

1     **SARS-CoV-2 infects lung epithelial cells and induces senescence and an inflammatory**  
2     **response in patients with severe COVID-19**

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32 senescence-associated secretory phenotype (SASP), angiotensin-converting-enzyme 2  
33 (ACE2), alveolar type-2 (AT2) cells, DNA damage, SenTraGor

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36 **Short title:** SARS-Cov-2 infected human alveolar cells exhibit features of cellular  
37 senescence

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41

42 **Abstract**

43

44 **Rationale:** SARS-CoV-2 infection of the respiratory system can progress to a life  
45 threatening multi-systemic disease, mediated via an excess of cytokines ("cytokine  
46 storm"), but the molecular mechanisms are poorly understood.

47 **Objectives:** To investigate whether SARS-CoV-2 may induce cellular senescence in lung  
48 epithelial cells, leading to secretion of inflammatory cytokines, known as the senescence-  
49 associated secretory phenotype (SASP).

50 **Methods:** Autopsy lung tissue samples from eleven COVID-19 patients and sixty age-  
51 matched non-infected controls were analysed by immunohistochemistry for SARS-CoV-2  
52 and markers of cellular senescence (SenTraGor, p16<sup>INK4A</sup>) and key SASP cytokines  
53 (interleukin-1 $\beta$ , interleukin-6). We also investigated whether SARS-CoV-2 infection of an  
54 epithelial cell line induces senescence and cytokine secretion.

55 **Measurements and Main Results:** SARS-CoV-2 was detected by immunocytochemistry  
56 and electron microscopy predominantly in alveolar type-2 (AT2) cells, which also  
57 expressed the angiotensin-converting-enzyme 2 (ACE2), a critical entry receptor for this  
58 virus. In COVID-19 samples, AT2 cells displayed increased markers of senescence  
59 [p16<sup>INK4A</sup>, SenTraGor staining positivity in 12 $\pm$ 1.2% of cells compared to 1.7 $\pm$ 0.13% in non-  
60 infected controls ( $p$ <0.001)], with markedly increased expression of interleukin-1 $\beta$  and  
61 interleukin-6 ( $p$ <0.001). Infection of epithelial cells (Vero E6) with SARS-CoV-2 *in-vitro*  
62 induced senescence and DNA damage (increased SenTraGor and  $\gamma$ -H2AX), and reduced  
63 proliferation (Ki67) compared to uninfected control cells ( $p$ <0.01).

64     **Conclusions:** We demonstrate that in severe COVID-19 patients, AT2 cells are infected  
65     with SARS-CoV-2 and show senescence and expression of proinflammatory cytokines. We  
66     also show that SARS-CoV-2 infection of epithelial cells may induce senescence and  
67     inflammation, indicating that cellular senescence may be an important molecular  
68     mechanism of severe COVID-19.

69

70

## 71 **Introduction**

72 The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes the  
73 Coronavirus disease 2019 (COVID-19) that primarily affects the respiratory system. The  
74 clinical course of the patients ranges from asymptomatic to a life-threatening respiratory  
75 failure accompanied by a multi-systemic inflammatory disease (1,2). Systemic disease  
76 may occur through a viral-mediated "cytokine storm" that consists of a variety of  
77 cytokines and chemokines (CXCL-10, CCL-2, IL-6, IL-8, IL-12, IL1 $\beta$ , IFN- $\gamma$ , TNF- $\alpha$ ) (3,4). The  
78 link between viral infection of cells and development of severe lung disease and systemic  
79 manifestations is still poorly understood. Viral infection results in the activation of  
80 complex innate and adaptive immune responses that are orchestrated sequentially,  
81 involving several cell types and inflammatory mediators (5,6). At the cellular level,  
82 intrinsic defence mechanisms are activated and outcomes range from complete recovery  
83 to cell death (7-11). An "intermediate" and essential cellular state that is overlooked, due  
84 to lack of efficient methodological tools, is cellular senescence (12,13).

85 Cellular senescence is a stress response mechanism that preserves organismal  
86 homeostasis. Senescent cells are characterized by prolonged and generally irreversible  
87 cell-cycle arrest and resistance to apoptosis (12,14). Additionally, they also exhibit  
88 secretory features collectively described, as the senescence-associated secretory  
89 phenotype (SASP) (12). SASP includes a variety of cytokines, chemokines, growth factors,  
90 proteases and other molecules, depending on the senescence type (12,15). They are  
91 released in the extracellular space as soluble factors, transmembrane proteins following  
92 ectodomain shedding, or as molecules engulfed within small exosome-like vesicles (16-  
93 18). Under physiological conditions, senescence is transiently activated and SASP

94 mediates the recruitment of immune cells for senescent cell clearance. In addition, other  
95 SASP factors promote tissue regeneration and repair, overall ensuring cellular/tissue  
96 homeostasis. On the contrary, persistence of senescent cells exerts harmful properties  
97 promoting tissue dysfunction and the maintenance of a “latent” chronic inflammatory  
98 milieu, via paracrine and systemic SASP (12,15).

99       There is little published evidence linking viral infection to cellular senescence (19-22).  
100 Given the significance of the “cytokine storm” in the progression of COVID-19 and the  
101 SASP secretion by senescent cells, we investigated whether cellular senescence occurs in  
102 COVID-19. We provide the first evidence supporting not only the evidence for senescence  
103 in COVID-19 infected lung cells, but also potential long-term adverse implications of this  
104 disease process.

105

106 **Materials and Methods**

107

108 **Lung tissue**

109 Formalin Fixed and Paraffin embedded autopsy lung tissue samples from eleven patients  
110 that died from COVID-19 (confirmed by RT-qPCR) and lung tissues resected prior to the  
111 COVID-19 outbreak, comprising a cohort of sixty previously published and new cases  
112 (negative controls) were analyzed (**Suppl. Table 1**) (23). Clinical sample collection and  
113 their experimental use were approved by the Commission Cantonale D'éthique de la  
114 Recherche, University of Lausanne, Switzerland (Ref 2020-01257), the Bio-Ethics  
115 Committee of University of Athens Medical School, Greece.

116

117 **Anti-SARS-COV-2 (G2) antibody generation**

118 Mice immunization and antibodies collection, selection and specificity determination are  
119 described in detail in **Suppl Information**. Transcriptome analysis of hybridomas and  
120 amino acid determination of selected clones are also provided in **Suppl Information**. Four  
121 clones, namely 479-S1, 480-S2, 481-S3 and 482-S4 are under patent application  
122 (**Gorgoulis V.G., Vassilakos D. and Kastrinakis N. (2020) GR-patent application no: 22-**  
123 **0003846810**).

124

125 **Cells and SARS-CoV-2 culture**

126 SARS-CoV-2 [isolate 30-287 (B.1.222 strain)] was obtained through culture in Vero E6  
127 cells (ATCC® CRL-1586), from an infected patient in Greece. The virus was recovered from  
128 a nasopharyngeal swab, rinsed in 1 ml saline and filtered twice through a 0.22 µm filter.

129 Virus stock was prepared by infecting fully confluent Vero E6 cells in DMEM, 10% fetal  
130 bovine serum (FBS), with antibiotics, at 37°C, 5% CO<sub>2</sub>. Virus stock was collected four days  
131 after inoculation, sequenced by NGS (**Suppl Information**) and the supernatant was frozen  
132 (-80°C) until use. Infections were carried out in 24-well plates, using SARS-CoV-2 at a 0.01  
133 MOI. Cells were either fixed with 4% paraformaldehyde or lysed with NucleoZOL  
134 (MACHEREY-NAGEL) 17 days post infection. Manipulations were carried out in a Biosafety  
135 level 3 facility.

136

137 **RNA extraction and Reverse-Transcription real-time PCR (RT-qPCR)** detection were  
138 performed as previously described (**Suppl Information**) (24).

139

#### 140 **Next Generation Sequencing (NGS)**

141 NGS was performed as previously described (25). Briefly, the Ion AmpliSeq Library Kit  
142 Plus was used to generate libraries following the manufacturer's instruction, employing  
143 the Ion AmpliSeq SARS-CoV-2 RNA custom primers panel (ID: 05280253, Thermo Fisher  
144 Scientific). Briefly, library preparation steps involved reverse transcription of RNA using  
145 the SuperScript VILO cDNA synthesis kit (Thermo Fisher Scientific), 17-19 cycles of PCR  
146 amplification, adapter ligation, library purification using the Agencourt\_AMPure XP  
147 (Beckman Coulter), and library quantification using Qubit Fluorometer high-sensitivity kit.  
148 Ion 530 Chips were prepared using Ion Chef and NGS reactions were run on an Ion  
149 GeneStudio S5, ion torrent sequencer (Thermo Fisher Scientific). Samples were run in  
150 triplicates.

151



152 **Immunocytochemistry and Immunohistochemistry**

153 ICC and IHC were performed according to published protocols (26). The following primary  
154 antibodies were applied overnight at 4°C: i) anti-SARS-CoV-2 (G2) monoclonal antibody  
155 (at a dilution 1:300), ii) anti-ACE-2 (Abcam), iii) anti- TTF-1 (Dako), iv) anti-CD68 (Dako)  
156 and v) anti-p16<sup>INK4A</sup> (Santa Cruz), vi) anti-IL-1 $\beta$  (Abcam) and vii) anti-IL-6 (R&D systems),  
157 viii) anti- phospho-histone (Ser 139) 2AX ( $\gamma$ H2AX) (Cell Signaling) and ix) anti-Ki67  
158 (Abcam)(**Suppl Information**).

159

160 **SenTraGor™ staining and double staining** experiments were performed and evaluated  
161 as previously described (26).

162

163 **Electron Microscopy**

164 Representative area from hematoxylin and eosin stained paraffin sections of the lung  
165 autopsy of COVID-19 patients and corresponding non COVID-19 controls were chosen  
166 under the light microscope and marked. Paraffin-embedded tissue was deparaffinized,  
167 rehydrated and fixed in 2.5% glutaraldehyde in PBS for 24h and post-fixed in 1% aqueous  
168 osmium tetroxide for 1h at 4°C. The tissue fragment was embedded in fresh epoxy resin  
169 mixture, stained with ethanolic uranyl acetate and lead citrate and observed with a FEI  
170 Morgagni 268 transmission electron microscope equipped with Olympus Morada digital  
171 camera.

172

173 **Statistical analysis**

174 The Wilcoxon paired non-parametric test was used to compare GL13 labelling indices and

175 levels of IL-6, IL-8 and IL-1 $\beta$  between two groups (non-COVID-19 and COVID-19 infected).

