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1 Cigarette smoke preferentially induces full length ACE2 exposure in primary human airway
2 cells but does not alter susceptibility to SARS-CoV-2 infection
3

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18
19

20 Abstract
21

22 Cigarette smoking has multiple serious negative health consequences. However, the
23 epidemiological relationship between cigarette smoking and SARS-CoV-2 infection is
24 controversial; and the interaction between cigarette smoking, airway expression of the ACE2
25 receptor and the susceptibility of airway cells to infection is unclear. We exposed

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26 differentiated air-liquid interface cultures derived from primary human airway stem cells to
27 cigarette smoke extract (CSE) and infected them with SARS-CoV-2. We found that CSE
28 increased expression of full-length ACE2 (flACE2) but did not alter the expression of a Type
29 I-interferon sensitive truncated ACE2 that lacks the capacity to bind SARS-CoV-2 or a panel
30 of interferon-sensitive genes. Importantly, exposure to CSE did not increase viral infectivity
31 despite the increase in flACE2. Our data are consistent with epidemiological data suggesting
32 current smokers are not at excess risk of SARS-CoV-2 infection. This does not detract from
33 public health messaging emphasising the excess risk of severe COVID-19 associated with
34 smoking-related cardiopulmonary disease.
35
36 150 words (Target 150)

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37 Introduction

38 SARS-CoV-2 is the causative agent of coronavirus disease 2019 (COVID-19). The SARS-CoV-2
39 envelope spike (S) protein is essential for virus attachment and cell entry via the main
40 cellular receptor - angiotensin converting enzyme 2 (ACE2) (Kuba et al. 2005; Wang et al.
41 2020; Daly et al. 2020; Li et al. 2003). Entry is further dependent on S-protein priming by
42 TMPRSS2 facilitating fusion of viral and cellular membranes (Hoffmann et al. 2020; Walls et
43 al. 2020; Shang et al. 2020).

44 ACE2 is a membrane-associated aminopeptidase expressed in a range of tissues including
45 vascular endothelia, ureteric epithelia and the small intestine (Harmer et al. 2002; Hamming
46 et al. 2004; Zou et al. 2020; Hikmet et al. 2020). In the renin-angiotensin-aldosterone system
47 (RAAS), it converts the vasoconstrictive hormone angiotensin-II to the vasodilator Ang 1-7
48 but has other physiological roles in glucose homeostasis and beta cell function (Jiang et al.
49 2014) (Niu et al. 2008; Bindom et al. 2010).

50

51 The physiological role for ACE2 at homeostasis in airway epithelial cells is unknown. In
52 preclinical murine models ACE2 was confirmed as the epithelial receptor for SARS-CoV-1
53 and was shown to confer protection against SARS-CoV-1 associated acute lung injury (Imai
54 et al. 2005; Kuba et al. 2005; Ren et al. 2006). However, the mechanism by which ACE2
55 mediates protection is unclear and may relate to its role in the pulmonary vascular
56 endothelium rather than the airway epithelium (Imai et al. 2005). In transcriptomic studies
57 of the human respiratory tract and lung, there is a proximal-distal ACE2 mRNA expression
58 gradient; expression is highest in the nasal epithelium and lower distally in the alveolar
59 epithelium, mirroring the permissiveness to SARS-CoV-2 infection (Sungnak et al. 2020;

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60 Lukassen et al. 2020; Ziegler et al. 2020; Hou et al. 2020) (Zamorano Cuervo and Grandvaux
61 2020). The distribution of ACE2 protein expression is less well characterised due to the
62 paucity of validated reagents but is consistent with the proximal-distal graded expression
63 pattern (Ortiz et al. 2020; Hikmet et al. 2020). Notably, ACE2 expression in the lung is not
64 altered by ACE inhibitors or angiotensin receptor blockers (Lee et al. 2020).
65
66 In addition, an N-terminally truncated (dACE2) isoform that is sensitive to interferon
67 stimulation or viral infection has been detected (Onabajo et al. 2020; Ng et al. 2020; Blume
68 et al. 2021; Shajahan et al. 2020). Importantly, dACE2 does not express the SARS-CoV-2
69 spike-protein binding domain and its relevance in SARS-CoV-2 infection and normal
70 physiology remains unclear (Onabajo et al. 2020; Blume et al. 2021).
71
72 The impact of ACE2 expression on COVID-19 incidence and severity is unclear (Chung et al.
73 2020). Much of the available information on ACE2 expression is from scRNA databases and
74 has not reported the relative expression of the two isoforms. Given the differential isoform
75 binding to SARS-CoV-2, this is likely to be an important issue.
76
77 Smoking has been associated with increased ACE2 expression in rodent models and in
78 studies on human subjects (Gebel et al. 2010; Hung et al. 2016; Yilin, Yandong, and Faguang
79 2015; Cai et al. 2020; Brake et al. 2020; Leung et al. 2020; Smith et al. 2020). Consistent with
80 this, higher ACE2 transcripts have been detected in the lungs of COPD patients (Cai et al.
81 2020; Jacobs et al. 2020; Leung et al. 2020; Smith et al. 2020) and in airway cells from
82 occasional or “social” smokers exposed to 3 cigarettes over a 24hr period (Aliee et al. 2020).

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83 Importantly, studies to date have not linked RNASeq data with protein and isoform
84 expression.
85
86 The epidemiological data regarding the association of smoking with COVID-19 is conflicting
87 and controversial (Farsalinos et al. 2020; Simons et al. 2020; Rossato et al. 2020; Hopkinson
88 et al. 2021; Grundy et al. 2020; Patanavanich and Glantz 2020). However, an extensive and
89 'living' meta-analysis undergoing regular updates as the evidence improves, suggests that
90 current smoking is not associated with an increased risk of SARS-CoV-2 infection (Simons et
91 al. 2020). Large surveys have suggested that chronic respiratory disease including COPD
92 (mainly a smoking-related disease in the UK) may be associated with an increased risk of
93 severe COVID-19 (Docherty et al. 2020; Williamson et al. 2020) . This may be consistent with
94 the observation in the living meta-analysis that former smokers were at an increased risk of
95 severe COVID-19 (Simons et al. 2020; Zhao et al. 2020).
96
97 Differentiated human airway epithelial cells grown at the air-liquid-interface (ALI) are the
98 optimal laboratory system in which to model the impact of smoking on the early stages of
99 SARS-CoV-2 infection (Blume et al. 2021; Purkayastha et al. 2020; Sachs, Finkbeiner, and
100 Widdicombe 2003). Purkayastha and colleagues recently reported brief exposure of
101 primary airway cultures (3 minute/day for four days) to "headspace" cigarette smoke (CS),
102 that is CS in a closed environment, to investigate the impact of CS on ACE2 expression and
103 SARS-CoV-2 infection (Purkayastha et al. 2020). A significant increase in ACE2 expression
104 was not detected in response to CS, in contrast to the available molecular epidemiological
105 data detailed above. However, they reported that CS exposure did increase viral infection at
106 48 hours.

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107

108 We now report our observations from experiments in which we exposed primary human
109 bronchial epithelial cells (HBECs) at ALI to cigarette smoke extract (CSE). We find that HBECs
110 upregulate ACE2 expression in response to CSE – consistent with the molecular
111 epidemiological evidence (Cai et al. 2020; Brake et al. 2020). However, this did not lead to
112 an increase in infection by SARS-CoV-2 despite preferential upregulation of full-length ACE2
113 receptor (flACE2) rather than the N-terminal truncated isoform in response to CSE exposure.
114 This suggests that in normal human bronchial epithelial cells physiological expression of
115 flACE2 does not limit viral infectivity. We go on to show that dACE2 is a Type I interferon-
116 sensitive gene in primary HBECs, and define the impact of CSE, nicotine and NRF2 agonists
117 on ACE2 isoform expression.

118

119 This study directly addresses one of the controversies in the link between smoking and
120 SARS-CoV-2. Our results are consistent with epidemiological evidence suggesting that
121 current smoking is not associated with a higher incidence of SARS-CoV-2 infection.

