

1 **SARS-CoV-2 infection activates dendritic cells via cytosolic receptors rather than extracellular TLRs**

2 Short title: SARS-CoV-2 infection required for immunity

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18

19 **Abstract**

20 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes coronavirus disease 2019  
21 (COVID-19), an infectious disease characterized by strong induction of inflammatory cytokines,  
22 progressive lung inflammation and potentially multi-organ dysfunction. It remains unclear whether  
23 SARS-CoV-2 is sensed by pattern recognition receptors (PRRs) leading to immune activation. Several  
24 studies suggest that the Spike (S) protein of SARS-CoV-2 might interact with Toll-like receptor 4 (TLR4)  
25 and thereby activate immunity. Here we have investigated the role of TLR4 in SARS-CoV-2 infection  
26 and immunity. Neither exposure of isolated S protein, SARS-CoV-2 pseudovirus nor a primary SARS-  
27 CoV-2 isolate induced TLR4 activation in a TLR4-expressing cell line. Human monocyte-derived  
28 dendritic cells (DCs) express TLR4 but not ACE2, and DCs were not infected by a primary SARS-CoV-2  
29 isolate. Notably, neither S protein nor the primary SARS-CoV-2 isolate induced DC maturation or  
30 cytokines, indicating that both S protein and SARS-CoV-2 virus particles do not trigger extracellular  
31 TLRs, including TLR4. Ectopic expression of ACE2 in DCs led to efficient infection by SARS-CoV-2.  
32 Strikingly, infection of ACE2-positive DCs induced type I IFN and cytokine responses, which was  
33 inhibited by antibodies against ACE2. These data strongly suggest that not extracellular TLRs but  
34 intracellular viral sensors are key players in sensing SARS-CoV-2. These data imply that SARS-CoV-2  
35 escapes direct sensing by TLRs, which might underlie the lack of efficient immunity to SARS-CoV-2 early  
36 during infection.

37

38 **Author summary**

39 The immune system needs to recognize pathogens such as SARS-CoV-2 to initiate antiviral immunity.  
40 Dendritic cells (DCs) are crucial for inducing antiviral immunity and are therefore equipped with both  
41 extracellular and intracellular pattern recognition receptors to sense pathogens. However, it is  
42 unknown if and how SARS-CoV-2 activates DCs. Recent research suggests that SARS-CoV-2 is sensed  
43 by extracellular Toll-like receptor 4 (TLR4). We have previously shown that DCs do not express ACE2,  
44 and are therefore not infected by SARS-CoV-2. Here we show that DCs do not become activated by  
45 exposure to viral Spike proteins or SARS-CoV-2 virus particles. These findings suggest that TLR4 and  
46 other extracellular TLRs do not sense SARS-CoV-2. Next, we expressed ACE2 in DCs and SARS-CoV-2  
47 efficiently infected these ACE2-positive DCs. Notably, infection of ACE2-positive DCs induced an  
48 antiviral immune response. Thus, our study suggests that infection of DCs is required for induction of  
49 immunity, and thus that intracellular viral sensors rather than extracellular TLRs are important in  
50 sensing SARS-CoV-2. Lack of sensing by extracellular TLRs might be an escape mechanism of SARS-CoV-  
51 2 and could contribute to the aberrant immune responses observed during COVID-19.

52

## 53 **Introduction**

54 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a novel coronavirus that causes  
55 coronavirus disease 2019 (COVID-19)(1). COVID-19 emerged in 2019 in Wuhan, China(2), and has since  
56 spread globally causing a pandemic. The symptoms of COVID-19 vary amongst individuals, ranging  
57 from mild respiratory symptoms to severe lung injury, multi-organ dysfunction and death(3-6).  
58 Increasing evidence suggests that disease severity depends not solely on viral infection, but also on an  
59 excessive host pro-inflammatory response, whereby high concentrations of pro-inflammatory  
60 cytokines result in an unfavorable immune response and induce tissue damage(7, 8). The events  
61 leading to excessive pro-inflammatory responses are not completely understood. Therefore, it is  
62 necessary to elucidate the mechanisms that are triggered by SARS-CoV-2 to induce innate and adaptive  
63 immune responses.

64 Innate immune cells express pattern recognition receptors (PRRs) that recognize pathogen-  
65 associated molecular patterns (PAMPs) and subsequently orchestrate an immune response against  
66 pathogens(9). Dendritic cells (DCs) are essential immune cells that function as a bridge between innate  
67 and adaptive immunity. DCs express various PRR families such as Toll-like receptors (TLRs) and  
68 cytosolic RIG-I-like receptors (RLRs) that are triggered upon virus interaction or infection(10). DCs are  
69 therefore essential during SARS-CoV-2 infection to sense infection and instruct T and B cells for  
70 efficient antiviral immune responses. However, it is unclear whether and how SARS-CoV-2 is sensed by  
71 DCs.

72 SARS-CoV-2 Spike (S) protein uses angiotensin converting enzyme 2 (ACE2)(11, 12) as receptor  
73 for infection. However, besides interacting with ACE2, recent *in silico* analyses suggest that the Spike  
74 (S) protein could also potentially interact with members of the TLR family, in particular TLR4(13, 14).  
75 TLR4 is abundantly expressed on DCs(15, 16), and therefore TLR4 signaling could be involved in  
76 induction of pro-inflammatory mediators. Other studies using cell lines and SARS-CoV-2 S protein  
77 support a potential interaction of TLR4 with the S protein(17-19). However, it remains unclear whether

78 infectious SARS-CoV-2 virus is sensed by TLR4 and whether this interaction induces DC activation and  
79 initiation of immunity.

80           Here, we have investigated how SARS-CoV-2 is sensed by human DCs. Neither recombinant S  
81 protein, SARS-CoV-2 pseudovirus nor a primary SARS-CoV-2 isolate induced immunity in TLR4-  
82 expressing cell lines or DCs, indicating that TLR4 or other extracellular TLRs are not involved in SARS-  
83 CoV-2 infection. However, ectopic expression of ACE2 on DCs led to infection by SARS-CoV-2 and  
84 induction of type I interferon (IFN) and cytokines. These data imply that intracellular PRRs rather than  
85 transmembrane TLRs are involved in instigating an immune response against SARS-CoV-2.

86

87 **Results**

88 *SARS-CoV-2 S protein does not trigger TLR4*

89 To assess whether TLR4 acts as a sensor of S protein of SARS-CoV-2, we treated a TLR4-expressing  
90 HEK293 cell line (293/TLR4) with SARS-CoV-2 recombinant S protein or S nanoparticles(20) and  
91 determined activation by measuring interleukin (IL)-8. Neither S protein nor S nanoparticles induced  
92 IL-8 secretion by 293/TLR4 cells, in contrast to the positive control LPS (Fig 1A). The parental 293 cells  
93 did not induce IL-8 upon treatment with S protein or S nanoparticle and LPS. These data suggest that  
94 S protein of SARS-CoV-2 does not trigger TLR4.

95 Primary monocyte-derived DCs express TLR4 but also other TLRs(21). We therefore exposed primary  
96 human DCs to SARS-CoV-2 S nanoparticles and assessed cytokine production by qPCR. Treatment of  
97 DCs with S nanoparticles did neither induce type I interferon (IFN) nor cytokines. (Fig 1B-E). The positive  
98 control LPS induced IFN $\beta$  (Fig 1B) and the interferon-stimulated gene (ISG) APOBEC3G (A3G) (Fig 1C)  
99 as well as cytokines IL-6 and IL-10 (Fig 1D, E). These data strongly suggest that S protein from SARS-  
100 CoV-2 does not trigger extracellular TLRs on DCs.

101

102 *SARS-CoV-2 virus particles do not trigger TLR4*

103 To assess whether TLR4 plays a role in SARS-CoV-2 entry and replication, we ectopically expressed  
104 ACE2 on 293 and 293/TLR4 cell lines and infected the cells with SARS-CoV-2 pseudovirus that expresses  
105 the full-length S glycoprotein from SARS-CoV-2 and contains a luciferase reporter gene(22). Infection  
106 was determined by measuring luciferase activity. SARS-CoV-2 pseudovirus infected ACE2-positive 293  
107 and 293/TLR4 cells but not the parental 293 and 293/TLR4 cells (Fig 2A). TLR4 expression did not affect  
108 infection, as infection was comparable between 293/ACE2 and 293/TLR4/ACE2 cells.

