1    **Proinflammatory responses in SARS-CoV-2 infected and soluble spike glycoprotein S1**

2    **subunit activated human macrophages**

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16      **Abstract**

17      Critically ill COVID-19 patients infected with SARS-CoV-2 display signs of generalized

18      hyperinflammation. Macrophages trigger inflammation to eliminate pathogens and repair tissue,

19      but this process can also lead to hyperinflammation and resulting exaggerated disease. The role of

20      macrophages in dysregulated inflammation during SARS-CoV-2 infection is poorly understood.

21      We used SARS-CoV-2 infected and glycosylated soluble SARS-CoV-2 Spike S1 subunit (S1)

22      treated THP-1 human-derived macrophage-like cell line to clarify the role of macrophages in pro-

23      inflammatory responses. Soluble S1 upregulated TNF-α and CXCL10 mRNAs, and induced

24      secretion of TNF-α from THP-1 macrophages. While THP-1 macrophages did not support

25      productive SARS-CoV-2 replication, virus infection resulted in upregulation of both TNF-α and

26      CXCL10 genes. Our study shows that S1 is a key viral component inducing inflammatory response

27      in macrophages, independently of virus replication. Thus, virus-infected or soluble S1-activated

28      macrophages may become sources of pro-inflammatory mediators contributing to

29      hyperinflammation in COVID-19 patients.

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32      **1.  Introduction**

33      Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is the causative agent of

34      Coronavirus Disease 2019 (COVID-19). Severely ill COVID-19 patients display lung tissue

35      damage associated with cell death and pathologic inflammation[1,2] linked to enhanced pro-

36      inflammatory cytokine and chemokine levels (*e.g.*, TNF-α and CXCL10)[3,4]. These pathologies are

37      compatible with dysregulated inflammatory response characteristic of cytokine release syndrome

38      or macrophage-activation syndrome[5] and generalized hyperinflammation[6]. These patients often

39    progress to respiratory failure due to complications from hyperinflammation and require

40    mechanical ventilation. Analysis of bronchoalveolar lavage fluid (BALF) from critically ill

41    COVID-19 patients revealed upregulation of inflammatory cytokine signatures, indicating influx

42    of active inflammatory macrophages in the airways[7,8]. Single-cell RNA sequencing also detected

43    SARS-CoV-2 RNA in BALF-associated macrophages[8], whereas virus antigen was detected in

44    subsets of tissue-resident and lymph node-associated macrophages[9]. Macrophages mediate

45    inflammatory responses following infection, migrating to affected tissue to eliminate invading

46    pathogens. These findings implicate macrophages in the exaggerated inflammatory response

47    during SARS-CoV-2 infection. In this study, we analyzed pro-inflammatory response against

48    SARS-CoV-2 and soluble SARS-CoV-2 spike glycoprotein S1 subunit in the human-derived THP-

49    1 macrophage cell-line.

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52    **2.   Results and Discussion**

53    *2.1. Soluble SARS-CoV-2 spike protein S1 subunit induces inflammatory response in THP-1*

54        *cells*

55    Reports using non-soluble SARS-CoV-2 trimeric spike (S) glycoprotein[10] and purified S1 subunit

56    produced in *E.coli*[11] suggest that the S protein activates inflammation in macrophages.  Prefusion

57    trimeric S is cleaved by cellular proteases[12] that dissociate the S1 subunit during virion assembly[13]

58    or after engagement of the ACE2 receptor[14]. Trimeric S is transient on the surface of virions, and

59    constructs designed to stabilize this conformation do not reflect the dynamic state of the S

60    glycoprotein and subunits in contact with cells. Importantly, dissociated S1 may remain engaged

61    to cell receptors and stimulate yet undefined effects. Furthermore, S1 is glycosylated at numerous

62    positions[15] that mediate functions such as shielding of viral epitopes. Glycosylation patterns are

63    not recapitulated in *E.coli* used to purify proteins and thus, non-glycosylated S1 produced in *E.coli*

64    may not reproduce the biological effects of SARS-CoV-2 S1.

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66    To clarify the role of S1 in proinflammatory responses in macrophages, we tested whether

67    glycosylated, soluble SARS-CoV-2 S1 produced in mammalian cells induced expression of pro-

68    inflammatory and antiviral cytokines. We evaluated expression of the pro-inflammatory cytokines

69    TNF-α, CXCL10 and IFN-γ[16,17] due to their association with hyperinflammation in COVID-19

70    patients, and the antiviral cytokine IFN-β since it restricts SARS-CoV-2 infection[16]. While S1 did

71    not induce gene expression of antiviral IFN-β (Fig.1A) or IFN-γ (Fig.1B) in THP-1 macrophages,

72    expression of proinflammatory TNF-α (Fig.1C) and CXCL10 (Fig. 1D) was upregulated following

