Engineering mesenchymal stromal cells with neutralizing and anti-inflammatory capability against SARS-CoV-2 infection

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The emergence of the novel human severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has led to the pandemic of coronavirus disease 2019 (COVID-19), which has markedly affected global health and the economy. Both uncontrolled viral replication and a proinflammatory cytokine storm can cause severe tissue damage in patients with COVID-19. SARS-CoV-2 utilizes angiotensin-converting enzyme 2 (ACE2) as its entry receptor. In this study, we generated ACE2 extracellular domain-Fc and single-chain variable fragment-interleukin 6 (IL-6) single-chain variable fragment against IL-6 receptor (scFv-IL6R)-Fc fusion proteins to differentially neutralize viruses and ameliorate the cytokine storm. The human ACE2 (hACE2)1–754 Fc fusion protein showed a potent inhibitory effect on pseudo-typed SARS-CoV-2 entry and a good safety profile in mice. In addition, scFv-IL6R-Fc strongly blocked IL-6 signal activation. We also established a mesenchymal stromal cell (MSC)-based hACE21–754-Fc and scFv-IL6R-Fc delivery system, which could serve as a potential therapy strategy for urgent clinical needs of patients with COVID-19.

INTRODUCTION

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) belongs to the genus Betacoronavirus and is closely related to the SARS-like coronavirus in bats. Phylogenetic analysis revealed that its genome sequence is approximately 80% similar to that of SARS-CoV. These two viruses exhibit ~74% amino acid sequence similarity at the receptor-binding domain (RBD) of the spike gene, suggestive of a possible common host receptor. Angiotensin-converting enzyme 2 (ACE2) has been recognized as a critical entry receptor for both SARS-CoV-2 and SARS-CoV.1,2 In comparison with the spike protein of SARS-CoV, the SARS-CoV-2 spike protein exhibits higher binding affinity to human ACE2 (hACE2); this difference may have contributed to the higher transmission rate of SARS-CoV-2,3,4 implying the importance of ACE2-dependent viral entry into cells. ACE2 is primarily expressed in lung alveolar epithelial type II cells, which are crucial for the gas-exchange function of the lungs.5,6 Severe lung injury in some patients with coronavirus disease 2019 (COVID-19) may stem from the dysfunction of these cells.7 ACE2 is also expressed in other organs such as the heart, liver, intestine, testis, kidney, and blood vessels,8–10 which may explain the multiple organ dysfunction syndrome (MODS) induced by SARS-CoV-2 infection.13 Apart from being the entry receptor of SARS-CoV, ACE2 facilitates tissue repair after lung injury.14

The interaction between the spike protein of SARS-CoV-2 and ACE2 is imperative for the viral entry into cells. Soluble ACE2 may act as a potential viral entry blocker by competing with membranous ACE2. Several studies have reported the antiviral effects of soluble ACE2-based therapeutics. Soluble ACE2, which removes the transmembrane domain from intact ACE2, is sufficient for neutralizing SARS-CoV-2 infection in vitro and in vivo.7,15 To enhance its half-life in vivo, soluble ACE2 was fused with an Fc fragment.16,17 Various approaches have been directed to enhance the affinity of soluble ACE2 toward the spike protein or the RBD of the spike protein as an attempt to improve its function as a competitive inhibitor of membranous ACE2.18–20 In addition, mutations were introduced for the inactivation of the enzymatic activity of soluble ACE2 to avoid any adverse cardiovascular effects caused by its catalytic activity.19,21 De novo protein design and computer-based peptide design have also been applied for engineering native soluble ACE2 to generate potential SARS-CoV-2 entry blockers.22–24 These studies highlight the importance of ACE2-based therapeutics for treating COVID-19. However, whether cell therapy can be used for the delivery of ACE2-based therapeutics is unclear.