109 Next we investigated whether SARS-CoV-2 pseudovirus activates TLR4. SARS-CoV-2 pseudovirus did  
110 neither induce IL-8 in parental 293 nor in 293/TLR4 cells (Fig 2B). Moreover, ACE2 expression did not  
111 induce activation as exposure of ACE2-positive 293 and 293/TLR4 cells to SARS-CoV-2 pseudovirus did

112 not lead to IL-8 production (Fig 2B). These data further support the findings that S protein from SARS-  
113 CoV-2 does not trigger TLR4 and also show that ACE2 does not affect TLR4 signaling.

114 Next, we treated either ACE2-positive or -negative 293 and 293/TLR4 cells with a primary SARS-  
115 CoV-2 isolate (hCoV-19/Italy) and determined infection and activation. Infection was determined by  
116 measuring virus particles in the supernatant by qPCR. As expected, both 293/ACE2 and 293/TLR4/ACE2  
117 cells were productively infected at similar levels by SARS-CoV-2, in contrast to ACE2-negative 293 and  
118 293/TLR4 cells (cutoff Ct values >30), (Fig 2C). Neither ACE2-positive nor -negative 293 and 293/TLR4  
119 cells expressed any IL-8 upon exposure to the primary SARS-CoV-2 isolate (Fig 2D). These data strongly  
120 suggest that TLR4 does not sense infectious SARS-CoV-2 virus particles.

121

#### 122 *Infectious SARS-CoV-2 does not activate DCs*

123 Subsequently, we examined whether SARS-CoV-2 pseudovirus induces DC maturation and cytokine  
124 production. DCs do not express ACE2 and we have previously shown that SARS-CoV-2 pseudovirus does  
125 not infect DCs(23). We investigated the maturation and cytokine production by DCs stimulated with  
126 SARS-CoV-2 pseudovirus. Exposure of DCs to SARS-CoV-2 pseudovirus did neither induce expression of  
127 costimulatory markers CD80 and CD86 nor maturation marker CD83, in contrast to LPS (Fig 3A-D).  
128 Moreover, SARS-CoV-2 pseudovirus did not induce any cytokines, in contrast to LPS (Fig 3E-H). These  
129 data indicate that the S protein expressed by SARS-CoV-2 pseudovirus does not activate DCs.

130 Next, we exposed DCs to a primary SARS-CoV-2 isolate and determined DC maturation and  
131 cytokine production. We have previously shown that DCs do not become infected by primary SARS-  
132 CoV-2(23). Exposure of DCs to the primary SARS-CoV-2 isolate did neither induce expression of CD80  
133 CD86, nor CD83, whereas LPS induced expression of CD83 and CD86 (Fig 4A-C).

134 Next we investigated cytokine induction by DCs after exposure to primary SARS-CoV-2 isolate or  
135 agonists for extracellular TLRs (TLR1/2, TLR2/6, TLR4, and TLR5). LPS, flagellin and LTA induced type I  
136 IFN responses as well as cytokines, whereas Pam3CSK4 only induced cytokines (Fig 4D-G). However,  
137 exposure of DCs to the primary SARS-CoV-2 isolate did not lead to induction of type I IFN responses

138 nor cytokines (Fig 4D-G). Therefore, these data strongly indicate that primary SARS-CoV-2 virus  
139 particles are not sensed by any extracellular PRRs on DCs such as TLR2, TLR4, and TLR5.

140

141 *Ectopic ACE2 expression on DCs results in SARS-CoV-2 infection and immune activation*

142 Next, we investigated whether infection of DCs after ectopic expression of ACE2 with primary SARS-  
143 CoV-2 isolate would induce immune responses. DCs do not express ACE2, but transfection with ACE2  
144 plasmid resulted in ACE2 mRNA and surface expression (Fig 5A-C). Next, both mock- and ACE2-  
145 transfected DCs were exposed to the primary SARS-CoV-2 isolate for 24h in presence or absence of  
146 blocking antibodies against ACE2. ACE2-expressing DCs were infected by SARS-CoV-2 and infection was  
147 blocked by antibodies against ACE2 (Fig 5D). Notably, infection of DCs with SARS-CoV-2 induced  
148 transcription of IFN $\beta$  (Fig 5E) as well as the ISG A3G (Fig 5F). Infection also induced pro-inflammatory  
149 cytokine IL-6 (Fig 5G). Both type I IFN responses and IL-6 were abrogated by blocking infection using  
150 ACE2 antibodies. Taken together, these data strongly indicate that infection is required to induce  
151 cytokine responses by DCs and suggest that intracellular PRRs rather than extracellular TLRs are  
152 involved in sensing SARS-CoV-2 and instigating immune responses against SARS-CoV-2.

153



154 **Discussion**

155 SARS-CoV-2 has established itself as a contagious human respiratory pathogen, which can trigger a  
156 robust inflammatory cytokine response(8). However, it remains largely unknown whether innate  
157 immune receptors are involved in the onset of immune responses against SARS-CoV-2. TLR4 has been  
158 suggested to play a role in sensing SARS-CoV-2 and inducing a strong immune response(13, 14). Here,  
159 our data suggest that SARS-CoV-2 by itself is not recognized by TLR4, as neither a TLR4-expressing 293  
160 cell line nor primary DCs were activated by exposure to recombinant S protein, SARS-CoV-2  
161 pseudovirus or primary SARS-CoV-2 virus particles. Ectopic expression of ACE2 on primary DCs allowed  
162 infection with primary SARS-CoV-2. Notably, productive infection of ACE2-positive DCs induced type I  
163 IFN and cytokine responses, which was abrogated by blocking ACE2. Our data therefore suggest that  
164 SARS-CoV-2 virus particles are not sensed by extracellular TLRs, including TLR4, but that infection via  
165 ACE2 is required.

166 Other studies have reported that S protein triggered TLR4, and also TLR2 and TLR6 are suggested to  
167 interact with SARS-CoV-2 S protein to induce pro-inflammatory responses(13, 14, 17-19, 24). However,  
168 neither a TLR4-expressing 293 cell line nor primary DCs were activated by recombinant S proteins. As  
169 it is possible that contaminations during the purification process of recombinant proteins might induce  
170 activation, we also investigated immune activation by SARS-CoV-2 pseudovirus and infectious primary  
171 SARS-CoV-2. However, neither TLR4-expressing 293 cells nor primary DCs were activated by  
172 pseudovirus or a primary isolate of SARS-CoV-2 as measured by cytokine production and DC  
173 maturation. Therefore, our data strongly suggest that S protein expressed by SARS-CoV-2 does not  
174 trigger TLR4. Since monocyte-derived DCs do not express ACE2, they are not infected by SARS-CoV-2  
175 and therefore exposure to primary SARS-CoV-2 will only allow sensing by extracellular PRRs that are  
176 expressed by DCs. Therefore, our data also imply that extracellular transmembrane TLRs do not sense  
177 SARS-CoV-2 virus particles.

178 Notably, ectopic expression of ACE2 on monocyte-derived DCs leads to infection and the production  
179 of cytokines, suggesting that replication of SARS-CoV-2 triggers cytosolic sensors. Indeed, studies

180 suggest that intracellular viral sensors such as RIG-I or MDA5 are involved in SARS-CoV-2 infection(25-  
181 27).

182 Interestingly, our data suggest that infection of immune cells and thus antigen presenting cells (APCs)  
183 is essential to induction of immunity. Therefore, it is important to identify ACE2-positive DC subsets  
184 and macrophages, since these APCs should be sensitive to infection and are therefore paramount in  
185 initiating adaptive immunity. In the absence of DC infection, epithelial cell infection and subsequent  
186 inflammation and tissue damage might account for initial immune activation and inflammation, and  
187 subsequent release of PAMPs and DAMPs might activate DCs(28). It remains unclear whether these  
188 secondary signals are able to correctly instruct DCs and this might underlie the strong inflammatory  
189 responses observed during COVID-19. Our finding that SARS-CoV-2 is not recognized by TLR4 might  
190 therefore be an escape mechanism leading to inefficient DC activation and subsequent aberrant  
191 inflammatory responses.

