

1 **Transcriptional profiling of immune and inflammatory responses in the context of**
2 **SARS-CoV-2 fungal superinfection in a human airway epithelial model**

3

4 Claire Nicolas de Lamballerie^{a#}, Andrés Pizzorno^{a#}, Julien Fouret^b, Lea Szpiro^a, Blandine
5 Padey^{a,b}, Julia Dubois^a, Thomas Julien^{a,c}, Aurélien Traversier^a, Victoria Dulière^{a,c}, Pauline
6 Bruna^{a,c}, Bruno Lina^{a,d}, Manuel Rosa-Calatrava^{a,c&}, Olivier Terrier^{a&*}

7

8 ^a CIRI, Centre International de Recherche en Infectiologie, (Team VirPath), Univ Lyon, Inserm, U1111,
9 Université Claude Bernard Lyon 1, CNRS, UMR5308, ENS de Lyon, F-69007, Lyon, France.

10 ^b Signia Therapeutics SAS, Lyon, France.

11 ^c VirNext, Faculté de Médecine RTH Laennec, Université Claude Bernard Lyon 1, Université de Lyon,
12 Lyon, France.

13 ^d Laboratoire de Virologie, Centre National de Référence des virus Influenza Sud, Institut des Agents
14 Infectieux, Groupement Hospitalier Nord, Hospices Civils de Lyon, Lyon, France.

15

16 *Correspondence to: olivier.terrier@univ-lyon1.fr (OT)

17 # CNdL and AP are co-first authors

18 & MRC and OT are co-last authors

19

20 **Abstract**

21 Superinfections of bacterial/fungal origin are known to affect the course and severity of
22 respiratory viral infections. An increasing number of evidence indicate a relatively high
23 prevalence of superinfections associated with COVID-19, including invasive aspergillosis, but
24 the underlying mechanisms remain to be characterized. In the present study, to better
25 understand the biological impact of superinfection we sought to determine and compare the
26 host transcriptional response to SARS-CoV-2 versus *Aspergillus* superinfection, using a model
27 of reconstituted human airway epithelium. Our analyses reveal that both simple infection and
28 superinfection induce a strong deregulation of core components of innate immune and
29 inflammatory responses, with a stronger response to superinfection in the bronchial epithelial
30 model compared to its nasal counterpart. Our results also highlight unique transcriptional
31 footprints of SARS-CoV-2 *Aspergillus* superinfection, such as an imbalanced type I/type III IFN,
32 and an induction of several monocyte- and neutrophil associated chemokines, that could be
33 useful for the understanding of *Aspergillus*-associated COVID-19 and but also management
34 of severe forms of aspergillosis in this specific context.

35 INTRODUCTION

36

37 The current pandemic of novel coronavirus disease 2019 (COVID-19), caused by severe acute
38 respiratory syndrome coronavirus 2 (SARS-CoV-2) began in Wuhan, Hubei province, China,
39 in December 2019. As of May 18, 2020, there have been more than 4,628,903 confirmed
40 COVID-19 cases in the world as reported by the WHO, including 312,009 deaths (WHO).
41 SARS-CoV-2 is a beta-coronavirus closely related to the severe acute respiratory syndrome
42 coronavirus-1 (SARS-CoV-1) and the Middle East respiratory syndrome coronavirus (MERS-
43 CoV) that emerged in 2003 and 2012, respectively. These viruses are also transmitted from
44 animals to humans and cause severe respiratory diseases in afflicted individuals.

45

46 In a short period of time, significant effort has been devoted to understanding the molecular
47 basis of the pathology associated with SARS-CoV-2 infection in an attempt to guide work on
48 treatment, vaccine and diagnostic test development. Numerous clinical studies have reported
49 the pathophysiology of COVID-19 has similar aspects to that initially described for SARS-CoV,
50 *i.e.* acute lung injury due to over-inflammation following early stages driven by infection and
51 viral replication (Chen et al., 2020; Guan et al., 2020; Huang et al., 2020; Mehta et al., 2020;
52 Zhu et al., 2020). Nevertheless, the particular underlying mechanisms of these exuberant
53 inflammatory responses in SARS-CoV-2 infection remain largely unknown and there is a need
54 to expand our knowledge of the host's response. In this context, several recent omics-based
55 approaches, including *in vivo* and *in vitro* transcriptional profiling studies, have highlighted
56 specific aspects of the signature of infection that could contribute to COVID-19 (Blanco-Melo
57 et al., 2020; Gordon et al., 2020; Messina et al., 2020; Xiong et al., 2020). Blanco-Melo and
58 colleagues, using transcriptional and serum profiling of COVID-19 patients, have notably
59 shown that the SARS-CoV-2 infection signature was defined by low levels of Type I and III
60 interferons juxtaposed to elevated chemokines and high expression of IL- 6 (Blanco-Melo et
61 al., 2020).

62

63 It is now well known that superinfections of bacterial/fungal origin can affect the course and
64 severity of respiratory viral infections. For example, the co-pathogenesis of viruses and
65 bacteria into the lung has been extensively studied, notably in the context of influenza
66 superinfection by bacteria such as *S. pneumoniae* (Bosch et al., 2013; McCullers, 2014;
67 Morens et al., 2008; Paget and Trottein, 2019). To date, there are limited data available on
68 superinfections associated with COVID-19, though superinfections were reported in 10%-20%
69 of SARS-CoV-2-infected adults admitted to Wuhan hospitals through the end of January 2020,
70 and notably in 50%-100% of those who died (Zhou et al., 2020). In intensive care units, COVID-
71 19 patients are at high risk of developing secondary infections, including fungal infections *e.g.*

72 invasive pulmonary aspergillosis (Lescure et al., 2020). Indeed, a recent study on the French
73 COVID-19 cohort reported that 33% of critically ill COVID-19 patients also showed invasive
74 aspergillosis (Alanio et al., 2020). However, while the reasons for increased vulnerability to
75 *Aspergillus* in COVID-19 patients remain undetermined, the putative contribution of *Aspergillus*
76 to SARS-CoV-2 related lung inflammation and COVID-19 pathophysiology also constitutes a
77 major unanswered question.

78
79 To better understand the biological impact of superinfection in the SARS-CoV-2 context we
80 sought to determine and compare the host transcriptional response to SARS-CoV-2 versus
81 that of a SARS-CoV-2 + *Aspergillus* superinfection. To reach this goal, we established a model
82 of SARS-CoV-2 infection and superinfection in reconstituted human airway epithelia (HAE),
83 based on previously published works (Nicolas de Lamballerie et al., 2019; Pizzorno et al., 2019,
84 2020). Our analysis reveals that both simple infection and superinfection induce a strong
85 deregulation of core components of innate immune and inflammatory responses, however, it
86 also highlights unique transcriptional footprints of the SARS-CoV-2 + *Aspergillus*
87 superinfection that provide valuable insight for the understanding not only of *Aspergillus*-
88 associated COVID-19 but also for the management of severe forms of aspergillosis.