Cell-based therapies, including immune cell and stem cell therapies, are currently being evaluated in the clinic for a wide range of diseases such as cancer, neurodegenerative disorders, and autoimmune diseases. As a representative of cell therapy, mesenchymal stromal cell (MSC)-based therapy is most widely used in clinics, with more than 1,138 registered clinical trials until 2020.25 MSCs can be obtained...
from different sources such as the bone marrow, cord blood, skin, Wharton’s jelly, and umbilical cord tissue. MSCs can self-renew and differentiate into adipocytes, chondrocytes, and osteocytes under specific in vitro culture conditions. The multi-potent differential potential, tissue repair ability, and anti-inflammatory properties of MSCs potentiate their clinical development for the treatment of hematopoietic failure, graft-versus-host diseases, and autoimmune diseases. In addition, MSCs exert paracrine functions by secreting soluble factors and releasing extracellular vesicles and possess the innate ability to transport cargos to inflammation sites, thus serving as promising drug-delivery vectors in preclinical models.

Approximately 5% of patients infected with SARS-CoV-2 develop severe lung injury symptoms, accompanied with overwhelmed immune activation. Some antiviral and anti-inflammatory drugs have been tested for treating COVID-19. However, these single-target drugs offer limited clinical benefits. Thus, drugs targeting the virus itself and ameliorating the tissue damage are urgently warranted to treat severe COVID-19. Here, we present an MSC-based cell therapy to simultaneously block SARS-CoV-2 entry and inhibit the virus-induced cytokine storm. This dual-targeting strategy provides a rapid and translational approach for COVID-19 treatment.

**RESULTS**

**ACE2-Fc could bind to the SARS-CoV-2 spike protein with high affinity**

SARS-CoV-2 is known to enter permissive cells through ACE2. The competitive, soluble extracellular domain of ACE2 might potentially act as a viral entry blocker. To test whether the ACE2 extracellular domain could block SARS-CoV-2 entry, we generated two recombinant ACE2-Fc fusion proteins comprising 1–615 or 1–740 amino acid residues of ACE2 fused to the human immunoglobulin G1 (IgG1) Fc (hFc) portion. These ACE21–615-hFc and ACE21–740-hFc fusion proteins bound to the SARS-CoV-2 spike protein at high affinity, as determined by flow cytometry. Data represent mean ± SD. Representative results of one from four repeated experiments are shown.

**ACE2-Fc could block the entry of pseudo-typed SARS-CoV-2 into ACE2* cells**

With the consideration of the potential risk of severe COVID-19 induced by SARS-CoV-2 infection, the research on SARS-CoV-2
must be conducted in laboratories with a biosafety level (BSL)-3 or higher. To overcome this hurdle in SARS-CoV-2 research, we established a pseudo-typed SARS-CoV-2 system by replacing vesicular stomatitis virus-G (VSV-G) with the SARS-CoV-2 spike protein to produce a pseudo-typed virus using the well-defined, third-generation lentivirus system (Figure 2A). We first checked the infection ability of this pseudo-typed virus in both ACE2+ and ACE2−/C0293 cells. Pseudo-typed SARS-CoV-2 could only infect ACE2+ 293 cells but not ACE2−/C0293 cells, suggestive of the SARS-CoV-2 spike, protein-mediated, ACE2-dependent entry process (Figure 2B). With the use of this pseudo-typed virus system, we tested the blocking efficacy of ACE2−/C0615-Fc and ACE2−/C0740-Fc. Indeed, ACE2−/C0740-Fc could strongly inhibit the entry of pseudo-typed SARS-CoV-2 into 293-ACE2 cells with a half-maximal inhibitory concentration (IC50) of ~0.68 nM (Figure 2C). Surprisingly, ACE2−/C0615-Fc had a very minor blocking effect on the entry of pseudo-typed SARS-CoV-2 despite its similar binding profile to S1-mFc, RBD-mFc, and 293-spike cells (Figures 1C–1E and 2D). To further test the specificity of the ACE2−/C0740-Fc-mediated blockade of the pseudo-typed SARS-CoV-2, we investigated the blocking effect of ACE2−/C0615-Fc on the entry of pseudo-typed VSV. Although ACE2−/C0740-Fc inhibited ~80% pseudo-typed SARS-CoV-2 infection, it did not affect the infection by pseudo-typed VSV (Figure 2E). To determine whether the ACE2−/C0740-Fc-mediated blockade could be applicable to other
cell types, we performed a similar assay on the lung epithelial A549-ACE2 cell line. Consistent with the data from 293-ACE2 cells, ACE2/C0740-Fc significantly blocked the entry of pseudo-typed SARS-CoV-2 into A549-ACE2 cells (Figure 2F). Therefore, ACE2/C0740-hFc blocked the pseudo-typed SARS-CoV-2 infection by competing with the membrane-anchored entry receptors.

