Peramivir, an anti-influenza virus drug, exhibits potential anti-cytokine storm effects

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Running title: Peramivir as a potential anti-cytokine storm agent

Abstract Coronavirus Disease 2019 (COVID-19) infected by Severe Acute Respiratory Syndrome Coronavirus -2 (SARS-CoV-2) has been declared a public health emergency of international concerns. Cytokine storm syndrome (CSS) is a critical clinical symptom of severe COVID-19 patients, and the macrophage is recognized as the direct host cell of SARS-CoV-2 and potential drivers of CSS. In the present study, peramivir was identified to reduce TNF- α by partly intervention of NF- κ B activity in LPS-induced macrophage model. In vivo, peramivir reduced the multi-cytokines in serum and bronchoalveolar lavage fluid (BALF), alleviated the acute lung injury and prolonged the survival time

17 in mice. In human peripheral blood mononuclear cells (hPBMCs), peramivir could also inhibit the release of TNF- α . Collectively, we proposed
18 that peramivir might be a candidate for the treatment of COVID-19 and other infections related CSS.

19
20 **KEY WORDS** cytokine storm syndrome; COVID-19; peramivir; acute lung injury; multi-cytokines

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Graphic Abstract

27 INTRODUCTION

28 Coronavirus disease 2019 (COVID-19) caused by Severe Acute Respiratory Syndrome Coronavirus -2 (SARS-CoV-2) has been reported to
 29 infect more than 12 million people worldwide killing 500 thousand people (updated on Jul 10th, 2020).¹ World Health Organization (WHO) has
 30 declared COVID-19 as a public health emergency of international concerns.² COVID-19 patients show typical clinical symptoms of fever,
 31 fatigue, dry cough and pneumonia.³⁻⁵ Excessive inflammatory response leads to acute respiratory distress syndrome (ARDS), coagulopathy, and
 32 septic shock which can be fatal in critical cases.³ Gross anatomy identifies the main pathological features including exudation and hemorrhage,
 33 epithelium injuries, infiltration of inflammatory immune cells and fibrosis in the lungs of fatal patients.^{3, 6-10}

34 A syndrome with a distinct cytokine storm was showed in a subgroup of patients with severe COVID-19, which has also been reported in
 35 SARS-CoV infected patients.¹¹⁻¹⁴ The cytokine storm refers to an uncontrolled excessive inflammatory response, spreading from a local lesion to
 36 the whole body through the systemic circulation.^{15, 16} The plasma concentrations of inflammation related cytokines such as interleukins (IL) -2,
 37 -6, -7, and -10, tumor necrosis factor- α (TNF- α), interferon- γ -inducible protein 10 (IP10), granulocyte-colony stimulating factor (G-CSF),
 38 monocyte chemoattractant protein -1 (MCP-1), and macrophage inflammatory protein 1 alpha (MIP-1 α) were significantly increased in the
 39 COVID-19 patients.^{3, 17, 18} Specially, the activation of alveolar macrophages was a characteristic abnormality.⁸

40 The leading cause of mortality is thought to be ARDS-induced respiratory failure, and patients generally receive supportive management in
 41 clinic practice.¹⁷ However, there is still no well-accepted effective treatment for COVID-19. The development of novel therapeutics has been
 42 mainly focused on antivirals^{19, 20} and vaccines.¹¹ In addition, the cytokine storm is increasingly being recognized as a key node for the patients
 43 deteriorating to severe COVID-19.^{3, 11, 17} Therefore, anti-inflammatory therapy has been considered as one of appropriate clinical adjuvant

44 treatment options, and the treatments include steroids (e.g., prednisone),²¹ selective cytokine blockade (e.g., tocilizumab),¹¹ JAK inhibition (e.g.,
45 Baricitinib),²² intravenous immunoglobulin, Chinese medicines and blood purification.^{11, 21}

46 Early in Feb, 2020, 75 of 99 COVID-19 patients received antiviral treatment including oseltamivir.²³ Besides, oseltamivir is noted to have
47 been widely used for confirmed or suspected COVID-19 cases in hospitals in China²⁴ and Thailand (NCT04303299). The other two clinical trials
48 (NCT04261270 and NCT04255017) involving oseltamivir in the treatment of COVID-19 are currently ongoing. However, the FDA-approved
49 neuraminidase inhibitors including oseltamivir, zanamivir and peramivir were ineffective against the SARS-CoV-2 virus in vitro.²⁵ There has
50 been no exact evidence to date that oseltamivir is effective in the treatment of COVID-19 in clinic.¹⁹

51 It is reported that oseltamivir exhibited the antiviral activity of reducing pulmonary viral load, thereby the cytokines production was
52 suppressed.²⁶ Macrophages play a vital role in both SARS-CoV-2 virus -induced lung lesions and the host cytokine-mediated response.⁸ In our
53 previous study, entecavir, a hepatitis B virus (HBV) inhibitor, was demonstrated to directly inhibit the release of cytokines in lipopolysaccharide
54 (LPS) -stimulated macrophage model,²⁷ which is a classic in vitro model to evaluate the anti-inflammatory activity of the drugs.²⁸ Herein, we
55 also examined whether these three neuraminidase inhibitors could inhibit the expression of inflammatory cytokines in LPS-stimulated
56 macrophages in the present study. The results showed peramivir had the best ability to inhibit TNF- α by ~70% among the three compounds.
57 Furthermore, we estimated the anti-cytokine storm effect and lung protection of peramivir in vivo. The anti-inflammatory effect of peramivir in
58 human peripheral blood mononuclear cells (hPBMCs) was also observed.

