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Little to no expression of angiotensin-converting enzyme-2 on most human peripheral blood immune cells but highly expressed on tissue macrophages

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Abstract

Angiotensin-converting enzyme-2 (ACE2) has been recognized as the binding receptor for the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Flow cytometry demonstrated that there was little to no expression of ACE2 on most of the human peripheral blood-derived immune cells including $CD4^+$ T, $CD8^+$ T, activated $CD4^+$/$CD8^+$ T, Tregs, Th17, NKT, B, NK cells, monocytes, dendritic cells, and granulocytes. There was no ACE2 expression on platelets and very low level of ACE2 protein expression on the surface of human primary pulmonary alveolar epithelial cells. The ACE2 expression was markedly upregulated on the activated type 1 macrophages (M1). Immunohistochemistry demonstrated high expressions of ACE2 on human tissue macrophages, such as alveolar macrophages, Kupffer cells within livers, and microglial cells in brain at steady state. The data suggest that alveolar macrophages, as the frontline immune cells, may be directly targeted by the SARS-CoV-2 infection and therefore need to be considered for the prevention and treatment of COVID-19.

KEYWORDS
angiotensin-converting enzyme-2 (ACE2), COVID-19, immune cells, lung, pathogenesis, SARS-CoV-2, macrophages

1 | INTRODUCTION

The epidemic of a new coronavirus infectious disease (COVID-19) is wreaking havoc worldwide, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Angiotensin-converting enzyme 2 (ACE2), with a multiplicity of physiological roles such as a negative regulator of the renin-angiotensin system, has been recognized as the entry receptor for SARS-CoV-2 infecting host cells [1], which is similar to the SARS-CoV. Other potential host cellular entry receptors have been tested or proposed for the viral invasion such as TMPRSS2 (transmembrane protease, serine 2) or cathepsin B/L [2], CD147 [3], O-acetylated sialic acid [4], antibody-dependent enhancement (ADE) pathway [5], and CD26 [6]. ACE2 may act as a limiting factor for viral infection at the initial stage [7]. The ACE2 expression has been mainly distributed in microvilli of the intestine and renal proximal tubules, gallbladder epithelium, testicular Sertoli cells and Leydig cells, glandular cells of seminal vesicle, and cardiomyocytes [8]. The human respiratory system is primarily affected by the SARS-CoV-2 infection. Using the polyclonal anti-serum-based immunohistochemistry, the expression of ACE2 was reported on type II alveolar epithelial cells [9]. However, a single-cell RNA profiling analysis showed that only 1.4% ± 0.4% of lung type II alveolar epithelial cells expressed ACE2 at RNA level [10]. Additionally, clinical autopsies

from SARS-CoV-infected patients demonstrated that there were major pathological changes in the lungs, immune organs, and small systemic blood vessels with vasculitis. The detection of SARS-CoV was primarily found in the lung and trachea/bronchus, but was undetectable in the spleen, lymph nodes, bone marrow, heart, and aorta [11]. This evidence highlights the overreaction of immune responses induced by viral infection which resulted in significant harm, as evidenced by pathogenesis of the lungs, immune organs, and small systemic blood vessels. Similar mechanisms have been proposed in the pathogenesis for SARS-CoV-2 infection [12]. The pathological study in COVID-19 patients revealed that the majority of infiltrated immune cells in alveoli were macrophages and monocytes, with minimal lymphocytes, eosinophils, and neutrophils [13]. Thus, there are fundamental knowledge gaps underlying the pathogenesis of COVID-19 that need to be clarified. To understand the immunopathology and advance the strategies for the prevention and treatment of COVID-19, we examined the levels of ACE2 expression on different types of immune cells. Our data demonstrated that the activated macrophages and alveolar tissue macrophages, among others, displayed high levels of ACE2, while most of the immune cells were negative or displayed very low expressions of ACE2. These data highlight the importance of macrophages in the pathogenesis and treatment of COVID-19.

2 | MATERIALS AND METHODS

2.1 | PBMC isolation

To detect the expression of ACE2 on different types of immune cells, human buffy coat blood units (n = 14; mean age of 49.07 ± 13.79; age range from 27 to 67 years old; eight males and six females) were purchased from the New York Blood Center (NYBC, New York, NY, USA). NYBC has received all accreditations for blood collections and distributions including institutional review board (IRB) approval and signed Consent Forms from donors. Human buffy coats were initially added to 40 ml chemical-defined serum-free culture X-VIVO 15™ medium (Lonza, Walkersville, MD, USA) and mixed thoroughly with 10 ml pipette. Next, they were used for isolation of peripheral blood-derived mononuclear cells (PBMC). PBMC were harvested as previously described [14]. Briefly, mononuclear cells were isolated from buffy coats blood using Ficoll-Paque™ PLUS (γ = 1.007, GE Healthcare, Chicago, IL, USA). Next, the red blood cells were removed using ACK Lysing buffer (Lonza, Walkersville, MD, USA). After three washes with saline, the whole PBMC were utilized for flow cytometry. To isolate monocytes, monocytes were purified from PBMC by using $CD14^+$ microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instruction, with purity of $CD14^+$ cells >90%.