73    exposure to S1. TNF-α was upregulated by 30-fold at 4h post treatment with S1 ($p < 0.05$) and

74    remained upregulated by two-fold at 16h post treatment (Fig. 1C). CXCL10 expression was

75    consistently upregulated by 3 to 8-fold in THP-1 macrophages exposed to S1 up to 16h post

76    treatment ($p<0.05$) (Fig. 1D). Although macrophages respond to IFN-γ by producing CXCL10,

77    they are not substantial sources of IFN-γ[18], which is mostly produced by lymphocytes to recruit

78    macrophages to infection sites[19]. Thus, SARS-CoV-2 S1 upregulated CXCL10 independently of

79    IFN-γ, similar to LPS[20] and TNF-α[21].

80    We further examined release of TNF-α from THP-1 macrophages treated with the SARS-CoV-2

81    S1 subunit. Treatment with S1 increased secretion of TNF-α in macrophages up to 30 times more

82    than vehicle-treated controls at 4h post treatment ($p< 0.05$) (Fig. 1E). These results demonstrated

83    that soluble S1 alone suffices to activate inflammatory response in macrophages independently of

84    full-length S protein, S-trimers and virus infection. Non-glycosylated S1 from *E.coli* induced TNF-

85  α secretion in murine macrophages[11]. Although these studies were conducted in mouse

86  macrophages[11], non-glycosylated S1 may expose sites able to trigger pro-inflammatory response,

87  which are cryptic in the glycosylated S1 protein. By utilizing physiologically relevant glycosylated

88  S1 derived from mammalian cells, we demonstrate its pro-inflammatory activity in human

89  macrophages.

90  *2.2. Inflammatory response in SARS-CoV-2 infected THP-1 macrophages in the absence of*

91  *productive viral replication*

92  Since SARS-CoV-2 S1 induced pro-inflammatory response in macrophages independently of

93  virus infection or replication (Fig. 1), we next evaluated pro-inflammatory response and viral

94  replication in THP-1 macrophages infected with SARS-CoV-2. As expected, infection with SARS-

95  CoV-2 in susceptible Vero E6 cells led to exponential increase of viral nucleocapsid (N) (Fig. 2A)

96  and S (Fig. 2B) genes. In contrast, expression of N (Fig. 2C) and S (Fig. 2D) genes was reduced

97  in THP-1 macrophages over the course of infection. Virus replication and release of infectious

98  progeny was determined by $TCID_{50}$ assay in supernatants from SARS-CoV-2 infected cells to

99  corroborate viral RNA findings. While infected Vero E6 cells supported robust release of

100 infectious virions due to productive replication, infectious virus production from infected THP-1

101 cells became undetectable after 8h post-infection (Fig. 2E). Finally, THP-1 macrophages did not

102 develop cytopathic effects (CPE) following SARS-CoV-2 infection, whereas Vero E6 cells

103 displayed progressive monolayer damage over the course of infection (Fig. 2F). These results

104 indicate that THP-1 human macrophages do not support productive replication of SARS-CoV-2.

105 Our study is in accord with a report showing non-productive replication of SARS-CoV-2 in human

106 monocyte-derived macrophages and DCs[22].

107    Despite non-productive replication, expression of TNF-α (Fig. 2G) and CXCL10 (Fig. 2H) in

108    THP-1 macrophages infected with SARS-CoV-2 was significantly upregulated by 2- and 3-fold at

109    4 and 24 hpi, respectively. Additionally, SARS-CoV-2 infection did not induce IFN-β (Fig.2I) or

110    IFN-γ (Fig. 2J) expression in THP-1 macrophages, whereas LPS upregulated both cytokines by

111    80- and 3-fold, respectively. Since both TNF-α and CXCL10 are key pro-inflammatory cytokines,

112    our results suggest low-grade pro-inflammatory response in THP-1 macrophages infected with

113    SARS-CoV-2 in the absence of productive virus replication. However, SARS-CoV-2 infection did

114    not prompt type-I interferon dependent antiviral response in these cells.

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116    Macrophages promote pro-inflammatory responses by producing cytokines and chemokines that

117    initiate and sustain inflammation. The role of macrophages in SARS-CoV-2 infection remains

118    unclear despite their function as pro-inflammatory cells and contribution to immune dysregulation.