122

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123

124 **Results**

125

126 **ACE2 is expressed on differentiated ciliated cells at homeostasis**

127 Previous studies have shown ACE2 expression increases following differentiation at the air-
128 liquid interface (ALI), but could be reversed if cultures were resubmerged (Jia et al. 2005).
129 We grew HBECs from Donor 1 at the air-liquid interface (ALI) for a minimum of 4 weeks to
130 produce a well-differentiated, pseudostratified mucociliary epithelium (**Figure 1a**). ACE2
131 mRNA expression was increased on differentiation, as was TMPRSS2, the main cellular
132 protease implicated in SARS-CoV-2 spike protein cleavage and FOXJ1, a key transcription
133 factor regulating airway ciliation. Full length ACE2 protein was not detectable in submerged
134 HBEC cultures but was readily detectable on differentiation (**Figure 1b-d**) emphasising the
135 importance of using differentiated HBECs to model airway infection. Confocal imaging
136 demonstrated apical ACE2 expression colocalising with markers of ciliated cells but not
137 goblet cells (**Figure 1 e,f**) (Sims et al. 2005).

138

139 **Cigarette smoke extract increases ACE2 expression in differentiated HBECs**

140

141 We then exposed differentiated ALI cultures from Donor 1 to 10% cigarette smoke extract
142 (CSE) for 48 h before harvesting cells (**Figure 2a**). CSE exposure induced a significant
143 increase in ACE2 (mRNA) and marked induction at the protein level relative to controls
144 (**Figures 2b** and **2c**). We evaluated ACE2 immunofluorescence after CSE exposure which was
145 consistent with increased apical ACE2 expression relative to control wells (**Supp Figure 1**).

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146 Increased ACE2 levels were also detected from differentiated ALI cultures derived from
147 Donor 2, a former smoker (61 pack-years) with COPD (**Supplementary Figure 2)**. Of note,
148 there was no evidence of cytotoxicity in response to CSE, as exposure did not cause an
149 increase in apoptosis or necrosis as shown by flow cytometric analysis (**Figure 2d;**
150 **Supplementary Figure 3**) or an obvious cytopathic effect on histology (**Figure 2e**).
151 Importantly, given prior data on the impact of nicotine on ACE2 expression in submerged
152 undifferentiated bronchial epithelial cells, (Russo et al. 2020), we found that nicotine did not
153 significantly alter the expression (mRNA) of either ACE2 or lead to a consistent change in
154 ACE2 protein expression (**Supplementary Figure 4**). CSE had a negligible effect on the
155 expression of the predominant nicotinic acetylcholine receptor expressed on airway
156 epithelial cells – CHRNA7 (**Figure 2f,g)**.
157

158 **HBECs exposed to CSE are not more susceptible to infection by SARS-CoV-2**
159

160 To determine whether CSE exposure would render the cells more susceptible to SARS-CoV-2
161 infection, differentiated ALI cultures (Donor1) were pre-treated with CSE for 48 h, then
162 inoculated with SARS-CoV2 for 3 h and harvested after 72 hours for flow cytometric
163 quantitation of infection or immunofluorescence (IF) (**Figure 3a**).
164

165 ACE2 colocalised with spike protein in infected wells (**Figure 3b**) consistent with ciliated cells
166 being more susceptible to infection and with prior reports (Sims et al. 2005; Schaefer et al.
167 2020). On some specimens infected cells appeared to be extruded from the epithelial
168 surface – as previously reported for SARS-CoV-1 and 2 (Sims et al. 2005).
169

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170 Importantly, despite the increased ACE2 levels following CSE exposure, there was no
171 significant difference in the total infected fraction or infected ciliated cell fraction between
172 control or CSE exposed cells (**Figure 3c**). Therefore, in this model CSE exposure did not
173 increase SARS-CoV-2 infection.
174
175 **Regulation of ACE2 isoform expression – impact of cigarette smoke**
176
177 The relationship between cigarette smoke exposure and viral infections has previously been
178 investigated using primary cells – for example cigarette smoke was associated with
179 increased rhinovirus infection in submerged cultures (Eddleston et al. 2011) or influenza A
180 infection in differentiated ALI cultures (Duffney et al. 2018). However, SARS-CoV-2, in
181 contrast to typical respiratory viruses, is associated with an attenuated cellular interferon
182 response (Blanco-Melo et al. 2020). Of note, a truncated isoform of ACE2 denoted as dACE2
183 was recently implicated as an interferon-sensitive gene (ISG), but importantly predicted to
184 not act as a receptor for SARS-CoV-2 based on the reported ACE2-Spike receptor binding
185 domain interaction (Blume et al. 2021; Onabajo et al. 2020; Ng et al. 2020; Li et al. 2005; Lan
186 et al. 2020).
187
188 We therefore explored the impact of cigarette smoke exposure on ACE2 isoform expression.
189 using recently described tools (**Figure 4a**) (Onabajo et al. 2020). We first assessed the
190 relative expression of flACE2 and dACE2 in submerged primary HBECs compared to
191 differentiated HBECs at ALI. dACE2 was upregulated on differentiation but modestly
192 compared to the full-length receptor (**Figure 4b**).
193

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194 We next tested isoform-specific expression of ACE2 exposed HBECs to Type I and Type II
195 interferons and CSE. IFN-α (Type I) but not IFN-γ (Type II) led to a transcriptional
196 upregulation of dACE2 (mRNA). Neither interferon significantly altered the expression of the
197 full-length transcript (**Figure 4c**). This extends the recently published data from
198 immortalised airway cells (Blume et al. 2021) and confirms that the N-terminus truncated
199 transcript (dACE2) is an interferon-sensitive isoform. Further, CSE did not significantly alter
200 the expression of a panel of interferon-sensitive genes (**Suppl Figure 5**). Therefore,
201 differentiated airway epithelial cells have the capacity to respond to Type I interferon and
202 CSE does not mimic that response.
203
204 CSE did not significantly alter the expression of dACE2 mRNA (**Figure 4d**). In agreement with
205 the transcriptional data, both N & C-terminus ACE2 antibodies (**Figure 4a**) showed that CSE
206 consistently upregulated flACE2 protein but had no impact on an ACE2 band migrating at
207 52kd - the predicted molecular weight of dACE2 (**Figure 4e and Suppl Figure 6**) (Blume et al.
208 2021). We conclude from these experiments that cigarette smoke does not activate
209 interferon signalling or ISGs in normal human bronchial epithelial cells and preferentially
210 upregulates flACE2 rather than dACE2.
211
212 **Antioxidants upregulate ACE2 in differentiated airway epithelial cells**
213
214 Nuclear factor erythroid 2–related factor 2 (NRF2) is the master transcriptional regulator of
215 the cellular antioxidant response and already a focus of therapeutic efforts to counteract
216 epithelial oxidative stress in COPD. NRF2 agonists have also been proposed as therapeutics
217 for COVID-19 (Olagnier et al. 2020). In our experiments, CSE treatment of ALI cultures led to

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218 the expected increase in NRF2 as well as ACE2 upregulation **(Figure 5a)**. Further, oltipraz, a
219 KEAP1 inhibitor and NRF2 agonist already in Phase III clinical trials, increased both ACE2
220 mRNA expression and flACE2 protein **(Figure 5b-d)**. This was a consistent finding in two
221 donors – a non-smoker (Donor 1) and an individual with COPD (Donor 2), (**Supplementary**
222 **Figure 7a**). Despite elevating flACE2 levels (**Supplementary Figure 7a)**, oltipraz pre-
223 treatment did not increase SARS-CoV2 infection (**Figure 5e**, **Supplementary Figure 7b**). Of
224 note, infection for these experiments was undertaken with the B1.1.7 variant. As others
225 have shown there is considerable inter-experiment variation when using SARS-CoV-2 to
226 infect in primary HBECs (Hou et al. 2020; Ravindra et al. 2021). (However, in three
227 experiments with multiple technical replicates from two donors (**Figure 5e**, **Supplementary**
228 **Figure 7b)**, there was no increase in infection with either CSE (consistent with **Figure 3C**) or
229 oltipraz. We also assessed the impact of combined treatment with oltipraz and CSE. Again,
230 despite induction of ACE2 (**Supplementary Figure 7a)**, there was no increase in SARS CoV-2
231 infectivity. Therefore, smoking and KEAP1 inhibition both increase flACE2 expression but
232 this does not lead to an increase in SARS-CoV-2 infectivity measure using either flow
233 cytometry or in situ immunofluorescence of differentiated bronchial epithelial cells at the
234 air-liquid interface.
235
236
237

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238 **Discussion**

239

240 The severity of COVID-19 is associated with increasing age and comorbidities including
241 obesity, diabetes mellitus and chronic respiratory disease. Separately, there is a long
242 established and direct link between smoking and serious adverse health outcomes,
243 including chronic respiratory diseases, cardiovascular disease and cancer.

