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COMMUNICATION TO THE EDITOR

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Further comments on the role of ACE-2 positive macrophages in human lung

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1 | INTRODUCTION

Since our previous publication [1], emerging evidence has demonstrated the actions of lung macrophages in the pathogenesis of COVID-19. However, we understand that there were some inconsistencies in the figures and some of the Data S1 in our original paper. Therefore, we wish to address these by adding Data S1.

2 | LITERATURE OVERVIEW ON ACE2 EXPRESSION ON HUMAN LUNG MACROPHAGES

The ACE2 expression on the surface of macrophages have been well established by studies such as ACE2 on alveolar macrophages [2–6], blood monocyte-derived macrophages [7–9], and macrophages of atherosclerotic carotid arteries [10]. Specifically, the ACE2 protein expression on alveolar macrophages has been demonstrated by different studies with lung tissues from normal human and COVID-19 patients (Table 1). Recently, Wang et al. demonstrated that alveolar macrophages derived from COVID-19 patients displayed ACE2 receptor, which can be infected by SARS-CoV-2 [4]. Furthermore, there was high ACE2 expression on normal lung macrophages that enables binding to the S protein [4]. Taken together, these publications support the conclusion and hypothesis regarding the action of alveolar macrophages in the pathogenesis of COVID-19 [1, 17–19].

There were inconsistent results regarding the ACE2 mRNA expression in alveolar macrophages reported by RNA-seq analysis. Contrasted with the reports [14–16], the ACE2 mRNA expression in alveolar macrophages were indicated by other groups [5, 11, 13]. In agreement with these studies, our current studies have demonstrated ACE2 protein expression on alveolar, lipopolysaccharide (LPS)-treated and other tissue macrophages [1]. These findings are consistent with other reports [2, 4–7]. For alveolar macrophages, there are different subpopulations with different phenotypes [20, 21]. To isolate macrophages, all procedures need to be performed on ice with cold phosphate buffered saline (PBS) to avoid the adherence of macrophages to vessels. Therefore, some macrophages may be potentially lost during the cDNA library preparations for bulk or single cell RNA-seq. The transcriptomic data from bulk or single cell RNA-seq requires further validation due to the poor correlation between transcriptomic analysis and protein abundance [22].

Hikmet et al. performed a body-wide analysis of ACE2 expression at the protein level [12] by using Lab Vision Autostainer 480S Module (Thermo Fisher Scientific, Freemont, CA). The analysis failed to identify ACE2 expressions in lung macrophages. These inconsistent results were potentially due to the procedure of antigen retrieval, which was essential to unmask the low-level or formalin cross-linked tissue antigens in formalin-fixed paraffin-embedded tissues, Antigens were normally retrieved using a pressure cooker and the citrate-based Antigen Unmasking Solution pH 6.0 (Catalog No. H-3300, Vector Laboratories, Burlingame, CA) [1].

TABLE 1 Summary of literatures on ACE2 expression in human lung macrophages

Literatures	ACE2$^+$Mϕ in human lung	Method	Antigen retrieval for IHC	Patient group
[5]	Yes	RNA-seq IHC	Yes	Ventilated patients and normal human lung tissues
[6]	Yes	IHC	Yes	Patients without recent pulmonary infection
[1]	Yes	IHC	Yes	Normal human lung tissues
[2]	Yes	IHC Western blot	Yes	Normal human lung tissues
[3]	Yes	IHC	Yes	Paraffin tissue sides from Biobank
[11]	Yes	scRNA-seq	-	Normal human lung tissues
[4]	Yes	IHC, Flow cytometry	Yes	COVID-19 patients and normal human lung tissues
[12]	No	IHC	N/A	Normal human lung tissues
[13]	Yes	scRNA-seq	-	Normal human lung tissues
[14]	No	scRNA-seq	-	Patients died from COVID-19
[15]	No	scRNA-seq	-	Healthy non-smoker
[16]	No	scRNA-seq	-	Normal human lung tissues

Abbreviations: IHC, immunohistochemistry; scRNA-seq, single cell RNA-seq.

