

1 Type I and Type III IFN Restrict SARS-CoV-2 Infection of Human Airway Epithelial

2 Cultures

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4 Abigail Vanderheiden^{a,b,c}, Philipp Ralfs^{b,c,d}, Tatiana Chirkova^{a,b}, Amit A. Upadhyay^{b,c,e},
5 Matthew G. Zimmerman^{a,b,c}, Shamika Bedoya^d, Hadj Aoued^b, Gregory M. Tharp^b,
6 Kathryn L. Pellegrini^b, Anice C. Lowen^d, Vineet D. Menachery^f, Larry J. Anderson^{a,b},
7 Arash Grakoui^{a,b,d}, Steven E. Bosinger^{b,c,e}, Mehul S. Suthar^{a,b,c,#}

8

9 ^aCenter for Childhood Infections and Vaccines (CCIV); Children's Healthcare of Atlanta;
10 Department of Pediatrics, Emory University School of Medicine, Atlanta, GA 30322
11 USA.

12 ^bEmory Vaccine Center, Emory University School of Medicine, Atlanta, GA 30329, USA.

13 ^cYerkes National Primate Research Center, Atlanta, GA 30329, USA.

14 ^dDepartment of Microbiology and Immunology, Emory University School of Medicine,
15 Atlanta, GA 30322, USA.

16 ^eDepartment of Pathology and Laboratory Medicine, Emory University School of
17 Medicine, Atlanta, GA 30322, USA.

18 ^fDepartment of Microbiology and Immunology, Institute for Human Infection and
19 Immunity, World Reference Center for Emerging Viruses and Arboviruses, University of
20 Texas Medical Branch, Galveston, TX, USA.

21 #Correspondence: Mehul S. Suthar (mehul.s.suthar@emory.edu)

22 Lead contact: Mehul S. Suthar (mehul.s.suthar@emory.edu)

23 Running head: Type I and III IFN restrict SARS-CoV-2 in HAE cells

24 **ABSTRACT**

25 The newly emerged human coronavirus, SARS-CoV-2, has caused a pandemic of
26 respiratory illness. The innate immune response is critical for protection against
27 Coronaviruses. However, little is known about the interplay between the innate immune
28 system and SARS-CoV-2. Here, we modeled SARS-CoV-2 infection using primary
29 human airway epithelial (pHAE) cultures, which are maintained in an air-liquid interface.
30 We found that SARS-CoV-2 infects and replicates in pHAE cultures and is directionally
31 released on the apical, but not basolateral surface. Transcriptional profiling studies
32 found that infected pHAE cultures had a molecular signature dominated by pro-
33 inflammatory cytokines and chemokine induction, including IL-6, TNF α , CXCL8. We
34 also identified NF- κ B and ATF4 transcription factors as key drivers of this pro-
35 inflammatory cytokine response. Surprisingly, we observed a complete lack of a type I
36 or III IFN induction during SARS-CoV-2 infection. Pre-treatment or post-treatment with
37 type I and III IFNs dramatically reduced virus replication in pHAE cultures and this
38 corresponded with an upregulation of antiviral effector genes. Our findings demonstrate
39 that SARS-CoV-2 induces a strong pro-inflammatory cytokine response yet blocks the
40 production of type I and III IFNs. Further, SARS-CoV-2 is sensitive to the effects of type
41 I and III IFNs, demonstrating their potential utility as therapeutic options to treat COVID-
42 19 patients.

43

44 **IMPORTANCE**

45 The current pandemic of respiratory illness, COVID-19, is caused by a recently
46 emerged coronavirus named SARS-CoV-2. This virus infects airway and lung cells
47 causing fever, dry cough, and shortness of breath. Severe cases of COVID-19 can
48 result in lung damage, low blood oxygen levels, and even death. As there are currently
49 no vaccines or antivirals approved for use in humans, studies of the mechanisms of
50 SARS-CoV-2 infection are urgently needed. SARS-CoV-2 infection of primary human
51 airway epithelial cultures induces a strong pro-inflammatory cytokine response yet
52 blocks the production of type I and III IFNs. Further, SARS-CoV-2 is sensitive to the
53 effects of type I and III IFNs, demonstrating their potential utility as therapeutic options
54 to treat COVID-19 patients.

55 INTRODUCTION

56 In December 2019, a novel human coronavirus, SARS-CoV-2, emerged in Wuhan,
57 China, causing an outbreak of severe respiratory disease (1, 2). In the span of several
58 months, SARS-CoV-2 rapidly escalated to a pandemic, with over 4 million infections
59 and 275,000 deaths worldwide (3). There are currently no vaccines or antivirals
60 approved for use in humans that can prevent or treat the infection. SARS-CoV-2
61 infection manifests as an upper and lower respiratory disease (named COVID-19 by the
62 World Health Organization), characterized by fever, dry cough, and shortness of breath.
63 SARS-CoV-2 targets lower respiratory tract cells, with one study finding 93% of their
64 patients' bronchial lavages were qRT-PCR positive for SARS-CoV-2 (4).
65 Correspondingly, lung abnormalities have been observed in several patients with
66 COVID-19, and severe infection can lead to respiratory failure and lung tissue
67 destruction (5). Severe disease has also been associated with a low level of
68 lymphocytes in the blood and high levels of pro-inflammatory cytokines, such as IL-6
69 and TNF- α (6). How the host innate immune system responds to SARS-CoV-2 infection
70 is not well understood.

71
72 The SARS-CoV-2 genome is 29.8 kb in length and predicted to contain 12 open reading
73 frames. This includes 15 putative non-structural proteins, envelope and capsid genes,
74 an RNA-dependent RNA polymerase (RDRP), and a spike protein (7, 8). SARS-CoV-2
75 virus is closely related to the β -coronavirus SARS-CoV, which caused an outbreak of
76 acute respiratory distress syndrome in China in 2003 (9). β -coronaviruses use the spike
77 protein receptor-binding domain to gain entry to target cells, and recent studies have

78 found that SARS-CoV-2 and SARS-CoV utilize the same receptor, ACE-2 (10). SARS-
79 CoV-2 entry was also found to require the expression of the cellular protease,
80 TMPRSS2 (10). ACE-2 and TMPRSS2 are expressed in epithelial tissue from the lung
81 and gut, with the highest expression in ciliated cells from the nasal cavity (11).
82 Consistently, previous studies have shown that SARS infects and replicates in primary
83 human airway epithelial (pHAE) cultures isolated from the nasal and tracheobronchial
84 regions (12).

85

86 Type I IFN is the first line of defense and is critical for blocking early virus replication,
87 spread, and tropism as well as promoting the adaptive immune response. Type I IFN
88 induces a systemic response that impacts nearly every cell in the host, while type III
89 IFNs are restricted to anatomic barriers and select immune cells (13). This selectivity is
90 due to the receptor expression patterns: type I IFN binds IFNAR1 and IFNAR2, which
91 are ubiquitously expressed. In contrast, type III IFN binds IFNLR1 and IL10-R β , which
92 are expressed preferentially on epithelial cells (13). Despite using different receptors,
93 Type I and III IFNs use similar downstream signaling complex (ISGF3) and induce
94 similar gene expression profiles. Type III IFN induces lower levels of ISG (interferon-
95 stimulated gene) expression, and at a slower rate than type I IFN (14, 15). Thus, type III
96 IFN produces a less inflammatory, localized response compared to type I IFN. The role
97 of type I and type III IFNs in restricting SARS-CoV-2 infection of lung epithelial cells has
98 not been studied.

99

100 In this study, we seek to address some of these unanswered questions about the innate
101 immune response to SARS-CoV-2. Here, we find that SARS-CoV-2 infects and
102 replicates in pHAE cultures and is released exclusively from the apical surface. We
103 performed transcriptional profiling and found that infection triggers a robust
104 inflammatory cytokine response characterized by the induction of IL-6, CXCL8, TNF- α ,
105 and IL-1 family cytokines. In contrast, we observed a lack of induction of type I IFN and
106 IFN-stimulated genes despite the expression of both type I and III IFN receptors. We
107 found that both pre- and post-treatment of pHAE cultures with type I or III IFNs reduced
108 SARS-CoV-2 replication and this was correlated with increased expression of IFN-
109 stimulated antiviral effector genes. Our studies demonstrate the utility of pHAE cultures
110 to model SARS-CoV-2 infection and identify type I and III IFNs as potential therapeutics
111 to restrict SARS-CoV-2 infection in COVID-19 patients.