244

245 During the pandemic, there has been intense interest in the link between cigarette smoking
246 and COVID-19. In terms of chronic respiratory disease, smoking is a major global cause of
247 COPD, and current smokers or individuals with COPD are more at risk of severe COVID-19
248 infections and death (Alqahtani et al. 2020; Leung et al. 2020; Zhao et al. 2020; Guo 2020;
249 Hopkinson et al. 2021; Simons et al. 2020). Molecular epidemiological studies have linked
250 COPD with an increased expression of ACE2, the main receptor for SARS-Co-V2 (Smith et al.
251 2020; Jacobs et al. 2020; Brake et al. 2020). Further, bulk and single cell RNA-Seq datasets
252 comparing smokers and never-smokers has consistently shown that cigarette smoking
253 (acute and chronic) leads to an increase in ACE2 expression, although these studies have not
254 discriminated between the two ACE2 isoforms that are now known to be expressed in the
255 airway (Cai et al. 2020; Zhang, Yue, et al. 2020; Aliee et al. 2020; Leung et al. 2020).

256

257 The elevated ACE2 expression in smokers has been linked with increased susceptibility to
258 infection (Purkayastha et al. 2020; Cai et al. 2020). However, the available clinical
259 epidemiological data suggests that smokers and non-smokers have similar risks of infection,
260 but that those smokers or ex-smokers with cardiorespiratory end-organ damage (COPD,

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261 cardiovascular disease) are more likely to have severe infections or die from COVID-19
262 (Williamson et al. 2020; Docherty et al. 2020; Simons et al. 2020; Grundy et al. 2020).
263
264 Although increased ACE2 in the conducting airways was suggested to increase susceptibility
265 to SARS-CoV-2 infection (Cai et al, 2020); others have suggested elevated ACE2 in the distal
266 airways or the pulmonary vasculature could be beneficial during the later stages of COVID-
267 19 and mooted upregulation of ACE2 as a rational therapeutic goal (Chaudhry et al. 2020;
268 Vaduganathan et al. 2020; Kuba et al. 2005; Monteil et al. 2020; Verdecchia et al. 2020).
269
270 We have now explored the link between smoking, ACE2 and SARS-CoV-2 infection *in vitro*
271 using differentiated primary human bronchial airway epithelial cells (HBECs) at the air-liquid
272 interface (ALI). Since these and similar nasal cells are the putative first sites of entry for
273 SARS-CoV-2, we sought to expose these *in vitro* human "airways" to SARS-CoV-2 with and
274 without cigarette smoke extract to understand the impact of smoking on the earliest stage
275 of *in vivo* infection.
276
277 We demonstrate that cigarette smoke induces ACE2 expression in HBECs using multiple
278 approaches – quantitative PCR, immunofluorescence and western blotting. This is consistent
279 with the molecular epidemiology data linking ACE2 expression and smoking. Given the
280 difficulties reproducing detection of ACE2 protein in clinical specimens (Aguiar et al. 2020;
281 Hikmet et al. 2020; Ortiz et al. 2020), it is particularly important to have demonstrated
282 expression of ACE2 protein and its localisation at the cell surface – where it has the capacity
283 to act as a receptor for SARS-CoV-2. Importantly, in our experiments, increased expression
284 of ACE2 in response to CSE does not significantly alter the percentage of cells infected. This

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285 is consistent with the epidemiological data suggesting smoking is not a major risk factor for
286 infection.
287
288 One explanation for the lack of impact of CSE on HBEC infection despite elevation of ACE2
289 was that it upregulates the truncated isoform of ACE2 – dACE2 - lacking the SARS-CoV-2
290 binding domain. However, we demonstrate that CSE mainly induces expression of the full-
291 length isoform, despite the cells retaining the potential to upregulate dACE2 in response to
292 IFNα.
293
294 Our results differ from recently published data that did not detect an increase in ACE2
295 mRNA/protein in response to cigarette smoke but nevertheless suggested that smoking
296 increases viral infection (Purkayastha et al. 2020). This discrepancy may reflect differences
297 in the smoking exposure protocols used. In our experiments we added cigarette smoke
298 extract to the basal chamber of the transwell while cells are maintained at ALI. This is a well-
299 established technique and has been used extensively in respiratory and cardiovascular
300 research (Schamberger et al. 2015; Ito, Ishimori, and Ishikawa 2018). Further, it
301 recapitulated the increase in ACE2 reported in clinical specimens. Purkayastha *et al* took an
302 alternative approach – exposure of the entire differentiated cell culture to smoke for a brief
303 period of 3 minutes/day for 4 days. This protocol was modelled on a study by Gindele et al
304 who adapted a customised, bespoke aerosol toxin exposure system to expose small airway
305 epithelial cells to relatively higher cigarette smoke doses for longer duration and at
306 repeated time-points during differentiation at ALI (Gindele et al. 2020). Their results
307 demonstrated a clear induction of ACE2 **(Supplementary Figure 8**) also reported by Smith et
308 al (Smith et al. 2020). In contrast, Purkayastha et al used a standard vacuum chamber with

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309 modest exposure doses and did not demonstrate ACE2 induction (Purkayastha et al. 2020).
310 Of note, neither exposure model led to CSE-associated toxicity.
311
312 There has been a keen interest in modulating ACE2 expression to influence the course of
313 COVID-19. This is a complex area, as noted by others (Michaud et al. 2020; Verdecchia et al.
314 2020; Zhang, Penninger, et al. 2020). One hypothesis is that reducing ACE2 expression in the
315 upper airways may protect against SARS-CoV-2 infection. This is consistent with
316 observations that transduction with ACE2 increases cellular susceptibility to infection albeit
317 in models where the overexpression of ACE2 is at unrepresentative supraphysiological levels
318 (McCray et al. 2007; Yang et al. 2007). It is critical to distinguish between the conducting and
319 distal airways (alveolar units) when considering the impact of ACE2 expression. These cell
320 types have different stem cell precursors, different functions and distinct responses to
321 specific stimuli (Stripp and Shapiro 2006). Any potential enthusiasm for reducing ACE2 in the
322 conducting airway where SARS-CoV-2 first infects cells must be tempered by a significant
323 body of evidence suggesting higher ACE2 may have a protective impact in the distal airway
324 and protect against acute lung injury/adult respiratory distress syndrome (Michaud et al.
325 2020; Verdecchia et al. 2020; Imai et al. 2005). Therefore, an ideal therapeutic in severe
326 COVID-19 may reduce ACE2 in the conducting airways but increase its expression in the
327 alveolar units. Although this is unlikely to be an achievable goal, our data demonstrating
328 that an elevation of flACE2 has no impact on cellular infection is consistent with the notion
329 that expression of flACE2 is not the key factor limiting SARS-CoV-2 infection of conducting
330 airway cells.
331

