1   **Novel Human Lung Tissue Model for the Study of SARS-CoV-2 Entry,**

2   **Inflammation and New Therapeutics**

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4   Judith Grau-Expósito[1]*****, David Perea[1]*****, Marina Suppi[1], Núria Massana[1], Ander Vergara[2], Maria

5   José Soler[2], Javier García-Pérez[3], José Alcamí[3,4], Anna Serrano-Mollar[5,6], Joel Rosado[7], Vicenç

6   Falcó[1], Meritxell Genescà[1]*****, and Maria J. Buzon[1]*****

7   [1]Infectious Diseases Department, Vall d'Hebron Research Institute (VHIR), Hospital Universitari

8   Vall d'Hebron, Universitat Autònoma de Barcelona, Barcelona, Spain. VHIR Task Force COVID-

9   19.

10   [2]Nephrology Research Department, Vall d'Hebron Research Institute (VHIR), Hospital

11   Universitari Vall d'Hebron, Universitat Autònoma de Barcelona, Barcelona, Spain. VHIR Task

12   Force COVID-19.

13   [3]AIDS Immunopathology Unit, National Center of Microbiology, Instituto de Salud Carlos III,

14   Madrid, Spain.

15   [4]Clinic HIV Unit, Hospital Clinic, IDIBAPS, Barcelona, Spain.

16   [5]Experimental Pathology Department, Institut d'Investigacions Biomèdiques de Barcelona,

17   Consejo Superior de Investigaciones Científicas (IIBB-CSIC), Institut d'Investigacions

18   Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain.

19   [6]Centro de Investigaciones Biomédicas en Red de Enfermedades Respiratorias (CIBERES),

20   Madrid, Spain.

21   [7]Thoracic Surgery and Lung Transplantation Department, Vall d'Hebron Institut de Recerca

22   (VHIR), Hospital Universitari Vall d'Hebron, Barcelona, Spain. VHIR Task Force COVID-19.

23   **Running title:** Human lung for the study of SARS-CoV-2

24   **Corresponding authors*:** Maria J. Buzon (mariajose.buzon@vhir.org) and Meritxell Genescà

25   (meritxell.genesca@vhir.org)

26   **Abstract**

27   The development of physiological models that reproduce SARS-CoV-2 infection in primary

28   human cells will be instrumental to identify host-pathogen interactions and potential

29   therapeutics. Here, using cell suspensions from primary human lung tissues (HLT), we have

30   developed a platform for the identification of viral targets and the expression of viral entry

31   factors, as well as for the screening of viral entry inhibitors and anti-inflammatory compounds.

32   We show that the HLT model preserves its main cell populations, maintains the expression of

33   proteins required for SARS-CoV-2 infection, and identifies alveolar type II (AT-II) cells as the most

34   susceptible cell targets for SARS-CoV-2 in the human lung. Antiviral testing of 39 drug candidates

35   revealed a highly reproducible system, and provided the identification of new compounds

36   missed by conventional systems such as VeroE6. Using this model, we also show that interferons

37   do not modulate ACE2 expression, and that stimulation of local inflammatory responses can be

38   modulated by different compounds with antiviral activity. Overall, we present a novel and

39   relevant physiological model for the study of SARS-CoV-2.

40   **Synopsis**
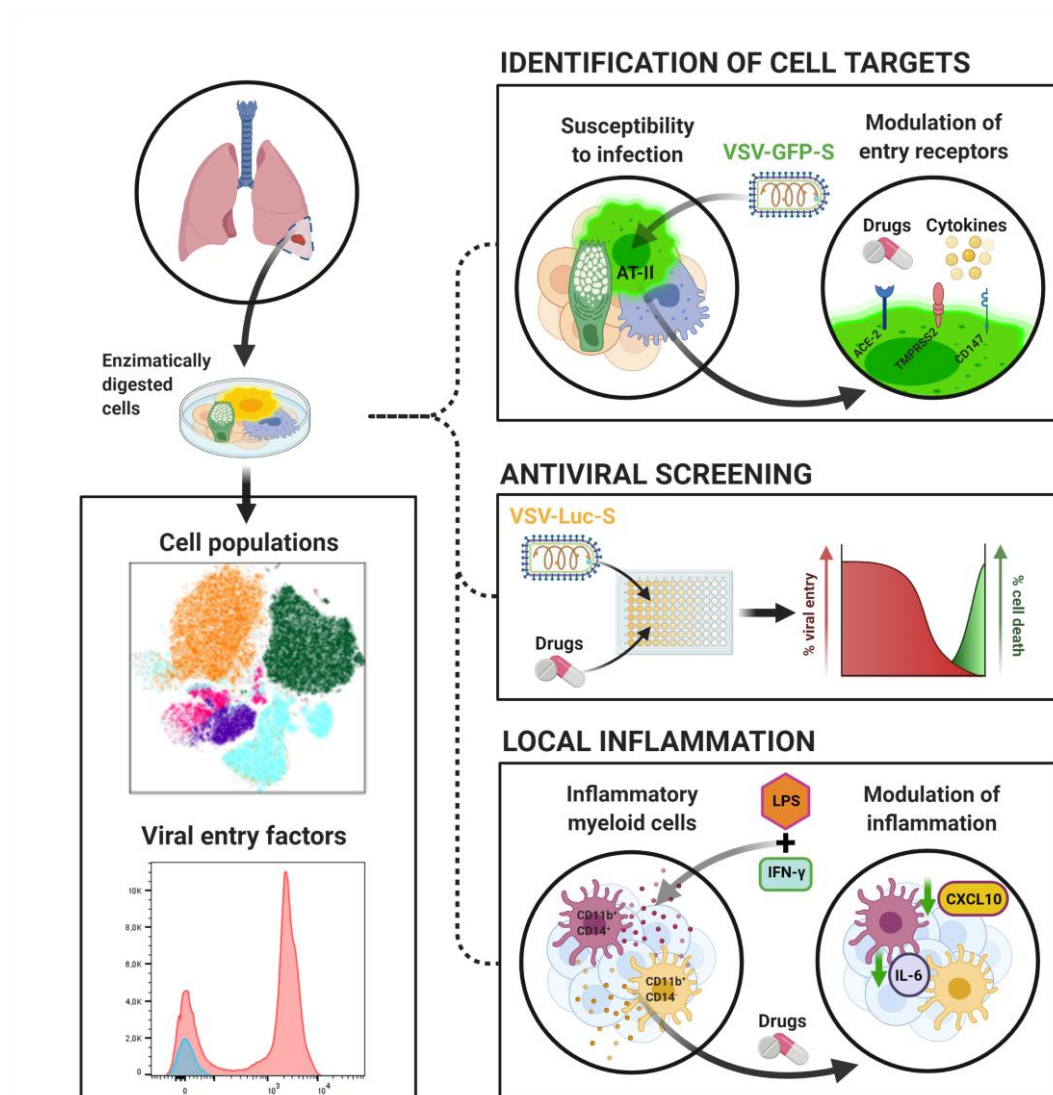
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42   *Ex vivo* physiological systems for the study of SARS-CoV-2-host interactions are scarce. Here, we

43   establish a novel model using primary human lung tissue (HLT) for the analysis of cell tropism

44   and identification of therapeutics.

45   • The HLT model preserves main cell subpopulations, including alveolar type-2 cells,

46   and expression of SARS-CoV-2 entry factors ACE2, CD147, and TMPRSS2.

47   • The HLT model is readily susceptible to SARS-CoV-2 entry.

48   • Antiviral testing in the HLT model allows the identification of new candidates missed

49   by conventional systems.

50   • Local inflammation is supported in the HLT model and offers the identification of

51   relevant anti-inflammatory compounds for SARS-CoV-2 infection.

52 **Introduction**

53   We are facing a global health emergency, the COVID-19 pandemic, caused by the novel SARS-

54   CoV-2 virus. There is currently no specific treatment to cure SARS-CoV-2 infection to limit disease

55   progression of associated COVID-19. Early clinical trials showed promising drug candidates;

56   remdesivir, a compound that inhibits the RNA synthesis of SARS-CoV-2, was superior to placebo

57   in shortening the time to recovery in adults who were hospitalized with COVID-19 [1], however,

58   the *Solidarity* trial failed to support this observation [2]. Recently, plitidepsin, a molecule that

59   targets the eEF1A host protein has shown potent preclinical efficacy against SARS-CoV-2 [3].

60   Importantly, the development of *Acute Respiratory Distress Syndrome* in severe COVID-19

61   patients has been linked to dysregulated inflammatory responses. In this regard, the

62   glucocorticoid dexamethasone decreased 28-day mortality among patients receiving invasive

63   mechanical ventilation, but no benefit was observed in patients without respiratory support [4].

64   Thus, no effective treatment is currently available for COVID-19, and the rapid identification of

65   antivirals with the potential to be easily transferred into the clinic could save human lives.

66   Screening of novel drug candidates is often performed using cell lines. In this sense, the most

67   widely used cell lines for SARS-CoV-2 studies are epithelial cells derived either from lung (Calu-

68   3), kidney (VeroE6), or colon (CaCo-2) [5]. These immortalized systems are highly reproducible

69   and easy to handle but lack physiological relevance. The differential gene expression profiling of

70   cell lines compared with primary cells from tissues might significantly affect important enzymes

71   involved in the viral replication cycle. For instance, the level of ACE2 expression, the main

72   receptor used by SARS-CoV-2 for viral entry, is shown to be different in several cell lines [6],

73   while only a small fraction of alveolar type II (AT-II) cells, the main target for SARS-CoV-2 in the

74   lung, express ACE2 [7, 8]. In addition, SARS-CoV-2 spike (S) glycoprotein, which is responsible for

75   viral entry into target cells, can be activated by several host proteases, such as furin,

76   transmembrane serine proteinase 2 (TMPRSS2) and cathepsin L, in a pH-dependent or

77   independent manner [9, 10]. Whereas in some cell lines S protein is activated by endosomal pH-

78   dependent protease cathepsin L, in airway epithelial cells viral entry depends on the pH-

79   independent TMPRSS2 protease [10]. Thus, it is currently not well defined if SARS-CoV-2 may

80   utilize multiple cell-type-specific host proteins for viral replication in primary target tissues and

81   therefore, the potency of therapeutics directed against these proteins may also differ.