176

177 **Results**

178

179 **Detection of SARS-CoV-2 in lung cells**

180 In order to detect SARS-CoV2 in lung tissue we developed monoclonal antibodies which  
181 react against the spike protein of SARS-CoV-2 and identified a high affinity antibody (G2)  
182 (**Suppl Figure 1, Suppl Figure 2, Suppl. Table 1A and Suppl. Table 1B**) (23). SARS-CoV-2  
183 was detected predominantly in alveolar type 2 (AT2) cells, which are identified by TTF-1  
184 positivity, and in sparse inflammatory cells (alveolar and tissue macrophages) in all  
185 COVID-19 patients (**Figure 1A, C**), ranging from <5 cells/4mm<sup>2</sup> tissue to >50 cells/4mm<sup>2</sup>  
186 tissue (**Suppl. Table 1A**). SARS-CoV-2 infected AT2 cells were occasionally large and  
187 appeared isolated (denuded or syncytial) or clustered (hyperplasia), exhibiting a variety  
188 of topological distribution (**Figure 1A**). These cells co-expressed the angiotensin-  
189 converting enzyme 2 (ACE2) receptor (**Figure 1B**), supporting SARS-Cov-2 infection being  
190 mediated by the ACE2 receptor (27). In addition, electron microscopy analysis in  
191 representative COVID-19 cases confirmed the presence of virus within AT2 cells (**Figure**  
192 **1Ci,ii**) and high magnification revealed virions in the proximity of the endoplasmic  
193 reticulum (**Figure 1Ciii,iv**) indicating their likely assembly and budding, as well as virions  
194 residing in cytoplasmic vesicles (**Figure 1Ciii,v-vi**), implying their transfer and release into  
195 the extracellular space.

196

197 **Senescence in SARS-CoV-2 infected cells**

198 A proportion of SARS-CoV-2 infected AT2 cells (range 8 to 21%) displayed a senescent  
199 phenotype, with positive staining for SenTraGor and p16<sup>INK4A</sup> (**Figure 2A-C**) (12,26-28). By

200 contrast lung tissues from age-matched non-COVID-19 cases with analogous co-  
201 morbidities (**Suppl Table 1**) showed significantly lower senescence (range 1-2%,  $p < 0.01$ ,  
202 Wilcoxon paired non-parametric test) (**Figure 2A-C**), suggesting that SARS-CoV-2 infection  
203 may induce senescence.

204 To functionally reproduce our hypothesis we infected Vero cells with a viral strain  
205 isolated from a COVID-19 patient. Vero cells is an established cellular system for viral  
206 propagation and studies, as apart from their high infectivity to SARS-CoV-2 they are  
207 among the few cell lines demonstrating SARS-CoV-2-mediated cytopathic effects, an  
208 essential aspect in diagnostics (29,30). Infection was carried out at a low MOI to mimic  
209 natural coronavirus infection (31). In line with our hypothesis, the infected cells following  
210 an initial surge of cell death reached an equilibrium demonstrating clear evidence of  
211 senescence, as compared to the non-infected control cells, 17 days post infection (**Figure**  
212 **3**). As Vero cells lack p16<sup>INK4A</sup> (32), the most likely trigger of senescence is DNA damage,  
213 as previously reported (12,33). DNA damage measured by  $\gamma$ -H2AX immunostaining, was  
214 evident in SARS-CoV-2 infected cells (**Figure 3A4**). It appears that genotoxic stress results  
215 from a vicious cycle imposed by the virus in host cells as it hijacks most intracellular  
216 protein machineries (11,34).

217

### 218 **Senescence associated secretory phenotype**

219 We found very high expression of both IL-1 $\beta$  and IL-6 by senescent AT2 cells in the lungs  
220 of COVID-19 patients while in the non COVID-19 control cases expression was very low in  
221 the few senescent AT2 cells detected ( $p < 0.001$ ) (**Figure 4A-C, Suppl Table 1**). As both  
222 cytokines are key components of the "cytokine storm", our findings suggest putative

223      implication of senescence via SASP in the poor clinical course of COVID-19 patients.

224      Likewise, SARS-CoV-2 senescent Vero cells displayed expression of SASP-related

225      cytokines, as assessed by our recently reported algorithmic assessment of senescence,

226      justifying the *in vivo* findings (**Figure 3B**).

227

228

229 **Discussion**

230 We have demonstrated the presence of SARS-CoV-2 in AT2 cells of patients who died  
231 from COVID-19 using a novel anti-viral antibody and confirmed by electron microscopy.  
232 We have shown for the first time that a proportion of SARS-CoV-2-infected AT2 cells  
233 acquire senescence features (as demonstrated by significantly increased staining with the  
234 novel senescence marker SenTraGor and increased p16<sup>INK4A</sup>). The finding that in age-  
235 matched non-COVID-19 cases the percentage of senescent cells was much lower (1-2%)  
236 than that of the COVID-19 clinical panel (8-21%), is strongly indicative that SARS-CoV-2  
237 triggers senescence (**Figure 2**). We therefore examined whether cellular infection with  
238 SARS-CoV-2 virus (B.1.222 strain) would induce cellular senescence in a susceptible cell  
239 line *in vitro* and found that in infected cells there was increased SenTraGor staining, as  
240 well as evidence of DNA damage measured by increased  $\gamma$ -H2AX expression. This strongly  
241 suggests that SARS-CoV-2 may attach to AT2 cells via ACE2 to infect these cells and  
242 through activation of the DNA damage response may induce cellular senescence (34). We  
243 also demonstrated that the cells infected with SARS-CoV-2 also show a high degree of  
244 expression of IL-1 $\beta$  and IL-6, both components of the SASP and implicated in systemic  
245 features of COVID-19 which is associated with a “cytokine storm” (3,4).

246 Senescent cells are in a state of cell cycle arrest but remain metabolically active and  
247 secrete a typical profile of inflammatory proteins known as the senescence-associated  
248 secretory phenotype (SASP). SASP components include the proinflammatory cytokines IL-  
249 1 $\beta$  and IL-6, which are elevated in COVID-19 patients that have acute respiratory distress  
250 syndrome (ARDS) or systemic inflammatory features. The SASP components could induce  
251 senescence in nearby cells (paracrine) or may spread senescence systemically

252 (endocrine), thus amplifying this chronic inflammation. It is likely that SARS-CoV-2  
253 spreads from epithelial cells in the lower airways to infect AT2 cells, which express ACE2,  
254 and cause local senescence and inflammation in the lung. The virus may then enter the  
255 circulation and senescence may subsequently spread systemically to affect other organs,  
256 leading to multi-organ failure and death (1,22)

257 An additional implication relates to the prolonged survival of senescent cells that are  
258 infected with the virus, as senescent cells are resistant to apoptosis (12,14). This may  
259 allow the virus to be hosted for longer periods compared to other cells with higher cell  
260 turnover, exposing its genome to host-mediated editing (35-38). Within this context, we  
261 recently reported abundance of the APOBEC enzymes, particularly G and H (RNA editing  
262 cytoplasmic variants), which are reported to play a pivotal role in viral RNA editing, in  
263 cells undergoing stress-induced senescence (24,39,40). In support to this notion, are the  
264 increased APOBEC 3G and 3H expression levels found in the infected Vero cells (**Suppl**  
265 **Figure 3**). Moreover, by conducting a detailed bioinformatic analysis of 423000 SARS-  
266 CoV-2 strains available in the GISAID database, we found that APOBEC signatures seem to  
267 potently determine the mutational profile of the SARS-CoV-2 genome (**Suppl Figure 4,**  
268 **Suppl Figure 5**).

269 A limitation of the study is the small sample size of examined COVID-19 lung autopsies,  
270 due to difficulty of getting access to this material. Another limitation due to the nature of  
271 the disease is that pathological features, such as senescence, can only be investigated  
272 within the context of cadaverous material, which represents the most severe outcome of  
273 the spectrum of COVID-19 clinical manifestations. Therefore, evaluation of senescence in

274 less severe conditions is not feasible. Findings have been observed in lung biopsies only  
275 and ideally should be also investigated in organs other than the lung.

276 Overall, SARS-CoV-2 induced senescence justifies the application of senotherapeutics  
277 not only as a therapeutic approach for the treatment of COVID-19 patients but also as a  
278 putative strategy to restrict mutational events that may favor the emergence of SARS-  
279 CoV-2 quasispecies (15,22,41). Senotherapies include senostatics that inhibit components  
280 of the cellular senescence pathways and senolytics, which induce senescent cells to  
281 become apoptotic (15,22). Several senolytic therapies have been shown to be effective in  
282 animal models of accelerated ageing diseases, including COPD, idiopathic pulmonary  
283 fibrosis, atherosclerosis and chronic kidney disease (12,15,22). A trial of senolytic therapy  
284 in patients with diabetic kidney disease demonstrated a reduction in senescent cells in  
285 the skin and reduced circulating SASP proteins, such as IL-1 $\beta$  and IL-6 (42). A clinical trial  
286 of a Senolytic compound (F) to inhibit progression to cytokine storm and ARDS in COVID-  
287 19 patients has been approved by the US Food and Drug Administration (FDA) and is  
288 anticipated to be soon launched (43).

289

290

#### 291 **Disclosure/Conflict of Interest**

292 The authors wish to declare no conflict of interest.

293

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458 **Figure Legends**

459

460 **Figure 1: Detection of SARS-CoV-2 in lung cells. A.** Representative images of SARS-CoV-2  
461 IHC staining in COVID-19 lung tissue. Competition with anti-peptide (S protein) showing  
462 specificity of the IHC staining. Representative negative, control IHC staining in non-  
463 COVID-19 lung tissues. Graph shows quantification of SARS-CoV-2 staining in the clinical  
464 samples (**Suppl Table 1**). **B.** Detection of SARS-CoV-2 in AT2 cells (confirmed by TTF-1  
465 staining) and in ACE-2 expressing cells. Double IHC staining for SARS-CoV-2 and TTF-1. **C.**  
466 Detection of SARS-CoV-2 by transmission electron microscopy (TEM) in a representative  
467 COVID-19 patient. Presence of SARS-CoV-2 within AT2 cells (**i,ii**) and of virions in the  
468 proximity of the endoplasmic reticulum (**iii,iv**) as well as in cytoplasmic vesicles (**iii,v-vi**).  
469 Corresponding scale bars are depicted. ICH: immunohistochemistry; AT2: alveolar type 2  
470 cells; ACE2: angiotensin-converting enzyme 2, \*\*\*Statistical significant:  $p < 0.001$ .

