A COMPENDIUM OF RECENT RESEARCH ON STEM CELL-BASED THERAPY FOR COVID-19

EDITED BY: Abdelkrim Hmadcha, Bernat Soria, Robert Chunhua Zhao, Tarik Smani and Israel Valverde

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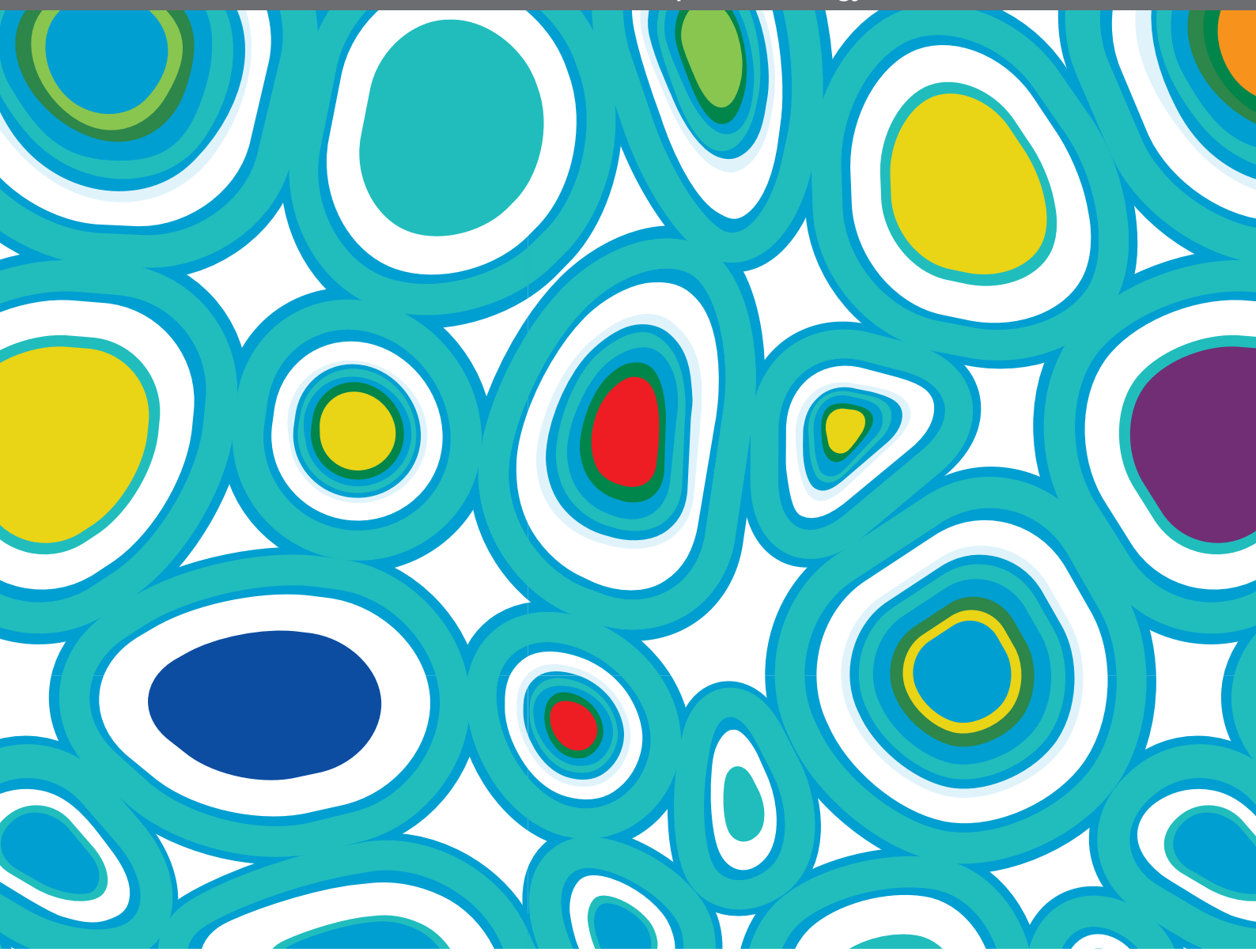

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A COMPENDIUM OF RECENT RESEARCH ON STEM CELL-BASED THERAPY FOR COVID-19

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EDITORIAL
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Editorial: A Compendium of Recent Research on Stem Cell-Based Therapy for Covid-19

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Keywords: advanced therapies, clinical trials, cell therapy, mesenchymal stromal cells, CAR T cells, COVID-19, cytokine storm, immunomodulaion

Editorial on the Research Topic

A Compendium of Recent Research on Stem Cell-Based Therapy for Covid-19

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INTRODUCTION

We have been dealing with an unprecedented global health crisis for nearly 2 years now. This started after an outbreak of atypical pneumonia of unknown etiology that was described in late December 2019 in China's Wuhan Province, the etiologic agent causing this pneumonia episode was identified as a novel coronavirus named "Severe Acute Respiratory Syndrome CoronaVirus-2" (SARS-CoV-2)" (Coronaviridae Study Group of the International Committee on Taxonomy of Viruses, 2020; Wu et al., 2020) and the disease was designated COronaVIrus Disease-2019 (COVID-19). The rapid expansion of COVID-19 cases in number and geographic distribution led the World Health Organization (WHO) to declare a global health emergency. The control of the disease was challenged by the lack of antiviral treatment and vaccines, by asymptomatic carriers and the rapid increase in infections worldwide; COVID-19 was officially classified and declared by WHO as a pandemic on March 11, 2020 (WHO, 2020).

COVID-19 affects people differently, the majority of infected individuals develop mild to moderate disease and recover without hospitalization, but a subgroup of patients progresses to severe disease, with a high mortality rate and limited treatment options. However, the clinical features of COVID-19 vary from asymptomatic forms to conditions involving multi-organ and systemic manifestations in terms of septic shock and multiple organ dysfunction syndrome (MODS). Common primary pathologic features of critical COVID-19 overlap with acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). While most infected individuals are usually asymptomatic or have mild symptoms, about 15% are affected by ARDS, of which 5% progress to multiple organ dysfunction syndrome or failure (Hui and Zumla, 2019).

COVID-19 involves direct attacks by SARS-CoV-2 on cells and secondary attacks on the body following activation of the immune system. Consequently, both the virus and the immune response

can cause damage to the body, leading to common complications or secondary infection. The spike protein (S-protein) of SARS-CoV-2 binds to its functional receptor angiotensin-converting enzyme 2 (ACE2), triggering endocytosis of virus particles to infect target cells (Li et al., 2003; Tay et al., 2020). Therefore, potential therapeutic candidates for studying the mechanisms of SARS-CoV-2 infection include the ACE2 receptor (Zhang et al., 2020).

Given that the exact pathogenesis of SARS-CoV-2 and the dynamics of the disease are not yet fully understood, nor are specific antiviral options available, makes the treatment of patients with COVID-19 still a challenge (Tay et al., 2020); therefore, the available treatment options are limited. Several antiviral drugs (Grein et al., 2020), corticosteroids (Wang et al., 2020; Rasheedi et al., 2021), convalescent plasma (Shen et al., 2020; Verma et al., 2020) and neutralizing monoclonal antibodies (Shanmugaraj et al., 2020) have been tested and have undergone different phases of clinical trials, but none have been approved for COVID-19. Another alarming development is the recurrence of infection in recovered and even vaccinated individuals, which challenges the efficacy of current treatments (Lan et al., 2020). Under this situation, research has been carried out at an unprecedented speed to achieve a vaccine. Although several vaccines are now available and millions of people have received their full dose, the process of assessing safety, efficacy is still challenged, and a longer follow-up is still required (Kadkhoda, 2021; Olliaro et al., 2021; Yan et al., 2021). While the efficacy of vaccines has focused on the reduction of the number of symptomatic cases, the durability of acquired immunity following vaccination, the protection against reinfection by SARS-CoV-2 and its emerging variants remain to be proven and even improved (Townsend et al., 2021).

Thus, there is an urgent need for the development of feasible, safe and effective therapies. Therefore, growing experimental and clinical evidence suggests that advanced therapies could provide a potential therapeutic alternative for COVID-19 caused by SARS-CoV-2 and its emerging variants. In particular, cell therapy has been proven to significantly improve the sequelae related to COVID-19. In this regard, MSCs are among the most frequently used cell type for cell therapy, and considerable efforts have been made to introducing this advanced cell-based therapy into clinical practice. MSCs have been established as promising candidate sources for cell-based therapy their broad pharmacological effects and due to their contributions to tissue and organ homeostasis, repair and support by self-renewal and multi-differentiation, as well as by their anti-inflammatory, anti-proliferative, immunomodulatory, pro-angiogenic, pleiotropic, tropic and trophic properties. Various diseases have been successfully treated by MSCs in animal models and hundreds of clinical trials related to the potential benefits of MSCs are ongoing or have been successfully concluded so far. MSCs also secrete a myriad of soluble factors and vesicles altogether contribute to tissues and organs support, repair, homeostasis and functionality. The efficacy of MSCs and their secretory factors has been proven in successfully reducing inflammation, dampening immune responses and repairing lung damage in various pre-clinical and clinical models (Hmadcha et al., 2009). As described in the review by Ligotti et al., the key roles of both immunosenescence and immunopathology in the outcome of SARS-CoV-2 infection are supported by the beneficial results obtained with infusion of MSCs that act by restoring immune homeostasis and contributing to lung repair. In addition, the potential of MSC-based therapy as an option for severe or critically ill COVID-19 patients has been explored (Leng et al., 2020; Sánchez-Guijo et al., 2020). Several studies focus on regenerative, immunomodulatory, and anti-inflammatory properties of mesenchymal stromal cells (MSCs) to reduce the manifestation of cytokine storm and to restore ARDS and ALI, exhibiting an important option to be applied to critical COVID-19 patients; or on MSCs secretome to treat COVID-19 pneumonia (Li et al., 2020; Liang et al., 2020; Meng et al., 2020; Tang et al., 2020; Lanzoni et al., 2021). The contribution of Arjmand et al., summarizes the research on cytokine storm, one of the key causes of MODS as a hallmark of COVID-19 severity, highlights the benefit of stem cell-based therapies to attenuate cytokine release syndrome and suggests emerging advantages of MSCs secretome and extracellular vesicles (EVs) as treatment approaches for COVID-19. Besides, 2 interesting hypotheses and theories are formulated on the one hand, Nazerian et al., hypothesize that the use of chimeric 8P9R peptide and soluble ACE2 using exosome-liposome hybrids in the form of a two-step phase-dependent therapeutic strategy could inhibit the viral intracellular pathway and also inhibit progression in cytokine storm in a personalized manner. Given that this strategy is sensitive to the inflammatory status of individuals, it may improve the outcome of COVID-19 mortality. On the other hand, Babajani et al., provided new shreds of evidence about mitochondrial dysfunction and its effects on the immune response in COVID-19 and hypothesized that *in vivo* and *in vitro* experimental transferring healthy mitochondria by MSCs to damaged cells can provide a new therapeutic approach for COVID-19.

Within the cell therapy approach, other research includes the use of hematopoietic stem cells derived from umbilical cord blood, bone marrow, or mobilized peripheral blood, as well as immune chimeric antigen receptor T-cell (CAR-T cells) (Vardhana and Wolchok, 2020). Of note, an interesting immunology and virology original paper by Li et al., provided data that can further improve development of vaccines and new therapies against COVID-19. In this regard, authors reported a series of potential epitopes on SARS-CoV-2 spike glycoprotein which are recognized by $CD4^+$ and $CD8^+$ T cells from patients recovered from COVID-19 in China. The authors found that $CD134^+$ and $CD137^+$ T cells can react to an epitope outside the spike receptor domain (RBD), isolated T-cell receptor (TCR) sequences from the immunoreactive T cells and also inserted into Jurkat and $CD4^+$ T cells to identify an epitope-specific TCR. Ferreras et al., for their part, characterized SARS-CoV-2-specific T-cell population within the $CD45RA^-$ memory T cells (either $CD4^+$ or $CD8^+$) from blood of convalescent donors. In addition, the authors have demonstrated that these cells can be easily, effectively, and rapidly isolated following a donor selection strategy based on IFN-γ expression after exposure with SARS-CoV-2-specific peptides and HLA antigen expression, thereby

obtaining clinical-grade CD45RA$^-$ memory T cells, without requirements for GMP conditions, allowing the establishment of a biobank of SARS-CoV-2 specific memory T cells to be used to treat moderate to severe cases of COVID-19 patients.

The understanding of the mechanism of infection and pathogenesis also have great appeal and are still limited. In this regard; the use of human pluripotent stem cells, both embryonic stem cells (hESCs) and induced stem cells (hiPSCs), to generate tissue-specific human organoids (lung, intestinal, liver, vascular, heart, and kidney organoids) may provide a next-generation cellular model for investigating viral infection and drug screening (Yang et al., 2020). In this context, Larijani et al., reviewed the currently available iPSC-derived cells, iPSC-derived organoid models and animal models for studying the pathophysiology mechanisms of COVID-19-associated disorders and discussed the challenges and limitations that need to be overcome to optimize modelling approaches for the disease, and proposed potential therapeutic advances identified in experimental studies. Relatedly, in their review Luo et al., recapitulated findings on the application of hiPSC-derived cell models and organoids for COVID-19 to understand the action of SARS-COV-2 on human cells, underlining the importance of developing these models for long-term experiments not only to study the pathology of SARS-CoV-2 infection, respiratory failure and dysregulation of organs and systems, but also, to mimic the natural host-virus interaction, to clarify viral infection mechanisms and to elucidate the *in vivo* conditions of viral life cycles and drug screening.

We would like to conclude referring to the opinion by Zhao CR and others, pioneer in using MSCs to improve the outcome in patients with COVID-19 pneumonia (Leng et al., 2020). Wang et al., bring the debate about the clinical use of MSCs and MSCs-derived EVs for combating COVID-19, the risk of their uncontrolled commercial application to the forefront and the lack of any suggestions on regulations and guidelines for regulatory agencies to adopt new policies to prevent the sale of unproven MSC-based treatments in COVID patients.

Here we assembled a compendium of 9 manuscripts (2 original research, 4 reviews, 2 hypothesis and theory and 1 opinion) that cover the scope of this research topic, thus highlighting some of the recent advances and progresses of preclinical and clinical research and discussing some of the critical aspects related to the application of stem cell for COVID-19. Altogether, the ultimate goal of all these strategies is to achieve a safe and controlled therapy for COVID-19.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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OPINION
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Combating COVID-19 With Mesenchymal Stem/Stromal Cell Therapy: Promise and Challenges

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There is no effective therapy for COVID-19 currently. Until an efficient vaccine is developed, therapeutic strategies that facilitate faster recovery in COVID-19 patients developing life-threatening complications are urgently needed. From this perspective, mesenchymal stem/stromal cells (MSCs) that have been used in the clinic for moderating the immune system in graft vs. host disease (GVHD) (Fisher et al., 2019), type 2 diabetes (Path et al., 2019), autoimmune diseases (Weiss and Dahlke, 2019), spinal cord injury (Shende and Subedi, 2017), and several other diseases deserve consideration for treating COVID-19. Importantly, MSCs lack angiotensin-converting enzyme-2 (ACE2) receptor, which is a receptor widely distributed on the surface of human cells, and required for the entry of coronavirus into host cells. Such property ensures that injected MSCs can accomplish immunomodulatory effects without being destroyed by the virus. Our clinical trial, which is also the first published report, showed that the intravenous injection of human umbilical cord-derived MSCs eased the cytokine storm syndrome (CSS) and significantly improved the outcome in severe COVID-19 patients (Leng et al., 2020), suggesting the promise of MSC therapy for saving lives of COVID-19 patients developing severe complications. Although we repeatedly emphasize that studies in a larger cohort of patients are urgently needed to validate this promising therapeutic intervention, some businesses are taking advantage of our findings and offer cell therapy for COVID-19 patients, using types of cells that might not have been tested vigorously for safety and efficacy in FDA-approved clinical trials. This unethical commercial use of MSCs is criticized by Leigh Turner in his article entitled "*Preying on Public Fears and Anxieties in a Pandemic: Businesses Selling Unproven and Unlicensed Stem Cell Treatments for COVID-19*" published in Cell Stem Cell (Turner, 2020), which we read with great interest and are inspired to write this opinion paper. We strongly agree that such businesses could pose significant risks to patients and detract efforts to advance evidence-based stem cell therapy for COVID-19. We must emphasize that there are no approved MSC-based approaches for the prevention or treatment of COVID-19 patients, although several FDA-approved clinical trials are ongoing at present. Furthermore, we would like to suggest some guidelines from regulatory agencies that might stop such businesses from selling stem cell therapy to COVID-19 patients. Such guidelines should: (1) require companies to provide detailed scientific information on the safety and efficacy of mesenchymal stem/stromal cells in preclinical trials; clear criteria of MSCs manufacture and quality control; scientific rationale of organizing a clinical trial using stem cell therapy for patients; the qualifications of the principal investigators and all the medical staff; registered information

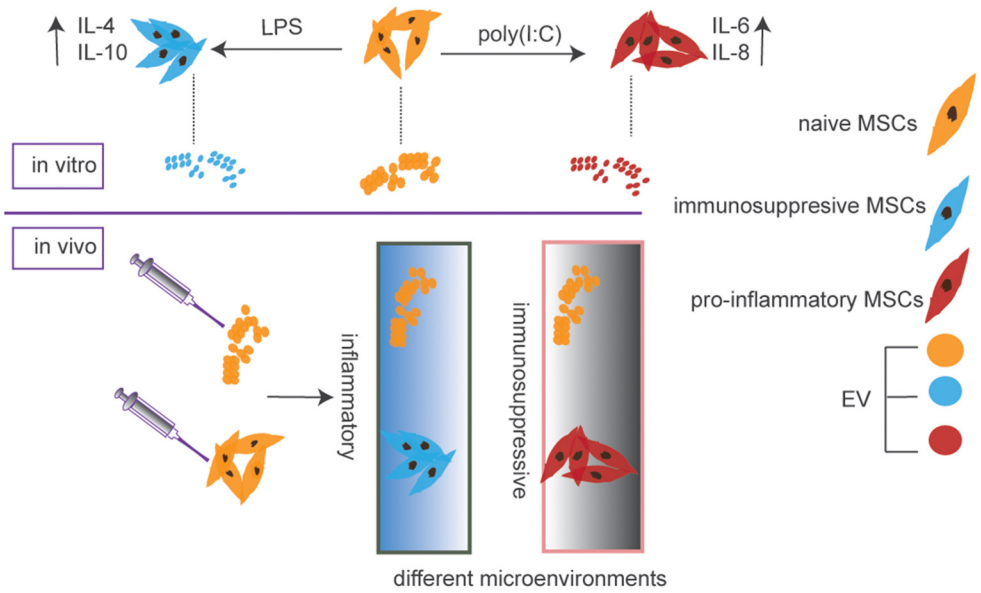

FIGURE 1 | EV cargos are determined by the status of parental MSCs and would not change after the injection, while MSCs could alter secretome after homing to sites of injury or inflammation due to their ability for plasticity depending on the microenvironment they come across.

in clinical trial https://clinicaltrials.gov/. (2) promote central or local government to establish an independent stem cell therapy committee that could update latest research outcomes or provide professional suggestions for patients. (3) encourage the public to report unproven stem cell therapy to the state administration so that the illegal company could be punished. The objective of these guidelines is to help patients and their caretakers to enroll only in randomized clinical trials conducted in renowned hospitals with approvals from the local Institutional Review Board (IRB) and the Federal Drug Administration (FDA). We think generating and implementing a new policy or law at the national level could stop such cell treatment to COVID-19 patients. Besides, the social platform, including media, should publicize and educate the public regarding dangers associated with cell treatment offered by businesses that are not FDA-compliant.

In addition to MSCs, some businesses are also selling MSC-derived extracellular vehicles (EVs) for COVID-19 therapy. EVs, nanosized vesicles secreted by virtually all cells for intercellular communication, carry a cargo comprising cytokines, growth factors, lipids, and microRNAs (Robbins and Morelli, 2014; Phinney and Pittenger, 2017; Kalluri and LeBleu, 2020). Studies in disease models have suggested that stem cell-derived EVs exert similar effects as their parental cells (Yanez-Mo et al., 2015; Kim et al., 2016). Because the therapeutic effects of MSCs have been attributed mainly to their secretome with a significant portion of which is disseminated through EVs (Rani et al., 2015; Keshtkar et al., 2018). MSC-derived EVs could be exploited for cell-free therapy. Compared to MSCs, EVs released from them have unique advantages such as more accessible storage and higher biosafety. However, the disadvantages include that MSCs could home to sites of injury or inflammation and change their secretome depending on the local microenvironment. Importantly, MSCs are relatively large cells with an estimated average size of around 30 μm in suspension (ranging from 16 to 53 μm) (Furlani et al., 2009; Leibacher and Henschler, 2016). This large size makes them easily trapped in lungs after intravenous administration, which might be a hurdle for the treatment of other diseases but a benefit for COVID-19 as lung is the major target organ of coronavirus. So, this specific and preferential pulmonary localization after administration is an advantage of MSCs compared to EVs in terms of treating COVID-19 or other lung diseases. On the other hand, the targeted delivery of EVs to intended tissues after an intravenous administration is currently challenging to achieve. More importantly, the cargo of EVs is determined by the status of parental MSCs as well as culture conditions (**Figure 1**), which do not change after interactions with the local microenvironment.

A very recent article reported the safety and efficacy of allogeneic bone marrow MSC-derived EVs in severe COVID-19 patients who were already on hydroxychloroquine and azithromycin treatment. The study was a prospective, non-randomized, open-label, investigation in which 24 SARS-CoV-2 PCR positive patients at a single hospital center received MSC-derived EVs (Sengupta et al., 2020). Although such EV treatment did result in 71% of patients recovering from COVID-19, it was unclear whether the recovery could be attributed to EVs as the study lacked a matching control group and the protein or the microRNA composition of EVs employed was not reported in the study. One should be extremely cautious with clinical trials using EVs as their cargo determines the

functional effects. Therefore, before MSC-derived EVs can be used in a clinical setting, standardized protocols for scaled-up production, isolation, functional evaluation, and batch-to-batch consistency need to be developed. These require a rigorous characterization of the composition of EVs generated in different batches using proteomics and small-RNA sequencing, the release criteria, and the biological properties. Also, the efficacy of EVs needs to be validated in animal models. While the MSC-derived EVs have the potential to replace MSC therapy for many conditions (Kim et al., 2016, 2020; Long et al., 2017), EV therapy does not seem ready in a short time.

In summary, we would like to emphasize that well-controlled, rationally designed clinical trials based on reliable scientific data are needed for both MSCs and MSCs-derived EVs for combating COVID-19.

AUTHOR CONTRIBUTIONS

SW, AS, and RZ conceived, researched, and wrote the manuscript with input from KJ. All authors contributed to the article and approved the submitted version.

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SARS-CoV-2-Specific Memory T Lymphocytes From COVID-19 Convalescent Donors: Identification, Biobanking, and Large-Scale Production for Adoptive Cell Therapy

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Syndrome coronavirus 2 (SARS-CoV-2) pandemic is causing a second outbreak significantly delaying the hope for the virus' complete eradication. In the absence of effective vaccines, we need effective treatments with low adverse effects that can treat hospitalized patients with COVID-19 disease. In this study, we determined the existence of SARS-CoV-2-specific T cells within CD45RA$^-$ memory T cells in the blood of convalescent donors. Memory T cells can respond quickly to infection and provide long-term immune protection to reduce the severity of COVID-19 symptoms. Also, CD45RA$^-$ memory T cells confer protection from other pathogens encountered by the donors throughout their life. It is of vital importance to resolve other secondary infections that usually develop in patients hospitalized with COVID-19. We found SARS-CoV-2-specific memory T cells in all of the CD45RA$^-$ subsets (CD3$^+$, CD4$^+$, and CD8$^+$) and in the central memory and effector memory subpopulations. The procedure for obtaining these cells is feasible, easy to implement for small-scale manufacture, quick and cost-effective, involves minimal manipulation, and has no GMP requirements. This biobank of specific SARS-CoV-2 memory T cells would be immediately available "off-the-shelf" to treat moderate/severe cases of COVID-19, thereby increasing the therapeutic options available for these patients.

Keywords: memory T cells (Tmem), adoptive cell therapy (ACT), COVID-19, lymphopenia, biobank

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INTRODUCTION

The new severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged as a worldwide pandemic in late 2019, causing an infectious disease known as COVID-19, with a wide and diverse range of symptoms. In most infected patients, the virus causes mild symptoms including fever and cough. In some cases, however, the virus causes a life-threatening disease with symptoms that include pneumonia, dyspnea and a hyperinflammatory process that includes cytokine storms and systemic immune thrombosis. Patients suffering from these symptoms require hospitalization and intensive treatment. A common feature of this severe disease is lymphopenia, which makes patients more vulnerable to co-infections and correlates with the severity disease (Qin et al., 2020; Zhao et al., 2020).

The first wave of the pandemic was contained with strong restrictive measures, social distancing, and healthcare interventions, although thousands of patients died. Far from disappearing, the SARS-CoV-2 pandemic has begun its second wave, thereby dimming the hopes for its complete eradication. The development of vaccines has vigorously pursued to generate active immunity through immunization (Thanh Le et al., 2020), but there is uncertainty as to the duration of the antibody-mediated immune response to COVID-19 (Li et al., 2008). Effective treatments are needed that can reduce symptoms severity and hospital stays and increase survival.

So far, the only treatment for COVID-19 is supportive. Antiviral therapy with lopinavir–ritonavir is ineffective in improving the outcomes hospitalized patients with COVID-19 (Cao and Hayden, 2020). Remdesivir has been recently approved to treat COVID-19, although its beneficial effect is still controversial (Jomah et al., 2020; Wang et al., 2020). Preliminary results with anti-inflammatory therapies such as dexamethasone (Recovery Collaborative Group et al., 2020) and mesenchymal stromal cells (Sanchez Guijo et al., 2020) have shown promising results for critically ill patients (World Health Organization [WHO] grade 6 and 7) (World Health Organization, 2020), but there are as of yet no effective antiviral therapies for stopping the progress of this disease in its early stages (WHO grade 1–4 moderate and severe) or even to prevent COVID-19.

The role of adaptive immunity in COVID-19 and the protective immunity conferred by T cells is still being characterized (Grifoni et al., 2020; Huang et al., 2020; Leung et al., 2020; Rodda et al., 2020; Sekine et al., 2020), and the role of memory T cells in conferring protection against SARS-CoV-2 has not yet been properly defined. The presence of memory T cells specific for another SARS coronavirus was found up to 11 years post-infection (Ng et al., 2016). This immunological memory creates a more rapid and robust secondary immune response to reinfections, which is determinant and constitutes the basis of adoptive cell therapy for viral infections in immunosuppressed patients in the context of allogeneic hematopoietic stem cell transplantation (HSCT). With this approach, the infusion of $CD45RA^-$ memory T cells considerably reduces the morbidity and mortality induced by viral reactivations by, for example, cytomegalovirus (CMV) and Epstein Barr virus (EBV) and simultaneously reduces the alloreactivity conferred by naïve $CD45RA^+$ T cells (Bleakley et al., 2014, 2015; Teschner et al., 2014; Triplett et al., 2015, 2018).

Memory T cells do appear when T cells recognize a pathogen presented by their local antigen-presenting cells. These T cells activate, proliferate, and differentiate into effector cells secreting compounds to control the infection. Once the pathogen has been cleared, most of the antigen-specific T cells disappear, and a pool of heterogeneous long-lived memory T cells persist (Mueller et al., 2013; Pennock et al., 2013). This population of memory T cells, defined as $CD45RA^-$ or $CD45RO^+$, is maintained over time conferring rapid and long-term immune protection against subsequent reinfections (Berard and Tough, 2002; Channappanavar et al., 2014).

In this study, we report the presence of a SARS-CoV-2 specific T-cell population within $CD45RA^-$ memory T cells from the blood of convalescent donors that can be easily, effectively, and rapidly isolated by CD45RA depletion. These specific SARS-CoV-2 $CD45RA^-$ memory T cells may be able to clear virally infected cells and confer T-cell immunity for subsequent reinfections. These cells can be stored for use in moderate and severe cases of COVID-19 patients requiring hospitalization, thereby representing an off-the-shelf living drug.

MATERIALS AND METHODS

Donors' Characteristics

The study included 6 COVID-19 convalescent donors and 2 healthy controls (**Table 1**). The convalescent donors were all tested for SARS-CoV-2 using reverse transcriptase polymerase chain reaction (RT-PCR) in nasopharyngeal samples between March and April 2020. The eligibility criteria included an age of 21–65 years, a history of COVID-19 with a documented positive RT-PCR test for SARS-CoV-2. At the time of this study, all donors tested negative for SARS-CoV-2. The median age of the convalescent donors was 37 years (range 23–41), 3 were women and 3 were men. The median duration until a negative PCR for SARS-CoV-2 was 13 days (range 5–17). Two of the donors presented with bilateral pneumonia but did not require hospitalization. Only 1 of the donors underwent treatment with oral hydroxychloroquine plus azithromycin plus lopinavir/ritonavir, while the other was treated with oral hydroxychloroquine plus azithromycin. The study enrolled two healthy donors who had not been exposed to COVID-19 patients and tested negative for anti-SARS-CoV-2 antibodies in June 2020. All participants granted their written consent, and the study was approved by the Hospital Institution Review Board (IRB number: 254/20).

Cell Processing and Detection of SARS-CoV-2-Specific Memory T Cells by Interferon-Gamma Assay

Peripheral blood mononuclear cells (PBMCs) from healthy donors and convalescent donors were isolated from their peripheral blood by density gradient centrifugation using Ficoll-Paque (GE Healthcare, Chicago, IL, United States). Briefly,

TABLE 1 | Participants' characteristics.

Number of donors	6
Mean age, years (range)	37 (23–41)
Sex (female/male)	3/3
Time to SARS-CoV-2-negative PCR, days (range)	12.83 (5–17)
Bilateral pneumonia, *n*(%)	2 (33.3%)
Time until samples taken from negative PCR, days (range)	14.70 (7–33)
Outpatients, *n* (%)	6 (100%)
Treatment	
Acetaminophen	4
Hydroxychloroquine + azithromycin + lopinavir/ritonavir	1
Hydroxychloroquine + azithromycin	1

the cells were rested overnight (o/n) at 37°C in TexMACS Medium (Miltenyi Biotec, Bergisch Gladbach, Germany) supplemented with 10% AB serum (Sigma-Aldrich, Saint Louis, MO, United States) and 1% penicillin/streptomycin (Sigma-Aldrich). The following day 1×10^6 cells were stimulated with pooled or individual overlapping SARS-CoV-2 peptides at a final concentration of 0.6 nmol/mL. For the positive control, 1×10^6 cells were stimulated in the presence of the plate-bound stimulator OKT3 at a final concentration of 2.8 μg/mL (mouse anti-human CD3 Clone OKT3, BD Biosciences). Cells with SARS-CoV-2 peptides and positive control were co-stimulated with CD28/CD49d at a final concentration of 5 μg/mL (anti-human CD28/CD49d Purified Clone L293 L25, BD Biosciences). Basal interferon gamma (IFN-γ) production by PBMCs was included as a background control in the absence of stimulation and co-stimulation. The peptide pools were short 15-mer peptides with 11 amino acid overlaps that can bind MHC class I and class II complexes and were therefore able to stimulate $CD4^+$ and $CD8^+$ T cells. The peptides cover the immunodominant sequence domains of the surface glycoprotein S, the complete sequence of the nucleocapsid phosphoprotein N and the membrane glycoprotein M (GenBank MN908947.3, Protein QHD43416.1, Protein QHD43423.2, Protein QHD43419.1; Miltenyi Biotec, Germany). After 5 h of stimulation, the cells were labeled with IFN-γ Catch Reagent (IFN-γ Secretion Assay-Detection Kit, human Miltenyi Biotec) containing bispecific antibodies for CD45 and IFN-γ, which were secreted by the stimulated target cells. After the secretion phase, the cell surface-bound IFN-γ was targeted using the IFN-γ PE antibody included in the kit.

Phenotype of Memory T Cells Containing SARS-CoV-2-Specific T Cells Determined by Flow Cytometry Assay

We analyzed the cell composition of the T cells specifically activated by SARS-CoV-2 in the IFN-γ assay by subtracting the basal cytokine response from the background control. We stained the cell surface for 20 min at 4°C using the following fluorochrome-conjugated antibodies titrated to their optimal concentrations: CD45RA FITC (BD Pharmingen), CD27 APC (BD Pharmingen), CD3 VioGreen (Miltenyi Biotec), CD4 PECy7 (BD Pharmingen), CD8 APC Cy7 (BD Pharmingen), and 7AAD (BD Horizon). For the Treg panel CD25 BV421 (BD Horizon) and CD127 PE-CF594 (BD Horizon), were used. For the activation panel, HLA-DR BV 421 (BD Pharmingen), CD69 BV421 (Biolegend), and CD25 BV421 (BD Horizon), were used. For the exhaustion panel PD1 AF700 (Biolegend) and NKG2A BV421 (Biolegend) were used. For the chemokine panel, CD103 BV421 (BD Horizon) and CCR7 PE-CF594 (BD Horizon) were used. Cell acquisition was performed using a Navios cytometer (Beckman Coulter), acquiring an average of 200,000 cells. The analysis was performed using FlowJo 10.7.1 (FlowJo LLC).

Interleukin-15 Stimulation of Memory T Cells

$CD45RA^-$ memory T lymphocytes from the convalescent donor were thawed and stimulated with interleukin (IL)-15 to obtain an activated phenotype. Cells were incubated in TexMACS Medium (Miltenyi Biotec, Germany) supplemented with 5% AB serum (Sigma-Aldrich, Saint Louis, MO, United States), 1% penicillin/streptomycin (Sigma-Aldrich, Saint Louis, MO, United States) and 50 ng/mL of IL-15 o/n and for 72 h. After that time the cells were harvested and the phenotypic assay was performed. The same culture without IL-15 was run in parallel as a control.

Donor Selection, Human Leukocyte Antigen Typing, and Large Clinical Scale $CD45RA^+$ T Cell Depletion

The criteria for selecting convalescent donors were as follows: (1) IFN-γ secretion upon activation with the three SARS-CoV-2-specific peptides (M, N, S) and, (2) the most frequent human leukocyte antigen (HLA) typing to cover most of the population. The HLA phenotype of the convalescent donor was performed at the Centro de Transfusión of the Comunidad of Madrid on two independent samples by SSO and NGS: A*02:01,A*24:02/B*44:02,B*51:01/C*16:02,C*16:04/DRB1*07:01,DRB1*11:03/DQB1*02:02,DQB1*03:01.

Non-mobilized apheresis was performed at the Bone Marrow Transplantation and Cell Therapy Unit of University Hospital La Paz (Madrid, Spain) using a CliniMACS Plus cell separation system (Miltenyi Biotec). The donor provided written informed consent, and the study was conducted according to the Declaration of Helsinki protocol and the guidelines of the local ethics committee (IRB number 5579). The Unit was responsible for complying with the requirements regarding the quality and safety of the donation, obtention, storage, distribution, and preservation of human cells and tissues under the Spanish specific regulation. Following apheresis, $CD45RA^+$ cells were depleted by immunomagnetic separation using a CliniMACS CD45RA Reagent and the CliniMACS Plus system (both from Miltenyi Biotec), following the manufacturer's instructions. $CD45RA^-$ cells were frozen using autologous plasma plus 5% dimethyl sulfoxide (DMSO) and stored. We were able to cryopreserve 30 aliquots at various doses according to the trial design. The viability, purity, phenotype and spectratyping of the $CD45RA^-$ fraction were analyzed by flow cytometry (FCM).

TCR Spectratyping

Most of the CDR3-encoding regions of the TCRV- and TCRV-γ genes were amplified using 2 V-J multi-primer sets for each locus and one additional multi-primer set covering D-J TCRV-β (Vitro, Master Diagnostica, Spain). Primers marked at their 5′ end with 6-FAM fluorochrome enabled the denatured fragment size analysis by capillary electrophoresis (ABI3130 DNA-analyzer) and Genemapper software (Thermo Fisher Scientific, United States).

Statistical Analysis

The quantitative variables are expressed as mean ± standard deviation (SD), while the qualitative variables are expressed as percentages (%). A two-tailed Mann–Whitney non-parametric test was used for comparison means for the non-paired samples using GraphPad Prism (version 8.0.0 for Windows, GraphPad Software, San Diego, CA, United States). A P-value < 0.05 was considered statistically significant.

RESULTS

Memory T Cells From Convalescent Donors Contain a SARS-CoV-2-Specific Population

We detected the presence of a SARS-CoV-2-specific population in both subsets of naïve $CD45RA^+$ and memory $CD45RA^-$ T cells in the PBMCs of the convalescent donors but not in the healthy controls (**Table 2** and **Figure 1**). The subsets also showed reactivity for the single peptides M, N, and S (data not shown). The mean $CD45RA^-CD3^+$ population in the convalescent donors was 90.01%. IFN-γ expression within the $CD45RA^-CD3^+$ population was 1.12%, whereas IFN-γ

TABLE 2 | Immunophenotypic characterization of the healthy controls and the convalescent donors after co-culture with the mixture of the three SARS-CoV-2 peptides (M, N, S).

Cell type	Subpopulation	C1	C2	Mean Pepx3	SD	D1	D2	D3	D4	D5	D6	Mean Pepx3	SD
$CD45RA^-$	% $CD45RA^-$	37.68	22.17	29.93	10.97	31.20	24.07	18.16	43.90	19.90	33.24	28.41	9.67
	IFN-γ+	0.00	0.00	0.00	0.00	0.77	0.99	3.00	0.30	0.90	0.87	1.14	0.94
$CD45RA^-$ CD3+	% CD3+	61.69	78.38	70.04	11.80	93.84	93.45	94.53	77.12	86.64	94.48	90.01	6.99
	IFN-γ+	0.00	0.00	0.00	0.00	0.69	0.79	3.03	0.34	1.01	0.83	1.12	0.96
$CD45RA^-$ CD8+	% CD8+	17.14	9.97	13.56	5.07	17.02	22.17	16.27	26.62	11.88	35.07	21.51	8.37
	IFN-γ+	0.00	0.00	0.00	0.00	0.38	0.46	3.45	0.12	0.70	0.00	0.85	1.29
	% CD8 + CM	87.86	87.29	87.58	0.40	86.52	65.72	76.30	88.00	90.01	42.50	74.84	18.28
	IFN-γ+	0.00	0.00	0.00	0.00	0.31	0.33	2.88	0.11	0.64	0.48	0.79	1.04
	% CD8 + EM	12.06	12.67	12.37	0.43	13.44	34.25	23.70	11.94	9.85	57.47	25.11	18.30
	IFN-γ+	0.00	0.00	0.00	0.00	0.00	0.79	5.58	0.00	0.00	0.00	1.06	2.23
$CD45RA^-$ CD4+	% CD4+	68.15	86.69	77.42	13.11	75.99	75.83	79.31	70.60	80.07	60.63	73.74	7.24
	IFN-γ+	0.00	0.00	0.00	0.00	0.81	0.91	2.93	0.43	1.11	1.32	1.25	0.87
	% CD4 + CM	89.09	81.50	85.30	5.37	70.76	86.05	90.34	82.88	91.94	90.78	85.46	7.96
	IFN-γ+	0.00	0.00	0.00	0.00	0.56	1.01	2.93	0.47	1.13	1.44	1.26	0.89
	% CD4 + EM	10.87	18.47	14.67	5.37	29.23	13.94	9.66	17.10	8.01	9.22	14.53	7.97
	IFN-γ+	0.00	0.00	0.00	0.00	1.37	0.39	3.17	0.21	1.35	0.98	1.25	1.06
CD45RA+	% CD45RA+	61.91	77.83	69.87	11.26	68.69	75.86	81.86	55.98	80.30	66.75	71.57	9.74
	IFN-γ+	0.00	0.02	0.01	0.00	0.67	0.93	6.47	0.10	0.52	0.32	1.50	2.45
CD45RA + CD3+	% CD3+	70.44	78.05	74.25	5.38	52.93	79.05	60.97	59.8	74.60	79.16	67.75	11.26
	IFN-γ+	0.02	0.01	0.02	0.00	0.40	0.27	1.25	0.06	0.23	0.17	0.40	0.43
CD45RA + CD8+	% CD8+	22.77	24.35	23.56	1.12	56.52	27.24	49.85	32.1	33.08	38.60	39.57	11.34
	IFN-γ+	0.00	0.00	0.00	0.00	0.13	0.46	1.69	0.09	0.41	0.21	0.50	0.60
	% CD8 + Naive	72.84	83.57	78.21	7.59	76.57	84.64	80.40	95.9	80.80	44.83	77.19	17.19
	IFN-γ+	0.00	0.00	0.00	0.00	0.17	0.22	0.51	0.10	0.29	0.15	0.24	0.15
	% CD8 + TEMRA	27.14	16.43	21.79	7.57	23.43	15.35	19.60	4.1	19.20	55.17	22.81	17.19
	IFN-γ+	0.00	0.00	0.00	0.00	0.07	1.81	7.02	0.00	1.37	0.29	1.76	2.68
CD45RA + CD4+	% CD4+	70.26	70.09	70.18	0.12	32.35	70.30	47.27	65.88	59.50	54.40	54.95	13.75
	IFN-γ+	0.00	0.00	0.00	0.00	0.47	0.14	0.55	0.06	0.22	0.09	0.26	0.21
	% CD4 + Naive	99.53	98.72	99.13	0.57	95.67	99.53	99.32	99.57	99.35	99.21	98.78	1.53
	IFN-γ+	0.00	0.00	0.00	0.00	0.21	0.13	0.52	0.07	0.21	0.09	0.21	0.16
	% CD4 + TEMRA	0.47	1.28	0.88	0.57	4.33	0.47	0.68	0.43	0.65	0.79	1.23	1.53
	IFN-γ+	0.00	0.00	0.00	0.00	5.77	0.00	2.30	0.00	0.00	0.00	1.35	2.35

The mean value after 5 h of exposure for the three SARS-CoV-2 peptides (M,N,S) is shown. SD, standard deviation; C, healthy controls; D, convalescent donors; Pepx3, mixture of the single SARS-Cov-2 peptides M,N,S.

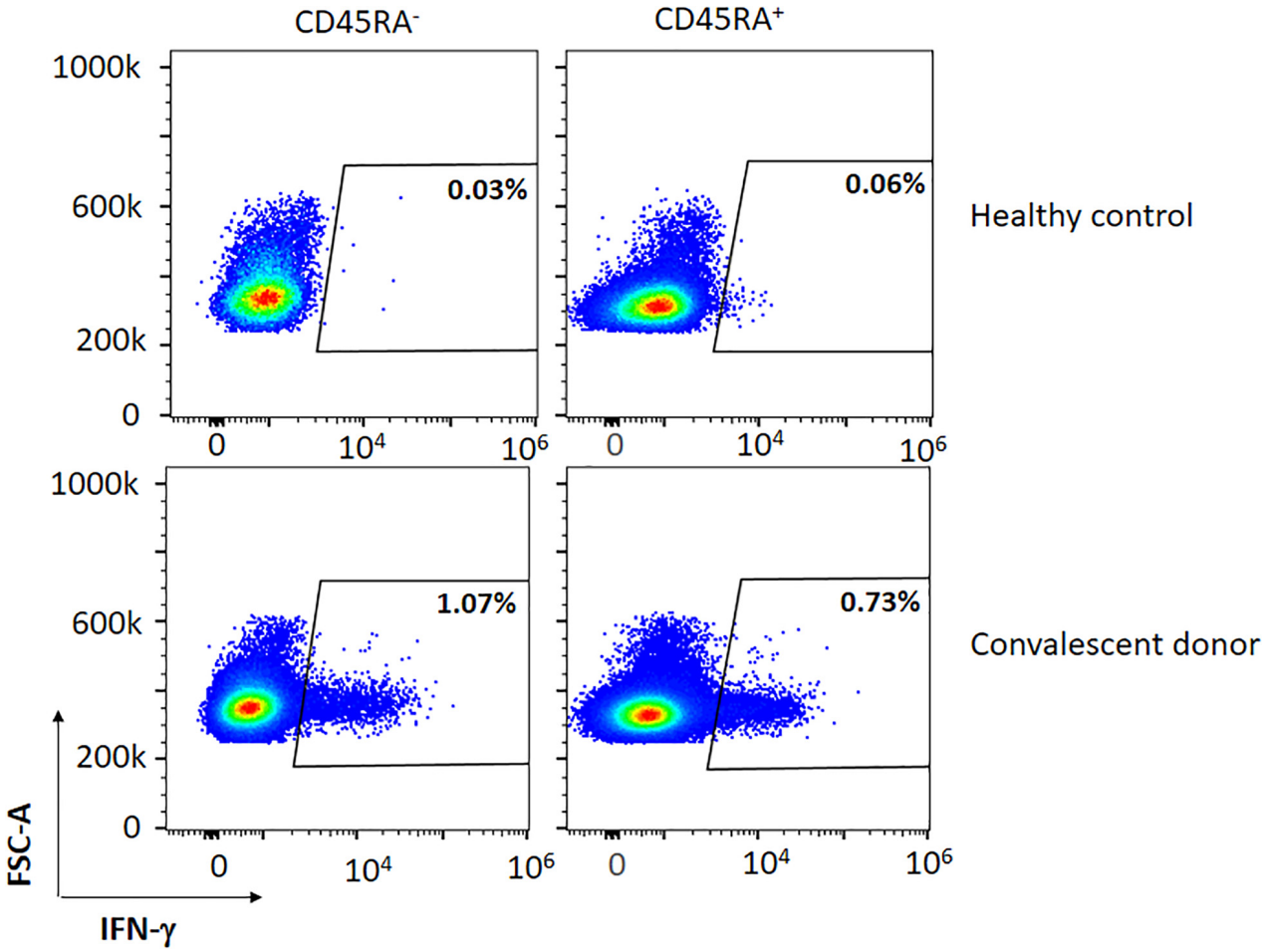

FIGURE 1 | Representative figure of the expression of IFN-γ in a convalescent and healthy donor within the CD45RA$^+$ and CD45RA$^-$ subpopulations after co-culture with the mixture of the three peptides (M, N, S).

expression within the CD45RA$^+$CD3$^+$ population was 0.40% (P = 0.065) (**Table 2**). We detected no IFN-γ expression in the healthy individuals. Despite the cohort's small size, we found no synergistic effect on the percentage of IFN-γ when the three peptides were mixed when compared with the single peptides (data not shown).

Identification of SARS-CoV-2-Specific CD4$^+$ T and CD8$^+$ T-Cell Responses Within CD45RA$^-$ Memory T Cells

We then sought to determine whether both CD8$^+$ and CD4$^+$ subsets contained specific SARS-CoV-2 T cells within the PBMCs of the convalescent donors. Among all subsets studied, we observed that CD45RA$^-$CD4$^+$ and CD45RA$^-$CD8$^+$ cells expressed 1.25 and 0.85% of IFN-γ, respectively (P = 0.132) (**Table 2**, **Figure 2**, and data not shown). Thus, all of the convalescent donors who recovered from COVID-19 generated CD4$^+$ T and CD8$^+$ T-cell responses against SARS-CoV-2 within the memory CD45RA$^-$ T-cell population.

We then analyzed the T central memory (CM) (CD45RA$^-$CD3$^+$CD27$^+$) and T effector memory (EM) (CD45RA$^-$CD3$^+$CD27$^-$) compartments. Although there were no significant differences, we observed responses to the SARS-CoV-2-specific peptides within all subpopulations. Within the CD4$^+$ and CD8$^+$ CM T-cell subsets, we detected a mean of 1.26 and 0.79% of IFN-γ$^+$ cells, respectively (P = 0.132). When examining the CD4$^+$ and CD8$^+$ EM T-cell subsets, we detected a mean of 1.25 and 1.06% of IFN-γ$^+$ cells, respectively (P = 0.108) (**Table 2** and **Figure 3**). These data demonstrate the presence of a population of memory T cells specific for SARS-CoV-2 within the CD45RA$^-$CD3$^+$ memory T cells.

Large-Scale CD45RA Depletion. Creation of an Off-the-Shelf Biobank of CD45RA$^-$ Memory T Cells From a COVID-19 Convalescent Donor

After depletion of the CD45RA$^+$ cells (as described in the Materials and Methods section), 99.8% of the cells were CD45RA$^-$CD3$^+$, and most of the CD45RO$^+$ cells were CD4$^+$

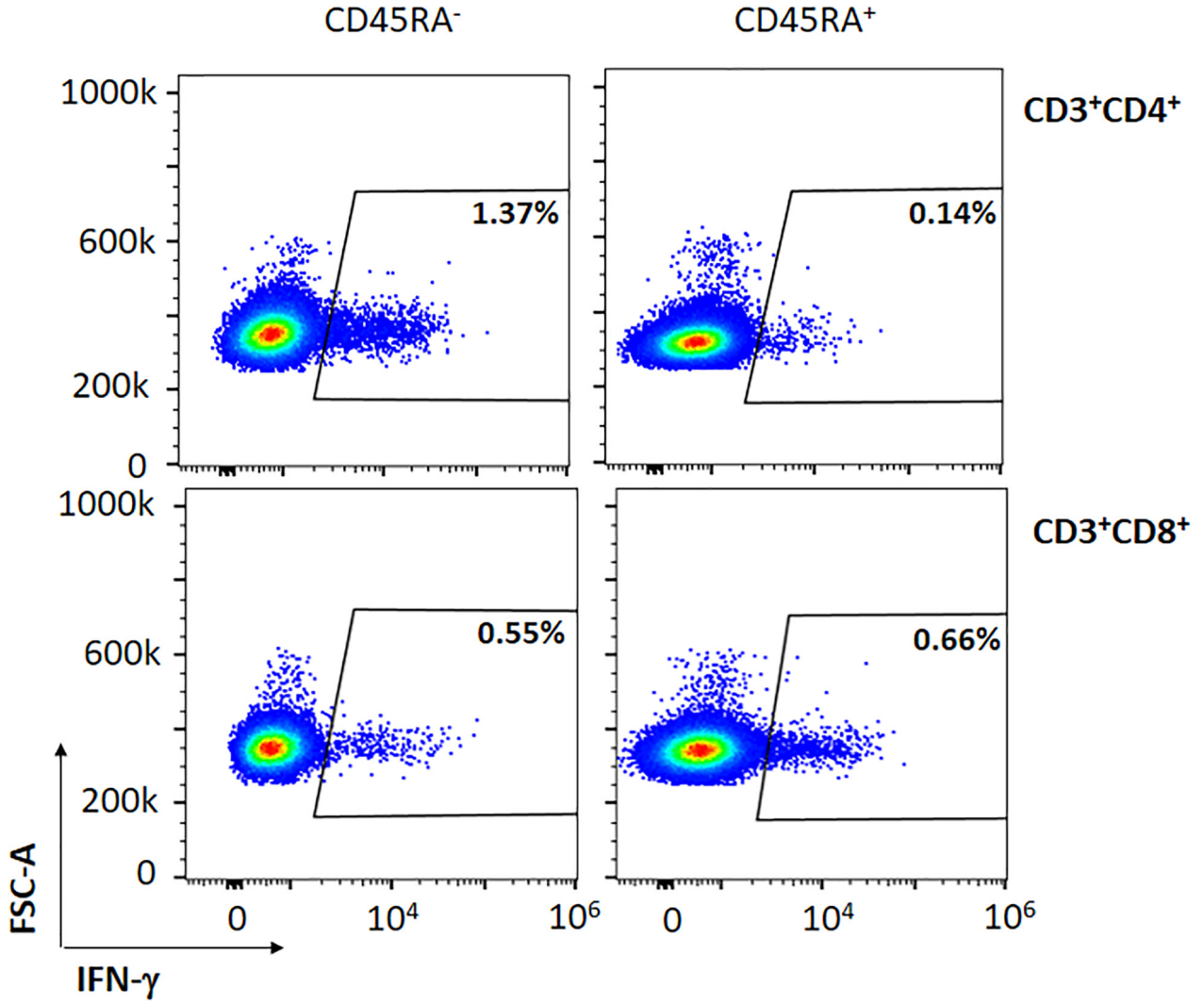

FIGURE 2 | Representative figure of the expression of IFN-γ in a convalescent donor within the CD45RA$^-$CD3$^+$CD4$^+$ and CD45RA$^-$CD3$^+$CD8$^+$ subpopulations after co-culture with the mixture of the three peptides (M, N, S).

with a high CD4/CD8 ratio. The viability of the cells after thawing was 98–99% (data not shown).

Identification of SARS-CoV-2-Specific CD4$^+$ T and CD8$^+$ T-Cell Responses Within CD45RA$^-$ Memory T Cells After CD45RA Depletion

After CD45RA depletion, the percentage of CD45RA$^-$CD3$^+$ cells were 80.05%. Within that population, 83.56% of the cells were CD4$^+$ and 14.37% were CD8$^+$ T cells. After exposure to the three SARS-CoV-2-specific peptides, the CD3$^+$, CD3$^+$CD4$^+$, and CD3$^+$CD8$^+$ subsets expressed IFN-γ at a rate of 0.36, 0.38, and 0.31%, respectively (**Table 3**).

Most of the CD4$^+$ and CD8$^+$ cells had a CM phenotype (89.1 and 61.6%, respectively). Both the CM and EM subpopulations expressed IFN-γ after exposure to the three peptides. Thus, we found that 0.38, 0.70, and 0.39% of the cells within the CD4$^+$ CM, CD8$^+$ CM, and CD4$^+$ EM subsets expressed IFN-γ, respectively. We found no specific IFN-γ expression within the CD8$^+$ EM subset (**Table 3**).

We then characterized in-depth the phenotypic expression of the activation, exhaustion, and Treg markers within the CD45RA$^-$ population of the convalescent donor. The percentage of Treg defined by CD127^{low}CD25$^+$ was 11.26%. We found that the CD45RA$^-$CD3$^+$ cells expressed the activation marker HLA-DR (19.40%) in both subsets of CD4$^+$ (16.70%) and CD8$^+$ cells (29.19%). After exposure to the three SARS-CoV-2-specific peptides, we observed that 0.93, 0.70, and 1.21% of the cells within the CD3$^+$HLA$^-$DR$^+$, CD4$^+$HLA$^-$DR$^+$, and CD8$^+$HLA-DR$^+$ populations expressed IFN-γ, respectively. The CD45RA$^-$CD3$^+$ cells also expressed the exhaustion marker NKG2A (1.88%), and this expression was 10.24% within the CD8$^+$ subset, whereas it was nearly undetectable in the CD4$^+$

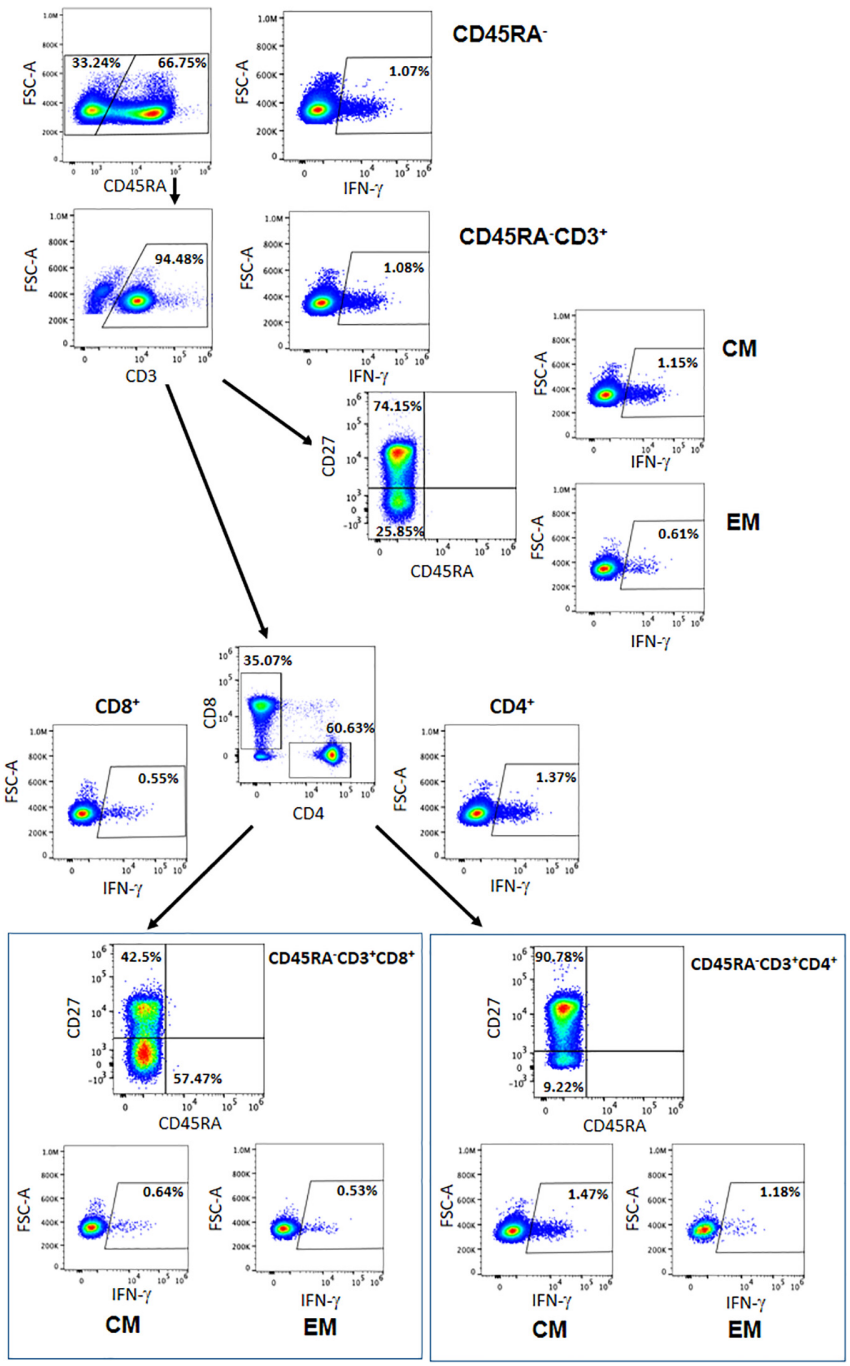

FIGURE 3 | Representative figure of the gating strategy of the different cells subsets within the CD45RA$^-$ population and the IFN-γ expressed. CM = central memory, EM = effector memory.

cells (0.14%). We found no IFN-γ expression within the NKG2A$^+$ population. In addition, 0.70% of the CD45RA$^-$CD3$^+$ cells expressed the exhaustion marker PD-1, and this expression was 1.43% within the CD4$^+$ subset and 0.13% within the CD8$^+$ cells. We found 0.62% of IFN-γ$^+$ cells within the CD45RA$^-$CD3$^+$CD4$^+$PD-1$^+$ population after exposure to the three peptides (**Table 3**).

CDR3 Use of TCR

The polyclonal distribution for both TCR-β and TCR-γ CDR3-encoding regions was almost identical among the controls and the CD45RA$^-$ population, whereas different oligoclonal fragments were observed in the CD45RA$^+$ population. Three of the oligoclonal fragments seen in the CD45RA$^+$ cells were also identified in the CD45RA$^-$ cells (**Figure 4**).

TABLE 3 | Phenotype of CD45RA memory T cells from a convalescent donor after large scale CD45RA depletion.