ACE2-Fc-mediated cytotoxicity against SARS-CoV-2 spike-positive cells is directed through the Fc portion

There are two functional portions of the ACE2/C0740-hFc fusion protein. The extracellular domain, ACE2/C0740, functions as a competitive binding factor to the membrane-anchored SARS-CoV-2 receptor ACE2, and the Fc portion mediates multiple functions by engaging Fc gamma receptor (FcγR)-positive cells and complements.34 To verify whether ACE2/C0740-hFc could induce death of SARS-CoV-2 spike-positive cells through antibody-dependent cell-mediated cytotoxicity (ADCC), we performed a natural killer (NK) cell and Raji-spike cell co-culture assay in the presence of ACE2/C0740-hFc. Given the high binding affinity of IgG1 to active FcγRs,35 ACE2/C0740-hFc induced significant ADCC and lysed more Raji-spike cells than Raji cells (Figure 3A). This observation highlights the potential lysis effect on virus-infected cells in vivo. We also tested whether ACE2/C0740-hFc could induce complement-dependent cytotoxicity (CDC). In the presence of complements, ACE2/C0740-hFc induced more lysis of spike-positive cells than that of spike-negative cells (Figure 3B). Taken together, ACE2/C0740-hFc could potentially eliminate virus-infected, spike-positive cells through both ADCC and CDC.

Safety profile of ACE2-C0740-hFc in vivo

ACE2 is a carboxypeptidase that potently degrades angiotensin II to angiotensin 1-7.36 ACE2 antagonizes the activation of the classical renin-angiotensin (RAS) system and protects against organ damage,
hypertension, diabetes, and cardiovascular diseases. Whether exogenous exposure to ACE2/C0740-hFc affects the normal physiological status is, however, unclear. First, we tested the in vivo half-life of hACE2/C0740-hFc in mice and found it to be approximately 8.22 h (Figure 4A). We subsequently collected different tissues and investigated the potential effect of ACE2/C0740-hFc on tissue damage. There were no differences in hematological features, body weight change, alanine aminotransferase (ALT), and immune cell infiltration in organs such as the heart, liver, spleen, lung, and kidney (Figures 4B–C4E). Although the enzyme activity of hACE2/C0740-hFc is well reported in mice, two potential factors may affect the evaluation of side effects, namely, the potential immune response against human protein hACE2 in mice and different interaction networks of hACE2 and mouse ACE2 (mACE2). To avoid these limitations, we performed similar experiments using mACE2/C0740-hFc and observed no adverse effects 7 days after treatment (Figures 4B–4E). Taken together, ACE2/C0740-hFc was well tolerated in mice without any obvious side effects.