471

472 **Figure 2: Senescence in SARS-CoV-2 infected cells. A.** Representative images of SARS-  
473 CoV-2, SenTraGor (senescence) and ACE-2 staining in serial sections of COVID-19 lung  
474 tissue. Double-immunostaining analysis (**2**) for SARS-CoV-2, SenTraGor (senescence),  
475 ACE-2 and p16<sup>INK4A</sup> in COVID-19 lung tissue. **B.** Representative results from serial staining  
476 for SARS-CoV-2, SenTraGor (senescence) and ACE-2, and double-staining experiment for  
477 SARS-CoV-2 and p16<sup>INK4A</sup> in non-COVID-19 lung tissue. **C.** Graphs depicting the increased  
478 levels of SenTraGor and p16<sup>INK4A</sup> in COVID-19 lung tissue. Corresponding scale bars are  
479 depicted. Statistical significance: \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ .

480

481 **Figure 3. SARS-CoV-2 infection, senescence and SASP expression in Vero cells. A.** SARS-  
482 CoV-2 presence (1), senescence induction (2), cellular proliferation (3) and DNA damage  
483 activation (4), with corresponding quantitative histograms, in Vero cells with and without  
484 SARS-CoV-2 infection. Double IHC staining for SARS-CoV-2 infection/senescence  
485 induction (5) and senescence induction/cellular proliferation (6). **B.** Graph depicting  
486 induction of SASP related cytokines following SARS-CoV-2 infection (mRNA expression).  
487 \*\*Statistical significant:  $p < 0.01$ .

488

489 **Figure 4: Senescence associated secretory phenotype (SASP) in COVID-19 lung tissues.**  
490 **A.** Representative staining results (at low and high magnification) of SenTraGor, IL-6, IL-  
491  $1\beta$  and TTF-1 in corresponding serial sections (1) and as double immunostaining analysis  
492 (2) of COVID-19 lung tissue. Original magnification: 400x. **B.** Representative staining  
493 results showing absence or minimal levels of SenTraGor, IL-6 and IL- $1\beta$  in age-matched  
494 non-COVID-19 control samples. Corresponding scale bars are depicted. IL-1  $\beta$ : Interleukin  
495  $1\beta$ ; IL-6: Interleukin-6; \*\*\*Statistical significant:  $p < 0.0001$ .

496

497 **Supplementary Information**

498 **Suppl Figure legends**

499

500 **Suppl Figure 1: SARS-CoV-2 antibody production and screening selection. A.** Workflow  
501 of the procedure for antibody production. **B.** Sequel of screening steps for antibody  
502 production and selection. **C.** Final screening step processes leading to the selection of G2  
503 monoclonal antibody (**Suppl Figure 2**).

504

505 **Suppl Figure 2:** Graph depicting the structure of G2 antibody as well as the DNA  
506 sequences of FRs and CDRs elements of variable regions.

507

508 **Suppl Figure 3:** Graph depicting abundance of APOBEC 3G and 3H expression levels in  
509 Vero cells by RT-qPCR analysis. \*Statistical significant:  $p < 0.05$ .

510

511 **Suppl Figure 4:** APOBEC consensus RNA 2D sequence and structure motifs. **A.** Mutation  
512 profile of SARS-CoV-2 genome exhibits APOBEC mutation signatures. Applying  
513 bioinformatics analysis, C to U mutations were found to be the most dominant (55%),  
514 suggesting an APOBEC driven signature. 56.8% of these mutations were confirmed to  
515 exert APOBEC binding characteristics. **B.** Depicts the APOBEC consensus 2D structure  
516 image as obtained from Beam software with statistics for the motif as shown in **(C)** and  
517 the position of the motif on the 120 nt window as presented in **(D)**. APOBEC average  
518 probability per base of being unpaired around a 120 nt region of the most probable C→U  
519 site. **E.** The average probability from all C→U sites as obtained from the RNAplfold

520 algorithm is depicted **(i)** while **(ii)** presents the consensus sequence motif relative to the  
521 average probability window.

522

523 **Suppl Figure 5: Infected senescent cells as a putative source for SARS-CoV-2 quasi-**

524 **species generation. A. (i)** Schematic layout presenting representative APOBEC sites from

525 the GISAID database analysis that overlap with the C→U sites of Vero cells after 17 days

526 of infection with the SARS-CoV-2 B.1.222 strain (see also panel **B**). The yellow bars show

527 the frequency of C→U substitutions when observing the GISAID database read counts

528 (green pileups) and with red is the C→U frequency when observing the SARS-CoV-2

529 genome, 17 days post infection. These representative sites are ranked as highest

530 relatively to the C→U counts as observed from the GISAID database. The genomic co-

531 ordinates of each C→U can be observed at the superimposed SARS-CoV-2 genome. On

532 the left of the graphs is the consensus motif when performing a motif analysis of all the

533 C→U sites of the SARS-CoV-2 genome, 17 days post infection. NGS reads were confirmed

534 in triplicate reads. **(ii)** Graph depicting frequency of nucleotide substitutions that

535 accumulated in the genome of B.1.222 strain following 17 days of infection. **(iii)** Pie chart

536 demonstrating that predominant C→U substitutions (65%) are APOBEC driven. **B.**

537 Additional locations of C→U substitutions observed in the genome of the SARS-CoV-2

538 progeny after 17 days of infection in Vero cells (relative to panel **A**).

539

540 **Material and Methods**

541

542 **RNA extraction and Reverse-Transcription real-time PCR (RT-qPCR) detection**

543 ***SASP cytokine and APOBEC G and H mRNA analysis***

544 RNA was extracted using the Nucleospin RNA kit (Macherey-Nagel #740955) according to  
545 the manufacturer's instructions. 1 µg RNA was used for cDNA preparation with  
546 Primescript™ RT Reagent Kit (Takara #RR037A). RT-qPCR was performed utilizing SYBR  
547 Select Master Mix (Life technologies #4472908) on a DNA-Engine-Opticon (MJ-Research)  
548 thermal cycler. Primer sequences employed were: *IL-1β* Fw: 5'-  
549 GGAAGACAAATTGCATGG-3', Rv: 5'-CCCAACTGGTACATCAGCAC-3'; *IL-6* Fw: 5'-  
550 AGAGGCACTGGCAGAAAAC-3', Rv: 5'-TGCAGGAACTGGATCAGGAC-3'; *IL-8* Fw: 5'-  
551 AGGACAAGAGCCAGGAAGAA-3', Rv: 5'-ACTGCACCTTCACACAGAGC-3'; *APOBEC3G* Fw: 5'-  
552 CCGAGGACCCGAAGGTTAC-3', Rv: 5'- TCCAACAGTGCTGAAATTCG-3'; *APOBEC3H* Fw: 5'-  
553 CGACGGCTTGAAAGGATAGAG-3', Rv: 5'- TGAGTTGTGTGTTGACGATGA-3'; B2M: β2-  
554 microglobulin (reference) gene Fw: 5'-TCTCTGGCTGGATTGGTATCT-3', Rv: 5'-  
555 CAGAATAGGCTGCTGTTCTATC-3' (1). Results, averaged from three independent  
556 experiments, are presented as n-fold changes after Sars-CoV-2 infection relatively to the  
557 non-infected condition, using the 2-ΔΔCT method.

558

559 ***Viral RNA detection***

560 RNA was extracted using the NucleoSpin Virus RNA purification kit (Macherey-Nagel  
561 #740.983) according to the manufacturer's instructions. RT-qPCR was performed utilizing  
562 the One Step PrimeScript III RT-PCR Kit (Takara # RR601B) on a Rotor-Gene Q 6000

563 (Qiagen) thermal cycler following the manufacturer's instructions and using the CDC N-  
564 gene directed primers [[https://www.cdc.gov/coronavirus/2019-ncov/lab/rt-pcr-panel-  
565 primer-probes.html](https://www.cdc.gov/coronavirus/2019-ncov/lab/rt-pcr-panel-primer-probes.html)].

566

## 567 **Anti-SARS-COV-2 antibodies**

### 568 ***Generation***

569 A series of monoclonal antibodies against SARS-CoV2 spike protein were produced  
570 according to a modified method of Koehler and Milstein (Koehler and Milstein,  
571 1975). Briefly, twelve BALB/c mice of 5 weeks of age were immunized intraperitoneally  
572 (i.p.) with 25µg of SARS-Cov2 protein (Trenzyme GmbH, Germany). All immunization and  
573 animal handling were in accordance with animal care guidelines as specified in EU  
574 Directive 2010/63/EU. After 5 cycles of immunization, mice were sacrificed, spleenocytes  
575 were collected and fused with P3X63Ag8.653 (ATCC® CRL1580™) following a modified  
576 method of Koehler and Milstein. Positive clones and antibody specificity were  
577 determined through extensive immunosorbent assays. Four clones, namely 479-S1, 480-  
578 S2, 481-S3 and 482-S4 are under patent application (**Gorgoulis V.G., Vassilakos D. and  
579 Kastrinakis N. (2020) GR patent application no: 22-0003846810**).