	Cell marker	% of cells after thawing	% IFN-γ^+	Fold change IL-15 (o/n)*	Fold changeIL-15 (72 h)**
$CD45RA^-$ subpopulation	$CD45RA^-$	99.85	0.36	1.00	0.99
	$CD45RA^-CD3^+$	80.05	0.36	1.05	0.99
	$CD45RA^-CD3^+CD4^+$	83.56	0.38	1.02	0.98
	$CD45RA^-CD3^+CD8^+$	14.37	0.31	0.84	1.10
	$CD45RA^-CD4^+$				
	$CD27^+$ (CM)	89.13	0.38	1.01	1.02
	$CD27^-$ (EM)	10.86	0.39	0.94	0.86
	$CD127^{low}CD25^+$ (Treg)	11.26	N/A	1.27	2.03
	$CD45RA^-$ $CD8^+$				
	$CD27^+$ (CM)	61.65	0.70	1.07	1.03
	$CD27^-$ (EM)	38.34	0.00	0.89	0.95
T-cell activation marker	$CD3^+$				
	HLA^-DR^+	19.40	0.93	1.15	2.63
	CD69 BV421 high	0.46	0.00	3.18	29.59
	$CD25^+$	60.68	N/A	1.50	8.52
	$CD4^+$				
	HLA-DR^+	16.70	0.70	1.16	2.42
	CD69 BV421 high	0.38	0.00	2.90	59.75
	$CD25^+$	66.10	N/A	1.48	8.58
	$CD8^+$				
	HLA-DR^+	29.19	1.21	1.15	1.89
	CD69 BV421 high	0.31	1.04	2.77	5.64
	$CD25^+$	9.60	N/A	4.42	36.42
T-cell exhaustion markers	$CD3^+$				
	$NKG2A^+$	1.88	0.00	0.73	1.39
	PD1 AF700	0.70	0.75	0.71	8.70
	$CD4^+$				
	$NKG2A^+$	0.14	0.00	1.24	0.56
	PD1 AF700	1.43	0.62	0.84	9.15
	$CD8^+$				
	$NKG2A^+$	10.24	0.00	0.76	1.22
	PD1 AF700	0.13	0.00	1.69	6.14
Chemokine receptor and integrin	$CD3^+$				
	CCR7	81.00	N/A	1.03	1.00
	CD103	2.18	N/A	0.74	1.24
	$CD4^+$				
	CCR7	88.13	N/A	1.00	0.97
	CD103	1.38	N/A	0.74	0.99
	$CD8^+$				
	CCR7	45.42	N/A	1.48	1.40
	CD103	5.96	N/A	0.89	1.39

**Fold change increase after overnight (o/n) treatment with IL-15. **Fold change increase after 72 h with IL-15. Fold change regarding the receptor expression has been calculated as the ratio between the value with IL-15 and the value from a control without IL-15. N/A,not available.*

Induction of an Activated Memory T-Cell Phenotype Within $CD45RA^-$ Memory T Cells After CD45RA Depletion

IL-15 is an essential cytokine for memory T cells that induces the activation, proliferation, and survival of T cells. After incubating the $CD45RA^-$ T cells with IL-15, we observed an increase in the activation markers HLA-DR, CD69, and CD25 after 72 h of incubation (2.63-fold, 29.59-fold, and 8.52-fold, respectively) when compared with the o/n incubation (1.15-fold, 3.18-fold, and 1.50-fold) (**Table 3**). The expression of the exhaustion markers NKG2A and PD-1 was also higher after 72 h of incubation (**Supplementary Figures 1–5**). We then examined the expression of chemokine CCR7 and integrin CD103, which are important for the homing of T-cells to the respiratory tract (Campbell et al., 2001; Uss et al., 2006). We observed that most of the $CD3^+CD4^+$ cells expressed $CCR7^+$ cells (88.13%), whereas the $CD3^+CD8^+$ subpopulation expressed 45.42% of $CCR7^+$ cells. As expected, CD103 expression was low in the peripheral blood (Uss et al., 2006). We detected an expression of 2.18, 5.96, and 1.38% in the

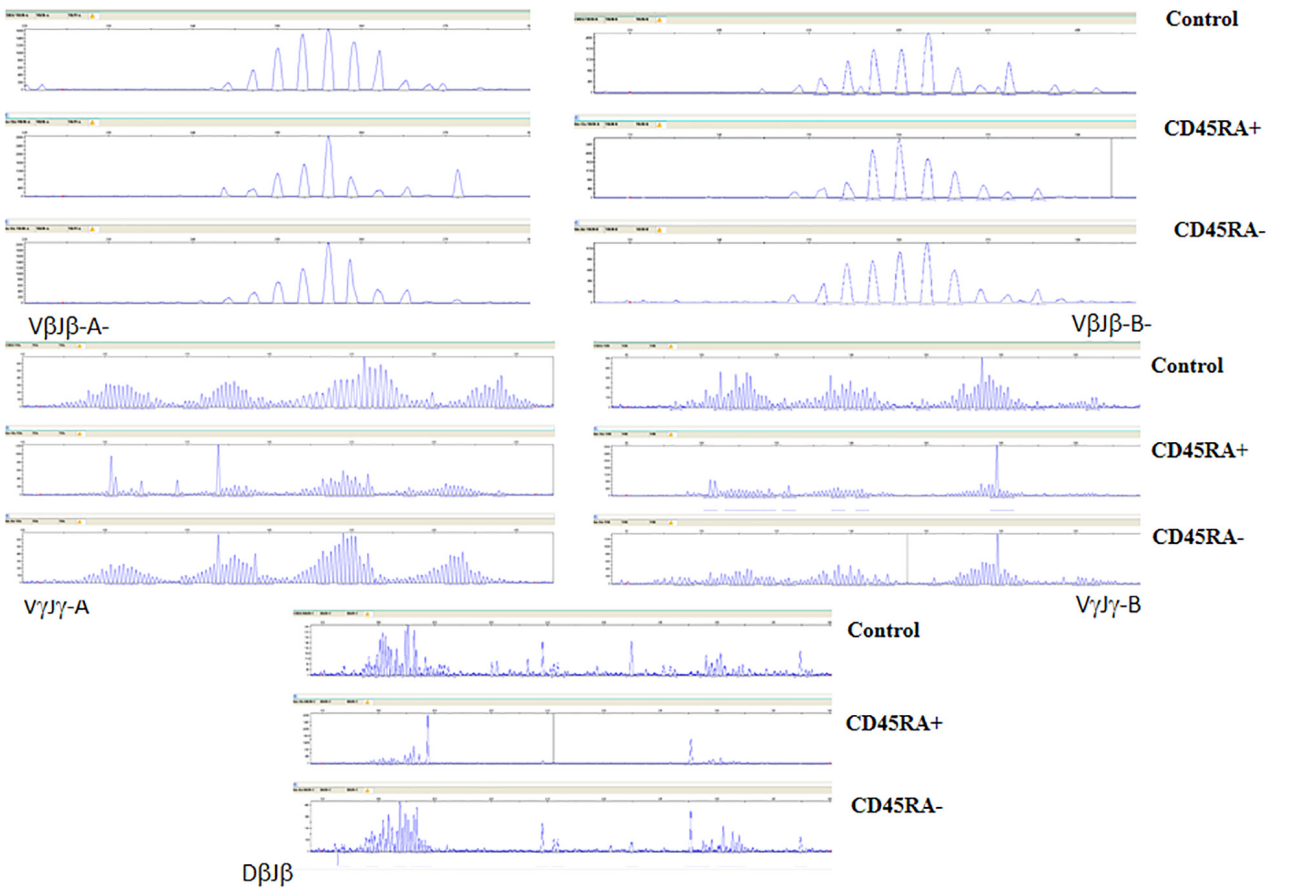

FIGURE 4 | TCR-β and -γ spectratyping for five multi-primer sets covering most of the CDR3-encoding regions. Electropherograms showing Gaussian distribution of polyclonal population (Control) is also mainly seen in CD45RA$^-$, with oligoclonal patterns in CD45RA$^+$. Some predominant fragments are shared in both convalescent donor populations.

CD3$^+$CD103$^+$, CD3$^+$CD8$^+$CD103$^+$, and CD3$^+$CD4$^+$CD103$^+$ compartments, respectively. Although the fold increase was not particularly remarkable in the CD45RA$^-$CD3$^+$ cells after 72 h of incubation (1.00-fold CCR7 and 1.24-fold CD103), the increase was higher (1.40) within the CD8$^+$ subpopulation (**Table 3** and **Supplementary Figures 6,7**).

DISCUSSION

In the absence of an effective vaccine and with the emergence of a second wave, there is an urgent need to find effective treatments for COVID-19. Here we report the presence of a SARS-CoV-2-specific T-cell population within the CD45RA$^-$ memory T cells of blood from convalescent donors. These cells can be easily, effectively, and rapidly isolated following a donor selection strategy based on IFN-γ expression after exposure with SARS-CoV-2-specific peptides and HLA antigen expression, thereby obtaining clinical-grade CD45RA$^-$ memory T cells from the blood of convalescent donors. These cells can be biobanked, thawed, and employed as a treatment for moderate to severe cases of COVID-19. These so-called "living drugs" retain the memory against SARS-CoV-2 and other pathogens the donors have encountered. Unlike plasma, where the concentration decreases after infusion, memory T cells expand and proliferate and should therefore have a more lasting effect.

In previous studies, this population of CD45RA$^-$ memory T cells showed no alloreactivity when compared with the CD45RA$^+$ counterpart (Fernández et al., 2017). These cells were mainly CD4+ (Fernández et al., 2019) and showed effectiveness against viral infections (Triplett et al., 2015). Phenotypically, we found that CD45RA$^-$ memory T cells were fully capable of producing IFN-γ in the presence of SARS-CoV-2-specific peptides. Both CD4$^+$ and CD8$^+$ CM and EM subsets were able to generate IFN-γ after exposure to the SARS-CoV-2 peptides, showing coverage of response. CD8$^+$ cytotoxic cells can kill virally infected cells by secreting cytokines; at the same time, CD4$^+$ T cells increase the ability of CD8$^+$ T cells to eliminate the virus. They have been shown to play an important role in controlling the viral replication of other viruses such as EBV and CMV (Juno et al., 2017). EM T cells are the first responders to infection, with a quick and strong response to pathogens, whereas CM T cells proliferate and create a new round of effector T cells (Pennock et al., 2013;

Farber et al., 2014). IL-15 is essential for the survival of memory $CD8^+$ and $CD4^+$ T-cell subsets, promoting the activation of $CD4^+$T cells, cytokine production and proliferation, and the maintenance of the memory population (Brincks and Woodland, 2010; Chen et al., 2014). After incubating the cells for 3 days with IL-15, we obtained a phenotype characteristic of an activated state, as shown by the fold increase in the activation markers HLA-DR, CD69, and CD25 and in the CCR7 and CD103 markers characteristic of the homing of T cells to the lymph nodes and mucosal tissues.

In our study, we observed no IFN-γ production by the SARS-CoV-2-specific T cells in healthy unexposed individuals, which agrees with the findings of Peng et al. (2020) but differs from other previously published data (Grifoni et al., 2020). This discrepancy could be due to the different detection methods employed and the small sample size.

Studies have shown the correlation between neutralizing antibodies and symptom severity, where antibody responses wane over time even in as short a period as 6–7 weeks after symptoms onset (Ibarrondo et al., 2020; Long et al., 2020; Seow et al., 2020). Importantly our data shows the presence of SARS-CoV-2 memory T cells in convalescent donors with mild symptoms, which has enormous implications for protection against further SARS-CoV-2 infections and in decreasing the severity of COVID-19. Further studies with larger cohorts are needed to determine the SARS-CoV-2 memory duration and thereby elucidate the long-term protection to SARS-CoV-2, as has been previously demonstrated for another coronavirus (Ng et al., 2016).

For proper T-cell recognition, both the donor and recipient need to share HLA alleles. Given the vast number of convalescent donors, finding the proper haploidentical donor based on HLA typing would not be difficult. Based on previously published data (Leung et al., 2020), this donor can cover around 93.6% of Spain's population. In accordance with the HLA donor-recipient match, we estimate that four donors will cover almost the whole of Spain's population (Leen et al., 2013; Leung et al., 2020). These cells are expected to remain in the patients until the host immune system is recovered. Previous experience with HSCT has shown that these cells can be detected in the host for weeks (Triplett et al., 2015). Besides data from the phase I clinical trial (unpublished) shows that we can detect donor chimerism for 3 weeks.

The procedure for obtaining the cells is easy to implement for small-scale manufacture, is quick and cost-effective, and involves minimal manipulation. AlsoCD45RA$^-$ memory T cell-based therapy is manufactured under the quality standards that apply to blood banks that perform HSCT daily with complex manipulations that are not considered advanced therapy medicinal product and can therefore be obtained without GMP condition requirements. The manufacturing of CD45RA$^-$ memory T cells is carried out in closed automated systems similar to clean rooms that guarantee an aseptic process for the administration to the patient. These factors make it feasible to create a biobank or stock from the blood of convalescent donors, which would be immediately available "off the shelf" for subsequent outbreaks, increasing the therapeutic options in the current SARS-CoV-2 pandemic. A clinical trial is currently assessing the safety of these cells for patients with moderate to severe COVID-19 (NCT04578210).

These cells could provide patients with (1) a pool of SARS-CoV-2-specific T cells that will respond quickly to the infection, (2) a pool of cells for patients with severe disease presenting with lymphopenia, and (3) a pool of specific memory T cells for other pathogens from the donors encountered during their life, which are vital for eliminating other secondary infections that usually develop in patients hospitalized with COVID-19 (Kim et al., 2020; Zhu et al., 2020).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Hospital Ramon y Cajal, Madrid, Spain. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CF, BS, and AP-M designed the study. CF, BP-M, and CM-D performed the *in vitro* experiments. JV, AB, and FG-S performed the HLA typing and TCR spectratyping. RD and AM performed the non-mobilized apheresis and cryopreservation of the cells. CM-C performed the statistics. CF, BP-M, BS, and AP-M wrote the first draft of the manuscript. All authors revised the manuscript, participated in the interpretation of the data and the approval and submission of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.620730/full#supplementary-material

Supplementary Figure 1 | HLD-DR expression in the CD45RA$^-$CD3$^+$, CD45RA$^-$CD3$^+$CD4$^+$, and CD45RA$^-$CD3$^+$CD8$^+$ populations from the convalescent donor. Cells were cultured with 50 ng/mL of IL-15 o/n and for 72 h.

Supplementary Figure 2 | CD69 expression in the CD45RA$^-$CD3$^+$, CD45RA$^-$CD3$^+$CD4$^+$, and CD45RA$^-$CD3$^+$CD8$^+$ populations from the convalescent donor. Cells were cultured with 50 ng/mL of IL-15 o/n and for 72 h.

Supplementary Figure 3 | CD25 expression in the $CD45RA^{-}CD3^{+}$, $CD45RA^{-}CD3^{+}CD4^{+}$, and $CD45RA^{-}CD3^{+}CD8^{+}$ populations from the convalescent donor. Cells were cultured with 50 ng/mL of IL-15 o/n and for 72 h.

Supplementary Figure 4 | NKG2A expression in the $CD45RA^{-}CD3^{+}$, $CD45RA^{-}CD3 + CD4^{+}$,and $CD45RA^{-}CD3^{+}CD8^{+}$ populations from the convalescent donor. Cells were cultured with 50 ng/mL of IL-15 o/n and for 72 h.

Supplementary Figure 5 | PD-1 expression in the $CD45RA^{-}CD3^{+}$, $CD45RA^{-}CD3^{+}CD4^{+}$, and $CD45RA^{-}CD3^{+}CD8^{+}$ populations from the convalescent donor. Cells were culture with 50 cultured/mL of IL-15 o/n and for 72 h.

Supplementary Figure 6 | CCR7 expression in the $CD45RA^{-}CD3^{+}$, $CD45RA^{-}CD3^{+}CD4^{+}$, and $CD45RA^{-}CD3^{+}CD8^{+}$ populations from the convalescent donor. Cells were cultured with 50 ng/mL of IL-15 o/n and for 72 h.

Supplementary Figure 7 | CD103 expression in the $CD45RA^{-}CD3^{+}$, $CD45RA^{-}CD3^{+}CD4^{+}$, and $CD45RA^{-}CD3^{+}CD8^{+}$ populations from the convalescent donor. Cells were cultured with 50 ng/mL of IL-15 o/n and for 72 h.

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HYPOTHESIS AND THEORY
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Targeted Mitochondrial Therapy With Over-Expressed MAVS Protein From Mesenchymal Stem Cells: A New Therapeutic Approach for COVID-19

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The SARS-CoV-2, the virus that causes COVID-19, has infected millions of people worldwide. The symptoms of this disease are primarily due to pulmonary involvement, uncontrolled tissue inflammation, and inadequate immune response against the invader virus. Impaired interferon (IFN) production is one of the leading causes of the immune system's inability to control the replication of the SARS-CoV-2. Mitochondria play an essential role in developing and maintaining innate cellular immunity and IFN production. Mitochondrial function is impaired during cellular stress, affecting cell bioenergy and innate immune responses. The mitochondrial antiviral-signaling protein (MAVS), located in the outer membrane of mitochondria, is one of the key elements in engaging the innate immune system and interferon production. Transferring healthy mitochondria to the damaged cells by mesenchymal stem cells (MSCs) is a proposed option for regenerative medicine and a viable treatment approach to many diseases. In addition to mitochondrial transport, these cells can regulate inflammation, repair the damaged tissue, and control the pathogenesis of COVID-19. The immune regulatory nature of MSCs dramatically reduces the probability of an immune rejection. In order to induce an appropriate immune response against the SARS-CoV-2, we hypothesize to donate mitochondria to the host cells of the virus. We consider MSCs as an appropriate biological carrier for mitochondria. Besides, enhancing the expression of MAVS protein in MSCs and promoting the expression of SARS-CoV-2 viral spike protein as a specific ligand for $ACE2^{+}$ cells will improve IFN production and innate immune responses in a targeted manner.

Keywords: COVID-19, mesenchymal stem cell, MAVS, S protein, SARS-CoV-2, mitochondria

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INTRODUCTION

The novel coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has caused a serious worldwide pandemic that has influenced different aspects of people's lives. The disease is rapidly spreading, with almost 130 million confirmed cases and 2.8 million deaths by the end of March 2021 (WHO Coronavirus (Covid-19) Dashboard, 2021). RNA-based next-generation sequencing and proteomics approaches have

revealed structural and non-structural proteins of the SARS-CoV-2. The S, N, M, and E genes encode structural proteins, while non-structural proteins are encoded by the open reading frame (ORF) regions (Huang et al., 2020). The virus enters the host cells by binding of its viral S protein to the angiotensin-converting enzyme 2 (ACE2) in different target organs, including the lung (type II surfactant-secreting alveolar cells), heart, kidney, brain, and intestine (Hoffmann et al., 2020). Initial manifestations of COVID-19 infection consist of fever, fatigue, myalgia, cough, dyspnea, and chest pain (Alimohamadi et al., 2020). Although most patients (85%) experience mild to moderate symptoms, some patients go through severe complications, including acute respiratory failure, acute respiratory distress syndrome (ARDS), septic shock, and acute cardiac injury (Dallan et al., 2020; Long et al., 2020). Despite the unclear underlying reasons for the life-threatening complications of COVID-19, severe inflammatory response due to high levels of pro-inflammatory mediators is theorized to be the leading cause of severe disease and death (Song et al., 2020). Following the identification and characterization of the SARS-CoV-2, a growing body of efforts has been made to suggest a curative approach for eliminating the complications of the disease. Considering the crucial role of the immune system in COVID-19 pathogenesis, targeting the cells and inflammatory mediators of the innate and adaptive immune system will provide interesting curative approaches to conquer the complications of the disease.

On the other hand, suggested therapeutic approaches should eliminate chronic and various complications of COVID-19, such as neurological, renal, cardiovascular, musculoskeletal, and long-term respiratory complications (Fraser, 2020; Kunutsor and Laukkanen, 2020; Heidarpour et al., 2021; Talasaz et al., 2021). Current treatments mainly consist of supportive care by invasive mechanical ventilation, antiviral drugs, and immunomodulatory agents (Rodríguez-Baño et al., 2021; Young et al., 2021). However, each of these therapies is only appropriate for a specific stage of the disease, and the effectiveness of these therapies is under question. Therefore, developing efficient therapies to control the COVID-19 complications is necessary.

SUPPORTING EVIDENCE FOR HYPOTHESIS

Interferons Impairment in COVID-19

Following cell infection by SARS-CoV-2, many immune and non-immune cells are activated in response to the virus, which altogether leads to the release of considerable amounts of inflammatory cytokines such as IL-1, IL-6, and TNF-α, which is called "cytokine storm." Ordinarily, it is expected that a well-coordinated immune response restricts the spread of the virus, whereas an excessive inflammatory response causes cytokine storm. It seems that this hyperinflammatory/immunodeficiency state is developed as a result of impaired innate immune response (Jamshidi et al., 2021). Type I interferons (IFNs), a group of innate immune cytokines, are secreted from virally infected cells and act on more than 7,000 genes that regulate critical cellular processes, including metabolism, survival, migration, and inhibition of virus replication and assembly (Wang and Fish, 2019). Many studies have proven the pivotal roles of IFNs in effective innate and adaptive immune responses against viral infections. In addition, the importance of IFNs in triggering antiviral immune responses during coronavirus infections is acknowledged (Lee and Ashkar, 2018; Banerjee et al., 2019).

It has been shown that severely impaired type I IFN response, marked as no IFN-β or low IFN-α, is the distinctive phenotype observed in critical COVID-19 patients. Impaired type I IFN response is associated with a higher viral load and uncontrolled inflammatory responses (Blanco-Melo et al., 2020; Hadjadj et al., 2020). In addition, some studies revealed that impaired IFN antiviral responses in the elderlies might emerge as a reason for poor prognosis in this group (Molony et al., 2018). Furthermore, the higher susceptibility of African-American populations to SARS-CoV-2 is attributed to the lower IFN-I production in response to the SARS-CoV-2 RNAs (Maiti, 2020).

In order to overcome impaired IFN-mediated immune response in COVID-19, previous studies proposed some solutions. For instance, it has been suggested to administer exogenous IFNs to improve the performance of the innate immune system. A retrospective cohort study in China revealed that early administration of inhaled aerosolized IFN-α2b results in lower death and shorter admission period. However, late administration of IFN-α2b is associated with the more extended admission period and higher mortality (Wang et al., 2020). In another study, forty-eight COVID-19 patients inhaled IFN-β1a, which led to a significant improvement on day 15 compared to the placebo group (Monk et al., 2021). Although administration of IFNs as an accepted therapeutic strategy improves immune responses, IFN therapy has faced controversial outcomes. It seems that the efficacy of administration of IFNs depends on the stage of the disease. In this line, using IFNs in the early stages generates desirable outcomes, whereas this strategy is ineffective in the severe or later stages of viral infection (Channappanavar et al., 2016).

As another solution, drug repurposing has been recently developed as an attractive topic due to lower costs and shorter timeline compared to standard drug discovery methods. Previously, it has been proposed that azithromycin, a macrolide antibiotic, can promote the response of type I IFN (IFN-β) and some virus recognition receptors in epithelial cells (Menzel et al., 2017; Li et al., 2019b). In this line, the antiviral efficacy of azithromycin on COVID-19 combined with hydroxychloroquine was showed *in vitro* (Damle et al., 2020). Trametinib, an anticancer drug, is another proposed drug against COVID-19, which acts via inhibition of mitogen-activated protein kinase (MEK) 1 and 2. It has been shown that MEK inhibitors triggered expression of some antiviral genes, including interferon regulatory factor 1 (IRF1), both at the mRNA and protein level, which results in improved IFN response (Lulli et al., 2017). However, drug repurposing is facing many challenges that may overweight its benefits. Serious adverse effects of some drugs, especially anticancer and immunomodulatory medications, lack of appropriate clinical trials and controversial reports regarding their effects are the main challenges.

It seems that targeting the IFN production pathway can improve the innate immune response, particularly in the early phases of COVID-19. It also prevents the pro-inflammatory overreactions or cytokine storm resulting from the rapid replication of SARS-CoV-2 in the absence of sufficient amounts of IFNs (Fung et al., 2020; Mozafari et al., 2020).

Mitochondrial-Mediated Impairment of the Immune Response in COVID-19

Different cellular organelles participate in coordinating the IFN production and innate immune responses. Mitochondria take part in regulating cell bioenergy, cell metabolism, apoptosis, and reactive oxygen species (ROS) production and consumption. Besides, mitochondria play an essential role in inducing innate antiviral immune reactions mainly through stimulating IFN production. Studies have shown that following mitochondrial dysfunction, endothelial cells release large amounts of pro-inflammatory mediators such as IL-1, IL-6, and TNF-alpha and enhance the expression of intercellular adhesion molecule-1 (ICAM-1) that cause monocyte infiltration and activation (Choi S.J. et al., 2018). In order to trigger innate immune responses, the pathogen-associated molecular patterns (PAMPs) are identified by pattern recognition receptors (PRRs) such as intercellular retinoic acid-inducible gene-I-like receptors (RLRs). There are various RLRs, such as melanoma differentiation-associated protein 5 (MDA5) and retinoic acid-inducible gene I (RIG-1), that are the principal activators of mitochondrial antiviral pathways. These receptors are expressed in both immune and non-immune cells and activate several pathways, including interferon production, after recognizing single-strand and double-strand RNAs of viruses (Banoth and Cassel, 2018).

It has been shown that 26 out of 29 viral proteins of the SARS-CoV-2 interact with a significant number of mitochondrial proteins that participate in critical cellular metabolic pathways. This study suggests that the SARS-CoV-2 infection could result in mitochondrial dysfunction (Gordon et al., 2020). Besides, mitochondrial dysfunction is related to the clinical evidence that some metabolic disorders such as obesity or diabetes exponentially increase severe complications and mortality risk of COVID-19 (Bertsimas et al., 2020). It has been indicated that immune responses are affected by sex in which women display more effective innate immune responses than men. The data is along with a much higher mortality rate of COVID-19 in men. It can be attributed to the exclusive natural selection of healthy mitochondria in females. In this manner, mitochondria with malfunctioning or mutations that can be harmful to the females are eliminated (Iessi et al., 2021). Taken together, it seems that mitochondrial dysfunction plays a critical role in severe complications of COVID-19.

It has been hypothesized that mitochondrial-targeted ubiquinone (MitoQ), a mitochondrial-targeted antioxidant, could play a potential role in COVID-19 treatment. The particular MitoQ structure promotes its targeted accumulation within the mitochondria and sequestering the ROS (Ouyang and Gong, 2020). However, eliminating ROS only affects the narrow aspect of mitochondrial dysfunction while the impairment of mitochondrion-mediated immune response is still not resolved.

MAVS as a Key Regulator of Innate Immune Response in COVID-19

Mitochondrial antiviral-signaling protein (MAVS), also known as IFN-β promoter stimulator I (IPS-1), is one of the essential messenger molecules for inducing the mitochondrial antiviral pathways, which was first described by Seth et al. in 2005 (Seth et al., 2005). MAVS consists of 540 amino acids and has three components, an N terminal caspase activation recruitment domain (CARD), a proline-rich domain, and a transmembrane C terminal domain (TM). Its gene is located on chromosome 20, and the protein itself is present in the outer membrane of the mitochondria (Wang et al., 2019). The binding of the virus antigens and RLRs complex to MAVS induces an innate immune response in the infected cells through two different mechanisms. The first mechanism is the activation of interferon regulatory factors (IRFs) and the nuclear factor kappa B (NF-κB) pathway that inhibits viral replication and assembly via enhancing the production of interferon-α and -β (Refolo et al., 2020). The second mechanism involves the activation of caspase-8 protein as a promoter of the internal pathway of apoptosis in the virus-infected cells that removes the infected cells and protects adjacent cells (El Maadidi et al., 2014).

The pivotal role of MAVS in IFN production makes it a valuable evading target for many viruses. In the coexistence of viruses and host cells, these pathogens have developed various strategies to conquer MAVS-mediated signaling. Cleavage of MAVS is one of the mechanisms involved in the dislocation of MAVS, resulting in the impairment of IFN-mediated immune response (Lu et al., 2020). For instance, it has been shown that different viruses such as cytomegalovirus (CMV), hepatitis C virus (HCV) and Zika virus can disrupt MAVS function and therefore impair IFN production (Choi H.J. et al., 2018; Ma et al., 2018; Yan et al., 2021). In this line, SARS-CoV-2 disrupts interferon response through damage to the MAVS protein, which leads to decreased expression of interferons in the early stages of infection and provides an excellent opportunity for the virus to multiply and spread (Lei et al., 2020). It is demonstrated that SARS-CoV-2 carries a protein gene called open reading frame-9b (ORF-9b) that inhibits the interferon response in the infected cells by inhibiting mitochondria and the MAVS signalosome. Additionally, it seems that disruption of mitochondrial energy production may induce lactic acid production and inhibit proper interferon response (Jiang et al., 2020). Besides, ORF-9b induces mitochondrial elongation by promoting ubiquitination and degradation of dynamin-like protein 1 (DNM1L) that participates in mitochondrial fission and maintenance (Shi et al., 2014).

It has been proposed to administer drugs that interact with ORFs and consequently prevent their role in virus replication and distribution. Regarding amino acid sequence searching, some chemical substances such as benzyl (2-oxopropyl) carbamate and 4-(dimethylamino) benzoic acid may prevent virus replication via targeting ORFs. However, they only inhibit a small range of viral

proteins, and their efficacy in the treatment of COVID-19 has not been evaluated yet (Chen and Zhong, 2020).

Mitochondrial Therapy and Mesenchymal Stem Cell

It is demonstrated that mitochondrial dysfunction plays a vital role in inhibiting antiviral response in the infected cells. It seems that restoring mitochondrial function and providing new healthy mitochondria could improve cell resistance against infection-related stress through regulating the bioenergy and innate immune response of the affected cells (Li et al., 2019a).

Some studies have suggested that mesenchymal stem cells (MSCs) are a viable option to transfer healthy mitochondria to infected cells. Mesenchymal stem cells are a group of non-hematopoietic stem cells that originate from a variety of adult tissues. Some of the characteristics of these cells, such as their ability to regenerate tissues, anti-inflammatory effects, immune-evading properties, and interaction with various intracellular or extracellular pathways, make them a new treatment for a variety of pathological conditions (Babajani et al., 2020). Mitochondrial transfer using the MSCs has been broadly evaluated in various organs, including cardiovascular, neurological, renal, and respiratory systems. MSCs have been utilized in ischemic vascular diseases to save human umbilical vein endothelial cells (HUVECs) with mitochondrial dysfunction. The mitochondrial donation recovered aerobic respiration and reduced apoptosis of ischemic endothelial cells (Liu et al., 2014). It is reported that mitochondria can move from the MSCs to neural cells and recover the bioenergetics and proliferation of the recipient cells (Babenko et al., 2018). It has been shown that mitochondrial transfer from MSCs to the respiratory epithelial cells of the asthma model can reduce epithelial cell apoptosis by reinstating mitochondrial function and regulating inflammation (Yao et al., 2018). In another study, transferring mitochondria to the stem cells improved oxidative phosphorylation and ATP production, resulting in increased proliferation, migration, and differentiation (Guo et al., 2020). Treatment of damaged renal cells with MSCs diminished ROS levels, downregulated mitochondrial apoptosis-related proteins and reduced cell apoptosis, shedding light on the beneficial effects of MSCs in regulating mitochondrial respiratory function (Geng et al., 2017). It was indicated that effective mitochondrial transfer is achieved by forming tunneling nanotubes (TNTs) between MSCs and the recipient cells, which allows direct intercellular communication (Jiang et al., 2016).

On the other hand, the possible benefits of MSCs administration in the treatment of COVID-19 have been widely studied in recent months (Rajarshi et al., 2020). MSCs reduce the complications of COVID-19 by affecting the pathological mechanisms of the disease. MSCs participate in immune regulation by modulating the cellular immunity and cytokine responses such as M1 to M2 macrophage alteration and activation of T reg cells (Zhang et al., 2019). They also enhance tissue repair in two different manners; first, by the paracrine effect on host cells of the tissue and second by differentiating to replace damaged cells (Li et al., 2017; Westhauser et al., 2017). Additionally, they regulate the function of the renin-angiotensin-aldosterone system (RAAS) by reducing angiotensin II accumulation in the alveoli. Angiotensin II plays an essential role in developing pulmonary fibrosis following the viral infection (Chen and Chou, 2018). MSCs increase the fluid clearance of alveoli and regulate the coagulation process, which reduces the chance of disseminated intravascular coagulation (DIC) and thrombosis (Wang et al., 2012).

HYPOTHESIS

We suggest that the *in vivo* transferring of mitochondria from manipulated MSCs that express viral S protein and have overexpressed MAVS protein could enhance the innate immune response and appropriate IFN production in a targeted manner. This hypothesis consists of three steps:

(1) It is possible to improve innate immune response by donating mitochondria with overexpressed MAVS protein as a crucial initiator of the immune response of the cell against the virus. Enhancing MAVS expression can compensate for the destructive effects of viral proteins such as ORF-9b on immune-related proteins of mitochondria, which results in IFN production in its due time. The appropriate action of mitochondria produces sufficient amounts of IFNs as a crucial innate immunity arm that inhibits replication and assembly of new viruses. It also eliminates damaged and infected cells by promoting apoptosis that limits infection. The proposed roles of donated mitochondria with over-expressed MAVS to the type II pneumocytes are shown in **Figure 1**.

(2) It is possible to utilize MSCs as a biological carrier for mitochondria to donate these organelles to the infected and damaged cells in COVID-19 cases. The immunomodulatory functions of MSCs enable us to transport considerable amounts of mitochondria to the damaged organs in an immune reaction-free condition. In addition to the carrier role of MSCs, these cells can provide extra benefits due to their inherent characteristics, such as immunomodulatory effect by controlling cytokine release, regulating RAAS function, increasing alveolar fluid clearance, and decreasing the chance of hypercoagulation (Jamshidi et al., 2021). The roles of MSCs in eliminating COVID-19 complications are shown in **Figure 2**.

(3) In order to make the donation process more targeted, we can use ACE2 specific ligands to target susceptible cells for infection. Viral S protein seems to be the best option for administration as a specific ACE2 ligand. The SARS-CoV-2 S protein is preserved among all human coronaviruses and participates in the virus recognition, attachment, and entry into the susceptible cells (Huang et al., 2020). Using S protein enables us to target all possible SARS-CoV-2 hosts ($ACE2^{+}$ cells) in various parts of the body, such as the lungs, oral mucosa, GI tract, kidney, and heart (Xu et al., 2020). Besides, SARS-CoV-2 S protein induces antibody production that brings about vaccine-like

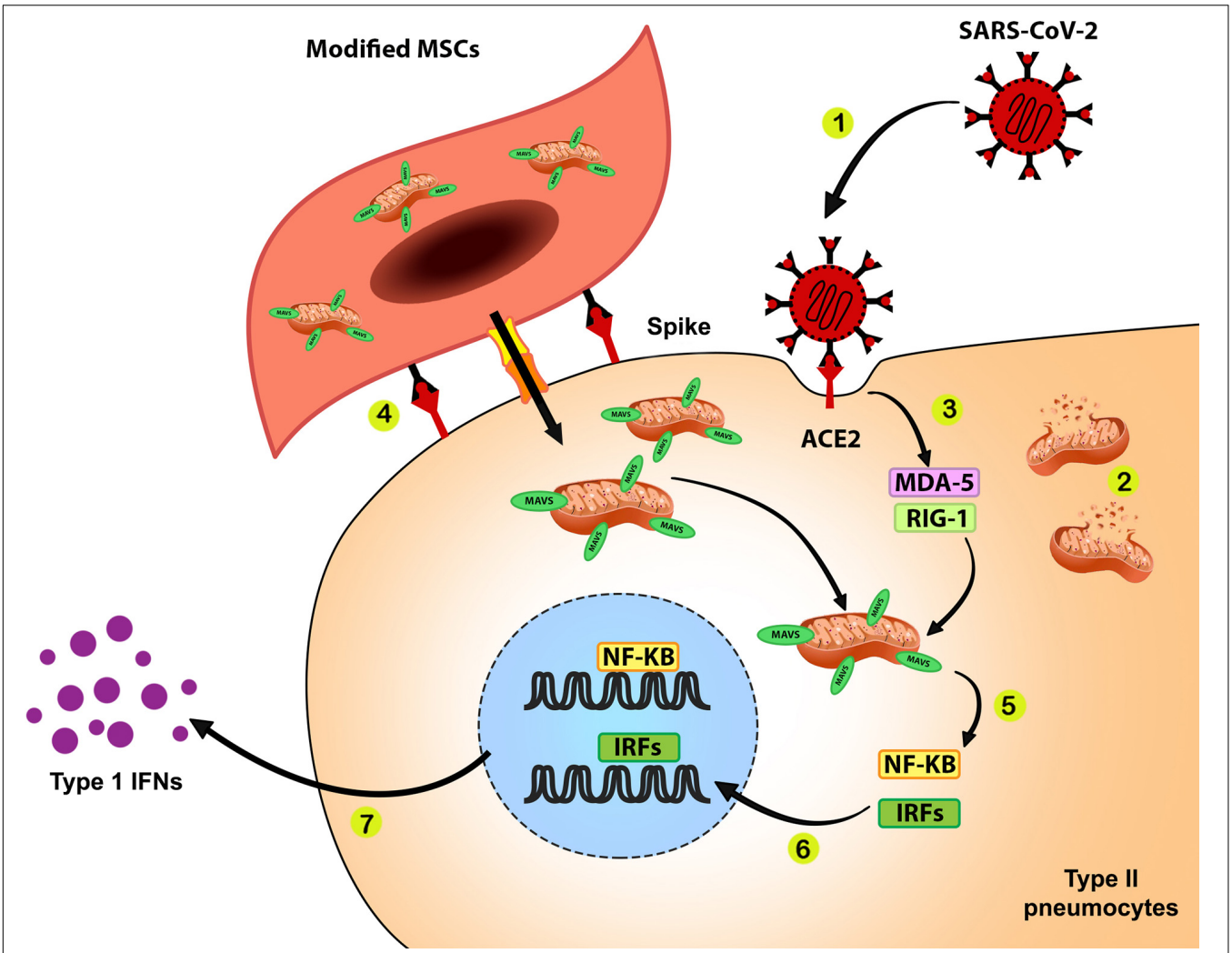

FIGURE 1 | Improving anti-SARS-CoV-2 response during mitochondrial therapy by donating mitochondria with over-expressed MAVS. **1.** SARS-CoV-2 infects ACE2 expressing cells such as surfactant-secreting type II alveolar cells via interaction between the viral spike protein and ACE2. **2.** Cell infection with SARS-CoV-2 results in mitochondrial dysfunction and disturbances of bioenergetics and innate immune response in infected cells. **3.** The RNA content of SARS-CoV-2 is recognized by pattern recognition receptors such as MDA-5 and RIG-1. **4.** MSCs that express surface spike protein as ACE2 ligand and containing mitochondria with overexpressed MAVS transfer these modified mitochondria to the surfactant-secreting type II alveolar cells. **5.** Binding of activated MDA-5 and RIG-1 to the MAVS on the outer membrane of healthy donated mitochondria would activate NF-κB and IRF transcription factors. **6.** NF-κB and IRF translocate into the cell nucleus and upregulate genes related to the innate immune response against SARS-CoV-2. **7.** Function of NF-κB and IRF will lead to the production of IFNs, which play a pivotal role in the antiviral response.

effects. It has been shown that the administration of viral S protein can provoke an immune response and produce anti-spike IgG (Keech et al., 2020). In another study, injection of manipulated MSCs, which expressed surface viral S proteins, into an animal model showed an effective antibody production (Liu et al., 2020).

EVALUATION OF THE HYPOTHESIS

This hypothesis can be evaluated by conducting *in vitro* and *in vivo* experimental studies to address the hypothesis's building block questions.

In vitro Studies

The viral vector containing the SARS-CoV-2 S protein gene will be transduced to MSCs using lentivirus transduction based on previous protocols (Meyerrose et al., 2008). Utilizing lentivirus vectors enables MSCs to produce S protein for a long period which raises the chance of targeted therapy of susceptible cells. The transduction success rate should be measured by GFP-labeled vectors. Administration of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay is recommended to evaluate MSCs viability after cell manipulation. Spike protein expression on the surface of modified MSCs can be studied by using specific antibodies against the spike protein and the flow cytometry method.

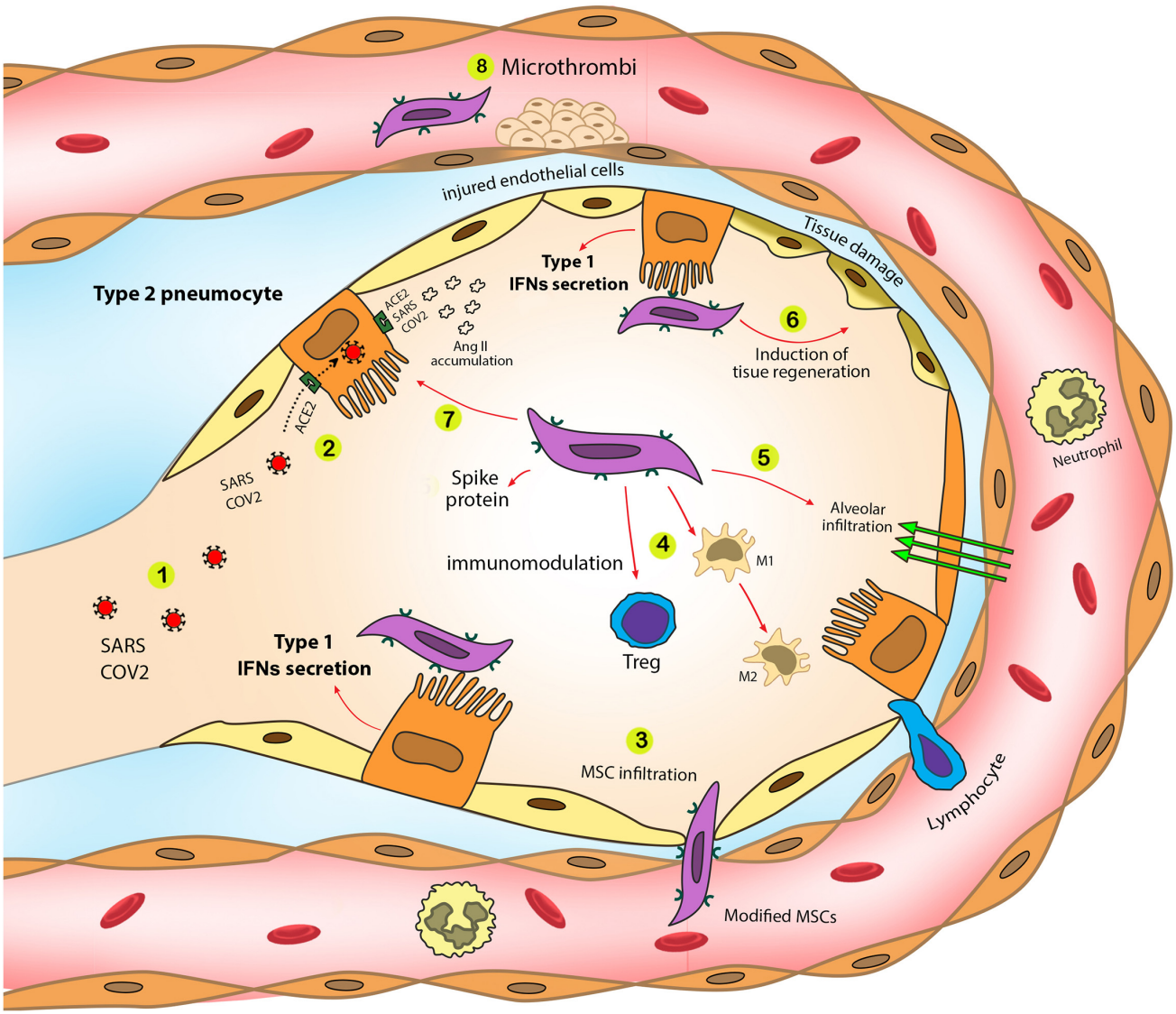

FIGURE 2 | SARS-CoV-2 pathogenic effects and the role of modified MSCs in the eradication of SARS-CoV-2 complications. Modified MSCs trigger type 1 IFN production in a targeted manner. Additionally, utilizing MSCs as mitochondrial carrier eliminates various COVID-19 complications and synergically improve mitochondrial therapy in COVID-19. **1.** SARS-CoV-2 enters alveoli through airways. **2.** SARS-CoV-2 infects type II pneumocytes through the ACE2 receptor. **3.** Modified MSCs entrap in the lungs and infiltrate into the alveoli. **4.** MSCs induce immunomodulatory effects that control cytokine storm during severe stages of COVID-19. Modified MSCs increase Treg cell activity and M1 to M2 macrophage switch, diminishing inflammatory mediator production by M1 macrophages. **5.** Modified MSCs increase fluid clearance exudation from infected alveoli. **6.** Modified MSCs can participate in tissue regeneration to eliminate chronic fibrosis due to COVID-19. **7.** SARS-CoV-2 infection results in angiotensin II accumulation and following lung fibrosis. MSCs can modulate in angiotensin II production that reduces lung fibrosis. **8.** MSCs modulate coagulopathies during COVID-19.

In order to enhance IFN production, the spike protein-expressing MSCs can be transfected with MAVS gene-containing plasmid by electroporation protocol based on previous protocols (Peister et al., 2004). The transfection success rate could be measured by GFP-labeled vectors, and MSCs viability after the manipulation should be assessed by MTT assay. Western blotting on cell lysates would evaluate MAVS expression and successful translation after plasmid transfection. Determining the subcellular localization of MAVS and accurate translocation of produced MAVS to the cell mitochondria is critical in this study. It has been shown that MAVS is localized in the mitochondrial outer membrane, and MAVS's mislocalization abolishes its activity (Seth et al., 2005). Evaluation of MAVS protein translocation to the mitochondrial outer membrane should be done by utilizing immunofluorescence assay and fluorescent-labeled antibodies for MAVS protein and mitochondrial outer membrane described by previous studies (Vazquez et al., 2017).

It is expected that MSCs transfer their mitochondria to the $ACE2^{+}$ cells. Thus, mitochondrial transfer from modified MSCs to the infected surfactant-secreting type II alveolar cells

should be evaluated using fluorescence labeling of mitochondria and FACS method after *in vitro* coculture of modified MSCs and SARS-Cov-2 infected type II pneumocytes. It is expected that mitochondrial transfer to the infected cells results in four changes: (1) reduction of oxidative stress, (2) enhancement of IFN production, (3) decreasing the apoptosis rate in the initial stages of SARS-CoV-2 infection, and (4) reduction of viral load in infected surfactant-secreting type II alveolar cells. IFN production can be measured by using the ELISA method. To assess the effects of mitochondrial transfer and improved IFN production on apoptosis of type II pneumocytes, annexin V/PI staining and flow cytometry is recommended. Viral replication in type II pneumocytes is expected to be interrupted after the intervention. Therefore, viral load should be measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

In vivo Studies

In vivo studies could include evaluating spike$^+$ MSCs distribution within the animal body, histopathological changes of the target organs of SARS-CoV-2, and measuring anti-SARS-CoV-2 IgM and IgG after the intervention. The animal model should be susceptible to SARS-CoV-2 infection with a sensitized respiratory tract. It is possible to transduce the model with adenovirus or adeno-associated virus that expresses human ACE2 protein (Muñoz-Fontela et al., 2020).

In order to assess spike$^+$ MSCs distribution in the body of the COVID-19 animal model, MSCs will be labeled with ultrasmall superparamagnetic iron-oxide nanoparticles (USPIO), and these cells could be tracked using MRI until 21 days after injection to the COVID-19 animal model (Crabbe et al., 2010). Histopathological changes of the lungs should be examined for inflammatory cell infiltration and fibrin deposition in the lung tissue of the COVID-19 animal model in treated and untreated groups at days 0, 2, 7, 10, 14, and 21 after the treatment by H&E staining and light microscopy. These changes should be compared with the untreated COVID-19 animal model. Anti-spike protein antibody titers would also be compared in treated and untreated groups. For this purpose, 20 days after the treatment, the blood samples will be obtained from both groups, and anti-spike antibody titers will be measured using ELISA.

DISCUSSION AND FUTURE DIRECTION

Most complications of COVID-19 come from the impaired innate and adaptive immune responses against SARS-CoV-2. This immunodeficiency/hyperinflammation state results in high viral load and cytokine storm. Considering the crucial role of mitochondria in inducing innate and adaptive immune responses by promoting IFN production and activating antiviral pathways, we suggested that transferring healthy mitochondria will improve innate immune response and prevent the distribution of the infection in the body. Besides, healthy mitochondria will improve cellular bioenergy that is impaired during metabolic syndrome and infection. In order to boost mitochondrial-mediated immune response, we suggest increasing the expression of mitochondrial antiviral-signaling protein (MAVS) protein as a pivotal player of the cellular innate immune response.

Targeted therapy of COVID-19 provides several benefits, including targeted mitochondria delivery to eligible cells, reduced side effects of direct mitochondria application, and a lesser number of administered mitochondria needed. It has been shown that the administration of mitochondria without any carrier causes inflammation. Considering the bacterial origin of mitochondria, the application of uncovered mitochondrial DNA, which is similar to bacterial DNA, induces toll-like receptor-9 (TLR9)-mediated inflammatory responses (Oka et al., 2012). Thus, designing appropriate carriers with the ability of targeted therapy improves the outcome. MSCs have been administered for targeted therapy in different pathological situations. These cells are immune evasive, which results in reducing the chance of immune rejection (Babajani et al., 2020). Additionally, MSCs and their exosomes possess innate therapeutic effects in different conditions, including cancer and COVID-19 (Golchin et al., 2020). The safety of clinical administration of MSCs was approved by many clinical studies (Thompson et al., 2020), and a growing body of studies are applying MSCs from different sources for the treatment of COVID-19 (available on www.clinicaltrials.gov). Additionally, it seems that expressing S protein, a ligand for ACE2, on the surface of MSCs will make the delivery of mitochondria more targeted. Besides, viral S protein might promote antibody production that induces vaccine-like effects (Kuate et al., 2007). Taken together, the anti-inflammatory features of spike expressing MSCs, along with their ability to eliminate complications of COVID-19, could make them valuable mitochondrial-targeted carriers for prevention and therapy in COVID-19.

Utilizing non-cellular or cellular carriers may provide some advantages and disadvantages. However, selecting a semi-biologic carrier that offers both cellular and non-cellular cargos benefits is the optimal aim in targeted mitochondrial therapy. As a miniature copy of the original cell, exosomes possess various beneficial functions of MSCs, including immunomodulation and regeneration (Jafari et al., 2021). Exosomes can be found in the cellular secretions that regulate several intercellular communications. Intercellular transfer of exosome-mediated mitochondria is reported in some studies (Maremanda et al., 2019). It has been reported that exosomes with encapsulated mitochondria can transfer these organelles from myeloid-derived regulatory cells (MDRCs) to CD4$^+$ T cells that resulted in alteration of pro-inflammatory function, bioenergetic, T cell differentiation and signaling of mitochondria in chronic inflammatory diseases (Hough et al., 2018). Administration of exosomes would provide some advantages over cells, including circumventing invasive cell harvesting, controlling dosage and potency, supply storable and easy-accessible sources, and extra stability (Yin et al., 2019). Thus, exosomes can be the future options for mitochondrial transfer-based therapies.

To the best of our knowledge, no studies have focused on administering MAVS overexpressed mitochondrial therapy to treat COVID-19. Therefore, it is essential to investigate the potential therapeutic effects of mitochondrial therapy for COVID-19.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

AB, PH-M, SA, and HN: conceptualization. AB, PH-M, and SA: investigation. AB, PH-M, and EJ: writing—original draft preparation. AB, PH-M, EJ, and HN: writing—review and editing. HN: supervision. All authors read and agreed to the submitted version of the manuscript.

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COVID-19 Pathology on Various Organs and Regenerative Medicine and Stem Cell-Based Interventions

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Severe acute respiratory syndrome-coronavirus 2, a novel betacoronavirus, has caused the global outbreak of a contagious infection named coronavirus disease-2019. Severely ill subjects have shown higher levels of pro-inflammatory cytokines. Cytokine storm is the term that can be used for a systemic inflammation leading to the production of inflammatory cytokines and activation of immune cells. In coronavirus disease-2019 infection, a cytokine storm contributes to the mortality rate of the disease and can lead to multiple-organ dysfunction syndrome through auto-destructive responses of systemic inflammation. Direct effects of the severe acute respiratory syndrome associated with infection as well as hyperinflammatory reactions are in association with disease complications. Besides acute respiratory distress syndrome, functional impairments of the cardiovascular system, central nervous system, kidneys, liver, and several others can be mentioned as the possible consequences. In addition to the current therapeutic approaches for coronavirus disease-2019, which are mostly supportive, stem cell-based therapies have shown the capacity for controlling the inflammation and attenuating the cytokine storm. Therefore, after a brief review of novel coronavirus characteristics, this review aims to explain the effects of coronavirus disease-2019 cytokine storm on different organs of the human body. The roles of stem cell-based therapies on attenuating cytokine release syndrome are also stated.

Keywords: coronavirus disease 2019, cytokine storm, mesenchymal stem cells, multi-organ failure, severe acute respiratory syndrome-coronavirus 2

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INTRODUCTION

In certain pathological conditions, such as a viral infection, the immune system may overproduce inflammatory cytokines. This situation sometimes can result in organ failure and death, known as a "cytokine storm." Today, the cytokine storm has drawn further consideration because of the new pandemic disease related to the novel coronavirus [severe acute respiratory syndrome- coronavirus 2 (SARS-CoV-2)] or coronavirus disease 2019 (COVID-19) (Tisoncik et al., 2012; Cena and Chieppa, 2020; Fajgenbaum and June, 2020; Puelles et al., 2020; Song et al., 2020). Herein, COVID-19 has warned us of the essential role of high host immunity and the detrimental outcomes of immune dysregulation. Indeed, the cytokine storm in COVID-19 is known to be one of the key causes of multiple-organ dysfunction syndrome (MODS) or multi-organ failure (MOF) as a hallmark of COVID-19 severity (Wang and Ma, 2008; Zaim et al., 2020; Kim et al., 2021). Many elderly individuals and those with comorbidities are more prone to developing a cytokine storm and dysfunctional immune reactions (Ciabattini et al., 2020; Nidadavolu and Walston, 2020; Tay et al., 2020). In severe cases, the involvement of assorted organs finally ends up in protracting the hospitalization time and raising the share of mortality (Rieg et al., 2020). Acute lung failure, acute kidney damage, acute liver failure, cardiovascular disease, and a broad range of hematological anomalies along with neurological disorders are characterized by MOF and MODS. Accordingly, since the MOF and MODS in COVID-19 subjects are important health issues, developing the application of modern therapeutic solutions can lead to ameliorate results and reduce mortality rates (Mokhtari et al., 2020; Sherren et al., 2020). Nowadays, cell therapy and regenerative medicine as one of the assuring and modern therapies have been able to promote the function of organs involved in various disorders and trigger their real healing reactions. Hereupon, different cells, specifically various stem cells, can be applied (Wong et al., 2013; Monsel et al., 2014; Mao and Mooney, 2015; Goodarzi et al., 2019). Therefore, the purpose of the current review is to highlight the effects of cytokine storms on different organs in COVID-19 individuals and how stem cells manage this phenomenon.

CHARACTERISTICS OF NOVEL CORONAVIRUS AND CORONAVIRUS DISEASE 2019

Since December 2019, SARS-CoV-2 infection has led to a highly contagious disease named COVID-19 (Hu et al., 2020; Sun P. et al., 2020; Yang et al., 2020). Coronaviruses, as a member of the Coronavirinae subfamily (in the Coronaviridae family), are positive-sense, single-stranded, non-segmented, and enveloped RNA viruses (Arjmand et al., 2020; Harrison et al., 2020; Zamanian Azodi et al., 2020). This novel betacoronavirus has been shown 79% of genome sequence identity to the previous betacoronaviruses, including severe acute respiratory syndrome-CoV (SARS-CoV) and Middle East respiratory syndrome-CoV (MERS-CoV), which also caused fatal respiratory illnesses (Hu et al., 2020). The genome sequence of SARS-CoV-2 contains 14 open reading frames (ORFs) that 16 non-structural proteins (nsp) are encoded by two-thirds of that. Moreover, nine accessory proteins (ORF) plus four structural proteins are encoded by the remaining one-third (Harrison et al., 2020). Transmembrane spike (s) glycoprotein mediates the coronavirus entry, which is mentioned to be the main antibody target (Walls et al., 2020). Indeed, each S protein of SARS-CoV-2 has two subunits, S1 and S2 domains. Receptor-binding domain (within the S1 domain) is used by the virus to bind to the angiotensin-converting enzyme 2 (ACE2) as the cellular receptor. It could also promote the effects of transmembrane protease serine type 2 (TMPRSS2) on cleaving S protein (Dong et al., 2020). After binding, viral–host cell membrane fusion is activated leading to the release of viral RNA into the cytoplasm (Ni et al., 2020). ACE2 and TMPRSS2 can be expressed by different organs and tissues in addition to the lungs including the heart, kidney, colon, esophagus, liver, brain, testis, and gallbladder, which suggests the extrapulmonary effects of SARS-CoV-2 (Dong et al., 2020). On the other hand, COVID-19, according to its severity, can be classified into four types, namely, mild, moderate, severe, and critical, with different manifestations in each group. Herein, fever, fatigue, dry cough, and diarrhea are mentioned as the most common symptoms (Wang Y. et al., 2020). Upper respiratory tract-related symptoms can be seen in the mild form of the disease. Moderate patients also have cough, shortness of breath, and tachypnea symptoms with no severe form of symptoms. Acute respiratory distress syndrome (ARDS), septic shock, sepsis, severe dyspnea, and tachypnea are the signs and symptoms of severe pneumonia seen in the severe form of the disease. Moreover, in some of the patients, a critical disease can be developed with the manifestations of respiratory failure, septic shock, and MODS or MOF (Hassan et al., 2020). Droplet and human-to-human transmission as the direct ways and contaminated objects/airborne contagion as indirect means are the ways that SARS-CoV-2 can spread (Lotfi et al., 2020). Herein, the incubation period of COVID-19 ranges from 0 to 24 days, which is averagely estimated at around 6.4 days (Wang Y. et al., 2020). The most common radiological characteristics of COVID-19 are ground-glass opacities in the lungs, patchy consolidation, interlobular involvement, and alveolar exudates (Sahin et al., 2020). Moreover, laboratory studies have shown increased lactate dehydrogenase, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), total bilirubin levels, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) as well as higher creatine kinase (CK) and D-dimer level. In addition, lymphocyte and eosinophil counts have shown lower levels as well as the levels of serum albumin and hemoglobin (Cascella et al., 2020; Velavan and Meyer, 2020). Additionally, as it was mentioned, MODS and systemic inflammatory response syndrome (SIRS) can be the results of SARS-CoV-2 infection in which a powerful cytokine storm has an important role (Sun X. et al., 2020).

CYTOKINE STORM

It has been found that different triggers, including infections and malignancy, can result in unregulated host immune responses leading to the activated pathways of cytokine production. Thus, cytokine storm can be introduced as a systemic inflammation leading to the production of inflammatory cytokines and activation of immune cells (Tang Y. et al., 2020). Different immunity dysregulation disorders recognized by systemic inflammation, constitutional symptoms, and MOF (as the possible result of MODS) can be mentioned in this definition, too. Cytokine storm in different conditions can be varying in onset and duration, which depends on the cause and administered therapeutic approaches. However, the clinical manifestations of it usually overlap (Grupp et al., 2013; Fajgenbaum and June, 2020). Additionally, higher infiltration of cytokines has been seen in lung tissues of patients, too. In this regard, COVID-19 patients with severe conditions have shown more elevated pro-inflammatory cytokines compared to those in moderate conditions. Thus, it can be also related to the poor prognosis of the disease. Taken together, this can indicate the contribution of cytokine storm to the mortality of COVID-19 (Tang Y. et al., 2020). Therefore, it is essential to monitor patients for cytokine storm signs (Henderson et al., 2020).

