Evaluation of SARS-CoV-2 Entry, Inflammation and New Therapeutics in Human Lung Tissue Cells

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

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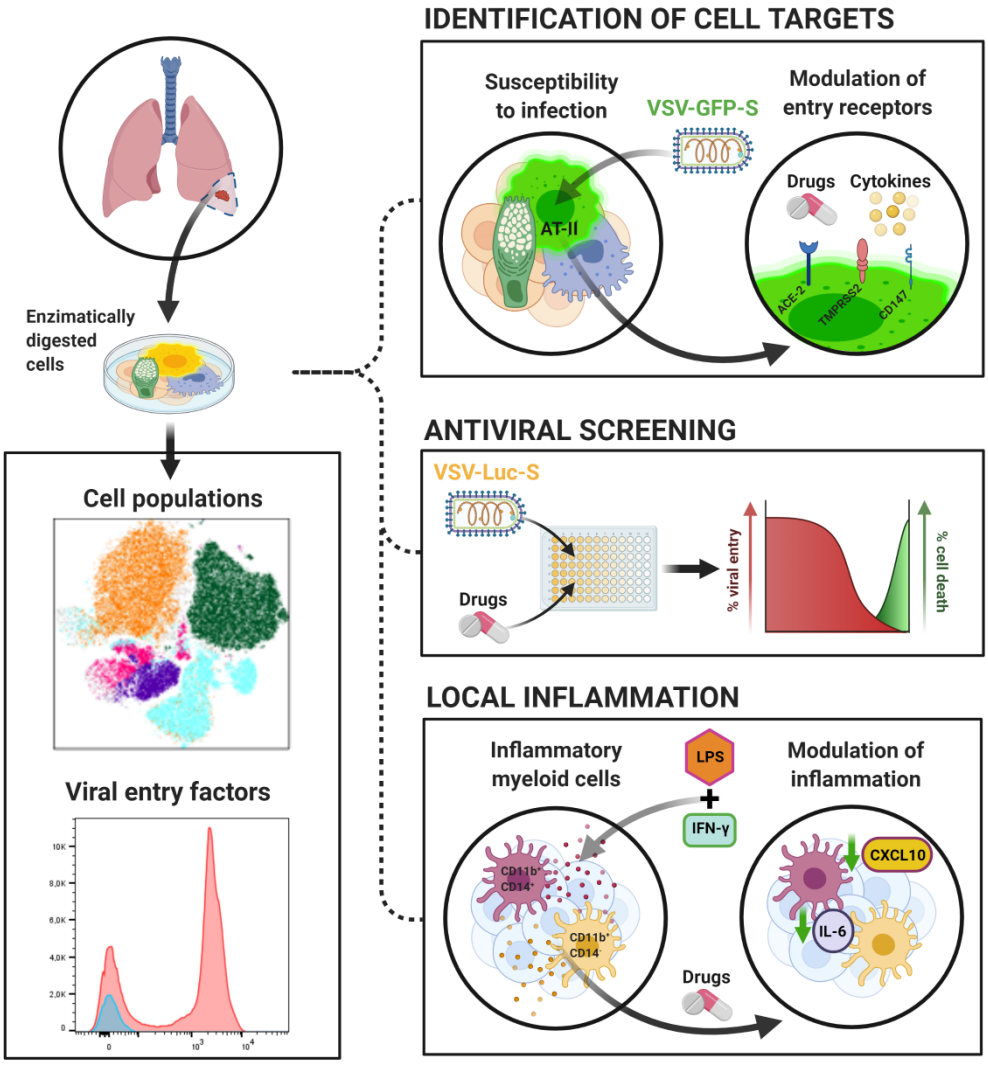
Abstract

The development of physiological models that reproduce SARS-CoV-2 infection in primary human cells will be instrumental to identify host-pathogen interactions and potential therapeutics. Here, using cell suspensions directly from primary human lung tissues (HLT), we have developed a rapid platform for the identification of viral targets and the expression of viral entry factors, as well as for the screening of viral entry inhibitors and anti-inflammatory compounds. The direct use of HLT cells, without long-term cell culture and *in vitro* differentiation approaches, preserves main immune and structural cell populations, including the most susceptible cell targets for SARS-CoV-2; alveolar type II (AT-II) cells, while maintaining the expression of proteins involved in viral infection, such as ACE2, TMPRSS2, CD147 and AXL. Further, antiviral testing of 39 drug candidates reveals a highly reproducible method, suitable for different SARS-CoV-2 variants, and provides the identification of new compounds missed by conventional systems, such as VeroE6. Using this method, we also show that interferons do not modulate ACE2 expression, and that stimulation of local inflammatory responses can be modulated by different compounds with antiviral activity. Overall, we present a relevant and rapid method for the study of SARS-CoV-2.

48 **Graphical Abstract**



49

50 **Highlights**

51 *Ex vivo* physiological systems for the study of SARS-CoV-2-host interactions are scarce. Here, we
52 establish a method using primary human lung tissue (HLT) cells for the rapid analysis of cell
53 tropism and identification of therapeutics.

54 - HLT cells preserve main cell subpopulations, including alveolar type-2 cells, and
55 expression of SARS-CoV-2 entry factors ACE2, CD147, TMPRSS2 and AXL.
56 - HLT cells are readily susceptible to SARS-CoV-2 infection without the need of cell
57 isolation or further cell differentiation.
58 - Antiviral testing in HLT cells allows the rapid identification of new drug candidates
59 against SARS-CoV-2 variants, missed by conventional systems.

60 • Local inflammation is supported in HLT cells and offers the identification of relevant
61 anti-inflammatory compounds for SARS-CoV-2 infection.

62 **Introduction**

63 Only one antiviral against SARS-CoV-2, remdesivir, has been approved for the treatment of
64 COVID-19 in adults and pediatric patients (12 years of age and older) requiring hospitalization
65 [1, 2]. Moreover, molnupiravir, a ribonucleoside analog that inhibits SARS-CoV-2 replication, has
66 shown activity in several preclinical models of SARS-CoV-2, including prophylaxis, treatment, and
67 prevention of transmission [3, 4]. Promising human clinical trials in non-hospitalized patients are
68 ongoing (NCT04575597), and it could be the first oral antiviral medicine approved for COVID-19.
69 Further, the development of *Acute Respiratory Distress Syndrome* in severe COVID-19 patients
70 has been linked to dysregulated inflammatory responses. In this regard, treatment with the
71 glucocorticoid dexamethasone decreased 28-day mortality among patients receiving invasive
72 mechanical ventilation, but little benefit was observed in patients without respiratory support
73 [5]. Despite these major advances in treatment options for COVID-19, the rapid identification of
74 new antivirals that could be easily transferred into the clinic is still of paramount importance,
75 particularly with the potential emergence of drug-resistant variants.

76 Screening of novel drug candidates is often performed using cell lines. In this sense, the most
77 widely used cell lines for SARS-CoV-2 studies are epithelial cells derived either from lung (Calu-
78 3), kidney (VeroE6), or colon (CaCo-2) [6]. These immortalized systems are highly reproducible
79 and easy to handle but lack physiological relevance. The differential gene expression profiling of
80 cell lines compared with primary cells from tissues might significantly affect important enzymes
81 involved in the viral replication cycle. For instance, the level of ACE2 expression, the main
82 receptor used by SARS-CoV-2 for viral entry, is variable among several cell lines [7], while only a
83 small fraction of alveolar type II (AT-II) cells, the main target for SARS-CoV-2 in the distal lung,
84 express ACE2 [8, 9]. In addition, SARS-CoV-2 spike (S) glycoprotein, which is responsible for viral
85 entry into target cells, can be activated by several host proteases, such as furin, transmembrane
86 serine proteinase 2 (TMPRSS2) and cathepsin L, in a pH-dependent or independent manner [10,
87 11]. Whereas in some cell lines S protein is activated by endosomal pH-dependent protease
88 cathepsin L, in airway epithelial cells viral entry depends on the pH-independent TMPRSS2
89 protease [11]. Thus, it is currently not well defined if SARS-CoV-2 may utilize multiple cell-type-
90 specific host proteins for viral replication in primary target tissues and therefore, the potency of
91 therapeutics directed against these proteins may also differ.

92 Further, inflammatory immune responses might also impact viral dynamics in the lung by
93 affecting the expression of entry receptors. In this sense, early studies discovered that ACE2 was
94 a human interferon-stimulated gene (ISG); IFN-β and IFN-γ were shown to strongly upregulate

95 the expression of ACE2 at the mRNA and cell surface protein levels, indicating that inflammatory
96 molecules could shape cell susceptibility to viral infection [12]. However, how anti-inflammatory
97 drugs may affect ACE2 expression and facilitate SARS-CoV-2 infection remains to be elucidated.
98 One study reported that the use of nonsteroidal anti-inflammatory drugs (NSAIDs), such as
99 ibuprofen, was linked to enhanced ACE2 expression in a diabetic-induced rat model [13] and
100 other reports raised alarms regarding the possible role of NSAIDs at increasing susceptibility to
101 SARS-CoV-2 infection [14, 15]. On the contrary, experimental and clinical evidence showed that
102 medium-to-low-dose glucocorticoids may play a protective role in COVID-19 by activating ACE2
103 and suppressing the associated cytokine storm [16]. Overall, the use of more relevant and
104 physiological models for the study of SARS-CoV-2 infection, the identification of drug candidates,
105 or the impact of new therapeutics on the disease, could significantly advance the successful
106 translation of the results into the clinic.

107 Primary epithelial cell cultures of nasal and proximal airway epithelium have been used to study
108 SARS-CoV-2 infection in the upper airways [9, 17-19]. Similarly, organ on-chip and organoid
109 models of AT-II cells have been successfully developed [18, 20]. While very useful, these models
110 require long-term culture (sometimes several weeks) combined with the addition of cytokines
111 that might change cell functionality [21]. The direct use of human lung tissue (HLT) cells offers
112 important advantages over other *in vitro* and *in vivo* approaches for several reasons; it mimics
113 the main site of viral replication in the lung, contains all heterogeneous cell components present
114 in the tissue (with greater functional complexity compared to cell lines), and the cells were not
115 subjected to long-term culture nor exposed to in vitro differentiation approaches. In the past,
116 similar lung models have been successfully established to study the effect of allergens and
117 inflammatory stimuli [22, 23]. Importantly, using HLT cells allow mimicking an inflammatory
118 local response that could be attenuated by anti-inflammatory drugs, providing a low/medium
119 throughput screening of anti-inflammatory candidates for the treatment of airway diseases [24].
120 Significantly, lung tissues not only can be infected with SARS-CoV-2, but also generate local
121 immune responses to viral infection [25]. Considering all these factors, here we aimed to
122 characterize a physiological human lung tissue system, which could be used for the study of
123 virus-host interactions and the identification of potential antiviral compounds and their capacity
124 to modify local inflammation.

125 ## Results

126 ### Characterization of HLT cells

127 Non-neoplastic lung parenchyma was obtained from hospitalized non-COVID19 patients
128 undergoing thoracic surgery. First, we optimized cell culture and digestion conditions, since the
129 methodology used for tissue processing can significantly impact cell-type yield, viability and
130 function of target cell populations. We focused on the preservation of EpCAM$^+$ cells expressing
131 HLA-DR [26, 27], which in adult alveolar parenchyma is characteristic of AT-II cells (thereafter
132 referred as enriched AT-II cells). We also aimed to preserve several hematopoietic cell subsets,
133 as shown in the flow cytometry gating strategy (**Figure S1A**). We observed that collagenase
134 outperformed liberase and trypsin at preserving the enriched population of AT-II cells, the main
135 SARS-CoV-2 target (**Figure S2A-B**). Among the hematopoietic cells present in lung parenchyma,
136 we identified CD3 T lymphocytes (which represented 7.39% ± 6.97 out of the total living cells),
137 myeloid dendritic cells (0.10% ± 0.06), monocytes/macrophages subsets (1.36% ± 1.27),
138 neutrophils (5.00% ±4.28) and alveolar macrophages (0.21% ± 0.16). Moreover, out of the non-
139 hematopoietic cells (CD45-), enriched AT-II and endothelial cells represented 1.11% ± 1.16 and
140 0.78% ± 0.81 of the total living cells, respectively. Based on EpCAM expression and lack of CD31
141 and CD45, other epithelial cells including AT-I cells represented 0.52% ± 0.46 out of the living
142 fraction (**Figure 1A**). All these populations have been previously identified in human lungs [25,
143 28, 29]. Of note, other abundant structural cell subsets were not defined by specific phenotypic
144 markers within the same panel. Moreover, the nature of AT-II cells in lung cell suspensions was
145 also addressed by staining with phosphatase alkaline (**Figure 1B**), which is expressed in AT-II cells
146 both *in vitro* and *in vivo* [30], and by detection of Surfactant Protein C, which is expressed
147 exclusively by fully differentiated AT-II cells [31, 32] (**Supplementary Figure 1B**).