3 | ISOTYPE-MATCHED CONTROLS SHOWED ACE2 EXPRESSION OF MACROPHAGES

There were some inconsistencies in the controls for three figures (e.g., Figure 1(A–C)) in our previous publication [1]. Figure 2C in our original publication [1] has been revised with their associated isotype-matched IgG controls in the overlay histograms (Figure 1(A)). The conclusion remains the same, with up-regulations of CD206 and CD209 on the IL-4-treated M2 macrophages, not on the LPS-treated M1 macrophages (Figure 1(A)) [1].

Figure 2D in our original publication [1] has been revised with two additional panels displaying the ACE2 expressions on M1 and M2 macrophages with their associated second Ab controls respectively (Figure 1(B)). The results were consistent with our previous data. The level of ACE2 expression was higher on the LPS-activated M1 macrophages than that of IL-4-treated M2 macrophages (Figure 1(B)) [1].

To confirm the ACE2 expression on M1 and M2 macrophages and to circumvent any autofluorescence in the green channel, we performed the immunocytochemistry by using a Cy5-conjugated donkey anti-mouse second antibody (Jackson ImmunoResearch Lab, catalog No. 711-175-150). Figure 2G in our original publication [1] has been revised with M1- and M2-associated second Ab controls respectively (Figure 1(C)). Using ImageJ software (version 1.8.0), quantification of fluorescence intensity demonstrated that there was a higher expression of ACE2 on M1 macrophages than on M2 macrophages (11.87 ± 4.0 vs. 3.79 ± 0.74, $p = 0.0017$) (Figure S1).

4 | HIGH EXPRESSION OF ACE2 ON PERIPHERAL BLOOD MONOCYTE-DERIVED M1 MACROPHAGES

To further confirm the ACE2 expression on M1 and M2 macrophages, flow cytometry was repeated among three additional donor-derived peripheral blood mononuclear cells (PBMC) (total $N = 6$, see following Figure 2(A–C)). Moreover, we performed the non-fluorescence immunocytochemistry with our established protocol [23]. After incubation with mouse anti-human ACE2 primary antibody, cells were stained with an ABC kit (Vector Laboratories, Burlingame, CA). The results further proved the higher expression of ACE2 on LPS-treated M1 macrophages (Figure 2(D)) than that of IL-4-treated M2 macrophages (Figure 2(E)).

5 | ACE2 EXPRESSION ON ALVEOLAR MACROPHAGES SHOWN BY ADDITIONAL IMMUNOHISTOCHEMISTRY STUDY IN LUNG TISSUE WITH RELEVANT CONTROLS

In figure 3A of our original publication [1], all paraffin tissue sections were purchased from BioChain Institute Inc (Newark, CA). For each slide, there were eight-tissue sections per slide, which were derived from eight normal adult humans respectively. All tissue samples were collected before the pandemic of COVID-19, without infections of SARS-CoV-2 and HIV.

The second Ab controls have been performed simultaneously for each tissue section from the same subject. Confocal microscopy showed background levels of fluorescence intensity in fluorescein isothiocyanate (FITC) and Cy3 channels in these tissue sections (Figure S2).

Additional immunohistochemistry confirmed that there was a high percentage of CD11b$^+$ macrophages among the donor-derived lung tissues (Figure 3). Most of ACE2$^+$-positive cells were co-localized with CD11b$^+$ macrophages. However, the levels of ACE2 expression were different among different CD11b$^+$ macrophages. Single immunostaining with CD11b and ACE2 Abs served as control respectively (Figure 3). The data further confirmed the ACE2 expression on alveolar macrophages.