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332 In COPD, NRF2 agonists would be a theoretically attractive way to induce the key
333 antioxidant response as well as modulate ACE2 expression. We show that oltipraz, an NRF2
334 agonist already in Phase 3 clinical trial for other indications, increased rather than decreased
335 ACE2 in the HBECs. Nicotine, a component of cigarette smoke, is also being actively assessed
336 in clinical trials as a potential protective agent in COVID-19 infection (ClinicalTrials.gov
337 Identifier NCT04583410), (Farsalinos et al. 2020). This was based on early epidemiological
338 observations suggesting smokers may be protected against SARS-CoV-2 infection as well as
339 preclinical studies on submerged bronchial epithelial cells suggesting nicotine may increase
340 ACE2 mRNA expression (Russo et al. 2020). It is uncertain how to translate observations on
341 ACE2 mRNA in undifferentiated airway cells into a clear understanding of the receptor
342 expression at the apical surface of airway epithelial cells. Our data show that nicotine does
343 not significantly alter ACE2 or CHRNA7 mRNA expression in differentiated HBECs after 48
344 hours treatment.
345
346 Our studies are limited by the focus on the conducting airways and therefore address the
347 initial phases of infection rather than the later stage at which individuals are admitted to
348 hospital and which are important for COVID-19 morbidity and mortality. Further, smoking
349 has important systemic impacts that cannot be modelled in ALI epithelial cultures. In that
350 context it is possible that CSE in the basal media more closely mimics the sustained systemic
351 effects of smoking. In terms of assaying cellular infection, differentiated airway ALI models
352 have profound advantages but it is possible that subtle changes in susceptibility to infection
353 are not detected because of the well-to-well variation typical of experiments using primary
354 cells.
355

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356 Overall, our data are entirely consistent with the documented epidemiology of SARS-CoV-2
357 infection. Individuals with chronic respiratory or cardiovascular disease associated are more
358 vulnerable to severe COVID-19. However, current smokers have a similar susceptibility to
359 SARS-CoV-2 infection as the general population. We show that the airway epithelial
360 response to cigarette smoke is associated with an increase in full length ACE2 – the key
361 SARS-CoV-2 receptor - but not an increased susceptibility to cellular infection. Therapeutic
362 strategies that increase ACE2 receptor expression in the conducting airways are unlikely to
363 increase cellular infection.
364
365
366

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367 **Methods**

368

369 **Primary human bronchial epithelial cell (HBEC) culture and other cell line culture**

370

371 Primary human bronchial epithelial cells (HBECs) derived from a non-smoking donor (Cat#
372 CC-2540, male; Lonza; Donor 1) or derived directly from a patient at Cambridge University
373 Hospitals NHS Trust (Research Ethics Committee Reference 19/SW/0152; Donor 2) were
374 expanded using PneumaCult™-Ex Plus Medium (Cat# 05040; Stemcell) supplemented with
375 Penicillin (100 I.U./ml)-Streptomycin (100 μg/ml). All experiments were performed using
376 cells at passage 3. All experiments in used cells expanded from Donor 1 except when stated
377 otherwise.

378

379 Cell lines including A549 (ATCC; Cat# CCL-185, male), Calu3 (ATCC; Cat# HTB-55, male)
380 HEK293T (ATCC; Cat# CRL-3216, female) (A549-ACE2, HEK293T-ACE2) have been used as
381 negative or positive controls. HEK293T lines were maintained in RPMI supplemented with
382 10% FBS, 2 mM L-glutamine, pH 7.5, and 1 mM sodium pyruvate at 37°C in a 5% CO2. A549
383 and Calu-3 cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) and
384 Eagle's Minimal Essential Medium (EMEM) respectively, supplemented as specified above.

385

386

387 **Air-Liquid Interface (ALI) Culture**

388

389 Briefly, 1 x 10^5 of expanded primary HBECs at passage 3 in 200 μl of supplemented
390 PneumaCult™-Ex Plus Media were seeded in the apical chamber of a 24-well Transwell®

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391 insert with 0.4μM pore (Cat# 353095, Falcon) pre-coated with Rat tail Type I collagen (Cat#
392 354236, Corning) with 500 μl of PneumaCult™-Ex Plus Media in the basolateral chamber.
393 The following day, both apical and basolateral chambers underwent a media change (200 μl
394 and 500 μl, respectively). After two days of submerged culture, media from the apical
395 chamber was removed to establish the air-liquid interface (ALI day 0) whilst media in the
396 basolateral chamber was replaced with 500 μl HBEC ALI differentiation medium
397 (PneumaCult™-ALI Medium, Cat# 05021; Stemcell). Basolateral media was changed every 2-
398 3 days and apical surface washed with warm PBS twice a week to remove any build-up of
399 mucous and secretions. Cultures were allowed to differentiate for at least 28 days before
400 being used for any experiments.
401
402 **Cigarette Smoke Extract (CSE) Generation and Treatment**
403
404 Cigarette smoke extract (CSE) was prepared, filter sterilised using 0.20μm filter and used
405 within 30 mins of generation. CSE was generated by smoking two Kentucky reference
406 cigarettes and bubbling the generated smoke through 25 ml ALI media at a rate of 100
407 ml/min. Each cigarette took roughly 6 mins to burn. This solution is regarded as "100% CSE"
408 and was diluted with ALI media to generate a 10% working solution. Cells were treated with
409 10% CSE for 48 hours before being treated with SARS-CoV-2, harvested or fixed for further
410 analysis.
411
412
413
414

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415 **SARS-CoV-2 infection**

416

417 The clinical isolate of SARS-CoV-2 viruses used in this study were SARS-CoV-
418 2/human/Liverpool/REMRQ0001/2020(Lineage B.29) (Chu et al. 2020) (Patterson et al.
419 2020) and SARS-CoV-2 England/ATACCC 174/2020 (Lineage B.1.1.7). Stocks were sequenced
420 before use and the consensus matched the expected sequence exactly. Viral titre was
421 determined by 50% tissue culture infectious dose (TCID50) in Huh7-ACE2 cells.

422

423 For infection, the indicated dose of virus was diluted in PBS to a final volume of 50 µL and
424 added to the apical chamber of the transwell of differentiated HBEC-ALI cultures for 2-3
425 hours prior to removal. At 72 hours post-infection HBEC-ALI apical surfaces were washed
426 once with PBS, dissociated with TrypLE, and fixed in 4% formaldehyde for 15 minutes. Fixed
427 cells were washed and incubated for 15 minutes at room temperature in Perm/Wash buffer
428 (BD #554723). Permeabilised cells were pelleted, stained for 15 minutes at room
429 temperature in 100 µL of sheep anti-SARS-CoV-2 nucleocapsid antibody (MRC-PPU, DA114)
430 at a concentration of 0.7 µg/mL, washed and incubated in 100 µL AF488 donkey anti-sheep
431 (Jackson ImmunoResearch #713-545-147) at a concentration of 2 µg/mL for 15 minutes at
432 room temperature. Stained cells were pelleted and fluorescence staining analysed on a BD
433 Fortessa flow cytometer.

434

435 **Immunofluorescence**

436 ALI cultures were washed three times with PBS and fixed using 4% paraformaldehyde (PFA)
437 for 15 minutes at room temperature before permeabilization with 0.3% Triton-X for 15

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438 minutes. Cells were blocked for 1 hour in 5% Normal goat serum/1% Bovine serum albumin
439 (BSA) at room temperature. Primary antibodies; anti-ACE2 antibody was initially Abcam
440 228349 but was discontinued part-way through this study and was then replaced with
441 21115-1-AP (Proteintech); Acetylated tubulin (T7451; Sigma); Muc5AC (MA5-12178;
442 Invitrogen), SARS-CoV / SARS-CoV-2 (COVID-19) spike antibody [1A9] (GTX632604; Genetex),
443 SARS-CoV-2 (COVID-19) Nucleocapsid antibody DA114 (MRC PPU) were added and
444 incubated at 4 degrees overnight. Following several washes with Phosphate Buffered Saline
445 and Tween-20 (PBS-T), cultures were incubated with secondary antibodies for 1 hour in the
446 dark at room temperature before being washed a further three times before Hoechst
447 staining (100 μg/ml) and mounting. Confocal images were captured using a Nikon C2
448 Confocal Microscope, magnification ×40 oil. Composite images were generated and
449 analysed using Fiji. Immunofluorescent images were also captured using a Cellomics
450 Arrayscan (ThermoFisher Scientific VTI) using 64 fields of view/transwell at x10
451 magnification and analysed using HCS. Studio 2.0 Client Software. Results are expressed as
452 percent of infected cells according to AF488 positive staining. For Figure 5E (Donor 1 only)
453 the controls were shared with a published experiment ( )