148 Next, we focus on the expression of previously identified proteins involved in viral entry.
149 Single-cell transcriptome studies have shown that ACE2, one of the main host cell surface
150 receptors for SARS-CoV-2 attachment and infection, is predominantly expressed by AT-II cells [9,
151 33]. Moreover, ACE2 expression wanes in distal bronchiolar and alveolar regions paralleled by
152 SARS-CoV-2 infectivity [9]. In human lung parenchyma-derived cells, we found ACE2 expression
153 mainly associated to the population enriched in AT-II cells (**Figure 1C**), a finding that was
154 confirmed by immunohistochemistry in concomitant tissue samples (**Figure 1D**). The percentage
155 of cells expressing ACE2 was rather small and varied between individuals (6.23% ± 3.47) (**Figure**
156 **1C**), as previously described [8]. We also studied the expression of CD147, which has been
157 reported as a route of SARS-CoV-2 infection *in vitro* and *in vivo* models [34]. CD147 was

158 ubiquitously expressed in several hematopoietic and non-hematopoietic cells (**Figure 1C**).
159 Importantly, 92.3% ± 2.4 of the population enriched in AT-II cells expressed CD147 (**Figure 1C**).
160 Similarly, and in agreement with other studies [35, 36], TMPRSS2 protease expression was also
161 identified in several subsets, including enriched AT-II cells (31.08% ± 7.06) (**Figure 1C**). When we
162 studied the double expression of ACE2 and TMPRSS2, or ACE2 and CD147, we found that only
163 1.31% ± 0.70 and 3.02% ± 1.84 of AT-II cells expressed both markers, respectively
164 (**Supplementary Figure 3B**). Last, we studied the expression of AXL, another candidate receptor
165 for SARS-CoV-2 entry in lung cells [37]. We detected high expression of AXL on myeloid dendritic
166 cells (45.27% ± 17.11), while only a fraction of the enriched AT-II cells (mean of 3.82% ± 4.71)
167 expressed AXL (**Supplementary Figure 3A**). Overall, we found that human lung cell suspensions
168 preserved critical populations and factors required for SARS-CoV-2 infection.

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

170 Next, we assessed if HLT cells were susceptible to viral infection. We generated pseudotyped
171 vesicular stomatitis virus (VSV) viral particles bearing the D614G form of the S protein of SARS-
172 CoV-2 and expressing either luciferase (VSV*ΔG (Luc)-S) or GFP (VSV*ΔG (GFP)-S) reporter genes
173 upon cell entry. As a control, we used the VSV-G virus, which has very broad cell tropism. As
174 expected, VeroE6 cells were highly susceptible to SARS-CoV-2 entry, as demonstrated for
175 VSV*ΔG (GFP)-S and VSV-ΔG (Luc)-S (**Figure 2A**). Of note, camostat, an inhibitor of the host
176 protease TMPRSS2, did not inhibit cell entry in this cell line, consistent with its lack of expression
177 of this protein (**Figure 2B**). Anti-ACE2 antibody blocked more than 90% of VSV*ΔG (Luc)-S, yet
178 was inactive for VSV-G (Luc) infection (**Figure 2B**). This observation has been widely reported
179 before [11, 38, 39], and identifies ACE2 as the main cell receptor required for viral entry in
180 VeroE6. Importantly for viral pathogenesis, it has been postulated that SARS-CoV-2 S protein
181 might downregulate ACE2 expression, as previously observed for SARS-CoV [40]. We
182 consistently observed a significant strong reduction in ACE2 expression after viral entry (**Figure
183 2C**).

184 We then evaluated the susceptibility of HLT cells to viral entry, using the same viral
185 constructs. HLT cells were readily infected with pseudotyped S particles (VSV*ΔG (Luc)-S and
186 VSV*ΔG (GFP)-S), with the natural donor variation representative of primary samples (**Figure
187 2D**). As expected [9], lung cells enriched with the AT-II phenotype were identified as the main
188 SARS-CoV-2 cell targets in steady conditions (**Figure 2E**). Blockade of ACE2 resulted in a donor-
189 dependent reduction of viral infectivity, ranging from 50 to 100% (**Figure 2F**). Camostat
190 significantly inhibited viral entry in all HLT assays, although the entry process was not always

191 completely abrogated (**Figure 2F**), suggesting that AT-II cells may become infected through the
192 use of alternative factors [41]. Similarly, the presence of an anti-CD147 antibody and the
193 recombinant protein AXL inhibited SARS-CoV-2 entry (**Figure 2F and Supplementary Figure 3D**).
194 Collectively, these data indicate that HLT cells are susceptible to SARS-CoV-2 viral entry, and that
195 ACE2, CD147, TMPRSS2 and AXL are important proteins required for viral entry in human lung
196 cells. Thus, these results support the value of the direct use of HLT cells to successfully study
197 SARS-CoV-2 viral entry, and related mechanisms, in a more physiological system compared to
198 immortalized cell lines.

199 **Antiviral assays in HLT cells**

200 To validate the HLT system as a platform for the rapid screening of antiviral candidates, we
201 assayed potential antiviral compounds, most of them previously identified by computational
202 methods with predicted ability to inhibit SARS-CoV-2 cell entry due to their interaction with S
203 protein or with the interface S-ACE2 [42]. A detailed description of the 39 selected drugs is
204 available in **Table S1**. HLT cells were exposed to VSV*ΔG (Luc)-S virus in the presence of 1/5
205 serial dilution of the different tested compounds. 20h post-exposure, antiviral activity and cell
206 viability were measured by luminescence. Cell viability for the individual HLT populations, before
207 and after SARS-CoV-2 infection, was measured by flow cytometry and is shown in
208 **Supplementary Figure 3C**. Antiviral results in HLT cells were systematically compared with
209 parallel testing in the cell line VeroE6. Among the 39 drugs that were evaluated in our study, 15
210 of them (38%) showed some antiviral activity against SARS-CoV-2 with EC_{50} values ranging from
211 0.37µM to 90µM (**Table S2**). From these, 25% had concordant results between both models
212 (**Figure 3**). Cepharanthine, a naturally occurring alkaloid reported to have potent anti-
213 inflammatory and antiviral properties, was one of the most potent antivirals identified in both
214 systems, with EC_{50} of 0.46µM and 6.08µM in VeroE6 and HLT cells, respectively (**Figure 3A and**
215 **B**). Of note, we observed cell toxicity at the highest concentrations ($CC_{50\ VeroE6}$= 22.3µM; $CC_{50\ HLT}$=
216 13.8µM), which translated in satisfactory *selectivity indexes* (SI= CC_{50} /EC_{50} of SI_{VeroE6} = 48.47 and
217 SI_{HLT} = 2.64 [43]. The anti-SARS-CoV-2 activity of hydroxychloroquine, a compound known to
218 interfere with endosomal acidification, which is necessary for cathepsin activity, has been
219 extensively reported [44, 45]. In our study, we observed that hydroxychloroquine was equally
220 effective at inhibiting viral entry in VeroE6 and HLT cells ($EC_{50\ VeroE6}$= 1.58µM; $EC_{50\ HLT}$= 3.22µM)
221 (**Figure 3A, B**), with no apparent cytotoxicity (**Figure 3C**). Ergoloid, an approved drug used for
222 dementia, and recently identified as a potential inhibitor of main protease of SARS-CoV-2 [46],
223 induced ~90% of viral entry inhibition in HLT cells at non-toxic concentrations (**Figure 3A-C**).
224 Indeed, *SI* for this compound was higher in the HLT model than in VeroE6 cells (SI_{VeroE6} = 3.9; SI_{HLT}

225 = 11.38). Similarly, ivermectin, a broad-spectrum anti-parasitic compound, showed very similar
226 antiviral potency in both models, however *SI* greatly differed between them (SI_{VeroE6} = 1.4; SI_{HLT} =
227 7.75). Additionally, we plot the individual EC_{50} values obtained from the different donors. We
228 show that the assay was reproducible (**Supplementary Figure 3E**), highlighting the suitability of
229 the HLT system for the rapid identification of antivirals.

230 We also detected discordant antiviral results between both models (**Figure 4**). Four drugs
231 inhibited SARS-CoV-2 entry in HLT cells without affecting cell viability, with no antiviral effect in
232 VeroE6 (**Figure 4A-C**). As expected and mentioned before, camostat, a TMPRSS2 inhibitor [47],
233 was not active in VeroE6 cells due to the lack of TMPRSS2 expression in this cell line. However,
234 camostat was highly active in HLT cells (EC_{50}=3.3µM). However, in a clinical trial with Covid-19
235 patients receiving camostat within the first 48h of admission, no significant benefit was
236 observed [48]. Yet, the potential benefit of camostat during the early phase of infection remains
237 to be addressed. Further, valaciclovir, an antiviral drug, presented some inhibitory potential at
238 100 µM only in HLT cells. Interestingly, phenformin, an antidiabetic drug and an mTOR inhibitor,
239 has been postulated as an inhaled drug candidate against influenza and coronavirus infections
240 [49]. Phenformin reduced the incidence of influenza infection in diabetic patients during the
241 1971 outbreak [49]. Here, we detected that phenformin significantly reduced viral entry at 20-
242 100µM only in HLT cells, supporting previous recommendations as inhaled treatment. Finally,
243 eriodictyol, a flavonoid used as a medicinal plant [50], demonstrated certain activity starting at
244 4µM. In contrast, quercetin induced some viral entry suppression only in VeroE6 cells and at
245 high concentrations (**Figure 4A-C**). To further demonstrate the suitability of our model to test
246 antivirals against other SARS-CoV-2 variants, we tested the inhibitory capacity of the drugs with
247 the lowest IC_{50} values, cepharanthine, camostat, ivermectin, hydroxychloroquine, and
248 ciclesonide using a pseudovirus containing the SARS-CoV-2 spike of the delta variant. Similar
249 inhibitory dynamics for all tested drugs were shown using both viral variants, indicating the
250 versatility of our model (**Figure 4D)**.

251 Last, we verified our main findings in HLT cells using a replication-competent SARS-CoV-2
252 virus. Although we initially also included ergoloid, we observed high toxicity in HLT cells (>60%
253 cell toxicity) at longer incubation times (48h), which precluded the execution of the antiviral
254 assay (**Supplementary Figure 3F**). HLT cells were infected with a SARS-CoV-2 clinical isolate and
255 viral genomes were measured in the supernatant of cell cultures by RT-PCR in the presence of
256 the different drugs. SARS-CoV-2 successfully replicated in HLT samples (**Figure 4E**), and viral
257 replication was partially inhibited by all five drugs (**Figure 4F**). These results are consistent with
258 previous reports describing the antiviral potency of camostat using precision-cut lung slices from

259 donors [47, 51]. Overall, these results indicate that HLT cells represent a reproducible and
260 relevant system for the screening of antivirals in a physiological model. This system not only
261 recapitulates the main antiviral activities observed in cell models, but also allows the
262 identification of new compounds missed by conventional systems.