Clinical Features and Laboratory Abnormalities

Generally, cytokine storm can cause fever in almost all of the patients, which can be in high grades in severe patients. Cytokine storm can promote tissue damage pathways that, along with acute-phase physiological changes and immune cell-mediated reactions, may lead to some other symptoms, too. Some of these symptoms are fatigue, anorexia, rash, arthralgia, myalgia, headache, diarrhea, and neuropsychiatric changes (confusion, delirium, aphasia, and seizure). It can also lead to disseminated intravascular coagulation, hemostatic imbalance, catastrophic hemorrhages, hypotension, vasodilatory shock, and even death. It has been found that spontaneous hemorrhage is related to hyperinflammation, low platelet levels, and coagulopathy. Dyspnea, tachypnea, cough, pulmonary edema, ARDS, and hypoxemia are also possible respiratory symptom-related events that can be accompanied by cytokine storm. Splenomegaly and hepatomegaly, in addition to cardiac damage, liver injury, and renal failure, can be developed due to the more severe conditions of cytokine storm (Lee et al., 2014; Templin et al., 2015; Fajgenbaum and June, 2020; Gao et al., 2021). On the other hand, some laboratory findings of patients with cytokine storm syndrome are pancytopenia, abnormalities in liver function tests, increased triglyceride and ferritin, and lower fibrinogen levels. The cerebrospinal fluid analysis may also show some abnormalities in patients who have neurological manifestations (Rosado and Gopal, 2019). Exhaustion of natural killer cells/T cells leading to lymphopenia are other characteristics of chronic COVID-19 condition. Studies have shown that lymphocyte count is decreased significantly in severe COVID-19, and thus, lymphopenia can predict the disease prognosis and its clinical outcomes (Tan et al., 2020; Zhao et al., 2020). Cytokines impose a positive effect on the immune system to recruit immune cells to the inflammation sites. It could develop inflammation and some organ damages. Some of the important cytokines that have roles in this extreme activation are growth factors (GF), chemokines, interleukins (IL), tumor necrosis factor (TNF), interferons (IFN), and colony-stimulating factors (CSF). These cytokines, according to their functions, can be placed in two groups of pro-inflammatory and anti-inflammatory factors. For instance, TNF, IFN-γ, IL-1β, and IL-12 are in the pro-inflammatory group of factors, whereas transforming growth factor beta (TGF-β), IL-4, and IL-13 are some anti-inflammatory factors (Song et al., 2020). The concept of cytokine storm for COVID-19 infection is derived from the data that showed that critically ill patients have higher levels of TNFα, IFN gamma-induced protein-10 (IP-10), and the chemokine (C-C motif) ligand-2 (CCL2), compared to the patients in mild or moderate stages of the disease (Castelli et al., 2020). In addition to IP-10 and TNF-α, severely ill patients have shown higher levels of IL-2, IL-10, IL-7, granulocyte colony-stimulating factor (G-CSF), macrophage inflammatory protein-1A (MIP-1A), and monocyte chemoattractant protein-1 (MCP-1), too (Fara et al., 2020). In this regard, lower CD4, CD8, and natural killer (NK) T cell levels and increased monocyte and macrophage levels in COVID-19 patients can explain the higher levels of chemicals in them (Dong et al., 2020). IL-6 is also an important inflammatory cytokine that is elevated during COVID-19 inflammatory condition. It is useful for disease monitoring and can indicate the severity of the disease in its initial phases (Aziz et al., 2020; Liu et al., 2020). Chimeric antigen receptor T cell (CAR-T) treatment technology is introduced as a proper approach for immunotherapy (in infections and disorders such as hematological cancers). It can lead to cytokine release syndrome (CRS) as an adverse effect that is mainly caused by IL-6 (Bonifant et al., 2016; Kishimoto, 2021). Taken together, a valid immunological feature of COVID-19 is CRS in which hyper inflammation can manifest by a disrupted immune activation. Herein, an important association has been found between mortality rates and severe inflammation (Fara et al., 2020).

Pathophysiological Features

In response to invading pathogens, the effective immune system is expected to return the body's homeostasis by recognizing invaders and responding to them proportionally. In order to obtain this homeostasis, sufficient cytokine production is required along with the avoidance of hyperinflammatory reactions. Because, although inflammation activates innate and adaptive immune systems, it can cause collateral damages in hyperinflammatory states (Fajgenbaum and June, 2020). Endothelial dysfunction, metabolic abnormalities, and MOD can occur as the results of higher cytokine levels. Herein, elevated levels of TNF and IL-1β as acute phase response cytokines and higher IL-8 and MCP-1 levels can cause a facilitated increase of IL-6 levels (which, in COVID-19 subjects, is considered as a main intermediary of viral cytokine storm and inflammation). IL-6 in combination with membrane-bound IL-6 receptor or soluble IL-6 receptor forms a complex that affects glycoprotein 130 (gp130).

Subsequently, it can lead to the regulated IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), and MCP-1 and thus the perpetuation of the inflammatory responses. This process could be done through the Janus kinases (JAK)/signal-transducer and activator of transcription (STAT) pathway. The acute phase responses driven by IL-6 and other cytokines result in elevated ferritin, CRP, complement, and also pro-coagulant factors. On the other hand, the hyperinflammatory reactions of cytokine storm can produce reactive oxygen species (ROS) that causes cell death and stimulation of NLR family pyrin domain containing3 (NLRP3) and nuclear factor-kappa B (NF-κB) (Bhaskar et al., 2020). Herein, it has been found that transcription factor NF-κB can drive induced cytokine storm (Khalil et al., 2020). NLRP3 inflammasome can induce the immune responses leading to the increased release of cytokines and circulating cell debris, named danger-associated molecular pattern molecules (DAMPs). This can trigger and amplify the innate immunity reactions and the complement cascade (ComC) whose activation can be related to the worsened prognosis of COVID-19 patients (Ratajczak and Kucia, 2020). Pulmonary-activated platelets through the formation of platelet–neutrophil complexes (PNCs) have an important contribution to systemic sepsis, too. Herein, they are mentioned to be considerable sources of cytokines and ROS. Moreover, the effects of PNCs on increasing neutrophil recruitment and development of proinflammatory/procoagulant environment and their contribution to ARDS have been also found (Morris et al., 2020).

Cytokine Storm and Multi-Organ Involvement in Coronavirus Disease 2019

Inappropriate reactions of host cells to a different group of acute insults can result in MODS, which is mentioned to be an important reason for mortality and morbidity in intensive care units (Wang and Ma, 2008). Indeed, in MODS condition, an autodestructive response of generalized inflammation can be the result of elevated pro-inflammatory cytokine levels (Aikawa, 1996). Hereupon, in COVID-19 infection, the hyperinflammatory responses along with severe acute respiratory syndrome direct effects are in association with disease complications (Zaim et al., 2020). The disease progression has been shown to be highly affected by extrapulmonary manifestations and its comorbidities. In this regard, multi-organ effects of COVID-19 have accompanied the infection since its emergence. Therefore, plausible organ injuries and comorbidities of COVID-19 require full attention in order to reduce death numbers (Sun X. et al., 2020; Wu T. et al., 2020; Zaim et al., 2020). Some important organ involvements of COVID-19 are explained separately in the next subtitles (**Figure 1**).

Cardiac Damage

Cardiovascular disease (CVD), as an important comorbidity accompanied by SARS-CoV-2 infection, can lead to a higher mortality rate than other associated comorbidities. Cytokine storm along with other mechanisms including thrombosis, endotheliosis, and lymphocytopenia can cause this cardiac damage or make it worsen (Unudurthi et al., 2020). Thus, the cardiovascular system can be involved in virus extrapulmonary effects with diverse manifestations of myocarditis, pericarditis, decompensated heart failure, cardiogenic shock, edema, myocardial fibrosis, and some other complications (Ellison-Hughes et al., 2020; Vitiello and Ferrara, 2020). The virus can cause its effects on the cardiac system through different ways of direct infection, binding to the functional receptors of ACE2, and immune damage. Cardiomyocyte infection by COVID-19 following by virus replication can lead to tissue degeneration, necrosis (Benítez-Guerra et al., 2020), and apoptosis (Heffernan et al., 2020). Indeed, the viral infection of COVID-19 can cause extracellular matrix remodeling leading to the production of fibrotic scars. These fibrotic lesions have some pathological effects that can even lead to death in patients including cardiac dysfunction and reduction of ejection fraction. Cardiac electrical conduction system can also experience some alterations that cause cardiac arrhythmias (Vitiello and Ferrara, 2020). The cardiac damage measured by increased levels of high-sensitivity troponin I has been mentioned to be found in about 20% of COVID-19 patients (Heffernan et al., 2020). Besides cardiac troponin I, increased levels of other laboratory cardiac markers have been seen in patients, such as higher levels of CK, creatine kinase-muscle/brain activity, myoglobin, alpha-hydroxybutyrate dehydrogenase (α-HBDH), N-terminal pro-brain natriuretic peptide (NT-proBNP), and AST (Benítez-Guerra et al., 2020). Cardiac markers are stated to be instrumental in cardiac damage diagnosis. In this regard, high-sensitivity troponin I is an appropriate marker for both diagnostic and prognostic approaches (Mishra et al., 2020). Early diagnosis of cardiac involvement is an essential step to utilize effective therapeutic approaches (Vitiello and Ferrara, 2020) due to the higher morbidity and mortality rates of patients with cardiac injury (Mishra et al., 2020).

Acute Respiratory Distress Syndrome

Acute respiratory distress syndrome is defined by an acute respiratory failure as a clinical syndrome in which arterial hypoxemia and dyspnea along with higher work of breathing can be presented. ARDS patients mostly need positive pressure ventilation and intubation. ARDS could be manifested in different disorders such as sepsis, pneumonia, major trauma, and aspiration (Matthay and Zemans, 2011). It has a broad range of causes and a wide spectrum of severity, imaging abnormalities, and other manifestations (Hariri and Hardin, 2020), which can overlap with some other conditions. Nonetheless, respiratory distress symptoms (for instance, dyspnea, and tachypnea); decreased arterial oxygen saturation; as well as epigastric pain, nausea/vomiting, hypotension, and fever are some mentionable symptoms (Parhizkar Roudsari et al., 2020). Hereupon, the associated symptoms of pneumonia-related COVID-19 range from asymptomatic (or mild upper respiratory tract infection) to severe forms of pneumonia, ARDS, and death (Xiao et al., 2020). Thus, ARDS is also an important and plausible comorbidity of COVID-19, the mentioned main cause of which is damages to the alveolar epithelial cells (Khalil et al., 2020). Cytokine storm, impaired IFN-I and IFN-III responses, and vasculopathy of COVID-19, as well as the host immune responses of COVID-19, can explain the underlying pathways of pneumonia-induced

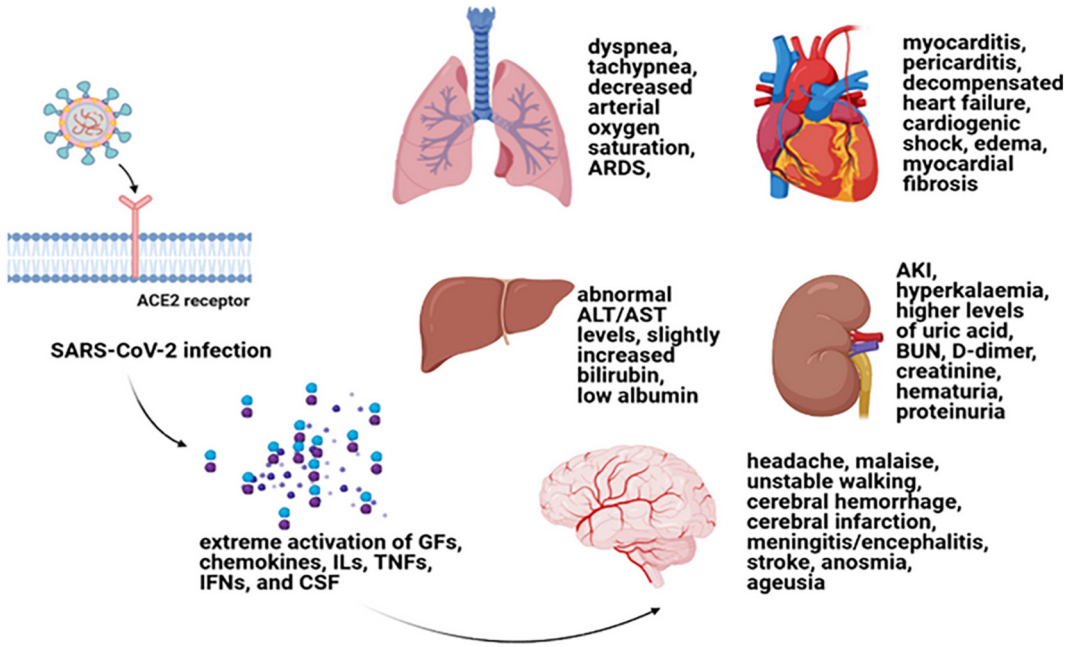

FIGURE 1 | Cytokine storm and multiple-organ damages. Cytokine storm is the term used for a systemic inflammation that can lead to the extreme activation of GFs, chemokines, ILs, TNFs, IFN, and CSF (Song et al., 2020). Cytokine storm can result in MODS in COVID-19 patients with a worse prognosis. Myocarditis, pericarditis, decompensated heart failure, cardiogenic shock, edema, myocardial fibrosis as some cardiovascular complications (Ellison-Hughes et al., 2020; Vitiello and Ferrara, 2020); respiratory distress symptoms including dyspnea and tachypnea and decreased arterial oxygen saturation (Parhizkar Roudsari et al., 2020); renal damage symptoms such as AKI, hyperkalemia, higher levels of uric acid, BUN, D-dimer, and creatinine as well as hematuria and proteinuria (Kunutsor and Laukkanen, 2020; Patel et al., 2020; Puelles et al., 2020); an abnormal ALT or AST levels along with slightly increased bilirubin and low albumin (for liver damage consequences) (Sun J. et al., 2020; Wu J. et al., 2020); and headache, malaise, unstable walking as well as cerebral hemorrhage and cerebral infarction, meningitis/encephalitis, stroke, anosmia, and ageusia as some nervous system damages (Alomari et al., 2020; Berger, 2020) can be mentioned as some of the important organ involvements of COVID-19 due to cytokine storm. GF, growth factor; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; CSF, colony-stimulating factor; MODS, multiple-organ dysfunction syndrome; TGF-β, transforming growth factor beta; AKI, acute kidney injury; BUN, blood urea nitrogen; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

ARDS. The pro-inflammatory cascades of SARS-CoV-2 infection due to cytokine storm has a mentionable link to macrophage activation syndrome (MAS), which is a life-threatening feature of autoimmune diseases and can be mimicked in several viral infections. It can cause the damaged cytolytic activity of NK cells and CD8 + T cells. High levels of IL-6 may reflect an over-exuberant inflammatory reaction as the result of cytokine storm and can drive these impairments associated with MAS (Torres Acosta and Singer, 2020). Because of the rapid development of ARDS, the high mortality rate, and the lower quality of life among survivors, finding novel and more effective therapeutic approaches is considerably required (Xiao et al., 2020).

Renal Damage

Renal damage also takes a part in the extrapulmonary effects of COVID-19 that has been observed in a significant population of COVID-19-infected patients. Autopsy studies have also shown renal involvement due to COVID-19 infection. In this regard, acute kidney injury (AKI) is a common finding that can be observed in up to 25% of critically ill patients of COVID-19 (with underlying comorbidities) (Patel et al., 2020). Needing for renal replacement therapy and electrolyte disturbance, such as hyperkalemia, are other common renal complications of COVID-19 (Kunutsor and Laukkanen, 2020). Renal damage can be manifested by higher levels of uric acid, blood urea nitrogen (BUN), D-dimer, and creatinine as well as hematuria and proteinuria (Puelles et al., 2020). Indeed, proteinuria can be the presentation of patients at hospital admission, and AKI mostly develops in critically ill patients at later stages of COVID-19. Thus, it could be a marker for MOD and the severity of the disease (Ronco et al., 2020). Taken together, it has been mentioned that renal damage is related to the severe forms of COVID-19 infection with fatal outcomes, and pre-existing CKD can lead to a higher incidence of AKI. A high expression of ACE2, TMPRSS2, and cathepsin L (CTSL) in the kidneys and direct cytopathic effects of the virus are possible causes of kidney involvement during the COVID-19 pandemic (Naicker et al., 2020; Puelles et al., 2020). Hypovolemia and ARDS-related AKI are other possible mechanisms of kidney injury associated with COVID-19. Moreover, cytokine storm in association with secondary hemophagocytic lymphohistiocytosis (sHLH) has a significant role in renal involvement of COVID-19. Hemodynamic changes, hypercoagulable state, and direct effects of cytokines (such as IL-6 and TNF) can be caused by hyperinflammatory states of cytokine storm that may lead to acute tubular necrosis (ATN) and tubulointerstitial nephritis

(TIN) (Ahmed et al., 2020). There are no specific therapeutic options for AKI-related COVID-19, and intensive care along with clinical experience are mainly supportive that declares the requirement for developing new approaches for the management of this involvement (Ronco et al., 2020).

Liver Damage

There are numerous studies that have shown liver involvement due to COVID-19 infection. Although the exact underlying mechanism of liver damage has not been founded yet, abnormal ALT or AST levels along with slightly increased bilirubin are common manifestations of liver dysfunction (Wu J. et al., 2020). Higher liver enzymes are found more commonly in males. In addition, severe cases have more elevated liver enzymes than milder cases of the disease. Low albumin is also an indicator of severe infection with a worse prognosis. According to the effects of cytokine storm on liver damage, the inflammation biomarkers such as CRP, serum ferritin, lactate dehydrogenase (LDH), IL-6, IL-2, and D-dimer were significantly higher in severely ill patients of COVID-19 (Sun J. et al., 2020). Results of an autopsy pathological study have shown moderate microvascular steatosis that was along with mild inflammation of the lobular portal zone, but direct killing influences of the virus were not observed (Wu J. et al., 2020). Taken together, direct viral effects on bile ducts, the role of cholangiocytes (for instance with their cell entry receptor of ACE2), and immune system activation particularly cytokine storm could be the probable causes for liver injury (Alqahtani and Schattenberg, 2020). On the other hand, drug-induced liver injury and hypoxia-induced damage are other possible mechanisms that can be hypothesized for liver damage because of SARS-CoV-2 infection (Kudaravalli et al., 2020; Wu J. et al., 2020). It should be mentioned that patients with a past medical history of liver diseases are more susceptible to liver damage from SARS-CoV-2 (Sun J. et al., 2020). However, more in-depth studies are required to demonstrate the causes of liver damage and to provide anti-COVID-19 treatments that particularly work on liver function (Vitiello et al., 2021).

Central Nervous System Damage

Besides the typical symptoms of COVID-19, some patients may show some neurological symptoms such as headache and malaise. Unstable walking as well as cerebral hemorrhage and cerebral infarction, meningitis/encephalitis, stroke, anosmia, and ageusia are other possible effects of SARS-CoV-2 infection on the nervous system (Alomari et al., 2020; Berger, 2020). Conducted studies have shown that neurological symptoms may be manifested in more than one-third of patients with COVID-19. However, they may be more commonly seen in severe infections (Niazkar et al., 2020). It has been shown that the virus can infect neurons and reduce synapse formation between them in which the olfactory route and blood–brain barrier could be the possible routes of invasion (Iadecola et al., 2020a; Marshall, 2020). *In vivo* studies done on human ACE2 transgenic mice found that an ACE2-dependent manner of neuronal infection

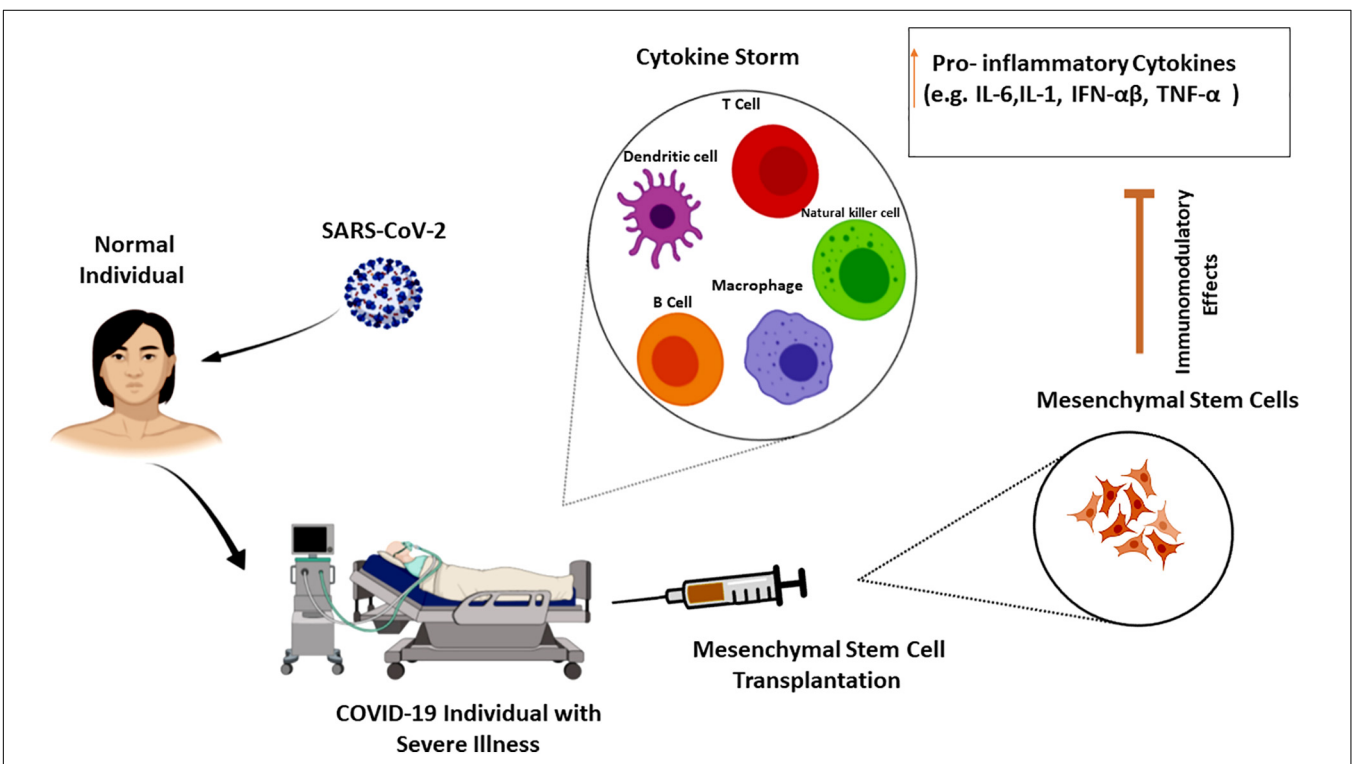

FIGURE 2 | Mesenchymal stem cells effects on cytokine storm. Cytokine storm as the hallmark of COVID-19 severity is the increasing level of pro-inflammatory cytokines that can lead to organ failure and death. The immune-regulatory effects of mesenchymal stem cells on various cell types of the innate and adaptive immune system can attenuate cytokine storm in severe COVID-19 cases (Lee and Song, 2018; Wang et al., 2018; Zhu et al., 2020).

can lead to neuronal death due to the organoids. Viral proteins and molecular complexes of damaged cells could enter the compromised blood–brain barrier, and after brain entry, they act as DAMPs and pathogen-associated molecular patterns (PAMPs). This can promote innate immune responses and express Toll-like receptors (TLR). It was found that these receptors can mediate the SARS-CoV pro-inflammatory effects that may result in higher cytokine production and impaired brain function (Iadecola et al., 2020a). Hypothalamic–pituitary–adrenocortical (HPA) axis can be also activated due to the unregulated cytokines in COVID-19, which can cause the autonomic nervous system and catecholamine/steroids release (Iadecola et al., 2020b). Despite all this, pathophysiological mechanisms underlying CNS-related COVID-19 infection should be found more precisely (Divani et al., 2020).

TREATMENTS FOR CYTOKINE STORMS

Overall, important strategies to avoid the development of cytokine storms and ameliorate the prognosis of infection include the reduction of viral load by targeted therapeutic approaches in the early stages of the disease (with no or moderate symptoms) and the regulation of inflammatory reactions via immune modulators (Florindo et al., 2020; Khadke et al., 2020; Pan et al., 2020; Tang L. et al., 2020; Ye et al., 2020). Herein, cytokine inhibition, blood purification medical care, corticosteroid therapies, and cell-based approaches are the foremost therapeutic strategies (Iannaccone et al., 2020; Ye et al., 2020). Accordingly, cytokine inhibition, e.g., by means of IL-6/IL-6R blockers, IL-1 family blockers, TNF-α blockers, and IFN-αβ blockers; blood purification medical care through the adsorption, plasma exchange, perfusion, and filtration of blood/plasma; corticosteroid therapies, which contribute to histone acetyltransferase (HAT) inhibition and histone deacetylase 2 (HDAC2) interest recruitment to be able to downregulate inflammatory genes; and cell-based approaches have powerful anti-inflammatory and immune-regulatory roles (Choudhary et al., 2020; Henriksen, 2020; Hojyo et al., 2020; Iannaccone et al., 2020).

REGENERATIVE MEDICINE AND CELL-BASED TREATMENTS

Cell therapy and regenerative medicine are marked as one of the most hopeful possible strategies for the regeneration of damaged or failed tissue and organs in the medical system. There are different approaches here, containing the use of cells from both autologous and allogeneic sources (Saberi et al., 2008; Aghayan et al., 2014a; Goodarzi et al., 2014, 2015). In other words, it encompasses a wide range of treatments via using various types of cells (e.g., T cells, NK lymphocytes, and different stem cells) with varying outcomes. In this context, the adoptive T-cell therapy or CAR T-cell therapy approach as a kind of immunotherapy has been shown to be effective against some infections and diseases. Herein, T cells from patient's own immune system (autologous source) are extracted and sent to a lab for genetic modification. The patient is then re-infused with the engineered cells (Maus et al., 2014; Bonifant et al., 2016; Maus and Levine, 2016; Seif et al., 2019). Despite the impressive effectiveness of CAR T-cell therapy in the treatment, it has a number of serious side effects including CRS and neurologic difficulties. CRS with an immediate onset tends to be a cytokine storm (Chen et al., 2019; Hong et al., 2021). Currently, T-cell therapy has also shown promise in immunosuppressed individuals as a preventive measure against COVID-19. Accordingly, investigators employed peripheral blood cells from convalescent subjects who had been endangered by the virus (Keller et al., 2020). Regulatory T cell-related strategies have been also suggested as considered treatment approaches for disease management according to their capacity for inactivation of innate/adaptive immunity through inhibitory molecules (Stephen-Victor et al., 2020). Additionally, transferring modified/unmodified antigen-specific T cells has shown promising results in the treatment of different disorders by reconstituting T cell subsets (effector/memory cells). In this context, adoptive T cell therapy by transferring T cell immune subsets is mentioned to have therapeutic benefits that can be the same as adult tissue stem cell features. However, high maintenance of memory T cells required and engraftment processes may create some limitations (Busch et al., 2016). In this regard, specific COVID-19-related T cells (within CD45RA- memory T cells) have been recognized that can be feasibly received by CD45RA depletion from convalescent donors. These cells can provide a population of cells for lymphopenia condition along with quick reactions to infection. COVID-19 CD45RA- memory T cells also provide immunity against secondary probable infections that may be found in COVID-19-hospitalized individuals (Ferreras et al., 2021). HLA-matched cytotoxic T cells isolated from convalescent patients are other promising approaches for the treatment of COVID-19 same to EBV-specific cytotoxic T cells, which were utilized for EBV + -related lymphomas (Hanley et al., 2020). Another promising candidate for significant advancement has been NK cell therapy. Hereupon, autologous or allogeneic origins may be used to create pure populations of NK cells. Using the allogeneic NK cells as a platform for CAR engineering has risen due to the limitations of autologous NK cells (such as decreased effector role and the demand for a patient-specific stock) (Veluchamy et al., 2017; Daher and Rezvani, 2018). Since a decrease in the number of NK cells can be linked to the severity of the COVID-19 infection, some clinical trials used engineered NK cells to help battle COVID-19 (Market et al., 2020; van Eeden et al., 2020). However, using NK cell also has a number of drawbacks that may hinder their effectiveness. Short lifespan (in the lack of cytokine support), low cell numbers, and vulnerability to the immunosuppressive situation, all of which could limit their trafficking and operation (Nayyar et al., 2019; Liu et al., 2021). In accordance with various introduced limitations, as the epicenter of regenerative medicine, mesenchymal stem cells (MSCs) have been widely investigated and applied, and also have appeared throughout this area as a strong and commonly used cell source. Their capacity to differentiate into diverse cell

TABLE 1 | Number of cell-based clinical trials for COVID-19 (https://clinicaltrials.gov/).

Clinical trial heading	Applied cell	Thetotal number of participants	State of the recruitment	ClinicalTrials.gov identifier	Locations
Mesenchymal Stem Cell Infusion for COVID-19 Infection	Mesenchymal stem cells	20	Recruiting	NCT04444271	Pakistan
Mesenchymal Stem Cell for Acute Respiratory Distress Syndrome Due for COVID-19 (COVID-19)	Mesenchymal Stem cells	10	Recruiting	NCT04416139	Mexico
Safety and Efficacy of Mesenchymal Stem Cells in the Management of Severe COVID-19 Pneumonia	Umbilical cord-derived mesenchymal stem cells	30	Not yet recruiting	NCT04429763	United States
Novel Coronavirus Induced Severe Pneumonia Treated by Dental Pulp Mesenchymal Stem Cells	Dental pulp mesenchymal stem cells	24	Not yet recruiting	NCT04302519	China
Mesenchymal Stem Cells in Patients Diagnosed With COVID-19	Mesenchymal stem cells	20	Recruiting	NCT04611256	United States
Use of Mesenchymal Stem Cells in Acute Respiratory Distress Syndrome Caused by COVID-19	Mesenchymal stem cells derived from Wharton's jelly of umbilical cords	9	Active, not recruiting	NCT04456361	United States
Efficacy of Infusions of MSC From Wharton Jelly in the SARS-Cov-2 (COVID-19) Related Acute Respiratory Distress Syndrome	*Ex vivo* expanded Wharton's jelly mesenchymal stem cells	30	Not yet recruiting	NCT04625738	France
Mesenchymal Stem Cell Therapy for SARS-CoV-2-related Acute Respiratory Distress Syndrome	Mesenchymal stem cells	60	Recruiting	NCT04366063	Iran
Novel Adoptive Cellular Therapy With SARS-CoV-2 Specific T Cells in Patients With Severe COVID-19	Adoptive T-cell therapy	8	Recruiting	NCT04351659	Singapore
Mesenchymal Stem Cells Therapy in Patients With COVID-19 Pneumonia	Mesenchymal stem cells	21	Completed	NCT04713878	Turkey
Part Two of Novel Adoptive Cellular Therapy With SARS-CoV-2 Specific T Cells in Patients With Severe COVID-19	SARS-CoV-2-specific T cells	18	Recruiting	NCT04457726	Singapore
A Study of Cell Therapy in COVID-19 Subjects With Acute Kidney Injury Who Are Receiving Renal Replacement Therapy	Allogeneic human mesenchymal stromal cells	22	Recruiting	NCT04445220	United States
Safety of T Regulatory Cell Therapy in Subjects With COVID-19 Induced Acute Respiratory Distress Syndrome	T regulatory cells	20	Not yet recruiting	NCT04737161	United States
Cell Therapy Using Umbilical Cord-derived Mesenchymal Stromal Cells in SARS-CoV-2-related ARDS	Umbilical cord Wharton's jelly derived human mesenchymal stromal cells	47	Active, not recruiting	NCT04333368	France
Treatment of Coronavirus COVID-19 Pneumonia (Pathogen SARS-CoV-2) With Cryopreserved Allogeneic P_MMSCs and UC-MMSCs	Cryopreserved placenta-derived mesenchymal stromal cells	30	Recruiting	NCT04461925	Ukraine
Study of Intravenous Administration of Allogeneic Adipose Stem Cells for COVID-19	Adipose-derived allogeneic mesenchymal stem cell	20	Recruiting	NCT04486001	United States
A Randomized, Double-Blind, Placebo-Controlled Clinical Trial to Determine the Safety and Efficacy of Hope Biosciences Allogeneic Mesenchymal Stem Cell Therapy (HB-adMSCs) to Provide Protection Against COVID-19	Allogeneic adipose-derived mesenchymal stem cells	100	Active, not recruiting	NCT04348435	United States
Mesenchymal Stromal Cell Therapy for Severe Covid-19 Infection	Bone marrow-derived mesenchymal stromal Cells	20	Recruiting	NCT04445454	Belgium
Treatment of COVID-19 Patients Using Wharton's jelly Mesenchymal Stem Cells	Umbilical cord Wharton's jelly derived human mesenchymal stem cells	5	Recruiting	NCT04313322	Jordan

(Continued)

TABLE 1 | Continued

Clinical trial heading	Applied cell	Thetotal number of participants	State of the recruitment	ClinicalTrials.gov identifier	Locations
A Phase I/II Study of Universal Off-the-shelf NKG2D-ACE2 CAR-NK Cells for Therapy of COVID-19	NK cells	90	Recruiting	NCT04324996	China
Safety and Efficacy of Allogeneic Human Dental Pulp Mesenchymal Stem Cells to Treat Severe COVID-19 Patients	Allogeneic human dental pulp mesenchymal stem cells	20	Recruiting	NCT04336254	China
Treatment With Human Umbilical Cord-derived Mesenchymal Stem Cells for Severe Corona Virus Disease 2019 (COVID-19)	Umbilical cord-derived mesenchymal stem cells	100	Completed	NCT04288102	China
Clinical Research of Human Mesenchymal Stem Cells in the Treatment of COVID-19 Pneumonia	Umbilical cord mesenchymal stem cells	30	Recruiting	NCT04339660	China
Cell Therapy Using Umbilical Cord-derived Mesenchymal Stromal Cells in SARS-CoV-2-related ARDS	Umbilical cord Wharton's jelly derived human mesenchymal stromal cells	47	Active, not recruiting	NCT04333368	France
Study of Human Umbilical Cord Mesenchymal Stem Cells in the Treatment of Novel Coronavirus Severe Pneumonia	Umbilical cord mesenchymal stem cells	48	Not recruiting	NCT04273646	China
Mesenchymal Stem Cell Treatment for Pneumonia Patients Infected With 2019 Novel Coronavirus	Mesenchymal stem cells	20	Recruiting	NCT04252118	China
Umbilical Cord (UC)-Derived Mesenchymal Stem Cells (MSCs) Treatment for the 2019-novel Coronavirus (n COV) Pneumonia	Umbilical cord mesenchymal stem cells	16	Recruiting	NCT04269525	China
NK Cells Treatment for Novel Coronavirus Pneumonia	NK cells	30	Recruiting	NCT04280224	China

lineages, migration, and cellular regulator secretion together with immunosuppressive and immunomodulatory potential of MSC secretome are the features that make them extremely valuable. On the other hand, their isolation is almost easy and does not have significant ethical concerns (Aghayan et al., 2014b; Larijani et al., 2014, 2015, 2021; Goodarzi et al., 2018a,b; Payab et al., 2018; Arjmand et al., 2019; Abedi et al., 2020; Tayanloo-Beik et al., 2021). These features makes them the most suitable stem cell approaches among many of them (Azmi et al., 2020). The umbilical cord, adipose tissue bone marrow, dental pulp, and menstrual blood are important sources of MSCs. MSCs derived from adipose tissue have been mentioned to have more interesting results initially, but the best source of stem cell is required to be found yet (Song et al., 2021). Moreover, through their impacts on T and B cells, macrophages, and dendritic cells, they help regenerate and refresh the condition (Lee and Song, 2018; Wang et al., 2018). Accordingly, by inhibiting the proliferation of T and B cells and by successful regulation of pro-inflammatory cytokines to optimize the microenvironment for intrinsic recovery, MSCs can reduce the cytokine storm. On the other hand, as the indirect effects to attenuate cytokine storm, they can restrict the innate immune system cell infiltration and consequently decrease the secretion of inflammatory cytokines (Ellison-Hughes et al., 2020; Gupta et al., 2020; Zhu et al., 2020; Jeyaraman et al., 2021; **Figure 2**). 6 days after MSC therapy, cytokine storm-related immunity cells were showed to have dwindled. Increased levels of lymphocytes and regulatory dendritic cells along with decreased CRP; IL-1, 6, and 12; IFN-γ; and TNF levels are also other results of this kind of therapy. Indeed, MSCs can provide antimicrobial peptides and anti-inflammatory cytokines (Leng et al., 2020; Rajarshi et al., 2020; Wang H.C. et al., 2020). Besides these anti-inflammatory features, secretion of IL-10 and some growth factors along with their regeneration and reparative capacity make them a potent therapeutic approach for lung repair and ARDS treatment in early stages (Azmi et al., 2020). MSC administration has been also shown to have benefits in sepsis and septic shock conditions regarding their capacity to normalize inflammatory biomarkers, oxygen saturation, and pulmonary improvements on CT imaging. For sepsis condition, umbilical cord-derived MSCs [especially from Wharton's jelly (WJ)], due to their effectiveness and acceptability, are mentioned to be the best source for MSCs (Laroye et al., 2020). In this respect, the US Food and Drug Administration (FDA) has recently confirmed the safety and efficacy of MSCs for widespread application in COVID-19 cases (Choudhery and Harris, 2020; Kavianpour et al., 2020). In addition to the mentioned benefits of MSC administration, there are still some challenges; MSC-related features regarding their dosage, route of administration, frequency, and homing into the damaged sites have provided some limitations. Remaining ethical concerns along with lack of standardized protocols in preparation and isolation processes are other challenges. One other important concern about MSC therapy is the side effect of increased hypercoagulability (Jeyaraman et al., 2021). Thus, according to the higher risk of thrombosis, cell-free therapies including MSC secretome

and MSC extracellular vesicles (EVs) seem to be interesting treatment approaches for COVID-19 that have shown no risk of mutagen/oncogenicity. Exosomes harbor different types of miRNAs/mRNAs and diverse protein components and have lower accompanied risk and decreased infection transmission. However, dosage, timing, and route of cell delivery are required to be known more clearly. Their capability for nebulized delivery and their longer storage periods also make them promising alternative therapeutic approaches (Maron-Gutierrez and Rocco, 2020; Kheirkhah et al., 2021). Therein, a number of cell-based clinical trials for COVID-19 are reviewed in **Table 1**.

CONCLUSION AND FUTURE SCOPE

Due to the prevalence and complications of COVID-19, including cytokine storm, which is followed by organ dysfunction or failure and death, finding the efficient approach to treat and improve patients is of great importance. Cell therapy is now a modern way of treating several diseases, and many experiments have been performed in recent months to use different types of cells to treat the COVID-19, i.e., the MSC transplantation (Parhizkar Roudsari et al., 2020). Manifold aspects associated with the MSC application (e.g., standard protocols for isolation and harvesting, selection of the proper source for isolation, the appropriate dosage, route, and the ideal timing of delivery) should be more discussed. Herein, in explaining the potential of MSCs, preclinical researches and continuing randomized trials will perform an important part to promote our knowledge about MSCs' fight against SARS-CoV-2 (Mastrolia et al., 2019; Al-Khawaga and Abdelalim, 2020). Because of possible accompanying side effects and limitations of stem cell-based therapeutic approaches, EVs are emerging alternative cellular treatments that have some advantages over MSCs (Maron-Gutierrez and Rocco, 2020). On the other hand, the importance of conducting more extensive studies to better understand the new coronavirus and its different variants cannot be ignored. This opens the door to researchers for designing more effective treatments. Accordingly, genetically modified MSCs have the ability to solve the challenges as a new sector and a developing field (Sage et al., 2016).

AUTHOR CONTRIBUTIONS

SA-M and PP wrote the first draft of the manuscript. FM-J, MR-T, and HA helped to study and gather information. FR and KG extensively edited the manuscript. BL participated in a critical review. BA helped to supervise the project and gave final approval of the version to be published. All authors contributed to the study's conception and design.

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GLOSSARY

SARS-CoV-2, severe acute respiratory syndrome coronavirus-2; COVID-19, coronavirus disease-2019; SARS-CoV, severe acute respiratory syndrome coronavirus; MERS-CoV, Middle East respiratory syndrome coronavirus; ORFs, open reading frames; nsp, non-structural proteins; S, spike; ACE2, angiotensin converting enzyme 2; TMPRSS2, transmembrane protease serine type 2; ARDS, acute respiratory distress syndrome; MODS, multiple-organ dysfunction syndrome; MOF, multi-organ failure; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CK, creatine kinase; SIRS, systemic inflammatory response syndrome; GF, growth factor; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; CSF, colony-stimulating factor; TGF-β, transforming growth factor beta; IP-10, IFN gamma-induced protein-10; CCL2, chemokine (C-C motif) ligand-2; MIP-1A, macrophage inflammatory protein-1A; MCP-1, monocyte chemoattractant protein-1; CRS, cytokine release syndrome; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; JAK: Janus kinases; STAT, signal-transducer and activator of transcription; ROS, reactive oxygen species; NF-κB, nuclear factor-kappa B; DAMPs, danger-associated molecular pattern molecules; ComC, complement cascade; PNCs, platelet–neutrophil complexes; CVD, cardiovascular disease; α-HBDH, alpha-hydroxybutyrate dehydrogenase; NT-proBNP, N-terminal pro-brain natriuretic peptide; MAS, macrophage activation syndrome; AKI, acute kidney injury; BUN, blood urea nitrogen; CTSL, cathepsin L; sHLH, hemophagocytic lymphohistiocytosis; ATN, acute tubular necrosis; TIN, tubulointerstitial nephritis; PAMPs, pathogen-associated molecular patterns; TLR, Toll-like receptors; HPA, hypothalamic–pituitary–adrenocortical; HAT, histone acetyltransferase; HDAC2, histone deacetylase 2; FDA, Food and Drug Administration; MSCs, mesenchymal stem cells; WJ, Wharton's jelly; EVs, extracellular vesicles; NK, natural killer; CAR, chimeric antigen receptor.

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HYPOTHESIS AND THEORY
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Developing Cytokine Storm-Sensitive Therapeutic Strategy in COVID-19 Using 8P9R Chimeric Peptide and Soluble ACE2

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Currently, the COVID-19 pandemic is an international challenge, largely due to lack of effective therapies. Pharmacotherapy has not yet been able to find a definitive treatment for COVID-19. Since SARS-CoV-2 affects several organs, treatment strategies that target the virus in a wider range are expected to be ultimately more successful. To this end, a two-step treatment strategy has been presented. In the first phase of the disease, when the patient is newly infected with the virus and the cytokine storm has not yet been developed, a chimeric peptide is used to inhibit virus entry into the host cell cytosol (by inhibiting endosomal pH acidification) and viral replication. After the virus entry and decrease of angiotensin converting enzyme 2 (ACE2) level, some people are unable to properly compensate for the ACE2 pathway and progress toward the cytokine storm. In the beginning of the cytokine storm, sACE2 protein is very effective in regulating the immune system toward the anti-inflammatory pathway, including M2 macrophages. Hence, the genes of 8P9R chimeric peptide and sACE2 would be inserted in an episomal vector with a separate promoter for each gene: the chimeric peptide gene promoter is a CMV promoter, while the sACE2 gene promoter is a NF-κB-sensitive promoter. The NF-κB-sensitive promoter induces the expression of sACE2 gene soon after elevation of NF-κB which is the main transcription factor of inflammatory genes. Thus, as the expression of inflammatory cytokines increases, the expression of sACE2 increases simultaneously. In this condition, sACE2 can prevent the cytokine storm by inhibiting the pro-inflammatory pathways. To deliver the designed vector to the target cells, mesenchymal stem cell-derived (MSC-derived) exosome-liposome hybrids are used. Herein, the strategy can be considered as a personalized clinical therapy for COVID-19, that can prevent morbidity and mortality in the future.

Keywords: 8P9R chimeric peptide, sACE2, cytokine storm, mesenchymal stem cells, exosomes, COVID-19, ARDS, NF-κB

INTRODUCTION

Acute respiratory disease caused by COVID-19 has become a public health emergency and is a major health problem causing respiratory tract infection which can range from an asymptomatic form to severe acute respiratory distress syndrome (ARDS). ARDS is one of the most common complications and also the major cause of respiratory failure in severe COVID-19 patients. Approximately 33% of hospitalized COVID-19 patients develop ARDS. The mortality rate in COVID-19-associated ARDS is 45%, and the prevalence of ARDS in patients who died from COVID-19 is about 90% (Tzotzos et al., 2020). COVID-19 can also lead to multi-organ dysfunction and failure in severe cases. Currently, the therapeutic strategies are mainly based on supportive care and non-specific antiviral drugs. Due to its devastating consequences, it is essential to identify a potential therapeutic option to prevent and repair the destruction caused by the disease.

The virus entry into the host cells is one of the major factors in viral infection which allows viruses to reach replication sites. SARS-CoV-2 uses the membrane protein angiotensin converting enzyme 2 (ACE2) as an entry receptor. The S glycoprotein of the virus composes of S1 subunit including RBD for binding to the membrane receptor and the S2 subunit for the fusion of the viral and cellular membrane (Yang et al., 2020). Coronaviruses enter the host cells via two routes: endocytic pathway is the main route and the other route is non-endosomal pathway. Hence, targeting the endocytic pathway could be a promising therapeutic strategy against coronaviruses. As SARS-CoV-2 entry requires acidification of endosomes and finally lysosomes, neutralizing the acidic environment of endosomes could impair viral entry pathways. Three groups of inhibitors have been used to block viral entry; they are lysosomotropic agents, endosomal–lysosomal protease inhibitors, and clathrin-mediated endocytosis inhibitors (Yang and Shen, 2020). These drugs are not specific for SARS-CoV-2 and may have some off-target effects. Therefore, designing a specific antiviral peptide to bind to the virus and neutralizing the endosomal pH and subsequent viral entry would be an interesting therapeutic option.

ACE2 is the main cell entry receptor for SARS-CoV-2. Following the entry of the virus into the host cell via the ACE2 receptor-mediated endocytosis, plasma membrane ACE2 levels are reduced. Decreased levels of membrane-bound ACE2 promote Ang II accumulation which is leading to increased over-activation in the ACE/Ang II/AT1 receptor axis and loss of ACE2/Ang-(1–7)/MasR axis. ACE2/Ang-(1–7)/MasR axis exerts anti-inflammatory effect. ACE2 regulate the balance of Ang II/Ang-(1–7) levels, resulting in exertion of protective effects of Ang-(1–7) and protection against destructive effects of Ang II. So, this imbalance in renin-angiotensin system (RAS) system may lead to systemic inflammation and consequence cytokine storm (Mahmudpour et al., 2020; Verdecchia et al., 2020). Due to the importance of ACE2/Ang II balance in development of cytokine storm, it can be considered as a potential therapeutic option.

Herein a novel strategy to overcome the above challenges in the treatment of COVID-19 is proposed. This strategy is a phase-dependent two-step therapeutic strategy which is sensitive to cytokine storm. In the first phase, the goal is to block the intracellular pathways via inhibiting the acidification of the endosomes environment which is resulted in the prevention of the virus escape to the cytosol and also prevention of the virus replication. For this purpose, 8P9R chimeric peptide would be used. This peptide specifically binds to the virus and enter only to the virus-infected cells.

Following the entry of the virus into the cell and internalization of the ACE2, the negative consequences of ACE2 depletion may occur. The second aim of the present study is to prevent these negative consequences only in individuals who are progressing into inflammatory state caused by depleted ACE2 and cytokine storm. This strategy can be considered as personalized therapy for COVID-19 patients. To achieve this goal, an engineered exosome-liposome hybrid containing an episomal vector is proposed. This vector contains a chimeric peptide gene and an ACE2 soluble form gene. Chimeric peptide gene expression is controlled by the CMV promoter, which is always active. But, the expression of the sACE2 gene is controlled by the nuclear factor kappa-light-chain-enhancer of activated B cells-sensitive (NF-κB-sensitive) promoter. NF-κB is a transcription factor for inflammatory-related genes. This transcription factor is selected to activate sACE2 gene expression in parallel with increased expression of inflammatory genes and progression into cytokine storm. sACE2 can perform its enzymatic activity in the extracellular space and also can block viruses as a decoy receptor.

SUPPORTING EVIDENCES FOR STRATEGY

Cell Entry Mechanism of SARS-CoV-2

The entry of SARS-CoV-2 into the host cell is the early stage of host cell infection and virus replication. Effective inhibition at this early stage of the disease is likely to lead to successful prevention and treatment. SARS-CoV-2 has four types of proteins, one of them is the spike surface protein which binds to the ACE2 receptor. After binding, the virus enters the endosome through endocytosis and travels along the intracellular pathway. The subsequent maturation and acidification of early endosomes to endolysosomes, and finally lysosomes, is required for fusion-activating S protein cleavage by cathepsin L. Several evidences on the roles of protease activators demonstrate that lysosomal cysteine protease Cathepsin L is crucial for SARS-CoV-2 entry (Muralidar et al., 2021). In lysosomes, cathepsin L (the last component of the intracellular pathway) fuses viral particles with lysosomal membrane and allows viral RNA to enter the cytosol and start the replication process. Activation of cathepsin L requires an acidic environment (optimal pH 5.0–5.5). Thus, the inhibition of lysosomal acidification by cysteine protease inhibitors, vacuolar H + ATPase inhibitors, and lysosomotropic inhibitors, has been shown to inhibit the fusion of viral particles bound to ACE2 with lysosomal membrane. Lysosomes with inhibited acidic pH are not able to activate cathepsin L and therefore viral escape does not occur in them (Blaess et al., 2020). Thus, effective inhibition at this early stage of the disease

is likely to lead to successful prevention and treatment of COVID-19.

Acidification is essential for endosomal maturation through which viruses escape from the endosomes. So, using drugs which dysregulate endosomal acidification would be useful to impair viral infection. Recent studies have reported that using drugs which negatively affects endosomal cholesterol release and increase endolysosomal cholesterol storage in combination with antiviral drugs would enhance antiviral activity due to synergistic interaction. Very recently, studies have shown the efficacy of the repurposed drugs that interfere with the SARS-CoV-2 cell entry mechanism in inhibiting viral entry and thus reducing the viral replication and load. Fluoxetine and itraconazole are drugs that were previously licensed for some other clinical uses. Schloer et al. (2020) and Zimniak et al. (2021) showed the efficacy of these host-directed drugs in fighting COVID-19 infection both alone and in combination with an antiviral drug, remdesivir. In another study, they investigated the *in vitro* antiviral activity of fluoxetine against SARS-CoV-2 in a model of acute infection and the level of infectious particles was lower than that in controls (Dechaumes et al., 2021). The mechanism through which fluoxetine exhibits its inhibitory effect in viral cell entry is that it prevents endolysosomal acidification by inactivating endolysosomal proton pump (v-ATPase) which leads to endolysosomal pH neutralization. In this condition, virus–endosome fusion and releasing of viral genome will not occur. Fluoxetine is a functional inhibitor of sphingomyelinase (FIASMA) which accumulates cholesterol in endolysosomes and causes a secondary reduction in the cholesterol content of other cell membranes which reduces the amount of cholesterol available for the virus to produce its envelope. This might also impact TMPRSS2 activity and/or virus-membrane fusion capacity, which might either reduce SARS-CoV-2 entry and/or shift the entry route in favor of an endosomal uptake (Schloer et al., 2021). In another study, Creeden et al. (2021) provided evidences that fluoxetine can inhibit IL-6 and NF-κB signaling, and therefore disrupt the production of pro-inflammatory cytokines like IL6 and IL6ST and mitigate the progression of cytokine storm. It has also been reported that using a standard dose of fluoxetine (21.6 mg daily) in 35 patients who were hospitalized for COVID-19 reduced risk of intubation and death (Hoertel et al., 2021). These evidences show that the combination of the proposed antiviral strategy with FDA-approved drugs can target the pivotal mechanisms of COVID-19 physiopathology and might provide a novel treatment for this disease.

Immune System Response and Cytokine Storm in COVID-19

Clinically, SARS-CoV-2 induced immune responses have two distinct phases. Protective phase in which immune responses especially adaptive immune responses have important roles in elimination of the virus at early stages of the disease. Damaging phase in which immune responses can also cause tissue damage via release of pro-inflammatory cytokines at severe stages (Duan et al., 2020). In severe conditions, excessive and uncontrolled production of pro-inflammatory cytokines including IL-6, IL-1, and TNF-α results in convergence of immune cells and leads to systemic inflammatory response, known as cytokine storm. These excessive reactions can progress into multi-organ failure, acute lung injury and in severe cases cause ARDS, which is one of the critical causes of death in COVID-19 patients (Ragab et al., 2020). One of the main components of the immune system which is involved in creating cytokine storm are macrophages. M1 macrophages are essential for inducing immune responses against viral infection but this function could be impaired due to infection by SARS-CoV-2. M2 macrophages are anti-inflammatory macrophages which are essential for suppression of inflammation and have tissue healing effects. So, switching of M1 macrophages to M2 phenotype is necessary to suppress inflammation. Inflammatory responses caused by the SARS-CoV-2 can aggregate alveolar macrophages. Following the activation of TLRs and PRRs, these resident macrophages are highly prone to polarization toward the M1 phenotype. Activation of M1 macrophages in large amount causes 'macrophage activation syndrome' (MAS), which is involved in SARS-CoV-2 induced ARDS. Therefore, switching macrophage polarization from M1 to M2 phenotype in the early phase of the disease can resolve inflammation and reduce excessive pro-inflammatory cytokines production within cytokine storm (Gracia-Hernandez et al., 2020; Kosyreva et al., 2021).

Acute respiratory distress syndrome cause mortality in 70% of sever COVID-19 cases (Hojyo et al., 2020). The immediate recognition and treatment of cytokine storm would improve the outcome of the disease. Thus, inhibiting the cytokine storm could be a potential therapeutic target to decrease mortality rate in severe COVID-19 patients (Ragab et al., 2020).

Role of ACE2 and RAS in COVID-19

In addition to the dysregulation of the immune system, dysfunction of the RAS due to the downregulation of ACE2 is associated with the mortality of COVID-19 patients. Both mechanisms are directly or indirectly associated with cytokine storm that promotes vascular hyperpermeability and vascular edema which is leading to hypercoagulation and eventually multi-organ damage (Choudhary et al., 2020). ACE2 has a crucial role in lung protection. In normal physiology of the body, ACE2 catalyzes the degradation of Ang-II into Ang-(1-7). Then, Ang-(1-7) binds to Mas receptors and exerts its positive effects, including anti-inflammatory and anti-fibrotic effects (South et al., 2020). Ang-(1-7) by acting via the Mas receptor inhibits NF-κB signaling pathway and interferes with inflammation (Issa et al., 2021). Ang-(1-7) reduces expression of pro-inflammatory cytokines, promotes anti-inflammatory M2 phenotype, and reduces pro-inflammatory M1 phenotype (de Carvalho Santuchi et al., 2019; Magalhaes et al., 2020). On the other hand, Ang-II induces pro-inflammatory and pro-fibrotic pathways through AT1R receptors. Ang-II causes vascular damage through inflammatory mechanisms. The high prevalence of thromboembolism in COVID-19 patients is due to endothelial cell damage as a result of increased inflammatory cytokines. ACE2 counteracts the negative effects of Ang-II by balancing the ACE2/Ang (1-7)/MasR and ACE/Ang-II/AT1R

pathways and prevents cytokine storm. Improper activation of macrophages leads to increased inflammation, activation of thrombotic pathways, Ang-II and bradykinin storm, which are the main causes of vascular inflammation (Banu et al., 2020).

ACE2 plays an important role in regulating inflammatory responses by lowering Ang-II levels. Following SARS-CoV-2 binding to ACE2 receptor, internalization of the receptor causes ACE2 levels to decrease. In SARS-CoV-2 infection, increased activity of the ACE-Ang-II axis relative to the ACE2-Ang-(1-7) axis causes acute lung damage. ACE2 downregulation leads to pulmonary vascular hyperpermeability and pulmonary edema, which eventually induce ARDS. ACE2 is expressed in the endothelial cells of blood vessels, epithelial cells of the lung, heart, intestine, and kidney, as well as in the CNS, so the virus can cause complications in various organs. Due to the increased vascular permeability, blood clot formation occurs (coagulation), which leads to multi-organ damage and ultimately death (Choudhary et al., 2020; Jafarzadeh et al., 2020; South et al., 2020).

Angiotensin converting enzyme 2 has been shown to have protective effects on various organs. ACE2/Ang-(1-7)/MasR pathway provides direct protection for lungs against destructive agents, improves insulin resistance in the liver, and reduces production of inflammatory cytokines and thus protects the kidneys against inflammation and fibrosis. On the other hand, the absence of ACE2 in the lung leads to inflammation, fibrosis and edema in lungs, oxidative stress and production of inflammatory cytokines and fibrosis in liver, and expression of hypoxia-related genes in cardiovascular system, increase in blood pressure and eventually heart disorders (Banu et al., 2020).

There are many reports that COVID-19 increases the incidence of stroke in patients. Damage of the cardiovascular and cerebrovascular system following SARS-CoV-2 infection may be attributed to the downregulation of ACE2. ACE2 downregulation impairs the balance of the RAS and results in uncontrolled hypertension, and may cause subsequent rupture of microaneurysms and aneurism of cerebral vessels. Downregulation of ACE2 also promotes inflammation, coagulation, and endothelial dysfunction. The main role of angiotensin II as a vasoconstrictor is well known. Downregulation of ACE2 augments this effect, causing uncontrolled hypertension, and may also induce rupture of cerebral microaneurysm in individuals with chronic hypertension (Rahmawati et al., 2021). Therefore, inhibiting the imbalance of RAS system may contribute to the prevention of the negative effects of the virus.

NF-κB Pathway Involved in Cytokine Storm

NF-κB is a major transcription factor that induces expression of various pro-inflammatory genes. NF-κB pathway plays a critical role in SARS-CoV-2 induced inflammation as it has been reported that hyper-activation of the NF-κB pathway implicates in the pathogenesis of the severe COVID-19 patients. The SARS-CoV-2 spike protein subunit 1 itself activates the pro-inflammatory NF-κB pathway and, in consequence, induces production of pro-inflammatory cytokines and chemokine and cause damage in lung epithelial cells (Kircheis et al., 2020).

The SARS-CoV-2 can cause a shift from protecting ACE2/RAS axis into the AngII/AT1R axis. The AngII-AT1R pathway triggers NF-κB/IL-6/STAT3 pathway which can activate the IL-6 amplifier via a positive feedback loop of NF-κB and STAT3 co-activation. IL-6 amplifier enhances production of pro-inflammatory cytokines, leading to cytokine storm (Hirano and Murakami, 2020). It has been shown that inhibition of NF-κB pathway can inhibit cytokine storm. It has demonstrated that inhibition of the nuclear translocation of the transcription factor NF-κB reduced the release of the pro-inflammatory cytokines and chemokine and interfered with their damaging effects such as multi-organ tissue damage and ARDS. Therefore, targeting the cytokine pathway via inhibiting NF-κB pathway have pronounced clinical effect in critical COVID-19 patients (Kircheis et al., 2020).

Chimeric 8P9R for Early Neutralization of SARS-CoV-2

P9R, which is a defensin-like peptide, is known for its antiviral activity against pH-dependent viruses (Zhao et al., 2020). PH-dependent viruses including rhinovirus, A(H1N1)pdm09 virus, A(H7N9) virus and coronaviruses (SARS-CoV, MERS-CoV, and SARS-CoV-2) are a group of viruses that a key step of their life cycle depends on endosomal acidification (Vigant et al., 2015). The positive charge of P9R can efficiently inhibit protons to move from cytosol into the endosome and therefore prevent endosomal acidification. Similar to P9R, well-known anti-malaria drugs chloroquine and hydroxychloroquine can also inhibit endosomal acidification in several viruses including zika virus (Li et al., 2017), enterovirus-A71 (Tan et al., 2018), and SARS-CoV-2 (Liu et al., 2020; Wang et al., 2020). Another drug that acts via disturbing endosomal acidification is niclosamide (an anti-parasitic drug) that has been proved to inhibit rhinovirus, influenza virus, and dengue virus (Jurgeit et al., 2012; Kao et al., 2018). Another interesting fact about P9R peptide is that it can also bind to viruses and inhibit their replication directly (Zhao et al., 2020). *In vivo* antiviral efficacy of P9R was also proved against influenza (H1N1)-infected mice (Zhao et al., 2020).

The drawback of P9R peptide is solely inhibition of the endosomal pathway. SARS-CoV-2 could enter and infect cells via two distinct mechanisms: The endosomal pathway and the TMPRSS2-mediated pathway. Although P9R inhibits the endosomal pathway efficiently, but it has not enough efficacy in inhibiting the TMPRSS2-mediated pathway. This suggests that using a multi-targeting drug or drug combination to block the both entry pathways of coronavirus infection might be more efficient in inhibiting viral replication in patients because different human cells could express ACE2 and TMPRSS2 separately or simultaneously. To solve this problem, 8P9R is suggested, which is a branched form of P9R. A new 2021 study has shown that branched P9R could crosslink viruses to enhance the antiviral activity (Zhao et al., 2021). In this study, Zhao et al. developed a dual-functional antiviral peptide 8P9R which could cross-link viruses to block viral entry on cell surface through

the TMPRSS2-mediated pathway and simultaneously inhibited endosomal acidification to block viral entry through endocytic pathway. While the single P9R lacks the ability to crosslink viruses to form big viral clusters, 8P9R suppresses SARS-CoV-2 infection more potently than P9R and shows significant antiviral activity against SARS-CoV-2 in hamsters and mice (Zhao et al., 2021).

