Neuraminidase inhibitors rewire neutrophil function in murine sepsis and COVID-19 patient cells

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ABSTRACT

Neutrophils overstimulation plays a crucial role in tissue damage during severe infections. Neuraminidase-mediated cleavage of surface sialic acid has been demonstrated to regulate leukocyte responses. Here, we report that antiviral neuraminidase inhibitors constrain host neuraminidase activity, surface sialic acid release, ROS production, and NETs released by microbial-activated human neutrophils. *In vivo*, treatment with Oseltamivir results in infection control and host survival in murine models of sepsis. Moreover, Oseltamivir or Zanamivir treatment of whole blood cells from severe COVID-19 patients reduces host NEU-mediated shedding of surface sialic acid and neutrophil overactivation. These findings suggest that neuraminidase inhibitors are host-directed interventions to dampen neutrophil dysfunction in severe infections.

Keywords: neuraminidase; sialic acid; sepsis; Oseltamivir; Zanamivir, neutrophil; SARS-CoV-2; COVID-19.

48

49 **INTRODUCTION**

50

51 Neutrophils are key components of the immune response against multiple pathogens[1].
52 However, during acute severe infections, such as sepsis and COVID-19, overactivated
53 neutrophils infiltrate vital organs and release many molecules including proteases,
54 reactive oxygen species (ROS), and neutrophil extracellular traps (NETs)[2,3]. While such
55 inflammatory mediators are essential to the control of infection, they can also damage
56 healthy cells[4]. Therefore, the function of neutrophils must be regulated to efficiently
57 clear microorganisms with minimal detrimental effects to the host.

58

59 A number of mechanisms controlling neutrophil activation have been described[5]. For
60 instance, the contents of sialic acid (Sia) have been demonstrated to regulate leukocyte
61 activation to microbial stimuli[6,7]. The dense array of Sia present in the glycocalyx of all
62 mammalian cells makes this monosaccharide a central molecule for many cellular
63 processes including: cell-cell interaction, signal transduction, and transendothelial
64 migration[8]. Neuraminidases (NEUs) are enzymes found in both pathogens and
65 mammalian hosts[9], which hydrolyze Sia residues linked to galactose, N-
66 acetylgalactosamine or polySia residues on glycoconjugates, thereby regulating many
67 physiological and pathological responses[10]. In human neutrophils, shedding of surface
68 Sia by microbial-derived NEUs leads to cellular activation, ROS production, and NETs
69 release[7,11–13]. Additionally, it has been demonstrated that LPS induces membrane-
70 associated NEU activation in murine or human macrophages and dendritic cells[14]. Upon

71 LPS binding to TLR4, NEU activity was shown to regulate NF-κB induction in
72 macrophages, suggesting a role for this enzyme during cellular activation[14].
73 Furthermore, in experimental Gram-negative sepsis or endotoxemia, NEU activity
74 mediated leukocyte dysfunction, associated with exacerbated inflammatory response
75 and high mortality rates[15,16]. As previous studies have demonstrated that pathogen-
76 derived NEU stimulate neutrophils[7,11,17], we investigated whether endogenous host
77 NEUs can be targeted to regulate neutrophil dysfunction observed in severe infections.
78
79 Here, we have identified host NEU activation as a positive regulator of microbial-
80 induced human neutrophil overactivation. Additionally, we have employed the antiviral
81 NEU inhibitors Oseltamivir and Zanamivir to explore this pathway and found that these
82 drugs fine-tune the neutrophil dysfunction observed in sepsis and COVID-19. Together,
83 our results show that NEU is a potential target for the control of neutrophil dysfunction
84 and presenting Oseltamivir or Zanamivir as adjunctive therapy for severe infections.

Results

LPS-induced surface Sia shedding in human neutrophils is mediated by NEU activity

As activated NEUs hydrolyze Sia residues linked to underlying galactose glycoconjugates[8], we employed a flow cytometry-based lectin binding assay to measure Sia levels on neutrophils after their activation. Alpha2-3-Sia is a major functional Sia linkage of surface glycans present on human neutrophils[18]. Therefore, we used the lectin MAL-II that binds selectively to α2-3- over α2-6-linked Sia[19]. LPS treatment of whole blood from healthy donors significantly reduces the binding of MAL-II on neutrophils ($CD66b^+$) when compared to untreated cells (**Supplementary Fig. 2A**). Next, cells were stained with Fc-chimera of Siglec-9, a sialic acid-binding protein that recognize Sia in α2-3 and α2-6 linkages[20]. Similarly, we observed that binding of Siglec-9-Fc (**Supplementary Fig. 2B**) is decreased on neutrophils treated with LPS, confirming a reduction of neutrophil Sia residues likely due to LPS-induced NEU activity in these cells. To test this hypothesis, we measured NEU activity in human leukocytes using the NEU substrate 4-MU-NANA[14] and validated the assay using NEU purified from *C. perfringens* (CpNEU) (**Fig. 1A-B)**. Both clinically available NEU inhibitors Oseltamivir and Zanamivir reduce CpNEU activity (**Fig. 1A-B)**. Using total leukocytes from healthy donors, we observed that LPS-induced NEU activity was significantly inhibited by Oseltamivir or Zanamivir (**Fig. 1C-D**). Moreover, these NEU inhibitors prevent LPS- or CpNEU-mediated reduction of MAL-II binding on neutrophils surface

108 (**Fig. 1E-H**). Together, these results show that LPS-induced host NEU activity
109 decreases Sia content on neutrophils, which can be inhibited by Oseltamivir and
110 Zanamivir.
111
112 **LPS-induced phagocytosis and killing of *E. coli* is modulated by NEU activity**
113
114 Bacteria uptake and killing are important functions of neutrophils[4]. We next investigated
115 whether host NEU regulates phagocytosis and killing of *E. coli.* Whole blood or total
116 leukocytes from healthy donors were preincubated with LPS or CpNEU, respectively,
117 and *E. coli* BioParticles® added to cells for 60 min. Ingested pHrodo *E. coli* by
118 neutrophils were analyzed by flow cytometry. As expected, we observed a significant
119 increase in MFI of pHrodo *E. coli* of unstimulated cells at 37 ^{o}C when compared to cells
120 at 4 ^{o}C (**Supplementary Fig. 3**). LPS (**Fig. 2A-C**) or CpNEU, used as a positive control
121 (**Fig. 2D-F**), but not heat-inactivated CpNEU, significantly enhances phagocytosis of *E.*
122 *coli*. Remarkably, these effects are inhibited by Zanamivir or Oseltamivir (**Fig. 2A-F**),
123 suggesting that LPS-enhanced phagocytosis involves a host NEU-dependent pathway.
124 Similarly, pretreatment of cells with LPS or CpNEU increases both the number of cells
125 with bacteria as well as the number of bacteria per cell (**Fig. 2G-J**). These effects were
126 also abolished when NEU inhibitors Oseltamivir and Zanamivir were added in the cell
127 cultures (**Fig. 2G-J**). Furthermore, LPS or CpNEU treatment enhances intracellular and
128 extracellular killing of *E. coli,* which are also inhibited by Oseltamivir or Zanamivir (**Fig.**
129 **2K-L**). These results suggest that NEU plays a critical role in LPS-stimulated
130 phagocytosis and killing responses of neutrophils.

131

132 **NEU blockade prevents neutrophil activation**

133 Shedding of cell surface Sia by mobilization of granule-associated NEU to the cell
134 surface has been associated with neutrophil activation[21]. Therefore, we analyzed
135 surface expression of CD66b and CD62L, two markers of human neutrophil activation[22–
136 24], and α2-3-Sia levels in LPS-exposed whole blood cultures. Both Oseltamivir and
137 Zanamivir inhibit LPS-induced shedding of α2-3-Sia (**Fig. 3A,B**) and CD62L (**Fig. 3D,E**)
138 or upregulation of CD66b (**Fig. 3G,H**) on neutrophils. Similarly, MAL-II preincubation,
139 which prevents hydrolysis of α2-3-Sia by NEU[25] by steric hindrance at the NEU
140 cleavage site, blocks LPS-induced neutrophil activation (**Fig. 3C,F,I).** These data show
141 that dampening NEU activity or blocking the hydrolysis of α2-3-Sia is sufficient to inhibit
142 human neutrophil activation by LPS. Similar results were observed in soluble CpNEU-
143 treated leukocytes (**Supplementary Fig. 4**). Next, we assessed whether NEU inhibitors
144 influenced LPS-stimulated ROS production and NETs release, key mediators of
145 bacterial killing and tissue injury[26]. We observed that neutrophils primed with LPS and
146 stimulated with PMA produce higher amounts of ROS when compared to unprimed cells
147 **(Fig. 3J-L)**. Both Oseltamivir and Zanamivir inhibit ROS release to levels similar to
148 unprimed cells. These results were also reproduced by the treatment of cells with
149 CpNEU (**Supplementary Fig. 5E-G**). Furthermore, Oseltamivir or Zanamivir
150 significantly inhibit LPS-induced NETs released by isolated neutrophils **(Fig. 3M)**.
151 Together, these data indicate that microbial-induced host NEU activity regulates
152 important neutrophil functions *in vitro*.

153

154 **Oseltamivir enhances survival rate of mice in clinically relevant models of sepsis**

155 Exacerbated neutrophil responses such as increased ROS production, NETs release,
156 and degranulation are associated with tissue injury and organ dysfunction[27]. By using
157 Oseltamivir as a therapeutic tool, we next explored the involvement of NEU activity *in*
158 *vivo* during experimental sepsis, a model of neutrophil dysfunction[3,28,29]. We first
159 induced sepsis by intraperitoneal administration of 1 x 10^7 CFU/mice of the Gram-
160 negative *E. coli* (ATCC 25922), which lacks NEU in its genome[30]. We used the dose of
161 10 mg/Kg of Oseltamivir by oral gavage (PO), which is the equivalent dose used in
162 humans (~7.5 mg/Kg)[31]. Oseltamivir pretreatment (2 hr before infection) plus post-
163 treatment (6 hr after infection, 12/12 h, PO, for 4 days) markedly boost host survival
164 (**Supplementary Fig. 6A**). Only a single dose of Oseltamivir before (2 hr) bacterial
165 administration was sufficient to reduce disease pathology. Oseltamivir significantly
166 decreases the number of neutrophils in the BAL and lung tissue 4 or 6 hr after infection
167 (**Supplementary Fig. 6B-C**). This pretreatment also augments the neutrophil migration
168 to the focus of infection, which is associated with an efficient control of infection
169 (**Supplementary Fig. 6D-F**). Furthermore, pretreatment with Oseltamivir decreases
170 BAL and plasma TNF and IL-17 levels (**Supplementary Fig. 6G-J**) and tissue injury
171 markers (AST, ALT, ALP and total bilirubin) (**Supplementary Fig. 6K-N**), as well as
172 prevents reduction of α2-3-Sia on peritoneal lavage SSC^{high}/GR-1^{high} cells
173 (**Supplementary Fig. 6O-P**). More importantly, the post-treatment efficacy of
174 Oseltamivir was also evaluated in survival of septic mice. Mice were IP challenged
175 with *E. coli* (1 x 10^7 CFU/mice) and treated 6 hr after infection with Oseltamivir for 4
176 days (10 mg/Kg, PO, 12/12h). Strikingly, in the post-treatment protocol, Oseltamivir

177 provides a significant improvement in the survival rate of septic mice (**Supplementary**
178 **Fig. 6Q**).
179
180 Next, we employed the CLP model to evaluate the effect of Oseltamivir in septic mice,
181 as it is considered the gold standard in preclinical sepsis[32]. Six hours after CLP, mice
182 were treated with Oseltamivir for 4 days (10 mg/Kg, PO, 12/12h). This treatment leads
183 to a small delay in the mortality rate of severe septic mice (**Fig. 4A**). Next, CLP septic
184 mice were treated with antibiotics because it is one of the standard interventions used in
185 clinical settings of sepsis[33]. Importantly, compared to the control animals, therapeutic
186 use of Oseltamivir plus antibiotics drastically improved survival rates of CLP mice
187 (87.5% experimental group vs 25% control group) (**Fig. 4B**). Forty-eight hr after surgery,
188 post-treated septic mice have a significant reduction of neutrophils in BAL and lungs,
189 improvement of neutrophil migration at the focus of infection, and reduced bacterial load
190 in PL and blood (**Fig. 4C-G**). Levels of TNF and IL-17 in PL and plasma and tissue
191 injury markers were also reduced in Oseltamivir treated mice (**Fig. 4H-O**). Additionally,
192 Oseltamivir also leads to a higher expression of α2-3-Sia on SSC^{high}/GR-1^{high} cells in PL
193 (**Fig. 4P-Q**) confirming blockade of NEU activity *in vivo*.
194
195 As respiratory tract infections, particularly pneumonia, are among the most common
196 sites of infection in sepsis[34], we intratracheally administered *K. pneumoniae* (ATCC
197 700603) into mice to address the effect of Oseltamivir. Post-treatment of mice with
198 Oseltamivir significantly improves survival of septic mice challenged with *K.*
199 *pneumoniae* (**Fig. 5A**). The increased host survival was accompanied by a decrease of

200 neutrophil migration in BAL, reduced levels of TNF and IL-17 and reduced levels of
201 tissue injury markers (**Fig. 5B-K**). Oseltamivir also prevents reduction of α2-3-Sia on
202 BAL SSC^{high}/GR-1^{high} cells (**Fig. 5L-M**). Together, these results show that host NEU
203 activation exacerbates inflammatory responses during sepsis and the use of Oseltamivir
204 improves disease outcome.
205
206 **Oseltamivir and Zanamivir rescue overactivated neutrophils from COVID-19**
207 **patients**
208
209 Similar to bacterial sepsis, recent evidence suggests that neutrophils fuel hyper-
210 inflammatory response during severe SARS-CoV-2 infection. Larger numbers of
211 circulating neutrophils have been associated with poor prognosis of COVID-19 patients
212 and analysis of lung biopsies and autopsy specimens showed extensive neutrophil
213 infiltration[2,35–41]. For instance, using single-cell analysis of whole blood from mild and
214 severe COVID-19 patients, Schulte-Schrepping *et al*. (2020) showed that neutrophils in
215 severe patients are highly activated, mainly characterized by the shedding of CD62L[42].
216 Confirming previous findings, we observed that neutrophils from active COVID-19
217 patients, but not from convalescent patients, displayed shedding of CD62L (**Fig. 6A**)
218 and upregulation of CD66b (**Fig. 6B**), indicating a high activation state of these cells.
219 Moreover, neutrophils from severe COVID-19 patients were found to present a
220 significant reduction of surface α2-3-Sia (**Fig. 6C)**, suggesting that NEU activity is
221 increased during severe COVID-19. Therefore, we ask whether neuraminidase
222 inhibitors can rescue neutrophil activation from COVID-19 patients. *Ex vivo* treatment of

223 whole blood with Oseltamivir or Zanamivir decreased neutrophil activation and restored
224 the levels of cell surface α2-3-Sia (**Fig. 6D-F**). **Fig. 6G** summarizes the effects of
225 Oseltamivir and Zanamivir on the surface levels of CD62L and α2-3-Sia by neutrophils,
226 where these treatments lead to a formation of two different clusters in cells from severe
227 COVID-19 patients. As soluble NEU enzymes are also present in plasma[15], we next
228 asked if plasma from COVID-19 patients can induce neutrophil response from healthy
229 donors. Indeed, stimulation of whole blood from healthy donors with fresh plasma from
230 severe, but not convalescent, COVID-19 patients leads to neutrophil activation (**Fig.
231 6H**), reduction of α2-3-Sia (**Fig. 6I**) as well as ROS production (**Fig. 6J,K**), which were
232 significantly reduced by Oseltamivir or Zanamivir (**Fig. 6H-J**). Additionally, we observed
233 that activity of NEU is increased in plasma from severe COVID-19 patients
234 (**Supplementary Fig. 7A)**. Serum glycoproteins from severe COVID-19 patients also
235 presented reduced levels of α2-3-Sia (**Supplementary Fig. 7B-E**) suggesting NEU
236 activation *in vivo*. Moreover, plasma samples from severe COVID-19 patients that were
237 heat-inactivated to inhibit soluble NEU activity **(Supplementary Fig. 7F**) still induces
238 neutrophil activation, suggesting that cellular NEU in conjunction with circulating factors
239 mediate NEU-dependent neutrophil activation in severe COVID-19. These results
240 highlight host NEU as a regulator of neutrophil activation in severe COVID-19 and
241 suggest this pathway as a potential host-directed intervention target to rewire neutrophil
242 responses during severe disease.