89

90

91 **RESULTS**

92

93 In order to identify similarities and differences between the host response to SARS-CoV-2
94 simple infection and *Aspergillus* superinfection, we sought to investigate the transcriptome of
95 human respiratory epithelial cells during infection, in comparison with non-infected cells. Aware
96 of the inherent limitations of experimental models using cell lines, we set up a model of
97 infection/superinfection in a physiological model of reconstituted human airway epithelium
98 (HAE). Developed from biopsies of nasal or bronchial cells differentiated in the air/liquid
99 interphase, these HAE models, that we previously used with different respiratory viruses
100 including SARS-CoV-2 (Nicolas de Lamballerie et al., 2019; Pizzorno et al., 2019, 2020)
101 reproduce with high fidelity most of the main structural, functional and innate immune features
102 of the human respiratory epithelium that play a central role during infection, hence constituting
103 an interesting surrogate to study airway disease mechanisms. We infected nasal or bronchial
104 HAE with SARS-CoV-2, and superinfection with *Aspergillus* was performed at 48 hours post-
105 infection (hpi), which we had previously defined as the peak of acute SARS-CoV-2 infection in
106 the HAE model (Pizzorno et al., 2020). Mock-infected, infected (CoV) and superinfected
107 samples (CoV+Asp) were harvested at 72 hpi (24 h after superinfection) to perform mRNA-
108 seq analysis (**Fig. 1A**). Both nasal and bronchial HAE models of superinfection were further

109 characterized and validated in terms of viral production, impact on trans-epithelial resistance
110 and apical release of IL-6, which we used as hallmarks of infection (**Fig. 2A**). Interestingly, in
111 contrast with nasal HAE, we observed a significant increase on the relative viral production
112 in the context of superinfection in bronchial HAE, associated with higher IL-6 levels and a
113 stronger negative impact on trans-epithelial resistance (**Fig. 1B**).

114
115 Differential expression analysis of mRNA-seq data compared to the mock-infected condition
116 identified 1638 and 454 differentially expressed genes (DEGs) in SARS-CoV-2-infected nasal
117 and bronchial HAE, respectively ($FC \geq 2$, Benjamini-Hochberg adjusted p-value < 0.01). In the
118 context of CoV+Asp superinfection, the number of DEGs was notably higher, with 2979 and
119 3235 genes in nasal and bronchial HAE, respectively (**Extended data. File 1**). Interestingly,
120 there was an important overlap between CoV infection and CoV+Asp superinfection
121 associated DEGs in both nasal and bronchial models. As illustrated in the Venn diagrams (**Fig.**
122 **1C**), more than 96% (nasal HAE) and 99% (bronchial HAE) of DEGs of the CoV signature
123 were also part of the CoV+Asp signature. To provide further functional interpretation of the
124 global transcriptional signatures, we performed a functional enrichment analysis on the CoV
125 and CoV+Asp nasal and bronchial models using the web-based DAVID toolkit. Gene Ontology
126 (GO), UniProt (KW) and Reactome (RC) terms were considered enriched when their
127 Bonferroni-adjusted corrected enrichment p-value was < 0.01 (**Fig. 1D**). As anticipated, a large
128 part of the most enriched and shared terms between all experimental conditions was related
129 to the host response to infection (Regulation of response to cytokine stimulus, regulation of
130 defense response, regulation of response to biotic stimulus) and also to cornification, which
131 regroups genes mostly involved in cell-death mechanisms (**Fig. 1D**). Interestingly, functional
132 enrichments specific to CoV infection or CoV+Asp superinfection were also highlighted. For
133 example, CoV infection but no superinfection induced the upregulation of a gene cluster
134 involved in Type I interferon pathway (**Fig. 1D**). Conversely, gene clusters harboring terms
135 associated with epithelium physiology and cilium movements (regulation of cellular component
136 movements, cell projection assembly, intraflagellar transport) but also with intra and extra-
137 cellular signaling (post-translational protein phosphorylation, protein localization to
138 extracellular membrane, peptide secretion) were exclusively enriched in the context of
139 CoV+Asp superinfection (**Fig. 1D**). In parallel, given the importance of the exacerbated
140 immune response observed mainly in severe COVID-19 cases, we further analyzed the
141 inflammation-related terms and identified similar, yet to a different extent, regulation patterns
142 for genes involved in the positive regulation of inflammatory response (GO:0050729) between
143 nasal and bronchial HAE. As exemplified in the heatmap (**Fig. 1D**), deregulated genes such
144 as calgranulin coding genes S100A12 and S100A9/A8 and downregulated genes such as
145 Vesicle-associated membrane protein coding genes VAMP7 or VAMP8 were identified as a

146 hallmark of CoV+Asp superinfection, in contrast to CoV infection (**Fig. 1D**). Similar
147 observations were performed using different terms related to inflammation (**Extended data**
148 **Fig. 1**), hence suggesting a very different inflammation signature resulting from superinfection.
149 Altogether, our results indicate that the CoV+Asp superinfection presents a transcriptomic
150 signature that recapitulates the overall signature of a simple CoV infection, but with both a
151 particularly distinct regulation of the inflammatory response and the additional regulation of
152 many biological processes related to the physiology of epithelia. Of note, we observed a
153 relatively similar global pattern of regulation between the nasal and bronchial HAE models,
154 differing primarily in the magnitude rather than the nature of the responses to CoV and
155 CoV+Asp superinfection.

156
157 In order to explore in more depth the transcriptomic signature of the superinfection, we then
158 performed a differential analysis of CoV+Asp signature using that of CoV infection as baseline.
159 This focused analysis highlighted 248 and 769DEGs in the CoV+Asp nasal and bronchial HAE,
160 respectively ($FC \geq 2$, adjusted p-value < 0.01), which allowed us to spot finer differences
161 between the two epithelium models. Indeed, the volcano plots in **Fig. 2A** and **B** representing
162 all DEGs induced by the CoV+Asp versus the CoV condition show interesting differences in
163 both the scale of deregulation and the nature of the most deregulated genes between the
164 upper and lower respiratory tract tissues. Interestingly, many genes involved in the regulation
165 of the inflammatory response such as HOX1, IL1B, IL1A, IL17C are found amongst the most
166 upregulated genes in the nasal HAE model (**Fig. 2A**). These genes are also upregulated in the
167 bronchial HAE model (**Extended data file 2**). To provide further functional interpretation of the
168 superinfection signature, we performed a functional enrichment analysis, using the same
169 strategy previously described. We only observed a limited number of enriched clusters of
170 downregulated genes, mostly in bronchial HAE, all of them being related to epithelial
171 physiology and cell movement (e.g. cytoskeleton-dependent intracellular transport, protein
172 localization to cilium, cilium movement GO terms, **Fig. 2C**). In line with this observation, our
173 analysis highlighted several clusters of enriched upregulated genes related to epithelial
174 physiology (e.g. locomotion, movement of cell or subcellular component GO terms, **Fig. 2C**).
175 On the other hand, terms related to signaling, host response and immunity were markedly
176 more upregulated in the bronchial HAE model (**Fig. 2C & Extended data Fig. 2**). In addition
177 to the gene enrichment profile shared between the nasal and bronchial models, our study also
178 highlighted several overlapping biological processes such as the cytokine signaling and IL10
179 signaling pathways (**Fig. 2C**), which is consistent with the most upregulated DEGs shown in
180 **Fig. 2A and Extended data file 2**. To better visualize these observations, we applied a protein-
181 protein interactions analysis using STRING network to investigate the DEGs corresponding to
182 several Reactome and GO terms (immune system process, cytokine signaling in immune

183 system, inflammatory response, interleukin-10 signaling) enriched in the bronchial and nasal
184 superinfection signatures along with their functional interactions (**Fig. 3A and 3B**). The two
185 interactome maps with mostly upregulated DEGs illustrate the strong functional
186 interconnections among several cytokines/chemokines (blue) and receptors (yellow) involved
187 in the immune and inflammatory responses occurring in the context of a SARS-CoV-2 +
188 *Aspergillus* superinfection. Interestingly, our analysis underlined the role of type III IFN (IFNL1,
189 INFL2 and INFL3) and several cytokine-coding genes such as CXCL2/CXCL8 (**Fig. 3A and**
190 **3B**) that are upregulated following simple SARS-CoV-2 infection and even more upregulated
191 in the context of superinfection, arguably illustrating an enhanced specific response to control
192 infection in both models.