Chimeric peptides are also well-known and they have been used for several purposes. Antibody-peptide chimers have been used in the treatment of cancer. The main application of this method is targeted internalization of toxic enzymes into tumor cells. Chimeric antibody-enzyme proteins help to increase tumor specificity, increase therapeutic potency, and reduce side effects compared to conventional cancer therapies (Andrady et al., 2011). Recently, application of chimeric proteins antibody-antiviral peptide for the treatment of MERS virus was reported (Wang et al., 2019). Thus, the use of a chimeric 8P9R-antispike peptide could efficiently inhibit the SARS-CoV-2 from entering the cells and replicating while providing more specificity for 8P9R to bind SARS-CoV-2.

Exosomes as a Vehicle and a Treatment

Mesenchymal stem cell (MSC)-derived exosomes have been used to modulate inflammatory responses and reduce multi-organ failure (Ghamari et al., 2020). Exosome-based therapies can be effective in treatment of severe pulmonary involvement in COVID-19 patients (Jamshidi et al., 2021). In ARDS, administration of MSC-derived exosomes reduces inflammation and increases regeneration of the alveolar epithelium and repairs the pulmonary endothelium. An animal study revealed that MSCs-derived exosomes detract extravascular lung fluid by 43% with a decline in pulmonary edema and permeability of lung (Dauletova et al., 2021). Modulation of the inflammatory response by exosomes is achieved primarily by decreasing inflammatory cytokines and neutrophil infiltration and subsequently by increasing anti-inflammatory cytokines and differentiating macrophages toward the M2 phenotype (Khalaj et al., 2020). In addition, exosomes stimulate the secretion of surfactant, which prevents the collapse of the alveolar wall. Treatment with exosomes has been shown to stop the progression of fibrosis (Rezakhani et al., 2021).

It should be noted that the advantage of using exosomes is that they can be loaded with different types of cargo, including drugs, mRNA, miRNA and protein, to improve the therapeutic potential (Babajani et al., 2020). Due to the specific structure of the exosomes, various drugs can be inserted into them; so, they can be used in a variety of diseases as drug delivery systems (Gardin et al., 2020). Exosomes containing spike protein have also been used as a new vaccine against SARS-CoV infections. Recently, administration of exosomes by inhalation was suggested in the treatment of respiratory diseases. This method has less pain and invasion, faster onset of action, and the possibility of using lower doses to achieve the same therapeutic effect compared to the oral or injection routes (Heo et al., 2019). Thus, exosomes could be used as a vehicle to transport the new proposed treatment to the cells and also as a regenerative strategy after the ARDS caused by COVID-19.

Soluble ACE2 Therapy for Balancing the RAS System

So far, therapies such as recombinant ACE2 and AT1R inhibitors have been used to treat COVID-19 patients. A 2020 study showed that human recombinant soluble ACE2 protein (hrsACE2) could significantly inhibit SARS-CoV-2-induced infection. They also showed that SARS-CoV-2, which can directly infect human organoids, was inhibited by hrsACE2 (Monteil et al., 2020). Also in 2020, the first use of hrsACE2 and a positive result was reported in a patient with COVID-19. Treatment with hrsACE2 caused the significant decrease of patient's Ang-II and increase of Ang-(1-7) and (1-9). The concentration of inflammatory cytokines also dropped dramatically. According to the results of this report, it seems that hrsACE2 can have protective enzymatic effects in COVID-19 patients (Zoufaly et al., 2020; Krishnamurthy et al., 2021). So, sACE2 may contribute to make a balance between Ang-II and Ang-(1-7) and also prevent inflammation caused by the virus.

STRATEGY

Considering the supporting evidences, there are two major phases in COVID-19 immunity. The first phase is elimination of the virus and inhibit disease progression into sever stages and the second phase is inflammatory state which leads to cytokine storm. So, in addition to boosting the immune responses, inhibiting viral replication can be helpful in the first phase and could reduce the burden on the immune system specially in patients who may lack innate immune responses. Moreover, inhibition of progression into cytokine storm in a precision way by using sensitivity to a critical inflammatory factor (NF-κB) in each person could use as a personalized therapeutic approach.

Here, for the first time, a two-step strategy which applies for both phases of the COVID-19 (viral clearance and inflammatory damaging phase) is introduced.

Using 8P9R chimeric peptide and sACE2 by utilization of exosome-liposome hybrids in the form of a phase-dependent two-step therapeutic strategy could inhibit the viral intracellular pathway and also inhibit progression into cytokine storm in a personalized manner. This strategy is sensitive to inflammatory state in individuals, so it can make improvements in COVID-19 mortality outcome. This strategy consists of two major phases:

1. In the first phase, it is possible to inhibit intracellular pathways which are crucial in viral replication by using a chimeric peptide that is specifically binds to the virus. 8P9R Chimeric Peptide specifically enters the virus-infected cells by binding to the virus and subsequently inhibit the acidification of the endosomes environment and thus prevent the virus escape into the cytosol and also inhibit virus replication. Therefore, the viral load would be reduced and the patients would not progress into hyper-inflammatory state. The proposed effects of 8P9R chimeric peptide in inhibiting the intracellular mechanisms are shown in **Figure 1**.

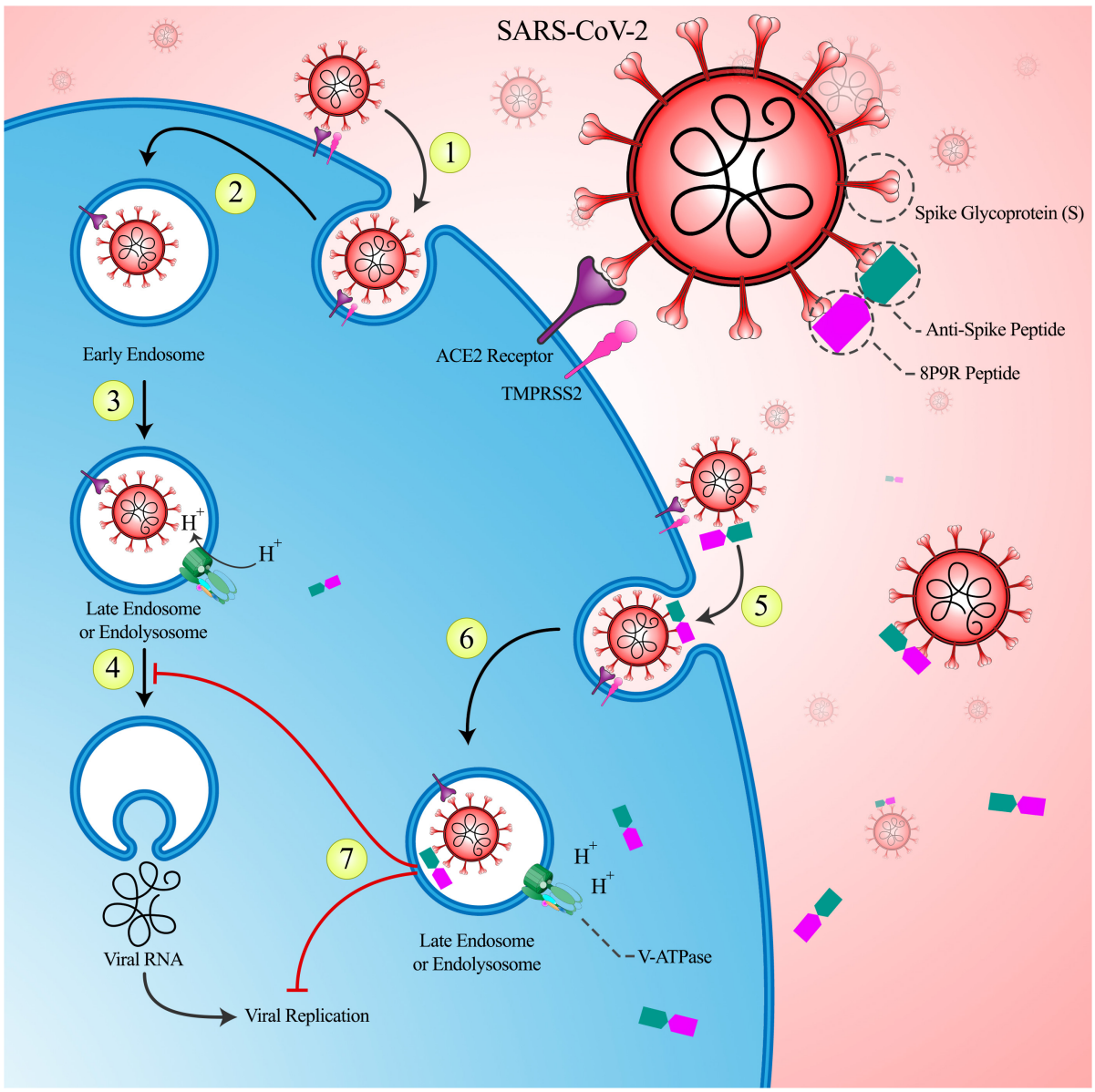

FIGURE 1 | The role of intervention in the primary phase of the COVID-19: (1) The SARS-CoV-2 virus enters the host cell via angiotensin converting enzyme 2 (ACE2) receptors with the help of TMPRSS-2. (2) Following the complete entrance of the virus, the early endosome forms. (3) The environment of the early endosome becomes acidic, as it matures and turns into late endosome. This acidification is caused by inward flow of protons through V-ATPase channels. (4) The acidification of endosome helps the virus to escape to the cytoplasm and move on with its replication. (5) The designed chimeric 8P9R peptide is able to attach to the spike protein of the virus. This attachment can prevent the viral ligand (spike protein) from bonding to its receptor and therefore neutralizes the virus. Thereafter, this peptide would internalize alongside with the virus to help the host cell to battle against the virus with the help of its inherent antiviral characteristics. (6) This peptide has a strong positive charge that interferes with the inward flow of protons and attenuates the acidic environment of endosome and thus prevents the viral escape. (7) Not only this peptide is able to prevent viral escape via attenuated endosomal acidification, but also it can directly interfere with viral replication and therefore decrease the viral load.

2. In the second phase, the goal is to prevent the negative consequences of the ACE2 downregulation in patients who are progressing into the hyper-inflammatory state. To achieve this goal, soluble form of ACE2 could be a potential therapeutic option. As mentioned above, ACE2/Ang II imbalance caused by the virus leads to a shift in immune system to an inflammatory state. This inflammatory state consists of macrophages switching to M1 phenotype and producing pro-inflammatory cytokines. As an important key, NF-κB is the main factor that regulates and activates inflammatory and immune responses such as inflammatory cytokine release and switching immune cells into inflammatory phenotype like M1 macrophages which are main mechanisms of creating cytokine storm

in COVID-19. Hence, using NF-κB-sensitive promoter to control the expression of sACE2 gene is an appropriate conditional switch that is sensitive to progression into inflammation. Indeed, it can induce expression of sACE2 just in parallel with the activating inflammatory pathways. Furthermore, sACE2 can also acts as a decoy receptor in addition to its enzymatic activity. This viral trapping can reduce viral load. The negative effects of the downregulation of ACE2 and the role of sACE2 in preventing the inflammatory state is shown in **Figure 2**.

In this paper, MSC-derived exosomes are suggested as a biological carrier for the designed vector. Due to low immunogenicity of the exosomes, the designed vector can be transferred in an immune reaction-free condition. In addition, the immunomodulatory and regenerative effects of these exosomes have been reported in COVID-19 patients (Jayaramayya et al., 2020; Pinky et al., 2021). To extend the exosomes capability and efficacy, preparing a liposome-exosome hybrid would be an appropriate option.

EVALUATION OF STRATEGY

To evaluate the strategy, first a specific liposome-exosome hybrid will be prepared to increase the function of the exosomes based on previous protocols (Sato et al., 2016). This specific hybrid contains a non-viral episomal vector containing GFP,

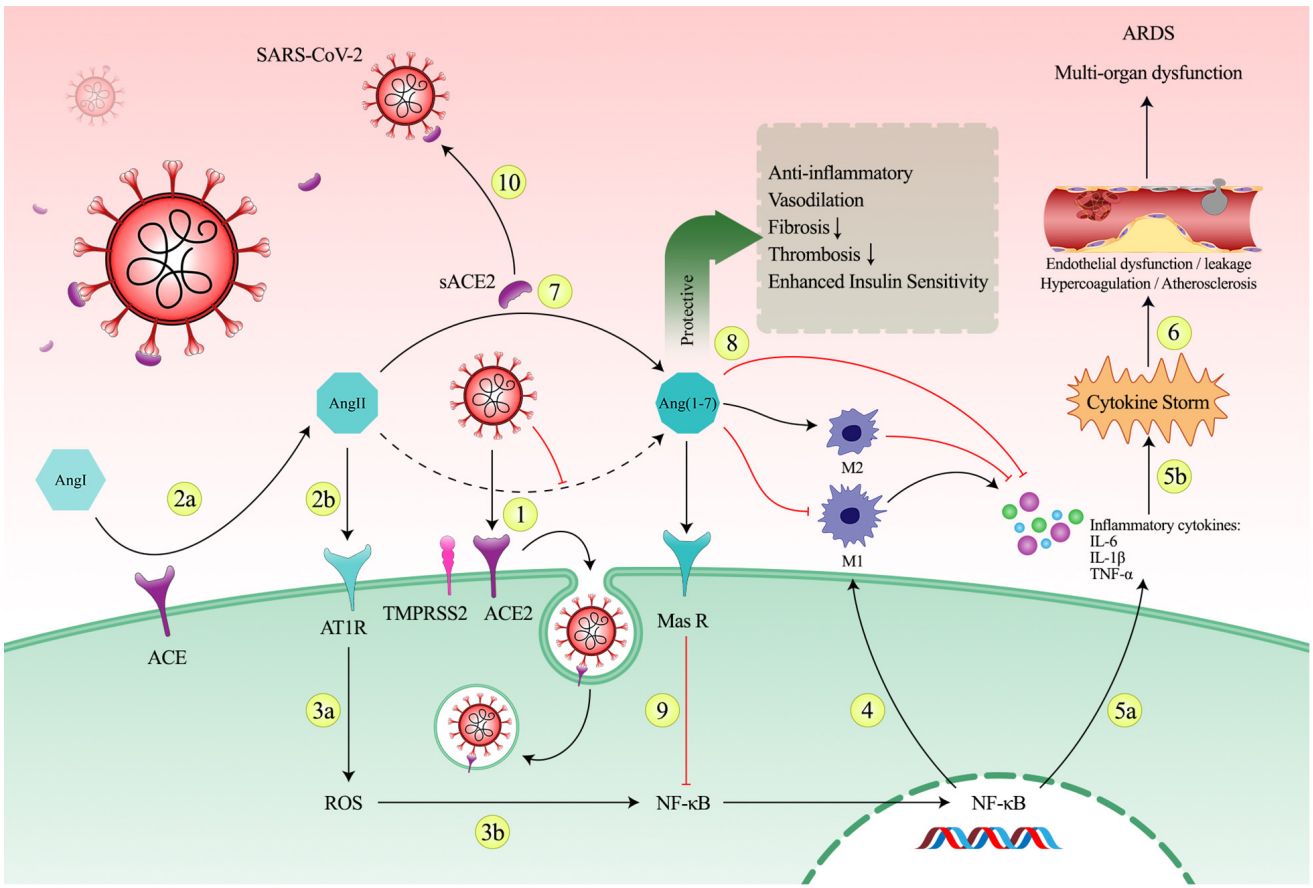

FIGURE 2 | The imbalance in the renin-angiotensin system (RAS) system caused by SARS-CoV-2 and the role of sACE2 in prevention of progression into the cytokine storm. (1) Following the entry of the virus into the host cell via the ACE2 receptor-mediated endocytosis, decreasing of the plasma membrane ACE2 levels and conversion of Ang II to Ang 1-7 occurs. (2a, 2b) Reduced ACE2 level in the cell membrane results in Ang II accumulation due to ACE enzymatic activity and in turn detrimental consequences of ACE2 depletion. Accumulation of Ang II may cause a shift in RAS axis toward the harmful arm of RAS system through AT1R over-activation. (3a, 3b) AngII/AT1R axis activation enhances ROS production which stimulates the nuclear translocation of the transcription factor NF-κB. (4) NF-κB is a key regulator of macrophage-driven inflammatory reaction and plays a critical role in polarization toward the pro-inflammatory M1 phenotype. (5a, 5b) Following activation of NF-κB, excessive release of pro-inflammatory cytokines, including IL-6, IL-1, and TNF-α, leads to cytokine storm. (6) The deleterious inflammation can cause endothelial dysfunction, endothelial leakage, hyper-coagulation, thrombosis, atherosclerosis, and eventually leads to tissue damage and sever multi-organ destruction. (7) In parallel with increased expression of inflammatory genes, sACE2 expression can make balance in RAS system as sACE2 can perform its enzymatic activity in the extracellular space. (8) Production of Ang 1-7 via enzymatic cleavage possesses counteracting effects against negative consequences of Ang II. Ang 1-7 has protective functions mainly in heart, kidney, brain, and lungs. Ang 1-7 promotes anti-inflammatory M2 phenotype and reduces pro-inflammatory M1 phenotype and reduces expression of pro-inflammatory cytokines. (9) Ang-(1-7) by acting via the Mas receptor inhibits NF-κB signaling pathways and interferes with inflammation. (10) sACE2 can also block viruses as a decoy receptor and this viral trapping reduces viral load.

CMV promoter, 8P9R, NF-κB sensitive promoter and sACE2 sequences. *In vitro* and *in vivo* studies will be conducted to determine the efficacy of suggested treatment.

In vitro Studies

Primary human lung epithelial cell and macrophage cell cultures are needed for *in vitro* experiments. In primary human lung epithelial cell culture, the efficacy of the chimeric 8P9R will be determined. To do so, at first the cell must be transfected with SARS-CoV-2 virus. After treating with vector-containing exosome-liposome hybrids, the viral load could be measured by VNT assay and cellular viability will be measured by TUNNEL assay.

On the other hand, macrophage cell culture can be used, as a critical part of both innate and adaptive immunity, to determine the anti-inflammatory effects of sACE2. Similar to epithelial cell culture, it is necessary to transfect macrophages with SARS-CoV-2 and treat them with vector-containing exosome-liposome hybrids. Then, the expression level of inflammatory cytokines such as IL-6, TNFα and IL-1β [via real-time-quantitative polymerase chain reaction (RT-qPCR) at mRNA level and western blot at protein level] and cellular viability should be measured by TUNNEL assay.

At both cell cultures, the transduction success rate of exosome-liposome hybrids (by expression of GFP protein) and expression of 8P9R and sACE2 (by RT-qPCR at mRNA level and western blot at protein level) must be approved at first.

It is expected that exosome-liposome hybrids transferring to cells results in two main changes: (1) reduction of viral load in infected cells and (2) reduction of inflammatory cytokines levels at both mRNA and protein levels.

In vivo Studies

For *in vivo* experiments, transgenic mice that are able to express human ACE2 receptors should be used as an animal model. First animals should be infected with SARS-CoV-2 virus. The route of exosome-liposome hybrids administration could be intravenous injection or nasal administration. Then the efficacy of vector-containing exosome-liposome hybrids in treatment of COVID-19 is evaluated. Similar to *in vitro* studies, the internalization of exosome-liposome hybrids should be apprived (via expression of GFP protein) and expression of 8P9R and sACE2 (via RT-qPCR at mRNA level and western blot at protein level). After administration of exosome-liposome hybrids, viral load (by RT-qPCR), expression of inflammatory cytokines such as IL-6, TNFα, and IL-1β (by RT-qPCR at mRNA level and western blot at protein level) and remission of clinical symptoms such as fever, respiratory distress can be measured.

Then, these changes should be compared with the untreated COVID-19 animal model. For this purpose, 20 days after the treatment, the blood samples will be obtained from both treated (both inhalation and I.V injection groups) and untreated groups, and viral load will be measured using RT-qPCR and the expression of inflammatory cytokines will be measured using RT-qPCR at mRNA level and western blot at protein level.

It is expected that exosome-liposome hybrids transferring to treated groups results in: (1) reduction of viral load, (2) reduction of inflammatory cytokines levels at both mRNA and protein level, (3) remission of clinical symptoms, and (4) reduction of mortality rate.

It must be investigated to find out which route of administration (inhalation vs. I.V injection) is more effective to overcome the disease and its consequences.

DISCUSSION AND FUTURE DIRECTION

After more than a year from the rise of COVID-19, several antiviral drugs and other pharmaceutical agents have been investigated as potential therapeutic candidates, but shockingly, most of them had been failed during clinical trials and yet supportive treatment strategies are the foremost critical portion of administration in serious cases of COVID-19 (CDC, 2021). Fortunately, the development of efficient vaccines against COVID-19 and initiation of global vaccination have fundamentally assisted with combating the virus, yet the number of daily casualties is significant (WHO, 2021). Then again, the constant emergence of new mutated variants of the virus has a bit compromised the absolute sense of relief caused by the vaccination, as there are certain mutations shown to be able to assist the virus with escaping from polyclonal human serum antibodies (Callaway, 2021; Greaney et al., 2021). Therefore, the lack of a proper treatment strategy, especially in severe cases, is still a global concern.

As mentioned earlier, the pathogenesis of the SARS-CoV-2 virus is extremely phase-dependent. "Phase-dependent" means that the immune response against the virus alters concurrently as the patient's condition deteriorates, this immunological alteration is the reason that this two-step therapeutic strategy has been suggested. In this strategy which can battle the virus in a personalized manner, a designed vector automatically evaluates the patient's condition and gives the appropriate response by expressing the right gene. In other words, two different goals are being pursued at the same time: (1) reducing the viral load and helping the immune system to combat the virus on arrival and (2) predicting and resolving the severe form of the disease including ARDS and multiple organ damage caused by unstrained cytokine storm.

In the primary phase of the disease, the virus enters the host's body and starts to infect host cells by its well-known receptor ACE2 (Yao et al., 2020). In this phase, a proper response of the immune system is crucial, so that the viral load decreases and the least possible number of cells become infected. Both innate and adaptive immune systems are recruited in this phase (Chowdhury et al., 2020). Regarding the adaptive immune system, neutralizing antibodies (from humoral immunity) can efficiently block the viral entry and limit the infection; in addition to their protective effects at the later stage of disease and their ability to prevent infection relapse (Chowdhury et al., 2020). Moreover, it has been shown that helper T cells and cytotoxic T cells (from cellular immunity) are extremely

important for the clearance of infected cells (Kumar et al., 2020). In the suggested treatment, chimeric 8P9R peptide acts in this phase and prevents both viral entry and proliferation. Following the attachments of the virus to ACE2, the viral entry occurs through either the TMPRSS2-mediated pathway or the endocytic pathway (Zhao et al., 2021). Chloroquine, which interferes with endocytic pathway through inhibiting endosomal inhibition, was introduced as a potential therapeutic agent in the very first beginning of the emergence of the disease, but unfortunately has been shown that has an adverse clinical efficacy and might be accompanied by cardiac side effects (Hashem et al., 2020; Axfors et al., 2021). Zhao et al., who have previously designed two antiviral peptides P9 (Zhao et al., 2016) and P9R (Zhao et al., 2020), recently introduced a novel cross-linking peptide 8P9R (Zhao et al., 2021). This cross-linking peptide exerts its antiviral effects through both inhibiting viral entry (via TMPRSS2 pathway) and interfering with endosomal acidification and viral escape to the cytosol (via endocytic pathway). They reported promising results on 8P9R peptide antiviral activity in their *in vitro* studies on hamsters and mice. Seeing these promising results, it is better to take advantage of 8P9R as the main defense tool in the first phase of the disease. It is also suggested that attaching an anti-spike peptide and developing a chimeric P89R that specifically attaches to the spike protein of the virus could lead to even better results. In other words, this chimeric peptide not only entails beneficial properties of 8P9R but also can directly attach to the spike protein of the virus and decrease the chance of its attachment to ACE2, and prevent viral entry. In addition, several repurposed drugs have been introduced which are able to prevent viral entry via different mechanisms. These repurposed drugs, including fluoxetine and itraconazole, which can dysregulate the endosomal cholesterol release and increase endolysosomal cholesterol storage have been suggested as synergistic combinatory therapeutic agents alongside with conventional antiviral therapies (Creeden et al., 2021; Dechaumes et al., 2021; Schloer et al., 2021). According to these promising results, using repurposed therapeutic agents as adjuvant therapy alongside with the two-step strategy seems to be very helpful with comprehensive blockade of viral entry.

In the second phase of the disease, when the disease becomes more severe, the protective role of the immune system is not prominent anymore and its unrestrained activity leads to cytokine storm, which is accompanied by a poor prognosis. ARDS, which is one of the main causes of death in COVID-19 patients, is significantly associated with cytokine storm syndrome (Kumar et al., 2020; Yao et al., 2020). It has been shown that, during ARDS, immune effector cells release very high amount of pro-inflammatory cytokines, and this uncontrolled activation of the immune system can lead to multi-organ (especially respiratory system) failure (Kumar et al., 2020). In this paper, for this phase of the disease, using sACE2 in addition to the baseline expression of chimeric 8P9R is suggested. The reason for choosing this agent is based on the pathophysiology of the virus and the response of the human body to it. By the expression of sACE2 which will be administered to the patients, the downregulated level of ACE2, that have been caused by viral entry and receptor internalization (Mahmudpour et al., 2020), is compensated and the ACE pathway (that can lead to cytokine storm) will not be upregulated anymore. In other words, we are counting on the enzymatic activity of the sACE2 to re-establish the impaired balance between ACE (leading to the production of inflammatory Ang 2 and activation of AT1 receptors) and ACE2 (leading to the production of anti-inflammatory Ang (1-7) and activation of MAS receptors) pathways. In addition, sACE2 can act as a decoy receptor, neutralize the virus inside the bloodstream, and prevent its entry to the host cell. Using recombinant sACE2 against SARS-CoV-2 is not a novel suggestion and this theory has been tested in the previous studies. Monteil et al. (2020) used Vero cells and engineered human blood vessel and kidney organoids to investigate the efficacy of clinical-grade recombinant soluble ACE2 (rsACE2) in SARS-CoV-2 blockade. In another study, Zoufaly et al. (2020) reported the first case of treatment with human rsACE2 in a patient with severe COVID-19. The SARS-CoC-2 virus rapidly disappeared from the serum, and later from the nasal cavity and lungs, following hrsACE2 therapy. However, due to the limitations of this study, the fact that this viral clearance was because of the treatment course or part of the natural history of the disease remains unclear. Recently, the therapeutic effect of combination therapy with remdesivir and rsACE2 was investigated on Vero E6 and kidney organoid and revealed that a strong additive effect can be reached at sub-toxic concentrations (Monteil et al., 2020). To make the treatment strategy personalized and sensitive to the condition of patients, using NF-κB-susceptible [a transcription factor for inflammatory cytokines (Kircheis et al., 2020)] promoter for the ACE2 gene is proposed, so that the expression of sACE2 will be proportionate to the level of the ACE2 downregulation.

At last, the reason for using MSC derived exosomes as carriers is that the immunomodulatory and regenerative effects of these exosomes have been confirmed in COVID-19 patients (Jayaramayya et al., 2020; Gupta et al., 2021). However, there is a potential limitation for using exosomes. The capacity of the MSC-derived exosomes is limited, therefore the vector, which entails several sequences, might not fit in the natural exosomes. To bypass this limitation, the MSC-derived exosomes can be hybridized with appropriate sized synthetic liposomes, similar to a method that Sato et al. (2016) for engineering the exosome-liposome hybrids.

According to the paradoxical response of the immune system to SARS-CoV-2 and its detrimental effect on the outcome of patients, developing an appropriate therapeutic approach is necessary. Therefore, a new two-steps strategy against COVID-19 targeting both viral entry and inflammatory state was introduced in this paper.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

YN, KV, AE, and HN: conceptualization and writing — review and editing. YN, KV, and AE: investigation and writing — original draft preparation. HN: supervision. All authors read and agreed to the submitted version of the manuscript.

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REVIEW
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Application of Human Induced Pluripotent Stem Cell-Derived Cellular and Organoid Models for COVID-19 Research

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The outbreak of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and its rapid international spread has caused the coronavirus disease 2019 (COVID-19) pandemics, which is a global public health crisis. Thus, there is an urgent need to establish biological models to study the pathology of SARS-CoV-2 infection, which not only involves respiratory failure, but also includes dysregulation of other organs and systems, including the brain, heart, liver, intestines, pancreas, kidneys, eyes, and so on. Cellular and organoid models derived from human induced pluripotent stem cells (iPSCs) are ideal tools for *in vitro* simulation of viral life cycles and drug screening to prevent the reemergence of coronavirus. These iPSC-derived models could recapitulate the functions and physiology of various human cell types and assemble the complex microenvironments similar with those in the human organs; therefore, they can improve the study efficiency of viral infection mechanisms, mimic the natural host-virus interaction, and be suited for long-term experiments. In this review, we focus on the application of *in vitro* iPSC-derived cellular and organoid models in COVID-19 studies.

Keywords: induced pluripotent stem cell, organoid, cellular model, COVID-19, SARS-CoV-2

INTRODUCTION

Since its outbreak in 2019, the coronavirus disease (COVID-19) pandemics have infected more than 190 million people and caused more than 4 million deaths[1]. COVID-19 is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), an enveloped positive-sense single-stranded RNA virus. It enters the host cells using angiotensin-converting enzyme 2 (ACE2) as the cell surface receptor and transmembrane serine protease 2 (TMPRSS2) as the effector to cleave its spike protein (Hoffmann et al., 2020). SARS-CoV-2 spreads mainly through the respiratory

[1]www.worldometers.info/coronavirus

tract (Lu et al., 2020). Respiratory failure is the most common cause of death in COVID-19 patients; meanwhile, severe fatal manifestations are also observed in other organs, such as the brain, heart, liver, intestines, and pancreas (Puelles et al., 2020). Therefore, it is of particular importance to find models that can imitate the natural host-virus interactions of SARS-CoV-2 in a variety of human cell types and organs, thus improving the study efficiency for identifying key molecular regulators and the underlying mechanisms of virus infection and disease progression.

There have been both animal models (e.g., transgenic mice expressing human ACE2 and non-human primates) and cell line models (e.g., African green monkey Vero E6 cells and human cancer cell lines) available for COVID-19 research (Takayama, 2020). However, animal models are quite costly and display very different physiological characteristics from human, and cell line models have limitations in reproducing the viral life cycle and the pathology of COVID-19 in different human organs and tissues that contain a variety of cell types (Liu et al., 2011). For example, the entry routes of SARS-CoV-2 vary between cell lines and human tissues, as do immune responses and host-virus interactions (Milewska et al., 2020). In addition, human cancer cells carry numerous tumor-associated mutations, such as P53 mutations, which could interfere the SARS-CoV-2 infection (Ma-Lauer et al., 2016). Therefore, there is an urgent need to establish more cost-efficient and human-relevant models for COVID-19 research.

The emergence of human induced pluripotent stem cells (iPSCs) has enabled derivation of functional human cells or organoids to model human diseases, including infectious diseases, to develop new therapeutic approaches and to promote drug discovery (Yu et al., 2007; Luo et al., 2015a, 2018), without ethical issues like the human embryonic stem cells (Luo et al., 2014). For example, functional liver organoids generated by human iPSCs have been developed as personalized models of hepatitis B virus (HBV) infection, which is a powerful long-term platform for both research and drug screening of HBV (Nie et al., 2018). Recently, iPSC-derived cellular and organoid models have been utilized to simulate SARS-CoV-2 infections in multiple organs, not only the lung, but also the heart, brain, liver, intestines, and pancreas (Jacob et al., 2020; Yu et al., 2020) (**Figure 1**). These studies have demonstrated that SARS-CoV-2 can infect and propagate in a variety of cell types, leading to transcriptional alternations that indicate inflammatory responses and changes in cell function (Marshall, 2020). The purpose of this review is to describe the usefulness of these iPSC-derived cellular and organoid models in simulating human cellular physiology and tissue microenvironment, and enabling the study of host-virus interaction and drug screening for the COVID-19 disease, thus leading to a more comprehensive understanding of the SARS-CoV-2 pathogenesis.

LUNG ORGANOIDS

Human iPSC-derived airway and alveolar organoids have been developed and used for studying the processes of SARS-CoV-2 infection and transmission in the lungs (Pei et al., 2020). With these organoid models, the researchers have determined the cellular tropism of the virus. Ciliated cells, club cells, and alveolar type II cells (AT2) cells, which are arranged from the proximal to the distal airway and terminal alveoli in sequence, have been confirmed as SARS-CoV-2-targeted cells in the study (Mulay et al., 2021). Moreover, the viral infection downregulates metabolic processes, particularly the lipid metabolism, which, together with the already known upregulation of immune responses, is another molecular feature of SARS-CoV-2-infected cells (Pei et al., 2020). On the other hand, infected SARS-CoV-2 can decrease the level of its target ACE2 on the host cell surface through a variety of mechanisms. A comprehensive analysis of these information might enable better understanding about the viral pathogenesis and discovery therapeutic targets for the treatment of COVID-19 (Pei et al., 2020).

In addition, the early immune responses to viral infections were investigated using iPSC-derived AT2 (iAT2) cells (Huang J. et al., 2020). The results showed that AT2 cells are a central component of the inflammatory signaling that responds to SARS-CoV-2 infection within the first 24 h, with NF-κB signaling predominating this response (Huang J. et al., 2020). These findings are consistent with those in newly purified primary AT2 cells infected with SARS-CoV-2 (Mulay et al., 2021). They also observed cellular stress, toxicity, iAT cell death, and significant loss of surfactant genes expression in their model (Huang J. et al., 2020). These findings may be clinically relevant, as similar results were found in lung autopsies of multiple individuals who died of COVID-19 (Bradley et al., 2020; Hou et al., 2020). Other researchers have previously shown that the primary AT2 cells can be infected with SARS-CoV in the body (Qian et al., 2013); it has also been recently shown that AT2 cells may help promote lung regeneration in COVID-19 survivors (Chen J. et al., 2020). The correlation between AT2 cells and SARS-CoV-2 infection was further emphasized.

Furthermore, the researchers have demonstrated that human iPSC-derived lung cells and organoids could serve as powerful platforms for discovering and testing anti-SARS-CoV-2 drugs (Huang J. et al., 2020; Pei et al., 2020). They have demonstrated that Remdesivir, a predrug nucleotide analog that inhibits virus replication (Eastman et al., 2020), CB6, a human neutralizing antibody (Shi R. et al., 2020), as well as TMPRSS2 protease inhibition could effectively inhibit the replication of SARS-CoV-2 in the iPSC-derived models. These results are consistent with those in fundamental studies using primary cell models and in clinical trials (Beigel et al., 2020; Wang M. et al., 2020). Therefore, iPSC-derived *in vitro* human models could be employed to identify and test therapeutic entities for the treatment of COVID-19.

Taken together, the above results demonstrated that lung cells and organoids derived from human iPSCs can be utilized as pathophysiological models to study the potential mechanisms of SARS-CoV-2 transmission and to identify and test COVID-19 therapeutic agents (Pei et al., 2020).

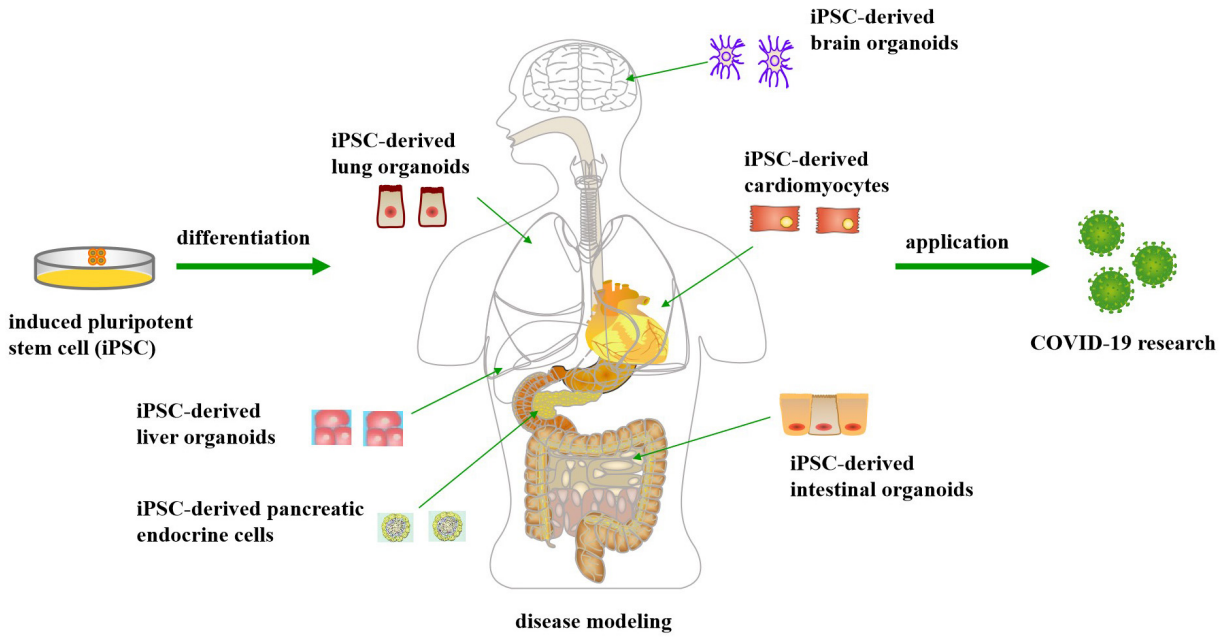

FIGURE 1 | Application of human induced pluripotent stem cell-derived cellular and organoid models for COVID-19 research.

CARDIOMYOCYTES

There is growing evidence that patients with COVID-19 exhibit severe heart complications, elevated biomarkers of heart damage, and cardiac function deterioration, including cardiovascular complications such as cardiomyopathy, acute myocardial infarction, arrhythmia, and heart failure, greatly increasing the risk of death (Aggarwal et al., 2020a; Bansal, 2020; Long et al., 2020; Madjid et al., 2020; Shi S. et al., 2020). Several compounds and antibodies, such as Remdesivir, Olumiant + Remdesivir, Casirivimab + Imdevimab, Bamlanivimab + Etesevimab, Sotrovimab and Tocilizumab are currently licensed drug for treatment of COVID-19 patients under emergency use authorization[2]. However, there is limited safety information on these recommended drugs, especially since heart toxicity caused by the drug can lead to lethal complications, including myocardial ischemia, arrhythmias, and heart failure. Therefore, it is critical to evaluate any potential adverse effects on the cardiovascular system associated with current COVID-19 medications to avoid fatal side effects (Aggarwal et al., 2020b).

Human iPSC-derived cardiomyocytes (CMs) can be utilized to recapitulate cardiac pathophysiology and are considered as one of the most promising sources for cardic disease modeling, heart repair and cardiac toxicology screening (Mitcheson et al., 1998; Lan et al., 2013; Moreno and Pearson, 2013; Sharma et al., 2014, 2017; Burridge et al., 2016). In this context, iPSC-CMs are recommended as a reliable method of heart toxicity examination in the comprehensive *in vitro* proarrhythmia assay (CiPA), as a non-clinical safety pharmacological paradigm, to circumvent the limitations of existing methods used in preclinical safety assessment of drugs (Gintant et al., 2016; Goineau and Castagné, 2017; Sala et al., 2017). Choi et al. (2020) have shown that iPSC-CMs acutely treated with Remdesivir show a risk of arrhythmia and changes in the electrophysiological properties of myocardial cells in a dose-dependent manner, indicating that overdose or drug accumulation may lead to noteworthy adverse heart reactions, such as prolonged QT interstitial periods. In addition, they have demonstrated that iPSC-CMs not only allow SARS-CoV-2 infection, but also support the propagation of infectious viral particles (Choi et al., 2020).

INTESTINAL ORGANOIDS

Up to 50% of COVID-19 patients develop gastrointestinal symptoms associated with longer duration and increased severity of the disease (Luo et al., 2020; Wang F. et al., 2020; Wei et al., 2020; Xiao et al., 2020). However, it remains debatable whether the virus found in the intestines is contagious, as few studies have examined infectious viruses in feces (Zang et al., 2020). In cell culture, primary intestinal cells are highly susceptible to SARS-CoV-2 and can produce infectious viral particles. Intestinal organoids can quickly grow from adult stem cells derived from cells of large intestine and small intestine biopsy tissue (Sato et al., 2011). Stem cell-derived intestinal organoids have similar characteristics with primary intestinal cells and have been widely used to study viral infection (Sato et al., 2011; Ettayebi et al., 2016).

Studies have employed human iPSC-derived intestinal organoids to study SARS-CoV-2 tropisms in different intestinal cell types. In both *in vivo* and iPSC-derived organoid models,

[2]www.fda.gov/emergency-preparedness-and-response/mcm-legal-regulatory-and-policy-framework/emergency-use-authorization#coviddrugs

ACE2 is strongly expressed in the small intestine, as well as TMPRSS2. In contrast, colon organoids have lower ACE2 expression (Zang et al., 2020). In intestinal organs, TMPRSS4 performs the same functions as TMPRSS2 to support virus entry (Zang et al., 2020). Experiments have shown that two groups of small intestinal organoid models can be infected with SARS-CoV-2 (Lamers et al., 2020; Zang et al., 2020). In these organoid models, SARS-CoV-2, like its closest relative SARS-CoV, mainly infects mature enterocytes and dividing cells (Lamers et al., 2020; Zang et al., 2020). On the other hand, studies have shown that when the SARS-CoV-2 viruses are cultured in gastric fluid in the large intestine and small intestine, they quickly lose their infectious power (Simoneau and Ott, 2020; Zang et al., 2020). Therefore, even though viral particles were found in the feces occasionally, it might not be the primary pathway for virus transmission.

Furthermore, human iPSC-derived intestinal organoids generate valuable pathological models for studying the underlying mechanisms of intestinal SARS-CoV-2 infection. It is reported that SARS-CoV-2 actively infects both proximally and distally patterned intestinal organoids, thus resulting in production of infectious viral particles and significant transcriptional alterations, such as upregulation of the interferon-related genes, in multiple epithelial cell types (Mithal et al., 2021). Another organoid study shows that SARS-CoV-2 can infect all intestinal cell types investigated except goblet cells, and disrupt intestinal integrity, which might be the cause of diarrhea and other gastrointestinal symptoms associated with COVID-19 (Krüger et al., 2021).

More importantly, these organoids can serve as a potential platform for organ-specific drug testing and drug screening. For example, the researchers found that Remdesivir therapy inhibits SARS-CoV-2 viral replication in the intestinal organoids (Krüger et al., 2021). Therefore, clinical treatment with this drug may prevent intestinal damage caused by SARS-CoV-2 and relieve intestinal symptoms.

BRAIN ORGANOIDS

Approximately 36.4% of the COVID-19 patients develop a variety of neurological complications, ranging from loss of smell, nausea, dizziness, and headache to encephalopathy and stroke (Mao et al., 2020). RNA of SARS-COV-2 was found in the brains of some patients (Helms et al., 2020; Moriguchi et al., 2020). The mechanisms of SARS-COV-2 disrupting the brain-blood barrier and infecting the central nervous system (CNS) draw great concerns (Li et al., 2020). Studies have shown that CNS infections may lead to the pathophysiological and clinical manifestations associated with COVID-19 (Steardo et al., 2020). Therefore, it is necessary to establish a suitable *in vitro* model to study nerve infection by SARS-CoV-2.

IPSC-derived brain organoids are valuable tools for investigating the biological properties of SARS-CoV-2 in the CNS (Bullen et al., 2020; Jacob et al., 2020). Studies using iPSC-derive brain organoids find that choroid plexus epithelial cells are the main target of SARS-CoV-2 infection in the CNS; meanwhile, neurons, and astrocytes are sparsely infected (Jacob et al., 2020; Pellegrini et al., 2020). This finding is consistent with the discovery that the choroid plexus region is one of the hotspots of ACE2 expression in the CNS under inflammatory status, and it is more susceptible to SARS-CoV-2 infection than other regions (Chen R. et al., 2020). After SARS-CoV-2 infection, increased cellular remodeling and inflammatory responses were observed in choroid plexus epithelial cells (Chen R. et al., 2020). This finding provides an evidence that SARS-CoV-2 infection of the choroid plexus leads to disruptions in blood-cerebrospinal fluid barrier (BCB) integrity. Researchers have proposed that BCB decomposition can promote entry of the virus as well as immune cells expressing cytokines into the cerebrospinal fluid and brain tissue, potentially causing nerve inflammation (Pellegrini et al., 2020).

Whether SARS-CoV-2 propagates in the CNS remains controversial. Some studies have reported successful SARS-CoV-2 replication in brain organoids (Zhang et al., 2020), while the others suggest that the viral replication and proliferation are less efficient in the brain organoids (Ramani et al., 2020). These opposite results may be due to differences in the methods of establishing brain organoid models and the multiplicities of infection (MOI) used in these studies. In the former study, the neural progenitor cell (NPC) population is also found to be a target of SARS-CoV-2 (Ramani et al., 2020). This is an important finding as NPCs are responsible for repairing brain lesions caused by degenerative diseases or malignancies (Zhu et al., 2013, 2014; Luo and Zhu, 2014; Luo et al., 2015b). The impaired NPC population might be the reason for late or incomplete recovery of neurological manifestations in COVID-19 patients. On the other hand, it should be noted that although human brain organoids represent valuable models for *in vitro* research on SARS-CoV-2 infection, they merely have simplified structures (e.g., vein systems and blood-brain barriers) like the developing fetal brain, and lack mature cells, particularly asteroid cells and astrocytes (Jacob et al., 2020; Ramani et al., 2020).

PANCREATIC ENDOCRINE CELLS

Single-cell RNA-seq analysis of primary human islets has indicated that both alpha cells and beta cells are positive for ACE2 and TMPRSS2 (Yang et al., 2020). Further validation experiments in humanized mouse model established by human iPSC-derived pancreatic endocrine cells confirm that both alpha cells and beta cells are susceptible to SARS-CoV-2 (Yang et al., 2020). Infected pancreatic endocrine cells display higher expression of pathways associated with apoptosis and viral infection, and lower expression of pathways associated with the normal functions of alpha cells and beta cells, thus leading to increased cell death and loss of cell identities (Yang et al., 2020). The infected cells are also expressing higher levels of chemokines, including CCL2, CXCL5, and CXCL6, and other degenerative factors and cytokines, which is similar with cells found in autopsies from COVID-19 patients (Blanco-Melo et al., 2020).

LIVER ORGANOIDS

More than 50% of COVID-19 patients have symptoms of viral hepatitis (Ong et al., 2020). Particularly, the proportion of patients with liver damage in patients with severe symptoms is much higher than that in patients with mild symptoms (Huang C. et al., 2020). However, due to the lack of suitable research models, it was unclear whether the liver damages were caused by a direct viral infection or by systemic dysfunctions, such as cytokine storms.

Relevant studies have deployed human organoids as tools to study the correlations of SARS-CoV-2 infection and liver damage at both cellular and molecular levels (Huch et al., 2015; Dutta and Clevers, 2017). Some studies have established human liver organoid models which are capable of preserving the ACE2 + /TMPRSS2 + cholangiocyte population in long-term 3-dimensional (3D) cultures (Yang et al., 2020; Zhao et al., 2020). Further, the studies have confirmed that the cholangiocytes in the human liver organoids are permissive to SARS-CoV-2 infection and supporting strong viral propagation (Yang et al., 2020; Zhao et al., 2020). Moreover, SARS-CoV-2 infection induces cell death in the host cholangiocytes. Thus, these studies supports that liver damage in COVID-19 patients might be caused by gallbladder decomposition and subsequent accumulation of bile acid due to viral infection (Yang et al., 2020; Zhao et al., 2020).

DISCUSSION AND FUTURE PERSPECTIVES

Most COVID-19 patients have mild respiratory symptoms; however, up to 20% of the patients develop severe pneumonia, leading to multi-organ failures and even death (Zhu N. et al., 2020). The development of iPSC technologies and the resulting differentiated cell models have dramatically accelerated studies of the pathogenesis of SARS-CoV-2 in various organs. Current studies have employed iPSC-derived cells and organoids, including iAT2 cells, cardiomyocytes, pancreatic endocrine cells, lung organoids, brain organoids, intestinal organoids, liver organoids, to investigate the underlying mechanisms of SARS-CoV-2 infection (Ardestani and Maedler, 2020; Bojkova et al., 2020; Huang J. et al., 2020; Jacob et al., 2020; Pei et al., 2020; Mithal et al., 2021). As COVID-19 could also cause kidney malfunctions (Chen N. et al., 2020), kidney organoids derived from iPSCs may be a potential research model as well (Phipson et al., 2019).

These iPSC-derived models are suited for leveraging the powers of the latest genetic tools, such as single-cell RNA-seq and CRISPR techniques, for COVID-19 research (Zhou et al., 2020). Single-cell RNA-seq techniques have been developed to investigate the viral tropisms and host transcriptional responses to viruses or external stimuli in complex organs and tissues (Zhu et al., 2018; Luo et al., 2019; Zhu D. et al., 2020). In the above studies, single-cell RNA-seq has been employed to screen for cell types that are positive of the SARS-CoV-2 receptor ACE2 and effector protease TMPRSS2, and to illustrate the transcriptional alternations after viral infection (Huang J. et al., 2020; Yang et al., 2020; Zang et al., 2020). Furthermore, the CRISPR system can be utilized to create genetically modified iPSC models for mechanism study of the candidate genetic factors (Luo et al., 2015c, 2016; Gkogkou et al., 2020; Kim et al., 2020; Zang et al., 2020; Yu, 2021). For example, with a CRISPR-engineered iPSC model, researchers have demonstrated that the single-nucleotide polymorphism rs4702, which is a common genetic variant located in the 3′ UTR of the protease FURIN, influences the SARS-CoV-2 permissiveness of alveolar and neuronal cells (Dobrindt et al., 2021).

On the other hand, there are a few limitations of iPSC-derived cellular and organoid platforms, such as inadequate complexity to reflect real tissue microenvironment and cell-cell interactions, and lack of real-time monitoring methods for 3D cultures. For examples, although the above studies have described the susceptibility of various cell types to SARS-CoV-2 infection with these iPSC derivatives, it is unclear whether these cell types are the primary targets for viral infection in COVID-19 without a more thorough analysis of samples from primary patients (Lamouroux et al., 2020). Besides, these iPSC-derived models are simplified ones compared to the fully functional and reacting human organs. In the future, these platforms should be exploited to produce more complex organoid models, including the immune system components that are missing from the current analysis (Yang et al., 2020).

CONCLUSION

In conclusion, human iPSC-derived cells and organoids can be used as ideal models for studying the mechanisms of viral infection and drug screening. Particularly, with the organoid models, the viral tropism and host responses of different cell types could be observed in a single system. These iPSC models help us better understand the pathogenesis of SARS-CoV-2 in different organs and systems, and provide powerful drug test and discovery platforms.

AUTHOR CONTRIBUTIONS

YL and DZ conceived the study. YL, MZ, and DZ prepared the figure. YL, MZ, YpC, YoC, and DZ wrote the manuscript. All authors read and approved the final manuscript.

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REVIEW
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Immunopathology and Immunosenescence, the Immunological Key Words of Severe COVID-19. Is There a Role for Stem Cell Transplantation?

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The outcomes of Coronavirus disease-2019 (COVID-19) vary depending on the age, health status and sex of an individual, ranging from asymptomatic to lethal. From an immunologic viewpoint, the final severe lung damage observed in COVID-19 should be caused by cytokine storm, driven mainly by interleukin-6 and other pro-inflammatory cytokines. However, which immunopathogenic status precedes this "cytokine storm" and why the male older population is more severely affected, are currently unanswered questions. The aging of the immune system, i.e., immunosenescence, closely associated with a low-grade inflammatory status called "inflammageing," should play a key role. The remodeling of both innate and adaptive immune response observed with aging can partly explain the age gradient in severity and mortality of COVID-19. This review discusses how aging impacts the immune response to the virus, focusing on possible strategies to rejuvenate the immune system with stem cell-based therapies. Indeed, due to immunomodulatory and anti-inflammatory properties, multipotent mesenchymal stem cells (MSCs) are a worth-considering option against COVID-19 adverse outcomes.

Keywords: COVID-19, cytokine storm, immunopathology, immunosenescence, stem cell transplantation

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INTRODUCTION

From December 2019 onwards, SARS coronavirus 2 (SARS-CoV-2) infection spread across the globe so rapidly that, on March 11, 2020, the World Health Organization (WHO) declared coronavirus disease-2019 (COVID-19) a global pandemic. While the pandemic is still in progress, the global scientific and medical community is focusing its resources on developing both anti-COVID-19 vaccines and therapeutic drugs, in order to gain control over the quick spread of this novel coronavirus.

SARS-CoV-2 is a *Betacoronavirus* of the *Coronaviridae* family that, like the other respiratory coronaviruses, is transmitted primarily via respiratory droplets, preferentially infecting lung alveolar epithelial cells using the human angiotensin-converting enzyme II (ACE2) as an entry

receptor and the transmembrane serine protease 2 (TMPRSS2), which cleaves viral spike, for priming (Hoffmann et al., 2020; Wan et al., 2020).

This virus may infect people regardless of age, sex, and ethnicity but with different symptomatic presentation and outcomes. Indeed, clinical manifestations can range from being mild to severe respiratory disease, with a mean incubation period of approximately 5 days before first symptoms onset (Lauer et al., 2020; Li Q. et al., 2020; McAloon et al., 2020).

Fever, fatigue and dry cough are the most common symptoms in patients with mild or moderate disease. Severe characteristic symptoms include pneumonia, dyspnoea and subsequently acute respiratory distress syndrome (ARDS) that requires intensive care unit (ICU) admission with intubation and mechanical ventilation and can be fatal. In some cases, infected patients can present as either asymptomatic or paucisymptomatic, with little or no clinical manifestations (Baj et al., 2020; Chen N. et al., 2020; Gao Z. et al., 2021).

Although the majority of COVID-19 cases are mild symptomatic with a moderate case-fatality rate (Fu L. et al., 2020), older patients are at higher risk of getting severe COVID-19 disease, including hospitalization, and death (Wu and McGoogan, 2020). Last April, there were 140,322,903 confirmed COVID-19 cases and 3,003,794 deaths reported worldwide (World Health Organization, 2021), while in Italy, 3,870,131 total cases of COVID-19 and 116,927 deaths have been registered (Ministero della Salute, 2021). As of March 31, 2021, almost 86% of SARS-CoV-2 positive Italian patients that died were patients over 70 years old, with a mean age of 81 years and a median of 82 years (range 0–109), more than 30 years higher than that registered in infected people (median age 47; 51.2% females, 48.8% males) (Epicentro. Istituto Superiore di Sanità, 2021). Disease severity in older patients is due to not only the viral infection but also to host-related factors that make the individual particularly vulnerable to more severe outcomes of the infection.

An optimal model for identifying the host factors involved in susceptibility and/or protection to SARS-CoV-2 infection is represented by the centenarians, i.e., subjects who have reached ten or more decades of life, escaping the common age-related diseases. Their ability to repair damages and respond well to stressors is due to a combination of "positive features," including intrinsic (genetic), extrinsic (environmental), and stochastic factors. Chance and life circumstances, nutrition, physical activity, and environmental exposures, pathogen burden and biology, with sex, genetic and epigenetic factors are all factors that may contribute to the longevity phenotype (Caruso, 2019). As a result, centenarians exhibit an optimal performance of the immune system, which contributes to their surprising tendency to recover from COVID-19 and complications (Lio et al., 2021). On the other hand, since the immune response to SARS-CoV-2 involves both innate and acquired immunity, dysregulation of the immune system has important consequences for the ability of the immune system to mount an effective response against the pathogen. Therefore, the remodeling of the immune response observed with aging can partly explain the age gradient in severity and mortality of COVID-19 (Cunha et al., 2020). In this context, the favorable prognosis and less severe signs on admission when hospitalized observed in young people may depend on an efficient acquired immune response against the virus (Gómez-Belda et al., 2021). On the other hand, the older immune system exhibits both cellular and humoral responses that are not efficient enough to limit infection and innate responses that are more prone to uncontrolled activation (Cunha et al., 2020). Various age-related quantitative and qualitative impairments have been reported in both innate and acquired immune responses, and they are commonly known as "immunosenescence." Reduction in the ability to respond to new antigens, attributed to insufficient production of naïve immune cells, and increased memory responses, due to the lifelong antigen-exposure during persistent viral infections, have been associated with immunosenescence (Brunner et al., 2011; Aiello et al., 2019). With advancing age, there is gradual atrophy of the thymus, responsible for the development and selection of immunocompetent T cells (Kurd and Robey, 2016). This phenomenon, called thymic involution, involves structural changes in tissue mass and cellularity, as well as functional decline, ultimately resulting in a significantly decreased thymic output of naïve T cells, important for the response to novel pathogens that have not yet been encountered, contributing to shrinkage of the T cell diversity repertoire (Palmer, 2013). Since SARS-CoV-2 is a novel coronavirus with seemingly no prior sensitization, the naïve T cell decline caused by thymic involution might contribute to the increased vulnerability to severe COVID-19 outcomes in the older population, as well as reduced vaccination efficiency (Genebat et al., 2021; Wang et al., 2021).

Another characteristic of the older immune system is the presence of a chronic, low-grade inflammation, called inflammageing (Franceschi and Campisi, 2014), associated with increased production of pro-inflammatory cytokines, acute phase proteins, and oxidative stress (Fulop et al., 2018; Aiello et al., 2019). Inflammageing is considered one of the main risk factors for many age-related degenerative diseases and is caused by several factors, including life-long antigenic stimulation of the innate immune system by chronic infections, senescent cells and their senescence-associated secretory phenotype (SASP) (De Martinis et al., 2005; Hearps et al., 2012; Franceschi and Campisi, 2014). As a consequence, there is an increase in serum inflammatory mediators with age, including interleukin (IL)-6, tumor necrosis factor (TNF)-α, IL-1, and C-reactive protein (CRP) (Franceschi and Campisi, 2014; Barbé-Tuana et al., 2020). This inflammatory environment could exacerbate the immune response to the SARS-CoV-2. It has previously been seen that a dysregulated and excessive inflammatory response is responsible for sub-optimal T cells, antibody responses and impaired virus clearance which ultimately lead to worsening of SARS-CoV-1 and MERS-CoV infections, discovered in November 2002 and June 2012 respectively, with approximately 80 and 50% genetic similarity with SARS-CoV-2 respectively (To et al., 2013; Channappanavar and Perlman, 2017; Rabaan et al., 2020). Similarly, in SARS-CoV-2 infection, uncontrolled inflammation leads to severe and irreversible multi-organ damage, especially on the cardiac, hepatic and renal systems (Tian et al., 2020).

In the early phases of infection occurring in the respiratory tract, SARS-CoV-2 infected cells produce damage-associated

molecular patterns (DAMP), such as ATP and nucleic acids, which are recognized by neighboring epithelial cells, endothelial cells and alveolar macrophages. This triggers the production of various pro-inflammatory cytokines and chemokines, allowing the recruitment of innate and acquired immune cells to the site of infection, including T cells (Tay et al., 2020; Ortega et al., 2021). In a healthy immune system, this initial inflammation recruits and activates virus-specific CD4+ and CD8+ T cells that kill the infected cells before the virus spreads, indirectly via induction of neutralizing antibody production o directly by inducing apoptosis, respectively. In a dysfunctional immune response, the accumulation of immune cells in the lungs can lead to the overproduction of pro-inflammatory cytokines, causing local cell and tissue damage with systemic implications (Tay et al., 2020). Considering that some cytokines can be both helpful in controlling the infection progression and harmful to the host, it is not easy to define a border between a regulated and a dysregulated response to a severe infection. Elevated levels of circulating cytokines, known as "cytokine storm," were reported in COVID-19 patients, with variability in onset and duration in the early stages of infection but with convergent and often overlapping late-stage clinical manifestations (Fajgenbaum and June, 2020).

SARS-CoV-2 can infect also monocytes and macrophages through ACE2, by receptor-mediated endocytosis, and independently on ACE2, by pattern recognition receptors (PPRs), mechanisms. That results in their activation and secretion of large amounts of pro-inflammatory mediators, which contribute to local tissue and systemic inflammation (Jafarzadeh et al., 2020; Moore and June, 2020). Thus, in a scenario where the SARS-CoV-2 infection itself causes an excessive and uncontrolled systemic release of pro-inflammatory cytokines, the inflammageing process can aggravate this condition, amplifying inflammatory events and favoring the insurgence of ARDS, multiple organ failure, and finally death in severe COVID-19 older patients (Meftahi et al., 2020). For these reasons, it could be suggested that early identification of individuals at higher risk of virus-induced cytokine storm could help to determine the most effective treatment to prevent irreversible organ damage contributing to the relatively high mortality in older COVID-19 patients.