243 **DISCUSSION**

244

245 Systemic inflammatory response may lead to unsuitable neutrophil stimulation, which is
246 associated with higher mortality rates in sepsis and sepsis-like diseases[43]. Therefore,
247 finding new therapeutic options to prevent neutrophil overstimulation while maintaining
248 their microbicidal abilities is hugely desired. Neuraminidase inhibitors are promising
249 drugs to fill this gap. Here we demonstrated that endogenous host NEUs mediate
250 exacerbated inflammatory responses by primary neutrophils. Clinically used viral NEU
251 inhibitors, Oseltamivir and Zanamivir, decrease human NEU activity and are effective in
252 prevent LPS-induced neutrophil responses or to rescue overactivation of neutrophils
253 from COVID-19 patients. In severe murine sepsis, therapeutic use of Oseltamivir fine-
254 tunes neutrophil migration results in bacterial clearance and high survival rates.

255

256 All of the four different isotypes of NEU described in mammals (NEU1, NEU2, NEU3
257 and NEU4) remove Sia from glycoproteins and glycolipids with specific substrate
258 preferences[44]. NEU1 cleaves preferentially α2-3-Sia and seems to be the most
259 important isoenzyme in immune cells. NEU1 is a lysosomal enzyme but it is also
260 present at the cell surface where it can regulate multiple receptors such as Fc gamma
261 receptor (FcγR), insulin receptor, integrin β-4, and TLRs[45]. While several stimuli were
262 described to induce NEU activity including LPS[14], PMA, calcium ionophore A23187,
263 fMLP[21], and IL-8[46], how NEUs are activated is poorly understood. However, NEU1
264 activation involves formation of a multicomplex of enzymes that stabilizes NEU1 in its
265 conformational active state[47]. Interestingly, NEU1 was found to be associated with

266 matrix metalloproteinase-9 (MMP9) at the surface of naive macrophages[48]. LPS binding
267 to TLR4 leads to activation of a G protein-coupled receptor (GPCR) via Gαi subunit and
268 MMP9 to induce NEU1 activity, which in turn removes α2-3-Sia from TLR4, allowing its
269 dimerization and intracellular signaling[25,48,49]. Although we have not formally addressed
270 whether the LPS-TLR4 pathway directly activates NEU function in human neutrophils,
271 our results employing MAL-II preincubation suggest desialylation is required for LPS-
272 mediated neutrophil responses. Thus, it is possible that NEU controls Sia levels in TLR4
273 molecules in human neutrophils as observed in macrophages and dendritic cells[25,49].
274 The effects of LPS on neutrophil responses observed here are in accordance with the
275 well-documented induction of ROS and NETs as well as in phagocytosis and bacterial
276 killing by these phagocytes[50–54]. Importantly, the upstream involvement of NEU
277 regulating LPS responses by neutrophils is in agreement with the previous
278 demonstration that TLRs stimulate these cells independent of gene transcription[50].
279 Together, our data suggests that NEU activation provides a fast response to enhance
280 microbial-induced neutrophil functions. Thus, we speculate that this could be an
281 evolutionary mechanism by which neutrophils quickly mobilize their microbicidal
282 mediators against pathogens.
283
284 Sialic acid removal from neutrophils surface markedly changes their adhesiveness,
285 chemotaxis, and migration[21,55–58]. In peritonitis- or pneumonia-induced sepsis in mice,
286 we observed that Oseltamivir prevented the massive neutrophil infiltration into
287 bronchoalveolar spaces or lung tissues, suggesting that regulation of neutrophil
288 migration by dampening NEU activity contributes to survival of septic mice. Interestingly,

289 we observed a divergent effect of Oseltamivir on neutrophil migration to the focus of
290 infection between peritonitis- and pneumonia-induced sepsis. This could be explained
291 by the different mechanisms involved in neutrophil migration to the peritoneal cavity and
292 lungs. While expression of CD62L and rolling of neutrophils to endothelium is necessary
293 for its migration to the peritoneal cavity, it seems to be not required for migration into the
294 lungs[3,59]. Moreover, systemic neutrophil activation leads to cell stiffening, resulting in
295 retention of neutrophils in the small capillaries of the lungs[60], which is frequently the first
296 organ impaired in non-pneumonia- and pneumonia-induced sepsis[61]. The role of NEU-
297 induced neutrophil activation suggested here is in agreement with previous
298 demonstration that NEU1 deletion in hematopoietic cells confers resistance to
299 endotoxemia[16]. Also, the sialidase inhibitor Neu5Gc2en protects endotoxemic irradiated
300 wild-type (WT) mice reconstituted with WT bone marrow but not WT mice reconstituted
301 with NEU1$^{-/-}$ bone marrow cells[16]. Similar to our finds, the treatment of mice with NEU
302 inhibitors increases host survival in *E. coli*-induced sepsis[15]. This outcome was
303 correlated with significant inhibition of blood NEU activity. Enhancement of soluble NEU
304 activity in serum decreases the Sia residues from alkaline phosphatase (APL) enzymes,
305 which are involved in the clearance of circulating LPS-phosphate during sepsis[15].
306
307 SARS-CoV-2 infection leads to mild illness in most of the patients, but ~20% of them
308 progress to severe disease with many characteristics resembling sepsis, including acute
309 respiratory distress syndrome (ARDS), cytokine storm, and neutrophil dysregulation[38,62–
310 64]. The transcriptional programs found in neutrophil subsets from blood and lungs of
311 severe, but not mild, COVID-19 patients are related to cell dysfunction, coagulation, and

312 NETs formation[39,42]. We observed that blood neutrophils from severe COVID-19 are
313 highly activated as demonstrated by reduced CD62L expression and increase of CD66b
314 expression, as previously reported[42]. We now add new information by showing that
315 neutrophils from severe, but not convalescent, COVID-19 patients have reduced
316 surface levels of α2-3-Sia, suggesting a relevant role of NEU for neutrophil activation
317 during COVID-19. More importantly, both the NEU inhibitors Oseltamivir and Zanamivir,
318 increased the α2-3-Sia content and rewired the overactivation of neutrophils from
319 severe COVID-19 patients. We speculate that the addition of NEUs competitive
320 inhibitors allowed the endogenous sialyltransferases to restore sialyl residues on
321 surface glycoconjugates. Fast changes of surface sialic acid levels by sialidases and
322 sialyltransferases seems to be an important mechanism to control neutrophil
323 response[55]. In neutrophils from healthy donors or COVID-19 convalescent patients,
324 Oseltamivir and Zanamivir did not interfere in resting state and had no effect on α2-3-
325 Sia content, suggesting that NEU has a low effect on surface Sia turnover on non-
326 activated neutrophils. How neutrophils are activated and the role of NEU in this process
327 remains to be defined in COVID-19, nevertheless, recent evidence showed that
328 neutrophils could be directly activated by SARS-CoV-2[2], cytokines[65], and alarmins[39,42]
329 such as calprotectin[39], a TLR4 ligand[66]. In addition, we now add new evidence by
330 showing that soluble NEU together with other circulating factors present in plasma from
331 severe COVID-19 patients also accounts for neutrophil activation.
332
333 Collectively, this work suggests that host NEU activation leads to shedding of surface
334 sialic acid with consequent neutrophil overstimulation, tissue damage, and high

335 mortality rates. On the other hand, NEU inhibitors-prevented shedding of sialic acid and
336 regulates neutrophil response, resulting in infection control and high survival rates
337 (working model in **Supplementary Fig. 8**). Taking into account that both drugs are
338 broadly used in humans with well-known toxic and adverse effects, our data suggest
339 Oseltamivir and Zanamivir could be repurposed for the treatment of sepsis or severe
340 infections such as COVID-19. Interestingly, a retrospective single-center cohort study
341 including 1190 patients with COVID-19 in Wuhan, China, showed that administration of
342 Oseltamivir was associated with a decreased risk of death in severe patients[67]. Our data
343 suggest that such encouraging results may be explained by the inhibition of NEU-
344 mediated neutrophil dysfunction *in vivo*. Nevertheless, randomized clinical trials with
345 clinically used NEU inhibitors in sepsis and COVID-19 are required to directly explore
346 this hypothesis.

347 **MATERIALS AND METHODS**

348

349 **Human blood samples**

350

351 Blood was collected from healthy donors (25 - 45 yr old, n=3-12) in endotoxin-free tubes
352 with K_3EDTA (Labor Import, Brasil). All participants gave their written informed consent
353 for blood collection after been informed on procedures. The research protocol followed
354 the World Medical Association Declaration of Helsinki and was approved by the
355 Institutional Review Board of the Federal University of Santa Catarina (CAAE
356 #82815718.2.0000.0121). Blood samples were also collected from severe COVID-19
357 (n=6) or convalescent COVID-19 (n=8) patients (25 to 89 yr old) admitted in the
358 Intensive Care Unit (ICU) or NUPAIVA (Research Center on Asthma and Airway
359 Inflammation) at the UFSC University Hospital from August to October 2020. Blood
360 samples from sex-matched healthy donors were used as controls. All patients or a close
361 family member gave consent for participation in the study, which was approved by the
362 UFSC IRB (CAAE #36944620.5.1001.0121). Supplementary Table 1 summarizes
363 patients clinical and laboratory records. These samples were used to analyze neutrophil
364 activation, surface α2-3-Sia as well as the effect of plasma under these parameters and
365 ROS production. Blood samples were also collected from severe COVID-19 (n=5)
366 patients (55 to 73 yr old) admitted at the Hospital Naval Marcílio Dias (HNMD). The
367 research was approved by the Research Ethics Committee (CEP) from Brazilian
368 National Health Council. All patients signed a free and informed consent form following
369 current legislation and the relevant ethical regulations approved by the Hospital Naval

370 Marcílio Dias (CAAE #31642720.5.0000.5256). These samples were used to analyze
371 the sialylation of plasma proteins. Supplementary Table 2 summarizes the clinical and
372 laboratory information of patients from this cohort.
373
374 **Evaluation of neutrophil activation, phagocytosis, killing, ROS, and NETs release**
375
376 Whole blood containing 1 x 10^6 leukocytes were incubated (37 ^oC, 5% CO_2) in the
377 presence or absence of Oseltamivir (100 µM, Sigma-Aldrich, San Luis, MO, USA),
378 Zanamivir (30 µM, Sigma-Aldrich), LPS (1 µg/mL, *E. coli* 0127:b8, Sigma-Aldrich), LPS
379 plus Oseltamivir or LPS plus Zanamivir for 90 min. Concentrations of Oseltamivir and
380 Zanamivir used here were chosen by concentration-effect experiments (10-100 µM
381 Oseltavimir and 1-30 µM Zanamivir) (data not shown). Since plasma is a rich source of
382 glycoconjugates, total leukocytes were used instead of whole blood to evaluate the
383 effect of isolated neuraminidase from *Clostridium perfringens* (CpNEU) on neutrophils.
384 Red blood cells (RBCs) were lysed by lysis buffer (0.15 M NH_4Cl; 0.1 mM EDTA; 12 mM
385 Na_2HCO_3) for 7 min, RT, followed by centrifugation (270 x g; 22°C; 7 min). Total
386 leukocytes (1 x 10^6 cells) were incubated (37 ^oC, 5% CO_2) in the presence or absence
387 of CpNEU (10 mU, Sigma-Aldrich), CpNEU plus Oseltamivir (100 µM) or CpNEU plus
388 Zanamivir (30 µM) for 60 min. Next, the following assays were performed. *Analysis of*
389 *neutrophil activation*. Leukocytes were then washed and resuspended in FACS buffer (2
390 mM EDTA/PBS). The mix of antibodies against CD66b (G10F5; BioLegend, San Diego,
391 CA, USA), CD62L (DREG-56; BioLegend), CD16 (3G8; BioLegend), isotypes or
392 *Maackia amurensis* Lectin II biotinylated (MAL-II, Vector Labs, San Diego, CA, USA)

