Supporting Information for

SHORT COMMUNICATION

Neutralization of SARS-CoV-2 pseudovirus using ACE2-engineered extracellular vesicles

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Methods

Animals

The Balb/c mice (female, 6–8 weeks) were procured from Shanghai Laboratory Animal Center (SLAC) Co., Ltd. (Shanghai, China). The mice had free access to water and food during the experimental period. All animal experiment procedures, complying with the animal experiment guidelines, have been approved by the Institutional Animal Care and Use Committee (IACUC) of the Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

Materials

Anti-ACE2 antibody (Abcam, UK); Anti-SARS-CoV-2 spike glycoprotein antibody (Abcam, UK); Plasmid Mini, Midi and Maxi Kits (Qiagen, Germany); Firefly Luciferase Reporter Assay Kit (Meilunbio, China); Human ACE2 protein (Sino Biological, China); Hoechst 33342 (Meilunbio, China).

Preparation of the EVs-ACE2

Prior to cell culture, FBS (fetal bovine serum) was centrifuged at 100,000×*g* for 2 h to deplete the serum-derived extracellular vesicles (EVs-free FBS). HEK293T and HEK293T-ACE2 cells, utilized for extracellular vesicle production, were cultured in Dulbecco's modified eagle medium (DMEM) with 10% EVs-free FBS for 48 h. Briefly, the EVs were isolated from the cell culture medium according to the method of a previous report.[42] After three centrifugations (300×*g* 10 min, 2000×*g* 10 min, and 10,000×*g* 30 min), the pellets, probably including cells, dead cells, cell debris, and large vesicles, were discarded. The supernatant was ultracentrifuged at 100,000×*g* for 70 min at 4℃ using the CP100NX ultracentrifuge (Hitachi, Japan). The EVs thus obtained were then resuspended using PBS and ultracentrifuged again at 100,000×*g* for 70 min. The purified EVs were collected for the subsequent experiments.

Characterization of the EVs

An NTA was performed using a Nanosight NS300 instrument (Malvern, UK). For transmission electron microscopy (TEM), the EVs were dropped onto the grids for 1 min, contrasted with 1% uranyl acetate, and dried. The micrographs were captured under a Talos L120C TEM (Thermo Scientific, Waltham, USA) at 120 kV. For the WB analysis, the EVs were lysed with a radioimmunoprecipitation assay buffer (RIPA) containing a protease inhibitor cocktail (100:1, v/v, Sigma-Aldrich, St. Louis, USA). The samples of EVs were analyzed using SDS-PAGE following a standard procedure. The blots were probed with antibodies specific to ACE2 (Rabbit, Abcam, UK). The bands were visualized on the ChemiDoc MP Imaging System (Bio-Rad, Hercules, USA). The freshly isolated EVs were stored at 4℃ in an EVs-free culture medium and the particle size changes in the test time frame were measured using NTA. The ACE2 expression in EVs-ACE2 was determined by the Coomassie brilliant blue method using human ACE2 protein (Sino Biological) as a standard (Bio-Rad, Hercules, CA). During the quantification procedure, a standard protein human ACE2 was separately loaded in 0.025, 0.5, 0.75, 1.0, 1.5 μg to five lanes. The standard protein was used to give a standard curve of known concentration. The whole EVs-ACE2 lysate was also separately loaded in 2.0, 5.0 μg to two lanes on the same gel. The content of ACE2 in each sample lane was then quantified using Image Lab software.

Production of the SARS-CoV-2 S-pseudovirus

To generate the SARSCoV-2 S-pseudovirus, the HEK293T cells were co-transfected with *pNL4-3.Luc.R-E*, *nCOV.his-spike-FL*, and a Golgi location *pTagRFP* plasmid using the transfection reagent PEI_{25k}. The HEK293T cells were seeded in the 12-well plates at a density of 5×10^5 cells per well and cultured for 20 h. The cells per well were treated with the PEI_{25k}/plasmids complex (1.3:1, w/w), including 1 μg PNL-4-3Luc.R-E, 0.5 μg pTagRFP, and a varying dose of

nCOV.his-spike-FL (0.25 μg, 0.33 μg, 0.5 μg, or 0.75 μg) in a fresh DMEM medium without FBS for 4 h at 37 ℃, and then replaced with a fresh medium.[22-24] Two days post transfection, the supernatant containing the SARS-CoV-2 S-pseudovirus were harvested and filtered through a membrane with 0.45-μm pore size. Subsequently, to investigate the best proportion of the three plasmids for the pseudovirus preparation, the HEK293T-ACE2 cells with a density of 5×10^5 cells/well were seeded in 12-well plates. After 24 h, the cells were incubated for 12 h with the pseudovirus produced by the different ratios of *pNL4-3.Luc.R-E*, *nCOV.his-spike-FL*, and *pTagRFP*. The cells were then washed with PBS three times and then used for the fluorescent imaging (CARL ZEISS, Germany).

Characterization of the S-pseudovirus

The level of the S protein was analyzed using a WB analysis with Anti-SARS-CoV-2 spike glycoprotein antibody (Abcam, UK). The median size and size distribution of the S-pseudovirus were analyzed using NTA.

WB analysis of ACE2 expression in various cell lines

The cells were seeded in 12-well plates at a density of 5×10^5 cells per well and incubated for 24 h. The cells were then collected and the levels of ACE2 analyzed by WB with Anti-ACE2 antibody (Abcam, UK), according to a standard procedure.