193

194

195 **DISCUSSION**

196

197 Invasive pulmonary aspergillosis (IPA), which typically occurs in an immunocompromised host,
198 represents an important cause of morbidity and mortality worldwide (Clancy and Nguyen,
199 2020). Superinfections were extensively documented in the case of influenza infections, with
200 the latter being usually described to “pave the way” for bacterial superinfections, but several
201 severe influenza cases have also been reported to develop invasive pulmonary aspergillosis
202 5/19/2020 9:59:00 AM. An increasing amount of evidence points towards a relatively high
203 prevalence of superinfections, including invasive aspergillosis, to be associated with COVID-
204 19 (Alanio et al., 2020; Lescure et al., 2020; Zhou et al., 2020). However, the underlying
205 mechanisms remain to be characterized. In the present study, we sought to better understand
206 the biological impact of superinfections by determining and comparing the host transcriptional
207 response to SARS-CoV-2 versus SARS-CoV-2 + *Aspergillus* superinfection. Collectively, our
208 results show a much stronger host response to superinfection in the bronchial epithelial model
209 compared to its nasal counterpart. In both models, functional analyses show that the SARS-
210 CoV-2 + *Aspergillus* superinfection signature reflects important changes in the expression
211 regulation of genes involved not only in epithelium physiology but also in the regulation of host
212 immune and inflammatory responses compared to that of the simple SARS-CoV-2 infection.

213

214 The reconstituted HAE model of infection/superinfection appears as a valuable support for the
215 study of respiratory viral infections and virus-host interactions in highly biologically relevant
216 experimental conditions. Previous results by our group using this model, constituted of fully
217 differentiated and functional human primary cells, have provided meaningful contributions to
218 the characterization of the kinetics of viral infection as well as on the tissue-level remodeling
219 of the cellular ultrastructure and local innate immune responses induced by SARS-CoV-2, in

220 line with our present observation (Pizzorno et al., 2020). Whereas no major differences in
221 terms of global superinfection signatures were observed between HAE models of nasal or
222 bronchial origin, the second part of our study highlighted more subtle differences between the
223 two models in terms of scale of deregulation (fold change and p-value), as well as in the nature
224 of the most deregulated genes (**Fig. 2C**). Ziegler and colleagues have recently reported that
225 differences of infectivity and consecutive host responses between different cell subsets (type
226 II pneumocytes, nasal goblet secretory cells) are linked to varying ACE2/TMPRSS2 levels,
227 ACE2 expression being linked to the IFN response (Ziegler et al., 2020). The discrepancies
228 we observed in the two HAE models could be explained by differences of cell type composition
229 that could be interesting to further explore using combinations of additional experimental
230 models, including ACE2/TMPRSS3 expression and single cell RNA-seq approaches.

231
232 Our analysis of the superinfection signature revealed an important role of physiology and
233 cilium-related genes, which could reflect an additional negative impact of *Aspergillus* infection
234 on the epithelium integrity (trans-epithelial resistance, **Fig. 1B**) and mucociliary clearance, in
235 good agreement with previous observations by our group following different types of viral
236 infection in HAE (Nicolas de Lamballerie et al., 2019). In the specific context of SARS-CoV-2
237 infection, the additional deleterious effect on epithelium integrity induced by aspergillosis might
238 contribute to the enhanced disease severity reported in the clinic while arguably increasing the
239 risk of additional bacterial or fungal superinfections, similarly to what has been described in
240 the case of other respiratory viral infections (Smith et al., 2013; Wu et al., 2016).

241
242 Another major finding of our study relates to the impact of infection/superinfections on
243 interferon and inflammatory responses, notably when compared to the limited transcriptomic
244 data available in literature to date. Using primary cells, Blanco-Melo and colleagues recently
245 reported that SARS-CoV-2 induces limited IFN-I and III responses (Blanco-Melo et al., 2020).
246 Our data are not entirely consistent with these findings. Whereas we also demonstrate a very
247 limited involvement of the type I interferon response in the context of simple SARS-CoV-2
248 infection -in contrast with data previously obtained with other viruses (Nicolas de Lamballerie
249 et al., 2019), our analysis highlights an important upregulation of crucial genes involved in the
250 type III interferon response. This response is even more pronounced in the context of
251 superinfection (**Fig. 3A and 3B**). Type III interferon (IFN- λ) is known to play a key role in innate
252 and adaptive mucosal immune responses to infection (Ye et al., 2019). Interestingly, IFN- λ has
253 been identified as a critical regulator of neutrophil activation to prevent fungal infection such
254 as invasive pulmonary aspergillosis (Espinosa et al., 2017). Our data suggest a divergence
255 between type I and type III interferon responses, with enhanced activation of the latter in the
256 context of fungal superinfection. This characteristic response could be associated with

257 increased neutrophil activation as an important first line of adaptive defense against these two
258 pathogens. This hypothesis is further supported by the observed induction of several
259 monocyte-and neutrophil associated chemokines, such as CCL2, CXCL2 and CXCL3 (**Fig. 3A**
260 and **B**). Interestingly, our results suggest a strong activation of adaptive immune response in
261 the context of SARS-CoV-2 infection, in line with currently available clinical data from COVID-
262 19 patients, which generally present high levels of circulating neutrophils (Chen et al., 2020;
263 Qin et al., 2020). These observations support an important role of neutrophil recruitment in the
264 response to COVID-19, more particularly in the context of fungal superinfection with an
265 exacerbation of pro-inflammatory response. In that sense, the upregulation of key genes
266 belonging to the IL-10 pathway observed in our analysis (**Fig. 2C**), previously demonstrated to
267 play a deleterious role in innate resistance to systemic aspergillosis (Clemons et al., 2000),
268 constitutes an illustration of how such “enhanced” state of inflammation could contribute to
269 increase severity.

270
271 In conclusion, our transcriptional profiling approach revealed unique features of the SARS-
272 CoV-2 + *Aspergillus* superinfection signature, characterized on one side by an “enhanced”
273 version of that induced by SARS-CoV infection, but also by specific changes on respiratory
274 tissue physiology, a distinct regulation of type I and type III interferons, and an over-induction
275 of inflammatory response. While we acknowledge that it would be rather bold to make a
276 statement on the possible severity associated with *Aspergillus* superinfection in a pre-existing
277 COVID-19 pathological context based solely on our results, our observations suggest that the
278 immunomodulation induced by SARS-CoV-2 infection could establish a favorable context for
279 the development of severe forms of aspergillosis .This could constitute an important aspect to
280 be considered for the immunological follow-up of COVID-19 patients with aspergillosis. On top
281 of that, the characteristic infection signatures described in this study provide valuable insight
282 in the perspective of possible future treatments targeting the COVID-19 inflammatory response,
283 which could result in counter-productive effects for the management of aspergillosis.

284 **MATERIALS AND METHODS**

285

286 **Reconstituted human airway epithelial model**

287 MucilAir™ HAE reconstituted from human primary cells obtained from nasal or bronchial
288 biopsies, were provided by Epithelix SARL (Geneva, Switzerland) and maintained in air-liquid
289 interphase with specific culture medium in Costar Transwell inserts (Corning, NY, USA)
290 according to the manufacturer's instructions. For infection experiments, apical poles were
291 gently washed twice with warm OptiMEM medium (Gibco, ThermoFisher Scientific) and then
292 infected directly with 150 µl dilution of virus in OptiMEM medium, at a multiplicity of infection
293 (MOI) of 0.1 (Pizzorno et al., 2020). For mock infection, the same procedure was followed
294 using OptiMEM as inoculum. Superinfection was performed in SARS-CoV-2 infected cells at
295 48h post-infection, with apical inoculation of 10 µl of *Aspergillus* in OptiMEM at a MOI of 1.
296 Samples were collected from apical washes or basolateral medium at 72h post infection.
297 Variations in transepithelial electrical resistance (TEER) were measured using a dedicated
298 volt-ohm meter (EVOM2, Epithelial Volt/Ohm Meter for TEER) and expressed as Ohm/cm².