192 It has been suggested that worsening of disease in COVID-19 patients coincides with the  
193 activation of the adaptive immune response, 1-2 weeks after infection(8). Since DCs have a bridging  
194 function to activate the adaptive immune response, it is important to study DCs in the context of  
195 COVID-19. Our research suggests that ACE2-negative DCs are not properly activated by infectious  
196 SARS-CoV-2. Moreover, our data suggest that SARS-CoV-2 is able to escape from extracellular TLRs that  
197 are one of the most important PRR families crucial for induction of innate and adaptive immunity, and  
198 further research will show whether the lack of TLR activation underlies observed inflammation during  
199 COVID-19.

200

201 **Materials and methods**

202 *Cell lines*

203 The Simian kidney cell line VeroE6 (ATCC® CRL-1586™) was maintained in CO<sub>2</sub> independent medium  
204 (Gibco Life Technologies, Gaithersburg, Md.) supplemented with 10% fetal calf serum (FCS), 2mM L-  
205 glutamine and penicillin/streptomycin. Culture was maintained at 37°C without CO<sub>2</sub>.

206 Human embryonic kidney cells (HEK293) were maintained in IMDM (Gibco) supplemented with 10%  
207 FCS and 1% penicillin/streptomycin (Invitrogen). HEK293 cells stably transfected with TLR4 cDNA  
208 (HEK/TLR4) were a kind gift from D. T. Golenbock(15). HEK293 and HEK/TLR4 cells were transiently  
209 transfected with pcDNA3.1(-)hACE2 (Addgene plasmid #1786) to generate HEK/ACE2 or  
210 HEK/TLR4/ACE2 cell lines. Transfection was performed using Lipofectamine LTX and PLUS reagent  
211 (Invitrogen) according to the manufacturer's protocol. After 24h, cells were split and seeded into flat-  
212 bottom 96-well plates (Corning) and left to attach for 24h, before performing further experiments.  
213 Cultures were maintained at 37°C and 5% CO<sub>2</sub>. Before infection with the SARS-CoV-2 isolate (described  
214 below), media was exchanged for CO<sub>2</sub>-independent media, since infection with a SARS-CoV-2 primary  
215 isolate occurs under CO<sub>2</sub> negative conditions. Human ACE2-expressing cell lines were analyzed for  
216 ACE2 expression via quantitative real-time PCR.

217

218 *Primary cells*

219 This study was performed in accordance with the ethical principles set out in the declaration of Helsinki  
220 and was approved by the institutional review board of the Amsterdam University Medical Centers,  
221 location AMC Medical Ethics Committee and the Ethics Advisory Body of Sanquin Blood Supply  
222 Foundation (Amsterdam, Netherlands). Human CD14+ monocytes were isolated from the blood from  
223 healthy volunteer donors (Sanquin blood bank) and subsequently differentiated into monocyte-  
224 derived dendritic cells (DCs). The isolation from buffy coats was done by density gradient  
225 centrifugation on Lymphoprep (Nycomed) and Percoll (Pharmacia). After separation by Percoll, the  
226 isolated monocytes were cultured in RPMI 1640 (Gibco) supplemented with 10% FCS, 2mM L-glutamin

227 (Invitrogen) and 10 U/mL penicillin and 100 ug/mL streptomycin, containing the cytokines IL-4  
228 (500 U/mL) and GM-CSF (800 U/mL) (both Gibco) for differentiation into DCs. After 4 days of  
229 differentiation, DCs were seeded at  $1 \times 10^6$  /mL in a 96-well plate (Greiner), and after 2 days of recovery,  
230 DCs were stimulated or infected as described below.

231 Alternatively, monocyte-derived DCs that were transfected with hACE2 were seeded at  $0.5 \times 10^6$   
232 cells/mL in a 6-well plate and transfection was performed with Lipofectamine LTX and PLUS reagents  
233 (Invitrogen) according to the manufacturer's instructions for primary cells. After 24h, cells were seeded  
234 at  $1 \times 10^6$ /mL in a 96-well plate and after 24h of recovery, they were infected with primary SARS-CoV-2  
235 isolate.

236

#### 237 *SARS-CoV-2 pseudovirus production*

238 For production of single-round infection viruses, human embryonic kidney 293T/17 cells (ATCC, CRL-  
239 11268) were co-transfected with an adjusted HIV-1 backbone plasmid (pNL4-3.Luc.R-S-) containing  
240 previously described stabilizing mutations in the capsid protein (PMID: 12547912) and firefly luciferase  
241 in the *nef* open reading frame (1.35ug) and pSARS-CoV-2 expressing SARS-CoV-2 S protein (0.6ug)  
242 (GenBank; MN908947.3)(22). Transfection was performed in 293T/17 cells using genejuice (Novagen,  
243 USA) transfection kit according to manufacturer's protocol. At day 3 or day 4, pseudotyped SARS-CoV-2  
244 virus particles were harvested and filtered over a 0.45  $\mu$ m nitrocellulose membrane (SartoriusStedim,  
245 Gottingen, Germany). SARS-CoV-2 pseudovirus productions were quantified by p24 ELISA (Perkin  
246 Elmer Life Sciences).

247

#### 248 *SARS-CoV-2 (primary isolate) virus production*

249 The following reagent was obtained from Dr. Maria R. Capobianchi through BEI Resources, NIAID, NIH:  
250 SARS-Related Coronavirus 2, Isolate Italy-INMI1, NR-52284, originally isolated January 2020 in Rome,  
251 Italy. VeroE6 cells (ATCC® CRL-1586™) were inoculated with the SARS-CoV-2 isolate and used for  
252 reproduction of virus stocks. CPE formation was closely monitored and virus supernatant was

253 harvested after 48 hours. Tissue culture infectious dose (TCID<sub>50</sub>) was determined on VeroE6 cells by  
254 MTT assay 48 hours after infection. Loss of MTT staining as determined by spectrometer is indicative  
255 of cell death.

256

#### 257 *Stimulation and infection*

258 HEK293 and transfected derivatives were left unstimulated or stimulated for 24h with 10 ng/mL  
259 lipopolysaccharide (LPS) from *Salmonella* (Sigma), 10 ug/mL isolated S protein, 10 ug/mL S  
260 nanoparticle, or with pseudotyped or authentic SARS-CoV-2, as specified below. DCs were left  
261 unstimulated, or stimulated with 10 ug/ml Pam3CSK4 (Invivogen), 10 ng/mL LPS from *Salmonella*  
262 *typhosa* (Sigma), 10 ug/mL flagellin from *Salmonella typhimurium* (Invivogen), 10 ug/mL lipoteichoic  
263 acid (LTA) from *Staphylococcus aureus* (Invivogen), pseudotyped virus or SARS-CoV-2. Blocking of ACE2  
264 was performed with 8 ug/mL anti-ACE2 (R&D systems) for 30 min at 37°C before adding stimuli.  
265 Monocyte-derived DCs do not express ACE2 and are therefore not infected. Therefore, pseudovirus  
266 stimulation was performed for 6h, after which the cells were lysed for mRNA analysis of cytokine  
267 production. DCs ectopically expressing ACE2 were stimulated for 24h with virus before the cells were  
268 lysed for mRNA analysis of cytokine production. Also, cells were stimulated for 24h and fixed for 30  
269 min with 4% paraformaldehyde, after which the expression of maturation markers was assessed with  
270 flow cytometry.

271 For the pseudovirus infection assays, HEK293 or 293/TLR4 cell lines and DCs were exposed to 95ng/mL  
272 and 191.05ng/mL of SARS-CoV-2 pseudovirus, respectively. Viral protein production was quantified  
273 after 3 days at 37°C by measuring luciferase reporter activity. Luciferase activity was measured using  
274 the Luciferase assay system (Promega, USA) according to manufacturer's instructions.