Engineered MSCs can be a potential drug-development strategy for COVID-19 treatment

The clinical translation of protein-based therapeutics can be time consuming owing to good manufacturing practices (GMP) production and quality control, which may not be feasible for the urgent need for treating COVID-19. Therefore, other drug-delivery systems should be considered to save time. MSCs have been widely used for treating autoimmune diseases and as vectors for protein drug delivery. We generated an MSC cell line stably expressing ACE2/C0740-hFc (MSC-ACE2/C0740-hFc) by lentiviral infection. As expected, this cell line persistently secreted ACE2/C0740-hFc into the culture supernatant (Figures 5A and S1). The concentration of ACE2/C0740-hFc in the supernatant was about 190 ng/mL after a 4-day culture. To determine whether ACE2/C0740-hFc in MSC-derived supernatant is bio-reactive, we first measured its ability to react with spike-positive 293 cells (Figure 5B). The MSC-secreted ACE2/C0740-hFc showed a binding strength similar to that of purified ACE2/C0740-hFc (Figure 5C). We next investigated whether ACE2/C0740-hFc from the MSC-derived supernatant could block pseudo-typed virus entry. Indeed, the MSC-derived supernatant showed a significant blockade effect on the entry of pseudo-typed SARS-CoV-2 into 293-ACE2 cells (Figure 5D). To test the possibility of using MSC-ACE2/C0740-hFc as a live drug in vivo, we injected these engineered MSCs into mice. The expression of ACE2-Fc in the serum lasted for more than 14 days after a single dose (Figure 5E). Further, we evaluated the bio-distribution of these MSCs, which were preferentially located in the lungs after 24 h of injection (Figure 5E). Thus, these MSCs are suitable for treating SARS-CoV-2-induced pneumonia. Therefore, the MSC-based ACE2/C0740-Fc delivery strategy provides a time-saving option for clinical treatment of SARS-CoV-2-induced disease.
MSCs expressing variable fragment-interleukin 6 (IL-6) single-chain variable fragment against IL-6 receptor (scFv-IL6R)-Fc can potentially inhibit SARS-CoV-2-induced immune damage

The proinflammatory cytokine storm can cause tissue damage in multiple disease models. Suppression of proinflammatory cytokines such as IL-6 has been shown to mediate therapeutic effects in autoimmune diseases and the chimeric antigen receptor (CAR)-T cell-induced cytokine storm.39,40 For instance, tocilizumab has been used to treat COVID-19 and improve patient outcome.41,42 With the consideration that MSCs are capable of rapidly producing proteins and preferentially migrating to inflammatory tissues, we aimed to generate MSCs expressing scFv-IL6R-Fc to suppress the SARS-CoV-2-induced cytokine storm. We first generated and purified scFv-IL6R-Fc protein and compared its activity with that of tocilizumab. scFv-IL6R-Fc showed a similar blockade effect on the IL-6-induced TF-1 cell proliferation compared with tocilizumab (Figure 6A). We then established an MSC cell line stably expressing scFv-IL6R-Fc. After 1 month of culture, this cell line was still capable of secreting scFv-IL6R-Fc. The concentration of scFv-IL6R-Fc in the supernatant was about 300 ng/mL after a 4-day culture (Figure 6B). To test the in vivo performance of the MSC-derived scFv-IL6R-Fc, we injected them intravenously into mice. Interestingly, the expression of scFv-IL6R-Fc in the serum lasted for more than 14 days after administration of a single dose (Figure 6C). Therefore, scFv-IL6R-Fc-expressing MSCs can be potentially used for blocking the SARS-CoV-2-induced cytokine storm and can be combined with virus-neutralizing MSC-ACE21–740-hFc to provide multi-layer protection against SARS-CoV-2 infection.

