

1 **In plain sight: the role of alpha-1-antitrypsin in COVID-19 pathogenesis and**
2 **therapeutics.**

3
4 Kasopefoluwa Y Oguntuyo^{1#}, Christian S Stevens^{1#}, Mohammed NA Siddiquey²,
5 Robert M Schilke², Matthew D Woolard², Hongbo Zhang², Joshua A Acklin¹, Satoshi
6 Ikegame¹, Chuan-Tien Huang¹, Jean K Lim¹, Robert W Cross³, Thomas W Geisbert³,
7 Stanimir S Ivanov², Jeremy P Kamil², and Benhur Lee¹

8

9 Affiliations:

- 10 1. Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York,
11 NY 10029
12 2. Department of Microbiology and Immunology, Louisiana State University Health
13 Shreveport, Shreveport, LA 71103
14 3. Department of Microbiology and Immunology, University of Texas Medical
15 Branch at Galveston, Galveston, TX 77555
16

17 #These authors contributed equally to this work.

18

19 Author contributions:

20 KYO, CSS and BL conceived and designed the study. KYO, CSS, MNAS, RMS, MDW,
21 HZ, and JAA collected data. SI, CTH, JKL, RWC, TWG, SSI, and JPK contributed
22 valuable reagents, data and/or tools. KYO and CSS analyzed the data and wrote the
23 original drafts of the paper. BL reviewed the draft, supported data analysis, and
24 provided invaluable direction throughout the conceptualization and execution of the
25 project. All authors had the opportunity to review the manuscript prior to submission and
26 JAA, SI, JKL, RWC, TWG, SSI, JPK provided valuable feedback during the editing
27 process.

28 **ABSTRACT**

29 Entry of SARS-CoV-2 is facilitated by endogenous and exogenous proteases. These
30 proteases proteolytically activate the SARS-CoV-2 spike glycoprotein and are key
31 modulators of virus tropism. We show that SARS-CoV-2 naïve serum exhibits significant
32 inhibition of SARS-CoV-2 entry. We identify alpha-1-antitrypsin (AAT) as the major
33 serum protease inhibitor that potently restrict protease-mediated entry of SARS-CoV-2.
34 AAT inhibition of protease-mediated SARS-CoV-2 entry in vitro occurs at concentrations
35 far below what is present in serum and bronchoalveolar tissues, suggesting that AAT
36 effects are physiologically relevant. Moreover, AAT deficiency affects up to 20% of the
37 population and its symptomatic manifestations coincides with many risk factors
38 associated with severe COVID-19 disease. In addition to the effects that AAT may have
39 on viral entry itself, we argue that the anti-inflammatory and coagulation regulatory
40 activity of AAT have implications for coronavirus disease 2019 (COVID-19)
41 pathogenicity, SARS-CoV-2 tissue restriction, convalescent plasma therapies, and even
42 potentially AAT therapy.

43

44 **KEYWORDS:** COVID-19, SARS-CoV-2, Alpha-1-antitrypsin, SERPINA1, alpha-2-
45 macroglobulin, TMPRSS2, protease, convalescent plasma

46

47 The COVID-19 pandemic that began in December 2019 has resulted in tens of millions
48 of cases with hundreds of thousands of deaths. The central conundrum of this
49 pandemic is the heterogeneity of disease severity in SARS-CoV-2 infected individuals.
50 The widespread disparities in outcomes has spurred global efforts to better understand
51 SARS-CoV-2 pathogenesis, investigate the factors contributing to the clinical course of
52 COVID-19, and develop viable therapeutics. One of the most promising therapeutic
53 targets is the Spike (S) glycoprotein of SARS-CoV-2, which bears the fusion machinery
54 necessary to mediate viral entry. Vaccines, monoclonal antibodies and convalescent
55 plasma therapy are all premised upon neutralizing SARS-CoV-2 spike (CoV2-S)
56 mediated entry. All three modalities are being developed at unprecedented speed.
57 Unfortunately, the lack of standardized virus neutralization assays (VNAs) or reporting
58 metrics have made it difficult to compare the efficacy across the proliferating number of
59 vaccine platforms and CoV2-S targeted treatment modalities.

60

61 **Standardizing a SARS-CoV-2 Viral Neutralization Assay**

62 In an accompanying study, we worked to develop a scalable, standardized VNA that
63 reflects the complex interplay between CoV2-S receptor interaction and proteolytic
64 activation. To that end we generated and validated VSV Δ G pseudotyped particles
65 bearing SARS-CoV-2 spike (CoV2pp).¹ We initially optimized infection conditions in
66 serum free media. Due to the role of proteolytic activation in SARS-CoV-2 entry, we
67 utilized exogenous trypsin as well as soybean trypsin inhibitor to maximize entry of the
68 CoV2pp while limiting cytotoxicity. These conditions were designed noting that
69 proteolytic activation of CoV2-S is required for the receptor-induced conformational

70 changes that result in virus-cell membrane fusion and viral entry. Several endosomal,
71 cell surface, and exogenous proteases, including furin, select cathepsins and
72 TMPRSSs, and trypsin-like proteases have been implicated in mediating these
73 cleavage events for SARS-CoV-2 and enhancing cellular entry (Fig. 1A).²⁻⁴ A similar
74 body of evidence also indicates that these proteases and others, such as elastase, play
75 critical roles in the productive processing of the S protein from SARS-CoV-1, MERS-
76 CoV, and other betacoronaviruses.⁵⁻⁷ In the case of MERS-CoV, proteolytic processing
77 of spike is capable of dictating cell tropism and correlates well with virulence.⁸ For
78 SARS-CoV-2, the role of cell surface protease-mediated entry is of sufficient importance
79 that TMPRSS2 overexpression can render some cell lines refractory to chloroquine
80 mediated inhibition of virus entry.⁹

81
82 To standardize our VNA, we initially utilized trypsin-treated CoV2pp for human serum
83 neutralization experiments. However, we observed that under these conditions, sera
84 from patients not exposed to SARS-CoV-2 was capable of neutralizing these
85 pseudoviruses, though, as expected, to a lesser extent than sera from patients that
86 tested positive for SARS-CoV-2 antibodies (Fig. 1B)¹.