59

60 MATERIALS AND METHODS

61 **Materials**

62 Compounds were purchased from TargetMol with a purity of > 98% (TargetMol). LPS (*E. coli* 0111:B4) was obtained from MilliporeSigma.

63 **Animal**

64 Male C57BL/6J mice (18-22 g) were purchased from the Changzhou Cavens Laboratory Animal Co., China. All mice were kept under an
65 automated 12 h dark-light cycle at a controlled temperature of 22°C ± 2°C and a relative humidity of 50%– 60% with free access to standard dry
66 diet and tap water. All animal experiments were carried out in adherence with the NIH *Guide for the Care and Use of Laboratory Animals*
67 (National Academies Press, 2011) and were approved by the Second Military Medical University Committee on Animal Care (EC11-055).

68 **CCS model**

69 CCS was induced by a single i.p. injection of LPS (15 mg/kg), as described previously.²⁹ After 4 h, mice were sacrificed and serum was collected.
70 After 8 h, the left bronchus was ligated and 1 mL saline was perfused into right lung lobe to collect BALF, and the left lung was fixed with
71 paraformaldehyde for histological analysis. Serum and BALF were further used for multi-cytokine analysis.

72 To investigate the effect of drugs on the survival time of CSS model mice, a single i.p. injection of saline (n=10), peramivir (20 and 60 mg/kg,
73 n=10) was performed at 1 h before i.p. injection of a lethal dose of LPS (30 mg/kg) to mice, respectively. After modeling, mice survival was
74 recorded every 2 h until 40 h.

75 **Preparation of the peritoneal macrophages**

76 The peritoneal macrophages were obtained from the mice after i.p. injection of 3 ml of 3% thioglycolate as described previously.²⁹ Briefly, the
77 mice were sacrificed, and the macrophages were isolated by lavage with 5 ml of RPMI 1640 (Gibco Life Technologies), washed twice with PBS

after 4 h of adherence, cultured in RPMI at 37°C and 5% CO₂, and finally stimulated with 100 ng/ml LPS to harvest supernatants. The isolated cells were used for cytokine analysis and cell viability assays.

Preparation of the hPBMCs

hPBMCs were obtained from freshly collected buffy coat fractions from healthy donors at the Tongren Hospital Affiliated to Shanghai Jiaotong University, China. Briefly, hPBMCs were isolated by centrifugation over a Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient at 800 g for 20 min at room temperature in a Sorvall RT6000B (DuPont, Wilmington, DE, USA). Most hPBMC isolates were adherence cells that mainly contained macrophages and monocytes. Isolated hPBMCs were cultured in RPMI 1640, 100 U/mL penicillin–streptomycin (Invitrogen Life Technologies), and 10% heat-inactivated fetal calf serum (Gibco Life Technologies). 3×10^5 cells were seeded in 96-well plates and incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO₂. hPBMCs were pretreated with peramivir at the concentrations of 2.5, 5 and 10 μM at 1 h before LPS (100 ng/ml) stimulation, and the supernatants were harvested at 6 or 12 h after LPS stimulation for cytokine analysis.

Cell viability assay

Cell Counting Kit-8 (TY0312, Dojindo Molecular Technology, Japan) was used to measure cell viability. Briefly, 10 μL of CCK-8 solution was added, and cells were incubated for 1 h at 37°C. Absorbance was measured using a Cytation 5 Cell Imaging Multi-mode Reader (BioTek Instruments, USA) at a wavelength of 450 nm.

Anti-inflammatory activity screening

We chose an in vitro model of LPS-stimulated peritoneal macrophages to induce TNF-α secretion, and screened potential anti-inflammatory molecules in the antiviral and antibacterial drug library. Briefly, 100 ng/ml LPS stimulated peritoneal macrophages for 4 h with simultaneous

incubation of compounds at a concentration of 10 μ M. The cell supernatant was diluted 10-fold and the TNF- α content was measured with a mouse TNF- α Elisa kit obtained from Invitrogen. The remaining cells were subjected to CCK8 assay to detect cytotoxicity.

NF- κ B luciferase activity assay

RAW264.7 cells stably transfected with an NF- κ B-responsive luciferase construct, kindly provided by Prof. An Qin (Shanghai Jiaotong University, China), were seeded in 96-well plates at a density of 2×10^5 cells per well, as previously described.³⁰ After 24 h, cells were pretreated with peramivir for 1 h and stimulated with LPS for 6 h. Cells were dealt with a luciferase assay system (Promega) and the luciferase activity was calculated using a Cytation 5 Cell Imaging Multi-mode Reader (BioTek Instruments, USA).

Western Blotting

Protein samples were separated by 10% SDS-PAGE, transferred to NC membrane and blocked with 5% non-fat milk in TBST. The membranes were washed with TBST and then incubated with the primary antibody for 6 hours at 4 degrees Celsius. The primary antibodies (1:1000) used were all from Cell Signaling Technology, USA and listed as follows: GAPDH antibody(#2118), stat3 antibody(#12640), phospho-stat3 antibody(#98543), SAPK/JNK antibody(#9252), phospho-SAPK/JNK antibody(#4668), p65 antibody(#4764), phospho-p65 antibody(#3033), IKK α antibody(#2682), phospho-IKK α/β antibody(#2697), I κ B α antibody(#4812), phospho-I κ B α antibody(#2859), p38 MAPK antibody(#8690), phospho-p38 MAPK antibody(#4511), Erk1/2 antibody(#4695) and phospho-Erk1/2 antibody(#4370). Then, the membranes were incubated in HRP-linked goat anti-rabbit IgG Antibody (1:10000, Cell Signaling Technology, USA, #7074) at room temperature for 1 hour and signals were detected by chemiluminescence (Bio rad, USA).