Inhibition of viral attachment by EVs-ACE2

To investigate the inhibition of pseudoviral attachment by EVs-ACE2, the cells were seeded in the 24-well plates at a density of 2.5×10^5 cells per well. After a 24 h culture, the S-pseudovirus were incubated with EVs-ACE2 or EVs-Control at 37 ℃ for 4 h (the quantity ratio of pseudovirus and EVs was 1:5), followed by culture with different cells at 37 ℃ for 12 h. Cell nuclei were then stained with Hoechst 33342 for 5 min, thoroughly washed with PBS three times to perform the fluorescent imaging (CARL ZEISS, Germany). For the quantitative measurements, the cells were digested, collected, and then analyzed using a flow cytometer (NovoCyte, Agilent, Santa Clara, USA). The S-pseudovirus without EV-pretreatment were used as a negative control.

Inhibition of viral infection by EVs-ACE2

To investigate the inhibition effect of pseudoviral infection by EVs-ACE2, the S-pseudovirus were also pretreated with the EVs-ACE2 4 h prior to adding to the cells. After 48 h of co-incubation, the cells were harvested and treated with 200 μL of the RIPA lysis buffer. After centrifugation at 12,000×*g* for 20 min, the luciferase activity of the supernatants was detected using the Luciferase Assay Kit (Meilunbio, Dalian, China), and the luminescence was measured using the EnSpire Multimode Plate Reader (PerkinElmer, Waltham, USA). The luminescence was normalized to the protein concentration of each sample, which was measured using a BCA Microplate Protein Assay Kit (Beyotime, Shanghai, China). Additionally, the quantification of luciferase expression in the transfected cells was determined by quantitative real-time polymerase chain reaction PCR (qRT-PCR). Total RNA was isolated from cells with Trizol (Tiangen, China). The reverse transcription was finished with the iScriptTM gDNA Clear cDNA Synthesis Kit (Bio-Rad, USA).

And, the real-time PCR was finished by using multiple kits (SYBR Premix Ex TaqTM, RR036A, Takara Bio). Furthermore, qRT-PCR reactions were finished in an ABI 7500FAST Sequence Detector System (ABI, USA). The luciferase forward and reverse primers were 5'-AATGTCCGTTCGGTTGGCAG-3' and 5'-GGCTGCGAAATGCCCATACT-3', respectively. And, the actin (the reference control) forward and reverse primers were 5'-GGTCATCACTATTGGCAACG -3' and 5'- ACGGATGTCAACGTCACACT-3', respectively.

In vivo inhibition test

The Balb/c mice were randomly divided into three groups. At the beginning of the experiment, the mice were placed in an animal anesthesia machine (EZSystem, USA) and 1% Isoflurane was used as an anesthetic. The mice received PBS (the blank control), the DiO-labeled EVs-ACE2 (60 μg), and an equal amount of the DiO-labeled EVs-Control (the negative control) via intranasal administration. Thirty minutes later, all of the mice were given 20 μL (12 μg) of the S-pseudovirus. Another 30 min later, they were sacrificed using a high dose of isoflurane. The nasal mucosa tissues of the mice were dissected and fixed with 4% paraformaldehyde for preparation of the cryosection slices with a thickness of 10 μm (CM1950, Leica, Germany). The tissue slices were imaged using a fluorescence microscope (Carl Zeiss, Germany). The overlap proportion was determined using ImageJ by calculating Pearson's value. The nasal mucosa tissues were also used for preparing the single-cell suspension for flow cytometry assay to detect the RFP signal. Additionally, the quantification of luciferase expression in the nasal mucosa tissues was determined by qRT-PCR. Total RNA was isolated from the nasal mucosa tissues with Trizol (Tiangen, China). The reverse transcription was performed using the iScriptTM gDNA Clear cDNA Synthesis Kit (Bio-Rad, USA).

WB analysis of the nasal mucosa tissue

The dissected nasal mucosa tissues were used to verify whether the ACE2 protein was expressed. After they were cut into pieces, 2 mL of the cell lysate was added, and they were placed on a shaker at 37 ℃ for digestion for 1 h. The lysate was then filtered using nylon mesh to remove the residual tissue. The total protein was determined using a BCA kit, and the samples were processed by SDS-PAGE and transferred to nitrocellulose membranes. The blots were probed with Anti-ACE2 and were visualized using a ChemiDoc MP Imaging System.

In vivo preliminary safety studies

The EVs-ACE2 or EVs-Control (60 μg in 20 μL PBS) were administered into the nasal cavity of the Balb/c mice, and the blood was collected via the orbital vein three days later. The serum was collected and analyzed using an automated hematology analyzer (XT-2000i, Sysmex, Kobe, Japan) for blood chemistry test, including alanine aminotransferase (ALT), total protein (TP), albumin (ALB), urea nitrogen (Urea), creatinine (CRE), calcium (Ca), phosphorus (P), potassium (K), and sodium (Na).

For the hematoxylin-eosin staining, the Balb/c mice were randomly divided into three groups. The mice received PBS (the blank control), EVs-ACE2 (60 μg), and the EVs-Control (60 μg) via nasal

administration. The animals were then humanely sacrificed 72 h post administration, and the major organs (heart, liver, spleen, lung, and kidney) were collected and fixed using 4% paraformaldehyde for histopathological examination.

Data analysis

Statistical analysis was performed using *t*-tests and one-way analysis of variance (ANOVA). Data were expressed as mean ± standard deviation (SD). Statistically, significant difference was defined as *P < 0.05, **P < 0.01, and ***P < 0.001.