DISCUSSION

The current COVID-19 pandemic has created a greater social and economic stir than its predecessors, SARS outbreak and Middle East Respiratory Syndrome (MERS) epidemic. Although some vaccines and neutralizing antibodies have been approved for clinical usage,43–50 their efficacy may be compromised by the frequent viral mutations.51,52 Therefore, safe and effective treatments for COVID-19 are urgently warranted. SARS-CoV-2 enters permissive cells mainly through ACE2.1 Neutralizing antibodies against the SARS-CoV-2 spike protein and soluble ACE2 receptors are potential drug candidates that can block the viral entry into cells.7,16 The acceptable stability, specificity, and manufacturing process have deemed neutralizing antibodies as promising candidates for drug development. Theoretically, these antibodies only affect the virus or virus-infected cells and thus, pose limited side effects in vivo. However, the high mutation rate may lead to the escape of the virus from neutralizing antibodies even after a single mutation. This hypothesis is supported by the observation that neutralizing antibodies against SARS-CoV have limited blocking effect on SARS-CoV-2 despite the high similarity in their RBD structures. This limitation can be avoided with a soluble ACE2-based neutralization strategy, as mutant viruses escaping soluble ACE2 will also lose the ability to infect ACE2-positive cells. One shortcoming with both
neutralizing antibodies and ACE2-Fc fusion protein is the antibody-dependent enhancement effect on virus infection. Further experiments are imperative to clarify these concerns. With the consideration of the biosafety issue, our study was performed using pseudo-typed viruses. Although the entry step of pseudo-typed viruses is similar to that of live viruses, further investigation with live virus and animal models will provide more information on the efficacy of ACE2-Fc-based therapy.

ACE2 is a carboxypeptidase that potently degrades angiotensin II to angiotensin 1-7. It plays a central role in the homeostatic control of cardiorenal action. ACE2 is a membrane protein, and its distribution is determined by the expressing cells. Soluble ACE2 is delivered to all possible tissues through the circulatory system, thereby raising the concern regarding its side effects on the cardiorenal system. Our preliminary mouse data showed no obvious side effects induced by ACE2-Fc treatment. Further investigation on the long-term side effects of ACE2 is needed in animal models before translation into clinical studies. Despite this limitation, there is one potential advantage of soluble ACE2 as a promising therapy. ACE2 has been reported to play a protective role in lung injury. SARS-CoV-2 infection leads to internalization of the ACE2 receptor, which may reduce ACE2 enzymatic activity and its subsequent protective effect on lung injury. Supplementation with soluble ACE2 may provide additional tissue repair effects beyond viral neutralization.

Given the important role of ACE2 in SARS-CoV-2 entry, multiple research groups have reported the applicability of soluble ACE2 as an effective entry blocker. In addition to using wild-type intact soluble ACE2, high-affinity ACE2 mutant, enzymatic dead mutation, or peptide inhibitors derived from ACE2 provide highly promising effects for blocking SARS-CoV-2 entry. Our data indicate that the Fc portion of ACE2-Fc can mediate weak ADCC and CDC to eliminate virus-infected, spike-expressing cells and may provide additional immune-regulatory functions for improving antiviral effects. We used MSC as a drug-delivery vector; its inflammation tissue tropism may provide an advantage of targeting the drug specifically to the virus-infected tissue. Furthermore, MSCs were engineered to secrete scFv-IL6R, which could sufficiently block the IL-6 signaling pathway and prevent the overwhelming inflammation in COVID-19.

To meet the urgent need for treating SARS-CoV-2-induced COVID-19, we have developed an MSC-based cell therapy strategy. MSCs have been widely used for treating autoimmune diseases for more than 20 years. There are ongoing clinical trials to evaluate the efficacy of MSCs for COVID-19 treatment, given their innate anti-inflammatory and tissue-repair activities. Here, we armed MSCs with virus neutralization and anti-cytokine storm capabilities to significantly boost their therapeutic effects. MSCs are preferentially located in inflammatory tissues, thus aiding in the targeted delivery of ACE2 fusion protein to SARS-CoV-2-infected tissues. This phenomenon significantly enhances the drug concentration in the targeted tissue and lowers the potential side effects. However, a plausible disadvantage of MSC-based therapy includes the challenges in regulating the dosage of soluble ACE2 secreted from cells. Further engineering of MSCs with on-off switchable promoters or suicide genes may provide a possible solution to overcome these hurdles.