2.2 | Flow cytometry

Phenotypic characterization of PBMC, monocytes, macrophages, and Th17 cells were performed by flow cytometry with associated markers (Table S1) including: PC5.5-conjugated anti-human CD3 mAb, ECD-conjugated anti-human CD3 mAb, Krome Orange-conjugated anti-CD8 mAb, APC-Alexa Fluor 750-conjugated anti-CD66b mAb, PC5.5-conjugated anti-human CD19 mAb, PC 5.5-conjugated anti-human HLA-DR mAb, Krome Orange-conjugated anti-CD14 mAb, APC-conjugated anti-human CD80, PE-conjugated anti-human CD123 mAb, PE-conjugated anti-human CD25 mAb, PE-Cy7-conjugated anti-human CD41 mAb, and FITC-conjugated anti-human CD42a mAb purchased from Beckman Coulter (Brea, CA, USA). The antibodies PB-conjugated anti-human CD3, and APC-conjugated anti-IL17A mAbs were purchased from Biolegend (San Diego, CA, USA). The antibodies APC-conjugated anti-human CD4 mAb, FITC-conjugated anti-human Hsp60 mAb, PE-Cy7-conjugated anti-human CD11c mAb, PE-Cy7-conjugated anti-human CD56 mAb, BV-421-conjugated anti-human CD127 mAb, PE-conjugated anti-RORγT, BV 421-conjugated anti-RORγT mAbs, PE-conjugated anti-human CD163, and BV421-conjugated anti-human CD209 were purchased from BD (Franklin Lakes, NJ, USA). PE-conjugated anti-IL17F was purchased from Invitrogen (Carlsbad, CA, USA). PE-Cy7-conjugated anti-human CD196 (CCR6) and PE-Cy7-conjugated anti-human MAVS mAbs were purchased from ThermoFisher Scientific (Waltham, MA, USA).

To detect an expression of ACE2 protein on different types of PBMC, macrophages, Th17 cells, platelets, or platelet-derived mitochondria, the indirectly labeled immunostaining with mouse anti-human ACE2 mAb (Novus Biologicals, Catalogue# NBP2-80035-100 μg, Clone #AC18F, Littleton, CO, USA) was utilized in combination with above lineage-specific fluorescence-labeled mAbs. Briefly, samples were first pre-incubated with human BD Fc Block (BD Pharmingen, Franklin Lakes, NJ, USA) to block non-specific binding for 20 min at room temperature, before being directly aliquoted for different antibody stainings. Cells were initially incubated with mouse anti-human ACE2 mAb at 1:50 dilution, at room temperature for 30 min. Next, cells were washed with PBS at 400 $g \times 5$ min and then stained with FITC-conjugated AffiniPure donkey or Cy5-conjugated AffiniPure donkey anti-mouse second Abs (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at 1:100 dilution for 30 min at room temperature. Cells only with second Ab staining served as control. After finishing the second Ab staining, cells were washed with 4 ml PBS to remove residual second Ab. Consequently, cells were immunostained with above lineage-specific fluorescence-labeled mAbs, as previously described [15-17]. Staining with propidium iodide (PI) (BD Biosciences, Franklin Lakes, NJ, USA) was used to exclude dead cells during the flow cytometry analysis.

To detect ACE2 expression on human pulmonary alveolar epithelial cells, the freshly isolated primary human pulmonary alveolar epithelial cells (HPAEpiC) were purchased from ScienCell Research Laboratories (Catalogue # 3200, Carlsbad, CA). Each vial contains $>1 \times 10^6$ cells in 1 ml volume from three healthy donors (two females and one male). HPAEpiC were directly prepared for flow cytometry after being thawed, without ex vivo cultures.

For ACE2-positive control, the recombinant adenoviral vectors expressing human ACE2 (Ad5-hACE2) under the control of the CMV promoter were obtained from BEI resources (NR-52390). The ACE2 expression was performed as previously described [18-20]. Briefly,

HEK293T cells at confluency of 80 ~ 90% in six-well plate were transduced with Ad5-hACE2 at a multiplicity of infection (MOI) = 0.5. After 24 h post-infection, infected cells were harvested and stained with ACE2 antibody, followed by Flow Cytometry.

For intracellular staining, cells were fixed and permeabilized according to the PerFix-nc kit (Beckman Coulter, Brea, CA, USA) manufacturer's recommended protocol. After staining, cells were collected and analyzed using a Gallios Flow Cytometer (Beckman Coulter, Brea, CA, USA), equipped with three lasers (488 nm blue, 638 red, and 405 violet lasers) for the concurrent reading of up to 10 colors. The final data were analyzed using the Kaluza Flow Cytometry Analysis Software (Beckman Coulter, Brea, CA, USA).

2.3 | Isolation of monocytes from PBMC and macrophage polarization

To generate monocyte-derived macrophages and detect ACE2 expression, monocytes were purified from human PBMC by using CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instruction, with the purity of $CD14^+$ cells >90%. The isolated monocytes were seeded in the six-well tissue culture-treated plate at 5×10^5 cells/ well in 2 ml X-VIVO 15 serum-free culture media at 37°C, 5% CO_2 condition. After 2-h culturing, the attached monocytes were washed twice with PBS to remove floating cells and cellular debris, followed by treatment with 50 ng/ml M-CSF [14, 15] (Sigma, Saint Louis, MO, USA) in X-VIVO 15 serum-free media at 37°C, 5% CO_2. After culturing for 7 days, the macrophages were treated with 1 μg/ml lipopolysaccharides (LPS) (Sigma, Saint Louis, MO, USA) or 40 ng/ml IL-4 (Biolegend, San Diego, CA, USA) for M1 and M2 polarization, respectively [21] for 24 h in X-VIVO 15 serum-free medium. Consequently, cells from different groups were collected for evaluations. To detect the ACE2 expressions on M1 and M2 macrophages, the immunocytochemistry was performed as previously described [17]. Briefly, M1 and M2 macrophages were fixed with 4% paraformaldehyde for 20 min at room temperature, blocked with 2.5% horse serum (Vector Laboratories, Burlingame, CA, USA) and human BD Fc Blocker for 20 min at room temperature, and then immunostained with mouse anti-human ACE2 monoclonal antibody (Novus Biologicals, Centennial, CO USA) at 1:200 dilution for 2 h at room temperature. Next, cells were stained with Cy5-conjugated AffiniPure donkey anti-mouse second Ab (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at 1:100 dilution at room temperature for 1 h. Only cells with second Ab staining served as negative control. Finally, cells were mounted by using mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA) and photographed with a Nikon Eclipse Ti2 inverted microscope, using NIS Elements Version 4.60 software.