ELISA

112 TNF- α released by mouse peritoneal macrophages was measured by a Mouse TNF- α ELISA Kit (Invitrogen, BMS607-3TEN) according to the
113 manufacturer's protocol. TNF- α released by hPBMCs were measured by Human TNF- α ELISA Kit (Youda, 1117202) according to the
114 manufacturer's protocol.

115 **Multi-cytokine measurement**

116 The serum levels of a total of 12 virus-related cytokines were measured by a bead-based immunoassay panel (Mouse Anti-Virus Panel, Cat No:
117 740622, Biolegend, USA). The BALF levels of a total of 12 inflammatory cytokines were measured by a bead-based immunoassay panel
118 (Mouse Inflammation Panel, Cat No: 740446, Biolegend, USA) on CytoFLEX Flow Cytometer (Beckman Coulter, USA) according to the
119 manufacturer's protocol.

120 **Immunofluorescence staining**

121 Isolated peritoneal macrophages in eight-well LabTek slides (PEZGS0816, Millipore, Billerica, Massachusetts, USA) were pretreated with
122 peramivir one hour before cell were simulated by LPS at a concentration of 1 μ g /ml for 30 min. Then cells were fixed in 4% paraformaldehyde,
123 blocked with 0.4% Triton X-100/2% bovine serum albumin at room temperature for 1 h, and then incubated with primary antibodies for p65
124 (CST, #8242, 1:400 dilution) overnight at 4 °C. After being washed with PBST 3 times, the samples were incubated with Alexa Fluor 488
125 (Beyotime, A0423, 1:500 dilution) for 1 h and washed again with PBS. Nuclei were stained with DAPI. Images were obtained by confocal
126 microscopy (TCS SP5, Leica, Solms, Germany).

127 **Histological analysis**

128 The left lung lobes of mice were fixed using formalin, and then the tissues were paraffin-embedded. Sections (5 μ m) of formalin-fixed tissues

were stained with haematoxylin and eosin (H&E) according to the manufacturer's instructions, and were photographed with a microscope (Olympus Corporation, Tokyo, Japan). The histological characteristics of the lung injury (including alveolar edema and hemorrhage, the number of infiltrating leukocytes, and the thickness of the alveolar wall and epithelium) were evaluated. Each histological characteristic was evaluated on a scale of 0 to 3 (0, normal; 1, mild; 2, moderate; 3, severe).

Statistical analysis

Data were expressed as means $\pm$ SEM. Statistical analyses used Student's *t*-test, two-way ANOVA or Kaplan-Meier Survival Analysis. GraphPad software was used for data analysis. Statistical significance was indicated as follows: **P* < 0.05, ***P* < 0.01, ****P* < 0.001, n.s. not significant.

RESULTS

Peramivir is an active anti-inflammatory agent without apparent cytotoxicity.

The three neuraminidase inhibitors (peramivir, oseltamivir, and zanamivir, Fig. 1a) were explored for their ability to inhibit TNF- α -induced by LPS in macrophages. They inhibit the elevation of TNF- α by 67.2%, 35.6% and 13.1% at 10 μ M, respectively (Fig. 1b). Furthermore, peramivir dose-dependently inhibited TNF- α release with the half-maximal inhibitory concentration (IC₅₀) as 4.3 μ M (Fig. 1c). Given that the inhibitory effect of the drugs on TNF- α might be achieved by cytotoxicity, we tested the cytotoxicity of peramivir in macrophages by a CCK-8 assay. It was demonstrated that no apparent toxicity was observed in the peramivir-treated macrophages at concentrations up to 40 μ M (Fig. 1d).

Peramivir inhibits LPS-induced cytokine storm in mice.

We used LPS-induced cytokine storm syndrome (CSS) mouse model to evaluate the in vivo inflammatory inhibitory activity. Peramivir was

146 administrated intraperitoneally (i.p.) at the concentration of 60 mg/kg at 1 h before LPS injection, and serum was collected at 4 h after LPS
147 injection for further experiment. Twelve cytokines in total were simultaneously measured using a mouse antiviral panel by flow cytometric bead
148 array. Compared with the control group, 7 cytokines including TNF- α , IL family (IL-6, IL-1 β , IL-12), chemokines (MCP-1), interferon family
149 (IFN- α , γ) were significantly decreased by the treatment (Table 1 and Fig. S1). The other 5 cytokines including IL-10, IP-10, chemokine (C-C
150 motif) ligand 5 (CCL-5), granulocyte-macrophage colony stimulating factor (GM-CSF), CXC chemokine ligand 1 (CXCL1) were slightly
151 downregulated without statistical significance.

152 Given that cytokines in bronchoalveolar lavage fluid (BALF) could directly represent the inflammation status in the lungs,^{7, 31} we examined
153 cytokines in BALF after 8 h of LPS stimulation. Twelve cytokines in total were simultaneously measured using a mouse inflammation panel by
154 flow cytometric bead array. Compared with the control group, TNF- α and IL-6 were significantly decreased by the treatment (Table 2 and Fig.
155 S2). IL-1 β , IL-10, IL-17A and MCP-1 showed downregulation without significant difference.