Also, it has been hypothesized that the accumulation of highly differentiated T cells with senescence-like features, together with inflammageing, may contribute to the destruction of lung tissue observed in severe COVID-19 patients (Akbar and Gilroy, 2020). Several studies have shown an expansion of senescent T cells in old individuals, driven by ongoing sub-clinical responses to chronic or persistent infections (Pawelec, 2018). These senescent T cells can express the natural killer cell receptors (NKRs) and can infiltrate into various tissues including the lung, acting as natural killer (NK) cells, thus promoting cytotoxicity against cells that expressed NKR ligands without prior antigen-specific priming (Pereira et al., 2020). According to this hypothesis, inflammation induced by SARS-CoV-2 infection in the lungs, hypothetically exacerbated by inflammatory monocytes infiltrated, would induce the expression of NKR ligands by epithelial cells of the host respiratory tract and lungs, and these may be recognized and killed by NK-like T cells (Akbar and Gilroy, 2020).

The severity of the disease, therefore, seems to depend primarily on the age and immune dysfunction. Nevertheless, these alone may still not be sufficient to explain the highest number of CFR in over 70 years population in South Korea, Spain, China, and Italy (Our World in data, 2021). Another important risk factor associated with the complications of COVID-19 is the presence of comorbidities, such as arterial hypertension, diabetes, and obesity, and older people are more likely to have these conditions (Guo L. et al., 2020; Li X. et al., 2020; Peña et al., 2020; Fresán et al., 2021). Many reports have confirmed the association between comorbidities and COVID-19 severity (Gao Y. D. et al., 2021), although it should be kept in mind that their high prevalence in the older population could be a confounding factor. The role of genetics is under scrutiny (Pojero et al., 2021).

In addition to all these risk factors, there is growing evidence of sex differences in severity and mortality of COVID-19. Specifically, when the fatality rate was stratified for gender, it was seen that males accounted for the majority of COVID-19 deaths with similar pieces of evidence in different countries (Alkhouli et al., 2020; Heras et al., 2020; Jin et al., 2020; Pradhan and Olsson, 2020; Sha et al., 2021). In Italy, the spread of COVID-19 has hit females (51.2%) slightly more than males (48.8%). However, data suggest that the mortality rate was higher for males than it was for females. Among all age groups, female Italian patients who died with complications associated with SARS-CoV-2 infection represented 43.9% and were older than men (median age: females 86 years, males 80 years). The mortality rate for female patients was 2.6%, while for male patients was 3.5% (Epicentro. Istituto Superiore di Sanità, 2021). This gender discrepancy of COVID-19–related morbidity and mortality can be attributed to a combination of biological sex differences, including differences in sex hormones involved in inflammatory processes and in expression levels of ACE2 and TMPRSS2, but literature data are not yet coherent (Gebhard et al., 2020; Haitao et al., 2020).

The role of sex hormones in immune responses has already been extensively investigated (Moulton, 2018; Márquez et al., 2020b; Fathi et al., 2021). Females show higher levels of estrogen and progesterone, with considerable fluctuations throughout the life span, while testosterone is more expressed in males, and these hormonal differences are assumed to play an important role in the innate and acquired immune responses (Fathi et al., 2021). For example, there is a large body of evidence for the role of estrogen in B cell development and activation and in various functions of T cells, in particular CD4+ T cells (T helper or TH) including differentiation, activation, cytokine production, homeostasis, and regulatory functions (Moulton, 2018). Estrogens have also been implicated in the homeostasis and activation of plasmacytoid dendritic cells (pDCs), key cells in antiviral immunity. Human female pDCs produce significantly higher interferon (IFN)-α levels in response to viral nucleic acids or Toll-like receptor (TLR) 7 agonists than pDCs derived from males, resulting in stronger secondary activation of CD8+ T cells (T cytotoxic or CTL) with impact on viral infection response

(Berghöfer et al., 2006; Meier et al., 2009). The stronger responses observed in females result in faster pathogen clearance and a better response to vaccines, but could also be responsible for greater susceptibility to autoimmune diseases, considering that 80% of systemic autoimmune disorders occurring in females (Klein and Flanagan, 2016). In comparison to estrogens, several studies reported that exposure to androgens induces suppression of immune reactivity, affecting different immune cell subsets (Gubbels Bupp and Jorgensen, 2018). Detailed analysis on the sex differences in immune phenotype in SARS-CoV-2 infection has detected that, although plasma levels of many inflammatory cytokines and chemokines were generally elevated in patients, IL-8, IL-18, and CCL5 levels were significantly higher in male compared to female patients (Takahashi et al., 2020), suggesting a high risk of the cytokine storm.

It has also been proposed that the high plasma levels of ACE2 observed in males may partly explain their increased susceptibility to SARS-CoV-2 infection (Sama et al., 2020), although another study reported no differences in ACE2 expression in various human tissues between males and females or between young and old people (Li M. Y. et al., 2020). Similarly, an association between increased COVID-19 severity in males and expression of TMPRSS2, which is involved in cellular entry of the virus, has been theorized. TMPRSS2 gene expression is modulated by the androgen receptor signaling and increases following exposure to androgens (Lin et al., 1999). The upregulated TMPRSS2 expression in response to androgens could explain the sex-discrepancy in COVID-19 outcomes (Mjaess et al., 2020).

Therefore, immunosenescence, inflammageing (i.e., immunopathology), comorbidities, as well as male gender, play an important role in contributing to increased vulnerability to severe SARS-CoV-2 infection outcomes in older adults. Thus, the heterogeneity of patients makes it difficult to identify a consensus COVID-19 immune signature. In addition, some variability in the parameters used to classify the severity of COVID-19 could contribute to making it more difficult to compare the findings. However, understanding the immunological status prior to encountering the novel virus and how this is affected during infection provides interesting targets for better understanding the pathogenesis and treatment of SARS-CoV-2 infection.

In this review, we provide a detailed assessment of how aging impacts the immune response to the virus, providing when possible a parallelism with COVID-19-induced immune changes, focusing on possible strategies to rejuvenate the immune system with stem cell-based therapies. Indeed, due to immunomodulatory and anti-inflammatory properties, multipotent mesenchymal stem cells (MSCs) are a worth-considering option against COVID-19 adverse outcomes (Moradinasab et al., 2021).

CYTOKINE STORM IN COVID-19 DISEASE

Several analyses of the cytokine profile from COVID-19 patients have correlated the cytokine storm with lung tissue damage, multi-organ failure, and disease severity. Cellular origins of this large amount of cytokines is difficult to identify since SARS-CoV-2 infection requires the engagement of several immune cells, including the innate macrophages, dendritic cells, natural killer cells, and T and B lymphocytes (Ragab et al., 2020). It was reported significantly increased plasma levels of pro-inflammatory cytokines, including IL-1β, IL-6, IL-7, IL-8, IL-9, IL-10, interferon (IFN)-γ, and TNF-α in patients with COVID-19 than in healthy adults, but similar levels of IL-5, IL12p70, and IL-15. In addition, severe cases admitted to the ICU have shown higher plasma concentrations of IL-2, IL-7, IL-10, TNF-α, and other cytokines and chemokines than non-ICU patients (Huang et al., 2020). Levels of IL-6, mediator of the acute inflammatory response, and C-reactive protein (CRP), non-specific marker of inflammation, in these patients continue to increase over time and is significantly associated with ARDS and death in COVID-19 patients (Herold et al., 2020; Ruan et al., 2020; Wu et al., 2020; Zhou F. et al., 2020). Also analysis in bronchoalveolar lavage (BAL) fluid, a source of information of the microenvironment on bronchioles and lung alveoli, have shown higher levels of inflammatory cytokines, including IL-8, IL-6, and IL-1β, in severe cases compared to patients with moderate COVID-19 infection (Liao et al., 2020). When compared to survivor recovered patients, remarkably higher serum levels of IL-2R, IL-6, IL-8, IL-10, and TNF-α at admission were found in COVID-19 deceased patients, with a rapid increase during hospitalization, reinforcing the idea that dynamics of these cytokines and related receptors were highly associated with disease outcome (Cui et al., 2020). Likewise, it is reasonable to assume that the appropriate production of anti-inflammatory cytokines can counterbalance the systemic inflammation that occurs after infection. In male centenarians, it has been observed overexpression of anti-inflammatory variants in immune/inflammatory genes that could protect them from damaging effects of the cytokine storm associated with COVID-19 disease (Lio et al., 2021). Thus, cytokine storm prevention and control may be a crucial strategy in the treatment of COVID-19 patients. In line with that, it was proposed different anti-inflammatory strategies, in order to avoid or at least alleviate the cytokine storm. Among these, the use of IL-37 and IL-38 was suggested as potential therapeutic cytokines capable of inhibiting IL-1β and IL-6 (Conti et al., 2020).

INNATE IMMUNITY: NATURAL KILLER (NK) CELLS

Natural killer cells are large granular lymphocytes, which play an important role in the host first line of defense against viral targets, via cell-mediated cytotoxicity mechanisms (CD56lowCD16+ subset) and secretion of pro-inflammatory cytokines (CD56hiCD16- subset). Among cytokines secreted by NK cells there are IFN-γ, with both antiviral and immune enhancing capabilities, TNF-α and granulocyte/macrophage colony-stimulating factor (GM-CSF) that can modulate the function of other innate and acquired immune cells (Mirandola et al., 2004; Paul and Lal, 2017). Thus, in addition

to directly lysing the infected cell, NK cells are able to activate and mobilize other immune cells, including DCs, involved in the early stages of infection. Evidence of the critical role of NK cells in limiting viral infection prior to the induction of acquired immune responses has been provided by clinical studies of individuals who are deficient in NK cells and/or their functions, leading to current infections by viral pathogens (Orange, 2013). Likewise, changes in NK cell count, phenotype, and functions during aging may have a direct impact on COVID-19 disease progression.

Although less consistently than T cells (see below), NK cell numbers are reduced in COVID-19 patients, particularly evident in severe cases compared with mild disease patients and healthy controls (Jiang et al., 2020; Zheng M. et al., 2020; Belaid et al., 2021; Taghiloo et al., 2021), and their functional exhaustion was correlated with disease progression (Zheng M. et al., 2020). Single-cell analyses of bronchoalveolar samples of patients with COVID-19 have shown the presence of NK cells in the immune cell population analyzed, suggesting NK cell trafficking into the site of infection (Chua et al., 2020; Liao et al., 2020). Cytotoxic CD56low NK cells were depleted mainly in patients requiring mechanical ventilation, whereas cytokine-secreting CD56hi NK cells were significantly depleted in all COVID-19 patients (Wilk et al., 2020; **Table 1**).

Conversely, an increase in peripheral NK cell frequency and absolute cell count has been observed with advancing age, with a decreasing fraction and cytokines production of CD56hi NK cell subset and an expansion of CD56low NK cells (Solana et al., 2014; Gounder et al., 2018). Whether this correlates with disease severity in the older person is unclear.

Additionally, NK cell cytotoxic and secretory functions are tightly regulated by a dynamic balance between activating and inhibitory signals from an arsenal of membrane receptors, including killer cell immunoglobulin-like receptors (KIRs), randomly generated during NK cell differentiation and maturation (Vidal et al., 2011). Specific KIR/ligand interactions seem to be associated with COVID-19 disease severity as demonstrated in a very recent paper (Bernal et al., 2021) and by preliminary results from our ongoing study (unpublished observations).

OTHER CELLS OF INNATE IMMUNITY: DENDRITIC CELLS (DCS), MONOCYTES, MACROPHAGES, NEUTROPHILS, AND MYELOID DERIVED SUPPRESSOR CELLS (MDSCS)

In addition to NK cells, there are other important players of the innate immune system that provide the host's first line of defense to the virus. Pattern recognition receptors (PRRs) expressed on innate immune cells, such as DCs and macrophages, recognize and bind surface viral epitopes, leading to anti-pathogen responses. The activation of these cells by the virus leads secretion of several inflammatory cytokines and chemokines, which activate and recruit other immune cells generating a positive feedback loop of inflammation (Pérez-Galarza et al., 2021). Aging alters also these actors involved in innate immunity in terms of both number/percentage and functionality, with different quantitative and qualitative consequences for DCs, monocytes, macrophages, neutrophils and myeloid derived suppressor cells (MDSCs) (Agrawal et al., 2017; Ventura et al., 2017; Oh et al., 2019; Feng et al., 2021). These changes in immune response may

TABLE 1 | Observed fluctuation in NK cells in adult COVID-19 patients.

NK cell subsets	Observation	Analyzed patients	Compared group(s)	References
CD16+CD56+	Reduction (%)	Deceased	Survivors	Cui et al., 2020
	Reduction (#)	Deceased	Survivors	Cui et al., 2020; Belaid et al., 2021
	Reduction (#)	Pneumonia patients	HD	Wang F. et al., 2020
	No change (#)	Severe$^{\eta}$	Mild	Wang F. et al., 2020
	Reduction (#)	Severe$^{\eta}$	Mild$^{\zeta}$ /moderate$^{\theta}$	Belaid et al., 2021
	Reduction (#)	Cases	HD	Jiang et al., 2020
CD16+	No change (%)	Cases	HD	Taghiloo et al., 2021
	Reduction (#)	Severe$^{\eta}$	Mild$^{\zeta}$	Taghiloo et al., 2021
CD56+	No change (%)	Cases	HD	Taghiloo et al., 2021
	Reduction (#)	Severe$^{\eta}$	Mild$^{\zeta}$	Taghiloo et al., 2021
CD56low	Reduction (%)	Critical$^{\iota}$	HD	Wilk et al., 2020
CD56hi	Reduction (%)	Cases	HD	Wilk et al., 2020
BAL NK cells	Reduction (%)	Severe$^{\eta}$ /Critical$^{\iota}$	Moderate$^{\theta}$	Liao et al., 2020
	Increase (%)	Moderate$^{\theta}$	HD	Liao et al., 2020

NK cell subset, peripheral blood NK cell subset analyzed in the reported studies; BAL, bronchoalveolar lavage; (%), as frequency values; (#), as absolute number; Cases, COVID-19 patients (irrespective of severity); HD, healthy donors.

$^{\zeta}$*Patients with clinical signs of pneumonia with one of the following: respiratory rate > 30 breaths/min, severe respiratory distress, or SpO_2 < 90% on room air.*

$^{\eta}$*Patients experiencing the following: respiratory failure, respiratory rate > 30 bpm, oxygen saturation < 93% at rest, arterial partial pressure of oxygen (PaO_2)/fraction of inspired oxygen (FiO_2) (PaO_2/FiO_2) ratio < 300 mmHg.*

$^{\theta}$*Fever, signs of airway disease, with or without a tomographic image indicating pneumonia.*

$^{\iota}$*Any of the following: requirement for mechanical ventilation, shock, or concomitant organ failure.*

have an important impact in first phases of viral replication. In the following sections, we will discuss specific alterations in the other innate immunity cells in COVID-19 patients recalling those observed in the older immune system.

Although a clear difference in terms of severity and mortality was demonstrated comparing female and male COVID-19 patients as anticipated, to the best of our knowledge no study deeply explored sex related differences in SARS-CoV-2 infection as regards innate immunity.

Dendritic Cells (DCs)

Dendritic cells are important immune cells, specialized to uptake, process and present antigen to naïve T cells, thus linking innate and acquired immunity. After pathogen internalization through recognition by PRRs, including TLRs, DCs process and present viral peptides to T cells via human leukocyte antigens (HLA)-T cell receptor (TCR) interaction, leading to activation of T cells.

Peripheral DCs are historically classified into three subsets based on their phenotype and functional characteristics: two subsets of myeloid DCs (mDCs) and one subset of plasmacytoid DCs (pDCs). The CD141+ mDC subset, named also myeloid (conventional) DC1 (cDC1), and CD1c+ mDCs, or cDC2, is both the primary source of IL-12, driving a TH1 response, but these cells are also able to secrete IL-6 and TNF-α. CD123+ pDCs, instead, produce high amounts of type I IFNs, critical for antiviral response (Collin et al., 2013; Mathan et al., 2013). An additional subset of DCs, called slanDCs, was identified by the use of M-DC8 monoclonal antibody, directed against the 6-sulfo LacNAc1 -slan- carbohydrate modification of *P*-selectin glycoprotein ligand-1. These cells are highly proficient in secreting inflammatory cytokines in both autoimmunity and infections (Schäkel et al., 1998; Dutertre et al., 2012; Günther et al., 2012; Hänsel et al., 2011, 2013; Tufa et al., 2014, 2016; Micheletti et al., 2016; Iannetta et al., 2019).

As regards the involvement of DCs in determining SARS-CoV-2 infection susceptibility and COVID-19 severity, experimental data demonstrated that TLR7 plays a critical role through viral single-stranded RNA recognition in the

TABLE 2 | Observed fluctuation in DCs and DC subsets in adult COVID-19 patients.

DC type	Observation	Analyzed patients	Compared group(s)	References
DCs	Reduction (%)	Hospitalized	HD	Zhou R. et al., 2020
	Reduction (%)	Convalescent	HD	Zhou R. et al., 2020
CD11c+ cDCs	Reduction (%)	Hospitalized	Convalescent	Zhou R. et al., 2020
	Reduction (%)/	Severe/Critical+	HD	Matic et al., 2020
	Reduction (%)/	Severe/Critical+	Mild/Moderate&	Matic et al., 2020
	No change (%)	Mild$	Convalescent	Neeland et al., 2021
	Increase (%)	Mild/Moderate&	HD	Matic et al., 2020
CD123-CD11c+ myeloid DCs	Reduction (%)	Pneumonia	HD	Zingaropoli et al., 2021
	Reduction (%)	ARDS	non-ARDS	Zingaropoli et al., 2021
CD11c+ slanDCs	Reduction (%)	Pneumonia	HD	Zingaropoli et al., 2021
AXL+SIGLEC6+ pre-DCs	Reduction (#)	Cases	HD	Kvedaraite et al., 2021
AXL+CD1c+ pre-DC2	Reduction (#)	Cases	HD	Kvedaraite et al., 2021
CLEC9A+ cDC1	Reduction (#)	Cases	HD	Kvedaraite et al., 2021
CLEC9A- cDC2*	Reduction (#)	Cases	HD	Kvedaraite et al., 2021
CD163-CD14- DC3	Reduction (#)	Cases	HD	Kvedaraite et al., 2021
CD163-CD14+ DC3	Reduction (#)	Cases	HD	Kvedaraite et al., 2021
CD163+CD14+ DC3	Reduction (#)	Cases	HD	Kvedaraite et al., 2021
CD5+DC2	Reduction (%)	Severe§	Moderate@	Kvedaraite et al., 2021
CD163-CD14- DC3	Reduction (%)	Severe§	Moderate@	Kvedaraite et al., 2021
AXL+CD1c+ pre-DC2	Increase (%)	Severe§	HD	Kvedaraite et al., 2021
CD123+CD11c- pDCs	Reduction (#)	Cases	HD	Kvedaraite et al., 2021
	Reduction (%)	ARDS	non-ARDS	Zingaropoli et al., 2021
	Reduction (%)	Pneumonia	HD	Zingaropoli et al., 2021
CD123+Lyn- pDCs	Reduction (%)/	Severe/Critical+	HD	Matic et al., 2020
	Reduction (%)/	Severe/Critical+	Mild/Moderate&	Matic et al., 2020
	Reduction (%)	Mild/Moderate&	HD	Matic et al., 2020

DC, dendritic cell; cDCs, conventional dendritic cells; pDCs, plasmacytoid dendritic cells; (%), as frequency values; (#), as absolute number; HD, healthy donors; ARDS, acute respiratory distress syndrome; Cases, COVID-19 patients (irrespective of severity).

/No statistically significant data provided.

+Fever or suspected respiratory infection with compromised respiratory function and worsening of respiratory symptoms with the necessity for mechanical ventilation.

&Mild clinical symptoms of upper respiratory tract viral infection and signs of pneumonia without need for supplemental oxygen.

**Including CD5+ DC2 and CD5– DC3.*

@Oxygen saturation -SO_2- between 90 and 94% or 0.5 to 3 L/min oxygen requirement at screening.

§Treated at the intensive care unit -ICU- or at a high-dependency unit.

$Including coryza, headaches, nausea, fever, cough, sore throat, malaise, headaches, and muscle aches.

endosomal compartments of pDCs (Moreno-Eutimio et al., 2020; Onofrio et al., 2020; Kim and Shin, 2021; Gabriele et al., 2021). A clinical study showed that loss-of-function variants in X-linked TLR7 genes were associated with impaired type I and II IFN responses in young males, suggesting that the difference in TLR7 gene dosage between males and females could explain, at least in part, the predisposition of males to developing severe COVID-19 (van der Made et al., 2020).

Numerical changes in DC subsets were investigated as putative factors influencing anti-SARS-CoV-2 responses, and various authors demonstrated that DCs tend to decrease in COVID-19 patients vs. healthy controls, but in some reports, data also reached a statistical significance comparing more severe cases with patients with a milder form of the disease (Matic et al., 2020; Zhou R. et al., 2020; Buttenschön and Mattner, 2021; Kvedaraite et al., 2021; Zingaropoli et al., 2021; **Table 2**).

A more detailed analysis of CD11c+ DCs accumulation/decrease as a function of both age and severity should be urgently performed in adult COVID-19 cases. In fact, non-plasmacytoid DCs are reported as numerically stable in the old people (Agrawal et al., 2017; Oh et al., 2019), but this trend seems to be reverted in severe COVID-19 patients, although an age specific stratification of the cases was not performed (**Table 2**; Matic et al., 2020; Zhou R. et al., 2020; Buttenschön and Mattner, 2021; Kvedaraite et al., 2021; Zingaropoli et al., 2021). Intriguingly, similar data were obtained comparing children in the acute phase of mild disease vs. their convalescent counterpart (Neeland et al., 2021), thus in the case of younger patients, milder disease is accompanied by CD11c+ DC fluctuations resembling those observed in adult severe cases. On the contrary, the contraction of CD11c+ DC pool was not documented comparing adult patients with mild symptoms vs. convalescent subjects (Neeland et al., 2021), and the same DC subset was even reported as increased in mild/moderate patients vs. healthy controls (Matic et al., 2020). Molecular mechanisms causing stability of CD11c+ DCs in older subjects seem to be compromised in both adults with severe disease and children with mild disease, and deserve further investigation.

Instead, plasmacytoid DCs tend to decrease in aging (Agrawal et al., 2017; Oh et al., 2019; Márquez et al., 2020b). A reduction in the plasmacytoid DCs was also reported in COVID-19 patients irrespective of severity (**Table 2**; Arunachalam et al., 2020; Matic et al., 2020; Kvedaraite et al., 2021; Zingaropoli et al., 2021).

During physiological aging processes, DCs exhibit multiple functional defects ranging from mitochondrial dysfunction to an impairment in antigen uptake/presentation and altered cytokine secretion (Giefing-Kröll et al., 2015; Agrawal et al., 2017; Ventura et al., 2017; Oh et al., 2019; Salminen et al., 2019). DCs showed some degree of immunophenotypic/functional alteration also in COVID-19. CD11c+ cDCs in hospitalized patients and convalescent (follow up) outpatients exhibited less surface expression of the co-stimulatory molecule CD86 than DCs in healthy subjects (Zhou R. et al., 2020; Buttenschön and Mattner, 2021). Similarly, expression of CD86 and HLA-DR was uniformly reduced on circulating DC precursor (pre-DC), pre-DC2, CD5+ DC2, CD163- CD14- DC3, CD163- CD14+ DC3, and CD163+ CD14+ DC3 in ICU or high dependency unit severe patients vs. healthy donors (Kvedaraite et al., 2021). Also, cDCs in both hospitalized patients and convalescent (follow up) outpatients showed resistance toward maturation stimuli, and an impaired ability to produce type I IFNs which was more prominent in hospitalized patients (Zhou R. et al., 2020; Buttenschön and Mattner, 2021), recalling a similar panel of IFN secretion defects documented in older individuals (Agrawal et al., 2017). Instead, the ability to answer to type I IFN signaling seemed to be preserved in COVID-19 patients, as demonstrated by the expansion of cell surface receptor tyrosine kinase (AXL) expressing fraction among DC1 pool and the reduced expression of c-kit as measured in DC1 (Rhodes et al., 2019; Kvedaraite et al., 2021). INF-signaling was also highly activated in BAL DC1 (Kvedaraite et al., 2021). However, myeloid DCs showed an impaired ability to answer to TLR stimulation (Arunachalam et al., 2020).

Activation of peripheral blood plasmacytoid DCs was made evident by the decreased expression of CD45RA in COVID-19 patients vs. healthy controls (Kvedaraite et al., 2021). Similarly, a reactive response in COVID-19 patients BAL plasmacytoid DCs was demonstrated by activation of cytokine and chemokine signaling pathways at a transcriptional level (Kvedaraite et al., 2021). However, another report documented an impairment in plasmacytoid DC function, with a reduced production of IFN-α and TNF-α after TLR stimulation (Arunachalam et al., 2020). Plasmacytoid DCs showed less HLA-DR in ICU or high dependency unit severe patients than in moderate cases (Kvedaraite et al., 2021).

Monocytes and Macrophages

Alterations of monocyte phagocytic and cytokine secreting functions were consistently documented in older individuals; however, numerical fluctuations do not affect all monocyte subsets. Based on the expression of the surface markers CD14 and CD16, circulating monocyte can be dived into three subsets: classical, intermediate, and non-classical monocytes, with CD14 decreasing from classical to non-classical subsets, and CD16 following an opposite expression pattern (Ziegler-Heitbrock et al., 2010).

CD14+ CD16– classical monocyte numbers do not change during aging, whereas CD14+ CD16+ non-classical monocytes tend to increase with age (Ventura et al., 2017; Oh et al., 2019; Feng et al., 2021). Despite these functional changes, monocytes seem to be key determinants of sex associated differences in immunosenescence, with a male superior ability in mounting inflammatory responses. In fact, men exhibit more chromatin accessibility at monocyte specific *loci* than women (Márquez et al., 2020a).

A sex specific study demonstrated that both male and female non-ICU patients exhibited a higher percentage of monocytes than male and female controls, respectively (Takahashi et al., 2020). While CD14+ CD16– classical monocyte frequency was overlapping among groups, CD14lowCD16- non-classical monocytes were detected with higher frequencies in non-ICU male patients vs. both male controls and female patients (Takahashi et al., 2020). However, such a gender related effect on non-classical monocytes was lost in all the studies pooling

male and female patients and controls together, leading to inconsistent results ranging from the absence of observed changes in monocyte frequency irrespective of severity to a statistically significant reduction in the case of more serious symptoms (**Table 3**). In addition, to explain the irreproducibility of results for both classical and non-classical monocytes, age dependent repercussions on monocyte dynamics in COVID-19 should be taken into account in the case of severity-based studies. In fact, lower percentages of both CD14+CD16– classical and CD14lowCD16+ non-classical monocytes were encountered in peripheral blood of infected children experiencing mild symptoms vs. convalescent children sampled 4–7 weeks following test results (Neeland et al., 2021).

The observed discrepancy among the available reports on classical and non-classical monocytes may also be a consequence of the lack of shared criteria to stratify SARS-CoV-2 infected subjects according to severity (**Table 3**; Wilk et al., 2020; Zhou R. et al., 2020; Kvedaraite et al., 2021; Neeland et al., 2021; Zingaropoli et al., 2021). In addition, from a more technical point of view, small differences in the immunophenotypic parameters evaluated to define monocytic subsets may be appreciated and may account for the lack of reproducibility of the observed data (**Table 3**).

Data about intermediate monocytes are also conflicting, requiring the inclusion of both gender- and age-related effects on disease severity in the study design. Percentage of CD14+CD16+ intermediate monocytes were reported as augmented in both male and female non-ICU patients vs. their sex matched controls, with more statistically significant results obtained for female subjects (Takahashi et al., 2020). On the contrary, a reduction in the frequency of peripheral blood CD14+CD16+ intermediate monocytes was detected in children with mild disease vs convalescent children sampled (Neeland et al., 2021). As reported in **Table 3**, a lower frequency of CD14++CD16+ intermediate monocytes was demonstrated in a cohort of patients with COVID-19 pneumonia vs. healthy donors (Zingaropoli et al., 2021). Instead, CD14+CD16+ intermediate monocytes were higher in both moderate and severe cases vs. healthy controls (Kvedaraite et al., 2021). No statistically significant difference was detected comparing contacts of SARS-CoV-2 cases with severe, mild and asymptomatic patients (Carsetti et al., 2020; **Table 3**). In none of these reports, a sex and age specific analysis was performed (Carsetti et al., 2020; Kvedaraite et al., 2021; Zingaropoli et al., 2021).

The effect of sex and age on COVID-19 patients' monocyte function was not dissected, although some preliminary data explored the behavior of monocyte subsets according to severity. As regards monocyte functionality, levels of CD14+CD16- classical and CD14+CD16+ intermediate monocyte HLA-DR and CD86 were reduced in ICU or high-dependency unit patients vs. both healthy controls and moderate cases of the disease, whereas the same markers were reduced on CD14lowCD16++ non-classical monocytes in severe patients vs. subjects with moderate disease only (Kvedaraite et al., 2021). Similar piece of evidence as regards HLA-DR expression emerged in another study (Arunachalam et al., 2020). Moreover, as previously observed for the mDCs, also COVID-19 patients CD14+ monocytes showed an impaired ability to answer to TLR stimulation, reinforcing the idea of an impaired innate response during COVID-19 disease (Arunachalam et al., 2020). However, classical monocytes from severe (according to the National Early Warning Score) COVID-19 cases showed type I IFN response, and exacerbation of TNF/IL-1β-driven inflammation (Lee et al., 2020; Kim C.W. et al., 2021). Consistently, in the lung environment, both BAL macrophages from severe patients (Liao et al., 2020; Kim C.W. et al., 2021) and airway macrophages from critical (according to WHO guidelines) cases (Chua et al., 2020) exhibited proinflammatory characteristics (Chua et al., 2020; Liao et al., 2020; Kim C.W. et al., 2021).

Neutrophils

The neutrophil number is preserved during aging, but the reduction in CD16 expression accounts for a notable functional decline (Ventura et al., 2017; Oh et al., 2019; Salminen et al., 2019; Cunha et al., 2020; Zimmermann and Curtis, 2020). Also, using animal models it was documented that lung neutrophils exhibit signs of exhaustion due to exposure to inflammatory mediators associated with advancing age (Ventura et al., 2017; Domingues et al., 2020).

Analysis of peripheral blood composition in COVID-19 cases revealed that in ICU patients neutrophil count was higher than that observed in non-ICU patients (Wang D. et al., 2020), and that neutrophil count was higher in deceased patients vs. survivors (Du et al., 2020; Fu Y. Q. et al., 2020).

SSChiCD16+ low density neutrophils, including migrating cells and immature elements in both healthy conditions and inflammation (Silvestre-Roig et al., 2019; Hassani et al., 2020), seem to exert a protective role in both adults and children. In fact, their accumulation was documented in PCR-negative adults and children exposed to SARS-CoV-2 in the household, with higher percentages recorded when sampling happened during the convalescence period (up to 7 weeks after positive test results) vs. exposure during the acute phase (Neeland et al., 2021).

No changes were recorded for neutrophils and activated CD63+ neutrophils comparing both adult SARS-CoV-2 positive and adult SARS-CoV-2 exposed subjects in acute and convalescent phase (Neeland et al., 2021). Instead, an increase in the percentage of CD63+ neutrophils was detected comparing infected children experiencing mild symptoms with both convalescent children sampled 4–7 weeks following test results and PCR negative children exposed to SARS-CoV-2 through household close contact with a positive in the acute phase (Neeland et al., 2021). Since expression of CD63 on neutrophils is a sign of activation (Silvestre-Roig et al., 2019), it would be worth deepening if more older individuals may experience a difference in neutrophil degranulation in comparison with younger patients, and if such a difference may be related to COVID-19 severity.

Myeloid Derived Suppressor Cells (MDSCs)

Myeloid-derived suppressor cells (MDSCs) are cells of the myeloid lineage exerting a suppressive function on other

TABLE 3 | Observed fluctuation in monocytic subsets in adult COVID-19 patients.

Monocytes	Observation	Analyzed patients	Compared group(s)	References
Classical CD14++CD16- monocytes	Reduction (%)	Hospitalized	HD	Zhou R. et al., 2020
	Reduction (%)	Convalescent	HD	Zhou R. et al., 2020
	No change (%)	Pneumonia	HD	Zingaropoli et al., 2021
	No change (%)	Mild[$]	Convalescent	Neeland et al., 2021
Classical CD14+CD16- monocytes	No change (%)	Severe[&]	Mild*	Carsetti et al., 2020
	No change (%)	Severe[&]	Asymptomatic	Carsetti et al., 2020
	No change (%)	Severe[&]	Contacts[+]	Carsetti et al., 2020
	No change (%)	Mild*	Asymptomatic	Carsetti et al., 2020
	No change (%)	Mild*	Contacts[+]	Carsetti et al., 2020
	No change (%)	Asymptomatic	Contacts[+]	Carsetti et al., 2020
CD14+CD16+ non-classical monocytes	Reduction (%)	Pneumonia	HD	Zingaropoli et al., 2021
	Reduction (%)	ARDS	Non-ARDS	Zingaropoli et al., 2021
CD14-CD16+ non-classical monocytes	Reduction (%)	Severe[&]	Mild*	Carsetti et al., 2020
	Reduction (%)	Severe[&]	Asymptomatic	Carsetti et al., 2020
	Reduction (%)	Severe[&]	Contacts	Carsetti et al., 2020
	No change (%)	Mild*	Asymptomatic	Carsetti et al., 2020
	No change (%)	Mild*	Contacts[+]	Carsetti et al., 2020
	No change (%)	Asymptomatic	Contacts[+]	Carsetti et al., 2020
CD14lowCD16+ non-classical monocytes	Reduction (%)	Mild[$]	Convalescent	Neeland et al., 2021
CD14lowCD16++ non-classical monocytes	Reduction (%)	Severe[§]	HD	Kvedaraite et al., 2021
CD14++CD16+ intermediate monocytes	Reduction (%)	Pneumonia	HD	Zingaropoli et al., 2021
CD14+CD16+ intermediate monocytes	Increase (%)	Moderate[@]	HD	Kvedaraite et al., 2021
CD14+CD16+ intermediate monocytes	Increase (%)	Severe[§]	HD	Kvedaraite et al., 2021
CD14+CD16+/– intermediate monocytes	No change (%)	Severe[&]	Mild*	Carsetti et al., 2020
	No change (%)	Severe[&]	Asymptomatic	Carsetti et al., 2020
	No change (%)	Severe[&]	Contacts[+]	Carsetti et al., 2020
	No change (%)	Mild*	Asymptomatic	Carsetti et al., 2020
	No change (%)	Mild*	Contacts[+]	Carsetti et al., 2020
	No change (%)	Asymptomatic	Contacts[+]	Carsetti et al., 2020

(%), as frequency values; HD, healthy donors; ARDS, acute respiratory distress syndrome, asymptomatic patients who tested positive but had no symptoms.
[$] *Including coryza, headaches, nausea, fever, cough, sore throat, malaise, headaches, and muscle aches.*
[§] *Treated at the intensive care unit -ICU- or at a high-dependency unit.*
[@] *Oxygen saturation -SO2- between 90 and 94% or 0.5 to 3 L/min oxygen requirement at screening.*
[&] *Pneumonia (fever, cough, dyspnoea, fast breathing) plus one of the following: respiratory rate > 30 breaths/min; severe respiratory distress; or SpO_2 < 90% on room air.*
**Patients requiring no hospitalization and experiencing symptoms like with fever, myalgia, and fatigue without obvious chest high resolution computed tomography findings for COVID-19.*
[+] *Contacts of SARS-CoV-2 confirmed cases who were negative by qPCR.*

immune cells through numerous molecular mechanisms (Salminen et al., 2019). MDSCs can be divided into two main branches, CD11b+CD33+CD15+CD14-HLA-DR- granulocytic MDSCs and CD11b+CD33+CD15-CD14+HLA-DRlo/- monocytic MDSCs, together with a further subset devoid of both granulocytic and monocytic markers (Salminen et al., 2019; Bergenfelz and Leandersson, 2020; Kramer and Abrams, 2020). Only the proportion of granulocytic MDSCs is reported as increased during aging (Salminen et al., 2019). However, to the best of our knowledge, no sexual dimorphism was described about distribution and function of MDSCs in individuals.

Expansion of functional granulocytic MDSCs was documented in COVID-19 patients vs. healthy donors, and was particularly evident in severe cases (Agrati et al., 2020) and in ICU patients (Sacchi et al., 2020). The percentage of monocytic myeloid derived suppressor cells (M-MDSCs) was higher in hospitalized patients vs. both convalescent (follow up) outpatients and healthy donors (Zhou R. et al., 2020; Buttenschön and Mattner, 2021).

ACQUIRED IMMUNITY: T CELLS

Among components of both innate and adaptive immune system, there seems to be more coherence in the information on T cell responses observed during COVID-19 disease. A central object of study in several reports, the information obtained to date on changes in cellular immunity, and discussed below in relation to age and sex, may help us to better understand the differences in infection outcomes observed.

Functionally, T cells are considered naïve until they encounter their cognate antigen in the periphery, and phenotypically they are CCR7+CD45RA+CD28+CD27+ cells. In the context of

SARS-CoV-2 infection, after encountering novel viral antigens, naïve T cells expand and differentiate into different types of effector cells, whose protective roles encompass TH-cell-mediated activation of B cells to produce virus-specific antibodies and elimination of virus-infected cells by CTLs. SARS-CoV-2-specific T cells have been reported in COVID-19 patients (Braun et al., 2020; Sekine et al., 2020). Following activation, the chemokine receptor CXCR3 is rapidly induced on naïve cells T cells and preferentially remains highly expressed on TH1 cells and effector CD8+ T cells (Groom and Luster, 2011). In contrast to innate immune cells, T cells develop a memory for repeated challenges. Indeed, following clearance of pathogen, most of effector T cells die but a small subset further differentiates into long-lived memory T cells that provide long-term protective immunity (Mahnke et al., 2013). So, in the event of a second contact with the virus, memory T cells rapidly mature into effector cells, responding to infection. Previously studies in mice have highlighted the importance of specific cell-mediated memory in protection from SARS-CoV-1 (Zhao J. et al., 2010; Channappanavar et al., 2014). It was shown that in some individuals exposure to SARS-CoV-2 has induced virus-specific T cell responses even in the absence of seroconversion, suggesting that individuals with no detectable antibodies may nonetheless be protected by cellular immunity (Gallais et al., 2021). This raises the question of whether T-cell responses would be more sensitive indicators of SARS-Co-V-2 exposure than antibody quantification of anti-nucleoprotein and anti-spike IgG or IgA levels in peripheral blood. However, individual variability in immune response must be taken into account, especially in view of the profound immune changes observed with aging. Thus, a thorough understanding of the role of T cells in the immune response to SARS-CoV-2 and how aging may impair their responsiveness is essential to gain insights into both the individual ability to respond to the first infection and the quality, magnitude, and durability of protective immunity against reinfection with SARS-CoV-2.

As this is an emerging pathogen to which the human population has never previously been exposed, although cross-reactive T cell recognition between circulating 'common cold' coronaviruses and SARS-CoV-2 has been suggested (Grifoni et al., 2020), the key step in the immune response is undoubtedly its recognition by naïve, and not memory, T cells. Early and efficient virus-specific CD8+ T cell responses and CD4+ T cell-dependent antibody responses of sufficient magnitude against SARS-CoV-2 would probably be protective (Zhao J. et al., 2016). However, age-related alterations in the T cell compartment could lead to a failure to develop protective immunity in old patients. As previously mentioned, thymic involution is the main driver of the reduced absolute numbers and percentages of peripheral naïve T cells observed in old individuals. Their homeostatic peripheral proliferation is thought to compensate for this loss but can lead to the outgrowth of certain T cell clones at the expense of others, with shrinkage of the T cell repertoire. As a consequence, the pool of naïve T cells decreases with age with quantitatively and qualitatively impairs de novo CD8+ T-cell responses (Briceño et al., 2016). In parallel, there is an expansion of the memory T cell pool, probably due to chronic or persistent infections, most commonly with Cytomegalovirus, which cause specific T cells to clonally expand through repetitive stimulation. One of the phenotypic characteristics of these persistent stimulated T cells is the progressive downregulation of the costimulatory receptor CD28 that is definitively lost in terminally differentiated T cells (Pangrazzi et al., 2020). These CD28– senescent T cells are characterized by low proliferative capacity, shortening of telomeres and express senescent markers, such as programmed cell death protein 1 (PD-1). These age-associated changes in T cell subset distribution, especially in CTL, critically affect primary immune responses against viruses (Pawelec, 2018; Aiello et al., 2019). A recent study performed by our group has highlighted differences in the lymphocyte subset distribution in both helper and cytotoxic compartments between females and males during aging (Ligotti et al., 2021). These immune gender differences during aging and in case fatality rates in COVID-19 disease support the need to incorporate investigation of biological factors underlying differences in immune responses to SARS-CoV-2 between females and males in order to identify targeted therapeutic interventions aimed to improve antiviral immune function (Bunders and Altfeld, 2020).

Despite differences in patient stratification, several studies have reported lymphopenia, affecting all lymphocyte subsets, in a significant proportion of patients with severe diseases, both males and females, such that it can be considered, together with hyper-cytokinaemia, a signature for severe COVID-19 infection and pneumonia (Bermejo-Martin et al., 2020). The occurrence of lymphopenia (lymphocyte count $< 0.8–1.1 \times 109$/L, depending on the cut-off value used) was reported in 85% (Yang et al., 2020), 72.3% (Liu K. et al., 2020; Zhan et al., 2020), 66.7% (Meng Y. et al., 2020), 63% (Huang et al., 2020), and 42.9% (Chen G. et al., 2020) of infected cases, with the highest percentages observed in critically ill patients. Specifically, significant decreases in absolute T cell counts, TH cells, and CTL cells were observed in the COVID-19 patients compared to healthy controls, but accentuated in the severe cases (Cui et al., 2020; Wang F. et al., 2020), indicating SARS-CoV-2 infection has a negative impact on T-cell mediated immunity. In older COVID-19 patients (median age 71 years), low lymphocytes count was a strong predictor of poor outcome while high lymphocyte levels were predictive of better outcome (Wang L. et al., 2020). This peripheral lymphopenia could reflect the recruitment of lymphocytes from the blood to the infected site in response to combinations of different chemokines expressed by airway and alveolar blood vessel endothelial cells (Alon et al., 2021). A meta-analysis of the mean difference in lymphocyte counts at admission between patients with good and with poor COVID-19 outcomes has shown that lymphopenia was significantly associated with severe COVID-19 and this association was affected by age but not by sex (Zhao Q. et al., 2020). However, some studies have suggested that the male sex is inversely associated with lymphocyte count, especially in patients with comorbidity (Meng Y. et al., 2020; Qin et al., 2020), although not always confirmed (Zhao G. et al., 2021). In the peripheral blood of SARS-CoV-2-infected patients, both CD4+ and CD8+ T cell blood counts are dramatically decreased compared to healthy controls, with the highest evidence observed in severe cases (de Candia et al., 2021). Nevertheless, male

COVID-19 patients have shown lower CD4+ T cell and higher CD8+ T cell proportions than female patients, indicating a possible more severe immune dysregulation (Zhao G. et al., 2021; **Table 4**).

Analysis of T cells in BAL of COVID-19 patients has identified lower CD8+ T cell levels in patients with severe infection than in patients with moderate infection, apparently against the hypothesis of sequestration on the site of infection. On the other hand, CD8+ T cells in BAL from patients with moderate infection have shown upregulation of genes involved in differentiation, activation, migration and cytokine-related pathways compared with severe cases (Liao et al., 2020; **Table 4**).

Despite the absolute counts of CD8+ T cells decreased, analysis of T cell activation has revealed a significant increment in the frequency and absolute numbers of activated (co-expressing HLA-DR and CD38) CD8+ T cells and increased multiple cytotoxic granules expression in COVID-19 pneumonia patients (De Biasi et al., 2020; Song et al., 2020; Chen Q. et al., 2021). Analysis of post-mortem lung tissue from 16 patients with COVID-19 has correlated the presence of abundant infiltrating activated CD8+ T cells (CD38, GZMA, GZMB, CCR5) with a specific immunopathological pattern characterized by low local expression of interferon stimulated genes, low viral counts, massive lung damage, and late death (Nienhold et al., 2020). Based on these pieces of evidence, it has been suggested that this aberrant activation of CTLs in patients with severe COVID-19 disease could play an important role in the pathogenesis of SARS-CoV-2 infection (Song et al., 2020). However, CD8+ T cells from older COVID-19 patients with mild disease do not show increased production of perforin and granzymes, probably due to high background levels of cytotoxic molecules (Westmeier et al., 2020; **Table 4**).

A significant increment in the frequency, but not in the absolute count, of activated CD4+ T cells or exhausted/senescent (PD-1+CD57+) peripheral CD4+ and CD8+ T cells was also observed (De Biasi et al., 2020). When gender differences were considered, this increase in percentages of activated and terminally differentiated T cells being significant in female but not in male patients compared with healthy controls, suggesting a more robust T cell response among female patients. Although these differences were observed in both CD4+ and CD8+ T cells, more significant differences were found in the cytotoxic subset (Chen and John Wherry, 2020; Takahashi et al., 2020; **Table 4**). The implications and mechanisms underlying increased CD8+ activity are unknown in the context of this novel virus but may play a role in driving the immunopathogenesis of COVID-19.

The characterization of the T cell subsets in a cohort of 39 COVID-19 with a median age of 64 years (range 35–94) has revealed that a decrease in both the percentage and the absolute number of naïve CD45RA+CCR7+ CD8+ T cells and in the absolute number of naïve CD45RA+CCR7+ CD4+ T cells (De Biasi et al., 2020; Belaid et al., 2021). A similar trend was observed as CCR7+CD45RA- central memory T cells, while the percentage, but not the absolute number of terminal effector CCR7-CD45RA+ T cells was higher, although with a decreased proliferation index suggesting a lack of clonal expansion after activation (De Biasi et al., 2020). Compared to hospitalized COVID-19 patients during acute disease, patients at 3–6 months of convalescence showed higher proportions of CD4+ and CD8+ CCR7–CD45RA+ effector T cells and reduced expression of the proliferation marker Ki-67. In male patients, disease progression was associated with higher age and lower CD8+ T cell response (IFN-γ production and activation), while these correlations were not seen in females. On the contrary, female patients showed greater T cell response, characterized by higher percentages of activated and terminally differentiated CD8+ T cells (Takahashi et al., 2020; **Table 4**).

CD4+ and CD8+ T cells in acute COVID-19 patients showed a substantial reduction of CXCR3 and CXCR5 expression, irrespective of disease severity, that is recovered upon convalescence (Shuwa et al., 2021). In another study, when compared to their younger counterparts and to age-matched healthy donors, over 80-year-olds COVID-19 patients showed a more pronounced reduction in CD8+ T cell count. Moreover, percentages of naïve CD45RO-CCR7+ CD28+ CD8+ T cells were markedly reduced in 29- to 79-year-old group of COVID-19 patients, suggesting an ongoing cytotoxic response during SARS-Cov-2 infection, but these differences were not evident in the over 80 group, probably due to the reduced pool of naïve T cells in the old individuals. Likewise, the increase in CD8+ CD45RO-CCR7- CD28- terminally differentiated effector and CD45RO+ CCR7- CD28- effector memory T cells was more evident in the younger group than in the 80- to 96-year-old age group (Westmeier et al., 2020). CD4+ memory responses to SARS-CoV-2 were detected in 100% of recovered patients, while 70% established CD8+ memory responses. Most of T cell reactivity to the viral Spike protein was dependent on CD4+ T cells and these responses were correlated with anti-SARS-Cov-2 IgG and IgA titres in COVID-19 cases (Grifoni et al., 2020). A sophisticated and detailed analysis of T cell immunophenotype in COVID-19 patients has revealed the heterogeneity of the immune response to SARS-CoV-2 infection ranging from robust CD8+ and/or CD4+ T cell activation and proliferation to minimal detectable responses compared to healthy donors (Mathew et al., 2020). This suggests that the relationship between acquired immunity and COVID-19 pathogenesis is complex and must be taken into account in the design of therapies and vaccines (**Table 4**).

Most analyses were not stratified by gender. It therefore remains to be established whether cell-mediated immune response may differ between the two sexes and whether this affects the durability of immunity and vaccine approaches. Moreover, given the multitude of well-documented age-related changes in the composition and responsiveness of the acquired immune system, further comparative studies between young and old are needed to investigate differences in virus-specific T cells responses. Indeed, any intervention aimed at improving immune status in old individuals should be targeted and individualized, especially in a context

TABLE 4 | Observed fluctuation in T cell subsets in adult COVID-19 patients.

T cell subsets	Observation	Analyzed patients	Compared group(s)	References
Total CD3+CD19– T lymphocyte	Reduction (%)	Deceased	Survivors	Cui et al., 2020
	Reduction (#)	Deceased	Survivors	Cui et al., 2020; Belaid et al., 2021
	Reduction (%)	Cases	Recovered	Mathew et al., 2020
	Reduction (%)	Cases	HD	Mathew et al., 2020
	Reduction (#)	Severe[η]	Mild[ζ] /moderate[θ]	Song et al., 2020; Belaid et al., 2021
	No change (%)	Severe[η]	Mild[ζ]	Song et al., 2020
CD3+CD4+ Helper T lymphocyte	Reduction (%)	Deceased	Survivors	Cui et al., 2020
	Reduction (#)	Deceased	Survivors	Cui et al., 2020
	Reduction (#)	Pneumonia patients	HD	Wang F. et al., 2020
	Reduction (#)	Severe[η]	Mild[ζ] /moderate[θ]	Song et al., 2020; Wang F. et al., 2020; Belaid et al., 2021; Taghiloo et al., 2021
	No change (%)	Severe[η]	HD	De Biasi et al., 2020
	Reduction (#)	Severe[η]	HD	De Biasi et al., 2020
	Reduction (%)	Cases	Recovered	Mathew et al., 2020
	Reduction (%)	Cases	HD	Mathew et al., 2020
	No change (%)	Severe[η]	Mild[ζ]	Song et al., 2020
CD3+CD8+ Cytotoxic T lymphocyte	Reduction (%)	Deceased	Survivors	Cui et al., 2020
	Reduction (#)	Deceased	Survivors	Cui et al., 2020; Belaid et al., 2021
	Reduction (#)	Pneumonia patients	HD	Wang F. et al., 2020
	Reduction (#)	Severe[η]	Mild[ζ] /moderate[θ]	Song et al., 2020; Wang F. et al., 2020; Belaid et al., 2021; Taghiloo et al., 2021
	No change (%)	Severe[η]	HD	De Biasi et al., 2020
	Reduction (#)	Severe[η]	HD	De Biasi et al., 2020
	Reduction (%)	Cases	Recovered	Mathew et al., 2020
	Reduction (%)	Cases	HD	Mathew et al., 2020
	No change (%)	Severe[η]	Mild	Song et al., 2020
CCR7+CD45RA+CD28+CD27+ CD4+ naïve	No change (%)	Severe[η]	HD	De Biasi et al., 2020
	Reduction (#)	Severe[η]	HD	De Biasi et al., 2020
CCR7-CD45RA+CD28+CD27+/- CD4+ central memory	No change (%)	Severe[η]	HD	De Biasi et al., 2020
	Reduction (#)	Severe[η]	HD	De Biasi et al., 2020
	No change (%)	Severe[η]	HD	De Biasi et al., 2020
	Reduction (#)	Severe[η]	HD	De Biasi et al., 2020
CCR7-CD45RA+CD28-CD27+/- CD4+ terminal effector	Increase (%)	Severe[η]	HD	De Biasi et al., 2020
	No change (#)	Severe[η]	HD	De Biasi et al., 2020
CCR7-CD45RA+CD27- CD4+ naïve terminal effector	Increase (%)	Cases	Recovered	Mathew et al., 2020
	Increase (%)	Cases	HD	Mathew et al., 2020
CD38+HLA-DR+ CD4+ activated	No change (#)	Severe[η]	HD	De Biasi et al., 2020
	Increase (%)	Severe[η]	HD	De Biasi et al., 2020
	Increase (%)	Mild[ζ]	HD	Song et al., 2020
	Increase (%)	Severe[η]	HD	Song et al., 2020
	Increase (%)	Deceased	Survivors	Belaid et al., 2021
	Increase (%)	Cases	Recovered	Mathew et al., 2020
	Increase (%)	Cases	HD	Mathew et al., 2020
	No change (%)	Severe[η]	Mild[ζ] /moderate[θ]	Song et al., 2020; Belaid et al., 2021
PD-1+CD57+ CD4+ exhausted/senescent	Increase (%)	Severe[η]	HD	De Biasi et al., 2020
	No change (#)	Severe[η]	HD	De Biasi et al., 2020
CCR7+CD45RA+CD28+CD27+ CD8+ naïve	Reduction (%)	Severe[η]	HD	De Biasi et al., 2020
	Reduction (#)	Severe[η]	HD	De Biasi et al., 2020
CCR7+CD45RA+ CD8+ naïve	Reduction (%)	Severe[η]	Mild[ζ] /moderate[θ]	Belaid et al., 2021
	Reduction (%)	Deceased	Survivors	Belaid et al., 2021
CCR7-CD45RA+CD28+CD27+/- CD8+ central memory	Reduction (%)	Severe[η]	HD	De Biasi et al., 2020
	Reduction (#)	Severe[η]	HD	De Biasi et al., 2020

(Continued)

TABLE 4 | Continued

T cell subsets	Observation	Analyzed patients	Compared group(s)	References
CCR7-CD45RA-CD28+/-CD27+/- CD8+ effector memory	No change (%)	Severe[η]	HD	De Biasi et al., 2020
	No change (#)	Severe[η]	HD	De Biasi et al., 2020
CCR7-CD45RA-CD8+ effector memory	Increase (%)	Deceased	Survivors	Belaid et al., 2021
CCR7-CD45RA+CD28-CD27+/- CD8+ terminal effector	Increase (%)	Severe[η]	HD	De Biasi et al., 2020
	No change (#)	Severe[η]	HD	De Biasi et al., 2020
CCR7-CD45RA+CD27- CD8+ naïve terminal effector	Increase (%)	Cases	Recovered	Mathew et al., 2020
	Increase (%)	Cases	HD	Mathew et al., 2020
CD38+HLA-DR+ CD8+ activated	Increase (%)	Severe[η]	HD	De Biasi et al., 2020
	Increase (#)	Severe[η]	HD	De Biasi et al., 2020
	Increase (%)	Mild[ς]	HD	Song et al., 2020
	Increase (%)	Severe[η]	HD	Song et al., 2020
	Increase (%)	Deceased	Survivors	Belaid et al., 2021
	Increase (%)	Severe[η]	Mild[ς]	Song et al., 2020
	Increase (%)	Cases	Recovered	Mathew et al., 2020
	Increase (%)	Cases	HD	Mathew et al., 2020
	No change (%)	Severe[η]	Mild[ς] /moderate[θ]	Belaid et al., 2021
PD-1+CD57+ CD8+ exhausted/senescent	Increase (%)	Severe[η]	HD	De Biasi et al., 2020
	No change (#)	Severe[η]	HD	De Biasi et al., 2020
BAL T cells	Reduction (%)	Severe[η] /Critical[ι]	Moderate[θ]	Liao et al., 2020
	Increase (%)	Moderate[θ]	HD	Liao et al., 2020

T cell subset, peripheral blood T cell subset analyzed in the reported studies; (%), as frequency values; (#), as absolute number; Cases, COVID-19 patients (irrespective of severity); HD, healthy donors; Recovered, non-hospitalized subjects who had recovered from SARS-CoV-2 infection.

[ς]*Patients with or without pneumonia admitted to general wards not requiring intensive care.*

[η]*Patients experiencing the following: respiratory failure, respiratory rate > 30 bpm, oxygen saturation < 93% at rest, arterial partial pressure of oxygen (PaO_2)/fraction of inspired oxygen (FiO_2) (PaO_2/FiO_2) ratio < 300 mmHg.*

[θ]*Fever, respiratory symptoms, and pneumonia evidenced by computed tomography.*

[ι]*Any of the following: requirement for mechanical ventilation, shock, or concomitant organ failure.*

such as COVID-19 that is fatal to a large proportion of this population.

ACQUIRED IMMUNITY: B LYMPHOCYTES

B cells function in the humoral immunity component of the acquired immune system, producing antibodies that are not secreted, but inserted into the plasma membrane where they serve as a part of B-cell receptors. When a naïve or memory B cell is activated by an antigen, it proliferates and may differentiate into a short living plasmablast, or a long living plasma cell (both known as antibody-secreting cells – ASCs) – it is not known if plasma cells represent a further maturation step of early plasmablasts (Jourdan et al., 2009; Khodadadi et al., 2019).

Immunosenescence impacts B lymphocytes at multiple levels. During aging, B cells show a decline in the number of newly produced clones flanked by a contraction of their repertoire, producing a variable level of accumulation of memory cells and an increase in the appearance of signs of exhaustion (Giefing-Kröll et al., 2015; Aiello et al., 2019; Crooke et al., 2019; Ma et al., 2019; Lian et al., 2020). The age associated decline in the amount of B cells appears more dramatic in men than in women, and menopausal related changes in B cell numbers are counteracted by hormone replacement therapy (Giefing-Kröll et al., 2015; Márquez et al., 2020a). Also, during aging women exhibit more pronounced chromatin accessibility at B cell *loci* than older male individuals, who suffer a downregulation of B cell-specific genes (Márquez et al., 2020a).

Changes in B cell distribution during COVID-19 affect the quality and intensity of SARS-CoV-2 elicited immune response, accounting for infection resolution and persistence of symptoms after viral clearance (Huang et al., 2021; Long et al., 2021; Shuwa et al., 2021; Wheatley et al., 2021). This was demonstrated also from a functional point of view, with both somatic hypermutation in B cell receptor (BCR) and higher antibody titres correlating with disease severity (Schultheiß et al., 2020; Okba et al., 2020). Thus, COVID-19 related B lymphocyte alterations determine not only disease severity but also the entity of long-term consequences and may have potential prognostic applications (Detsika et al., 2021; Kim C.W. et al., 2021; Kos et al., 2021; Shuwa et al., 2021).

Total B Cells

During aging a contraction of both number and percentage of peripheral B cells was described multiple times, with a more visible reduction in older males (Bulati et al., 2014; Giefing-Kröll et al., 2015; Hazeldine and Lord, 2020; Lian et al., 2020; Márquez et al., 2020b,a; Vellas et al., 2020). In COVID-19

the analysis of B cell changes according to a gender-based stratification of patients revealed that only the frequency of B cells was higher in both non-ICU male and female patients than in controls, reaching a statistically significant result narrowly when the comparison was performed between the two female groups (Takahashi et al., 2020). However, no sex or age correlated differences in B cell counts of COVID-19 patients were documented (Jurado et al., 2020; Takahashi et al., 2020; Jin et al., 2021).

In gender pooled reports about the total number and percentage of B cells in COVID-19+ subjects, data vary from a study to another on the basis of both the choice of the confronted parameter (number of cells rather than percentage) and the adopted criteria to stratify the analyzed subjects according to severity (Carsetti et al., 2020; Sosa-Hernández et al., 2020; Detsika et al., 2021; Kos et al., 2021; Shuwa et al., 2021; Xiong et al., 2021). Numerous papers are available documenting the invariability, the decrease and the increase of B cell numbers and frequency in various categories of COVID-19 patients (**Table 5**). This lack of homogeneity mirrors the absence of an agreement about the stratification of COVID-19 cases according to severity. In addition, the selected statistical analysis strategy, especially in the presence of multiple comparisons, may "mask" some statistically significant results, requiring a larger sample size or a different statistical approach to reveal relevant differences among the compared categories (Jurado et al., 2020; Sosa-Hernández et al., 2020; Woodruff et al., 2020; d'Alessandro et al., 2021; Newell et al., 2021; Shuwa et al., 2021). As a result, the abundance of confounding reports slows down the extrapolation of prognostic and predictive pieces of information from published reports.

Despite these contradictory data, a recent metanalysis demonstrated a statistically significant decrease in B cells in severe disease cases vs. non severe disease group (Akbari et al., 2020).

TABLE 5 | Observed fluctuation in B cells in adult COVID-19 patients.