87
88 **Uncovering AAT: a heat-labile, CoV2pp neutralizing factor in SARS-CoV-2 naïve**
89 **serum**

90 To pre-empt the variable neutralizing effect of seronegative serum, we diluted our
91 trypsin-treated CoV2pp in DMEM containing 10% fetal bovine serum (FBS). However,
92 while this provided an easy solution in standardizing our assay for “out-of-the-box” use

93 when sent to multiple labs, it left the question of “why” unanswered. This neutralizing
94 effect of SARS-CoV-2 naïve human sera was ameliorated through heat-inactivation by
95 incubation at 56°C for 1hr (Fig. 1B), and also appeared to be specific to CoV2-S
96 mediated entry as VSV-G pseudotyped particles (VSV-Gpp) were unaffected. Thus, the
97 serum neutralizing factor(s) was unlikely to be cross-reactive antibodies to seasonal
98 coronaviruses or complement *per se*. We therefore suspected a heat-labile serum
99 factor(s) capable of inhibiting trypsin.¹⁰

100

101 To validate these observations, we tested a panel of non-heat-inactivated human sera
102 for neutralization activity against CoV2pp and VSV-Gpp. SARS-CoV-2 seropositive and
103 seronegative sera were diluted in SFM (Supplemental Fig. 1A). Here, we observed
104 neutralization of trypsin-treated CoV2pp by both seronegative and seropositive sera
105 (Fig. 2A and Supplemental Fig. 1B). Importantly, VSV-Gpp was not inhibited by the
106 samples tested (Fig. 2B and Supplemental Fig. 1C), once again showing that this effect
107 was CoV2pp-specific. At the same time, an external group at Louisiana State University
108 Health Shreveport (LSUHS) independently observed neutralization by seronegative
109 sera under similar experimental conditions using our CoV2pp (Fig. 2C, Supplemental
110 Fig. 1D and 1E). Remarkably, in both groups, SARS-CoV-2 naïve sera inhibited
111 CoV2pp entry by 90-97% (Fig. 2A and 2C, left panels). Nonetheless, seropositive
112 patient sera showed inhibition orders of magnitude beyond this threshold, suggesting
113 antibody mediated inhibition of CoV2pp entry (Fig. 2A and C, right panels). Additionally,
114 using the identical serum samples in Fig. 2C, collaborators at the University of Texas
115 Medical Branch at Galveston (UTMB) observed modest, but significant, neutralization of

116 live virus by seronegative sera as assayed by a plaque reduction neutralization assay
117 (Fig. 2D and Supplemental Fig. 1F).

118

119 Upon making these repeated observations, we searched the literature for highly
120 abundant and heat labile products in serum and identified alpha-1-antitrypsin (AAT) and
121 alpha-2-macroglobulin (A2M) as potential candidates.¹⁰ These blood products are
122 typically present in human serum at high concentrations (1.1-2.2 mg/mL for AAT and 2-
123 4 mg/mL for A2M) and have been described to inhibit both exogenous and endogenous
124 proteases.¹¹⁻¹³ Despite the careful characterizations of the role endogenous and
125 exogenous proteases play in SARS-CoV-2 entry, there have been limited
126 characterizations of the role *in vivo* protease inhibitors play in modulating SARS-CoV-2
127 entry. A2M and AAT alone are responsible for approximately 10% and 90% of serum
128 antiprotease capacity, respectively.¹⁴

129

130 A2M functions to inhibit a broad range of proteases, such as serine and cysteine
131 proteases. In addition to protease inhibitory functions, A2M also inhibits thrombin to
132 prevent coagulation and binds to growth factors and cytokines. No clinical conditions
133 have yet been associated with low plasma levels of A2M.¹¹ On the other hand, AAT is a
134 protease inhibitor that irreversibly binds serine proteases and plays additional roles in
135 the regulation of inflammation and coagulation.¹⁵ Notably, decreased plasma
136 concentrations of or function of AAT have been associated with liver and lung disease,
137 particularly pulmonary emphysema due to unregulated neutrophil elastase activity.¹²

138 Mutations leading to these conditions are highly prevalent as nearly 20% of individuals
139 have non-wildtype AAT alleles.¹³

140

141 To assess whether AAT and/or A2M alone could inhibit trypsin-treated CoV2pp entry,
142 we added each at the time of infection and observed potent entry inhibition by AAT and
143 modest inhibition by A2M, with IC₅₀s of 14.47µg/mL and 54.20µg/mL, respectively (Fig.
144 3A, left panel). Importantly, neither protein inhibited VSV-Gpp (Fig. 3A, right panel).
145 Albumin, the most abundant protein in blood, showed no significant reduction of entry of
146 either CoV2pp or VSV-Gpp (Fig. 3A), which underscores that the inhibitory effects of
147 AAT and A2M on CoV2-S mediated entry was specific.

148

149 While these findings suggest that AAT, and to a lesser extent A2M, can inhibit
150 exogenous trypsin-like proteases known to enhance SARS-CoV-2 entry, tissue
151 restriction of SARS-CoV-2 infection is also mediated by proteases at the cell surface.^{2,3}
152 Therefore, we sought to investigate whether either protein could inhibit TMPRSS2, an
153 endogenous serine protease implicated in SARS-CoV-2 pathogenicity. We previously
154 engineered two ultra-permissive 293T clones stably expressing ACE2 (clone 5-7) or
155 ACE2+TMPRSS2 (clone F8-2). Each of these lines was capable of highly efficient
156 CoV2pp entry in the absence of trypsin pre-treatment. To assess entry inhibition by AAT
157 and A2M, we performed VNAs in both clones using CoV2pp without any trypsin pre-
158 treatment but diluted in standard media (DMEM+10% FBS). Here, we observed that
159 AAT inhibited CoV2pp entry into TMPRSS2 expressing F8-2 clones, but not the 5-7
160 clones (Fig. 3B). For both cell lines, A2M and albumin both displayed no entry inhibition

161 at the concentrations tested. As additional controls, we observe potent inhibition of
162 CoV2pp by sRBD in both cell lines and, as expected, Nafamostat mesylate, a serine
163 protease inhibitor, inhibited entry in only the TMPRSS2-expressing F8-2 clones
164 (Supplemental Fig. 2). Notably, AAT and Nafamostat inhibition of CoV2pp entry into
165 TMPRSS2-expressing F8-2 cells approached a maximal inhibition of ~80%, suggesting
166 that SARS-CoV-2 can enter via other pathways noted in Fig. 1A.

167

168 Together, these observations show that not only can SARS-CoV-2 naïve sera potently
169 inhibit protease-mediated entry of SARS-CoV-2, but AAT and A2M appear to be
170 potential regulators of protease-mediated entry by SARS-CoV-2. In concert with the
171 large body of literature about AAT and the proteolytic processing of coronavirus spike
172 proteins, we are led to three distinct, but important hypotheses. The first is that AAT and
173 A2M may play a biologically relevant role in tissue restriction in SARS-CoV-2 infection.
174 The second is that, if the first hypothesis is true, variant AAT genotypes could influence
175 the relative severity of COVID-19. And thirdly, treatment with the already FDA-approved
176 AAT, could play a role in simultaneously controlling viral burden as well as aberrant
177 immune responses.