275 For the primary SARS-CoV-2 infection assays, HEK293 or HEK/TLR4 cell lines and DCs were exposed to  
276 the SARS-CoV-2 isolate (hCoV-19/Italy) at different TCIDs (100 and 1000; MOI 0.0028-0.028) for 24  
277 hours at 37°C. After 24 hours, cell supernatant was taken and DCs were lysed for isolation of viral RNA.  
278 Also, the HEK293/ACE2 and HEK/TLR4/ACE2 cell lines were exposed to the SARS-CoV-2 isolate (hCoV-

279 19/Italy) at TCID 100 (MOI 0.0028) for 24 hours at 37°C. After 24 hours, the cells were washed 3 times  
280 and new media was added. After 48h, cell supernatant was harvested and the cells were lysed to  
281 investigate productive infection.

282

### 283 *RNA isolation and quantitative real-time PCR*

284 Cells exposed to SARS-CoV-2 pseudovirus were lysed and mRNA was isolated with the mRNA Catcher™  
285 PLUS Purification Kit (ThermoFisher). Subsequently, cDNA was synthesized with a reverse-  
286 transcriptase kit (Promega). RNA of cells exposed to SARS-CoV-2 WT was isolated with the QIAamp  
287 Viral RNA Mini Kit (Qiagen) according to the manufacturer's protocol. cDNA was synthesized with the  
288 M-MLV reverse-transcriptase kit (Promega) and diluted 1 in 5 before further application. PCR  
289 amplification was performed in the presence of SYBR green (ThermoFisher) in a 7500 Fast Realtime  
290 PCR System (ABI). Specific primers were designed with Primer Express 2.0 (Applied Biosystems). The  
291 ORF1b primers used were as described before(29). The normalized amount of target mRNA was  
292 calculated from the Ct values obtained for both target and household mRNA with the equation  $N_t =$   
293  $2^{Ct(GAPDH)-Ct(target)}$ . The following primers were used:

294 GAPDH: F\_CCATGTTTCGTCATGGGTGTG; R\_GGTGCTAAGCAGTTGGTGGTG; TLR4:

295 F\_CTGCAATGGATCAAGGACCAG; R\_CCATTCGTTCAACTCCACCA; ACE2:

296 F\_GGACCCAGGAAATGTTTCAGA; R\_GGCTGCAGAAAGTGACATGA; ORF1b:

297 F\_TGGGGTTTTACAGGTAACCT; R\_AACACGCTTAACAAAGCACTC; IL-8: F\_TGAGAGTGGACCACACTGCG;

298 R\_TCTCCACAACCCTCTGCACC; IFNB: F\_ACAGACTTACAGGTTACCTCCGAAAC;

299 R\_CATCTGCTGGTTGAAGAATGCTT; APOBEC3G: F\_TTGAGCCTTGAATAATCTGCC;

300 R\_TCGAGTGTCTGAGAATCTCCCC; IL-6: F\_TGCAATAACCACCCCTGACC;

301 R\_TGCGCAGAATGAGATGAGTTG; IL-10: F\_GAGGCTACGGCGCTGTCAT; R\_CCACGGCCTTGCTCTTGTT

302

303 *ELISA*

304 Cell supernatants were harvested after 24h of stimulation and secretion of IL-8 was measured by ELISA  
305 (eBiosciences) according to the manufacturer's instructions. OD450 nm values were measured using a  
306 BioTek Synergy HT. Supernatant containing SARS-CoV-2 pseudovirus was inactivated with 0.1% triton  
307 and supernatant containing SARS-CoV-2 was inactivated with 1% triton before performing ELISA.

308

### 309 *Flow cytometry*

310 For cell surface staining, cells were incubated in 0.5% PBS-BSA (phosphate-buffered saline containing  
311 0.5% bovine serum albumin (BSA; Sigma-Aldrich)) containing antibodies for 30 min at 4°C. Single-cell  
312 measurements were performed on a FACS Canto flow cytometer (BD Biosciences) and FlowJo V10  
313 software (TreeStar) was used to analyze the data. The antibody clones used are: CD86 (2331 (FUN-1),  
314 BD Pharmingen), CD80 (L307.4, BD Pharmingen), CD83 (HB15e, BD Pharmingen), ACE2 (AF933, R&D  
315 systems), goat-IgG (AB-2535864, ThermoFisher Scientific), donkey-anti-goat (A-21447, ThermoFisher  
316 Scientific). For each experiment, live cells were gated on FSC and SSC and analyzed further with the  
317 markers mentioned.

318

### 319 *Statistics*

320 Graphpad Prism v8 (GraphPad Software) was used to generate all graphs and for statistical analyses.  
321 Statistics were performed using a Student's *t* test for pairwise comparisons. Multiple comparisons  
322 within groups were performed using an RM one-way analysis of variance (ANOVA) with a Tukey's  
323 multiple comparisons test, or two-way ANOVA with a Tukey's or Šidák's multiple comparisons test  
324 where indicated.  $p < 0.05$  were considered statistically significant.

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329

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338

339 **Author Contributions**

340 LEHvdD and MBJ designed experiments; LEHvdD, MBJ, JE, and JLvH performed the experiments; PJMB,  
341 MB, ACvN, NAK, MJvG and RWS contributed essential research materials and scientific input. LEHvdD,  
342 MBJ and TBHG analyzed and interpreted data; LEHvdD, MBJ and TBHG wrote the manuscript with input  
343 from all listed authors. TBHG supervised all aspects of this study.

344

345 **Conflicting interests**

346 All authors declare no commercial or financial conflicts of interest.

347

348 **Data availability**

349 The data generated during this study are available from the corresponding author on reasonable  
350 request.

351



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421

422

423 **Figure legends**

424 **Fig 1: S protein and SARS-CoV-2 S nanoparticle do not trigger TLR4**

425 (A) 293 cells or 293/TLR4 cells were exposed to LPS, SARS-CoV-2 S protein or S nanoparticles for 24h.  
426 IL-8 production was determined by ELISA. (B-E) Primary dendritic cells were exposed to LPS or SARS-  
427 CoV-2 S nanoparticles for 8h. Expression of IFN $\beta$  (B), A3G (C), IL-6 (D) and IL-10 (E) was determined  
428 with qPCR. Data show the mean values and SEM. Statistical analysis was performed using (A) two-way  
429 ANOVA with Šidák's multiple comparisons test, or (B-E) one-way ANOVA with Tukey's multiple  
430 comparisons test. (A) \*\*\*\*p<0.0001 (n=3). (B-E) \*\*\*p<0.001; \*\*p<0.01; \*p<0.05 (n=6).

431

432 **Fig 2: SARS-CoV-2 virus particles do not trigger TLR4**

433 (A-B) ACE2-positive and -negative 293 and 293/TLR4 cells were exposed to SARS-CoV-2 pseudovirus  
434 and infection was determined after 3 days by measuring luciferase activity (A), and IL-8 production was  
435 measured after 24h by ELISA (B). (C-D) ACE2-positive and -negative 293 and 293/TLR4 cells were  
436 exposed to a primary SARS-CoV-2 isolate and infection was determined after 24h by measuring the  
437 viral gene ORFb1 expression in supernatant by qPCR (C) and IL-8 production was measured after 24h  
438 by ELISA (D). Data show the mean values and SEM. Statistical analysis was performed using two-way  
439 ANOVA with Šidák's (A) or Tukey's (B-D) multiple comparisons test. (A-D) \*\*\*\*p<0.0001; \*\*p<0.01 (A-  
440 B; n=3 in triplicates) (C-D; n=3). RLU = relative light units.

441

442 **Fig 3: SARS-CoV-2 pseudovirus does not activate dendritic cells**

443 (A-D) Primary DCs were exposed to LPS or SARS-CoV-2 pseudovirus and maturation and cytokine  
444 production was determined after 24h and 6h respectively. (A) Representative histogram of CD86  
445 expression. (B-D) Cumulative flow cytometry data of CD80 (B), CD86 (C), and CD83 (D) expression. (E-  
446 H) mRNA levels of IFN $\beta$  (E), A3G (F), IL-6 (G) and IL-10 (H) were determined with qPCR. Data show the  
447 mean values and SEM. Statistical analysis was performed using one-way ANOVA with Tukey's multiple

448 comparisons test. (B-D) \*\*\*\* $p < 0.0001$ ; \*\*\* $p < 0.001$ ; \* $p < 0.05$  (B-C;  $n=5$ ) (D;  $n=4$ ). (E-H) \*\*\* $p < 0.001$ ;  
449 \*\* $p < 0.01$ ; \* $p < 0.05$  ( $n=5$ ). MFI = mean fluorescence intensity.