263 **Impact of inflammation and anti-inflammatory drugs on ACE2 expression and SARS-CoV-2**
264 **viral entry**

265 Since SARS-CoV-2 viral infection rapidly induces an inflammatory response, we wondered if
266 certain components of this response could modulate ACE2 expression, potentially increasing
267 viral binding of SARS-CoV-2 and thus, enhancing infection. Further, ACE2 has been previously
268 identified as an ISG or a component of the IFN-signaling pathway [12, 52], and a recent
269 investigation showed that cultured human primary basal epithelial cells treated with IFN-α2 and
270 IFN-γ led to upregulation of ACE2 [12]. Moreover, IL-1β and IFN-β upregulated ACE2 in large
271 airway epithelial cell cultures [9]. Thus, considering that type I interferons represent a first line
272 of defence against viral infections and that several cytokines are rapidly induced and associated
273 with disease severity in COVID-19 patients [53], we tested the effects of different molecules on
274 ACE2 expression in HLT cells. In initial experiments, we tested three different doses of a wider
275 range of molecules (including tumor necrosis factor (TNF), IL-6 and IFN-γ that were subsequently
276 discarded), which were used to select doses and compounds of interest. Finally, the effect of
277 IFN-α2, IFN-β1, IL-1β, IL-10 and GM-CSF on ACE2 expression was evaluated in HLT cells. Cells
278 were then treated with selected immune stimuli and cultured for 20h, when the expression of
279 ACE2 in enriched AT-II cells was evaluated by flow cytometry. The only significant change we
280 observed was for IL-1β stimulation, which decreased the fraction of AT-II cells expressing ACE2
281 (**Figure 5A**). No other significant changes were observed, indicating that relevant inflammatory
282 stimuli, besides IL-1β, have a limited impact on ACE2 expression in AT-II cells.

283 Moreover, it is currently not well documented if anti-inflammatory drugs could modulate
284 ACE2 expression, and consequently, impact susceptibility to SARS-CoV-2 infection [54]. Several
285 glucocorticoids have shown to impart activating effects on ACE2 expression in cell lines; cortisol
286 showed the strongest effect on ACE2 activation, followed by prednisolone, dexamethasone, and
287 methylprednisolone [16]. Moreover, NSAIDs, compounds that inhibit cyclooxygenase-1 and 2
288 mediating the production of prostaglandins, which play a role in inflammatory responses, have
289 been linked to ACE2 upregulation [16]. Here, we use HLT cells to study the effect of several anti-
290 inflammatory drugs on both ACE2 expression and SARS-CoV-2 viral entry. 1/5 dilutions of
291 ibuprofen, cortisol, dexamethasone and prednisone were added to HLT cells for 20h. Overall, no

292 effect on ACE2 expression was observed after the addition of these anti-inflammatory drugs
293 (**Figure 5B**). Consequently, tested anti-inflammatory compounds showed no major impact of the
294 viral entry assay; however, high concentrations of prednisone and dexamethasone showed a
295 partial reduction of viral entry in HLT cells, without any apparent impact on VeroE6 cells (**Figure**
296 **5C**). Thus, selected anti-inflammatory drugs had limited impact on ACE2 expression within
297 enriched AT-II cells from the HLT model, as well as in SARS-CoV-2 viral entry.

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

299 Last, we were interested in modelling the anti-inflammatory properties of several drugs in
300 HLT cells. Based on their previous antiviral potency, we selected cepharanthine, ergoloid,
301 camostat, ivermectin, hydroxychloroquine and ciclesonide for further evaluation. Of note, some
302 of these drugs have been previously identified as immunomodulators with anti-inflammatory
303 effects (**Table S1**). However, their direct impact on inflammatory molecules directly secreted by
304 human lung cells have not been evaluated. HLT cells were stimulated with lipopolysaccharides
305 (LPS) and IFN-γ in the presence of these antivirals and, 20h after, the expression of IL-6 and
306 CXCL10, a potent pro-inflammatory cytokine and a chemokine respectively, were intracellularly
307 measured by flow cytometry. IL-6 and CXCL10 were selected as molecules significantly increased
308 in severe patients during acute infection, with a prediction value for hospitalization [55]. A
309 representative flow cytometry gating strategy is shown in **Figure S4**. As shown in **Figure 6A,** two
310 major subpopulations of myeloid cells contributed to the upregulation of CXCL10 and IL-6
311 expression upon stimulation. Myeloid $CD11b^{+}CD14^{+}$ were the cells with a greater response, with
312 50% of these cells expressing IL-6 and 30% expressing CXCL10 after stimulation (**Figure 6B**).
313 Using this model of local inflammation, we tested the capacity of the selected compounds to
314 attenuate this response. We observed that camostat had the most potent effect, which
315 significantly reduced the expression of CXCL10 in $CD11b^{+}CD14^{-}$ and of IL-6 in $CD11b^{+}CD14^{+}$
316 myeloid subsets (**Figure 6B**). Ergoloid, which has not been linked to modulation of inflammation
317 before, significantly reduced the expression of cytokines in $CD11b^{+}CD14^{+}$ myeloid cells, and
318 cepharanthine reduced IL-6 production within this same subset. In contrast, ciclesonide induced
319 CXCL10 secretion in $CD11b^{+}CD14^{-}$ myeloid cells (**Figure 6B**). Altogether, our results validate the
320 use of HLT cells as a relevant method for the identification of anti-inflammatory compounds
321 impacting specific pro-inflammatory cell populations located in the lung parenchyma.

322 **Discussion**

323 The emergency created by the fast spread of SARS-CoV-2 infection worldwide required a
324 quick response from physicians treating these patients, who adapted to the rapid knowledge
325 being generated by both clinical practice and basic research. However, up to date, only one
326 antiviral drug against SARS-CoV-2 has been approved for clinical use. New antivirals are urgently
327 needed, and the choice of the cell and animal models used to test the efficacy of drugs will
328 impact its rapid translation into the clinics. Here, we propose the use of human lung tissue (HLT)
329 cells as a method that can be safely performed in a BSL2 facility, which allows i) the identification
330 of cell targets and expression of viral entry factors, ii) the impact of inflammation on host-
331 pathogen interactions and iii) a rapid medium-high throughput drug screening of entry inhibitors
332 against SARS-CoV-2 variants and local anti-inflammatory candidates.

333 Using pseudotyped viral particles expressing the SARS-CoV-2 spike, we first corroborated that
334 a fraction of $CD45^-$ $CD31^-$ $HLA\text{-}DR^+$ and $EpCam^+$ is enriched in AT-II cells and are the primary cell
335 target in lung tissue in steady conditions. This agrees with several studies using different
336 approximations [12, 36, 56] and validates our primary model for viral tropism identification.
337 While cell lines have been traditionally used for the screening of potential antiviral compounds
338 due to their reproducibility, as well as being quick and user-friendly assays, they lack
339 physiological relevance. Similarly, entry receptors and viral factors have been identified using
340 immortalized cell lines [11, 57], and cell targets for SARS-CoV-2 in tissues have been mainly
341 determined by analyzing the expression of viral entry factors in RNA-seq datasets [58] or using
342 replication-competent SARS-CoV-2 isolates in BSL3 facilities [59]. Importantly, these studies
343 have identified AT-II cells as main viral targets for SARS-CoV-2 infection in the lungs, and the
344 molecules ACE2, CD147, TMPRSS2 and AXL as important factors for viral entry [11, 34, 37, 60].
345 However, the development of more refined and translational *ex vivo* models of SARS-CoV-2
346 entry will not only have implications for understanding viral pathogenesis, but also will be useful
347 for the characterization of cell targets under specific conditions or for the identification of
348 potential antivirals blocking viral entry in primary cells. The direct use of HLT cells allows the
349 maintenance of cell type diversity and it may represent a significant advantage over previous
350 models [18, 20, 61].

351 Moreover, we showed that the HLT cells can be successfully used for drug screening
352 purposes, not only against the D614G virus but also against the delta variant. We tested 39 drugs
353 and compared the results with antiviral testing in VeroE6 cells. Not surprisingly, we showed
354 discordant results between both methods. Indeed, we found that 33.3% of the tested

355 compounds had discordant results between HLT and VeroE6 cells; 26.66% of drugs showed some
356 antiviral effect in HLT but no activity was detected in VeroE6, and 6.67% showed only antiviral
357 effects in VeroE6 cells. Among other reasons, the differential expression of several key proteins
358 needed for viral entry, might explain current discrepancies between cell types. Importantly,
359 using HLT cells, we identified several compounds with antiviral activity; cepharanthine showed
360 an EC_{50} of 6.08µM and concordantly, it was recently identified in a high throughput screening as
361 one of the most potent drugs against SARS-CoV-2 [62], likewise several other studies have
362 pointed towards this drug as a potent entry and post-entry SARS-CoV-2 inhibitor [63]. Instead,
363 for hydroxychloroquine, an early report suggested no antiviral activity in human lung cells due
364 to different expression of the required proteases for viral entry [64]. Furthermore, clinical trials
365 failed to show effectiveness of this drug as a treatment for COVID-19 [65-67]. A strong
366 dependency of SARS-CoV-2 on TMPRSS2 for viral entry, rather than on cathepsin L, was
367 identified as a possible mechanistic explanation for its failure *in vivo* [68]. In our study, however,
368 we observed that this drug was equally effective at inhibiting viral entry in VeroE6 and HLT cells,
369 and also was effective when using replication competent viral isolates in HLT cells. Concordantly,
370 in differentiated air-liquid interface cultures of proximal airway epithelium and 3D organoid
371 cultures of alveolar epithelium, hydroxychloroquine significantly reduced viral replication [69].
372 The multiple mechanisms of action postulated for hydroxychloroquine action, including
373 interference in the endocytic pathway, blockade of sialic acid receptors and restriction of pH
374 mediated S protein cleavage at the ACE2 binding site [70], could help to explain its antiviral effect
375 in primary lung cells. Similarly, we identified ivermectin as an effective antiviral in HLT cells. Of
376 note, ivermectin received limited attention as a potential drug to be repurposed against COVID-
377 19 based on its limited ability to reach lung tissue *in vivo* [71]. Further, a clinical trial failed to
378 show a reduction in the proportion of PCR-positive patients seven days after ivermectin
379 treatment [72].

380 Importantly, HLT cells also provide a platform for testing anti-inflammatory drugs and the
381 modulation of viral entry factors with drug candidates and immunomodulatory stimuli. We
382 showed that IL-1β was able to reduce ACE2 expression in the fraction of enriched AT-II cells, in
383 contrast to other cytokines induced during SARS-CoV-2 infection like IFN-α2, IFN-β1, IL-10 and
384 GM-CSF, which did not impact ACE2 protein production. In primary epithelial cells derived from
385 healthy nasal mucosa, Ziegler et al. [12] showed a significant induction of ACE2 transcripts after
386 IFN-α2 and IFN-γ stimulation, as well as and in a human bronchial cell line treated with either
387 type I or type II IFN. Moreover, the authors showed that influenza A virus infection
388 increased ACE2 expression in lung resections [12], strongly suggesting that ACE2 was an

389 ISG. However, following studies showed that ACE2 transcription and protein production was not
390 responsive to IFN. Instead, they described a new RNA isoform, MIRb-ACE2, that was highly
391 responsive to IFN stimulation, but importantly, encoded a truncated and unstable protein
392 product [73, 74]. These results highlight the need to validate scRNA-seq data with orthogonal
393 approaches, such as the confirmation of protein expression levels in relevant systems. In HLT
394 cells, we quantified ACE2 protein expression and importantly, focused our analysis on putative
395 AT-II cells, the main SARS-CoV-2 targets in lung parenchyma. Also, in agreement with our results,
396 a primary human bronchial epithelial cell model, type I (β), II (γ), or III (λ1) IFNs did not induced
397 ACE2 expression [75]. Moreover, a study performed by Lang et al [76], showed that IFN-γ and
398 IL-4 downregulate the SARS-CoV receptor ACE2 in VeroE6 cells, and similarly, stimulation of
399 A549 cells with IFN-α, IFN-γ, and IFN-α+IFN-γ did not identify ACE2 as an ISG [77].