82   Further, inflammatory immune responses might also impact viral dynamics in the lung by

83   affecting the expression of entry receptors. In this sense, early studies discovered that ACE2 was

84   a human interferon-stimulated gene (ISG); IFN-β and IFN-γ were shown to strongly upregulate

85   the expression of ACE2 at the mRNA and cell surface protein levels, indicating that inflammatory

86   molecules could shape cell susceptibility to viral infection [11]. However, how anti-inflammatory

87   drugs may affect ACE2 expression and facilitate SARS-CoV-2 infection remains to be elucidated.

88   One study reported that the use of nonsteroidal anti-inflammatory drugs (NSAIDs), such as

89   ibuprofen, was linked to enhanced ACE2 expression in a diabetic-induced rat model [12] and

90   other reports raised alarms regarding the possible role of NSAIDs at increasing susceptibility to

91   SARS-CoV-2 infection [13, 14]. On the contrary, experimental and clinical evidence show that

92   medium-to-low-dose glucocorticoids may play a protective role in COVID-19 by activating ACE2

93   and suppressing the associated cytokine storm [15]. Overall, the use of more relevant and

94   physiological models for the study of either SARS-CoV-2 infection, the identification of drug

95   candidates, or the impact of new therapeutics on the disease could significantly advance the

96   successful translation of the results into the clinic.

97   Primary epithelial cell cultures of nasal and proximal airway epithelium have been used to study

98   SARS-CoV-2 infection in the upper airways [8, 16-18]. Similarly, organ on-chip and organoids

99   models of AT-II cells have been successfully developed [17, 19]. While very promising, these

100 models still miss certain aspects of cellular complexity present in primary tissues [20]. In this

101 regard, establishing a human lung tissue (HLT) model could offer a physiologically relevant

102 alternative to *in vitro* and *in vivo* approaches for several reasons; it mimics the main site of viral

103 replication in the lung, and contains all heterogeneous cell components present in the tissue

104 with greater functional complexity compared to cell lines. Although still representing a

105 reductionist *ex vivo* approach, it allows controlled experimental conditions. In the past, similar

106 lung models have been successfully established to study the effect of allergens and

107 inflammatory stimuli [21, 22]. Importantly, the HLT model allows mimicking an inflammatory

108 local response that could be attenuated by anti-inflammatory drugs [23]. Furthermore, it has

109 been used to provide low/medium throughput screening of anti-inflammatory candidate drugs

110 for the treatment of airway diseases [23]. Significantly, lung tissues not only can be infected with

111 SARS-CoV-2, but also generate local immune responses to viral infection [24]. Considering all

112 these factors, here we aimed to develop and characterize a physiological and reproducible

113 human lung tissue model, which could be used for the study of virus-host interactions,

114 identification of potential antiviral compounds, as well as help investigating the role of

115 inflammation in relation to SARS-CoV-2 infection.

116   **Results**

117   **Characterization of the HLT model**

118   To establish the HLT model, non-neoplastic areas from lung tissue resections were obtained

119   from hospitalized non-COVID19 patients undergoing thoracic surgery. First, we optimized cell

120   culture and digestion conditions, since the methodology used for tissue processing can

121   significantly impact viability and function of target cell populations. Thus, we tested the

122   preservation of AT-II, defined mainly by EpCAM and HLA-DR expression [25], and of several

123   hematopoietic cell subsets, after tissue digestion with different enzymes. Flow cytometry gating

124   strategy and cell markers used for identification of main populations are shown in **Figure S1A.**

125   We observed that collagenase outperformed liberase and trypsin at preserving AT-II cells, the

126   main SARS-CoV-2 target (**Figure S2A-B**). Among the hematopoietic cells present in the lung

127   tissue we identified CD3 T (which represented 1.94% ± 0.59 out of the total live cell fraction),

128   myeloid dendritic cells (0.03% ± 0.007), monocytes/macrophages subsets (0.42 ± 0.14%),

129   neutrophils (1.80% ± 0.53) and alveolar macrophages (0.05% ± 0.01). Moreover, non-

130   hematopoietic cells such as AT-II and endothelial cells represented 0.24% ± 0.05 and 0.5% ± 0.15

131   out of the total live cell fraction, respectively (**Figure 1A**). All populations have been previously

132   identified in human lungs [24, 26, 27]. Moreover, the nature of AT-II cells in lung cell suspensions

133   was confirmed by staining with phosphatase alkaline (**Figure 1B**), which has been shown to be a

134   reliable marker of type II cell phenotype both *in vitro* and *in vivo* [28]. Next, we focus on the

135   expression of previously identified proteins involved in viral entry. Single-cell transcriptome

136   studies have shown that ACE2, one of the main host cell surface receptors for SARS-CoV-2

137   attachment and infection, is predominantly expressed by AT-II cells [8, 29]. Moreover, ACE2

138   expression wanes in distal bronchiolar and alveolar regions paralleled by SARS-CoV-2 infectivity

139   [8]. In human parenchyma lung cells, we found ACE2 expression mainly in AT-II cells (**Figure 1C**),

140   a finding that was confirmed by immunohistochemistry (**Figure 1D**). Percentage of AT-II cells

141   expressing ACE2 was rather small and varied between individuals (6.29% ± 1.00) (**Figure 1C**), as

142   previously described [7]. We also studied the expression of CD147 on lung cells, which has been

143   reported as a novel route of SARS-CoV-2 infection *in vitro* and *in vivo* models [30]. CD147 was

144   ubiquitously expressed in several hematopoietic and non-hematopoietic cells (**Figure 1C**).

145   Importantly, 92.3% ± 1.14 of AT-II cells expressed CD147 (**Figure 1C**). Similarly, and in agreement

146   with other studies [31], TMPRSS2 protease expression was identified in AT-II cells (21.33% ±

147   7.52) (**Figure 1C**). Overall, we found that human lung suspensions preserved critical cell

148   populations and the expression of factors required for SARS-CoV-2 infection.

149   **Susceptibility of HLT cells to SARS-CoV-2 viral entry**

150   Next, we assessed if HLT cells were susceptible to viral infection. We generated pseudotyped

151   vesicular stomatitis virus (VSV) viral particles bearing the S protein of SARS-CoV-2 and expressing

152   either luciferase (VSV*ΔG (Luc)-S) or GFP (VSV*ΔG (GFP)-S) reporter genes upon cell entry. As a

153   control, we used the VSV-G virus, which has very broad cell tropism. As expected, VeroE6 cells

154   were highly susceptible to SARS-CoV-2 entry, as demonstrated for VSV*ΔG (GFP)-S and VSV-ΔG

155   (Luc)-S (**Figure 2A**). Anti-ACE2 antibody blocked more than 90% of VSV*ΔG (Luc)-S, but was

156   inactive for VSV-G (Luc) infection (**Figure 2B**). This observation has been widely reported before

157   [10, 32, 33], and identifies ACE2 as the main cell receptor required for viral entry in VeroE6.

158   Importantly for viral pathogenesis, it has been postulated that SARS-CoV-2 S protein might

159   downregulate ACE2 expression, as previously observed for SARS-CoV [34]. We consistently

160   observed a significant strong reduction in ACE2 expression after viral entry (**Figure 2C**).

161   We then evaluated the susceptibility of HLT cells to viral entry, using the same viral

162   constructs. HLT cells were readily infected with pseudotyped S particles (VSV*ΔG (Luc)-S and

163   VSV*ΔG (GFP)-S), with the natural donor variation representative of primary samples (**Figure

164   2D**). As expected [8], lung cells compatible with an AT-II phenotype were identified as the main

165   cell targets in steady conditions (**Figure 2E**). Blockade of ACE2 resulted in a donor-dependent

166   reduction of viral infectivity, ranging from 50 to 100% (**Figure 2F**). Camostat, a TMPRSS2

167   inhibitor, significantly inhibited viral entry in all HLT assays, although the entry process was not

168   always completely abrogated (**Figure 2F**), suggesting that AT-II cells may become infected

169   through the use of alternative factors [35]. Similarly, the presence of an anti-CD147 antibody

170   inhibited SARS-CoV-2 entry in AT-II cells (**Figure 2F**). Collectively, these data indicate that HLT

171   cells are highly susceptible to SARS-CoV-2 viral entry, and that ACE2, CD147 and TMPRSS2 are

172   important proteins for viral entry in human lung cells. Overall, these results support the value of

173   the HLT model to successfully study SARS-CoV-2 viral entry, and related mechanisms, in a more

174   physiological system.