178

179 **The suspected roles of protease inhibitors as uncharacterized players in COVID-** 180 **19 pathogenesis and therapy.**

181 In an accompanying paper, we reported that a factor, or factors, in SARS-CoV-2
182 seronegative sera is capable of inhibiting trypsin-mediated entry of CoV2pp. Here, we
183 pinpoint AAT and A2M as highly abundant serum factors that inhibit the effect of

184 exogenous proteases on CoV2pp entry. Moreover, at the concentrations tested, we
185 show that AAT, but not A2M, can inhibit TMPRSS2-mediated entry of CoV2pp at sub-
186 physiologic concentrations. Two recent pre-peer review reports support these findings.
187 Azouz et al utilized a fluorescent cleavage assay in a screen that identified four small
188 molecules and AAT as inhibitors of TMPRSS2 enzymatic activity.¹⁶ Wettstein et al
189 report neutralization of SARS-CoV-2 by polypeptides extracted from pooled
190 bronchoalveolar lavage (BAL) fluids and, after fractionation and mass spectrometry,
191 identify AAT as a component driving this activity.¹⁷ Although previously reported, the
192 identification of AAT in the BAL confirms that it can diffuse into lung tissues and
193 suggests that it can be present at the site of SAR-CoV-2 infection and replication. They
194 also show that while the fraction containing AAT inhibits >99% of infection, there is
195 another set of fractions that inhibit just under 90% of infection. We speculate that these
196 fractions may contain A2M which we show to be a less potent inhibitor of trypsin-
197 mediated CoV2pp entry, perhaps due to the requirement of a tetramer to trap two
198 proteases (Fig. 3A).¹¹ Interestingly, this lack of potency may be offset by its broader
199 protease inhibition potential, particularly for cysteine proteases such as Cathepsin B
200 and L, which have been reported to play a role in endosomal mediated SARS-CoV-2
201 entry. Additionally, elastase—a serine protease released by neutrophils—has been
202 previously reported to play a role in enhancing SARS-CoV-1 and MERS entry.⁷
203 Although the role of elastase in SARS-CoV-2 entry has not been elucidated, elevation of
204 neutrophil counts in BAL and serum have been consistently associated with severe
205 COVID-19 cases.^{18–20} In spite of its name, AAT has a stronger binding affinity to

206 elastase than trypsin, and this is borne out by the clinically significant sequelae
207 associated with AAT deficiency.¹⁵

208

209 Though the findings presented here focus on protease inhibitors' ability to inhibit
210 protease-mediated SARS-CoV-2 entry, the inhibitors play significant additional roles.

211 While others have speculated that neutrophil elastase should be considered as a target
212 for potential COVID-19 prophylactics, AAT was not noted specifically.²¹ AAT, as an

213 acute phase protein, has been characterized to play roles in modulating inflammation by

214 inhibiting elastase among other factors. Elastase is critical for the formation of neutrophil

215 extracellular traps (NETs) in acute pneumonia, which can amplify inflammatory

216 responses if not resolved by AAT. Runaway pulmonary inflammation and NETosis is an

217 emerging theme in COVID-19 pathogenesis.²² AAT is also known to modulate activities

218 that result in downstream IL-6 inhibition, which is heavily implicated in COVID-19

219 pathogenicity.^{12,23} Seeking to capitalize on these anti-inflammatory roles and the already

220 well established use of recombinant AAT to treat AAT deficiency, McElvaney et al

221 recently reported the initiation of a clinical trial for AAT treatment of COVID-19 based on

222 their published work.²⁴ Moreover, AAT has regulatory roles in the coagulation cascade²⁵

223 and, via elastase inhibition, could inhibit NET-triggered immunothromboses.²⁶ Notably,

224 inflammatory dysregulation and coagulopathies have been reported to play a role in the

225 disparate COVID-19 severities between patients.²⁷

226

227 The potent neutralization of protease-mediated cellular entry by SARS-CoV-2 along with

228 the wide range and prevalence of functionally different AAT genotypes implies a

229 potential role for AAT in variable COVID-19 severity. Of particular note, nearly 20% of
230 individuals are either heterozygous or homozygous for non-wildtype alleles, many of
231 which have been described to result in reduced levels of AAT in the blood or reduced
232 AAT function.¹³ Although many mutations have been characterized, new mutations that
233 impair abundance or function are still being identified. For example, AAT variants such
234 as G373R or the R223C (F allele) have been identified and reported to have wildtype
235 levels in the blood, but impaired inhibitory activity against neutrophil elastase.^{28,29} We
236 speculate that this CoV2pp VNA assay may be capable of serving as a scalable means
237 by which one could screen for deficient AAT functionality and not simply abundance.
238 Considering that widespread screening of AAT is rarely performed in the absence of
239 emphysema, it is reasonable to expect that there may be more unidentified mutations
240 that impair abundance or function of AAT, which may subsequently result in aberrant
241 response to SARS-CoV-2 infection. This undesired response by individuals with
242 functional AAT deficits may enable effective viral entry, dysregulated inflammation,
243 and/or coagulopathies (Fig. 4). It also raises the possibility that AAT may represent a
244 novel therapeutic approach in the fight against SARS-CoV-2.²⁵

245

246 However, in the case of convalescent plasma therapies, the presence of AAT in blood
247 plasma from donors may play a beneficial role as non-immunoglobulin products are
248 currently not excluded from transfused plasma. In line with this, a recent meta-analysis
249 from Joyner et al suggests that convalescent plasma therapy is beneficial to its
250 recipients.³⁰ While the purported benefits are attributed to the presence of neutralizing
251 antibodies, the authors also acknowledge that “other biological mechanisms” may

252 contribute to these observations. Additionally, a recent bioRxiv report shows modest
253 benefits from the transfer of standard IVIG, suggesting that neutralizing antibodies are
254 not the only serum components that may play a role in alleviating the burden of COVID-
255 19 in patients.³¹ In its myriad roles, the transfer of AAT may provide additional benefits
256 to convalescent plasma recipients by inhibiting SARS-CoV-2 entry, restraining
257 inflammation and/or moderating coagulation.

258

259 In sum, these findings highlight the importance of protease inhibitors in restricting
260 exogenous and/or endogenous protease-mediated enhancement of SARS-CoV-2 entry.
261 We also speculate that the diversity of AAT genotypes, the complex regulation of its
262 activity, and its myriad roles in inflammation and coagulation, implicates functional AAT
263 levels in COVID-19 pathogenicity. There is an urgent need to address:

- 264 1) The biologically relevant roles AAT, A2M, or other proteases play in tissue
265 restriction in SARS-CoV-2 infection.
- 266 2) The impact of variant AAT genotypes on the relative severity of COVID-19.
- 267 3) Whether treatment with, the already FDA-approved, AAT, may be able to play
268 a role in simultaneously controlling viral burden as well as aberrant immune
269 responses and if simultaneous treatment with A2M should be considered
270 given their ability to form inhibitor complexes.³²

271

272 **METHODS**

273 Maintenance and generation of isogenic cell lines:

274 Vero-CCL81, parental 293T, and isogenic 293T cells were cultured in DMEM with 10%
275 heat inactivated FBS at 37°C in the presence of 5% CO₂. Isogenic 293T cell clones 5-7
276 and F8-2 were generated by lentivirus transduction to stably express ACE2 only or
277 ACE2 and TMPRSS2, respectively. ACE2 expression was under puromycin selection
278 and TMPRSS2 was under blasticidin selection as previously described.