The MSC delivery method is critical for the successful clinical treatment of COVID-19 and determines treatment efficacy and safety.
Previous studies have shown that the in vivo persistence of MSCs is limited,\textsuperscript{61–63} which restricts their potency as a delivery vector. Further optimization to reduce immunogenicity and enhance survival of MSCs in vivo is critical to improve their efficacy. With the consideration of the safety profile of intravenously infused MSCs, some severe COVID-19 patients are in a hypercoagulable-procoagulant state and are at high risk of thrombosis and embolization.\textsuperscript{64} Some non-bone marrow-derived MSCs express high levels of the procoagulant tissue factor, which might induce severe thrombosis and embolization when intravenously infused in patients with COVID-19.\textsuperscript{64–66} Pre-screening for the expression of the procoagulant tissue factor of MSCs and the hemocompatibility test before infusion is critical for preventing this adverse effect.

The limitations of our study include the lack of the use of the SARS-CoV-2 virus and immune-competent animal models. Although pseudo-viruses are widely used to dissect the viral-entry mechanism, they are not capable of replication in host cells; this may cause overestimation of the viral-entry blockade effect of ACE2-1.\textsuperscript{740}-Fc. Furthermore, the deficiency of non-obese diabetic (NOD)-Prkdcscid severe combined immunodeficiency (scid)Il2g\textsuperscript{tm1Wjl} (NSG) mice in adaptive immune cell components may be favorable for MSC persistence in vivo. When allogeneic MSCs are used in the clinic, their clearance by the recipient’s adaptive immune response reduces both the delivered drug concentration and its half-life. Further optimization of the in vivo persistence of MSCs and improvement of transgene expression will be critical before their clinical application. Owing to the lack of BSL-3 laboratory conditions, we did not evaluate the side effects of ACE2-1.\textsuperscript{740}-Fc in virus-infected hosts. As ACE2-1.\textsuperscript{740}-Fc showed cytotoxicity against spike-expressing cells, it may cause tissue damage upon virus infection. This should be further investigated using appropriate virus-infected models. It would also be interesting to test whether ACE2-1.\textsuperscript{740}-Fc is effective against the mutated SARS-CoV-2 strain.

Taken together, our data indicate that ACE2-Fc can serve as a potential therapeutic agent for COVID-19 treatment with a relatively good safety profile. MSC-based viral blockade and inflammation control can provide a rapid alternative for multi-layer protection against SARS-CoV-2 infection.

MATERIALS AND METHODS

Mice

C57BL/6j mice were purchased from Beijing Vital River Laboratory Animal Technology (Beijing, China). NSG equivalent M-NSG mice were obtained from the Shanghai Model Organisms Center (Shanghai, China). All mice were maintained under specific pathogen-free conditions. Animal care and use were in accordance with institutional and NIH protocols and guidelines, and all studies were approved by the Animal Care and Use Committee of Shanghai Jiao Tong University.

Cell lines and reagents

Lenti-X 293 cells were purchased from Clontech. A549 cells were obtained from the American Type Culture Collection. Raji cells were kindly provided by the Stem Cell Bank, Chinese Academy of Sciences (Shanghai, China). TF-1 cells were kindly provided by the Cell Resource Center, Peking Union Medical College (Beijing, China). Human NK cells and MSCs were kindly provided by Shanghai Longyao Biotechnology (Shanghai, China). Plasmids encoding the SARS-CoV-2 spike protein and ACE2 were obtained from Molecular Cloud (Nanjing, China) and sub-cloned into a pCDH-EF (System Bioscences) lentiviral vector plasmid with a puromycin-resistance marker. To establish SARS-CoV-2 spike- or ACE2-expressing cell lines, Lenti-X 293, A549, or Raji cells were infected with a SARS-CoV-2 spike- or ACE2-expressing lentivirus. After selection with puromycin, the pooled resistant cells were identified by flow cytometry. The cell culture medium was supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. Lenti-X 293 as well as A549 cells and their derivatives were cultured in complete Dulbecco’s modified Eagle’s medium (DMEM). Raji cells and their derivatives were cultured in complete Roswell Park Memorial Institute (RPMI) medium. Human AB serum was purchased from Gemini Bio-products (West Sacramento, CA, USA).