2.4 | Immunohistochemistry

Paraffin tissue sections from normal adult humans (n = 8, aged from 21 to 87 years old, three males, five females) were purchased from BioChain Institute Inc (Catalog No: T8234431, Newark, CA, USA), including brain, lung, liver, spleen, and kidney tissue sections. Immunostaining was performed as previously described with minor modifications [22]. To block non-specific staining, sections were incubated in a buffer containing 2.5% horse serum (Vector Laboratories, Burlingame, CA, USA) and human Fc Block (BD Pharmingen, Franklin Lakes, NJ, USA) for 20 min at room temperature. Tissue sections were initially immunostained with anti-ACE2 rabbit polyclonal antibody (Abcam, Cambridge, MA, USA) and rat anti-human CD11b monoclonal antibody (mAb) (Biolegend, San Diego, CA, USA) at 1:100 dilution for 2 h at room temperature. Next, tissue sections were stained with FITC-conjugated AffiniPure donkey anti-rat second Ab and Cy3-conjugated AffiniPure donkey anti-rabbit second Ab (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at 1:100 dilution at room temperature for 1 h. For every experiment, only tissue sections with second Ab staining served as negative control. Finally, the slides were mounted by using mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA) and photographed with a Nikon A1R confocal microscope on a Nikon Eclipse Ti2 inverted base, using NIS Elements Version 4.60 software. DIC images were photographed with Photometrics CoolSNAP™ DYNO CCD camera.

2.5 | Statistics

Statistical analyses were performed with GraphPad Prism 8 (Version 8.0.1) software. The normality test of samples was performed by the Shapiro–Wilk test. Statistical analyses of data were performed by the two-tailed paired Student's t-test to determine statistical significance between untreated and treated groups. The Mann–Whitney U test was utilized for non-parametric data. Values were given as mean ± SD (standard deviation). Statistical significance was defined as $p < 0.05$, with two sided.

3 | RESULTS

3.1 | Little to no expression of ACE2 on most human peripheral blood-derived immune cells

To explore the direct action of SARS-CoV-2 on immune cells, we examined the ACE2 expressions on different types of immune cells from human peripheral blood (n = 14). They were characterized and gated with cell type-specific surface markers [23]: $CD3^+$ for T cells, $CD3^+CD4^+$ for $CD4^+$ T cells, $CD3^+CD8^+$ for $CD8^+$ T cells, $CD11c^+CD14^-$ for myeloid dendritic cells (mDC), $CD14^-CD123^+$ for plasmacytoid DC (pDC), $CD14^+$ for monocytes, $CD19^+$ for B cells, $CD4^+CD25^+CD127^{low/-}$ for regulatory T cells (Tregs), $CD3^+CD56^+$ for NKT cells, $CD3^-CD56^+$ for NK cells, and $CD3^-CD66b^+$ for granulocytes (Figure 1(A)–(c)). Using the recombinant adenoviral vectors expressing human ACE2 (Ad5-hACE2)-transfected 293 T cells as positive control [20] (Figure 1(D)), flow cytometry demonstrated that there were no expressions of ACE2 on most types of immune cells, or with a background level (< 5%)

(Figure 1(E)–(G)). The percentages of ACE2$^+$ cells for NK and NKT cells were only 1.06% ± 1.22% and 2.01% ± 1.17%, respectively (n = 11) (Figure 1(F)), and 0.31% ± 0.27% for ACE2$^+$CD4$^+$ T cells. The activated CD4$^+$HLA-DR$^+$ T cells displayed only 2.15% ±1.34% of ACE2$^+$ cells (n = 11). The activated CD8$^+$HLA-DR$^+$ was only 2.72% ± 1.64% (n = 10) (Figure 1(F)). We performed statistical analysis among different cell populations and found that the percentages of ACE2$^+$ NKT cells, activated CD4$^+$HLA-DR$^+$ T cells, activated CD8$^+$HLA-DR$^+$ T cells, and ACE2$^+$ B cells were markedly higher than that of CD4$^+$ T cells (p < 0.0001, p < 0.0001, p < 0.0001, p = 0.0015, respectively)