175   **Antiviral assays in the HLT model**

176   To validate the HLT model as a platform for the screening of antiviral candidates, we assayed

177   potential antiviral compounds, most of them previously identified by computational methods

178   with predicted ability to inhibit SARS-CoV-2 cell entry due to their interaction with S protein or

179   with the interface S-ACE2 [36]. A detailed description of the 39 selected drugs is available in

180   **Table S1**. HLT cells were exposed to VSV*ΔG (Luc)-S virus in the presence of 1/5 serial dilution

181   of the different tested compounds. 20h post-exposure, antiviral activity and cell viability were

182 measured by luminescence. Results in HLT cells were systematically compared with antiviral

183 testing performed in the cell line VeroE6. Among 39 drugs that were evaluated in our study, 16

184 (41%) showed some antiviral activity against SARS-CoV-2 with EC_{50} values ranging from 0.5µM

185 to 66µM (**Table S2**). We observed that 23% of drugs had concordant results between both

186 models (**Figure 3**). Cepharanthine, a naturally occurring alkaloid reported to have potent anti-

187 inflammatory and antiviral properties, was one of the most potent antivirals identified in both

188 systems, with EC_{50} of 0.4µM and 6.1µM in VeroE6 and HLT cells, respectively (**Figure 3A and B**).

189 Of note, we observed cell toxicity at the highest concentrations ($CC_{50\ VeroE6}$= 22.3µM; $CC_{50\ HLT}$=

190 13.8µM), which translated in a satisfactory *selectivity index* (SI) in VeroE6 cells, but close to the

191 standard threshold of 2 in HLT cells (SI_{VeroE6} = 55.7; SI_{HLT} = 2.2) [37]. The anti-SARS-CoV-2 activity

192 of hydroxychloroquine, a compound known to interfere with endosomal acidification necessary

193 for the proteolytic activity of cathepsins, has been extensively reported [38, 39]. In our study,

194 we observed that hydroxychloroquine was equally effective at inhibiting viral entry in VeroE6

195 and HLT cells ($EC_{50\ VeroE6}$= 1.58µM; $EC_{50\ HLT}$= 9.26µM) (**Figure 3A, B**), with no apparent cytotoxicity

196 (**Figure 3C**). Ergoloid, an approved drug used for dementia, and recently identified as a potential

197 inhibitor of SARS-CoV-2 main protease [40], induced a ~90% viral entry inhibition in HLT cells at

198 non-toxic concentrations (**Figure 3A-C**). Indeed, *SI* for this compound was higher in the HLT

199 model than in VeroE6 cells (SI_{VeroE6} = 3.9; SI_{HLT} = 11.38). Similarly, ivermectin, a broad-spectrum

200 anti-parasitic compound, showed very similar antiviral potency in both models, however *SI*

201 greatly differed (SI_{VeroE6} = 1.4; SI_{HLT} = 8). Additionally, we also calculated the *Coefficient of*

202 *Variation (CV)* of the antiviral assays as a measure of relative variation in 50% Effective

203 Concentration (EC_{50}) values. We show that the *CV* varied from 0.9 to 15.36% in HLT cells (**Figure**

204 **3D**). The low *CV* obtained highlights the suitability of the HLT model for the identification of

205 antivirals.

206 We also detected discordant antiviral results between both models (**Figure 4**). Indeed, 5

207 drugs inhibited SARS-CoV-2 entry in HLT cells without affecting cell viability, but no effect was

208 observed in VeroE6 (**Figure 4A-C**). As expected, camostat, a TMPRSS2 inhibitor [41], was not

209 active in VeroE6 cells due to lack of TMPRSS2 expression in this cell line. However, camostat was

210 highly active in HLT cells (EC_{50}=2.7µM). Indeed, a clinical trial testing its potential to prevent

211 SARS-CoV-2 transmission is ongoing (NCT04625114). Further, sulindac, a nonsteroidal anti-

212 inflammatory drug, and valaciclovir, an antiviral drug, presented some inhibitory potential at

213 100 µM only in HLT cells. Interestingly, phenformin, an antidiabetic drug and an mTOR inhibitor,

214 has been postulated as an inhaled drug candidate against influenza and coronavirus infections

215 [42]. Phenformin reduced the incidence of influenza infection in diabetic patients during the

216   1971 outbreak [42]. Here, we observed that phenformin significantly reduced viral entry starting

217   at 4µM only in HLT cells, supporting previous recommendations as inhaled treatment. Finally,

218   eriodictyol, a flavonoid used as a medicinal plant [43], demonstrated certain activity at 20-

219   100µM. In contrast, luteolin induced potent viral entry suppression only in VeroE6 cells at high

220   concentrations (**Figure 4A-C**). Overall, these results indicate that the HLT is a reproducible and

221   highly relevant model for antiviral testing in a physiological system that recapitulates main

222   antiviral activities observed in cell models, and offers the identification of new compounds

223   missed by conventional systems.

224   **Impact of inflammation and anti-inflammatory drugs on ACE2 expression and SARS-CoV-2**

225   **viral entry**

226   Since SARS-CoV-2 viral infection rapidly induces an inflammatory response, we wondered if

227   certain components of this response could modulate ACE2 expression, potentially allowing

228   increased viral binding of SARS-CoV-2 and thus, enhancing infection. Further, ACE2 has been

229   previously identified as an ISG or a component of the IFN-signaling pathway [11, 44], and a

230   recent investigation showed that cultured human primary basal epithelial cells treated with IFN-

231   α2 and IFN-γ led to upregulation of ACE2 [11]. Moreover, IL-1β and IFN-β upregulated ACE2 in

232   large airway epithelial cell cultures [8]. Thus, considering that type I interferons represent a first

233   line of defence against viral infections and that several cytokines are rapidly induced and

234   associated with disease severity in COVID-19 patients [45], we tested the effects of different

235   molecules on ACE2 expression in our model. In initial experiments, we tested three different

236   doses of a wider range of molecules (including tumor necrosis factor (TNF), IL-6 and IFN-γ), which

237   were used to select doses and compounds of interest. Finally, the effect of IFN-α2, IFN-β1, IL-

238   1β, IL-10 and GM-CSF on ACE2 expression was evaluated in the HLT model. Cells were then

239   treated with selected immune stimuli and cultured for 20h, when the expression of ACE2 in AT-

240   II cells was evaluated by flow cytometry. The only significant change we observed was for IL-1β

241   stimulation, which decreased the fraction of AT-II cells expressing ACE2 (**Figure 5A**). No other

242   significant changes were observed. Overall, we show that relevant inflammatory stimuli have a

243   limited impact on ACE2 expression in AT-II cells, while the effect and impact of IL-1 β on viral

244   infection should be further explored.

245   It is currently not well documented if anti-inflammatory drugs could modulate ACE2

246   expression, and consequently, might impact susceptibility to SARS-CoV-2 infection [46]. Several

247   glucocorticoids have shown to impart activating effects on ACE2 expression in cell lines; cortisol

248   showed the strongest effect on ACE2 activation, followed by prednisolone, dexamethasone, and

249 methylprednisolone [15]. Moreover, NSAIDs, compounds that inhibit cyclooxygenase-1 and 2

250 mediating the production of prostaglandins, which play a role in inflammatory responses, have

251 been linked to ACE2 upregulation [15]. Here, we took advantage of our HLT model to study the

252 effect of several anti-inflammatory drugs on both ACE2 expression and SARS-CoV-2 viral entry.

253 1/5 dilutions of ibuprofen, cortisol, dexamethasone and prednisone were added to HLT cells for

254 20h. Overall, no effect on ACE2 expression was observed after the addition of these anti-

255 inflammatory drugs (**Figure 5B**). Consequently, the antiviral assay showed no major impact of

256 anti-inflammatory compounds on viral entry; however, high concentrations of prednisone and

257 dexamethasone showed a partial reduction of viral entry in HLT cells, without any apparent

258 impact on VeroE6 (**Figure 5C**). Thus, we show that selected anti-inflammatory drugs have limited

259 impact on ACE2 expression within AT-II cells present in the HLT model, as well as in SARS-CoV-2

260 viral entry.

261 **Anti-inflammatory properties of selected compounds**

262 Last, we were interested in modelling the anti-inflammatory properties of several drugs in

263 our HLT model. Based on their antiviral potency in HLT cells, we selected cepharanthine,

264 ergoloid, camostat, ivermectin, hydroxychloroquine and ciclesonide for further evaluation. Of

265 note, some of these drugs have been previously identified as immunomodulators with anti-

266 inflammatory effects (**Table S1**). However, their direct impact on inflammatory molecules

267 directly secreted by human lung cells has not been evaluated. HLT cells were stimulated with

268 lipopolysaccharides (LPS) and IFN-γ in the presence of these antivirals and, 20h after, the

269 expression of IL-6 and CXCL10, a potent pro-inflammatory cytokine and chemokine respectively,

270 was intracellularly measured by flow cytometry. As shown in **Figure 6A,** two major

271 subpopulations of myeloid cells contributed to the upregulation of CXCL10 and IL-6 expression

272 upon stimulation. Myeloid CD11b$^+$CD14$^+$ were the cells with a greater response, showing 50%

273 of cells expressing IL-6 and 30% expressing CXCL10 after stimulation (**Figure 6B**). Using this

274 model of local inflammation, we tested the capacity of the selected compounds to modify this

275 response. We observed that camostat had the most potent effect, which significantly reduced

276 the expression of CXCL10 in CD11b$^+$CD14$^-$ and of IL-6 in CD11b$^+$CD14$^+$ myeloid subsets (**Figure**

277 **6B**). Ergoloid, which has not been linked to modulation of inflammation before, significantly

278 reduced the expression of cytokines in CD11b$^+$ CD14$^+$ myeloid cells, and cepharantine reduced

279 IL-6 production from this subset. Finally, ciclesonide induced CXCL10 secretion in CD11b$^+$CD14$^-$

280 myeloid cells (**Figure 6B**). Altogether, our results validate the HLT model as a relevant method

281 for the identification of anti-inflammatory compounds impacting specific pro-inflammatory cell

282 populations from the lung parenchyma.