393 coupled to Streptavidin (Biolegend), plus human BD Fc Block™ (BD Pharmingen™) and
394 Fixable Viability Stain (FVS, BD Horizon™, San Jose, CA, USA) were added to
395 leukocytes for 30 min at 4 °C. Cells were washed, resuspended in FACS buffer,
396 acquired in a FACSVerse cytometer and analyzed using FlowJo software (FlowJo LLC).
397 Approximately 100.000 gated events were acquired in each analysis. *Phagocytosis*
398 *assays*. After RBCs lysis, 1 x 10^6 leukocytes were incubated at 37 °C (5% CO_2) or at 4
399 °C (control) with 100 µg/mL pHrodo™ Red *E. coli* BioParticles® (Thermo Fisher,
400 Waltham, MA, USA) for 60 min and the MFI of neutrophils (FVS^-/$CD66b^+$ cells) with
401 ingested bioparticle was analyzed by FACS. Total leukocytes were also incubated with
402 1 x 10^6 CFU of live *E. coli* (ATCC 25922) for 90 min (37 °C, 5% CO_2). Next, the cells
403 were washed twice (2 mM EDTA/PBS), fixed (FACS buffer/PFA 2%) and the
404 percentage of neutrophils with bacteria or the percentage of neutrophils with ≥3 bacteria
405 was analyzed by light microscopy using Differential Quick Stain Kit (Laborclin, Brazil).
406 *Bacterial killing*. Total leukocytes (1 x 10^6) were incubated (37 °C, 5% CO_2) with 1 x 10^6
407 CFU of live *E. coli* for 180 min. The samples were centrifuged (270 g, 7 min, 4 °C) and
408 10 µL of supernatant were diluted until 10^6 and spread onto agar brain-heart infusion
409 (BHI, Kasvi, Brazil) to quantify the viable extracellular bacteria. The pellets were washed
410 twice with PBS/2 mM EDTA (270 g, 7 min, 4 °C), the leukocytes were lysed with 2%
411 Triton-X, washed (PBS, 2000 g, 15 min, 4 °C), resuspended in PBS and 10 µL of
412 samples were diluted until 10^6 and spread onto agar BHI. Plates were incubated
413 overnight at 37 °C and viable bacteria were expressed as mean ± SEM of CFU/mL.
414 *ROS assay*. After RBCs lysis, 1 x 10^6 leukocytes were incubated at 37 °C (5% CO_2) with
415 10 µM of cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA,

416 ThermoFisher) for 5 min. Next, cells were stimulated or not with phorbol 12-myristate
417 13-acetate (PMA) for 10 min, fixed, washed twice with PBS/2 mM EDTA (270 g, 7 min,
418 4 ^{o}C) and analyzed by FACS. *NETs assay*. NETs quantification was performed as
419 previous described[68] on the supernatant of isolated neutrophils. Briefly, an anti-MPO
420 antibody bound to a 96-well flat-bottom plate captured the enzyme MPO (5 µg/ml;
421 Abcam), and the amount of DNA bound to the enzyme was quantified using the Quant-
422 iT™ PicoGreen® kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's
423 instructions. Fluorescence intensity (Ex 488 nm/Em 525 nm) was quantified in a
424 FlexStation 3 Microplate Reader (Molecular Devices, San Jose, CA, USA). *Neutrophil*
425 *isolation*. Human circulating neutrophils were isolated by Percoll density gradients[69].
426 Briefly, four different gradients, 72%, 65%, 54%, and 45%, were used to isolate human
427 circulating neutrophils. After centrifugation at 600 g for 30 min at 4 °C, the cell layer at
428 the 72% gradient interface was collected as the neutrophil fraction. The erythrocytes
429 were removed by lysis, and cell pellets were resuspended in RPMI 1640. Isolated
430 neutrophils (1 x 10^6/well) were treated with Oseltamivir, Zanamivir or medium 1 h before
431 the stimulus with PMA (50 nM) or LPS (10 µg/mL). After 4 hr of stimuli (37 ^{o}C, 5% CO_2),
432 the supernatant was collected to measure the levels of NETs.
433

434 **Neutrophil responses with plasma from COVID-19 patients**

435

436 Whole blood samples from sex-matched healthy donors (n = 7) were incubated for 2 h
437 (37 °C, 5% CO_2) with 7% of fresh plasma from healthy donors, severe or convalescent
438 COVID-19 patients or heat-inactivated plasma (56 °C, 30 min) from severe COVID-19

439 patients in the presence or absence of Oseltamivir (100 μM) or Zanamivir (30 μM).
440 Surface levels of α2-3-Sia and CD66b and ROS production were assessed on
441 neutrophils by FACS.
442
443 **Neuraminidase kinetics assay**
444
445 After RBCs lysis, 0.5 x 10^6 leukocytes were resuspended in HBSS and added to 96-well
446 flat-bottom dark plate (SPL Life Sciences, South Korea) on ice. Then, 4-
447 Methylumbelliferyl-N-acetyl-α-D-Neuramic Acid (4-MU-NANA, Sigma-Aldrich) substrate
448 (0.025 mM) was added followed by medium, or LPS (1 μg/mL), LPS plus Oseltamivir
449 (100 μM), LPS plus Zanamivir (30 μM). CpNEU (10 mU), CpNEU plus Oseltamivir or
450 CpNEU plus Zanamivir were used as positive controls of the assay. The volume was
451 completed to 200 μL with HBSS, followed by reading on the Spectramax® Paradigm®
452 instrument starting 3 min after and every 5 min for 55 min at 37 ºC. Sialidase activity
453 was also assessed in heat-inactivated or fresh plasma from severe COVID-19 patients
454 in the presence or absence of Oseltamivir (100 μM) or Zanamivir (30 μM) using Tecan
455 Infinite 200 multi-reader. The fluorescent substrate 4-MU-NANA formation was detected
456 at ex 350 nm/em 450 nm.
457
458 **Mice**
459
460 The care and treatment of the animals were based on the Guide for the Care and Use
461 of Laboratory Animals[70] and all procedures followed the ARRIVE guidelines and the

462 international principles for laboratory animal studies[71]. C57BL/6 (Jackson Laboratory,
463 Bar Harbor, ME, USA) mice (8–10 weeks old) and Swiss mice (10–12 weeks old) were
464 housed in cages at 21 ± 2°C with free access to water and food at the Animal Facility of
465 the Department of Microbiology, Immunology, and Parasitology and Department of
466 Pharmacology from UFSC, respectively. A total of 228 mice were used in this study.
467 Protocols were approved by the Animal Use Ethics Committee of UFSC (CEUA
468 #8278290818).
469
470 ***E. coli-, Klebsiella pneumoniae-* and CLP-induced sepsis**
471
472 *E. coli* (ATCC 25922, Manassas, VA, USA) or *K. pneumoniae* (ATCC 700603) were
473 used to induce severe sepsis in mice. Naive mice were intraperitoneal (IP) challenged
474 with 100 µL of 1 x 10^7 CFU of the *E. coli* suspension. A group of *E. coli*-septic mice was
475 randomly pretreated (2 hr before infection) and post treated by *per oral* (PO, 12/12 hr)
476 via with saline or Oseltamivir phosphate (10 mg/kg, Eurofarma, Brazil) for 4 days to
477 survival analysis. Another group was pretreated 2 hr before infection with a single dose
478 of Oseltamivir phosphate (10 mg/kg, PO) and the pathophysiological response was
479 analyzed at 4 and 6 hr after infection. *E. coli*-septic mice were also randomly
480 posttreated (6 hr after infection, 12/12 hr) with saline or Oseltamivir phosphate (PO, 10
481 mg/kg) for 4 days to survival analysis. For pneumonia-induced sepsis, mice were
482 anesthetized with isoflurane (3–5 vol%) and placed in supine position. A small incision
483 was made in the neck where the trachea could be localized and a *K. pneumoniae*
484 suspension (4 x 10^8 CFU/50 µL of PBS) was injected into the trachea with a sterile 30-

485 gauge needle. Skin was sutured and animals were left for recovery in a warm cage.
486 After 6 hr of infection and then 12/12 hr mice were treated with Oseltamivir phosphate
487 (PO, 10 mg/kg) for survival analysis. In another set of experiments, pneumonia was
488 induced and mice were treated 6 hr after infection with a single dose of Oseltamivir
489 phosphate (10 mg/kg, PO) for material collection and analysis of pathophysiological
490 response 24 hr after infection.
491 Cecal ligation and puncture (CLP)-induced sepsis were performed as previously
492 described[28]. Mice were anesthetized with xylazine (2 mg/kg, IP, Syntec, Brazil) followed
493 by isoflurane (3–5 vol%, BioChimico, Brazil), a 1 cm midline incision was made in the
494 anterior abdomen, and the cecum was exposed and ligated below the ileocecal junction.
495 The cecum was punctured twice with an 18-gauge needle and gently squeezed to allow
496 its contents to be released through the punctures. Sham-operated (Sham) animals
497 underwent identical laparotomy but without cecal ligation and puncture. The cecum was
498 repositioned in the abdomen, and the peritoneal wall was closed. All animals received 1
499 mL of 0.9% saline subcutaneous (SC) and 100 μL of tramadol (5 mg/kg, SC, Vitalis,
500 Brazil) immediately after CLP. CLP-septic mice were randomly treated (starting 6 h after
501 infection, PO) with 100 μL of saline or Oseltamivir phosphate (10 mg/kg, 12/12 hr) for 4
502 days. In another set of experiments, CLP mice were randomly IP treated (6 hr after
503 infection, 12/12 hr) during 4 days with 100 μL metronidazole (15 mg/kg, Isofarma,
504 Brazil) plus ceftriaxone (40 mg/kg, Eurofarma, Brazil) and Oseltamivir phosphate (10
505 mg/kg) or saline by PO to survival analysis or treated for 36 hr to analyze the
506 pathophysiological response at 48 hr after CLP.

507 **Neutrophil migration**

508 The animals were euthanized in a CO_2 chamber, the bronchoalveolar lavage (BAL) and
509 peritoneal lavage (PL) were performed and the number of neutrophils was determined
510 at 4 and 6 hr after *E. coli*, 24 hr after *K. pneumoniae* infection or 48 hr after CLP
511 surgery, as described[28]. Next, mice were perfused with PBS/EDTA (1 mM) and the
512 lungs were harvested. Lungs were passed through 40-µm nylon cell strainers and
513 single-cell suspensions were centrifuged in 35% Percoll® solution (315 mOsm/kg,
514 Sigma-Aldrich) for 15 min at 700 g to enrich leukocytes populations. Pelleted cells were
515 then collected, and erythrocytes were lysed. Single-cell suspensions from individual
516 mice were determined using a cell counter (Coulter ACT, Beckman Coulter, Brea, CA,
517 USA) or with a haemocytometer. Differential counts were also determined on Cytospin
518 smears stained using Differential Quick Stain Kit (Laborclin, Brazil). Blood samples were
519 collected by heart puncture and tubes containing heparin for further analysis.
520 Neutrophils from LP or BAL were also stained with anti-Ly-6G/Ly-6C (GR-1, RB6-8C5;
521 BioLegend) and MAL-II to be further analyzed by FACS, as previously described.
522 Analysis was carried out in SSC^{high}/GR-1^{high} cells.

523 **Bacterial counts**

524 The bacterial counts were determined as previously described[28]. Briefly, the BAL, PL or
525 blood were harvested and 10 µL of samples were plated on Muller-Hinton agar dishes
526 (Difco Laboratories, Waltham, MA, USA) and incubated for 24 hr at 37 °C. PL or BAL
527 samples were diluted until 10^6.

528 **ELISA**

529 TNF (R&D Systems, Minneapolis, MN, USA) and IL-17 (XpressBio Life Sciences
530 Products, Frederick, MD, USA) levels in plasma, PL or BAL were determined by ELISA
531 kits according to the manufacturer's instructions.

532 **Tissue injury biochemical markers**

533 Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline
534 phosphatase (ALP) activities, and the levels of total bilirubin were determined in plasma
535 samples by commercial kits (Labtest Diagnóstica, Brazil). The procedures were carried
536 out according to the manufacturer's instructions.

537

538 **Lectin blotting of serum glycoproteins**

539 To evaluate if severe COVID-19 changes the α2-3 sialylation of serum glycoproteins,
540 serum samples from four healthy donors and five severe COVID-19 patients were
541 blotted with MAL-II as previously described[72]. Samples were diluted in SDS-PAGE
542 sample buffer and heated at 100 °C for 5 min. Twelve µg of total protein were resolved
543 in 10% SDS-PAGE and transferred onto nitrocellulose membranes (Millipore,
544 Burlington, MA, USA). Membranes were blocked (TBST + 5% BSA) overnight at 4 ^{o}C
545 and incubated for 2 hr with 1 µg/mL of biotin-conjugated MAL-II, washed and incubated
546 for 40 min with alkaline-phosphatase (ALP)-conjugated streptavidin (Southern Biotec,
547 Birmingham, AL, USA) diluted 1:10.000. Both MAL-II and streptavidin-ALP were diluted
548 in TBST (5% BSA plus 150 mM of $CaCl_2$). Membranes were then revealed with

549 BCIP/NBT (5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium) color
550 development substrate (Promega, Brazil). The lane intensities were quantified using the
551 Image J software. In parallel, the resolved SDS-PAGE were stained with Coomassie
552 Brilliant blue R 250 (Merck KGaA, Germany) to compare the protein profile between the
553 different samples.

554

555 **Statistical analysis**

556 The data are reported as the mean or median ± SEM of the values obtained from two to
557 seven independent experiments. Each experiment using human samples was
558 performed using three to five samples from healthy donors or one to three samples from
559 severe or convalescent COVID-19 patients. We used five mice per experimental group
560 except for survival analyses in which twelve to twenty mice were used. The mean or
561 median values for the different groups were compared by analysis of variance (ANOVA)
562 followed by Dunnett and/or Tukey post-tests. Bacterial counts were analyzed by the
563 Mann–Whitney *U*-test or unpaired *t*-test using a parametric test with Welch's correction.
564 Survival curves were plotted using the Kaplan–Meier method and then compared using
565 the log-rank method and Gehan-Wilcoxon test. Data was analyzed using GraphPad
566 Prism version 8.00 for Mac (GraphPad Software, USA). A $P < 0.05$ was considered
567 significant.

568 **Abbreviations**

569 4-MU-NANA 4-methylumbelliferyl-N-acetyl-α -D-Neuramic Acid

570 ALP alkaline phosphatase

571 ALT alanine aminotransferase

572 ARRIVE Animal Research: Reporting of In Vivo Experiments

573 AST aspartate aminotransferase

574 ATCC American Type Culture Collection

575 BAL bronchoalveolar lavage

576 BHI brain–heart infusion

577 CD cluster of differentiation

578 CFU colony forming units

579 CLP cecal ligation and puncture

580 CM-H2DCFDA cell-permeant 2',7'-dichlorodihydrofluorescein diacetate

581 CpNEU *Clostridium perfringens* neuraminidase

582 DANA 2,3-dehydro-2-deoxy-N-acetylneuraminic acid

583 EDTA ethylenediamine tetra-acetic acid

584 FACS fluorescence-activated cell sorting

585 FcγR Fc gamma receptor

586 fMLP N-formyl-Met-Leu-Phe

587 FVS fixable viability stain

588 HBSS Hanks' balanced salt solution

589 IgG immunoglobulin G

590 IP intraperitoneal pathway

591 LPS lipopolysaccharide

592 MAL-II *Maackia amurensis* lectin II

593 MFI median fluorescence intensity

594 NETs neutrophil extracellular traps

595 NEU neuraminidases

596 Neu5Ac2en 2-deoxy-2, 3-didehydro-D-N-acetylneuraminic acid

597 PBS phosphate buffered saline

598 PFA paraformaldehyde

599 PL peritoneal lavage

600 PMA 12-myristate 13-acetate

601 PMN polymorphonuclear

602 PO oral pathway

603 RBCs red blood cells

604 ROS reactive oxygen species

605 SARS-CoV-2 severe acute respiratory syndrome coronavirus 2

606 SC subcutaneous pathway

607 Sia sialic acids

608 Siglecs sialic acid-binding immunoglobulin-like lectins

609 TLR toll-like receptor

610 COVID-19 coronavirus disease 2019

611 WT wild-type

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615

616 **Authors' contributions**

617 All named authors meet the criteria of Nature Medicine for authorship of this manuscript,
618 take responsibility for the work's integrity as a whole, and have given final approval for
619 publication.