B cells	Observation	Analyzed patients	Compared group(s)	References
Total peripheral blood B cells	No change (#)	Intubated	Non-intubated	Detsika et al., 2021
	No change (#)	Intubated (admission)	Intubated (recovery)	Detsika et al., 2021
	No change (#)	Severe^{+} (admission)	Mild* (admission)	Jurado et al., 2020
	No change (#)	Moderate$^{\&}$ (admission)	Mild* (admission)	Jurado et al., 2020
	No change (#)	Severe^{+} (admission)	Moderate$^{\&}$ (admission)	Jurado et al., 2020
	No change (#)	Critical§	Severe$^{£}$	Liu Y. et al., 2021
	No change (#)	Cases	HD	d'Alessandro et al., 2021
	No change (#)	Severe$^{>}$	Non-severe$^{<}$	d'Alessandro et al., 2021
	Reduction (#)	Deceased	Survivors	Cantenys-Molina et al., 2021; Kos et al., 2021; Xiong et al., 2021
	Reduction (#)	Critical§	Moderate$^{@}$	Liu Y. et al., 2021
	Reduction (#)	Severe°	Non-severe∧	Zhang W. et al., 2020
	Reduction (#)	Composite$^{\alpha}$	Non-composite$^{\beta}$	Zhang W. et al., 2020
	Increase (#)	ICU	NCU	Kos et al., 2021
	Increase (#)	ICU	NCU (non-COVID-19)	Kos et al., 2021
	Increase (#)	ICU	ICU (non-COVID-19)	Kos et al., 2021
	No change (%)	Convalescent	HD	Newell et al., 2021
	No change (%)	Severe$^{>}$	Non-severe$^{<}$	d'Alessandro et al., 2021
	No change (%)	Severe^{+} (admission)	Mild* (admission)	Jurado et al., 2020
	No change (%)	Severe^{+} (discharge)	Mild* (discharge)	Jurado et al., 2020
	No change (%)	Moderate$^{\&}$ (admission)	Mild* (admission)	Jurado et al., 2020
	No change (%)	Moderate$^{\&}$ (discharge)	Mild* (discharge)	Jurado et al., 2020
	No change (%)	Severe^{+} (admission)	Moderate$^{\&}$ (admission)	Jurado et al., 2020
	No change (%)	Severe^{+}(discharge)	Moderate$^{\&}$(discharge)	Jurado et al., 2020
	Reduction (%)	ICU	NCU	Kos et al., 2021
	Reduction (%)	ICU	NCU (non-COVID-19)	Kos et al., 2021
	Reduction (%)	ICU	ICU (non-COVID-19)	Kos et al., 2021
	Reduction (%)	Severe$^{\gamma}$	Mild$^{\delta}$	Shuwa et al., 2021
	Reduction (%)	Severe$^{\gamma}$	Moderate$^{\varepsilon}$	Shuwa et al., 2021
	Reduction (%)	Severe$^{\zeta}$	Asymptomatic	Carsetti et al., 2020
	Increase (%)	Cases	HD	Woodruff et al., 2020
	Increase (%)	Severe$^{\eta}$	Mild/moderate$^{\theta}$	Sosa-Hernández et al., 2020
	Increase (%)	Cases	Convalescent	Files et al., 2021

(Continued)

TABLE 5 | Continued

B cells	Observation	Analyzed patients	Compared group(s)	References
Bone marrow and spleen total B cells	Reduction (#)	Deceased	Controls[$]	Ihlow et al., 2021
BAL B cells	Reduction (%)	Severe[/]	Mild[/]	Kim C.W. et al., 2021
	Increase (%)	Mild[/]	HD[/]	Kim C.W. et al., 2021
	Increase (#)	Severe[/]	Mild[/]	Kim C.W. et al., 2021
	Increase (#)	Severe[/]	HD[/]	Kim C.W. et al., 2021

BAL, bronchoalveolar lavage; (%), as frequency values; (#), as absolute number; Cases, COVID-19 patients (irrespective of severity); ICU, intensive care unit patients; HD, healthy donors; NCU, normal care unit patients; NCU (non-COVID-19), normal care unit patients treated for other reasons than COVID-19; ICU (non-COVID-19), intensive care unit patients treated for other reasons than COVID-19; asymptomatic, patients who tested positive but had no symptoms.

**Clinical mild symptoms with no abnormal radiological findings.*

[+] Non-severe pneumonia.

[&] As defined by the physician in charge or meeting at least one of the following criteria: acute respiratory distress, shock, admission to the intensive care unit -ICU-; patients with a fatal outcome were also included in this group.

[§] With any of the following: (1) respiratory failure requiring mechanical ventilation; (2) shock; or (3) other organ failure requiring monitoring and treatment in ICU.

[Ł] Individuals experiencing any of the following: (1) respiratory distress, respiratory rate ≥ 30 breaths/min; (2) oxygen saturation on room air ≤ 93% at rest; (3) oxygenation index $PaO_2/FIO_2 \leq 300$ mmHg; or (4) lung infiltrates > 50% within 24–48 h.

[$] Age matched subjects who died for cardiac conditions.

[>] ICU admission, mechanical ventilation, or high-flow oxygen therapy.

[<] Patients who were not experiencing ICU admission, mechanical ventilation, or high-flow oxygen therapy.

[@] Fever and other respiratory tract symptoms with pneumonia on chest computed tomography.

[°] Respiratory rate ≥ 30/min, a pulse oxygen saturation ≤ 93% on room air, oxygenation index ≤ 300 mmHg, respiratory failure for which invasive ventilation was necessary, signs of shock (circulatory failure), organ failure and ICU care.

[∧] Mild symptoms + normal radiology findings in both lungs, and fever, cough, and other respiratory symptoms + radiological signs of pneumonia.

[α] Patients experiencing admission to the ICU, mechanical ventilation, or death.

[β] Patients who did not experience admission to the ICU, mechanical ventilation, or death.

[γ] Patients with > 10L or 60% supplemental oxygen, managed in ICU.

[δ] Patients with < 3L or 28% supplemental oxygen.

[ε] Patients with < 10L or <60% supplemental oxygen, requiring non-invasive ventilation (NIV) or continuous positive airway pressure (CPAP).

[ζ] Patients with clinical signs of pneumonia with one of the following: respiratory rate > 30 breaths/min, severe respiratory distress, or SpO_2 < 90% on room air.

[η] Patients experiencing the following: respiratory failure, respiratory rate > 30 bpm, oxygen saturation < 92% at rest, arterial partial pressure of oxygen (PaO_2)/fraction of inspired oxygen (FiO_2) (PaO_2/FiO_2) ratio < 300 mmHg.

[θ] Fever, signs of airway disease, with or without a tomographic image indicating pneumonia.

[/] No stratification criteria neither statistically significance data provided.

Transitional and Naïve B Cells

Immunosenescence is associated with a decrease in CD27-IgD+ naïve B lymphocytes and the consequential reduction in B cell repertoire diversity (Cancro et al., 2009; Crooke et al., 2019; Ma et al., 2019; Oh et al., 2019). To the best of our knowledge, no naïve B cell specific molecular and numerical analysis was performed as a function of age and gender in COVID-19, and naïve B cells are reported as mostly unchanged in their frequency irrespective of disease severity (**Table 6**; Sosa-Hernández et al., 2020; Long et al., 2021; Shuwa et al., 2021). However, frequency of CD19+CD27loIgM+IgD+ mature naïve B cells seemed to be correlated with seroconversion, considering that percentages recorded for this B subset were higher in patients with high seroconversion index values vs. subjects with low seroconversion index values (seroconversion indices were estimated by summing the *Z*-scores for each of the four seroconversion assays used by the authors – see Galbraith et al., 2021 for details) (Galbraith et al., 2021).

On the contrary, transitional B cells tend to remain stable in adults (Perez-Andres et al., 2010; Blanco et al., 2018) and may exert immunomodulatory functions producing IL-10 (Zhou Y. et al., 2020). In COVID-19 literature, data about transitional B cell frequency according to disease severity are not fully homogeneous, despite the majority of data suggest that a reduction in transitional B cells may contribute to more severe disease (**Table 6**; Sosa-Hernández et al., 2020; Woodruff et al., 2020; Shuwa et al., 2021).

Some of the lack of reproducibility (leading to the same methodological considerations) that we emphasized in the previous paragraph may be also encountered in those studies dissecting alterations of transitional and naïve B cells in COVID-19 patients. In addition, a shared agreement about the terms by which expressing the immunophenotypic features of the described subset would be extremely beneficial to ensure the comparability of data (**Table 6**).

Memory B Cells

Dysregulation of B cell production, survival and turnover in older subjects affects the ability to respond to new antigens (thus influencing response to vaccination) and may cause the accumulation of class switched memory cells (IgD-CD27+), although the eventuality of such an effect is still a matter of debate (Ventura et al., 2017; Aiello et al., 2019; Crooke et al., 2019; Hazeldine and Lord, 2020). Also, age-associated B cells (ABCs), defined as CD11b+CD11c+CD21low/-T-bet+, increase with age, and accounts for a reduction in the generation of new B cells, the release of pro-inflammatory cytokines and autoantibody production in autoimmune diseases (Rubtsova et al., 2015; Hagen and Derudder, 2020; Pietrobon et al., 2020; Sachinidis et al., 2020; Kim C.W. et al., 2021).

TABLE 6 | Observed fluctuation in naïve and transitional B cells in adult COVID-19 patients.

B cell subset	Observation	Analyzed patients	Compared group(s)	References
CD27-IgD+ naïve	No change (%)	Severe[γ]	Moderate[ε]	Shuwa et al., 2021
	No change (%)	Severe[γ]	Mild[δ]	Shuwa et al., 2021
	No change (%)	Severe[γ]	HD	Shuwa et al., 2021
	No change (%)	Moderate[ε]	Mild[δ]	Shuwa et al., 2021
	No change (%)	Moderate[ε]	HD	Shuwa et al., 2021
	No change (%)	Mild[δ]	HD	Shuwa et al., 2021
	No change (%)	Cases	Recovered	Mathew et al., 2020
	No change (%)	Cases	HD	Mathew et al., 2020
	No change (%)	Recovered	HD	Mathew et al., 2020
	Increase (%)	Severe[κ]	Prepandemic controls	Acosta-Ampudia et al., 2021
IgD+CD38–/+ naïve	No change (%)	Severe[η]	Critical[ι]	Sosa-Hernández et al., 2020
	No change (%)	Severe[η]	Mild/moderate[θ]	Sosa-Hernández et al., 2020
	No change (%)	Severe[η]	HD	Sosa-Hernández et al., 2020
	No change (%)	Critical[ι]	Mild/moderate[θ]	Sosa-Hernández et al., 2020
	No change (%)	Critical[ι]	HD	Sosa-Hernández et al., 2020
	No change (%)	Mild/moderate[θ]	HD	Sosa-Hernández et al., 2020
CD21+CD27– naïve	No change (%)	Recovered	HD	Long et al., 2021
Naïve	Increase (%)	Mild[/]	HD	Huang et al., 2021
	Increase (%)	Moderate[/]	HD	Huang et al., 2021
	Increase (%)	Cured[/]	HD	Huang et al., 2021
CD24hiCD38hi transitional	Reduction (%)	Severe[γ]	HD	Shuwa et al., 2021
	Reduction (%)	Severe[γ]	Convalescent	Shuwa et al., 2021
CD24+CD38hi transitional	No change (%)	Severe[η]	HD	Sosa-Hernández et al., 2020
	Reduction (%)	Severe[η]	Mild/moderate[θ]	Sosa-Hernández et al., 2020
	Reduction (%)	Critical[ι]	Mild/moderate[θ]	Sosa-Hernández et al., 2020
	Increase (%)	Mild/moderate[θ]	HD	Sosa-Hernández et al., 2020
CD27-CD38int CD24+ transitional	Reduction (%)	ICU	Outpatients	Woodruff et al., 2020

B cell subset, peripheral blood B cell subset analyzed in the reported studies; (%), as frequency values; (#), as absolute number; Cases, COVID-19 patients (irrespective of severity); ICU, intensive care unit patients; Recovered, non-hospitalized subjects who had recovered from SARS-CoV-2 infection; HD, healthy donors; Outpatients, outpatients with milder disease.

[γ]*Patients with > 10 L or 60% supplemental oxygen, managed in ICU.*

[δ]*Patients with < 3L or 28% supplemental oxygen.*

[ε]*Patients with < 10L or <60% supplemental oxygen, requiring non-invasive ventilation (NIV) or continuous positive airway pressure (CPAP).*

[ζ]*Patients with clinical signs of pneumonia with one of the following: respiratory rate > 30 breaths/min, severe respiratory distress, or SpO_2 < 90% on room air.*

[η]*Patients experiencing the following: respiratory failure, respiratory rate > 30 bpm, oxygen saturation < 92% at rest, arterial partial pressure of oxygen (PaO_2)/fraction of inspired oxygen (FiO_2) (PaO_2/FiO_2) ratio < 300 mmHg.*

[θ]*Fever, signs of airway disease, with or without a tomographic image indicating pneumonia.*

[ι]*Any of the following: requirement for mechanical ventilation, shock, or concomitant organ failure.*

[κ]*Respiratory distress, i.e., ≥30 breaths/min. in resting state, oxygen saturation of 90% or less on room air; or arterial partial pressure of oxygen (PaO_2)/fraction of inspired oxygen (FiO_2) of 300 or less.*

[/]*No stratification criteria provided.*

As regards B cell homeostasis in COVID19+ patients, to the best of our knowledge only one age focused study explored the effect of immunosenescence on memory B cell dynamics, demonstrating that percentage of CD21+CD27+ memory B cells exhibited a direct correlation with patients age (Kuri-Cervantes et al., 2020). Data from both COVID-19 patients (Lenti et al., 2020; Ogega et al., 2021; Shuwa et al., 2021) and recovered COVID-19 subjects (Long et al., 2021) showed no effects in terms of memory B cell accumulation, resembling those reports documenting a similar scenario during aging (Crooke et al., 2019). These findings were not confirmed by all the available studies, mainly because of differences in the patient stratification criteria, insufficient sample size and inconsistency in the evaluated immunophenotypic features that were used to detect the desired subset (**Table 7**; Carsetti et al., 2020; Lenti et al., 2020; Mathew et al., 2020; Sosa-Hernández et al., 2020; Acosta-Ampudia et al., 2021; Shuwa et al., 2021).

Only depletion of peripheral IgM+ memory B cells seems to be a hallmark of SARS-CoV-2 infection (Carsetti et al., 2020; Lenti et al., 2020). Intriguingly, the percentage of IgM+ and switched memory B cells showed an inverse correlation with symptoms duration in convalescent patients, with no age or gender related influence (Newell et al., 2021).

As expected in immunocompetent subjects, seroconversion is positively associated with frequency of switched memory B cells, IgM+ memory B cells, CD19+CD27hiIgM-IgD+ c-delta switched memory B cells, and CD19+ CD11c+Tbet+ ABCs, whose percentages are higher in patients with a high

seroconversion index vs. patients with a low seroconversion index (Galbraith et al., 2021).

Late memory CD27-IgD- Double negative (DN) B cells are exhausted memory cells that actively contribute to the inflammatory status associated with aging by secreting TNF-α and IL-6 (Bulati et al., 2014; Crooke et al., 2019; Fraussen et al., 2019; Ma et al., 2019; Hagen and Derudder, 2020; Pietrobon et al., 2020).

DN B lymphocytes reached a statistically significant expansion in both COVID-19 patients and convalescent subjects vs. controls (**Table 7**; Mathew et al., 2020; Shuwa et al., 2021). However, at an individual patient level, DN B cell fraction tends to decrease

TABLE 7 | Observed fluctuation in B cells in adult COVID-19 patients.

B cells	Observation	Analyzed patients	Compared group(s)	References
CD21+CD27+ memory	Reduction (%)	Severe$^{\rho}$	HD	Kuri-Cervantes et al., 2020
CD27+IgD+ unswitched memory	No change (%)	Severe$^{\gamma}$	Moderate$^{\varepsilon}$	Shuwa et al., 2021
	No change (%)	Severe$^{\gamma}$	Mild$^{\delta}$	Shuwa et al., 2021
	No change (%)	Severe$^{\gamma}$	HD	Shuwa et al., 2021
	No change (%)	Moderate$^{\varepsilon}$	Mild$^{\delta}$	Shuwa et al., 2021
	No change (%)	Moderate$^{\varepsilon}$	HD	Shuwa et al., 2021
	No change (%)	Mild$^{\delta}$	HD	Shuwa et al., 2021
	Increase (%)	Severe$^{\kappa}$	Prepandemic controls	Acosta-Ampudia et al., 2021
	Reduction (%)	Severe$^{\eta}$	HD	Sosa-Hernández et al., 2020
	Reduction (%)	Critical$^{\iota}$	HD	Sosa-Hernández et al., 2020
	Reduction (%)	Cases	Recovered	Mathew et al., 2020
	Reduction (%)	Cases	HD	Mathew et al., 2020
IgD+IgM+ unswitched memory	No change (%)	Severe	Mild	Ogega et al., 2021
	No change (%)	Severe	HD	Ogega et al., 2021
	No change (%)	Mild	HD	Ogega et al., 2021
CD27+IgD- switched memory	No change (%)	Severe$^{\gamma}$	Moderate$^{\varepsilon}$	Shuwa et al., 2021
	No change (%)	Severe$^{\gamma}$	Mild $^{\delta}$	Shuwa et al., 2021
	No change (%)	Severe$^{\gamma}$	HD	Shuwa et al., 2021
	No change (%)	Moderate$^{\varepsilon}$	Mild $^{\delta}$	Shuwa et al., 2021
	No change (%)	Moderate$^{\varepsilon}$	HD	Shuwa et al., 2021
	No change (%)	Mild$^{\delta}$	HD	Shuwa et al., 2021
	Reduction (%)	Severe$^{\eta}$	HD	Sosa-Hernández et al., 2020
	Reduction (%)	Cases	Recovered	Mathew et al., 2020
	Reduction (%)	Cases	HD	Mathew et al., 2020
CD27-IgM- switched memory	Reduction (%)	Severe$^{\xi}$	Mild$^{\pi}$	Carsetti et al., 2020;
CD38+/–IgM–IgD– switched memory	No change (%)	Severe$^{\lambda.}$	Mild$^{\mu}$	Ogega et al., 2021
	No change (%)	Severe$^{\lambda.}$	HD	Ogega et al., 2021
	No change (%)	Mild$^{\mu}$	HD	Ogega et al., 2021
	No change (#)	Cases	HD	Lenti et al., 2020
	No change (#)	Cases	Hypofunction$^{\nu}$	Lenti et al., 2020
	No change (#)	Cases	Splenectomy$^{\zeta}$	Lenti et al., 2020
CD27+IgD-IgM-CD38+ switched memory	Increase (%)	Severe$^{\kappa}$	Prepandemic controls	Acosta-Ampudia et al., 2021
IgM+ memory	Reduction (%)	Severe$^{\xi}$	Asymptomatic	Carsetti et al., 2020
	Reduction (%)	Severe$^{\xi}$	Mild$^{\pi}$	Carsetti et al., 2020
	Reduction (%)	Cases	HD	Lenti et al., 2020
IgM-IgG- memory	Increase (%)	Severe$^{\xi}$	Asymptomatic	Carsetti et al., 2020
	Increase (%)	Severe$^{\xi}$	Mild$^{\pi}$	Carsetti et al., 2020
CD27-IgD- double negative memory	Increase (%)	Severe$^{\gamma}$	HD	Shuwa et al., 2021;
	Increase (%)	Convalescent	HD	Shuwa et al., 2021;
	Increase (%)	Cases	Recovered	Mathew et al., 2020
	Increase (%)	Cases	HD	Mathew et al., 2020
CD38–/+CD24–CD21+CD11c– DN1	Reduction (%)	Severe$^{\eta}$	Mild/moderate$^{\theta}$	Sosa-Hernández et al., 2020
	Reduction (%)	Critical $^{\iota}$	Mild/moderate $^{\theta}$	Sosa-Hernández et al., 2020
CD38–CD24-CD21–CD11c+ DN2	Increase (%)	Severe$^{\eta}$	Mild/moderate$^{\theta}$	Sosa-Hernández et al., 2020
	Increase (%)	Severe$^{\eta}$	Critical$^{\iota}$	Sosa-Hernández et al., 2020

(Continued)

TABLE 7 | Continued

B cells	Observation	Analyzed patients	Compared group(s)	References
CD38–/+CD24–CD21–CD11c– DN3	Increase (%)	Severe[η]	HD	Sosa-Hernández et al., 2020
	Increase (%)	Critical[ι]	Mild/moderate[θ]	Sosa-Hernández et al., 2020
	Increase (%)	Critical[ι]	HD	Sosa-Hernández et al., 2020

B cell subset, peripheral blood B cell subset analyzed in the reported studies; BAL, bronchoalveolar lavage; (%), as frequency values; (#), as absolute number; Cases, COVID-19 patients (irrespective of severity); ICU, intensive care unit patients; Recovered, non-hospitalized subjects who had recovered from SARS-CoV-2 infection; HD, healthy donors; Outpatients, outpatients with milder disease; ARDS, acute respiratory distress syndrome; NCU, normal care unit patients; NCU (non-COVID-19), normal care unit patients treated for other reasons than COVID-19; ICU (non-COVID-19), intensive care unit patients treated for other reasons than COVID-19; Asymptomatic, patients who tested positive but had no symptoms.

[ρ]included subjects were in the following condition(s): high flow nasal cannula–noninvasive, ventilator (non-ARDS), mild to severe ARDS, and ECMO.

[γ]Patients with > 10L or 60% supplemental oxygen, managed in ICU.

[δ]Patients with < 3L or 28% supplemental oxygen.

[ε]Patients with < 10L or < 60% supplemental oxygen, requiring non-invasive ventilation (NIV) or continuous positive airway pressure (CPAP).

[ζ]Patients with clinical signs of pneumonia with one of the following: respiratory rate > 30 breaths/min, severe respiratory distress, or SpO_2 < 90% on room air.

[η]Patients experiencing the following: respiratory failure, respiratory rate > 30 bpm, oxygen saturation < 92% at rest, arterial partial pressure of oxygen (PaO_2)/fraction of inspired oxygen (FiO_2) (PaO_2/FiO_2) ratio < 300 mmHg.

[θ]Fever, signs of airway disease, with or without a tomographic image indicating pneumonia.

[ι]Any of the following: requirement for mechanical ventilation, shock, or concomitant organ failure.

[κ]Respiratory distress, i.e., ≥ 30 breaths/min. in resting state, oxygen saturation of 90% or less on room air; or arterial partial pressure of oxygen (PaO_2)/fraction of inspired oxygen (FiO_2) of 300 or less.

[λ]Hospitalized patients.

[μ]Ambulatory patients.

[ν]Spleen hypofunction patients.

[ξ]Patients who underwent splenectomy for trauma.

[π]Patients requiring no hospitalization and experiencing symptoms like with fever, myalgia, and fatigue without obvious chest high resolution computed tomography findings for COVID-19.

from acute disease to convalescent status (Newell et al., 2021; Shuwa et al., 2021). A keen characterization of DN B cell subsets, revealed that the increase of DN B cells in severe and critical patients may be attributable to an augmented CD38–CD24–CD21–CD11c+ DN2 and CD38–/+CD24–CD21–CD11c– DN3 fraction (**Table 7**; Sosa-Hernández et al., 2020).

As regards SARS-CoV-2 specific memory, widening evidences demonstrated that generation of SARS-CoV-2 memory B cells initiates early after infection/symptoms onset and is durable (Hartley et al., 2020; Abayasingam et al., 2021; Byazrova et al., 2021; Carsetti et al., 2021; Dan et al., 2021; Gaebler et al., 2021; Long et al., 2021; Ogega et al., 2021; Sakharkar et al., 2021; Sherina et al., 2021; Sokal et al., 2021; Tong et al., 2021). It remains to be deepened if sex may play a role in influencing the time of onset of B cell memory in COVID-19 subjects.

Plasma Blasts and Antibody Secreting Cells

Aging hampers the proper differentiation of B cells into plasma cells (Ventura et al., 2017; Crooke et al., 2019; Hazeldine and Lord, 2020). This phenomenon seems to be avoided during SARS-CoV-2 infection, since the plasmblast fraction of B cells was reported as augmented in all patients whereas levels tend to be restored in convalescent and recovered patients (Mathew et al., 2020; Sosa-Hernández et al., 2020; Long et al., 2021; Shuwa et al., 2021; Wildner et al., 2021; **Table 8**). This piece of data reflects an infection related reactive expansion, given that the percentage of plasma cells positively correlates with the fraction of IgG+ and IgA+ positive B cells (Shuwa et al., 2021). The increase in plasma cells showed a direct correlation with an oligoclonal expansion of antibody clones (Kuri-Cervantes et al., 2020). Reported evidences for COVID-19 seem to recall the contraction of the B cell repertoire which was demonstrated in older people, although the involvement of the same molecular mechanisms remains to be elucidated (Aiello et al., 2019; Crooke et al., 2019; Ma et al., 2019; Lian et al., 2020). Obviously, plasma cells exhibited a significant association with seroconversion, with frequencies being higher in patients with high seroconversion indices vs. those with low ones (Galbraith et al., 2021).

Similar results were replicated at the lung level. In fact, the total number and proportion of BAL plasma cells was increased in COVID-19 patients (according with disease severity) vs. healthy controls (Kim C.W. et al., 2021).

B Cell Trafficking Phenotype

B cell trafficking phenotype exhibits profound modification during aging (Bulati et al., 2014).

During acute COVID-19, B cells showed a reduced expression of CXCR3, CXCR5 and integrin β7 according to disease severity, with CXCR3 and CXCR5 level normalization in convalescent subjects (Shuwa et al., 2021).

With a detailed characterization of B cell subsets, it was documented that CXCR5 expression was reduced on naïve, plasmablasts, and memory B cells, including DN memory B cells (Kuri-Cervantes et al., 2020; Mathew et al., 2020). This is a very intriguing piece of data, since CXCR5 did not show any statistically significant difference in its expression on B lymphocyte subsets comparing young and old subjects (Bulati et al., 2014), thus it is not expected to be differentially modulated in COVID-19 as a consequence of aging.

BAL B lymphocytes from patients with mild disease express CCR6 and CXCR3, whereas BAL B cells from patients with

TABLE 8 | Observed fluctuation in ASCs and plasmablasts in adult COVID-19 patients.

ASCs and plasmablasts	Observation	Analyzed patients	Compared group(s)	References
CD19+/–IgM–IgD–CD38+/+ ASCs	No change (%)	Severe$^{\lambda}$	Mild$^{\mu}$	Ogega et al., 2021
	No change (%)	Severe$^{\lambda}$	HD	Ogega et al., 2021
	No change (%)	Mild$^{\mu}$	HD	Ogega et al., 2021
CD19+CD21–CD27+CD38+/high ASCs	No change	Recovered$^{\varsigma}$	HD	Long et al., 2021
CD27hiCD38hi plasmblasts	Increase (%)	Severe$^{\gamma}$	Convalescent	Shuwa et al., 2021
	Increase (%)	Severe$^{\gamma}$	HD	Shuwa et al., 2021
	Increase (%)	Moderate$^{\varepsilon}$	Convalescent	Shuwa et al., 2021
	Increase (%)	Moderate$^{\varepsilon}$	HD	Shuwa et al., 2021
	Increase (%)	Mild$^{\delta}$	HD	Shuwa et al., 2021
	Increase (%)	Convalescent	HD	Shuwa et al., 2021
CD27+CD38+ plasmablasts	Increase (%)	Cases	Recovered$^{\rho}$	Mathew et al., 2020
	Increase (%)	Cases	HD	Mathew et al., 2020
	Increase (%)	Cases	HD	Wildner et al., 2021;
	Increase (%)	ICU	Outpatients	Woodruff et al., 2020
	Increase (%)	ICU	HD	Woodruff et al., 2020
CD27+ CD38hi ASC/plasmablasts	Increase (%)	Severe$^{\eta}$	HD	Sosa-Hernández et al., 2020
	Increase (%)	Critical$^{\iota}$	HD	Sosa-Hernández et al., 2020
	Increase (%)	Mild/moderate$^{\theta}$	HD	Sosa-Hernández et al., 2020
CD38+CD24– plasmablasts	Increase (%)	Severe$^{\zeta}$	Asymptomatic	Carsetti et al., 2020
	Increase (%)	Severe$^{\xi}$	Mild$^{\pi}$	Carsetti et al., 2020
	Increase (%)	Severe$^{\xi}$	Contacts$^{\sigma}$	Carsetti et al., 2020

ASCs, antibody secreting cells; (%), as frequency values; Cases, COVID-19 patients (irrespective of severity); ICU, intensive care unit patients; HD, healthy donors; Outpatients, outpatients with milder disease; Asymptomatic, patients who tested positive but had no symptoms.

$^{\gamma}$*Patients with > 10 L or 60% supplemental oxygen, managed in ICU.*

$^{\delta}$*Patients with < 3 L or 28% supplemental oxygen.*

$^{\varepsilon}$*Patients with < 10 L or <60% supplemental oxygen, requiring non-invasive ventilation (NIV) or continuous positive airway pressure (CPAP).*

$^{\zeta}$*Patients with clinical signs of pneumonia with one of the following: respiratory rate > 30 breaths/min, severe respiratory distress, or SpO_2 < 90% on room air.*

$^{\eta}$*Patients experiencing the following: respiratory failure, respiratory rate > 30 bpm, oxygen saturation < 92% at rest, arterial partial pressure of oxygen (PaO_2)/fraction of inspired oxygen (FiO_2) (PaO_2/FiO_2) ratio < 300 mmHg.*

$^{\theta}$*Fever, signs of airway disease, with or without a tomographic image indicating pneumonia.*

$^{\iota}$*Any of the following: requirement for mechanical ventilation, shock, or concomitant organ failure.*

$^{\kappa}$*Respiratory distress, i.e., ≥30 breaths/min. in resting state, oxygen saturation of 90% or less on room air; or arterial partial pressure of oxygen (PaO_2)/fraction of inspired oxygen (FiO_2) of 300 or less.*

$^{\lambda}$*Hospitalized patients.*

$^{\mu}$*Ambulatory patients.*

$^{\nu}$*Spleen hypofunction patients.*

$^{\xi}$*Patients who underwent splenectomy for trauma.*

$^{\pi}$*Patients requiring no hospitalization and experiencing symptoms like with fever, myalgia, and fatigue without obvious chest high resolution computed tomography findings for COVID-19.*

$^{\rho}$*Non-hospitalized subjects who had recovered from SARS-CoV-2 infection.*

$^{\varsigma}$*Adults with a prior positive COVID-19 PCR test who met the definition of recovery based on the guideline from the Chinese Center for Disease Control and Prevention.*

$^{\sigma}$*Contacts of SARS-CoV-2 confirmed cases who were negative by qPCR.*

severe disease express more CXCR4 vs patients with mild disease and healthy subjects (Kim C.W. et al., 2021). Given that both CCR6 and CXCR4 are involved in B cell circulation to lymph nodes, whereas CXCR3 (whose gene is located on X chromosome)[1] rules B cell attraction to inflammation sites (Kunkel and Butcher, 2003), it would be worth deepening if BAL B cells from COVID-19 exhibit different B cell trafficking features according to both severity and gender. Also, in severe patients, BAL plasma cells had an increased expression of CCR2 and CCR10 (Kim C.W. et al., 2021). CCR2 is normally expressed on normal plasma cells, is renowned for mediating homing aberrant plasma cells in multiple myeloma and is downregulated during B cell maturation, while CCR10 is highly expressed by IgA producing plasma cells (Vande Broek et al., 2003; Flaishon et al., 2004; Shirakawa et al., 2008; Hu et al., 2011). Also, in terminally differentiating B cells CCR10 expression may be induced by 1,25-Dihydroxyvitamin D3, a known modulator of B cell homeostasis that showed a correlation with COVID-19 severity (Martens et al., 2020; Notz et al., 2021).

Functional Considerations About B Cells

As previously mentioned, inflammageing -defined as the balance between inflammatory and anti-inflammatory mechanisms pending toward inflammation- is a typical hallmark of an aging immune system (Ventura et al., 2017; Hazeldine and Lord, 2020).

[1]https://www.genecards.org/cgi-bin/carddisp.pl?gene=CXCR3&keywords=cxcr3 (accessed July 22, 2021).

COVID-19 offers the chance to improve the knowledge about the deregulation of molecular mechanisms ruling the production of inflammatory mediators. In fact, cytokine storm, characterized by increased circulating cytokine levels, with potential life-threatening acute systemic inflammation and secondary organ dysfunction, is a prominent feature of SARS-CoV-2 infection especially in severe cases (see section "Cytokine storm in COVID-19 disease").

As expected, after TLR9 agonist CpGB stimulation the percentage of IL-6+ B cells increased in acute COVID-19 patients vs. healthy controls and convalescent patients irrespective of possible chest X-ray abnormality (Shuwa et al., 2021). On the contrary, the percentage of IL-10+ B cells was significantly lower in patients with persistent lung pathology vs. healthy controls and normal chest X-ray subjects (Shuwa et al., 2021). However, analyzed at an individual patient level, the frequency of IL-10+ B cells increased from acute phase to convalescent status (Shuwa et al., 2021).

Transcriptomic analysis revealed that B cell immune answers are predominant in BAL fluid (Cavalli et al., 2020), and that BAL B cells from severe COVID-19 patients were highly activated by TNF-α signaling (Kim C.W. et al., 2021). B-cell activating factor (BAFF) receptor and transmembrane activator and calcium modulator and cyclophilin ligand interactor were expressed by all patients, but in severe patients there was an upregulation of apoptosis related gene transcription, probably mediated by BAFF signaling (Kim C.W. et al., 2021).

Study of peripheral B lymphocytes revealed a pattern of activation/exhaustion, characterized by increased frequency of CD69 and CD95 in hospitalized subjects vs. healthy controls and convalescent patients, and by an increase in PD1 frequency in non-hospitalized (convalescent) individuals vs. both healthy controls and COVID-19 hospitalized patients (Files et al., 2021). This may mirror the establishment of efficient mechanisms modulating strength and durability of B cell responses in convalescent subjects, given that both CD69 and CD95 are markers of lymphocyte activation (Catlett and Bishop, 1999; Vazquez et al., 2009), whereas PD1 is an immune modulator (Wang et al., 2019). Also, CD95 expression is increased on CD27+ B cells from older individuals vs. younger donors (Chong et al., 2005), and might be involved in age related differences in the regulation of anti-SARS-CoV-2 immune responses.

MESENCHYMAL STEM CELL TRANSPLANTATION IN COVID-19 PATIENTS: RATIONALE AND RESULTS

Mesenchymal stem cells, also known as mesenchymal stromal cells (MSCs) (Viswanathan et al., 2019), are heterogeneously multipotent stem cells that can be isolated from a variety of sources, including umbilical cord, human tissues like bone marrow and adipose tissue (using the stromal vascular fraction – SVF), and menstrual blood (Blaber et al., 2012; Kallmeyer and Pepper, 2015; Elgaz et al., 2019; Copcu, 2020; Gentile and Sterodimas, 2020a,b; Jeyaraman et al., 2020; Juárez-Navarro et al., 2020; Lin et al., 2020; Barros et al., 2021; Gentile, 2021; Mazini et al., 2021; Xu et al., 2021).

Mesenchymal stem cells are universally renowned for their unique immunomodulatory properties; once led into circulation, they are able to reach inflammation sites (Kallmeyer and Pepper, 2015), where they exert an inhibitory function on neutrophils, monocytes, dendritic cells, NK, B and T lymphocytes (Elgaz et al., 2019; Juárez-Navarro et al., 2020; Kim H. et al., 2020; Moradinasab et al., 2021). MSC ruled immunomodulation is performed by direct cell–cell interaction (through the expression of molecules like B7H1, PD-L1, and PD-L2) (Elgaz et al., 2019; Lin et al., 2020) as well as by a paracrine action, mediated by vehiculation of anti-inflammatory mediators through extracellular vesicles (Schulman et al., 2018; Martin-Rufino et al., 2019; Gowen et al., 2020; Juárez-Navarro et al., 2020; Kim H. et al., 2020; Lin et al., 2020; O'Driscoll, 2020; Wang J. et al., 2020; Gentile, 2021; Raghav et al., 2021; Su et al., 2021) and by cytokine (IL-10, transforming growth factor-β, TNF-stimulated gene 6 protein, IFN-γ) and soluble factor indoleamine-pyrrole 2,3-dioxygenase, prostaglandinE2, nitric oxide) secretion (Schulman et al., 2018; Elgaz et al., 2019; Martin-Rufino et al., 2019; Juárez-Navarro et al., 2020; Lin et al., 2020; Shetty, 2020; Gentile, 2021; Raghav et al., 2021). MSC polarization toward an anti-inflammatory phenotype is exacerbated by TLR stimulation elicited by pathogen components, like viral RNA (Waterman et al., 2010). Also, MSCs retain a differentiation potential making them ideal contributors to tissue repair (Blaber et al., 2012; Kallmeyer and Pepper, 2015; Elgaz et al., 2019; Copcu, 2020; Jeyaraman et al., 2020; Juárez-Navarro et al., 2020; Rogers et al., 2020).

As suggested by successful MSC use in a number of pathological scenarios involving uncontrolled immune activation with consequent tissue damage, like for example H9N2 induced acute lung injury, ARDS, autoimmune diseases and graft-versus-host disease (Waterman et al., 2010; Elgaz et al., 2019; Coelho et al., 2020; Yen et al., 2020; Barros et al., 2021; Musial and Gorska-Ponikowska, 2021; Su et al., 2021), the undeniable immunomodulatory and inflammation relieving properties of MSCs make them putative candidates as rejuvenating factors for the older immune system, on the basis of the documented ability to reduce pro-inflammatory mediators and to promote the expansion of regulatory lymphocyte subsets (Yeo et al., 2021). Also, MSC transplantation can contribute to the amelioration of aging frailty (Schulman et al., 2018; Florea et al., 2019; Sun et al., 2019), but MSC may exhibit age related deteriorating functions linked to inflammageing, especially in terms of alterations in number and characteristics of extracellular vesicles, DAMP production, excessive IL-6 release, loss of MSC ability to shift monocyte polarization toward M2, and triggering of ineffective hemopoiesis (Tsuruhara et al., 2017; Lee and Yu, 2020). Potency also seems to be affected by aging in bone marrow derived MSCs (BM-MSCs), but not in MSC obtained from adipose tissue (Rogers et al., 2020).

Assuming also that MSCs are negative for ACE2 and TMPRSS2 SARS-CoV-2 receptor complex (Leng et al., 2020; Moradinasab et al., 2021), MSC transplantation or infusion of MSC derived extracellular vesicles in the context of a deregulated

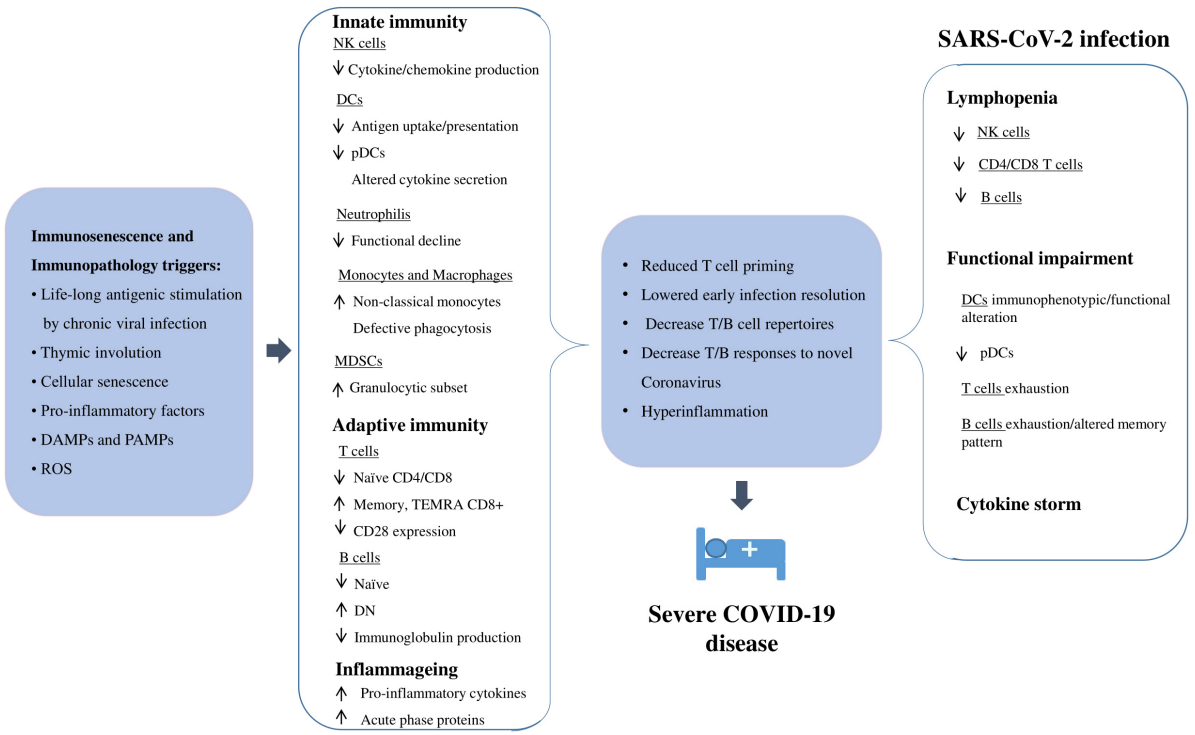

FIGURE 1 | A schematic view of how immunopathology and immunosenescence could contribute to severe COVID-19 in older people. PAMPs, pathogen associated molecular patterns; ROS, reactive oxygen species. See text for other acronyms.

immune response, cytokine storm and lung injury elicited during COVID-19 (especially in older people) appeared as a promising strategy (Gentile and Sterodimas, 2020a,b; Gorman et al., 2020; Monguió-Tortajada et al., 2020; Qin and Zhao, 2020; Rogers et al., 2020; Tsuchiya et al., 2020; Gentile, 2021; Raza et al., 2021).

Following the first paper by Leng et al. (2020) (see below), a number of data extrapolated from ongoing and concluded clinical studies on COVID-19 patients using both BM-MSCs and umbilical cord MSCs (UC-MSCs), together with reports employing MSCs from other sources or MSC vesicles, with positive results have been published so far (Al-Khawaga and Abdelalim, 2020; Barkama et al., 2020; Gentile et al., 2020; Golchin et al., 2020; Gorman et al., 2020; Liang et al., 2020; Lin et al., 2020; Meng F. et al., 2020; Sánchez-Guijo et al., 2020; Sengupta et al., 2020; Shu et al., 2020; Gentile, 2021; Jamshidi et al., 2021; Kouroupis et al., 2021; Moradinasab et al., 2021; Payares-Herrera et al., 2021; Sharma and Zhao, 2021).

In that paper, Leng et al. (2020) showed that MSCs cured or significantly improved the functional outcomes of seven patients without observed adverse effects. After treatment, the peripheral lymphocytes were increased, the C-reactive protein as well as TNF-α decreased, while IL-10 increased, and the overactivated cytokine-secreting immune cells CXCR3+CD4+ T cells, CXCR3+CD8+ T cells, and CXCR3+ NK cells disappeared in 3–6 days. In addition, a group of CD14+CD11c+CD11bmid regulatory DC cell population dramatically increased. Therefore, the intravenous transplantation of MSCs was safe and effective for treatment in patients with COVID-19 pneumonia, especially for patients in critically severe condition.

It was then demonstrated that both BM- and UC-MSCs showed to be able to improve COVID-19 survival rates (Shu et al., 2020; Häberle et al., 2021; Lanzoni et al., 2021; Moradinasab et al., 2021), symptoms (Liang et al., 2020; Meng F. et al., 2020; Shu et al., 2020; Tang et al., 2020; Zengin et al., 2020; Hashemian et al., 2021; Moradinasab et al., 2021), pulmonary functions (Liang et al., 2020; Meng F. et al., 2020; Shu et al., 2020; Zengin et al., 2020; Häberle et al., 2021; Moradinasab et al., 2021), inflammatory marker levels (CRP, TNF-α, neutrophil extracellular traps, IL-1RA, IL-5, IL-6, IL-18, IL-27, IL-17E/IL-25, IL-17F, CXCL-1, neutrophil-to-lymphocyte ratio, D-dimer) (Guo Z. et al., 2020; Liang et al., 2020; Tang et al., 2020; Zengin et al., 2020; Zhang Y. et al., 2020; Hashemian et al., 2021; Lanzoni et al., 2021; Moradinasab et al., 2021) and lymphopenia (Guo Z. et al., 2020; Liang et al., 2020; Zhu Y. et al., 2020) after few days of treatment. Placenta derived MSCs (Barkama et al., 2020), adipose derived MSCs (Sánchez-Guijo et al., 2020) and menstrual blood derived MSCs (Xu et al., 2021) showed to have similar effects.

In the context of the possible therapeutic use of MSCs to promote lung regeneration in COVID19, adipose and bone marrow derived MSCs are able to contribute to lung regeneration in animal models, by differentiation into type 2 alveolar epithelial cells and their immunomodulatory potential; in the case of adipose derived MSC, the success of both mechanisms is

facilitated by their large availability (Copcu, 2020; Behnke et al., 2020; Gentile and Sterodimas, 2020a,b; Rogers et al., 2020). The same regenerative properties are shown by SVF, whose cellular components secrete pro-angiogenetic and immune-modulating mediators (Gentile and Sterodimas, 2020a,b; Gentile, 2021).

As regards the preference for autologous rather than allogenic alternatives, the choice is influenced by limited time availability as well as intrinsic dependence of MSC properties on the age of the donor; in fact, given that also MSC experience senescence (Lee and Yu, 2020), the choice of allogenic MSCs or more senescence resistant adipose derived MSC may be recommendable in older patients (Rogers et al., 2020; Moradinasab et al., 2021).

Molecular mechanisms triggered by MSC infusion and by exosome derived MSCs in COVID-19 patients are currently under investigation. As regards immune cell homeostasis, overactivated cytokine-secreting immune cells CXCR3+CD4+ T cells, CXCR3+CD8+ T cells, and CXCR3+ NK cells disappeared soon after the MSC transplantation (Leng et al., 2020), with a general increase in both percentage and count of B lymphocytes, NK, CD3+, CD4+ and CD8+ cells (Liang et al., 2020; Sengupta et al., 2020; Tang et al., 2020; Zhang Y. et al., 2020; Zhu Y. et al., 2020) up to the decrease of neutrophil-to-lymphocyte ratio (Liang et al., 2020). This is nice food for thought, since MSC immunomodulation activity is exerted through inhibition of B, CD4+ and CD8+ T cell proliferation (Budoni et al., 2013; Joel et al., 2019; Moradinasab et al., 2021). Instead, MSC action on neutrophils is mainly functional rather than "numerical" (Li and Hua, 2017; Joel et al., 2019; Lin et al., 2020; Moradinasab et al., 2021), but the available data demonstrate that in more severe COVID-19 cases and in deceased patients neutrophils are also more abundant other that involved in cytokine storm establishment (see sections "Introduction" and "Neutrophils"). It remains to be demonstrated that MSC elicited an increase in COVID-19 T and B cell numbers may be related to an increase in the proportion of Tregs or Bregs, as observed in other contexts (Joel et al., 2019; Liu J. et al., 2020; Moradinasab et al., 2021) or if the apoptotic rate in neutrophils may be increased specifically in MSC treated patients. MSCs hamper B cell differentiation into plasma cells (Tsuruhara et al., 2017; Moradinasab et al., 2021); the same effect on COVID-19 patients and eventual consequences on anti-SARS-CoV-2 antibody production deserve further investigation. In addition, MSCs alter B cell trafficking phenotype, including downregulation of CXCR4 and CXCR5 (Joel et al., 2019), that are differentially regulated in COVID-19 (see section "B Cell Trafficking Phenotype section"). Molecular changes elicited by MSC influencing recirculation of B cells to lungs in the case of SARS-CoV-2 infection are still unexplored. Finally, given DN B cell increase in both immunosenescence and COVID-19 (see section "Memory B cells"), the potential rejuvenating effect of MSC on the exhausted subset, like DN B cells, might represent a putative mechanism of action contributing to the amelioration of these inflammatory conditions.

Similarly, (as mentioned above) CD14+CD11c+CD11bmid regulatory DC cell population increased after MSC transplantation, (Leng et al., 2020). The origin of CD14+CD11c+CD11bmid regulatory DC cells deserve to be assessed, because (I) CD11c+ DCs are reported as reduced in COVID-19 patients and show multiple functional alterations [see section "Dendritic cells (DCs)"] (II) the exploration of such an ontological pattern would put some more light on monocyte dynamics. CD11c is highly expressed by conventional DC2, powerful stimulators of naïve T cells (Rhodes et al., 2019) reduced in COVID-19 [see section "Dendritic cells (DCs)"] and by monocyte derived DCs (Delirezh et al., 2011; Boyette et al., 2017; Rhodes et al., 2019; Chometon et al., 2020). Despite this detail, no functional defects in conventional DC2 or monocyte derived DCs generation were studied in SARS-CoV-2 infected individuals so far. Data for better IL-1β, IL-6, and TNF-α producer classical macrophages (CD16-) are instead more commonly settled on detecting no variation in their frequency (see section "Monocytes and macrophages"). Since all monocytes are able to differentiate into macrophages, but (I) classical monocytes have a superior ability to differentiate into monocyte derived DCs (expressing CD11c) and (II) the relationship between conventional DC2 and monocytes is controversial (Delirezh et al., 2011; Boyette et al., 2017; Rhodes et al., 2019; Chometon et al., 2020), it would be worth discovering what molecular pathways are activated in classical monocytes by interacting with MSCs that may influence their immunophenotypic and functional faith.

Also, DCs exhibit a number of functional alterations in COVID-19, including reduced expression of CD86 and HLA-DR, whose upregulation is specifically hampered by MSCs during DC maturation (Joel et al., 2019). It remains to be assessed if this effect of MSCs on DCs is summed to that elicited by the microenvironment in SARS-CoV-2 infected tissue.

Together, these results underscore the role of MSCs in improving COVID-19 patient outcomes via maintenance of immune homeostasis, i.e., improving immunosenescence and relieving immunopathology.

CONCLUSION

Summarizing clinical findings discussed in the present paper, it is clear that the synergistic effects of immunosenescence and inflammageing (i.e., immunopathology) in older individuals have an important impact on their immune responses to SARS-CoV-2 infection (**Figure 1**) and should be taken into account whenever looking for factors influencing mortality rates in COVID-19 (Salimi and Hamlyn, 2020; Bajaj et al., 2021; Chen Y. et al., 2021). Changes in innate immune responses and the failure to trigger an effective acquired immune response (i.e., immunosenescence), in combination with a higher pro-inflammatory status (i.e., immunopathology) should explain why older people do not appropriately control viral replication and the potential clinical consequences triggered by a cytokine storm, also reducing the chances of proper recovery after infection resolution (Salimi and Hamlyn, 2020; Bajaj et al., 2021; Chen Y. et al., 2021). This awareness is critical to the implementation of any strategy aimed at improving protective immunity and vaccine efficacy against SARS-CoV-2 in the older population

(Bajaj et al., 2021). Regarding the gender differences in SARS-CoV-2 infection outcome reported in the present review, most studies are observational only and do not take into account that males and females may have several pre-existing conditions affecting the chance of successful aging, hence involving different responses to virus infection (Leng and Margolick, 2020; Mauvais-Jarvis et al., 2020; Sampathkumar et al., 2020; Ligotti et al., 2021; Lio et al., 2021). The key roles of immunosenescence and immunopathology in the outcome of SARS-CoV-2 infection are further supported by the beneficial results obtained with MSC infusion that, as previously discussed, act restoring immune homeostasis and contributing to lung repair (Gentile and Sterodimas, 2020a,b; Gorman et al., 2020; Monguió-Tortajada et al., 2020; Qin and Zhao, 2020; Rogers et al., 2020; Tsuchiya et al., 2020; Gentile, 2021; Raza et al., 2021). Therefore, the enhancement of the efficacy of the acquired immune response and the relief of the pro-inflammatory status should be an important issue both for SARS-CoV-2 infection resolution as well as for the appropriate generation of immunity upon vaccination.

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REVIEW
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Recent Advances of COVID-19 Modeling Based on Regenerative Medicine

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Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) has caused a pandemic since December 2019 that originated in Wuhan, China. Soon after that, the world health organization declared Coronavirus disease-2019 a global health concern. SARS-CoV-2 is responsible for a lethal respiratory infection as well as the involvement of other organs due to its large tropism spectrum such as neurologic, cardiovascular, endocrine, gastrointestinal, and renal systems. Since the behavior of the virus is not fully understood, a new manifestation of the infection is revealed every day. In order to be able to design more efficient drugs and vaccines to treat the infection, finding out the exact mechanism of pathogenicity would be necessary. Although there have been some big steps toward understanding the relevant process, there are still some deficiencies in this field. Accordingly, regenerative medicine (RM), can offer promising opportunities in discovering the exact mechanisms and specific treatments. For instance, since it is not always possible to catch the pathophysiology mechanisms in human beings, several modeling methods have been introduced in this field that can be studied in three main groups: stem cell-based models, organoids, and animal models. Regarding stem cell-based models, induced pluripotent stem cells are the major study subjects, which are generated by reprogramming the somatic stem cells and then directing them into different adult cell populations to study their behavior toward the infection. In organoid models, different cell lines can be guided to produce a 3D structure including liver, heart, and brain-like platforms. Among animal models, mice are the most common species in this field. However, in order for mice models to be permissive to the virus, angiotensin-converting enzyme 2 receptors, the main receptor involved in the pathogenicity of the virus, should be introduced to the host cells through different methods. Here, the current known mechanism of SARS-CoV-2 infection, different suggested models, the specific

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response toward different manipulation as well as challenges and shortcomings in each case have been reviewed. Finally, we have tried to provide a quick summary of the present available RM-based models for SARS-CoV-2 infection, as an essential part of developing drugs, for future therapeutic goals.

Keywords: COVID-19, SARS-CoV-2, model, stem cell, induced pluripotent stem cells, regenerative medicine, organoid

INTRODUCTION

Emerging from Wuhan, China, severe acute respiratory syndrome Coronavirus-2(SARS-CoV-2) has introduced a new pandemic to the world. Coronavirus disease-2019(COVID-19) is the new deadly viral infection in the family of human coronaviruses including SARS and Middle East Respiratory Syndrome (MERS). It is more contagious than the former ones and has caused considerable mortality and morbidity. Due to the lack of an effective treatment, the number of patients is rising constantly (Akhmerov and Marbán, 2020; Basiri et al., 2020; Becker, 2020; Golchin et al., 2020; Yang et al., 2020).

Scientists' knowledge of this infection is rapidly growing, for instance, the function of the angiotensin-converting enzyme2 (ACE2) receptor in SARS-CoV-2 tropism and mechanism of infection is partially understood (Harmer et al., 2002; Becker, 2020; Yu et al., 2020). The ACE2 receptor is expressed on a large proportion of human cells such as lung parenchyma, the heart, kidney, and gastrointestinal tract. It is believed that the ACE2 receptor plays a significant role in the presentation of many symptoms: acute respiratory distress syndrome (ARDS), diarrhea, etc. (Yiangou et al., 2020). Although many biotechnology companies have developed different vaccines and millions of people have been vaccinated to date, their effectiveness is still under question and longer follow-up is needed. Among various attempts and intensive research on possible strategies, constructing regenerative medicine (RM) based platforms has been investigated for a novel therapeutic approach that can provide the information needed for understanding the virus behavior and its pathogenesis (Ramezankhani et al., 2020; Yang et al., 2020). RM is about repairing, regenerating, and restoring the missing function of organs or tissues, thus it can be beneficial for studying the interactions between the virus and host cells and therefore, the pathophysiology, which can lead to the development of new drugs and vaccines (Atala et al., 2020; Basiri et al., 2020; Yang et al., 2020). As an example of therapeutic attempts, cell therapies, especially mesenchymal stem cell (MSC) therapy, have undergone investigations. MSCs have promising features like immunomodulatory effects, which are helpful in treating SARS-CoV-2 infection (Basiri et al., 2020; Golchin et al., 2020). At the time of writing this review, around 80 clinical trials are registered in *www.clinicaltrials.gov* that have utilized cell- based strategies including stem cells (mostly MSCs) and their derivatives (e.g., exosomes), memory T cells, and natural killer (NK) cells for treating COVID-19 and its related organ injuries. However, there are limited data on MSC therapy in pre-clinical studies, especially on models of lung injury of COVID-19 (Khoury et al., 2020; Ramezankhani et al., 2020; Ferreras et al., 2021; Park et al., 2021; Pérez-Martínez et al., 2021). Disease modeling (*in vitro, in vivo*, or both) is considered to be one of the major components of RM. Indeed, to elucidate the infection mechanisms and its manifestation in the human body, proper and reliable models are of urgent need. Accordingly, many animal models, different types of stem cells, and the cell-based cultures and organoids [a 3 dimensional (3D) structure in extracellular matrix (ECM)] have

Abbreviations: SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus-2; MERS, Middle East Respiratory Syndrome; RM, Regenerative Medicine; hESCs, Human Embryonic Stem Cells; hiPSCs, Human Induced Pluripotent Stem Cells; MSCs, Mesenchymal Stem Cells; NSCs, Neural stem cells; NPCs, Neural progenitor cells; NK, Natural killer cells; AT2s, Type2 Alveolar Epithelial Cells; ARB, Angiotensin Receptor Blocker; hrsACE2, Human Recombinant Soluble ACE2; ARDS, Acute Respiratory Distress Syndrome; HSV1, Herpes Simplex virus-1; IFN, Interferon; HLA, Human Leukocyte Adhesion; hs-cTnI, Highly sensitive troponin-I; Cyr61, Cysteine-rich protein 61; CCN1, CCN family number 1; GSIs, γ-Secretase Inhibitors; GFP, Green Fluorescent Protein; SFTPC, Surfactant Protein-C; QNHC, Quinacrine Dihydrochloride; MPA, Mycophenolic Acid; CLDN1, claudin1; SLC10A2, solute carrier family 10 member 2; CFTR, Cystic Fibrosis Transmembrane Conductance Regulator; RLU,Relative Luciferase Units; ECM, Extracellular Matrix; SCID, Severe Combined Immune Deficiency; NOD, Non-obese Diabetic; NSG, NOD-SCID iL2RG^{null}; 3D, 3 dimensional; hACE2, Human Angiotensin-Converting Enzyme 2; hPSC, Human Pluripotent Stem Cell; PDGFb, Platelet Derived Growth Factor Subunit B; KRT18, Cytokeratin 18; TMPRSS2, Transmembrane Serine Protease 2; RIG-I, Retinoic acid-Inducible Gene-I-like; PAMPs, Pathogen-Associated Molecular Patterns; Ang-1, Angiopoietin-1; KGF, Keratinocyte Growth Factor; BNP, Brain Natriuretic Peptide; MAS, Macrophage Activation Syndrome; ORF, Open Reading Frame; GM-CSF, Granulocyte-Macrophage Colony-Stimulating Factor; CXCL, C-X-C motif Chemokine Ligand; CXCL10/IP-10, C–X–C motif chemokine Ligand 10/Interferon γ-induced Protein 10 kDa; S protein, Spike; BMI, Body Mass Index; PD, Programmed cell Death protein; CD, Cluster of Differentiation; NSP, Non-structural Protein; M protein, Membrane; E protein, Envelope; N protein, Nucleocapsid; HCQ, Hydroxychloroquine; LPS, Lipopolysaccharide; DEX, Dexamethasone; TNF-a, Tumor Necrosis Factor Alpha; IL, Interleukin; NC, No Change; ARCoV, A vaccine candidate with the component of a mRNA sequence encoding the receptor binding domain of the virus which is encapsulated in lipid nanoparticles; RANTES, CCL5; Exo MSC-NTF, MCS-derived exosomes producing neurotrophic factor; AKI, Acute Kidney Injury; NIH, National Institutes of Health; WHO, World Health Organization; FDA, Food and Drug Administration; RAS, Renin–Angiotensin System; SIRS, Severe Inflammatory Response Syndrome; BBB, Blood- Brain Barrier; TAT, Thrombin Antithrombin complex; ABSCs, Airway Basal Stem Cells; CS, Cigarette Smoke; APC, Antigen Presenting Cells; STAT-1, Signal Transducer And Activator Of Transcription 1; JAK, Janus kinase; CRISPR/Cas9, Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated protein9; MASCp6, Mouse-Adapted Strain At Passage 6; Ifnar1-/- mice, C57BL/6 mice with a genetic ablation of their type I interferon receptors; Il28r-/- mice, C57BL/6 mice with a genetic ablation of their type III interferon receptors; WT, Wild Type; STAT2-/- hamster, lacking type I and III IFN signaling; IL28R hamster, lacking IFN type III signaling; ISG, Interferon-Stimulated Genes; GI, Gastrointestinal; PMN, Polymorphonuclear.

been used frequently to model diseases including neurological, cardiac, and metabolic disorders (Sterneckert et al., 2014; Doss and Sachinidis, 2019; Atala et al., 2020; Basiri et al., 2020; Sun et al., 2020; Payab et al., 2021). To model COVID-19, some of the most common animal species are mice, rats, and hamsters. For instance, mice have been genetically engineered to express the human ACE2 gene. In addition, there have been attempts toward using ACE2 expressing stem cells in RM platforms (Atala et al., 2020; Yang et al., 2020). Moreover, since SARS-CoV-2 affects multiple systems and organs, RM has played a role in analyzing the infection mechanism and host response by generating organoids of the lung, kidney, cardio, intestine which replicate the critical features of organs and model the disease *in vitro* (Atala et al., 2020; Basiri et al., 2020; Sun et al., 2020). In this review, a brief report of the known pathophysiology of SARS-CoV-2 infection in different organs is presented. Then, an extended review of recent advances toward designing models of COVID-19 is provided to help researchers find the best and most appropriate model. This article has tried to present the available data on modeling strategies of this new infection and their pros and cons for designing future effective therapeutic strategies.