400 A feasible explanation for the decrease of ACE2 protein production upon IL-1 β treatment is
401 that IL-1β activates disintegrin and metalloproteinase domain-containing protein 17 (ADAM17)
402 [78], which mediates the shedding of ACE2 [79]. Although this effect would seem positive to
403 reduce SARS-CoV-2 infection, ACE2 is a lung-protective factor, as it converts Angiotensin (Ang)
404 II to Ang-(1–7); while Ang II promotes harmful effects in the lung, e. g. fibrosis, vasoconstriction,
405 inflammation, endothelial dysfunction, edema, and neutrophil accumulation[80], Ang-(1-7) has
406 counter-regulatory effects protective of lung injury. Moreover, Ang-(1–7) plays an essential role
407 in hemostasis, as it favors anti-thrombotic activity in platelets [81]. In any case, treatment of
408 COVID-19 patients with respiratory insufficiency and hyper inflammation with IL-1 inhibitors was
409 associated with a significant reduction of mortality [82], indicating that at least during severe
410 COVID-19 the overall effect of IL-1β is detrimental. While the reduction of ACE2 expression in
411 AT-II cells by IL-1β may be of interest, it needs to be determined if in combination with other
412 cytokines rapidly induced during viral respiratory infection [83], this effect would remain.
413 Further, glucocorticoids and NSAIDS have been linked to ACE2 upregulation previously [16]. In
414 contrast, we did not observe any significant impact of ibuprofen, cortisol, dexamethasone and
415 prednisone on ACE2 protein expression. These results are concordant with a recent report
416 showing that suppression of cyclooxygenase (COX)-2 by two commonly used NSAIDs, ibuprofen
417 and meloxicam, had no effect on ACE2 expression, viral entry, or viral replication in a mouse
418 model of SARS-CoV-2 infection [84]. Moreover, dexamethasone incompletely reduced viral
419 entry. This observation partially agrees with a study using lung cells previously treated with
420 dexamethasone, which showed significant suppression of SARS-CoV-2 viral growth [25].

421 We additionally show the interest of the HLT model to test local inflammation and evaluate
422 potential anti-inflammatory drugs. The culture of diverse cell subsets localized in the lung

423 parenchyma, without further cell separation, allows the detection of inflammatory responses
424 generated by different resident subpopulations, which is a significant advantage over
425 monoculture. Several resident-myeloid subsets, together with newly recruited ones, may
426 contribute to the rapid cytokine storm detected in COVID-19 patients [85-87]. Thus, the
427 identification of antiviral drugs that can also limit the extent of these initial pro-inflammatory
428 events may offer added value to the overall therapeutic effect of a given drug. In this sense, we
429 observed that camostat significantly reduced the expression of proinflammatory molecules IL-6
430 and CXCL10 in several myeloid $CD11b^{+}$ subsets. Concordantly, in a previous study using primary
431 cultures of human tracheal epithelial cells infected with H1N1 virus, camostat also reduced the
432 concentrations of the cytokines IL-6 and TNF in cell supernatants [88], suggesting a potent anti-
433 inflammatory potential. In contrast, ivermectin did not affect the expression of cytokines in our
434 model. However, ivermectin was previously shown to have protective anti-inflammatory effects
435 in mice, reducing the production of TNF, IL-1 and IL-6 *in vivo* and *in vitro* [89]. Of note, in our
436 system we optimized the detection of changes for the intracellular expression of IL-6 and CXCL10
437 by local myeloid cells, and thus, how these intracellular changes reflect total production in
438 supernatant needs further evaluation.

439 Finally, it is also important to note the potential limitations of the model. First, we did not
440 maintain the cells in an air-liquid interface, which may alter cell function. Other limitations
441 include the limited availability of human lung samples, inter-patient variation (age, smoking,
442 etc.), the effects on lung biology of the medical condition instigating surgery, and the exact
443 location of the sample resection, which may affect the proportion of cell subsets such as AT-II.
444 However, this variability is what shapes the HLT into a more physiological and relevant model in
445 comparison to current methods based on immortalized cell cultures. Besides the interest of the
446 different readouts from HLT cell-based system as proposed here, our results highlight drugs with
447 antiviral activity together with immunomodulatory properties, which could increase the benefit
448 of a given treatment during COVID-19 disease progression. For instance, camostat,
449 cepharanthine and ergoloid were three of the most potent drugs inhibiting SARS-CoV-2 entry,
450 and remarkably, also exerted a significant anti-inflammatory effect on myeloid cells. Clinical
451 trials with camostat, ergoloid and cepharanthine, ideally administrated during early infection,
452 should shed light on their use as both antivirals and anti-inflammatory compounds.

453 **Materials and methods**

454 **Cells and virus**

455 VeroE6, isolated from kidney epithelial cells of an African green monkey, were grown in
456 DMEM medium supplemented with 10% fetal bovine serum (FBS; Gibco) 100 U/ml penicillin,
457 and 100 µg/ml streptomycin (Capricorn Scientific) (D10) and maintained at 37°C in a 5% CO_2
458 incubator.

459 The spike of the SARS-CoV-2 virus (D614G variant) was generated (GeneArt Gene Synthesis,
460 ThermoFisher Scientific) from the codon-optimized sequence obtained by Ou et al. [41] and
461 inserted into pcDNA3.1D/V5-His-TOPO (pcDNA3.1-S-CoV2Δ19-G614). The spike of the SARS-
462 CoV-2.SctΔ19 B.1.617.2 (delta) virus was generated (GeneArt Gene Synthesis, ThermoFisher
463 Scientific) from the full protein sequence of the original SARS-CoV-2 isolate Wuhan-Hu-1 (WH1)
464 modified to include the mutations specific to the delta variant (VOC-21APR-02: T19R, 157-
465 158del, L452R, T478K, D614G, P681R, D950N). These plasmids present the mutation D614G and
466 a deletion in the last 19 amino acids from the original spike. Pseudotyped viral stocks of
467 VSV*ΔG(Luc)-S were generated following the protocol described by Whitt [90] with some
468 modifications. Briefly, 293T cells were transfected with 3µg of the plasmid encoding the SARS-
469 CoV-2 spike. Next day, cells were infected with a VSV-G-Luc virus (MOI=1) (generated from a
470 lentiviral backbone plasmid that uses a VSV promoter to express luciferase) for 2h and gently
471 washed with PBS. Cells were incubated overnight in D10 supplemented with 10% of I1
472 hybridoma (anti-VSV-G) supernatant (ATCC CRL-2700) to neutralize contaminating
473 VSV*ΔG(Luc)-G particles. Next day, the resulting viral particles were collected and titrated in
474 VeroE6 cells by enzyme luminescence assay (Britelite plus kit; PerkinElmer), as described
475 previously [91].

476 **Lung tissue**

477 Lung tissues were obtained from patients without previous COVID-19 history and a recent
478 negative PCR test for SARS-CoV-2 infection undergoing thoracic surgical resection at the
479 Thoracic Surgery Service of the Vall d'Hebron University Hospital. Study protocol was approved
480 by the Ethical Committee (Institutional Review Board number PR(AG)212/2020). Non-neoplastic
481 tissue areas were collected in antibiotic-containing RPMI 1640 and immediately dissected into
482 approximately 8-mm^3 blocks. These blocks were first enzymatically digested with 5 mg/ml
483 collagenase IV (Gibco) and 100 µg/ml of DNase I (Roche) for 30 min at 37ºC and 400 rpm and,
484 then, mechanically digested with a pestle. The resulting cellular suspension was filtered through

485 a 70µm-pore size cell strainer (Labclinics) and washed twice with PBS. Pellet recovered after
486 centrifugation was resuspended in fresh medium (RPMI 1640 supplemented with 5% FBS, 100
487 U/ml penicillin, and 100 µg/ml streptomycin) and DNase I to dissolve cell aggregates, and the
488 resulting cell suspension was then filtered through a 40µm-pore size cell strainer (Labclinics).
489 Cell number and viability were assessed with LUNA™ Automated Cell Counter (Logos
490 Biosystems). For cell phenotyping the following antibodies were used: anti-CD31 (PerCP-Cy5.5,
491 BioLegend), anti-CD11b (FITC, BioLegend), anti-CD11c (Pe-Cy7, BD Biosciences), anti-E-cadherin
492 (Pe-CF594, BD Biosciences), primary goat anti-ACE2 (R&D systems), anti-CD14 (APC-H7, BD
493 Biosciences), anti-CD45 (AF700, BioLegend), anti-EpCAM (APC, BioLegend), anti-CD3 (BV650, BD
494 Biosciences), anti-CD15 (BV605, BD Biosciences) and anti-HLA-DR (BV421, BioLegend). For ACE2
495 detection, after surface staining, cells were stained with secondary donkey anti-goat IgG (PE,
496 R&D Systems) for 30 min at 4 ºC. Cell viability was determined using an AQUA viability dye for
497 flow cytometry (LIVE/DEAD fixable AQUA, Invitrogen). In some experiments, instead of CD11b
498 or CD15, we used a primary rabbit anti-TMPRSS2 or anti-CD147 (BV605, BD Biosciences),
499 respectively. For TMPRSS2 detection, after ACE2 staining with the appropriate secondary
500 antibody, cells were washed twice with PBS 1% NMS (normal mouse serum) and then stained
501 with a secondary goat anti-rabbit IgG (AF488, Thermofisher) for 30 min at 4ºC. For SPC detection,
502 after surface staining with a primary rabbit anti-SPC antibody (Biorbyt) and instead of ACE2
503 staining, cells were stained with a secondary donkey anti-rabbit IgG (PE, Biolegend) for 30 min
504 at 4 ºC. After fixation with PBS 2% PFA, cells were acquired in an LSR Fortessa (BD Biosciences),
505 and data were analyzed using the FlowJo v10.6.1 software (TreeStar).

506 **Cytospin and alkaline phosphatase staining**

507 Cytospin preparations were obtained from freshly isolated human lung cells at an
508 approximate density of 150,000 cells/slide, and air-dried during 15 min. Cells were stained with
509 alkaline phosphatase, as an enzyme marking epithelial type II cells, following manufacturer's
510 instructions (Alkaline phosphatase Kit, Sigma). The intensity of pink stain reflects the amount of
511 alkaline phosphatase in positive cells.

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

513 Human lungs were maintained in 10% formalin for 24 hours and then embedded in paraffin.
514 Paraffin-embedded lungs were cut into 4 µm sections. After removing the paraffin, endogenous
515 peroxidases were inactivated in an aqueous solution containing 3% H_2O_2 and 10% methanol and
516 antigen retrieval was performed heating the samples in citrate buffer (10mM citric acid, pH 6.0).

517 The sections were then blocked in bovine serum albumin (5%), incubated with anti-ACE2
518 antibody (R&D Systems cat. nº AF933, dilution 1:100) and with biotinylated secondary antibody
519 against goat IgGs (Vector Laboratories cat. nº BA-9500, dilution 1:250). Proteins were visualized
520 using the ABC Peroxidase Standard Staining Kit (ThermoFisher) followed by 3,3'-
521 Diaminobenzidine (DAB) Enhanced Liquid Substrate System (Sigma Aldrich). Counterstaining
522 was done with hematoxylin.