454

455

456 **qRT-PCR**

457 RNA was extracted using RNeasy Mini Kit (Qiagen) according to manufacturer's instructions
458 and quantified using a NanoDrop Spectrophotometer (ThermoFisher). cDNA synthesis was
459 performed using a High-Capacity cDNA Reverse Transcription Kit (ThermoFisher). qRT-PCR

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460 was performed using Fast SYBR® Green Mix (ThermoFisher) alongside the following primers
461 used for detecting expression of genes of interest: ACE2 Forward (5'-3'):
462 CGAAGCCGAAGACCTGTTCTA, Reverse (5'-3'): GGGCAAGTGTGGACTGTTCC; dACE2 Forward
463 (5'-3'): GGAAGCAGGCTGGGACAAA, Reverse(5'-3'): AGCTGTCAGGAAGTCGTCCATT; TBP
464 (House keeper) Forward (5'-3'): AGTGAAGAACAGTCCAGACTG, Reverse (5'-3'):
465 CCAGGAAATAACTCTGGCTCAT; TMPRSS2 Forward (5'-3'): CTGCTGGATTTCCGGGTG, Reverse
466 (5'-3') TTCTGAGGTCTTCCCTTTCTCCT; FOXJ1 Forward (5'-3'):
467 TGGATCACGGACAACTTCTGCTA, Reverse (5'-3') CACTTGTTCCAGAGACAGGTTGTGG; MUC5B
468 Forward (5'-3'): CCTGAAGTCTTCCCCAGCAG, Reverse (5'-3') GCATAGAATTGGCAGCCAGC.
469 Samples were run in technical triplicates on a StepOne machine and relative differences in
470 expression were determined using the comparative ΔC_T method and TBP used as the
471 endogenous house-keeping control.
472

473 **Western Blotting**

474 Recombinant Anti-ACE2 antibodies (Cat# ab108209; Abcam: N-terminal) and (Cat# ab15348;
475 Abcam: C-terminal), alpha-tubulin (Cat# sc-32293; Santa-Cruz) and beta-actin (Cat# sc-
476 69879; Santa-Cruz) were used for ACE2/dACE2, alpha-tubulin detection and beta-actin,
477 respectively.
478

479 **Apoptosis detection**

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480 ALI cultures exposed to CSE or control media were washed three times with PBS and
481 detached from the transwell membrane with accutase. Apoptotic cells was detected
482 by concurrent staining with annexin V–APC and PI (Cat# 88-8007-72, eBioscience) and
483 their far-red and red fluorescence was measured by flow cytometry (Fortessa LSR, BD).

484

485 **Quantification and Statistical Analysis**

486

487 Statistical analyses of mRNA expression assays and infection quantification data were
488 performed using Prism 8 software (GraphPad Software). P values were calculated using a
489 two-tailed, Mann Whitney *U*-test unless stated otherwise. P values were noted as follows:
490 ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Error bars represent the mean +/-
491 standard error of the mean unless stated otherwise.

492

493

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520
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522
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524 Figure Legends

525 **Figure 1. ACE2 expression increases upon differentiation in HBECs cultured at the air-**
526 **liquid interface (ALI).**

527 **A.** Schematic representation of ALI experimental set-up using HBECs. Cell type specific
528 markers are shown in parentheses. **B.** HBEC *TMPRSS2* and *ACE2* expression (RNA) both
529 increase when cultured for 28 days at the ALI compared to submerged, non-differentiated
530 cell culture. Expression of the transcription factor required for ciliation, *FOXJ1* is also
531 significantly upregulated. RT-qPCR data presented as log2 relative fold-change in expression
532 compared to submerged HBECs from n=5 independent experiments (Mann Whitney, **, $P <$
533 0.01). Error bars represent mean and the standard error of the mean. **C.** ACE2 protein is also
534 increased during differentiation. A549 cells overexpressing ACE2 are used as a positive
535 control. Representative western blot from 3 independent experiments. ACE2 antibody
536 Ab228349 was used. **D.** ACE2 expression (red fluorescence, antibody Ab228349) is
537 upregulated on differentiation. **E**) ACE2 (red, Antibody used 21115AP) is expressed apically
538 on the epithelial cell surface, predominantly colocalising with ciliated cells (acetylated
539 tubulin, green fluorescence). **F) ACE2** (red, antibody 21115AP) does not colocalise with
540 goblet cells (MUC5AC, green fluorescence). Scale bars on fluorescent images = 100μm. All
541 experiments in Figure 1 used cells expanded from Donor 1.

542

543 **Figure 2. Exposure to cigarette smoke extract (CSE) increases ACE2 expression at both**
544 **the RNA and protein level**

545 **A.** Schematic showing ALI culture experimental set-up with CSE. **B.** CSE exposure (10%)
546 for 48 h increases *ACE2* expression in differentiated HBECs relative to untreated controls.
547 RT-qPCR data presented as log2 relative fold-change in expression from n=8 independent
548 experiments (Mann-Whitney, ***, $P < 0.001$). **C.** CSE exposure (10%) for 48 h also

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549 increases ACE2 protein expression relative to untreated control. Calu-3 are presented as the
550 positive control. Representative western blot from 3 independent experiments. ACE2
551 antibody – ab15348. **D.** CSE exposure does not induce apoptosis in differentiated HBECs at
552 ALI relative to control samples as analysed by flow cytometric analysis using AnnexinV
553 (AnV) and Propidium iodide (PI) staining see also Supplementary Figure 3. Data represents 2
554 independent experiments using cells from Donor 1. **E.** H&E staining of sectioned
555 differentiated ALI HBEC cultures from Donor 1 with and without CSE exposure (x20
556 magnification). **F**. Treatment of differentiated HBECs at ALI with 1 uM Nicotine for 48 h
557 does not induce *ACE2* expression. **G**. CSE exposure does not significantly alter *CHRNA7*
558 expression **G**. RT-PCR data for **F** and **G** is presented as log2 relative fold-change in
559 expression from n=3-8 independent experiments (Mann-Whitney, ns).
560

561 **Figure 3. HBECs exposed to CSE are not more susceptible to infection by SARS CoV2**
562 **A.** Schematic representation of SARS-CoV-2 infection of CSE exposed HBEC ALI cultures.
563 **B.** SARS-CoV-2 infection was detected using an antibody specific to the viral spike protein
564 (S2 domain). Representative immunofluorescent images showing 72 h post SARS-CoV-2
565 infection; expression of viral spike protein (green) primarily co-localised with ACE2 (red)
566 expressing cells. White arrowheads indicate co-localisation of markers in merged imaged. **C.**
567 Flow cytometry quantification of ciliated and non-ciliated cells infected with $8x10^3$ TCID50
568 of B.29 lineage SARS-CoV-2 following control/CSE exposure (n=12); each dot represents an
569 individual transwell from 2 independent experiments, bars represent mean values (Mann
570 Whitney; ns, non-significant).
571

572 **Figure 4. A short isoform of ACE2 is upregulated during HBEC differentiation and**
573 **interferon-alpha stimulation but not CSE exposure at ALI.**