620

621 ROF, FCA, DM, RS, DSM, AB, MSM and FS designed experiments and analyzed data;
622 ROF, FAC, CFS, DM, CWW, CLB, AAS, JA, LFF, CCN, NMP, PCS, FRF, FAS, LA, NH,
623 AB and FS performed experiments; SB, AM, MSM, FQC and RM contributed with
624 critical reagents/tools/clinical samples; ROF, FS, AB wrote the manuscript.

625

626 **Competing Interests statement**

627 The authors declare that no conflict of interest exists.

628

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799 **Figures and Figure Legends**
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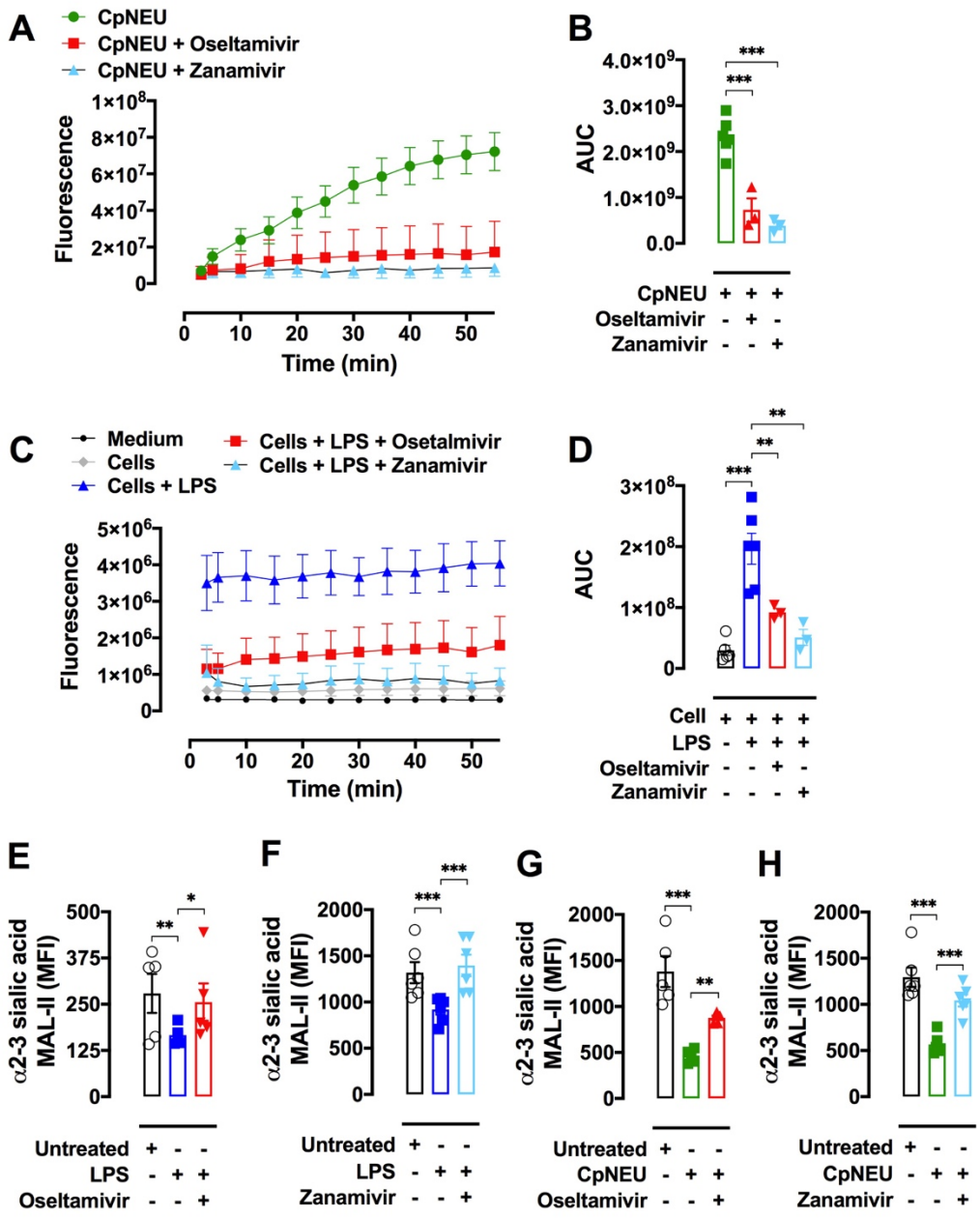

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803 **Figure 1. LPS stimulates NEU activity in human leukocytes.** Neuraminidase isolated
804 from *Clostridium perfringens* (CpNEU) was used to validate the NEU activity assay.
805 CpNEU (0.012 UI) was added in a 96-well flat-bottom dark plate on ice in the presence
806 or not of its inhibitors Oseltamivir phosphate (100 μM) or Zanamivir (30 μM). Next, the
807 substrate 4-MU-NANA (0.025 mM) was added and the fluorescent substrate was read 3
808 min after at 37 ºC (**A**). The area under the curve (AUC) values are shown in **B**. Total

809 leukocytes resuspended in HBSS were added in a plate on ice and 4-MU-NANA
810 substrate (0.025 mM) was added followed by the addition of medium, LPS (1 μg/mL),
811 LPS plus Oseltamivir (100 μM) or LPS plus Zanamivir (30 μM). The fluorescent
812 substrate was read 3 min after at 37 ºC (**C**). Raw data were subtracted from the control
813 group containing only HBSS (medium) and expressed as AUC values (**D**). Whole blood
814 containing 1 x 10^6 leukocytes from healthy donors were stimulated or not with LPS (1
815 μg/mL, 90 min, 37 ^oC, 5% CO_2), LPS plus Oseltamivir (100 μM), or LPS plus Zanamivir
816 (30 μM). Total leukocytes (1 x 10^6) were incubated with CpNEU (10 mU, 60 min, 37 ^oC,
817 5% CO_2), CpNEU plus Oseltamivir (100 μM), or CpNEU plus Zanamivir (30 μM).
818 Leukocytes were stained with MAL-II to detect α2-3 sialic acids (**E-F**). The MFI was
819 analyzed on $CD66b^+$ cells using the gate strategies shown in Supplementary Fig. 1. *P
820 < 0.05; **P < 0.01; ***P < 0.001. This figure is representative of three independent
821 experiments (n= 3-6) and data are shown as mean ± SEM. LPS = lipopolysaccharide;
822 MAL-II = *Maackia amurensis* lectin II; CpNEU = neuraminidase.
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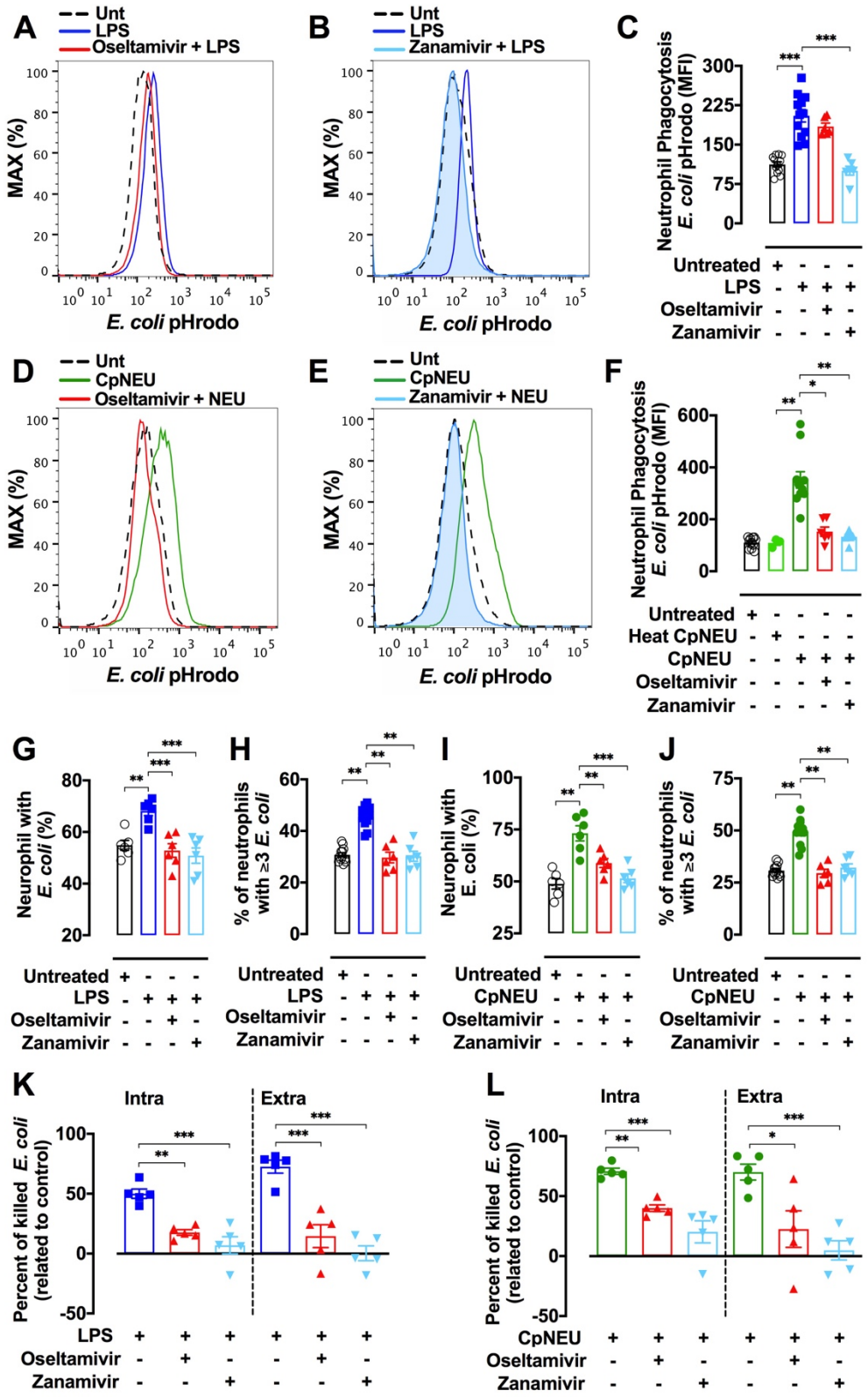

824

825 **Figure 2. LPS increases phagocytosis and killing of *E. coli* in a NEU-dependent**
826 **manner.** Whole blood from healthy donors containing 1 x 10^6 leukocytes were exposed

827 (37 ^{o}C, 5% CO_2) or not to LPS (1 µg/mL, 90 min), LPS plus Oseltamivir (100 µM), or
828 LPS plus Zanamivir (30 µM) (**A-C; G-H; K**). Total leukocytes (1 x 10^6) were exposed or
829 not to CpNEU (10 mU, 60 min, 37 ^{o}C, 5% CO_2), CpNEU plus Oseltamivir (100 µM), or
830 CpNEU plus Zanamivir (30 µM) (**D-F; I-J; L**) and the phagocytosis and killing assays
831 were performed. Leukocytes were incubated with *E. coli* pHrodo BioParticles® (100
832 µg/mL) for 60 min at 37 °C to assess phagocytosis in viable CD66b$^+$ cells (**A-F**) (as
833 gated in Supplementary Fig. 1). Live *E. coli* was used to evaluate phagocytosis by light
834 microscopy or to assess the killing by leukocytes. Cells were stimulated as described
835 above and 1 x10^6 leukocytes were incubated at 37 °C with *E. coli* (1 x10^6 CFU) for 90
836 min for phagocytosis or for 180 min for killing assays. The percentage of cells with
837 ingested bacteria (**G; I**) and the number of bacterial particles per cell (**H; J**, ≥3 particles
838 per cell) were evaluated. The killing of *E. coli* was evaluated by spreading 10 µL of
839 supernatant (extracellular killing) or 10 µL of the intracellular content in agar medium
840 and the CFU were counted. Killing *E. coli* was expressed as the rate of fold change
841 compared to the unprimed (untreated) cells (**L**). Symbols represent individual donors
842 and data are shown as mean ± SEM from pooled data of two to three independent
843 experiments (n = 3-12). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Unt = untreated; LPS =
844 lipopolysaccharide; CpNEU = neuraminidase.
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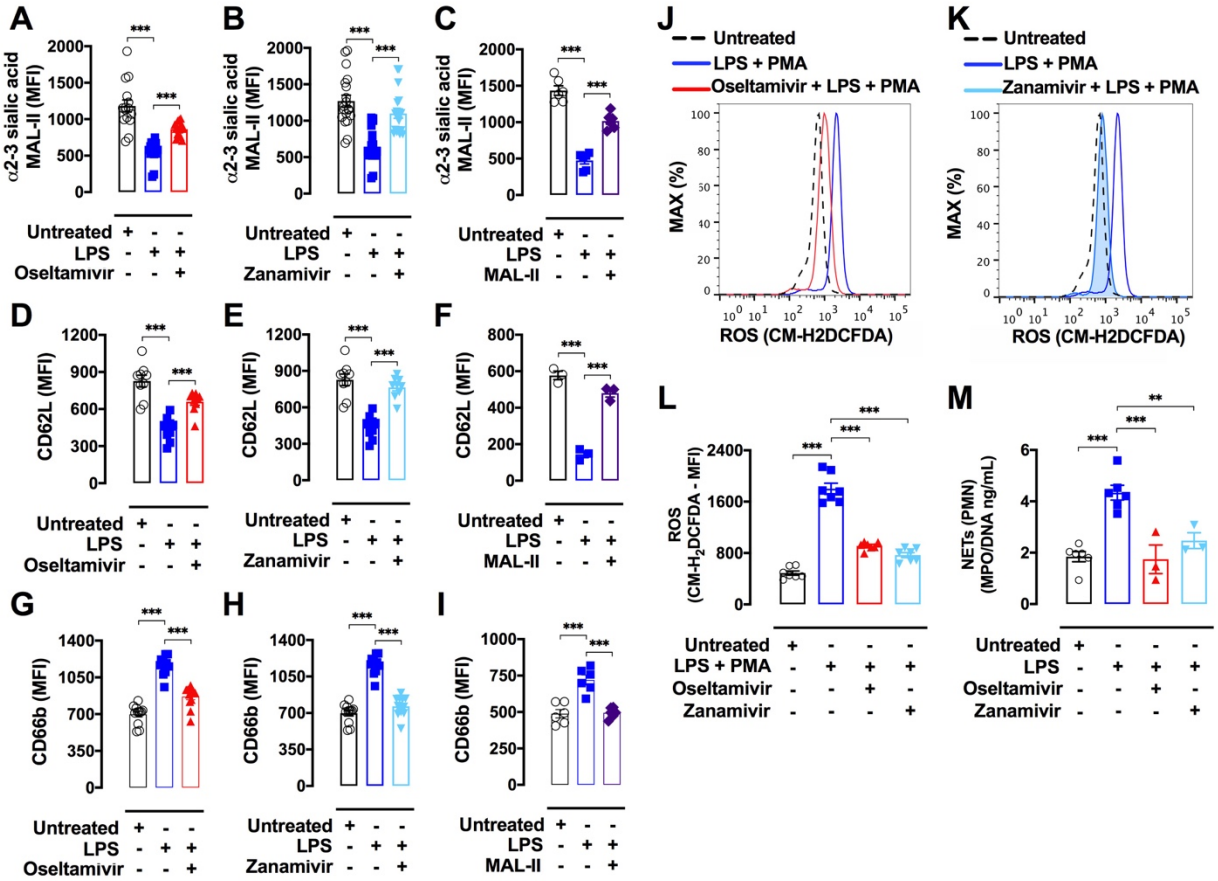