119    Our study shows that although THP-1 macrophages do not support productive virus replication,

120    infection with SARS-CoV-2 upregulates expression of pro-inflammatory mediators linked to

121    generalized hyperinflammation in COVID-19 patients[4,23]. Our study identifies the SARS-CoV-2

122    soluble S1 subunit as a viral factor involved in activation of pro-inflammatory response in

123    macrophages. The soluble S1 subunit triggers pro-inflammatory response in non-infected

124    macrophages and therefore, interaction with the S1 subunit suffices to induce this response

125    independently of virus infection. Extracellular soluble S1 originating from infected lung epithelial

126    cells may interact with uninfected macrophages to stimulate inflammation. Shedding of

127    dissociated S1 has been shown during expression of the entire S protein on the surface of

128    pseudoviruses[14] and in mammalian cells[24]. Our study raises the possibility that cell-free soluble S1

129    engages with non-infected macrophages to induce low-grade pro-inflammatory response. SARS-

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130     CoV-2 infection in THP-1 macrophages does not lead to CPE (indicative of cell death) or

131     productive viral replication, while low-grade inflammatory response remains upregulated for at

132     least 24 hours after SARS-CoV-2 exposure (Fig. 2H). Therefore, virus-infected or S1-activated

133     macrophages may become sources of pro-inflammatory mediators contributing to

134     hyperinflammation in COVID-19 patients. Our study provides evidence for contribution of human

135     macrophages to inflammation-associated immunopathology of SARS-CoV-2 by two possible

136     mechanisms (graphical abstract) – a) virus infection of macrophages, and b) interaction of soluble

137     S1 protein with uninfected macrophages.

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139     **3. Materials and Methods**

140     *Cells and virus:* Cell cultures were maintained at 37C in a 5% $CO_2$ atmosphere. Vero E6 cells

141     (ATCC; catalog no. CRL-1586) were cultured in DMEM medium (Gibco 12430062)

142     supplemented with 10% FBS, 100 IU/ml Penicillin and 100 µg/ml Streptomycin. Human

143     monocyte-like cells, THP-1 cell line (ATCC; catalog no. TIB-202), were cultured in RPMI 1640

144     medium (Gibco 21870076) supplemented with 10% FBS, 10mM HEPES, 1mM sodium pyruvate,

145     50µM of beta-mercaptoethanol, 100 IU/ml Penicillin and 100 µg/ml Streptomycin. SARS-CoV-

146     2 isolate USA-WA1/2020 (BEI resources catalog no. NR-52281) was propagated in Vero E6 cells

147     to generate a virus stock with a titer of 1.76 x$10^6$ 50% tissue culture infective doses ($TCID_{50}$)/mL.

148     All the SARS-CoV-2 titrations were performed by $TCID_{50}$ assay on Vero E6 cells and titers were

149     calculated by the method of Reed and Muench. Work with infectious virus was performed in

150     biosafety cabinets within a biosafety containment level 3 facility. Personnel wore powered air

151     purifying respirators during all procedures (MAXAIR Systems, Irvine, CA).

152    *Cell infection:* THP-1 monocyte-like cells were seeded in the presence of phorbol 12-myristate

153    13-acetate (PMA, 100ng/mL) to induce differentiation into macrophages. After 24 hours of

154    incubation, undifferentiated cells were washed away and remaining differentiated macrophages

155    were incubated in fresh media without PMA for an additional 24 hours. THP-1 cells were either

156    mock infected with supernatants from non-infected Vero E6 cells or inoculated at an MOI of 0.5

157    for 1 hour at 37C with virus stock generated in Vero E6 cells. Cells were washed once with PBS

158    and incubated at 37°C in complete media for the indicated times. Vero E6 cells were seeded and

159    incubated for 24 hours before virus infection at an MOI of 0.1 following the same procedure as

160    with THP-1 cells. After indicated times, culture supernatants were collected for titration assays

161    (TCID$_{50}$) and RNA was extracted from infected cells using RNeasy Plus Mini Kit (Qiagen; catalog

162    no. 74134) following manufacturer's instructions. RNA extracted from THP-1 cells treated with

163    100ng/mL of LPS (Invivogen, catalog no. tlrl-eklps) for 4 hours was used as a control.

164

165    *Cell treatment*: THP-1 monocyte-like cells were seeded in presence of phorbol 12-myristate 13-

166    acetate (PMA, 100ng/mL) and allowed to differentiate as described above. Cells were treated with

167    8nM (0.6μg/mL) of recombinant soluble SARS-CoV-2 Spike S1 protein purified from HEK293

168    cells (SinoBiological; catalog no. 40591-V08H-B) or an equivalent volume of vehicle control for

169    specified times. Cell culture supernatants were collected for ELISA assays and RNA was extracted

170    from infected cells using Trizol (ThermoFisher; catalog no. 15596026) following manufacturer's

171    instructions.