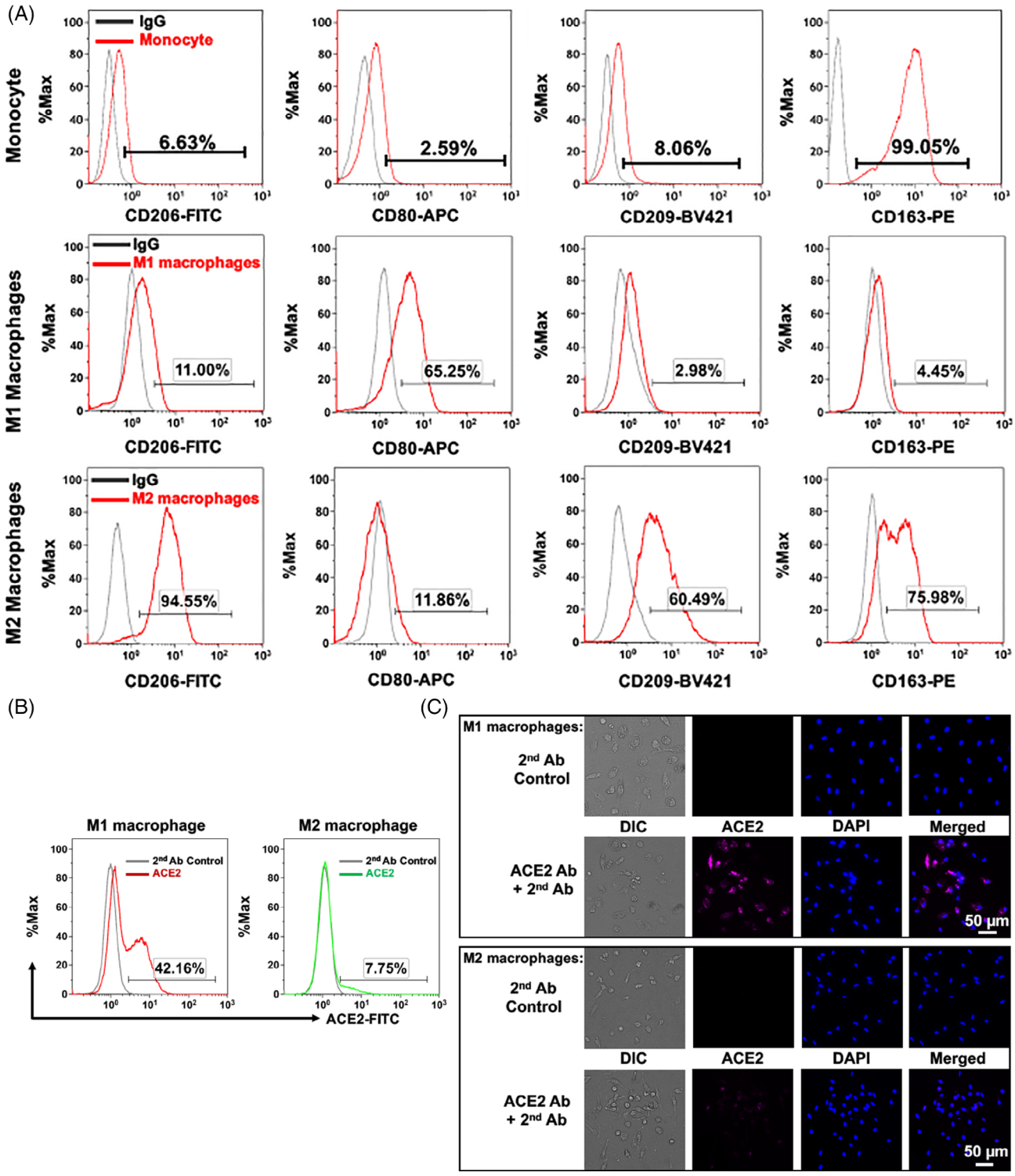

FIGURE 1 Examine ACE2 expression on M1 and M2 macrophages. (A) Phenotypic characterization of M1/M2 with their associated markers. Isotype-matched IgGs served as negative controls. Data were representative from one of two PBMC preparations. This figure is an amended figure 2C from the previous publication [1]. (B) Overlay histogram shows the high expression of ACE2 on M1 macrophages (red, left) in comparison with M2 macrophages (green, right). The second Ab staining served as negative control (gray). This figure is an amended figure 2D from the previous publication [1]. (C) Fluorescence microscopy shows high expression of ACE2 on M1 macrophages. This figure is an amended figure 2G from the previous publication [1]. Representative images were from one of immunostaining with six experiments

(A)