299

300 **Pathogens**

301 All experiments involving clinical samples and the manipulation of infectious SARS-CoV-2
302 were performed in biosafety level 3 (BSL-3) facilities, using appropriate protocols. The
303 BetaCoV/France/IDF0571/2020 SARS-CoV-2 strain used in this study was isolated directly
304 from a patient sample as described elsewhere (Pizzorno et al., 2020). Viral stocks were
305 prepared and quantified in Vero E6 cells (TCID₅₀/mL). *Aspergillus niger* (ATCC 16404) was
306 quantified on maltose extract agar plates (CFU/ml).

307

308 **mRNA sequencing**

309 Total RNA was extracted using the RNeasy Mini Kit (QIAGEN, ref 74104) with DNase
310 treatment, following the manufacturer's instruction. 500 µg total RNA was used to prepare
311 polyA-enriched RNA-seq libraries using the KAPA mRNA Hyper Prep Kit (Roche, ref.
312 KK8581/KK8581). Those libraries were prepared separately for each sample with 11
313 amplification cycles, then all libraries were equimolar-pooled before sequencing. Paired-end
314 sequencing (2×100bp) was performed with Illumina NovaSeq 6000 sequencing platform on a
315 SP flowcell (Illumina, ref: 20040326).

316

317 **RNA-Seq data trimming**

318 Raw reads were first cleaned with Cutadapt 2.8 (Martin, 2011) to trim adapters
319 (AGATCGGAAGAGCACACGTCTGAACTCCAGTCA and
320 AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT respectively for the first and the second

321 reads) and low-quality ends (i.e terminal bases with phred quality score below 30). Only reads
322 longer than 75bp and with less than 20% of N (undetermined base) after trimming were kept
323 for further analysis.

324

325 **Transcript abundance quantification**

326 The Kallisto 0.46.1 software was used for reference-indexing and transcript abundance
327 estimation (Bray et al., 2016). The reference transcriptome based on NCBI RefSeq annotation
328 release 109.20191205 and genome assembly build GRCh37.p13 were chosen for this analysis
329 (Maglott et al., 2005). Default options were used for Kallisto except for reads orientation that
330 was specified. The **Extended Data Table 2** provides a summary statistics of the pseudo-
331 alignment process.

332

333 **Differential expression analysis**

334 Differential expression analysis was performed with R 3.6.3. First abundances at transcript-
335 level were imported using tximport 1.14.0 (Soneson et al., 2015). Only coding mRNA
336 transcripts were considered. Gene-level effective lengths were obtained by weighted mean
337 with their expression values in FPKM (Fragment Per Kilobase Million) weights. Raw counts
338 were also computed at gene-level by sum and used as input for DESeq2 1.26.0 (Love et al.,
339 2014). Normalization within DESeq2 used first gene-level effective length and then the size
340 factor that was estimated using the function 'estimateSizeFactors'. Differential expression
341 testing was performed using default parameters. P-values were adjusted using the Benjamini-
342 Hochberg method (Benjamini and Hochberg, 1995) after the independent filtering implemented
343 by default in DESeq2. A gene was considered differentially expressed if both the adjusted p-
344 value is below 0.01 and the induced change in expression is at least a two-fold increase (for
345 up-regulated genes) or a two-fold decrease (for down-regulated genes). Finally, the NCBI gene
346 IDs were mapped to Uniprot IDs using Uniprot cross-references
347 (<https://www.uniprot.org/database/DB-0118>) (Breuza et al., 2016; Maglott et al., 2005). First,
348 Uniprot entries associated to several gene IDs were removed. Indeed, we are not focused on
349 gene fusion products or read-through transcripts in this study. In case of a gene ID associated
350 to several Uniprot entries, only the best entry based on the reviewed status and the annotation
351 score was kept; if those values were not discriminant enough, only duplicated entries with a
352 reviewed status and an annotation score of 5 were kept. The protein-protein interaction (PPI)
353 network was analyzed with STRING 11.0 and visualized with Cytoscape 3.8.0.

354

355 **In silico functional analysis**

356 Based on mapped Uniprot IDs, gene set enrichment was performed using the parent-child
357 union method as previously described (Grossmann et al., 2007). In this method each functional

358 term, called child, is considered relatively to its parents to compute the probability of
359 enrichment (p-value). We considered three functional databases with parent-child relations:
360 Reactome (Fabregat et al., 2018), Uniprot Keyword (restricted to “Biological process” and
361 “Disease”) (Breuza et al., 2016) and Gene ontology (Restricted to “Biological process”)
362 (Ashburner et al., 2000; The Gene Ontology Consortium, 2015). Different files (available online:
363 URLs were all accessed the 04/02/2020) were parsed to define Uniprot ID associations with
364 terms and identify child to parents relations within each functional database; URLs are
365 specified in **Extended Data Table 1**. As previously defined (Grossmann et al., 2007), the
366 p_min probability represents how well a child term can be enriched relative to its parents. The
367 higher this probability is, the less informative is the child term relative to its parents. This is
368 also a statistic that can be used for independent filtering to reduce the p-value adjustment
369 burden. Therefore, all terms with a p_min probability higher than 10e-4 were filtered out before
370 p-values adjustment using Bonferroni method (Dunn, 1961). This multiple-testing correction
371 procedure was handled separately for each functional database and analyzed gene list. A
372 minimum adjusted p-value of 0.05 has been set for significant enrichment.

373

374 **Acknowledgments**

375 The authors would like to thank Epithelix (Switzerland) and IGENSEQ sequencing core facility
376 (Institut du Cerveau ICM, Paris) for their help. This study was funded by CNRS, and Mérieux
377 research grants. The sponsors had no role in study design, collection, analysis and
378 interpretation of data, manuscript writing, or in the decision to submit the article for publication.

379 **REFERENCES**

380

381 Alanio, A., Delliere, S., Fodil, S., Bretagne, S., and Megarbane, B. (2020). High prevalence of
382 putative invasive pulmonary aspergillosis in critically ill COVID-19 patients. *MedRxiv*
383 2020.04.21.20064915.

384 Blanco-Melo, D., Nilsson-Payant, B.E., Liu, W.-C., Uhl, S., Hoagland, D., Møller, R., Jordan,
385 T.X., Oishi, K., Panis, M., Sachs, D., et al. (2020). Imbalanced Host Response to SARS-CoV-
386 2 Drives Development of COVID-19. *Cell*.

387 Bosch, A.A.T.M., Biesbroek, G., Trzcinski, K., Sanders, E.A.M., and Bogaert, D. (2013). Viral
388 and Bacterial Interactions in the Upper Respiratory Tract. *PLOS Pathog* 9, e1003057.

389 Bray, N.L., Pimentel, H., Melsted, P., and Pachter, L. (2016). Near-optimal probabilistic RNA-
390 seq quantification. *Nat. Biotechnol.* 34, 525–527.

391 Breuza, L., Poux, S., Estreicher, A., Famiglietti, M.L., Magrane, M., Tognolli, M., Bridge, A.,
392 Baratin, D., Redaschi, N., and UniProt Consortium (2016). The UniProtKB guide to the human
393 proteome. *Database J. Biol. Databases Curation* 2016.

394 Chen, N., Zhou, M., Dong, X., Qu, J., Gong, F., Han, Y., Qiu, Y., Wang, J., Liu, Y., Wei, Y., et
395 al. (2020). Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus
396 pneumonia in Wuhan, China: a descriptive study. *The Lancet* 395, 507–513.

