein C and protein S is required for this effect.³⁰ Protein C also promotes TFPI activity.³⁵ Interestingly, membrane-bound protein S also contributes to anti-inflammatory efferocytic clearance by mediating membrane dynamics between macrophages and epithelial cells. Decreased lung epithelial *PROS1* expression may further exacerbate COVID-19 related pathology through diverse mechanisms.⁵² While protein S is canonically known to be produced in the liver along with protein C, biologically significant amounts of protein S are known to be produced in the lung, kidney, and gonads where it play key roles in the regulation of tissue homeostasis.²⁵

Additionally, within the Regulation of Blood Coagulation GO Term, several genes regulating the activity of plasminogen were identified. PLAU (encoding Urokinase) and PLAT (encoding tissue plasminogen activator) are significantly increased in SARS-CoV-2 NHBEs (Figures 3C and 3D). While PLAU is responsible for both the activation of plasminogen and tissue remodeling, research has also shown that upregulation of PLAU in the context of lung epithelia can induce increased tissue factor expression and coagulation in spite of its plasminogen activation.³⁶ Infected NHBEs also significantly upregulate SERPINB2, which encodes the protein Plasminogen Activator Inhibitor 2 (PAI-2) (Figure 3E). PAI-2 inhibits Urokinase and tissue plasminogen activator via proteolytic inactivation of plasminogen activators.⁵³ PAI-2 is commonly cytoplasmic, but membrane permeabilizing epithelial cell death in COVID-19 patients may drive the secretion of cytoplasmic proteins such as PAI-2 during SARS-CoV-2 infection.⁵⁴ The expression of *PLAUR*, a receptor localizing activated urokinase to the extracellular membrane, is also significantly increased in SARS-CoV-2 infected NHBEs (Figure 3F). The localized activity of PAI-2 may significantly inhibit the effect of PLAU/PLAUR in complex and thereby contribute to the formation of pulmonary embolisms and distal coagulopathies.

Regulation of blood coagulation by cells isolated from the BALF of COVID-19 Patients

We next hypothesized that NHBE differentially expressed genes enriched in the Regulation of Blood Coagulation (GO:0030193) GO term would be similarly active in COVID-19 patient BALF. We expected to see this recapitulation of the observed NHBE phenotype in patient BALF due to the pulmonary epithelial cell fraction of cells in the BALF cell mixture. Plotting BALF expression data for this gene list revealed a clear and consistent pattern of transcriptional regulation as well (Figure 4A). In addition, plotting of the expression data of

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BALF differentially expressed genes in the Regulation of Blood Coagulation GO term revealed further regulation of the coagulation cascade (Figure 4B).

Many of these expression signatures recapitulate the activity of *in vitro* SARS-CoV-2 infected NHBE cells. These include upregulation of pro-coagulation genes such as *F3* (TF), *SERPINA10*, and *SERPINB2*, and downregulation of anti-coagulation genes such as *PROS1* and *PLAUR*, and *PLAT*. Additionally, *PROCR*, encoding a receptor augmenting the inhibitory activity of protein S and protein C, was suppressed in COVID-19 patient BALF. Unlike the NHBE expression profile, there is increased *TFPI* and *PLAT* expression in COVID-19 patient BALF, indicating some antagonism of hyper-coagulation. However, coagulopathies observed in some COVID-19 patients indicate that this signaling can be insufficient to prevent hypercoagulation.



Figure 4: The gene expression profile of differentially enriched genes from RNA isolated from the BALF of **COVID-19 patients**. (A) Heatmap presentation of select differentially expressed genes presented in figure 2 (NHBE cells infected with SARS-CoV-2). The expression data presented in the heat map demonstrates the expression profile of those genes in BALF derived samples. Bolded genes represent differentially expressed genes as calculated by DESeq2 (P.adj < .05). (B) Heatmap presentation of an expanded selection of all differentially genes within the regulation of Blood Coagulation GO Term (GO:0007596) from BALF derived heathy control and patient samples.

Analysis of coagulation pathway gene expression in PBMCs



Figure 5: The gene expression profile of differentially enriched genes from RNA isolated from PBMCs of COVID-19 patients. The genes included in this heatmap were identified as enriched in the regulation of blood coagulation GO term for NHBE cells infected with SARS-CoV-2. No genes are bolded, as none were found to be differentially expressed as calculated by DESeq2 (P.adj < .05). The expression data presented in the heat map demonstrates the expression profile of these genes in PBMC derived samples. To determine gene expression regulating the extrinsic coagulation cascade or plasminogen activation system was changed in circulating immune cells, we analyzed transcriptomes of COVID-19 patient purified PBMCs as described in Xiong *et al.*⁴⁰ PantherDB Functional enrichment analysis found no enrichment of genes regulating or effecting coagulation in PBMCs from COVID-19 patients. (Figure 5). The primary publication of these datasets describes expected induction of hyper-inflammation and the immune cell death. We concluded it is unlikely that circulating immune cells during SARS-CoV-2 infection are driving coagulopathies via the coagulation cascade or plasminogen activation system.