112 RESULTS

113 Human bronchial airway epithelial cells are permissive to SARS-CoV-2 infection.

114 To understand the response of airway epithelial cells to SARS-CoV-2 infection, we
115 utilized pHAE cultures isolated from the bronchial or tracheal region. These cells were
116 cultured using an air-liquid interface model to create a polarized, pseudostratified
117 epithelial layer. This culture system excellently re-capitulates the unique features of the
118 human respiratory tract, including mucus production and coordinated cilia movement
119 (16). pHAE cultures were infected on the apical side with SARS-CoV-2 at an MOI of 0.1
120 and 0.25 (as determined on VeroE6 cells). In our study, we used a low cell culture-
121 passaged and sequence-verified SARS-CoV-2 strain, nCoV2019/WA, which was
122 isolated in January 2020 from nasopharyngeal and oropharyngeal swab specimens
123 collected three days post-symptom onset (17). Using two different measurements, we
124 demonstrate that SARS-CoV-2 infects and replicates in pHAE cultures. On the apical
125 surface, we detected infectious SARS-CoV-2 beginning 24 hours post-infection (p.i.)
126 and increased through 48 hours p.i. as measured by plaque assay (**Fig. 1A**). In
127 contrast, we were unable to detect infectious SARS-CoV-2 virus on the basolateral side
128 at any timepoint or MOI, suggesting the directional release of the virus from pHAE
129 cultures. Next, we confirmed the presence of viral RNA in the cells by qRT-PCR with
130 primer/probes that anneal to the SARS-CoV-2 RNA-dependent RNA polymerase
131 (RDRP). We observed an increase in viral RNA between MOI 0.1 and 0.25 at 48 hours
132 p.i. (**Fig. 1B**). Combined, these findings demonstrate that pHAE cultures are permissive
133 for SARS-CoV-2 infection.

134

135 **SARS-CoV-2 infection prompts a pro-inflammatory response in pHAE cultures.**

136 We next evaluated the innate immune response to SARS-CoV-2 infection. To this end,
137 we performed bulk mRNA-sequencing analysis on differentiated pHAE cultures infected
138 with SARS-CoV-2 (MOI= 0.25) at 48 hours p.i. Following infection, we observed 1,039
139 differentially expressed genes (DEG) ($P < 0.01$; 1.5-fold change cut-off), with 458
140 upregulated (**Fig. 2A** in red) and 581 downregulated (**Fig. 2A** in blue) DEGs. We
141 detected viral transcripts spanning most of the viral genome, although there was minor
142 variation between the three replicates (**Fig 2B**).

143

144 Next, we investigated genes associated with pro-inflammatory cytokine/chemokine
145 production and signaling. Several genes showed increased expression in SARS-CoV-2
146 infected cells as compared to mock-infected cells (**Fig 3A**; purple highlighted genes,
147 **Supplementary Table 1**). We then performed Gene Set Enrichment Analysis (GSEA)
148 using the Hallmarks data set from MSigDB. Compared to mock, SARS-CoV-2 infected
149 cells had significant enrichment for TNF α signaling, with a normalized enrichment score
150 (NES) of 1.53 and p-value < 0.0001 , and IL-6-STAT3 related signaling (NES= 1.34, $p <$
151 0.04) (**Fig. 3B**). Correspondingly, SARS-CoV-2 infected cells had increased transcript
152 expression of IL-6, TNF α and other cytokine genes, including the IL-17 family (IL17C,
153 IL23A), and the IL-1 family (IL18, IL1B) (**Fig. 3C**). Several chemokines were also
154 upregulated in SARS-CoV-2 infected cells, including molecules that promote monocyte
155 migration (CCL4, CCL5) and neutrophil migration (CXCL8, CXCL6). We also analyzed
156 genes associated with barrier immunity, which includes genes related to the production
157 of mucus and anti-microbial/-viral peptides (AMPs) (**Fig. 3D**; highlighted in orange).

158 While these genes are expressed in mock and infected cells, we observed little to no
159 change in gene expression and only a few genes approached threshold levels
160 (**Supplementary Table 2**). This suggests that SARS-CoV-2 does not alter mucus and
161 AMP production in pHAE cultures.

162

163 To determine the transcriptional regulatory network induced in SARS-CoV-2 infected
164 cells, we performed cis-regulatory sequence analysis using iRegulon to predict
165 regulatory nodes. iRegulon identifies enrichment of transcription factor binding motifs
166 within a DEG list (18). Consistent with pathway enrichment for pro-inflammatory
167 cytokines, our analysis identified NF- κ B and ATF-4 as top predicted transcriptional
168 regulators following SARS-CoV-2 infection (**Fig. 3E**). NF- κ B regulates a substantial
169 portion of the DEGs, with a network of 312 DEGs. This network includes some of the
170 top upregulated genes, such as IL-6 and CXCL8. ATF-4 has a slightly smaller enriched
171 network of 219 DEGs, that includes CHAC1, which is a key marker of the unfolded
172 protein response (**Fig. 3E; Supplementary Table 3**) (19, 20). The identification of NF-
173 κ B as a key transcriptional node is consistent with our observations that SARS-CoV-2
174 triggers cytokine induction. ATF-4 is associated with the promotion of a cellular stress
175 response. Thus, we used GSEA to investigate if ATF4 corresponds to an enrichment of
176 cellular stress pathways in SARS-CoV-2 infected cells (21). Compared to mock, SARS-
177 CoV-2 infected pHAE cultures showed an enrichment of genes related to the unfolded
178 protein response, such as ASNS, CHAC1, and STC2 (NES= 1.39, p-value< 0.0001)
179 (**Fig. 3F**). CEBP plays a critical role in maintaining epithelial barriers by regulating the
180 epithelial to mesenchymal transition (22). Accordingly, GSEA also revealed enrichment

181 of genes associated with the transition from epithelial to mesenchymal tissue (**Fig. 3F**).
182 Overall, the transcriptional profiling of infected pHAE cultures revealed that the normal
183 homeostatic functions are disrupted and they induce a pro-inflammatory phenotype
184 characterized by NF- κ B signaling, ER stress response and IL-6 production.

185

186 **SARS-CoV-2 does not induce IFN production or signaling in pHAE cultures.** Viral
187 sensing by pathogen recognition receptors (PRRs) is a key initial step in responding to
188 viral infections. Activation of PRR pathway triggers a signaling cascade that induces
189 expression of type I IFN, interferon-regulatory factor (IRF)-dependent and IFN-
190 dependent stimulated genes. First, we determined that SARS-CoV-2 induces minimal
191 type I or III IFNs at the transcript level (**Fig. 4A**). In both mock and SARS-CoV-2
192 infected samples, there was no detectable IFN α of any subtype, and low induction of
193 IFN β 1 and IFN λ 1, with normalized read counts less than 10 (**Fig. 4A**). Next, we
194 determined whether this was due to the lack of expression of molecules required for the
195 induction of IFN transcription, such as the RIG-I-like receptor signaling pathway. We
196 found that several genes within this pathway, including RIG-I, MDA5, TBK1, TRAF6,
197 IRF-3, and IRF-7 are expressed at baseline but show little to no induction in response to
198 SARS-CoV-2 (**Fig. 4B**; highlighted in green; **Supplementary Table 4**).

199

200 We observed little to no change in expression for several genes related to type I IFN
201 signaling, including transcription factors and antiviral effector genes, such as IFIT2,
202 IFIT3, IFITM1, OAS1 and MX1 (**Fig. 4C-D**; highlighted in pink; **Supplementary Table**
203 **5**). We next evaluated the expression of both the type I IFN (IFNAR1 and IFNAR2) and

204 type III IFN (IFNLR1 and IL10R β) receptors. At the transcript level, the type I and III IFN
205 receptors are present in pHAE cultures, but the expression of these receptors does not
206 change with infection (**Fig. 4E**). Combined, these data demonstrate that SARS-CoV-2
207 infection does not induce a type I or III IFN response in pHAE cultures, but these cells
208 express the signaling components to respond to type I or III IFN signaling.