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173    *Reverse Transcription Quantitative PCR (RT-qPCR):* For cellular genes, total RNA (500ng) was

174    used for cDNA synthesis using High-Capacity cDNA Reverse Transcription Kit (Applied

175　Biosystems; catalog no. 4368814) following manufacturer's instructions. Approximately

176　20ng of cDNA were used as template for qPCR reactions using SSOAdvanced Universal

177　SYBR Green Supermix following manufacturer's instructions (BioRad; catalog

178　no.1725271). Following primers were used for detection of cellular genes by qPCR:

179

180　GAPDH　　Fw:5'-ACAACTTTGGTATCGTGGAAGG-3';

181　　　　　　Rv: 5'-GCCATCACGCCACAGTTTC-3'

182　TNF-α　　Fw:5'-CCTCTCTCTAATCAGCCCTCTG-3';

183　　　　　　Rv:5'- GAGGACCTGGGAGTAGATGAG-3'

184　IL6　　　Fw: 5'- ACTCACCTCTTCAGAACGAATTG-3';

185　　　　　　Rv: 5'- CCATCTTTGGAAGGTTCAGGTTG-3'

186　IFN-γ　　Fw: 5'- TCGGTAACTGACTTGAATGTCCA-3';

187　　　　　　Rv: 5'- TCGCTTCCCTGTTTTAGCTGC-3'

188　IFN-β　　Fw: 5'- GCTTGGATTCCTACAAAGAAGCA-3';

189　　　　　　Rv: 5'-ATAGATGGTCAATGCGGCGTC-3'

190　CXCL10　Fw: 5'-GTGGCATTCAAGGAGTACCTC-3';

191　　　　　　Rv: 5'- GCCTTCGATTCTGGATTCAGACA-3'

192

193　qPCR reactions were performed in a CFX96 Touch Real-Time PCR Detection System

194　(Biorad, CA). Relative gene expression of target genes was determined using the average

195　Ct for technical replicates normalized to GAPDH. Fold change over mock-infected cells

196　was determined using $2^{-ddCt}$ method.

197

198   For quantification of viral RNA, total RNA (250ng or 500ng) was used for cDNA synthesis using

199   SuperScript IV VILO Master Mix (Invitrogen, catalog no. 11756050) using the manufacturer's

200   instructions. cDNA was diluted to 1:10 and used as a template for qPCR reactions using PowerUp

201   SYBR Green Master Mix (Applied Biosystems, catalog no. A25742). Primers for detection of

202   SARS-CoV-2   genes[25]   N   (Fw:5'-CAATGCTGCAATCGTGCTAC-3';   Rv:5'-

203   GTTGCGACTACGTGATGAGG-3') and S (Fw:5'- GCTGGTGCTGCAGCTTATTA-3';

204   Rv: 5'- AGGGTCAAGTGCACAGTCTA-3') were used for qPCR, along with GAPDH

205   primers listed above. qPCR reactions were performed using a StepOnePlus Real-Time PCR

206   System (Applied Biosystems, CA), Ct values were determined using Design and Analysis 2.5.0

207   (Applied Biosystems), and normalized to GAPDH RNA levels using $2^{-dCt}$.

208

209   *Enzyme-linked immunosorbent assay (ELISA):* Human TNF alpha Uncoated ELISA Kit

210   (ThermoFisher, catalog no. 88-7346) was used to determine secretion of TNF-α in culture

211   supernatants of cells treated with SARS-CoV-2 S1 subunit. Cytokine concentration was calculated

212   according to manufacturer's instructions.

213

214   *Statistical analysis:* Two-Way ANOVA adjusted by Sidak's multiple comparison test was

215   performed to evaluate relative expression from RT-qPCR data and ELISA from three experimental

216   groups compared at multiple time points. A *p* value of <0.05 was considered significant for all

217   statistical tests. All statistical tests were performed using GraphPad Prism v6.01 (CA, USA).

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221 **Authorship**

222 T.A.M., K.C., K.H. and L.G.M. performed the experiments. T.A.M., K.C. and S.B. contributed to

223 the experimental design, data analyses and interpretation, preparation of figures and tables, and

224 prepared the manuscript. All authors have approved the final manuscript as submitted and agree

225 to be accountable for all aspects of the study.

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234 **Conflict of Interest Disclosure:** Authors have no conflicts of interest to declare.

235

236 <u>**References**</u>
237
238 1.   Guan, W. *et al.* Clinical Characteristics of Coronavirus Disease 2019 in China. *N. Engl. J.*

239      *Med.* **382**, 1708–1720 (2020).

240 2.   Zhang, H. *et al.* Histopathologic Changes and SARS-CoV-2 Immunostaining in the Lung

241      of a Patient With COVID-19. *Ann. Intern. Med.* **172**, 629–632 (2020).

242 3.   Qin, C. *et al.* Dysregulation of Immune Response in Patients With Coronavirus 2019

243      (COVID-19) in Wuhan, China. *Clin. Infect. Dis.* **71**, 762–768 (2020).

244 4.   Del Valle, D. M. *et al.* An inflammatory cytokine signature predicts COVID-19 severity

11

245    and survival. *Nat. Med.* **26**, 1636–1643 (2020).