156 **Peramivir effectively attenuates acute lung injury and prolong the survival in LPS-induced mice.**

157 The histological examinations of two COVID-19 death cases both showed alveolar damage with cellular fibromyxoid exudates, pulmonary
158 edema and interstitial mononuclear inflammatory infiltrates.^{7, 31} The mice injected with LPS (i.p.) exhibited similar pathological features to
159 ARDS, such as infiltration of inflammatory cells (black arrow), congestion (green arrow) and edema within thickened alveolar (yellow arrow)
160 (Fig. 2a). In contrast, the alveolar structures of mice in the peramivir treated group were relatively intact, inflammatory cell infiltrations were
161 significantly reduced, and mild alveolar thickening and less bleeding points or congestion were observed (Fig. 2a). Lung injury scores were
162 calculated (Fig. 2b) to show significant protective effects of peramivir (score = 2.6 ± 0.6) to the lung tissues compared with that of control group

(score = 4.8 ± 0.33). The survival time was prolonged in mice treated with peramivir in a dose-dependent manner after an i.p. injection of a lethal dose of LPS (30 mg/kg) compared with that in control mice (Fig. 2c).

Peramivir decreases NF- κ B transcriptional activity in RAW264.7 and the peritoneal macrophages.

NF- κ B is an important transcriptional regulator in cells that participated in inflammatory responses, of which the activation induces the expression of multiple genes and production of cytokines consequently leading to cytokine storm.³² Peramivir dose-dependently inhibited LPS-induced NF- κ B transcriptional activity in the RAW264.7 cells with a NF- κ B reporter luciferase system (Fig. 2d). Furthermore, we detected some key factors in inflammation responses by western blot and found that the LPS-induced activation of NF- κ B pathway (phosphorylation of p65) and MAPKs (phosphorylation of p38 and Erk1/2) were inhibited after peramivir intervention (Fig. 2e). Immunofluorescence images of LPS-stimulated peritoneal macrophages treated with or without peramivir indicated that LPS-induced nuclear translocation of p65 was attenuated by peramivir (Fig. 2f).

Peramivir inhibits multi-cytokine releases in LPS-induced human peripheral blood mononuclear cells (hPBMCs).

Considering the translational value of peramivir in clinical practices, the release of TNF- α was tested in LPS-induced hPBMCs, which were obtained from two healthy donors. Peramivir (Fig. 3a, b and Fig. S3) significantly counteracted the level of TNF- α at 6 h and 12 h in a time- and dose-dependent manner without apparent toxicity.

DISCUSSION

The three neuraminidase inhibitors bear similar pharmacophoric side chains (black, Fig. 1a) and different core scaffolds (red, Fig. 1a). Peramivir

180 has a five-membered cyclopentanol ring, while oseltamivir and zanamivir have six-membered ring, which might be a critical part for the
181 anti-inflammatory activity in the chemical structure of view.

182 Peramivir, an intravenous neuraminidase inhibitor, was approved for the emergency use in severe influenza in 2009 by the FDA. The antiviral
183 effect of peramivir on influenza has been described previously,³³ nevertheless, few studies have paid attentions to the anti-inflammatory activity
184 of peramivir. The activation of the immune system is attributed to the virus-induced cytokine response.^{34, 35} Inhibition of these cytokines can
185 potentially control the severity of the virus-induced inflammatory complications and finally reduce the mortality.^{34, 35} The inflammatory
186 cytokines and influenza pathogenicity has been well correlated.^{36, 37} In mouse model of H1N1 influenza, peramivir inhibits the levels of TNF- α ,
187 IL6 and IFN- γ in the lung tissue.³⁸ And compared to oseltamivir, peramivir shows more obvious anti-inflammatory effect.³⁹ The
188 anti-inflammatory effect of peramivir in vivo may be due to its antiviral symptomatic treatment, while we tried to investigate whether peramivir
189 can directly inhibit the release of cytokines by inflammatory immune cells.

190 As the neuraminidase is not expressed in the SARS-CoV-2 virus, oseltamivir, zanamivir and peramivir were ineffective against the virus in
191 vitro.²⁵ In a clinical study, 75% of the COVID-19 patients received antiviral treatment including oseltamivir and 5 of them simultaneously
192 infected with SARS-CoV-2 and influenza were recovered after treating with oseltamivir.²³ We hypothesized that these neuraminidase inhibitors
193 might have other effects including anti-inflammation, indicating the adjuvant therapeutic value of neuraminidase inhibitors in COVID-19.

194 Viruses and bacteria induce immune cell activation and release of cytokines are Toll-like receptors (TLRs) dependent.⁴⁰ The induction of
195 inflammatory cytokines depends on the activation of NF- κ B although they recognize different subtypes of TLRs.⁴¹ We assumed that
196 SARS-CoV-2 may activate NF- κ B on cytokine storm similar to that of SARS-CoV.⁴¹ LPS activates immune cells such as monocytes and

macrophages, causing the synthesis and release of inflammatory cytokines.²⁸ TNF- α is one of the central cytokines involved in inflammation initiation and amplification in virus infections,⁴² and is reported to be elevated in critical COVID-19 cases,³ suggesting it as a proper indicator for in vitro drug screening.