397 Clancy, C.J., and Nguyen, M.H. (2020). COVID-19, superinfections and antimicrobial
398 development: What can we expect? *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.*

399 Clemons, K.V., Grunig, G., Sobel, R.A., Mirels, L.F., Rennick, D.M., and Stevens, D.A. (2000).
400 Role of IL-10 in invasive aspergillosis: increased resistance of IL-10 gene knockout mice to
401 lethal systemic aspergillosis. *Clin. Exp. Immunol.* 122, 186–191.

402 Dunn, O.J. (1961). Multiple Comparisons among Means. *J. Am. Stat. Assoc.* 56, 52–64.

403 Espinosa, V., Dutta, O., McElrath, C., Du, P., Chang, Y.-J., Cicciarelli, B., Pitler, A., Whitehead,
404 I., Obar, J.J., Durbin, J.E., et al. (2017). Type III interferon is a critical regulator of innate
405 antifungal immunity. *Sci. Immunol.* 2.

406 Gordon, D.E., Jang, G.M., Bouhaddou, M., Xu, J., Obernier, K., White, K.M., O’Meara, M.J.,
407 Rezelj, V.V., Guo, J.Z., Swaney, D.L., et al. (2020). A SARS-CoV-2 protein interaction map
408 reveals targets for drug repurposing. *Nature* 1–13.

409 Grossmann, S., Bauer, S., Robinson, P.N., and Vingron, M. (2007). Improved detection of
410 overrepresentation of Gene-Ontology annotations with parent child analysis. *Bioinforma. Oxf.*
411 *Engl.* 23, 3024–3031.

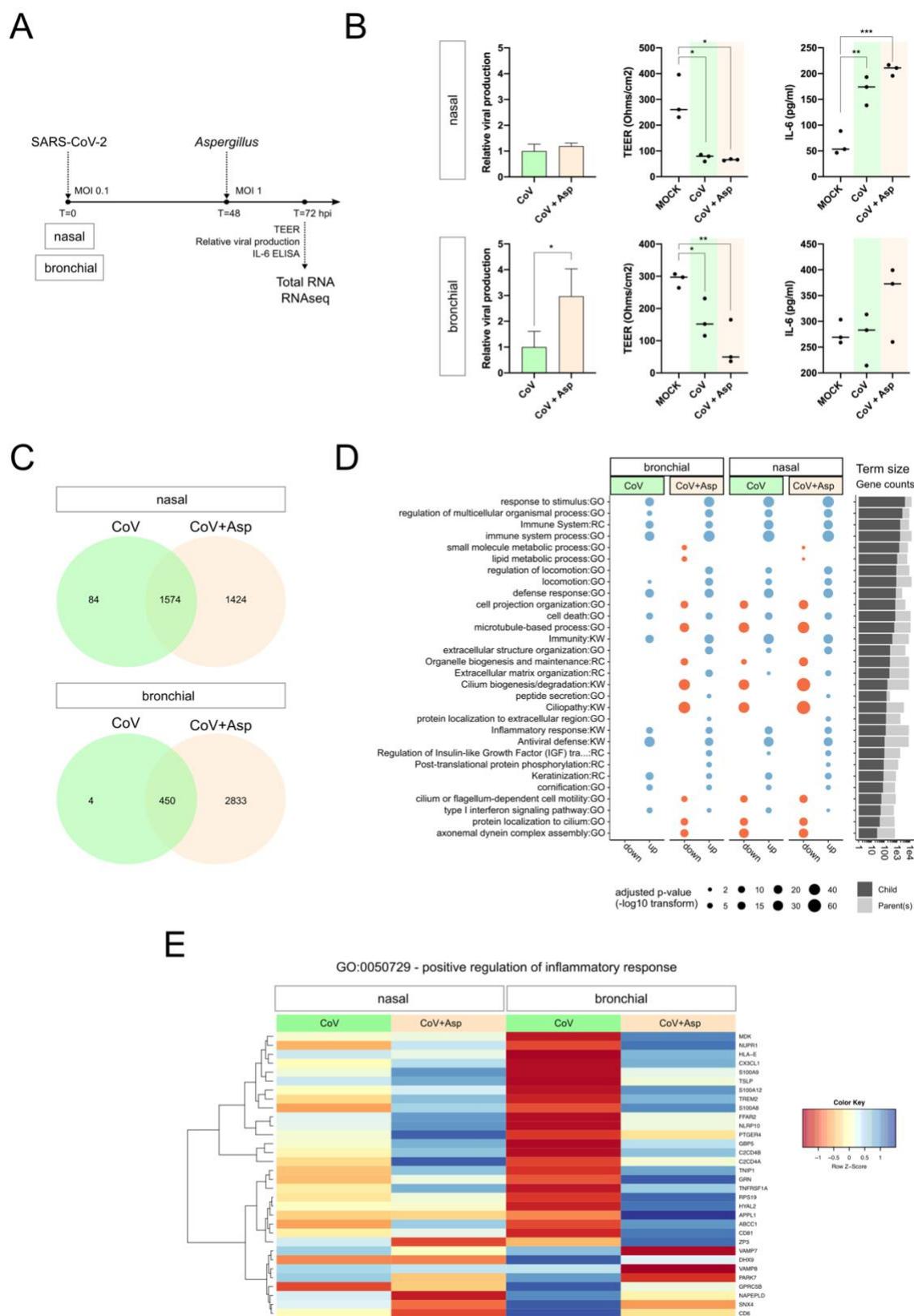
412 Guan, W., Ni, Z., Hu, Y., Liang, W., Ou, C., He, J., Liu, L., Shan, H., Lei, C., Hui, D.S.C., et al.
413 (2020). Clinical Characteristics of Coronavirus Disease 2019 in China. *N. Engl. J. Med.* 382,
414 1708–1720.

415 Huang, C., Wang, Y., Li, X., Ren, L., Zhao, J., Hu, Y., Zhang, L., Fan, G., Xu, J., Gu, X., et al.
416 (2020). Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China.
417 *Lancet Lond. Engl.* 395, 497–506.