209

210 **Pre-treatment with type I and type III IFN restrict SARS-CoV-2 replication.** To
211 determine whether SARS-CoV-2 is sensitive to type I and III IFNs, we pre-treated the
212 basolateral side of pHAE cultures with IFN β 1 or IFN λ 1 (100 IU/mL) for 24 hours prior to
213 infection. The next day, pHAE cultures were infected on the apical side with SARS-CoV-
214 2 (**Fig. 5A**). As compared to untreated cells, we observed significantly reduced viral
215 RNA in type I (3-fold less) and III (3-fold less) IFN treated cells by 24 hours p.i. (**Fig.**
216 **5B**). We next evaluated infectious virus release and found that pHAE cultures pre-
217 treated with type I or III IFNs significantly reduced (14-fold and 12-fold respectively)
218 SARS-CoV-2 viral burden by 24 hours p.i., resulting in greater than 90% reduction in
219 virus replication as compared to untreated SARS-CoV-2 infected cells (**Fig. 5C**).

220

221 To better understand how type I and III IFN signaling promotes restriction of SARS-
222 CoV-2 replication, we performed qRT-PCR analysis of SARS-CoV-2 infected, treated
223 and untreated cells. Both type I and III IFN treatment upregulated ISGs in uninfected
224 and infected pHAE cells. This included innate immune sensors RIG-I and MDA5, and
225 the IFIT family of antiviral effector genes (**Fig. 5D**). Changes in transcription factors
226 were also observed with increases in IRF-7 and to a lesser extent IRF-1. Treatment with

227 type I and III IFNs upregulated ISGs regardless of infection status, but SARS-CoV-2
228 infected samples had larger fold changes compared to mock-infected cells. In SARS-
229 CoV-2 infected samples, treatment with type I IFN induced higher expression of certain
230 ISGs (IFIH1, IFIT2) than type III IFN (**Fig. 5D**). These findings demonstrate that pre-
231 treatment with type I or III IFN increases antiviral effector expression and restricts
232 SARS-CoV-2 infection of pHAE cultures.

233

234 **Treatment of SARS-CoV-2 infected pHAE cultures with type I and III IFN reduces**
235 **viral burden.** We next evaluated the antiviral potential of type I and III IFNs in a
236 therapeutic model of infection. In this case, pHAE cultures were infected with SARS-
237 CoV-2 (MOI= 0.5) and, at 24 hours p.i., we treated the basolateral side with IFN β 1 or
238 IFN λ 1 (100 IU/mL). Infectious virus release was measured before and after treatment
239 (**Fig. 6A**). Twenty-four hours after treatment (48 hours p.i.) there was no significant
240 difference in viral burden between treatments. However, by 72 hours p.i., treatment with
241 both type I and III IFN had reduced SARS-CoV-2 viral levels 50-fold compared to
242 untreated samples, resulting in a 98% reduction in viral burden at 72 hours p.i. (**Fig.**
243 **6A**). Analysis of RNA at 72 hours p.i. confirmed that both IFN β 1 and IFN λ 1 treatment
244 reduced viral RNA compared to untreated cells, 12-fold and 20-fold, respectively (**Fig.**
245 **6B**). Similarly, to our pre-treated samples (**Fig. 5**), treatment after infection with type I
246 or III IFNs upregulated ISGs, such as RIG-I, MDA5, and IFIT family members,
247 compared to untreated (**Fig. 6C**). Type I and III IFN increased expression of IRF
248 transcription factors, IRF-7 and IRF-1 compared to mock. Combined, therapeutic

249 treatment with type I or III IFNs was effective at reducing viral burden, and upregulating
250 ISGs in SARS-CoV-2 infected pHAE cultures.

251

252 **DISCUSSION**

253 In this study, we found that human pHAE cultures, which model the air-liquid interface of
254 the lung, are permissive to SARS-CoV-2 infection and the virus is unilaterally released
255 from the apical surface. Transcriptional profiling revealed that SARS-CoV-2 infected
256 pHAE cultures trigger a pro-inflammatory response driven by ATF-4 and NF- κ B. This
257 analysis also suggested that the normal cellular functions of pHAE cultures were
258 disrupted during SARS-CoV-2 infection, as evidenced by the enrichment of ER stress
259 pathways. Despite having a baseline expression of viral sensing pathway components,
260 pHAE cultures did not produce type I or III IFN in response to SARS-CoV-2 infection,
261 and there was little to no induction of antiviral effector genes. However, pHAE cultures
262 are sensitive to the effects of type I and III IFN, as pre-treatment with exogenous IFN
263 was able to reduce SARS-CoV-2 burden significantly. Therapeutic administration of
264 type I and III IFNs was also effective at restricting viral replication, and induced
265 upregulation of ISGs in pHAE cultures. Thus, demonstrating that SARS-CoV-2 is
266 susceptible to both type I and type III IFN, and identifying them as potential antiviral
267 treatments.

268

269 The baseline expression of molecules such as RIG-I, MDA5, IRF-3 and IRF-7 in pHAE
270 cultures suggests that these cells are capable of producing type I and III IFN. In other
271 models of viral infection, pHAE cultures produce a robust IFN response (14, 23).
272 Previous studies with SARS-CoV, showed that it is able to block the production of type I
273 IFN, by inhibiting the phosphorylation and nuclear translocation of IRF-3, and interfering
274 with STAT signaling (24-29). However, studies of both *in vitro* and *in vivo* SARS-CoV
275 infection found that delayed production of type I IFN did occur eventually (30, 31).

276 Follow-up studies assessing whether SARS-CoV-2 uses similar mechanisms to block
277 type I and III IFN production will be essential for understanding viral antagonism of
278 innate immune signaling pathways during infection.

279
280 pHAE cultures did not produce IFN in response to SARS-CoV-2 infection. Interestingly,
281 SARS-CoV-2 was sensitive to the effects of both type I and III IFNs, resulting in reduced
282 viral burden concomitant with an upregulation of ISGs. Lung epithelial cells are highly
283 polarized, and often have differential localization of receptors, such as ACE-2, on the
284 apical vs basolateral side (32). Thus, an interesting follow-up study would be to
285 compare the differential effects of cytokine application to the apical side. While the
286 reduction in viral load was similar between both IFNs, type I IFN was able to induce
287 higher expression than type III IFN (~ 2-fold) of most ISGs in pre-treated pHAE cultures.
288 However, in our post-treated samples, this difference disappeared. Studies of the
289 differential effect of type I and III IFN in other viral infection models have shown
290 differences in both magnitude and timing of ISG and IRF transcription factor expression.
291 Type I IFN responses often peak early then decline sharply, while type III IFN
292 responses take longer to initiate and are more sustained (14, 15). Detailed analysis of
293 the transcriptional response to type I and III IFNs noted these differences are driven
294 largely by IRF-1 (33). Correspondingly, we did see higher induction of IRF-1 in type I
295 IFN treated pHAE cultures compared to type III, during SARS-CoV-2 infection. Thus,
296 while type I and III IFNs have similar effects on SARS-CoV-2 replication, type III IFN
297 might have a more localized, less inflammatory effect on the immune response.
298 Dissecting the signaling pathways of type I and type III IFN could provide a clearer

299 understanding of the innate immune response in airway epithelial cells and inform the
300 application of these cytokines as therapeutics.