Production of ACE2 and spike fusion protein

For hACE2-1.\textsuperscript{740}-hFc, hACE2-1.\textsuperscript{615}-hFc, mACE2-1.\textsuperscript{740}-hFc, S1-mFc, and RBD-mFc fusion protein production, DNA sequences encoding the indicated proteins (Figure S1) were cloned into the pCDH-EF vector (System Biosciences, Mountain View, CA, USA). Briefly, plasmids containing the indicated fusion protein were transiently transfected into Lenti-X 293 cells using polyethyleneimine. From days 1 to 5 after transfection, the medium was refreshed with DMEM supplemented with 1% FBS. All supernatants containing the recombinant proteins were collected and pooled. Purification was carried out using a Diamond Protein A column according to the manufacturer’s protocol (Bethyl, Shanghai, China).

Neutralization assay with pseudo-typed SARS-CoV-2

Lenti-X 293 cells were transfected with lentivirus package component plasmids, Gap/pol (Addgene; #12251), RSV-Rev (Addgene; #12253), pCDH-EF-infrared-fluorescent protein (IRFP)-luciferase (luc), and pcDNA3.1(+)-2019-nCoV-spike-P2A-EGFP (Molecular Cloud; #MC_0101087). Supernatants containing lentivirus particles were collected 48 and 72 h post-transfection by ultracentrifugation for direct usage or concentration. The viral titer (transduction units per milliliter) was determined by flow cytometry analysis of transduced Lenti-X 293-ACE2 cells. In the viral neutralization assay, hACE2-1.\textsuperscript{740}-hFc and hACE2-1.\textsuperscript{615}-hFc were serially diluted to indicated concentrations in complete DMEM. Diluted hACE2-1.\textsuperscript{740}-hFc and hACE2-1.\textsuperscript{615}-hFc were incubated with pseudo-typed lentiviral particles for 15 min at room temperature (20°C–25°C), inoculated on 293-ACE2 or A549-ACE2 monolayers in 96-well plates in the presence of 10 μg/mL polybrene, and further incubated at 37°C for 48 h. For A549-ACE2 infection, the samples were subjected to 60 min spinning at 1,258 x g at 32°C to improve the infection efficiency. Near-IRFP reporter activity was measured using Cytofleex S (Beckman Coulter). The percentage of infectivity was calculated as...
the ratio of IRFP readout in the presence of fusion protein normalized to that in the absence of fusion protein. The IC_{50} value was determined using a four-parameter logistic regression model (GraphPad Prism version 8).

ELISA and flow cytometry analysis of hACE2_{1-740}-hFc and hACE2_{1-615}-hFc
ELISA plates (Jet Biofil, Guangzhou, China) were coated with 2 μg/mL of RBD-mFc at 4°C for overnight. Plates were washed thrice with phosphate-buffered saline (PBS) containing 0.05% Tween 20 and blocked with 2% FBS in PBS at room temperature for 1 h. Samples with diluted, hACE2_{1-740}-hFc and hACE2_{1-615}-hFc were added to the wells, and the plates were incubated for 1 h at room temperature. Plates were then washed thrice and incubated with an alkaline phosphatase (AP)-conjugated goat anti-hFc secondary antibody (Jackson ImmunoResearch) diluted 1:2,000 in a blocking buffer for 1 h at room temperature. AP activity was measured at 405 nm using p-nitrophenyl phosphate (Guangzhou Howei Pharmaceutical Technology, Guangzhou, China) on an ELISA plate reader (Molecular Devices). Plates were then washed thrice and incubated with an alkaline phosphatase (AP)-conjugated goat anti-hFc secondary antibody (Jackson ImmunoResearch) diluted 1:2,000 in a blocking buffer for 1 h at room temperature. AP activity was measured at 405 nm using p-nitrophenyl phosphate (Guangzhou Howei Pharmaceutical Technology, Guangzhou, China) on an ELISA plate reader (Molecular Devices). The half-maximum effective concentration (EC_{50}) binding values were calculated using GraphPad Prism (version 8).