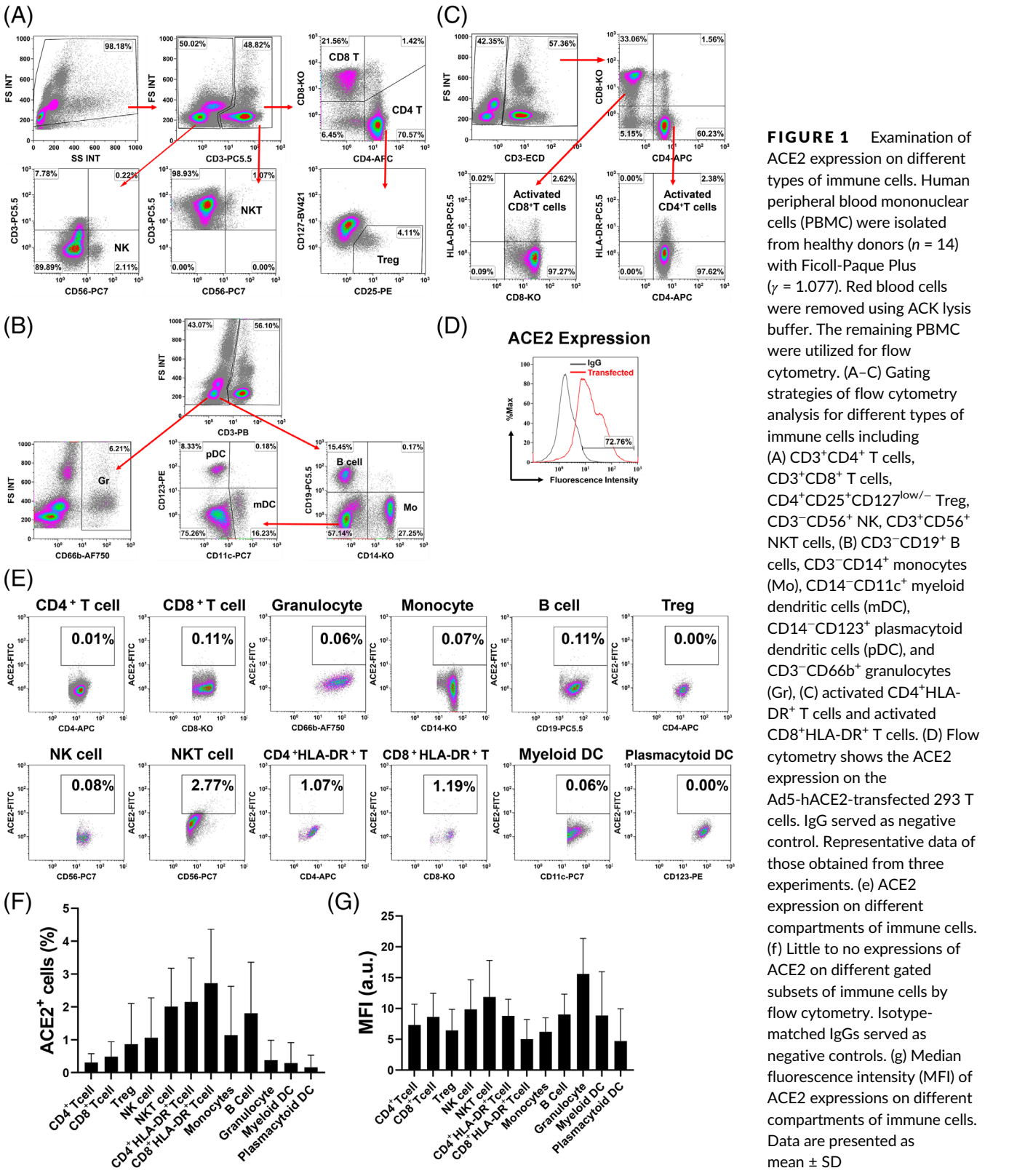

FIGURE 1 Examination of ACE2 expression on different types of immune cells. Human peripheral blood mononuclear cells (PBMC) were isolated from healthy donors (n = 14) with Ficoll-Paque Plus (γ = 1.077). Red blood cells were removed using ACK lysis buffer. The remaining PBMC were utilized for flow cytometry. (A–C) Gating strategies of flow cytometry analysis for different types of immune cells including (A) CD3$^+$CD4$^+$ T cells, CD3$^+$CD8$^+$ T cells, CD4$^+$CD25$^+$CD127$^{low/-}$ Treg, CD3$^-$CD56$^+$ NK, CD3$^+$CD56$^+$ NKT cells, (B) CD3$^-$CD19$^+$ B cells, CD3$^-$CD14$^+$ monocytes (Mo), CD14$^-$CD11c$^+$ myeloid dendritic cells (mDC), CD14$^-$CD123$^+$ plasmacytoid dendritic cells (pDC), and CD3$^-$CD66b$^+$ granulocytes (Gr), (C) activated CD4$^+$HLA-DR$^+$ T cells and activated CD8$^+$HLA-DR$^+$ T cells. (D) Flow cytometry shows the ACE2 expression on the Ad5-hACE2-transfected 293 T cells. IgG served as negative control. Representative data of those obtained from three experiments. (e) ACE2 expression on different compartments of immune cells. (f) Little to no expressions of ACE2 on different gated subsets of immune cells by flow cytometry. Isotype-matched IgGs served as negative controls. (g) Median fluorescence intensity (MFI) of ACE2 expressions on different compartments of immune cells. Data are presented as mean ± SD

(Figure 1(F)). However, there were no marked differences in the values of median fluorescence intensity (MFI) among different cell populations (Figure 1(G)). The data suggest that SARS-CoV-2 virus may not directly attack blood immune cells lacking the ACE2 expression.

3.2 | No expression of ACE2 on Th17 cells

T-helper type 17 (Th17) cells are important pathogenic mediators for several autoimmune diseases, potentially contributing to the pathogenesis of COVID-19. RORγt (retinoic acid receptor-related orphan nuclear receptor gamma t) belong to nuclear hormone receptors (NHRs) and act as a crucial transcription factor for the differentiation and function of Th17 cells both in humans and mice [24]. Using RORγt, interleukin-17A (IL-17A), IL-17F, and CCR6 as specific Th17 markers [24], the purity of $IL17A^+ROR\gamma T^+$ Th17 cells was 19.91% ± 2.75% (Figure S1(A)). The percentage of $IL17A^+IL17F^+$ Th17 cells was 20.19% ± 2.94% (Figure S1(A)). The purity of $IL17A^+CCR6^+$ Th17 cells was 20.14% ± 2.86% (Figure S1(A)). The gated Th17 cells failed to express ACE2 (Figure S1(B), 0.47% ± 0.65%, $n = 4$). These data imply no direct interaction between Th17 cells and SARS-CoV-2.