Compared with oseltamivir, peramivir shows better inhibition of TNF- α in vitro. The reduction in TNF- α , IL-1 β , IL-6, IL-12, IFN- α , IFN- γ and MCP-1 levels promoted by peramivir might play an important role in reducing stress due to the overactivated immune system and preventing organ damage of infected mice. This hypothesis was confirmed by pathological examination and lung index evaluation, which found that treatment with peramivir alleviated the severity of LPS-associated pneumonia and prolonged the survival time for mice. The lethal lung pathology caused by LPS was due to the excessive cytokine response that was primarily produced by the activated macrophages.²⁸ Peramivir inhibited the levels of TNF- α and IL-6 in the BALF of LPS-induced mice. Furthermore, peramivir attenuated TNF- α induced by LPS both in mouse peritoneal macrophages and hPBMCs, which confirmed that peramivir can inhibit the inflammatory cytokine response mediated by macrophages.

In conclusion, the modulatory function of peramivir on LPS-induced inflammatory cytokines might contribute to the additional beneficial effect of the drug in antiviral therapy. This study provides evidence for the therapeutic value of peramivir for the potential application as an anti-inflammatory agent against cytokine dysregulation.

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218

219 **AUTHOR CONTRIBUTIONS**

220 L. S., C.-L. Z., W.-N. Z, Z.-B. W. conceived and designed the experiments; L. S., Y. T., D.-G. C., C.-X. Z., Z.-B. W. participated in the
221 experiments; L. S., Y. T., Z.-B. W. analyzed the data; L. S., C.-L. Z., Z.-B. W. wrote the manuscript; all the authors provided the final approval
222 of the manuscript.

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224 **DECLARATION OF COMPETING INTEREST**

225 The authors declare no competing interests.

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227 **REFERENCES**

228 [1] WHO, Coronavirus disease 2019 (COVID-19) situation report – 172. Jul 10, 2020., in, 2020.

229 [2] WHO. Coronavirus disease (COVID-19) Pandemic, 2020.

- 230 [3] Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J, Wei Y, Wu W, Xie X, Yin W, Li H, Liu M,
231 Xiao Y, Gao H, Guo L, Xie J, Wang G, Jiang R, Gao Z, Jin Q, Wang J, Cao B. Clinical features of patients infected with 2019 novel coronavirus
232 in Wuhan, China, Lancet 2020;**395**: 497-506.
- 233 [4] Young BE, Ong SWX, Kalimuddin S, Low JG, Tan SY, Loh J, Ng OT, Marimuthu K, Ang LW, Mak TM, Lau SK, Anderson DE, Chan KS,
234 Tan TY, Ng TY, Cui L, Said Z, Kurupatham L, Chen MI, Chan M, Vasoo S, Wang LF, Tan BH, Lin RTP, Lee VJM, Leo YS, Lye DC, Singapore
235 Novel Coronavirus Outbreak Research T. Epidemiologic Features and Clinical Course of Patients Infected With SARS-CoV-2 in Singapore,
236 JAMA 2020;10.1001/jama.2020.3204.
- 237 [5] Holshue ML, DeBolt C, Lindquist S, Lofy KH, Wiesman J, Bruce H, Spitters C, Ericson K, Wilkerson S, Tural A, Diaz G, Cohn A, Fox L,
238 Patel A, Gerber SI, Kim L, Tong S, Lu X, Lindstrom S, Pallansch MA, Weldon WC, Biggs HM, Uyeki TM, Pillai SK, Washington State -nCo
239 VCIT. First Case of 2019 Novel Coronavirus in the United States, N Engl J Med 2020;**382**: 929-936.
- 240 [6] Assiri A, Al-Tawfiq JA, Al-Rabeeah AA, Al-Rabiah FA, Al-Hajjar S, Al-Barrak A, Flemban H, Al-Nassir WN, Balkhy HH, Al-Hakeem RF,
241 Makhdoom HQ, Zumla AI, Memish ZA. Epidemiological, demographic, and clinical characteristics of 47 cases of Middle East respiratory
242 syndrome coronavirus disease from Saudi Arabia: a descriptive study, Lancet Infect Dis 2013;**13**: 752-761.
- 243 [7] Xu Z, Shi L, Wang Y, Zhang J, Huang L, Zhang C, Liu S, Zhao P, Liu H, Zhu L, Tai Y, Bai C, Gao T, Song J, Xia P, Dong J, Zhao J, Wang FS.
244 Pathological findings of COVID-19 associated with acute respiratory distress syndrome, Lancet Respir Med
245 2020;10.1016/S2213-2600(20)30076-X.
- 246 [8] Bian X, Shi Z, Chen R, Cai J, Wang C, Xie J, Zhao L, Fei X, Zhang H, Tan Y, Zhou L, Liu Z, Ren Y, Yuan L, Zhang Y, Zhang J, Liang L,
247 Chen X, Liu X, Wang P, Han X, Weng X, Chen Y, Yu T, Zhang X. Alveolar Macrophage Activation and Cytokine Storm in the Pathogenesis of