279

280 Production of VSV Δ G pseudotyped particles and neutralization studies:

281 Detailed protocols for the production and use of standardized VSVpp (CoV2pp and
282 VSV-Gpp) are given in Oguntuyo and Stevens et al.¹ Briefly, 293T producer cells were
283 transfected to express the viral surface glycoprotein of interest, infected with VSV Δ G-
284 rLuc-G* reporter virus, then virus supernatant collected and clarified 2 days post
285 infection prior to use. Trypsin treated CoV2pp were treated as previously described.¹ All
286 pseudotyped viruses (PsV) were aliquoted prior to storage at -80°C and tittered prior to
287 usage for neutralization experiments. Neutralization experiments were performed by
288 diluting the appropriate PsV with a 4-fold serial dilution of Albumin (Sigma-Aldrich,
289 A8763), alpha-1-antitrypsin (Sigma-Aldrich, SRP6312), alpha-2-Macroglobulin (Sigma-
290 Aldrich, SRP6314) or Nafamostat mesylate (Selleckchem, S1386). SARS-CoV-2
291 soluble RBD (sRBD) with human IgG-Fc was produced by the Lee Lab using a
292 recombinant Sendai virus expression platform (manuscript in preparation). De-identified
293 patient sera were obtained via institutional biobanks that allowed use for research

294 purposes. All infections were processed for detection of Renilla luciferase activity at
295 20hrs post-infection, and luminescence was read on the Cytation3 (BioTek).

296

297 Plaque reduction neutralization titration (PRNT) by sera of SARS-CoV-2:

298 Neutralization experiments with live virus were performed by incubating sera with 50-
299 100 PFU of SARS-CoV-2 for one hour at 37°C. All sera were diluted in serum free
300 DMEM. Serial dilutions started at a four-fold dilution and went through seven three-fold
301 serial dilutions. The virus-serum mixture was then used to inoculate Vero E6 cells for
302 one hour at 37°C and 5% CO₂. Cells were overlaid with EMEM medium (no FBS) and
303 1.25% Avicel, incubated for 3 days, and plaques were counted after staining with 1%
304 crystal violet in formalin.

305

306 **ACKNOWLEDGMENTS**

307 The authors acknowledge the following funding: KYO and CS were supported by Viral-
308 Host Pathogenesis Training Grant T32 AI07647; KYO was additionally supported
309 by F31 AI154739. BL acknowledges flexible funding support from NIH grants R01
310 AI123449, R21 AI1498033, and the Department of Microbiology and the Ward-Coleman
311 estate for endowing the Ward-Coleman Chairs at the ISMMS. JPK and SSI
312 acknowledge funding from a LSUHS COVID-19 intramural grant. JPK and SSI
313 acknowledge additional funding from NIH grants AI116851 and AI143839, respectively.
314 BL wishes to dedicate this paper to Ernest L Robles-Levroney, the first graduate student
315 BL had the privilege to train. Ernie Robles-Levroney was dedicated teacher, role model

316 and trailblazer who passed away unexpectedly during the course of writing this

317 manuscript.

318

319 **FIGURES / FIGURE LEGENDS**

320

321 **Figure 1. Overview of SARS-CoV-2 entry and inhibition of trypsin treated CoV2pp**
322 **entry by COVID seronegative sera. (A)** Overview of SARS-CoV-2 entry. Three modes
323 of entry are displayed: (1) Entry mediated by endosomal proteases, such as Cathepsin
324 B, (2) entry mediated by cell surface proteases, such as TMPRSS2, and (3) entry
325 mediated by exogenous proteases, such as trypsin. This model was created in
326 Biorender. **(B)** Inhibition of trypsin treated CoV2pp entry by COVID seronegative sera.
327 Presented is a schematized version of the results presented in supplemental Figure 3A
328 of our previous publication. For this experiment, sera samples were incubated with
329 trypsin treated CoV2pp for 30mins prior to addition to Vero-CCL81 cells. Both the
330 CoV2pp and the sera samples were diluted in serum free media. The grey lines
331 represent COVID seronegative sera, they purple lines are COVID seropositive sera, and
332 the dashed lines are samples that were heat inactivated (HI) for 1hr at 56°C prior to use
333 for CoV2pp neutralizations.

334

335 **Figure 2. Negative patient sera inhibit exogenous protease mediated**
336 **enhancement of CoV2pp. (A)** SARS-CoV-2 seronegative sera inhibit trypsin treated
337 CoV2pp. Seronegative or seropositive samples were first identified based on IgG
338 antibodies against Spike (Supplemental Fig. 1). The indicated sera diluted in serum-free
339 DMEM were incubated with a pre-titered amount of CoV2pp prior to spinoculation on
340 Vero-CCL81 cells as described.¹ Sera were not heat inactivated before use in our
341 neutralization assays. Normalized infection data at the highest and lowest dilutions

342 tested are shown as % maximal infection (media only) with results from seronegative
343 (left) and seropositive (right) sera plotted on linear and log scale, respectively. Data
344 points represent the mean of neutralizations performed in triplicate. Dilutions shown
345 were compared using a paired t test (ns, not significant; **, $p < 0.01$; ***, $p < 0.005$, and
346 ****, $p < 0.0001$). Full neutralization curves are shown in Supplemental Fig. 1. **(B)**
347 SARS-CoV-2 seronegative sera do not inhibit VSV-Gpp. Experiment performed and
348 presented as in Fig. 2A. **(C)** Inhibition of trypsin treated CoV2pp entry by SARS-CoV-2
349 seronegative sera independently observed. Collaborators in a different state
350 independently performed the identical experiment described in Fig. 1A with their own
351 cohort of seropositive and seronegative samples. Data shown are means from technical
352 quadruplicates/sample/dilution and presented exactly as for Fig. 2A. **(D)** Live SARS-
353 CoV-2 is modestly inhibited by seronegative sera. Sera samples presented in Fig. 2C
354 were utilized for plaque reduction neutralization experiments (PRNT) with live virus (left
355 panel) as described in the materials and methods. Presented here are the mean of one
356 experiment done in technical duplicates and error bars show SEM. Data presented as in
357 Fig. 2A.

358

359 **Figure 3. Alpha-1-antitrypsin (AAT) and alpha-2-macroglobulin (A2M) inhibit**
360 **protease mediated enhancement of CoV2pp entry. (A)** AAT and A2M inhibit trypsin-
361 mediated enhancement of CoV2pp entry. Trypsin treated CoV2pp (left panel) and
362 standard VSV-Gpp (right) were diluted in serum free media, then used to infect Vero-
363 CCL81 cells in the presence of the indicated concentrations of albumin, AAT, or A2M.
364 Data are from two independent experiments and are presented as percent relative

365 infection where each concentration was normalized to the lowest concentration of the
366 test reagent used. Data fit as described in Fig. 1A. **(B)** AAT inhibits TMPRSS2-mediated
367 enhancement of CoV2pp entry. CoV2pp not treated with trypsin were diluted in
368 DMEM+10% FBS and utilized to infect 293T-ACE2+TMPRSS2 clone F8-2 (left panel) or
369 293T-ACE2 clone (5-7) in the presence of the indicated concentrations of A2M, AAT, or
370 Albumin. Data points are means +/- SEM a representative experiment performed in
371 triplicates, but otherwise presented as described as in Fig. 3A.