580

### 581 ***RNA sequence determination and amino acid prediction***

582 RNA was collected from biological duplicates of generated hybridomas as described  
583 elsewhere (2). RNA samples were processed according to manufacturer's instructions,  
584 using the following kits: NEBNext® Poly(A) mRNA Magnetic Isolation Module (E7490S),  
585 NEBNext® Multiplex Oligos for Illumina® (Index Primers Set 1, NEB7335) and

586 NEBNext® Ultra™ II Directional RNA Library Prep with Sample Purification Beads  
587 (E7765S). After successful QC (RNA 6000 Nano bioanalyzer, Agilent) and quantity  
588 measurements (Qubit™ RNA HS Assay Kit, Thermofisher), 1ug was used for mRNA  
589 selection, cDNA construction, adaptor ligation and PCR amplification (11 cycles),  
590 according to the manufacturer's protocol:  
591 ([https://international.neb.com/products/e7760-nebnext-ultra-ii-directional-rna-library-](https://international.neb.com/products/e7760-nebnext-ultra-ii-directional-rna-library-prep-kit-for-illumina#Product%20Information)  
592 [prep-kit-for-illumina#Product%20Information](https://international.neb.com/products/e7760-nebnext-ultra-ii-directional-rna-library-prep-kit-for-illumina#Product%20Information)). The 479-G2-ATCACG index from NEB  
593 E7335 was used. The final libraries were analyzed with Agilent High Sensitivity DNA Kit on  
594 an Agilent bioanalyzer, quantitated (Qubit dsDNA HS Assay Kit, Thermofisher) and, after  
595 multiplexing, were run using a NextSeq 500/550 Mid Output Kit v2.5 (150 cycles), paired  
596 end mode on a NextSeq550 (Illumina) at final concentration 1,3pM with 1% PhiX Control  
597 v3.  
598 Fastq files were demultiplexed with Flexbar (3). Quality control of the Fastq files was  
599 assessed with FastQC tools (4). Adapter sequences were removed with Cutadapt program  
600 (5) with the following parameters: quality trimming was set to 20 and the minimum  
601 allowed nucleotide length after trimming was 20 nucleotides using --pair-filter=any to  
602 apply the filters to both paired reads. A two way alignment mode was followed to  
603 identify the antibody clone. More precisely alignments were performed with Bowtie2 (6)  
604 with parameters set as following: -D 20 -R 3 -N 1 -L 20 -i S,1,0.50 --no-mixed --no-  
605 discordant against an index made from IMGT database <http://www.imgt.org/> having  
606 downloaded all mouse and human IG genes. Also this mode of alignments was executed  
607 for quality control and visualization of the aligned reads spanning the IG gene segments  
608 on the genome browser. The second mode refers to the determination and

609 reconstruction of the clones. This was performed with MiXCR suite (7). At first,  
610 alignments against the IG repertoire were performed with kaligner and visualization of  
611 alignments was assessed. It was observed that the use of kaligner gave better results  
612 with higher clone hits regarding the VH and VL segments. Full assembly of the clones was  
613 performed. A full report of the number of reads and assembly of CDR and FR clones is  
614 provided in clones479\_S1kalign.txt. The clones with the highest number of reads and  
615 coverage across the V,D,J segments were considered. The reported matched sequences  
616 were also checked with IgBlast tool <https://www.ncbi.nlm.nih.gov/igblast/>. In addition,  
617 after the assembly of the amino acid reconstruction of the FR and CDR regions of the full  
618 variable fragment for both the Heavy and Light antibody chains, a 3D visualization was  
619 also determined via folding the V protein fragment with iTasser suite (8). The above  
620 analysis has been extensively described in **Gorgoulis VG, Vassilakos D and Kastrinakis N.**  
621 **(2020) GR patent application no: 22-0003846810.**

622

### 623 **Immunocytochemistry (ICC)-Immunohistochemistry (IHC)**

624 Method: ICC and IHC were performed according to previous published protocols (9). In  
625 brief, 3 µm thick sections from formalin-fixed paraffin embedded (FFPE) lung tissues were  
626 employed. Antigen retrieval was heat-mediated in 10 mM citric acid (pH 6.0) for 15  
627 minutes. The following primary antibodies were applied: i) the anti-SARS-CoV-2 (G2)  
628 monoclonal antibody (dilution 1:300), ii) anti-ACE-2 [Rabbit polyclonal antibody Abcam,  
629 Cat.no: ab15348 (dilution 1:200)], iii) anti- TTF-1 [rat monoclonal antibody Dako, Clone  
630 8G7G3/1, Cat.no: M3575 (Ready-to-Use)], iv) anti-CD68 [mouse monoclonal antibody  
631 Dako, Clone PG-M1, Cat.no: M0876 (dilution 1:50)] and v) anti-p16<sup>INK4A</sup> [mouse



632 monoclonal antibody Santa Cruz, clone: F-12, Cat.no.:sc-1661. (dilution 1:100)], vi) IL-1 $\beta$   
633 [Rabbit polyclonal antibody Abcam, Cat.no: ab2105 (dilution 1:150)] and vii) IL-6 [mouse  
634 monoclonal antibody R&D systems, clone: Clone: 6708, Cat.no:MAB206 (dilution 1:100)],  
635 all overnight at 4°C. Development of the signal was achieved using the Novolink Polymer  
636 Detection System (Cat.no: RE7150-K, Leica Biosystems). Specimens were counterstained  
637 with hematoxylin.

638 Negative Controls for the anti-SARS-CoV-2 (G2) monoclonal antibody: *i) Biological*,  
639 comprising previously published and new lung tissue samples from a cohort of 50 cases  
640 that underwent surgery prior to COVID-19 outbreak. *ii) Technical*: a. Omission of the G2  
641 primary monoclonal antibody, b. Blocking of the G2 primary monoclonal antibody using  
642 the corresponding S-protein (Cat.no.P2020-029, Trenzyme) in a 1:10 (G2/Spike protein)  
643 ratio and c. Two slides per case were employed for each staining or control experiment.

644 Evaluation of G2 staining: Cells were considered positive irrespective of the staining  
645 intensity. Two different semi-quantitative IHC evaluation approaches, previously  
646 described were adopted (10,11) According to the first, the number of G2 positive cells  
647 per 4mm<sup>2</sup> was encountered and scored according to the following criteria: (+) for positive  
648 staining in <5 cells per 4 mm<sup>2</sup>, (+) for positive staining in 5–50 cells per 4mm<sup>2</sup> and (+++)  
649 for positive staining in >50 cells per 4 mm<sup>2</sup> (10). Regarding the second one, the number  
650 of G2 positive cells per whole slide was estimated and subsequent scores were assessed:  
651 (+) between one and five positive cells per whole slide (scattered cells), (++) more than  
652 five cells per whole slide but no foci (isolated cells) and (+++) more than 10 cells in one  $\times$   
653 20 field (with foci) (11). For IL-6 and IL-1 $\beta$ , the percentage of immunopositive cells was

654 encountered (12). Evaluations were performed blindly by four experienced pathologists  
655 (KE, PF, CK and VG) and intra-observer variability was minimal ( $p \leq 0.05$ ).

656

657 **Bioinformatic analysis for identification of mutational signatures in the SARS-CoV-2**  
658 **genome**

659 ***Screening for mutational signatures in the SARS-CoV-2 genome***

660 To investigate the mutational properties on the SARS-CoV-2 genome we downloaded from  
661 GISAID database (<https://www.gisaid.org/>) 423.000 available strains that were  
662 distributed globally. These strains were aligned with the Wuhan first assembly  
663 NC\_045512, obtained from NCBI (<https://www.ncbi.nlm.nih.gov/sars-cov-2/>), with  
664 Bowtie aligner (13) using the following command:

```
665 /bowtie2-2.4.2-sra-linux-x86_64/bowtie2-align-s --wrapper basic-0 -x Covncbiref -p 4 -D  
666 20 -R 3 - N 1 -L 20 -i S,1,0.50 -f allCov19.fa
```

667 In order to identify the mutations we have created an “in-house” script using *calmd*  
668 function from SAMtools (14) , based on the analysis of deciphering mutations from the  
669 proteome occupancy profile study (15). We applied the following commands for minus  
670 and reverse stranded reads:

```
671 samtools sort accepted_hits.bam -o accepted_hitsort.bam
```

```
672 samtools rmdup -s accepted_hitsort.bam rmdupsorted.bam
```

```
673 #forward library
```

```
674 samtools view -h -f 0x0010 rmdupsorted.bam | samtools calmd -S -
```

```
675 ~/Desktop/Bioinformatics/NCBI.fa /dev/stdin |
```

```
676 /media/covid_meth/deademinatation_covid19/get_edit_stat.pl      '-'      >
677 Mapping_editStatus.bed
678 samtools view -h -F 0x0010 rmdupsorted.bam | samtools calmd -S -
679 ~/Desktop/Bioinformatics/NCBI.fa /dev/stdin |
680 /media/covid_meth/deademinatation_covid19/get_edit_stat.pl      '+'      >>
681 Mapping_editStatus.bed
682 #reverse library
683 samtools view -h -F 0x0010 rmdupsorted.bam | samtools calmd -S -
684 ~/Desktop/Bioinformatics/GRCh37/hg19.fa /dev/stdin |
685 /media//covid_meth/deademinatation_covid19/get_edit_stat.pl    '-'      >
686 Mapping_editStatus2.bed
687 samtools view -h -f 0x0010 rmdupsorted.bam | samtools calmd -S -
688 ~/Desktop/Bioinformatics/GRCh37/hg19.fa /dev/stdin |
689 /media//covid_meth/deademinatation_covid19/get_edit_stat.pl    '+'      >>
690 Mapping_editStatus2.bed
691 The scripts bellow were used in order to determine the counts per type of mutation and
692 filter for C→U or G→A mutations in respect with the strand orientation of the alignments.
693 Bedtools (16) have also been used to obtain the fasta sequences and the windows around
694 the C→U sites.
695 #sort reads
696 sort -k1,1 -k2,2n -k3,3nMapping_editStatus2.bed| uniq -c >
697 Mapp_editstat_APOBECcounts.bed sort -k1,1 -k2,2n -k3,3nMapping_editStatus2.bed|
```

```
698  uniq -c > Mapp_editstat_APOBECcounts.bed sed -i 's/^ *//g'
```

```
699  Mapp_editstat_APOBECcounts.bed
```

```
700  #obtain the fasta
```

```
701  fastaFromBed -s -fi ~/Desktop/Bioinformatics/GRCh37/hg19.fa -
```

```
702  bedMapp_editstat_APOBECcounts.bed -tab -foAPOBEC_counts1fa.bed
```

```
703  #get mutation type C→U or A→G
```

```
704  perl fixmutstat.pl APOBEC_counts1fa.bed APOBEC_counts1facorrect
```

```
705  Based on the filtered candidate sites with a frequency of mutations above than 5 reads
```

```
706  we have obtained windows of  $\pm 60$  nucleotides and folded the RNA sequences from these
```

```
707  regions with Vienna RNA fold algorithm (17) to determine the RNA 2D structure. SHAPE
```

```
708  reactivities from SHAPE-seq data (18) were used to guide the RNA folding.
```

```
709  RNAfold --noPS --shape=forViennatest.SHAPE.txt --shapeConversion=S -g <
```

```
710  forViennatest..fa
```

```
711  >> test.txt.
```

```
712  To decipher the candidate motifs we counted the frequency of letters  $\pm 5$  nucleotides
```

```
713  from the most frequent deademinated nucleotide. The frequency for each letter was
```

```
714  determined via a perl script which extracts all possible k-mers and their frequencies. Next
```

```
715  these k-mers, based on their frequency, were plotted with Web-logo motifs (19). In
```

```
716  addition position-weight matrices (PWM) for each letter around the deademinated RNA
```

```
717  nucleotide were extracted.
```

```
718  Our analysis on motifs and RNA structure for choosing the candidate APOBEC sites based
```

```
719  on publicly available known studies (20-22) that also demonstrate similar characteristics
```

```
720  regarding the motif specific APOBEC signature and RNA structure. From our analysis we
```

721 determined a CCT/A enrichment around regions of open hairpin structures agreeing with  
722 the results from the literature.