283   **Discussion**

284   The emergency created by the rapid spread of SARS-CoV-2 infection worldwide required a

285   quick response from physicians treating these patients, who adapted to the rapid knowledge

286   being generated by both clinical practice and basic research. However, up to date, no specific

287   drugs against SARS-CoV-2 have been approved for clinical use. In this sense, the choice of the

288   cell and animal models used to test the efficacy of antivirals will impact its rapid translation into

289   the clinics. Here, we have developed a novel human lung tissue (HLT) model that can be safely

290   performed in a BSL2 facility, which allows i) the identification of cell targets and expression of

291   viral entry factors, ii) the impact of inflammation on host-pathogen interactions and iii) a rapid

292   medium-high throughput drug screening of SARS-CoV-2 entry inhibitors and local anti-

293   inflammatory candidates.

294   While cell lines have been traditionally used for the screening of potential antiviral

295   compounds due to their reproducibility, as well as being quick and user-friendly assays, they lack

296   physiological relevance. Similarly, entry receptors and viral factors have been identified using

297   immortalized cell lines [10, 47], and cell targets for SARS-CoV-2 in tissues have been mainly

298   determined by analyzing the expression of viral entry factors in RNA-seq datasets [48] or using

299   replication-competent SARS-CoV-2 isolates in BSL3 facilities [49]. Importantly, these studies

300   have identified AT-II cells as main viral targets for SARS-CoV-2 infection in the lungs, and the

301   molecules ACE2, CD147 and TMPRSS2 as the main factors for viral entry [10, 30, 50]. However,

302   the development of more refined and translational *ex vivo* models of SARS-CoV-2 entry will not

303   only have implications for understanding viral pathogenesis, but also will be useful for the

304   characterization of cell targets under specific conditions and the identification of potential

305   antivirals blocking infection of primary cells. In our HLT model, the maintenance of cell type

306   complexity in their *in vivo* proportions may represent a significant advantage over previous

307   models [17, 19, 51]. Using pseudotyped viral particles expressing SARS-CoV-2 spike, we first

308   corroborated that AT-II cells are the primary cell target in lung tissue in steady conditions. This

309   agrees with several studies using different approximations [11, 52, 53] and validates our primary

310   model for viral tropism identification.

311   Moreover, we showed that the HLT model can be successfully used for drug screening

312   purposes. We tested 39 drugs and compared the results with antiviral testing in VeroE6 cells.

313   Not surprisingly, we showed discordant results between both models. Indeed, we found that

314   37.5% of the tested compounds had discordant results between HLT and VeroE6 cells; 31.25%

315   of drugs showed some antiviral effect in HLT but no activity was detected in VeroE6, and 6.2%

316 showed only antiviral effects in VeroE6 cells. Among other reasons, the differential expression
317 of several key proteins needed for viral entry, might explain current discrepancies between
318 models. Importantly, using the HLT model, we identified several compounds with antiviral
319 activity; cepharanthine showed an EC_{50} of 6.1μM and concordantly, it was recently identified in
320 a high throughput screening as one of the most potent drugs against SARS-CoV-2 [54], likewise
321 several other studies have pointed towards this drug as a potent entry and post-entry SARS-CoV-
322 2 inhibitor [55]. Instead, for hydroxychloroquine, an early report suggested no antiviral activity
323 in human lung cells due to different expression of the required proteases for viral entry [56]. In
324 our study, however, we observed that this drug was equally effective at inhibiting viral entry in
325 VeroE6 and HLT cells. Nevertheless, clinical trials failed to show effectiveness of this drug as a
326 treatment for COVID-19 [57-59]. Recently, a strong dependency of SARS-CoV-2 on TMPRSS2 for
327 viral entry, rather than on cathepsin L, was identified as a possible mechanistic explanation for
328 its failure *in vivo* [60]. Similarly, we identified ivermectin as an effective antiviral in HLT cells. Of
329 note, ivermectin received limited attention as a potential drug to be repurposed against COVID-
330 19 based on its limited ability to reach lung tissue *in vivo* [61]. Further, a clinical trial failed to
331 show a reduction in the proportion of PCR-positive patients seven days after ivermectin
332 treatment [62].

333 Importantly, the HLT model also provides a platform for testing anti-inflammatory drugs and
334 the modulation of viral entry factors with drug candidates and immunomodulatory stimuli. We
335 showed that IL-1β was able to reduce ACE2 expression in AT-II cells, in contrast to other
336 cytokines induced during SARS-CoV-2 infection like IFN-α2, IFN-β1, IL-10 and GM-CSF, which did
337 not impact ACE2 protein production. In primary epithelial cells derived from healthy nasal
338 mucosa, Ziegler et al. [11] showed a significant induction of ACE2 transcripts after IFN-α2 and
339 IFN-γ stimulation, as well as and in a human bronchial cell line treated with either type I or type
340 II IFN. Moreover, the authors showed that influenza A virus infection increased ACE2 expression
341 in lung resections [11], strongly suggesting that ACE2 was an ISG. However, following studies
342 showed that ACE2 transcription and protein production was not responsive to IFN. Instead, they
343 described a new RNA isoform, MIRb-ACE2, that was highly responsive to IFN stimulation, but
344 importantly, encoded a truncated and unstable protein product [63, 64]. These results highlight
345 the need to validate scRNA-seq data with orthogonal approaches, such as the confirmation of
346 protein expression levels in relevant systems. In the HLT model, we quantified ACE2 protein
347 expression and importantly, focused our analysis on AT-II cells, the main SARS-CoV-2 targets in
348 lung parenchyma. Also, in agreement with our results, a primary human bronchial epithelial cell
349 model, type I (β), II (γ), or III (λ1) IFNs did not induced ACE2 expression [65]. Moreover, a study

350 performed by Lang et al [66], showed that IFN-γ and IL-4 downregulate the SARS-CoV receptor

351 ACE2 in VeroE6 cells, and similarly, stimulation of A549 cells with IFN-α, IFN-γ, and IFN-α+IFN-γ

352 did not identify ACE2 as an ISG [67].

353 A feasible explanation for the increase of ACE2 protein production upon IL-1 β treatment is

354 that IL-1β activates disintegrin and metalloproteinase domain-containing protein 17 (ADAM17)

355 [68], which mediates the shedding of ACE2 [69]. Although this effect would seem positive to

356 reduce SARS-CoV-2 infection, ACE2 is a lung-protective factor, as it converts Angiotensin (Ang)

357 II to Ang-(1–7); while Ang II promotes harmful effects in the lung, e. g. fibrosis, vasoconstriction,

358 inflammation, endothelial dysfunction, edema, and neutrophil accumulation[70], Ang-(1-7) has

359 counter-regulatory effects protective of lung injury. Moreover, Ang-(1–7) plays an essential role

360 in hemostasis, as it favors anti-thrombotic activity in platelets [71]. In any case, treatment of

361 COVID-19 patients with respiratory insufficiency and hyper inflammation with IL-1 inhibitors was

362 associated with a significant reduction of mortality [72], indicating that at least during severe

363 COVID-19 the overall effect of IL-1β is detrimental. While the reduction of ACE2 expression in

364 AT-II cells by IL-1β may be of interest, it needs to be determined if in combination with other

365 cytokines rapidly induced during viral respiratory infection [73], this effect would remain.

366 Further, glucocorticoids and NSAIDS have been linked to ACE2 upregulation previously [15].

367 In contrast, we did not observe any significant impact of ibuprofen, cortisol, dexamethasone and

368 prednisone on ACE2 protein expression. These results are concordant with a recent report

369 showing that suppression of ciclooxigenase (COX)-2 by two commonly used NSAIDs, ibuprofen

370 and meloxicam, had no effect on ACE2 expression, viral entry, or viral replication in a mouse

371 model of SARS-CoV-2 infection [74]. Moreover, dexamethasone incompletely reduced viral

372 entry. This observation partially agrees with a study using lung cells previously treated with

373 dexamethasone, which showed significant suppression of SARS-CoV-2 viral growth [24].

374 In addition, we used the model for testing local inflammation and potential anti-

375 inflammatory drugs. Several resident and recruited myeloid subsets may contribute to the rapid

376 cytokine storm detected in COVID-19 patients [75-77]. Thus, the identification of antiviral drugs

377 that can also limit the extent of these initial pro-inflammatory events may offer added value to

378 the overall therapeutic effect of a given drug. In this sense, we observed that camostat

379 significantly reduced the expression of proinflammatory molecules IL-6 and CXCL10 in several

380 myeloid CD11b^{+} subsets. Concordantly, in a previous study using primary cultures of human

381 tracheal epithelial cells infected with H1N1 virus, camostat also reduced the concentrations of

382 the cytokines IL-6 and TNF in cell supernatants [78], suggesting a potent anti-inflammatory

383   potential. In contrast, ivermectin did not affect the expression of cytokines in our model.
384   Differing from previous results, ivermectin was shown to have anti-inflammatory effects in mice
385   and *in vitro* using murine macrophages; ivermectin reduced the production of TNF, IL-1 and IL-
386   6, and suppressed LPS-induced NF-κB translocation[79]. Of note, our HLT model of inflammation
387   is optimized up to detect changes in the intracellular expression of cytokines by local myeloid
388   cells, and thus, how these intracellular changes reflect total cytokine production in supernatant
389   needs further evaluation.