Figure 3. LPS-induced human neutrophil response involves NEU activity. Whole blood from healthy donors containing 1 x 10^6 leukocytes were stimulated or not with LPS (1 µg/mL, 90 min, 37 °C, 5% CO_2), LPS plus Oseltamivir (100 µM), LPS plus Zanamivir (30 µM), or LPS plus MAL-II (1 µg/mL, MAL-II promotes steric hindrance at the NEU cleavage site and prevent sialic acid cleavage). Leukocytes were marked with MAL-II to detect α2-3 sialic acids (**A-C**) or stained with the cell activation markers CD62L (**D-F**) and CD66b (**G-I**). After RBCs lysis leukocytes were incubated with 5 µM CM-H2DCFDA fluorescent probe for 15 min. PMA (10 µM) was used to stimulate ROS production for 10 min (**J-L**). Supplementary Fig. 5 showed ROS production in additional control groups. The MFI was analyzed on $CD66b^+$ cells using the gate strategies shown in Supplementary Fig. 1. Isolated neutrophils were treated with Osetamivir (100 µM) or Zanamivir (30 µM) 1 h before the stimulus with LPS (10 µg/mL) for 4 h. The concentration of NETs was evaluated by MPO-DNA PicoGreen assay on supernatants

863 of cells (**M**). Symbols represent individual donors and data are shown as mean ± SEM
864 from pooled data of two to three independent experiments (n = 7) except for F and M
865 that was made once with n=3. ***$P < 0.001$; **$P < 0.01$. Unt = untreated; CM-H2DCFDA
866 = 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester; LPS =
867 lipopolysaccharide; PMA = phorbol 12-myristate 13-acetate; ROS = reactive oxygen
868 species; NETs = neutrophil extracellular traps; RBCs = red blood cells; MAL-II =
869 *Maackia amurensis* lectin II; PMN = polymorphonuclear leukocytes.

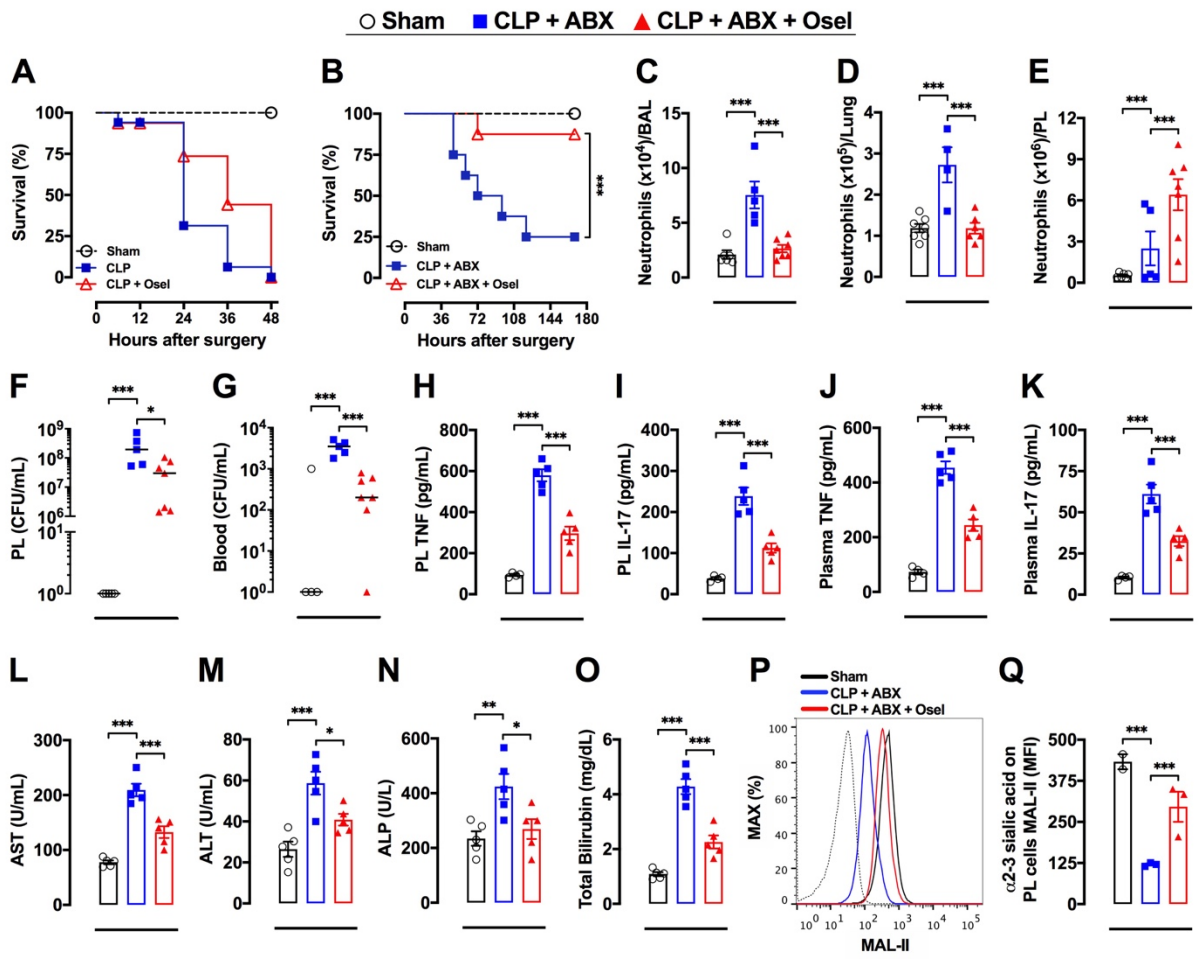


Figure 4. Oseltamivir enhanced host survival in CLP-induced sepsis. Severe sepsis was induced by the cecal ligation and puncture (CLP) model. Mice were randomly treated (starting 6 hr after infection, 12/12 h, PO, for 36 hr, n=16) with saline or Oseltamivir phosphate (10 mg/kg) and their survival rates were monitored over 48 hr (**A**). In another set of experiments, CLP mice were randomly IP treated (started 6 hr after infection, 12/12 hr) during 4 days with 100 µL metronidazole (15 mg/kg)/ceftriaxone (40 mg/kg) (ABX) plus saline or Oseltamivir phosphate (10 mg/kg) by PO and their survival rates (n=12) were monitored over 168 hr (**B**). Also, mice were subjected to CLP and treated with ABX + saline or ABX + Olsetamivir as described in B and euthanized 48 hr after surgery to evaluate the number of neutrophils in BAL (**C**), lung tissue (**D**), and peritoneal lavage (PL) (**E**); TNF (**H**), IL-17 (**I**), and CFU (**F**) were also determined in PL. Blood CFU (**G**) and plasmatic levels of TNF (**J**), IL-17 (**K**), AST (**L**), ALT (**M**), ALP (**N**) and total bilirubin (**O**) were also evaluated 48 hr after surgery.

885 The amount of surface α2-3 sialic acids were assessed by MAL-II staining in
886 SSC^{high}/Gr-1^{high} cells in PL and analyzed by FACS, as shown by the representative
887 histograms (**P**) and MFI (**Q**); dotted line = unstained cells. The results are expressed as
888 percent of survival (n=16), mean or median (only for FACS data) ± SEM. *$P < 0.05$; ***P
889 < 0.001. These experiments were repeated 3 times for survival analysis and twice for
890 other parameters (n=3-7). ABX = antibiotics (metronidazole/ceftriaxone); Sham = sham-
891 operated. Osel = Oseltamivir; AST = alanine aminotransferase; ALT = aspartate
892 aminotransferase; ALP = alkaline phosphatase; CFU = colony-forming units.

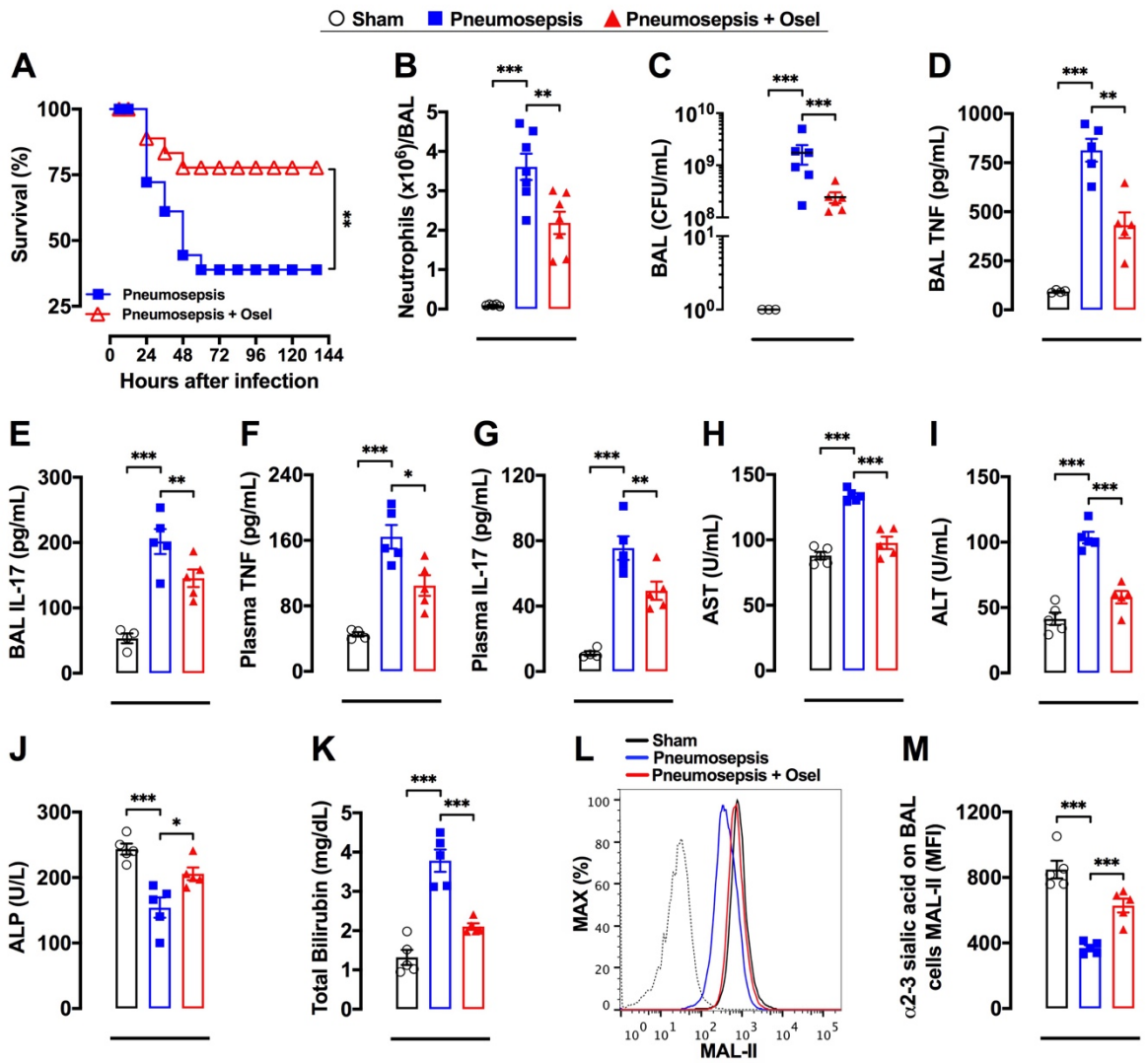


Figure 5. Oseltamivir enhanced mice survival in *K. pneumoniae*-induced sepsis. Sepsis was induced by intratracheal administration of *K. pneumoniae* and mice were randomly treated (starting 6 hr after infection, 12/12 hr, PO, n=20) with saline or Oseltamivir phosphate (10 mg/kg) and survival rates were monitored for 144 hr (**A**). In similar set of experiments, septic mice (n=6-7) were treated 6 hr after infection with a single dose of Oseltamivir phosphate (10 mg/kg, PO) and mice were euthanized 24 hr after infection to determine the number of neutrophils (**B**) and CFUs (**C**), and levels of TNF (**D**) and IL-17 (**E**) in BAL. Plasma levels of TNF (**F**), IL-17 (**G**), AST (**H**), ALT (**I**), ALP (**J**) and total bilirubin (**K**) were also evaluated 24 hr after infection. The amount of surface α2-3 sialic acids were assessed by MAL-II staining in SSC^{high}/Gr-1^{high} cells in BAL and analyzed by FACS, as shown by the representative histograms (**L**) and MFI

905 (**M**); dotted line = unstained cells. The results are expressed as percent of survival,
906 mean or median (only for FACS data) ± SEM. *$P < 0.05$; *$P < 0.01$; ***$P < 0.001$. Sham
907 = sham-operated mice; Osel = Oseltamivir; AST = alanine aminotransferase; ALT =
908 aspartate aminotransferase; ALP = alkaline phosphatase.