723

#### 724 ***Verification of APOBEC specific motifs by applying machine learning***

725 To filter and obtain scores for each APOBEC specific candidate site we have also applied a  
726 machine learning scheme using convolutional neural networks having as input the  
727 sequence and RNA structure around the candidate strongest APOBEC sites with high  
728 frequency that also demonstrate a high potential for APOBEC binding.

729

#### 730 ***APOBEC consensus RNA 2D sequence and structure motifs***

731 To determine the consensus RNA structure properties we have used the Vienna RNA  
732 folding output dot bracket notation, which performed the folding based on the icSHAPE  
733 reactivities as input to BEAM program (23). In addition, the binding sites from (19) have  
734 been used to decipher the structure for the hg19. The structure properties for the  
735 APOBEC sites on the hg19 have been folded using SHAPE (24) and DMS (25) data to guide  
736 the RNA folding. Secondary RNA structure motifs regarding the hg19 have been  
737 determined using BEAM software. Beam software was used as follows:

738 First the dot bracket notation is translated in a 24 letter language for structure called  
739 Bear:

```
740 java -jar encoder.jar APOBEC.db APOBEC.fb
```

```
741 java -jar /$basepath3/BEAM_release_1.5.1.jar -f APOBEC.fb -w 15 -W 30 -M 5
```

742 Next, then consensus motifs are extracted, using as maximum threshold to output the  
743 top 5 motifs with maximum width of motif set to 30 nucleotides.

744

745 **APOBEC average probability per base of being unpaired around a 120 nt region of the**  
746 **most probable C→U site**

747 Furthermore RNAplfold has been used to extract the probabilities per base of being  
748 unpaired having the following parameters

749 *RNAplfold -W 30 -L 15 -u 1 --shape=forViennatest.SHAPE.txt --shapeMethod=D --*  
750 *shapeConversion=O -g <forViennatest..fa >> test.txt*

751 The output per sequence is a *\_lunp* file where from these files the smoothed geometric  
752 mean per base pair is extracted and plotted having the candidate deaminated site,  
753 located in the center of the 120 nucleotide window. The plots are done in R using fit and  
754 polygon functions from the standard R bioconductor packages. The confidence intervals  
755 per base are calculated as  $CI = \bar{x} \pm z \frac{s}{\sqrt{n}}$  where  $z = 0.95$  % confidence,  $s$ =standard deviation  
756 and  $n$  are the number of sequences  $\sim 3500$ .

757 In order to accomplish docking of the APOBEC with the RNA substrates we have used  
758 SimRNA suite (26) to determine the RNA 3D structure properties of the SARS-CoV-2 RNA  
759 around the candidate C→U sites. DARS-RNP potential (27) has been used using PDB files  
760 from crystallographic data for APOBEC3G with PDB code 6bux and 6k3j (28) from the PDB  
761 database (<https://www.rcsb.org/>) for docking the RNAs as obtained from SimRNA with  
762 APOBEC. Scores were ranked and the RNAs with docking scores higher than 1 standard  
763 deviation over the mean were used for the extraction of consensus motifs both in terms  
764 of structure and sequence. Regarding the sequence motif a window of  $\pm 10$  nt around the  
765 high docking score was obtained and according to the *k-mer* distribution PWM matrices  
766 are extracted and plotted with web-logo.

767 The SimRNA commands to extract the 3D RNA structure are the following:

768 ./SimRNA -s 3D\_testVf.fa -c config2.dat -S 3D\_test.struct -o tRNAs python2

769 trafl\_extract\_lowestE\_frame.py tRNAs.trafl"

770 ./SimRNA\_trafl2pdb tRNAs-000001.pdb tRNAs\_minE.trafl : AA" perl configpdb2.pl

771 tRNAs\_minE-000001\_AA.pdb tRNAs.config"

772 The configuration file for the 3D simulations is set as:

773 *NUMBER\_OF\_ITERATIONS 160000*

774 *TRA\_WRITE\_IN\_EVERY\_N\_ITERATIONS 16000*

775 *INIT\_TEMP 1.35*

776 *FINAL\_TEMP 0.90*

777 *BONDS\_WEIGHT 1.0*

778 *ANGLES\_WEIGHT 1.0*

779 *TORS\_ANGLES\_WEIGHT 0.0*

780 *ETA\_THETA\_WEIGHT 0.40*

781 *SECOND\_STRC\_RESTRAINTS\_WEIGHT 1.0*

782 *FRACTION\_OF\_NITROGEN\_ATOM\_MOVES 0.10*

783 *FRACTION\_OF\_ONE\_ATOM\_MOVES 0.45*

784 *FRACTION\_OF\_TWO\_ATOMS\_MOVES 0.44*

785 *FRACTION\_OF\_FRAGMENT\_MOVES 0.01*

786 In order to visualize the properties that might determine the binding of APOBEC, the

787 power of integrated gradients tools (29) were used to obtain the motifs. Our analysis is

788 based upon an already developed method DeepRipe (30) adding an extra module to also

789 incorporate the RNA structure information. The classifier has been trained to distinguish

790 such motifs which are characteristic for APOBEC binding.