523 **Antiviral screening assay**

524 The complete list of compounds tested in this study, including information about its clinical
525 use, product reference and vendors is shown in **Table S1**. Duplicates of five-fold serial dilutions
526 of 39 antiviral compounds were tested in both VeroE6 cell line and in human lung tissue (HLT)
527 cells using at least 2 different donors. For VeroE6, five-fold serial dilutions of the compounds,
528 ranging from 100µM to 0.25nM, were prepared in D10 in a 96-well flat-bottom plates. VeroE6
529 cells were added at a density of 30.000 cells/well and incubated with the drug for at least 1 h
530 before infection. Subsequently, cells were infected with 1,500 $TCID_{50}$ of VSV*ΔG(Luc)-S virus. In
531 parallel, drug cytotoxicity was monitored by luminescence. To evaluate the antiviral activity of
532 drugs in HLT cells, five-fold serial dilutions of the compounds, ranging from 100µM to 0.8µM or
533 6.4nM, were prepared in R10 in a 96-well conic-bottom plates. HLT cells were added at a density
534 of 300,000 cells/well and incubated with the compound for at least 1h before infection. Then,
535 MOI 0.1 of VSV*ΔG(Luc)-S virus were added to wells, and plates were spinoculated at 1,200g
536 and 37ºC for 2h. After infection, fresh medium was added to the wells and cell suspensions were
537 transferred into a 96-well flat-bottom plate. Cells were then cultured overnight at 37°C in a 5%
538 CO_2 incubator. Each plate contained the following controls: no cells (background control), cells
539 treated with medium (mock infection) and cells infected but untreated (infection control). After
540 20h, cells were incubated with Britelite plus reagent (Britelite plus kit; PerkinElmer) and then
541 transferred to an opaque black plate. Luminescence was immediately recorded by a
542 luminescence plate reader (LUMIstar Omega). To evaluate cytotoxicity, we used the CellTiter-
543 Glo® Luminescent kit (Promega), following the manufacturer's instructions. Data was
544 normalized to the mock-infected control, after which EC_{50} and CC_{50} values were calculated using
545 Graph-Pad Prism 7.

546 **Drug validation with replication competent SARS-CoV-2**

547 These experiments were performed in a BSL3 facility (Viral Vector Production Unit,
548 Universitat Autònoma de Barcelona, UAB). The SARS-CoV-2 virus was isolated from a

549 nasopharyngeal swab from an infected patient hospitalized at the Vall d'Hebron Hospital.
550 VeroE6 cells were cultured on a cell culture flask (25 cm^2) at 1.5×10^6 cells overnight prior to
551 inoculation with 1 mL of medium from a Deltaswab VICUM® tub containing the swab. Cells were
552 cultured for 1h at 37°C and 5% CO_2. Afterwards, DMEM containing 2% FCS were added to the
553 cells and incubated for 48h. Cells were assessed daily for cytopathic effect and the supernatant
554 was recollected and subjected to viral titration in VeroE6 by plaque assay.

555 For antiviral drug validation, HLT samples were incubated with different drugs at 20µM for
556 at least 1h before infection. Tested drugs were camostat, cepharanthine, ergoloid,
557 hydroxychloroquine, ivermectin and ciclesonide. Then, cells were infected with a MOI 0.5 of the
558 SARS-CoV-2 viral isolate, and the plate was incubated for 2h at 37ºC and 5% CO2. After infection,
559 samples were extensively washed with PBS 1X to eliminate residual virus and suspended in fresh
560 media containing antiviral drugs and transferred into a new plate. 24 or 48h post infection, 140µl
561 of supernatant was collected in tubes containing 140µl of DNA/RNA Shield (Zymo Research) for
562 SARS-CoV-2 inactivation. For each experiment, a negative control, cells treated with only
563 medium, and a positive control, cells incubated in the presence of the virus alone, were included.
564 Percentage of viral infection was calculated by RT-PCR. Briefly, viral RNA from the supernatant
565 was extracted using the QIAamp Viral RNA Mini Kit (Qiagen), following the manufacturer's
566 instructions. RNA was reverse transcribed with SuperScript III (Invitrogen), in accordance with
567 the instructions provided by the manufacturer, and cDNA was quantified by qPCR using the
568 2019-nCoV CDC RUO Kit (IDT, catalog #10006713) for the detection of viral RNA of the
569 nucleocapsid region N1 from the SARS-CoV-2 (N1 forward 5'-GACCCCAAAATCAGCGAAAT-3' and
570 N1 reverse 5'-TCTGGTTACTGCCAGTTGAATCTG-3'; N1 probe 5'-FAMACCCCGCAT/ZEN/TACGTTT
571 GGTGGACC-3IABkFQ-3'). Copies of SARS-CoV-2 RNA were quantified using a standard (2019-
572 nCoV_N_Positive Control from IDT, catalog #10006625). Samples were run on a 7000 SDS
573 instrument (Applied Biosystems).

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

575 VeroE6 and lung cells were incubated with five-fold serial dilutions of selected anti-
576 inflammatory compounds (ranging from 100µM to 0.8µM) for 20h. Tested drugs included
577 cortisol, ibuprofen, prednisone and dexamethasone. Lung cells were also incubated with the
578 following cytokines: GM-CSF (100 ng/ml, Immunotools), IL-1β (10 ng/ml, Immunotools), IL-10
579 (100 ng/ml, Immunotools), IFN-β (100 U/ml, Immunotools), or IFN-α2 (100 U/ml, Sigma Aldrich).
580 For determination of ACE2 expression, the following surface staining antibodies were used:
581 primary goat anti-ACE2 (R&D Systems), anti-CD45 (AF700, BioLegend), anti-EpCAM (APC,

582 BioLegend), and anti-HLA-DR (BV421, BioLegend). For ACE2 detection, cells were then stained
583 with secondary donkey anti-goat IgG (PE, R&D Systems) for 30 min at 4 ºC. A Fluorescent Minus
584 One control (FMO) without primary anti-ACE2 antibody was used as a control. Cell viability was
585 determined using an AQUA viability dye for flow cytometry (LIVE/DEAD fixable AQUA,
586 Invitrogen). After fixation with PBS 2% PFA, cells were acquired in an LSR Fortessa (BD
587 Biosciences) and analyzed using the FlowJo v10.6.1 software (TreeStar).

588 **Immunomodulatory capacity of selected drugs**

589 HLT cells were cultured in a round-bottom 96-well plate containing 20 µM of cepharanthine,
590 ergoloid, ciclesonide, hydroxychloroquine, ivermectin, or camostat mesylate alone or in
591 combination with the stimuli LPS (50 ng/ml) and IFN-γ (100 ng/ml). For each patient, a negative
592 control, cells treated with only medium, and a positive control, cells incubated in the presence
593 of LPS and IFN-γ, were included. Immediately, brefeldin A (BD Biosciences) and monensin (BD
594 Biosciences) were added to cells and cultured overnight at 37 ºC in 5% CO_2. Next day, cellular
595 suspensions were stained with the following antibodies: anti-CD11b (FITC, BioLegend), anti-
596 CD69 (PE-CF594, BD Biosciences), anti-CD14 (APC-H7, BD Biosciences), anti-EpCAM (APC,
597 BioLegend), anti-CD3 (BV650, BD Biosciences), anti-CD45 (BV605, BioLegend), and anti-HLA-DR
598 (BV421, BioLegend). Cells were subsequently fixed and permeabilized using the
599 Cytofix/Cytoperm™ kit (BD Biosciences) and intracellularly stained with anti-IL-6 (PE-Cy7,
600 BioLegend), and anti-CXCL10 (PE, BioLegend). Cell viability was determined using an AQUA
601 viability dye for flow cytometry (LIVE/DEAD fixable AQUA, Invitrogen). After fixation with PBS
602 2% PFA, cells were acquired in an LSR Fortessa (BD Biosciences), and data were analyzed using
603 the FlowJo v10.6.1 software (TreeStar).

604 **Statistical analyses**

605 Statistical analyses were performed with Prism software, version 6.0 (GraphPad). A P value
606 <0.05 was considered significant.

607 **Author contributions**

608 Conceptualization, MJ.B. and M.G.; Sample Collection, J.R.; Methodology J.G-E, D.P, M.S, N.
609 M, A.V, MJ.S, J.G-P, J.A, J.B, B.T, A.S.M and V.F; Formal Analysis, J.G-E., D.P, M.S, M.G and MJ.B;
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611 Acquisition, M.G and MJ.B.; all authors revised the manuscript; Supervision, M.G and MJ.B.

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843

844 **Figure legends**

845 **Figure 1. Phenotyping of human lung cells.** (**A**). *t*-distributed Stochastic Neighbor
846 Embedding (t-SNE) representation displaying the major cell clusters present in the $CD45^+$ and
847 $CD45^-$ $EpCAM^+$ fractions of a representative human lung tissue. The vertical bars in the right
848 panel show the frequency of each subset relative to live cells. All cell subsets were identified as
849 shown in Figure S1A. mDCs, myeloid dendritic cells; enriched AT-II, enriched fraction in alveolar
850 type 2. (**B**). Phosphatase alkaline positive AT-II cells (pink staining) were detected in a cytospin
851 obtained from human lung tissue cells and observed at 10x. Lower panel shows a high
852 magnification (40x) of the black square. Scale bars are 100 µm and 10 µm in top and bottom
853 panels, respectively. (**C**). *t*-distributed Stochastic Neighbor Embedding (tSNE) representation for
854 ACE2, CD147 and TMPRSS2 expression in $CD45^+$ and $CD45^-EpCAM^+$ fractions from a
855 representative lung tissue. Right graphs show the percentage of expression of each entry factor
856 in the different cell subpopulations, which were identified as in Figure 1A with some
857 modifications for the identification of myeloid cells and neutrophils (From big cells:
858 monocytes/macrophages, $CD11c^+$HLA-DR^+ $CD14^+$; Alveolar macrophages and mDCs, $CD11c^+$ HLA-
859 DR^+ $CD14^-$; Neutrophils, $CD11c^-$ HLA-DR^- $CD14^-$ $CD3^-$). (**D**). Images of ACE2 immunohistochemical
860 staining in human lung tissue sections at 40x magnification, counterstained with haematoxylin
861 (top) or without (bottom). Black arrows indicate staining of ACE2 in AT-II cells (upper panel).
862 Mean±SEM is shown for all graphs.

863 **Figure 2. Susceptibility of VeroE6 and the HLT model to SARS-CoV-2 viral entry.** VeroE6 and
864 HLT cells were infected with two different viral constructs (GFP and Luciferase) expressing the
865 spike protein upon viral entry; VSV*ΔG(GFP)-Spike and VSV*ΔG(Luc)-Spike. (**A**) Representative
866 flow cytometry plots of VeroE6 cells infected with VSV*ΔG(GFP)-Spike or the background form
867 VSV*ΔG(GFP)-empty (left panel); and luciferase activity (RLUs; relative light units) at 20h post-
868 infection with the pseudotyped VSV*G(Luc)-G, the VSV*ΔG(Luc)-Spike or the background form
869 VSV*ΔG(Luc)-empty (right panel). (**B**) Percentage of viral entry after treatment with anti-ACE2
870 antibody (10µg/ml) and camostat (100µM) in VeroE6 cells infected with the pseudotyped virus
871 expressing the control G protein or the spike from SARS-CoV-2. (**C**) A flow cytometry plot
872 showing ACE2 expression in GFP^+ VeroE6 cells. Right graph shows mean fluorescence intensity
873 (MFI) of ACE2 in both infected and uninfected fractions, based on GFP expression. (**D**)
874 Representative flow cytometry plots of HLT cells infected with the viral construct expressing the
875 spike protein (VSV*ΔG(GFP)-Spike) or the background form (VSV*ΔG(GFP)-empty) (left panel);
876 and luciferase activity (RLUs; relative light units) at 20h post-infection with the VSV*ΔG(Luc)-
877 Spike or the background form VSV*ΔG(Luc)-empty (right panel). Infection was measured as the

878 percentage of GFP or RLUs, respectively. (**E**) Susceptible HLT cells to viral entry (identified as
879 GFP^+ cells) compatible with an AT-II phenotype, determined by the co-expression of HLA-DR and
880 EpCAM in the $CD45^-CD31^-$ fraction of live cells. (**F**) Bar plots showing the percentage of viral entry
881 inhibition on HLT cells in the presence of anti-ACE2 antibody (15µg/ml), camostat (100µM) or
882 anti-CD147 antibody (25µg/ml) after cell challenge with VSV*ΔG(Luc)-Spike (left graph) or
883 VSV*ΔG(GFP)-Spike (right graph). Mean±SEM is shown for all graphs. Data in panel 2C were
884 analyzed by a Wilcoxon matched-pairs test; *p<0,05. Data in panel 2F were analyzed by one
885 sample t-test; *p<0.05, **p<0.01, **p<0.0001

886 **Figure 3. Antiviral assays with concordant results between models**. (**A**). Percentage of viral
887 entry in VeroE6 and HLT cells exposed to VSV*ΔG(Luc)-Spike in the presence of cepharanthine,
888 ergoloid, hydroxychloroquine, hypericin, licofelone, ivermectin, ciclesonide, quercetin,
889 vidarabine and celecoxib. Drugs were used at concentrations ranging from 100µM to 0.256nM,
890 in VeroE6, and to 0.8µM in lung cells. Non-linear fit model with variable response curve from at
891 least three independent experiments in replicates is shown (red lines). Cytotoxic effect on
892 VeroE6 cells and HLT exposed to drug concentrations in the absence of virus is also shown (green
893 lines). (**B**). EC_{50} values of each drug in VeroE6 and HLT cells. (**C**). CC_{50} values of each drug are
894 shown for VeroE6 and for HLT cells.