301

302 In response to SARS-CoV-2 infection, pHAE cultures primarily produced pro-
303 inflammatory cytokines, which will promote localized edema, fever, and the recruitment
304 of immune cells into the lung. Many of the cytokines induced by SARS-CoV-2 infection,
305 such as CXCL6, CXCL8 and CXCL5 are chemotactic factors that primarily recruit
306 neutrophils (34). However, the recruitment of neutrophils may be of limited use in
307 controlling SARS-CoV-2, as neutrophils are not particularly effective against intracellular
308 pathogens, and have been associated with poor prognosis in other models of
309 respiratory disease (35). Several cytokines primarily associated with a T_H17 response
310 (IL-23, IL-17) were also highly upregulated in SARS-CoV-2 infected pHAE cultures.
311 While T_H17 cells are important for barrier immunity, they are most potent against
312 extracellular pathogens, and have the potential to cause chronic inflammation (36). IL-6,
313 neutrophil infiltration, and T_H17 immunity have all been previously implicated in the
314 development of fibrotic tissue in the lung (37). Additionally, SARS-CoV-2 infected pHAE
315 cultures were highly enriched in genes associated with the unfolded protein response
316 and the epithelial to mesenchymal transition, which are both pathways directly linked to
317 the formation of fibrotic tissue (38-40). The RNA-Seq data suggests that SARS-CoV-2
318 may be producing a sub-optimal immune response in pHAE cultures, as evidenced by a
319 preference for the production of cytokines whose primary function is in the defense
320 against extracellular pathogens, and the complete lack of type I and III IFN signaling.
321 Furthermore, this dysregulated immune response combined with an increase in ER

322 stress may be promoting the formation of fibrotic epithelial tissue during SARS-CoV-2
323 infection. Additional studies are needed to explore the specific role these cytokines play
324 during SARS-CoV-2 infection of the lung, and their implications in the formation of
325 fibrotic tissue.

326

327 In this study, we characterized the innate immune response to SARS-CoV-2 infection
328 using an *in vitro* model of the air-lung epithelial barrier. We found that pHAE cultures are
329 permissive to SARS-CoV-2, but mount a weak innate antiviral response, that is notably
330 lacking type I and III IFN production, signaling, and induction of ISGs. Instead, the
331 innate immune response signature of infected pHAEs is dominated by pro-inflammatory
332 cytokines and chemokines that will recruit neutrophils and monocytes. Most importantly,
333 therapeutic treatment of pHAE cultures with type I and III IFNs are able to reduce
334 SARS-CoV-2 viral burden. Overall, these data suggest that pHAE cultures mount a
335 misdirected innate immune response to SARS-CoV-2 infection, but the early
336 administration of type I or III IFN could potentially decrease virus replication and
337 disease.

338

339

340 **MATERIALS AND METHODS:**

341 **Viruses and cells.** SARS-CoV-2 (2019-nCoV/USA_WA1/2020) was isolated from the
342 first reported case in the US (17). A plaque purified passage 4 stock was kindly
343 provided by Dr. Natalie Thornburg (CDC, Atlanta, GA). Viral titers were determined by
344 plaque assay on VeroE6 cells (ATCC). Vero cells were cultured in complete DMEM
345 medium consisting of 1x DMEM (Corning Cellgro), 10% FBS, 25 mM HEPES Buffer
346 (Corning Cellgro), 2 mM L-glutamine, 1mM sodium pyruvate, 1x Non-essential Amino
347 Acids, and 1x antibiotics.

348

349 **Quantification of infectious virus.** For plaque assays, 10-fold dilutions of viral
350 supernatant in serum-free DMEM (VWR, #45000-304) were overlaid on VeroE6
351 monolayers and adsorbed for 1 hour at 37°C. After adsorption, 0.8% Oxoid Agarose in
352 2X DMEM supplemented with 10% FBS (Atlanta Biologics) and 5% sodium bicarbonate
353 was overlaid, and cultures were incubated for 72 hours at 37°C. Plaques were
354 visualized using crystal violet staining (70% methanol in ddH₂O). For focus-forming
355 assays, 10-fold dilutions of viral supernatant on VeroE6 cells were incubated with a
356 methylcellulose overlay (1.0% methylcellulose in 2X DMEM) for 24 hours at 37°C.
357 Methylcellulose was then removed, cells were fixed with 2%-PFA and permeabilized
358 with 0.1% BSA-Saponin in PBS. Cells were incubated with an anti-SARS-CoV-2 spike
359 protein primary antibody conjugated to biotin (generously provided by Dr. Jens
360 Wrammert, Emory University) for 2 hours at room temperature (RT), then with avidin-
361 HRP conjugated secondary antibody for 1 hour at RT. Foci were visualized using True
362 Blue HRP substrate and imaged on an ELI-SPOT reader.

363

364 **Generation of pHAE cultures.** pHAE cultures were kindly provided by Dr. C. U. Cotton
365 (Case Western Reserve University) and cultured, as described previously (16). Briefly,
366 after initial expansion in F media supplemented with ROCK inhibitor (Selleck Chemical
367 LLC), bronchial lung specimens were seeded on Transwell permeable support inserts
368 (Costar-Corning), cultured until confluent then transferred to an air/liquid interface.
369 Cultures were differentiated for 3 weeks and maintained in DMEM/Ham's F12 media
370 differentiation media supplemented with 2% of Ultrosor G (Pall Corp., France), until they
371 had TEER measurements greater than 1000 Ω and were deemed ready for use.

372

373 **SARS-CoV-2 infection of pHAE cultures.** Prior to infection, the apical side of the
374 pHAE cultures was washed 3 times with PBS. Virus was diluted to the specified MOI in
375 PBS and allowed to adsorb for 1 hour at 37°C. After adsorption, the apical side was
376 washed 3 times with PBS to remove excess virus, and the basolateral media was
377 changed. To collect viral supernatant, PBS was added to the apical side and incubated
378 for 30 minutes at 37°C. Treatment of pHAE cultures with type I or type III IFN was
379 performed by adding 100 IU/mL of human IFN β or human IFN λ 1 (PBL Assay Science).

380

381 **RNA sequencing and bioinformatics.** pHAE cultures were infected at MOI= 0.25 for
382 48 hours. RNA was harvested from mock and infected pHAE cultures (n=3) by gently
383 scraping the Transwell insert with a plunger to dislodge the cells, which were then lysed
384 by treatment with RNA lysis buffer for > 5 minutes. Total RNA was extracted using the

385 Zymo Quick-RNA MiniPrep kit according to the manufacturers protocol. mRNA
386 sequencing libraries were prepared by the Yerkes Genomics Core
387 (http://www.yerkes.emory.edu/nhp_genomics_core/). Libraries were sequenced on an
388 Illumina NovaSeq 6000 and mapped to a merged reference of the hg38 human
389 reference genome. Viral genes were mapped to the FDAARGOS_983 strain of the
390 2019-nCoV/USA-WA1/2020 SARS-CoV2 isolate (GenBank Accession: MT246667.1),
391 using STAR v2.7.3a (41). Reads were normalized and differentially expressed genes
392 were analyzed using DESeq2. Gene set enrichment analysis was performed using the
393 software provide by the Broad Institute and the MSigDB database. Pathway analysis
394 was performed using Cytoscape software. The raw data of all RNA sequencing will be
395 deposited into the Gene Expression Omnibus (GEO) repository and the accession
396 number will be available following acceptance of this manuscript.

397

398 **Quantitative reverse transcription-PCR (qRT-PCR).** RNA was extracted from the
399 pHAEC cultures using the method described above. Purified RNA was reverse
400 transcribed into cDNA using the high-capacity cDNA reverse transcription kit (Thermo
401 Fisher, 43-688-13). RNA levels were quantified using the IDT PrimeTime Gene
402 Expression Master, and Taqman gene expression Primer/Probe sets. All qPCR was
403 performed in 384- well plates and run on a QuantStudio5 qPCR system. To quantify
404 viral RNA, SARS-CoV-2 RDRP specific primers (F:GTGARATGGTCATGTGTGGCGG ,
405 R:CARATGTTAAASACACTATTAGCATA) and probe (56-
406 FAM/CAGGTGGAA/ZEN/CCTCATCAGGAGATGC/3IABkFQ) were used. The following
407 Taqman Primer/ Probe sets were used in this analysis: Gapdh (Hs02758991_g1), IFIT2

408 (Hs01922738_s1), IFIT3 (Hs01922752_s1), DDX58 (Hs01061436_m1), IFIH1
409 (Hs00223420_m1), OAS1 (Hs00973637_m1), IRF1 (Hs00971960_m1), IRF7
410 (Hs01014809_g1), MX1 (Hs00895608_m1). C_T values were normalized to the reference
411 gene GAPDH and represented as fold change over mock.