293-spike cells were incubated with indicated samples containing ACE2_{1-740}-hFc or ACE2_{1-615}-hFc at 4°C for 30 min, washed thrice with 2% FBS in PBS, and incubated with 1:200 diluted Alexa Fluor 647-conjugated goat anti-hFc antibodies (Jackson ImmunoResearch). Samples were analyzed on a CytoFLEX S (Beckman Coulter), and data were analyzed using FlowJo software (Tree Star).

Lentivirus production
Lentivirus was produced by transient transfection of Lenti-X 293 with a four-plasmid system. Supernatants containing lentivirus particles were collected 48 and 72 h post-transfection and used to establish stable cell lines.

In vitro ADCC and CDC assays
Raji and Raji-spike cells were labeled with 5 or 50 μM carboxyfluorescein succinimidyl amino ester (CFSE), respectively, according to the manufacturer’s protocol (MedChemExpress, Shanghai, China). Approximately 2 × 10^4 CFSE-labeled Raji and Raji-spike cells were co-cultured with 4 × 10^5 primary NK cells or supplemented with 10% human AB serum in the presence of various concentrations of hACE2_{1-740}-hFc. After 48 h, the cells were analyzed by flow cytometry. The ratio of CFSE<sup>high</sup>/CFSE<sup>low</sup> was used to indicate the relative death of Raji-spike cells versus Raji cells.

In vivo characterization of hACE2_{1-740}-hFc
Pharmacokinetics were determined in C57BL/6 mice after a single injection of 100 μg ACE2_{1-740}-hFc. Serum concentrations at indicated time points were determined by ELISA using immobilized RBD-mFc. C57BL/6 mice received a single injection of 100 μg of ACE2_{1-740}-hFc. 7 days later, hematology, body weight, and hematoxylin and eosin (H&E) staining of the indicated organs were analyzed. H&E staining was performed by Servicebio (Wuhan, China).

Bio-distribution of MSC-ACE2-Fc
M-NSG mice were intravenously injected with 5–7.5 × 10^7 MSC-ACE2-Fc through retro-orbital sinus. On days 1, 7, and 14 after injection, the heart, liver, spleen, lung, and kidney were collected. Genomic DNA was isolated from these organs using an E.Z.N.A. Tissue DNA Kit I (Omega Bio-tek). The relative distribution of human MSCs was quantified by real-time polymerase chain reaction (PCR) using primers (5’-aagcataaggtcgtagagagat-3’ and 5’-cagagtctgatggtagtacatg-3’) against the human IL2RG gene, which was absent in M-NSG mice.

TF-1 proliferation assay
TF-1 cells were seeded in flat-bottomed, 96-well plates at 10,000 cells/well in the presence of 1, 10, or 50 ng/mL IL-6 (Sino Biological, Beijing, China). The cells were then incubated with 0.1, 1, or 10 μg/mL of scFv-IL6R-Fc or tocilizumab (Roche) for 48 h. Following incubation, cell proliferation was measured by the Cell Counting Kit (CCK)-8 assay (Dojindo Molecular Technologies, Shanghai, China) according to the manufacturer’s protocol.

Statistical analysis
Data are expressed as mean ± standard deviation (SD) or standard error of the mean (SEM). The data of two groups were compared using a two-tailed unpaired Student’s t test. Statistically significant differences are indicated by *p < 0.05, **p < 0.01, and ***p < 0.001.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2021.05.004.

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AUTHOR CONTRIBUTIONS
X.Y. designed the project. X.Z., P.H., H.W., Y.X., M.L., F.L., H.Z., Q.D., H.L., X.Q., J.L., X.W., and X.Y. performed the experiments. X.Z. and X.Y. analyzed the results and wrote the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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