895 **Figure 4. Antiviral assays with discordant results between models**. (**A**). Percentage of viral
896 entry in VeroE6 and HLT cells exposed to VSV*ΔG(Luc)-Spike in the presence of luteolin,
897 eriodictyol, phenformin, camostat, sulindac, and valaciclovir. Drugs were used at concentrations
898 ranging from 100µM to 0.256 nM, in VeroE6, and to 0.8µM in lung cells. Non-linear fit model
899 with variable response curve from at least three independent experiments in replicates is shown
900 (red lines). Cytotoxic effect on VeroE6 cells and HLT exposed to drug concentrations in the
901 absence of virus is also shown (green lines). (**B**). EC_{50} values of each drug in VeroE6 and HLT cells.
902 (**C**)**.** CC_{50} values of each drug are shown for VeroE6 and HLT cells. (**D**). Percentage of viral entry
903 in HLT cells exposed to VSV*ΔG(Luc)-Spike-delta and VSV*ΔG(Luc)-Spike-D614G variants in the
904 presence of ivermectin, camostat, hydroxychloroquine, cepharanthine and ciclesonide. Drugs
905 were used at concentrations ranging from 100µM to 0.8µM. (**E**) Number of viral genomes/µl at
906 24h and 48h after infection with replication-competent SARS-CoV-2. (**F**). Percentage of viral
907 replication in the presence of 20µM of camostat, cepharanthine, hydroxychloroquine,
908 ivermectin and ciclesonide after infection with replication-competent SARS-CoV-2. Mean±SEM
909 is shown. Data in panel 4F were analyzed by one sample t-test; *p<0.05, **p<0.01.

910 **Figure 5. Impact of inflammation and anti-inflammatory drugs on SARS-CoV-2 viral entry**
911 **and ACE2 expression.** Both models, HLT cells and VeroE6 cells, were incubated in the presence
912 of different anti-inflammatory drugs to evaluate the modulation of ACE2 expression by flow
913 cytometry and the antiviral effect by luminescence. (**A**). HLT cells were treated with different
914 stimuli for 20h and the percentage of protein expression (left) or the mean fluorescence
915 intensity (MFI, right) of ACE2 receptor was evaluated in the enriched AT-II fraction by flow
916 cytometry. (**B**) Modulation of ACE2 protein expression was assessed by flow cytometry in both
917 models, Vero E6 and HLT cells, in the presence of different concentrations of each anti-
918 inflammatory drug, ranging from 100µM to 0.8µM. Percentage of ACE2 expression was
919 quantified in AT-II cells from at least six independent lung samples, and in VeroE6 cells from 2
920 independent experiments. (**C**). Cytotoxic effect on Vero E6 and HLT cells exposed to
921 VSV*ΔG(Luc)-Spike in the presence of different concentrations of the anti-inflammatory drugs
922 prednisone, cortisol, ibuprofen and dexamethasone. Drugs were used at a concentration
923 ranging from 100µM to 0.256 nM, in VeroE6, and to 0.8µM in lung cells. Non-linear fit with
924 variable response curve from at least two experiments in replicates is shown (red lines).
925 Cytotoxic effect on Vero E6 cells and HLT cells exposed to different concentrations of drugs in
926 the absence of virus is also shown (green lines). Mean±SEM are shown and statistical
927 comparisons with the control medium were performed using the Wilcoxon test. *p<0.05.

928 **Figure 6. Anti-inflammatory effect of compounds with antiviral activity against SARS-CoV-**
929 **2.** HLT cells were cultured in the presence of 20µM of cepharanthine, ergoloid mesylate,
930 ciclesonide, hydroxychloroquine sulfate, ivermectin or camostat mesylate, alone or in
931 combination with the stimuli LPS (50 ng/ml) and IFN-γ (100 ng/ml). (**A**) *t*-distributed Stochastic
932 Neighbor Embedding (t-SNE) representations displaying the major cell clusters present in live
933 $CD45^+$ myeloid gate, based on FSC and SSC, of a representative human lung tissue in baseline
934 conditions and after stimulation with LPS and IFN-γ. Two major subsets of myeloid cells are
935 shown ($CD11b^+$ $CD14^+$, in blue-green, and $CD11b^+$ $CD14^-$, in orange). The expression of CXCL10
936 and IL-6 among the different populations is shown in maroon and green, respectively. (**B**)
937 Expression of CXCL10 and IL-6 was measured in HLT cells in response to stimuli in the presence
938 of selected drugs in both myeloid subpopulations, $CD11b^+$ $CD14^+$ (left panel) and $CD11b^+$ $CD14^-$
939 (right panel). HQ, hydroxychloroquine. Mean±SEM are represented and statistical comparisons
940 with the control medium were performed using the One sample t test. *p<0.05, **p<0.01.

941 **Figure S1. Gating strategy for the identification of cell subpopulations in the human lung**
942 **tissue model**. (**A**) General gating strategy used to identify different cell subsets in lung samples.
943 A gate based on FSC vs. SSC was followed by doublet and dead cells exclusion. From live $CD45^-$

944 cells, endothelial cells ($CD31^+$, purple) and epithelial cells ($EpCAM^+$, grey) were gated, and within
945 epithelial cells, AT-II cells ($EpCAM^+$ and $HLA\text{-}DR^+$, pink) were identified. Out of live $CD45^+$ cells
946 and based on FSC vs. SSC, we identified a lymphocyte population in which we distinguished
947 between non-T lymphocytes (turquoise) and T cells (dark green) based on CD3 expression; and
948 big cells, where we identified three major subsets based on their expression of CD11b and CD11c
949 and, subsequently, CD14 and HLA-DR markers. We identified alveolar macrophages (blue),
950 monocytes (violet), myeloid dendritic cells (mDCs, fuchsia) and neutrophils (orange). (**B**)
951 Representative flow cytometry plots showing Surfactant Protein C (SPC) staining and its
952 respective fluorescence minus one (FMO) control. (**C**) Representative flow cytometry plots
953 showing ACE2 staining and its respective fluorescence minus one (FMO) control.

954 **Figure S2. Optimization of lung tissue enzymatic digestion visualized by *t*-distributed**
955 **Stochastic Neighbor Embedding (tSNE).** (**A**) Representative tSNE maps showing concatenated
956 flow cytometry standard files for three different protocols based on different digestion enzymes
957 (collagenase, liberase or trypsin) from total live cells (upper), $CD45^+$ cells (middle) and $CD45^-$ cells
958 (lower). (**B**) Bar plots showing cell-type composition (count) analyzed by flow cytometry for each
959 tissue protocol.

960 **Figure S3.** (**A**) *t*-distributed Stochastic Neighbor Embedding (tSNE) representation for AXL
961 expression in $CD45^+$ and $CD45^-EpCAM^+$ fractions from a representative lung tissue. Right graphs
962 show the percentage of expression of the AXL entry factor in the different cell populations,
963 which were identified as in Figure 1A. (**B**) Percentage of enriched AT-II cells co-expressing the
964 entry factors ACE and CD147 (in blue), and ACE and TMPRSS2 (in purple). (**C**) Frequency of each
965 subset relative to live cells at 0h and 24h with and without the presence of virus . All cell subsets
966 were identified as shown in Figure S1A. (**D**) Bar plots showing the percentage of viral entry
967 inhibition on HLT cells in the presence of anti-ACE2 antibody (15µg/ml) or recombinant human
968 AXL (50µg/ml) after cell challenge with VSV*ΔG(Luc)-Spike. Data were analyzed by one sample
969 t-test; *$p<0.05$, **$p<0.01$. (**E**) EC_{50} values for in the HLT model obtained from 3 different lung
970 donors and performed in replicates. (**F**) Cells from 1 donor were cultured with 20 µM of selected
971 drugs for 48h, and cell toxicity was measured using the CellTiter-Glo® Luminescent kit
972 (Promega), following the manufacturer's instructions. Data was normalized to the untreated
973 control. Mean±SEM is shown for all graphs.

974 **Figure S4. Gating strategy for the identification of anti-inflammatory effects of selected**
975 **compounds.** General gating strategy used to evaluate the expression of inflammatory molecules
976 in lung samples. A gate based on FSC vs. SSC was followed by doublet and dead cells exclusion.

977 From live $CD45^+$ cells and based on FSC vs. SSC, we identified lymphocyte population and big
978 cells, in which we identified two subsets based on their expression of CD11b and CD14: myeloid
979 $CD11b^+CD14^+$ cells (blue-green) and myeloid $CD11b^+CD14^-$ cells (orange) are shown.