390   Finally, it is also important to note the potential limitations of the model, including the limited
391   availability of human lung samples, inter-patient variation (age, smoking, etc.), the effects on
392   lung biology of the medical condition instigating surgery, and the location of the sample
393   resection which may affect the proportion of cell subsets such as AT-II. However, this variability
394   is what shapes the HLT into a highly physiological and relevant model in comparison to current
395   methods based on immortalized cell cultures. Besides the interest of the model proposed here,
396   our results highlight drugs with antiviral activity in HLT cells together with immunomodulatory
397   properties, which could increase the benefit of this treatment during COVID-19 disease
398   progression. For instance, camostat, cepharantine and ergoloid were three of the most potent
399   drugs inhibiting SARS-CoV-2 entry, and remarkably, also exerted a significant anti-inflammatory
400   effect on myeloid cells. Clinical trials with camostat, currently ongoing, ergoloid and
401   cepharatine, will shed light on their use as both antivirals and anti-inflammatory compounds.

402   **Materials and methods**

403   **Cells and virus**

404   VeroE6, isolated from kidney epithelial cells of an African green monkey, were grown in

405   DMEM medium supplemented with 10% fetal bovine serum (FBS; Gibco) 100 U/ml penicillin,

406   and 100 µg/ml streptomycin (Capricorn Scientific) (D10) and maintained at 37°C in a 5% CO_2

407   incubator.

408   The spike of the SARS-CoV-2 virus was generated (GeneArt Gene Synthesis, ThermoFisher

409   Scientific) from the codon-optimized sequence obtained by Ou et al.[35] and inserted into

410   pcDNA3.1D/V5-His-TOPO (pcDNA3.1-S-CoV2Δ19-G614). This plasmid presents the mutation

411   D614G and a deletion in the last 19 amino acids from the original spike. Pseudotyped viral stocks

412   of VSV*ΔG(Luc)-S were generated following the protocol described by Whitt [80] with some

413   modifications. Briefly, 293T cells were transfected with 3µg of the plasmid encoding the SARS-

414   CoV-2 spike. Next day, cells were infected with a VSV-G-Luc virus (MOI=1) (generated from a

415   lentiviral backbone plasmid that uses a VSV promoter to express luciferase) for 2h and gently

416   washed with PBS. Cells were incubated overnight in D10 supplemented with 10% of I1

417   hybridoma (anti-VSV-G) supernatant (ATCC CRL-2700) to neutralize contaminating

418   VSV*ΔG(Luc)-G particles. Next day, the resulting viral particles were collected and titrated in

419   VeroE6 cells by enzyme luminescence assay (Britelite plus kit; PerkinElmer), as described

420   previously [81].

421   **Lung tissue**

422   Lung tissues were obtained from patients without previous COVID-19 history and a recent

423   negative PCR test for SARS-CoV-2 infection undergoing thoracic surgical resection at the

424   Thoracic Surgery Service of the Vall d'Hebron University Hospital. Study protocol was approved

425   by the Ethical Committee (Institutional Review Board number PR(AG)212/2020). Non-neoplastic

426   tissue areas were collected in antibiotic-containing RPMI 1640 and immediately dissected into

427   approximately 8-mm^3 blocks. These blocks were first enzymatically digested with 5 mg/ml

428   collagenase IV (Gibco) and 100 µg/ml of DNase I (Roche) for 30 min at 37ºC and 400 rpm and,

429   then, mechanically digested with a pestle. The resulting cellular suspension was filtered through

430   a 70µm-pore size cell strainer (Labclinics) and washed twice with PBS. Pellet recovered after

431   centrifugation was resuspended in fresh medium (RPMI 1640 supplemented with 5% FBS, 100

432   U/ml penicillin, and 100 µg/ml streptomycin) and DNase I to dissolve cell aggregates, and the

433   resulting cell suspension was then filtered through a 40µm-pore size cell strainer (Labclinics).

434   Cell number and viability were assessed with LUNA™ Automated Cell Counter (Logos

435   Biosystems). For cell phenotyping the following antibodies were used: anti-CD31 (PerCP-Cy5.5,

436   BioLegend), anti-CD11b (FITC, BioLegend), anti-CD11c (Pe-Cy7, BD Biosciences), anti-E-cadherin

437   (Pe-CF594, BD Biosciences), primary goat anti-ACE2 (R&D systems), anti-CD14 (APC-H7, BD

438   Biosciences), anti-CD45 (AF700, BioLegend), anti-EpCAM (APC, BioLegend), anti-CD3 (BV650, BD

439   Biosciences), anti-CD15 (BV605, BD Biosciences) and anti-HLA-DR (BV421, BioLegend). For ACE2

440   detection, after surface staining, cells were stained with secondary donkey anti-goat IgG (PE,

441   R&D Systems) for 30 min at 4 ºC. Cell viability was determined using an AQUA viability dye for

442   flow cytometry (LIVE/DEAD fixable AQUA, Invitrogen). In some experiments, instead of CD11b

443   or CD15, we used a primary rabbit anti-TMPRSS2 or anti-CD147 (BV605, BD Biosciences),

444   respectively. For TMPRSS2 detection, after ACE2 staining with the appropriate secondary

445   antibody, cells were washed twice with PBS 1% NMS (normal mouse serum) and then stained

446   with a secondary goat anti-rabbit IgG (AF488, Thermofisher) for 30 min at 4ºC. After fixation

447   with PBS 2% PFA, cells were acquired in an LSR Fortessa (BD Biosciences), and data were

448   analyzed using the FlowJo v10.6.1 software (TreeStar).

449   **Cytospin and alkaline phosphatase staining**

450   Cytospin preparations were obtained from freshly isolated human lung cells at an

451   approximate density of 150,000 cells/slide, and air-dried during 15 min. Cells were stained with

452   alkaline phosphatase, as an enzyme marking epithelial type II cells, following manufacturer's

453   instructions (Alkaline phosphatase Kit, Sigma). The intensity of pink stain reflects the amount of

454   alkaline phosphatase in positive cells.

455   **ACE2 immunohistochemical staining in human lung tissue sections**

456   Human lungs were maintained in 10% formalin for 24 hours and then embedded in paraffin.

457   Paraffin-embedded lungs were cut into 4 µm sections. After removing the paraffin, endogenous

458   peroxidases were inactivated in an aqueous solution containing 3% H_2O_2 and 10% methanol and

459   antigen retrieval was performed heating the samples in citrate buffer (10mM citric acid, pH 6.0).

460   The sections were then blocked in bovine serum albumin (5%), incubated with anti-ACE2

461   antibody (R&D Systems cat. nº AF933, dilution 1:100) and with biotinylated secondary antibody

462   against goat IgGs (Vector Laboratories cat. nº BA-9500, dilution 1:250). Proteins were visualized

463   using the ABC Peroxidase Standard Staining Kit (ThermoFisher) followed by 3,3'-

464   Diaminobenzidine (DAB) Enhanced Liquid Substrate System (Sigma Aldrich). Counterstaining

465   was done with hematoxylin.

466   **Antiviral screening assay**

467   The complete list of compounds tested in this study, including information about its clinical

468   use, product reference and vendors is shown in **Table S1**. Duplicates of five-fold serial dilutions

469   of 39 antiviral compounds were tested in both VeroE6 cell line and in human lung tissue (HLT)

470   cells using at least 2 different donors. For VeroE6, five-fold serial dilutions of the compounds,

471   ranging from 100µM to 0,25nM, were prepared in D10 in a 96-well flat-bottom plates. VeroE6

472   cells were added at a density of 30.000 cells/well and incubated with the drug for at least 1 h

473   before infection. Subsequently, cells were infected with 1,500 $TCID_{50}$ of VSV*ΔG(Luc)-S virus. In

474   parallel, drug cytotoxicity was monitored by luminescence. To evaluate the antiviral activity of

475   drugs in HLT cells, five-fold serial dilutions of the compounds, ranging from 100µM to 0.8µM or

476   6.4nM, were prepared in R10 in a 96-well conic bottom plates. HLT cells were added at a density

477   of 300,000 cells/well and incubated with the compound for at least 1h before infection. Then,

478   MOI 0,1 of VSV*ΔG(Luc)-S virus were added to wells, and plates were spinoculated at 1,200g

479   and 37ºC for 2h. After infection, fresh medium was added to the wells and cell suspensions were

480   transferred into a 96-well round-bottom plate. Cells were then cultured overnight at 37°C in a

481   5% CO_2 incubator. Each plate contained the following controls: no cells (background control),

482   cells treated with medium (mock infection) and cells infected but untreated (infection control).

483   After 20h, cells were incubated with Britelite plus reagent (Britelite plus kit; PerkinElmer) and

484   then transferred to an opaque black plate. Luminescence was immediately recorded by a

485   luminescence plate reader (LUMIstar Omega). To evaluate cytotoxicity, we used the CellTiter-

486   Glo® Luminescent kit (Promega), following the manufacturer's instructions. Data was

487   normalized to the mock-infected control, after which EC_{50} and CC_{50} values were calculated using

488   Graph-Pad Prism 7.

489   **Modulation of ACE2 expression by anti-inflammatory drugs and immune stimuli**

490   VeroE6 and lung cells were incubated with five-fold serial dilutions of selected anti-

491   inflammatory compounds (ranging from 100µM to 0.8µM) for 20h. Tested drugs included

492   cortisol, ibuprofen, prednisone and dexamethasone. Lung cells were also incubated with the

493   following cytokines: GM-CSF (100 ng/ml, Immunotools), IL-1β (10 ng/ml, Immunotools), IL-10

494   (100 ng/ml, Immunotools), IFN-β (100 U/ml, Immunotools), or IFN-α2 (100 U/ml, Sigma Aldrich).