450

451 **Fig 4: Primary SARS-CoV-2 isolate does not activate dendritic cells**

452 (A-G) Primary DCs were exposed to LPS or primary SARS-CoV-2 isolate and DC maturation was  
453 measured after 24h by flow cytometry. Cumulative flow cytometry data of CD80 (A), CD86 (B), and  
454 CD83 (C) expression. (D-G) Primary DCs were exposed to different TLR agonists or primary SARS-CoV-  
455 2 isolate and mRNA levels of IFN $\beta$  (D), A3G (E), IL-6 (F) and IL-10 (G) were determined with qPCR. Data  
456 show the mean values and SEM. Statistical analysis was performed using one-way ANOVA with Tukey's  
457 multiple comparisons test. (A-C) \*\* $p < 0.01$ ; ns = non-significant (A-B;  $n=7$ ) (C;  $n=6$ ). (D-G) Data are  
458 compared to the unstimulated condition, \* $p < 0.05$ ; ns = non-significant ( $n=5$ ). MFI = mean fluorescence  
459 intensity.

460

461 **Fig 5: Ectopic expression of ACE2 on DCs results in infection and induction of immune responses**

462 (A-C) Ectopic expression of ACE2 on primary DCs was determined by qPCR and flow cytometry. (A)  
463 Cumulative qPCR data of ACE2 expression on DCs. (B) Representative histogram of ACE2 expression on  
464 DCs. (C) Cumulative flow cytometry data of ACE2 expression. (D-G) ACE2-positive and -negative DCs  
465 were exposed to primary SARS-CoV-2 isolate in presence or absence of blocking antibodies against  
466 ACE2. Infection (D) and mRNA levels of IFN $\beta$  (E), A3G (F), and IL-6 (G) were determined with qPCR. Data  
467 show the mean values and SEM. Statistical analysis was performed using (A, C) unpaired student's t-  
468 test or (D-G) one-way ANOVA with Tukey's multiple comparisons test. (A, C) \*\*\*\* $p < 0.0001$ ; \*\* $p < 0.01$   
469 ( $n=9$ ). (D-G) \*\*\*\* $p < 0.0001$ ; \*\*\* $p < 0.001$ ; \*\* $p < 0.01$ ; \* $p < 0.05$ ; ns = non-significant; (D-F;  $n=9$ ) (G;  $n=7$ ).  
470 MFI = mean fluorescence intensity.