3.3 | No expression of ACE2 on platelets

Increasing clinical evidence demonstrated the coagulation abnormalities in COVID-19 subjects including disseminated intravascular coagulopathy (DIC) and low levels of platelet count. To determine whether platelets were directly targeted by SARS-CoV-2 or trigged by viral inflammatory reactions, we examined the ACE2 expression on the highly-purified $CD41b^+CD42a^+$ platelets from human peripheral blood (Figure S2(A), $n = 6$). Flow cytometry established that there was no ACE2 expression on platelets (Figure S2(B),(C), 0.58% ± 0.42%, $n = 6$), highlighting that the coagulopathy may be indirectly caused by the viral inflammation.

Our previous work established that platelets could release mitochondria contributing to the immune modulation and islet β-cell regeneration [25]. To explore the mitochondrial function in viral infection, flow cytometry indicated that while the purified platelet-derived mitochondria did not express ACE2 (Figure S2(D)), they strongly display the mitochondrial antiviral-signaling protein (MAVS) with the percentage of MitoTracker Deep $Red^+HSP60^+MAVS^+$ mitochondria at 96.02% ± 2.74% (Figure S2(E), $n = 3$). These data suggest that platelets may have potential to improve antiviral immunity through the releasing mitochondria.

3.4 | High expression of ACE2 on the activated type 1 macrophages (M1)

Macrophages have been characterized with type 1 macrophages (M1, inflammatory) and type 2 macrophages (M2, anti-inflammatory), according to their phenotypic differences such as spindle-like morphology and high expression CD206 and CD209 on M2 macrophages [15]. Initially, flow cytometry established that the purified $CD14^+$ monocytes from human peripheral blood failed to express ACE2 (1.56% ± 1.91%, $n = 6$) (Figure 2(A)). Macrophages were then generated in the presence of 50 ng/ml macrophage colony-stimulating factor (M-CSF) with the percentage of spindle-like cells at 63.25% ± 8.85% ($n = 4$). To evaluate the ACE2 expressions on macrophages, M1 and M2 macrophages were activated by the treatment with lipopolysaccharide (LPS) [14] and interleukin-4 (IL-4), respectively [21]. Phase contrast image showed significant differences in the morphology between two groups (Figure 2(B)). LPS-treated M1 macrophages exhibited pseudopod-like protrusions compared to the spindle form of IL-4-treated M2 macrophages (Figure 2(B)). Phenotypic characterization established that upregulations of CD206 and CD209 on the IL-4-treated M2 macrophages, not on the LPS-treated M1 macrophages (Figure 2(C)). Flow cytometry demonstrated that the level of ACE2 expression was higher on the LPS-activated M1 macrophages than that of IL-4-treated M2 macrophages (Figures 2(D)–(F)). This finding was further confirmed by the confocal microscopy and image analysis (Figure 2(G)). Therefore, the data suggest the upregulation of ACE2 expression on the activated M1 macrophages.

3.5 | Expression of ACE2 on tissue macrophages

To detect the expression of ACE2 on tissue macrophages, we initially performed double-staining with human macrophage marker CD11b through the immunohistochemistry in the brain, lung, liver, kidney, and spleen tissues of normal adult human donors. Using an expression of ACE2 in the kidney as a positive control (Figure 3(A), fifth panel), the data revealed that most ACE2 expressions were co-localized with the $CD11b^+$ tissue macrophages in the brain, lung and liver (Figure 3 (A)), which are known as microglia in brain (Figure 3(A), top panel), dust cells (alveolar macrophages, Figure 3(A), second and third panels) and Kupffer cells (Figure 3(A), fourth panel), respectively. Most splenocytes failed to exhibit ACE2 (Figure 3(A), sixth panel). Unexpectedly, there was little to no expression of ACE2 on the alveolar epithelial cells (Figure 3(A), second and third panels).

To further detect the distribution of ACE2 expression on alveolar epithelial cells, we utilized the primary human pulmonary alveolar epithelial cells (HPAEpiC) to define their level of ACE2 expression. Flow cytometry proved the low level (9%) of ACE2 expression on human pulmonary alveolar epithelial cells (Figure 3(B)). Therefore, these data indicate the high expression of ACE2 on tissue macrophages with low level ACE2 expression on alveolar epithelial cells.

4 | DISCUSSION

The human pulmonary system is primarily targeted by SARS-CoV-2. Our current studies demonstrated little to no expression of ACE2 on both primary human pulmonary alveolar epithelial cells and alveolar