412

413 **Statistical Analysis.** Statistical analyses were performed using Graphpad Prism 8,
414 ggplot2 R package, and GSEA software. Statistical significance was determined as
415 P<0.05 using a student's t-test (when comparing two groups lacking paired
416 measurements) or a one-way ANOVA test (when comparing more than two groups
417 lacking paired measurements), All comparisons were made between treatment or
418 infection conditions with time point matched, uninfected and untreated controls.

419

420

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439

440 **Author contributions:**

441 A.V., P.R., and M.S. contributed to the acquisition, analysis, and interpretation of the
442 data, T.C. M.Z. and A.G. contributed to the acquisition and interpretation of the data,
443 A.U., K.P., and S.E.B. contributed to the acquisition and analysis of the data, S.B., A.L.,

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445 S.E.B. contributed to the interpretation of the data, as well as the conception and design
446 of the work. A.V., P.R., A.G., and M.S. contributed to the acquisition, analysis, and
447 interpretation of the data, as well as the conception and design of the work, and writing
448 the manuscript.

449

450 **Declaration of Interest:** The authors declare no competing interests.

451

452

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- 600
- 601

602 **FIGURE LEGENDS**

603 **Figure 1. pHAE cultures are permissive to SARS-CoV-2 infection.** A) Healthy,
604 differentiated pHAE cultures were infected by adsorption to the apical side at the
605 indicated MOI. The supernatant was collected from the apical or basolateral side of the
606 epithelial layer, and the virus was measured by plaque assay. B) Viral RNA was
607 measured by probing for the SARS-CoV-2 RDRP RNA at 48 hours p.i. by qRT-PCR. C_T
608 values are represented as relative fold change over mock (\log_{10}). All experiments were
609 repeated twice with biological triplicates.

610

611 **Figure 2. Bulk RNA-Seq analysis of SARS-CoV-2 infected pHAE cultures.** pHAE
612 cultures were infected apically with SARS-CoV-2 (MOI= 0.25) for 48 hours, at which
613 point mock and SARS-CoV-2 infected (n=3) samples were harvested for bulk RNA-Seq
614 analysis. A) Volcano plot demonstrating DEGs. Lines indicate cut-offs; p-value <0.01,
615 fold-change <-1.5 or >1.5. Highlighted in red are the most highly upregulated genes (p-
616 value <0.001, fold-change >1.5), in blue are most highly downregulated genes (p-value
617 <0.001, fold-change < -1.5). B) Normalized read counts (\log_2) of SARS-CoV-2 RNA
618 products, using the MT246667.1 reference sequence.

619

620 **Figure 3. SARS-CoV-2 infection promotes a pro-inflammatory and ER stress**
621 **response in pHAE cultures.** pHAE cultures were infected apically with SARS-CoV-2
622 (MOI= 0.25) for 48 hours, at which point mock and SARS-CoV-2 infected (n=3) samples
623 were harvested for bulk RNA-Seq analysis. For global DEG analysis see figure 2. A)

624 Volcano plot with all DEGs in grey and the indicated gene set highlighted (purple= pro-
625 inflammatory signaling). B) GSEA plots of the enrichment score plotted against gene
626 rank. Individual gene hits are indicated by the solid black line below the enrichment
627 score curve. NES and p-value are indicated on the plot. Gene sets are from the
628 Hallmarks gene set from MSigDB. C) Heatmap illustrating z-scores for the indicated
629 genes in mock and SARS-CoV-2 infected samples D) Volcano plot illustrating barrier
630 immunity associated genes in orange. E) Network map illustrating regulatory nodes for
631 our DEGs. F) GSEA plots for the indicated gene sets.

632

633 **Figure 4. pHAE cultures fail to upregulate type I or III IFNs in response to SARS-**
634 **CoV-2 infection.** RNA-Seq analysis of the IFN response, for global DEG analysis see
635 figure 2. A) Normalized read counts (\log_2) in mock (white) and SARS-CoV-2 infected
636 (grey samples) of type I and type III IFN. Volcano plots illustrating all genes in grey and
637 B) genes associated with IFN production in green C) genes associated with IFN
638 signaling in pink. D) Bar graphs indicating the normalized read count (\log_2) for
639 interferon-stimulated genes and E) type I and type III IFN receptors for mock and
640 SARS-CoV-2 infected samples.

641

642 **Figure 5. Pre-treatment with type I or III IFNs restricts SARS-CoV-2 replication in**
643 **pHAE cultures.** pHAE cultures were pre-treated from the basolateral side with IFN β 1 or
644 IFN λ 1 (100 IU/mL) for 24 hours. Cultures were then infected apically (MOI= 0.25) and
645 harvested at 24 hours p.i. A) Experimental schematic. B) SARS-CoV-2 viral burden in

646 untreated, IFN β 1, and IFN λ 1 treated cultures as assessed via focus forming assay.
647 Percent reduction was calculated as the percent of the untreated sample at 24 hours p.i.
648 qRT-PCR analysis was performed at 24 hours p.i. for C) viral RNA, or D) ISGs. qRT-
649 PCR data are represented as fold change over mock, untreated pHAEC samples. Data is
650 representative of two independent experiments performed in biological triplicate. All
651 data were analyzed using one-way ANOVA. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

652

653 **Figure 6. Post-treatment with type I or III IFNs decreases viral burden in pHAEC**
654 **cultures.** pHAEC cultures were infected with SARS-CoV-2 (MOI = 0.5) apically. Twenty-
655 four hours p.i., cultures were treated from the basolateral side with IFN β 1 or IFN λ 1 (100
656 IU/mL). 72 hours p.i. (48 hours post-treatment), cultures were harvested for qRT-PCR
657 analysis. A) SARS-CoV-2 burden was assessed via FFA for the apical side of
658 untreated, IFN β 1, and IFN λ 1 treated cultures. Percent reduction was calculated for the
659 72-hour timepoint. qRT-PCR analysis at 72 hours p.i. as compared to mock, untreated
660 samples, measuring B) viral RNA, or C) ISGs. qRT-PCR data are represented as fold
661 change over mock. Results are representative of two independent experiments
662 performed in triplicate. Growth curves were analyzed using a two-way ANOVA, qRT-
663 PCR data were analyzed using one-way ANOVA. * = $p < 0.05$, ** = $p < 0.01$, *** =
664 $p < 0.001$.

665

666

667 **SUPPLEMENTAL FIGURE LEGENDS**

668 **Supplemental Table 1. Pro-inflammatory associated gene expression in pHAE**
669 **cultures.** List of the pro-inflammatory genes surveyed in pHAE cultures. Fold-changes
670 (\log_2) are calculated over mock, with the corresponding p-value.

671
672 **Supplemental Table 2. Barrier immunity associated with gene expression in pHAE**
673 **cultures.** List of the barrier immunity associated genes surveyed in pHAE cultures.
674 Fold-changes (\log_2) are calculated over mock, with the corresponding p-value.

675
676 **Supplemental Table 3. NF-kB, ATF-4 and CEBPB associated genes.** List of genes
677 promoted by the NF-kB, ATF-4 and CEBPB transcription factors. Fold-changes (\log_2)
678 are calculated over mock, with the corresponding p-value.

679
680 **Supplemental Table 4. Genes associated with the induction of interferon in pHAE**
681 **cultures.** List of the PRR signaling genes surveyed in pHAE cultures. Fold-changes
682 (\log_2) are calculated over mock, with the corresponding p-value.

683
684 **Supplemental Table 5. Interferon stimulated and IFN receptor-associated genes in**
685 **pHAE cultures.** List of the genes associated with IFN signaling and ISGs surveyed in
686 pHAE cultures. Fold-changes (\log_2) are calculated over mock, with the corresponding p-
687 value.