Infection of human lung epithelial cells with influenza A virus does not impact coagulation pathway gene expression



Figure 6: The gene expression profile of differentially enriched genes from RNA isolated from NHBE cells infected with PR8 IAV. The genes included in this heatmap were identified as enriched in the regulation of blood coagulation GO term for NHBE cells infected with SARS-CoV-2. The expression data presented in the heat map demonstrates the expression profile of these genes in NHBE cell cultures that are mock infected or infected with PR8 IAV at a multiplicity of infection of 3. Bolded genes represent differentially expressed genes as calculated by DESeq2 (P.adj < .05).

To determine if these transcriptional changes are specific for SARS-CoV-2 or are more generalizable to respiratory viruses that infect the lung epithelium, we analyzed data sets from IAV infected NHBE cells. Heatmap plotting of genes found to be differentially expressed in NHBE cells during SARS-CoV-2 infection (Figure 1A), did not reveal notable signatures during IAV infection (Figure 6). Most critically, the master regulator of the coagulation cascade, Tissue Factor (F3), is not differentially expressed in the context of IAV infection. This would significantly lessen the haemostatic impact of suppressed plasminogen activation. These findings are consistent with the lesser degree of coagulopathy associated with IAV in the clinic, and further support the notion that coagulopathies during SARS-CoV-2 infection are independent of systemic inflammation common to both infections. COVID-19 associated coagulopathies may be triggered by changes in lung epithelial transcription uniquely induced by SARS-CoV-2 infection.

Analysis of COVID-19 patient BALF single cell RNA-seq

In vitro bulk RNA-seq data from SARS-CoV-2 infected NHBEs and COVID-19 patient BALF suggest that pulmonary epithelial cells contribute to the induction of COVID-19 associated coagulopathy through the expression of key genes regulating the extrinsic coagulation cascade and the plasminogen activation system. However, to further confirm these results we analyzed single cell RNA-sequencing of COVID-19 patient BALF samples.

We accessed COVID-19 patient BALF scRNA-seq data generated and published by Liao et al.⁴² These data provided strong evidence that pulmonary epithelial cells in COVID-19 patients are transcribing signals that likely contribute to diverse COVID-19 associated coagulopathies. (Figure 7) Figure 7A shows a Uniform Manifold Approximation Projection (UMAP) plot representing the cell type specific clustering of the Liao *et al.* data. The subset identified as epithelial cells comprised \sim 5% of the total cells sequenced and clustered away from all other immune cell subtypes identified. Figure 7B represents a dot plot depiction expression data from this epithelial cell subset only, with patient samples stratified by disease severity. Figure 7C depicts the UMAP feature plots highlighting the cell types expressing each gene and the intensity of that expression. From these data, we can conclude that lung epithelial cells in COVID-19 patients are significantly upregulating genes tissue factor, the master regulator driving the extrinsic coagulation cascade. Further, while there is an upregulation of the tissue factor's cognate inhibitor, tissue factor pathway inhibitor, the degree of upregulation is smaller and restricted to fewer than half as many cells. Additionally, during severe COVID-19, protein S expression is entirely lost in many pulmonary epithelial cells where it is expressed at homeostasis. Cells that continue to express the transcript do so at much lower levels. These data also validate that pulmonary epithelial cells are significantly upregulating the expression of proteins which suppress the anticoagulant effect of plasminogen. These changes include the upregulation of plasminogen activator inhibitors such as SERPIN protease inhibitors and the upregulation of PLAUR, a plasminogen urokinase localizing protein. However, it is important to note that while these expression patterns were observed in vitro and in patient pulmonary epithelial cells, significant expression changes regarding the plasminogen activation system were also observed in macrophages and neutrophils that would likely also contribute to a procoagulative state.



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Figure 7: scRNA-seq analysis of select extrinsic coagulation cascade and plasminogen regulating genes in all epithelial cells from BALF samples described in Liao et al. Refer to Liao et al for a full description of the epithelial cell markers used for identification. (A) Seurat generated Dot Plot summarizing a selection of genes regulating coagulation. Dot color is representative of the degree of relative expression for each gene and dot size is representative of the precent of cells expressing each gene. (B) Seurat generated UMAP plot depicting cell clustering for all identified cell types in Liao et al. See Liao et al. for a full description of settings used for the Seurat UMAP computation. (C) Seurat generated feature plots showing the distribution and relative expression of mRNA for selected genes regulating coagulation in cells isolated from bronchoalveolar lavage fluid.