495   For determination of ACE2 expression, the following surface staining antibodies were used:

496   primary goat anti-ACE2 (R&D Systems), anti-CD45 (AF700, BioLegend), anti-EpCAM (APC,

497   BioLegend), and anti-HLA-DR (BV421, BioLegend). For ACE2 detection, cells were then stained

498   with secondary donkey anti-goat IgG (PE, R&D Systems) for 30 min at 4 ºC. A Fluorescent Minus

499   One control (FMO) without primary anti-ACE2 antibody was used as a control. Cell viability was

500   determined using an AQUA viability dye for flow cytometry (LIVE/DEAD fixable AQUA,

501   Invitrogen). After fixation with PBS 2% PFA, cells were acquired in an LSR Fortessa (BD

502   Biosciences) and analyzed using the FlowJo v10.6.1 software (TreeStar).

503   **Immunomodulatory capacity of selected drugs**

504   HLT cells were cultured in a round-bottom 96-well plate containing 20 µM of cepharanthine,

505   ergoloid mesylate, ciclesonide, hydroxychloroquine, ivermectin, or camostat mesylate alone or

506   in combination with the stimuli LPS (50 ng/ml) and IFN-γ (100 ng/ml). For each patient, a

507   negative control, cells treated with only medium, and a positive control, cells incubated in the

508   presence of LPS and IFN-γ, were included. Immediately, brefeldin A (BD Biosciences) and

509   monensin (BD Biosciences) were added to cells and cultured overnight at 37 ºC in 5% CO_2. Next

510   day, cellular suspensions were stained with the following antibodies: anti-CD11b (FITC,

511   BioLegend), anti-CD69 (PE-CF594, BD Biosciences), anti-CD14 (APC-H7, BD Biosciences), anti-

512   EpCAM (APC, BioLegend), anti-CD3 (BV650, BD Biosciences), anti-CD45 (BV605, BioLegend), and

513   anti-HLA-DR (BV421, BioLegend). Cells were subsequently fixed and permeabilized using the

514   Cytofix/Cytoperm™ kit (BD Biosciences) and intracellularly stained with anti-IL-6 (PE-Cy7,

515   BioLegend), and anti-CXCL10 (PE, BioLegend). Cell viability was determined using an AQUA

516   viability dye for flow cytometry (LIVE/DEAD fixable AQUA, Invitrogen). After fixation with PBS

517   2% PFA, cells were acquired in an LSR Fortessa (BD Biosciences), and data were analyzed using

518   the FlowJo v10.6.1 software (TreeStar).

519   **Statistical analyses**

520   Statistical analyses were performed with Prism software, version 6.0 (GraphPad). A P value

521   <0.05 was considered significant.

522   **Author contributions**

523   Conceptualization, MJ.B. and M.G.; Sample Collection, J.R.; Methodology J.G-E, D.P, M.S, N.

524   M, A.V, MJ.S, J.G-P, J.A, A.S.M and V.F; Formal Analysis, J.G-E., D.P, M.S, M.G and MJ.B; Writing-

525   Original Draft J.G-E and MJ.B; Writing- Review & Editing, J.G-E, M.G. and MJ.B; Funding

526   Acquisition, M.G and MJ.B.; all authors revised the manuscript; Supervision, M.G and MJ.B.

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730   **Figure legends**

731   **Figure 1. Phenotyping of human lung cells.** (**A**). *t*-distributed Stochastic Neighbor

732   Embedding (t-SNE) representation displaying the major cell clusters present in the CD45$^+$ and

733   CD45$^-$EpCam$^+$ fractions of a representative human lung tissue. The vertical bars in the right panel

734   show the frequency of each subset relative to live cells. All cell subsets were identified as shown

735   in Figure S1A. mDCs, myeloid dendritic cells; AT-II, alveolar type 2. (**B**). Phosphatase alkaline

736   positive AT-II cells (pink staining) were detected in a cytospin obtained from human lung tissue

737   cells and observed at 10x. Lower panel shows a high magnification (40x) of the black square.

738   Scale bars are 100 µm and 10 µm in top and bottom panels, respectively. (**C**). *t*-distributed

739   Stochastic Neighbor Embedding (tSNE) representation for ACE2, CD147 and TMPRSS2

740   expression in CD45$^+$ and CD45$^-$EpCam$^+$ fractions from a representative lung tissue. Right graphs

741   show the percentage of expression of each entry factor in the different cell subpopulations,

742   which were identified as in Figure 1A with some modifications for the identification of myeloid

743   cells and neutrophils (From big cells: monocytes/macrophages, CD11c$^+$HLA-DR$^+$CD14$^+$; Alveolar

744   macrophages and mDCs, CD11c$^+$ HLA-DR$^+$ CD14$^-$; Neutrophils, CD11c$^-$ HLA-DR$^-$ CD14$^-$ CD3$^-$). (**D**).

745   Images of ACE2 immunohistochemical staining in human lung tissue sections at 40x

746   magnification, counterstained with haematoxylin (top) or without (bottom). Black arrows

747   indicate staining of ACE2 in AT-II cells (upper panel). Mean±SEM is shown for all graphs.

748   **Figure 2. Susceptibility of VeroE6 and the HLT model to SARS-CoV-2 viral entry.** VeroE6 and

749   HLT cells were infected with two different viral constructs (GFP and Luciferase) expressing the

750   spike protein upon viral entry; VSV*ΔG(GFP)-Spike and VSV*ΔG(Luc)-Spike. (**A**) Representative

751   flow cytometry plots of VeroE6 cells infected with VSV*ΔG(GFP)-Spike or the background form

752   VSV*ΔG(GFP)-empty (left panel); and luciferase activity (RLUs; relative light units) at 20h post-

753   infection with the pseudotyped VSV*G(Luc)-G, the VSV*ΔG(Luc)-Spike or the background form

754   VSV*ΔG(Luc)-empty (right panel). (**B**) Percentage of viral entry after treatment with anti-ACE2

755   antibody (10µg/ml) in VeroE6 cells infected with the pseudotyped virus expressing the control

756   G protein or the spike from SARS-CoV-2. (**C**) A flow cytometry plot showing ACE2 expression in

757   GFP$^+$ VeroE6 cells. Right graph shows mean fluorescence intensity (MFI) of ACE2 in both infected

758   and uninfected fractions, based on GFP expression. (**D**) Representative flow cytometry plots of

759   HLT cells infected with the viral construct expressing the spike protein (VSV*ΔG(GFP)-Spike) or

760   the background form (VSV*ΔG(GFP)-empty) (left panel); and luciferase activity (RLUs; relative

761   light units) at 20h post-infection with the VSV*ΔG(Luc)-Spike or the background form

762   VSV*ΔG(Luc)-empty (right panel). Infection was measured as the percentage of GFP or RLUs,

763   respectively. (**E**) Susceptible HLT cells to viral entry (identified as GFP$^+$ cells) compatible with an

764   AT-II phenotype, determined by the co-expression of HLA-DR and EpCAM in the $CD45^-CD31^-$

765   fraction of live cells. (**F**) Bar plots showing the percentage of viral entry inhibition un HLT cells in

766   the presence of anti-ACE2 antibody (15µg/ml), camostat (100µM) or anti-CD147 antibody

767   (25µg/ml) after cell challenge with VSV*ΔG(Luc)-Spike (left graph) or VSV*ΔG(GFP)-Spike (right

768   graph). Mean±SEM is shown for all graphs. Data in panel 2C were analyzed by a Wilcoxon

769   matched-pairs test; *p<0,05.

770   **Figure 3. Antiviral assays with concordant results between models**. (**A**). Percentage of viral

771   entry in VeroE6 and HLT cells exposed to VSV*ΔG(Luc)-Spike in the presence of cepharanthine,

772   ergoloid, hydroxychloroquine, hypericin, licofelone, ivermectin, ciclesonide, quercitin,

773   vidarabine and celecoxib. Drugs were used at concentrations ranging from 100µM to 0.256nM,

774   in VeroE6, and to 0.8µM in lung cells. Non-linear fit model with variable response curve from at

775   least two independent experiments in replicates is shown (red lines). Cytotoxic effect on VeroE6

776   cells and HLT exposed to drug concentrations in the absence of virus is also shown (green lines).

777   (**B**). EC_{50} values of each drug in VeroE6 and HLT cells. (**C**). CC_{50} values of each drug are shown for

778   VeroE6 and for HLT cells. (**D**). EC_{50}'s coefficient of variation was calculated for ergoloid,

779   hydroxychloroquine and cepharanthine in the HLT model. Data includes two independent

780   experiments using different donors in replicates.

781   **Figure 4. Antiviral assays with discordant results between models**. (**A**). Percentage of viral

782   entry in VeroE6 and HLT cells exposed to VSV*ΔG(Luc)-Spike in the presence of luteolin,

783   eriodictyol, phenformin, camostat, sulindac, and valaciclovir. Drugs were used at concentrations

784   ranging from 100µM to 0.256 nM, in VeroE6, and to 0.8µM in lung cells. Non-linear fit model

785   with variable response curve from at least two independent experiments in replicates is shown

786   (red lines). Cytotoxic effect on VeroE6 cells and HLT exposed to drug concentrations in the

787   absence of virus is also shown (green lines). (**B**). EC_{50} values of each drug in VeroE6 and HLT cells.

788   (**C**). CC_{50} values of each drug are shown for VeroE6 and HLT cells.

789   **Figure 5. Impact of inflammation and anti-inflammatory drugs on SARS-CoV-2 viral entry**

790   **and ACE2 expression.** Both models, HLT cells and VeroE6 cells, were incubated in the presence

791   of different anti-inflammatory drugs to evaluate the modulation of ACE2 expression by flow

792   cytometry and the antiviral effect by luminescence. (**A**). HLT cells were treated with different

793   stimuli for 20h and the percentage of protein expression (left) or the mean fluorescence

794   intensity (MFI, right) of ACE2 receptor was evaluated in the AT-II fraction by flow cytometry. (**B**)

795   Modulation of ACE2 protein expression was assessed by flow cytometry in both models, Vero

796   E6 and HLT cells, in the presence of different concentrations of each anti-inflammatory drug,

797 ranging from 100μM to 0.8μM. Percentage of ACE2 expression was quantified in AT-II cells from

798 at least six independent lung samples, and in VeroE6 cells from 2 independent experiments. (**C**).