791



## 792 **Supplementary References**

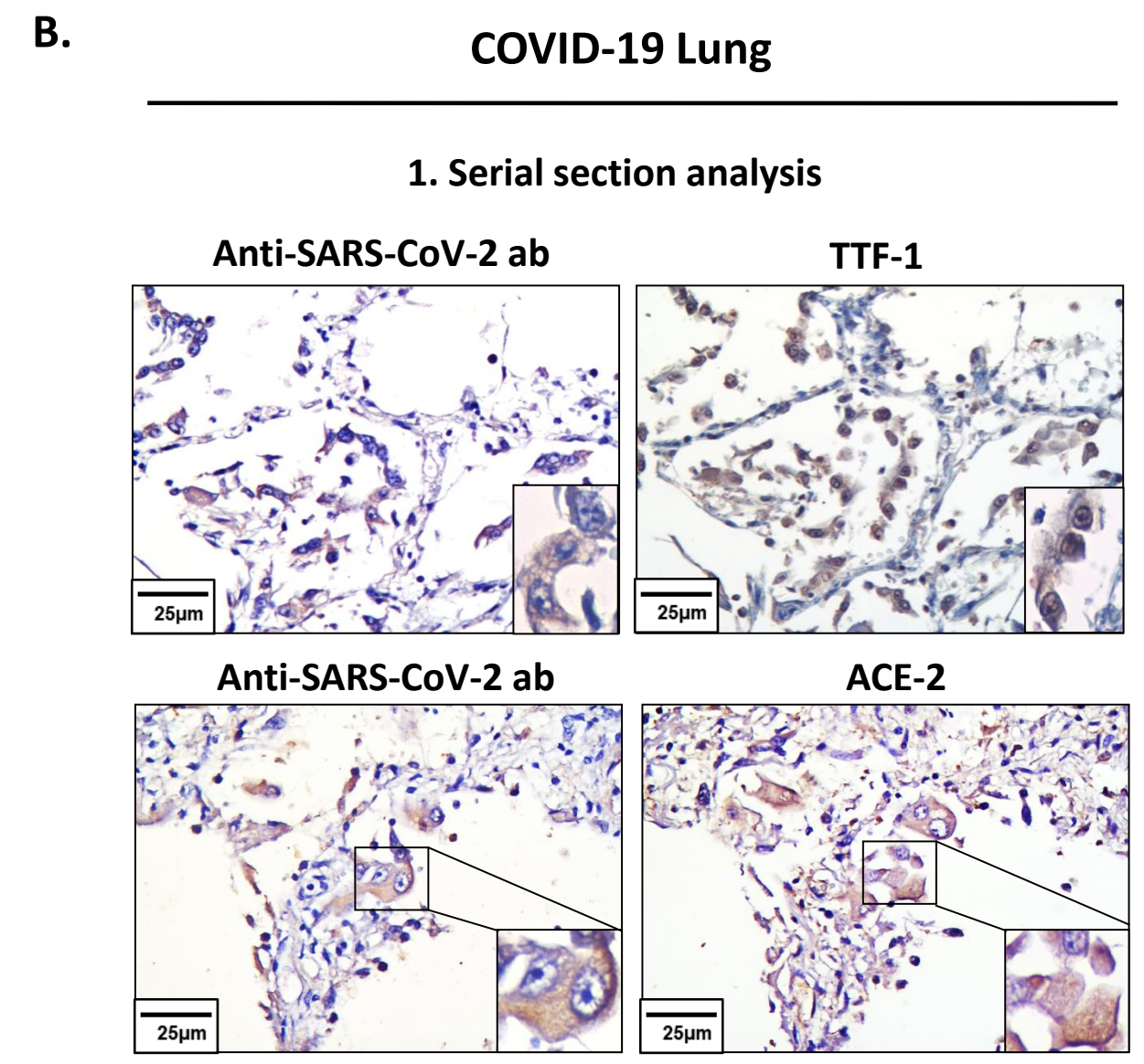
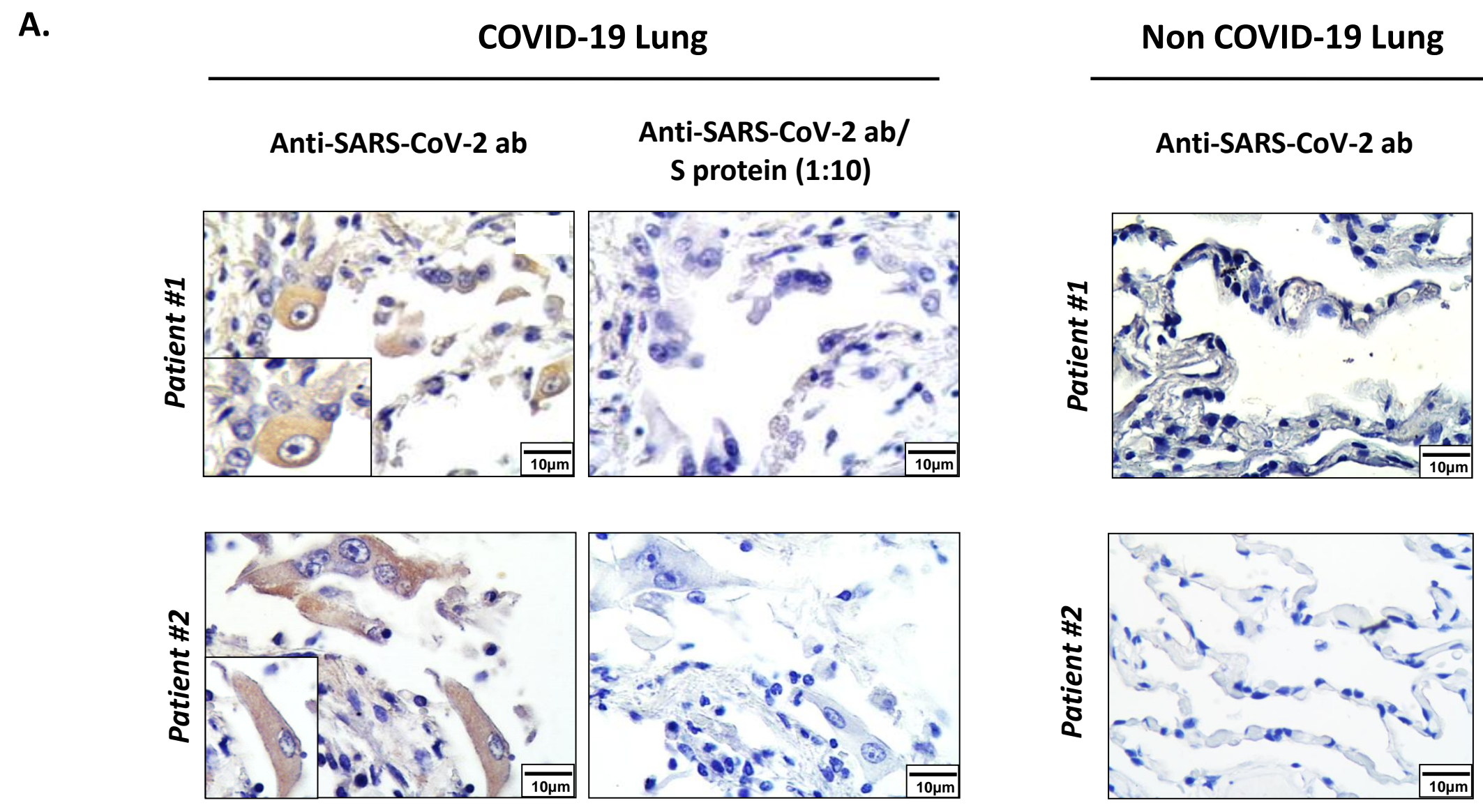
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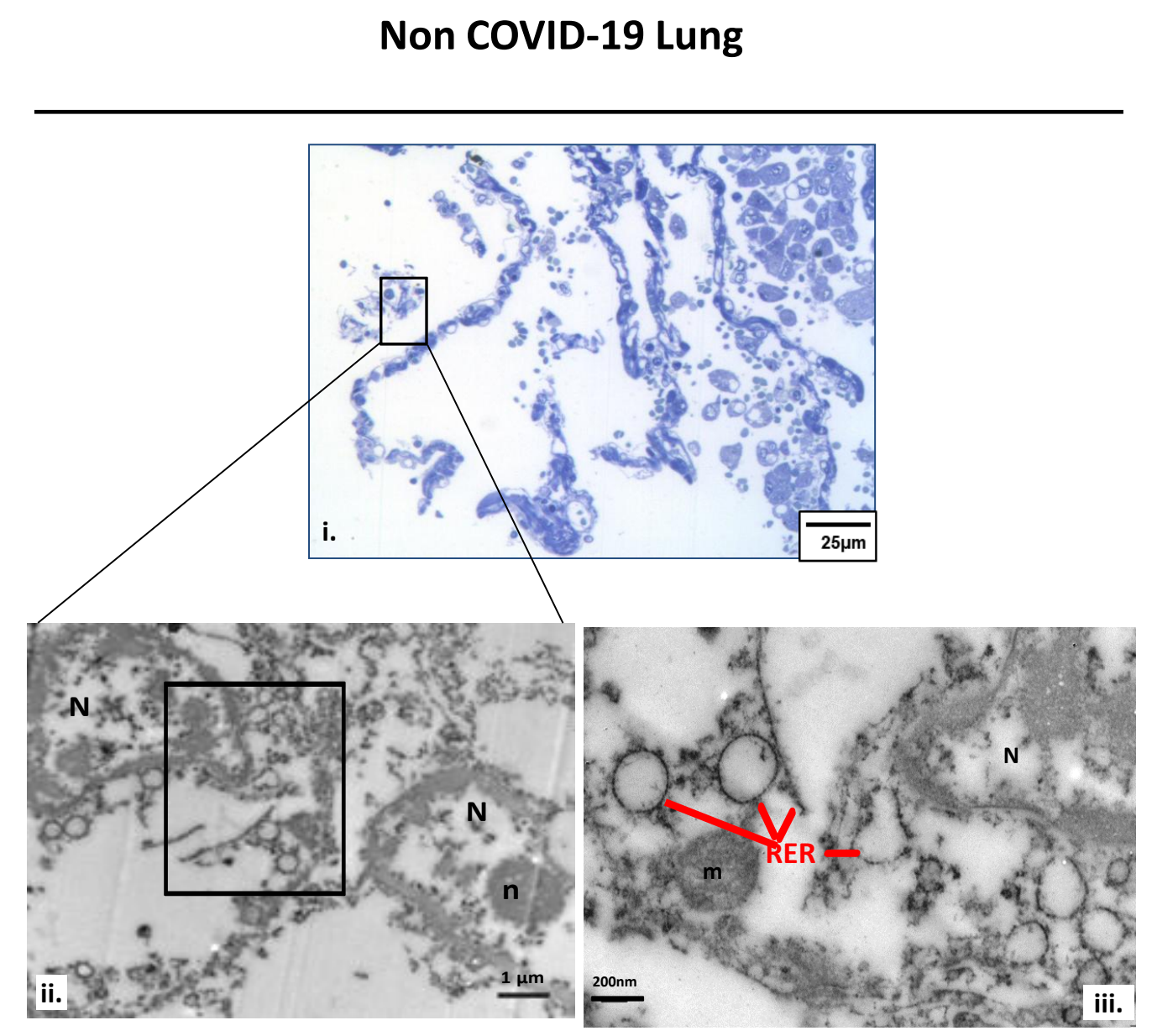
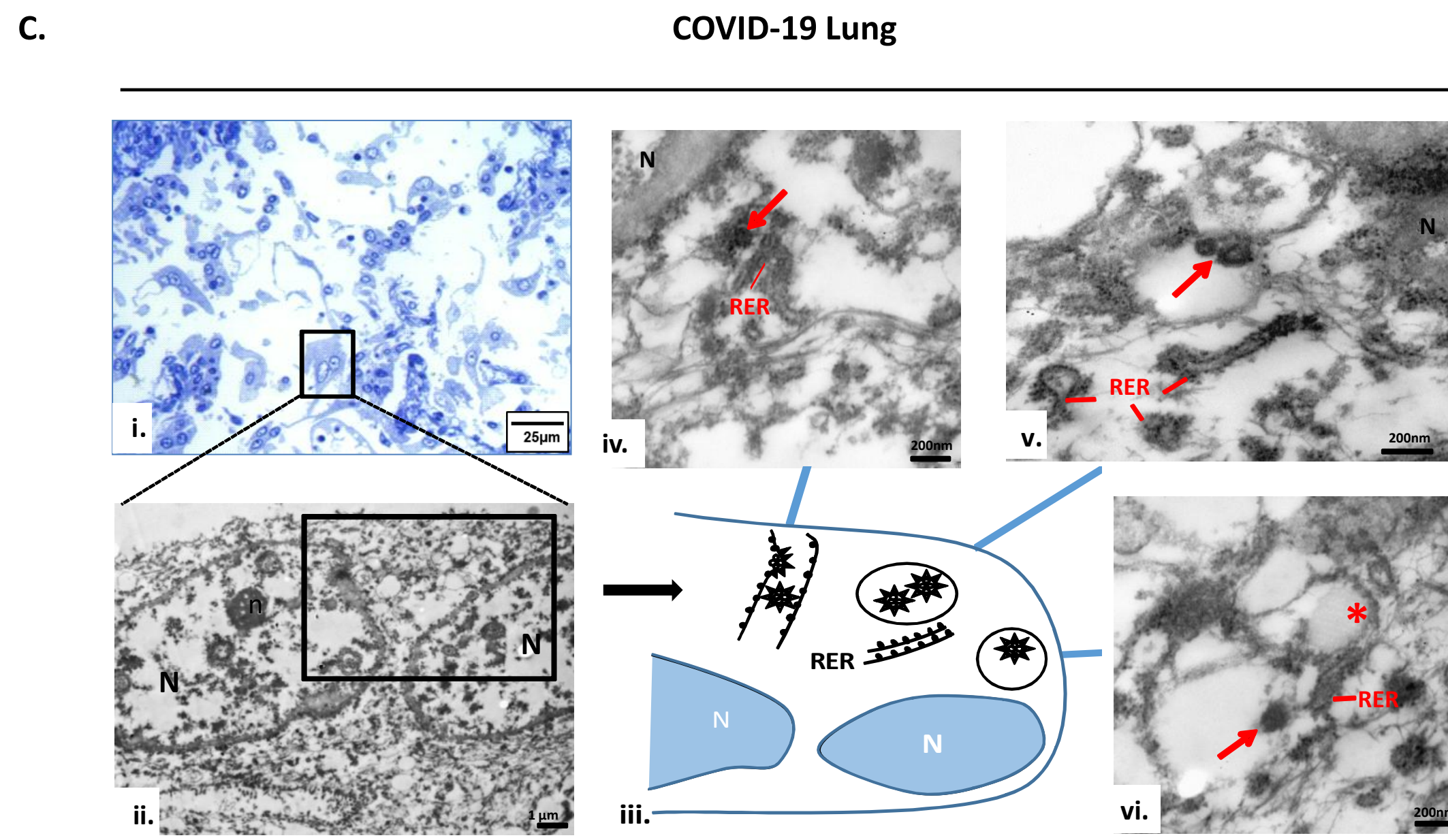
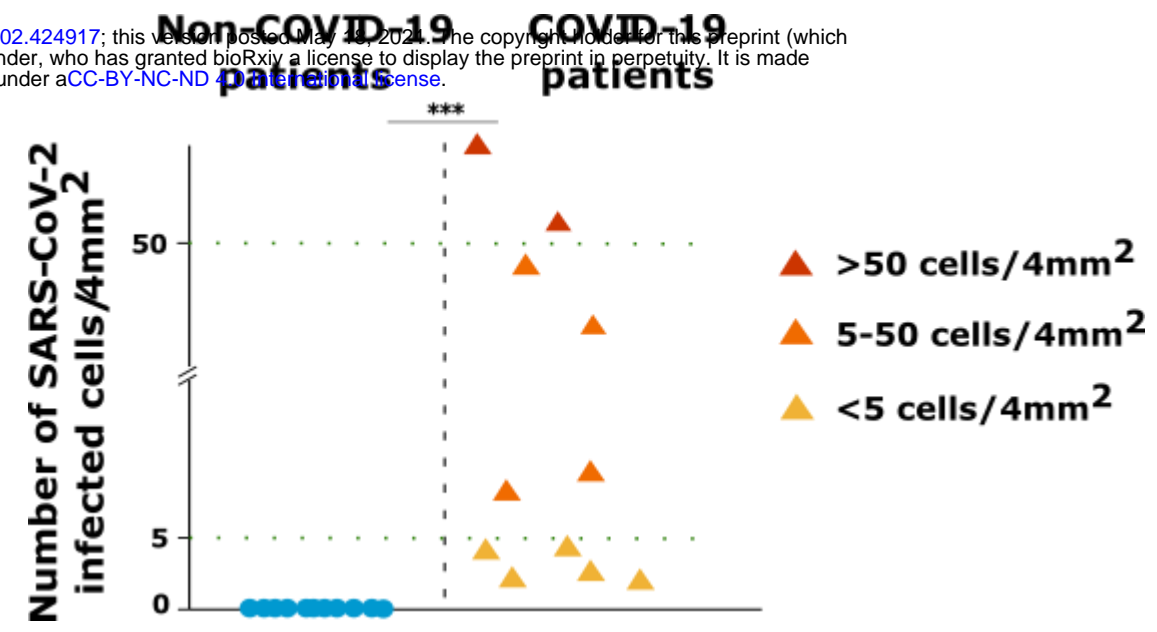
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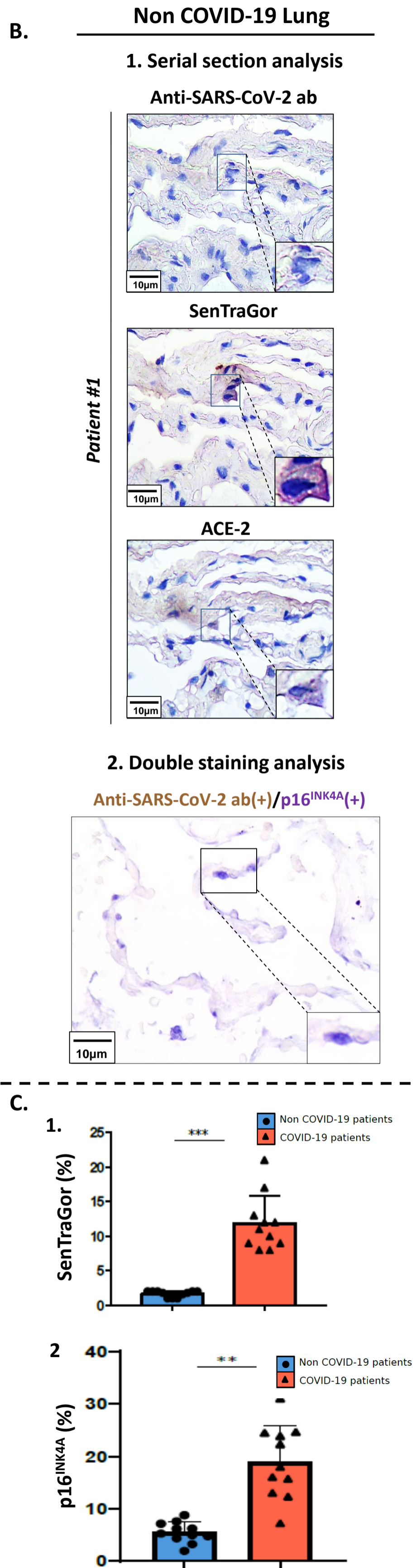
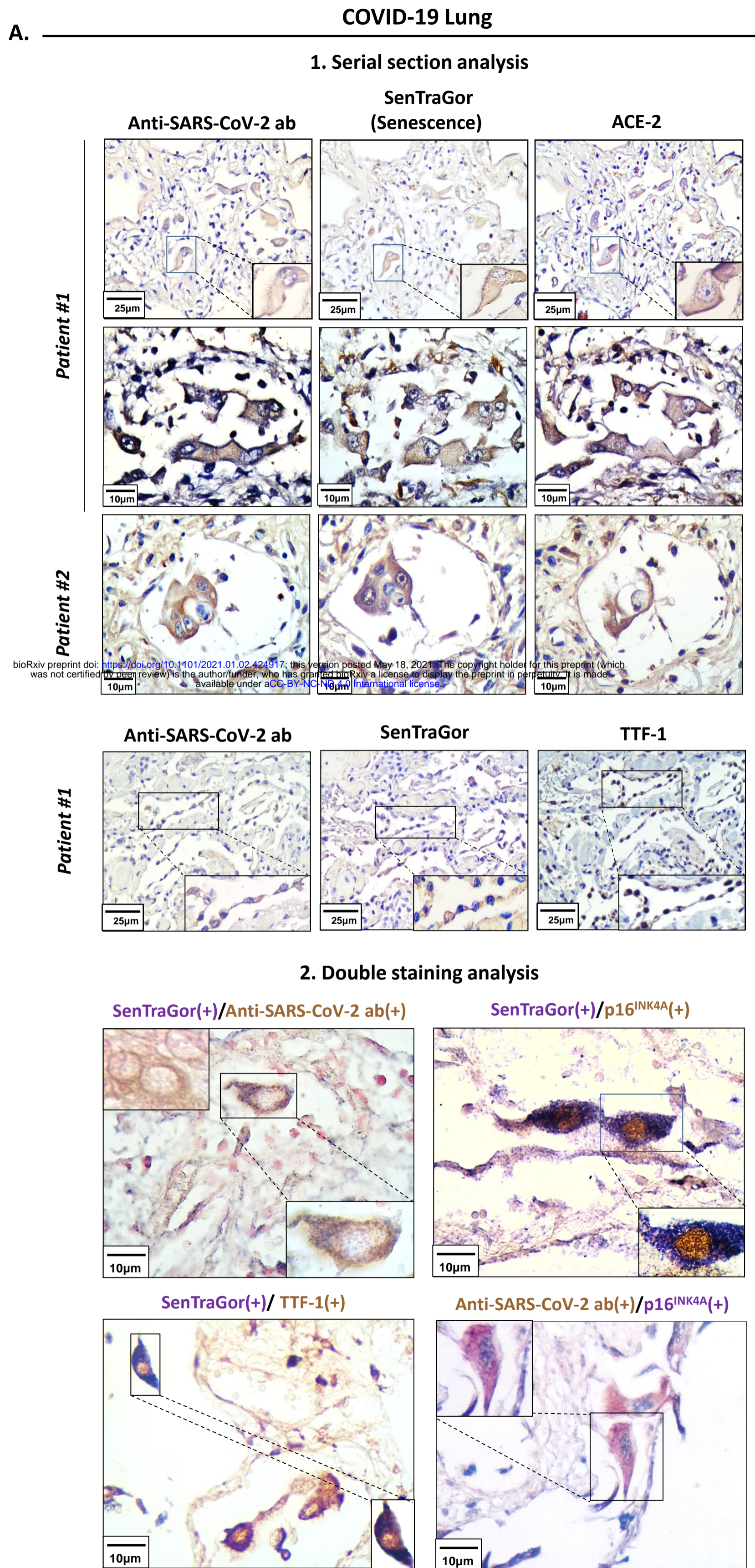
**FIGURE 1**