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574 **A.** Schematic of full-length ACE2 (flACE2) and the truncated isoform (dACE2) detailing
575 position of antibody binding epitopes for immunofluorescence and western blot analysis. The
576 location of primers used to distinguish ACE2 and dACE are also shown. **B.** HBECs
577 differentiated at ALI upregulate a short isoform of ACE2 (dACE2) as well as the full length
578 ACE2. RT-PCR data shows log2 relative fold-change in expression from n=7 independent
579 experiments (Mann-Whitney **, $P < 0.01$). **C.** d*ACE2* is specifically sensitive to interferon-
580 alpha stimulation (24 h) but not interferon-gamma at 24 h. Full-length ACE2 shows no
581 modulation with interferon treatment at 24 h. RT-PCR data shows log2 relative fold-change
582 in expression from n=3-6 independent experiments (Mann-Whitney, * $p<0.05$). **D**. 48 h
583 exposure of CSE does not promote an increase in dACE2 mRNA. RT-PCR data shows log2
584 relative fold-change in expression from n=7 independent experiments (Mann-Whitney, ns).
585 **E**. Western blot is representative of 5 independent experiments and shows the impact of 48 h
586 exposure of CSE on flACE2/dACE2 expression. Also see Supplementary Figure 6. ACE2
587 antibody used ab15348.
588
589 **Figure 5. CSE and oltipraz increase ACE2 and NRF2 expression but not SARS-CoV-2**
590 **infection.**
591 **A.** CSE exposure induces NRF2 mRNA expression at 48 h. **B**. The NRF2 agonist Oltipraz
592 increases NRF2 mRNA **C.** Oltipraz increases flACE2 mRNA expression. D. Oltipraz
593 increases flACE2 protein expression (ACE2 antibody Ab15348). RT-PCR data shows log2
594 relative fold-change in expression from n=7 independent experiments (Mann-Whitney, ** P
595 < 0.01, * $p<0.05$). Representative western blot from 3 independent experiments. **E.**
596 Fluorescent image analysis of whole transwell ALI cultures show that for Donors 1 and 2,
597 CSE or NRF2 agonists, alone or in combination, did not result in increased infection. Each
598 datapoint represents a single well and infection was with 1×10^4 TCID50 of B.1.1.7 SARS-

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599 CoV-2. Representative microscopy montages show the entire ALI transwell. Scale bar: 500
600 μm. Infection was quantitated and presented as percentage of cells infected for each
601 condition.
602

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Figure 1

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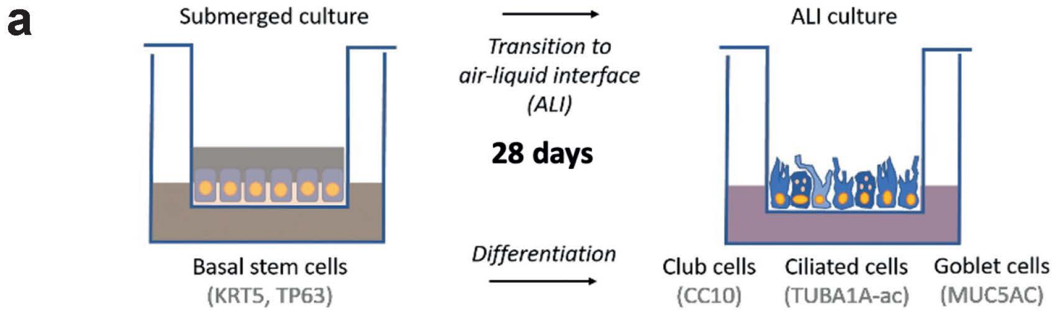

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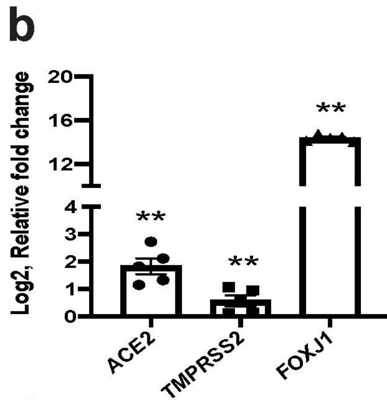

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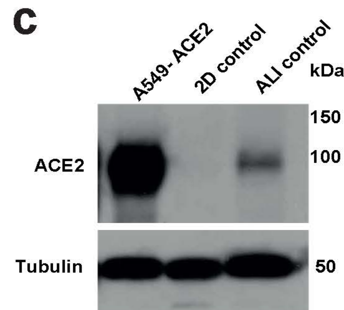

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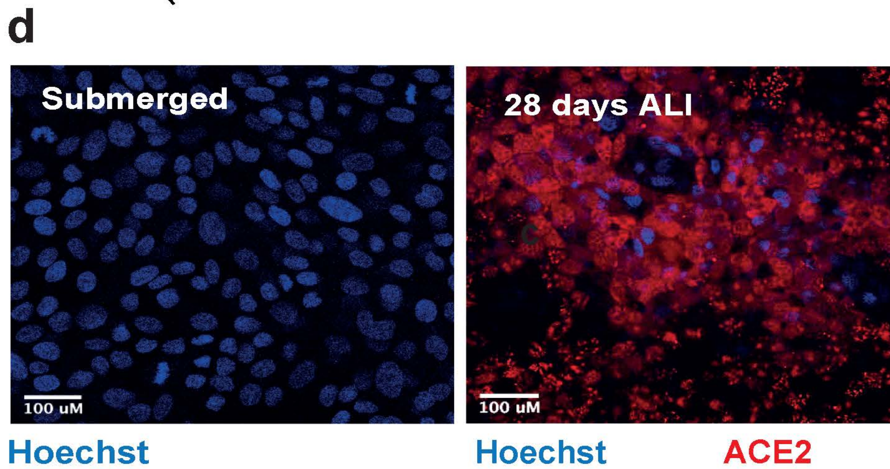

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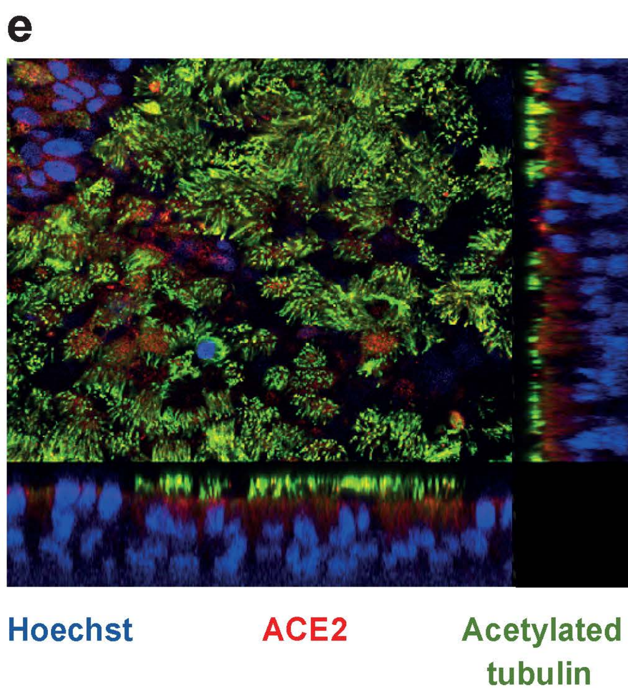

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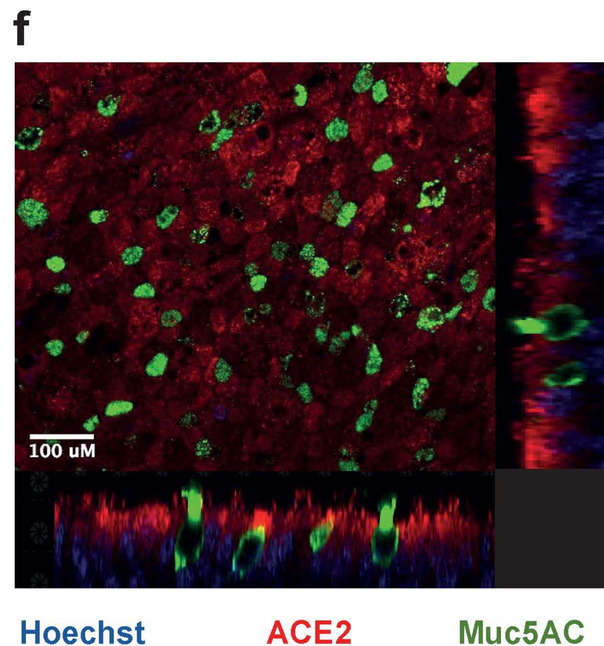