Figure 1

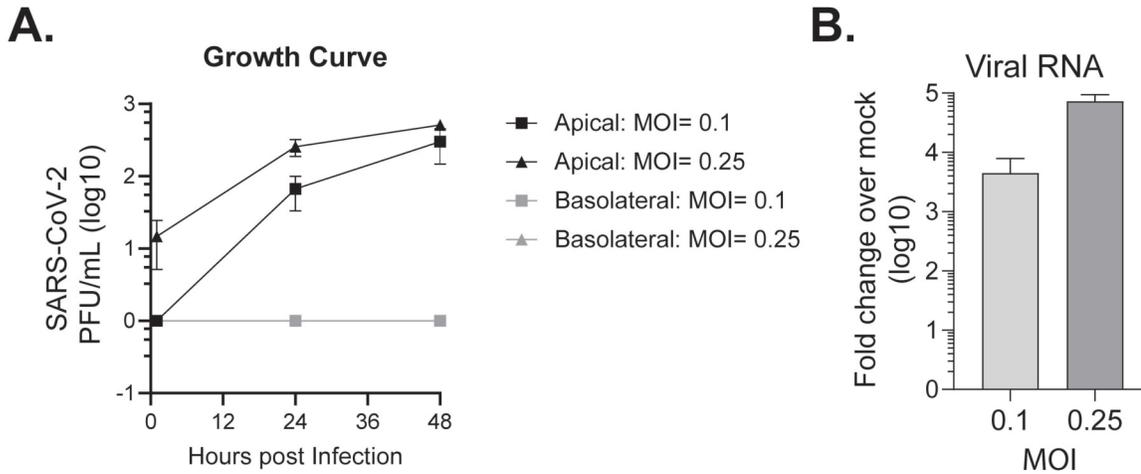
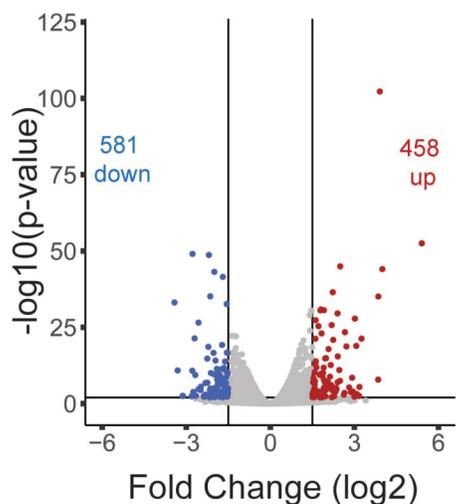


Figure 1. pHAE cell cultures are permissive to SARS-CoV-2 infection. A) Healthy, differentiated pHAE cell cultures were infected by adsorption to the apical side at the indicated MOI. The supernatant was collected from the apical or basolateral side of the epithelial layer, and the virus was measured by plaque assay. B) Viral RNA was measured by probing for the SARS-CoV-2 RDRP RNA at 48 hours p.i. by qRT-PCR. CT values are represented as relative fold change over mock (log10). All experiments were repeated twice with biological triplicates.

Figure 2

A.



B.

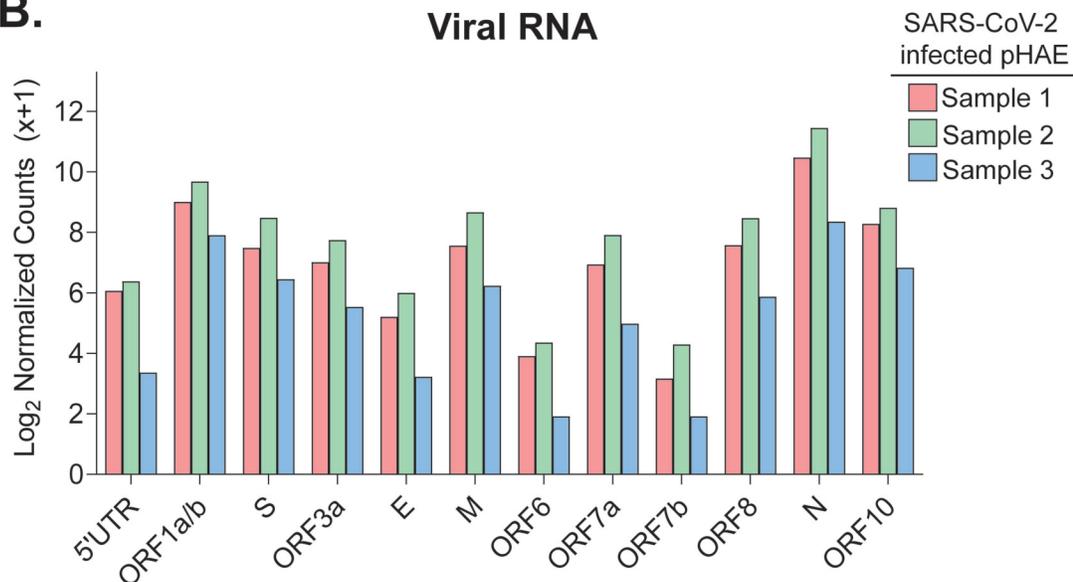


Figure 2. Bulk RNA-Seq analysis of SARS-CoV-2 infected pHAE cell cultures. pHAE cell cultures were infected apically with SARS-CoV-2 (MOI= 0.25) for 48 hours, at which point mock and SARS-CoV-2 infected (n=3) samples were harvested for bulk RNA-Seq analysis. A) Volcano plot demonstrating DEGs. Lines indicate cut-offs; p-value <0.01, fold-change <-1.5 or >1.5. Highlighted in red are the most highly upregulated genes (p-value <0.001, fold-change >1.5), in blue are most highly downregulated genes (p-value <0.001, fold-change < -1.5). B) Normalized read counts (log₂) of SARS-CoV-2 RNA products, using the MT246667.1 reference sequence.