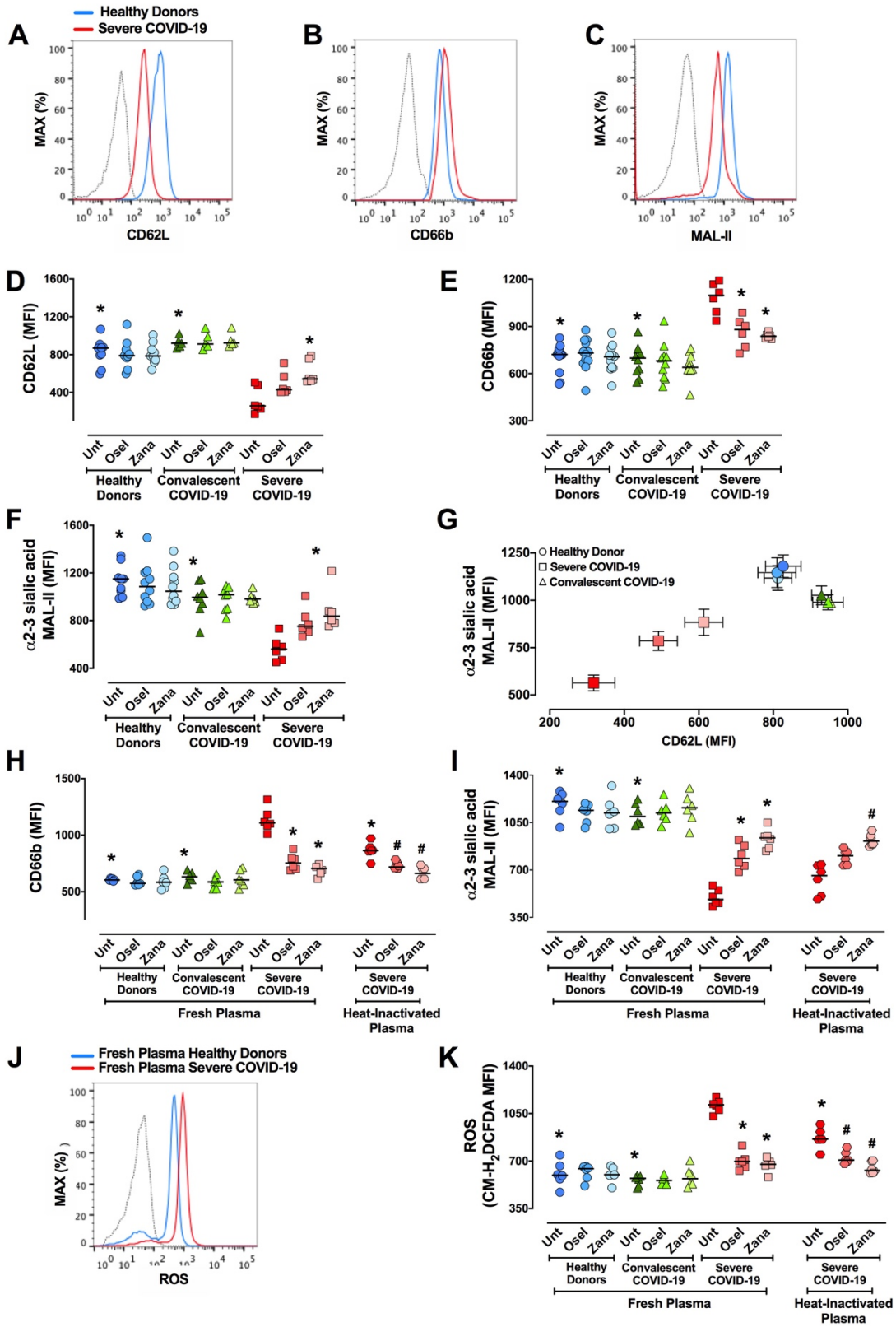


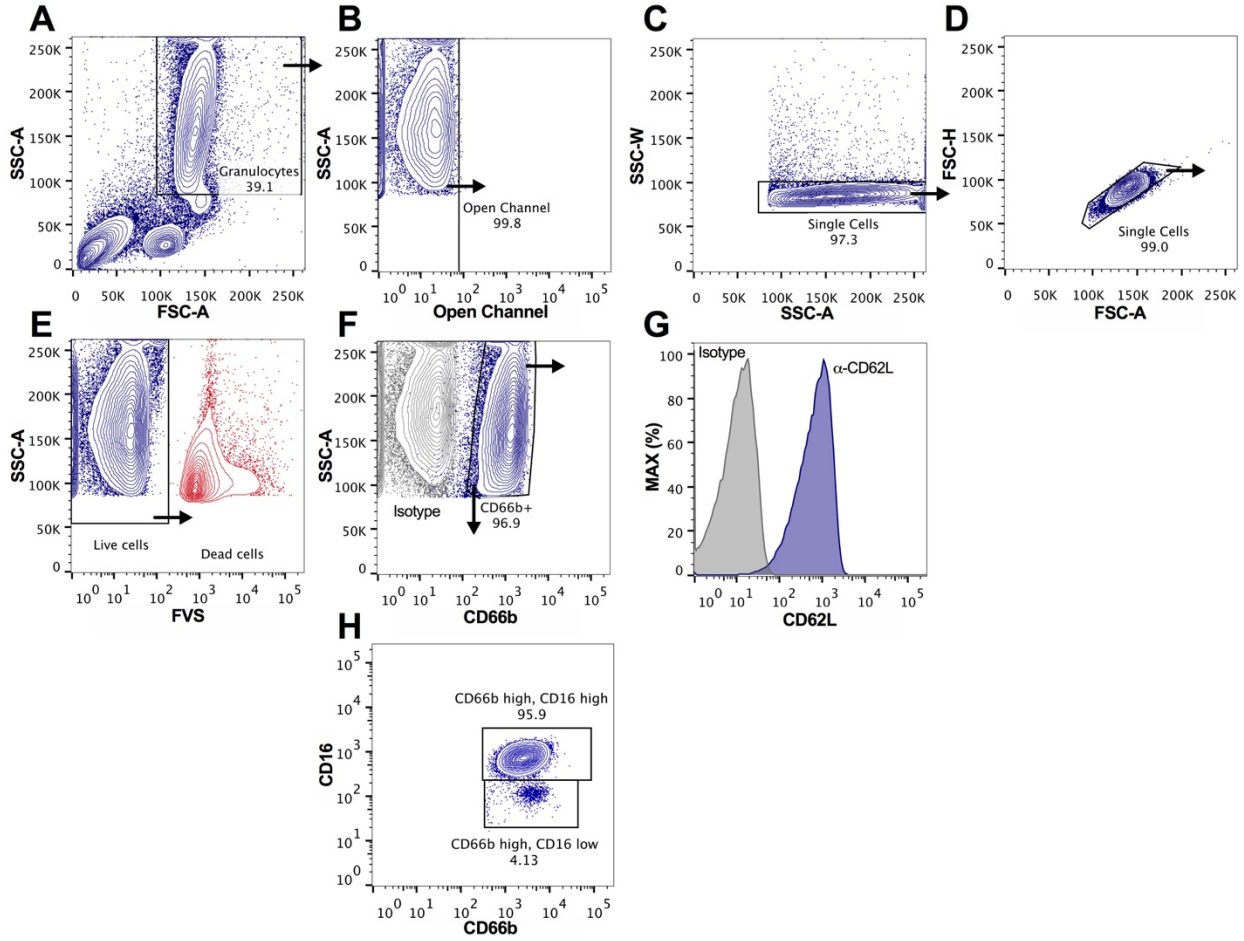
Figure 6. Oseltamivir and Zanamivir decrease neutrophil activation and increase α2-3 sialic acid levels in active, but not convalescent neutrophils from COVID-19

912 **patients.** Whole blood from healthy donors (n= 10), severe COVID-19 patients (n= 6) and
913 convalescent COVID-19 patients (n= 8) were treated or not with Oseltamivir (100 μM) or
914 Zanamivir (30 μM) and total leukocytes were stained with the cell activation markers
915 CD62L (**A and D**), CD66b (**B and E**) and MAL-II to detect α2-3 sialic acids (**C and F**).
916 Correlation of surface levels of α2-3 sialic acids vs CD62L (**G**). Blood samples from
917 healthy donors (n = 7) were incubated for 2 h (37 °C, 5% CO_2) with 7% of fresh plasma
918 from healthy donors, severe or convalescent COVID-19 patients or with 7% of heat-
919 inactivated plasma from severe COVID-19 patients in the presence or absence of
920 Oseltamivir (100 μM) or Zanamivir (30 μM) and levels of CD66b (**H**), surface α2-3-Sia
921 (MAL-II) (**I**), and ROS production (**J, K**) were assessed by FACS. The MFI was analyzed
922 on $CD66b^+$ cells using the gate strategies shown in Supplementary Fig. 1. Symbols
923 represent individual donors and data are shown as scatter dot plot with line at median
924 from pooled data of two to seven independent experiments. The statistical significance
925 between the groups was assessed by ANOVA followed by a multiple comparisons test of
926 Tukey. The accepted level of significance for the test was $P<0.05$. * was significantly
927 different when compared with Untreated Severe COVID-19; # was significantly different
928 when compared with Untreated Heat-Inactivated Plasma from Severe COVID-19. Osel =
929 Oseltamivir; Zana = Zanamivir.
930

931 **Supplementary Data**

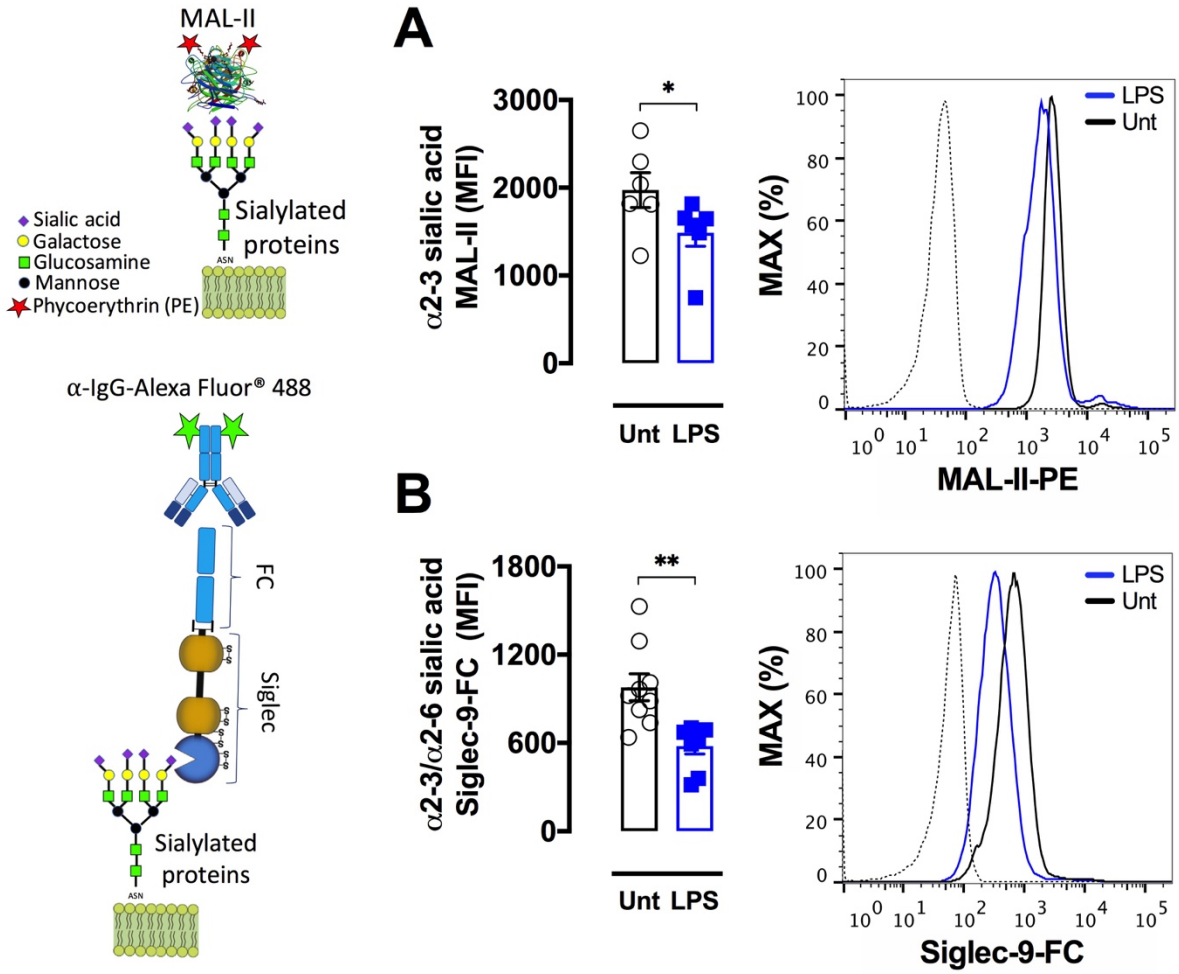
932 **Supplementary Figures and Figure Legends**

933

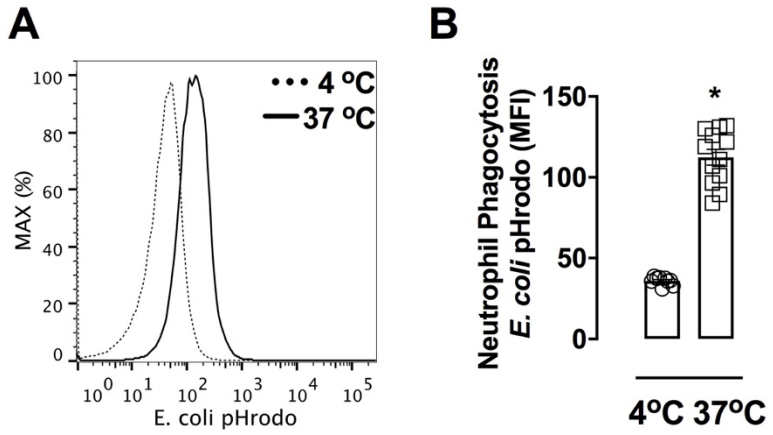


934

935 **Supplementary Fig. 1. Representative gate strategy used for neutrophils analysis.**
936 A classic forward-scatter (FSC) vs side-scatter (SSC) characteristic dot plot was used to
937 select neutrophils population (**A**) from peripheral blood collected from healthy donors
938 and patients. Autofluorescent (**B**) and doublets (**C-D**) were excluded and live cells were
939 selected (**E**). CD66b^{+} positive cells (**F**) were gated and the MFI of surface markers, such
940 as CD62L (**G**) were assessed. Around 96% of CD66b^{+} cells are mature neutrophils
941 (CD66b-high/CD16-high) and 4% of CD66b^{+} cells are CD66b-high/CD16-low, which is
942 suggestive of immature neutrophils or eosinophils (**H**). Approximately 100.000 gated
943 events were collected in each analysis. The analysis was performed in a FACSVerse
944 using FACSuite software (BD Biosciences) and FlowJo software (FlowJo LLC).