246  5.    Giamarellos-Bourboulis, E. J., Netea, M. G. & Rovina, N. Clinical and Translational

247    Report Complex Immune Dysregulation in COVID-19 Patients with Severe Respiratory

248    Failure. doi:10.1016/j.chom.2020.04.009.

249  6.    Morris, S. B. *et al.* Case Series of Multisystem Inflammatory Syndrome in Adults

250    Associated with SARS-CoV-2 Infection — United Kingdom and United States, March–

251    August 2020. *MMWR. Morb. Mortal. Wkly. Rep.* **69**, 1450–1456 (2020).

252  7.    Liao, M. *et al.* Single-cell landscape of bronchoalveolar immune cells in patients with

253    COVID-19. *Nat. Med.* **26**, 842–844 (2020).

254  8.    Grant, R. A. *et al.* Circuits between infected macrophages and T cells in SARS-CoV-2

255    pneumonia. *Nature* 1–8 (2021) doi:10.1038/s41586-020-03148-w.

256  9.    Wang, C. *et al.* Alveolar macrophage dysfunction and cytokine storm in the pathogenesis

257    of two severe COVID-19 patients. *EBioMedicine* **57**, 102833 (2020).

258  10.   Zhao, Y. *et al.* SARS-CoV-2 spike protein interacts with and activates TLR41. *Cell Res.*

259    (2021) doi:10.1038/s41422-021-00495-9.

260  11.   Shirato, K. & Kizaki, T. SARS-CoV-2 spike protein S1 subunit induces pro-inflammatory

261    responses via toll-like receptor 4 signaling in murine and human macrophages. *Heliyon* **7**,

262    e06187 (2021).

263  12.   Hoffmann, M., Kleine-Weber, H. & Pöhlmann, S. A Multibasic Cleavage Site in the Spike

264    Protein of SARS-CoV-2 Is Essential for Infection of Human Lung Cells. *Mol. Cell* **78**,

265    779-784.e5 (2020).

266  13.   Sasaki, M. *et al.* SARS-CoV-2 variants with mutations at the S1/ S2 cleavage site are

267    generated in vitro during propagation in TMPRSS2-deficient cells. *PLoS Pathog.* **17**,

268    e1009233 (2021).

269    14.    Zhang, L. *et al.* SARS-CoV-2 spike-protein D614G mutation increases virion spike

270           density and infectivity. *Nat. Commun.* **11**, 1–9 (2020).

271    15.    Shajahan, A., Supekar, N. T., Gleinich, A. S. & Azadi, P. Deducing the N- and O-

272           glycosylation profile of the spike protein of novel coronavirus SARS-CoV-2.

273           *Glycobiology* **30**, 981–988 (2020).

274    16.    Yoshikawa, T., Hill, T., Li, K., Peters, C. J. & Tseng, C.-T. K. Severe Acute Respiratory

275           Syndrome (SARS) Coronavirus-Induced Lung Epithelial Cytokines Exacerbate SARS

276           Pathogenesis by Modulating Intrinsic Functions of Monocyte-Derived Macrophages and

277           Dendritic Cells. *J. Virol.* **83**, 3039–3048 (2009).

278    17.    Chen, N. *et al.* Epidemiological and clinical characteristics of 99 cases of 2019 novel

279           coronavirus pneumonia in Wuhan, China: a descriptive study. *Lancet* **395**, 507–513

280           (2020).

281    18.    Sanceau, J., Wijdenes,', J., Revel, M. & Wietzerbin, J. IL-6 and IL-6 receptor modulation

282           by IFN-γamma and tumor necrosis factor-alpha in human monocytic cell line (THP-1).

283           Priming effect of IFN-γamma. *J. Immunol.* **147**, 2021 (1991).

284    19.    Schmolka, N. *et al.* Epigenetic and transcriptional signatures of stable versus plastic

285           differentiation of proinflammatory γδ T cell subsets. *Nat. Immunol.* **14**, 1093–1100

286           (2013).

287    20.    Bandow, K. *et al.* LPS-induced chemokine expression in both MyD88-dependent and -

288           independent manners is regulated by Cot/Tpl2-ERK axis in macrophages. *FEBS Lett.* **586**,

289           1540–1546 (2012).

290    21.    Harris, D. P., Bandyopadhyay, S., Maxwell, T. J., Willard, B. & DiCorleto, P. E. Tumor

13

291        necrosis factor (TNF)-α induction of CXCL10 in endothelial cells requires protein

292        arginine methyltransferase 5 (PRMT5)-mediated nuclear factor (NF)-κB p65 Methylation.

293        *J. Biol. Chem.* **289**, 15328–15339 (2014).

294   22.   Zheng, J. *et al.* Severe Acute Respiratory Syndrome Coronavirus 2–Induced Immune

295        Activation and Death of Monocyte-Derived Human Macrophages and Dendritic Cells. *J.*

296        *Infect. Dis.* **XX**, 1–11 (2020).