DISCUSSION:

The combined analysis of data from Blanco-Melo et al, Xiong et al, and Liao et al, collectively demonstrate how pulmonary epithelial cells may drive transcriptional responses promoting COVID-19 associated coagulopathies, including induction of the extrinsic coagulation cascade without compensatory inhibitory signals and the suppression of the plasminogen activation system via the upregulation of plasminogen inactivation proteins and localization factors. In-vitro NHBE infection data from Blanco-Melo et al. further demonstrate that COVID-19 induced transcriptional changes driving the extrinsic coagulation cascade and suppressing the plasminogen activation system are not similarly induced during IAV infection. These data suggest the possibility coagulopathy in COVID-19 patients may be more prevalent relative to IAV patients due to the transcriptional changes SARS-CoV-2 induces during infection of the pulmonary epithelium. Additionally, through replication of the infection model performed by Blanco-Melo et al., we were able to demonstrate that the changes in mRNA detected through next generation sequencing were consistent with an increase in the levels of secreted and cellular bound TF during NHBE infection. The increase in TF protein levels were significantly greater than the detected increase in mRNA levels, providing further evidence that epithelial cell derived

tissue factor is a likely contributor to COVID-19 associated coagulopathy in the pulmonary space. It is also possible that such epithelial derived signals may contribute to COVID-19 associated coagulopathies in beyond the lung, but further experimental evidence is needed to confirm a possible mechanistic link. Understanding these epithelial derived signals and their interaction with other pro-coagulant signals that SARS-CoV-2 may induce (such as endothelial cell dysfunction, platelet hyper-activation, liver dysfunction, or inflammation-thrombosis circuits), will enable the scientific community to devise more effective ways to mitigate COVID-19 and other coronavirus induced coagulopathies in the future.

There is mounting evidence that coagulation defects are a significant contributor to COVID-19 pathology. Reports of COVID-19 associated coagulopathies have proliferated globally, including acute pulmonary embolism in the microvasculature of the lung, as well as cerebral, renal, and bowel localized embolic disease.^{8,11,12} Acute pulmonary thromboembolism presents in 30% of severe clinical COVID-19 patients by pulmonary CT angiography, which is also associated with elevation of serum D-dimer. D-dimer is a by-product of fibrinolysis degrading crosslinked fibrin clots.¹³ Clinicians also reported that biomarkers of coagulation (clot strength, platelet and fibrinogen contributions to clot strength, and elevated d-dimer levels) are significantly increased with COVID-19 associated ARDS.¹⁴A diverse spectrum of proinflammatory mediators shown to be dramatically upregulated in COVID-19 and other coronavirus pathologies are also known to contribute to TF induced hypercoagulability.⁵⁵ Recent work by Stefely et al. found marked increases in the levels of Factor V activity associated with severe COVID-19 disease.⁵⁰ Factor V was the most strongly associated parameter with disease severity across all measurements included in the study. Such increases directly indicate that the induction of the extrinsic coagulation cascade is a hallmark of severe COVID-19 disease, and the data aggregated here suggest that pulmonary epithelial cells are contributing factors in COVID-19 associated coagulopathies.

It is important to note that many other cell types likely play key roles in the induction of COVID-19 associated coagulopathies. Much work is still needed to determine how epithelial cell derived tissue factor and other pro-coagulant signals induced by SARS-CoV-2 infection contributes to the induction of COVID-19 pulmonary coagulopathies. It also remains an open question if epithelial cell derived pro-coagulant signals contributes to the induction of systemic coagulopathy through distal interactions with other cell types. Other important players in regulating the coagulation cascade are vascular endothelial cells.⁵⁶ The possibility of direct endothelial cell infection by SARS-CoV-2 has been proposed as a possible driver of hypercoagulation in COVID-19. ⁵⁷ However, there has been a lack of confirmatory data from human patients regarding productive endothelial cell infection by SARS-CoV-2 over the course of the pandemic. For instance, Immunohistochemical analysis failed to detect reactivity in post-mortem staining of COVID-19 patient pulmonary endothelial cells.⁵⁸ Additionally, recent *in-vitro* studies from two independent research teams found that primary endothelial cells and pluripotent stem cells differentiated into endothelial cells were not susceptible to direct infection with SARS-CoV-2.^{59,60}. Indirect damage of endothelial cells by systemic inflammation or factors derived from infected epithelial cells during SARS-CoV-2 induced ALI is a much more likely driver of these signals. To our knowledge, there are currently no RNA-sequencing datasets with infected endothelial cell cultures or tissue available. However, such data sets would be invaluable in characterizing endothelial cells responses to epithelial derived coagulation signals or endothelial induction of SARS-CoV-2 associated coagulopathies. Additionally, significant amounts of coagulation regulatory proteins including protein C and protein S are synthesized in the liver.⁶¹

Liver coagulopathies and dysfunction has also been associated with COVID-19, ^{62,63} and the possibility that liver dysfunction in COVID-19 is contributing to coagulation should also be investigated. Recent work has also shown that platelets activation may be involved in the induction of coagulopathy during COVID-19.^{64(p2)} It is very likely that the induction of SARS-CoV-2 mediated coagulopathies is dependent on the interaction of several tissue types driving the extrinsic coagulation system and platelet activation while suppressing fibrinolysis via plasminogen.