Supplementary Fig. 2. LPS reduces surface α2-3 sialic acids from human neutrophils. Whole blood containing 1 x 10^6 leukocytes from healthy donors were stimulated with 1 µg/mL LPS for 90 min and α2-3 sialic acid contents were assessed by staining cells with biotinylated *Maackia Amurensis* Lectin II (MAL-II) (**A**) followed by streptavidin-phycoerythrin (PE) incubation. Siglec-9 ligands (**B**) were labeled by incubation of chimeric protein containing Siglec-9 sialic acid-Ig binding domain fused to a human IgG-Fc portion (Siglec-9-Fc). Siglec-Fc-9 were incubated with α-IgG1-Alexa Fluor 488 before adding the probe to cells. The MFI was analyzed on CD66b$^+$ cells using the gate strategies shown in Supplementary Fig. 1. *P < 0.05. Symbols represent individual donors and data are shown as mean ± SEM from pooled data of two to three independent experiments (n=6-9). Unt = untreated cells; LPS = lipopolysaccharide; dotted line = unstained cells.



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960
961 **Supplementary Fig. 3. Phagocytosis of *E. coli* pHrodo bioparticles at 4 °C and 37**
962 **°C.** Total leukocytes (1 x10^6) were incubated with *E. coli* pHrodo bioparticles (100
963 µg/mL) for 60 min at 4°C or 37 °C and the phagocytosis in viable CD66b$^+$ cells was
964 assessed. Symbols represent individual donors and data are shown as mean ± SEM
965 from pooled data of three to four independent experiments (n = 9-12). *P < 0.001.
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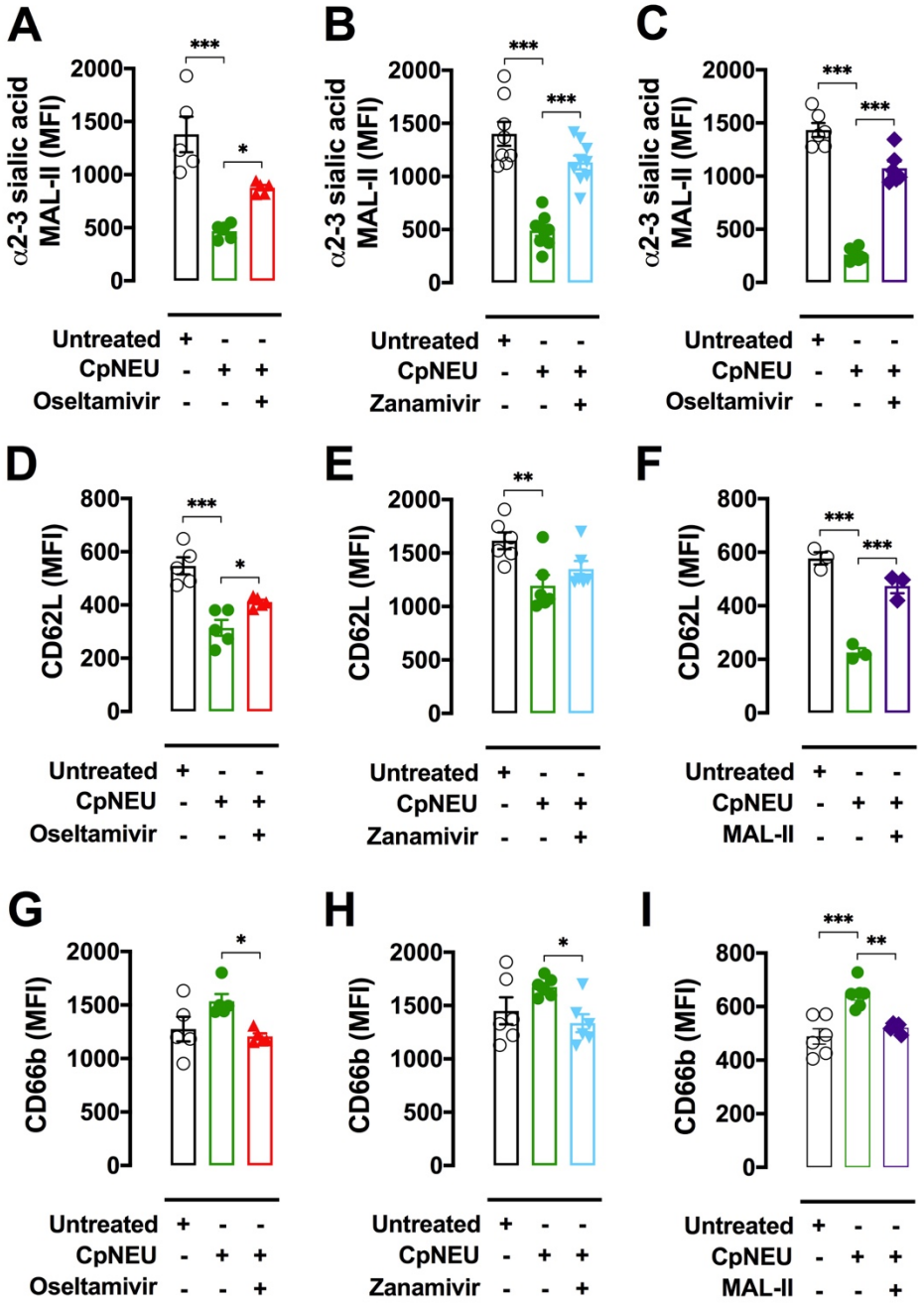

967

968 **Supplementary Fig. 4. CpNeu-induced human neutrophil activation.** Total
969 leukocytes (1 x 10^6) were incubated or not with CpNEU (10 mU, 60 min, 37 ^oC, 5%
970 CO_2) CpNEU plus Oseltamivir (100 μM), CpNEU plus Zanamivir (30 μM) or CpNEU plus
971 MAL-II (1 μg/mL). Leukocytes were stained with MAL-II to detect α2-3 sialic acids (**A-C**)
972 or with cell activation markers CD62L (**D-F**) and CD66b (**G-I**). The MFI was analyzed on
973 $CD66b^+$ cells. Symbols represent individual donors and data are shown as mean ± SEM

974 from pooled data of two to three independent experiments (n = 5-9) except for F that
975 was made once with n=3. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. MAL-II = *Maackia*
976 *amurensis* lectin II; CpNEU = neuraminidase *Clostridium perfringens*.
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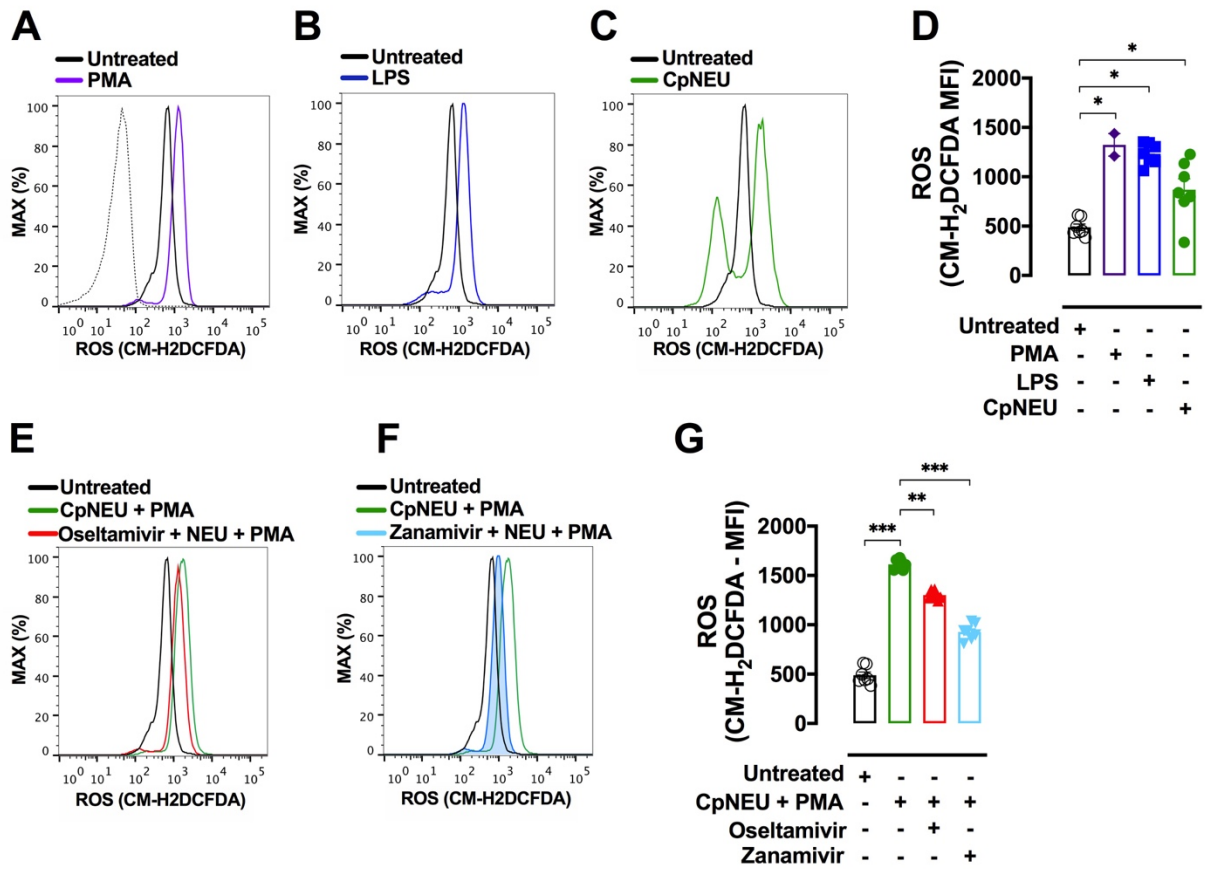
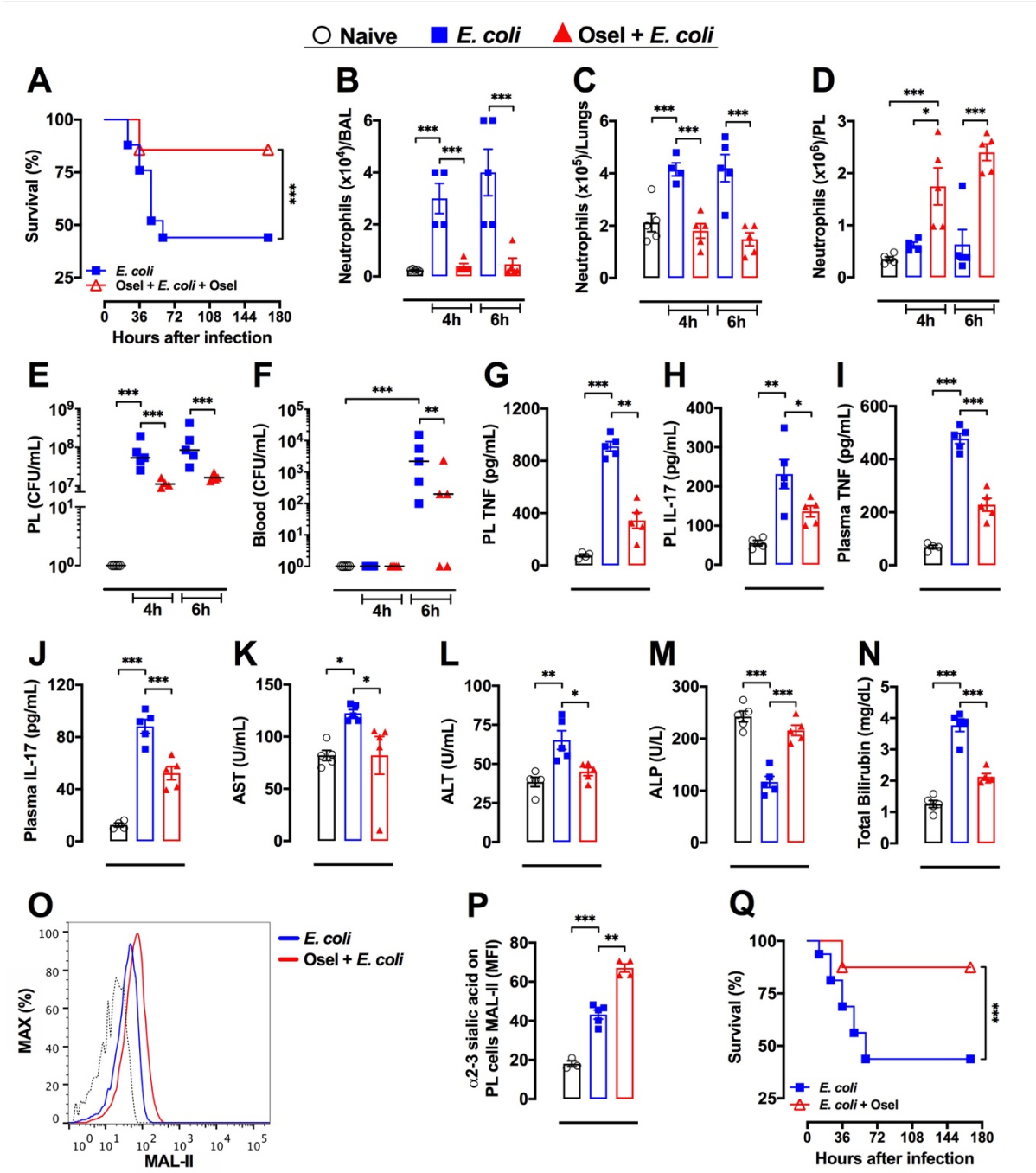
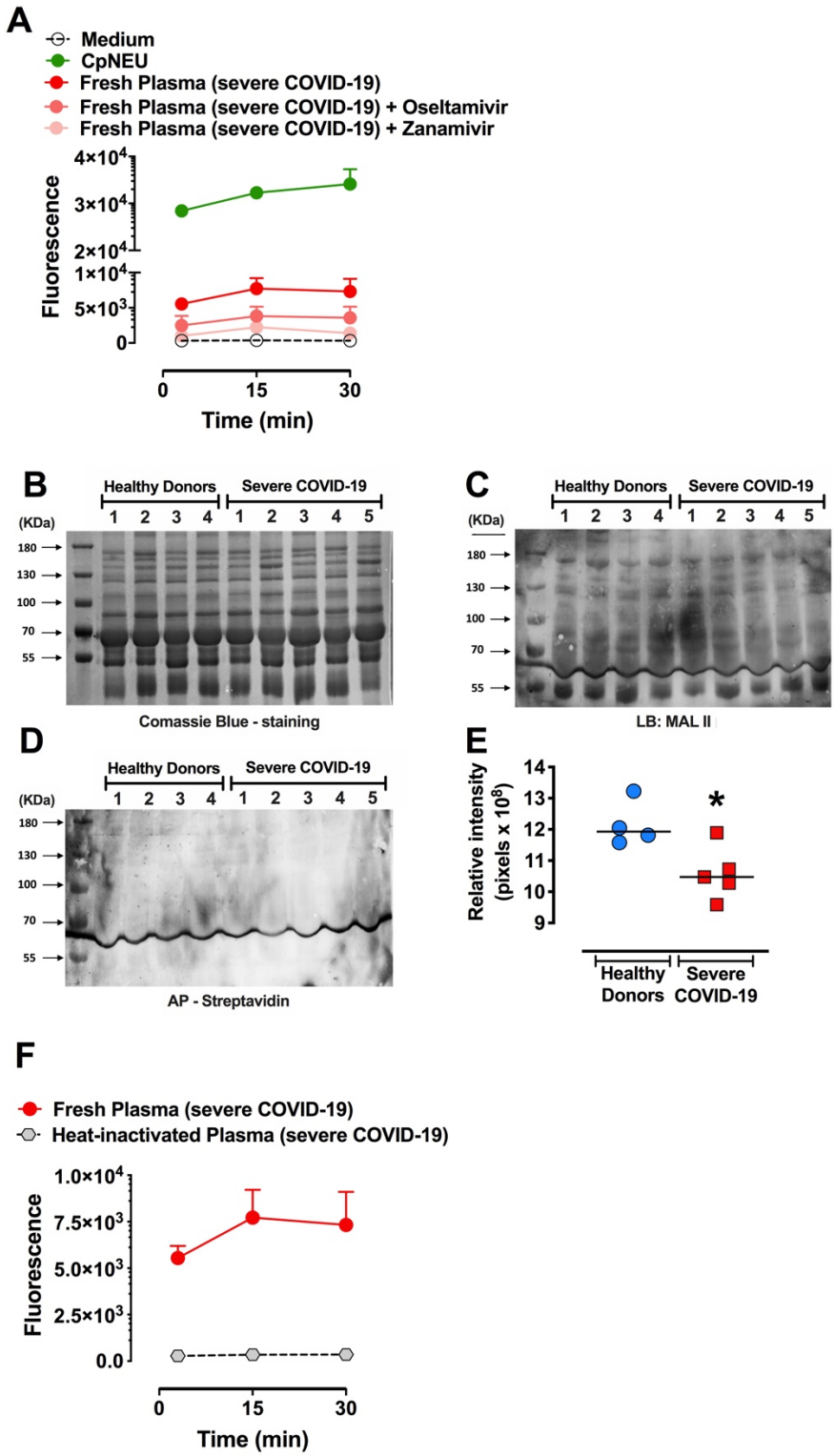