471

472

Fig 1

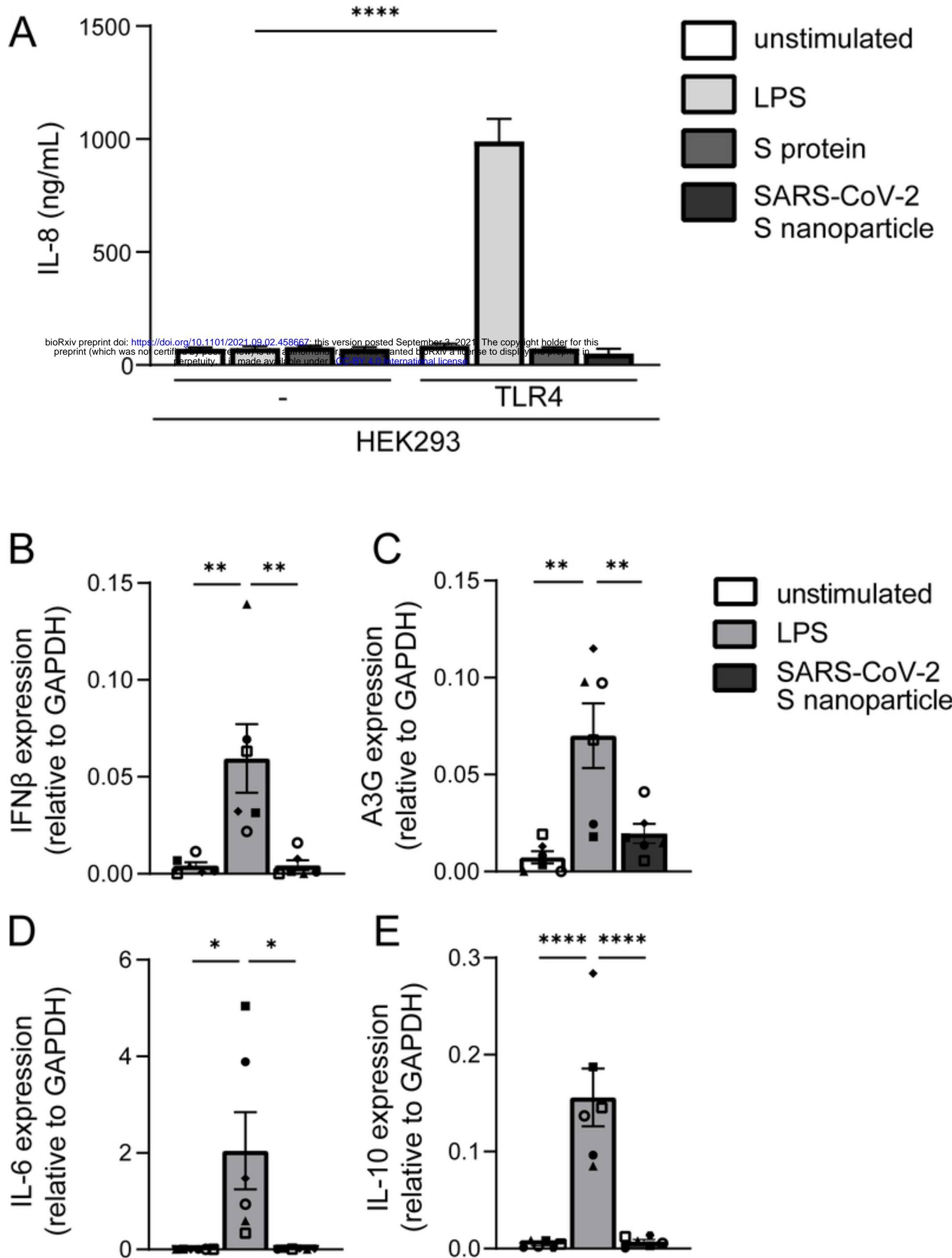


Figure 1

Fig 2

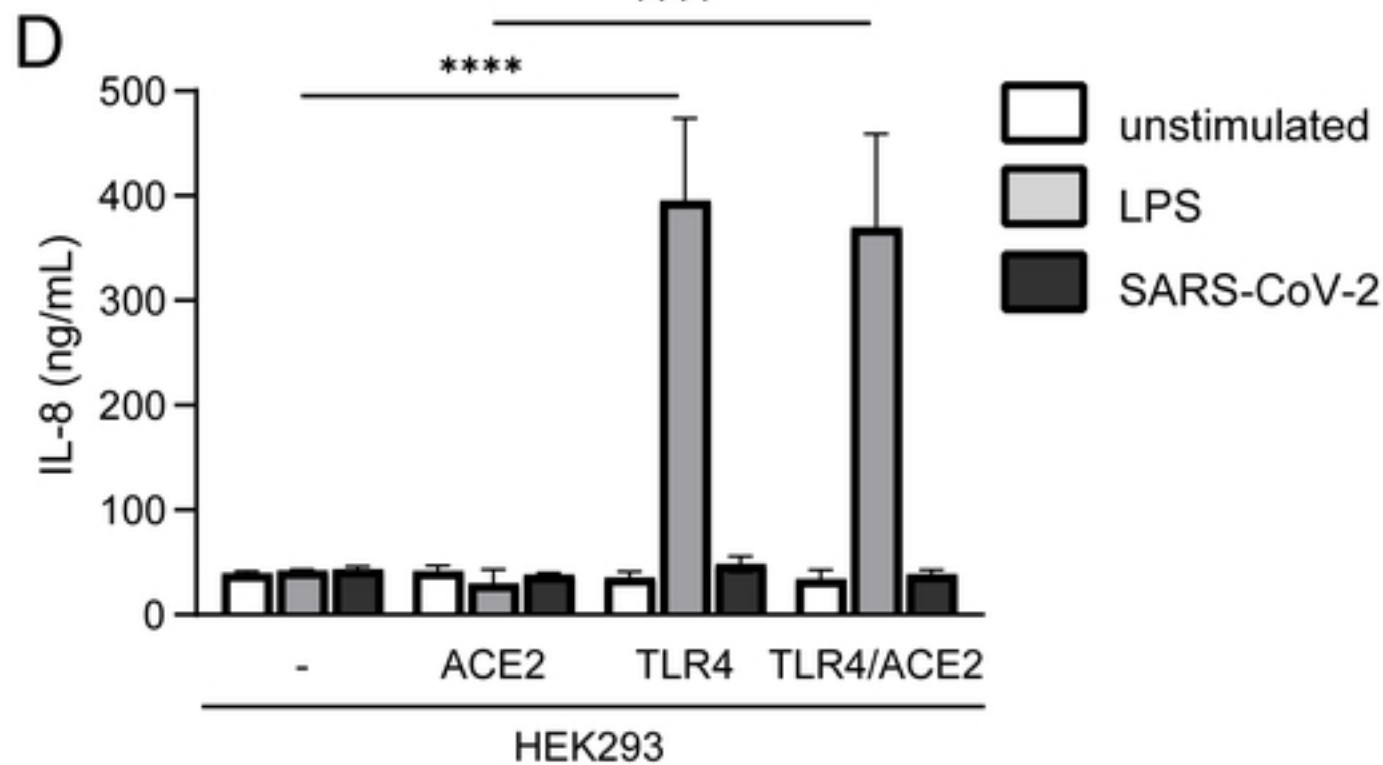
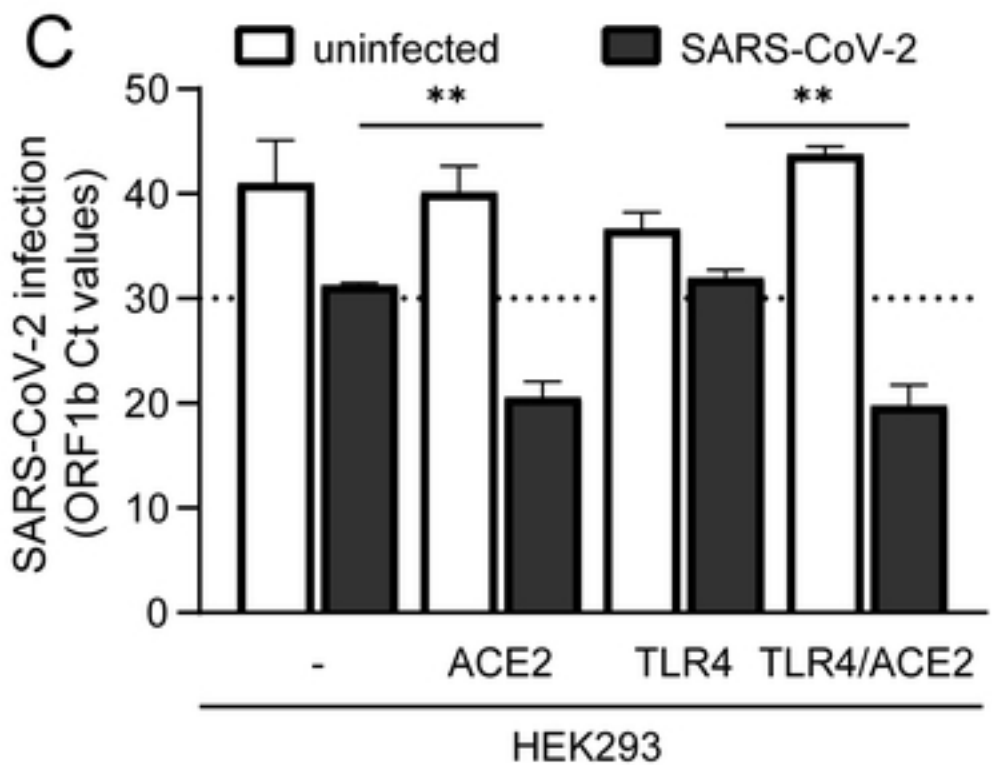
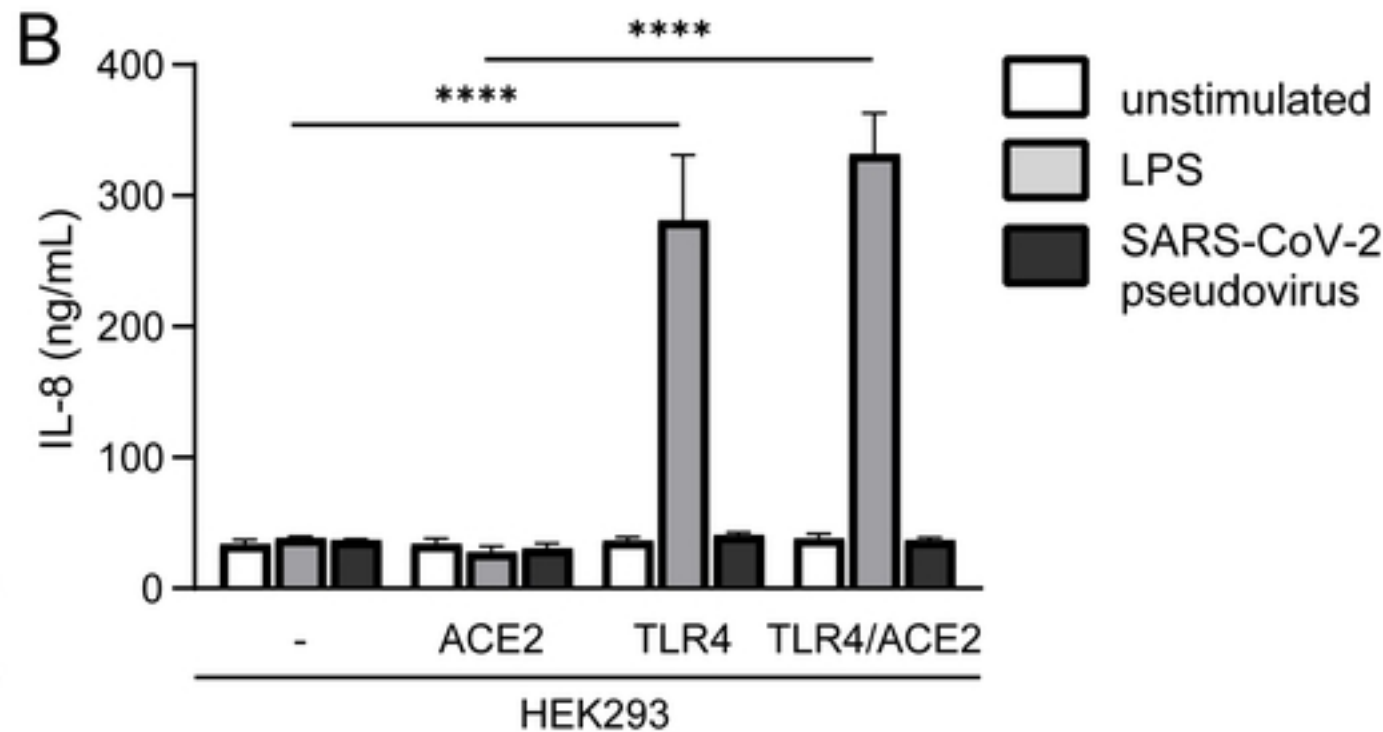
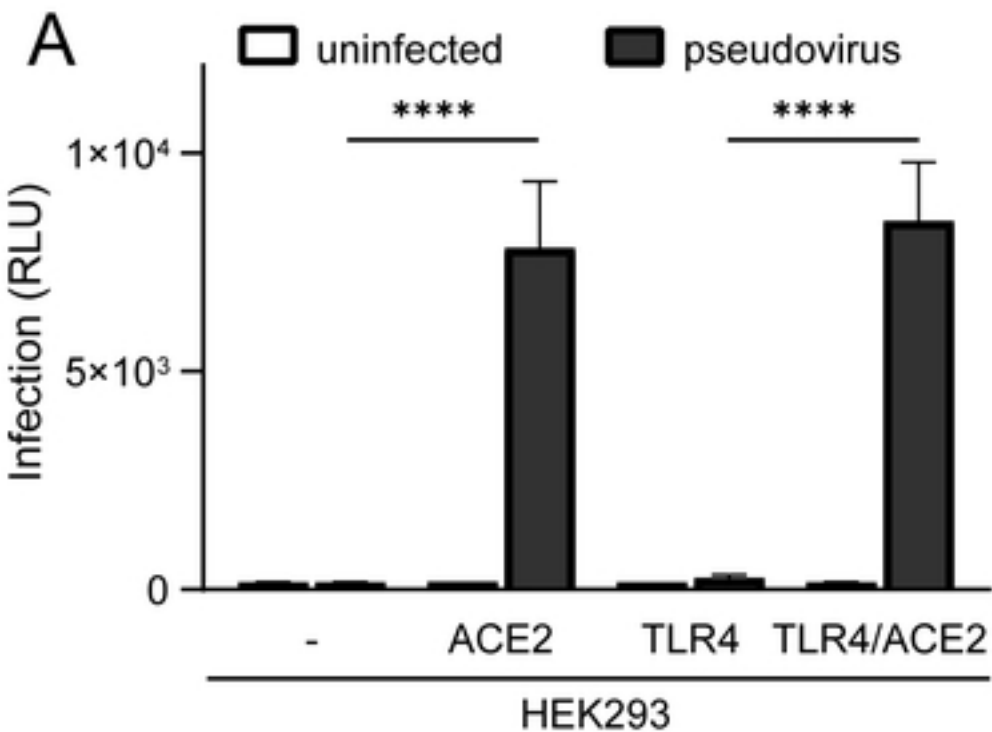
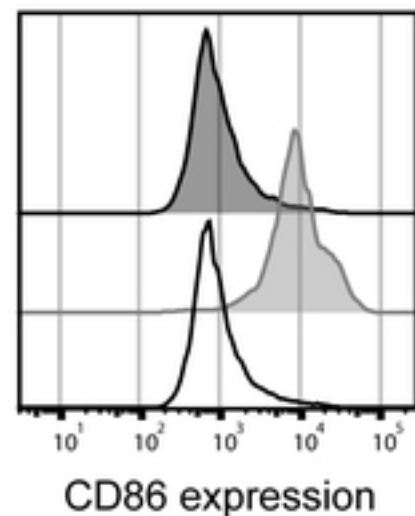