372

373 **Figure 4. Putative relationship between alpha-1-antitrypsin function and SARS-**
374 **CoV-2.** Differential alpha-1-antitrypsin (AAT) abundance and/or function may result in a
375 differential response to infection by SARS-CoV-2. Due to our observations that AAT can
376 inhibit CoV2pp protease-mediated entry, we expect that in the presence of functional
377 AAT (light blue, right) there is only modest amounts of SARS-CoV-2 protease-mediated
378 entry relative to those with AAT functional deficiencies (orange, left). In addition to its
379 entry effects, we speculate that normal AAT abundance and/or function may reign in an
380 otherwise dysregulated immune response. Moreover, AAT's role in regulation of the
381 coagulation cascade may further prevent the development of coagulopathies. For the
382 latter, a normal immune response to infection is indicated by a single up arrow and the
383 absence of coagulopathy is indicated by a dash. Figure generated in Biorender.

384

385 **Supplemental Figure 1. Spike ELISA data and full neutralization curves. (A)** Spike
386 ectodomain ELISAs for JBA and JBB samples. Our seronegative and seropositive
387 samples were utilized. ELISAs performed as previously described^(ref) and shown are the

388 OD490 values from the 1:100 sera dilution. Shown are the median and interquartile
389 range. **(B)** Full neutralization curves from trypsin treated CoV2pp. Data points are mean
390 +/- SEM from experiment done in triplicates. **(C)** Full neutralization curves from VSV-
391 Gpp. Presented are the means of an experiment in technical triplicate with error bars
392 showing SEM. **(D)** Spike ectodomain ELISAs from LSA and LSB samples. Twelve
393 seropositive and twelve seronegative samples were utilized. Shown are the OD450
394 values from the 1:100 sera dilution. **(E)** Full neutralization curves from LSU CoV2pp
395 neutralization are shown here. **(F)** Live virus full neutralization curves. Live virus
396 neutralizations performed as described in the Methods and the same samples as in
397 Supplemental Fig. 1E were used. Presented here are the means of one experiment
398 done in technical duplicate and error bars show SEM and data were fit using variable
399 slope, 4-parameter logistics regression curve (robust fitting method).

400

401 **Supplemental Figure 2. Nafamostat mesylate inhibits CoV2pp entry into**
402 **TMPRSS2 expressing cells.** CoV2pp were mixed with a serial dilution of either
403 Nafamostat or sRBD prior to infection of isogenic cells stably expressing
404 ACE2+TMPRSS2 (clone F8, left panel) or ACE2 (clone 5-7, right panel). Presented
405 here are the results of an experiment done in technical triplicates. Error bars show SEM
406 and data were fit using variable slope, 4-parameter logistics regression curve (robust
407 fitting method).

408 **REFERENCES**

409

- 410 1. Oguntuyo, K. Y. and Stevens, C. *et al.* Quantifying absolute neutralization titers
411 against SARS-CoV-2 by a standardized virus neutralization assay allows for
412 cross-cohort comparisons of COVID-19 sera. *medRxiv* (2020).
- 413 2. Hoffmann, M. *et al.* SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2
414 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* **181**, 271-280.e8
415 (2020).
- 416 3. Zhu, F. C. *et al.* Proteolytic activation of the SARS-coronavirus spike protein:
417 Cutting enzymes at the cutting edge of antiviral research. *J. Virol.* **395**, 379–397
418 (2020).
- 419 4. Shang, J. *et al.* Cell entry mechanisms of SARS-CoV-2. *Proc. Natl. Acad. Sci.*
420 **117**, 11727–11734 (2020).
- 421 5. Shulla, A. *et al.* A Transmembrane Serine Protease Is Linked to the Severe Acute
422 Respiratory Syndrome Coronavirus Receptor and Activates Virus Entry. *J. Virol.*
423 **85**, 873–882 (2011).
- 424 6. Menachery, V. D. *et al.* Trypsin Treatment Unlocks Barrier for Zoonotic Bat
425 Coronavirus Infection. *J. Virol.* **94**, (2019).
- 426 7. Belouzard, S., Madu, I. & Whittaker, G. R. Elastase-mediated Activation of the
427 Severe Acute Respiratory Syndrome Coronavirus Spike Protein at Discrete Sites
428 within the S2 Domain. *J. Biol. Chem.* **285**, 22758–22763 (2010).
- 429 8. Park, J. E. *et al.* Proteolytic processing of Middle East respiratory syndrome
430 coronavirus spikes expands virus tropism. *Proc. Natl. Acad. Sci.* **113**, 12262–

- 431 12267 (2016).
- 432 9. Hoffmann, M. *et al.* Chloroquine does not inhibit infection of human lung cells with
433 SARS-CoV-2. *Nature* (2020). doi:10.1038/s41586-020-2575-3
- 434 10. Sriram Vemuri, C. Tony Yu, and N. R. Formulation and Stability of Recombinant
435 Alpha-1-Antitrypsin. in *Stability and Characterization of Protein and Peptide Drugs*
436 (eds. Wang, Y. J. & Pearlman, R.) **5**, 263–285 (Springer US, 1993).
- 437 11. Rehman, A. A., Ahsan, H. & Khan, F. H. Alpha-2-macroglobulin: A physiological
438 guardian. *J. Cell. Physiol.* **228**, 1665–1675 (2013).
- 439 12. Blanco, I. *Alpha-1 Antitrypsin Biology. Blanco's Overview of Alpha-1 Antitrypsin*
440 *Deficiency 1*, (Elsevier Inc., 2017).
- 441 13. Bornhorst, J. A., Greene, D. N., Ashwood, E. R. & Grenache, D. G. α 1-Antitrypsin
442 phenotypes and associated serum protein concentrations in a large clinical
443 population. *Chest* **143**, 1000–1008 (2013).
- 444 14. de Serres, F. & Blanco, I. Role of alpha-1 antitrypsin in human health and
445 disease. *J. Intern. Med.* **276**, 311–335 (2014).
- 446 15. Strnad, P., McElvaney, N. G. & Lomas, D. A. Alpha 1 -Antitrypsin Deficiency. *N.*
447 *Engl. J. Med.* **382**, 1443–1455 (2020).
- 448 16. Azouz, N. P., Klingler, A. M. & Rothenberg, M. E. Alpha 1 Antitrypsin is an
449 Inhibitor of the SARS-CoV2–Priming Protease TMPRSS2. *bioRxiv* (2020).
450 doi:10.1101/2020.05.04.077826
- 451 17. Wettstein, L. *et al.* Alpha-1 antitrypsin inhibits SARS-CoV-2 infection. *bioRxiv*
452 (2020). doi:10.1101/2020.07.02.183764
- 453 18. Zhou, Z. *et al.* Heightened Innate Immune Responses in the Respiratory Tract of