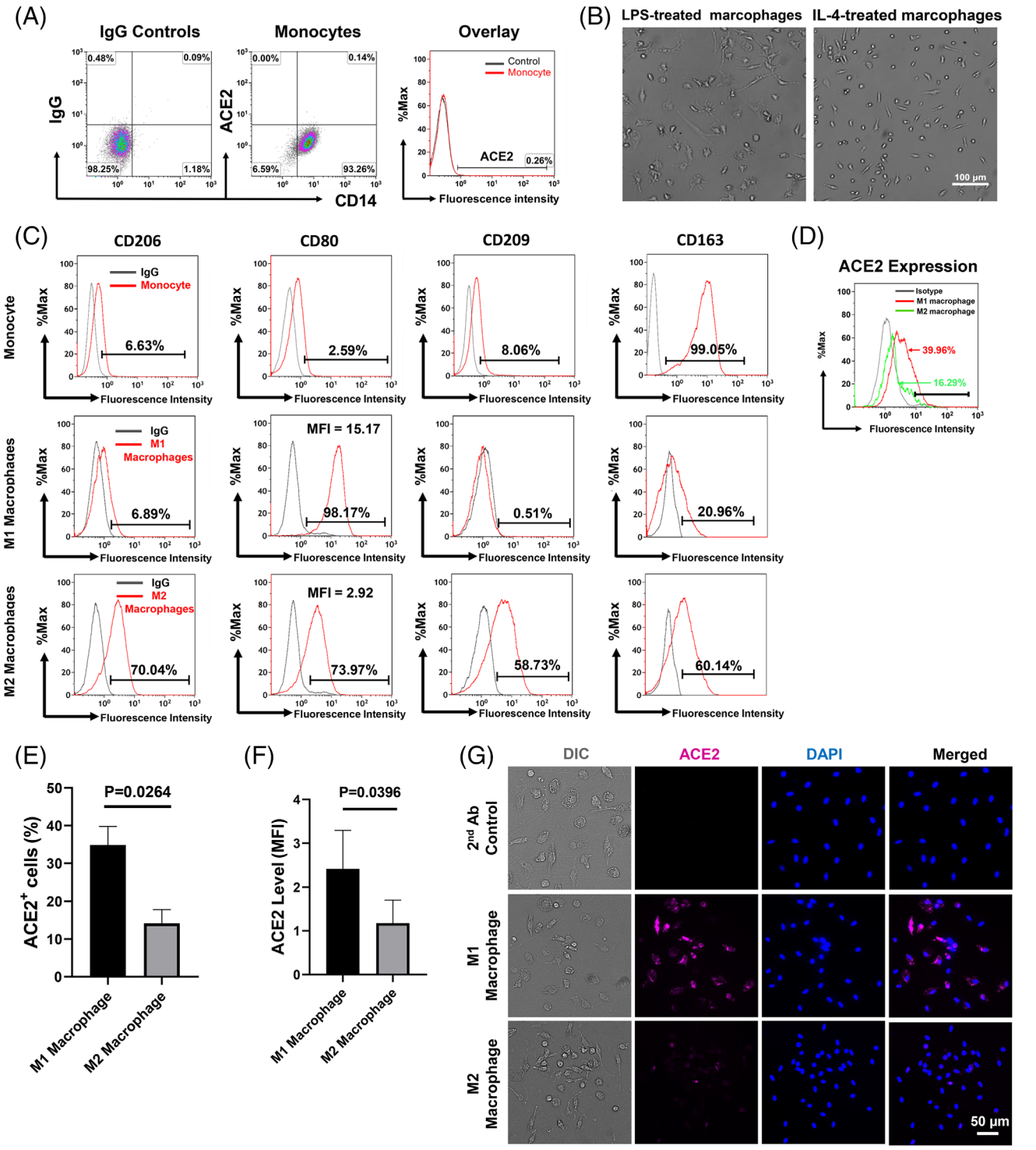

FIGURE 2 Different levels of ACE2 expressions on M1 and M2 macrophages. (A) The freshly purified $CD14^+$ monocytes from human peripheral blood failed to express ACE2. Isotype-matched IgG served as control. (B–G) The purified $CD14^+$ monocytes were initially seeded in the tissue culture-treated six-well plate at 5×10^5 cells/well and cultured in X-VIVO 15 serum-free media with 50 ng/ml M-CSF [14], at 37°C, 5% CO_2 conditions. After 7 days, macrophages were treated with 1 μg/ml LPS or 40 ng/ml IL-4 for 24 h, respectively. (B) Morphological change of macrophages after the treatment LPS (n = 4, left). IL-4-treated macrophages served as control with maintaining the morphology of spindle-like cells (right). (C) Phenotypic characterization of M1/M2 with their associated markers. Isotype-matched IgGs served as negative controls. Data were representative from one of two PBMC preparations. (D) Overlay histogram shows the high expression of ACE2 on M1 macrophages (red) in comparison with M2 macrophages (green). The isotype second Ab staining served as negative control (grey). (E) M1 macrophages display higher percentage of $ACE2^+$ cells than that of M2 macrophages. Data are presented as mean ± SD; n = 3. (F) M1 macrophages display higher level of ACE2 median fluorescence intensity (MFI) than that of M2 macrophages. Data are presented as mean ± SD; n = 3. (G) Fluorescence microscopy shows high expression of ACE2 on M1 macrophages. Representative images were from one of immunostaining with six experiments