Figure 2

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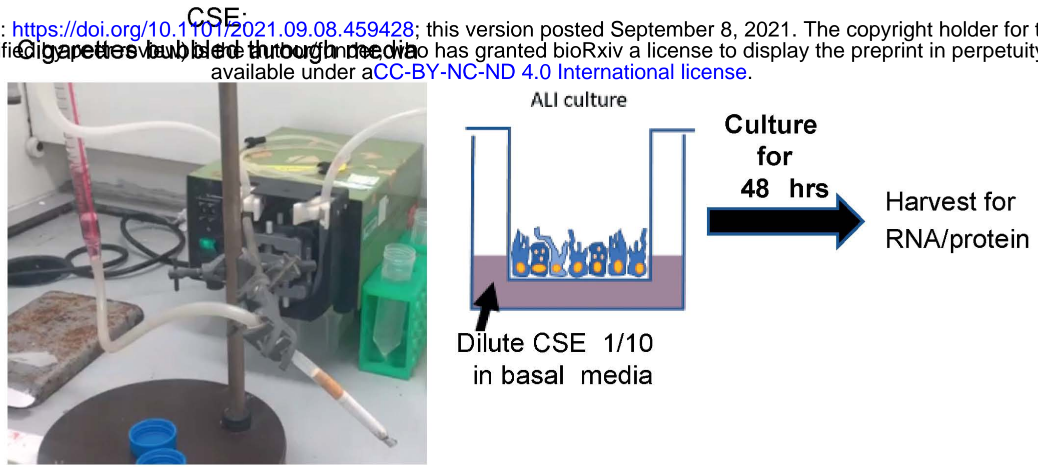

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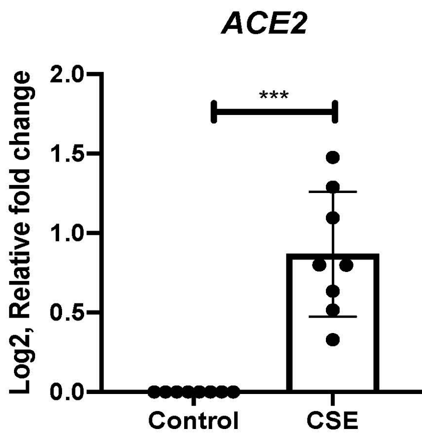

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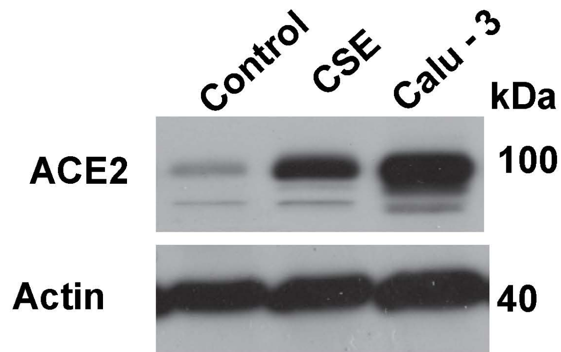

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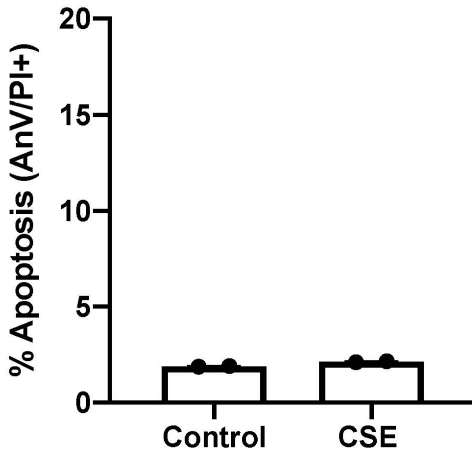

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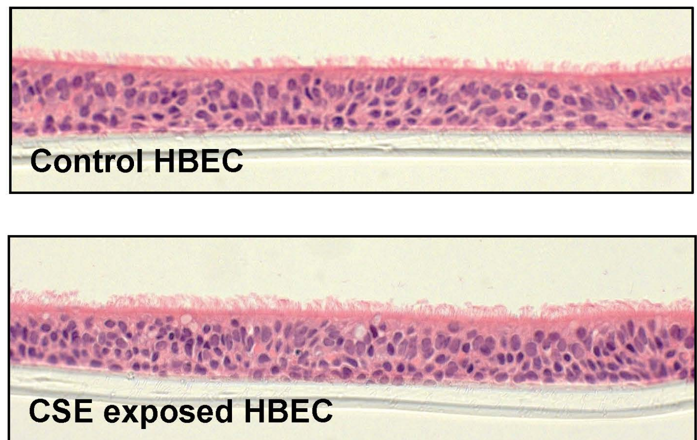

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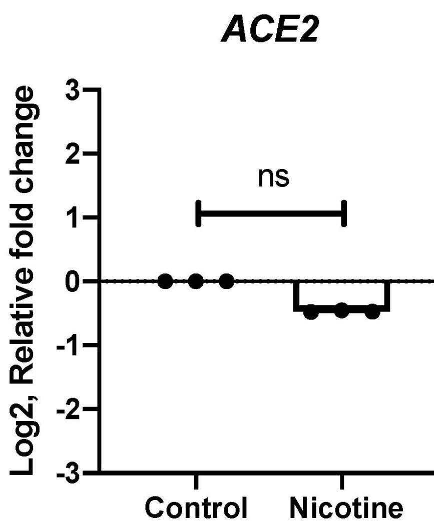

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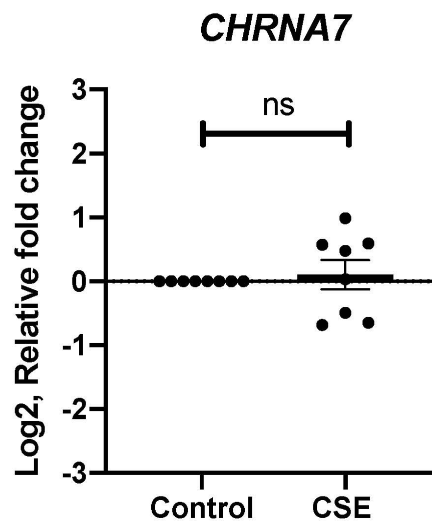

Figure 3

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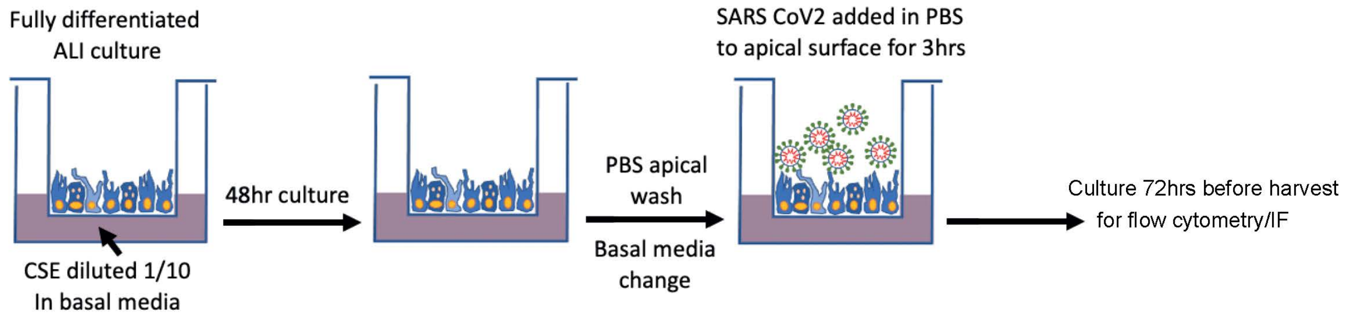