- 454 COVID-19 Patients. *Cell Host Microbe* **27**, 883-890.e2 (2020).
- 455 19. Lucas, C. *et al.* Longitudinal analyses reveal immunological misfiring in severe
456 COVID-19. *Nature* (2020). doi:10.1038/s41586-020-2588-y
- 457 20. Liao, M. *et al.* Single-cell landscape of bronchoalveolar immune cells in patients
458 with COVID-19. *Nat. Med.* **26**, 842–844 (2020).
- 459 21. Mohamed, M. M. A., El-Shimy, I. A. & Hadi, M. A. Neutrophil Elastase Inhibitors: A
460 potential prophylactic treatment option for SARS-CoV-2-induced respiratory
461 complications? *Crit. Care* **24**, 311 (2020).
- 462 22. Barnes, B. J. *et al.* Targeting potential drivers of COVID-19: Neutrophil
463 extracellular traps. *J. Exp. Med.* **217**, (2020).
- 464 23. Grifoni, E. *et al.* Interleukin-6 as prognosticator in patients with COVID-19. *J.*
465 *Infect.* (2020). doi:10.1016/j.jinf.2020.06.008
- 466 24. McElvaney, O. J. *et al.* Characterization of the Inflammatory Response to Severe
467 COVID-19 Illness. *Am. J. Respir. Crit. Care Med.* (2020).
468 doi:10.1164/rccm.202005-1583OC
- 469 25. Janciauskiene, S. & Welte, T. Well-Known and Less Well-Known Functions of
470 Alpha-1 Antitrypsin. Its Role in Chronic Obstructive Pulmonary Disease and Other
471 Disease Developments. *Ann. Am. Thorac. Soc.* **13**, S280–S288 (2016).
- 472 26. Middleton, E. A. *et al.* Neutrophil Extracellular Traps (NETs) Contribute to
473 Immunothrombosis in COVID-19 Acute Respiratory Distress Syndrome. *Blood*
474 (2020). doi:10.1182/blood.2020007008
- 475 27. Jose, R. J. & Manuel, A. COVID-19 cytokine storm: the interplay between
476 inflammation and coagulation. *Lancet Respir. Med.* **8**, e46–e47 (2020).

- 477 28. Cook, L., Burdon, J. G. W., Brenton, S., Knight, K. R. & Janus, E. D. Kinetic
478 characterisation of alpha-1-antitrypsin F as an inhibitor of human neutrophil
479 elastase. *Pathology* **28**, 242–247 (1996).
- 480 29. Laffranchi, M. *et al.* Characterisation of a type II functionally-deficient variant of
481 alpha-1-antitrypsin discovered in the general population. *PLoS One* **14**, e0206955
482 (2019).
- 483 30. Joyner, M. J. *et al.* Early Safety Indicators of COVID-19 Convalescent Plasma in
484 5,000 Patients. *medRxiv* (2020). doi:10.1101/2020.05.12.20099879
- 485 31. Sakoulas, G. *et al.* Intravenous Immunoglobulin (IVIG) Significantly Reduces
486 Respiratory Morbidity in COVID-19 Pneumonia: A Prospective Randomized Trial.
487 *medRxiv* (2020). doi:10.1101/2020.07.20.20157891
- 488 32. Dejgaard, S., Ortapamuk, O. & Özer, I. The Trypsin-Inhibitory Efficiency of Human
489 α 2 -Macroglobulin in the Presence of α 1 -Proteinase Inhibitor: Evidence for the
490 Formation of an α 2 -Macroglobulin- α 1 -Proteinase Inhibitor Complex. *J. Enzyme*
491 *Inhib.* **14**, 391–405 (1999).
- 492

Figure 1. Overview of SARS-CoV-2 entry and inhibition of trypsin treated CoV2pp entry by COVID-19 seronegative sera.