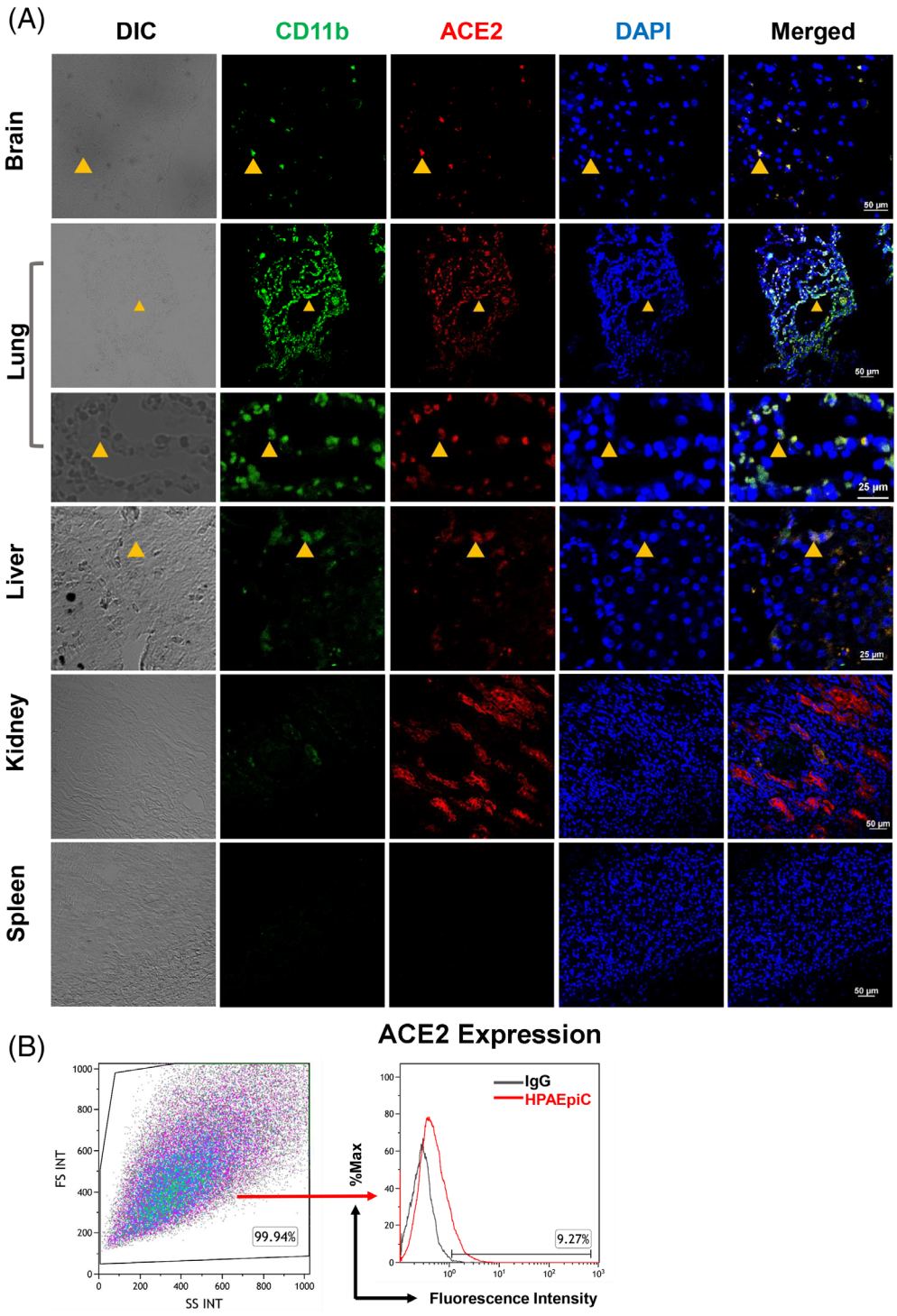

FIGURE 3 Expression of ACE2 on human tissue macrophages. (A) Expression of ACE2 was co-localized with the CD11b^{+} tissue macrophages (pointed by yellow triangles) in the brain, lung, and liver, which are known as microglia in brain (top panel), dust cells (alveolar macrophages, second and third panels) and Kupffer cells (fourth panel), respectively. There was little to no expression of ACE2 on alveolar epithelial cells. Paraffin tissue sections were derived from normal adult human donors (Biochain Institute). Tissue sections stained with second Abs served as negative control. (B) Analysis of ACE2 expression on the primary human pulmonary alveolar epithelial cells (HPAEpiC) by flow cytometry. The second Ab staining served as negative control (gray) for ACE2 immunostaining (red). The total cell population (left panel) was gated for flow cytometry analysis

epithelial cells of paraffin lung tissue sections, which is consistent with previous reports [8]. Notably, we found that high expression of ACE2 was colocalized with human tissue macrophages of the lung (alveolar macrophages) and liver (Kupffer cells) and microglial cells in brain at steady state, and up-regulated on the LPS-activated M1 macrophages. However, most immune cells in the steady-state human peripheral blood samples were negative for ACE2 expression, including the freshly isolated CD14^{+} monocytes. It implies that the circulating monocytes may differentiate into alveolar macrophages after migration into the pulmonary tissues with an up-regulation of ACE2 expression. These alveolar macrophages act as the front line immune cells defending against the SARS-CoV-2 viral infection. These data highlight the importance of alveolar macrophages during the pathogenesis of lung damage in COVID-19 subjects. Liao et al. reported that there were abundant proinflammatory macrophages in the bronchoalveolar lavage fluid of severe COVID-19 patients [26]. Additionally, Feng et al. reported that the viral nucleocaspid protein (NP) of SARS-CoV-2 was found in ACE2^{+}CD169^{+} macrophages, but

not in CD3$^+$ T cells and B220$^+$ B cells through postmortem examinations of COVID-19 patients' spleens and lymph nodes [27]. Based on these evidence, we propose that lung macrophages may be directly targeted by the SARS-CoV-2 and play a critical role in the initiation and development of COVID-19 (Figure 4). Post viral infection, the SARS-CoV-2 may either (1) be directly cleared by the healthy macrophages with asymptomatic or mild clinical symptoms or (2) destroy the dysfunctional macrophages and evoke the immune system with cytokine storm, leading to severe clinical symptoms such as high fever, hypoxia and acute respiratory distress syndrome (ARDS) (Figure 4). This perspective may advance the understanding of the clinical course of COVID-19 and facilitate the development of prevention and treatment strategies.

Recently, Ural et al. (2020) reported that alveolar macrophages displayed the phenotype of type 1 macrophages [28], while an interstitial subset of CD169$^+$ lung-resident macrophages, primarily located around the airways in close proximity to the sympathetic nerves of the bronchovascular bundle, exhibits the characteristics of type 2 macrophages and anti-inflammatory effects [28]. Therefore, these two types of macrophages play an essential role in the immune surveillance and maintenance of homeostasis of the pulmonary system. Considering all current approaches for the prevention and treatment of COVID-19, there are no therapies, either being tested or at the beginning of the pipeline, that directly focus on the modulation of macrophages. To this respect, it is critical to protect and restore the functions of alveolar macrophages (or other tissue macrophages) through immune modulations for the prevention and treatment of COVID-19, leading to being potentially beneficial to correct the viral inflammation, effectively ameliorate anti-viral immunity, efficiently reduce the viral load, improve clinical outcomes, expedite the patient recovery, and decline the rate of mortality in patients after being infected with SARS-CoV-2.