980

Figure 1

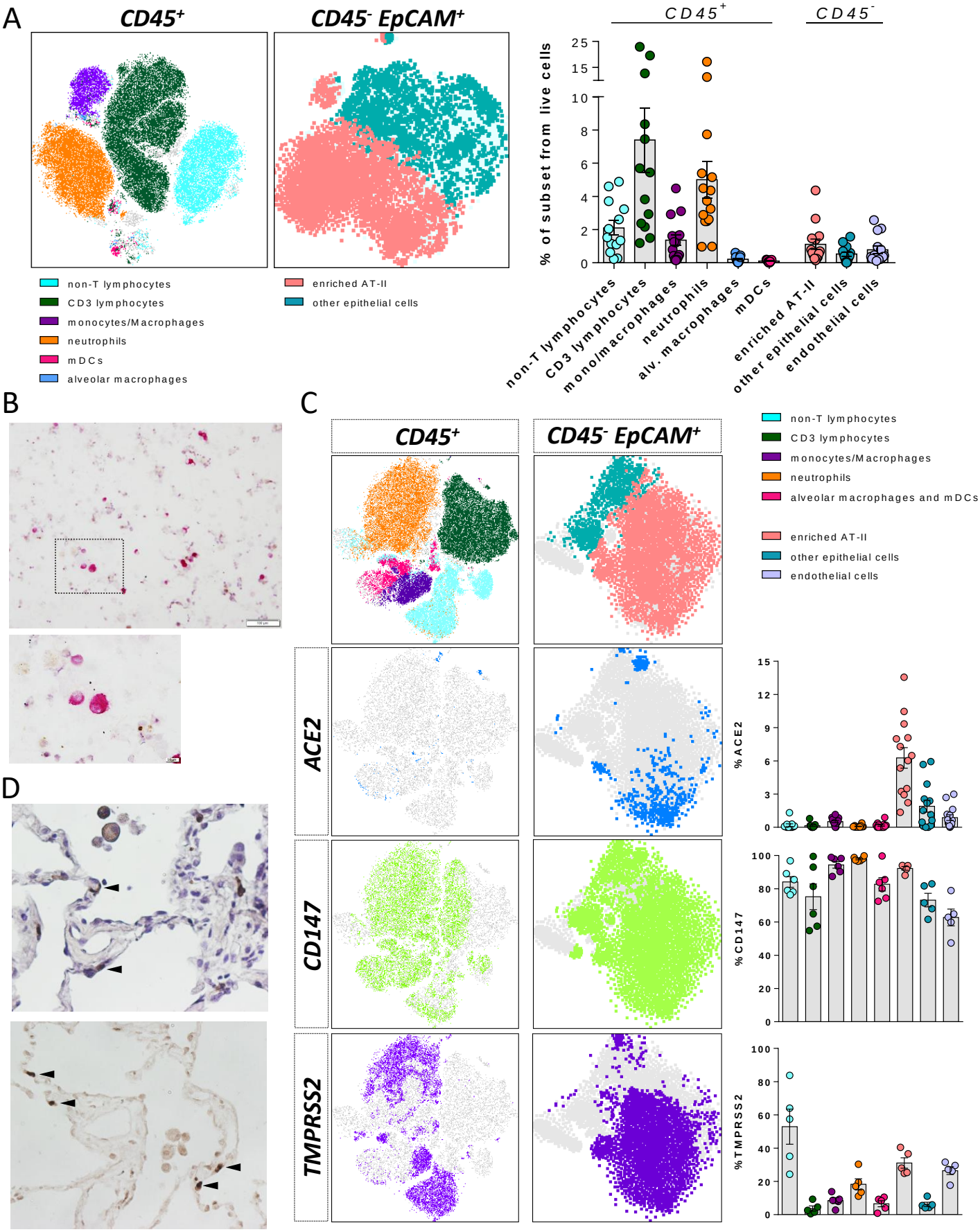
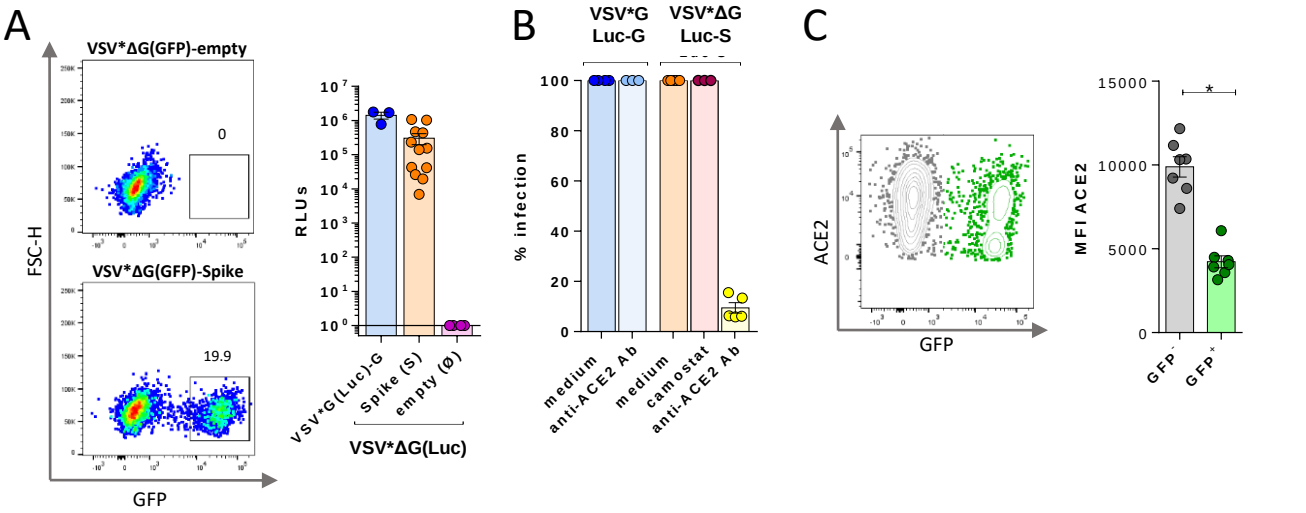

A
CD45+
CD45- EpCAM+
non-T lymphocytes
CD3 lymphocytes
monocytes/Macrophages
neutrophils
mDCs
alveolar macrophages
enriched AT-II
other epithelial cells
CD45+
CD45-
% of subset from live cells
non-T lymphocytes
CD3 lymphocytes
mono/macrophages
neutrophils
alv. macrophages
mDCs
enriched AT-II
other epithelial cells
endothelial cells
B
C
CD45+
CD45- EpCAM+
non-T lymphocytes
CD3 lymphocytes
monocytes/Macrophages
neutrophils
alveolar macrophages and mDCs
enriched AT-II
other epithelial cells
endothelial cells
ACE2
% ACE2
CD147
% CD147
TMPRSS2
% TMPRSS2
D

Figure 2

VeroE6



HLT cells

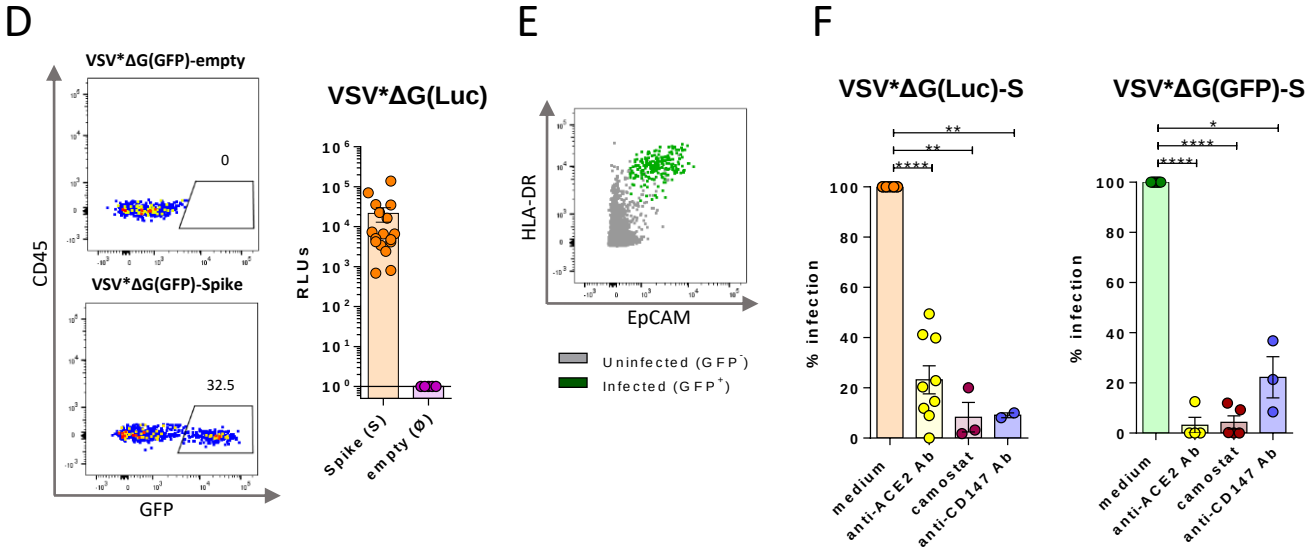
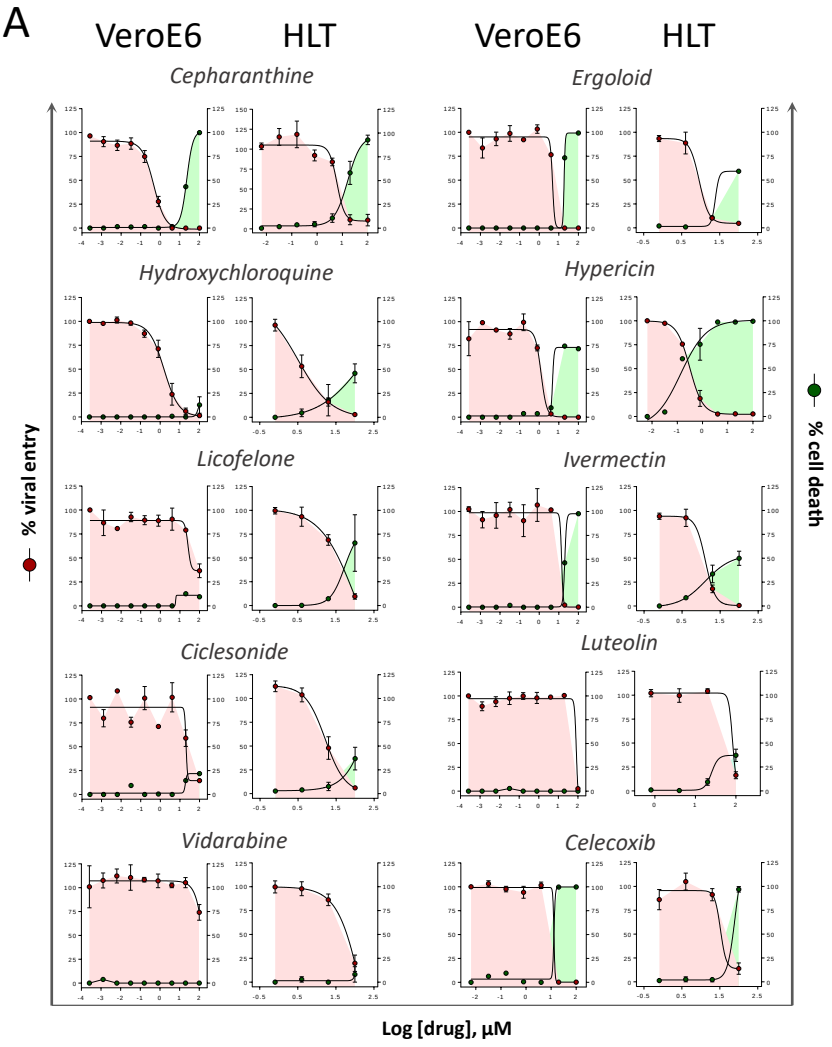


Figure 3

A



B

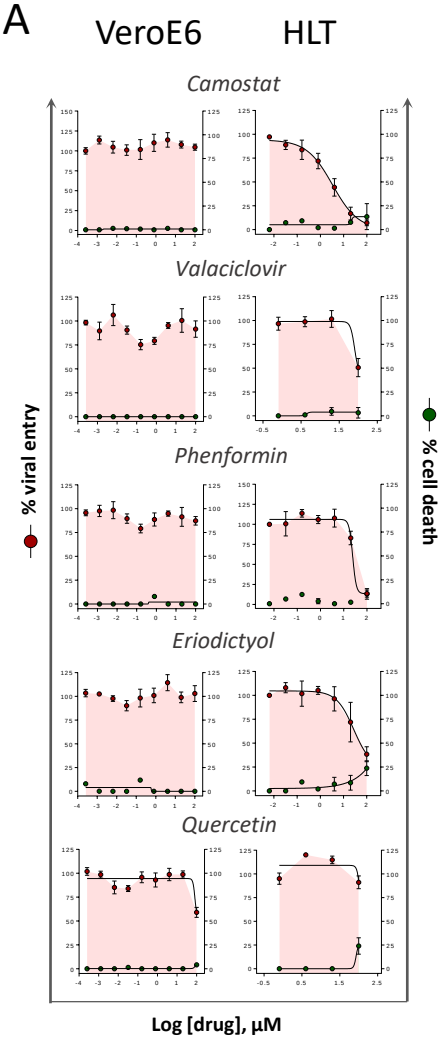
	VeroE6	HLT
Drugs	EC_{50} (µM)	
Cepharanthine	0.46	6.08
Ergoloid	4.78	9.17
Hydroxychloroquine	1.58	3.22
Hypericin	1.24	0.31
Licofelone	87.50	32.16
Ivermectin	13.94	12.98
Ciclesonide	20.52	16.41
Luteolin	~70.7	~82.39
Vidarabine	>100	51.60
Celecoxib	12.99	55.72