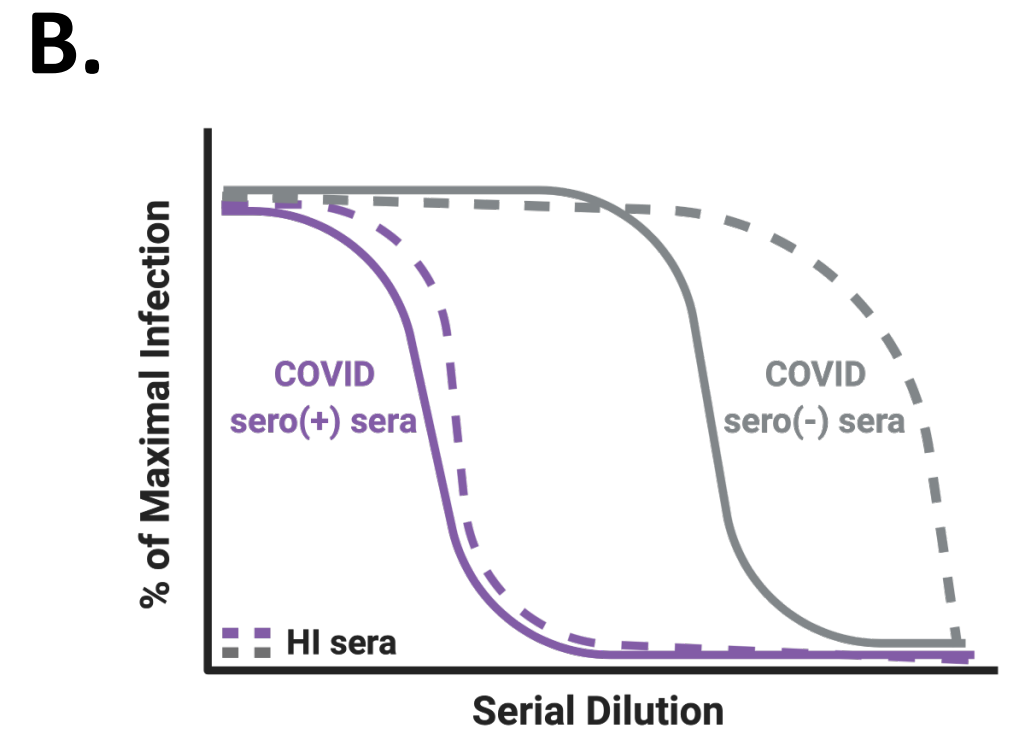
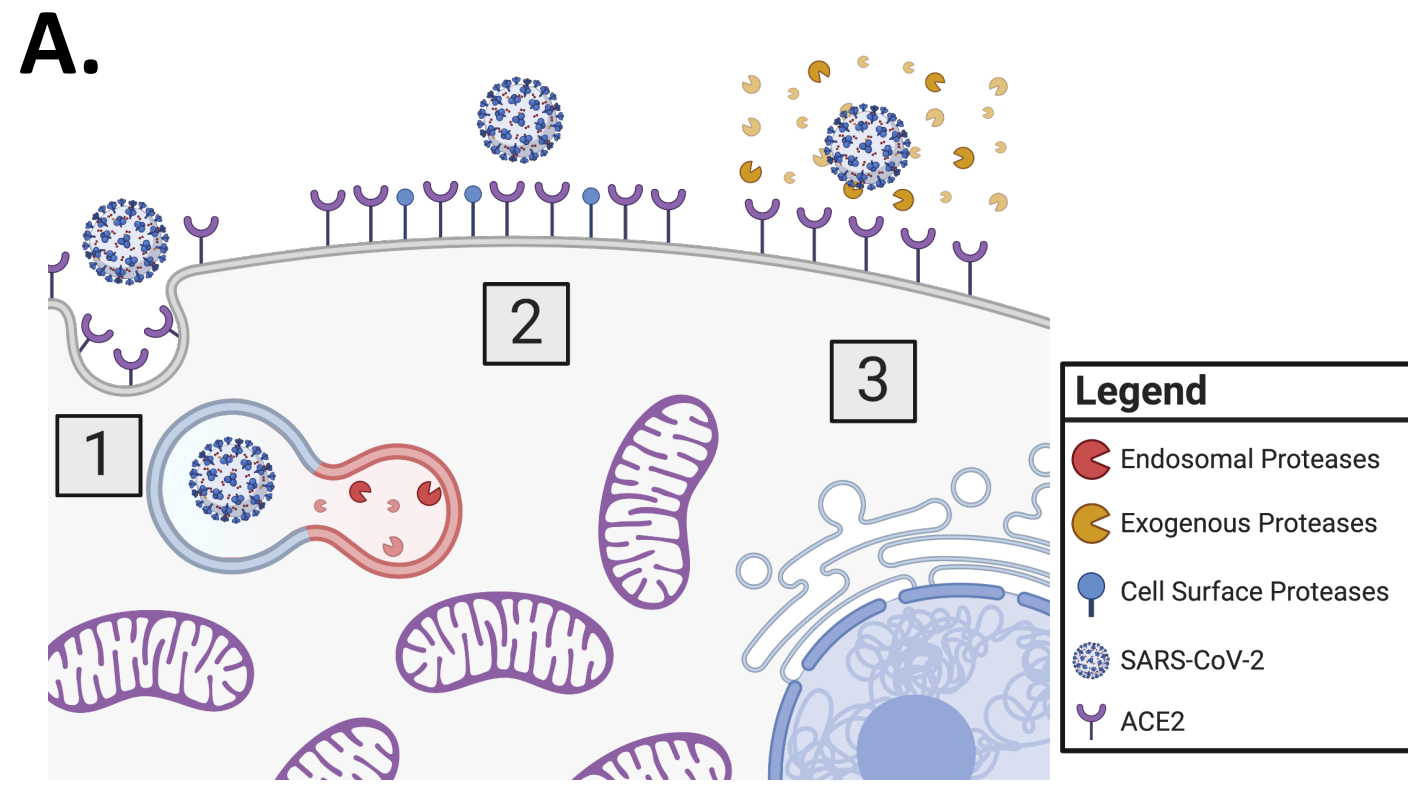
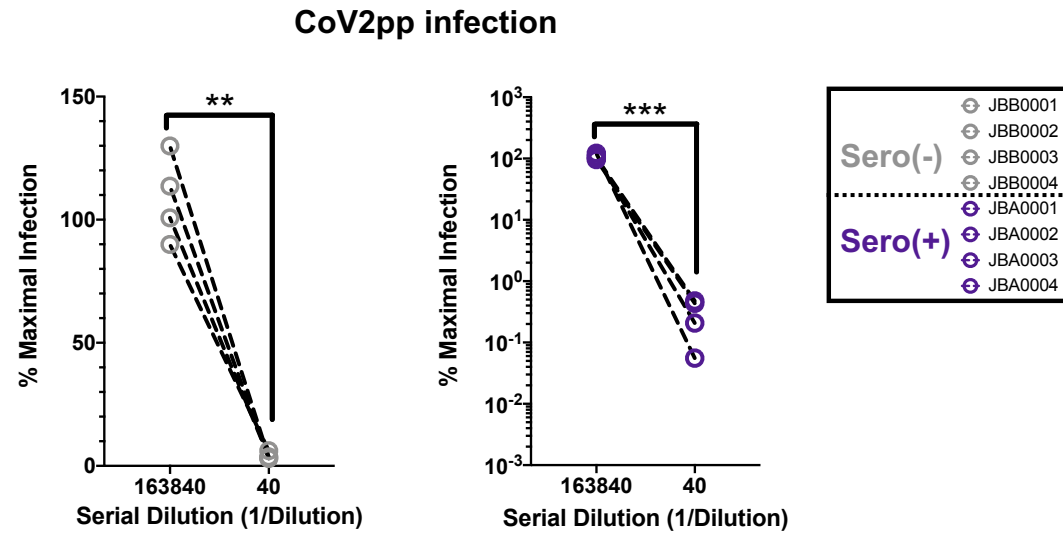
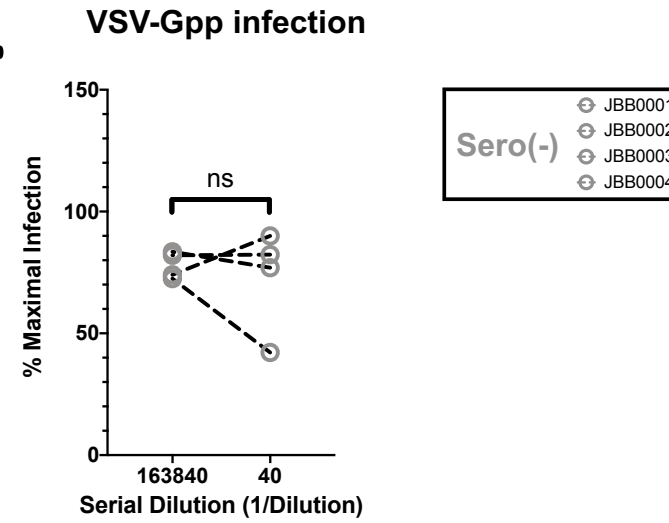


Figure 2. Negative patient sera inhibit exogenous protease mediated enhancement of CoV2pp.

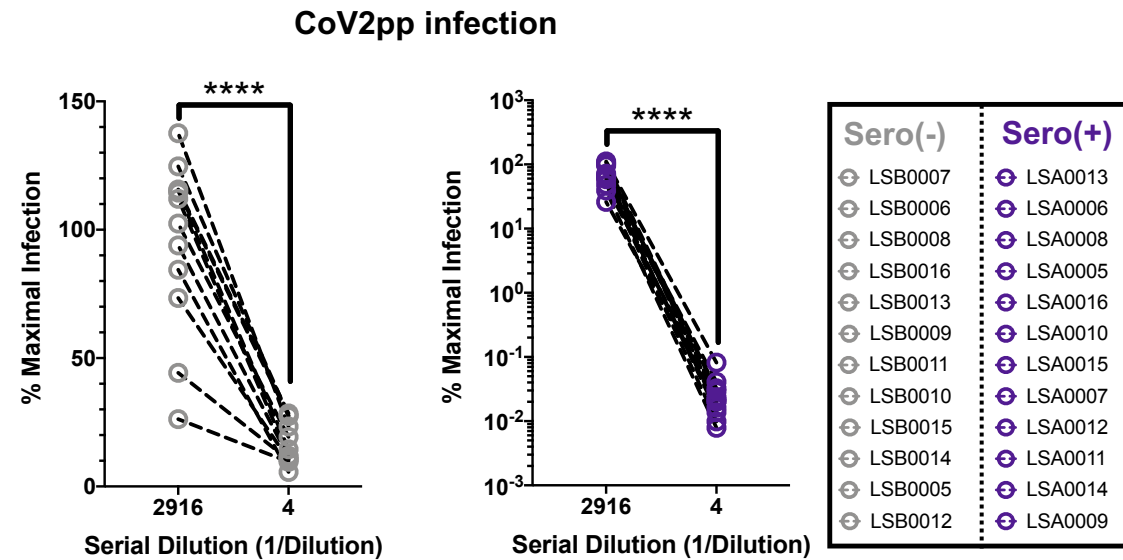
A.