- 248 Severe COVID-19, Research Square 2020;10.21203/rs.3.rs-19346/v1.
- 249 [9] Qin C, Zhou L, Hu Z, Zhang S, Yang S, Tao Y, Xie C, Ma K, Shang K, Wang W, Tian DS. Dysregulation of immune response in patients with
250 COVID-19 in Wuhan, China, Clin Infect Dis 2020;10.1093/cid/ciaa248.
- 251 [10] Conti P, Ronconi G, Caraffa A, Gallenga CE, Ross R, Frydas I, Kritas SK. Induction of pro-inflammatory cytokines (IL-1 and IL-6) and
252 lung inflammation by COVID-19: anti-inflammatory strategies, J Biol Regul Homeost Agents 2020;**34**.
- 253 [11] Mehta P, McAuley DF, Brown M, Sanchez E, Tattersall RS, Manson JJ. COVID-19: consider cytokine storm syndromes and
254 immunosuppression, Lancet 2020;10.1016/s0140-6736(20)30628-0.
- 255 [12] Hui DSC, Zumla A. Severe Acute Respiratory Syndrome: Historical, Epidemiologic, and Clinical Features, Infect Dis Clin North Am
256 2019;**33**: 869-889.
- 257 [13] Rockx B, Baas T, Zornetzer GA, Haagmans B, Sheahan T, Frieman M, Dyer MD, Teal TH, Proll S, van den Brand J, Baric R, Katze MG.
258 Early upregulation of acute respiratory distress syndrome-associated cytokines promotes lethal disease in an aged-mouse model of severe acute
259 respiratory syndrome coronavirus infection, J Virol 2009;**83**: 7062-7074.
- 260 [14] Smits SL, de Lang A, van den Brand JM, Leijten LM, van IWF, Eijkemans MJ, van Amerongen G, Kuiken T, Andeweg AC, Osterhaus AD,
261 Haagmans BL. Exacerbated innate host response to SARS-CoV in aged non-human primates, PLoS Pathog 2010;**6**: e1000756.
- 262 [15] Tisoncik JR, Korth MJ, Simmons CP, Farrar J, Martin TR, Katze MG. Into the eye of the cytokine storm, Microbiol Mol Biol Rev 2012;**76**:

263 16-32.

264 [16] Channappanavar R, Perlman S. Pathogenic human coronavirus infections: causes and consequences of cytokine storm and
265 immunopathology, *Semin Immunopathol* 2017;**39**: 529-539.

266 [17] Ruan Q, Yang K, Wang W, Jiang L, Song J. Clinical predictors of mortality due to COVID-19 based on an analysis of data of 150 patients
267 from Wuhan, China, *Intensive Care Med* 2020;10.1007/s00134-020-05991-x.

268 [18] Wei H, Xu X, Tian Z, Sun R, qi Y, Zhao C, Wang D, Zheng X, Fu B, Zhou Y. Pathogenic T cells and inflammatory monocytes incite
269 inflammatory storm in severe COVID-19 patients, *Natl Sci Rev* 2020;10.1093/nsr/nwaa041.

270 [19] Lu H. Drug treatment options for the 2019-new coronavirus (2019-nCoV), *Biosci Trends* 2020;**14**: 69-71.

271 [20] Li G, De Clercq E. Therapeutic options for the 2019 novel coronavirus (2019-nCoV), *Nat Rev Drug Discov* 2020;**19**: 149-150.

272 [21] New coronavirus pneumonia prevention and control program (7th ed.) (in Chinese). in, 2020.

273 [22] Richardson P, Griffin I, Tucker C, Smith D, Oechsle O, Phelan A, Stebbing J. Baricitinib as potential treatment for 2019-nCoV acute
274 respiratory disease, *Lancet* 2020;**395**: e30-e31.

275 [23] Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, Qiu Y, Wang J, Liu Y, Wei Y, Xia J, Yu T, Zhang X, Zhang L. Epidemiological and clinical
276 characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study, *Lancet* 2020;**395**: 507-513.

- 277 [24] Ding Q, Lu P, Fan Y, Xia Y, Liu M. The clinical characteristics of pneumonia patients co-infected with 2019 novel coronavirus and
278 influenza virus in Wuhan, China, J Med Virol 2020;10.1002/jmv.25781.
- 279 [25] Li H, Wang YM, Xu JY, Cao B. [Potential antiviral therapeutics for 2019 Novel Coronavirus], Zhonghua Jie He He Hu Xi Za Zhi 2020;**43**:
280 170-172.
- 281 [26] Lee N, Wong CK, Chan MCW, Yeung ESL, Tam WWS, Tsang OTY, Choi KW, Chan PKS, Kwok A, Lui GCY, Leung WS, Yung IMH,
282 Wong RYK, Cheung CSK, Hui DSC. Anti-inflammatory effects of adjunctive macrolide treatment in adults hospitalized with influenza: A
283 randomized controlled trial, Antiviral Res 2017;**144**: 48-56.
- 284 [27] Su L, Tu Y, Kong DP, Chen DG, Zhang CX, Zhang WN, Zhuang CL, Wang ZB. Drug repurposing of anti-infective clinical drugs: discovery
285 of two potential anti-cytokine storm agents, Pharmacol Res 2020; Under review.
- 286 [28] Nathan C. Points of control in inflammation, Nature 2002;**420**: 846-852.
- 287 [29] Zhang S, Tu Y, Sun YM, Li Y, Wang RM, Cao Y, Li L, Zhang LC, Wang ZB. Swiprosin-1 deficiency impairs macrophage immune response
288 of septic mice, JCI Insight 2018;**3**.
- 289 [30] Zhu S, Soutto M, Chen Z, Peng D, Romero-Gallo J, Krishna US, Belkhiri A, Washington MK, Peek R, El-Rifai W. Helicobacter
290 pylori-induced cell death is counteracted by NF-kappaB-mediated transcription of DARPP-32, Gut 2017;**66**: 761-762.
- 291 [31] Luo W, Yu H, Gou J, Li X, Sun Y, Li J, Liu L. Clinical Pathology of Critical Patient with Novel Coronavirus Pneumonia (COVID-19),

292 Preprints 2020: 2020020407.

293 [32] Chen X, Zhou L, Peng N, Yu H, Li M, Cao Z, Lin Y, Wang X, Li Q, Wang J, She Y, Zhu C, Lu M, Zhu Y, Liu S. MicroRNA-302a
294 suppresses influenza A virus-stimulated interferon regulatory factor-5 expression and cytokine storm induction, J Biol Chem 2017;**292**:
295 21291-21303.