Also, other molecular factors increased with SARS-CoV-2 infection, including phosphatidylserine exposure, interferon expression, ICAM expression, angiotensin II expression, and complement activation, are known to "decrypt" TF from its inactive form on the surface of tissue cells and endothelial cells.³⁰ Such "coagulation-inflammation-thrombosis" circuit feedback loops coupled with the multiple zymogen activation mediated feedback loops within the extrinsic blood coagulation cascade, could significantly contribute to the induction of COVID-19 coagulopathy in patients.³⁰

Coagulation cascade induction by the epithelia is thought to be necessary during ARDS or ALI, and may be protective.⁶⁵ However, when it becomes dysregulated it can be damaging. ARDS is often associated with increased biomarkers of coagulation and fibrinolysis. Further, pulmonary edema fluids as well as plasma from patients with acute lung injury contain lesser amounts of anti-coagulant protein C and higher amounts of plasminogen activator inhibitors. Some proportion of these key coagulation inhibitors are likely secreted from epithelial and endothelial pulmonary cells and acting on the local environment, in addition to proteins in the circulatory system.^{15–17}

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Other genes such as podoplanin and endothelin-1, which are known to drive vasoconstriction and disseminated intravascular coagulation respectively in infection contexts, are known to be significantly upregulated during SARS-CoV-2.^{66–69} In the lung podoplanin is expressed by epithelial cells and endothelin is expressed by epithelial and endothelial cells.^{70–72}

The role of the lung epithelium in coagulation defects has not been fully explored, however some prior research illuminates its possible contribution. Lung epithelial cell lines have been shown to have increased expression of TF after incubation with pulmonary edema fluid from ARDS patients.⁷³ In addition, mouse models demonstrate that lung epithelial-derived TF may play an important role in tissue protection during ALI caused by LPS.⁷⁴ *In vitro* experiments with human epithelial cells indicate that TF may also contribute to basal cell survival.⁷⁵ Taken together these data suggest that while induction of the extrinsic coagulation cascade by lung epithelial cells may aid host responses during some stages of infection, but SARS-CoV-2 infection can trigger changes that drive systemic and local coagulopathies.

Lytic regulated cell death of respiratory epithelial cells, particularly via pyroptosis, may contribute to COVID-19 pathogenesis.⁷⁶ During lytic cell death intracellular contents typically isolated within cell membranes are released. Proteins such as TF, plasminogen activating inhibitors, and pro-coagulant factors may be released into the pulmonary space or circulation due to COVID-19 induced lytic cell death of epithelial cells. Such factors likely drive paracrine signaling to nearby endothelial cells in the lung, which could further exacerbate coagulation systemically via the secretion of activated coagulation cascade zymogens and thrombin into the blood. It is also possible that such factors could also enter the blood stream directly near damaged endothelial tissues in the lung, potentially contributing to the induction of systemic coagulopathies observed in severe COVID-19 patients. Epithelial derived hyper-coagulation

factors and plasminogen inhibitors may also drive local pulmonary hyper-coagulation and exacerbate pulmonary tissue destruction during SARS-CoV-2 infection.

Further investigation of pulmonary endothelial, epithelial, and immune cell responses to SARS-CoV-2 will be essential for unraveling the mystery of COVID-19 induced coagulopathy. Identifying the cellular factors that drive SARS-CoV-2 induced coagulopathy is essential, both for understanding foundational SARS-CoV-2 biology and optimizing clinical practices. Understanding the transcriptional regulation of the coagulation cascade in the lung epithelium, a particularly druggable target cell type, may help develop therapeutic strategies to mitigate this serious complication of coronavirus infection.

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Online Data Supplement

Supplemental Data 1: Full DESeq2 results output from the analysis of NHBEs infected with SARS-CoV-2 by bulk RNA-seq. Refer to Blanco-Melo et al. for a full description of the methods used to generate these data.

Supplemental Data 2: Full DESeq2 results output from the analysis of NHBEs infected with PR8 IAV by bulk RNA-seq. Refer to Blanco-Melo et al. for a full description of the methods used to generate these data.

Supplemental Data 3: Full DESeq2 results output from the analysis of COVID-19 patient derived BALF by bulk RNA-seq. Refer to Xiong et al. for a full description of the methods used to generate these data.

Supplemental Data 4: Full DESeq2 results output from the analysis of COVID-19 patient derived PBMCs by bulk RNA-seq. Refer to Xiong et al. for a full description of the methods used to generate these data.