- 418 Lescure, F.-X., Bouadma, L., Nguyen, D., Parisey, M., Wicky, P.-H., Behillil, S., Gaymard, A.,
419 Bouscambert-Duchamp, M., Donati, F., Le Hingrat, Q., et al. (2020). Clinical and virological
420 data of the first cases of COVID-19 in Europe: a case series. *Lancet Infect. Dis.*
- 421 Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and
422 dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15, 550.
- 423 Maglott, D., Ostell, J., Pruitt, K.D., and Tatusova, T. (2005). Entrez Gene: gene-centered
424 information at NCBI. *Nucleic Acids Res.* 33, D54–D58.
- 425 Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing
426 reads. *EMBnet.Journal* 17, 10–12.
- 427 McCullers, J.A. (2014). The co-pathogenesis of influenza viruses with bacteria in the lung. *Nat.*
428 *Rev. Microbiol.* 12, 252–262.
- 429 Mehta, P., McAuley, D.F., Brown, M., Sanchez, E., Tattersall, R.S., and Manson, J.J. (2020).
430 COVID-19: consider cytokine storm syndromes and immunosuppression. *The Lancet* 395,
431 1033–1034.
- 432 Messina, F., Giombini, E., Agrati, C., Vairo, F., Bartoli, T.A., Moghazi, S.A., Piacentini, M.,
433 Locatelli, F., Kobinger, G., Maeurer, M., et al. (2020). COVID-19: Viral-host interactome
434 analyzed by network based-approach model to study pathogenesis of SARS-CoV-2 infection.
435 *BioRxiv* 2020.05.07.082487.
- 436 Morens, D.M., Taubenberger, J.K., and Fauci, A.S. (2008). Predominant role of bacterial
437 pneumonia as a cause of death in pandemic influenza: implications for pandemic influenza
438 preparedness. *J. Infect. Dis.* 198, 962–970.
- 439 Nicolas de Lamballerie, C., Pizzorno, A., Dubois, J., Julien, T., Padey, B., Bouveret, M.,
440 Traversier, A., Legras-Lachuer, C., Lina, B., Boivin, G., et al. (2019). Characterization of
441 cellular transcriptomic signatures induced by different respiratory viruses in human
442 reconstituted airway epithelia. *Sci. Rep.* 9, 11493.
- 443 Paget, C., and Trottein, F. (2019). Mechanisms of Bacterial Superinfection Post-influenza: A
444 Role for Unconventional T Cells. *Front. Immunol.* 10.
- 445 Pizzorno, A., Terrier, O., Nicolas de Lamballerie, C., Julien, T., Padey, B., Traversier, A.,
446 Roche, M., Hamelin, M.-E., Rhéaume, C., Croze, S., et al. (2019). Repurposing of Drugs as
447 Novel Influenza Inhibitors From Clinical Gene Expression Infection Signatures. *Front. Immunol.*
448 10.
- 449 Pizzorno, A., Padey, B., Julien, T., Trouillet-Assant, S., Traversier, A., Errazuriz-Cerda, E.,
450 Fouret, J., Dubois, J., Gaymard, A., Lescure, F.-X., et al. (2020). Characterization and
451 treatment of SARS-CoV-2 in nasal and bronchial human airway epithelia. *BioRxiv*
452 2020.03.31.017889.
- 453 Qin, C., Zhou, L., Hu, Z., Zhang, S., Yang, S., Tao, Y., Xie, C., Ma, K., Shang, K., Wang, W.,
454 et al. (2020). Dysregulation of immune response in patients with COVID-19 in Wuhan, China.
455 *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.*
- 456 Smith, C., Kulkarni, H., Robert, H., Williams, G., Andrew, P., Easton, A., and O’Callaghan, C.
457 (2013). Influenza virus infection of human ciliated respiratory epithelial cells in culture. *Eur.*
458 *Respir. J.* 42, P4406.

- 459 Sonesson, C., Love, M.I., and Robinson, M.D. (2015). Differential analyses for RNA-seq:
460 transcript-level estimates improve gene-level inferences. *F1000Research* 4, 1521.
- 461 Wu, N.-H., Yang, W., Beineke, A., Dijkman, R., Matrosovich, M., Baumgärtner, W., Thiel, V.,
462 Valentin-Weigand, P., Meng, F., and Herrler, G. (2016). The differentiated airway epithelium
463 infected by influenza viruses maintains the barrier function despite a dramatic loss of ciliated
464 cells. *Sci. Rep.* 6, 39668.
- 465 Xiong, Y., Liu, Y., Cao, L., Wang, D., Guo, M., Jiang, A., Guo, D., Hu, W., Yang, J., Tang, Z.,
466 et al. (2020). Transcriptomic characteristics of bronchoalveolar lavage fluid and peripheral
467 blood mononuclear cells in COVID-19 patients. *Emerg. Microbes Infect.* 9, 761–770.
- 468 Ye, L., Schnepf, D., and Staeheli, P. (2019). Interferon- λ orchestrates innate and adaptive
469 mucosal immune responses. *Nat. Rev. Immunol.* 19, 614–625.
- 470 Zhou, F., Yu, T., Du, R., Fan, G., Liu, Y., Liu, Z., Xiang, J., Wang, Y., Song, B., Gu, X., et al.
471 (2020). Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan,
472 China: a retrospective cohort study. *Lancet Lond. Engl.* 395, 1054–1062.
- 473 Zhu, N., Zhang, D., Wang, W., Li, X., Yang, B., Song, J., Zhao, X., Huang, B., Shi, W., Lu, R.,
474 et al. (2020). A Novel Coronavirus from Patients with Pneumonia in China, 2019. *N. Engl. J.*
475 *Med.* 382, 727–733.
- 476 Ziegler, C.G.K., Allon, S.J., Nyquist, S.K., Mbanjo, I.M., Miao, V.N., Tzouanas, C.N., Cao, Y.,
477 Yousif, A.S., Bals, J., Hauser, B.M., et al. (2020). SARS-CoV-2 Receptor ACE2 Is an
478 Interferon-Stimulated Gene in Human Airway Epithelial Cells and Is Detected in Specific Cell
479 Subsets across Tissues. *Cell*.
- 480