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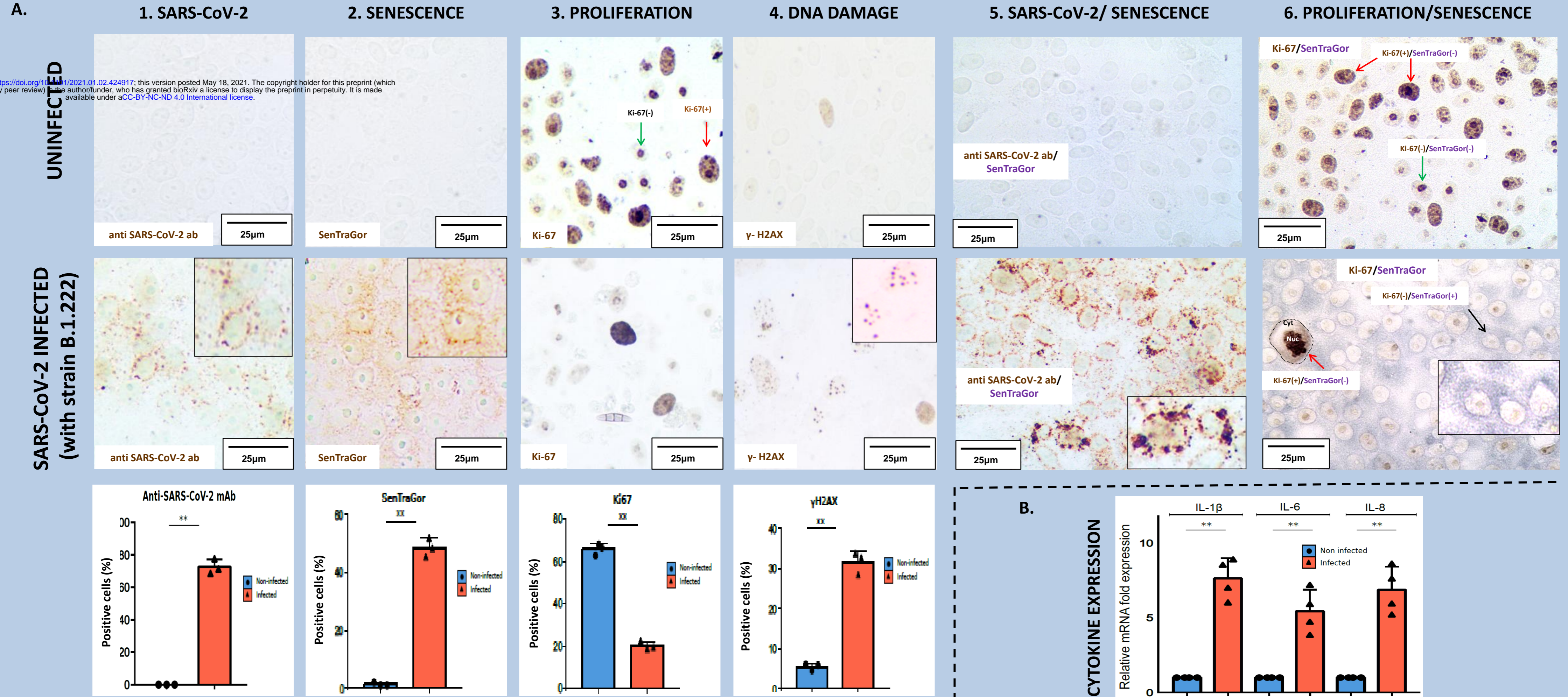
**FIGURE 2.**