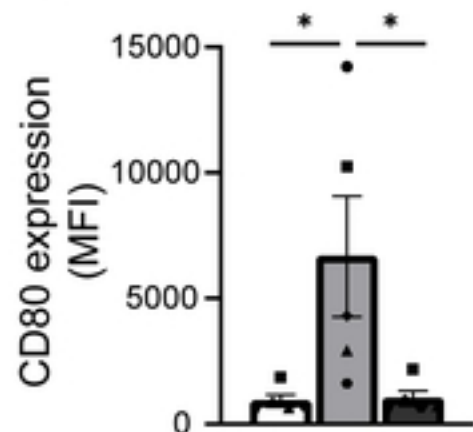
Figure2

Fig 3

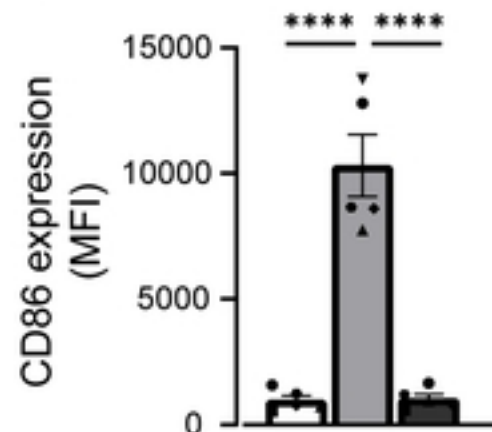
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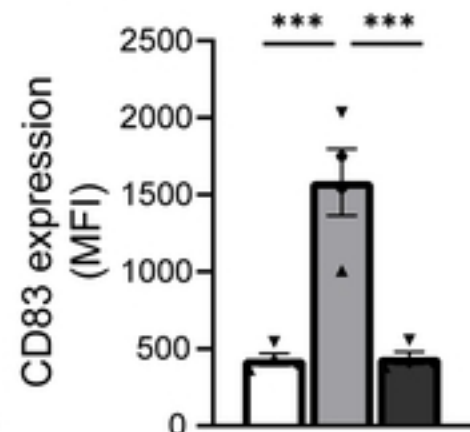
B



C

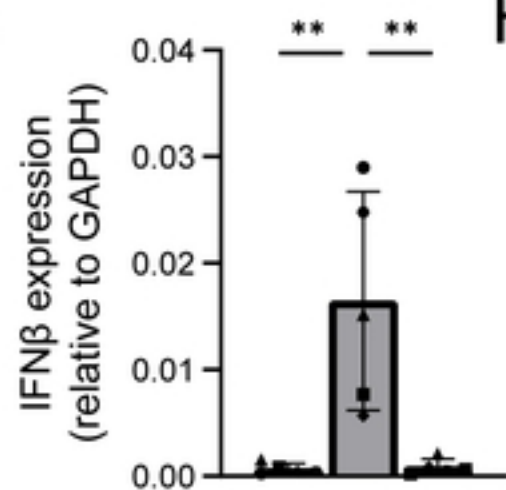


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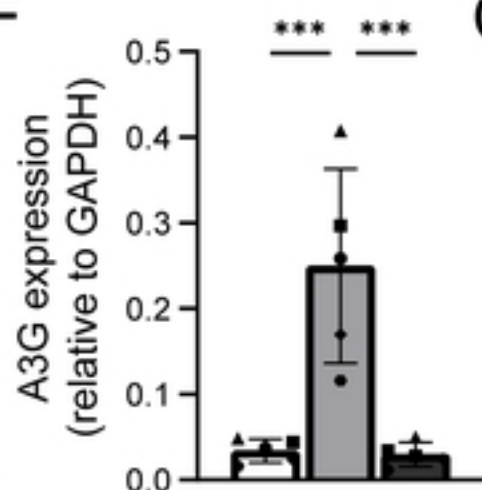


□ unstimulated  
 ■ LPS  
 ■ SARS-CoV-2 pseudovirus

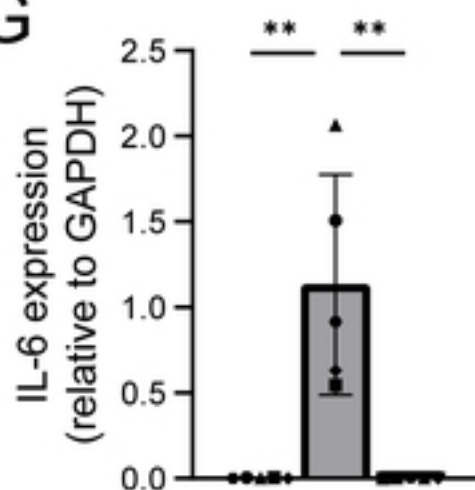
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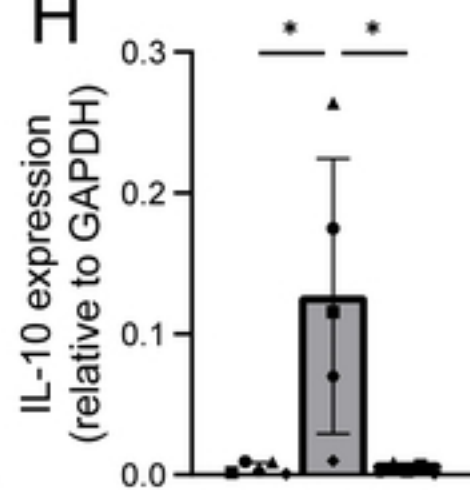
F