297   23.   Li, S. *et al.* SARS-CoV-2 triggers inflammatory responses and cell death through caspase-

298        8 activation. *Signal Transduct. Target. Ther.* **5**, 1–10 (2020).

299   24.   Zhang, J. *et al.* Structural impact on SARS-CoV-2 spike protein by D614G substitution.

300        *Science.* **372**, 525–530 (2021).

301   25.   Park, M., Won, J., Choi, B. Y. & Lee, C. J. Optimization of primer sets and detection

302        protocols for SARS-CoV-2 of coronavirus disease 2019 (COVID-19) using PCR and real-

303        time PCR. *Exp. Mol. Med.* **52**, 963–977 (2020).

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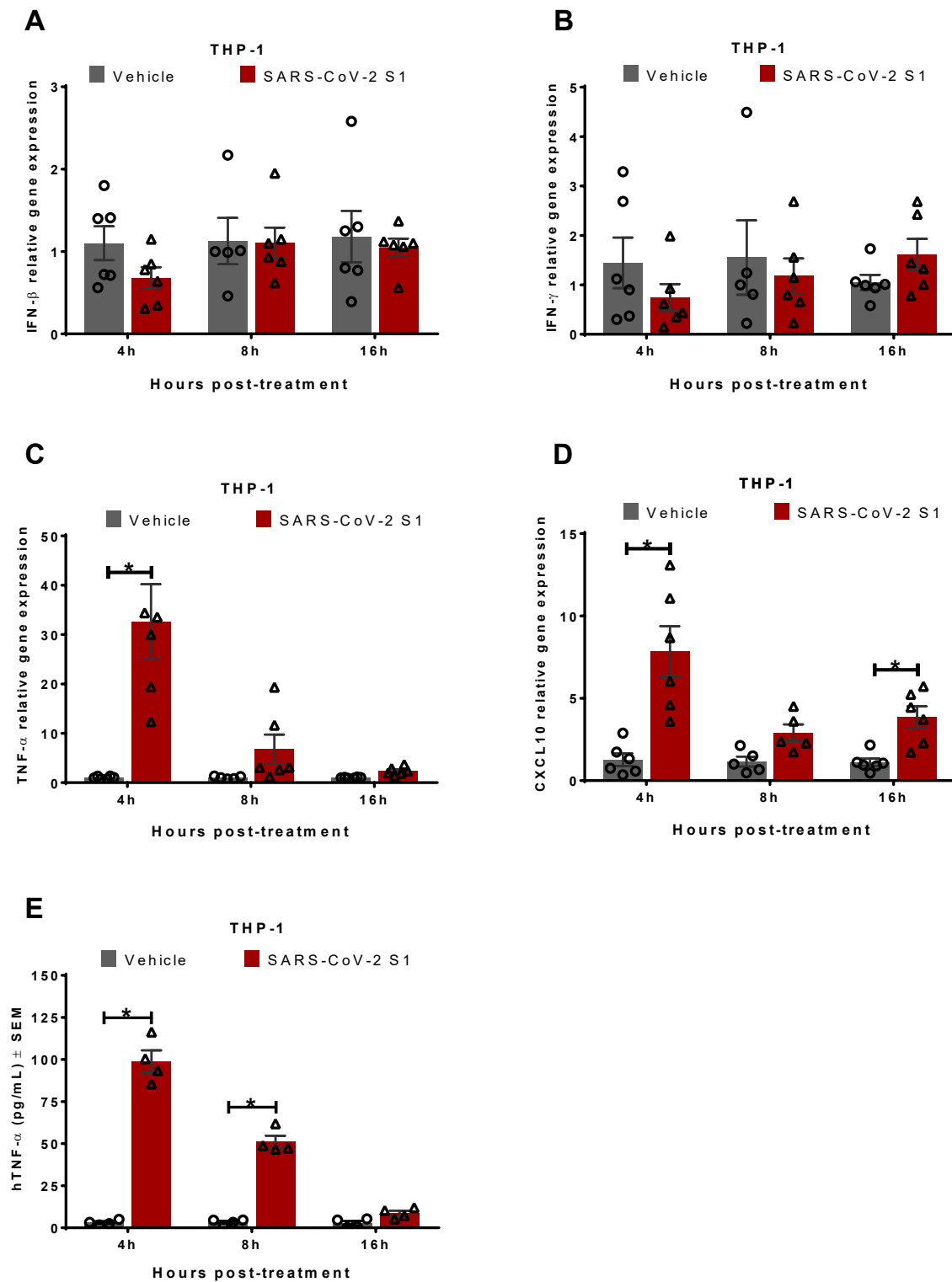308    **<u>Figure Legends</u>**