Figure 3

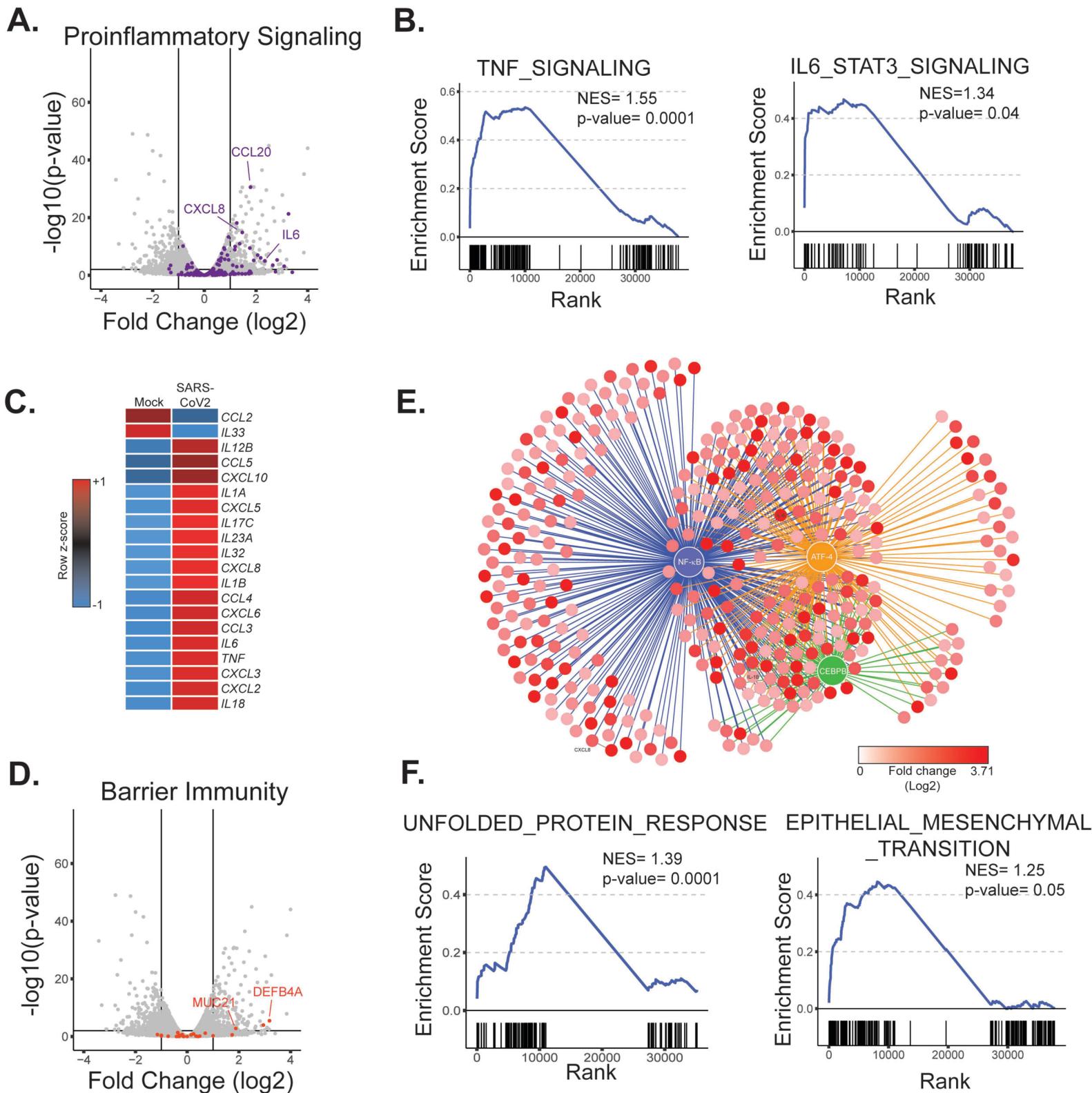


Figure 3. SARS-CoV-2 infection promotes a pro-inflammatory and ER stress response in pHAEC cell cultures. pHAEC cell cultures were infected apically with SARS-CoV-2 (MOI= 0.25) for 48 hours, at which point mock and SARS-CoV-2 infected (n=3) samples were harvested for bulk RNA-Seq analysis. For global DEG analysis see figure 2. A) Volcano plot with all DEGs in grey and the indicated gene set highlighted (purple= pro-inflammatory signaling). B) GSEA plots of the enrichment score plotted against gene rank. Individual gene hits are indicated by the solid black line below the enrichment score curve. NES and p-value are indicated on the plot. Gene sets are from the Hallmarks gene set from MSigDB. C) Heatmap illustrating z-scores for the indicated genes in mock and SARS-CoV-2 infected samples D) Volcano plot illustrating barrier immunity associated genes in orange. E) Network map illustrating regulatory nodes for our DEGs. F) GSEA plots for the indicated gene sets.

Figure 4

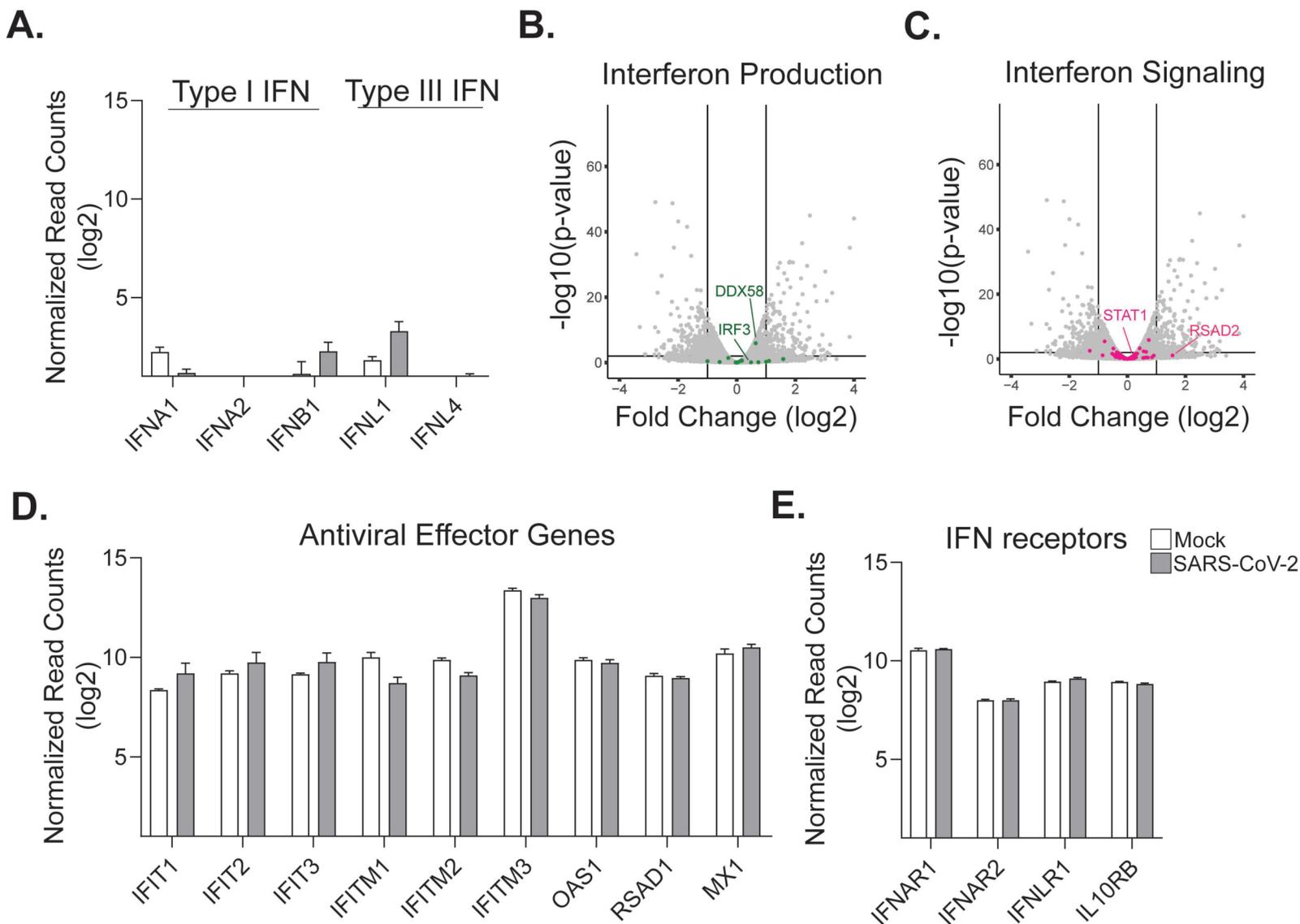


Figure 4. pHAEC cell cultures fail to upregulate type I or III IFNs in response to SARS-CoV-2 infection.

RNA-Seq analysis of the IFN response, for global DEG analysis see figure 2. A) Normalized read counts (log₂) in mock (white) and SARS-CoV-2 infected (grey samples) of type I and type III IFN. Volcano plots illustrating all genes in grey and B) genes associated with IFN production in green C) genes associated with IFN signaling in pink. D) Bar graphs indicating the normalized read count (log₂) for interferon-stimulated genes and E) type I and type III IFN receptors for mock and SARS-CoV-2 infected samples.