296 [33] Bantia S, Kellogg D, Parker C, Upshaw R, Ilyushina NA, Babu YS. A single intramuscular injection of neuraminidase inhibitor peramivir
297 demonstrates antiviral activity against novel pandemic A/California/04/2009 (H1N1) influenza virus infection in mice, Antiviral Res 2011;**90**:
298 17-21.

299 [34] Sladkova T, Kostolansky F. The role of cytokines in the immune response to influenza A virus infection, Acta Virol 2006;**50**: 151-162.

300 [35] Tumpey TM, Basler CF, Aguilar PV, Zeng H, Solorzano A, Swayne DE, Cox NJ, Katz JM, Taubenberger JK, Palese P, Garcia-Sastre A.
301 Characterization of the reconstructed 1918 Spanish influenza pandemic virus, Science 2005;**310**: 77-80.

302 [36] Pommerenke C, Wilk E, Srivastava B, Schulze A, Novoselova N, Geffers R, Schughart K. Global transcriptome analysis in
303 influenza-infected mouse lungs reveals the kinetics of innate and adaptive host immune responses, PLoS One 2012;**7**: e41169.

304 [37] Tate MD, Pickett DL, van Rooijen N, Brooks AG, Reading PC. Critical role of airway macrophages in modulating disease severity during
305 influenza virus infection of mice, J Virol 2010;**84**: 7569-7580.

306 [38] Zhang Y, Yao J, Qi X, Liu X, Lu X, Feng G. Geniposide demonstrates anti-inflammatory and antiviral activity against pandemic

- 307 A/Jiangsu/1/2009 (H1N1) influenza virus infection in vitro and in vivo, *Antivir Ther* 2017;**22**: 599-611.
- 308 [39] Tanaka A, Nakamura S, Seki M, Iwanaga N, Kajihara T, Kitano M, Homma T, Kurihara S, Imamura Y, Miyazaki T, Izumikawa K, Takeya
309 H, Yanagihara K, Kohno S. The effect of intravenous peramivir, compared with oral oseltamivir, on the outcome of post-influenza pneumococcal
310 pneumonia in mice, *Antivir Ther* 2015;**20**: 11-19.
- 311 [40] Creagh EM, O'Neill LA. TLRs, NLRs and RLRs: a trinity of pathogen sensors that co-operate in innate immunity, *Trends Immunol* 2006;**27**:
312 352-357.
- 313 [41] Liao QJ, Ye LB, Timani KA, Zeng YC, She YL, Ye L, Wu ZH. Activation of NF-kappaB by the full-length nucleocapsid protein of the
314 SARS coronavirus, *Acta Biochim Biophys Sin (Shanghai)* 2005;**37**: 607-612.
- 315 [42] Malaviya R, Laskin JD, Laskin DL. Anti-TNFalpha therapy in inflammatory lung diseases, *Pharmacol Ther* 2017;**180**: 90-98.
- 316 [43] Chen G, Wu D, Guo W, Cao Y, Huang D, Wang H, Wang T, Zhang X, Chen H, Yu H, Zhang X, Zhang M, Wu S, Song J, Chen T, Han M, Li
317 S, Luo X, Zhao J, Ning Q. Clinical and immunologic features in severe and moderate forms of Coronavirus Disease 2019, *medRxiv*
318 2020;10.1101/2020.02.16.20023903.
- 319

Figure Legends

Fig. 1 Identification of peramivir as anti-inflammatory agents. **a** Chemical structures of peramivir, oseltamivir and zanamivir. **b** Peramivir showed the strongest TNF- α inhibitory effect compared with oseltamivir and zanamivir. **c** The dose-response curves for the TNF- α inhibitions of peramivir exhibited IC₅₀s of 4.3 μ M. **d** Cell viabilities of macrophages with peramivir treatment at different concentrations.

Fig. 2 Peramivir effectively attenuates acute lung injury and prolong the survival in LPS-induced mice. **a** Representative images of lung H&E staining of control, and peramivir treatment groups. Black, green and yellow arrows indicated infiltration of inflammatory cells, congestion and edema within thickened alveolar, respectively. Scale bars, 100 or 200 μ m as indicated. **b** Lung injury scores of control and peramivir treatment groups (n=5). * P < 0.05. **c** Survival time of LPS-induced CSS in control, and peramivir (20, 60 mg/kg) groups (n=10). Kaplan–Meier analysis was performed. * P < 0.05, ** P < 0.01. **d** RAW264.7 cells were co-cultured with peramivir at concentrations of 2.5, 5 and 10 μ M at 1 h before LPS stimulation. The activity of NF- κ B luciferase was upregulated in all groups after 8 h, and there was a significant decline in cells co-cultured with peramivir in a dose-dependent manner. *** P < 0.001. **e** The activation of the NF- κ B, MAPK and STAT pathway in LPS-stimulated macrophages after the treatment of peramivir. **f** p65 nuclear translocation in LPS-stimulated macrophages after the treatment of peramivir (blue, DAPI; green, p65; cyan, cyan). Scale bars, 10 μ m as indicated.