309    **Figure 1.** <u>SARS-CoV-2 soluble Spike protein S1 subunit (S1) induces inflammatory response in</u>

310    <u>THP-1 macrophages</u>. THP-1 cells were treated with purified recombinant soluble S1 protein

311    (0.6ug/mL, 8nM) or vehicle for indicated times. RT-qPCR was used to detect relative gene

312    expression of cytokines IFN-β (A), IFN-γ (B), TNF-α (C), and CXCL10 (D). (E) Secretion of

313    TNF-α was determined by ELISA assays in supernatants from THP-1 cells treated with purified

314    recombinant soluble S1 protein. Error bars denote the standard error of the mean (SEM) from 3

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315   biologically independent experiments. *$p < 0.05$, **$p < 0.01$ determined by Two-way ANOVA

316   adjusted by Sidak's multiple comparison test.

317

318   **Figure 2.** THP-1 macrophages express cytokines following SARS-CoV-2 infection in the absence

319   of productive virus replication. RT-qPCR was used to detect SARS-CoV-2 N (A) and S (B) viral

320   genes in mock infected or SARS-CoV-2 infected Vero E6 cells. RT-qPCR was used to detect N

321   (C) and S (D) viral genes in mock infected or SARS-CoV-2 infected THP-1 macrophages. E)

322   Culture supernatants from mock infected or SARS-CoV-2 infected THP-1 macrophages and Vero

323   E6 cells were analyzed by $TCID_{50}$ assay to determine infectious virus production. F) Bright field

324   microscopy photographs of Vero E6 (MOI=0.1) and THP-1 (MOI=0.5) macrophages infected with

325   SARS-CoV-2 for indicated times. RT-qPCR was used to detect relative gene expression of

326   cytokines TNF-α (G), CXCL10 (H), IFN-β (I) and IFN-γ (J) in mock infected or SARS-CoV-2

327   infected THP-1 macrophages. LPS-treated macrophages (100ng/mL, 4 hours) were used as

328   controls. Error bars denote the standard error of the mean (SEM) from 2 to 3 biologically

329   independent experiments. Hpi= hours post infection. *$p < 0.05$ determined by Two-way ANOVA

330   adjusted by Sidak's multiple comparison test.

331

332   **Figure 3.** Proposed contribution of SARS-CoV-2 and soluble S1 in inducing inflammatory

333   responses by macrophages. SARS-CoV-2 virions and soluble S1 proteins shed from productively

334   infected epithelial cells trigger proinflammatory responses by macrophages, which then contribute