481 **FIGURES**

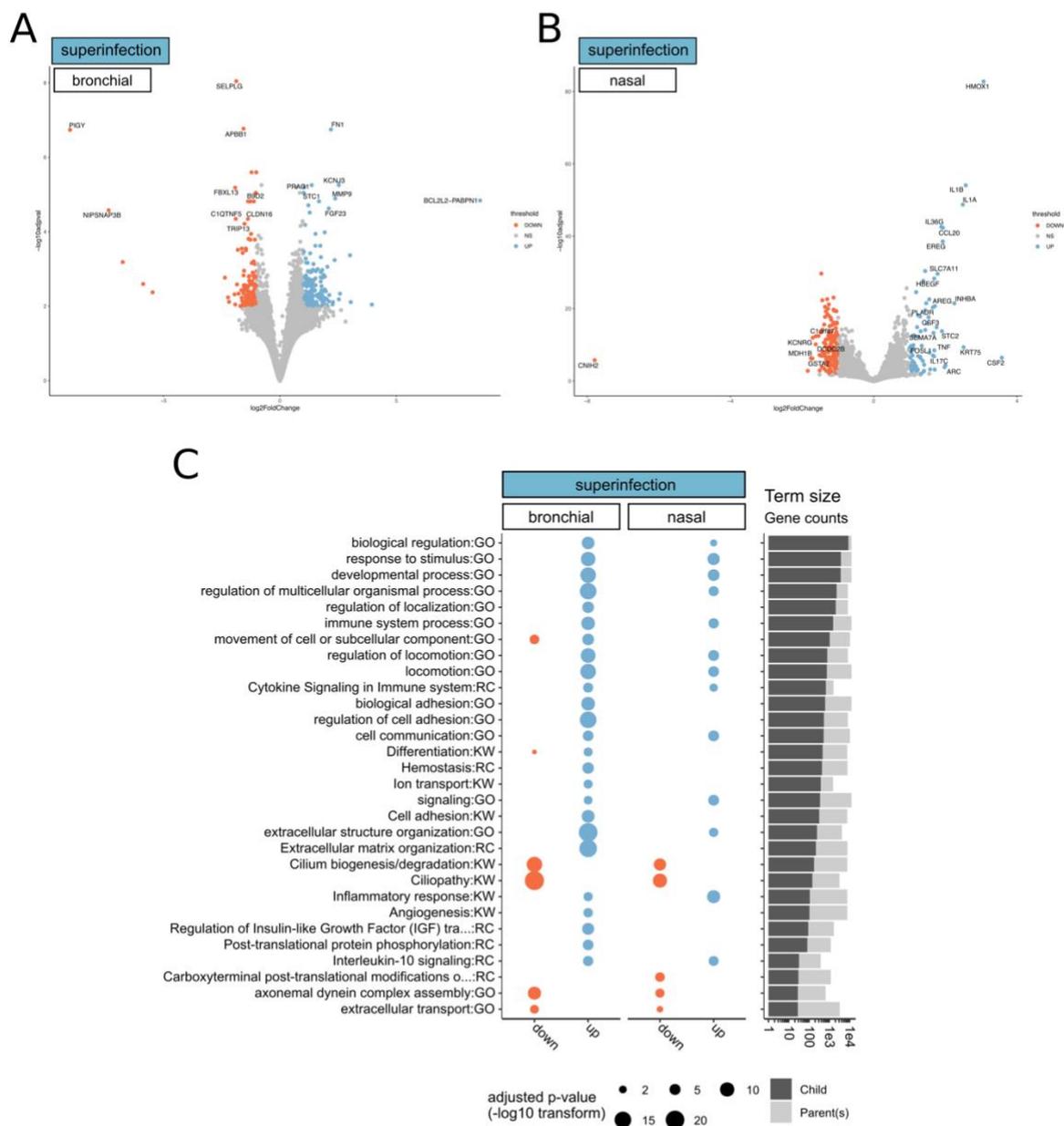


482

483

484 **Figure 1**

485 (A) Overview of experimental strategy (B) At 72h post-infection, for both nasal and bronchial
486 HAE model, the relative viral production (intracellular) was determined using RTqPCR, and
487 the impact of infection on epithelium integrity was monitored by measure of the transepithelial
488 resistance (TERR Ohms/cm₂). IL-6 was measured at the apical using a specific ALISA assay.
489 (C) Nasal and bronchial gene signature overlap for SARS-CoV2 or SARS-CoV-2+*Aspergillus*
490 infected conditions vs. Mock. We contrasted the significantly differentially expressed gene lists
491 corresponding to CoV vs. Mock and CoV+Asp vs. Mock in nasal and bronchial HAE. Only
492 genes above threshold ($\log_2(\text{FC}) > 1$ or < -1 compared to the mock-infected condition and
493 Benjamini-Hochberg adjusted p value < 0.01) were considered. (D) Overview of functional
494 enrichment results for SARS-CoV2 or SARS-CoV-2+*Aspergillus* infected conditions vs. Mock.
495 Considering (CoV vs. Mock) and (CoV+Asp vs. Mock) for both bronchial and nasal HAE,
496 significantly up- or down- regulated gene lists (x-axis) were tested for significant enrichment
497 using the parent-child strategy (see methods). If below the threshold (0.01), the adjusted p-
498 values corresponding to different terms (y-axis) are represented by point sizes (see legend).
499 After clustering terms based on gene occurrences (binary distance & Ward algorithm) in 15
500 metagroups, only the top 2 (lowest adjusted p-value) were represented here. For the complete
501 list of significant terms, see supplementary figure S1. The bar plot on the right represents the
502 sizes of enriched terms (called child) in comparison to the size of their parents (see methods
503 for definitions). (E) Positive regulation of the inflammatory response in nasal and bronchial
504 HAE. We extracted the list of proteins associated with the term GO:0050729 and visualized
505 the scaled log transformed expression results in a heatmap. Each row represents a gene and
506 each column is an experimental condition. The color key indicates the scaled expression levels
507 vs. Mock (red, low; blue, high).



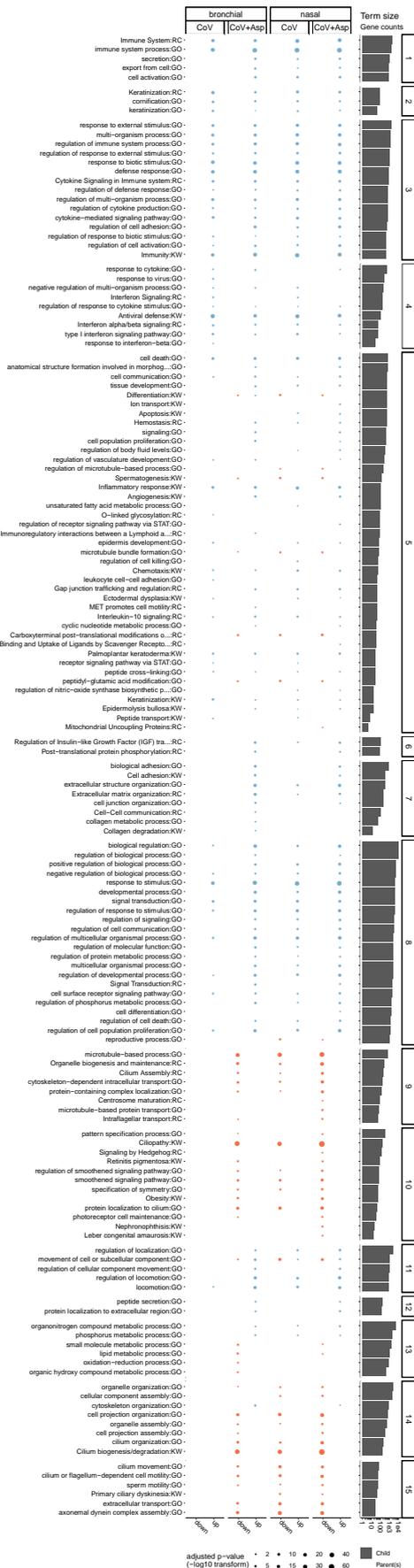
509 **Figure 2**

510 (A) & (B) Volcano plots of differentially expressed genes from (A) nasal and (B) bronchial tissue
511 superinfected by *Aspergillus*. We considered significantly modulated genes (CoV+Asp vs. CoV)
512 for both bronchial and nasal HAE ($\log_2(\text{FC}) > 1$ or < -1 compared to the CoV condition and
513 Benjamini-Hochberg adjusted p-value < 0.01). The color scale represents the log-transformed
514 fold change values and ranges from low (red) to high (blue). (C) Overview of functional
515 enrichment results of SARS-CoV-2+*Aspergillus* superinfection vs. SARS-CoV2 infection.
516 Considering (CoV+Asp vs. CoV) for both bronchial and nasal epithelium type, significantly up-
517 or down- regulated gene lists (x-axis) were tested for significant enrichment using the parent-
518 child strategy (see methods). If below the threshold (0.01), the adjusted p-values
519 corresponding to different terms (y-axis) are represented by point sizes (see legend). After
520 clustering terms based on gene occurrences (binary distance & Ward algorithm) in 15
521 metagroups, the top 2 in terms lowest p-value were represented here. For the complete list of
522 significant terms, see **Extended Data File 2**. The bar plot on the right represents the sizes of
523 enriched terms (called child) in comparison to the size of their parents (see methods for
524 definitions).

527 **Figure 3**

528 (A) & (B) Network visualization of immunity-associated proteins in superinfected HAE. We
529 selected the 76 and 191 genes significantly differentially expressed (vs. CoV) in our study
530 (respectively in nasal (A) and bronchial (B) HAE) associated with the Reactome “Interleukin-
531 10 signaling” (R-HSA-6783783) and “cytokine signaling in immune system” pathway (R-HAS-
532 1280215), the Uniprot keyword “inflammatory response” (KW-0395) and the Gene Ontology
533 term “immune system process” (GO:0002376). The circles indicate genes modulated in our
534 study in the superinfection context. The predicted associations are materialized by the dark
535 lines. Cytokines/chemokines were highlighted in blue and receptors in yellow.