**Figure 3**

**Vero cells**

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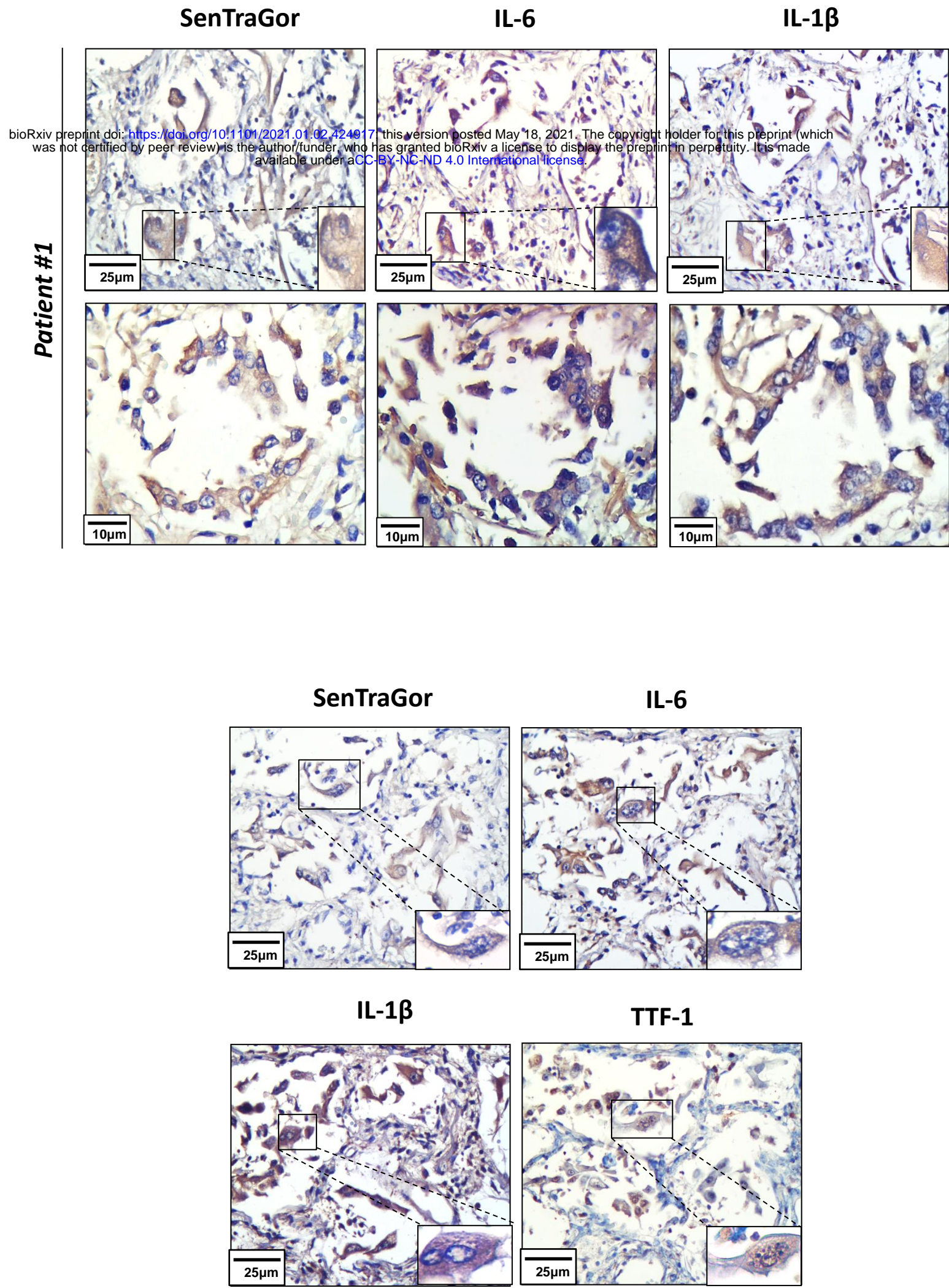
**FIGURE 4.**

**COVID-19 Lung**

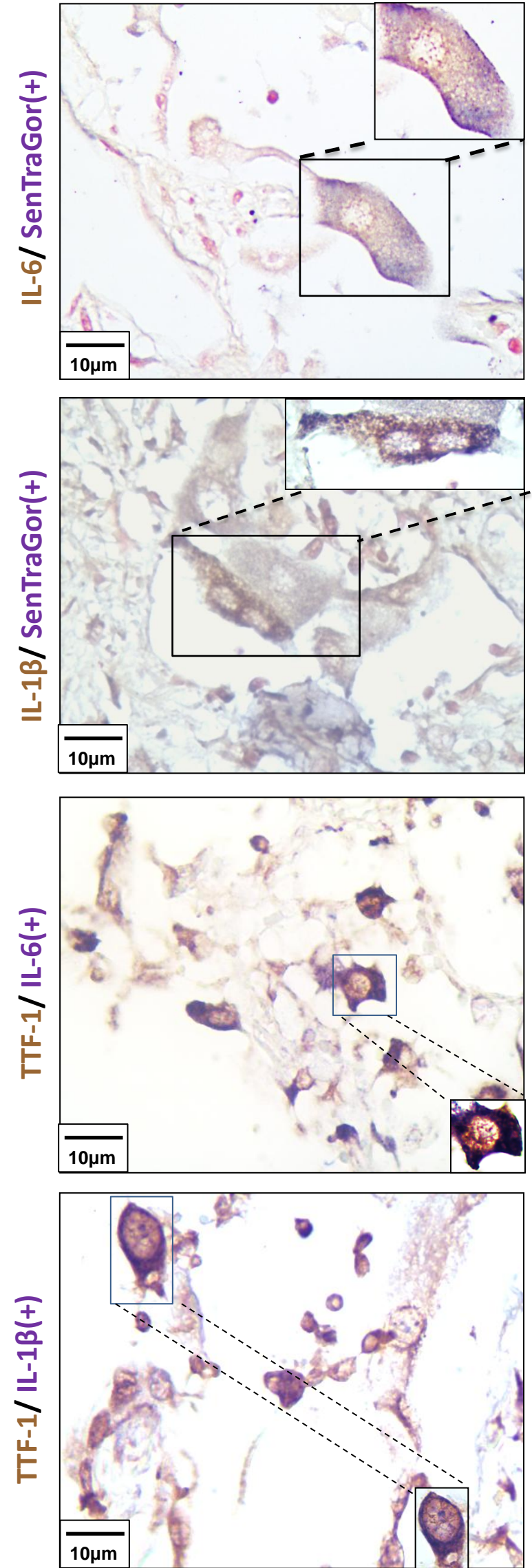
**Non COVID-19 Lung**

**A.**

**1. Serial section analysis**

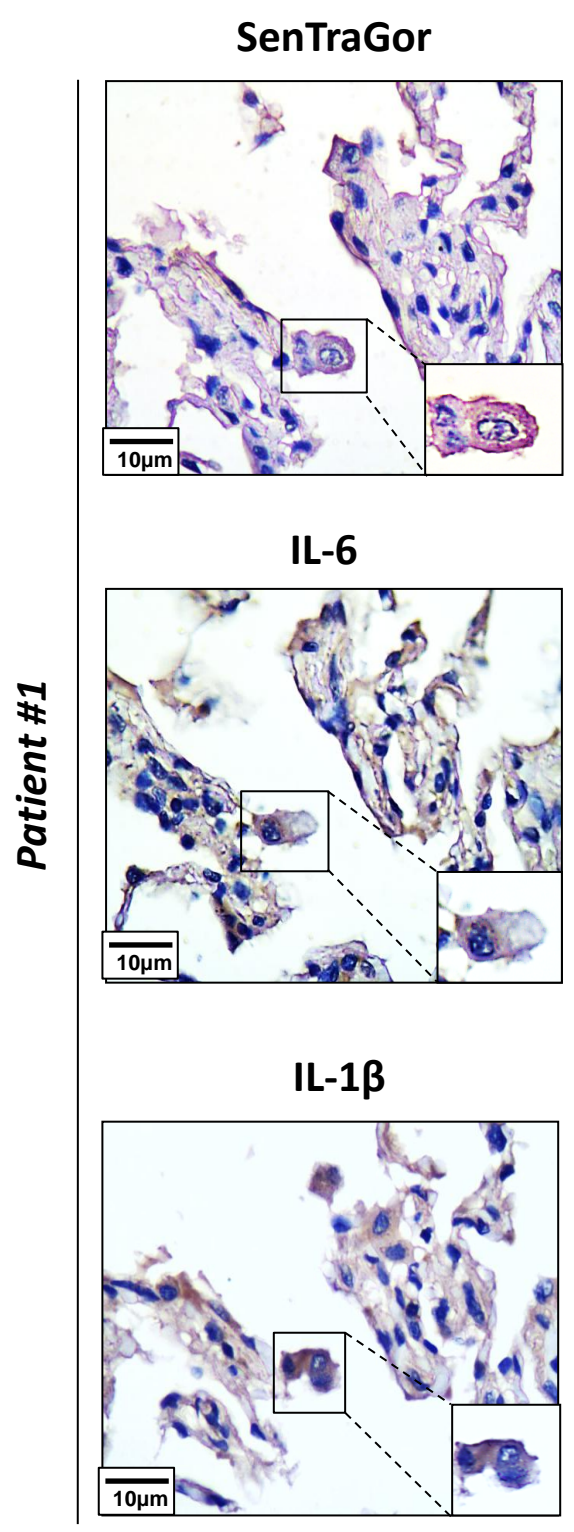


**2. Double staining analysis**



**B.**

**Serial section analysis**



**C.**