799 Cytotoxic effect on Vero E6 and HLT cells exposed to VSV*ΔG(Luc)-Spike in the presence of

800 different concentrations of the anti-inflammatory drugs prednisone, cortisol, ibuprofen and

801 dexamethasone. Drugs were used at a concentration ranging from 100μM to 0.256 nM, in

802 VeroE6, and to 0.8μM in lung cells. Non-linear fit with variable response curve from at least two

803 experiments in replicates is shown (red lines). Cytotoxic effect on Vero E6 cells and HLT cells

804 exposed to different concentrations of drugs in the absence of virus is also shown (green lines).

805 Mean±SEM are shown and statistical comparisons with the control medium were performed

806 using the Wilcoxon test. *p<0.05.

807 **Figure 6. Anti-inflammatory effect of compounds with antiviral activity against SARS-CoV-**

808 **2.** HLT cells were cultured in the presence of 20μM of cepharanthine, ergoloid mesylate,

809 ciclesonide, hydroxychloroquine sulfate, ivermectin or camostat mesylate, alone or in

810 combination with the stimuli LPS (50 ng/ml) and IFN-γ (100 ng/ml). (**A**) *t*-distributed Stochastic

811 Neighbor Embedding (t-SNE) representations displaying the major cell clusters present in live

812 CD45$^+$ myeloid gate, based on FSC and SSC, of a representative human lung tissue in baseline

813 conditions and after stimulation with LPS and IFN-γ. Two major subsets of myeloid cells are

814 shown (CD11b$^+$ CD14$^+$, in blue-green, and CD11b$^+$ CD14$^-$, in orange). The expression of CXCL10

815 and IL-6 among the different populations is shown in maroon and green, respectively. (**B**)

816 Expression of CXCL10 and IL-6 was measured in HLT cells in response to stimuli in the presence

817 of selected drugs in both myeloid subpopulations, CD11b$^+$ CD14$^+$ (left panel) and CD11b$^+$ CD14$^-$

818 (right panel). HQ, hydroxychloroquine. Mean±SEM are represented and statistical comparisons

819 with the control medium were performed using the One sample t test. *p<0.05, **p<0.01.

820 **Figure S1. Gating strategy for the identification of cell subpopulations in the human lung**

821 **tissue model**. (**A**) General gating strategy used to identify different cell subsets in lung samples.

822 A gate based on FSC vs. SSC was followed by doublet and dead cells exclusion. From live CD45$^-$

823 cells, endothelial cells (CD31$^+$, purple) and epithelial cells (EpCAM$^+$, grey) were gated, and within

824 epithelial cells, AT-II cells (EpCAM$^+$ and HLA-DR$^+$, pink) were identified. Out of live CD45$^+$ cells

825 and based on FSC vs. SSC, we identified a lymphocyte population in which we distinguished

826 between non-T lymphocytes (turquoise) and T cells (dark green) based on CD3 expression; and

827 big cells, where we identified three major subsets based on their expression of CD11b and CD11c

828 and, subsequently, CD14 and HLA-DR markers. We identified alveolar macrophages (blue),

829 monocytes (violet), myeloid dendritic cells (mDCs, fuchsia) and neutrophils (orange). (**B**)

830   Representative flow cytometry plots showing ACE2 staining and its respective fluorescence

831   minus one (FMO) control.

832   **Figure S2. Optimization of lung tissue enzymatic digestion visualized by *t*-distributed**

833   **Stochastic Neighbor Embedding (tSNE).** (**A**) Representative tSNE maps showing concatenated

834   flow cytometry standard files for three different protocols based on different digestion enzymes

835   (collagenase, liberase or trypsin) from total live cells (upper), CD45$^+$ cells (middle) and CD45$^-$ cells

836   (lower). (**B**) Bar plots showing cell-type composition (count) analyzed by flow cytometry for each

837   tissue protocol.

838   **Figure S3. Gating strategy for the identification of anti-inflammatory effects of selected**

839   **compounds.** (**A**) General gating strategy used to evaluate the expression of inflammatory

840   molecules in lung samples. A gate based on FSC vs. SSC was followed by doublet and dead cells

841   exclusion. From live CD45$^+$ cells and based on FSC vs. SSC, we identified lymphocyte population

842   and big cells, in which we identified two subsets based on their expression of CD11b and CD14:

843   myeloid CD11b$^+$CD14$^+$ cells (blue-green) and myeloid CD11b$^+$CD14$^-$ cells (orange) are shown.
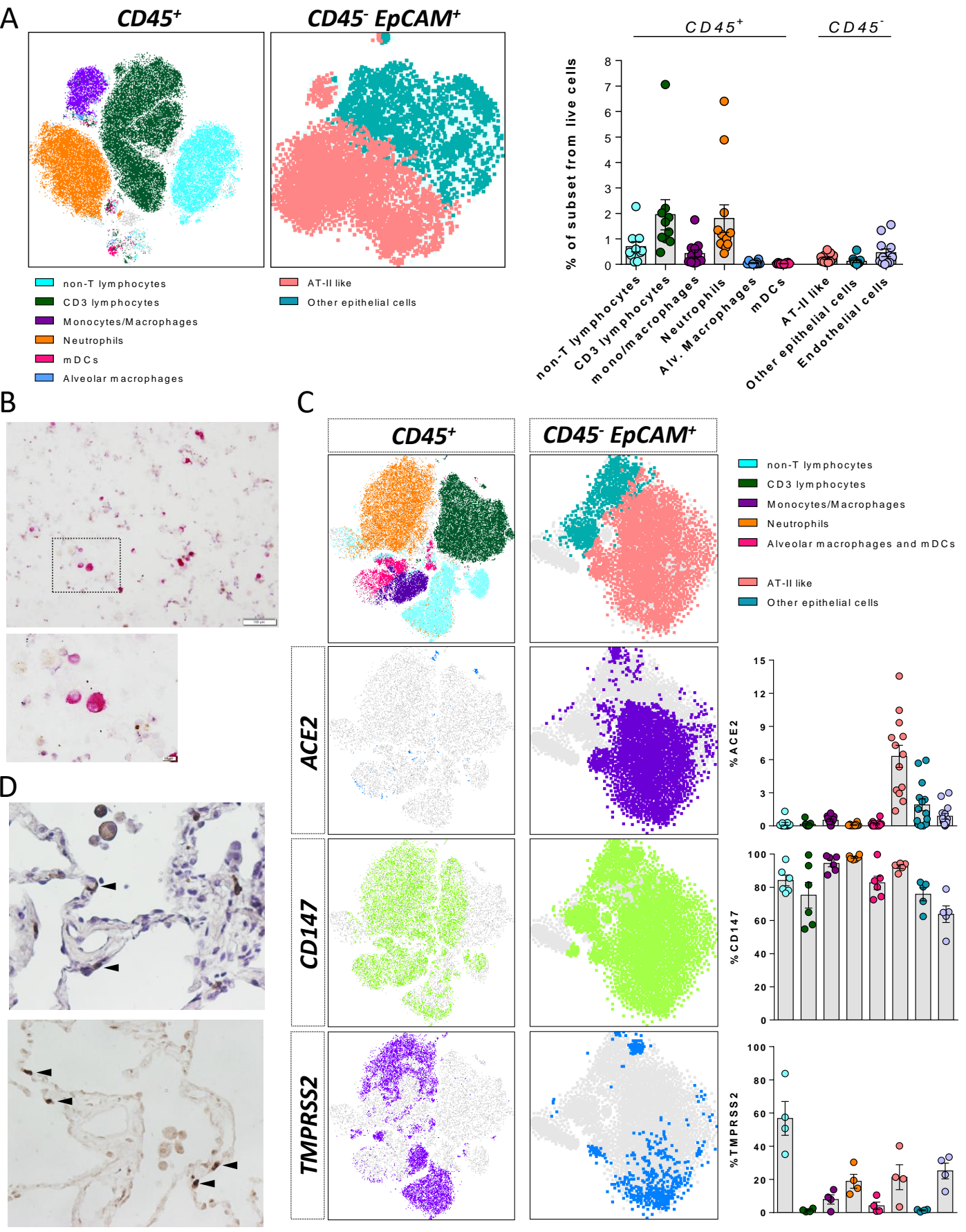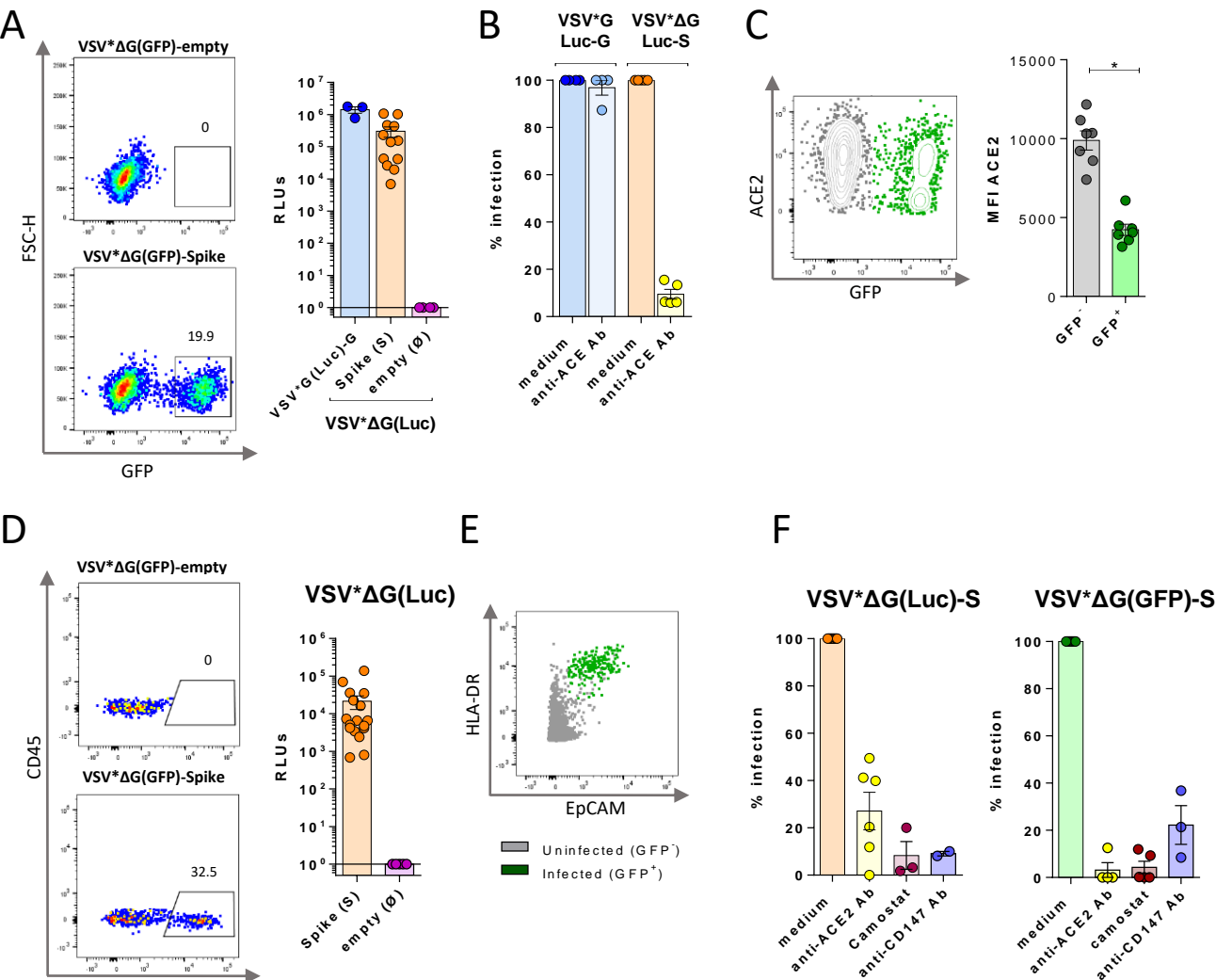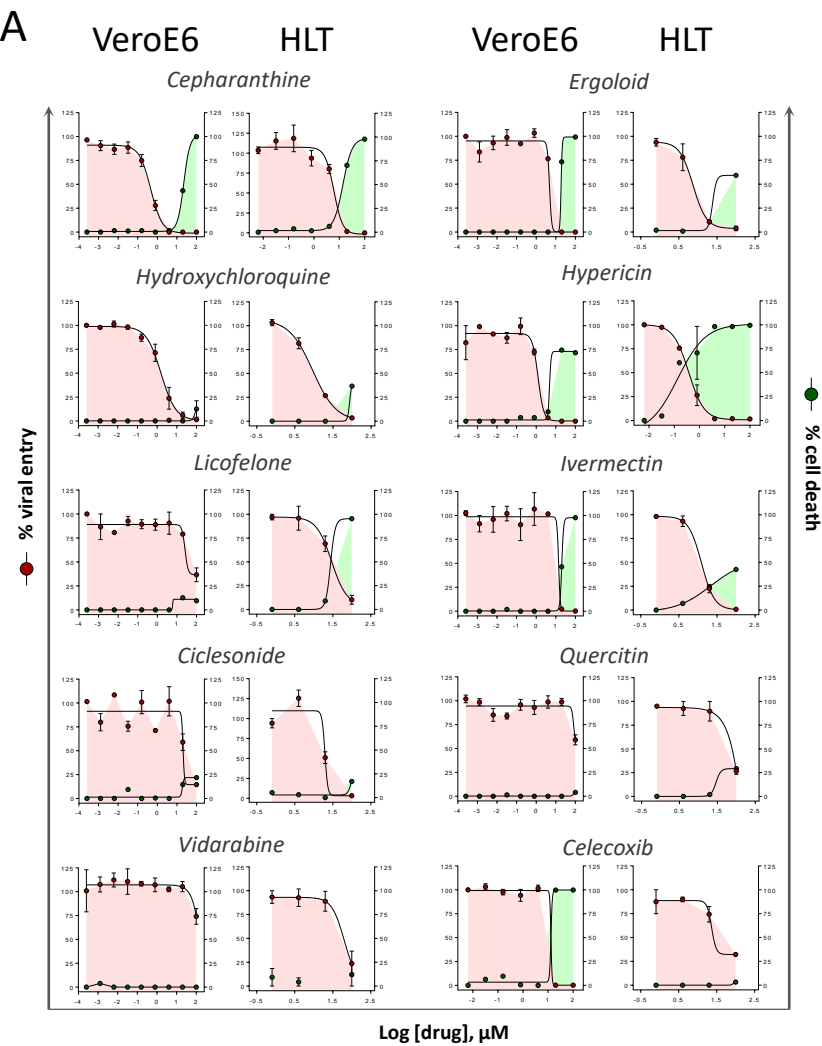
Figure 1

Figure 2

Figure 3

A

VeroE6   HLT   VeroE6   HLT

Cepharanthine   *Ergoloid*

Hydroxychloroquine   *Hypericin*

Licofelone   *Ivermectin*

Ciclesonide   *Quercitin*

Vidarabine   *Celecoxib*

% viral entry   % cell death

Log [drug], µM

B

	VeroE6	HLT
Drugs	EC$_{50}$ (µM)	
Cepharanthine	0.4	6.1
Quercitin	>100	78
Ergoloid	4.7	7.7
Ciclesonide	20.5	19.6
Licofelone	87.5	29.3
Hydroxychloroquine	1.58	9.2
Ivermectin	13.9	12.4
Hypericin	1.2	0.37
Vidarabine	>100	66
Celecoxib	13	~25

C

	VeroE6	HLT
Drugs	CC$_{50}$ (µM)	
Cepharanthine	22.3	13.8
Quercitin	>100	>100
Ergoloid	18.6	~87.7
Ciclesonide	>100	>100
Licofelone	>100	29.4
Hydroxychloroquine	>100	>100
Ivermectin	20.23	>100
Hypericin	4.8	0.13
Vidarabine	>100	>100
Celecoxib	13	>100

D

Coefficient of Variation of EC$_{50}$

Ergoloid   Hydroxychloroquine   Cepharanthine

Figure 4

A

VeroE6   HLT   VeroE6   HLT

% viral entry

% cell death

Luteolin   Eriodictyol

Phenformin   Camostat

Sulindac   Valaciclovir

Log [drug], µM

B
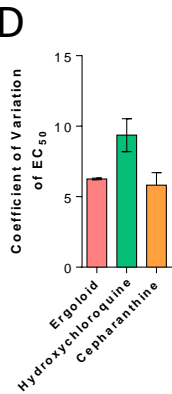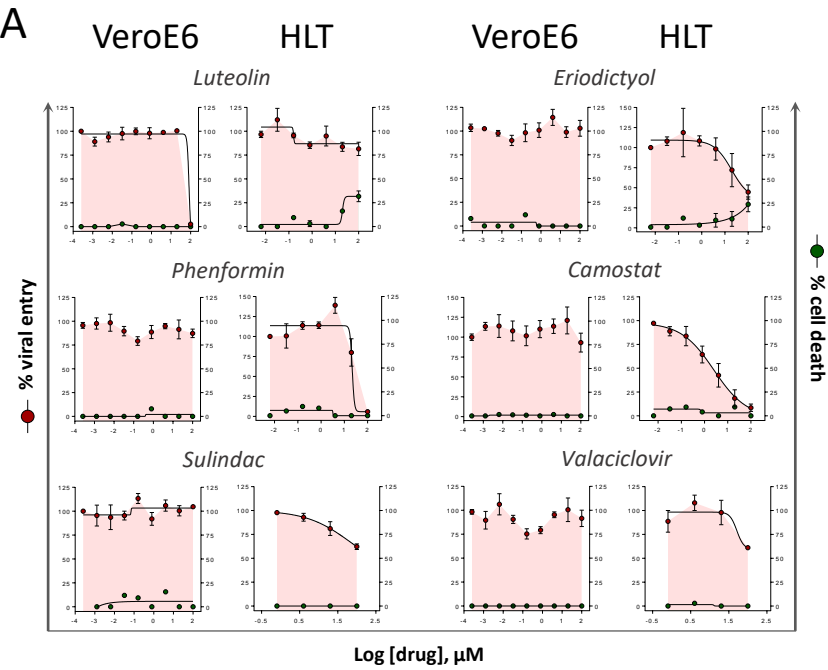
	VeroE6	HLT
Drugs	EC$_{50}$ (µM)	
Eriodictyol	no effect	90
Luteolin	~70.7	no effect
Camostat	no effect	2.7
Phenformin	no effect	~22.45
Sulindac	no effect	>100
Valaciclovir	no effect	>100

C

	VeroE6	HLT
Drugs	CC$_{50}$ (µM)	
Eriodictyol	>100	>100
Luteolin	>100	>100
Camostat	>100	>100
Phenformin	>100	>100
Sulindac	>100	>100
Valaciclovir	>100	>100

Figure 5

Figure 6

A

B