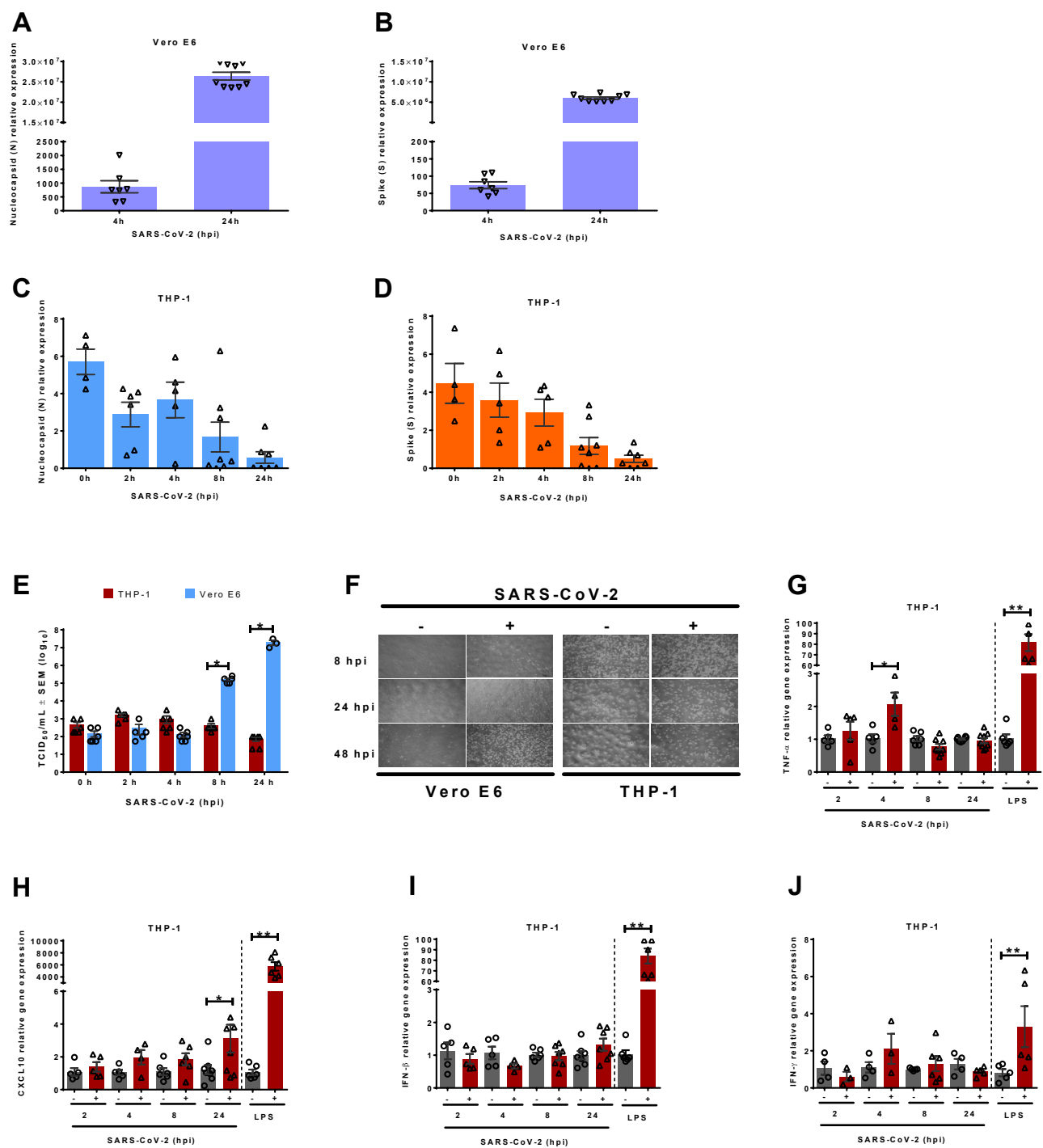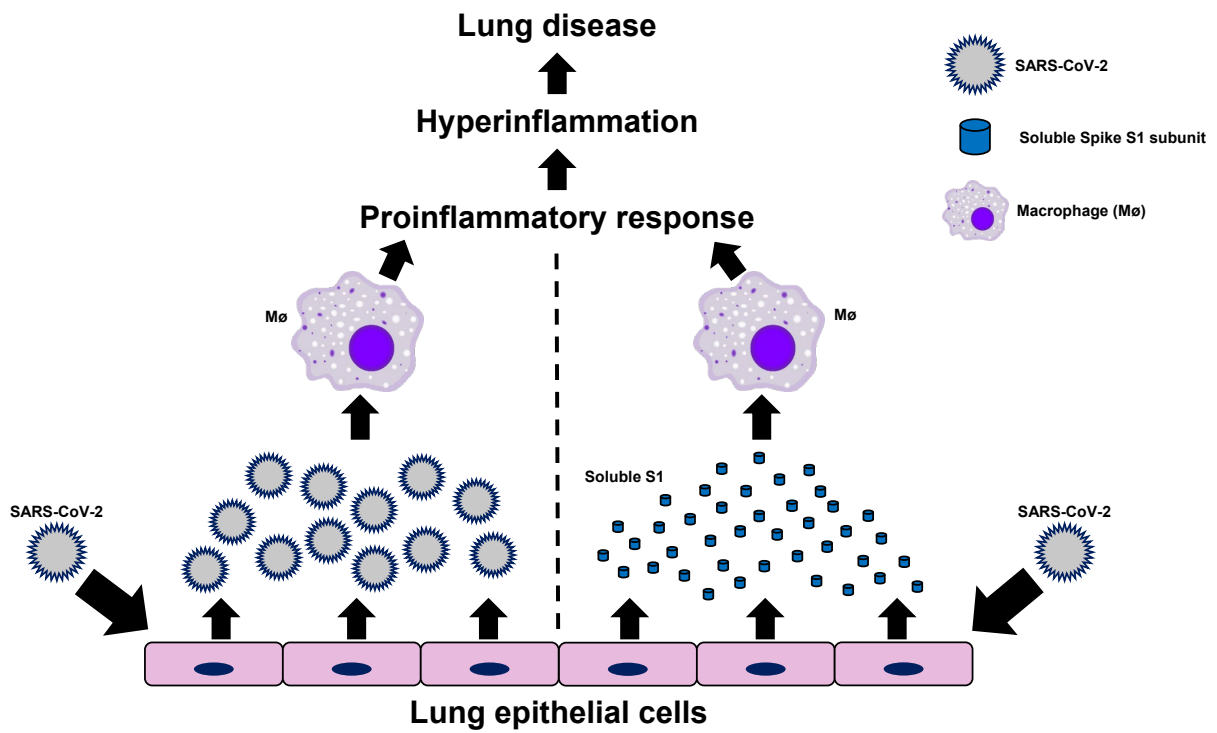335   to hyperinflammation and lung disease associated with COVID-19.

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338 **Figure 1**



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341    **Figure 2**



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345   **Figure 3**



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