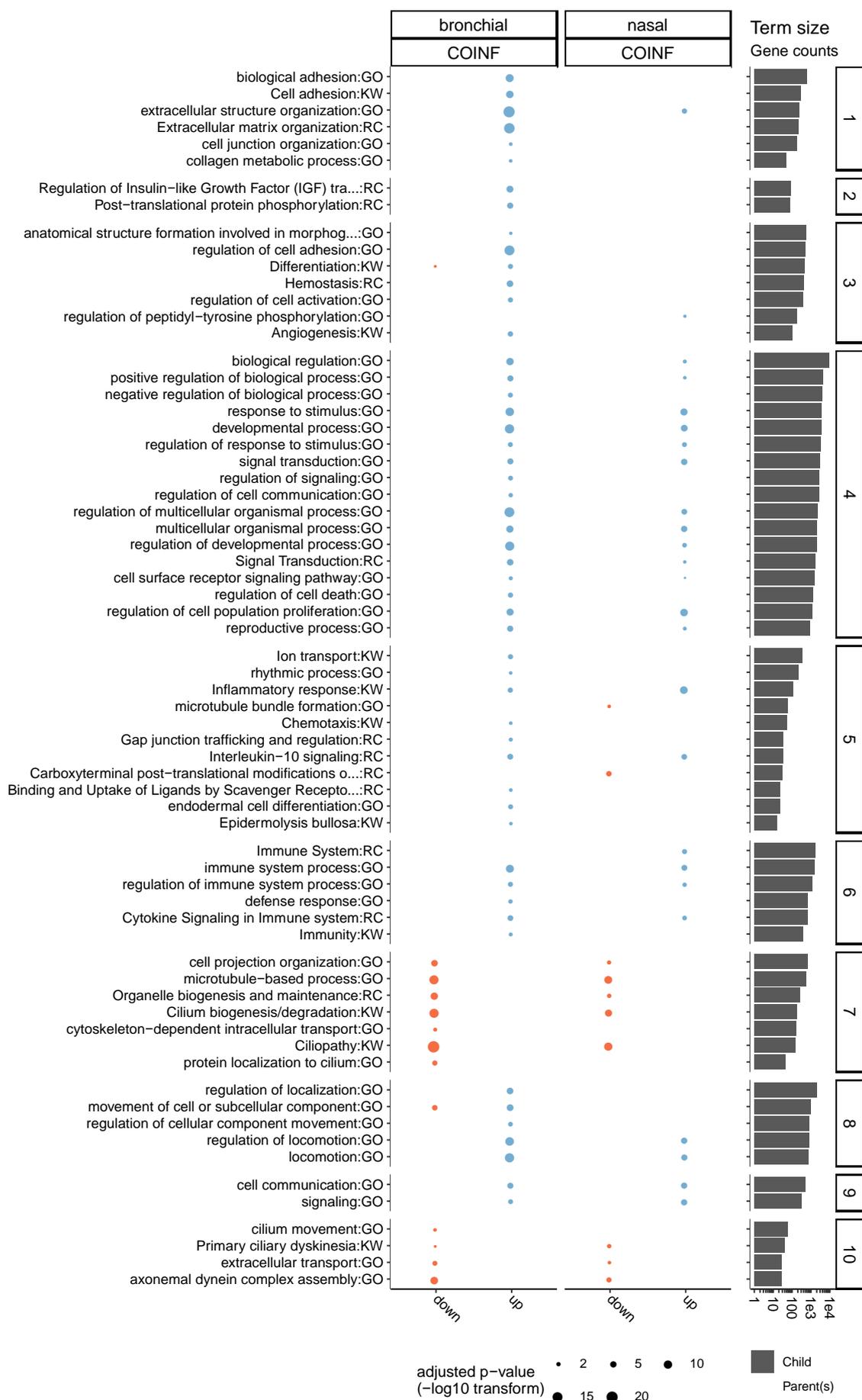
536 Extended Data



537

538 **Extended Data Figure 1**

539 Complete view of functional enrichment results for SARS-CoV2 or SARS-CoV-2+Aspergillus
540 infected conditions vs. Mock. Considering (CoV vs. Mock) and (CoV+Asp vs. Mock) for both
541 bronchial and nasal epithelium type, significantly up- or down- regulated gene lists (x-axis)
542 were tested for significant enrichment using the parent-child strategy (see methods). If below
543 the threshold (0.01), the adjusted p-values corresponding to different terms (y-axis) are
544 represented by point sizes (see legend). Terms were clustered based on gene occurrences
545 (binary distance & Ward algorithm) in 15 metagroups. The bar plot on the right represents the
546 sizes of enriched terms (called child) in comparison to the size of their parents (see methods
547 for definitions).



549 **Extended Data Figure 2**

550 Complete view of functional enrichment results of SARS-CoV-2+*Aspergillus* superinfection vs.
551 SARS-CoV2 infection. Considering (CoV+Asp vs. CoV) for both bronchial and nasal epithelium
552 type, significantly up- or down- regulated gene lists (x-axis) were tested for significant
553 enrichment using the parent-child strategy (see methods). If below the threshold (0.01), the
554 adjusted p-values corresponding to different terms (y-axis) are represented by point sizes (see
555 legend). Terms were clustered based on gene occurrences (binary distance & Ward algorithm)
556 in 10 metagroups. The bar plot on the right represents the sizes of enriched terms (called child)
557 in comparison to the size of their parents (see methods for definitions).

558

559 **Extended Data File 1 & 2:**

560 Extended data files will be available upon request olivier.terrier@univ-lyon1.fr

561

Database	Information type	URLs
Reactome	Details (name,Id)	https://reactome.org/download/current/ReactomePathways.txt
Reactome	gene-term associations	https://www.uniprot.org/uniprot/?sort=score&desc=&compress=no&query=organism:%22Homo%20sapiens%20[9606]%22&fil=&format=tab&force=yes&columns=id,entry%20name,length,genes(PREFERRED),reviewed,database(Reactome)
Reactome	Topology	https://reactome.org/download/current/ReactomePathwaysRelation.txt
Uniprot Keywords	Topology	http://ftp.ebi.ac.uk/pub/databases/interpro/ParentChildTreeFile.txt
Uniprot Keywords	Details (name,Id)	http://ftp.ebi.ac.uk/pub/databases/interpro/entry.list
Uniprot Keywords	gene-term associations	https://www.uniprot.org/uniprot/?sort=score&desc=&compress=yes&query=organism:%22Homo%20sapiens%20[9606]%22&fil=&format=tab&force=yes&columns=id,entry%20name,length,database(InterPro),genes(PREFERRED),reviewed
Gene Ontology	Topology and Details (name,Id)	 keywords/uniprot_keywords.obo.gz">https://www.uniprot.org/keywords/?sort=&desc=&compress=yes&query=&fil=&format=obo&force=yes' > keywords/uniprot_keywords.obo.gz
Gene Ontology	gene-term associations	https://www.uniprot.org/uniprot/?query=organism%3A%22Homo%20sapiens%20%5B9606%5D%22&sort=score&columns=id%2Centry%20name%2Ckeywords%2Cgenes(PREFERRED)%2Cdatabase(GeneID)%2Creviewed&format=tab

562

563 **Extended Data Table 1**

564 List of URLs parsed to build the databases used for enrichment analysis. Three types
 565 of information were used (i) details about terms meaning term ID and complete name
 566 and eventually category (e.g Biological process or Molecular function for Gene
 567 Ontology); (ii) gene-term associations; (iii) Topology and especially child to parent's
 568 relations to apply the parent-child method.

Epithelium type	sample	Percentage of fragments aligned	Millions of fragments aligned
Bronchial	CoV+Asp	22,30%	9,6
	CoV+Asp	25,60%	5,7
	CoV+Asp	25,20%	7,1
	CoV	61,50%	13,8
	CoV	66,30%	15,9
	CoV	46,00%	11,5
	Mock	80,30%	19,1
	Mock	78,60%	20,9
	Mock	79,20%	18,5
	Nasal	CoV+Asp	45,40%
CoV+Asp		47,00%	15,1
CoV+Asp		40,90%	9,5
CoV		53,00%	15,2
CoV		53,00%	14,4
CoV		57,10%	15,7
Mock		82,20%	21,7
Mock		80,70%	22,1
Mock		80,40%	19,2

569

570

571 **Extended Data Table 2**

572 Statistics of RNA-Seq fragment pseudo-alignment to the human transcriptome.