Figure 5

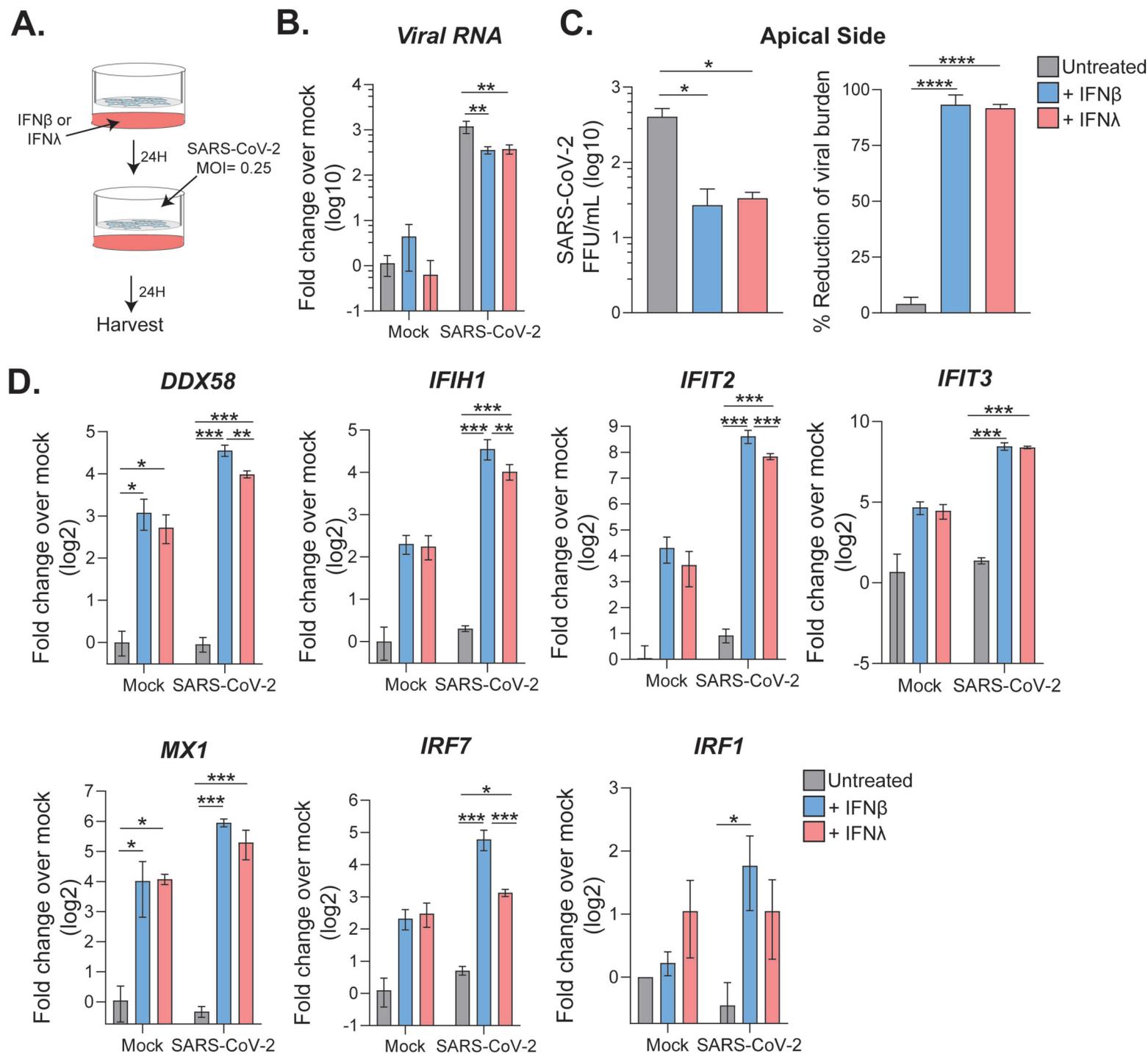


Figure 5. Pre-treatment with type I or III IFNs restricts SARS-CoV-2 replication in pHAEC cell cultures. pHAEC cell cultures were pre-treated from the basolateral side with IFN β 1 or IFN λ 1 (100 IU/mL) for 24 hours. Cultures were then infected apically (MOI= 0.25) and harvested at 24 hours p.i. A) Experimental schematic. B) SARS-CoV-2 viral burden in untreated, IFN β 1, and IFN λ 1 treated cultures as assessed via focus forming assay. Percent reduction was calculated as the percent of the untreated sample at 24 hours p.i. qRT-PCR analysis was performed at 24 hours p.i. for C) viral RNA, or D) ISGs. qRT-PCR data are represented as fold change over mock, untreated pHAEC samples. Data is representative of two independent experiments performed in biological triplicate. All data were analyzed using one-way ANOVA. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Figure 6

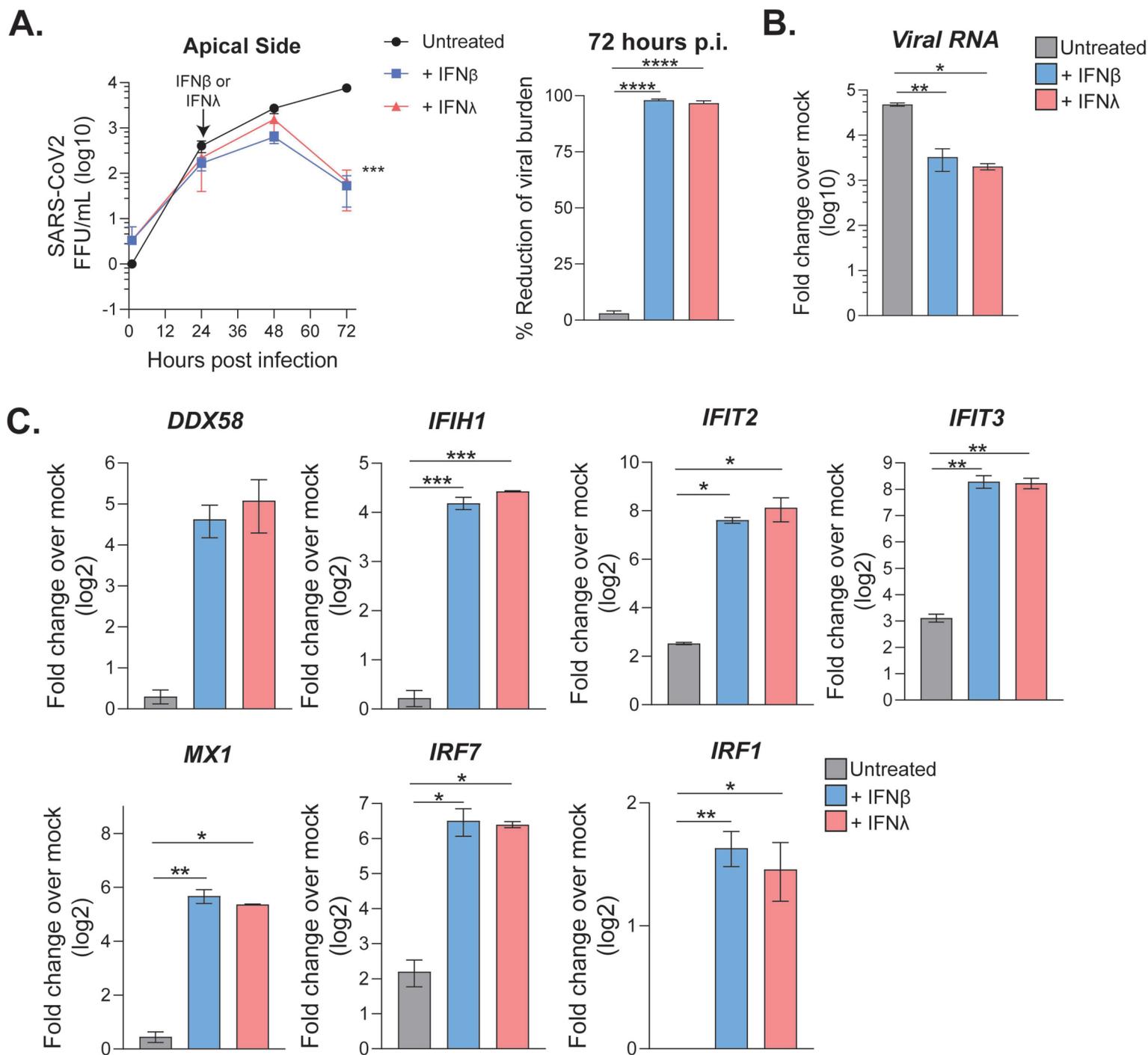


Figure 6. Post-treatment with type I or III IFNs decreases viral burden in pHAE cell cultures. pHAE cell cultures were infected with SARS-CoV-2 (MOI = 0.5) apically. Twenty-four hours p.i., cultures were treated from the basolateral side with IFN β 1 or IFN λ 1 (100 IU/mL). 72 hours p.i. (48 hours post-treatment), cultures were harvested for qRT-PCR analysis. A) SARS-CoV-2 burden was assessed via FFA for the apical side of untreated, IFN β 1, and IFN λ 1 treated cultures. Percent reduction was calculated for the 72-hour timepoint. qRT-PCR analysis at 72 hours p.i. as compared to mock, untreated samples, measuring B) viral RNA, or C) ISGs. qRT-PCR data are represented as fold change over mock. Results are representative of two independent experiments performed in triplicate. Growth curves were analyzed using a two-way ANOVA, qRT-PCR data were analyzed using one-way ANOVA. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.