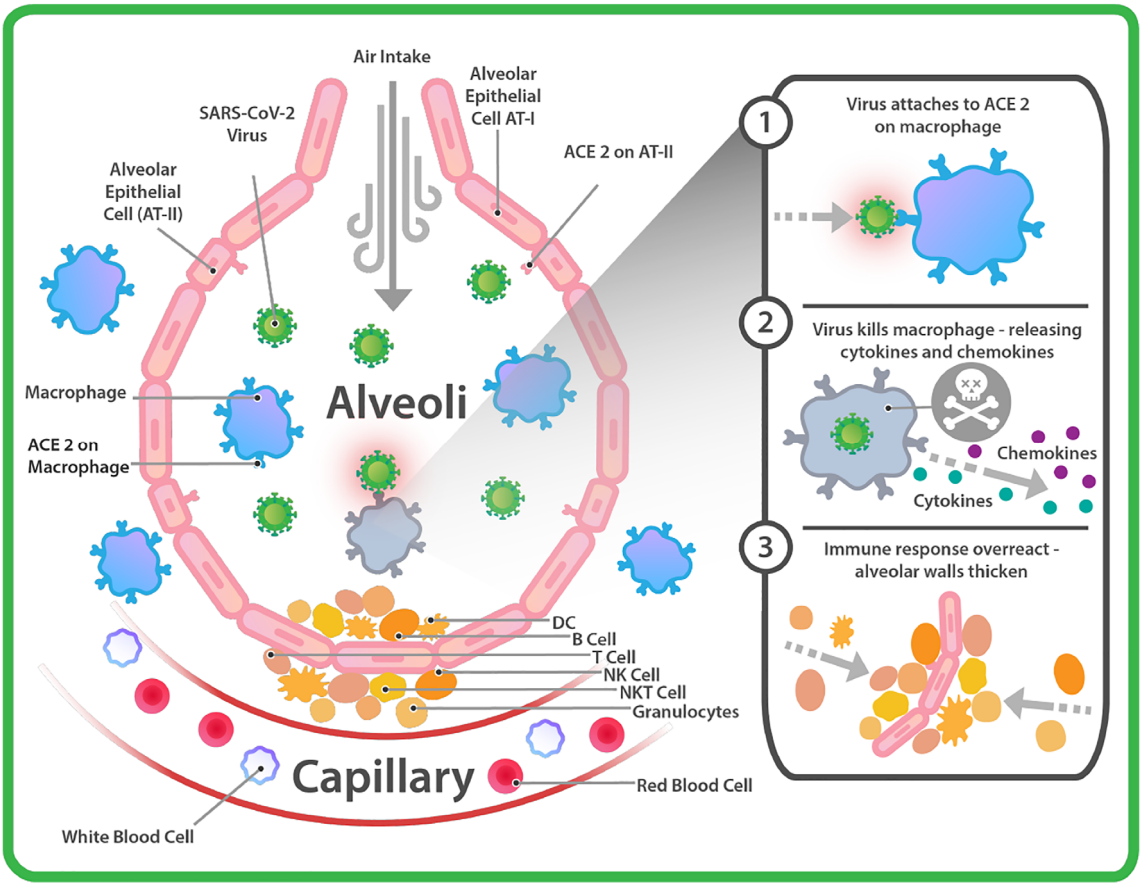

FIGURE 4 Outline the proposed mechanism underlying the pathogenesis of COVID-19. ACE2 protein was primarily displayed on alveolar macrophages of lung, with no or low expression on alveolar type 1 (AT-I) and type 2 (AT-II) epithelial cells. Upon entering the pulmonary alveoli, healthy alveolar macrophages may directly kill the virus, with asymptomatic or mild clinical symptoms. At this earlier stage 1, the infected alveolar macrophages may alternatively recruit other immune cells to build up the antiviral immunity through releasing cytokines (e.g., IL-1, IL-6, IL-12, and TNFα) and chemokines (e.g., CXCL1 and CXCL2 to recruit granulocytes, CXCL10 to recruit T cells, NK cells, and DCs). For instance, the recruited CD4$^+$ T cells may secret interferon (IFN)-γ to strengthen the antiviral immunity of alveolar macrophages and minimize the viral load. However, if this first line of defense is broken, the more cytokines and chemokines are released from the dead cells or dead-cell engulfed macrophages (stages 2 and 3), the more immune cells are infiltrated into pulmonary systems, leading to patients experiencing a rapid deterioration and the development of ARDS with high fatality in the clinic

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AUTHOR CONTRIBUTIONS

Xiang Song: Data curation; formal analysis; methodology; writing-review and editing. **Wei Hu:** Data curation; formal analysis; writing-review and editing. **Haibo Yu:** Data curation; formal analysis; writing-review and editing. **Laura Zhao:** Writing-original draft; writing-review and editing. **Yeqian Zhao:** Visualization; writing-review and editing. **Xin Zhao:** Resources; writing-review and editing. **Hai-Hui Xue:** Resources; writing-review and editing. **Yong Zhao:** Conceptualization; formal analysis; funding acquisition; supervision; validation; writing-original draft; writing-review and editing.

CONFLICT OF INTEREST

All authors have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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