MECHANISM OF SARS-COV-2 INFECTION

Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) is a highly transmissible virus that demonstrates a broad spectrum of tissue tropism, a quality that justifies its level of contagiousness (Harrison et al., 2020; Khoury et al., 2020). Due to its multi-organ tropism, a spectrum of pathologic symptoms from neurological, cardiac, pancreatic, digestive, and renal to the most common one, respiratory signs can be presented (Alsaad et al., 2020; Puelles et al., 2020; Qin and Zhao, 2020; Simoneau and Ott, 2020; Wiersinga et al., 2020; Yang et al., 2020). The multi-organ tropism is due to the ACE2 receptor expression on different cells around the body. In the following part, a brief description of the chain of events when SARS-CoV-2 attacks different organs of the body is presented. Knowing this mechanism helps us understand the foundation of each model designing better.

Lung

In respiratory tract involvement, SARS-CoV-2 causes symptoms from viral pneumonia to ARDS of the upper and lower respiratory tracts (Khoury et al., 2020; Simoneau and Ott, 2020; Youk et al., 2020). ARDS diagnosis is confirmed based on Berlin 2012 criteria: (1) presence of a clinical insult as well as new or worsening respiratory symptoms. (2) bilateral opacities on the imaging that are not fully described as effusion, collapse, or nodule. (3) respiratory failure due to edema that is not due to cardiac failure or volume overload and (4) impaired oxygenation that is described in three levels of mild, moderate, and severe each with its specific cut-offs (Gibson et al., 2020). Since the beginning of the pandemic in 2019, there have been some huge steps toward discovering the mechanism of action and virulence of the virus in order to improve the efficacy of vaccines and drug candidates (Ramezankhani et al., 2020; Zheng et al., 2021). SARS-CoV-2 is composed of four main structural proteins including Spike (S), Membrane (M), Envelope (E), and Nucleocapsid (N). In the mechanism of virus infection, the very first step of pathogenicity is the entrance to the parenchymal lung cells by the interaction between ACE2 receptors and virus surface S protein (Harrison et al., 2020; Li et al., 2020; Ramezankhani et al., 2020; Shang et al., 2020; Simoneau and Ott, 2020; Yang et al., 2020). After that, viral S protein undergoes lysis by transmembrane serine protease 2 (TMPRSS2) of the host cells. This gives the virus genome an opportunity to be translated to viral polymerase and proteases by the host ribosomes (Li et al., 2020; Simoneau and Ott, 2020). The products of this process are viral RNA and structural proteins (Fehr and Perlman, 2015; Li et al., 2020; Simoneau and Ott, 2020). The range of SARS-CoV-2 effects on the respiratory system varies from pneumonia to ARDS. The clinical definition was provided in the previous part. In SARS-CoV-2 infection, the pathological features of ARDS are associated with diffuse alveolar changes such as hyaline membrane formation, interstitial thickening, edema, and fibroblasts proliferation. The associated mechanisms are discussed in the following (Gibson et al., 2020). One of them is the direct cytopathic effect (Ramezankhani et al., 2020). Studies have shown that SARS-CoV-2 causes apoptosis and necrosis in ACE2 positive cells (airway epithelial cells in this case) via cytopathic effect. In addition, cilia movement cessation is another pathologic effect in the airways. Direct virus entrance in some other ACE2 positive cells is one of the possible suggested mechanisms of cell damage (Ramezankhani et al., 2020; Wang et al., 2020). Considering the immunologic effects, the host retinoic acid-inducible gene-I-like (RIG-I) or Toll-like receptors recognize viral pathogen-associated molecular patterns (PAMPs) (Simoneau and Ott, 2020). In the cellular immune system, antigen presenting cells (APC) activate CD4 + cells as well as CD8 + T cells, which destroy infected cells directly. Furthermore, a part of the pro-inflammatory cytokine production (such as interleukin (IL)-1, IL-6, IL-12, interferon(IFN)-γ, and Tumor Necrosis Factor(TNF)-a) is induced by CD4 + T cells, that leads to recruitment of innate immune cells (Costela-Ruiz et al., 2020; Ramezankhani et al., 2020). The uncontrolled production of inflammatory cytokines can also cause cytokine storm, which then leads to ARDS (Molinaro et al., 2020; Ramezankhani et al., 2020). The cytokine storm increases vascular permeability leading to edema as one of the items in ARDS criteria due to damaging the endothelial cell junctions (tight junction protein and the zonula occludens) as well as letting proteins, neutrophils, erythrocytes, and platelets pass into the interstitial tissue. This process is compatible with opacities seen in imaging. Then with the progression of alveolar accumulation with exudate, ventilation to perfusion mismatch is exaggerated and deteriorates impaired oxygenation that results in the progression toward ARDS (Molinaro et al., 2020; Ramezankhani et al., 2020; Suri et al., 2021). Another reason for the hyperinflammatory state is macrophage hyperactivation and neutrophil infiltration. macrophage hyperactivation causes macrophage activation syndrome (MAS) and the latter results in necroinflammation (Cao, 2020; Otsuka and Seino, 2020; Ramezankhani et al., 2020). Interferons (mainly IFN-1) are another group of antiviral factors produced because of antiviral

immune response (Boudewijns et al., 2020; Busnadiego et al., 2020; Lei et al., 2020; Simoneau and Ott, 2020). Memory T cells mostly show an immune response to SARS-CoV-2 structural proteins, especially S proteins. Accordingly, structural proteins could be considered as vaccine candidates (Ng et al., 2016; Ramezankhani et al., 2020). SARS-CoV-2 interferes with immune system antiviral actions by using different methods such as causing cell apoptosis and cytopathic effects (Simoneau and Ott, 2020). In addition, SARS-CoV-2 contains enzymes that modify viral and host cell RNAs. This mechanism makes the virus able to escape antiviral receptor detection. Different mechanisms have been proved to be associated with immune system evasion by SASR-CoV-2. SARS-CoV-2 possesses several proteins such as the NSP family, ORF proteins, M, N that are important in evading the immune system through several ways like cleaving host mRNA, inhibiting inflammatory cytokines production, sequestering viral RNA, inducing apoptosis or direct cytopathic effects, and inhibiting host proteins translation. Discussing these mechanisms in detail is beyond the scope of this article (Kasuga et al., 2021). Different factors have been known to be associated with poor prognosis of ARDS in COVID-19 patients, lymphopenia and cytokine storm are the two most important factors (Pourbagheri-Sigaroodi et al., 2020). Cell reduction in lymphopenia happens in T CD4 + and 8 + as well as B cells. The expression of CD38 and human leukocyte adhesion (HLA)-DR in T cells as the hallmark of T cell activation during viral pneumonia, was significantly higher in COVID-19 patients (Hue et al., 2020). PD-1 expression demonstrates immune dysfunction during sepsis. A study showed that during the pathogenesis of the infection, CD8 + T cells express lower levels of PD-1 in comparison to non-COVID ARDS patients (Hue et al., 2020). This finding supports the previous claim that immune system hyper activation is one of the associated mechanisms in ARDS development and probably forecasts its severity during COVID-19 infection (Hue et al., 2020). Higher levels of IL-10, CXCL10/IP-10, granulocyte-macrophage colony-stimulating factor (GM-CSF), and CX3CL1 as well as more severe viral shedding in the examination of nasopharyngeal swabs were also reported in COVID-ARDS patients (Hue et al., 2020). It highlights that immune dysregulation and more severe viral shedding are other negative prognostic factors of COVID-ADRS (Hue et al., 2020). Systemic inflammatory response, as well as multi-organ failure, can affect the susceptibility of developing ARDS. Comorbidities like old age, hypertension, diabetes mellitus, elevated BMI, cardiac and chronic lung diseases are associated with progression to ARDS in COVID-19 infection (Suri et al., 2021). On the other hand, some studies have reported a more severe phenotype of the disease in immunocompromised patients (Belsky et al., 2021). Therefore, we can conclude that hyper inflammatory response of a competent immune system and the cytopathic effect of the virus in the absence of an effective immune system are two possible underlying factors of developing ARDS. However, this deduction is not complete due to the lack of sufficient knowledge in the case of COVID-19 pathogenicity, which reflects the need for designing effective models in order to facilitate studying viral mechanisms of action in more detail (Remy et al., 2020).

Kidney

Renal involvement in COVID-19 infection is manifested most commonly as Acute Kidney Injury (AKI) which makes the patient prone to other complications (Robbins-Juarez et al., 2020; Arikan et al., 2021). Several pathophysiologic mechanisms have been suggested to be the cause of COVID-19 renal manifestations, which are discussed briefly in the following point. The first one is systemic hemodynamic instability (Legrand et al., 2021). In this state, due to decreased cardiac output or renal venous congestion, renal perfusion is disrupted. The underlying reasons for decreased cardiac output are mentioned under the cardiovascular system subtitle. On the other hand, venous dilation increases renal interstitial and tubular pressure that lead to a hypoxic situation as well as compromising the glomerular filtration rate (Legrand et al., 2021). Another underlying reason is cytokine storm, a life-threatening situation during which the immune system is highly activated and inflammatory cytokines are extensively released, causing organ failure. In addition to systemic inflammation, cytokine release can happen locally in renal tissue. During COVID-19 infection, renal cells start to release inflammatory cytokines such as TNF and FAS that cause renal dysfunction by direct cell injury (Legrand et al., 2021). The virus can also induce cell damage through the cytopathic effect that invades renal cells directly. On the other hand, SARS-CoV-2 inhibits type I IFN production which leads to increased viral replication as well as immune dysregulation. Out-of-control complement release is another underlying reason for hyper-inflammatory state during SARS-CoV-2 infection that induces tissue injury. Adaptive immunity dysfunction such as T cell, plasmacytoid dendritic cell, eosinophil, and natural killer cell depletion has also been reported during the course of infection. The last mechanism is endothelial damage and micro-thrombi formation, the pathophysiology of which is discussed under the "cardiovascular system subtitle" (Legrand et al., 2021).

Eye

According to studies, COVID-19 infection does not cause specific retinal involvement, however, conjunctivitis has been reported in some cases with positive PCR (Pirraglia et al., 2020; Seah and Agrawal, 2020). On the other hand, patients with eye comorbidities are at risk of advanced or uncontrolled forms of the disease because of disrupted ophthalmic care delivery during the pandemic (Pujari et al., 2021). However, several ophthalmic symptoms have been reported in animal studies such as retinitis, conjunctivitis, anterior uveitis, chorditis, retinal detachment and, optic neuritis (Seah and Agrawal, 2020). The underlying vacuities during the COVID-19 infection can cause these symptoms (Seah and Agrawal, 2020). Tissue inflammation is also directly induced by viral replication in the retina that causes immune cells to infiltrate and pro-inflammatory cytokines to be released (Seah and Agrawal, 2020). An autoimmune nature of the infection is also suggested in the studies due to the autoantibodies that are produced during the infection against retinal cells that lead to degradation of photoreceptors, ganglion cells, and neuroretina (Seah and Agrawal, 2020). ACE2 protein, which is necessary for the virus entrance, is expressed in the

aqueous humor but more studies are needed in order to explore its expression in other structures such as conjunctiva or cornea (Seah and Agrawal, 2020).

Gut

Patients with COVID-19 can show several gastrointestinal symptoms such as diarrhea, nausea, vomiting, gastrointestinal bleeding, and abdominal pain (Zhang J. et al., 2020). ACE2 receptor, as well as TMPRSS2, are highly expressed in the gastrointestinal system, thus viral entrance and replication happen extensively in the gut after the respiratory system. Tissue inflammation underlies these symptoms during infection (Steardo et al., 2020; Zhang J. et al., 2020). High levels of fecal calprotectin and IL-6 as inflammatory factors support this claim (Zhang J. et al., 2020). Hyper-inflammatory syndromes such as hemophagocytic lymphohistiocytosis and cytokine storm also are accused of organ failure in the gastrointestinal system like other body organs (Zhang J. et al., 2020). However, based on studies human defensin-5 protein that plays an important role against SARS-CoV-2 in the gut, can be increased during the inflammation (Zhang J. et al., 2020).

Liver

Abnormal liver transferase levels can be detected in about 15–43% of COVID-19 patients with more probability in severe cases (Zhang C. et al., 2020). Acute liver injury has also been reported in some studies (Zhang C. et al., 2020). Liver injury can happen during systemic inflammation that is caused by inflammatory cytokine release during the course of infection. High levels of Th17 and 2, IL-2,6,7,10, TNF-a, granulocyte-colony stimulating factor, IFN-inducible protein-10, monocyte chemotactic protein 1and macrophage inflammatory protein 1 alpha in COVID-19 patients support this idea (Li and Fan, 2020). Liver dysfunction can also happen through direct viral invasion in the infection (Zhang C. et al., 2020). In addition, stress- induced liver injury is another pathologic event that can be caused by hypoxia-reoxygenation, activation of oxidative stress mechanisms, intestinal endotoxemia, and activation of the sympathetic nervous and adrenocortical system in COVID-19 patients (Li and Fan, 2020). As cholangiocytes highly express ACE2 receptors, they are suggested to be one of the main cells responsible for liver injury in COVID-19 infection (Zhang C. et al., 2020). However, ACE2 is poorly expressed in the liver, suggesting that there are other entrance paths of the virus to infect the cells (Steardo et al., 2020). Drug toxicity is also mentioned to be another reason for liver dysfunction (Li and Fan, 2020; Zhang C. et al., 2020).

Brain

Neurological symptoms of SARS-CoV-2 infection are categorized into two different groups, the first one is anosmia and ageusia that are reported in mild cases and the second group of symptoms consists of mental confusion and cognitive impairment in severe cases (Chigr et al., 2020). ACE2 receptors in the CNS are specifically expressed in the brain stem, subfornical organ, paraventricular nucleus, the nucleus of tractus solitaries, and rostral ventrolateral medulla which are responsible for respiratory and cardiovascular systems regulation, therefore a part of these systems dysfunction can be explained by this claim (Chigr et al., 2020; Steardo et al., 2020). There are different entrance paths to the nervous system. Nasal inoculation of SARS-CoV-2 can infect CNS via the olfactory bulb; other paths are bloodstream (blood-brain barrier (BBB) disruption) and vagus nerve that carries the virus from the respiratory system (Chigr et al., 2020; Steardo et al., 2020). Like other organs, tissue inflammation affects the nervous system. Microglia and astrocyte activation support this issue (Steardo et al., 2020). Astrocytes can be attacked by the virus, cells that play an important role in forming BBB, so by astrocyte involvement, the neuro-infection expands. This situation happens when a systemic cytokine storm is triggered by SARS-CoV-2 (Steardo et al., 2020). BBB disruption leads to neuroinflammation that causes neuronal death (Steardo et al., 2020). Hence, the cognitive disorder, behavioral and personality changes that are reported in severe cases can be explained by this mechanism (Steardo et al., 2020). Persistent neuro-inflammation along with hypoxia is accompanied by more severe presentations such as delirium (Steardo et al., 2020).

Cardiovascular System

As mentioned before, cardiac function can be compromised during SARS-CoV-2 infection leading to decreased cardiac output and in advanced stages, multi-organ dysfunction. Cardiovascular injury can happen through different mechanisms. The first one as was mentioned before is systemic inflammation which involves the cardiovascular system as well as other tissue (Petrovic et al., 2020). Low level of inflammation is the reason for non-specific viral infection symptoms but in severe cases, if the severe inflammatory response syndrome (SIRS) criteria are met, hemodynamic disorders such as shock, disseminated intravascular coagulopathy, and multi-organ failure will lead to cardiac dysfunction (Petrovic et al., 2020). In rare cases, myocardial injury due to hyper-inflammation is reported during COVID-19 infection that compromises cardiac function. Renin–angiotensin system (RAS) activation in the first stages of SIRS to reverse the situation, is responsible for increasing blood pressure through different mechanisms, one of them is vasoconstriction. At first, this condition helps the cardiovascular system to reverse the situation but by the time, hypertension will increase the burden on the cardiovascular system until the heart cannot compensate for the situation (Petrovic et al., 2020). In addition, viral entrance through ACE2 down- regulates its production. This leads to an imbalance between ACE2 and angiotensinogen II levels that is another reason for cardiovascular failure (Petrovic et al., 2020). Hypercoagulable state of the body in COVID-19 as well as plaque instability can exaggerate cardiovascular failure. Hyper-inflammation is one of the triggers of hypercoagulability by disrupting the hematopoietic system (Petrovic et al., 2020). It also plays a negative role in inducing plaque instability. Elevated levels of catecholamines as a result of inflammation may cause plaque rupture that can lead to acute coronary syndrome (Petrovic et al., 2020). Plaque rupture, by exposing its content (foamy macrophage) as well as smooth muscles, induces micro- thrombi formation that can move to other organs and cause organ dysfunction (Petrovic et al., 2020). IL-6, an

inflammatory cytokine, can also be released by smooth muscles and worsen the situation.

MODELING COVID-19

As the pandemic goes on, the necessity to discover the mechanisms of virulence and injury to cells and tissues becomes more and more critical (Sun et al., 2020; Youk et al., 2020). Since it is not always possible to directly investigate the pathophysiology mechanisms in human beings, several modeling methods have been introduced including organoids, different types of stem cells, and animals (Boudewijns et al., 2020; Li et al., 2020; Shpichka et al., 2020; Sun et al., 2020). Several types of 3D-designed organoids derived from body organs that are targets of SARS-CoV-2 organotropism are discussed. Stem cell models can be categorized into two main groups induced pluripotent stem cells (iPSCs) and non- iPSCs. Various types of animal models, each with the quality of being infected by the virus in an innate manner or by being induced through different methods are discussed extendedly under the subtitle. These models can have several functions other than helping the scientists understand different aspects of COVID-19 pathogenesis such as testing the efficacy of drug and vaccine candidates. Based on a recent update of the National Institutes of Health (NIH) COVID-19 treatment guideline, there are some antiviral, immunosuppressant, and antimalarial drugs that are used during the infection. Remdesivir -the only Food and Drug Administration (FDA)-approved COVID-19 drug- and dexamethasone are approved by the NIH guideline (Covid-19 Treatment Guidelines Panel, 2021). The convalescent plasma is suggested for the emergency cases. In addition, ivermectin, nitazoxanide, hydroxychloroquine, chloroquine, azithromycin, lopinavir/ritonavir, and other HIV Protease inhibitors are some other drugs that are not approved by the guideline. Remdesivir suppresses RNA transcription by inhibiting RNA polymerase function. Chloroquine as an antimalarial drug inhibits viral cell-binding by preventing the ACE-2 receptor to be glycosylated. Both Chloroquine and Hydroxychloroquine have immunomodulatory features as well as inhibiting viral fusion because of increasing endosomal pH and preventing viral genome to be released. Azithromycin as a synergistic drug has antiviral and anti-inflammatory effects. However, neither of them has shown efficacy in lowering viral load in respiratory tracts clinically. Ivermectin as an antiparasitic drug inhibits a specific type of intracellular transporting proteins of infected cells that are used by the virus to spread infection. But, it has not shown a significant clinical advantage in trials. HIV Protease inhibitors were suggested to inhibit COVID-19 proteases but trials did not support that clinically. Nitazoxanide is an antiparasitic drug that suppresses specific enzymes of the infected cell that are hijacked by the virus for processing viral proteins. Clinical trials do not confirm that in COVID-19 patients. Different types of anti-SARS-CoV-2 monoclonal antibodies have been proved to be effective in mild to moderate cases that are at the risk of COVID severity. Some other drugs are under evaluation such as colchicine, fluvoxamine, and other immunomodulators (Covid- 19 Treatment Guidelines Panel, 2021). COVID-19 specific T lymphocytes as a plasma subset of convalescent donors have shown some progress in this field (Ferreras et al., 2021). Furthermore, RM has made some steps toward COVID-19 treatment. As previously mentioned, the efficacy of using MSCs in the treatment of the infection has been shown in different clinical studies (Sánchez-Guijo et al., 2020). However, there is still much to be done for the suggested medical, immunologic and RM treatments to be approved by valid organizations. Modeling can extensively advance this field of study.

Stem Cell Modeling

Induced Pluripotent Stem Cells-Derived Models

Although embryonic stem cells (ESCs) have been used in many clinical trials and studies as a valuable source in different fields of regenerative medicine, downsides such as ethical concerns and immune rejection following allogeneic transplantation led to the development of iPSCs. These stem cells are produced by reprogramming adult somatic cells and like ESCs, can differentiate into any type of somatic cells. IPSC discovery resulted in enormous progress in research areas such as biomedicine, drug discovery, diseases pathophysiology and etiology, cell therapy, and generally, regenerative and personalized medicine (Doss and Sachinidis, 2019). Moreover, hiPSCs have been recently used as beneficial models of infectious diseases. For instance, hiPSC-derived hepatocytes have been infected with hepatitis B virus, and hiPSC-derived cardiomyocytes have been studied for cardiomyopathy of Chagas disease. Another example is the study of hiPSC-derived neural progenitor cells (NPCs) infection with Zika virus and Herpes Simplex virus-1(HSV1). Accordingly, different types of cells derived from iPSCs have been investigated for SARS-CoV-2 infection (Nolasco et al., 2020). Type2 alveolar epithelial cells (AT2s) play a crucial role in the lung by producing surfactant and differentiating into type 1 alveolar epithelial cells (AT1s). However, their proliferation capacity is poor in *in vitro* cultures. On the other hand, hiPSC-derived AT2s have greater proliferative potential. AT2s derived from hiPSCs (either in an organoid form or *in vitro* culture at the air-liquid interface) have provided a valuable tool for studying and modeling the effects of SARS-CoV-2 infection on these cells and presenting the changes in cellular and molecular mechanisms including loss of surfactant gene expression, cellular toxicity and stress, and viral entry via TMPRSS2 (Anderson and Francis, 2018; Abo et al., 2020; Nolasco et al., 2020). In addition to lung infection, SARS-CoV-2 can cause systemic inflammation and infect other systems and organs like CNS, digestive tract, and liver (Kase and Okano, 2020; Simoneau and Ott, 2020). One of the proposed mechanisms of viral migration is endothelium infection. Although direct infection of endothelial cells by SARS-CoV-2 is reported, there are controversial results from studying hiPSC-derived endothelial cells. Results of these studies showed that the infection of these cells by SARS-CoV-2 pseudo virus entry was lower than other cells like cardiomyocytes and even no infection was detected while they expressed ACE2 (Nolasco et al., 2020; Yang et al., 2020). Therefore, one of the hypotheses

for the actual pathogenesis is *in vivo* up-regulation of ACE2 in response to systemic changes and interferon stimulation, as ACE2 is an interferon-stimulated gene (ISG). This hypothesis needs to be tested by models of endothelial cells from iPSCs. Besides, COVID-19 is known to bring a hypercoagulable state upon patients-probably not in a similar way to other infections-presumably by cytokine storm and endothelial dysfunction. Therefore, hiPSC-derived endothelial cells can help understand the possible novel mechanisms of hypercoagulation in this vascular-thrombotic disease (Nolasco et al., 2020). There have been concerns about cardiac manifestations of COVID-19 since myocardial injury and arrhythmias are reported in some patients. ACE2 is expressed in cardiomyocytes that makes them susceptible to SARS-CoV-2 infection. It is found that patients with heart failure exhibit more ACE2 expression that can make them more prone to severe conditions. Also, high levels of highly sensitive Troponin-I (hs-cTnI) lead to a poor prognosis of the disease. It is not completely clear whether all cardiovascular changes are directly due to the virus infection or are results of other organ dysfunctions like impaired pulmonary function. Accordingly, different studies have used cardiomyocytes derived from hiPSCs and hESCs for modeling this condition and drug screening (e.g., ACE inhibitors or ARBs, remdesivir, and chloroquine). It is found that the cells are susceptible to viral infection and replication that causes apoptosis and contractility alterations, including ceased beating. In addition, the infection induces changes in gene expression like down-regulating ACE2 expression and the genes associated with mitochondrial function (Choi et al., 2020; Nolasco et al., 2020; Sharma et al., 2020). ACE2 has well-known protective effects for the heart by inhibiting overload of angiotensin II. Studies have shown that SARS-CoV-2 causes translocation of ACE2, which results in its suppression, and also the virus increases brain natriuretic peptide (BNP) expression. Hence, the infection results in dysregulation of angiotensin balance and leads to inflammation and thrombosis as well as increasing some inflammatory cytokines. It can be concluded that both the direct cytopathic effect and the immune response of the host are responsible for the cardiac injury (Wong et al., 2020). Different neurological disorders in patients with COVID-19 (especially those with comorbidities) are reported such as headache, loss of smell and taste, meningitis, and acute hemorrhagic necrotizing encephalopathy (Kase and Okano, 2020; Moriguchi et al., 2020; Poyiadji et al., 2020; Simoneau and Ott, 2020). It seems that SARS-CoV-2 can have a direct pathogenic effect on CNS cells in addition to causing cytokine storms, but the exact mechanism remains unclear. Therefore, many iPSC-derived models have been used to understand this mechanism (Simoneau and Ott, 2020). Cysteine-rich protein 61(Cyr61) or CCN family number 1(CCN1) is a virulent factor that is known to be enhanced in SARS-COV-2 infection. Further, ACE2 expression is high in some parts of the brain like the thalamus and choroid plexus. To investigate the potential role of these receptors for CNS infection, the expression of ACE2 and CCN1 was examined in neural stem cells (NSCs) and NPCs derived from hiPSCs. The expression of these SARS-CoV-2 targets not only was seen in hiPCS-derived NSC/NPCs but also was found in the young neurons differentiated from these cells, thus SARS-CoV-2 can infect them. Moreover, this COVID-19 model was used to find a therapeutic approach. Pretreatment of hiPSCs-derived NSC/NPCs (neurosphere) with γ-secretase inhibitors (GSIs), DAPT, and compound 34, which inhibit signaling, was performed. The expression of CCN1 was significantly suppressed in neurospheres. Hence, GSIs can be used to treat CNS disorders of COVID-19, though further investigation is required (Kase and Okano, 2020). Some other studies of iPSC-derived brain organoids are addressed in the next section.

Other Stem Cell Models

In addition to the iPSC-derived models discussed, we reviewed models derived from other types of stem cells. For instance, scientists have used cultured human airway basal stem cells (ABSCs) as a MSC-based model of SARS-CoV-2 infection (Purkayastha et al., 2020). It was confirmed that the viral load in ABSCs that were exposed to cigarette smoking (CS) was about 2-3 folds higher than in the mock-exposed group. The result changed after 72 h due to cell apoptosis as well as patients' genetic differences. Other effects of CS on the respiratory tract are decreased ciliated cells as well as the increased level of ABSCs as a part of a repair response stimulated by the specific exposure. None of them would happen in a normal process of virus pathogenicity due to repair mechanism inhibition. An increased rate of apoptosis is also another finding during SARS-CoV-2 infection that is confirmed by ABSCs model. This process is reinforced by CS. This model also can be applied to investigate the effect of interferon therapy on innate immune system activity state. Based on the findings of ABSC models, down-regulation of gene expression including those associated with immune responses and metabolic processes would be another result of the infection, however, genes involved in interferon signaling and chromatin organization seem to be the exceptions. Altogether, ABSCs can be a suitable MSC model in order to study the virus mechanism of action leading to acute lung injury in humans, on which the effect of environmental factors such as CS can be studied (Purkayastha et al., 2020).

Organoid Models

Regenerative medicine has provided organoids that have many advantages in the field of disease modeling in comparison to *in vivo* and other *in vitro* models. Organoids can be generated either from adult stem cells or PSCs. Establishing organoids from PSCs like iPSCs and ESCs needs the media containing growth factors for culturing in a way that mimics the process of developing a particular structure in an embryo. On the other hand, organoids derived from adult stem cells are formed by providing a 3D matrix and growth factors for the resident stem cells of our targeted tissue (Atala et al., 2020; van der Vaart et al., 2021). This novel technology of artificially developed 3D structures is more accessible and a faster tool than animal models. Moreover, they mimic the relevant niche and maintain the genetic profile and physiological characteristics of their original tissue. Hence, since organoids have contributed to gaining more insight into the pathophysiology of different

organ dysfunctions and investigating therapeutic approaches, they can serve as valuable models for investigating the SARS-CoV-2 infection and its treatment strategies (Atala et al., 2020; Mahalingam et al., 2021).

Lung

The primary cause of mortality in this pandemic is lung disease; therefore respiratory models including airway and alveolar organoids were used to study SARS-CoV-2. As an example of airway organoids, Lamers et al. generated 2D bronchoalveolar-like organoids in the air-liquid system and small airway 2D cultures from human small airway stem cells. The bronchoalveolar model included alveolar, basal, and rare neuroendocrine cells which were grown from 3D lung bud tip progenitor organoids. The models were infected by the virus and AT2 like cells were targeted in bronchoalveolar type while in small airway model, ciliated cells were known as the main targets of SARS-CoV-2. Moreover, to test drug screening, the bronchoalveolar model was treated with IFN-λ1. The results showed reduced viral replication and infection. Despite the advantages of studying the pathogenesis using the airway organoids, establishing more specialized alveolar systems are essential (Salahudeen et al., 2020; Lamers et al., 2021; van der Vaart et al., 2021). ARDS is one of the most severe clinical presentations of COVID-19. As previously described, AT2s have a pivotal role in lung involvement and ARDS. However, modeling AT2s *in vitro* has been challenging due to limitations like rapid dedifferentiation, loss of phenotype, and requirement of supporting fibroblasts (Huang et al., 2020; Katsura et al., 2020). Accordingly, some recent studies have developed new models of AT2s derived from iPSCs. Huang et al. generated iPSC-derived AT2s cultured in 2D air-liquid interface and 3D epithelial spheres expressing surfactant protein-C (SFTPC). The 2D cultures were used to adapt the model with the physiopathology of SARS-CoV-2 infection as it happens at the apical membrane of the cells, 2D cultures can simulate the mechanism more accurately than the 3D types. ACE2 and TMPRSS2 expression was confirmed by immunofluorescence staining and the SARS-CoV-2 infection was indicated by localizing the viral particles in different spaces like the lamellar body and tubular myelin. After the infection, the epithelial-intrinsic innate immune response including inflammatory phenotype and NF-κB signaling (1day post-infection), decreased expression of surfactant gene, cellular stress and toxicity, moderate IFN responses, and iPSC-derived AT2 death (4 days post-infection) was observed. Moreover, camostat mesylate (aTMPRSS2 inhibitor) and remdesivir administration resulted in reduced infection, which indicates therapeutic potentials (Huang et al., 2020). Katsura et al. (2020) established a novel alveosphere culture for AT2s from primary lung tissue that expressed ACE2 and TMPRSS2, and was permissive to the infection. Similar findings were reported after the SARS-CoV-2 infection including up-regulation of inflammatory signaling, cell death, surfactant loss, and the IFN response. In addition, pre-treatment of the alveospheres with IFNs revealed a prophylactic effect and reduced viral titers. One of the differences between these two studies is the time of IFN response; in the alveosphere model, the IFN pathway was detected 48 h post-infection while in the iPSC-derived model, it occurred 4 days after the infection. Another 3D culture technique was developed for hAT2s from healthy donor lungs by Youk et al., which established the cellular polarity and made the AT2s stable, although it did not completely present the full alveoli. It demonstrated transcriptional changes after SARS-CoV-2 infection, IFN response, and ISGs expression at 3 days post-infection (Youk et al., 2020). By comparing the details and results of the mentioned studies, some models have considerable advantages. For instance, 2D structures showed more virus titer than the 3D organoids, which can be more useful for testing the antiviral agents. Further, in 3D organoids, the apical side of the cells is inside the model while in the 2D culture, especially the bronchoalveolar-like model, they are exposed to the air. Hence, they are suggested to be more relevant and suitable for studying the virus pathogenesis (Lamers et al., 2021). Besides, Han et al. generated an *in vivo* model of lung organoids. The organoids were developed from human pluripotent stem cells (hPSCs) and the presence of AT2-like cells and the expression of ACE2, and TMPRSS2 were validated. To make the *in vivo* model, progenitor cells of the lung were transplanted subcutaneously into non-obese diabetic (NOD) severe combined immune deficiency (SCID) gamma (NSG) iL2RG^{nul} mice and produced structures with AT2-like cells expressing ACE2. To test the infection, the xenograft was inoculated with SARS-CoV-2 entry virus. The RNA analysis confirmed the infection, and the infected organoids showed enhanced chemokine signaling compared to the mock-infected group. Moreover, the inhibitory effects of some FDA-approved drugs including imatinib, mycophenolic acid (MPA), quinacrine dihydrochloride (QNHC), and chloroquine were investigated and a significant reduction of infection and virus entry was observed. In addition, the transplanted mice were treated with imatinib mesylate, MPA, or QNHC before virus inoculation that decreased the luciferase staining. Conclusively, lung organoids were introduced as useful candidates for modeling the drug discovery of COVID-19 (Han et al., 2021).

It is evident that organoids can contribute to understanding the mechanism of this new infection. The airway models can be studied for virus shedding and mild forms of the disease and the alveolar models are more relevant for research on severe stages and complications of COVID-19 (van der Vaart et al., 2021).

Liver

A liver organoid is produced by differentiation of hiPSCs into definitive endoderm and then, inducing liver cells (mainly albumin$^+$cells). The immunostaining showed ACE2 expression in most albumin$^+$ hepatocytes. Next, using the SARS-CoV-2 pseudo-entry virus showed that the organoid is permissive to the infection that is similar to adult primary hepatocyte and cholangiocyte organoids. The results were consistent with the reports of COVID-19-related hepatitis, which was observed in some patients (Yang et al., 2020; Yu et al., 2020). In order to investigate the pathogenesis of SARS-CoV-2 infection in the liver, human liver ductal organoids were generated from liver bile duct-derived progenitor cells in a long-term 3D culture system. The immunostaining indicated ACE2$^+$ and TMPRSS2$^+$ cholangiocytes and the examination of SARS-CoV-2 genomic

RNAs showed susceptibility of the cells to infection and increased viral load. Besides, the significant decrease in the viral load 24 h after the infection was due to enhanced expression of apoptotic factors and cholangiocytes' death. Since the main function of cholangiocytes is the transportation of bile acid, the tight junction between them is necessary for the collection and excretion of bile acid from hepatocytes into bile ducts. In SARS-CoV-2 infection, the expression of genes involved in cell junction organization such as claudin1 (CLDN1), a bile acid transportation like solute carrier family 10 member 2(SLC10A2) and cystic fibrosis transmembrane conductance regulator (CFTR) is decreased. Hence, it causes liver damage by impairing cholangiocyte function and accumulation of bile acid. In addition, the organoid can be used for drug discovery to prevent liver injury of COVID-19 (Zhao et al., 2020; Youhanna et al., 2021).

Eye

Although it is reported that eyes are affected in COVID-19, there is no available evidence of retinal involvement (Ahmad Mulyadi Lai et al., 2021). ACE2 and TMPRSS2 are known to be expressed in the retinal organoid (Mahalingam et al., 2021). Accordingly, hiPSC-derived retinal organoids and a retinal monolayer culture dissociated from the organoid were utilized. Real-time quantitative PCR (qPCR) and immunofluorescence staining showed ACE2 and TMPRSS2 expression both in the organoid and monolayer cultures. Additionally, SARS-CoV-2 pseudovirus containing the GFP coding sequence was employed to study the susceptibility of the organoids and the monolayer cultures to the infection. Detecting GFP signals demonstrated that SARS-CoV-2 could infect them and enhance the expression of some genes related to the apoptosis pathway and inflammation response. On the other hand, in a pre-print study, Makovoz et al. has produced hPSC-derived whole eye organoids. The results showed that the expression of ACE2 and TMPRSS2 in the cornea was high and the corneal organoids were permissive for virus entry and infection. Limbus was found to be the most susceptible part which exhibited a significant NF-κB mediated inflammatory response. However, it is not clear whether the inflammation is due to direct infection or the virus spreading to the eye through systemic infection (Makovoz et al., 2020; Yu, 2021). Altogether, it seems that the eye is a route of infection; hence the organoids and monolayer cultures represent useful models of studying the SARS-CoV-2 mechanism of pathogenesis (Ahmad Mulyadi Lai et al., 2021).

Kidney

One of the most common organ damages in COVID-19 patients is AKI. Moreover, ACE2 is significantly expressed in convoluted tubules but it is not clear whether the kidney injury is due to direct cytopathic effects of the virus or immunopathogenic damage (Xia et al., 2020; Mahalingam et al., 2021). Hence, 2D conditional reprogramming (CR) and 3D organoid of human kidney proximal tubule epithelial cells (KPTECs) were established as novel platforms. The DNA repair ability and lineage function, and expression of specific function markers were maintained in CR KPTECs. Interestingly, the ACE2 expression was detected in both models with a higher level (around two-fold) in the 3D organoid. The permissiveness of the reprogrammed cells and the models for SARS-CoV-2 infection was estimated by reading relative luciferase units (RLU) after the pseudovirus infection. Hence, these cells have served as physiological platforms to model SARS-CoV-2 infection, investigate the kidney response, and use them for drug discovery (Xia et al., 2020). Further, one of the proposed treatments of COVID-19 is human recombinant soluble ACE2 (hrsACE2) which has undergone phases 1 and 2 clinical trials. The potential inhibitory effect of hrsACE2 was tested in kidney organoids developed from hESCs. A mixture of hrsACE2 and particles of SARS-CoV-2 was used for infecting the kidney organoid and qRT-PCR indicated a dose-dependent reduction in the level of SARS-CoV-2infection and viral load (Monteil et al., 2020).

Gut

Gastrointestinal symptoms of COVID-19 are one of the most familiar and prevalent effects of the infection that are present in around 50% of patients (Krüger et al., 2021). For improving research quality, different intestinal and colonic organoids were produced. For instance, a human small intestinal organoid (hSIO) was developed from primary gut epithelial stem cells. Cultured hSIOs were exposed to both SARS-CoV and SARS-CoV-2 and qRT-PCR assessment showed infected enterocyte lineage cells with both viruses. Further, mRNA sequence analysis indicated that SARS-COV-2 caused ISGs and cytokines induction and up-regulation of ACE2 stronger than SARS-COV (Lamers et al., 2020). In another study, human intestinal organoids were derived from PSCs. ACE2 and TMPRSS2 expression was seen in the intestinal cells except for goblet cells, which lacked ACE2 expression. The viral RNA levels exhibited infection of the organoid cells (not goblet cells). Additionally, the inhibitory effects of remdesivir, famotidine, and EK1 (a peptidic pancoronavirus fusion inhibitor) were investigated. The results showed that remdesivir blocked SARS-CoV-2 replication in a dose dependent manner; EK1 decreased the number of infected cells. However, famotidine did not show any inhibitory effect in the intestinal organoids. Thus, human intestinal organoids derived from PSCs represented a valuable physiological model for experimental studies (Krüger et al., 2021). Further studies indicated ACE2 and TMPRSS2 expression as well as SARS-COV-2 permissiveness of colonic organoid derived from hPSCs. For studying COVID-19 *in vivo*, the colon organoids were transplanted under the kidney capsule of NSG mice. In addition to ACE2 detection, infecting the organoid with SARS-CoV-2 via intra-xenograft inoculation confirmed the permissiveness and the viral infection of the organoids. Next, the inhibitory effects of some FDA-approved drugs were tested. Colonic organoids were treated with imatinib, QNHC, and MPA and then infected with SARS-CoV-2. They all resulted in decreased viral RNA level and nucleocapsid protein expression that means blocking the infection. Taken together, the colonic organoids have introduced an experimental model for drug discovery (Han et al., 2021).

Cardiovascular System

The endothelial damage of COVID-19 is a matter of great importance that needs further investigation. Not only As previously mentioned, hiPSCs derived endothelial cells express ACE2, yet they seem not to be infected with SARS-CoV-2 since the pseudo-entry virus resulted in low luciferase activity (Yang et al., 2020; Yiangou et al., 2020). On the other hand, capillary organoids derived from iPSCs exhibited SARS-CoV-2 replication that was revealed by analyzing qRT-PCR. Moreover, as discussed in kidney organoids, Monteil et al. revealed that the infection was reduced by adding hrsACE2 (Monteil et al., 2020). Collectively, there is a possibility that the injury of the endothelium is not due to direct infection but the paracrine effects that should be studied in different relevant models (Yiangou et al., 2020).

Brain

In spite of the reports on finding SARS-CoV-2 in CSF and brain, the exact prevalence of CNS infection and the mechanism of virus entry and pathogenesis are not fully understood (Pellegrini et al., 2020; Song et al., 2021). In a hiPSC-derived brain organoid, SARS-CoV-2 infected the neurons and induced cell death despite low levels of ACE2 expression. However, a productive replication of the virus was not detected. Besides, SARS-CoV-2 caused aberrant Tau localization and phosphorylation. Tau is a protein that is responsible for stabilizing the microtubules of neurons and promoting axonal growth. Tau dysfunction is known to be involved in Alzheimer's disease. While it is mainly localized at the axons, the infected neurons showed an increased level of Tau into the cell somas. Moreover, it was revealed that virus entry caused tau phosphorylation at the T231 site, which is remarkable for aberrant phosphorylation leading to neural toxicity. Although tau hyper phosphorylation and abnormalities in the infected neurons were detected, it is not completely clear whether they are the direct effects of the virus or the results of neural stress. Hence, more investigation is suggested (Ramani et al., 2020). Another study developed dorsal forebrain organoids from hESCs and reported that ACE2 was expressed in the organoid with a significant decreased level in the progenitor and stem cells. The neurons were found susceptible to the spike-containing SARS-CoV-2 pseudo-entry virus but increasing the viral load did not elevate the virus infectivity (Yi et al., 2020). Besides, the BBB and blood-CSF-barrier (B-CSF-B) are known as major obstacles to the entry of pathogens like SARS-CoV-2. B-CSF-B, which is simpler than BBB, consists of an epithelial layer of the choroid plexus, which has interactions with blood. In addition, it participates in the immune response by producing some inflammatory cytokines in the CSF. To test the choroid plexus role in SARA-CoV-2 neurotropism, the hPSC-derived brain organoids, which represented the choroid plexus and the cortical tissue, were developed. ACE2 and TRMPSS2 expression were detected in the choroid plexus and not in the neural progenitors. Next, the organoids were incubated with both SARS-CoV-2 spike pseudo virus and live SARS-CoV-2. The results showed that the epithelial cells of the choroid plexus were susceptible to the virus infection and its replication while the neural region exhibited little to no infection. Moreover, at 4 days post-infection, considerable damage to the barrier integrity and a decrease in the internal fluid (CSF leakage) were reported (Pellegrini et al., 2020). Similar findings were reported by Jacob et al. who generated monolayer cultures of neurons, astrocytes, and microglia as well as organoids of the cerebral cortex, hippocampus, hypothalamus, midbrain, and choroid plexus (Jacob et al., 2020). The results showed no infection in the monolayer culture of microglia and sparse infection in cortical neurons and astrocytes. Additionally, introducing SARS-CoV-2 to the multiple organoids indicated a limited infection rate in neurons and astrocytes, which did not exhibit a significant increase, days after the infection. Notably, the choroid plexus regions in some organoids like the hippocampus showed greater numbers of infected epithelial cells. For more investigation, hiPSCs were grown to differentiate into the choroid plexus organoid, in which a productive infection was observed. SARS-CoV-2 led to enhanced cell death, inflammation, and changes in the barrier and secretory function (Jacob et al., 2020).

The number of studies using iPSC-derived cells and organoid models of COVID-19 associated disorders is rising. Most of these studies are discussed in the text and some other researches and pre-print literature are depicted in **Figure 1**. Also brief data on the pros and cons of each stem cell or organoid model check **Table 1**.

Animal Models

As previously discussed, COVID-19 is a new scientific field of study with noticeable gaps that have to be covered especially in testing the efficacy of new therapeutic methods in the field of clinical as well as regenerative medicine. Several animal models can be used with similarities to the human body in some aspects. These species also have been used for testing drug/vaccine candidates of clinical medicine and proved their efficacy, so it is implied that they can be suitable models for regenerative medicine investigations. Different species have been used in this field among which, mice are the most common (Sun et al., 2020). The problem is that mice are resistant to SARS-CoV-2 therefore this issue is resolved by introducing the human ACE2 (hACE2) receptor via an adenovirus into the cells (Atala et al., 2020; Zheng et al., 2021). In a study, cytokeratin 18 (KRT18) promoter, which is an epithelium specific gene, was used for the host cells to express hACE2 (Chow et al., 1997; Zheng et al., 2021). This process causes pneumonia along with weight loss, severe pulmonary pathology, and SARS-CoV-2 replication in the lungs (Sun et al., 2020; Zheng et al., 2021). In some other studies, Lipopolysaccharide has been used to simulate the hyperinflammatory state caused by SARS-COV-2 infection (Molinaro et al., 2020). Mice-based studies confirmed the role of the interferon I (IFN-I) pathway in the clearance of virus as well as the role of interferon II (IFN-II) signaling through signal transducer and activator of transcription 1 (STAT-1) in diminishing clinical severity and hyperinflammatory state of the respiratory system (Sun et al., 2020). In an experiment of Ad5-hACE2 mice, the application of plasma from recovered patients improved the disease severity in addition to increasing the clearance rate of the virus as well as remdesivir (Sun et al., 2020). Leukosomes have been suggested to promote the efficacy of anti-inflammation drugs when applied as a drug delivery system (Molinaro et al., 2020). Ferret is another animal

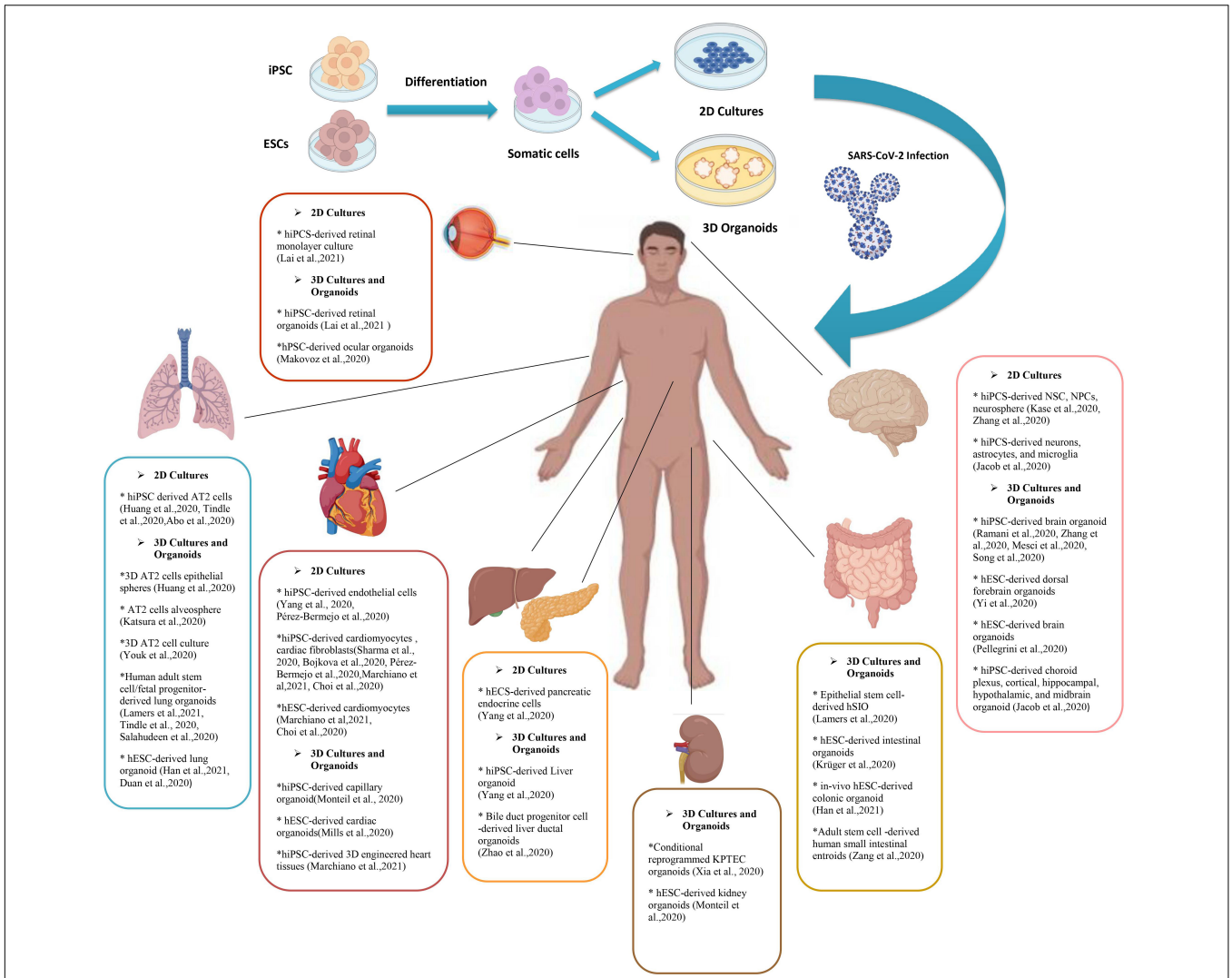

FIGURE 1 | A Summary of pluripotent stem cell (PSC) derived 2d and 3D models of SARS-CoV-2 infection. Human induced pluripotent stem cells (hiPSCs) and human embryonic stem cells (hESCs) have been used to produce adult somatic cells of different organs. Culturing these cells can become either 2D models of the cells or tissue or the novel model of 3D organoids. Various studies have used PSCs (and some adult stem cells) to investigate the SARS-CoV-2 infection mechanism and its manifestations. This figure provides a brief review of currently available researches on different stem cell-based models to discover the SARS-CoV-2 mechanism of action and potential therapeutic strategies. PSCs, Pluripotent stem cell; hESCs, Human embryonic stem cells; hiPSCs, Human induced pluripotent stem cells; SARS-CoV-2, Severe acute respiratory syndrome Coronavirus-2; NSCs, Neural stem cells; NPCs, Neural progenitor cells; AT2s, Type2 alveolar epithelial; 2/3-D, 2/3 dimensional; KPTECs, Kidney proximal tubule epithelial cells; hSIO, Human small intestinal organoid.

model of SARS-COV-2 infection, which is prone to SARS-COV *per se* and presents symptoms like cough, rhinorrhea, and lower physical activity (ter Meulen et al., 2004; Park et al., 2020). Positive samples of infectious virus only could be collected through upper respiratory tract washes, and not other collected specimens such as a rectal swab, which was positive of low copy numbers of non-infectious virus (Shi et al., 2020). Then, the application of suggested drugs such as lopinavir-ritonavir, Hydroxychloroquine (HCQ), emtricitabine-tenofovir, and human monoclonal antibodies led to lower severity of clinical presentations (ter Meulen et al., 2004; Park et al., 2020). Another study showed that as well as ferrets, cats, and with less susceptibility, dogs are prone to SARS-CoV-2. In a study using cats as animal models, it was proved that the infection transmits in an airborne state among cats (Shi et al., 2020). Besides, in a study of Syrian hamsters as one of the permissive species to SARS-CoV-2, the STAT-2 signaling mechanism has been considered as one of the mechanisms involved in the virus pathogenesis as well as its protective role as part of the immune system (Boudewijns et al., 2020). Each discussed model has some advantages that make it appropriate to be used as a human body simulator but some disadvantages that make investigations more challenging. However, assessing these disadvantages opens new doors toward developing more efficient animal models. Several types of species models, specific manifestations of each after virus inoculation, and results of suggested drugs/vaccine candidates have been

TABLE 1 | Pros and cons of stem cell and organoid models of Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2).

Type of model		Pros	Cons	References
Stem cell models	MSC-based models	- Lower cost in contrast to lung organoid models	- Various differentiation capacity of stem cells from different donors - variation in the number of infected cells in each set of cultures - Limited sample size due to the challenges and costs of obtaining human tissue - Lack of inflammatory cells in the cultures - Offering only short-term culture capabilities in contrast to the stability and indefinite growing capabilities of organoid models	Purkayastha et al., 2020; Chugh et al., 2021
	IPSC-derived models	- Generating various differentiated cells with same genetic background -Mimicking the biology of host-virus interaction - Patient (genotype)-specific - Suitable for gene editing - The susceptibility to become more complex via co-culturing - Easy to keep in culture and maintain	- Labor/time consuming and higher cost culture - Possible variations among paths of differentiations - Some limitations in representing the exact *in vivo* manifestation of infected tissues	Nolasco et al., 2020; Chugh et al., 2021
Organoid models	Brain	- Allowing the scientist to explore neurotoxic effect of COVID-19 - A suitable platform to study the antiviral effects of anti COVID-19 drugs through preserving BBB from viral damage	-Absence of vascularization as in human brain - Requiring modification for making long-term observation possible	Chugh et al., 2021
	Gut	- Containing all proliferative and differentiated cell types of the *in vivo* epithelium - Reflecting the high susceptibility of gastrointestinal system to be infected *in vivo*, by representing the high rate of enterocytes infection as the most common cell type pf intestinal system.	- Weakness in showing the patient's defense mechanisms against digestive system infection during COVID-19 (intestinal flora and lymphatic system)	Lamers et al., 2020; Yu, 2021
	Kidney	- Effectively representing COVID-19 associated AKI - Showing the efficacy of combination therapy using Remdesivir with human recombinant soluble ACE2 in reducing virus entry and replication.	- Cannot mimic the exact features of native renal tissue	Chugh et al., 2021
	Cardiovascular system	- Suitable for exploring the efficacy of new drug candidates in reversing cardio-toxic effects of the virus - Appropriate for validating the potential role of specific genetic variants in COVID-19 pathology	- The impossibility of mimicking arrhythmia and myocardial infarction - The need for careful adjusting of drug doses because of higher drug levels in the organoid in comparison to human blood due to the absence of metabolic organs in the cardiovascular organoids	Yiangou et al., 2020; Yu, 2021
	Lung	- Allowing efficient viral replication - Suitable for exploring interactions between human cells and viruses and the response of lung stem cells to SARS-CoV-2 - Can be used to test drugs targeting a wide range of viruses	- The absence of stroma and immune cells - The absence of a definite culturing protocol in order to prevent bias	Chugh et al., 2021; Lamers et al., 2021; Mallapaty, 2021; Yu, 2021
	Liver	- Investigated liver tissue damage of SARS-CoV-2 *ex vivo*. - Mimicking host-virus interaction due to retaining the biology of individual tissues such as preserving human-specific ACE2 + /TMPRSS2 + population of cholangiocytes	- Inability to reflect the cellular complexity of human hepatobiliary system for instance specific immune cell subsets -Lack of non-parenchymal cells -Presenting immature liver phenotype	Zhao et al., 2020; Lui et al., 2021; Youhanna et al., 2021; Yu, 2021

SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; AKI, Acute kidney injury; MSC, Mesenchymal Stem Cell; IPSC, Induced pluripotent stem cells; BBB, Blood Brain Barrier; ACE2, Angiotensin-converting enzyme 2; TMPRSS2, Transmembrane protease; serine 2.

TABLE 2 | Animal models of Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-COV-2) and the results of tested drug/vaccine candidates.

Animal model	Method of model designing	Presented symptoms	Tested drugs/vaccine candidates	Results	References
Ferrets	Readily permissive to the virus	-↑Body temperatures -Infectious virus shedding through upper respiratory tract washes - Loss of appetite	-Lopinavir-ritonavir -HCQ -Emtricitabine-tenofovir	-↓Overall clinical scores than control group -↓Virus titer in emtricitabine-tenofovir-treated group	ter Meulen et al., 2004; Park et al., 2020; Shi et al., 2020
			Prophylactic human monoclonal antibody (against SARS-CoV)	-↓Viral replication in the lung -↓Viral induced lung pathology	
Outbred domestic cats	Readily permissive to the virus	-Infectious virus shedding through nasal turbinate, soft palates, tonsils, tracheas, lungs -Massive lesions in the nasal and tracheal mucosa epitheliums and lungs			Shi et al., 2020
hACE2 BALB/c mice	-Introducing hACE2 via adenovirus -Introducing KRT18	-Severe pneumonia -vasculitis -Severe brain involvement in some cases -Anosmia -Weight loss	-Plasma of recovered patients -Poly I:C -Remdesivir	-Protection against lethal severity -Accelerating virus clearance	Sun et al., 2020; Zheng et al., 2021
BALB/c mice	Intraperitoneal injection of LPS	Severe lung damage (ARDS)	DEX-loaded leukosomes	-↑Therapeutic activity of dexamethasone -↓Inflammatory response	Molinaro et al., 2020; Zhang N. N. et al., 2020; Kaspi et al., 2021
			ARCoV vaccine candidate	-↑Innate immune cells in the muscles in IM injection -↑Neutralizing antibodies and IgG -↑Cellular immunity (CD4 + or8 + T cells) -↑IFN-II, TNF-a, IL-2 -NC in IL-4 and IL-6	
			Exo MSC-NTF	-↓Alveolar wall thickness, fibrin presence, and neutrophil accumulation -↑Oxygenation saturation -↓Proinflammatory cytokines (IFN-II, IL-6, TNF-a, RANTES) - ↓ TF, TAT	
C57BL/6 mice (Ifnar1-/- or Il28r-/-)		- Mild lung pathology (both) -↑Viral replication (Ifnar1-/-) -↑Intra-alveolar hemorrhage, peribronchiolar inflammation (Ifnar1-/-)	Plasma of recovered patients	-3-10-fold↓in viral loads -NC in histopathological features -↓Akt1 mRNA levels -↑DDX58, cGAS mRNA levels	Yang et al., 2020
Immunocompetent BALB/c mice	Inoculated with MASCp6 virus	-Moderate pneumonia and inflammatory responses in the lung and trachea	ARCoV vaccine candidate	-Full immunization	Zhang N. N. et al., 2020
Macaca fascicularis	Readily permissive to the virus		ARCoV vaccine candidate	-↑IgG -↑IFN-II -↑IL-4 + /CD4 + cell response(no difference between vaccinated and placebo group)	Zhang N. N. et al., 2020

(Continued)

TABLE 2 | (Continued)

Animal model	Method of model designing	Presented symptoms	Tested drugs/vaccine candidates	Results	References
Syrian hamsters (WT)	Readily permissive to the virus	-Bronchopneumonia and inflammatory response, neutrophil infiltration, multifocal necrotizing bronchiolitis and edema in the lungs	-VHH-72-Fc -Plasma of recovered patients	-↓Viral loads in the lung (VHH-72-Fc)	Boudewijns et al., 2020
Syrian hamsters (STAT2-/- or IL28R-a-/-)	Readily permissive to the virus	-↓ISG expression -↓Antiviral response -NC in viral RNA levels in the lung (more infectious virus in STAT2-/-) -↑Proinflammatory cytokines in the lung -↓Lung pathology and PMN cells infiltration (STAT2-/-) -Bronchopneumonia and peribronchiolar inflammation (IL28R-a-/-)			Yang et al., 2020

hACE2, human Angiotensin-Converting Enzyme 2; KRT18, cytokeratin 18; HCQ, Hydroxychloroquine; LPS, lipopolysaccharide; DEX, dexamethasone; ARCoV, a vaccine candidate with the component of a mRNA sequence encoding the receptor binding domain of the virus which is encapsulated in lipid nanoparticles; IFN-II, interferon II; TNF-a, tumor necrosis factor alpha; IL-2, interleukin-2; NC, no change; Exo MSC-NTF, MCS-derived exosomes producing neurotrophic factor; RANTES, CCL5; TF, Tissue Factor; TAT, Thrombin Antithrombin complex; MASCp6, mouse-adapted strain at passage 6; Ifnar1-/- mice, C57BL/6 mice with a genetic ablation of their type I interferon receptors; Il28r-/- mice, C57BL/6 mice with a genetic ablation of their type III interferon receptors; WT, wild type; STAT2-/- hamster, lacking type I and III IFN signaling; IL28R hamster, lacking IFN type III signaling; ISG, interferon-stimulated genes; GI, gastrointestinal; PMN, polymorphonuclear; VHH-72-Fc, SARS-CoV-2-specific single-domain antibody Fc fusion construct.

provided in **Table 2**. Also, pros and cons of each species model are accessible in the **Figure 2**.