Sample No.	ACE2 expression on M1 % (MFI)	ACE2 expression on M2 % (MFI)
P408	30.12 (1.95)	16.28 (1.12)
P409	34.46 (1.86)	9.96 (0.69)
P518	39.96 (3.43)	16.29 (1.73)
P539	42.16 (1.92)	7.75 (1.16)
P540	19.90 (1.23)	9.73 (1.01)
P541	24.98 (1.33)	11.66 (1.08)

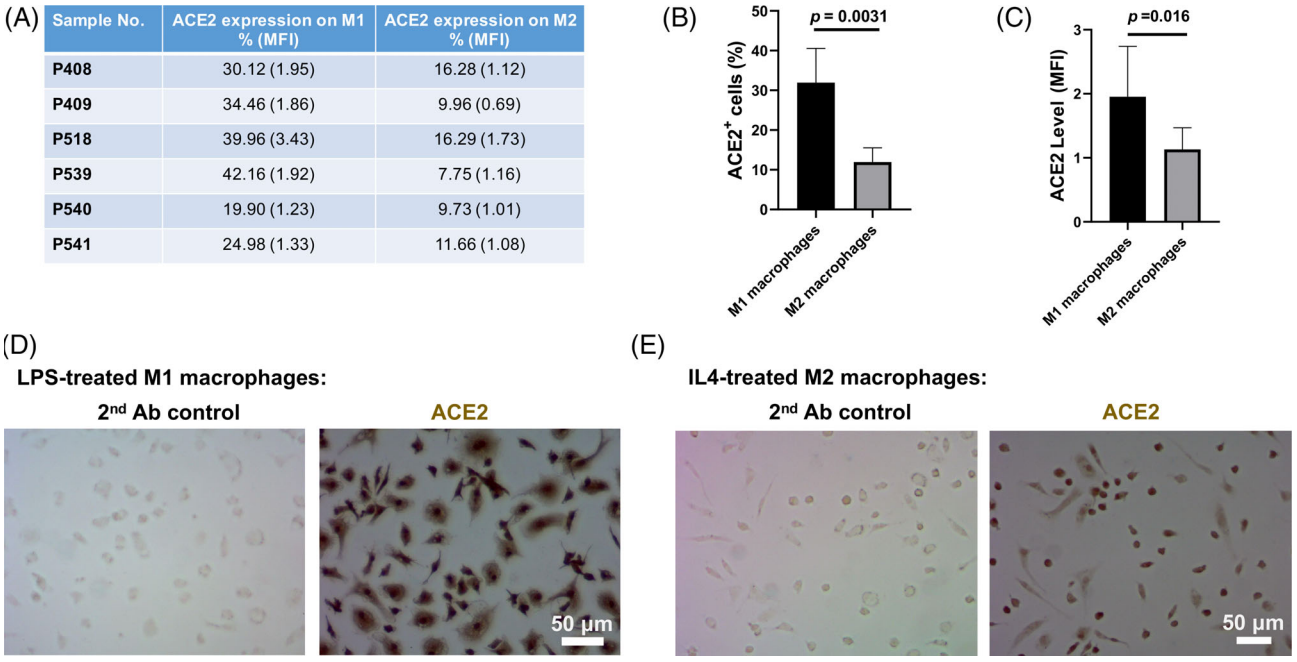

FIGURE 2 Examine the ACE2 expression on peripheral blood monocyte-derived M1 and M2 macrophages. The purified $CD14^+$ monocytes were initially seeded in the tissue culture-treated six-well plate at 5×10^5 cells/well and cultured in X-VIVO 15 serum-free media with 50 ng/ml M-CSF at 37°C, 5% CO_2 conditions. After 7 days, macrophages were treated with 1 μg/ml LPS or 40 ng/ml IL-4 for 24 h, respectively, and followed by flow cytometry (A–C) with 5000 cellular events per sample ($n = 6$). (A) Flow cytometry showed the expression level of ACE2 on M1 and M2 macrophages from six different donors, with the percentage (%) and median fluorescence intensity (MFI, in parentheses). (B) M1 macrophages display higher percentage of $ACE2^+$ cells than that of M2 macrophages. Data are presented as mean ± SD; $n = 6$. (C) M1 macrophages display higher level of ACE2 MFI than that of M2 macrophages. Data are presented as mean ± SD; $n = 6$. (D) Expression of ACE2 on LPS-treated M1 macrophages. Staining with second Ab served as negative control. (E) Expression of ACE2 on IL-4-treated M2 macrophages. Staining with second Ab served as negative control. Original magnification, ×100