Supplementary Fig. 5. ROS production in neutrophils stimulated with LPS, CpNEU, or PMA. Whole blood from healthy donors containing 1 x 10^6 leukocytes were exposed or not to LPS (1 µg/mL, 90 min) (**B and D**). Total leukocytes (1 x 10^6) were incubated or not with CpNEU (10 mU, 60 min) (**C-D**), CpNEU plus Oseltamivir (100 µM) or CpNEU plus Zanamivir (30 µM) (**E-G**). Leukocytes were incubated with 5 µM CM-H2DCFDA fluorescent probe for 15 min and PMA (10 µM) was used to stimulate ROS production for 10 min (**A and E-G**). The MFI was analyzed on CD66b$^+$ cells**.** Symbols represent individual donors and data are shown as mean ± SEM from pooled data of two independent experiments (n = 2-6). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. C = control; CM-H2DCFDA = 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester; LPS = lipopolysaccharide; CpNEU = neuraminidase *Clostridium perfringens*; PMA = phorbol 12-myristate 13-acetate.



Supplementary Fig. 6. Oseltamivir improved the outcome of *E. coli*-induced sepsis. Sepsis was induced by intraperitoneal (IP) administration of 1 × 10^7 CFU/mice *E. coli* (ATCC 25922). Mice were randomly pretreated *per oral* (PO) via (2 hr before infection) and posttreated (6 hr after infection, 12/12 hr, PO, for 4 days) with Oseltamivir phosphate (Osel, 10 mg/Kg) or saline and their survival rates were monitored over 168 hr (**A,** n=16). In another set of experiments (n=3-5) mice were randomly pretreated (2 hr

before infection) with Oseltamivir phosphate (10 mg/Kg, PO) and the number of neutrophils in bronchoalveolar lavage (BAL, **B**) and in lung tissue (**C**) was counted. In peritoneal lavage (PL) infiltrating neutrophils counts (**D**), TNF (**G**), IL-17 (**H**) and the number of colony-forming units (CFU) in PL (**E**) or blood (**F**) were determined 4 or 6 hr after infection. Plasma levels of TNF (**I**), IL-17 (**J**), AST (**K**), ALT (**L**), ALP (**M**) and total bilirubin (**N**) were evaluated. The amount of surface α2-3 sialic acids were also assessed in PL SSC^{high}/Gr-1^{high} cells as shown by the representative histograms (**O**) or MFI (**P**); dotted line = unstained cells. Mice were also randomly posttreated (starting 6 hr after infection, 12/12 hr, PO, for 4 days) with saline or Oseltamivir phosphate (10 mg/Kg) and their survival rates were monitored over 168 hr (**Q**). The results are expressed as percent of survival (n=16), mean or median (only for FACS data) ± SEM. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. These experiments were repeated 3 times for survival analysis and twice for other parameters. Osel = Oseltamivir; AST = alanine aminotransferase; ALT = aspartate aminotransferase; ALP = alkaline phosphatase; MAL-II = *Maackia amurensis* lectin II.



Supplementary Fig. 7. NEU activity is increased in plasma from severe COVID-19 patients. NEU activity was evaluated in fresh plasma from severe COVID-19 patients in the presence or absence of Oseltamivir (100 μM) or Zanamivir (30 μM) (**A**) and in heat-

1018 inactivated plasma from COVID-19 patients (**F**). Neuraminidase isolated from
1019 *Clostridium perfringens* (CpNEU) was used to validate the NEU activity assay. Twelve
1020 µg of total serum proteins from healthy donors (n=4) or severe COVID-19 patients (n=5)
1021 were separated by SDS-PAGE 10% and stained with coomassie blue (**B**). Lectin
1022 blotting (LB) were performed with biotin-conjugated MAL-II for staining α2-3 sialic acid-
1023 containing proteins **(C)** or membrane were incubated with AP-Streptavidin without MAL-
1024 II (as a control) **(D).** The intensity of lectin staining of each lane was evaluated and
1025 normalized to the total proteins on corresponding gel lanes **(E)**; *p=0.02 Unpaired t test -
1026 Welch's correction**.** MAL-II = *Maackia amurensis* lectin II; CpNEU = neuraminidase
1027 *Clostridium perfringens*.
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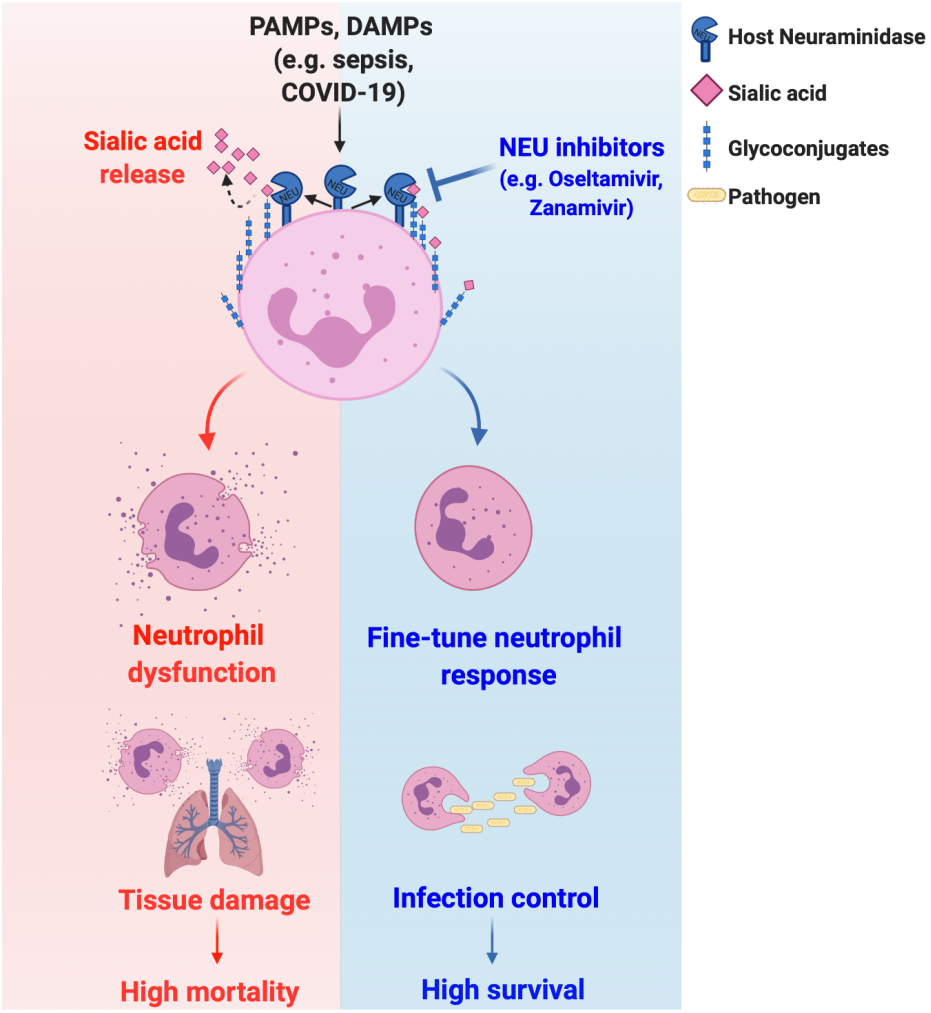

Supplementary Fig. 8. Working model. PAMPs and DAMPs in severe diseases such as sepsis and COVID-19 lead to neuraminidase activation with shedding of surface sialic acid and neutrophil overactivation, resulting in tissue damage and high mortality rates. On the other hand, neuraminidase inhibitors (e.g., Oseltamivir, Zanamivir) prevent the sialic acid release to regulates neutrophil response, resulting in infection control and high survival rates.

1040 **Supplementary Table 1.**

1041

1042 **Supplementary Table 1. Clinical information of samples from UFSC University**
1043 **Hospital, SC, Brazil**

1044

	Severe COVID-19	***Convalescent COVID-19***
Characteristic		
Gender (Male/Female)	3/2	7/5
Age (years)	44.4 (25;65)	53.2 (36;80)
Weight (kilos)	90.0 (62.2;127)	85.1 (51.8;150)
Height (m)	1.72 (1.65;1.8)	1.7 (1.55;1.83)
Cough	4 (80%)	8 (66.7%)
Dyspnea	4 (80%)	5 (41.7%)
Chest Pain / Oppression	1 (20%)	4 (33.3%)
Asthenia	2 (40%)	3 (16.7%)
Myalgia	3 (60%)	3 (16.7%)
Anosmia	1 (20%)	5 (41.7%)
Ageusia / Dysgeusia	1 (20%)	4 (33.3%)
Fever	3 (60%)	3 (16.7%)

Paresthesia	0 (0%)	0 (0%)
Headache	0 (0%)	2 (16.7%)
Diarrhea	0 (0%)	1 (8.3%)
Diabetes mellitus	3 (60%)	4 (33.3%)
Systolic BP (mmHg)	132.2 (100;150)	126.4 (100;177)
Diastolic BP (mmHg)	84.2 (80;90	78.55 (60;102)
Heart rate (bpm)	96.4 (80;115)	86.4 (72;115)
Respiratory frequency (bpm)	20.8 (18;25)	20.8 (11;29)
Length of hospitalization (days)	6.6 (4;10)	8.3 (3;16)
Mechanical ventilation	0 (0%)	3 (16.7%)
Mechanical ventilation time (days)	0 (0%)	7.3 (5;9)
Saturation (O2%)	90.4 (85;97)	93.3 (86;99)
SOFA Score	0.17 (0;1)	0.6 (0;2)
Laboratory data		
Hemoglobin (g/dL)	13.5 (11;15)	13.65 (11.3;15.7)
Hematocrit (%)	40 (33.4;47.1)	40.2 (34.8;45.5)

Leukocytes (/µL)	7988 (4070;12930)	8098 (5330;10660)
Neutrophils (/µL)	5731 (3355;10098)	6448.5 (3923;9135)
Lymphocytes (/µL)	1526 (451;3728)	955.8 (208;1687)
Monocytes (/µL)	369 (253;483)	473.2 (168;961)
Platelets (x10^3/µL)	235 (115:314)	238 (117;436)
PCR (mg / dL)	52.5 (22;78.7)	90.7 (15;214)

1048 **Supplementary Table 2. Clinical information of**
1049 **samples from Hospital Naval Marcílio Dias, RJ,**
1050 **Brazil**

Characteristic	*Severe COVID-19*
Gender (Male/Female)	2/3
Age (years)	66 (55-73)
Cough	4 (80%)
Dyspnea	4 (80%)
Chest Pain / Oppression	0 (0%)
Asthenia	1 (20%)
Myalgia	2 (40%)
Anosmia	2 (40%)
Ageusia / Dysgeusia	0 (0%)
Fever	4 (80%)
Paresthesia	0 (0%)
Headache	0 (0%)
Diarrhea	3 (60%)

Diabetes mellitus	3 (60%)
Systolic BP (mmHg)	141.4 (108;166)
Diastolic BP (mmHg)	74.8 (51;100)
Heart rate (bpm)	83.8 (57;102)
Respiratory frequency (bpm)	24 (14-40)
Length of hospitalization (days)	32.8 (10;90)
Mechanical ventilation	5 (100%)
Saturation (O2%)	87.3 (83;91)

Laboratory data	
Hemoglobin (g/dL)	11.92 (7.9;13.3)
Hematocrit (%)	35.72 (23;41.6)
Leukocytes (/μL)	7900 (5400;12100)
Neutrophils (/μL)	5724.8 (4266;7502)

Lymphocytes (/μL)	983.8 (430;1694)
Monocytes (/μL)	575.8 (216;1452)
Platelets (x10^3/μL)	172.8 (109;219)
PCR (mg / dL)	15.8 (5.16;26.8)

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