Challenges and Limitations

Despite all advantages of the models which were discussed, there are different challenges in the experimental researches on SARS-CoV-2 infection. IPSCs have been widely studied especially for generating organoids, to model the new pandemic disease, but there are some limitations that should be mentioned. For instance, there are some reports that during the reprogramming process, some of the epigenetic memory of donor cells is retained in iPSCs that can result in biased differentiation to target cells. Moreover, there are risks of tumorigenicity in the case of cell transplantation which are contaminated with undifferentiated iPSCs. However, novel protocols for differentiation and the purification process as well as using molecules that cause selective death of these cells have decreased the risks but more follow-up is needed. Besides, differentiating somatic cells from hiPSCs is considered to be expensive and time-consuming (Doss and Sachinidis, 2019; Nolasco et al., 2020). Notable bottlenecks of modeling using iPSC-derived cells are lack of maturity and exhibiting embryonic-like characteristics, especially in late-onset diseases. Some proposed ways to overcome these drawbacks are treatment with mitochondrial stress inducers to provoke aging and direct differentiation of somatic cells like fibroblasts to the relevant cells like neurons that share similar cell markers. On the other hand, novel technological advances like gene editing, organ-on-chip, and "-omics" methodologies can improve the iPSC-based therapeutic approaches (Doss and Sachinidis, 2019). Organoids, as a product of advancements in the fields of PSCs and cell therapy, ECM biology, and tissue engineering, have played a critical role in experimental studies. In the field of RM, organoids have been presented for transplantation instead of organs like the liver, as they recapitulate the main aspects of the organs. Moreover, they represent a step forward to drug screening and a promising tool for personalized medicine. However, there are some obstacles both in the future transplant process and the current modeling area of this novel tool. Some of the challenges and disadvantages of different organoids and iPSC-derived cells are addressed in **Table 1**. The shape of the organoids, their size, and cell composition and maturation are not completely under control. Additionally, modeling complex and chronic diseases needs a suitable microenvironment and is based on advanced technology in bioengineering (Prior et al., 2019). Major limitations of organoids in studying SARS-CoV-2 infection include their simplicity and lack of immune cells. Therefore, the studies mainly focus on intrinsic pathways. For instance, SARS-CoV-2 causes epithelial damage and inflammatory response in the lung. Hence, the absence of immune cells and stroma are known as major challenges and limitations of lung organoids (Chugh et al., 2021). It is suggested that the organoids can be introduced to immune cells in culture to investigate extrinsic mechanisms of actions (Katsura et al., 2020; Yang et al., 2020; Youk et al., 2020). Accordingly, hESCs were utilized in a study which is pre-print at the time of writing this review, to generate a co-culture of lung cells and macrophages. The results showed a reduction of SARS-CoV-2 N protein expression after adding macrophages, though N protein was detected in the macrophages *per se*. More studies are required to find the exact role of macrophages, and also other parts of the immune system in the pathogenesis of this virus (Duan et al., 2020; Simoneau and Ott, 2020). In another pre-print study, a mixture of the upper airway and alveolar cells derived from hiPSCs was developed. It showed that the airway cells are more prone to infection

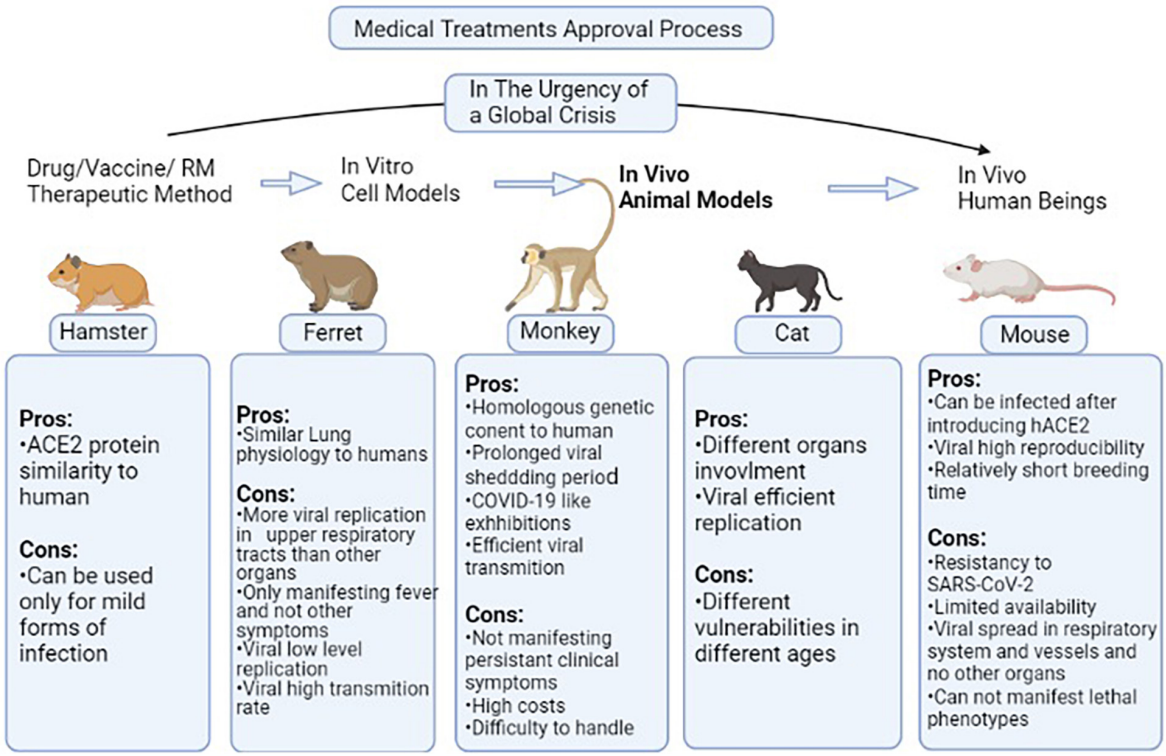

FIGURE 2 | Pros and cons of each species model discussed in **Table 1**. In non-critical situations, the routine process of a medical treatment to be approved begins from *in vitro* level of experiment and passes toward human experiments through passing the animal model testing stage. In the situation of global crisis such as the COVID-19 pandemic, the *in vitro* and animal model testing stages are not much focused on and the candidate therapeutic methods are directly applied to volunteers. But this gap has to be filled as soon as possible in order to reduce the drug/vaccines/Regenerative Medicine (RM) therapies rate of error. This figure provides the pros and cons of different species models in the case of studying animal models used in this field (Kumar et al., 2020; Rahim and Muhammad, 2020; Zheng et al., 2021). ACE2, Angiotensin-converting enzyme 2; hACE2, human-ACE2; SARS-COV2, Severe acute respiratory syndrome coronavirus 2; RM, Regenerative Medicine.

than alveolar cells in a manner that was similar to the patients' lungs. Further, these novel models can be served as beneficial platforms for drug discovery (Simoneau and Ott, 2020; Tindle et al., 2021). In addition, since endothelial dysfunction and vascular impairment like thrombosis and microangiopathy have been found in patients' lungs, adding endothelial cells to lung organoids may lead to generating more relevant models (Lamers et al., 2021). Due to the complicated behavior of SARS-CoV-2, the involvement of multiple organs, and their interactions with different types of cells and stem cells, more advanced technologies like organ-on chips or complex organoids can be more beneficial (Chugh et al., 2021). Among challenges in brain organoids, the absence of vasculature in choroid plexus organoids and the presence of not fully mature neurons in cortical organoids, which affects the susceptibility to infection, would be worth mentioning (Pellegrini et al., 2020). Moreover, the results of studying SARS-CoV-2 infection in astrocytes, microglia, and cortical neurons are controversial and to some extent, inconsistent. Since these cells have a critical role in CNS homeostasis, developing brain organoids containing these kinds of cells could be useful to take a step forward (Ramani et al., 2021). Besides, the current technology of organoids cannot manifest the communication between different organs and immunological responses in COVID-19 infection. In this context, animal models are more advantageous (Yu, 2021). As one of the most necessary major phases in manufacturing biomedical products, the ideal animal models should be able to simulate the disease in humans and its manifestations as similar to humans as it is possible. For COVID-19 modeling, the animal model must be permissive to the infection. Transgenic mice that express hACE2 are one of the best animal models, yet their availability is limited. Another mouse model is the adeno-associated virus delivery-based model, which expresses ACE2 in non-relevant cells and makes the interpretation of the analyzed data quite hard. Hamsters can model SARS-CoV-2 infection but they can only be used to study the mild forms of the disease because the lung pathology resolves to normal after 14 days post-infection (Kumar et al., 2020). As it was mentioned before, an important point about modeling this disease is that additional risk factors like advanced age and chronic diseases such as diabetes and obesity are associated with increased morbidity and mortality. These comorbidities cause alterations including the dysregulation in body homeostasis and repair mechanisms that make the infection progress to its severe and lethal forms. Hence, developing models with mentioned features to provide a platform for shifting the disease from a curable infection to a multi-organ failure state and ARDS can be

beneficial. Accordingly, the results of using aged animals such as aged mice and old macaques showed higher levels of lung injury and inflammation. Other modified models for obesity (e.g., ob/ob and high-fat diet-induced obesity mice), diabetes (e.g., STZ-treated and NOD mice), and immune deficiency/impairment (e.g., SCID and STAT1-knockout mice) might be more easily available to analyze the progression of the severe status of SARS-CoV-2 infection (Croy et al., 2007; Cleary et al., 2020; Payab et al., 2021). Another advantage of using proposed animal models is the possible ability to test the efficacy of interventions and treatments in different stages of the disease. When animal models which can develop multi-organ failure are used, suggested therapies for the mild to severe conditions of the infection can be tested in a pre-clinical setting. Therefore, the safety issues of the drugs and even prophylactic agents might be explored better (Cleary et al., 2020). Collectively, an appropriate model which develops the disease, the body response, and the comorbidities for a comprehensive understanding of the pathogenesis and possible treatments is still elusive. Though, available models have led to invaluable information on the disease and different vaccines (Cleary et al., 2020; Kumar et al., 2020).

CONCLUSION AND CLOSING REMARKS

The COVID-19 pandemic has placed a huge burden on people, communities, and health facilities all around the world. Great attempts toward understanding the disease and finding treatments and vaccines to fight against SARS-COV-2 are being made. Giant clinical trials such as the world health organization (WHO)'s Solidarity clinical trial and the randomized evaluation of COVID-19 (RECOVERY) trial of the United Kingdom are global platforms that are trying to identify the treatments of SARS-CoV-2 infection (No Author, 2020). Current available approved drugs for treating patients with COVID-19 are remdesivir, dexamethasone, and immunomodulators including tocilizumab and sarilumab (IL-6 inhibitors), baricitinib (a Janus kinase (JAK) inhibitor), for hospitalized patients, and anti-SARS-CoV-2 monoclonal antibodies like sotrovimab and casirivimab for non-hospitalized adults. In addition to these drugs, RM-based approaches including transplantation of progenitor or stem cells, tissues, and exosomes have also gained attention. Several trials of cell therapy are investigating the potential therapeutic effects of stem cells (Asgharzade et al., 2021; Covid-19 Treatment Guidelines Panel,2021; Rodriguez-Guerra et al., 2021). Besides, RM has been considered to be a promising field for drug and vaccine discovery and exploring the pathogenesis of the virus through disease modeling. Stem cells (especially iPSCs) and organoids, as major components of RM, have been recognized as useful models of SARS-CoV-2 infection. Various PSC-derived cell types (e.g., AT2s, cardiomyocytes, and neurons) were generated to study the SARS-CoV-2 effect on human organs and systems. Regarding the extensive data to support the value and benefits of hiPSC-derived somatic cells to model different diseases as well as various kinds of viruses, several studies on SARS-CoV-2 infection have used this RM-based model. Developing hiPSC-derived AT2s has clarified some aspects of the viral pathogenesis in the lungs. Moreover, endothelial cells derived from hiPSCs are known as a practical tool for studying the hypercoagulable state, which is caused by SARS-CoV-2 (Atala et al., 2020; Nolasco et al., 2020). The unique ability of SARS-CoV-2 to cause myocardial injury in a considerable percentage of patients has inspired scientists to explore the pathogenic mechanism. Research on hiPSCs has revealed that the virus directly affects the cardiomyocytes and actively replicates in these cells. Additionally, as mentioned before, the virus causes down-regulation of ACE2 expression, in contrast to other tissues like the intestine (Lamers et al., 2020; Wong et al., 2020). In addition, the antiviral effect of remdesivir and chloroquine was assessed in cardiomyocytes derived from hiPSCs and hESCs. Remdesivir exhibited stronger antiviral activity than chloroquine. However, it induced cardiotoxicity by causing QT prolongation and reducing cell viability at a higher concentration than the estimated peak plasma concentration. The results not only introduce the cardiomyocytes derived from hPSCs as a powerful experimental model for drug screening but also advise close monitoring of patients undergoing treatment with remdesivir, especially those with chronic heart diseases or severe form of COVID-19 (Choi et al., 2020). As well as hPSCs, organoids have acted as both *ex vivo* and *in vivo* models for lung injury in COVID-19. Transplantation of lung organoids into NSG mice and testing some proposed drugs have made the models more realistic and experimental (Han et al., 2021). Other organoid models including small and large intestine, kidney, retina, liver, pancreas, heart, and brain organoids are used and discussed in this review. For example, brain organoids and iPSC-derived neural cells have revealed some insights into the neurotropism of SARS-CoV-2. The number of these studies is still limited and the results are controversial, but they have been helpful in finding some of the underlying causes of neurological defects and also, testing some drugs. Based on currently available data, infecting the choroid plexus is a major component of brain damage. Different studies have suggested impairment of synaptogenesis, viral toxicity in NSC/NPCs, and neural cell death to explain the neurological symptoms. However, it is not clear whether neurons, astrocytes, or microglia are infected by the virus or not. There are reports of sparse to no infection in microglia and cortical neurons, yet more investigation is required (Jacob et al., 2020; Kase and Okano, 2020; Zhang B. Z. et al., 2020; Ramani et al., 2021).

In addition to the role of organoids in exploring damages of tissue and somatic cells, they are accepted as valuable *ex vivo* models for studying stem cells. Accordingly, reports have revealed that the stem cells can be affected by SARS-CoV-2 as it decreases the population of resident stem cells in the lung. Some studies have reported that one type of pulmonary stem cell expresses ACE2, which can lead to its loss. Besides, intestinal organoid-based studies showed that both quiescent and active intestinal stem cells express ACE2. Therefore, investigating the exact role of the signaling pathways and susceptibility of stem cells to SARS-CoV-2 are essential in terms of finding potential treatments (Chugh et al., 2021).

The application of organoids in studying new variants of SAR-CoV-2 is another novel advantage of this model. The new

variants including Delta and Lambda are more transmissible and can escape from the immune system and to some extent, the current vaccines. Organoid systems can provide a rapid platform for comparison of these strains and their susceptibility to vaccines and drugs (Mohammadi et al., 2021; van der Vaart et al., 2021). Modifying the organoids by using novel technologies like clustered regularly interspaced short palindromic repeats and CRISPR-associated protein9 (CRISPR/Cas9) and biomaterials as well as combining different cell types can result in more applicable and reliable pre-clinical models (Yu, 2021).

Furthermore, different animal models have helped researchers investigate body response and treatment options. Mice, hamsters, ferrets, and primates are the most common animal models and their characteristics were addressed previously. The size, rapid growth, and breeding of mice have made them favorable animal models in preclinical studies. On the other hand, the problem of their resistance to SARS-CoV-2 infection has raised some issues. The application of hACE2 transgene-dependent expression and adeno-associated virus delivery methods has helped scientists generate promising useful models. Nonetheless, there are still concerns about the similarity of hACE2 expression and distribution to humans and its related possible differences in modeling the SARS-CoV-2 infection mechanism. Hence, more specified manipulation of the relevant locus in mice may develop more accurate models. In contrast to mice, hamsters are known to be permissive to SARS-CoV-2. This model can be used for the mild form of COVID-19 and exploring the successful inflammation resolving mechanisms for therapeutic targets (Cleary et al., 2020; Kumar et al., 2020).

This review has discussed currently available models and their challenges in the paradigm of RM and proposed potential therapeutic approaches discovered in the experimental studies. Despite previous advances in this field, there are still shortcomings highlighted under the "CHALLENGES AND LIMITATIONS" section, which should be defeated in order to optimize COVID-19 RM modeling. For example, further exploration of gene editing or "omics" to improve the limitations of iPSC-based modeling such as immaturity and epigenetic memory retaining problems is suggested. However, economic efficiency should be considered in applying these new technologies. Altogether, considering the impossibility of studying the detailed mechanism of pathogenicity and the sequence of suggested drugs or vaccine candidates in human beings, these big steps toward RM in the SARS-CoV-2 field of study should be continued.

AUTHOR CONTRIBUTIONS

BL participated in the study design and drafting the manuscript. NF-H, MA, and AT-B contributed to the drafting of the manuscript. MR-T and HA provided final approval of the version to publish. BA supervised the project from the scientific view of point and advised on study design. All authors read, provided feedback, and approved the final manuscript.

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T Cell Immunity Evaluation and Immunodominant Epitope T Cell Receptor Identification of Severe Acute Respiratory Syndrome Coronavirus 2 Spike Glycoprotein in COVID-19 Convalescent Patients

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A better understanding of the role of T cells in the immune response to Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is helpful not only for vaccine development but also for the treatment of COVID-19 patients. In this study, we determined the existence of SARS-CoV-2-specific T cells in the blood of COVID-19 convalescents. Meanwhile, the specific T cell response in the non-RBD region was stronger than in the RBD region. We also found that SARS-CoV-2 S-specific reactive $CD4^+$ T cells exhibited higher frequency than $CD8^+$ T cells in recovered COVID-19 patients, with greater number of corresponding epitopes presented. Importantly, we isolated the SARS-CoV-2-specific $CD4^+$ T cell receptors (TCRs) and inserted the TCRs into allogenic $CD4^+$ T cells. These TCR-T cells can be activated by SARS-CoV-2 spike peptide and produce IFN-γ *in vitro*. These results might provide valuable information for the development of vaccines and new therapies against COVID-19.

Keywords: SARS-CoV-2, COVID-19, T cell epitope, TCR, TCR-T

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INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been identified as the cause of coronavirus disease 2019 (COVID-19). As of June 17, 2021, more than 3 million deaths and 176 million cases have been reported worldwide.[1] Vaccines play an important role in the battle against COVID-19, and multiple vaccines have been rolled out in countries (Anderson et al., 2020; Polack et al., 2020; Voysey et al., 2021; Xia et al., 2021). The level of neutralizing antibody has been

[1] https://coronavirus.jhu.edu/map.html

reported to decrease both in most of the asymptomatic and symptomatic groups during the early convalescent phase (Long et al., 2020), with no high concentration of neutralizing activity found even in most recovered plasma samples (Li et al., 2020; Robbiani et al., 2020), suggesting that the effective window for the neutralizing antibody induced by vaccines may be relatively limited.

According to the research of severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV), T cell immunity plays a decisive part in the disease recovery and long-lasting protection (Zhao et al., 2010, 2016; Channappanavar et al., 2014a; Ng et al., 2016). T cells can clear the virus even in the absence of antibodies and when the innate immune system is not activated (Zhao et al., 2010). Meanwhile, the depletion of $CD4^+$ T cells during SARS-CoV infection will delay virus clearance (Chen et al., 2010). Furthermore, T cells help clean the SARS-CoV and, at the same time, form a long-lasting memory response to it (Fan et al., 2009; Ng et al., 2016). SARS-CoV-2 can induce virus-specific T cells in the absence of seroconversion (Sekine et al., 2020; Gallais et al., 2021). Hence, for SARS-CoV-2, T cell-mediated cellular immunity may present certain beneficial advances over antibodies, and a strong and extensive T cell response may be essential for continuous immune protection against SARS-CoV-2. T cell-mediated cellular immunity largely depends on the recognition of its antigen presented by the major histocompatibility complex (MHC) through its T cell receptors (TCRs; Hu et al., 2021). Therefore, it is crucial to obtain SARS-CoV-2-specific TCRs with high functionalities for the immunological protection of this highly mutated coronavirus.

The current study aimed to investigate the T cells' response to SARS-CoV-2 spike protein (S protein) and identify the S-specific TCR. We found a stronger and broader SARS-CoV-2 S protein-specific $CD4^+$ T cell response in most COVID-19 convalescent patients. Specifically, we have identified an immunodominant epitope B65 within the S protein, which presents a unique value for the peptide vaccine development. Further, we found an S-specific TCR targeting the immunodominant peptide B65. We also showed that normal $CD4^+$ T cells constructed with B65-targeted TCRs exhibit functional reactivity against SARS-CoV-2 S. Our study provided valuable information for developing potential vaccines and new treatment strategies against COVID-19.

MATERIALS AND METHODS

COVID-19 Convalescent Patients and Healthy People

Blood samples of COVID-19 convalescent patients were collected from the Yongchuan Hospital of Chongqing Medical University and the Third Affiliated Hospital of Chongqing Medical University. Healthy people's blood samples, which were never exposed to SARS-CoV-2 or chronic infections, include hepatitis B or C, HIV, and Syphilis, which were collected from the Center of Immunology Research, Chongqing Medical University. This project was approved by the ethics committee of Chongqing Medical University (20200310). Informed consent was obtained from all individual donors before this research.

Peripheral Blood Mononuclear Cell Isolation

Whole blood was diluted with an equal volume of Dulbecco's phosphate-buffered saline (PBS) with 2% fetal bovine serum (FBS, Gibco) and mixed gently. The diluted samples were pipetted down the side of the SepMate^{TM}-50 tube (Stemcell Technologies, Vancouver, BC, Canada) with added Lymphoprep^{TM} (Stemcell). To separate peripheral blood mononuclear cells (PBMCs), the diluted blood samples with density gradient medium were centrifuged at 1,200 × *g* for 10 min with the brake applied at room temperature. PBMCs were washed twice using PBS supplemented with 2% FBS (between the washes, spinning at 1,000 g, for 7 min at RT, and discarding the supernatant) and were cryopreserved until subsequent analysis.

Enrichment of IFN-γ-Secreting and $CD4^+$T Cells

For the enrichment of IFN-γ-secreting T cells, peptide pools (5 μM/peptide) for 6 h were used to stimulate the PBMCs of recovered COVID-19 patients. Then, IFN-γ-secreting T cells were caught using IFN-γ Secretion Assay Cell Enrichment and Detection Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD4^+$T cells were selected by EasySep Human $CD4^+$ T Cell Enrichment Kit (Stemcell).

T Cell Receptor Sequencing and Analysis

T cell receptor sequencing and analysis were performed as previously described (Hu et al., 2021). In short, RT PCR and nested PCR were used to amplify the TCRα and TCRβ chain genes. Then, the PCR products were analyzed by sequencing (Tsingke, Guangzhou, China). The IMGT/V-QUEST tool was used to analyze the TCR repertoire.

Cell Culture

Peripheral blood mononuclear cells and $CD4^+$Jurkat cells were cultured in RPMI 1640 (Gibco) containing 10% FBS, 2 mM GlutaMAX^{TM}, 25 mM HEPES, and 10 μg/ml gentamicin (Gibco, Grand Island, NY, United States) and 55 μM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, United States). 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% FBS, 2 mM GlutaMAX^{TM}, 1 mM sodium pyruvate (Gibco), and MEM NEAA supplement (Gibco). IFN-γ-secreting T cells were expanded using irradiated (50-Gy) allogeneic PBMCs at a ratio of 1:100. The cells were cultured in a mixture medium [complete media: stocktickerAIM-V media (Gibco) = 1:1] containing 30 ng/ml OKT3 antibody (Miltenyi Biotec) and 3,000 IU/ml IL2 (PeproTech). All cells were cultured for 14 days before use.

Construction of Lentivirus Vectors and Cell Infection

Lentivirus vector construction and lentivirus production were established as previously described (Hu et al., 2021). To generate

TCR-transduced-CD4$^+$ T and TCR-transduced-CD4$^+$ Jurkat cells, CD4$^+$ T cells were stimulated with 1 μg/ml OKT3 antibody and 1 μg/ml anti-CD28 antibody (Miltenyi Biotec) and cultured in a complete medium containing 100 IU/ml IL-2 for 48 h. CD4$^+$ Jurkat cells and stimulated CD4$^+$ T cells were seeded into 24-well-plates and incubated with 500 μl lentivirus and 500 μl RPMI 1640 for 6 h. Next, 500 μl complete medium was added to each well. After 24 h, the original medium was replaced with a 2-ml complete medium and incubated for 48 h. Then, the cells were harvested and used for subsequent assays.

Stimulation of Peripheral Blood Mononuclear Cells With Peptides and Cocultured Experiment

Peripheral blood mononuclear cells were stimulated with peptide (5 μM) 24 or 48 h after resting overnight in complete medium. Supernatants of 24 h were collected for enzyme-linked immunosorbent assay (ELISA) to detect cytokine. Stimulated cells of 48 h were used to test SARS-CoV-2 specific T cells by flow cytometry. For a cocultured experiment: PBMCs as presenting cells were labeled with a 5-μM peptide for 2 h at 37°C. The PBMCs were washed twice with PBS before coculturing with TCR-transduced CD4$^+$ T or TCR-transduced-CD4$^+$ Jurkat cells for 24 or 6 h, respectively. Flow cytometry was used to check the activated cells.

Flow Cytometry Analysis

The immunological phenotypes of PBMCs were assessed by flow cytometry using the following surface markers: BV510-anti-CD3, PerCP-Cy5.5-anti-CD4, FITC-anti-CD8, PE-anti-CD19, APC-anti-CD45RA, BV412-anti-CCR7, BV605-anti-CD56, PE-anti-CD14, APC-anti-CD16 (BioLegend). The IFN-γ-secreting T cells were detected using PE-anti-IFN-γ (Miltenyi Biotec) and BV510-anti-CD3. After stimulation with peptide for 48 h, the SARS-CoV-2 specific T cells were tested applying BV510-anti-CD3, PerCP-Cy5.5-anti-CD4, FITC-anti-CD8 and BV421-anti-CD137, and APC-anti-CD134 (BioLegend, San Diego, CA, United States). The activations of TCR-transduced-CD4$^+$ Jurkat and TCR-transduced-CD4$^+$ T cells were assayed using PerCP-Cy5.5-anti-CD4, APC-anti-mTCRβ, PE-anti-CD69, PE-anti-IFN-γ, and BV421-anti-CD137. In brief, cells were stained with various Abs at room temperature for 20 min. Then, the cells were washed with PBS and stained for 15 min using LIVE/DEAD viability dye (Thermo Fisher Scientific, Waltham, MA, United States). Data were acquired using the FACSCelesta cytometer and analyzed by FlowJo software. For single-cell acquisition, CD3$^+$CD4$^+$CD134$^+$CD137$^+$T cells were sorted into 96-well PCR plates (Bio-Rad) at a single-cell level using FACSAria III.

IFN-γ ELISA Assays

IFN-γ capture antibody (2 μg/ml, BioLegend) and IFN-γ detection antibody (1 μg/ml, BioLegend) were used to detect cytokine. The ELISA plate (Corning) was coated overnight with a capture antibody at 4°C and washed three times with PBS containing 0.05% Tween 20 (PBST), then blocked with 3% BSA at 37°C for 1 h. Then, supernatants were added to the ELISA plate (50 μl/well) and incubated at 37°C for 1 h. After washing with PBST for five times, the detection antibody was added and incubated at 37°C for 30 min. The plate was incubated with streptavidin-ALP (1:1,000) at 37°C for 30 min after washing with PBST five times. Next, the plate was washed with PBST six times, followed by incubation with 50 μl pNPP solution (Mabtech, Stockholm, Sweden) at 37°C for 40 min. Finally, the plate was analyzed *via* the Varioskan LUX Multimode Microplate Reader at 405 nm.

Statistical Analysis

GraphPad Prism 8.0 was used for statistical analyses. Data are shown as the mean ± SD. The unpaired *t*-test and Spearman's test were used for data analysis. $p < 0.05$ was considered statistically significant.

RESULTS

Immunological Phenotypes of Recovered COVID-19 Patients

Peripheral blood samples from 10 recovered individuals of COVID-19 were collected to study the T cells' immune response to SARS-CoV-2. All participants were diagnosed with mild COVID-19, including four men and six women, ranging from 32 to 59 years old, with a median age of 47. From symptom onset or positive PCR results to discharge, the recovery time is 7–26 days, with a median recovery time of 18 days. As a control group, we collected 10 healthy people who had not been infected with SARS-CoV-2.

To characterize cellular immunological phenotypes, we isolated PBMCs from the convalescent patients and the healthy controls for subsequent multicolor flow cytometry analysis. The proportions of B cells, monocytes, and natural killer (NK) cells were similar in the two groups (**Figures 1A–C**). Meanwhile, no significant differences were observed in the frequencies of CD3$^+$, CD4$^+$, and CD8$^+$ T cells between the recovered group and the control group (**Figures 1D–G**). We also assess the subtype of CD4$^+$ and CD8$^+$ T cells. The frequencies of CD4$^+$ central memory T cells (T_{CM}), CD8$^+$ T_{CM}, and CD8$^+$ effector T cells (T_{EF}) were relatively higher in the recovered group than in the control group (**Figures 1H,I**).

Severe Acute Respiratory Syndrome Coronavirus 2 Spike Glycoprotein-Reactive T Cells in Recovered COVID-19 Patients

To study SARS-CoV-2-specific T cell response, we selected S protein for being a key protein for viral entry affecting viral infections and being a target for vaccines (Anderson et al., 2020; Polack et al., 2020; Wrapp et al., 2020; Voysey et al., 2021; Weinreich et al., 2021; Xia et al., 2021). To this end, we designed 317 peptides containing 15 amino acids (aa), with 11-aa overlaps, to span the entire S protein. These peptides were divided into 32 mixed pools (Mix), each consisting of

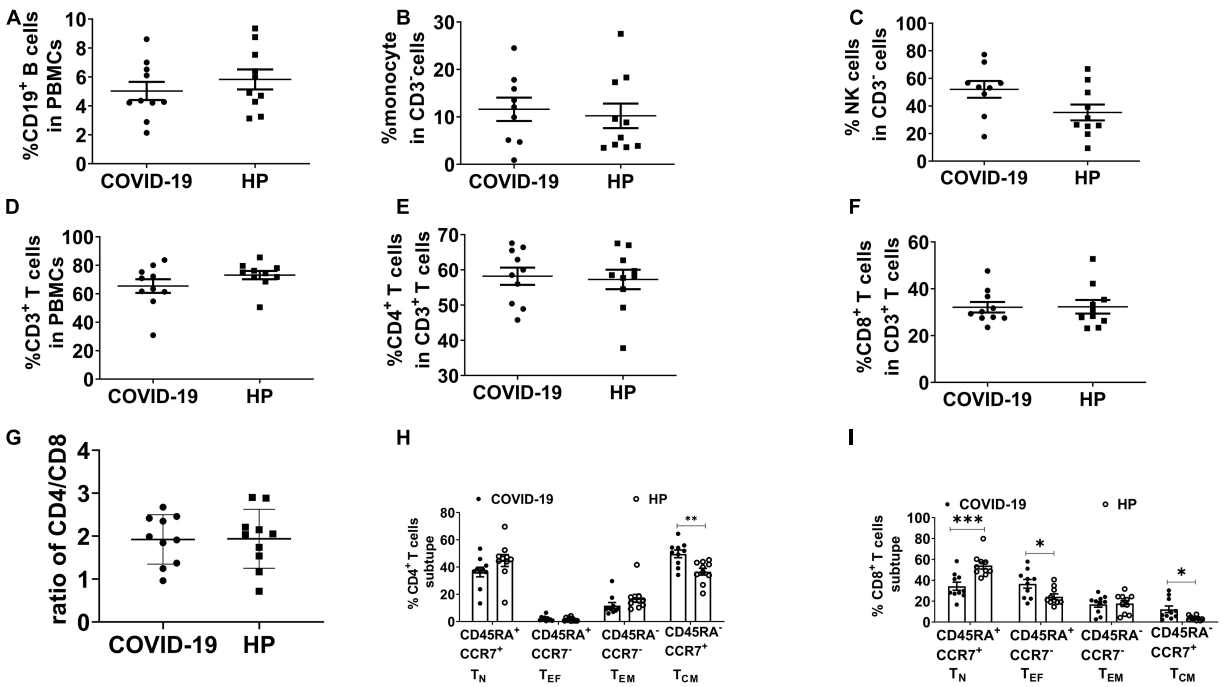

FIGURE 1 | Immunophenotyping of PBMC in recovered COVID-19 patients and HP. Multicolor FC was used to detect the frequency of **(A)** B cells ($CD3^-CD19^+$), **(B)** monocytes ($CD3^-CD16^-CD14^+$), **(C)** NK cells ($CD3^-CD56^+CD16^+$), **(D)** $CD3^+$ T cells, **(E)** $CD4^+$ T cells ($CD3^+CD4^+CD8^-$), and **(F)** $CD8^+$ T cells ($CD3^+CD4^-CD8^+$). **(G)** The ratio of CD4/CD8. **(H,I)** T_{CM} ($CD45RA^-CCR7^+$), T_N ($CD45RA^+CCR7^+$), T_{EF} ($CD45RA^+CCR7^-$), and T_{EM} ($CD45RA^-CCR7^-$) in 10 recovered COVID-19 patients and 10 HP. FC: flow cytometry; PBMCs: peripheral blood mononuclear cells; COVID-19: Coronavirus Disease-2019; HP, healthy people; T_{CM}, central memory T cells; T_N, naïve T cells; T_{EF}, effector T cells; T_{EM}, effector T memory cells. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

10 peptides. The N-terminal of the S protein is designated as S-I, containing non-receptor-binding domain (non-RBD, S-I-N; Mix21-31) regions and the RBD (Mix15-20), while the C-terminal of the S protein is called SII (Mix32-46) (**Figure 2A**). To increase the amount of SARS-CoV-2-specific T cells, we performed rapid expansion *in vitro*. As shown in **Figure 2B**, the expansion process successfully increased the frequencies of SARS-CoV-2 specific T cells in 75% samples, although with only a slight effect on the samples of C10 and C28. Then, we determined the activation of the specific T cell populations by ELISA for the IFN-γ secretion post *in vitro* restimulation with different peptide pools for 24 h. We found that T cells specifically responding to multiple regions of the S protein do exist in the PBMCs of all recovered COVID-19 patients. These specific T cells demonstrated different reactivities to different regions of SARS-COV-2 S, which were 100% for S-I-N, 75% for RBD, and 87.5% for S-II. Of note, the RBD region accounted for the weakest T cell response from the majority of patient samples, including two that showed barely detectable levels of IFN-γ (**Figure 2C**). Further, we analyzed the magnitude and breadth of T cell reactivity to different S protein regions in detail. Collected T cell responses to individual Mix were compared, and we found that both S-I-N and S-II corresponded to larger numbers of peptide pools with better potential to induce IFN-γ secretion, despite individual variations (**Figures 2D,E**). To investigate the clinical relevance of such T cell reactivity, we used Spearman's linear correlation and found no significant correlation between the magnitude and breadth of T cell responses and patient age or the time of recovery (**Figures 2F–I**). Overall, these results indicated that the PBMCs of recovered COVID-19 patients contained specific populations of T cells with high reactivity to SARS-CoV-2 S protein, of which the RBD region might induce a relatively lower level of T cell responses.

Identification of Severe Acute Respiratory Syndrome Coronavirus 2 S Protein-Specific $CD4^+$ and $CD8^+$ T Cell Epitopes

To better characterize the specific epitopes for SARS-CoV-2 S-reactive T cells, single peptides from each positive mixture pool were used to stimulate T cells from recovered COVID-19 patients. A total of 11 peptides containing SARS-CoV-2 T cell epitopes were recognized by recovered COVID-19 patients PBMCs, 2 from S-I-N, 3 from RBD, and 6 from S-II (**Figures 3A,B** and **Table 1**). The T cells from C8 and C11 participants recognized 7 and 6 peptides, respectively (**Figure 3B** and **Table 1**). Furthermore, we determined whether these 11 positive peptides had preferential correspondence to $CD4^+$ or $CD8^+$ T cells. Flow cytometry analysis showed that the frequency of SARS-CoV-2-specific CD4 + T cells was higher than that of $CD8^+$ T cells (**Figure 3C**). Specifically, peptide B65 from S-I-N was recognized in seven out of eight participants (87.5%) and contained at least two epitopes recognized by $CD4^+$ T cells or

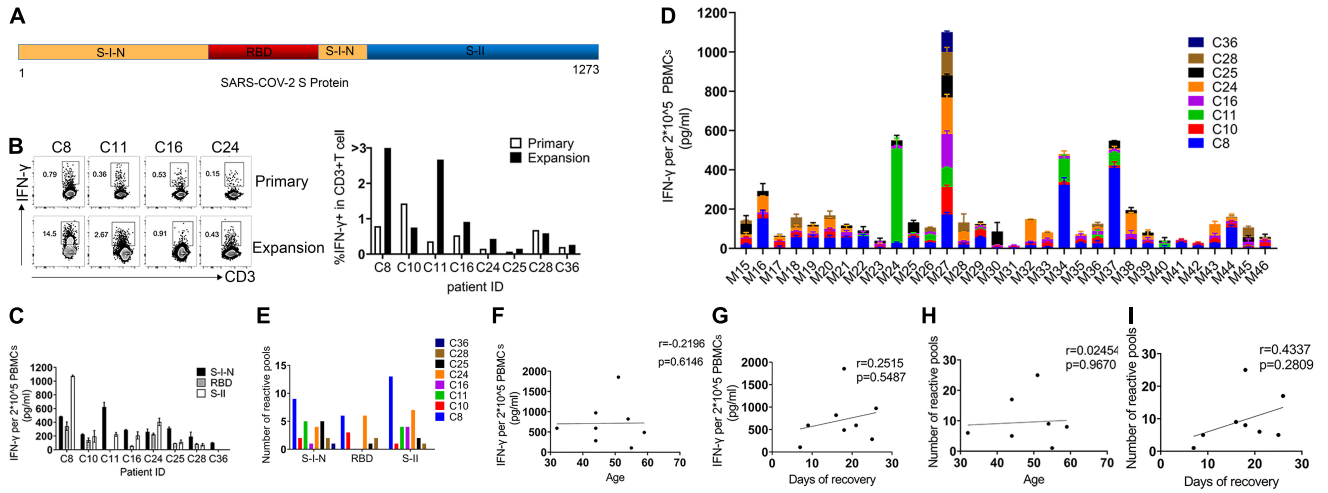

FIGURE 2 | Severe Acute Respiratory Syndrome Coronavirus 2 spike glycoprotein-reactive T cells in recovered COVID-19 patients. **(A)** The structural of SARS-COV-2 spike glycoprotein: the N-terminal of S protein is called S-I, including RBD and non-RBD regions (S-I-N), and the C-terminal of the S protein is called S-II. **(B)** FC was used to detect the frequency of SARS-CoV-2 S-specific T cells ($CD3^+IFN\text{-}\gamma^+$) before and after expansion. **(C)** The IFN-γ production in PBMCs of each individual was detected by ELISA after stimulation with SARS-COV-2 S peptides (24 h). A total of 2×10^{-5} PBMCs were stimulated with complex Mix pools of different regions of SARS-COV-2 S protein: S-I-N (Mix21-31), RBD (Mix15-20), or S-II (Mix32-46). **(D)** The magnitude of T cell response for each individual to a single Mix pool. **(E)** The breadth of T cell response for each individual to different regions of SARS-COV-2 S protein. The breadth of T cell response was calculated by the number of positive Mix pools. The number of positive mix pools = IFN-γ/DMSO > 2. **(F–I)** The correlation analyses of magnitude and breadth of T cell responses versus patients' age **(F,H)** and the time of recovery **(G,I)**. The quantitative results are shown as the mean ± SD of at least two independent experiments. SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; RBD, receptor-binding domain; FC, flow cytometry; PBMCs, peripheral blood mononuclear cells; IFN-γ, interferon-γ; ELISA, enzyme linked immunosorbent assay; Mix, mixture.

$CD8^+$ T cells (**Figure 3D** and **Table 1**). Moreover, we found that 10 of 11 peptides induced the activation of $CD4^+$ T cells, while only four peptides induced the activation of $CD8^+$ T cells (**Table 1**). Two patients, C8 and C11, whose samples were reactive to more peptides than the rest of the participants, contain both $CD4^+$ and $CD8^+$ SARS-CoV-2 S-specific T cells (**Table 1**). These results indicated that the $CD4^+$ subtypes predominantly managed the positive T cell responses to SARS-CoV-2 S protein.

Meanwhile, we evaluate the cross-reactivity of the PBMCs from healthy people to these positive peptides *via* IFN-γ ELISA assays. Only the B250 peptide was found to induce a relatively higher level of T cell responses from 2 of 10 healthy donors (**Figure 3E**). Due to the reported similarity between SARS-CoV-2 and the common cold coronavirus (Mateus et al., 2020; Nelde et al., 2021), we examined the homology of the positive peptides to a few coronaviruses. We found that 10 of 11 positive peptides exhibited the highest degree of homology to SARS-CoV, followed by MERS-CoV, whereas B38 was specific to SARS-CoV-2 S protein (**Figure 3F**). Also, the majority of these positive peptides share a certain extent of sequence similarities to the human common cold coronaviruses 229E, HKU1, NL63, and OC43. Among them, the sequence of B250 showed a high resemblance to all coronaviruses examined. Notably, the immunodominant peptide B65 showed approximately 60% sequence similarity to SARS-CoV and 40% to MERS-CoV, while it presented relatively low homology levels to the four common cold coronaviruses (**Figure 3F**). These findings demonstrated the existence of SARS-CoV-2 S-reactive T cells in healthy people, and such cross-reactivity was highly likely due to the sequence similarity between the S protein and those of the human common cold coronaviruses.

Reactivity of B65-Specific $CD4^+$ TCR-T Cells to B65 Peptide *in vitro*

To identify the TCRs responsible for the specific T cell reaction to the immunodominant peptide B65, we sorted the $CD134^+CD137^+CD4^+$ T cell population from the C11 sample at a single-cell level. All TCRs were examined by amplifying single-cell TCR genes and analyzing the TCR sequences by IMGT. Finally, a total of seven dominant TCRs were established in B65-specific TCR repertoire (**Figure 4A**). The top three dominant TCR clones (TCR1-3) were transduced into $CD4^+$ Jurkat and the allogenic $CD4^+$ T cells. As shown in **Figure 4B**, TCR1 and TCR2 were expressed in $CD4^+$ Jurkat and $CD4^+$ T cells both at high levels. To determine whether B65 could be recognized by the engineered cells, we loaded this peptide to PBMCs, which act as antigen-presenting cells, followed by a coculture with TCR-transduced $CD4^+$ Jurkat or $CD4^+$ T cells. The elevated expression of CD69 suggested that B65 can activate both TCR1- and TCR2-transduced Jurkat cells (**Figure 4C** left). Also, the increased levels of CD137 expression and IFN-γ secretion demonstrated that B65 could induce the activation of TCR1-transduced $CD4^+$ T, while TCR2 expressing $CD4^+$ T was not responsive (**Figure 4C** right). These results showed that we successfully identified a specific $CD4^+$ TCR against B65. In conclusion, we presented a dominant epitope of SARS-CoV-2 S from the PBMCs of COVID-19 convalescent

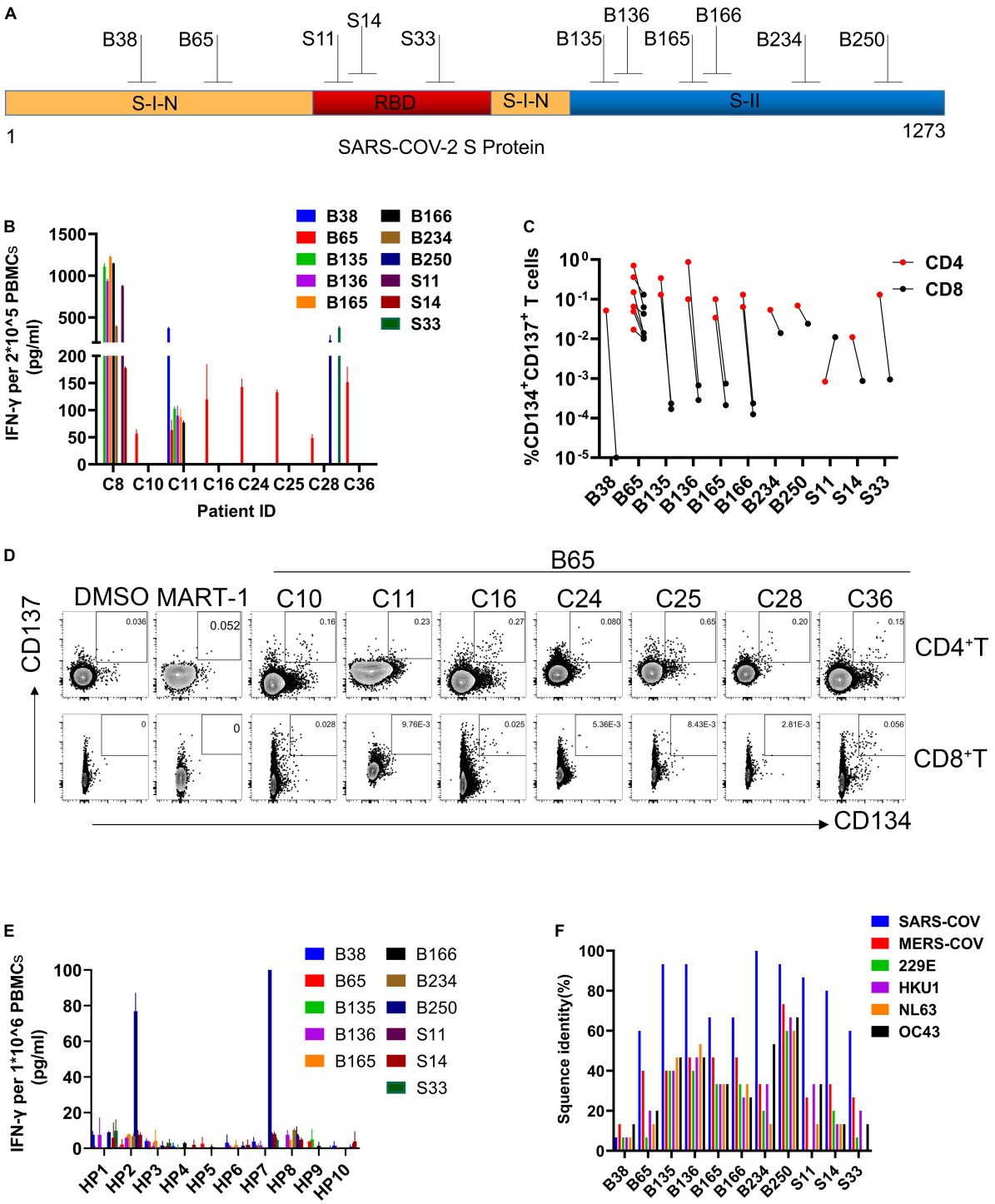

FIGURE 3 | Identification of SARS-CoV-2 S protein-specific $CD4^+$ and $CD8^+$ T cell epitopes. **(A)** The structure of SARS-COV-2 spike glycoprotein. **(B)** IFN-γ was determined by ELISA. 2×10^{-5} PBMCs for each participant were stimulated with a single peptide from positive mix pools (24 h). **(C)** The frequency of reactive CD4 + and CD8 + T cells to each peptide in participants. **(D)** FC plot example. FC was used to detect the reactive CD4 + T cells (CD3 + CD4 + CD8-CD134 + CD137 +) or CD8 + T cells (CD3 + CD4-CD8 + CD137 + CD134 +) in PBMCs stimulated by positive peptide or MART-1 (negative control) (48 h). **(E)** IFN-γ ELISA assays were used to evaluate the cross-reactivity of HP to peptide. 1×10^{-6} PBMCs for each HP were stimulated with each peptide (24 h). **(F)** The proportion of sequence identity of SARS-CoV-2 spike glycoprotein to the spike glycoproteins of SARS-COV, MERS-COV, and human common cold coronaviruses 229E, HKU1, NL63, and OC43. IFN-γ, interferon-γ; ELISA, enzyme-linked immunosorbent assay; PBMCs, peripheral blood mononuclear cells; FC, flow cytometry; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SARS-COV, severe acute respiratory syndrome coronavirus; MERS-COV, Middle East respiratory syndrome coronavirus (MERS-CoV); MART-1, melanoma-associated antigen.

TABLE 1 | Peptides containing T cell epitopes.

	Peptide	position	Amino acid sequence	CD4 + /CD8 + T cell response	Participants
S-I-N (*N* = 2)	B38	149–163	NKSWMESEFRVYSSA	CD4	C11[a]
	B65	257–271	GWTAGAAAYYVGYLQ	CD4/CD8	C10[c], C11[a], C16[c] C24[a], C25[a], C28[a], C36[c]
RBD (*N* = 3)	S11	359–373	SNCVADYSVLYNSAS	CD8	C8[b]
	S14	371–385	SASFSTFKCYGVSPT	CD4	C8[a]
	S33	447–461	GNYNYLYRLFRKSNL	CD4	C28[a]
S-II (*N* = 4)	B135 B136	745–759 749–763	DSTECSNLLLQYGSF CSNLLLQYGSFCTQL	CD4 CD4	C8[a], C11[a] C8[a], C11[a]
	B165	865–879	LTDEMIAQYTSALLA	CD4	C8[a], C11[a]
	B166 B234	869–883 1141–1155	MIAQYTSALLAGTIT LQPELDSFKEELDKY	CD4 CD4/CD8	C8[a], C11[a] C8[c]
	B250	1205–1219	KYEQYIKWPWYIWLG	CD4/CD8	C28[c]

[a]*CD4*$^+$*T cell response.* [b]*CD8*$^+$*T cell response.* [C]*both CD4*$^+$ *and CD8*$^+$*T cell response.*

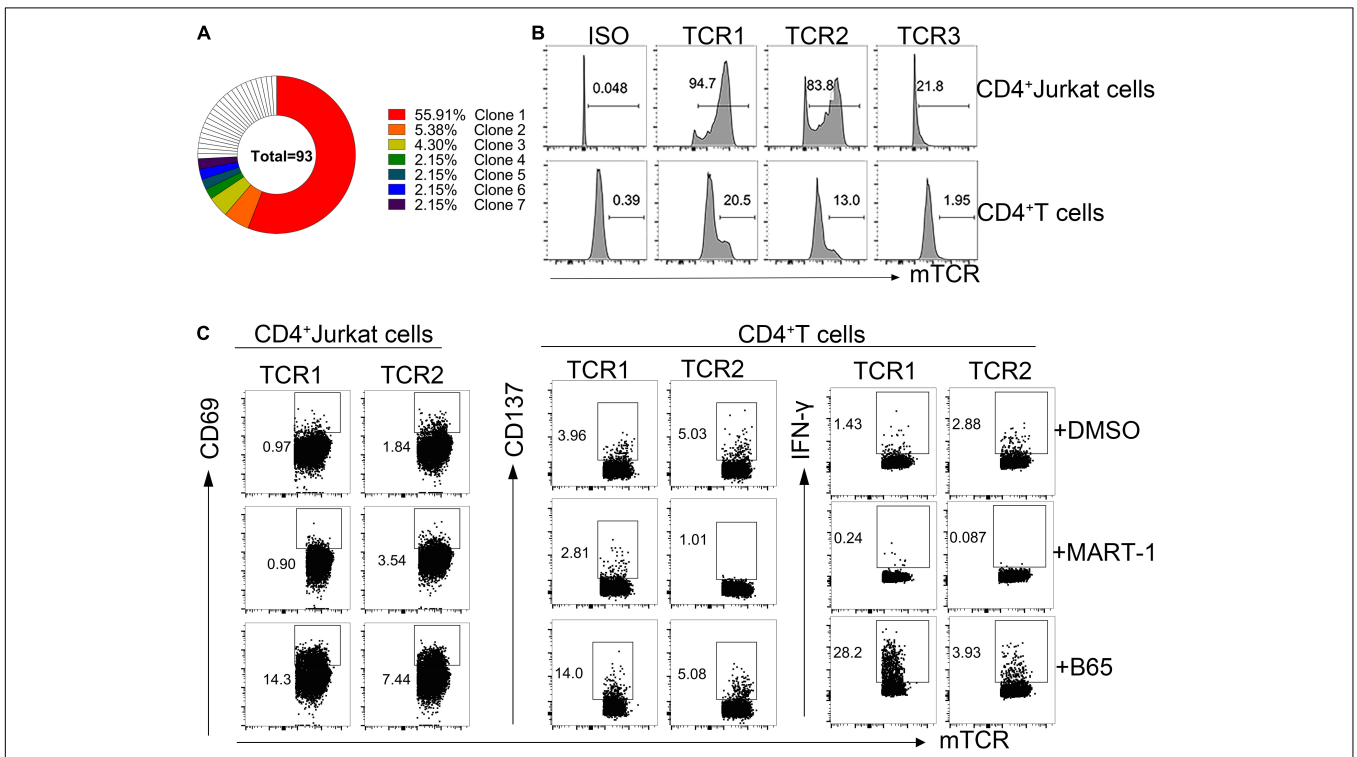

FIGURE 4 | Reactivity of B65-specific CD4$^+$ TCR-T cells to B65 peptide *in vitro*. **(A)** MGT/V-Quest was used to analyze the TCR repertoire of B65-specific CD4$^+$ T cells. Different colors mean different TCR clones. White indicates unique clones. **(B)** The expression of TCR in CD4$^+$ Jurkat or CD4$^+$ T cells was detected by FC. **(C)** TCR-transduced CD4$^+$ Jurkat or CD4$^+$ T cells were cocultured with PBMCs plus B65 or MART-1 (negative control) for 6 or 24 h, respectively. The level of activation marker of CD69 or CD137 and IFN-γ in TCR-transduced CD4$^+$ Jurkat or CD4$^+$T cells was determined by FC, respectively. FC, flow cytometry; IFN-γ, interferon-γ; TCR, T cells receptors; PBMCs, peripheral blood mononuclear cells; MART-1, melanoma-associated antigen.

patients, represented by the peptide B65, together with its specific functional TCR for CD4$^+$ T cells.

DISCUSSION

This study found that SARS-CoV-2 S-specific reactive CD4 + T cells exhibited a higher frequency than CD8$^+$ T cells in recovered COVID-19 patients, with a greater number of corresponding epitopes presented. Importantly, we identified a peptide B65 within the S-I-N region containing immunodominant epitopes for CD4$^+$ and CD8$^+$ T cells, and it could induce a strong T cell response in convalescent PBMCs. Further, we successfully obtained the CD4$^+$ TCRs recognizing B65 and confirmed that the constructed B65 TCR-T could display strong functional activity against SARS-CoV-2 S. Our findings provide vital information

for understanding the cellular immunity against SARS-CoV-2 and for the development of effective strategies for COVID-19 treatment.

Compared with healthy people, COVID-19 convalescent patients were shown with similar T cells, yet they contained a significantly larger proportion of T_{CM} cells in their PBMCs. This finding was in line with the previous discovery that the majority of SARS-CoV-2-specific T cells exhibited T_{CM} phenotypes (Peng et al., 2020). Due to the limitation of sample size, we did not determine the specificity of these T_{CM} cells. Nevertheless, the overall robust response to the S protein indicated that recovered patients from COVID-19 might exhibit resistance to SARS-CoV-2-antigen reexposure. Moreover, we presented the evidence that both the frequency and epitopes of SARS-CoV-2-specific $CD4^+$ T cells were higher than those of $CD8^+$ T cells, which were consistent with recent findings in COVID-19 convalescents (Grifoni et al., 2020; Sekine et al., 2020). Given the divergent role of $CD4^+$ T cells in regulating both humoral immunity and cellular immunity, we suspect that the relatively mild disease severity of these COVID-19 patients included in our study may benefit from a stronger $CD4^+$ T cell response to SARS-CoV-2. This was supported by the recent finding that the number of SARS-CoV-2 S-specific $CD4^+$ T cells was positively correlated with the anti-S RBD IgG titers and magnitude of SARS-COV-2 S-specific $CD8^+$ T cells (Grifoni et al., 2020).

Knowledge obtained from studying SARS-CoV indicates that B cells and antibodies are comparatively short-lived (1–2 years), whereas memory T cells exhibit a longer life span (>6–17 years) (Channappanavar et al., 2014b; Zhao et al., 2016; Lin et al., 2020). Therefore, T cell immunity can be used to evaluate the timely effectiveness of vaccines and as a reflection of the long-term protection provided by vaccines. In this study, we evidenced that B65 within the S-I-N region of SARS-CoV-2 S could be recognized by the PBMCs of almost all participants tested, indicating that T cell responses and neutralizing antibodies might favor different regions of the S protein. To be noted, B65 does not contain any amino acids corresponding to the mutations of the highly infectious SARS-CoV-2 variants, such as B.1.1.7, B.1.351, B.1.28, and B.1.617 (**Supplementary Material**). Hence, B65 may serve as a valuable addition for assessing specific T cell responses post-vaccination, and it can also be used as a potential candidate for peptide vaccine development to trigger effective T cell response.

The T cell immune response plays an essential role in intracellular viral clearance (Yang et al., 2020; Weinreich et al., 2021). However, in COVID-19 patients, the total number of $CD4^+$ and $CD8^+$ T cells was significantly reduced, and their phenotype exhibited exhaustion markers, which usually lead to the functional impairment of T cells (Diao et al., 2020; Zheng H. Y. et al., 2020; Zheng M. et al., 2020). Hence, improving virus-specific T cell response may be the key to recovering COVID-19 patients, especially for severe cases. Adoptive T cell therapies have been proven to be an effective treatment approach against malignant tumors and viral infections (Leen et al., 2006). Studies have shown that there were SARS-CoV-2-specific memory T cells in COVID-19 convalescents (Ferreras et al., 2021; Perez-Martinez et al., 2021), and the symptoms were significantly improved in patients with SARS-CoV-2 after being treated with SARS-CoV-2-specific memory T cells (Perez-Martinez et al., 2021). This result indicated that adoptive T cell therapies are an effective treatment for COVID-19. This study presented an S-specific TCR targeting the immunodominant peptide B65, with functional significance against SARS-CoV-2. Future studies with an enhanced transfection efficiency of TCRs and corresponding detailed functional verification *in vivo* may significantly increase the efficacy of SARS-CoV-2-specific $CD4^+$ TCR-T cells and help us to better evaluate this potential therapeutic method for treating COVID-19 patients.

Both TCR and HLA are the restriction factors for TCR-T therapies. Previous work identified 30 HLA class I and 45 HLA class II alleles of HLA-restricted epitopes, which cover the most common specificities in the general worldwide population (Grifoni et al., 2021). To cover most populations, more TCR should be acquired based on these epitopes. Especially, to avoid host versus graft reaction (HVGR) and graft versus host reaction (GVHR), the patient autologous T cells can be used to construct TCR-T cells when treating COVID-19.

In conclusion, we confirmed that SARS-CoV-2 S protein induces strong and broad $CD4^+$ T cell responses in the majority of COVID-19 convalescent patients. Further, we presented an immunodominant epitope B65 which might be a valuable candidate for peptide vaccine development. Importantly, SARS-CoV-2-specific-TCR-T cells could be a new approach of COVID-19 treatment.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Chongqing Medical University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

AJ designed the study. XH, QC, MS, CH, SC, TL, YW, YH, JH, and SL were responsible for collecting the samples. LL, XH, QC, MS, CH, SC, and JZ performed the experiments. LL, XH, and QC performed the single-cell TCR cloning. LL and QC performed the data analysis. AJ, LL, and QC wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.696662/full#supplementary-material

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