6 | DISCUSSION

SARS-CoV-2, also known as COVID-19, has been spreading globally for over 16 months, with over 169 million confirmed cases and over 3.5 million deaths. Significant progress has been made in understanding SARS-CoV-2 origin, transmission, the clinical course of infection, and vaccination. However, a definitive pathogenesis and treatment for COVID-19 are still lacking. Human pulmonary system is primarily organ targeted by SARS-CoV-2 through ACE2, which has been recognized as the primary entry receptor for SARS-CoV-2 infecting host cells [24]. Current work and previous studies [1–6] demonstrated the ACE2 protein expression on lung macrophages, highlighting that lung macrophages can be directly attacked by SARS-CoV-2 during the pathogenesis of COVID-19, which include the initial strain Wuhan-Hu-1 (D614 and its early variant D614G) and several fast-spreading variants (e.g., B.1.1.7 lineage in the United Kingdom, B.1.351 lineage in South Africa, B.1.1.28 lineage in Brazil, and B.1.617 in India) [25–27]. Therefore, it is critical to improve lung macrophage function, eradicate the viral infections, and stop the transmission.

Lung macrophages, as essential antigen-presenting cells, can be derived from yolk sac and bone marrow-derived circulating monocytes [28]. To date, overreactions of monocytes/macropahges have been recognized in the hyperinflammation or cytokine storm of severe COVID-19 [18, 19, 29]. No pharmacological interventions have as yet shown significant efficacy in the treatment of severe COVID-19 patients [30, 31]. Although the United States Food and Drug Administration (FDA) has approved remdesivir under an emergency-use authorization for the treatment of adults and children with severe COVID-19, mortality rates among patients have remained high. Conventional immune suppressions by blocking cytokines and their receptors (e.g., IL-1, IL-6, or TNFα) or JAK inhibitors or steroids also have not significantly improved outcomes in severe patients, possibly because they impair both innate and adaptive anti-viral immunity, potentially increasing viral dissemination and making patients vulnerable to other infections. Finally, passive immunization with convalescent plasma from recovered COVID-19 subjects showed a good safety profile for treating hospitalized COVID-19 patients, yet clinical efficacy still remains to be determined [32]. These clinical trials highlight the challenges in conquering COVID-19, but underscore the urgent need for novel approaches, such as focusing on the functional modulation of monocytes/macrophages. To this respect, the US FDA-approved phase 2 clinical trial (ClinicalTrials.gov Identifier No:

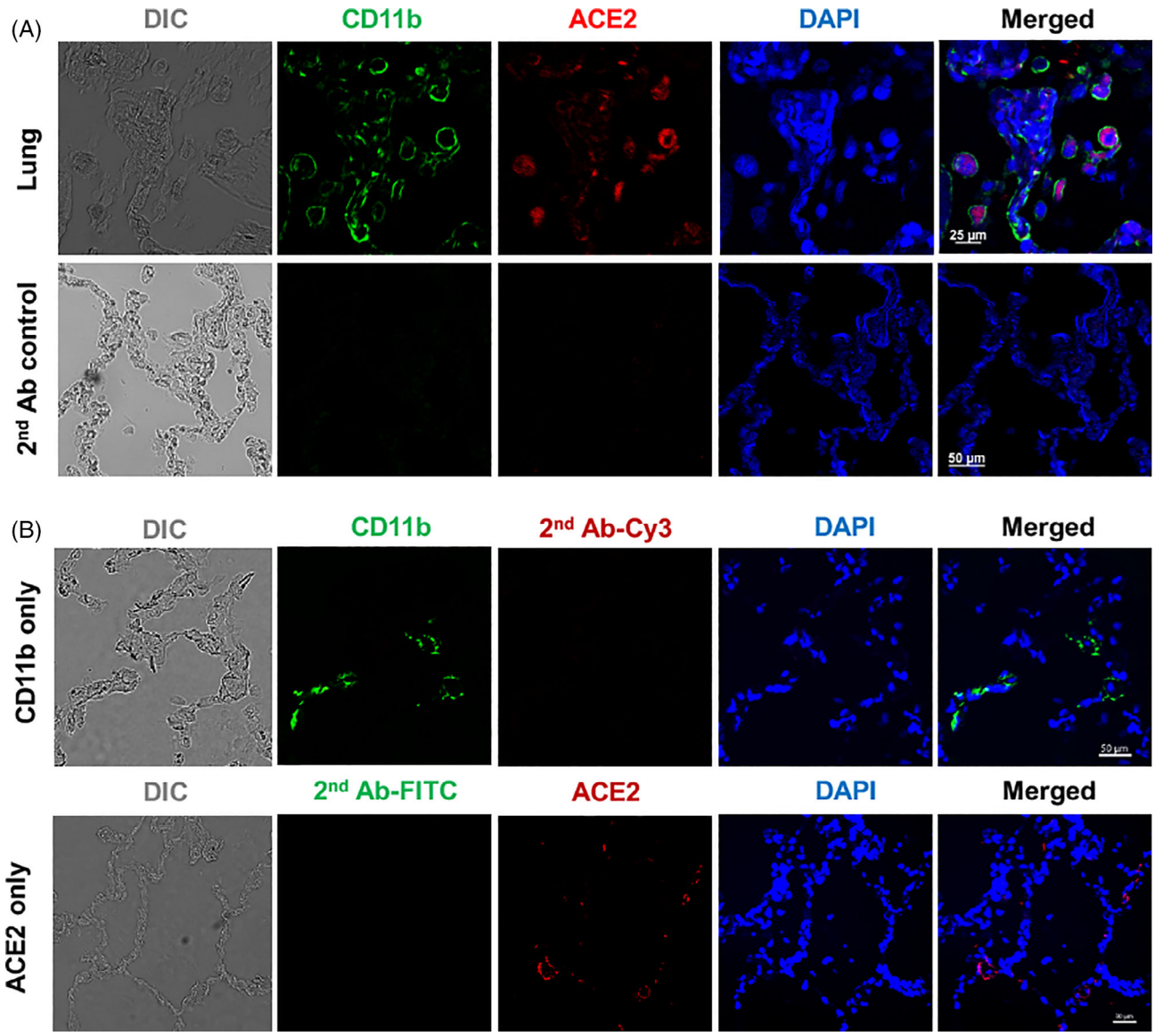

FIGURE 3 Immunohistochemistry shows ACE2 expression on alveolar macrophages. (A) Expression of ACE2 was co-localized with the $CD11b^{+}$ alveolar macrophages. Lung tissue sections stained with second abs served as negative control (bottom panel, scale bar 50 μm.) for CD11b (green) and ACE2 (red) staining (top panel, scale bar, 25 μm). (B) Single immunohistochemistry with CD11b Ab and ACE2 Ab respectively in lung tissue sections. Lung tissue sections stained with second abs served as negative controls. Scale bar, 50 μm

NCT04299152) of Stem Cell Educator® therapy, a closed-loop system through the immune education of cord blood-derived stem cells (CB-SC) on patient immune cells including monocytes [33–35], may offer a novel approach to target alveolar macrophages and promote M2 macrophage polarization through the modulation of blood monocytes by CB-SC and CB-SC-released exosomes [36, 37]. Additionally, there are clinical trials to block the monocyte/M1 macrophage-associated inflammatory cytokines (e.g., anti-IL-6, anti-IL-6 receptor, anti-IL-1β, and anti-TNF) in COVID-19 subjects [18]. A preliminary study showed the beneficial effects in severe COVID-19 patients after the treatment with tocilizumab (anti-IL-6 receptor Ab) [38]. Thus, it is expected that targeting macrophages may lead to producing comprehensive immune modulation at both the local (lung) and systemic levels and effectively improve clinical outcomes.

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PEER REVIEW

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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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