C

	VeroE6	HLT
Drugs	CC_{50} (µM)	
Cepharanthine	22.37	16.15
Ergoloid	18.67	~100
Hydroxychloroquine	>100	~100
Hypericin	4.80	0.14
Licofelone	>100	~53.61
Ivermectin	20.23	~100
Ciclesonide	>100	>100
Luteolin	>100	>100
Vidarabine	>100	>100
Celecoxib	12.98	~100

Figure 4

A



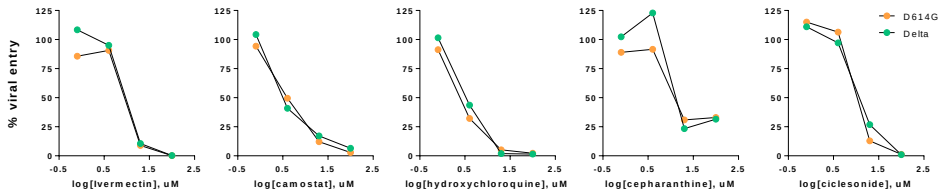
B

	VeroE6	HLT
Drugs	EC_{50} (µM)	
Camostat	no effect	3.30
Valaciclovir	no effect	>100
Phenformin	no effect	38.16
Eriodictyol	no effect	90
Quercetin	>100	no effect

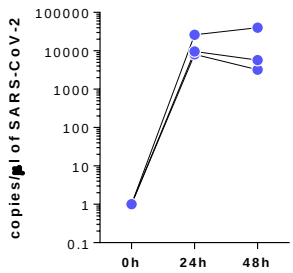
C

	VeroE6	HLT
Drugs	CC_{50} (µM)	
Camostat	>100	>100
Valaciclovir	>100	>100
Phenformin	>100	>100
Eriodictyol	>100	>100
Quercetin	>100	>100

D



E



F

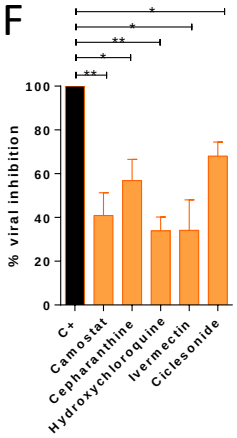
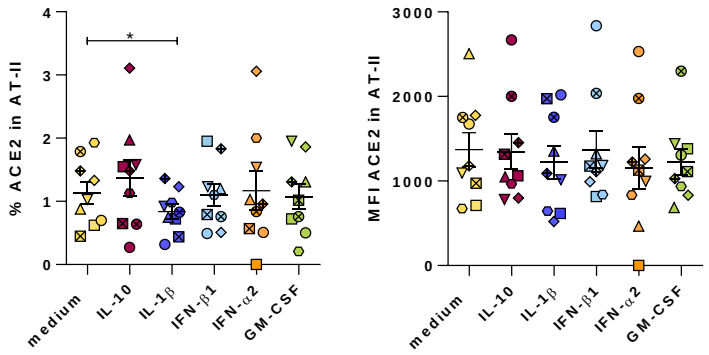
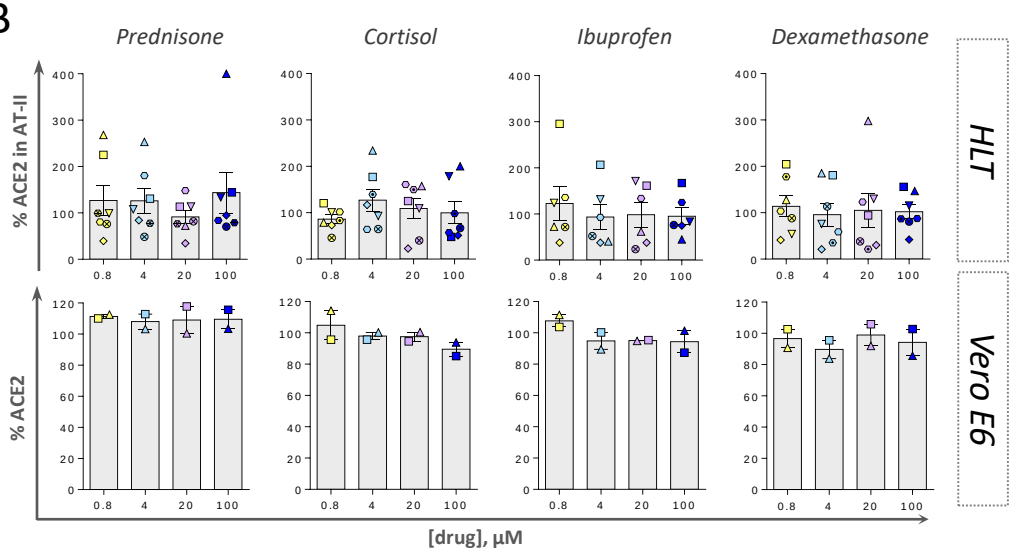


Figure 5

A


% ACE2 in AT-II
MFI ACE2 in AT-II
medium
IL-10
IL-1β
IFN-β1
IFN-α2
GM-CSF

B


Prednisone
Cortisol
Ibuprofen
Dexamethasone
% ACE2 in AT-II
% ACE2
HLT
Vero E6
[drug], µM

C

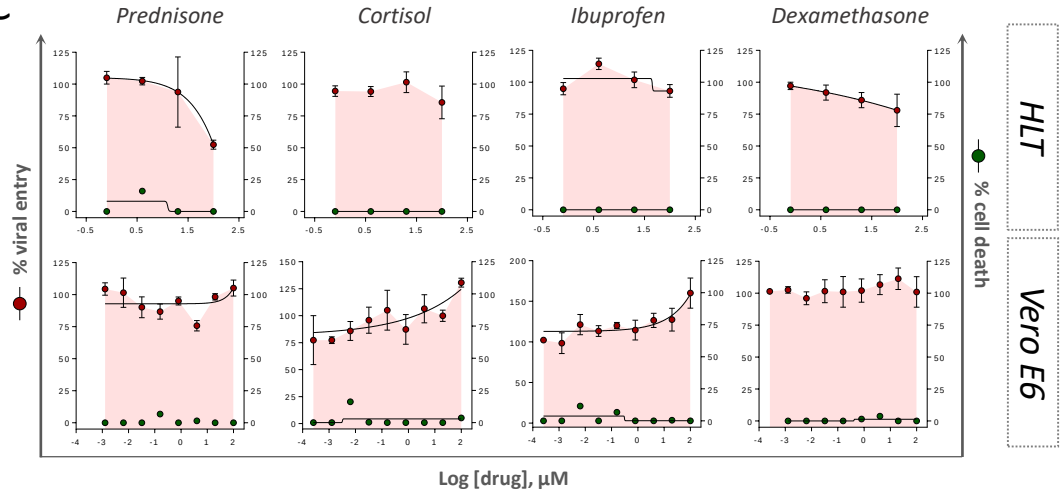
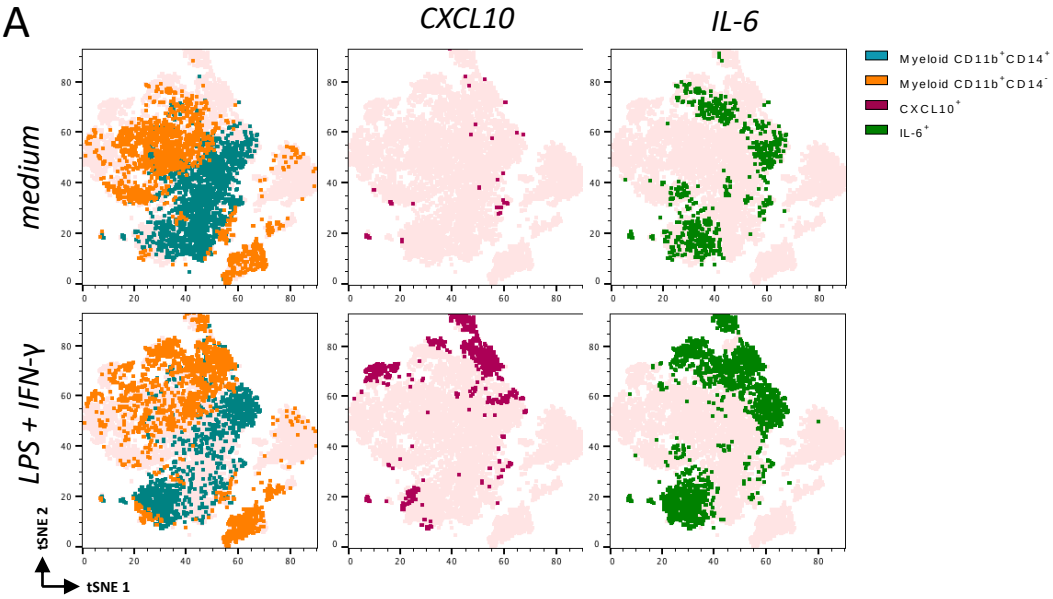
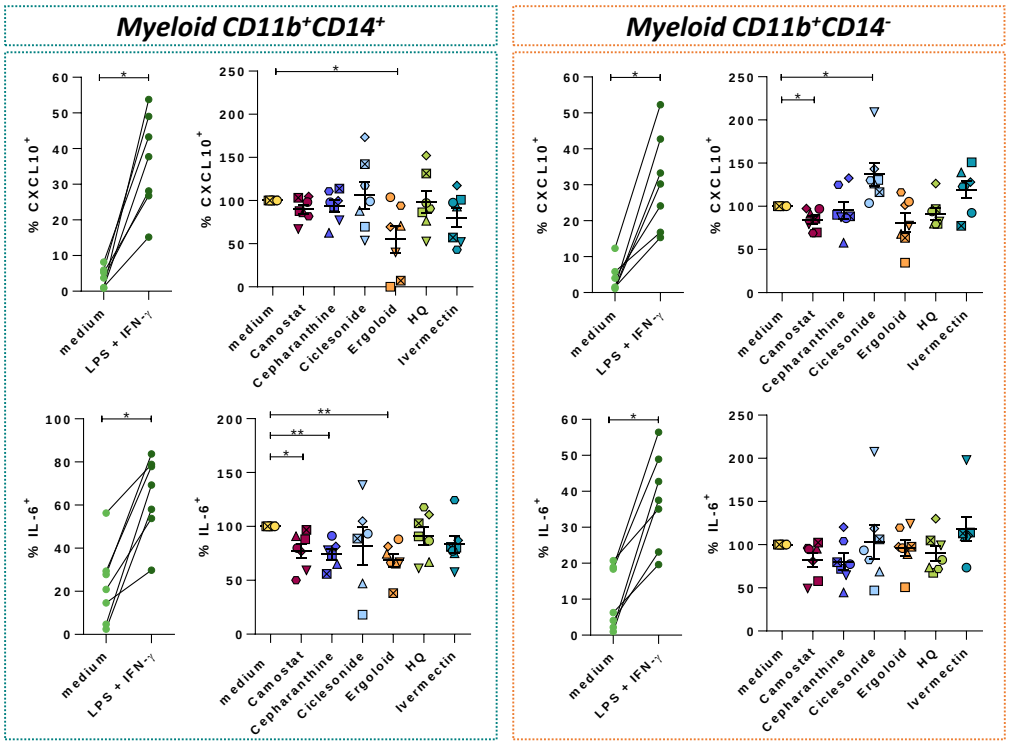

Prednisone
Cortisol
Ibuprofen
Dexamethasone
% viral entry
% cell death
HLT
Vero E6
Log [drug], µM

Figure 6

A


CXCL10
IL-6
medium
LPS + IFN-γ
Myeloid CD11b+CD14+
Myeloid CD11b+CD14-
CXCL10+
IL-6+
tSNE 2
tSNE 1

B


Myeloid CD11b+CD14+
Myeloid CD11b+CD14-
% CXCL10+
% IL-6+
medium
LPS + IFN-γ
Camostat
Cepharanthine
Ciclesonide
Ergoloid
HQ
Ivermectin