Fig. 3 Peramivir inhibits cytokine release in LPS-induced hPBMCs from a health donor. TNF- α concentration was elevated by LPS stimulation. **a** and **b** Peramivir reduced TNF- α and IL-10 release in a time (6 and 12 h)- and dose (2.5, 5, 10 μ M)-dependent manner. Peramivir showed no toxicity toward hPBMCs. * P < 0.05, ** P < 0.01, *** P < 0.001.

Table 1. Clinical feature and experimental results of cytokines tested in mouse serum.

337 **Table 2.** Clinical feature and experimental results of cytokines tested in mouse BALF.

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Table 1. Clinical feature and experimental results of cytokines tested in mouse serum

Cytokines	Elevated in COVID-19 ^{3, 49}	Higher in Severe Cases ^{3, 49}	Control (pg/ml)	Peramivir (pg/ml)	<i>P</i>	Trend
TNF- α	Yes	Yes	1716.55 $\pm$ 82.10	1181.45 $\pm$ 47.58	<0.001	↓
IL-6	Yes	No	101572.3 $\pm$ 5186.61	80316.8 $\pm$ 3464.71	0.002	↓
IFN- α	No Data	No Data	649.52 $\pm$ 17.15	393.0 $\pm$ 16.59	<0.001	↓
IFN- γ	Yes	No	1521.02 $\pm$ 154.27	516.95 $\pm$ 60.44	<0.001	↓
IL-1 β	Yes	No	578.21 $\pm$ 50.23	275.12 $\pm$ 28.97	<0.001	↓
IL-10	Yes	Yes	1048.84 $\pm$ 61.75	926.39 $\pm$ 66.67	0.25	—
IL-12	No	No	237.15 $\pm$ 7.88	119.17 $\pm$ 7.16	<0.001	↓
CXCL1	No Data	No Data	31944.11 $\pm$ 6590.53	24037.25 $\pm$ 3011.44	0.44	—
IP10	Yes	Yes	44400.48 $\pm$ 2953.97	39370.0 $\pm$ 1970.93	0.27	—
MCP-1	Yes	Yes	35984.98 $\pm$ 1082.87	26563.21 $\pm$ 1257.91	<0.001	↓
CCL-5	No Data	No Data	12179.03 $\pm$ 1014.57	9986.45 $\pm$ 455.71	0.19	—
GM-CSF	Yes	No	234.27 $\pm$ 12.01	190.45 $\pm$ 9.93	0.06	—

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Table 2. Clinical feature and experimental results of cytokines tested in mouse BALF

Cytokines	Elevated in COVID-19	Higher in Severe Cases	Control (pg/ml)	Peramivir (pg/ml)	P	Trend
TNF- α	No Data	No Data	26.39 $\pm$ 4.53	15.67 $\pm$ 2.56	0.03	↓
IL-6	No Data	No Data	10176.96 $\pm$ 2354.05	3916.37 $\pm$ 994.83	0.03	↓
IFN- β	No Data	No Data	30.92 $\pm$ 5.65	46.02 $\pm$ 9.69	0.23	—
IFN- γ	No Data	No Data	67.02 $\pm$ 20.29	100.06 $\pm$ 75.45	0.35	—
IL-1 α	No Data	No Data	180.92 $\pm$ 50.29	186.23 $\pm$ 53.49	0.95	—
IL-1 β	No Data	No Data	28.99 $\pm$ 5.99	22.21 $\pm$ 2.30	0.32	—
IL-10	No Data	No Data	43.11 $\pm$ 12.99	29.82 $\pm$ 7.20	0.38	—
IL-17A	No Data	No Data	26.81 $\pm$ 11.91	17.21 $\pm$ 4.12	0.47	—
IL-23	No Data	No Data	9.49 $\pm$ 1.87	11.45 $\pm$ 2.85	0.60	—
IL-27	No Data	No Data	22.16 $\pm$ 3.39	20.60 $\pm$ 4.25	0.79	—
MCP-1	No Data	No Data	900.08 $\pm$ 285.4	383.04 $\pm$ 71.79	0.12	—
GM-CSF	No Data	No Data	7.50 $\pm$ 1.38	10.08 $\pm$ 2.17	0.38	—

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347 **Figure 1**

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349 **Figure 2**

Figure 3

Supplementary Figures

Figure S1

Fig. S1 Effects of peramivir on different cytokines in mice serum. Compared with the control group, TNF- α , IL family (IL-6, IL-1 β , IL-12), chemokines (MCP-1), interferon family (IFN- α , γ) were significantly decreased by the treatment. **P < 0.01, ***P < 0.005.

Figure S2

Fig. S2 Effects of peramivir on different cytokines in mouse bronchoalveolar lavage fluid (BALF). Compared with the control group, TNF-α and IL-6 were significantly decreased by the treatment. *P < 0.05.

366 Figure S3

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368 **Fig. S3 Peramivir inhibits TNF-α release in LPS-induced hPBMCs from the second health donor.** TNF-α concentration was elevated by LPS stimulation. a and

369 b Peramivir reduced TNF-α release in dose (2.5, 5, 10 μM)-dependent manner. Peramivir showed no toxicity toward hPBMCs. *P < 0.05, **P < 0.01, ***P < 0.005.