G



H



□ unstimulated  
 ■ LPS  
 ■ SARS-CoV-2 pseudovirus

Figure3



Fig 4

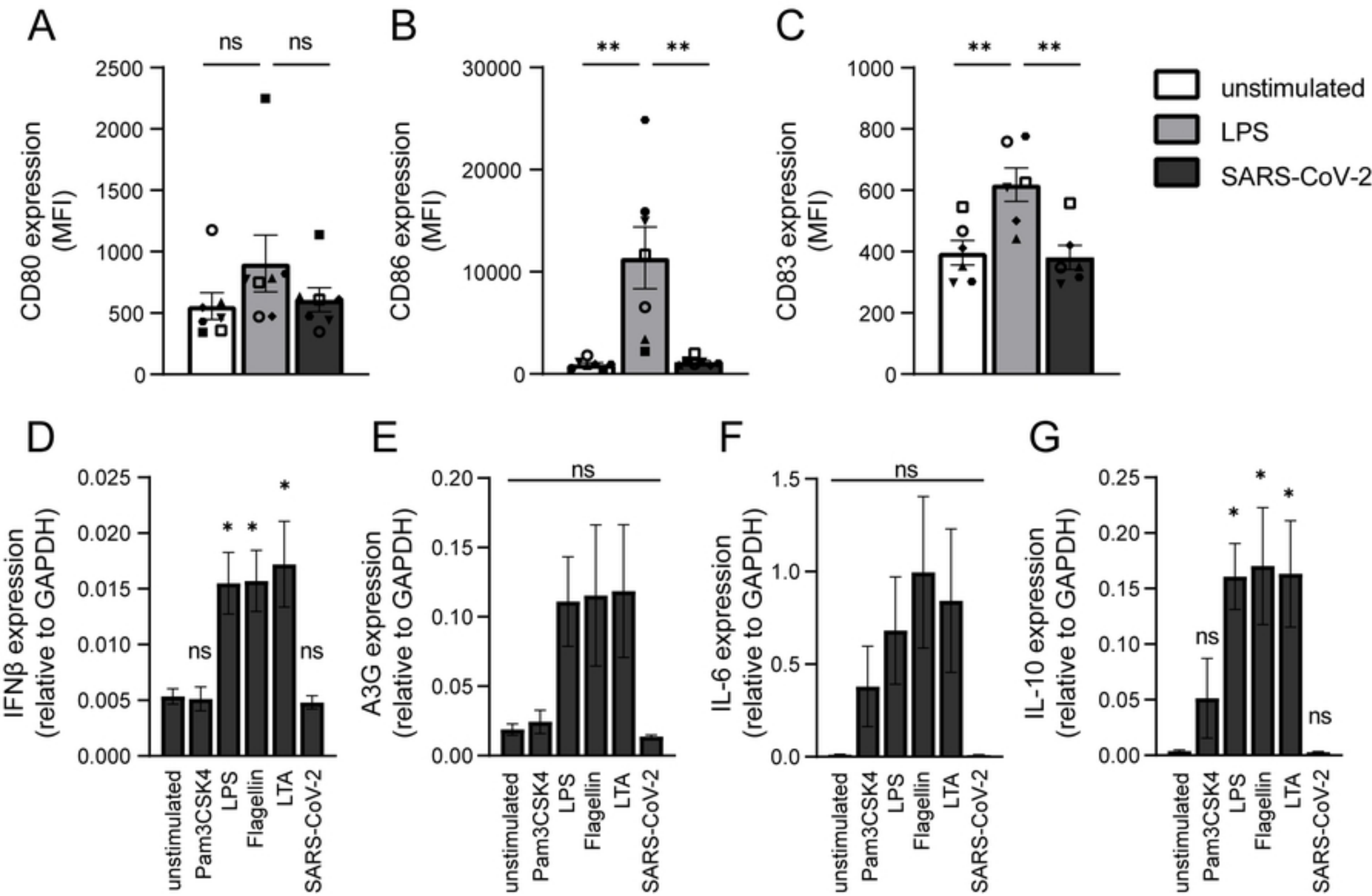


Figure4



Fig 5

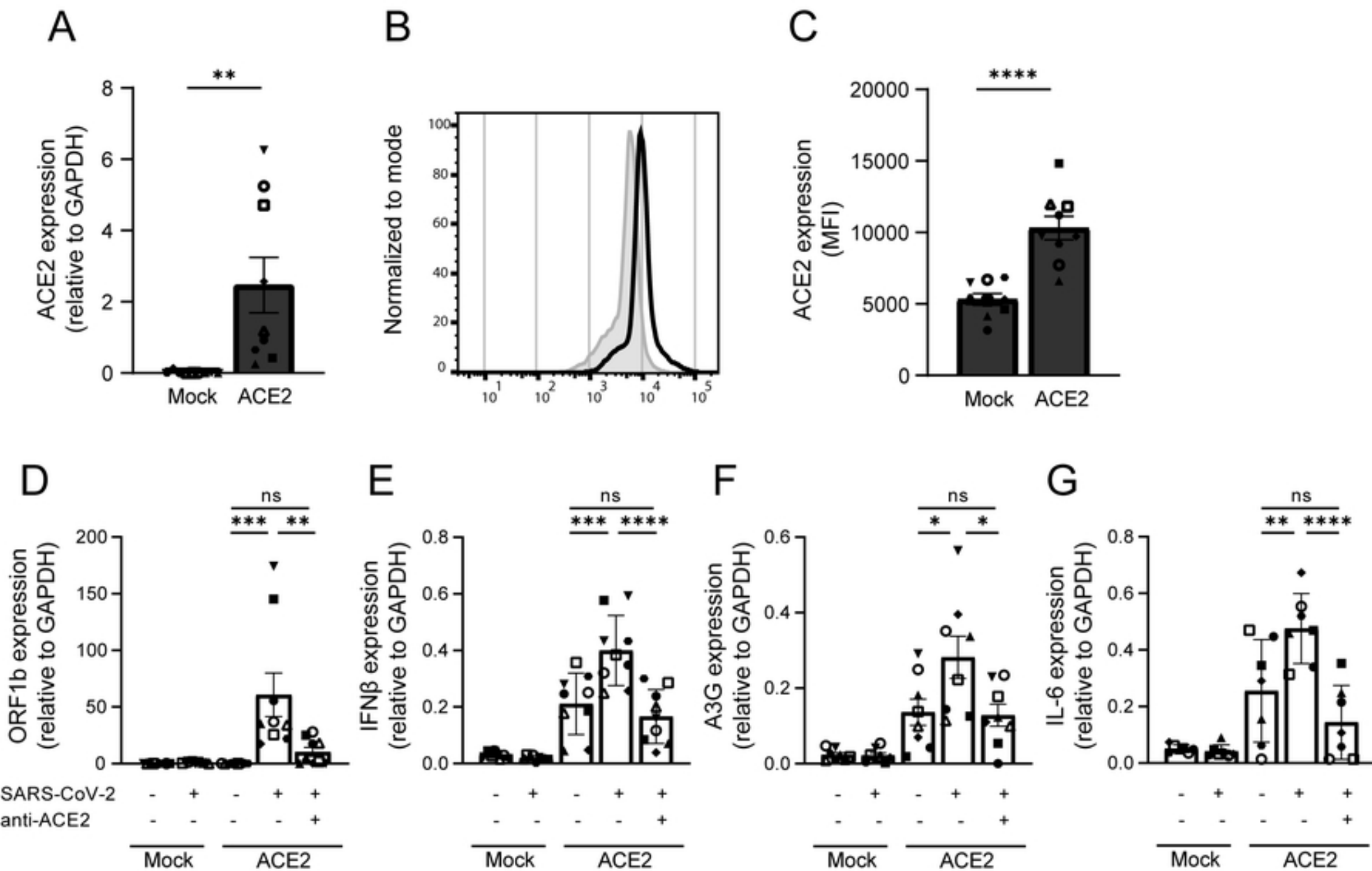


Figure5