B.



C.



D.

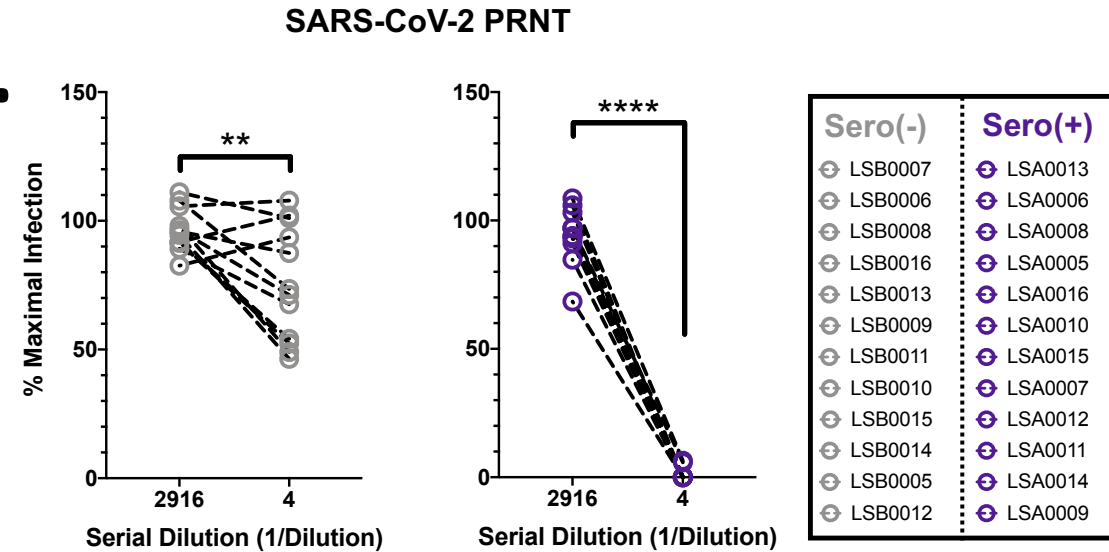
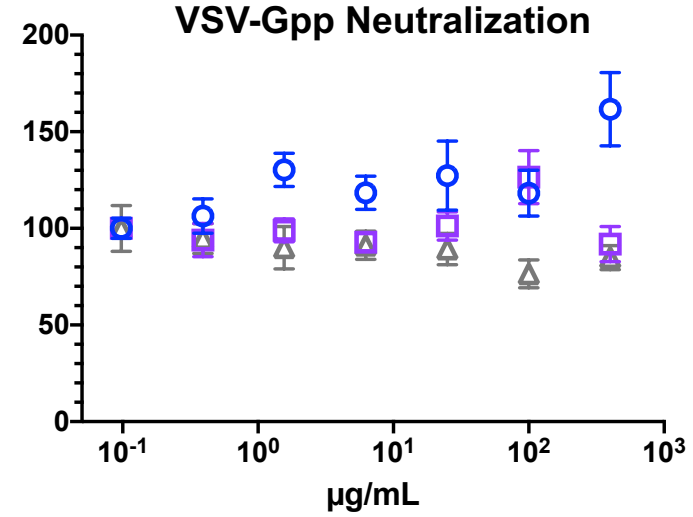
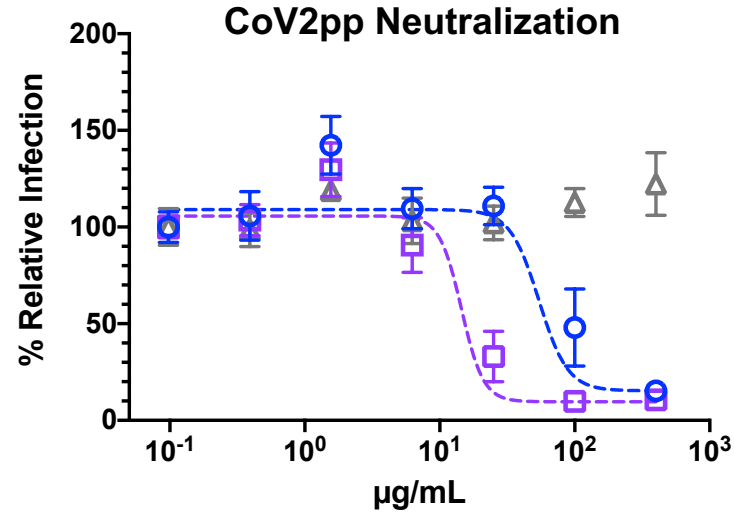


Figure 3. Alpha-1-antitrypsin (AAT) and alpha-2-macroglobulin (A2M) inhibit protease mediated enhancement of CoV2pp entry.

A.



B.

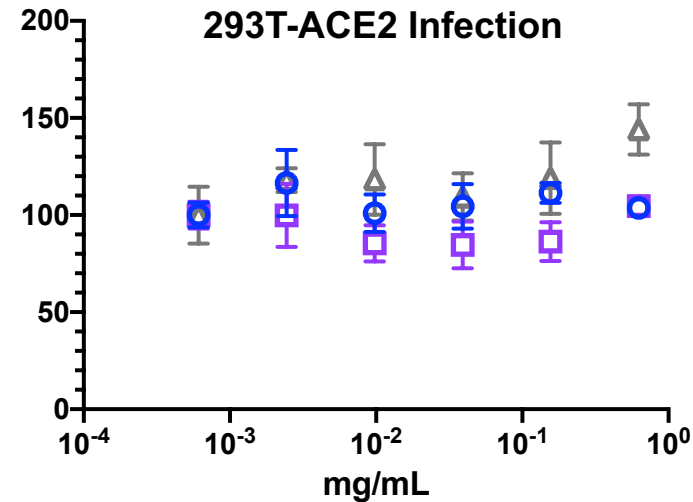
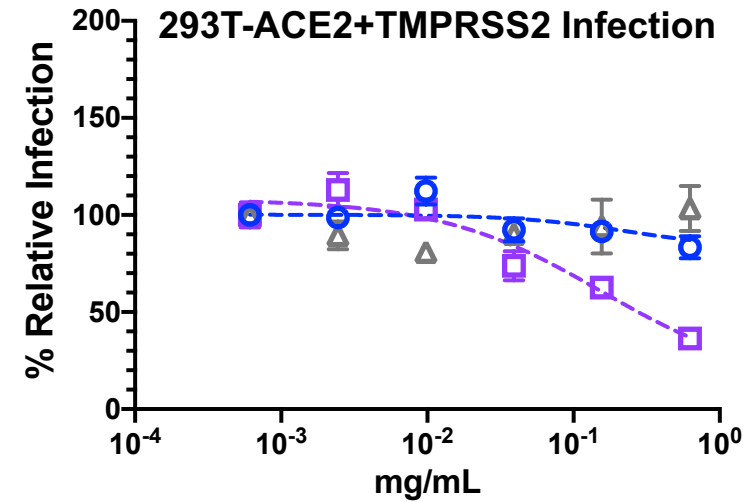


Figure 4. Putative relationship between alpha-1-antitrypsin function and SARS-CoV-2.