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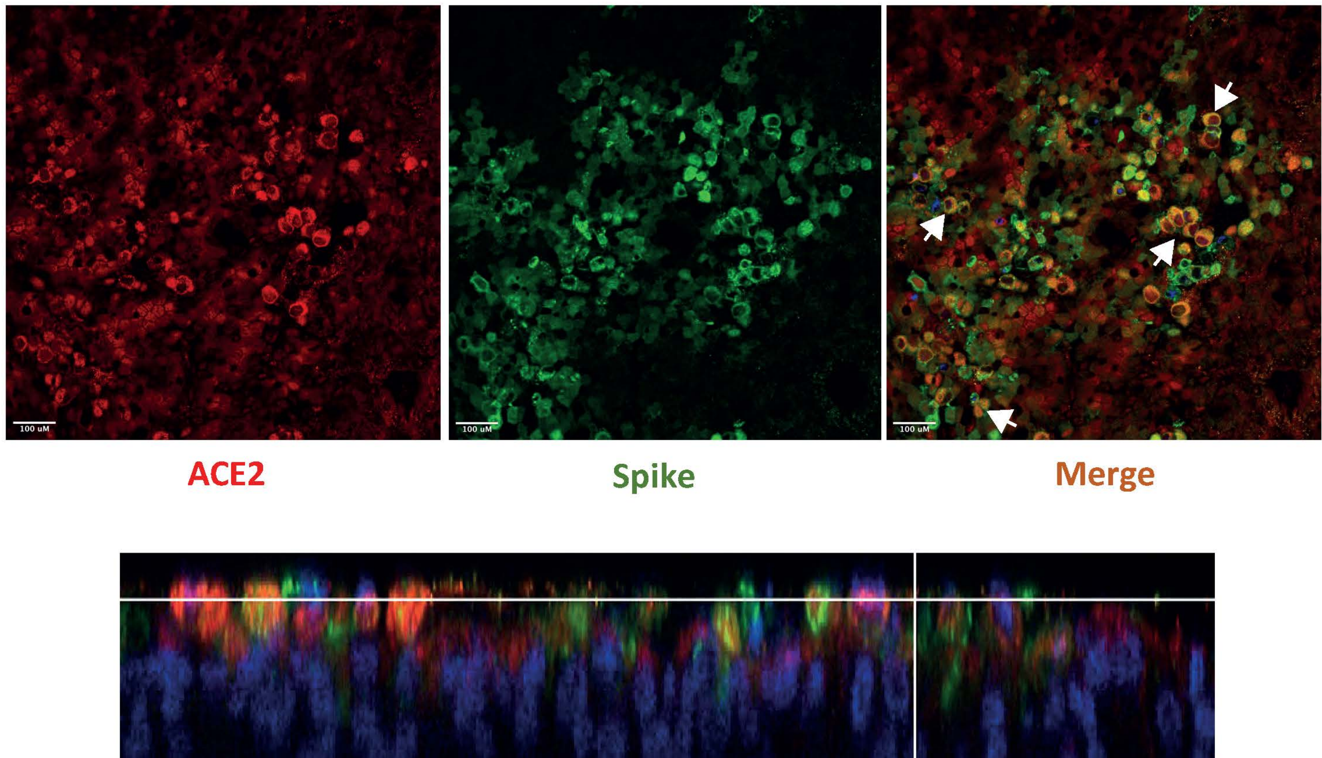

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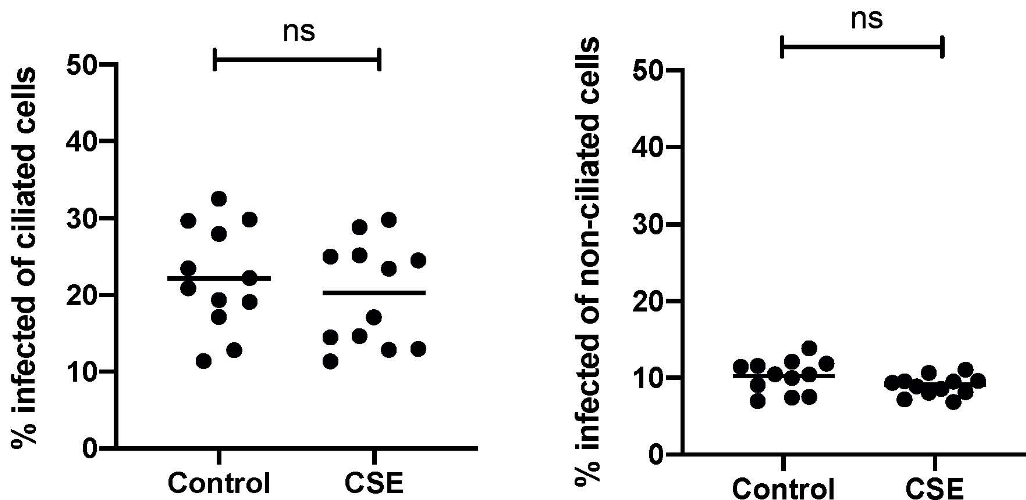

Figure 4

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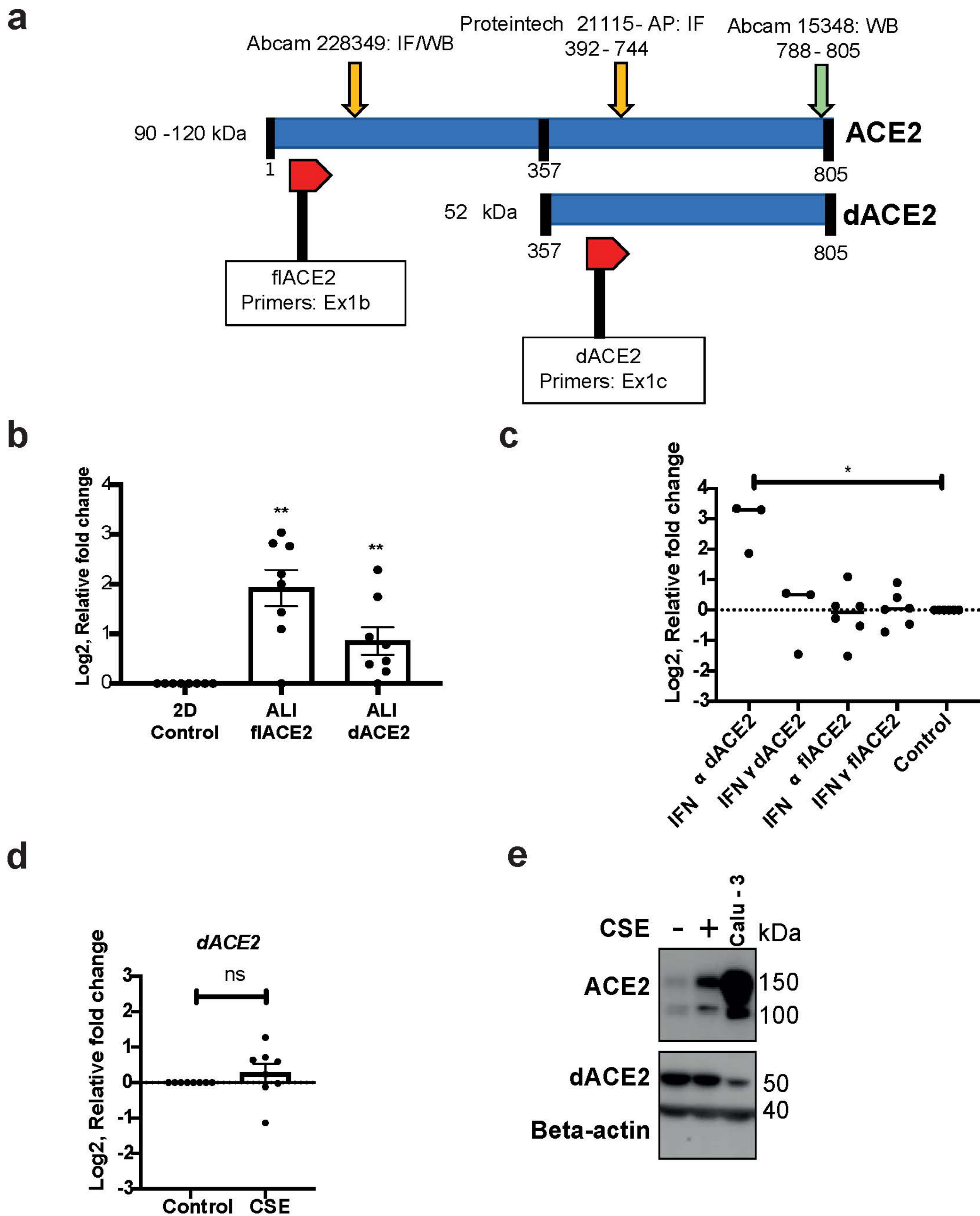

Figure 5

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